Orchid Conservation Techniques Manual

First International Orchid Conservation Congress - Training course
September 2001

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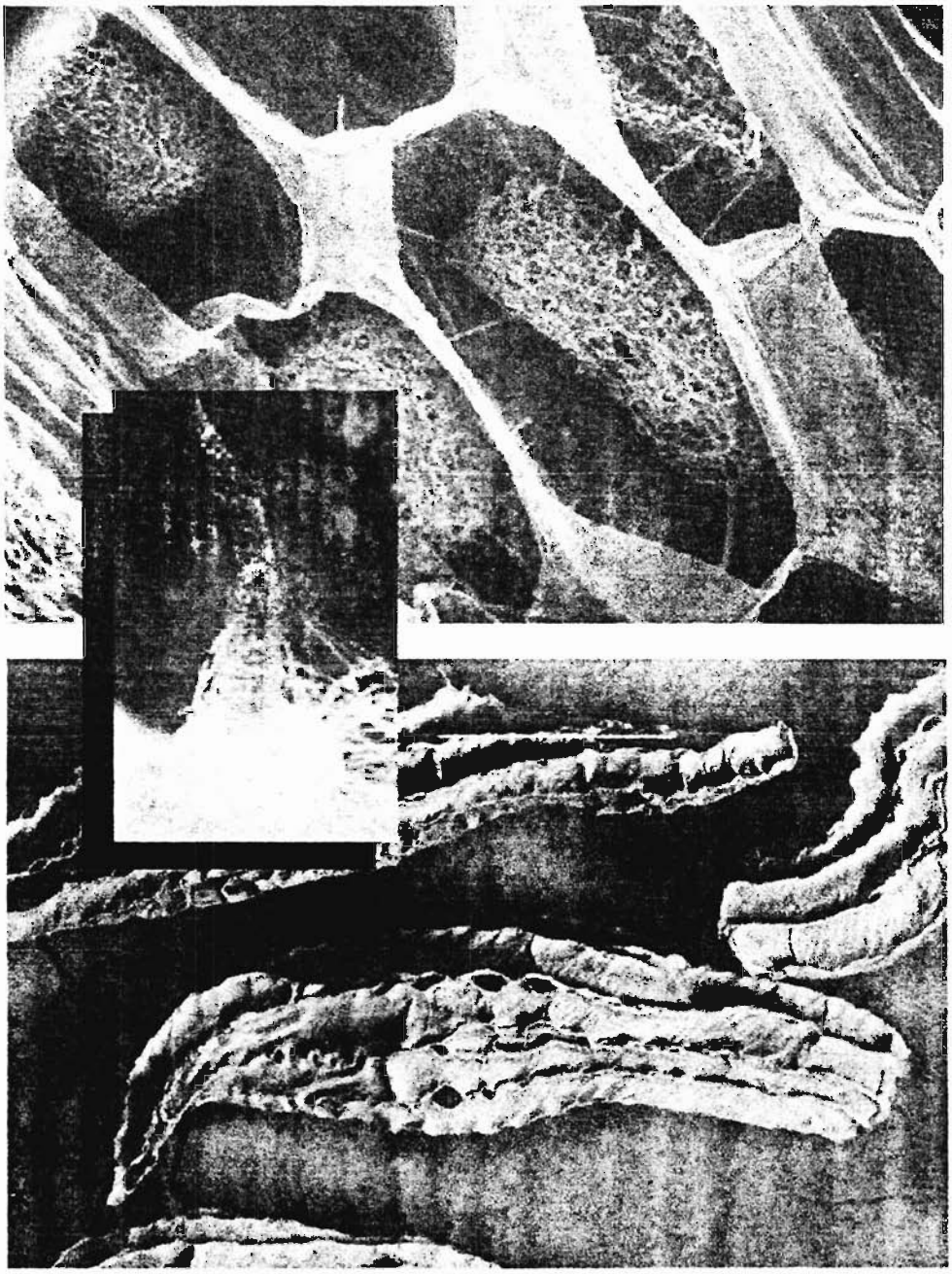

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Plant Science, Kings Park & Botanic Garden
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Front Cover: ESEM of fungal peloton visible in LS of *Prassophylum fimbriata* root. SEM image of *Caladenia arenicola* seed. Symbiotic seedling of *Thelymitra manginii*.

Table of Contents

Acknowledgment: Portions of Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) *Working with Mycorrhizas in Forestry and Agriculture.* (Pirie Printers: Canberra) have been used in this manual.

Orchid Conservation Training Course - September 20-22, 2001

Topic	Presenters	Time[2]	Notes
Thursday September 20, 8:30 am – DAY 1 Kings Park & Botanic Gardens Laboratory			
1. Welcome, Introduction, Presentation of Resources and Group Photo	Kingsley Dixon		
1.1.Laboratory manual with comprehensive bibliography of orchid conservation techniques		0.30	Manual with diagrams, recipes and bibliography
1.2. Orchid Research Kit		0.10	Tools, mounting media, stains
1.3. Laboratory safety and microscope use		0.10	Care of equipment
1.4. Intro to media preparation, axenic methods	Eric Bunn & KPBG staff	0.30	Talk and demos.
Morning tea		**0.15**	
2. Genetic Diversity and Sampling Issues	S. Krauss, Robyn	1.00	Talk and demo
3. Isolation of orchid fungi			
3.1. Field trip to local bushland	KPBG staff, students	0.45	Collect material, seed baits
LUNCH		**4**	
3.2. Isolation of orchid fungi and care of isolates	KPBG staff, students	2.00	Isolate fungi from peletons and surface sterilised materials
Afternoon tea		**0.15**	
4. Propagation and Care of Seedlings			
4.1. *In vitro* methods, seed germination, cryopreservation, etc.	KPBG staff Margaret Ramsay	1.30	Demos. and lab tour
4.2. Nursery management	Bob Dixon,	0.45	Talk and nursery tour
	Total	**8**	
Friday September 21, 8:30 am – DAY 2 University of Western Australia Teaching Laboratory			
5. Fungi			Video microscope demos
5.1. Identification of soil fungi	Prof. Sivasithamparam	1.30	Talk + demos of orchid fungi
Morning tea		**0.15**	
5.2. Advanced methods of fungal identification	A/Prof B. Dell & guests	1.00	Talk and demo
5.3. Demonstrations of advanced microscopy techniques using orchid materials	Prof. J. Kuo and graduate students	1.00	UWA Microscopy and Microanalysis Centre
LUNCH		**4**	
6. Microscopic Methods			
6.1. Introduction to methods	Mark Brundrett	0.30	Slide presentation
6.2. Sectioning, clearing, staining	KPBG staff	1	Hands-on staining, sectioning
Afternoon tea		**0.15**	
6.3. Useful histology techniques	Mark Brundrett	1	Present results in groups
6.4. Viability testing	Margaret Ramsay, etc.	1.00	Demos and activities
	Total	**8.30**	
Saturday September 22, 9:00am – DAY 3 University of Western Australia Teaching Laboratory			
7. Orchid Propagation & Restoration Case Studies			
7.1. Warm growing terrestrials	Kingsley Dixon etc.	0.30	Talk and demo
7.2. Cool growing terrestrials	Margaret Ramsay	0.30	Talk
7.3. Slipper orchids	as above	0.30	Talk
Morning tea		**0.15**	
7.4. Tuber induction	Nika Debeljak	0.15	Demo
7.5. Translocation & out-planting	Andrew	0.30	Short presentations
7.6. Habitat management	Mark & Andrew	0.30	Factors threatening orchids
7.7. Recovery plans	Andrew	0.30	Presentation and discussion
LUNCH		**4.3**	
3.2. View and discuss results of fungus isolation trials		0.30	
8. General Discussion Session	Shelagh Kell*	1.00	*moderator
9. Free time			
10. Conclusions and Presentation of Certificates			Workshop dinner
	Total	**6.00**	
Pollination biology	Colin Bower	1.30	? Monday night 5-6:30, Hyatt

typically fleshy, few in number and lasting for a single growing season of around six months. Some terrestrial orchids, such as the genera *Caladenia* and *Pterostylis* have roots that are highly reduced or absent (Ramsay *et al.* 1986). The roots of many terrestrial orchids form fleshy organs, know as tubers, for nutrient storage and pernating over unfaverable conditions (Dixon 1991). The tuber contains an apical bud which elongates forming the new stem which also gives rise to new roots. Some species of terrestrial orchids produce more than one replacement tuber each year and thus tend to form colonies (asexual reproduction) but more typically only a single replacement tuber is formed and increases in population size is the result of seedling recruitment (sexual reproduction). Stems of orchids are very varied and can be almost vestigial or long and swollen. Typically the stems of terrestrial orchids are succulent or fleshy (Hoffman and Brown 1992; Jones 1993).

Orchid seeds are minute and lack many of the structural components that are found in seeds of most other Angiosperms. Seeds are generally less than 2 mm in length and less than one mm in width (Arditti 1992; Arditti and Ghani 2000). The most significant difference is the lack of cell differentiation and food reserves such as endosperm. There are small amounts of lipids stored in seeds. The testa of orchid seeds often have a lipophilic covering (Arditti 1979).

Mature orchid seeds have a simple embryo made up of parenchyma cells containing lipid and protein bodies (Manning and van Staden 1987; Rasmussen 1990; Richardson *et al.* 1992). For more detailed accounts of seed structure see the review by Peterson *et al.* (1998). A distinctive characteristic of orchid seed is the absence of a differentiated shoot apical meristem or root apical meristem at maturity (Veyret 1974).

Mycorrhizas

Mycorrhizas (fungus-roots) are symbiotic associations between specialised soil fungi and plants involved in nutrient transfer (see Brundrett (2001) for a full definition). Orchids have mycorrhizal associations that are morphologically different from other types of mycorrhizas and involve a phyllogenetically distinct group of soil fungi (Hadley 1982; Rasmussen 1995; Currah *et al.* 1997; Smith and Read 1997).

In the early 1900's, investigators first succeeded in germinating orchids *in vitro* and in observing the process of infection in embryos and seedlings (Bernard 1903; Burgeff 1909). Opinions differed as to the nature of the relationship between orchid and fungi based on structures observed. Orchid mycorrhizas are characterised by the presence of dense hyphal coils in cortical cells of the root, stem or protocorms of orchid species (Harley and Smith 1983; Masuhara and Katsuya 1994). Early histological researchers recognised two types of orchid mycorrhizas (i) tolypophagy, which occurs in most species, and (ii) ptyophagy, found in a few highly mycotrophic species (eg. *Gastrodia).* Ptyophagy has been interpreted as deformation and lysis (also called digestion) of the intracellular hyphal tips through which fungal cell contents are released (Burgeff 1959), but this requires confirmation by observations using modern techniques such as electron microscopy. In

most associations, the endophyte forms well-defined hyphal coils, known as pelotons, in infected cells before hyphal digestion takes place. Successive waves of peloton formation, digestion and re-infection of the same cells have been reported (Burgeff 1959; Smith and Read 1997). Transfer of metabolites across the host-fungus interface rather than digestion is now considered to be the primary mode of nutrient transfer in all types of mycorrhizas, but these fluxes have not been measured in orchids (Smith *et al.* 1990). The digestion of fungal hyphae within plant cells occurs in all types of mycorrhizas where fungi occupy plant cells (Smith and Read 1997). This process is considered to be a general defense response to fungal invasion and a means of regulating the metabolic cost of association to the plant (Brundrett 2001).

There are considerable variations in the distribution of mycorrhizas within roots or stems of orchids. Mycorrhizal colonisation has been reported to be sporadic in most epiphytes, but is generally more widespread and consistent in terrestrial orchids (Burgeff 1959; Rasmussen 1995). Different genera of terrestrial orchids can have distinctive colonisation patterns within their roots or stems (Ramsay *et al.* 1986). These mycorrhizal infection patterns in the whole plant (ie. root, collar, stem, rhizome, etc) may be associated with particular fungal types (Ramsay *et al.* 1986). Colonisation patterns of mycorrhizal fungi within plants are primarily determined by host cell properties, but morphological features can also be correlated with the presence of certain fungi (Brundrett 2001).

Orchid Fungi

Identity and Specificity

The fungi that are known to form orchid mycorrhizas are Basidiomycetes, and most, formerly belonged to the form-genus *Rhizoctonia* (Sneh *et al.* 1991; Currah *et al.* 1997). Orchid rhizoctonias are recognised by the general feature of the mycelium in culture, the presence in most isolates of short inflated segments which resemble spores and the formation of loose aggregates of hyphae regarded as poorly developed sclerotia or resting bodies (Hadley 1982). Isolates which sometimes form sexual stages in culture (a difficult and poorly repeatable procedure) belong to the basidiomycete genera *Ceratobasidium, Ceratorhiza, Epulorhiza, Sebacina, Thanatephorus,* and *Tulasnella* (Warcup and Talbot 1967; 1971; Currah *et al.* 1997). A diverse assemblage of fungi belonging to other groups have also been isolated from orchid roots, but may not all be beneficial (Currah *et al.* 1997).

The question of specificity within the Orchidaceae has been a point of contention for many years, Knudson believed there were low levels of specificity for tropical epiphytic species. Conversely, Burgeff (1909; 1959), who worked with many terrestrial orchids, thought that there was strong specificity in the association between orchid and fungus. Burgeff (1909) distinguished seven categories of seed/fungus relations, ranging from fully incompatible to fully compatible, that have proved to be adequate for describing the full range of

interactions. Additional evidence available today suggests that both these hypotheses are correct (Arditti 1992). Culture and symbiosis tests have shown that there may be numerous races or strains, varying in physiological features and morphology within the spectrum of one species or a group of similar isolates. For example, Harvais and Hadley (1967) isolated 244 *Rhizoctonia* strains from *Dactylorhiza purpurella* and other north British orchids. These belonged to 15 main groups, but most groups were rarely obtained from more than one habitat, with the exception of *R. repens* which was widespread. Curtis (1939) argued that ecological distribution of the fungi was related to habitat rather than host. Harvais and Hadley (1967) also showing that *D. purpurella* was symbiotic with nearly all isolates tested. However, there are other orchids, such as *Goodyera repens* that is usually infected with one fungus (*Ceratobasidium cornigerum)* as an adult (Hadley 1982). These observations may explain why *G. repens* occurs in more restricted habitats than *D. purpurella.* Many other orchids that have been studied in detail seem to have fairly specific fungal associates that vary less between habitats (eg. Warcup 1981; Ramsay *et al.* 1987; Currah *et al.* 1997; Sen *et al.* 1999).

Smreciu (1989) showed that *Platanthera hyperborea* seeds germinated and protocorms developed on an asymbiotic culture medium as well as on Warcup's medium with several fungal endophytes. Richardson (1992) found that the terrestrial orchid *P. hyperborea* germinated with *Rhizoctonia cerealis* or *Ceratorhiza goodyerae-repentis* and suggested, with supporting evidence from (Smreciu 1989) and Masuhara and Katsuya (1989), that orchids from the genus *Platanthera* may not be highly specific as to it's fungal endophytes. Different fungal types have been shown to specifically associate with genera of Western Australian orchids (Ramsay *et al.* 1986; 1987). For example in sterile culture on potato dextrose agar, all *Caladenia* species examined use a mucoid, slow growing, cream-coloured fungus whereas *Pterostylis* species require a fast growing fluffy, white endophyte for *in vitro* seed germination and seedling growth.

Most orchid fungus specificity studies have been based on germination tests conducted under sterile conditions and assume the same fungi will form effective associations with adult plants (eg. Warcup 1980; Ramsay *et al.* 1986). However, these studies show the importance of mycorrhizal fungi to germination, but not necessarily those important to the survival of adult plants. Seed of some orchids will not germinate with fungi isolated from adult plants (Masuhara *et al.* 1993; Zelmer *et al.* 1997). It has been reported that host-fungus specificity is generally lower at the time of germination than it is during seedling development *in vitro* (Muir 1989), and seems to decrease again as the plant reaches the photoaoutotrophic phase when many distantly related fungi may be encountered as endophytes (Warcup 1981; Alexander and Hadley 1983). Thus, the fungus associated with seed germination may not stay with the orchid throughout its life, but there are also many examples where fungi isolated from adult orchids promote successful germination (see Arditti (1992) for examples). The establishment of healthy seedlings with leaves, not merely the cracking of the testa should be regarded as the decisive criterion of host-fungus compatibility (Peterson *et al.* 1998).

For example, Warcup (1971; 1973) tested factorial combinations of orchid seed-fungal isolates in germination tests to examine the level of specificity in several Australian terrestrial orchid genera. He found that *Diuris* spp. contained only *Tulasnella calospora* (*Rhizoctonia repens*) while *Caladenia* spp. were associated with *Sebacina vermifera*. There also were high levels of host-fungus specificity within *Diuris* and *Pterostylis*. Ramsay *et al.* (1987) used hyphal anastomosis groups to categorise fungai associated with the genus *Pterostylis* and showed that distribution of fungal types may be linked to particular habitat types. For example, anastomosis group (AG) 8 is only recorded for hot, arid habitats, AG3 semi-arid sites (eg exposed rock habitats) and AG4 is only associated with *Pterostylis* which grow in introduced pine plantations. Conversely, vicarious fungal groups such as AG2 isolates associate with *Pterostylis* species across a range of habitats. Sympatric species of *Pterostylis* have also been found to use different AG's.

Orchids may be able to live in symbiosis with one or several different fungi, but the relative importance of co-occurring fungi is unknown. Co-occurring orchid species with different fungi may minimise competition for the same nutrient resource, if different species of fungi access different soil resources. In general, the diversity of orchid fungi associating with a particular orchid species appears to be much lower than for other types of mycorrhizal fungi (ie. most studies have reported one or two fungi per species). The greatest implication of high-host fungus specificity and low fungal diversity to orchids would be to restrict orchids to certain habitats where these specific fungi occur.

Distribution in Substrates

The spatial distribution of orchid fungi is largely unknown. Organic matter, which is the ultimate resource for the mycotrophic nutrition, is unevenly distributed in the soil and populations of other mycorrhizal fungi occur in discrete patches in soils (eg. Brundrett and Abbott 1995). Thus it is reasonable to assume that the inoculum of orchid fungi in soils is also patchy. Competition between orchid siblings for available mycelia occurs in *in vitro* cultures (Alexander and Hadley 1983; Rasmussen *et al.* 1989; Tsutsui and Tomita 1989). Some species of orchids tend to grow in clusters, while others tend to be widely spaced, although this depends to a large extent on a differential tendency to produce vegetative offshoots, interaspecific competition among seedlings could also influence spacing patterns.

Understanding the distribution of orchid mycorrhizal fungi within soil or other substrates is important for attempts to return orchids to the field and in understanding the distribution of orchids. The patchy distribution of orchids may be influenced by the presence or absence of the specific mycorrhizal fungi that are essential for the survival of the orchid. A seed burial technique devised by Rasmussen and Whigham (1993) allows the distribution of effective orchid endophytes to be assessed *in situ* in natural habitats.

Early work by Curtis (1939) and Harvais and Hadley (1967) suggests that fungi associated with orchids may be more widespread in the soil than their hosts. A number of isolates

have been isolated from soil in areas where orchids were absent (Warcup and Talbot 1967). *Ceratobasidium cornigerum* and *Tulasnella calospora* appear to have worldwide distribution which may also apply to other species. Perkins *et al.* (1995) observed that different combinations of mycorrhizal fungi occurred in the roots of *Microtis parviflora* across three sites indicating that other factors as well as host specificity was important in determining which fungi were mycorrhizal with *M. parviflora* at any particular site. Environmental factors may vary considerably between each site. Also the density of each fungus in the soil may vary temporally and spatially (Perkins and McGee 1995). *In situ* studies of orchid mycorrhiza are discussed further in Chapter 4.

Mycorrhizal Dependency and Nutrition

Knudson's (1922) experiments first demonstrated that seedlings did not have an absolute requirement for mycorrhizas, as they could take up nutrients directly if available in suitable forms. However, their have been numerous demonstrations of the benefits provided to orchids by their fungi which establish that most terrestrial orchids have an obligate requirement for mycorrhizas when growing in natural habitats (Arditti 1992; Rasmussen 1995; Peterson *et al.* 1998). Further evidence is provided by observations of the passage of phosphorus compounds, traced by radioactive isotopes, from the site of feeding through the external mycelium to the endophytic hyphae (Smith 1966; 1997; Alexander *et al.* 1984; Alexander and Hadley 1985).

Further evidence of the effectiveness of orchid mycorrhizas is provided by the occurrence of many orchid species in habitats with extremely infertile soils with low accessibility of minerals, or with extremely high or low pH, which often have a loose texture and high humus content (eg. Sheviak 1974).

The situation with epiphytic orchids is less clear, as their protocorms often become photosynthetic at an early stage and adult plants have limited and sporadic fungal colonisation, which may indicate decreasing mycorrhizal dependency (Rasmussen 1995). A recent study of germinating epiphyte seeds demonstrated that mycorrhizal fungi can also assist in water uptake (Yoder *et al.* 2000).

Orchid mycorrhizas differ from typical ECM or VAM associations, as orchid fungi can provide a source of energy as well as mineral nutrients to their host plants (Rasmussen 1995). These carbon compounds presumably are derived from the breakdown of organic substances in the surrounding substrate. The transfer ^{14}C from orchid fungus to the host plant has been demonstrated (Alexander and Hadley 1985), but the ultimate source of this carbon has not been determined. Radioactive carbon has been traced from fungi to seedlings but not from seedlings to fungi. The plant may supply essential vitamins or amino acids to fungi in some cases (Leake 1994). However, there is no real evidence that fungi receive substantial benefits from any of their associations with orchids and often grow without any assistance from orchids.

Mycorrhizal associations where fungi do not seem to receive any benefits from plants have been called epiparasitic, myco-heterotrophic, cheating associations, or exploitative associations (Leake 1994; Taylor and Bruns 1999; Brundrett 2001). This is the reverse of most relationships between higher plants and fungi. In this review these associations are designated as exploitative mycorrhizas, reflecting the nature of the relationship from the fungal perspective. The Orchidaceae is reported to contain species with differing levels of dependence on mycorrhizas, extending from fully-autotrophic members, presumably with mutualistc mycorrhizal associations to fully-heterotrophic members with exploitative associations. However, the nature of mycorrhizal associations of many of these plants has not been investigated and their nutritional dependency on fungi has been assumed whenever other explanations are lacking.

Little is known about the other ecological roles of fungi that associate with green orchids and some of these fungi may have an adverse impact on other plants. For example, epiphytic orchids sometimes appear to have a detrimental effect on trees (Ruinen 1953; Johansson 1977), but this could result from a correlation between epiphyte abundance and tree decline due to other factors. There are some cases where *Rhizoctonia* isolates from orchids have been identified as pathogens in roots of other plants (Warcup 1985; Zelmer *et al.* 1996), or parasites of VAM fungi (Williams, 1985). However, most orchids have fairly specific associations with *Rhizoctonia* strains that are not known to be pathogens of other plants (Warcup 1981; Ramsay *et al.* 1987; Muir 1989; Currah *et al.* 1997; Sen *et al.* 1999). There currently is insufficient information to safely say whether autotrophic orchids normally have associations with unspecialised soil fungi or specialised mycorrhizal fungi. We know very little about the other roles of orchid fungi in soils. This knowledge is essential for us to develop an understanding of the biology and ecology of these beautiful and fascinating plants.

Orchid Seed Germination

This section focuses on terrestrial orchids, as epiphytes typically are propagated by asymbiotic means. As discussed below, there is strong evidence for vital of role orchid fungi in the propagation and growth of terrestrial orchids. This contrasts, with epiphytic orchids where mycorrhizal fungi may have less importance after seed germination.

The minute seeds of terrestrial orchids have very little stored nutrient reserves available to support seedling development (Arditti and Ghani 2000). These limited reserves and the subterranean germination of many terrestrial species, result in the general belief that mycorrhizal fungi are normally essential for seed germination. Studies comparing the effectiveness of symbiotic and asymbiotic germination have usually shown that symbiotic germination was more rapid and effective than asymbiotic germination (see Peterson *et al.* (1998) for examples of orchid seed germination). In some terrestrial orchids successful germination was only achieved by symbiotic germination (Hadley 1982). Muir (1989) screened a wide range of fungal isolates for their capacity to promote germination and

growth of European species of *Orchis, Ophrys, Dactylorrhiza* and *Serapias*. He found that rare species were compatible with fewer isolates of fungi than common species in the same genus. In some cases, different fungi are responsible for the growth of mature plants than those responsible for germination. Little information is available on the *in situ* germination of orchid seed (Masuhara and Katsuya 1994; Zelmer and Currah 1997; Rasmussen and Whigham 1993; Currah *et al.* 1997).

Orchid conservation

The phenomenal diversity of orchids and various threatening processes (Table 1.1) have resulted in many threatened orchid species (IUCN 1996).

Table 1.1. World-wide conservation threats to orchids (IUCN 1996)

Threat	Cause
Habitat destruction, modification and fragmentation	Logging Agriculture and plantations Habitat fragmentation Urban development Mining
Collecting from natural habitat	Horticultural trade Amateur collection Consumable orchids

The alteration or destruction of an orchids habitat will threaten each orchid species to a different degree according to geographical distribution, habitat specificity, and population size. A species rarity may be determined based on these criteria (Rabinowitz *et al.* 1986). Orchid species which are rare are intrinsically more prone to extinction than those which are common as there is always going to be naturally occurring catastrophes (eg. intense fires, floods, or unusually severe mesoclimatic variations).

Understanding more about a species biology will enable us to more effectively direct conservation efforts. Many of the endangered plant species in Western Australia now require assistance if there is to be no further loss of species diversity. The translocation of a species to the field to rebuild depleted orchid populations is a option available to land managers (ANPC, 1997). Careful life history studies may provide invaluable information for future management of Western Australia's endangered flora.

Orchids are well researched taxonomically, but relatively little is known of their conservation biology and importantly, methods for management and translocation. Unlike other plants, terrestrial orchids are unique in their highly specialised pollination

mechanisms and habitat requirements. They also require advanced technology for large-scale propagation. These methods include: (i) Collection of seed and fungal symbionts which are effective for germination, (ii) Development of effective propagation methods for the target species; and (iii) Translocation of orchids to safe sites.

Symbiotic associations are generally considered to be essential throughout the Orchidaceae. However, we have a very limited understanding of the ecology of the orchid, their associated symbionts, or the interactions between them. The majority of current knowledge on orchid fungus and host plant interactions is based on *in vitro* studies using fungal isolates from mature orchid plants. However, in recent years researchers have demonstrated the ability to unlock some of the mysteries of orchid mycorrhizas under field (*in situ*) situations.

There still remains large amounts of research to be carried out throughout the world in order to unlock many of the secrets remaining in orchid biology and the biology of associated fungi.

Techniques presented in this course manual will be required when investigating these associations further.

-Chapter 2-
Media preparation

There are large numbers of media routinely used for orchid propagation by asymbiotic protocols. A comprehensive list is presented in Rasmussen (1995) and Hicks (2000). Common media used for dealing with the symbiotic germination of Australian terrestrials are detailed below. These are the main media used in this workshop.

Asymbiotic

Notes on media formulations

There are several media formulations that are commercially available. All are (or should be) the product of experimentation to optimize performance. Some formulas are publicly available, others are proprietary, and not published. Mother flask media are used for germinating seeds, and replate media are used to grow the resulting protocorms to a size where they may be deflasked successfully.

A commonly employed medium is P-6668, sold by Sigma-Aldrich Chemical Company. It is also sold as P-668 (PhytoTech Labs). It is inexpensive, reliable, readily available, and the formula is published. Its primary drawback is that it is too strong for many species, and must be used at a lower rate. It is very flexible, and may be used for replate by combining it with banana. A commercial formula, P-1056 (Sigma-Aldrich), is P-6668 plus 30 grams of a 50/50 mix of banana powder and malto dextrin. It is simple enough to purchase P-6668/P-668 and add ~50 grams of banana baby food or banana pulp to make a suitable replate medium. If using raw bananas, the fruits should be as mature as possible. They may be frozen to turn them to mush, rather than manually pulping them.

In the same way that P-6668/P-668 may be used for replate flask by adding banana pulp, other "complex" additives may be used in a similar manner. Replate media may call for the addition of banana, pineapple, or coconut liquid, all of which contain high levels of reducing sugars among other things. This contrasts with mother flask that contains only sucrose, a disaccharide (which is probably partially converted to glucose and fructose during autoclaving in the mildly acidic solution). Fructose is also available in virtually pure form as honey.

For some cold-tolerant terrestrial species, special additives such as tree sap from birches, potato cubes, and other complex components have been shown to benefit germination or development of seedlings. It is important to keep a close eye on developing cultures. If protocorms on mother flask start to turn from green to other colors, they should be replated as quickly as possible to fresh replate media before they die. Further, if protocorms or seedlings are not doing well on a given medium, do not hesitate to move them to something new to see if growth will improve.

Pa5
(Dixon 1989)

Used for asymbiotic germination of terrestrial orchids.

To approximately 750 ml of deionised water add the following stocks.

Stock Solution[2]	
Solution Ia	25 ml
Solution Ib	25 ml
Solution II	50 ml
L & S minor minerals	2.0ml
2 see below for stock solution recipes	
Other	
Biotin	0.1 ml
Nicontinic acid	1.0 ml
Pryridoxine HCL	1.0 ml
Calcium pantothenate	1.0 ml
Thiamine HCL	1.0 ml
Ascorbic acid	1.0 ml
Myo-inositol	0.1g
Sucrose	20 g
Agar	8.0g
Coconut water	50 ml
pH	5.6

In a microwave proof jug, add the above components (except the coconut water) and bring up to 950 ml with deionised water. Mix and adjust pH to 5.6. Dissolve agar in microwave for approximately 10 min then pour into two 500ml stock bottles and autoclave for 20min at 121°C. Cool enough to touch in a 50°C water bath before adding filter sterilised coconut water. Mix and pour into petri dishes in the laminar flow. Allow Pa5 plates to cool before replacing lids.

Preparation of stock solutions

Stock solution	**Component**	**g/L**	**mM**
Solution Ia	Ca (NO3)2.4H2O	40	169
Solution Ib	(NH4)2SO4	10	76
	KCL	10	134
	MgSO4.7H2O	10	40
	FeNaEDTA	1.54	6.3

Solution II	K2HPO4	5	29
	Citric Acid	1.8	9

Symbiotic

For each medium amounts added to one liter of de-ionised water. Powdered agar (Leiner Davis Gelatin, Grade J3) is used here.

Seed germination

Oat meal agar (OMA)
(Clements and Ellyard 1979; Dixon 1989)

Rolled oats	2.5g
Agar	8.0g
pH	5.5

In a microwave proof jug, add the rolled oats and agar and bring up to 1000 ml with deionised water. Mix and adjust pH to 5.5. Dissolve agar in microwave for approximately 10 min then pour into two 500ml stock bottles and autoclave for 20min at 121°C. Cool enough to touch in a 50°C water bath before pouring into petri dishes in the laminar flow. Allow OMA plates to cool before replacing lids.

Fungal isolation and culture

Fungi isolating medium (FIM)
(Clements and Ellyard 1979; Dixon 1989)

Sodium nitrate		0.3g
Potassium dihydrogen orthophosphate		0.2g
Magnesium sulphate		0.1g
Potassium chloride		0.1g
Yeast extract		0.1g
Sucrose		2.5g
Agar		8.0g
Streptomycin sulphate	(stock)[1]	10.0ml
pH		6.8

1 add after autoclaving, when medium has cooled to 50°C, using sterile filtration.

In a microwave proof jug add all components, except streptomycin sulfate (see above note[1]) and agar and bring up to 990 ml with deionised water. Mix and adjust pH to 6.8. Dissolve components in microwave for approximately 10 min then pour equal volumes into two 500ml stock bottles and autoclave for 20min at 121°C. Cool enough to touch in a 50°C water bath before adding the streptomycin sulphate in sterile conditions. Pour into petri dishes in the laminar flow. Allow FIM plates to cool before replacing lids.

Stock streptomycin solution

Dissolve 1g of Streptomycin sulphate in 70ml of deionised water. Store in the dark at 4°C for up to one month.

1/5 Potato dextrose agar (PDA)

PDA powder	6.8g
Agar	6.0g
pH	6.8

Add PDA powder and agar to 1000ml deionised water in a microwave proof jug. Adjust pH to 6.8. Microwave on high until the agar has dissolved and pour into 500ml media bottles before autoclaving at 121°C for 20 min.

-Chapter 3-
Genetic Techniques for Orchid Conservation

There is currently a bewildering array of genetic techniques available for the analysis of variation at the population and individual level. These notes provide a very brief introduction to some of these. References are listed where more detailed information can be found. Particular attention is paid to the PCR based multi-locus DNA fingerprinting technique AFLP (Amplified Fragment Length Polymorphism), which is the predominant technique being used at Kings Park for practical outcomes in conservation, restoration and native plant breeding.

Some of the markers available include: allozymes, RFLP, RAPDs, DAF, AFLP, SCARs, SAMPL, microsatellites (SSRs), minisatellites, as well as DNA sequencing

This array of markers can be broken down into a few main categories:

Co-dominant markers – both alleles in a diploid organism are visualized by a genetic marker system such that homozygous and heterozygous genotypes are detected. Examples include allozymes, RFLPs and microsatellites. At the phenotype level, the gene product of both alleles is detected, producing two bands on a gel at a given locus.

Dominant markers – only one allele in a diploid organism is visualized by a genetic marker system such that only two genotypes are detected, either band presence or band absence. At the phenotype level, the gene product of only one allele (in either homozygous or heterozygous form) is detected. Examples include RAPDs and AFLP.

Of these, a distinction can be made between **PCR and non-PCR based techniques.** Non-PCR techniques include allozymes and RFLP. PCR techniques include RAPD, AFLP and micorsatellites.

PCR (Polymerase Chain Reaction) utilizes the enzyme DNA polymerase within a complex reaction mix to make millions of copies of DNA from just one copy, allowing visualization on an electrophoresis gel. Four steps are involved:
1. Denature: The reaction mix is heated to 94oC to separate the double stranded DNA template into single stranded molecules.
2. Annealing: Lower temperature (40-70oC) to allow short DNA molecules called "primers" (8 to 20 base pairs in length) to "anneal" to their complementary strands.
3. Extension: Increase temperature to 72oC to allow the copying of single stranded DNA commencing at the primer-template complexes. DNA polymerase is the catalyst for this extension.
4. Repeat steps 1-3 for 20 to 50 cycles to amplify the regions of DNA flanked by the primers exponentially.

The use of heat stable DNA polymerases which survive the lengthy exposure to high temperatures required by PCR, and the development of thermocyclers capable of cycling temperatures quickly and accurately have facilitated the automation of this process and revolutionised the analysis of genetic variation at the population level.

The principle genetic techniques for conservation include:

Allozymes: Alternate forms of enzymes encoded by different alleles at the same locus. Allozymes are prepared by homogenizing tissue to produce a solution of proteins which is electrophoresed through a gel (starch or cellulose acetate). Specific enzyme products are then visualized by the use of a stain facilitatiing the enzymatic reaction. For example, Alcohol Dehydrogenase is scored by the use of alcohol, which is converted to aldehyde that binds with a dye for visualisation of bands. Alleles of different sizes have different mobilities.

RFLP (Restriction Fragment Length Polymorphism): Polymorphisms at specific sites in the DNA sequence revealed by cutting DNA with restriction enzymes, electrophoresis, blotting these products to a membrane which is then probed with radioactive DNA allowing visualisation of bands of different size.

RAPDs (Random Amplified Polymorphic DNA): Polymorphisms obtained by amplification of DNA fragments by PCR using "arbitrary" primers. The primers are typically 10 bases long and serve as both forward and reverse amplification. The DNA fragments are electrophoresed in an agarose gel and visualised with the use of ethidium bromide and a UV light source.

Microsatellites (or Simple Sequence Repeats – SSRs): Tandem repeats of very short nucleotide motifs (1-6 bases long) eg. (CA)17 or (AAG)12. Specific primers amplify these sites by PCR, with visualization by electrophoresis on agarose gels or on acrylamide gels using an automated sequencer and fluorescent labeling.

AFLP (Amplified Fragment Length Polymorphism). A powerful new PCR-based multi-locus DNA fingerprinting technique with high reproducibility and wide application. A number of steps are involved – Restriction of the DNA with restriction enzymes such as EcoRI and MseI; Ligation of adapters to the ends of these fragments; A pre-selective PCR, using and EcoRI primer and an MseI primer, each with a 1 base extension beyond the adapter site; A selective PCR amplification, using EcoRI and MseI primers with a 2,3 or 4 base extension beyond the adapter site – the EcoRI primer is fluorescently labeled, enabling electrophoresis and visualisation on an automated DNA sequencer; Scoring of fingerprints using dedicated software such as GeneScan and Genotyper. Individual fragments are accurately sized by the inclusion of an internal size standard of a different wavelength (colour). Further details are provided below.

Genetic variation generated by all these techniques is visualised by some form of electrophoresis. **Electrophoresis** is the migration of particles through a gel medium under the influence of an electric current. Proteins or DNA fragments of different size migrate through the gel at different rates. Visualisation of these products is by the use of stains such as ethidium bromide, or by the use of fluorescent dyes tagged to DNA when using an automated sequencer.

Scoring and analysis of data

As mentioned, **co-dominant markers** such as allozymes and microsatellites allow us to distinguish between heterozygotes and homozygotes at a single locus (site). Heterozygotes produce 2 bands on an electrophoresis gel, one for each allele. Homozygotes will produce a single band as both alleles are identical. Often, these homozygous bands will be double the intensity of a heterozygote band. Consequently, within a population at a single locus, we are able to determine the number of alleles, the frequency of each allele, the observed heterozygosity, the expected heterozygosity based on Hardy-Weinberg equilibrium, and how populations differ in their allele frequencies. At multiple loci, we can calculate the percentage of polymorphic loci and the average number of alleles per locus. We can also calculate the proportion of the total variation within hierarchies such as within and between populations. This structuring of genetic variation can be used, under certain assumptions, to infer historical levels of gene flow. Computer programs for the calculation of population genetic statistics are given below. These include the use of microsatellite variation for the characterisation of population bottlenecks, paternity analysis and individual relatedness.

For **dominant markers** such as RAPDs and AFLP, heterozygotes cannot usually be distinguished from homozygotes. Consequently, these gels are typically scored according to the presence or absence of fragments of a particular size. Population statistics derived from these data include the percentage of markers that are polymorphic per population. Estimates of expected heterozygosity can be made given certain assumptions. However, the disadvantage of dominance is overcome, especially with AFLP, by the generation of a very high number of markers per individual. As a consequence, unique DNA fingerprints can be generated for genetically distinct individuals. As a consequence, AFLP markers often allow the unambiguous assignment of paternity to offspring in natural populations. The presence/absence data matrix can be converted to an estimate of genetic distance between individuals using a distance measure such as euclidean distance, which can then be analyzed by way of a clustering analysis, or better still, by an ordination technique such as multi-dimensional scaling. This "genetic map" summarises the genetic distance between all individuals analysed. For example, we are using this sort of analysis to delineate genetic provenance for species targeted for restoration. The presence/absence data matrix is also suitable for a partitioning of variance analysis, for example, using AMOVA (Analysis of Molecular Variance), which is analogous to a conventional ANOVA, and is available in the Arlequin program, or in Genalex (see computer programs below).

TECHNICAL NOTES

1. DNA EXTRACTION

Perhaps the most critical step for molecular analysis, and AFLP in particular, is the extraction of clean, high molecular weight DNA. Here are some of the DNA extraction techniques we use in the lab at Kings Park.

1a. DNAZOL®

DNAzol is currently our main extraction procedure because it is quick, cheap and effective. It has worked well for us on a wide range of taxa. It is a new technique based on a novel guanidine detergent lysing solution which permits selective precipitation of DNA from a cell lysate.

DNAzol®- available from Invitrogen (formerly Life Technologies)

Grind plant tissue (ca. 100mg) in liquid nitrogen using a mortar and pestle (or micropestle and eppendorf).

Add 300ul Plant DNAzol and mix well.

Add 1-2ul RNAse and incubate at 25 degrees for 1.5hrs

Add 300ul chloroform and mix vigorously. Shake for 5 – 15mins

Centrifuge at 13,500rpm for 10 minutes, transferring the supernatant to a new tube

Add 225ul cold 100% ethanol. Mix by inversion and stand for 5 minutes

Centrifuge at 10,000rpm for 4 minutes

Discard supernatant. Wash pellet with 300ul DNAzol Wash (1 volume DNAzol and 0.75 volume 100% ethanol). Stand for 5 minutes

Centrifuge at 10,000rpm for 4 minutes

Pour of supernatant and re-wash pellet with 70% ethanol (twice)

Discard supernatant and dry off pellet. Dissolve in 70ul TE (or ddwater)

1b. CTAB EXTRACTION PROCEDURE (See Doyle reference below)

Grind leaf material in liquid nitrogen until a powder is obtained

Add 500ul CTAB Extraction Buffer. Incubate at 65 degrees for 15-20 minutes

Centrifuge at 11,500rpm for 10 minutes, decant supernatant and add 200ul 5M potassium acetate (pH 4.8). Mix gently and place in freezer for 10 minutes

Thaw and centrifuge at 11,500rpm for 10 mins at 4 degrees

Pour off supernatant noting the volume and then add equal amounts of chloroform to the sample. Mix well and leave on shaker for 10 – 60 minutes

Spin at 8000rpm for 10 mins, transferring top layer to a new tube. Repeat Step 5 if top layer cloudy.

Add 330ul cold isopropanol and mix gently to precipitate DNA. Place in freezer 5-30mins

Spin at 3000rpm for 15 mins to collect DNA. (CAREFUL : Pellet will not be solid

To separate the DNA from polysaccharides dissolve pellet & viscous layer in 100ul DI water and then add 400ul 2.5M NaCl.

Precipitate DNA by adding 1 ml 95% cold ethanol

Centrifuge at 6000rpm for 10 mins discarding the supernatant

Wash pellet with 1 ml 70% ethanol (to remove salts), decant liquid

Dry and resuspend DNA in 100ul 0.1TE.

RNAse with 1-2ul of enzyme at 37 degrees for 60 mins

1c. Jobes et al (1995 – see references) extraction procedure

An excellent alternative to a CTAB procedure to efficiently remove polyphenolics, polysachharides, and RNA. It is based on the use of PVP to bind polyphenolic compounds, a high molar concentration of sodium chloride to inhibit co-precipitation of polysaccharides and NDA, and an improved method for the removal of RNA by selective precipitation with lithium chloride. However, it is much more time consuming than either DNAzol or the QIAGEN DNeasy procedure, and requires the use fo some nasty reagents (phenol, chloroform, lithium chloride).

1d. QIAGEN DNeasy

We have also used the Qiagen DNeasy kit (cat # 69104) with generally very good results. It has the features of being very quick and does not require chloroform or phenol. However, this option is more expensive, at around $5 per sample. By comparison, the DNAzol extraction works out at around $1.50 per sample.

2. AFLP PROTOCOL

Using the core reagent kit available from Invitrogen (formerly Life Technologies) cat # 10482-016

see also:
PE Applied Biosystems. 1996. AFLP™ Plant Mapping Protocol. Part Number 402083, Revision B. PE Applied Biosystems, Foster City, California.

2.1 Restriction digest of genomic DNA

Add the following to a 1.5ml eppendorf tube creating a master mix.

5x Reaction Buffer	2.5 ul
EcoRI/MseI	1 ul

Aliquot master mix, then add 200 – 500 ng sample DNA (up to a maximum volume of 9ul – make up difference with H20) giving a total of 12.5ul.

Mix gently and briefly spin down. Incubate at 37 degrees for 2 hours.

Incubate the mixture at 70 degrees for 15 minutes. Place tubes on ice

2.2 Ligation of adapters

Add to the above,

Adapter ligation solution	12 ul

T4 DNA ligase	0.5ul

Mix gently and briefly spin down. Incubate at 20 degrees for 2 hours or o/nite.

Perform 1:10 dilution of the ligation mixture as follows :

Take 5 ul of reaction mixture and transfer to a new tube. Add 45 ul 0.1 TE buffer, mix well. Label tubes (2C)

2.3 Pre-selective PCR reaction

Add the following to a 1.5ml eppendorf as a master mix.

Pre-amp primer mix I (from Invitrogen cat # 10792-018)	10 ul
10x PCR Buffer (no MgCl)	1.25 ul
1.5mM MgCl	0.8 ul
Taq (0.3 units)	0.06 ul

Aliquot 12.1 ul of master mix into each PCR well

Add 1.25 ul of diluted DNA from step 2.

Pre-selective PCR program: 20 cycles of 94oC for 30s, 56oC for 60secs, 72oC for 60s.

Perform a 1:50 dilution by taking 3 ul of pre-selective PCR product and add to a new tube containing 147 ul 0.1 TE.

Label tubes (3C)

2.4 Selective PCR reaction

Add the following to a 1.5 ml eppendorf as a master mix per reaction.

10x PCR buffer (no MgCl)	1 ul
1.5mM MgCl	0.6 ul
Taq (0.25 units)	0.05 ul
Water	3.10 ul

Aliquot 4.75 ul into each PCR well.

Primer mix:

m-primer	2.25 ul
e-primer (labeled)	0.25 ul

Add 2.5 ul of primer mix (final concentration 15ng m-primer and 7.5ng e-primer) to each PCR well containing the master mix. Note that we include the dNTPs in the m-primer to give a final concentration of 0.2mM of each.

+ 2.5 ul of your "3C" template DNA.

Selective PCR:
A touchdown PCR commencing with one cycle 94oC for 30 sec, 70oC for 2 mins, and 72oC for 2 mins. In subsequent cycles, the annealing temperature is reduced 1oC steps to 61oC, followed by 23 cycles at 61oC. A single step of 60oC for 30mins follows, before holding at 4oC.

2.5 Electrophoresis on an ABI Prism 377 Automated Sequencer

Formamide Master Mix

	36 lanes	48 lanes
Formamide	30 ul	40 ul
Rox 500	8 ul	12 ul
Dye	14 ul	20 ul

Aliquot 1.3 ul into each tube, then add 2.5 ul of each selective PCR product.

Mix, spin down, 90 degree water bath for 2 minutes, then ice. Load 2 ul (36 lanes) or 1.2 ul (48 lanes) onto gel. Remove paper comb 5 minutes after commencement of run.

Electrophoresis performed using a 5% acrylamide gel using an ABI Prism 377 automated sequencer.

A Few Useful References

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Some computer programs for genetic data analysis

ARLEQUIN	http://anthropologie.unige.ch/arlequin
GDA	http://alleyn.eeb.uconn.edu/gda/
POPGENE	http://www.ualberta.ca/~fyeh/index.htm
TFPGA	http://herb.bio.nau.edu/~miller
ANALYSE	http://helios.bto.ed.ac.uk/evolgen/Mac/Analyse/index.html
GENALEX	rod.peakall@anu.edu.au

a web link to many programs can be found at:
http://www.biology.lsu.edu/general/software.html

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-Chapter 4-
ORCHID SEED SOWING TECHNIQUES

SEEDS

In order to maximise the genetic diversity of plants produced, the preferred material for propagation is normally seeds. As the principal mechanism for orchid seed dispersal is by wind, each capsule may contain thousands of seed. Terrestrial orchids have gained the reputation for being difficult to germinate. While this is true to some extent, it is becoming clear that they do not form a homogenous group in regard to their requirements for germination and seedling development and that although some problems have been resolved, there still remain many challenges.

The most important factor to consider when propagating from seeds is the quality of that seed. This may appear an obvious statement but much effort and money has been put into projects where complicated techniques were used unsuccessfully, the problems lying with poor seed quality.

Pollination

Small populations may have problems with attracting pollinators and there may be self-incompatibility mechanisms that prevent production of seed. If collecting from naturally pollinated populations, guidelines should be used on the proportion collected (collection from several plants ,taking no more than 10% of total, not collecting when there are very few individuals, are guidelines commonly used).

If natural seed is low, then hand pollination may be desirable and will usually have to be carried out in any case for plants held in *ex situ* collections as the pollinators are often not around or have no access to plants. Cross pollination may also be advantageous and decisions are increasingly being informed by conservation genetics studies. The extent that controlled crosses take place depends on the objectives behind sowing. Priorities for re-introduction and for cultivation of plants for trade may be quite different. More seed may be collected from hand pollinated plants without reducing the reproductive potential of the wild plants.

Pollination is usually carried out using a toothpick or fine forceps, making the most of the stickiness of the pollenias

It may be desirable in some cases to test the efficacy of the pollen using the ' hanging drop' techniques described by Light and MacConaill (1996)

Collection

As scientists frequently rely on field botanists, reserve managers etc to collect capsules, it is important to give clear instructions. These might include guidelines on when to collect capsules (what degree of maturity), average time for capsules to mature, type of container, packaging. Emphasis is given to not using polythene bags or other airtight containers such as film canisters as they will retain moisture and seeds are difficult to remove due to problems with static. In addition, it is important to inform them not to put seed in the refrigerator. On receiving seeds, all known information should be recorded (donor, provenance, harvest date, condition of seeds) and herbarium voucher specimens made where possible.

COLLECTION OF ORCHID SEED FOR PROPAGATION

1. As a guideline, the quantity of natural set seed to be collected from a wild population should be no more than 10% of the seed available on the day of collection. Where there are only a few individuals , no seed should be taken. Rare species that are hand pollinated will result in more capsules than would naturally occur in the wild and therefore more seed may be collected without reducing the reproduction potential of the wild plants.

2. The appropriate time to collect orchid seed pods is when they are turning brown, prior to the onset of dehiscence (just before the capsule begins to split). At this stage the seed will be fully developed, but the risk of contamination by fungal and bacterial spores will be reduced as the seeds will not have been exposed to the elements.
The time required for the seed capsules to mature will vary according to species and weather conditions. As they will ripen faster following dry and/or sunny periods, it is advisable to check their progress frequently if possible and err on the side of immaturity when collecting.

3. Usually the capsules ripen at different times on an inflorescence according to when they have flowered and it is advisable to collect individual capsules from a number of plants, whilst leaving the rest of the capsules to ripen further. If more than one pod per plant is ripe then they can be pooled.

4.For preference, please collect seed pods by placing a glass vial over the capsule and cutting it off near to the flowering stem. Place a plug of cotton wool in the vial neck and pack it in a padded envelope. Please **do not put too many capsules in each vial** as the condensation produced can result in mouldy and unusable pods.

5.Paper photography 'negative' envelopes may also be used but **do not use polythene bags , or other airtight containers** as these will retain moisture and possibly harm the seed. Orchid seed are extremely difficult to recover from plastic bags and vessels (eg film cannisters) due to their dust-like nature and problems with static. In addition, **do not put seed into the refridgerator**; the high relative humidity will be extremely damaging.

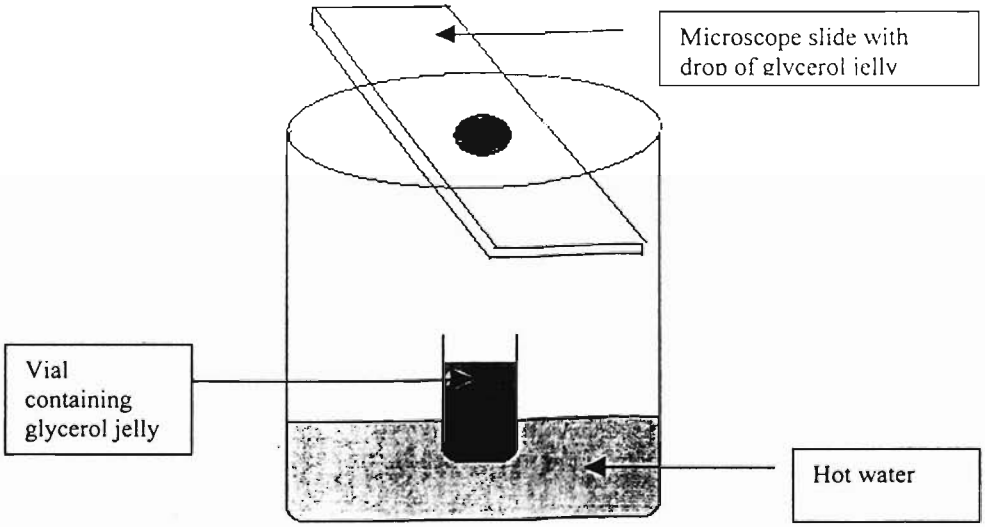
Capsules should be labelled with the following information:
Species; Locality; Date of pollination (if applicable); Date of collection; Collector.

MAKING A MICROSCOPE SLIDE OF ORCHID SEED

It is useful to make a microscope slide of all the seed you intend to sow.
If the seed received is unknown, colour shape and size of the seed can help to distinguish the genera.
Sometimes seeds may appear normal but may contain no embryo. Making a slide is useful to record this and may indicate that the seed is not worth sowing.

- Fill a large beaker with very warm water
- Place vial containing glycerol jelly in the hot water to melt.
- Position a microscope slide over the top of the beaker and with a pasteur pippette, place a drop of gycerol on the slide.
- Drop a very small amount of orchid seed into the drop of glycerol jelly and stir with a mounted needle
- Drop on a circular cover slip (this can be ringed with nail varnish to create a permanent slide)
- Observe under microscope

The stains : Alcian blue (carbohrydrate) or Coomassie Brilliant Blue (proteins) can be added to the glycerol jelly but these are not essential. These stains were originally used to stain pollen. Orchid seed embryos can usually be seen without staining.



MATURE v. IMMATURE SEED?

Where possible mature seed should be collected and sown, the main advantage of this being that mature seed can be stored and banked for later use / as reserve. It is not possible to store immature seed (although research continues).

For mature seed, the most appropriate time to collect orchid seed pods is just prior to the onset of dehiscence. At this stage the seed will be fully developed but the risk of contamination by fungal and bacterial spores will be reduced as the seed will not have been exposed to the elements.

Seed sowing techniques for immature seed

If there are problems with obtaining germination of mature seed because of possible inhibitors / dormancy, then this can often be overcome by collection of 'green' capsules before the seed is fully mature. Green capsules are relatively straightforward to sow. The disadvantages of using immature seed capsules, besides not being able to store the seed, are that the risk of carrying over viruses is greater and that the seed has to be sown at once, all in one session. Judging the ripeness of the capsule in the field is also difficult and requires some experience (some collectors judge this by the amount of resistance and springiness of the capsule when squeezed!) However if the capsule is collected too early, some maturation will take place over a few weeks.

Seed sowing methods for mature seed

Sowing techniques for mature seed can be divided into two main groups – those where the sterilant is in direct contact with the seeds and those where the contact is indirect – through the use of filter paper or other physical separation.

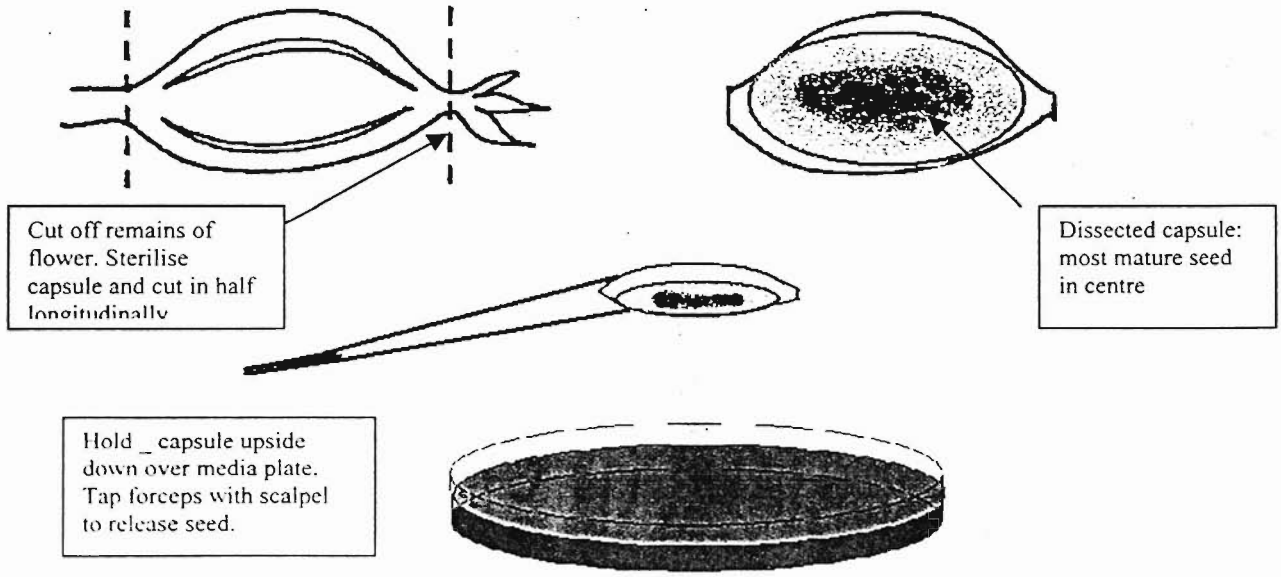
Direct contact of the seeds in bleach solution mean that lower concentrations can be used and for shorter time periods. Orchid seed tend to float on the surface of solutions due to their hydrophobic testas and because air is usually trapped in the seed. The many variations to seed sowing techniques are often strategies to deal with this and include the use of surfactants to break surface tension, vacuum infiltration, and the use of blood rotators, shakers and stirrers to agitate seed. The other main problem is collecting and dispensing seed from the vessel once it is sterilised. Solutions to this include specially designed test tubes with internal glass filters, use of filtration systems and collection with pipette.

With all of these sterilisation methods, transfer of seeds to Petri dish or flask is best carried out using aseptic techniques in a Laminar Flow bench with a HEPA (high efficiency particulate) filter . Increased 'qualified amateur' interest in sowing orchid seeds has led to the development and adaptation of techniques for use in the home and low tech environments. While interest is mainly concentrated on epiphytic tropical taxa, there is increasing expertise being developed for temperate terrestrial species, particularly *Cypripediums*. Many stages of seed sowing, transfer and media preparation can take place in kitchen/ low tech lab. Aaron Hicks (2000) has prepared a book with descriptions of the use of glove boxes, working over steam etc.

SOWING SEED FROM A GREEN CAPSULE

- Cut the capsule from the plant using a sterilised scalpel blade. Use a new blade for each capsule to prevent viral infection. Capsules can be stored for a couple of days before sowing but are better sown immediately.
- Carefully trim off any dried flower that is still attached.
- Using a soft toothbrush, scrub the capsule carefully with detergent.
- Soak capsule in 1% solution of bleach for 10 minutes.
- In sterile air bench remove capsule from bleach with sterile tweezers and place on Petri dish/cutting surface.
- Take capsule in tweezers, dip into 100% alcohol or meths and pass capsule through flame (do not hold for long in flame, just sufficient to ignite the alcohol).
- Allow flames to go out and capsule and tweezers to cool . Repeat twice.
- Place capsule on sterile cutting surface, cut longitudinally down the capsule to give 2 halves.
- Gently lift up a capsule half with forceps and hold upside down over a media plate.Tap the forceps with a scalpel - seed should fall from the capsule. Spread evenly and thinly over the plate.
- Turning the capsule upwards, tease apart seed with scalpel and then repeat sowing action .
- Continue until all seed sown and then scrape out all contents.

The most mature seed occurs in the centre of the capsule so it is useful to write on the plates the order of sowing. This will give a rough indication of the maturity of the seed. Some growers cut a capsule into 3 along the seed dispersal vents.
Seed of very immature seed capsules cannot be stored. However green capsules may contain fully mature seed. In this case, a proportion of the seeds may be sown directly and the remaining seeds stored.



One of the main differences in techniques required for epiphytic and terrestrial orchid seeds is that while epiphytic seeds usually require only sufficient sterilisation time to disinfest, terrestrial seeds may require additional sterilisation periods or different treatment (eg acid or chilling) in order to degrade the seed coat or to break dormancy.

Sterilants

The sterilants normally used are sodium hypochorite (NaOCl) and calcium hypoclorite (CaOCl). Hypochlorite solutions have at least 2 effects on orchid seeds: sterilisation and removal of suberin in the integuments, thus enhancing diffusion and permeability for water. The testa is degraded by alkinisation and oxidation. The amount of suberin varies with different species. Generally darker integuments mean more suberisation and the need for a longer sterilisation time or stronger hypochlorite solution to make them permeable. Progressive oxidation of the seed coat brings about higher permeability but above the optimum duration will have a toxic effect on the embryos resulting in death or injured cells.

Sodium hypochlorite is usually the sterilant of choice where disinfestation alone is sufficient. It is important to check on available chlorine on the container as this varies considerably. At RBGKew, dilutions of between 1% and 5% of a NaOCl solution containing 13% available chorine are commonly used. Higher concentrations would be required if using household bleach that typically contains about 5% available chlorine. Calcium hypochlorite is more commonly used for longer sterilisation times (up to 24 hours) as damage to the embryo is less than sodium hypochlorite used for the same time period (NaOCL is a stronger oxidising agent)

The standard preparation of a saturated solution of calcium hypochlorite that is quoted in many papers is 10g Calcium hypochlorite powder in 140ml of water. This was calculated for a powder containing 24 % available chlorine to give a final solution with available chlorine of 1.7% .
Calcium hypochorite powder is now obtainable in a wide range of concentrations of available chlorine up to 75%. This has led to concerns that researchers using the standard preparation of 10g/140ml are inadvertently making up much higher concentrations than anticipated and thus causing damage to the seeds. Thus it is important to check the container for the estimated available chlorine content and calculate the amount required accordingly.
In the paper of Van Waes and Debergh (1986), a concentration of 5%w/v is used. The Calcium hypochlorite used at Kew contains approximately 35% available chlorine which used at the concentration of 5g per 100ml (5%) gives 1.7% available chlorine

Acids
An alternative treatment to long soaks in Ca(OCl) to breakdown the testa is the use of 0,5-2% Sulphuric acid (H_2SO_4) for 5-15 minutes as a pre-treatment before sterilising in hypochlorite (Malmgren 1993, 1996). The concentration and duration of acid pre-treatment have to be finely adjusted for each species as does the time in hypochlorite which is much reduced (eg 7 minutes instead of 45 for *Anacamptis pyramidalis).*

SEED SOWING—PACKET METHOD

The main advantage of using an indirect contact method such as use of filter paper packets (Mitchell, 1989) is in ease of handling. It is also a useful technique where only small amounts of seed are available as none are wasted
Higher concentrations of sterilant have to be used along with surfactant in order for the sterilant to penetrate the paper. It is not suitable for long soaks as the filter paper may disintegrate. It is important to use a grade of filter paper that will withstand soaking (eg Whatman 54, Hardened).

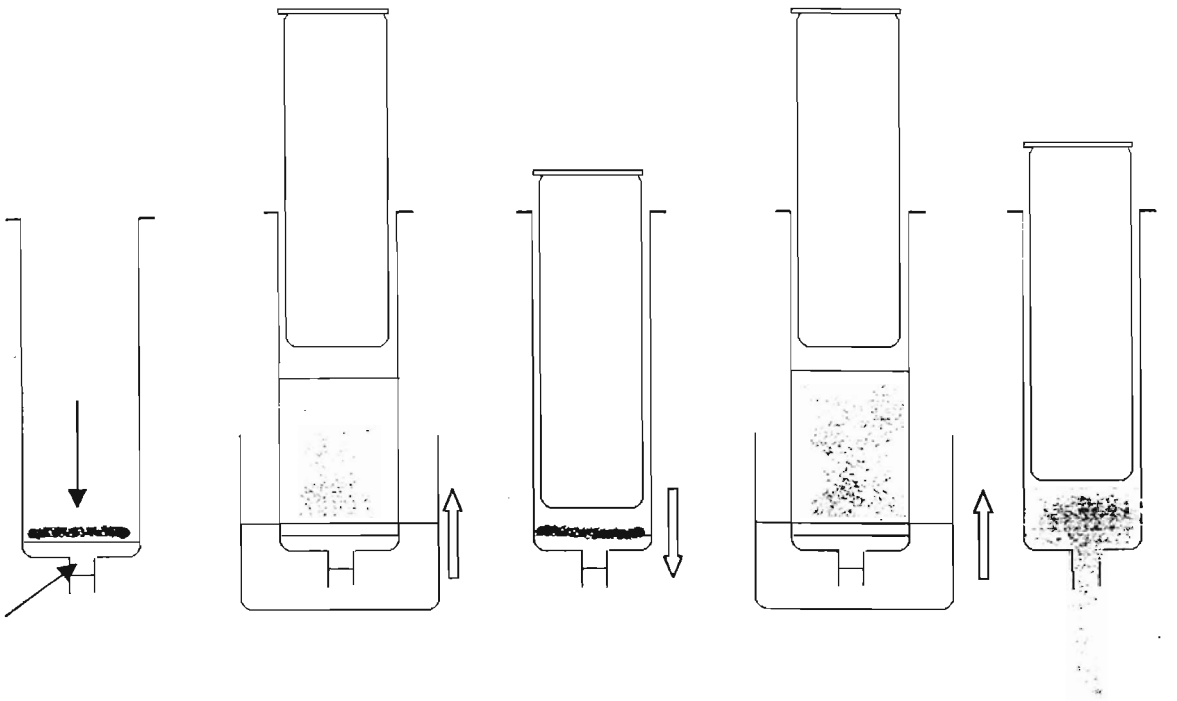
- Place a small amount of seed in the centre of the filter paper (square approx 45mm x 45 mm or 42.5 circle)
- Fold the filter paper to form a small packet (see Figure below)
- Alternatively, prepare to stage D and then inserting a spatula , open up packet slightly and place seed in the packet
- Secure with steel staple at edge of packet. (seed should be at base of packet, at opposite end to staple)
- Do not put too many seeds in a packet as the sterilant may not be able to penetrate – make several packets instead.
- Place packets in distilled water with forceps, squeezing out any air
- Transfer packets to beaker /screw top bottle of sterilising solution, squeezing them with forceps again to expel any remaining air (to prevent packets floating). Do not place more than 5 packets in a beaker, it is better to use several vessels.
- Place the beaker on a magnetic stirrer (the staple acts as a stirring bar) and allow to agitate for 10-50 minutes according to treatment. Alternatively use a small bottle containing 1 or 2 packets and gently shake by hand.
- Move the beaker to sterile area/ flow bench , remove packets from sterilant using forceps and place in sterile distilled water (in separate tubes or screw top bottles). Agitate for 5 minutes
- Repeat rinsing step twice more
- Remove the packet from the water and squeeze with large forceps to remove excess water.
- Cut off the staple and open up packet with forceps.
- Holding the filter paper seed side downwards , lightly dab the filter paper against the medium – the seeds should adhere to the surface. If seeds are sticking to the filter paper gently rubbing the back of the filter with forceps can assist transfer. With practice, seeds can be sown very evenly and thinly.

SEED SOWING: SYRINGE METHOD

As all sterilization and rinsing of seed takes place in same syringe, it is possible to carry out this procedure at an open bench , only requiring clean environment (cabinet or working over steam) for final sowing stage)

- Use 10ml or 20 ml glass syringe and small beaker to support it.

- Take small piece of cotton wool, twist end and place in bottom of syringe, with point in tip (it needs to be compacted enough to act as filter during sterilizing and rinsing stages but loose enough to dislodge for sowing stage)
- Add a spatula of seed to the syringe
- Replace plunger and draw up sterilizing solution (1%-2% NaOCl).
- Tap syringe to remove air bubbles and disperse seeds , invert syringe and depress plunger sufficient to dispel all air
- Agitate by tipping syringe backwards and forwards for minimum of 10 minutes
- Force out sterilizing solution pushing plunger to bottom of syringe
- Pull plunger to top of syringe and rinse plunger with sterile water
- To rinse seeds, draw up sterile water, agitate briefly and force out water using plunger.
- Repeat rinsing process 2 or 3 times.
- Dislodge cotton wool plug (by tapping or pushing sterile needle up syringe tip , draw up small amount (1-2 ml) of sterile water
- Disperse seeds in solution then squirt seeds onto medium. Excess water can be sucked back into syringe.
- Alternatively seeds may be sown by removing cotton wool with forceps and dabbing on surface of medium, also by dabbing end of plunger



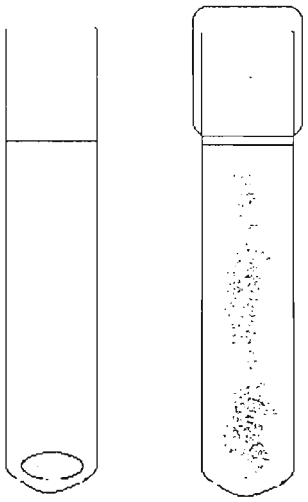
SEED SOWING – DIRECT STERILIZATION IN TEST –TUBE

This method can be used in combination with the Tetrazolium viability staining technique to obtain a direct comparison of viability testing and germination rates (seed having undergone same sterilization treatment)

- Place small amount of seeds in test-tube (either sealed with a screw on lid or rubber bung)
- Add sterilant with wetting agent (to aid contact with seed and aid sinking)
- Seal with stopper
- Shake test-tube for 5-15 minutes so seeds have good contact with the sterilant
- For longer periods of sterilization (especially for terrestrial orchid seeds), equipment such as a blood rotator will allow gentle constant agitation .
- 1-2 minutes before sterilization period is completed, stop agiatation to allow seed to settle.
- Seeds will either settle at the bottom or form a ring around the top of the test-tube.
- Use pipette (sterile disposable plastic pasteur pipettes are useful) to carefully remove sterilizing solution from above the seeds.
- It is not critical to remove all the sterilant as any remaining will be progressively diluted through the rinsing steps.
- Rinsing step: using another sterile pipette, add sterile distilled water , seal and agitate.
- Allow seed to settle and remove water with pipette
- Repeat rinsing step
- In the final rinse leave about 1ml of water.
- Shake briefly to suspend seeds then promptly tip test-tube contents onto Petri dish/flask (or use pipette to suck up suspension and dispense).
- Spread seed over plate with sterilized spatula (excess water can be removed with the pipette)

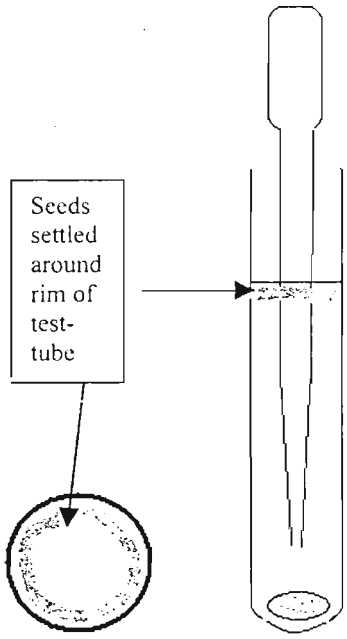
A Buckner funnel with filter paper can be used to separate seeds from sterilizing/ rinsing solutions as described in Thompson (1996) and Hicks(2000)
Seed collected on the filter paper can be sown by dabbing the filter paper on the surface of the medium.

STERILIZATION STEP



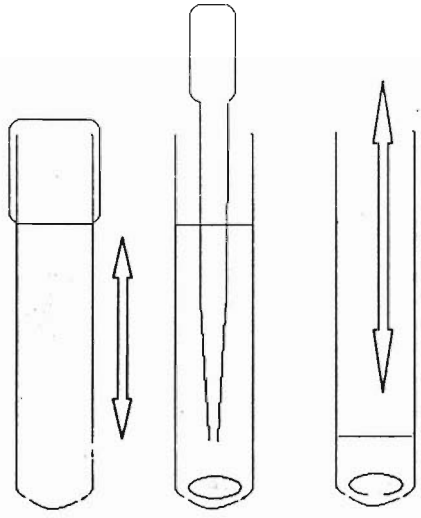
Add seeds and sterilant. Seal with stopper

Agitate then allow seeds to settle



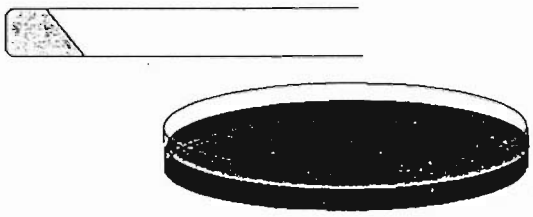
Carefully remove sterilant with pipette without disturbing seeds

RINSING STEP



Add sterile water and agitate

Allow seeds to settle, remove water with pipette Repeat rinsing step



Re-suspend seeds in 1ml of final rinse then pour on plate.

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Notes on different genera

Aron Hicks

P-6668 (Sigma) and P-668 (PhytoTech) are equivalent. W2.5 and W3 refer to Western Labs media W2.5 and W3. P-1056 is produced by Sigma, and is equivalent to P-6668 plus 30 grams of a 50/50 mix of banana powder and malto-dextrin. P-0785 is produced by PhytoTech.

Subfamily Epidendroideae, tribe Epidendreae, subtribe Laeliinae.

Cattleya, epidendrum, laelia, and allied genera all germinate well on P-668 and W2.5, and grow well on P-1056. Growth may be somewhat poorer on P-0785.

Subfamily Epidendroideae, tribe Maxillarieae, subtribe Oncidiinae.

Oncidiums germinate readily on P-668, and grow well on P-1056.

Subfamily Epidendroideae, tribe Vandeae, subtribe Sarcanthinae.

Phalaenopsis and vandas germinate well on P-668, and grow well on P-1056. Phalaenopsis may prefer calcium hypochlorite over sodium hypochlorite for disinfection (no personal experience).

Subfamily Epidendroideae, tribe Gongoreae, subtribe Stanhopeinae.

Coryanthes, gongora, peristera, and stanhopea germinate and grow very well on P-668, and grow very well on P-1056. The seeds of this group may be very short-lived (weeks), but some live for years. Seeds are difficult to effectively disinfect once contaminated as they are large, airy seeds that are difficult to wet thoroughly for disinfection. Although subsequent growth on P-1056 is usually very good, members of this tribe are prone to succumb to iron deficiency, which shows up as yellowed leaves with green veins. Doubling the quantity of iron-EDTA chelate in the formula overcomes this difficulty, as does adding sterile iron-water solution to developed flasks.

Subfamily Epidendroideae, tribe Dendrobieae, subtribe Dendrobiinae.

Dendrobiums germinate well on P-668, and grow well on P-1056.

Subfamily Cypripedioideae.

Paphiopedilums and phragmipediums germinate best on W2.5, and grow better on W3. Some may require highly specific media, and a little tinkering to produce results. Parameters include banana, coconut, or pineapple content (i.e., monosaccharide content), media strength, and pH (some members of these genera may like it higher: check to determine if species naturally occurs on limestone or other carbonate rocks). Cypripediums require highly specific disinfection and germination regimes. Germination of some species may require long (>1 hour) exposure to chlorine solutions to inspire germination. Media may have to be formulated with amino acids as nitrogen source; this consideration may be of value for other terrestrial genera. Long disinfection time is thought to be a function of chlorine, and not high pH, burning up germination inhibitors,

and may be sidestepped through green capsule technique (harvesting 2-3 weeks before maturation).

Alternate Methods

Disinfection of Orchid Seed for Flasking

There are many techniques that may be employed for disinfection of orchid seed. The technique and variations thereof that are described here are those we have developed over several years to maximize seed recovery and efficacy of disinfection.

Before performing any disinfection, several tools must be prepared. Replate forks, consisting of stainless steel microspatulas (Sigma S9147) are wrapped in foil and autoclaved. Whatman 25mm Disposable Filter Funnels (Whatman # 1922-1004) are prepared with 2.5 cm disks of Whatman #4 filter paper, wrapped in foil, and autoclaved. Vacutainer test tubes (7 mL capacity) are cleaned with soap and water, wrapped in foil, and autoclaved. As tubes are reused, this serves to destroy any seed remaining as well as to reduce contamination from stray organisms inside the tubes. Finally, an autoclavable wash bottle (500 mL capacity) is filled halfway with distilled water, the lid unscrewed to prevent loss of contents, wrapped in foil, and autoclaved. When cool, all the supplies are moved to the sterile flasking workspace.

When ready to sow, seeds are deposited in the sterile test tube, and 2 drops of a wetting agent solution (consisting of 2 drops "Palmolive" dishwashing detergent in 100 mL of distilled water) are added. More is added if the quantity of seed is large, or the seed tends to float. The tube is filled at least halfway with a disinfection solution consisting of 5100 ppm of DCCA (Dichloroisocyanuric acid, Sigma D2536). The solution is made with 5.1 grams of the fresh solid, dissolved in 1 liter of water. Tightly stoppered and refrigerated in clean plastic bottles, this solution lasts at least 1 year. The solution is mildly acidic, producing more hypochlorous acid than hypchlorite ion, making it a better disinfectant. The tube is stoppered, taking care not to contaminate the part of the stopper that enters the tube.

Seed is disinfected for a period of time proportional to the following parameters. Contamination often occurs when the seed has not been handled with respect or been exposed to poor storage conditions. Seed is sterile when it leaves the capsule; everything we do to it between then and when we disinfect it for sowing increases the chance of contamination. Treated correctly, we can minimize these inevitable losses. The following sliding scale is as follows:

	Low Risk	**High Risk**
Seed size	Small seeds	Large, fluffy seeds (stanhopea, etc.)
Seed storage	Stored promptly, when dry	Stored late, or stored wet
Detritus	Seed is clean, free of debris	Lots of chaff, capsule parts, other material
Handling	Only clean paper, sterile tools	Handled with dirty tools, on bad surfaces

These risks are relative. No static figures can be given for how long seed should be disinfected. However, clean seed that was harvested from a mature capsule with steady moisture removal over a relatively short period of time, and stored in clean paper may require only 5 minutes of disinfection. On the other hand, seed that was collected, barely sorted from its capsule material, and shipped in an envelope while it was still moist, collected on a piece of used magazine paper, may be burgeoning with spores and microbes. Over half an hour of disinfection may be required, and still not produce clean cultures.

We routinely disinfect dried seed for 22-25 minutes with 5100 ppm DCCA, and our mother flask contamination rate runs around 20% of flasks. I do not know if this is regarded as high or not; as we often deal with seed that has changed hands repeatedly, or been shipped thousands of miles, I do not consider it to be unacceptable. If not heavily contaminated, sowing more than one flask increases the chances of producing at least one non-contaminated culture.

During disinfection, the seeds are agitated periodically; the tubes are stood upside down for at least 1/3 of the time, and the stoppers removed, sprayed with chlorine-based disinfectants, and returned to ensure the stopper is thoroughly disinfected.

As most of the seed we deal with is dried, it often floats. Disinfection of floating seed is made more difficult in that the surfaces may be difficult to thoroughly wet. To encourage wetting, we often employ the use of a small hand-held vacuum pump ("Mityvac," often used for testing engine parts) to reduce the pressure in the test tubes. The partial vacuum is applied and released several times, until the seeds start to sink. Gently agitating the seeds while releasing the vacuum tends to remove more air bubbles, and wet the seeds better. The pump can produce 20-25" Hg vacuum at the most. It does not appear to damage the seeds.

When the disinfection time is up, the seeds and solution are dumped into the Whatman funnels. Any funnel capable of being autoclaved will work. Whatman #4 paper captures even the tiniest of seeds. Coffee filter paper may also work if Whatman is not available. Allow the funnel to drain, and then use the wash bottle of sterile water to deliver sterile distilled water to wash the seeds.

The small quantity of sterile, wet seeds may be sown in one of a number of ways. More water may be added, and the seeds and water washed into the waiting flask. Alternatively, a sterile tool may be used to recover the seeds, and deposit them on the media. A hole may be poked in the bottom of the filter with a sterile tool, and the seeds washed into the flask with a stream of sterile water. For the Whatman filter funnels, the funnel may be disassembled, and the filter paper removed with a sterile tool; this paper can be placed inside the flask, and seeds will usually germinate right on top of the paper. Seeds can also be wiped or washed off so they contact media directly.

For the media, either P-6668 (Sigma) or P-668 (PhytoTech) is used, along with at least one other media (Western W2.5). In the manner described below, seeds can be sown on multiple media with ease.

Media is prepared one liter at a time, as per instructions. With P-6668/P-668, the media is often too hot or strong for all species to do well. As a very general rule, species with smaller roots require weaker media. When in doubt, use weaker media. P-6668/P-668 may be used at _, 2/3, or _ strength as easily as full label strength.

Media is heated to 70-80 degrees C, and poured into each of 40 baby food jars (25 mL per jar). Lids are put on loosely, and jars are stacked into the pressure cooker,

and autoclaved for 12 minutes. While still hot, jars are removed, the lids tightened, and swirled once or twice to make sure the charcoal and agar are dispersed evenly.

When cool, jars are placed in large bins, according to their lot number and formula. When ready for flasking, it is easy enough to pick out the appropriate number of baby food jars, and sow the same species on as many different media as you wish. As a result, it is possible to sow a given species with relative ease on 2 or more media to determine which formulation works best. A baby food jar can support 100-1000 protocorms for a month with ease. No vent is required at this stage. When lids are fitted tightly, contamination is low (well below 1% per month, unopened), indicating that the seal is effective.

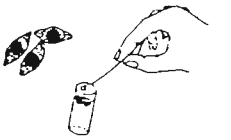
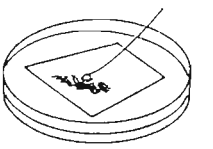
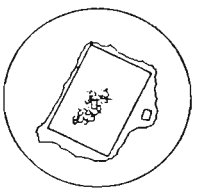
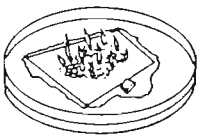
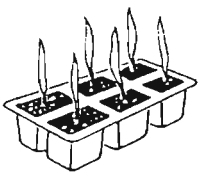
For those that prefer green capsule technique, I follow the procedure outlined below.

Ensure that capsules have not opened. Look for small cracks along the suture lines, holes from handling or insects, etc. Capsules are then placed in a 25mm culture tube, along with ~1 cc wetting agent solution as described above, and filled _ full or more (for long capsules) with 5100 ppm DCCA (as above). The tube is stoppered, and gently sloshed back and forth intermittently for at least 30 minutes. The capsule is not scrubbed or otherwise mechanically manipulated other than to rock the tube back and forth, and stand it upside down to ensure the stopper is sterilized.

After 30 minutes, the capsule is removed and placed on a piece of sterile aluminum foil. A sterile razor blade is used to remove the end of the capsule, and a sterile tool is inserted to remove seeds, which are then delivered to the media.

This technique works very well, with a negligible contamination rate. Seed is delivered to multiple flasks, and sterile technique is employed assiduously.

Overview of symbiotic propagation of terrestrial orchids.

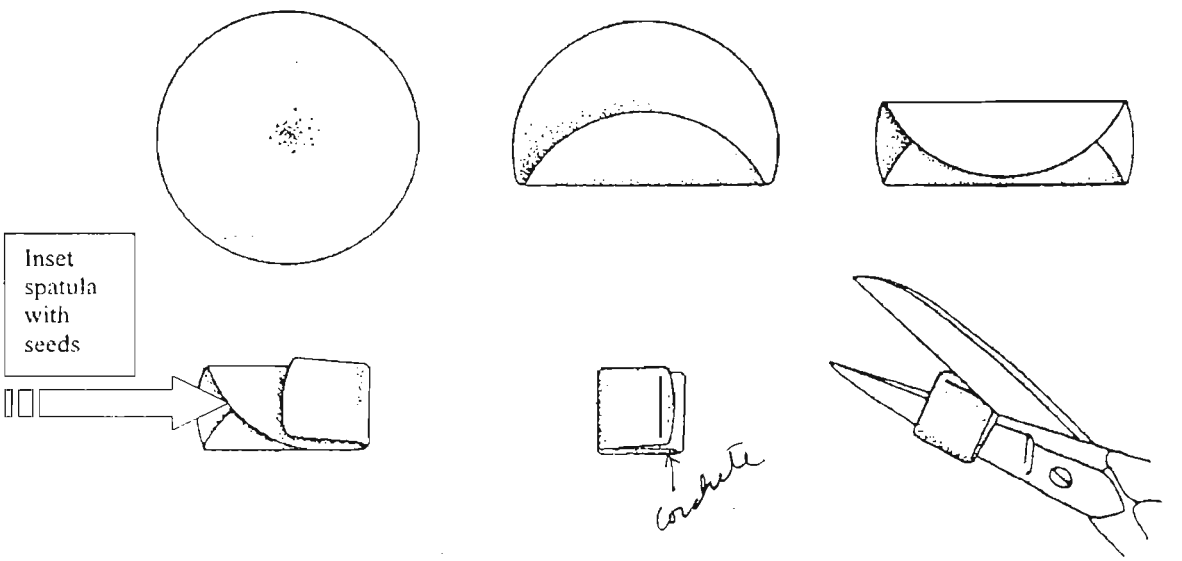
Stage	Procedure	Diagram
Seed Collection	The collection of seed from field plants is an important step for the production of orchid seedlings in the laboratory.	
Seed Sterilisation	It is necessary to kill any bacteria or fungi on the seed prior to placing it on the sterile germination medium. This is done by washing the seed in a weak bleach solution.	
Seed Sowing	Once the seed is sterile it is rinsed in sterile water to remove any bleach residue and spread evenly over filter paper placed onto the germination plates.	
Fungal inoculation	Before the seed will germinate it needs to be dinnoculated with the correct helper fungus issolated from growing plants of the same species as the seed. The fungus grows across the germination plates infecting the seed stimulating seed germiantion.	
Seed Germination	After a period of upto 8 weeks the germinated seed will have devloped leaves and need to be transfered to other growing conditions.	
Growing on	The transfer of orchid seedlings to soil is one of the main areas of concentration of the Western Power Orchid Rescue Program. Initial results appear promising.	

SEED SOWING—PACKET METHOD

The main advantage of using an indirect contact method such as use of filter paper packets (Mitchell, 1989) is in ease of handling. It is also a useful technique where only small amounts of seed are available as none are wasted

Higher concentrations of sterilant have to be used along with surfactant in order for the sterilant to penetrate the paper. It is not suitable for long soaks as the filter paper may disintegrate. It is important to use a grade of filter paper that will withstand soaking (eg Whatman 54, Hardened).

- Place a small amount of seed in the centre of the filter paper (square approx 45mm x 45 mm or 42.5 circle)
- Fold the filter paper to form a small packet (see Figure below)
- Alternatively, prepare to stage D and then inserting a spatula , open up packet slightly and place seed in the packet
- Secure with steel staple at edge of packet. (seed should be at base of packet, at opposite end to staple)
- Do not put too many seeds in a packet as the sterilant may not be able to penetrate – make several packets instead.
- Place packets in distilled water with forceps, squeezing out any air 5% bleach @ 20 min
- Transfer packets to beaker /screw top bottle of sterilising solution, squeezing them with forceps again to expel any remaining air (to prevent packets floating). Do not place more than 5 packets in a beaker, it is better to use several vessels.
- Place the beaker on a magnetic stirrer (the staple acts as a stirring bar) and allow to agitate for 10-50 minutes according to treatment. Alternatively use a small bottle containing 1 or 2 packets and gently shake by hand.
- Move the beaker to sterile area/ flow bench , remove packets from sterilant using forceps and place in sterile distilled water (in separate tubes or screw top bottles). Agitate for 5 minutes
- Repeat rinsing step twice more
- Remove the packet from the water and squeeze with large forceps to remove excess water.
- Cut off the staple and open up packet with forceps.
- Holding the filter paper seed side downwards , lightly dab the filter paper against the medium – the seeds should adhere to the surface. If seeds are sticking to the filter paper gently rubbing the back of the filter with forceps can assist transfer. With practice, seeds can be sown very evenly and thinly.



METODO 1
- [illegible] + algodón  → no refrigerar
- Esterilizar: metanol 20 — sólo no reduce todos los contaminantes
  Hipoclorito — cloruro disponible < 30%
  13 15!
- Cortar las puntas
- Pasar x alcohol y llama (no quemar!)
- Partir en 2, espacio en petri.
- Usar material lo + fresco posible.
- Semillas maduras: secar en ... ca. hs ...% H₂O
  Los refrigerar. crioperservar

METO 2.
- maduras
- desinfectar c/ bleach
- probl: flota y no queda en contacto total, y son muy pequeñas
- filtro
  Adv. ↓ pérdida de semillas, y se pueden guardar todos hs la siembra
  bleach ↑ ... xq' pasa el filtro papel

- gasas / algodón → bleach, agitar, buen contacto
  → agua x 2-3 enjuague

-Chapter 5-
Orchid Fungi Isolation

For terrestrial orchids only underground parts are infected with the appropriate fungus. Typically stem collars or roots contain fungal pelotons. Following the collection of material from the field, soil is removed by repeated washing under running tap water. Rubbing the material between fingers assists in the removal of soil and other contaminates. Two methods are used to obtain sterile pelotons for initiation of fungal cultures.

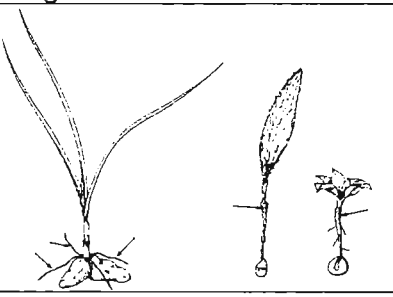
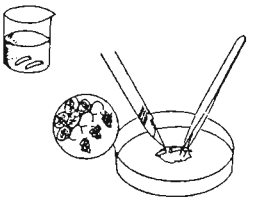
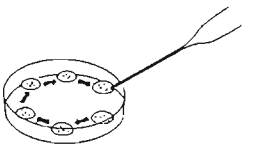
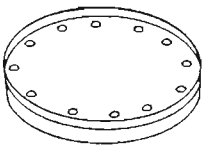
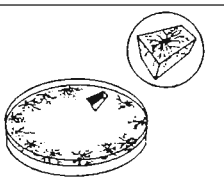
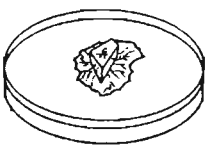
A. Surface sterilisation
Tissues are surface sterilised in a 0.5% sodium hypochlorite solution for 5 – 10 minutes. Roots or collars are then rinsed twice in sterile water. The outer layer (epidermis) of cells is removed using a dissecting microscope and either pelotons are extracted from cortical cells or tissue blocks are cut and plated onto fungi isolating media (FIM).

B. Peloton rinsing
This method uses no chemical sterilents. After the removal of as much surface contaminates as possible under running tap water the infected tissue is placed through three vigorous washes in sterile water. As above outer cell layers are removed further reducing contaminant levels. Pelotons are then extracted using a dissecting microscope and passed through a series of 5-6 rinses in sterile droplets of water. Pelotons are moved through the washes using a glass capillary. These can be constructed by stretching a fine pasteur pipete heated in a flame. Individual pelotons can then be plated onto FIM. Approximately 50 pelotons are extracted from each orchid to ensure a selection of isolates is obtained. When investigating fungal diversity more pelotons may be required. This method is commonly used at Kings Park and is outlined below.

Isolate Purification
FIM plates are stored in the dark at room temperature. Examination of pelotons every few days is required until hyphae are observed growing from individual pelotons. For fast growing isolates hyphae are present after 2-3 days. However, for slow growing fungi hyphae may not appear for 7-10 days or longer.

Bacterial contamination can be removed by repeated subculturing on FIM with antibiotics such as streptomycin sulphate (see media preparation for further details). It may be necessary to subculture heavily contaminated cultures to partial or window plates. These are incomplete plates ie. Not completely covered with FIM. A small cube containing the contaminated isolate is placed onto the floor of the petri dish approximately 10mm from the FIM. Hyphae grow across the surface of the plastic leaving bacteria behind. Generally a bacteria free isolate can then be subcultured to new FIM plates.

Overview of fungal isolation

Stage	Procedure	Diagram
Plant Collection	The collection of plant material containing fungi is different depending on the habit of the target species. Typically infected tissue is similar within genera.	
Cleaning material	To remove surface contaminates material is rinsed under running tap water removing soil. Rinsing in sterile water or using a sterilising agent will further reduce contaminates. Pelotons are extracted in the laminar flow.	
Rinsing pelotons	After pelotons are dissected from cells they are rinsed approximately six times in sterile water. Pelotons can be transferred through the rinses using a fine pasteur pipet.	
Plating out of pelotons	Individual pelotons can then be transferred to FIM and incubated at 18°C to 22°C.	
Subculturing of isolates	After two to ten days hyphae can be observed growing from the pelotons. Subculturing at this stage should be carried out to obtain a pure isolate.	
	Fungal isolates can be maintained on PDA, oat agar or FIM. Monitoring for contamination or dust mite infestation should be carried out regularly.	

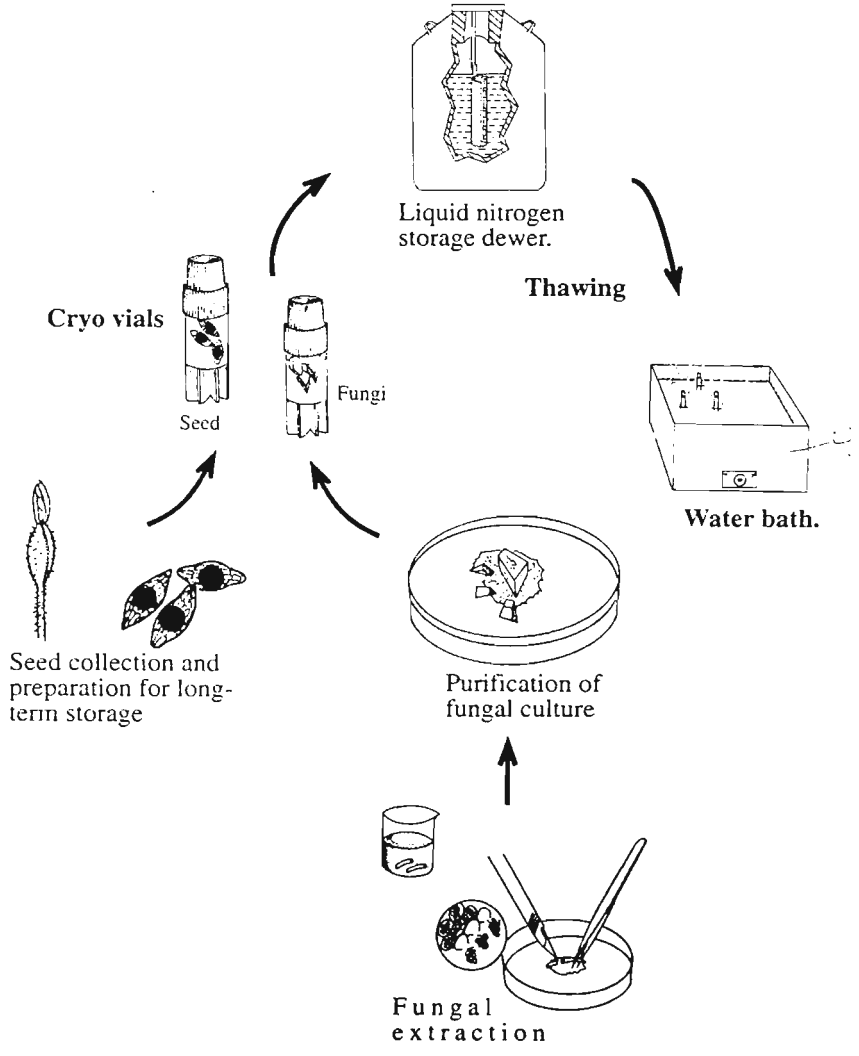
Culture storage

For the short-term, fungal isolates can be maintained on FIM plates, Potato Dextrose Agar plates (see media preparation for details) or other media. However, repeated subculturing of isolates may lead to a reduction in the isolates capacity to germinate seed. Also petri dished tend to be easily infected by dust mites thus contaminating cultures. A range of fungi storage protocols can be used once tested with specific fungal isolates. These include storage in sterile water, under oil, or in plastic tubes sealed with Petroleum Jelly.

The long-term storage of fungi is best achieved using the ultra-low temperatures associated with liquid nitrogen (-196°C). Here isolates are protected from mite infestations or genetic change. This method is outlined in chapter 6.

-Chapter 6-
Cryo-storage of orchid seed and mycorrhiza

Presented here is an overview of the methods currently used at Kings Park for the cryopreservation of terrestrial orchid seed and associated fungi. It is recommended that new taxa be tested prior to committing entire seed lots to liquid nitrogen storage.

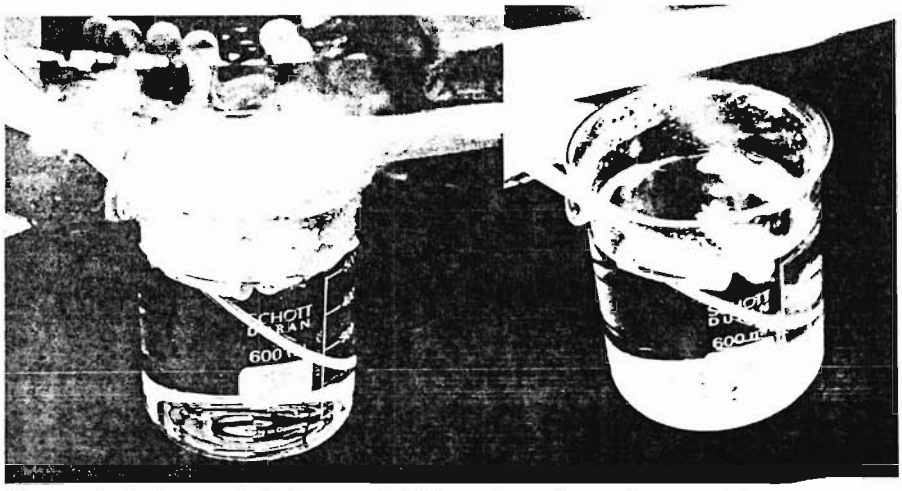


Cryostorage of orchid mycorrhiza

1. Select actively growing cultures on 1/4 Potato Dextr[illegible] /-gar medium.
3. Label and prepare sterile cryo vials.
4. Place several (five) 3mm[illegible] blocks of innoculum into e[illegible] cryo vial.
5. Tighten caps firmly.
6. Plunge into liquid nitroge[illegible]
7. Complete records and add details to database.

To remove from Liquid nitrogen.

1. Remove vials from storage and rapidly thaw in 40°C [illegible]r bath for 2-3 minutes.
2. Plate out agar blocks onto Fungi Isolating Medium.
4. Monitor cultures for signs of growth or contamination



Two ml vials containing orchid fungi undergoing rapid thawing in 40°C water bath.

Cryostorage of orchid seed

1. Collect seed and remove any remaining capsule mate[illegible] or plant debris.
2. Dry seed over silica gel for 24 hours.
3. Place seed in 2 ml cryo vial.
4. Label each cryo-vial with appropriate code.
5. Plunge into liquid nitrogen.

To remove seed from liquid nitrogen.

1. Rapidly thaw seed in 40°C water bath.
2. Sow seed using suitable methods.

-CHAPTER 7-
Microscopic Techniques

7.1. Microscope use and care

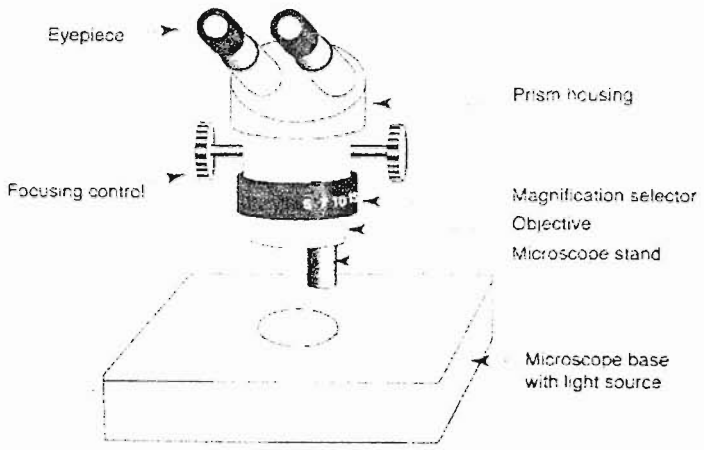
This chapter contains information on methods commonly used to sample roots and to examine their mycorrhizal associations. Brief introductory notes on microscope use are provided below.

1. *Microscopes should be separate from areas where samples are prepared to minimise exposure to dust and liquids.* It is advisable to have a clear plastic platform made to protect the base of the microscope for liquid samples. All parts of the microscope must be kept free of dust and cleaned carefully when they become dirty.

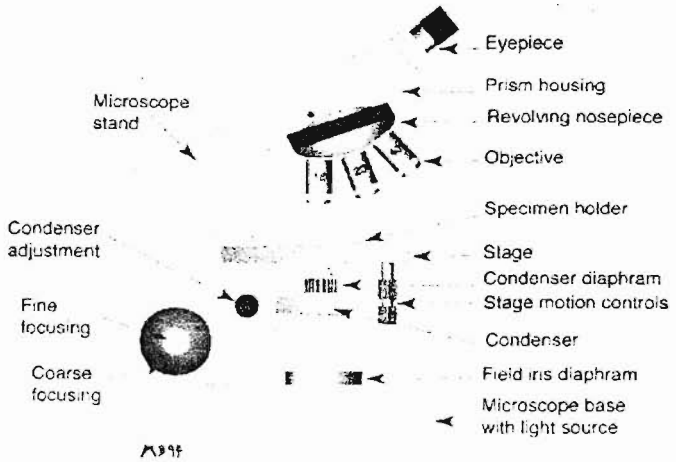
2. Great care must be taken when examining liquid preparations to avoid contact with objectives. Immersion oil must be used very carefully to avoid damage to other objectives. Objective lenses are also easily damaged by abrasion.

3. The microscope condenser must be carefully adjusted so that illumination of the specimen is uniform and sufficiently bright (Kohler illumination). Raise the condenser until the image of it's iris diaphragm is in focus, centre that image and then open the iris beyond the field of view of the objective.

A. Dissecting microscope



B. Compound microscope



7.2. Clearing and staining mycorrhizal roots

Mycorrhizal associations are not visible when fresh roots are observed, because internal structures are obscured by the natural pigments and cell contents within roots. Clearing procedures use hot KOH to remove cell contents and cell wall pigments. Fungal structures are revealed by the use of stains which bind to fungal hyphae such as Trypan blue or chlorazol black E in lactoglycerol (Phillips & Hayman 1970, Brundrett *et al.* 1984).

Safety warnings ! ! ! ! ! !

1. 10 % w/v **KOH** is used to clear roots. **Care should be taken to avoid skin contact with this caustic chemical !** Use gloves, safety glasses etc.
2. **Chlorazol black E and Trypan blue are suspected to be carcinogens !** (as are many other biological stains — Coombes & Haveland-Smith 1982). Use gloves to protect your hands when using stain solutions and take care to avoid breathing dust, or getting any into your eyes, when handling dye powders.
3. **Formalin-based preservatives** are bad for your health. Preservation of plant samples with 50% ethanol works as well and is much safer.
4. Refer to safety data sheets for the latest information on the chemicals you use.

Equipment and reagents

- 50% ethanol (v/v) root preservative
- 10 % KOH (w/v potassium hydroxide) dissolved in water. *This is an exothermic reaction — use a heat resistant container!*
- 0.03 % w/v Chlorazol black E in (CBE) in lactoglycerol (1:1:1 lactic acid, glycerol and water). Dissolve CBE in water before adding equal volumes of lactic acid and glycerol.
- 0.05 % w/v Trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water)
- 50 % Glycerol-water (v/v) solution for destaining and storage of stained roots
- food grade lactic acid and glycerol are adequate and can be purchased in bulk
- autoclave (121°C), water bath, or oven (60-90°C) to heat root samples for clearing and staining
- autoclave-resistant glass jars, or tubes to hold samples
- fine nylon mesh screen (±100 µm) to prevent root loss when changing solutions
- fine forceps and dissecting needles to transfer roots
- plastic vials with tight-sealing lids for storage of stained samples in 50% glycerol
- Clear plastic dishes with inscribed grid lines to measure colonization
- Fine screen (100 µm) with nylon mesh for transferring roots from solutions
- Fine forceps and probes for manipulating roots
- Microscope slides, long cover slips and PVLG mountant (see below)
- Plastic vials with tight-sealing lids for storage of samples
- Dissecting microscope with a transmitted light illumination- a clear plastic panel over the microscope base is recommended to provide a stable platform and for protection from spilled liquids
- Compound microscope with an eyepiece crosshair

A. Processing material

Clearing and staining procedures require root samples that have been washed free of soil.

1. Root samples 1-2 g or smaller are placed in 10% KOH, taking care to avoid contact with this corrosive chemical. It is imperative that KOH or staining solution volumes are sufficient for the amount of roots being processed and that roots are not tightly clumped together. It is best to chop roots into 2-4 cm long segments before clearing them or sub-divide large volumes of roots.

2. An autoclave using a liquids cycle of 15-20 min. at 121°C is efficiently clears most roots. Samples containing old roots, roots with abundant phenolics, or field-collected roots often require longer clearing times (25-60 min.). Samples should be in autoclave-resistant glass containers that are less than 1/3 full, or they may overflow in the autoclave. Wide containers work better than tall-narrow tubes.

3. Roots can also be cleared by heating KOH in a water bath at 60-90°C. Long times may be required (from 5 hrs. to several days).

4. Cleared samples are captured on a fine sieve and rinsed with water before transferring them into the staining solution.

5. Cleared material is stained with CBE or trypan blue in a lactoglycerol solution (Brundrett *et al.* 1984) (Bevege 1968, Phillips & Hayman 1970).

6. Samples are stained by heating for several hours at 90°C, or by leaving them in the solution for several days. The staining solution may be reused several times if filtered through folded cheesecloth or 50 µm nylon screen after each use (to remove plant fragments). Staining solutions become translucent when too weak for further use.

7. Post-clearing bleaching with alkaline hydrogen peroxide (0.5 % NH_4OH and 0.5 % H_2O_2 v/v in water) effectively removes excessive remaining pigments (Bevege 1968, Kormanik & McGraw 1982).

8. Samples should be destained in 50 % glycerol (CBE) or lactoglycerol (Trypan blue) for several days prior to observation, to allow excess stain to leach from roots.

10. Roots stained with CBE can be stored in 50 % glycerol, but trypan blue staining is less permanent, unless samples are stored in lactoglycerol or the staining solution.

11. Semi-permanent slides of stained roots can be made with PVLG mountant (see below).

12. Interference-contrast microscopy substantially enhances the contrast of stained fungal structures when photographs are taken.

13. Vital staining procedures that measure succinate dehydrogenase activity can be used to confirm that mycorrhizal fungus hyphae which are enumerated are metabolically active (Schaffer et al. 1993, Tisserant et al. 1993).

14. Modifications to standard clearing and staining procedures have been proposed for safety reasons. Grace & Stribley (1991) suggest that methyl blue or aniline blue, can be used as less toxic replacements for chlorazol black E, or trypan blue. However, there is insufficient evidence to confirm that these dyes are non-toxic — so they must also be handled carefully. A lower concentration of KOH (2.5%) can be used to reduce the risk of injury (Koske & Gemma 1989).

15. The stain acid fuchsin can be used, in combination with fluorescence microscopy, to stain fungal structures in roots (Merryweather & Fitter 1991).

PVLG mountant (Koske & Tessier 1983)

polyvinyl alcohol*	8.33 g
distilled water	50 ml
lactic acid	50 ml
glycerine	5 ml

* 24-32 centripose viscosity polyvinyl alcohol is dissolved in water by heating (90° C) overnight.

B. Measuring colonization by mycorrhizal fungi

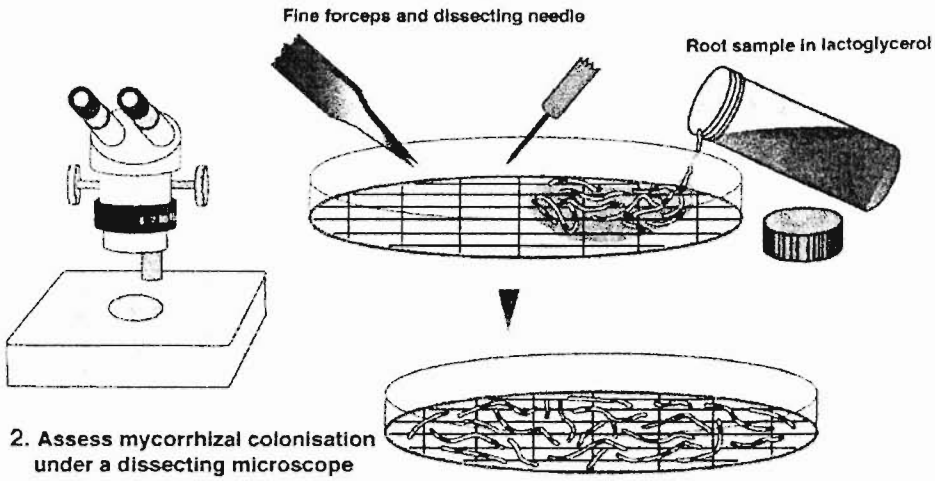
The most frequently used root and mycorrhizal colonization measuring procedure is the grid line intersection method (Newman 1966, Giovannetti & Mosse 1980).

1. Randomly dispersed samples in a 9-cm diameter Petri plate with grid lines (see below). The dispersed sample is scanned along the grid lines using a dissecting microscope to quantify segments of plant material which are designated as *colonised* or *nonmycorrhizal.*

2. Randomly selected pieces designated as mycorrhizal should also be mounted on microscope slides and viewed with a compound microscope.

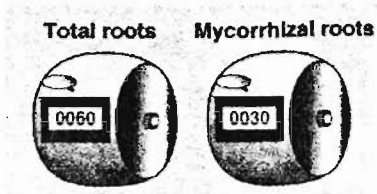
THE GRIDLINE INTERSECTION METHOD

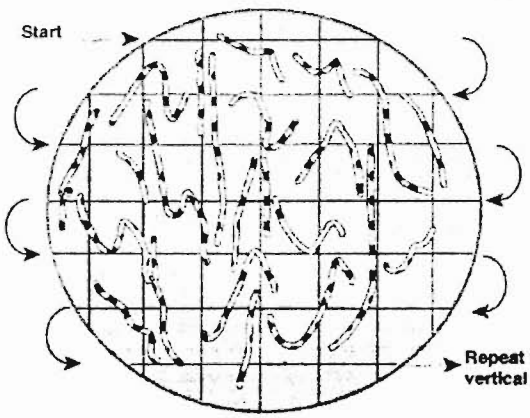
1. Randomly disperse cleared and stained roots in dish with grid lines



2. Assess mycorrhizal colonisation under a dissecting microscope

3. Follow all horizontal and vertical lines. Count intersects with roots and mycorrhizas separately





Horizontal lines

1 / 1
3 / 7
4 / 9
6 / 10
2 / 7
4 / 6
1 / 2

Key to roots

Mycorrhizal

Nonmycorrhizal

Vertical 2 / 2 2 / 4 1 / 2 0 / 3 3 / 4 0 / 1 1 / 2

Total = 30/60 = 50% root length colonised

7.3. Sectioning fresh plant material

Observations of fresh plant material reveals the natural pigmentation, or refringence of unstained root sections and even more detail is produced by uv-induced autofluorescence of semi-thin hand sections. This procedure works best with fresh (turgid) roots, but success is also possible with alcohol-preserved roots.

Equipment

- Sharp 2-edged razor blades (**USE WITH CARE**)
- Material to hold plant material during sectioning
  - Parafilm™ squares and plastic Petri dish lids
  - styrofoam or pith pieces
- Dissecting needle, fine forceps or paint brush to manipulate sections
- Stains, section holders, slides, coverslips, etc.

1. Cross sections of plant tissue immobilised by Parafilm can be made using a sharp razor blade (Frohlich 1984). The best results are obtained when the blade is drawn repeatedly across a bundle of roots to produce numerous sections (see below). Many sections of variable thickness should be produced rapidly, as it is impossible to precisely regulate the thickness of sections.

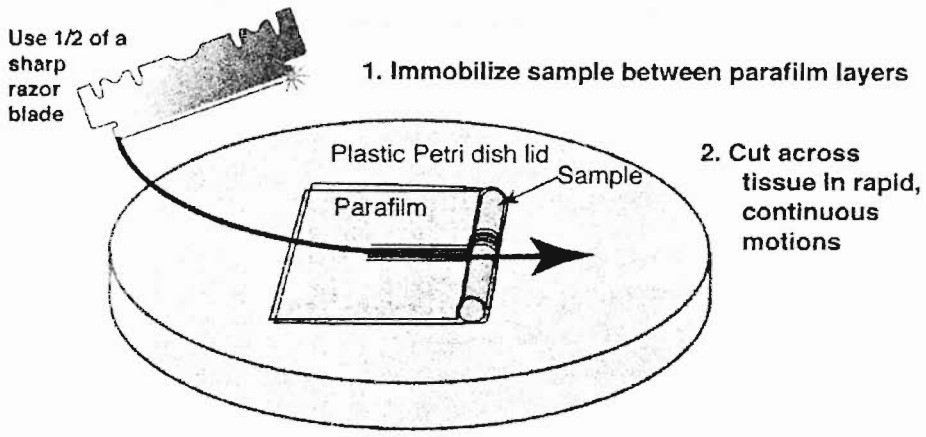
2. Thin, uniform sections are then selected with fine forceps or a pipette while observing sections floating in water under a dissecting microscope. Selected sections can be placed in sample holders, or transferred between solutions with a pipette.

3. Similar results can be obtained using styrofoam pieces, pith, or other similar materials to immobilise roots during hand sectioning.

4. Root hand sections can be cleared in a similar manner to whole roots (see above), but require shorter exposure to hot KOH. Sections placed in a small glass vial using a wide-mouthed pipette are cleared in 10% KOH for 4-12 hours at 60-90° C.

5. Sections are stained with the Trypan blue of Chlorazol black E by heating them (60° C) for 2-3 hours, or by leaving them in the stain for a day. Sections are then rinsed with water after the clearing and staining steps, using a pipette to transfer solutions.

6. Stained root sections can be mounted on microscope slides in 50% glycerine, or a semi-permanent water based media such as PVLAG (Section 7.2). Interference contrast microscopy can be used to greatly enhance the contrast of stained sections.



7.4 Histochemical Staining

Only a few of the most commonly used methods are presented here. Further information is available in standard botanical microtechnique and histology references such as O'Brien & McCully (1981) and Brundrett *et al.* (1994).

A. Vital staining

Hand sections can be placed in mesh-bottomed, multi-chambered section holders (Brundrett *et al.* 1988), which can be made for use in the following staining procedures.

1. The vital stain fluorescein diacetate can be used to indicate living fungal hyphae and spores under ultraviolet light with a fluorescence microscope (Schubert *et al.* 1987, Hamel *et al.* 1990). Material is mounted in a 50 µg/ml (w/v) solution in water or buffer (made by first dissolving 5 mg of FDA in 1 ml of acetone).

B. Nuclear staining

Fluorescence staining of fungal nuclei and septa

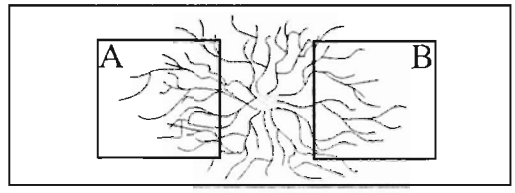
Dye solutions
Prepare a stock solution by adding 10mg of Hoechst Dye to 25 ml of distilled water and heat in a 37°C water bath until disolved. Store at 4°C.

Buffer solutions at the correct pH (7.8) are prepared with 0.1M KH_2PO_4 and 0.1 M NaOH and at pH 10.5 with 0.025M H_3BO_3 and 0.1M NaOH.

Stains are prepared by adding 0.6 ml of the stock dye solution to 50 ml of buffer solutions.

Slide Preparation
Immerse sterile glass slides in potato-dextrose agar (PDA) and place slides on glass rods in a 90 mm petri dish containing sterile filter paper moistened with sterile distilled water.

Place a 1 mm cube of agar bearing mycelium in the middle of the slide. Incubate petri dishes in the dark until the colony diameter reaches approximately 15 - 20 mm.



Slide Staining
Following incubation slides are placed in the air-flow of a laminar flow until the agar surface dries. A drop of each stainging solution at the correct pH is added to each side of the colony and cover slips placed over the drops.

Slides are observed under a fluorescene microscope. Stained nuclei and septa can be observed.

Other method

. The florescent nuclear stain DAPI (4',6-diamidino-2-phenylindole - 5 µg/ml w/v in water or buffer) effectively stains nuclei in mycorrhizal fungi (Cooke *et al.* 1987, Balestrini *et al.* 1992).

C. Seed viability staining

The most commonly used chemical stains used to determine potential viability of orchid seeds are triphenyltetrazolium chloride (TTC) or Fluorescein diacetate (FDA). These tests are particularly useful if storing and sowing seeds over several years. For example, stored *Liparis* seeds dropped from 70 % to 30 % stained embryos (using TTC test) over 5 years with corresponding drop in germination rate. Their other main advantage is in their use to test seed that has not germinated to determine whether the problem lies with the seed or if viability is high , different germination techniques should be considered.

The triphenyl tetrazolium chloride (TTC) staining procedure, developed by Lakon (1949) is the most widely used biochemical assay for evaluation of the viability of seeds. As compared to direct germination assays, this assay has the advantages of being rapid and suitable for controlled conditions. This test has been successfuly used with epiphytic tropical orchids (Singh ,1981) and Van Waes and Debergh (1986) adapted this assay for several Western European terrestrial orchid species.
Trials by Lauzer et al (1994) on *Cypripedium* indicate that germination of mature seed was significantly lower than the percentage of embryo staining with TTC . However in our experience, TTC staining can give a rough indication of germination and is a useful guide , especially for old stored seed, as to whether the seed is worth sowing.

Temperate terrestrial seed coats require pre-treatment with Ca(OCl) solution (see section below on sterilants) in order for staining with TTC to occur. A 24 hour soak in sterile water is required following the hypochlorite pre-treatment in order to remove hypochlorite which can hinder reduction of TTC in viable embryos.

Preparation Of Ca(OCl) solution

For a 5% weight per volume solution.
In a fume cupboard, wearing protective mask, gloves and eye protection weigh out 5g calcium hypochlorite powder in small plastic beaker. Still in the fume cupboard, add a little of the 100ml of water to the powder and mix together using a pestle (Ca(OCl) is difficult to dissolve).
Stir in the rest of the water and 1 ml Tween -80 (1% v/v). Allow to stir thoroughly for 10 minutes, then filter. Use filtered solution immediately.

Preparation of the TTC solution

In 100ml sterile distilled water, dissolve 1g of 2,3,5-triphenyltetrazolium chloride (TTC). Adjust pH to 7 with 0.1M NaOH. Store in the dark at 20 +/- 2 C (foil wrapped brown glass bottle at room temperature). TTC is toxic.

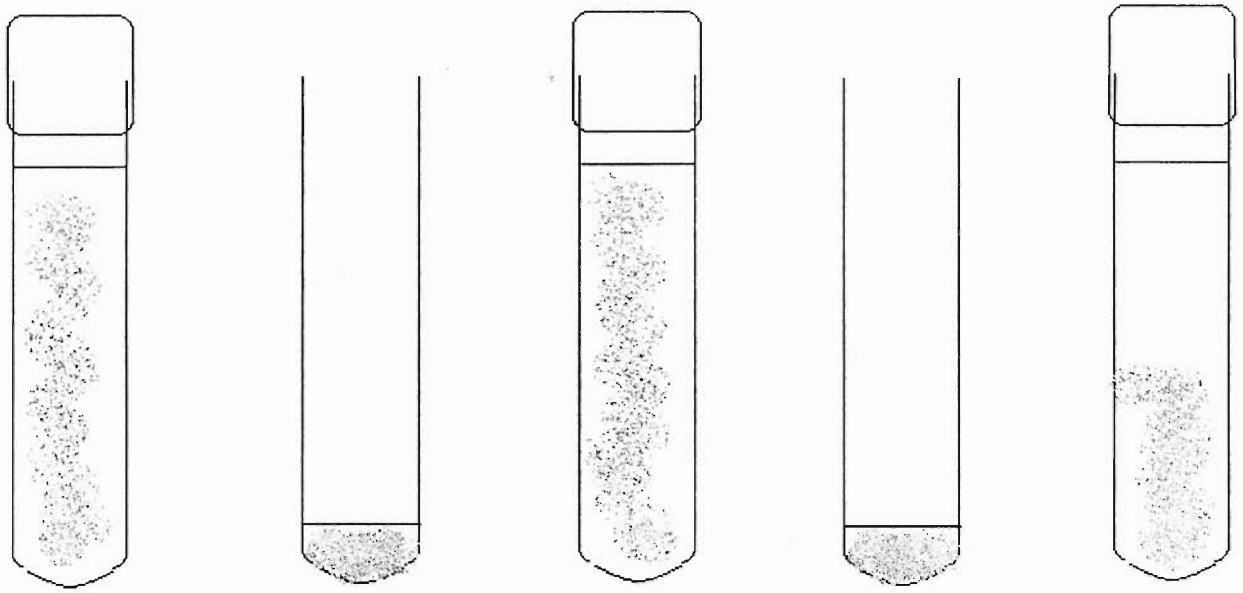
Viability testing:

1)Pretreatment of the seeds in 5% Ca(OCl)(w/v) + 1% Tween 80 (v/v): the optimum duration of the pretreatment to be determined for each species
2) Soak in sterile water for 24 h
3) Apply TTC test

- Place a small amount of seeds in 10ml of Ca(OCl) solution in pyrex test tube with screw cap. gently shake for appropriate time (12-18 hrs Cypripedium, 45-60 minutes Spiranthes, 3-8 hrs for most other Genera)
- Seeds will probably sink to bottom of tube as air in testa will have been replaced with liquid.
- Pipette off bleach solution and replace with sterile water. Change water at least twice and then leave seeds in water for a minimum of 24 hours.
- Pipette off water and replace with 10ml of 1% TTC solution . Place tubes in darkness at 30C for 24 hrs (at Kew we use covered waterbath but incubator can be used)
- Pipette off TTC solution and wash seeds 3 times for 5mins each in sterile distilled water.
- Apply 0.001% Malachite green solution for 5 minutes (optional , useful where testa very bleached).

6)Assess result:

- Pipette off most of water then empty out seeds on filter paper circle divided in four equal parts .
- Rinse out remaining seeds with a small amount of water and disperse seeds evenly over surface.
- Using microscope, score :total number of seeds; number of seeds which have embryos; number of embryos which are stained red. Calculate percentage viability by dividing the number of embryos stained red by the total number of seeds counted and multiplying by 100.
- Determine the mean and standard deviation of the 4 replicates.
- Marking a grid on the filter paper and using a tally counter will aid scoring.



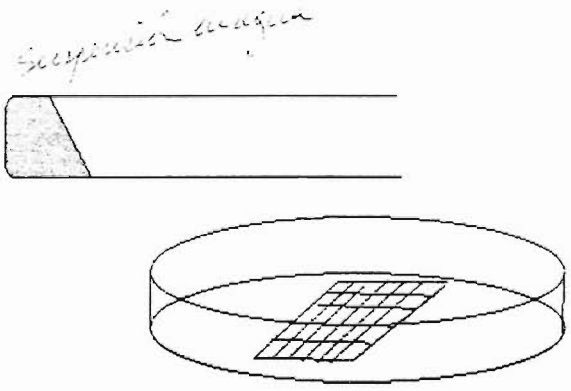
10ml 5%CaOCl
1-18 hours

Remove
CaOCl

Sterile
Distilled
water 24 hrs

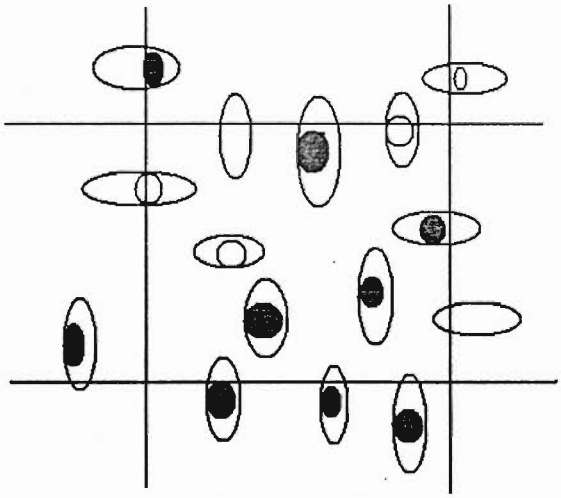
Remove
water

Tetrazoliu
m 24 hrs
30°C



Wash seeds 3 x 5 mins
Sterile distilled water

Retain 1ml of last rinse,
shake to suspend seed,
pour onto filter paper
marked with grid



Score number of
completely coloured
(red) embryos

7.5. Advanced Microscopy

a. SEM

Preparation of material for critical point drying and SEM

1. Fixation & vacuum infiltration

a. cut fresh tissues (not >2cm cube) in 2.5-5% gluteraldehyde in 0.05M phosphate buffer pH 7.0
b. transfer specimens into the vials containing the fixative and ID card (write in pencil).
c. vacuum infiltration for 3-5 mins. & then leave in fixative for 24hrs or longer if necessary.

2. Dehydration

- Replace the fixative in the vial with the buffer for 30 mins. Following this replace buffer with fresh buffer for another 30 mins.
- Replace the buffer with 50% acetone for 24 hours or longer.
- Replace the 50% acetone with 75% acetone for 24 hours or longer.
- Replace the 75% acetone with 90% acetone for 24 hours or longer.
- Replace the 90% acetone with 95% acetone for 24 hours or longer.
- Replace the 95% acetone with 100% acetone for 24 hours or longer.
- Replace the 100% acetone with fresh 100% acetone for 24 hours or longer. You may leave specimen in this stage for storage till you are ready to do the critical point drying but ensure that the acetone does not dry out.

3. Critical Point Drying & SEM examination:

- Specimens will undergo critical point drying prior to being mounted on stubs with carbon tape and coated with Au + C coating.
- Coated specimens were viewed with the Philips 505 Scanning Electron Microscope.

b. Processing/Embedding schedule for GMA

Preparation of material for embedding in GMA.

Procedure	Protocol	Duration
Fixative [1,a]	add small pieces of material to fixative	1 hr to 1 wk
Buffer wash [2]	2 times	15 min
Methoxyethanol	2 times	12 hr
Ethanol	2 times	12 hr
Propan-1-ol	2 times	12 hr
Butan-1-ol	2 times	12 hr
GMA [a]	once	24 hr
GMA [a]	transfer to foil tray	24 hr
Heat at 60°C to cure	transfer pots to oven, remove air, add argon	24 hr

Notes:
1. Fixative: 2.5% Glutaraldehyde (pH 6-7) in 0.025 M phosphate buffer.
2. Phosphate buffer: 0.05M $NaH_2PO_4.2H_2O$ (MW 156.01) / 0.05M Na_2HPO_4 (MW 141.96)
a. Vacuum infiltrate.

Glutaraldehyde solution (Fixative) becomes acidic with prolonged standing. If pH is below 3 and liquid is tinted (honey-coloured) discard.

To raise the pH level of 25% stock glutaraldehyde, combine small amounts with activated carbon and filter. Several applications of carbon may be necessary.

GMA Resin notes

Purified GMA (resin)
Caboxax 200/PEG200 (plasticiser)
Benzoyl peroxide (catalyst)

Mix ingredients together after following notes (i) and (ii).
Stir 1-2 hr at room temperature, do not heat. Polymerise at 60°C under anaerobic conditions.

Notes:
(i) The GMA should be as close to pH 7 as possible. From the bottle it is pH 3. To adjust pH, mix with activated charcoal (4 g per 100 ml) and stir for 1-2 hr. Filter through Whatman No. 1. The more acidic the GMA, the more the resin will take-up any cationic stains (i.e. Toluidine blue).

(ii) Benzoyl peroxide is an explosive substance when dried, therefore it is stored in the fridge in 25% water. Weigh out the required dry weight plus an additional 25% to compensate for the weight of water.
DO NOT DRY!!!!!

-Chapter 8-
Propagation

Seed Germination

Standard symbiotic germination protocols using fungi isolated from host taxa that are known to be effective in germination assays are used to produce protocorms suitable for transfer to soil (Fig. 8.1A). Germination plates should be initiated three months prior to the onset of the growing season so that seedlings are ready for transfer to the glasshouse during the cooler months of the year and to align seedling growth with natural growth phases.

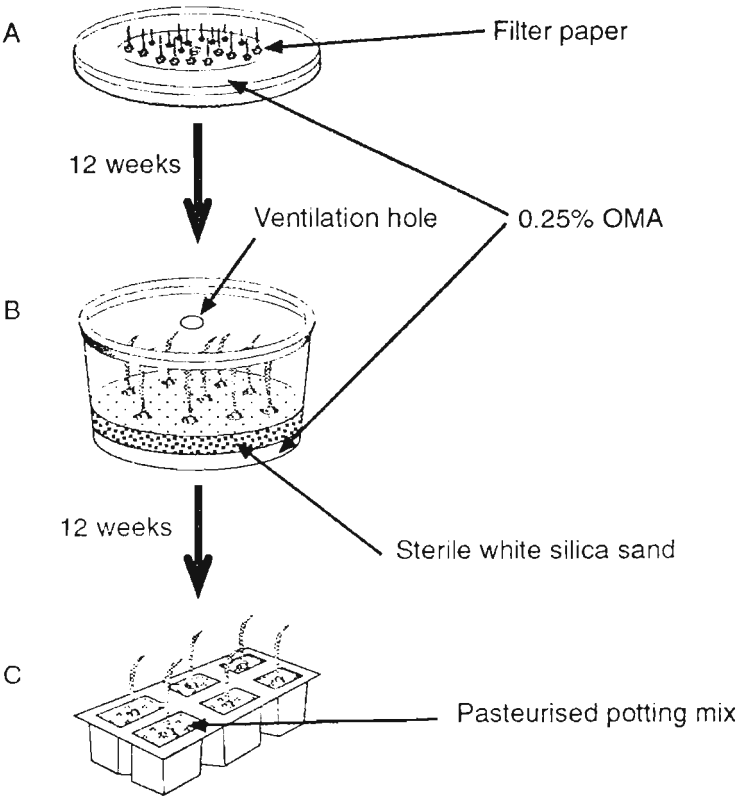


Fig. 8.1. Procedure for the establishment of terrestrial orchids in soil. A, symbiotic germination plate with appropriate fungal isolate; B, sand/agar overlay and C, symbiotic seedlings transferred to pasteurised potting mix in the glasshouse.

Growth Containers

Disposable, polycarbonate containers (60 mm high x 100 mm diameter) are used for the transfer of germinants from symbiotic germination plates. Protocorms, with a green leaf, at stages three and four (Ramsay *et al.* 1986), are transferred to a sand/agar layer, as shown

in Figure 8.1B. Approximately 60 ml of 0.25 % oat meal agar (OMA) (Clements and Ellyard 1979) is added to each container and a lid attached prior to autoclaving for 20 mins at 121°C. Once the OMA has solidified a 10 mm layer of double sterilised (121°C for 20 min; rest 24 hr; 121°C for 20 min) white silica sand is placed over the OMA under sterile conditions. Moisture from the agar is allowed to permeate the sand prior to planting. Containers are ventilated using millipore discs placed over a 6 mm diameter hole in the lid. Growth containers with seedlings are incubated at 22°C with a 16/8hr light/dark regime.

Inoculation of containers
Symbiotic seedlings provided the source of fungal inoculumn for the containers.

Transfer to Soil
Following incubation in containers for three months, containers with seedlings can be placed in the glasshouse for one week prior to removing the lids. Seedlings are hardened for a further 5 days before being transferred to soil. Seedlings are then planted in individual compartments (35 x 30 x 50 mm) in plastic seedling trays (Fig. 8.1C) and covered with a clear plastic dome (Yates® humidity cover) with variable ventilation provided by six, 2 cm diameter holes. Covers can be removed after 10 days with the vents closed for the first three days. Seedlings should be monitored for common glasshouse pests such as scarid fly larvae (see Chapter 9.3 for control measures).

The soil mix and seedlings should be synchronised with natural conditions outside the glasshouse and allowed to dry off during at onset of the dry season. Tuber development can be assessed at this stage. Watering is commenced with the first rains of the season. Tuber survival was confirmed by the emergence of a leaf.

In situ *seed sowing*
Seed is sown to inoculated field sites after the break of the season and the onset of cooler conditions. To assist sowing seed can be thoroughly mixed with 80 g of sterile white silica sand and spread evenly over the soil surface. Mulch is kept moist by watering with rainwater. These methods can be applied to soil in the glasshouse or natural bushland sites. Although seedlings developed and produced a tuber most seedlings failed to survive summer drought conditions. Research using artificial watering may improve seedling establishment rates.

Seedling establishment
Seeds are germinated and transferred to growth containers in the laboratory before being transferred to the glasshouse as described above (Fig. 8.1). Symbiotic seedlings in containers are removed from the glasshouse 24 weeks after sowing and hardened under shade outside the glasshouse for one week prior to transfer to field sites. Planting should

occur after the break of the season (onset of rain). Seedlings are watered using rainwater as required.

Outplanting by tuber transfer

Dormant juvenile tubers of terrestrial orchids produced following the methodology described above can be planted to field sites at the break of the season. Three 3 mm cubes of OMA culture media colonised with appropriate fungi are placed in small holes (1 - 2 cm deep) adjacent to translocated tubers to act as a source of inoculum. Disturbance to each site is minimal as dormant tubers are inserted into the ground using a small cylindrical tool approximating the size of the tubers.

Overview of flora conservation in Western Australia

Andrew Brown, Department of Conservation & Land Management

- **Area-based Wildlife Management Programs.** In order to determine their conservation status surveys are conducted on all plant taxa known from just one or two collections in the WA herbarium. Recommendations for listing as Threatened flora or placement into Priorities 1-4 are then made in a published Wildlife Management Program. Over 80 orchid taxa are listed. This document also lists recovery actions for each taxon.
- **Threatening processes.** Most threatened orchid taxa in WA are threatened due to past clearing, disease such as dieback (does not directly affect orchid flora but causes habitat change) or salinity. Continuing threats include degraded habitat, weeds, roadwork, lack of pollinators, herbicide overspray, fertiliser runoff, trampling and picking by visitors, grazing (native and introduced animals), insects, flooding from rising water tables and a lack of suitable disturbance events such as fire. Also birds, e.g. ravens have been seen nipping off flowers.
- **Listing** (DRF, priority 1-4). Currently 350 plant taxa (including 21 presumed extinct) are listed as threatened in WA, 31 are orchids. These are protected by legislation, particularly the Wildlife Conservation Act, Section 23f. Protection covers all lands including private.
- **Ranking.** Once an orchid taxon is listed as Threatened flora it is run past IUCN and CALM Policy 50 criteria and placed into one of three categories depending on threat:

a) Critically Endangered (CR) – will become extinct if recovery actions are not implemented immediately. Twelve orchid taxa are rated CR.
b) Endangered (EN) - less threatened but likely to become CR if recovery actions are not put into place. Fifteen orchid taxa are rated EN.
c) Vulnerable (VU) - rare but not in immediate threat. Four orchid taxa are rated VU.

- **Interim Recovery Plans (IRPs)** – These are produced for taxa ranked as CR. The plans are designed to run for three years and address only those actions deemed necessary to prevent extinction. Funding for recovery actions comes from various sources, e.g. State, Commonwealth (NHT), WWF and the Landscope Conservation Visa Card.
- **Recovery Plans (RPs)** – These are implemented once an IRP has run its course (if the taxon is still regarded as CR). These plans run for five years, and address the long-term recovery of a species. Most recommended research (pollination biology, seed dispersal, germination requirements including associated fungi, fire studies, genetics etc) listed in IRPs is done during the implementation of RPs.
- **Recovery Teams (RTs)** - Area-based RTs have been set up in the southwest of WA. The teams oversee the implementation of recovery actions for a number of threatened plant taxa, particularly those with IRPs and RPs. Implementation involves the participation of people from government departments, local government, community groups and landowners.
- **Orchids.** The 31 orchid taxa listed as Threatened in WA are found mainly in the highly cleared farming and suburban areas. Threats vary between populations, and include

lack of or highly degraded habitat, weeds, roadwork and herbicide overspray. IRPs have been written for all orchid taxa ranked as CR.

- The timing of weeding has been important for some species, as sometimes there is only a narrow window of opportunity available before the orchids emerge. Orchid emergence times have been noted and made available to me by orchid enthusiasts.
- **Example of two CR orchid taxa** (*Caladenia elegans* and *Pterostylis* sp. Northampton)

a) **Description and history.** Both species discovered by Stan Fink in 1976. Flowering occurs in late July-mid August. Seven populations of *C. elegans* are known and four populations of *P.* sp. Northampton. IRPs have been written for both taxa. Pollination and genetic research has been completed for *C. elegans.*

b) **Habitat.** Remnant mixed shrubland in winter-wet areas. Soils are clay-loams. Most populations are on narrow, degraded road reserves adjacent to cleared farmland.

c) **Threats.** Weeds, roadwork, drainage, erosion, pigs, grazing, lack of pollinators in some populations, lack of suitable disturbance. Herbivore control is one of the most important recovery means. Several populations are now fenced to exclude rabbits and kangaroos. Pigs are a major problem in some areas (rooting out orchids in search for roots etc). Staff from the Agricultural Protection Board have been catching and shooting pigs.

d) **Pollination biology**. The pollinator for *C. elegans* is a native Thynnid wasp; for *P.* sp. Northampton it is a small native Dipteran (fly). *How are they attracted?* Pheromones for *C. elegans*; unknown for *P.* sp. Northampton. The mechanisms used by *C. elegans* are glandular hairs that emit pheromones and a homing array of labellum glands; for *P.* sp. Northampton an irritable labellum temporarily traps the insect against the stigma and anthers. It is important to understand the requirements of the pollinators. Habitat reconstruction has been successfully implemented to encourage pollinators back into the area of one population of each of these species.

e) **Fire Regimes** (suitable and inappropriate). Hot summer fire results in increased flowering of *C. elegans* in the first year but has little effect on *P.* sp. Northampton at that time. Subsequently, however, there is an increase in the numbers of flowering plants of *P.* sp. Northampton (years 2-4) which lasts until associated native shrub species crowd out plants. During this time the number of flowering plants of *C. elegans* diminishes. Many associated plants, e.g. *Thryptomene saxicola* and *Grevillea pinifolia* produce strong new growth following fire, resulting in better flowering, stronger insect activity and increased orchid pollination. Occasional summer fire is therefore important for promoting increased flowering and seed set for the two species. Smoke/fire treatment has also been used successfully to encourage natural germination of native vegetation in weedy areas on roadsides. A danger with fire is in its timing (summer), when it may be a threat to adjacent landowners. Fire also increases the vigour of introduced weeds.

Discussion

- Fire is not only a stimulant for flowering of many orchid taxa but also the vector causing change in habitat structure. Many native plants germinate following fire and for several years can create a dense thicket of seedlings. For some orchid species the change is beneficial but for others it may take many years for populations to regain their original size. The recommended minimum frequency of fires is 12 - 15 years. It is important to

consider the pollinator food plants, and whether their desired fire frequency coincides with that of the orchids.

- Smoke water treatment in situ with dormant orchid plants has had limited success in promoting flowering, although orchid flowering is often stimulated by summer fire.
- Where new orchid populations have been established from translocations, wasps and other insects from nearby remnant vegetation have in some cases visited and pollinated the flowers.
- For orchid species pollinated by Thynnid wasps, juvenile wasps probably parasitise the larvae of beetle, indicating that beetle larvae may be a limiting factor. It appears that each Thynnid species parasitises only one species of beetle. The beetle has only been identified for 14 out of 1000 Australian thynnid species. Only 20 - 25 % of Australian thynnid wasps are described. It is important to distinguish orchid-related Thynnids from others present. An orchid pollinator can be identified by taking a flower away from its colony on a warm still day. In WA the use of auto-trigger photography (infrared) to identify pollinators was unsuccessful, as other insects triggered the camera.
    - There is a need to ensure synchronicity of flowering for both wasp food plant and orchid. *Caladenia elegans* requires the presence of a *Thryptomene* species on which the Thynnid feeds; however in most cases it is presumed that Thynnids have general food plant requirements.

-CHAPTER 9-

GROWING ORCHIDS

9.1. The Glasshouse

Glasshouse experiments with orchids allow plant physiology, plant mineral nutrition, mycorrhizal associations, or plant responses to environmental factors to be studied under more controlled conditions than occur in the nursery of field. Basic equipment for glasshouse experiments is listed here.

Requirements
Growing conditions
light levels and ventilation must be adequate
cooling and/or heating may be required
raised benches are needed to prevent contamination
water must rapidly drain away
a clean, dust free working environment is essential
Equipment
clean, sterile pots
suitable potting mix
system to pasteurise or fumigate mixes to remove harmful organisms
suitable fertilisers
pure, uncontaminated water
pest control measures, if required

Growth substrates

Specific soils from field sites or a suitable potting mix are required. Unwanted microbes should be removed by steam pasteurisation (2 x 60 min at 70°C on consecutive days), or by using one of the other methods discussed below.

Watering plants

Large plants may need to be watered accurately every day to limit differences in water supply between treatments.

Nutrient supply

It is essential that fertilizers are accurately and uniformly applied to plants. Specific orchid formulations are commercially available.

Environmental conditions

The glasshouse environment should be monitored to ensure light and temperature conditions are favourable to plant growth. These conditions should be reported when publishing results. Standardising growing conditions will increase the accuracy of experiments.

Measuring plants and harvesting experiments

The optimal duration of experiments will depend on the growth rate of plants and soil nutrient levels. Height measurements can be used to reveal growth trends and determine when to stop the experiment. Once the experiment is over, plants can be harvested by cutting off shoots, which are weighed to determine fresh weight then dried to determine dry weight and by washing roots free from soil.

9.2. The Nursery

A wide range of practices can be encountered in nurseries producing orchids. Here we are addressing requirements for Western Australian terrestrial orchids, however, the basic principles/issues also apply to epiphytic orchids. Key issues for the management of plants in nurseries include nursery hygiene, the choice and preparation of potting mixes, the use of fertilisers. The composition, pH, moisture content and temperature of the rooting media, fumigation and pasteurisation practices, and the use of biocides and fungicides can be manipulated to provide conditions which are optimal for plant production.

A. Potting mix components

No single ingredient can provide all the characteristics required to grow seedlings effectively, so nurseries generally combine several of the substrates described below to make a potting mix. These ingredients can be mixed with soil or sand, but are most often combined to make a "soil-less" potting mixture.

Peat

Peat is a major component of many potting mixtures providing an organic substrate for root growth. There are many variations in "peat" formulations used by nursery managers and these include local cladium sedge type, shredded coconut, etc. However, the traditional peat derived from sphagnum moss has the best physical and chemical formulation being low in available nutrients and pH. For container mixes peat is often used as a 50% mix with other components such as vermiculite and perlite.

Sand or aggregate

These are used mainly to assist drainage and add weight to the mix and reduce shrinkage in dry mixes during dormancy.

Vermiculite and perlite

These relatively cheap and readily available components are bulking agents that retain moisture while ensuring a well drained mix. They also help prevent the growing medium from settling and compacting. Both are lightweight and pH neutral. Perlite lacks any buffering capacity and is nutritionally inert, while vermiculite has a higher buffering capacity and can provide Mg and K for seedling growth (Goh & Haynes 1977). These are rarely used with our terrestrial orchids. Algal growth can be a problem with these ingredients.

Composted tree bark,

The best components are *Allocasurina* bark and branchlets (needles) and pine needles. Pine bark is rarely used.

Composted wood products

Sawdust and fine wood chips are used as natural substitutes for peat if supplies are limited, or prohibitively expensive. Some types of wood can be toxic to root growth and preliminary testing of these products is essential prior to their use. Aged and leached wood products are preferred (i.e. composts). The main ingredient used in Western Australia is Jarrah sawdust (*Eucalyptus margenata*) which is cheap and readily available.

Composts

Compost is manufactured by combining plant material such as straw, wood products, plant residues and animal wastes in a pile, and maintaining it in a moist state, to allow partial microbial decomposition (Hoitink 1980, Hardy &

Sivasithamparam 1989). The final product is a humus like soil amendment relatively high in nitrogen with a much reduced C:N ratio, relative to raw wood products. Compost has a high water retention quality, low weight and mixes well with sand, soil, perlite or vermiculite (Liegal & Venator 1987). Potting mixes based on composted eucalypt bark have been found to suppress pathogens such as *Phytophthora* spp. (Hoitink 1980, Hardy & Sivasithamparam 1991). The main materials composted for terrestrial orchids are Jarrah sawdust, *Allocasuarina* bark/branchlets and pine needles. No animal wastes are used.

Mixtures

A number of mixes have been use over a long period of time, however, these are usually based on organic ingredients sourced from natural ecosystems which are unsustainable. Other organic and inorganic materials are being trialed (Nika). Generally mixes must be open, free draining and contain some organic matter for beneficial fungi. Some common mixes used successfully for Western Australian terrestrial orchids are given.

Mix one	*Proportions*
Coarse sand	4.5 parts
Loam	4.0 parts
Peatmoss	1.5 parts

Mix two	
Coarse sand	4.0 parts
Rich loam	2.0 parts
Wood chips (well leached to remove tannins)	2.0 parts
Composted leaf litter	2.0 parts

Mix three	
Coarse sand	2.5 parts
Coastal plain sand	5.0 parts
Loam	1.0 parts
Peatmoss	1.5 parts

B. Sterilising potting mixes

Potting mixes with sphagnum moss peat, perlite or vermiculite as major components often do not require sterilisation (pasteurisation) whereas mixes with sawdust, bark, raw humus, or soil should be sterilised (pasturised) to reduce the potential threat from pathogens and other microorganisms which would limit mycorrhizal fungal growth on the root and eliminate weed seed. It is best to sterilise (pasteurise) potting mixes when they are warm and moist. Peat, however, can be steamed when it is almost dry. Steam treatment, when carried out at 70°C for 30 minutes, is preferred because it is non-toxic, and only partially sterilises mixes, eliminating harmful microorganisms and weed seed, while some beneficial microbes remain (Baker & Roistacher 1957). It is important that much of the saprophytic microflora remains. An additional goal in mycorrhizal nurseries is to eradicate less efficient strains of mycorrhizal fungi, such as *Thelephora* spp., which can reduce the success of controlled inoculation programs. A wide variety of steam pasteurisation equipment is available, but may be too expensive for small size nurseries. Temperatures much over 70°C can be disadvantageous in potting mixes containing some types of acid soil because the release of toxic amounts of Mn can cause Fe deficiency in containerised plants (Sonneveld 1979).

A cheaper alternative to the use of steam is solar pasteurisation where moist potting mixes are covered by, or enclosed in, clear polyethylene sheeting and exposed to direct sunlight for a period of time. Temperature under the polyethylene sheet can rise to over 60°C during the summer months, and may be higher in the tropics. Solarisation results in continuous or repeated exposure to sublethal temperature which will either kill the majority of the pests, weed seeds, and pathogens, or weaken them so that they cannot survive (Stapleton *et al.* 1985, Porter & Merriman 1985). It is advisable that during this period air temperatures are high to obtain temperatures that control major root diseases (Porter & Merriman, 1985).

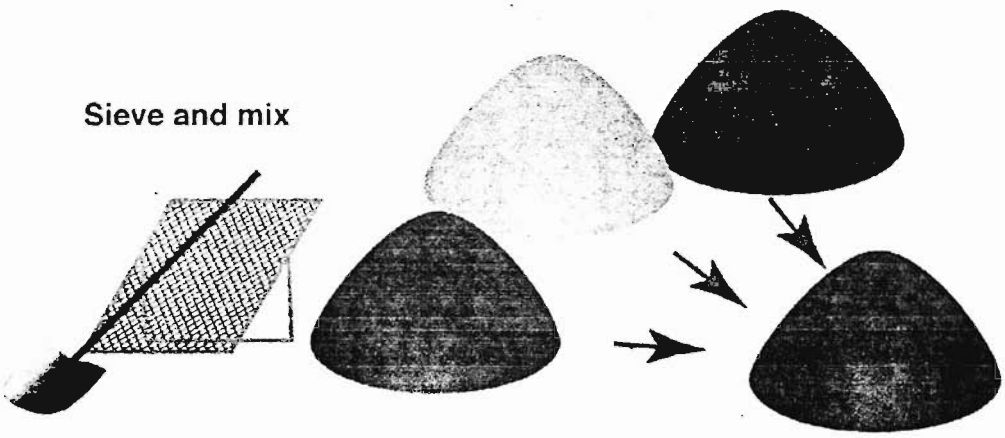
Chemical sterilisation with fumigants can be simple and effective, but precautions indicated on canisters must be followed and advice sought from the local agricultural pest control agency. Methyl bromide-chloropicrin (MBC) fumigant is the more widely used, providing effective sterilisation of soils. In Western Australia only licensed operators are allowed to apply fumigants.

WARNING

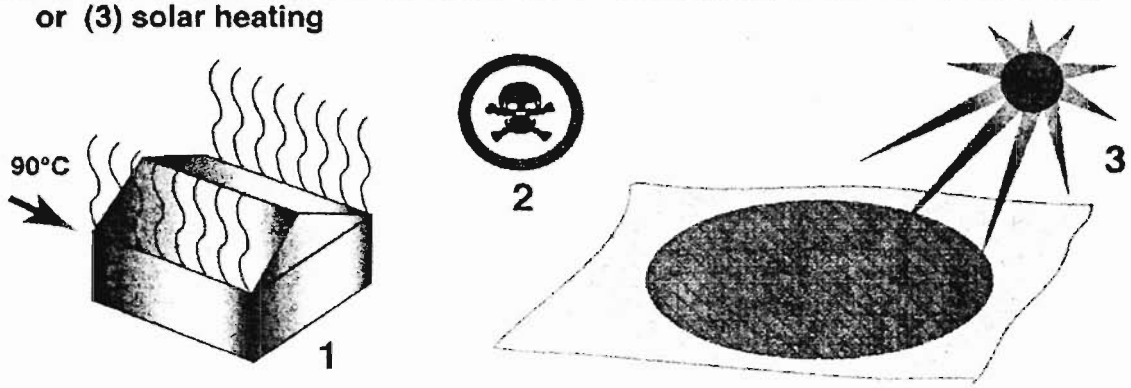
Chemical sterilants are highly hazardous materials. Extreme care is essential in the handling and application of these chemicals and the instructions for use indicated on labels must be strictly followed.

POTTING MIXES

A. Combine ingrdients to make a potting mix with appropriate pH, nutrient level, drainange and moisture holding capcity



B. Pasteurise potting mix using (1) steam heating, (2) chemical fumigation, or (3) solar heating



9.3. Disease and Pest Control

Orchid seedlings are usually grown in pots in the nursery. Disease and pest management is simplified if seedlings are grown in containers which are raised off the ground, since they are not exposed to soil pests and pathogens and will be well aerated. Intensive production of plants in nurseries creates opportunities for pests and diseases to flourish if not checked. These are exacerbated under conditions of poor hygiene, or where plants are stressed due to poor management (e.g. over or under watering, inappropriate potting mixes, etc.). Nurseries require appropriate strategies for controlling pests and diseases which consider the interactions between the causal organism, the containerised mycorrhizal plant and environmental factors (integrated pest management). Chemicals are widely used to control fungal diseases (fungicides), insect pests (insecticides) and nematodes (nematicides) in nurseries. In addition, some nurseries may use herbicides to control weeds. **No chemicals should be used without adequate safety procedures and correct training.**

Kings Park and Botanic Garden nursery is an 'accredited nursery' and therefore adheres to the conditions required under the Nursery Industry Accreditation Scheme Australia (NIASA).

However, for research purposes a small 'unaccredited area' is reserved for this purpose where it is necessary to use materials, especially soil, from natural ecosystems which cannot be pasteurised or fumigated.

The major orchid pests in Kings Park nursery and their control methods are as follows:

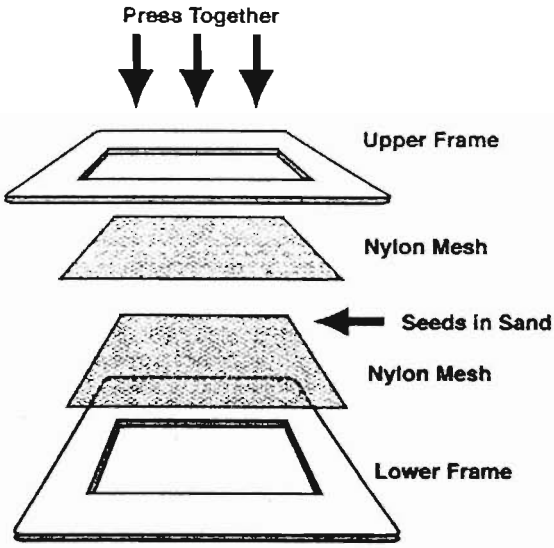
- Scarid fly larvae (eat the roots of protocorms/seedlings) drench with Mesurol 750® (Methiocarb) or VectoBac GAS® (*Bacillus thuringiensis* subsp. *israelensis*).
- Slugs and snails are also controlled by Measurol 750® or various types of baits.

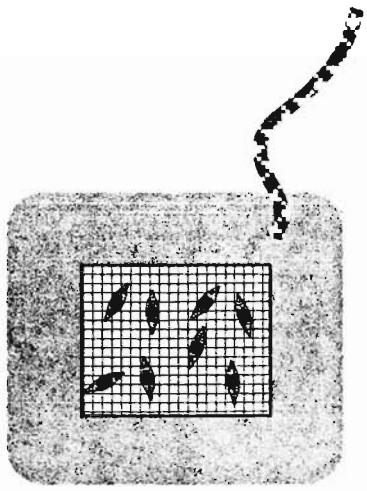
-CHAPTER 10-
BAITING MYCORRHIZAL FUNGI IN SOILS

Inoculum potential is defined as the energy for growth of an organism at the surface of its host, and is a consequence of the numbers of active propagules of that organism and their nutritional status (Garrett 1956). Understanding the distribution of orchid mycorrhizal fungi within soil or other substrates is important for attempts to return orchids to the field and in understanding the distribution of orchids. The patchy distribution of orchids may be influenced by the presence or absence of the specific mycorrhizal fungi essential for the survival of the orchid. Orchid dispersal may be a function of mycorrhizal distribution, seed dispersal and conditions suitable for the germination of orchid seed and the establishment of orchid plants. Studies into the in situ germination of orchid seed in field sites have shown that good germination can be obtained.

A. The slide frame baiting method

A seed burial technique devised by Rasmussen and Whigham (1993) allows the distribution of effective orchid endophytes to be assessed *in situ* in natural habitats. In a similar study in Western Australia, where seeds remained in the soil throughout the growing season demonstrated tuber development from protocorms, confirming that effective fungi were present (Batty et al. 2001). In this study the successful germination of orchid seeds was found to be higher in close proximity to adult plants of the same species.

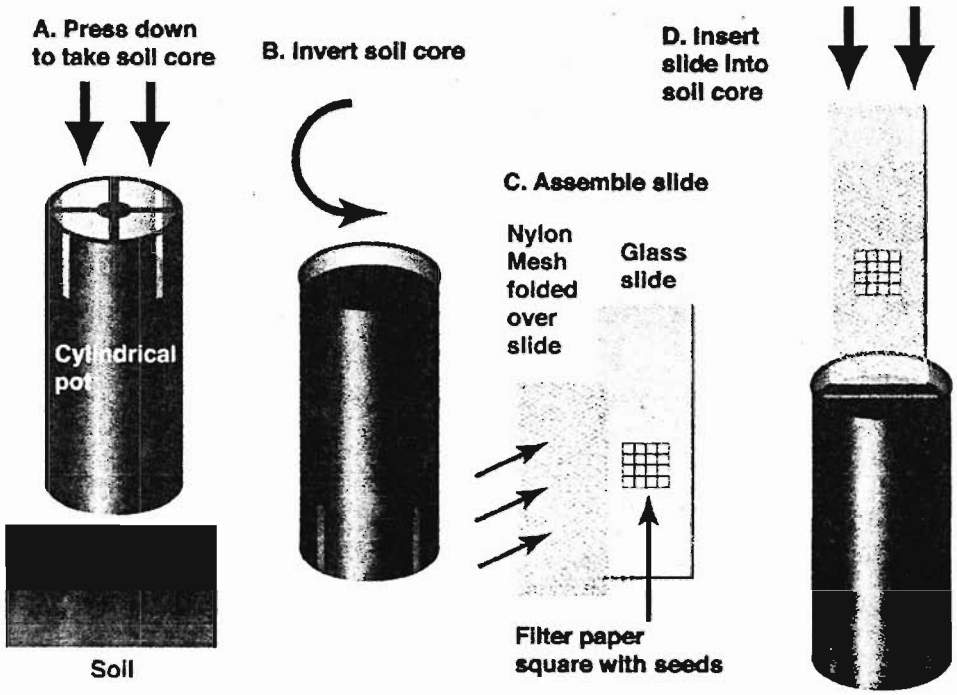




Orchid seed and sterile silica sand are placed between the two pieces of nylon mesh and held in place by slide frames. The quantity of seed placed in each seed bait will depend on the quantity of seed available. For rare taxa fewer seed can be used. To assist in locating the seed baits in the field a length of nylon string can be attached to the slide frame by a hot glue gun or other quick drying glue. Frames can be made up in advanced and stored (for a few weeks) at room temperature until required.

B. Intact soil core baiting

This method is currently under development in our laboratory and only a brief introduction is provided here. Seeds adhering to a damp square of membrane filter paper (see below) are placed on one or both sides of a standard glass slide and covered with a folded piece of nylon mesh. Two or more taxa of orchids can be as bait plants in the same cores by placing their seeds on opposite sides of the glass slide or on separate squares of membrane filter. The glass slide with seeds is then inserted into a slit cut into the soil (use only gentle pressure when inserting the glass slide, or it may break and cut you!!). We have used this technique to compare seed germination in soil obtained from under different orchid taxa.



C. Seed germination over soil

This technique allows non-destructive observation of seed germination using field collected soil samples or potting mix inoculated with a particular fungus. It is a modification of a method devised to observe germination of fungus spores in contact with the soil solution (Brundrett & Juniper 1995).

1. Sieved soil is, watered to approximately 2x field capacity with de-ionised water packed into 100 mm square Petri dishes (approx. 100 g soil / dish)is overlaid with one layer of nylon mesh (pore size 50 µm).

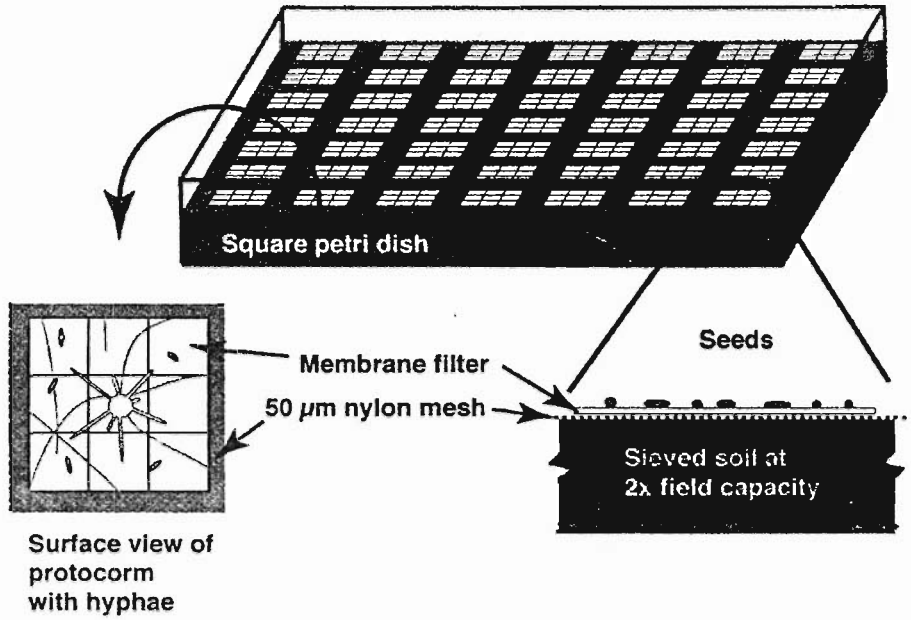
2. Square (1 cm x 1 cm) pieces of membrane filter are cut then arranged on the mesh (see Fig. below). The filter squares and nylon mesh can be sterilized in 70% ethanol and rinsed with distilled water before use. It is usually not necessary to surface sterilize the seeds.

3. Petri dishes are sealed and incubated in the dark at an appropriate temperature. At weekly intervals, the dishes are placed under a dissecting microscope with

fibre-optic illumination to observe the number of seeds at different stages of germination.

4. Seeds at different germination stages can be mounted on slides to make anatomical observations.

Germination of orchid seeds on membrane filter squares



- Seed germination on membrane filters:
  - — squares cut from nitrocellulose membrane filters (0.45 μm)
  - — Petri dishes (square 10 x 10 cm)
  - — 11 x 11 cm pieces of nylon mesh (50 μm)
  - — steamed sandy soil

Appendix

Symbiotic Orchid Seed Germination References

1. Tan, T.K., *et al.*, *Infection of Spathoglottis plicata (Orchidaceae) seeds by mycorrhizal fungus.* Plant Cell Reports, 1998. **18**(1-2): p. 14-19.

Spathoglottis plicata seeds were encapsulated in 4-mm-diameter capsules of alginate-chitosan or alginate-gelatin and infected with the mycorrhizal fungus Rhizoctonia AM9. The encapsulated seeds were placed directly on Rhizoctonia culture. About 66% of the seeds encapsulated in sucrose-free chitosan-alginate established a symbiotic relationship with the mycorrhizal fungus after co-culturing for 2 weeks. The highest percentage of infection observed was about 84%. Addition of sucrose or using gelatin-alginate for encapsulation reduced the percentage of infection by about half. The growth of Rhizoctonia AM9 in sucrose-free alginate, chitosan and gelatin was found to be minimal. The advantages of germinating orchid seeds, encapsulated in sucrose-free polymers, through mycorrhizal infection is discussed.

2. Zelmer Carla, D., L. Cuthbertson, and S. Currah Randy, *Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms.* Mycoscience, 1996. **37**(4): p. 439-448.

The identity and ecological role of fungi in the mycorrhizal roots of 25 species of mature terrestrial orchids and in 17 species of field incubated orchid seedlings were examined. Isolates of symbiotic fungi from mature orchid mycorrhizas were basidiomycetes primarily in the genera Ceratorhiza, Epulorhiza and Moniliopsis; a few unidentified taxa with clamped hyphae were also recovered. More than one taxon of peloton-forming fungus was often observed in the cleared and stained mycorrhizas. Although Ceratorhiza and Epulorhiza strains were isolated from the developing protocorms, pelotons of clamped hyphae were often present in the cleared protocorms of several orchid species. These basidiomycetes are difficult to isolate and may be symbionts of ectotrophic plants. The higher proportion of endophytes bearing clamp connections in developing seeds than in the mycorrhizas is attributed to differences in the nutritional requirements of the fully mycotrophic protocorms and partially autotrophic plants. Most isolates of Ceratorhiza differed enzymatically from Epulorhiza in producing polyphenol oxidases. Dual cultures with thirteen orchid isolates and five non-orchid hosts showed that some taxa can form harmless associations with non-orchid hosts. It is suggested that most terrestrial orchid mycorrhizas are relatively non-specific and that the mycobionts can be saprophytes, parasites or mycorrhizal associates of other plants.

3. Van Der Kinderen, G., *Observations on in situ germination of Epipactis helleborine (L.) Crantz.* Lindleyana, 1995. **10**(4): p. 223-231.

Germination of Epipactis helleborine was studied under natural conditions, using a new technique of controlled soil incubation in situ. The influence of temperature, incubation depth and seed viability was examined in relation to the germination process. A long imbibition period, starting 3 months after seed dispersal, was observed. Its duration was equal regardless of the depth at which seeds were incubated. The proportion of seeds involved in the process was higher than suggested by biochemical viability testing, which proved to be unreliable. Differentiation of embryos to protocorms started almost one year after seed dispersal. A cold period preceded this stage of development and may have broken seed dormancy, but differentiation was observed only after fungal infection. After this, a marked depth-dependent difference in rate of development was observed.

4. Quay, L., A. McComb Jen, and W. Dixon Kingsley, *Methods for ex vitro germination of Australian terrestrial orchids.* Hortscience, 1995. **30**(7): p. 1445-1446.

Seeds of two Australian terrestrial orchid species (Caladenia latifolia R.Br. and Diuris magnifica D. Jones) were germinated in a potting mix of Allocasuarina fraseriana (Miq.) L. Johnson leaf mulch and perlite (1:1). The potting mix was irradiated (7 Gy for 14 hours), steam pasteurized (70C for 30 minutes) or

nontreated, and inoculated with the appropriate mycorrhizal fungus for each species, a sterile red fungus (SRF), or both. Protocorm formation and green shoots were evident at 8 and 10 weeks, respectively, after seed sowing. The highest mean number of seedlings was 84 for C. latifolia and 234 for D. magnifica per 270-ml container in pasteurized potting mix inoculated with mycorrhizal fungi and SRF. Shoots were longest after 20 weeks (28 mm for C. latifolia and 52 mm for D. magnifica, respectively) in pasteurized potting mix inoculated with mycorrhizal fungi only. Germination was absent in control treatments without mycorrhizal fungi; with SRF only; or in nonsterile potting mix with mycorrhizal fungi, SRF, or both.

5. Oddie, R.L.A., K.W. Dixon, and J.A. McComb, *Influence of substrate on asymbiotic and symbiotic in vitro germination and seedling growth of two Australian terrestrial orchids.* Lindleyana, 1994. **9**(3): p. 183-189.

Agar, Gelrite, Agrosoke, ultrafine perlite and Allocasuarina leaf mulch were tested as in vitro substrates for the germination and growth of two Australian terrestrial orchids, Elythranthera brunonis and Diuris longifolia. When cultured asymbiotically, seeds of both species showed more germination and growth on medium gelled with Gelrite than on medium gelled with agar. Elythranthera seeds cultured on ultrafine perlite gave a similar level of growth and germination as on Gelrite but, for Diuris seedlings ultrafine perlite was superior to Gelrite. Agrosoke and Allocasuarina leaf mulch supported no germination in either species. Symbiotic germination of both species was more rapid than asymbiotic germination. Elythranthera seeds cultured symbiotically on Gelrite showed more growth than those on agar or ultrafine perlite. A higher strength of Gelrite and a lower strength of agar promoted more germination and growth of symbiotic D. longifolia seedlings. E. brunonis produced tubers after 20 weeks growth, with more tubers being produced on Gelrite than on agar or ultrafine perlite.

6. Johnson Stephen, R., *Symbiotic seed germination in relation to potential naturalization ability of Bletilla Striata (Orchidaceae).* Lindleyana, 1994. **9**(2): p. 99-101.

Orchid species may vary in their specificity for symbionts. Species with less restricted fungal relationships and wide range of possible pollinators may possess the ability to naturalize outside their native habitats. Unsterilized seeds of Bletilla striata were collected from garden grown plants in 1988. Unsterilized seeds were placed onto a non sterile commercially available potting soil and enclosed in plastic chambers to maintain high relative humidity. Five chambers were inoculated with leaf mold from the rhizosphere of Cypripedium acaule and another five were not. Seed germination was greater than 60%, mortality was less than 20% in the C. acaule leaf mold treatment and in the treatment lacking C. acaule leaf mold, seed germination was 34% with a 45% mortality of seedlings. Seedlings developed from protocorms in less than 1 month. This suggests that Bletilla striata is not highly specific in response to fungal symbionts and may therefore possess some naturalization ability.

7. Wang, H. and J.-T. Xu, *A cytochemical study of acid phosphatase in the mycorrhizal cells of Gastrodia elata seedling.* Acta Botanica Sinica, 1993. **35**(10): p. 772-778.

A cytochemical study has been made to examine the activity of acid beta-glycerophosphatase in the mycorrhizal cells of the seedling of Gastrodia elata Bl. using thin sectioning technique in which sections were embedded in glycol mathacrylate (GMA). After the seedling was invaded by the hyphae of Mycena osmundicola Lange, two different kinds of infected cells were formed in its root cortex: the outer 1-2 cell layers namely the hyphae-containing cells (or host cells) contained many coiled hyphae pelotons; the inner comparatively large cell layer or fungus-digesting cells contained a few straight hyphae. Localization of acid phosphatase in hyphae-containing cells showed that only a few senescent hyphae retained the enzyme activity and the plant cells did not release hydrolytic enzyme. So it is considered that the hyphal lysis in hyphae-containing cell may be due to autolysis. In contrast, higher acid phosphatase activity was visualized in many vesicles and small vacuoles of the fungus-digesting cells. When a hypha entered a fungus-digesting cell through a hyphae-containing cell, a number of enzyme granules (i.e. enzyme-containing vesicles) gathered around it. Later on the enzyme granules expanded gradually and became small enzyme vacuoles of 1.6-2.0 mu-m in diameter. Still later the small enzyme vacuoles fused with each other to form a large vacuole in which a part of an invading hypha was enclosed and gradually digested by hydrolytic enzymes. Finally, the digesting vacuole changed into a residual body containing

Taxonomy

hyphal characteristics

↓

Rhizoctonia

↓

nuclear status

↙ ↘

multi- bi-

↓

Sexual spores

↓

Teleomorphs

↓

Anastomosis groups
up to 12

— produce las micorrizas de orquídeas

↓

Zymogram groups
(hosts?)

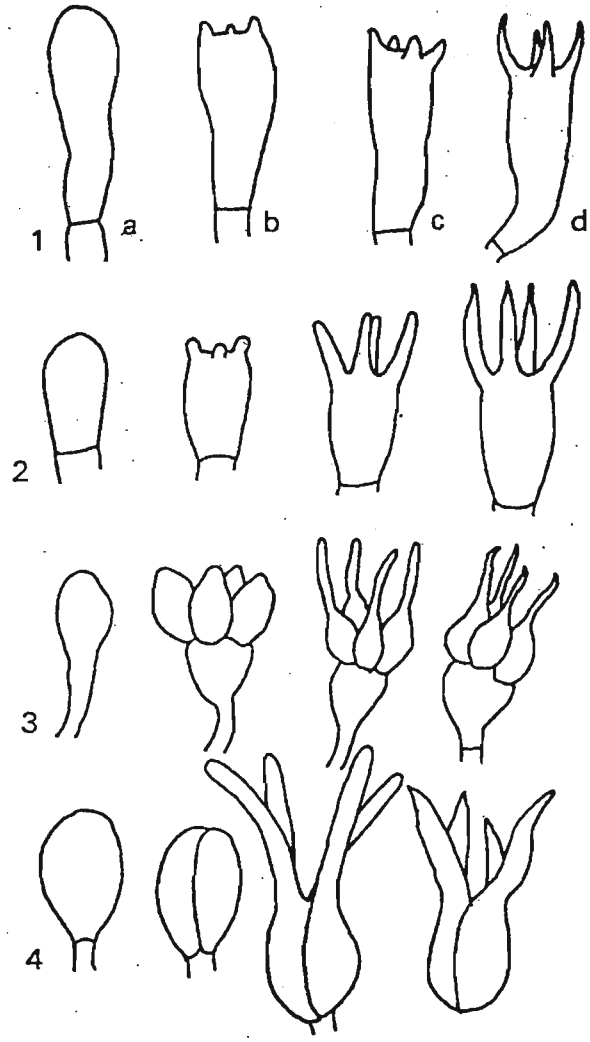


FIGURE 1. Various types of basidia found in teleomorph genera associated with *Rhizoctonia*-like anamorphs. 1: *Athelia*; 2: *Thanatephorus*; 3: *Tulasnella;* 4: *Exidia*. a: protobasidium, b: basidium with protosterigmata; c: basidium with secondary protosterigmata; d: mature basidium, sterigmata with spiculum.

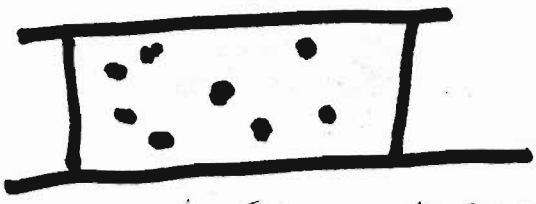
Rhizoctonia

a basidiomycete (produces basidiospores)

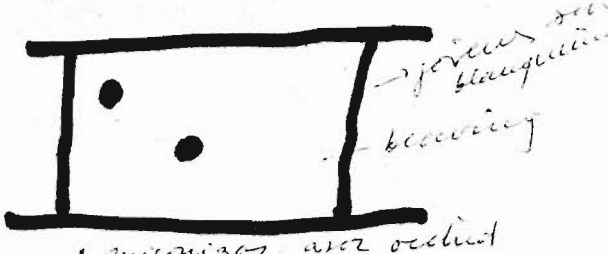
no fruiting body visible to naked eye

belong to Ceratobasidiaceae

multinucleate



binucleate



Predominantly belongs to *Thanetophorus*

Common in *Ceratobasidium*

Mainly pathogenic. Some exceptions (eg. AG12)

Mainly non-pathogenic in orchids

Both can produce:

(a) typical hyphae
(b) sexual (basidio-) spores
(c) sclerotia (resting structure)

Figure 3.7 **Mycelial characteristics of *Rhizoctonia solani* AG-11 on PDA after 21 days of incubation in a growth cabinet without light maintained at 20°C. Note the floccose aerial mycelia and the sclerotia embedded in PDA in 9 cm Petri dishes.**

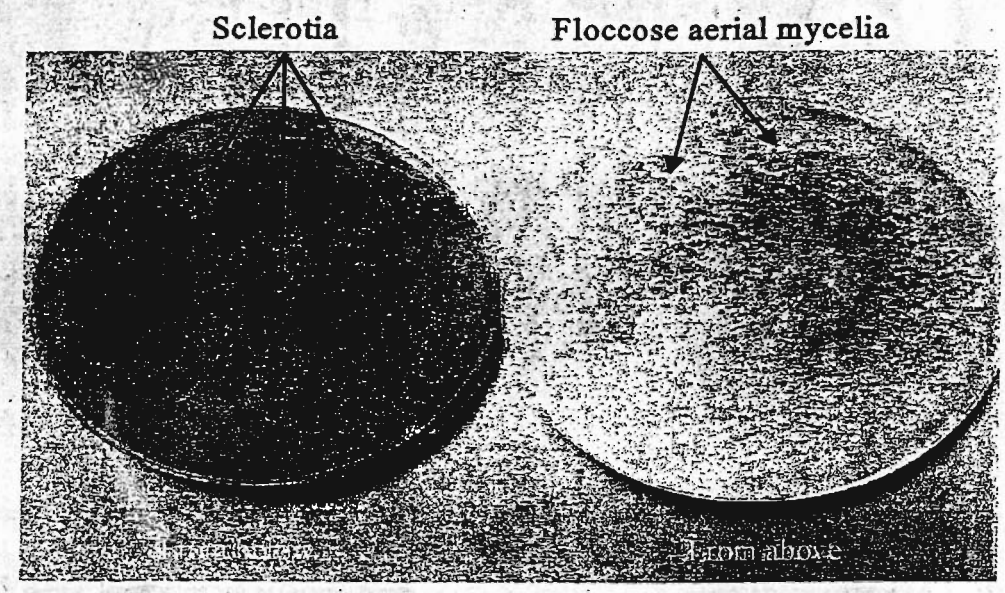
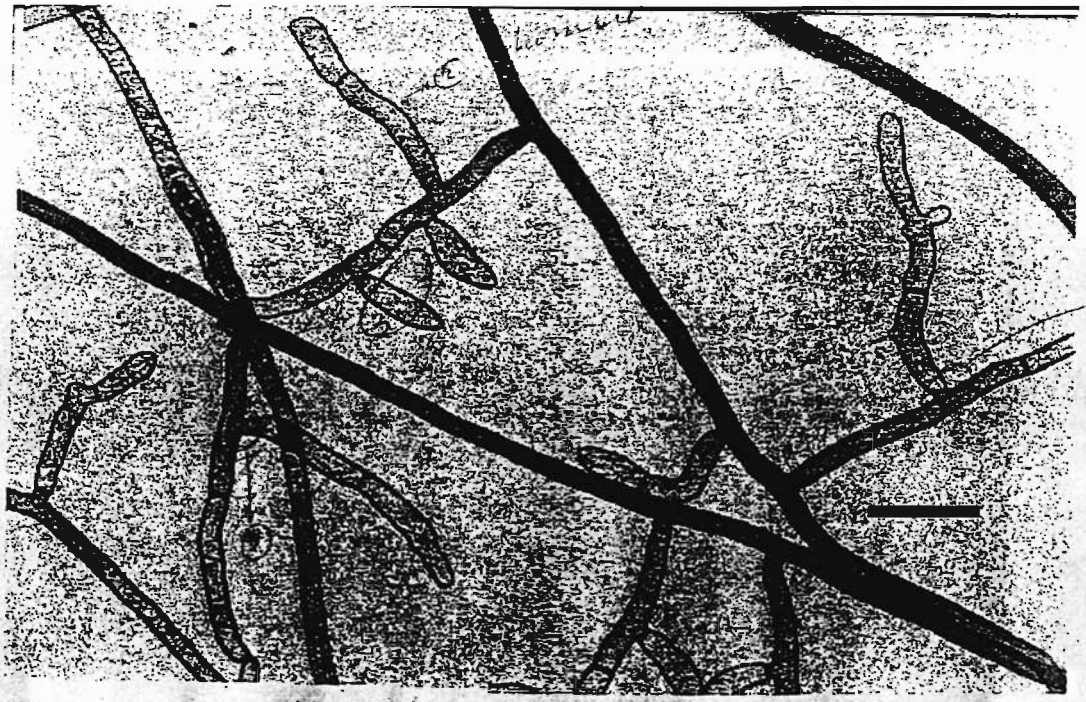


Figure 3.8 **Characteristic hyphae of *Rhizoctonia solani* AG-11 after 36 h at 20°C on 1.5% water agar. The hyphae were stained with Toluidine blue *in situ* in a 9 cm Petri dish. Bar = 37.5 µm.**



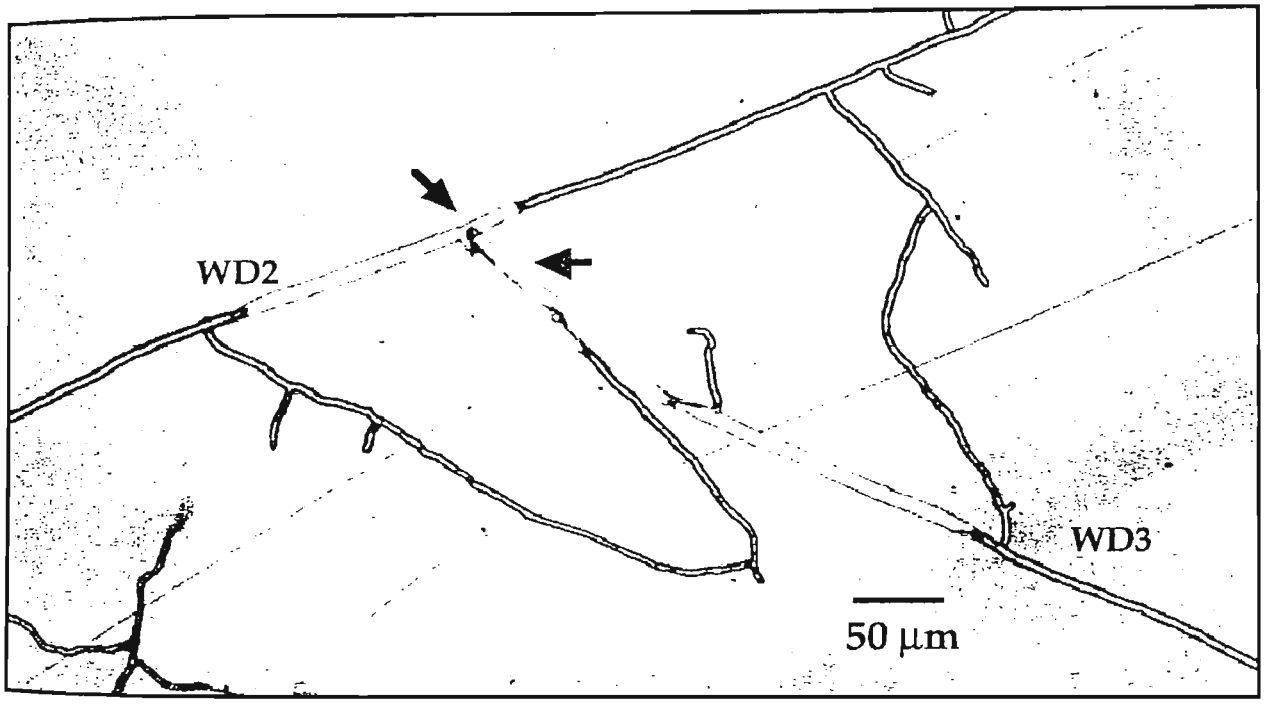
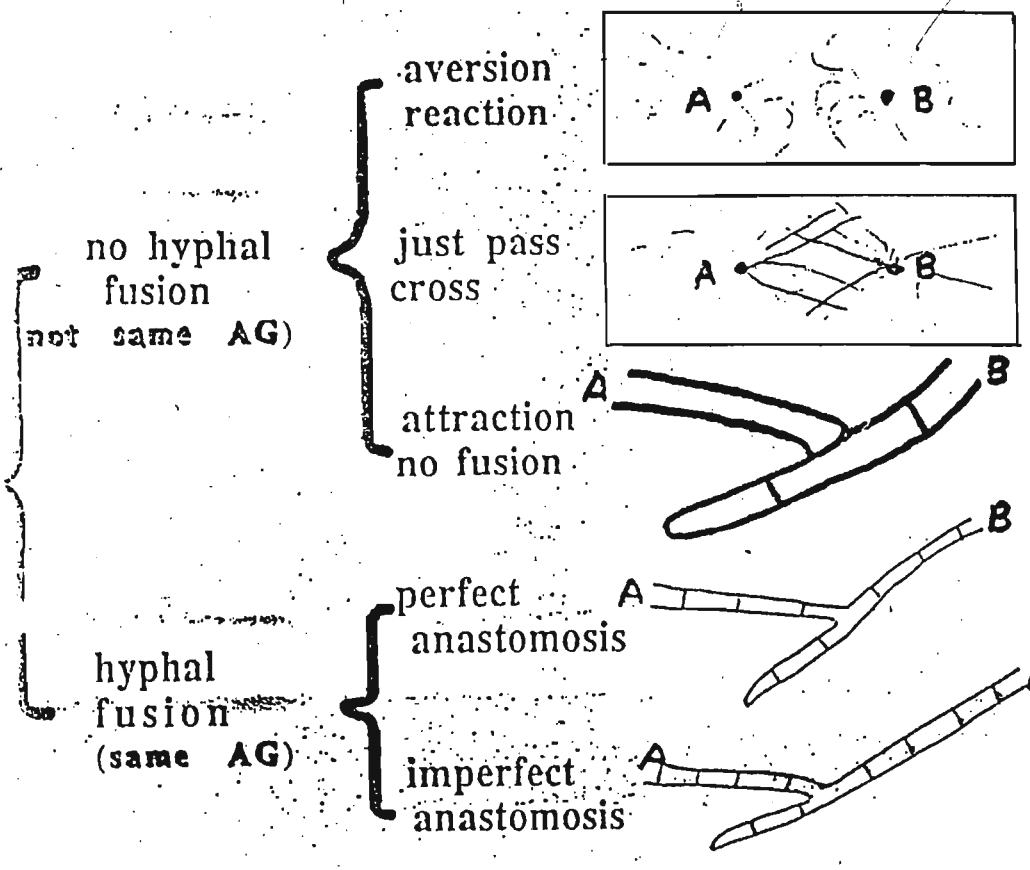


Figure 3.10 C2 anastomosis reaction between hyphae belonging to isolate pairing WD2/WD3 (site of fusion marked by arrow)

Rhizoctonia

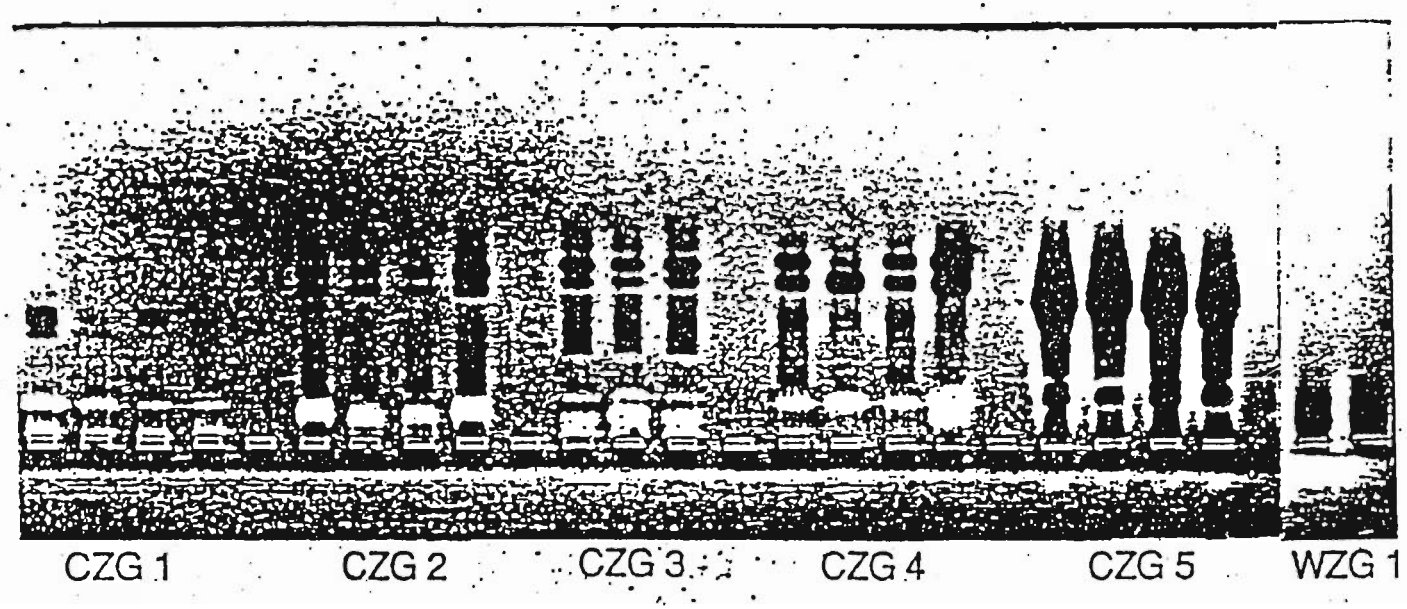
Anastomosis grouping



Rhizoctonia

Pectic enzymes

Zymogram grouping (ZG)



Bare-patch of cereals
ZG1

minal or intercalary in hyphae, often also in conidia, hyaline, smooth-walled or roughened, 5–15 μm diam. Sclerotial pustules present in some isolates, pale to green or deep violet.

F. oxysporum is one of the most variable *Fusarium* species. The nine species and numerous varieties distinguished in sect. *Elegans* by Wollenweber and Reinking (6424) were combined into one by Snyder and Hansen (5459) and this concept has been generally accepted, apart from the fact that *F. redolens* Wollenw. (q.v.) is now mostly treated as distinct (1943). — Chlamydospores, which are essential for survival in the soil are not readily formed

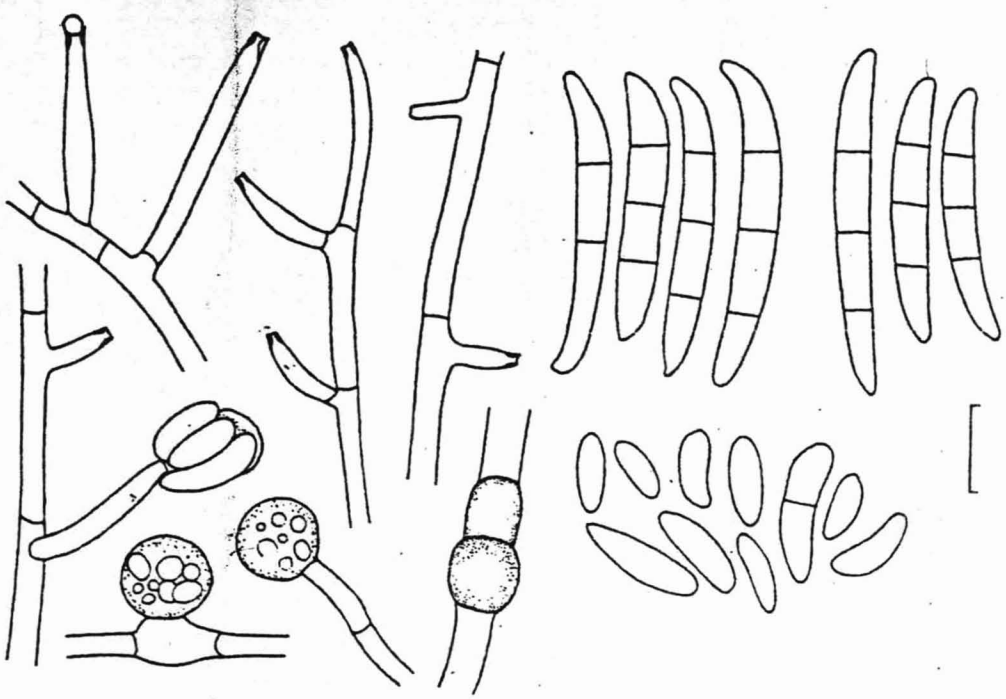


FIG. 134. *Fusarium oxysporum*, short phialides, macro- and micro-conidia, terminal and intercalary chlamydospores. CBS 242.59.

by all isolates, but may be induced by transferring agar squares to distilled water, soil extract or to a two-salt medium (640, 5571); their formation is also favoured by traces of a C source (4682), a low C/N ratio (897), or even the herbicide trifluralin (5738). — The phytopathological literature on more than 100 pathotypes (640, 2046) has not been considered in this compilation. — The teleomorph is still unknown (2044), despite assertion to the contrary (827) and the formation of hyphal anastomoses as a possible mechanism of genetic recombination has been investigated (2495). — In TEM and light-microscopic studies the time course of mitosis and the ultrastructure of the mitotic nucleus have been investigated (65). The majority of cells are uni- or binucleate, but up to five nuclei have been observed (397, 514, 2496); individual nuclei contain 12 chromosomes (641, 4669). Light intensity and concentration of the N source largely influence the colony habit (826). Single conidial isolations can be segregated in an unstable orange type and a stable purple type which shows a number of distinct characteristics (3256). — The fine structure of hyphae (2109) and chlamydospores (2110, 2111, 5571) were investigated by TEM. Numerous large micro-bodies have been observed in hyphae within host tissues but not in agar-grown hyphae (6292). — Serological methods (5774) and amino acid composition analyses (5897)

An improved technique for fluorescence staining of fungal nuclei and septa

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Abstract

Nuclei and septa of fungal mycelium grown on glass slides coated with potato-dextrose agar stained very clearly with fluorescent Hoechst Dye 33258 in buffer of pH 7.8 and 10.5, respectively. The technique is simple, rapid, and gives excellent and clear staining.

Introduction

Methods of staining fungal nuclei and septa using normal light microscopy (Tu and Kimbrough 1973; Herr 1979; Bandoni 1979; Burpee *et al.* 1980) cannot guarantee stable and satisfactory results. However, techniques for fluorescence microscopy (Lemke *et al.* 1975; Mogford 1979; Kangatharalingam and Ferguson 1984; Yamamoto and Uchida 1982) are complicated and time consuming. Most of the current methods of staining involve transferring hyphae from the growth media to glass slides before staining them. This often causes difficulties in obtaining young hyphae (especially for those species which produce few or no aerial hyphae), and spreading the mycelium out on the slide.

During the course of a project to determine the presence of dolipore septa and number of nuclei in cells of *Rhizoctonia* spp., a simplified fluorescence staining method was developed which was also found to be suitable for all other fungal species tested.

Methods

Test cultures A total of 266 fungal isolates was tested. They were 18 isolates of *Thanatephorus cucumeris* (Frank) Donk belonging to ten anastomosis groups (AG 1-1B, 2-1, 2-2 III, 2-21V, 3, 4, 5, 6, 8, and BI), 13 isolates of binucleate *Rhizoctonia* from nine AG groups (AG A, Ba, C, D, E, F, G, I, and K), 228 isolates of *Rhizoctonia* spp. isolated from wheat roots in Western Australia, two isolates of *Phomopsis leptostromiformis* (Kühn) Bubak (Type A and Type B, both isolated from *Lupinus angustifolius* L.), and one isolate each of *Trichoderma harzianum* Rifai (C135, isolated from composted eucalypt bark mix), *Fusarium oxysporum* Sclecht (isolated from a wheat field soil), *Gaeumannomyces graminis* (Sacc.) Arx & Oliver var. *tritici* Walker (isolated from roots of *Triticum aestivum* L.), *Phytophthora cinnamomi* Rands (IMI 165644, A1 mating type, isolated from *Banksia prionotes* L.) and *Pythium irregulare* Buism. (isolated from *Triticum aestivum* cv. Gamenya).

Preparation of the dye solutions A stock solution was prepared by adding 10 mg of Hoechst Dye 33258 (CALBIOCHEM, La Jolla, CA 92037, USA) to 25 mL of distilled water and then heating in a 37°C water bath to dissolve the dye. This stock solution was stored at 3°C.

Buffer solutions of pH 6.8, 7.0, 7.2, 7.4, 7.6, 7.8, and 8.0 were made with 0.1 M KH_2PO_4 and 0.1 M NaOH, and pH 9.5, 10.0 and 10.5 solutions made with 0.025 M H_3BO_3 and 0.1 M NaOH (Robinson 1978). Staining solutions were prepared by adding 0.6 mL of the stock dye solution to 50 mL of each of the buffer solutions.

Preparation of slides Glass slides were immersed in 75% ethanol, drained and flamed. Before cooling, each slide was immersed and coated with Difco potato-dextrose agar (PDA), then placed on glass rods in 90 mm diameter plastic Petri dishes containing sterile filter paper moistened with sterile distilled water. A small block (1 mm^2) of agar (PDA) bearing mycelium of each fungus was transferred to the middle of each slide. The Petri dishes were then incubated in darkness at 25°C for 48 h.

Staining procedures After incubation, the slides were placed in a laminar flow cabinet with hyphae exposed to the air-flow for approximately 10 min. When the agar coating had dried, a drop of each staining solution at the appropriate pH was added to each side of the colony, and cover slips placed promptly over each of the drops. This technique allows two stainings to be made on one slide (Figure 1). Slides were observed under a fluorescence

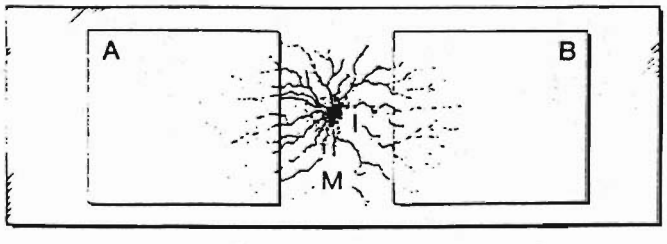

Figure 1 An illustration of the method used for staining mycelium grown on a glass slide coated with PDA. A = staining with pH 7.8 dye solution. B = staining with pH 10.5 dye solution. I = inoculum plug. M = fungal mycelium.

microscope (Leitz, Wetzlar, Germany) with filter No. A (Code No. 513596), and a HBO 200 W high pressure mercury arc lamp as a light source.

Results and Discussion

Rhizoctonia spp. were tested with dye solutions ranging in pH from 6.8 to 10.5. The nuclei stained best in pH 7.8 dye solution and septa were also clear (Figures 2 and 3). However, the septa stained best, with sharp brightness of the prominant septal pore apparatus, in the pH 10.5 dye solution (Figure 4). The stainings were very clear and of good contrast. As both stains were accomplished on one slide, and the technique was simple and reliable, it was possible to examine 15 to 20 samples of *Rhizoctonia* spp. per hour.

The staining of nuclei with pH 7.8 dye solution and septa with pH 10.5 solution proved to be equally successful for all the other fungal species. As these species belong to different genera, the technique is likely to be of general applicability.

In contrast to the method proposed by Kangatharalingam and Ferguson (1984), our technique is applicable to and convenient for fungal species which do not produce profuse aerial hyphae. Because the hyphae are stained *in situ*, it is possible to observe the nuclei and septa of hyphae at different growth stages. In addition, all the hyphae under the cover slip are stained equally well, providing a large area for observation. Another advantage of our technique is that the staining time is reduced and slides can be viewed instantly.

The septa were stained clearly enough for identification of the dolipore in dried preparations. Although the septa on slides not subjected to drying in the laminar flow cabinet were stained more clearly than in dried preparations (Figure 5), the nuclei in non-dried slides stained poorly with all of the staining solutions tested.

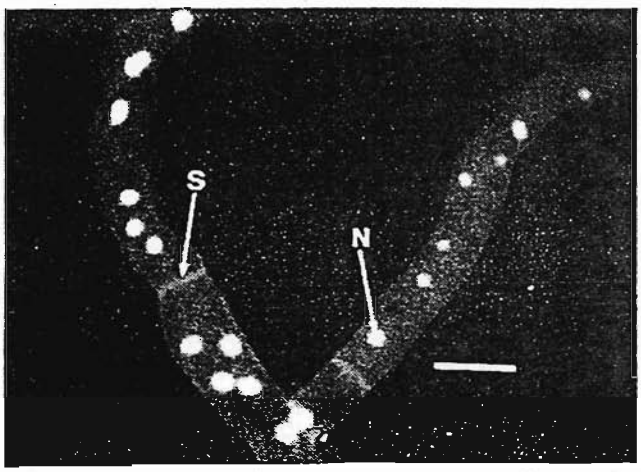

Figure 2 Stained nuclei (N) and septa (S) of *Thanatephorus cucumeris*, AG 1–1B, with pH 7.8 dye solution. Bar = 10 μm.

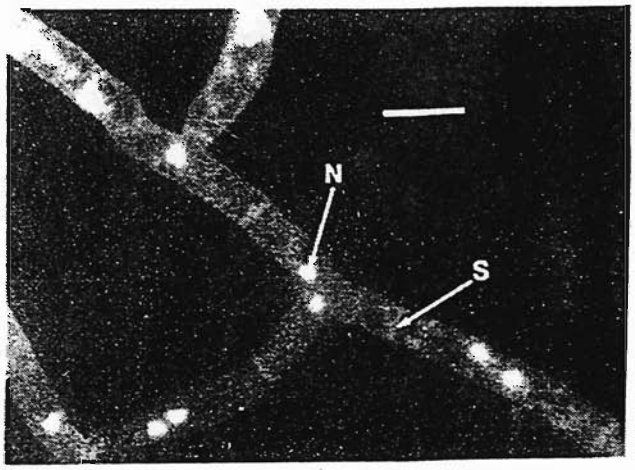

Figure 3 Stained nuclei (N) and septa (S) of *Ceratobasidium cornigerum* with pH 7.8 dye solution. Bar = 10 μm

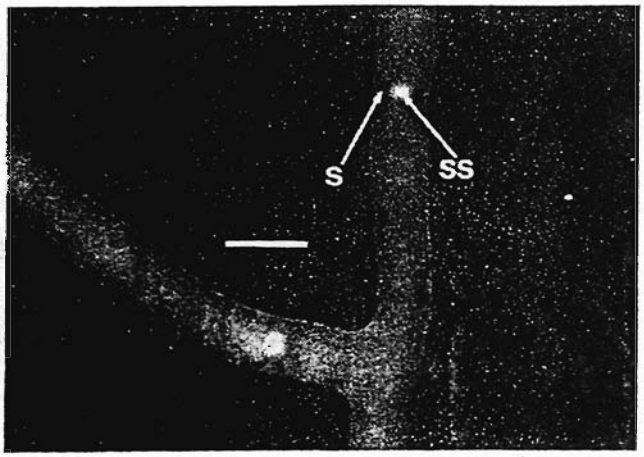

Figure 4 Septum (S) and septal swelling (SS) of *Thanatephorus cucumeris*, AG 8, stained with pH 10.5 dye solution. Bar = 10 μm.

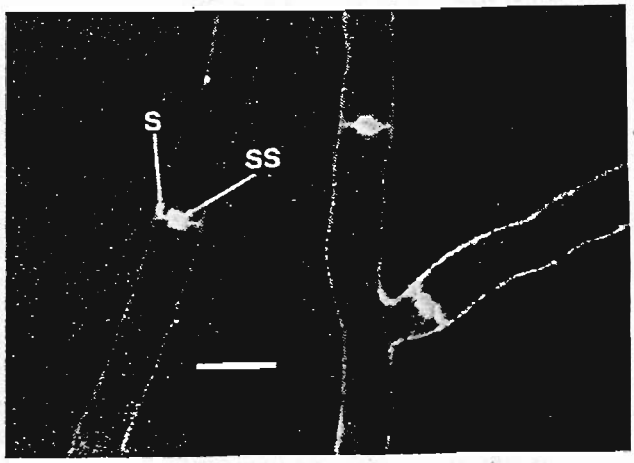

Figure 5 Septum (S) and septal swelling (SS) of *Thanatephorus cucumeris*, AG 8, from a non-dried slide stained with pH 10.5 dye solution. Bar = 10 μm.

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PRINCIPLES OF FLORA CONSERVATION

WILDFLOWER SOCIETY OF WESTERN AUSTRALIA (INC)

SEPTEMBER, 1991

To provide focus and leadership in enjoying, knowing, growing and conserving Australian plants and their habitats.

PREAMBLE

The activities of human beings necessarily interact with the natural world. Since the industrial revolution, human activities have modified our global environment giving us uncertainties of climate change, acid rain, ozone depletion, extensive loss of natural ecosystems, disease and pollution to mention a few.

The loss of species is an advance sign of the accelerated destruction of natural ecosystems. We need to address this decline urgently. We all need to manage our activities and developments with much greater care and sensitivity towards other living things.

A key part is keeping people in contact with natural vegetation. This opportunity still exists in the Wildflower State of W.A. unlike the situation in most of Europe. But much needs to be done to educate the community, politicians and decision makers if we are to halt the loss of bushland and the decline in biodiversity in our own backyard.

The Wildflower Society of W.A. believes that the conservation of our remaining bushland heritage is of paramount importance. The philosophy of conservation of the beautiful and unique wildflowers of the West is encapsulated in these ten principles of flora conservation. They were officially adopted by the Society at its 1991 State Conference.

WILDFLOWER SOCIETY OF WESTERN AUSTRALIA (INC.)
REVEGETATION POLICY

INTRODUCTION

A major aim of the Wildflower Society is the preservation of native flora. The natural Western Australian landscape is a valuable asset worthy of protection and conservation, thus retaining our sense of place.

Land clearing in Western Australia has resulted in extensive loss of native vegetation, habitat and landscape amenity. In the Perth Metropolitan area and the wheatbelt only small patches of remnant vegetation remain. Many species of plants and animals are either extinct or threatened with extinction. Our unique Western Australian landscape has been modified and often bears little resemblance to that which existed before European settlement.

Clearing of native vegetation in rural areas has caused a rise in water table levels resulting in salinisation and waterlogging of soil. This has led to the continual degradation of remnant vegetation and loss of productive agricultural land. This scenario is likely to increase dramatically in the short term.

Revegetation of rural catchments is one of the key strategies for controlling rising water tables and salinity. While commercial species (e.g. blue gums and oil mallees) are being used and are valuable to primary industry, indigenous (local native) vegetation can re-establish the Western Australian landscape amenity and provide habitat for our endangered fauna.

The aim of this policy is to provide a set of standards and guidelines to encourage the preservation and regeneration of indigenous vegetation.

PRINCIPLES OF REVEGETATION

OBJECTIVES

1. To promote the use of local native species in revegetation projects.
2. To encourage as far as possible the reconstruction of the vegetation community indigenous to the locality.
3. To ensure that revegetation provides the basis for a sustainable functioning ecosystem.
4. To encourage the establishment of native vegetation corridors throughout urban and rural landscapes

POLICY STATEMENTS

1.1 Revegetation projects should use local native species, either grown from seed collected from the locality or by direct broadcasting of locally collected seed.

1.2 Local seed orchards need to be established to provide a suitable source of seed from local provenance species for regeneration projects.

1.3 Seed orchards should be designed to both maintain genetic integrity of each species and the intraspecific variation within each species, so that inbreeding depression and hybridisation can be prevented.

2.1 Retention of remnant native bushland and natural regeneration is preferable to revegetation. An expressed intention of rehabilitation should not be taken as an excuse for clearing.

2.2 On sites adjacent to native bushland local provenance species only should be used. Use of the same species from other locations can interfere with the genetic integrity of local species. Use of non local plants may also increase the threat of weed invasion.

2.3 Sites that are modified or degraded in areas remote from remnant vegetation may need different species for revegetation. Examination of flora growing in similar climatic and soil conditions should provide guidance.

3. Projects should aim to re-establish the biological, genetic and structural diversity of local native vegetation to ensure the provision of diverse fauna habitats. This will be more sustainable and will buffer the ecosystem against environmental fluctuations.

4. Where practicable, revegetation programs should be planned to link remnant vegetation in the region to provide flora and fauna corridors.

Policy as passed 1996 state Conference, 29th June 1996.

WILDFLOWER SOCIETY OF WESTERN AUSTRALIA (INC.)
PRINCIPLES OF SEED COLLECTION AND USAGE

PREAMBLE

For many years the Society has sold seeds to both Society members and the general public. Society members have donated time to collecting, selecting, preparing and distributing wildflower seed for sale. In times when wildflower seed was only available through specialist bodies these seed banks allowed Society members and the wider community access to a variety of seeds of our wildflowers in quantities suitable for home gardens at low cost. For many years this was a core activity of the Society and raised a large proportion of the Society's consolidated funds. Today wildflower plants and seeds are much more readily available to the home gardener through nurseries and seed merchants. Also revegetation programmes and bushland restoration have created a wildflower seed industry calling for the collection of tonnes of seed. This seed industry is regulated through the Department of Conservation and Land Management. As the peak community group concerned with flora conservation in the state the Wildflower Society serves on advisory groups related to this industry. The Society's Flora Conservation Principles are the basis for our advice on these groups and our own everyday activities. These factors and the continued loss of bushland areas in the state have resulted in the need to develop a Wildflower Society Seed Policy that complements our Principles of Flora Conservation.

This policy recognises that the Society has a role in supplying seed for home gardens and a commitment to ensure that seed sources do not compromise the preservation of our bushland.

PRINCIPLES OF SEED COLLECTION AND USAGE

1. CULTIVATION

Cultivation in gardens gives people the opportunity to care about, study and enjoy our flora and lessen human impact on our environment. The commercial collection of seed from the bushland is incompatible with flora conservation. All commercial production of seed should therefore necessarily be based on cultivation.

2. SEED ORCHIDS

Seed for cultivation in gardens and for most revegetation projects should be sourced from cultivated plants in seed orchards. Seed orchards should be designed to both maintain the genetic integrity of each species and intraspecific variation within each species. Seed collected from bushland can be used to establish orchards.

3. REVEGETATION

Revegetation projects range from habitat reconstruction for nature conservation to land reclamation and the appropriate source of seed will depend on the nature of the project. Habitat reconstruction that is adjacent to areas of bushland requires seed of local provenance. The more distant the revegetation is from natural populations the less stringent is this requirement and seed should then be sourced from seed orchards.

4. RESTORATION

Bushland restoration aims at maintaining the ecological integrity and evolutionary processes in bushland areas. Seed required for restoration work should be of local provenance, being seed collected from plants growing in the same community and position in the landscape within a reasonable distance of the bushland area being restored.

Policy as passed at the 1995 State Conference, 24th June 1995.

PRINCIPLES OF FLORA CONSERVATION

1. HERITAGE

Wildflowers are our natural heritage held in trust for future generations. We have no right to destroy species.

2. ECOLOGY

Natural bushland is quintessential to this heritage. Human beings cannot reproduce the complexity and diversity of species and their ecology which have evolved over millions of years.

3. RESPONSIBILITY

Human beings are the ultimate custodians of life. We have the ability to destroy life, so must therefore assume the responsibility to preserve life. This responsibility lies with all levels of society; individual, community, government, and corporate.

4. INTRINSIC VALUE

All bushland has intrinsic value. The value of the life of any species cannot be measured in dollars.

5. BOTANY

The systematic botanical surveying of our flora and vegetation are essential. The undescribed flora requires urgent but rigorous scientific attention.

6. PRESERVATION

(i) Flora conservation embraces the preservation of the ecology of vegetation types. Reserves, whether public or private, should be ecologically viable and interconnected to allow the continued movement of species and hence future evolution and adaptation and longterm survival. Nevertheless, all reserves are worthy of preservation.

(ii) The ecological integrity of reserves is inviolate. The notion of exploration and mining in reserves is scientifically and morally unjustifiable.

7. RARE AND ENDANGERED SPECIES

Natural habitats of rare and endangered species must be preserved. Cultivation in gardens does not facilitate continuing biodiversity and species evolution.

8. HUMAN IMPACT

Human actions are changing the world and have impact on the flora. Changes include the Greenhouse Effect, acid rain, large scale land clearance and dieback. The greatest potential for survival in the face of environmental change lies with the most genetically diverse and healthy natural populations.

9. EDUCATION

The community has a right to know about, care about and enjoy our floral heritage. Community involvement and education are conducive to caring and enjoyment. Our children have the same rights. Furthermore, all children have the right of access to bushland within walking distance of home and school.

10. CULTIVATION

Cultivation of wildflowers in gardens enables people to study and enjoy our flora, and lessen impact on our environment. The commercial picking of wildflowers and collection of seed from bushland is incompatible with flora conservation. The commercial production of wildflowers and seed is therefore necessarily based on cultivation.

FURTHER INFORMATION

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OFFICE TELEPHONE: (09) 383 7979 – 24 HOUR TELEPHONE SERVICE

METROPOLITAN BRANCHES	MEETING DETAILS
Perth	Naturalists' Hall, Merriwa St., Nedlands. 8pm 2nd Tuesday of month
Northern Suburbs	Kingsley Hall, Kingsley Drive Kingsley, 8pm 3rd Tuesday of month
Murdoch	Murdoch University, Science & Computing Centre (car park A) 7.45pm 1st Wednesday of month
Eastern Hills	Octagonal Hall, McGlew Road Glen Forrest. 7.45pm 4th Friday of month
Darling Range	Kalamunda High School, Canning Road 7.30pm 4th Tuesday of month
Armadale-Kelmscott	History House, Jull St. Armadale 7.45pm 2nd Thursday of month.

COUNTRY BRANCHES	MEETING DETAILS
Albany	Resources Centre, Serpentine Rd Albany 2nd Friday of Month
Geraldton	The Residency, Marine Terrace Geraldton 3rd Monday of month
Lesueur	Meeting place rotated Pres. Don Williams ph. 096 523 035
Manjimup	Warren Community Centre (Opp. Woolworths) 3rd Monday of month.
Mukinbudin	Pres. Ms W Bayly ph. 090 487 060
York	Pres. J. Tonkinson ph. 096 411 602
Affiliated Groups	Broome, Busselton, Carnarvon, Denmark, Esperance, Mandurah, Mullewa, Nickol Bay
District Contacts	Brookton, Cranbrook, Kalbarri, Kalgoorlie Pingaring, Ravensthorpe, Tambellup (Details from Office)

THE WILDFLOWER SOCIETY IS A MEMBER OF THE
ASSOCIATED SOCIETIES FOR GROWING AUSTRALIAN PLANTS (ASGAP),
AND AS SUCH IT SUPPORTS AND PROMOTES THE STUDY OF ALL AUSTRALIAN PLANTS.
NATURALLY IT HAS A PARTICULAR INTEREST IN THE FLORA OF WA.

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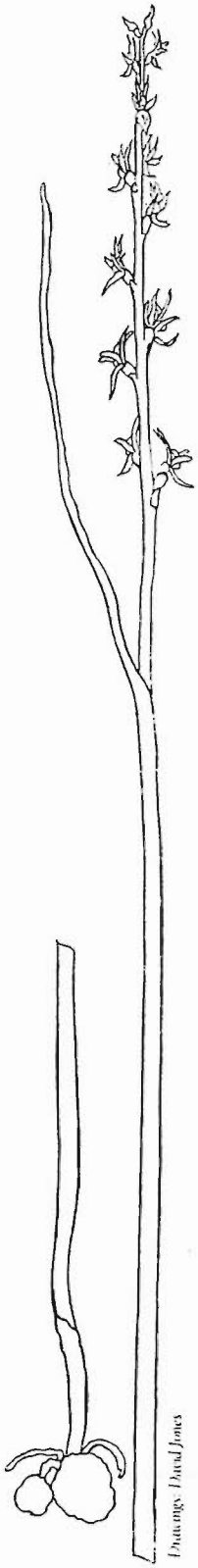
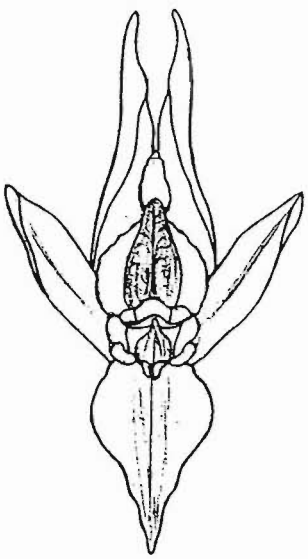
Tarengo Leek Orchid

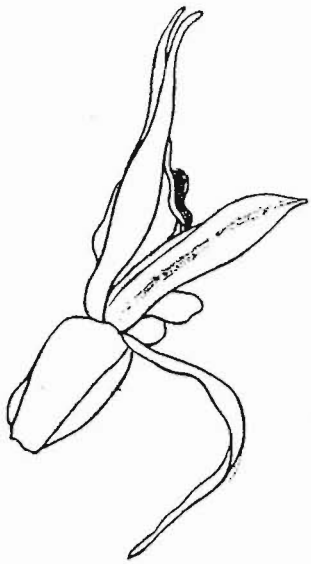
This is the endangered Tarengo Leek Orchid (*Prasophyllum petilum*). It is known from only three small areas in the Boorowa and Canberra regions. The NSW National Parks and Wildlife Service is keen to find other sites where this orchid occurs. If you think you may have seen this plant, please contact the NPWS at the address given on the other side of this leaflet.

Natural Heritage Trust



Drawings: David Jones





About the Tarengo Leek Orchid

This small ground orchid is only noticeable in the springtime (October – December) when it is in flower. At this time, a flowering stalk reaching up to 30cm high breaks through its single, cylindrical leaf. Along the flowering stalk are five to twenty small, cream or pinkish-green, sweetly scented flowers. After flowering and fruiting the plant dies back to its underground tuber.

Where to look

The only known areas where the Tarengo Leek Orchid has been found are native grasslands or grassy woodlands on relatively fertile soils. The main native grasses are Kangaroo Grass (*Themeda australis*), Snow Grass (*Poa* species), Redleg Grass (*Bothriochloa macra*) and Wallaby Grass (*Austrodanthonia* species). Native grasslands like this usually only persist in areas that are rarely, or only occasionally grazed by stock. The sites may be seasonally moist or boggy.

Why is it important?

The Tarengo Leek Orchid occurs in areas that are good for farming and grazing. It was probably much more common before European settlement, but is now so rare that it could easily go extinct if we don't take care of it.

Remember

If you find this plant on your property, it is because your land is managed in a way that is conducive to the orchid's survival. It is unlikely that you would be required to significantly change how you are managing your land because of the orchid and you may be able to contribute to its continued survival and our understanding of its habitat requirements.

If you think you've found one

If you find a flower that you think may be a Tarengo Leek Orchid, compare it carefully with the photograph and drawing on this leaflet. Does it have a single, onion-like leaf with a flower spike breaking through part way up, and creamy or pinkish-green scented flowers.

If so, or if you would like more information, please contact:

Stephen Clark, Threatened Species Unit, NPWS Southern Directorate
PO Box 2115 Queanbeyan NSW 2620. Ph: (02) 6298 9700 email: stephen.clark@npws.nsw.gov.au

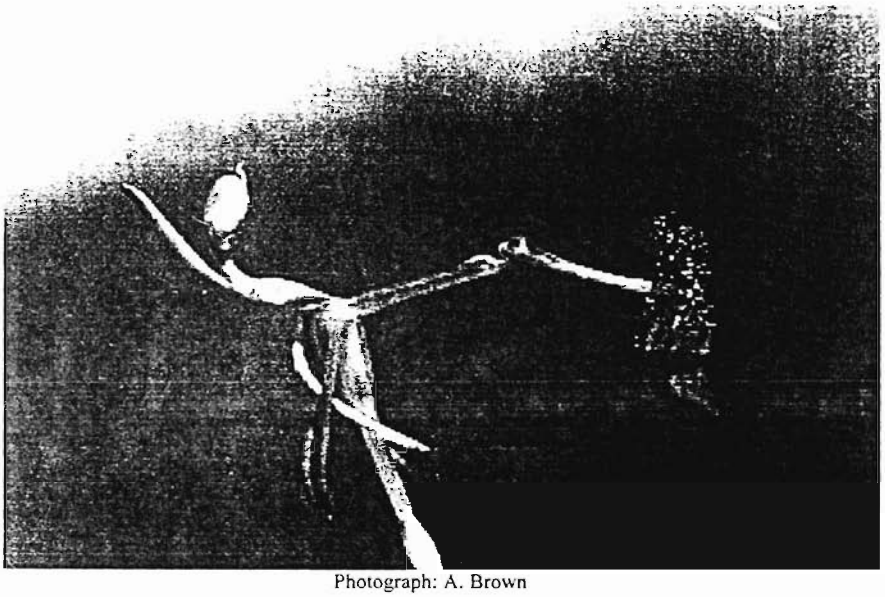
LATE HAMMER ORCHID

(*DRAKAEA CONFLUENS* MS)

INTERIM RECOVERY PLAN

2001-2003

Robyn Phillimore and Andrew Brown



Photograph: A. Brown

January 2001

Department of Conservation and Land Management
Western Australian Threatened Species and Communities Unit
PO Box 51, Wanneroo, WA 6946

Natural Heritage Trust

Department of Conservation and Land Management

Conserving the nature of WA

THE GOVERNMENT OF WESTERN AUSTRALIA

FOREWORD

Interim Recovery Plans (IRPs) are developed within the framework laid down in Department of Conservation and Land Management (CALM) Policy Statements Nos. 44 and 50.

IRPs outline the recovery actions that are required to urgently address those threatening processes most affecting the ongoing survival of threatened taxa or ecological communities, and begin the recovery process.

CALM is committed to ensuring that Critically Endangered taxa are conserved through the preparation and implementation of Recovery Plans or Interim Recovery Plans and by ensuring that conservation action commences as soon as possible and always within one year of endorsement of that rank by the Minister.

This Interim Recovery Plan will operate from January 2001 to December 2003 but will remain in force until withdrawn or replaced. It is intended that, unless the taxon is no longer threatened with extinction, this IRP will be replaced by a full Recovery Plan after three years.

This IRP was approved by the Director of Nature Conservation on 12 April, 2001. The provision of funds identified in this Interim Recovery Plan is dependent on budgetary and other constraints affecting CALM, as well as the need to address other priorities.

Information in this IRP was accurate at January 2001.

SUMMARY

Scientific Name: *Drakaea confluens* ms
Common Name: Late Hammer Orchid
Family: Orchidaceae
Flowering Period: September to November
CALM Regions: South Coast, Central Forest
CALM Districts: Albany, Mornington
Shires: West Arthur, Gnowangerup

Recovery Teams

Albany District Threatened Flora Recovery Team (ADTFRT); Central Forest Region Threatened Flora Recovery Team (CFRTFRT)

Illustrations and/or further information: Brown, A., Thomson-Dans, C. and Marchant, N. (Eds) (1998) *Western Australia's Threatened Flora*. Department of Conservation and Land Management, Western Australia; Hoffman, N. and Brown, A. (1998) *Orchids of South-west Australia*. Revised 2nd edition with supplement. University of Western Australia Press, Nedlands; Stoutamire, W.P. (1975) Australian Terrestrial Orchids, Thynnid Wasps and Pseudocopulation. *American Orchid Society Bulletin*, 43:13-18.

Current status: *Drakaea confluens* ms was declared as Rare Flora in October 1996 and was ranked Critically Endangered (CR) in December 1997. It currently meets World Conservation Union (IUCN, 1994) Red List Category 'CR' under criteria B1+2c due to the severe fragmentation of populations and a decline in the area, extent and quality of habitat. The main threats are inappropriate fire regimes, grazing, firebreak maintenance, recreational activities, weed invasion and disease.

Habitat requirements: *Drakaea confluens* ms is endemic to Western Australia where it is found in two widely separated areas, northeast of Boyup Brook and in the Stirling Range National Park. It grows in deep sandy soil in mixed jarrah (*Eucalyptus marginata*) and Banksia (*Banksia attenuata*) woodland (Hoffman and Brown, 1998). It is currently known from eight extant populations with a total of approximately 180 plants and from two populations in which there are no extant plants.

Critical habitat: The critical habitat of *Drakaea confluens* ms comprises the area of known populations, adjacent areas of similar habitat within 200 metres of populations, corridors of remnant vegetation that link populations, and other nearby occurrences of suitable habitat that are not currently known to contain populations of the species but which may be suitable for translocations.

Existing Recovery Actions: The following recovery actions have been or are currently being implemented:

1. Appropriate land managers have been informed of the species' location and their legal obligations.
2. Declared Rare Flora (DRF) markers have been installed at Population 9.
3. Dashboard stickers and posters that illustrate DRF markers and describe their purpose have been produced and distributed.
4. CALM and members of the WA Native Orchid Study and Conservation Group (WANOSCG) have undertaken many surveys for the species.
5. Populations 4 and 5 were fenced to exclude stock.
6. Control of kangaroos to reduce grazing at Populations 4 and 5 has been ongoing by the landowner.
7. Action is underway to have the tenure and vesting of land containing Population 7 changed to that of conservation.
8. An experimental burn was undertaken on private property (Population 4) in April 2000.
9. The Albany District and Central Forest Region Threatened Flora Recovery Teams are overseeing the implementation of this IRP.
10. CALM staff from the Albany District and Central Forest Region Office's regularly monitor populations.

IRP Objective: The objective of this Interim Recovery Plan is to abate identified threats and maintain or enhance *in situ* populations to ensure the long-term preservation of the taxon in the wild.

Recovery criteria

Criteria for success: The number of individuals within populations and/or the number of populations have increased.

Criteria for failure: The number of individuals within populations and/or the number of populations have decreased.

Recovery actions

1. Coordinate recovery actions.
2. Develop and implement a fire management strategy.
3. Collect seed and tissue culture material.
4. Monitor and control vertebrate grazing.
5. Conduct further surveys.
6. Undertake weed control.
7. Apply phosphite as required.
8. Monitor the impact of phosphite application.
9. Notify and liaise with relevant land owners.
10. Monitor populations.
11. Protect populations on private land.
12. Obtain biological and ecological information.
13. Promote awareness.
14. Incorporate recovery actions into the Interim Management Guidelines (IMG's) for a new conservation park
15. Write a full Recovery Plan.

1. BACKGROUND

History

Drakaea confluens ms was first recognised as being distinct by E. Chapman[1], who discovered the species growing in bushland on his farm near Boyup Brook. Further collections of the species, housed at the Western Australian Herbarium, were made by in 1983 by CALM staff from an area near Mondurup Peak in the Stirling Range National Park. Although the Boyup Brook populations flower every year, only one of the Stirling Range populations has been seen since despite numerous subsequent surveys.

Following surveys in the Boyup Brook and Stirling Range National Park areas by CALM staff and orchid enthusiasts, one new population was found in Haddleton Nature Reserve by Mr Chapman in 1990 and another near Lake Ngartiminny by staff from CALM's Collie District in 1992. A survey by members from the Western Australian Native Orchid Study and Conservation Group (WANOSCG) in 1999 also resulted in the discovery of a new population in Camel Lake Nature Reserve north of the Stirling Range. In October 2000 another new population was discovered by orchid enthusiasts in the Stirling Range National Park. Despite these surveys, most *D. confluens* ms populations consist of very few mature plants with only around 180 plants known in total.

In 1999, fire burnt the area containing Population 8 of *Drakaea confluens* ms and the area adjacent to Population 9, within Stirling Range National Park and Camel Lake Nature Reserve. Regeneration of the site will be monitored. Population 1 was also burnt in 1983 and Population 2 in 1996, both in Stirling Range National Park.

Description

Drakaea confluens ms grows 15 to 30 cm high. It has a single greyish-green, heart-shaped leaf, held flat to the ground. The leaf is one to two centimetres across and may either be smooth or covered with short hairs. The flower stem, up to 30 cm long, supports a single flower which is two to four centimetres long and three to five millimetres wide (Brown *et al.*, 1998; Hoffman and Brown, 1998).

Drakaea confluens ms grows with other hammer orchids such as *D. livida* and *D. glyptodon* but begins flowering when they are finishing. *D. confluens* ms is distinguished easily from related species of hammer orchid by its leaf which if often covered in short dense hairs, and flower which has a two coloured labellum with a straight, rather than upturned, apex (Hoffman and Brown, 1998).

Distribution and habitat

Drakaea confluens ms is endemic to Western Australia where it is found in two widely separated areas, northeast of Boyup Brook and in the Stirling Range National Park. It grows in deep sandy soil in mixed jarrah (*Eucalyptus marginata*) and Banksia (*Banksia attenuata*) woodland (Hoffman and Brown, 1998). The vegetation type at Population 9 differs slightly from that of other populations, consisting mostly of *Melaleuca* and mallee *Eucalyptus* over heath. The species is known from eleven populations and a total of approximately 180 mature plants.

Associated species include *Melaleuca preissiana*, *Leptospermum erubescens*, *Phlebocarya ciliata*, *Hibbertia subvaginata*, *Acacia extensa*, *Drakaea livida*, *D. glyptodon*, *Kunzea ericifolia*, *Jacksonia furcellata*, *Xanthorrhoea preissii*, *Melaleuca scabra*, *Calytrix flavescens*, *Petrophile linearis*, *Adenanthos obovatus*, *Kunzea recurva*, *Xanthorrhoea platyphylla*, *Melaleuca striata*, *Lambertia inermis*, *Conospermum floribundum*, *Regelia inops*, *Paracaleana nigrita*, *Persoonia longifolia*, *Allocasuarina fraseriana* and *Gompholobium scabrum*.

Critical habitat

Critical habitat is habitat identified as being critical to the survival of a listed threatened species or community. Habitat means the biophysical medium or media: (a) occupied (continuously, periodically or occasionally) by an organism or group of organisms; or (b) once occupied (continuously, periodically or occasionally) by an organism, or group of organisms, and into which organisms of that kind have the

[1] Eric Chapman, Orchid enthusiast Boyup Brook

potential to be reintroduced. (*Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act)).

The critical habitat for *Drakaea confluens* ms comprises:

- The habitat of known populations.
- Similar habitat within 200 metres of known populations (these provide potential habitat for natural recruitment).
- Corridors of remnant vegetation that link populations with other nearby areas of apparently suitable habitat that do not currently contain the species.
- Areas of similar habitat that may be used for future translocation.

Explanatory Note: Adjacent uncleared vegetation linked to the known habitat of the species and additional occurrences of the habitat are potential areas for the species and provide opportunities for reintroduction, re-invasion and translocation. They may also provide habitat for the orchid's pollinator.

Biology and ecology

Drakaea is a small genus of nine species, all of which are endemic to the south-west of Western Australia (Hoffman and Brown, 1998). They are commonly known as hammer orchids because of unusual hammer-like shape of the labellum found in all species. The labellum approximates a female Thynnid wasp in scent and appearance and attracts male wasps. The male wasp attempts to fly away holding the labellum but because it is hinged the wasp comes into contact with the column picking up or depositing pollen (Stoutamire, 1974).

Preliminary observations of pollinator activity by staff of the Botanic Garden and Parks Authority (BGPA) suggest hand pollination is required to secure further seed and to improve natural recruitment (personal observation K. Dixon[2]).

The flowering time of *Drakaea confluens* ms differs between Stirling Range populations, which start flowering in September, and Boyup Brook populations, which begin flowering in October.

It is likely that the orchid would be killed by fire if burnt during its active growing period (late April to late November) but that fires during its dormancy period (December to early April) would cause no damage to plants. An experimental burn was undertaken in April 2000 at Population 4 to assess fire response and to develop operational scale fire regimes. The area will continue to be closely monitored.

Like other *Drakaea* species, *D. confluens* ms colonises disturbed areas, such as old firebreaks. Once the canopy cover becomes enclosed, the orchid gradually disappears. However, continued disturbance, such as annual grading of firebreaks, is known to kill plants of *Drakaea* species.

Threats

Drakaea confluens ms was declared as Rare Flora in October 1996 and was ranked Critically Endangered (CR) in December 1997. It currently meets World Conservation Union (IUCN, 1994) Red List Category 'CR' under criteria B1+2c due to the severe fragmentation of populations and a decline in the area, extent and quality of habitat. The main threats are inappropriate fire regimes, grazing, firebreak maintenance, recreational activities, weed invasion and disease.

- **Inappropriate fire** during late Autumn, Winter and Spring can adversely affect the viability of populations by killing flowering plants and preventing seed set. Fire during the summer when plants are dormant has no detrimental effect. Most orchid species emerge from the soil by mid-April and dehisce their seed by late November. The optimum time for fire is therefore from December to mid April. Proliferation of weeds is often a consequence of burning. These produce an annual fuel load that is usually more combustible than the original native vegetation.

[2] Kingsley Dixon, Botanic Garden and Parks Authority

- **Grazing** of *Drakaea confluens* ms by kangaroos (*Macropus fuliginosus*) has been recorded at Populations 4, 5 and 7. Grazing and trampling may damage *D. confluens* plants thereby limiting natural recruitment.

- **Firebreak maintenance** may threaten Population 9 (see Biology and ecology). The land manager is aware of the location of the population and Declared Rare Flora markers are installed to prevent possible damage.

- **Weed invasion** is a threat to Population 5. Weeds suppress early plant growth by competing for soil moisture, nutrients and light. They also exacerbate grazing pressure and increase the fire hazard due to the easy ignition of high fuel loads, which are produced annually by many grass weed species (Lynch 1987; Saunders *et al.* 1987; Taylor 1987). In addition to the proximity of a weed seed source, effects include increased wind speed, modified hydrology and altered disturbance regimes, including fire.

- **Disease** may indirectly threaten all populations. Although it is unlikely that *Drakaea confluens* ms is susceptible to dieback, the habitat is highly susceptible and therefore impacts directly on the species. Deaths due to Dieback results in the removal of the upper canopy cover and change the vegetation structure of the lower canopy. Species that are resistant to dieback (eg, sedges) take over, resulting in a thickening of the canopy floor. Hammer orchids usually grow in open sandy patches between shrubs and cannot survive under a thick canopy. Sites within the Stirling Range National Park have been assessed for the presence of dieback. Populations 1 and 2 contain linear infections of dieback near an adjacent road, and Population 8 is dieback free.

Summary of population information and threats

Pop. No. & Location	Land Status	Year/No. plants	Condition	Threats
1. E of Mondurup Peak	National Park	1983 2 1999 0	Healthy	Disease, inappropriate fire
2. E of Mondurup Peak	National Park	1983 1 1999 0	Healthy	Disease, inappropriate fire
4. SSW of Darkan	Private Property	1983 100+ 1999 81 (15)	Healthy	Grazing, inappropriate fire
5. SSW of Darkan	Private Property	1998 34 1999 41	Healthy	Grazing, weeds, disease, inappropriate fire
6. SSW of Cordering	Nature Reserve	1998 11 1999 4	Healthy	Inappropriate fire, disease
7a. NW of Lake Ngartiminny	Unallocated Crown Land (Proposed Conservation Park)	1999 53	Healthy	Powerline maintenance, disease, inappropriate fire
7b. NW of Lake Ngartiminny	Unallocated Crown Land (Proposed Conservation Park)	1998 1 1999 0	Healthy	Grazing, inappropriate fire, disease
7c. NW of Lake Ngartiminny	Unallocated Crown Land (Proposed Conservation Park)	1999 2	Healthy	Grazing, inappropriate fire, disease
8. E of Mondurup Peak	National Park	1983 2 2000 8	Healthy	Disease, inappropriate fire
9. Camel Lake	Nature Reserve	2000 1	Healthy	Firebreak maintenance activities, inappropriate fire, disease
10. N Stirling Range Dve	National Park	2000 6	Healthy	Disease, inappropriate fire
11. Porongurups	Private Property	2000 1	Healthy	Inappropriate fire

Note: Numbers in brackets refers to vegetative plants. Population 3 has been confirmed as *Drakaea isolata* ms.

Guide for decision-makers

Section 1 provides details of current and possible future threats. Development in the immediate vicinity of the population or within the defined critical habitat of *Drakaea confluens* ms will require assessment. Developments should not be approved unless the proponents can demonstrate that they will not have a negative impact on the species, and its habitat or potential habitat or have the potential to spread or amplify dieback disease caused by the plant pathogen *Phytophthora cinnamomi*.

2. RECOVERY OBJECTIVES AND CRITERIA

Objectives

The objective of this Interim Recovery Plan is to abate identified threats and maintain or enhance *in situ* populations to ensure the long-term preservation of the species in the wild.

Criteria for success: The number of individuals within populations and/or the number of populations have increased.
Criteria for failure: The number of individuals within populations and/or the number of populations have decreased.

3. RECOVERY ACTIONS

Existing recovery actions

Appropriate land managers have been informed of the species' location and their legal obligations.

Declared Rare Flora (DRF) markers have been installed at Population 9. Awareness of the significance of Declared Rare Flora (DRF) markers is being promoted to Shires and landowners through the distribution of dashboard stickers and posters. These illustrate DRF markers, describe their purpose and provide a contact telephone number if a marker is encountered during works.

CALM staff and members of WANOSCG have undertaken surveys for the species with several new populations being discovered.

Populations 4 and 5 have been fenced to exclude stock.

Control of kangaroos to reduce grazing at Populations 4 and 5 has been implemented by the landowner.

Population 7 is currently on unallocated Crown land but has been identified under the Regional Forest Agreement as a proposed conservation park. Action is currently being undertaken to have the tenure and vesting changed.

An experimental burn was undertaken at Population 4 in April 2000 by staff from CALM's Central Forest Region and the private property owner. A 5 m by 2 m transect was burnt in an area that had contained seven *Drakaea confluens* ms plants in the past (none seen recently). A nearby same-sized area where four *D. confluens* ms plants had been seen was left unburnt as a control. The burn aimed to assess the use of fire as a population management tool. The site is being monitored to see if plants of *D. confluens* appear and to assess its effect on associated plant species.

The Albany District and Central Forest Region Threatened Flora Recovery Teams (ADTFRT, CFRTFRT) are overseeing the implementation of this IRP and will include it in their annual report to CALM's Corporate Executive and funding bodies.

Staff from the Albany District and Central Forest Region CALM Offices regularly monitor all populations.

Future recovery actions

Where populations occur on lands other than those managed by CALM, permission has been or will be sought from the appropriate land managers prior to recovery actions being undertaken.

1. Coordinate recovery actions

The ADTFRT and CFRTFRT are overseeing the implementation of recovery actions for *Drakaea confluens* ms and will include information on progress in their annual report to CALM's Corporate Executive and funding bodies.

Action: Coordinate recovery actions
Responsibility: CALM (Albany District, Central Forest Region) through the ADTFRT and CFRTFRT
Cost: $500 per year

2. Develop and implement a fire management strategy

A fire management strategy that defines fire control measures, and fire frequency and timing will be developed in consultation with relevant authorities and land managers.

Action: Develop and implement a fire management strategy
Responsibility: CALM (Albany District, Central Forest Region) through the ADTFRT and CFRTFRT
Cost: $2,400 in first year and $1,000 in subsequent years.

3. Collect seed and tissue culture material

Neither seed nor tissue culture material has been collected for *Drakaea confluens* ms. Due to the low number of plants and threats from habitat degradation, the recovery of the species in the long-term may require *ex situ* conservation techniques. From field observations it appears that little natural pollination occurs and it is likely that hand pollination of the orchid is required to increase seed set. If it is not possible to collect adequate quantities of viable seed, other more costly *ex situ* conservation methodologies may

need to be investigated. These can involve living collections or storage of tissue culture material. If resources are limited these techniques will need to be carefully prioritised in relation to *in situ* conservation.

Action: Collect seed and tissue culture material
Responsibility: CALM (Albany District, Central Forest Region, BGPA) through the ADTFRT and CFRTFRT
Cost: $3,300 per year

4. Monitor and control vertebrate grazing

Monitoring the impact of kangaroos on Populations 4, 5 and 7 should continue and, if necessary, protection or control measures implemented.

Action: Monitor populations
Responsibility: CALM (Central Forest Region) through the CFRTFRT
Cost: $600 per year.

5. Conduct further surveys

Further surveys will be conducted during the species' flowering period (October to November). Local volunteers such as members of Naturalists clubs, WANOSCG and wildflower societies will be encouraged to help in surveys supervised by CALM staff.

Action: Conduct further surveys
Responsibility: CALM (Albany District, Central Forest Region) through the ADTFRT and CFRTFRT
Cost: $5,000 per year

6. Undertake weed control

Weeds are a threat to Population 5. The following actions will be implemented:

1. Appropriate herbicides will be selected after determining which weeds are present.
2. Invasive weeds will be controlled by hand removal or spot spraying around *Drakaea confluens* ms plants when weeds first emerge.
3. Weed control will be scheduled to coincide with spraying of other threatened flora populations within the district.

The tolerance of associated native plant species to herbicides at the site of *Drakaea confluens* ms is not known and weed control programs will be undertaken in conjunction with research.

Action: Undertake weed control
Responsibility: CALM (Central Forest Region, CALMScience) through the CFRTFRT
Cost: $700 per year

7. Apply phosphite as required

Populations 1 and 2, which contain linear infections of *Phytophthora cinnamomi*, will be sprayed with Phosphite. The extent and impact of dieback on other populations of *Drakaea confluens* ms will also be assessed and the requirements for dieback treatment evaluated.

Action: Apply phosphite as required
Responsibility: CALM (Albany District, Dieback Disease Coordinator) through the ADTFRT
Cost: $2,900 in first and third years.

8. Monitor the impact of phosphite application

Following the application of phosphite, monitoring its impact on *Drakaea confluens* ms and its effect on *Phytophthora cinnamomi* is required.

Action: Monitor the impact of phosphite application

Responsibility: CALM (Albany District, Dieback Disease Coordinator) through the ADTFRT
Cost: $1,000 per year

9. Notify and liaise with relevant land owners

The owners of private property containing Population 11 will need to be officially notified of its presence. Staff from CALM's Central Forest Region and Albany District will continue liaison with owners of other properties that contain populations of *Drakaea confluens* ms to ensure that they are not damaged or destroyed accidentally. Due to the potential susceptibility of the habitat of this species to dieback, the need for dieback hygiene procedures will be included in information provided to landowners.

Action: Liaise with relevant landowners
Responsibility: CALM (Albany District, Central Forest Region) through the ADTFRT and CFRTFRT
Cost: $1,400 per year

10. Monitor populations

Annual monitoring of factors such as habitat degradation (including the impact of dieback), population stability (expansion or decline), weed invasion, pollination activity, seed production, recruitment, and longevity is essential.

Action: Monitor populations
Responsibility: CALM (Albany District, Central Forest Region) through the ADTFRT and CFRTFRT
Cost: $3,100 per year

11. Protect populations on private land

Ways of achieving protection of the land on which Populations 4 and 5 occur will be investigated. Possible methods of achieving future conservation management include covenanting and land purchase.

Action: Protect populations on private land
Responsibility: CALM (Central Forest Region) through the CFRTFRT
Cost: To be determined

12. Obtain biological and ecological information

Increased knowledge of the biology and ecology of the species will provide a scientific basis for management of *Drakaea confluens* ms in the wild. Investigations will include:

1. A study of the soil seed bank dynamics and the role of various factors including disturbance (eg fire), competition, and rainfall, grazing in recruitment and seedling survival.
2. Determination of reproductive strategies, phenology and seasonal growth.
3. Investigation of the mating system and pollination biology.
4. Investigation of population genetic structure, levels of genetic diversity and minimum viable population size.
5. Investigation of the impacts of dieback disease and control techniques on *Drakaea confluens* ms and its habitat.

Action: Obtain biological and ecological information
Responsibility: CALM (CALMScience, Albany District, Central Forest Region) through the ADTFRT and CFRTFRT
Cost: $18,200 per year

13. Promote awareness

The importance of biodiversity conservation and the need for the long-term protection of *Drakaea confluens* ms in the wild will be promoted to the public through the local print and electronic media and through poster displays. An information sheet that includes a description of the plant, its habitat type, threats and management actions will be produced. Formal links with local naturalist groups and interested individuals will also be encouraged.

A poster for *Drakaea confluens* ms will be produced and distributed. It will include photographs of the species and its habitat, a description of the plant, and a description of its habitat, threats and management actions. The exact location of the species will remain confidential.

Action: Promote awareness
Responsibility: CALM (Albany District, Central Forest Region, Corporate Relations) through the ADTFRT and CFRTFRT
Cost: $1,100 in first year and $900 in subsequent years

14. Incorporate recovery actions into the Interim Management Guidelines (IMGs) for a new conservation park

Recovery actions for *Drakaea confluens* ms will need to be addressed in the IMG for the new conservation park (Population 7c).

Action: Incorporate recovery actions into the IMGs for a new conservation park
Responsibility: CALM (Central Forest Region) through the CFRTFRT
Cost: $400 in third year

15. Write a full Recovery Plan

At the end of the second-year of this IRP, the need for further recovery will be assessed. If *Drakaea confluens* ms is still ranked Critically Endangered at that time a full Recovery Plan will be developed that prescribes actions required for the long-term recovery of the species.

Action: Write a full Recovery Plan
Responsibility: CALM (WATSCU, Albany District, Central Forest Region) through the ADTFRT and CFRTFRT
Cost: $18,100 in third year

4. TERM OF PLAN

This Interim Recovery Plan will operate from January 2001 to December 2003 but will remain in force until withdrawn or replaced. It is intended that, if the taxon is still ranked Critically Endangered, this IRP will be replaced by a full Recovery Plan after three years.

5. ACKNOWLEDGMENTS

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Gillian Stack	Former Project Officer, WA Threatened Species and Communities Unit
Kim Williams	Program Leader Nature Conservation, CALM Central Forest Region

We would like to thank the staff of the W.A. Herbarium for providing access to Herbarium databases and specimen information, and CALM's Wildlife Branch for their extensive assistance.

6. REFERENCES

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7. TAXONOMIC DESCRIPTION

Hoffman, N. and Brown, A. (1998) *Orchids of South-west Australia*. Revised 2nd edition with supplement. University of Western Australia Press, Nedlands.

Drakaea confluens ms grows 15 to 30 cm high. Its uniformly green leaf is often covered in short dense hairs and is one to two centimetres long and one to two centimetres wide. The single flower is two to four centimetres long and three to five millimetres wide. The two-coloured labellum is distinctive and has a straight rather than upturned apex.

Hopper and Brown Taxanomic paper in ed

Drakaea confluens Hopper and A.P. Brown, sp. nov.

Differs from *D. livida* J. Drummond in its uniformly green scabrous leaf, and its two-coloured labellum lamina lacking an upturned apex. Differs from *D. isolata* in its larger flowers and two-coloured labellum with conspicuous spots and its enlarged abdomen in side view with a steep curve up to a short tail.

D. confluens has a labellum similar in structure to that of *D. isolata*, and also has a similar minutely papillate scabrous leaf. However, *D. confluens* differs in its larger flowers and two-coloured labellum with conspicuous spots and its enlarged abdomen in side view with a steep curve up to a short tail. It differs from *D. livida* in its scabrous minutely papillate leaf and in the labellum lamina with a straight or slightly upturned tail.

Derivation of name. Named from the Latin *confluens* (confluent, running together), alluding to the labellum which has features of both *D. livida* (e.g. conspicuous spots) and of *D. elastica* (e.g. straight or slightly upturned tail).

some metabolic waste. The above results suggest that fungus-digesting cells can actively release hydrolytic enzymes by lysosomal vesicles to digest the invading hyphae, but such function is not present in the hyphae-containing cells, the role of which may be attributed to attracting and controlling the invading hyphae.

8. Beyrle, H.F. and S.E. Smith, *Excessive carbon prevents greening of leaves in mycorrhizal seedlings of the terrestrial orchid Orchis morio.* Lindleyana, 1993. **8**(2): p. 97-99.

Symbiotic germination of seeds of the terrestrial orchid Orchis morio L. on liquid oatmeal medium resulted in rapid development of protocorms and the formation of roots and leaves within six weeks. In daylight, the leaves developed chlorophyll and became green. With the addition of starch or sucrose (1%) plantlets developed longer roots, but light-induced chlorophyll synthesis was inhibited. Orchid mycorrhiza is discussed as a mechanism of heterotrophic carbon assimilation with the exploitation of the carbohydrate pool of the mycorrhizal fungus by the orchid as the main feature.

9. Malmgren, S., *Large-scale asymbiotic propagation by seed of Anacamptis, Ophrys, Orchis and other European orchids with entire tubers.* Svensk Botanisk Tidskrift, 1993. **87**(4): p. 221-234.

It is now possible to propagate a large number of temperate terrestrial orchids on several different growing media with asymbiotic methods. However, several species of Ophrys, Orchis, Anacamptis, and similar orchids with entire tubers, as well as species of Cypripedium, seem to be very sensitive to inorganic nitrogen, especially nitrate, in the medium. However, a simple medium, where an amino-acid solution is the sole nitrogen source, has been used with great success. This solution, as well as the vitamin source, are standardized solutions used normally as pharmaceutical substances in hospital medical care. Most terrestrial orchids seem to be possible to propagate on a large scale on this medium, or very small variations of it (amino acids 0.4-1 g; saccarose 10-20 g/1000g). Concerning germination a combined treatment with H-2SO-4 and NaClO is described. Several species, which are otherwise problematic, germinate very well with this method, even from mature seeds. Mycorrhiza symbiosis is not necessary for survival after transfer into the soil. Survival is just a question of plant size and experienced plantlet nursing.

10. Weber, T. and J. Bach Thomas, *Partial purification and characterization of membrane-associated 3-hydroxy-3-methylglutaryl-coenzyme A lyase from radish seedlings.* Zietschrift fuer Naturforschung Section C Journal of Biosciences, 1993. **48**(5-6): p. 444-450.

We solubilized from radish membranes and purified by 154-fold 3-hydroxy-3-methylglutaryl-CoA lyase (HMGL, EC 4.1.3.4) catalyzing the conversion of 3-hydroxy-3-methylglutaryl-(HMG-)CoA into acetyl-CoA and acetoacetate. The apparent molecular mass under non-denaturating conditions is 70 kDa. The enzyme has a broad pH optimum around 8.0 and its activation energy as determined from the linear part of an Arrhenius plot is 137.1 kJ/mol. The K-m with respect to (S)-HMG-CoA is 40 mu-M. The enzyme is extremely unstable and rapidly loses activity even when kept on ice, but retains some activity over several weeks when stored at -80 degree C.

11. Masuhara, G.K.K., *Fungal Coil Formation of Rhizoctonia-Repens in Seedlings of Galeola-Septentrionalis Orchidaceae.* Botanical Magazine Tokyo, 1076. **104**(1076): p. 275-281.

Protopcorms or protocorms with roots of an achlorophyllous orchid Galeola septentrionalis were inoculated with isolates of Rhizoctonia repens, R. solani, and Rhizoctonia spp. The seedlings were infected with eight of twelve isolates of R. repens. Fungal coils were formed in the cells, which was suggestive of a symbiotic association. The other isolates caused soft rot or no infection to the protocorms or the protocorms with a root.

12. Uetaka, Y.K.K.O.A., *Penetration and Peloton Formation By Binucleate Rhizoctonia Ag-C On Symbiotic Germination of Spiranthes-Sinensis Seeds.* Reports of the Tottori Mycological Institute, 1990. 28: p. 307-316.

Penetration time, site, and procedure, and peloton formation of binucleate Rhizoctonia AG-C during the symbiotic germination of Spiranthes sinensis (Orchidaceae) were observed using a light and a scanning electron microscopes. The fungus invaded from the side opposite to the meristem of embryo with single hyphae. The embryo initiated germination just after the fungal invasion, emerged from the testa, and became a protocorm. Hypae pennetrated subepidermal and inner cortical parenchymal cells and formed pelotons, but did not penetrate meristematic cells. Pelotons in subepidermal parenchymal cells were stained strongly with safranin, and were not digested, whereas pelotons in the inner cortical parenchymal cells were stained weakly with safranin, and were finally digested. Secondary infection occured to the cells containing digested pelotons. Emergence of hyphae from the epidermis and hairs of protocorms was observed.

13. Smreciu, E.A.C.R.S., *Symbiotic Germination of Seeds of Terrestrial Orchids of North America and Europe*. Lindleyana, 1989. **4**(1): p. 6-15.

Symbiotic and asymbiotic germination of seeds of north temperature terrestrial orchids (10 North American species: Amerorchis rotundifolia, Calypso bulbosa, Coeloglossum viride, Corallorhiza maculata, C. trifida, Cypripedium calceolus, Goodyera repens, Platanthera hyperborea, P. obtusata, and P. orbiculata and 10 European species: Dactylorhiza maculata, D. sambucina, Epipactis palustris, E. purpurata, Gymnadenia conopsea, G. odoratissima, Neottia nidus-avis, Nigritella nigra, and Orchis morio) were tested on a synthetic medium and on a cellulose medium inoculated with one of six rhizoctonia strains isolated from orchid mycorrhizae and various pathogenic sources. Some development, ranging from embryo swelling to shoot development, was observed for 17 orchids. Embryos of Corallorhiza maculata, C. trifida, and Epipactis purpurata failed to develop further. Ceratobasidium cereale stimulated germination of 11 orchids but tended to function as a pathogen. Rhizoctonia anaticula and Sistotrema sp. stimulated germination of several orchid species. Sistotrema formed pelotons in protocorm cells similar to those found in mature orchid mycorrhizae. Thanatephorus pennatus, Ceratobasium obscurum, and Rhizoctonia anaticula were not effective in promoting seed germination or protocorm development in most of the orchids.

14. Manning, J.C.V.S.J., *The Development and Mobilization of Seed Reserves in Some African Orchids*. Australian Journal of Botany, 1987. **35**(3): p. 343-353.

The development, final appearance and digestion of seed reserves in a number of genera of the Orchidaceae (tribe Orchideae) has been studied comprehensively, using ultrastructural and histochemical techniques complemented by gas chromatographic analysis of free sugars. Mature seeds of Disa, Disperis and Huttonaea contain substantial reserves of lipid and protein in the embryo. The protodermal cells of Disperis also contain protein-carbohydrate bodies. Free sugars are present but starch occurs only in immature seeds. Glyoxysomes are absent and lipolysis does not occur in seeds incubated without an external source of sucrose, and although a little starch is formed it is apparently synthesised from endogenous sucrose reserves. In the presence of exogenous sucrose, however, proteins are hydrolysed and glyoxysomes appear. Substantial quantities of starch are formed in such seeds. From these observations it is apparent that orchid seeds are unable to utilise endogenous reserves of lipid unless simple sugars are supplied to the medium but can utilise the free sugars present in the embryo. Resultant conclusions on the role of mycorrhizae in the germination of orchid seeds are discussed.

15. Tsutsui, K.T.M., *Symbiotic Germination of Spiranthes-Sinensis Associated With Some Orchid Endophytes*. Journal of the Faculty of Agriculture Hokkaido University, 1986. **62**(4): p. 440-452.

With 7 Rhizoctonia isolates obtained from 4 orchid species out of 10, symbiotic capability to Spiranthes sinensis seeds was tested on oat medium. A binucleate Rhizoctonia AG-C isolate obtained from Gymnadenia camtschatica was the most effective symbiont to this orchid and germinated seeds grew large enought to transplant within 3 months after seeding. Two binucleate Rhizoctonia isolates, possibly Rhizoctonia repens, obtained from Spiranthes sinensis per se were symbiotic to this orchid but fat less effective. And there was a considerable difference between the two in their effectiveness. The effect of culture media on the symbiosis was considerably large but it could not change the essential nature of the symbiosis. For this orchid the medium containing mineral salts, 1% cellulose, 0.1% sucrose and 0.02-

0.05% yeast extract was better than the oat medium for symbiotic culture. The addition of mycelial powder or culture filtrate of the compatible fungus to non-symbiotic culture did not stimulate the germination or protocorm growth. Although Spiranthes sinensis was found to germinate non-symbiotically on a wide range of media irrespective of light conditions, the protocorm growth was very slow and it required more than 16 months from seeding to attain sufficient growth for transplanting.

16. Markovina, A.L. and P.A. McGee, *Comparison of symbiotic and asymbiotic seed germination and plantlet development in Sarcochilus (Vandeae; Orchidaceae).* Lindleyana. [print], 2000. **15**(2): p. 68-72.

In vitro symbiotic and asymbiotic seed germination and plantlets development of Sarcochilus species and hybrids were compared. Of four fungi isolated from pelotons from Sarcochilus olivaceus Lindl. and S. falcatus R.Br., three germinated a similar proportion of seeds following inoculation on a commercial asymbiotic medium. The fourth isolate overgrew the seed. One fungus, tentatively identified as Ceratorhiza sp., stimulated growth to the extent that some seedlings were large enough for ex vitro growth 13 weeks after germination. Mycorrhiza formation in the root-like organs of one-year-old asymbiotically germinated plantlets of a hybrid of Sarcochilus resulted in faster growth rates than of asymbiotic plantlets growing on a commercial medium. Again, one fungus stimulated growth of leaves and roots significantly more than the others. We suggest that symbiotic techniques have a role in commercial production of plants.

17. Wood Christopher, B., W. Pritchard Hugh, and P. Miller Angela, *Simultaneous preservation of orchid seed and its fungal symbiont using encapsulation-dehydration is dependent on moisture content and storage temperature.* Cryo Letters, 2000. **21**(2): p. 125-136.

Seeds of Dactylorhiza fuchsii (common spotted orchid) and Anacamptis morio (green-winged orchid) were encapsulated in alginate beads with hyphae of the basidomycete fungus Ceratobasidium cornigerum. Pre-treatment of beads for 18 h with sucrose at an optimum concentration of 0.75 M decreased the desiccation rate in a flow of sterile air (c. 23 degreeC, 30% RH) and increased seed and fungal survival after up to 16 h drying. Pre-treated and 16-h dried beads were transferred to cryo-vials and subsequently stored at a range of low temperatures for up to 30 d. Neither embryo growth of both orchids nor fungal development was detrimentally affected by 1 d storage at -196 degreeC when the beads were pre-dried to c. 20% moisture content. Encapsulated D. fuchsii seed and compatible fungus had Itoreq 5% and Itoreq 45% viability when beads of the same moisture content were stored for 1 d at -20degreeC and -70degreeC respectively. In contrast, viability of the seed and the fungus remained unchanged during 30 days storage at -196degreeC but was progressively lost at 16degreeC over the same interval. The results indicate opportunities for the use of simultaneous cryopreservation as a conservation tool for diverse taxa.

18. McKendrick, S.L., *et al.*, *Symbiotic germination and development of myco-heterotrophic plants in nature: ontogeny of Corallorrhiza trifida and characterization of its mycorrhizal fungi.* New Phytologist, 2000. **145**(3): p. 523-537.

The processes of symbiotic germination and seedling development were analysed in the myco-heterotrophic orchid C. trifida, seeds of which were buried in 'packets' either adjacent to or at varying distances from adult plants in defined communities of ectomycorrhizal tree species. Germination occurred within eight months of burial under Betula-Alnus and within seven months under Salix repens. It was always associated with penetration of the suspensor by a clamp-forming mycorrhizal fungus. Four distinct developmental stages were defined and the rates of transition through these stages were plotted. There was no evidence of a relationship between extent of germination or rate of development and the presence of naturally distributed plants of C. trifida at the spatial scale of 1 m. The best germination and the most rapid rate of development of C. trifida seedlings occurred in a Salix repens community located at a considerable distance from any extant C. trifida population. Determination of internal transcribed spacer (ITS) RFLPs and of gene sequences of the fungi involved in symbiotic germination and growth of C. trifida, revealed them to belong exclusively to the Thelephora-Tomentella complex of the Thelephoraceae. These fungi are known also to be ectomycorrhizal associates of trees. It is hypothesized

that the rate of growth of the C. trifida seedlings is determined by the ability of the fungal symbionts to transfer carbon from their ectomycorrhizal co-associates

19. Takahashi, K., I. Ogiwara, and N. Hakoda, *Seed germination of Habenaria (Pecteilis) radiata (Orchidaceae: Orchideae) in vitro.* Lindleyana, 2000. **15**(1): p. 59-63.

Characteristics of seed germination in Habenaria (Pecteilis) radiata in vitro were studied with respect to effects of seed age, culture media, light conditions, and mycorrhizal fungal isolates. The germination percentage of seeds maintained under 10-15 deg C, 30-50% humidity conditions for seven months was unaffected by light conditions, culture media, and fungal isolates, and the seeds had a high germination percentage 28 days after sowing. Seeds sown in October immediately after harvesting had a lower germination percentage than those sown in May of the following year, and germination percentage for these also varied with light conditions, culture media, and fungal isolate. For seeds sown in October, those cultivated on Hyponex medium had a higher germination percentage than those cultivated on oatmeal medium, and those in lighted conditions had the higher germination percentage. Additionally, the existence of a fungal isolate (isolate 9240; Ceratobasidium sp.) that does not promote early growth but does assist in germination was recognized

20. Umata, H., *Germination and growth of Erythrorchis ochobiensis (Orchidaceae) accelerated by monokaryons and dikaryons of Lenzites betulinus and Trametes hirsuta.* Mycoscience, 1999. **40**(4): p. 367-371.

Four sib-monokaryons and two reconstituted dikaryons of L. betulinus and T. hirsuta [Coriolus hirsutus], accelerated the seed germination of E. ochobiensis, an achlorophyllous orchid. All isolates of L. betulinus and 3 isolates of C. hirsutus induced the development of plants from germinated seeds. Although three monokaryotic isolates of C. hirsutus failed to induce the development of plants, the reconstituted dikaryons induced the development

21. Zettler, L.W., J.C. Burkhead, and J.A. Marshall, *Use of a mycorrhizal fungus from Epidendrum conopseum to germinate seed of Encyclia tampensis in vitro.* Lindleyana, 1999. **14**(2): p. 102-105.

In vitro symbiotic seed germination and early development of the Florida butterfly orchid, Encyclia tampensis, is described. Seeds of E. tampensis, a commercially exploited epiphytic species, were inoculated with a mycorrhizal fungus originally isolated from another epiphytic orchid, Epidendrum conopseum. Seeds inoculated with the fungus germinated (99.6%) within 21 days, whereas seeds incubated in the absence of the fungus failed to germinate. About 2% of the seedlings initiated leaves after 13 weeks of incubation. Evidence for the orchid-fungal symbiosis consisted of the observation of coils of fungal hyphae (pelotons) in cortical cells of leaf-bearing seedlings

22. Sarma, C.M. and K. Satinder, *Application of Rhizoctonia solani for establishment of in vitro raised Arundina graminifolia (D. Don) Hochr. seedlings under natural conditions.* Journal of the Orchid Society of India, 1998. **12**(1): p. 77-78.

The establishment of plantlets of A. graminifolia in terms of survival rate and growth in different growing media was studied. Plant survival and growth were best in a medium composed of charcoal, bricks, sand, leaf mould and cow dung (1:1:1:1:1). Treatment with Rhizoctonia solani enhanced percentage survival

23. Fan, L., S. Guo, and J. Xu, *Interaction between protocorms of Gastrodia elata (Orchidaceae) and Mycena dendrobii in symbiotic germination.* Mycosystema, 1999. **18**(2): p. 219-225.

Symbiotic germination tests between seeds of G. elata and M. dendrobii demonstrated that M. dendrobii stimulated seed germination. Many seeds germinated and formed protocorms which were colonized by fungal hyphae. The hyphae were commonly distributed in stipecell, subepidermal parenchyma cells (SEP) and inner cortical parenchyma cells (ICP), and were separated from protocorm cell cytoplasma by electron-lucent materials and the protocorm cell plasma membrane. The hyphae formed pelotons in SEP.

but in ICP they were digested and lysed. Protocorm cells containing degenerated hyphae were frequently recolonized by hyphae, and hyphal digestion and the reinfection of protocorm cells occurred repeatedly throughout the protocorm growth

24. Tomita, M. and S. Konno, *A preliminary report on the symbiotic germination of nine Japanese terrestrial orchids.* Journal of the Japanese Society for Horticultural Science, 1998. **67**(5): p. 696-698.

To develop an effective propagation method, a symbiotic culture (using 20 fungal strains) was attempted in 9 species of Japanese terrestrial orchids. In all orchid species tested, seeds germinated in symbiotic culture with Rhizoctonia isolates; no seeds germinated in symbiotic culture with non-Rhizoctonia isolates. Seeds of Goodyera biflora var. macrantha, G. foliosa var. laevis and G. hachijonesis var. matsumurana, when inoculated binucleate Rhizoctonia strains, germinated and exhibited virtually the same TTC [tetrazolium test] results as embryos. Those in a symbiotic culture with binucleate Rhizoctonia grew well compared with seeds on a non-symbiotic culture. The 6 other orchid species exhibited lower germination rates and reduced TTC test results. There were numerous effective fungal strains with 5 orchid species with the exception of Cypripedium macranthos var. speciosum, which developed a symbiotic relationship with only one of the fungal isolates tested. The binucleate Rhizoctonia fungal group were suitable for promoting symbiotic germination and growth of Aorchis cyclochila, Dactylorhiza aristata and Gymnadenia camtschatica; Amitostigma kinoshitae and Ponerorchis graminifolia var. graminifolia responded better to R. repens

25. Zettler, L.W. and C.J. Hofer, *Propagation of the little club-spur orchid (Platanthera clavellata) by symbiotic seed germination and its ecological implications.* Environmental & Experimental Botany, 1998. **39**(3): p. 189-195.

Propagation of the auto-pollinated terrestrial orchid, P. clavellata, using symbiotic seed germination is described. Seeds from 3 populations in the southern Appalachians (Tennessee, South Carolina and Georgia. USA) were inoculated with mycorrhizal fungi (Epulorhiza spp.) in vitro. Seed germination and protocorm (seedling) developmental stages were evaluated up to 1 year after sowing. Seeds from Tennessee and South Carolina had significantly higher percentage germination (76.4 and 81.5%, respectively) than seeds from Georgia (16.2%). More advanced seedlings suitable for transfer to the soil were obtained from the Georgia seed lot. A single fungal isolate (E. inquilina) originally obtained from roots of P. clavellata, promoted advanced seedling development; 3 other isolates from obtained from P. ciliaris, P. cristata and P. integrilabia were ineffective. The ecological role of this orchid with its associated fungi is discussed with respect to the other cohabiting Platanthera species

26. Umata, H., *In vitro germination of Erythrorchis ochobiensis (Orchidaceae) in the presence of Lyophyllum shimeji, an ectomycorrhizal fungus.* Mycoscience, 1997. **38**(3): p. 355-357.

In vitro germination of a myco-heterotrophic orchid, E. ochobiensis, was tested in the presence of ectomycorrhizal fungi, L. shimeji and Tricholoma fulvocastaneum. L. shimeji stimulated the germination after incubation for 1.5 months. Although most germinated seeds did not grow further after 3 months, several seeds developed into small protocorms but showed amorphous profiles. Fungal mycelia were observed in the germinated seeds and protocorms, but pelotons were not detected. Since the seeds did not germinate axenically, it is suggested that the fungus has the ability to stimulate germination

27. Zelmer, C.D., L. Cuthbertson, and R.S. Currah, *Fungi associated with terrestrial orchid mycorrhizas, seeds and protocorms.* Mycoscience, 1996. **37**(4): p. 439-448.

The identity and ecological role of fungi in the mycorrhizal roots of 25 species of mature terrestrial orchids and in 17 species of field incubated orchid seedlings were examined. Isolates of symbiotic fungi from mature orchid mycorrhizas were basidiomycetes primarily in the genera Ceratorhiza, Epulorhiza and Moniliopsis; a few unidentified taxa with clamped hyphae were also recovered. More than 1 taxon of peloton-forming fungus was often observed in the cleared and stained mycorrhizas. Although Ceratorhiza and Epulorhiza strains were isolated from the developing protocorms, pelotons of clamped hyphae were often present in the cleared protocorms of several orchid species. These basidiomycetes were difficult to

isolate and may be symbionts of ectotrophic plants. The higher proportion of endophytes bearing clamp connections in developing seeds than in the mycorrhizas is attributed to differences in the nutritional requirements of the fully mycotrophic protocorms and partially autotrophic plants. Most isolates of Ceratorhiza differed enzymatically from Epulorhiza in producing polyphenol oxidases. Dual cultures with 13 orchid isolates and 5 non-orchid hosts showed that some taxa can form harmless associations with non-orchid hosts. It is suggested that most terrestrial orchid mycorrhizas are relatively non-specific and that the mycobionts can be saprophytes, parasites or mycorrhizal associates of other plants

28. Umata, H., *Seed germination of Galeola altissima, an achlorophyllous orchid, with aphyllophorales fungi.* Mycoscience, 1995. **36**(3): p. 369-372.

Seed germination tests on G. altissima were carried out with 5 aphyllophorales fungi: Erythromyces crocicreas, Ganoderma australe, Loweporus tephroporus, Microporus affinus and Phellinus sp. All 5 species were effective for seed germination of the orchid. E. crocicreas, which has hitherto been regarded as the only endomycorrhizal fungus of the orchid, was confirmed to be effective for further development of the orchid

29. Rasmussen, H.N., *Germination biology and mycotrophy of terrestrial orchids. Studies of north temperate species 1986-1993.* SP Rapport Statens Planteavlsforsog, 1995(31).

Results of in vitro and field studies and a literature survey on the germination and growth of orchids in association with mycorrhizas are discussed

30. Rasmussen, H.N., *Terrestrial orchids. From seed to mycotrophic plant.* Cambridge University Press, Cambridge, UK, 1995. **332**: p. 50.

Terrestrial orchid biology is surveyed, from seed dispersal to the established plant, including the role of mycorrhizas, using observations from field and in vitro experiments. There are chapters on seed properties, development, survival and germination, mycorrhizas and mycorrhizal fungi, underground organs, abiotic factors in growth and development, life history and phenology, and propagation. The final chapter reviews the life history, endophytes, role of mycorrhizas and propagation of 36 genera and their species. There is a list of references and appendices of media and names and synonyms. The book is intended for both physiologists and those involved in orchid horticulture

31. Kinderen, G.v.d., *Observations on in situ germination of Epipactis helleborine (L.) Crantz.* Lindleyana, 1995. **10**(4): p. 223-231.

Germination of E. helleborine seeds was studied under natural conditions, using a new technique of controlled soil incubation in situ. The influence of temperature, incubation depth and seed viability was examined in relation to the germination process. A long imbibition period, starting 3 months after seed dispersal, was observed. Its duration was equal regardless of the depth at which seeds were incubated. The proportion of seeds which proved to be viable in the soil incubation test was higher than suggested by biochemical viability testing, which proved to be unreliable. Differentiation of embryos to protocorms started almost one year after seed dispersal. A cold period preceded this stage of development and may have broken seed dormancy, but differentiation was observed only after mycorrhizal infection. After this, a marked depth-dependent difference in rate of development was observed

32. Uetake, Y., A. Ogoshi, and N. Ishizaka, *Cytochemical localization of malate synthase activity in the symbiotic germination of Spiranthes sinensis (Orchidaceae) seeds.* Transactions of the Mycological Society of Japan, 1993. **34**(1): p. 63-70.

Activity of malate synthase was demonstrated in microbodies (glyoxysomes) in asymbiotic seeds 4, 10, 15 and 25 d after sowing on oat powder agar, and in symbiotic seeds at 4 d after sowing and inoculation with binucleate Rhizoctonia AG-C on the same medium. In symbiotic culture, the reaction products in microbodies decreased in the embryo after 5 d and were absent from or present in limited amounts in the

protocorms at 10 d and in young seedlings 15 d after sowing and inoculation. Lipid bodies were degraded slowly in asymbiotic seeds, but rapidly in infected cells of symbiotic seeds. These results suggest that fungal invasion caused changes in the metabolism of lipid reserves in the orchid embryo cells

33. Zettler, L.W., F.V. Barrington, and T.M. McInnis, Jr., *Developmental morphology of Spiranthes odorata seedlings in symbiotic culture.* Lindleyana, 1995. **10**(3): p. 211-216.

Symbiotic seed germination and developmental morphology of S. odorata protocorms (seedlings) were investigated using 2 orchid endophytes isolated from the North American terrestrial orchids S. odorata and Platanthera ciliaris. Seeds germinated during the second week of incubation in both symbiotic and asymbiotic culture; however, seedling development in the absence of a fungus was arrested after 35 days. Fungal-infected seedlings produced leaf primordia in darkness, but further leaf (shoot) development occurred only after exposure to white light. Seedlings infected with the P. ciliaris endophyte continued to develop, leading to the soil-establishment of 5.6% of the originally-sown embryos, 299 days following fungal inoculation. Seedlings that initiated the formation of leaf primordia represented the most critical stage in the early development of this species, with light and fungi both playing key roles. Although this species is routinely propagated asexually through vegetative cloning, the continued implementation of symbiotic techniques for this and other temperate terrestrial orchids is advocated here, at least until the reproductive potential of native orchids is fully understood

34. Rasmussen, H.N. and D.F. Whigham, *Seed ecology of dust seeds in situ: a new study technique and its application in terrestrial orchids.* American Journal of Botany, 1993. **80**(12): p. 1374-1378.

A method is described by which seeds of terrestrial orchids are sown and retrieved in the field under almost natural conditions. For the first time it is possible to conduct a quantitative study of orchid germination in situ and observe seasonal growth and mortality of seedlings. The technique has also enabled the relation between the site where the seeds are sown, the availability of an appropriate fungus to infect the seeds, and seedling establishment in the soil to be investigated. Five local species were studied. Corallorrhiza odontorhiza, Goodyera pubescens and Galearis spectabilis all began to germinate in May-June, after 23-30 weeks in the soil. These species differed in their dependency on infection at germination time, but none of the seedlings developed beyond the point of rupturing the testa except when infected. Seeds of Liparis liliifolia and Tipularia discolor did not germinate within the first 12 months of the experiment. The implications and potential uses of this field sowing technique for further studies and for other kinds of minute seeds are discussed

35. Zettler, L.W. and T.M. McInnis, Jr., *Light enhancement of symbiotic seed germination and development of an endangered terrestrial orchid (Platanthera integrilabia).* Plant Science, 1994. **102**(2): p. 133-138.

Seeds of the endangered orchid P. integrilabia were exposed to 1 of 3 light treatments: 7 days initial darkness followed by 16 h light/day, 16 h light/day for 7 days followed by darkness, and 16 h light/day throughout. Alternatively, a continuous dark treatment in symbiotic and asymbiotic culture was provided. Seeds exposed to a photoperiod of 16 h/day or an initial 7 days of darkness followed by 16 h light/day were largely inhibited from germinating. Seeds exposed to 16 h light/day during the first 7 days after fungal inoculations followed by darkness had a significantly higher percentage germination (44%) than the other treatments, including inoculated seeds in continuous darkness (20.3%). Seedling (protocorm) development (i.e., formation of leaf primordia) was also enhanced by initial light compared with seedlings under continuous darkness. It is speculated that light exposure followed by darkness occurs naturally, starting with the shedding of seeds from capsules to their immersion in a substrate where germination occurs. The beneficial effects of light in this study argues in support of light usage to stimulate germination and seedling development of temperate terrestrial orchids

36. Wilkinson, K.G., *et al., Effect of IAA on symbiotic germination of an Australian orchid and its production by orchid-associated bacteria.* Plant & Soil, 1994. **159**(2): p. 291-295.

Seven isolates of orchid-associated bacteria (OAB) belonging to 5 species were tested for their effect on mycorrhiza-assisted germination of the terrestrial orchid Pterostylis vittata. Growth regulator standards were also tested to evaluate their potential roles in the germination and development of the orchid. Strains of Pseudomonas putida, Xanthomonas maltophilia and Bacillus cereus promoted symbiotic germination, whereas certain strains of P. putida and an Arthrobacter species reduced it. Symbiotic germination was enhanced by IAA, inhibited by GA<sub(3)> and suppressed by kinetin. Each species of OAB produced IAA, although the conditions of growth affected the production of the auxin. IAA was not produced by the mycorrhizal fungus from P. vittata under the test conditions. Enhancement of symbiotic germination development may have resulted either from the production of IAA by the OAB and/or by the induction of endogenous growth regulators in the orchid by the metabolites of the bacterium and/or mycorrhizal fungus

37. Zettler, L.W. and T.M. McInnis, Jr., *Symbiotic seed germination and development of Spiranthes cernua and Goodyera pubescens (Orchidaceae: Spiranthoideae).* Lindleyana, 1993. **8**(3): p. 155-162.

The symbiotic germination and subsequent development of seeds of 2 North American terrestrial orchids was studied using a combination of 3 seed storage temperatures (6, 22 or -7 deg C) and 3 orchid mycorrhizal endophytes isolated from Platanthera ciliaris, P. integrilabia and S. cernua. Germination percentages were not significantly enhanced by fungus inoculation. Seeds germinated in all treatments including those in asymbiotic culture (non-inoculated). Seed germination percentages of G. pubescens increased in direct proportion to increasing seed storage temperatures. The opposite was true for S. cernua seeds, which when chilled remained viable in storage for at least 9 months. Development of G. pubescens was arrested following the appearance of the first true leaves after incubation in vitro under a 16-h photoperiod. Three of 196 S. cernua seedlings in soil flowered 787 days after germination. All but 3 of the 196 seedlings were derived from chilled seeds inoculated with a P. ciliaris endophyte. A mortality rate of 4.1% for seedlings established in soil was recorded 704 days following germination. The inoculation of chilled S. cernua seeds with a P. ciliaris endophyte appears to be a feasible means of cultivating this species

38. Ozkoc, I. and M. Dalci, *The effect of some fungi upon germination and development of Orchis laxiflora Lam. (Orchidaceae) seeds on two different media.* Doga Turk Biyoloji Dergisi, 1993. **17**(1): p. 23-28.

Eleven mycorrhizal fungal isolates from various countries were compared in 2 oat-based media. Seed germination and seedling development in both media were best with the foreign isolates No. 624 and F 418, followed by F 397. Two Turkish isolates were found to be ineffective

39. Guo, S.X. and J.T. Xu, *Studies on the changes of cell ultrastructure in the course of seed germination of Bletilla striata under fungus infection conditions.* Acta Botanica Sinica, 1990. **32**(8): p. 594-598.

The effect of fungal [mycorrhizal] infection on ultrastructural changes occurring during germination of B. striata seeds observed by EM, is reported

40. Richardson, K.A., R.L. Peterson, and R.S. Currah, *Seed reserves and early symbiotic protocorm development of Platanthera hyperborea (Orchidaceae).* Canadian Journal of Botany, 1992. **70**(2): p. 291-300.

Seeds of this North American terrestrial orchid consist of a thin testa and a simple embryo without a cotyledon. Studies showed that epidermal and parenchyma cells of the embryo contained lipid and protein as storage reserves. Many of the protein bodies had globoid crystals identified by their P, Ca<sup(2+)>, Mg<sup(2+)>, and K<sup(+)> content. Germination occurred with either Rhizoctonia cerealis or Ceratorhiza goodyerae-repentis as the fungal symbiont on Warcup's medium. The fungus entered through dead suspensor cells and triggered protocorm development and concomitant utilization of lipid and protein reserves. Fungal hyphae formed pelotons with protocorm cells initially, and these went through stages of vacuolation and collapse. Some hyphae stored small numbers of polyphosphate

bodies. Clumps of degenerated hyphae were usually encased within material that stained positive with aniline blue, presumably callose

41. Rasmussen, H.N., *Seed dormancy patterns in Epipactis palustris (Orchidaceae): requirements for germination and establishment of mycorrhiza.* Physiologia Plantarum, 1992. **86**(1): p. 161-167.

Some terrestrial orchid species, including E. palustris, are considered extremely difficult to germinate and cultivate in vitro. Observations of orchids germinating in nature are very few, and the timing and requirements for seedling establishment are unknown for most species. Seeds of E. palustris were incubated in vitro with an unidentified fungal strain isolated from plants in the field, but germination was poor unless several other conditions were also met. These conditions were scarification of the testa in Ca(OCl)<sub(2)>, an initial incubation for several weeks at 20 deg C, and a subsequent cold stratification for 8-12 weeks at 4-8 deg . With these pretreatments, germination responses exceeded 50% after incubation for 4 weeks at 20 deg . Healthy protocorms with normal organ development were only produced by symbiotic culture following this lengthy seed preparation. The findings suggest that under natural conditions the seeds need some after-ripening and the testa needs to be partially decomposed before germination. The requirement for chilling suggests that germination of seeds in situ occurs in spring

42. Masuhara, G., K. Katsuya, and K. Yamaguchi, *Potential for symbiosis of Rhizoctonia solani and binucleate Rhizoctonia with seed of Spiranthes sinensis var. amoena in vitro.* Mycological Research, 1993. **97**(6): p. 746-752.

Rhizoctonia isolates obtained from non-orchid sources were tested for symbiotic ability with seeds of S. sinensis var. amoena in vitro. All or some isolates of R. solani anastomosis group (AG)-1, AG-2-1, AG-2-2, AG-3, AG-4, AG-5, AG-6, AG-7, AG-8 and AG-BI induced symbiotic germination of the orchid seeds. In binucleate Rhizoctonia anastomosis groups, also all or some isolates of AG-A, AG-Ba, AG-Bb, AG-B, AG-C, AG-D, AG-E, AG-F, AG-G, AG-H, AG-I, AG-K, AG-L, AG-M, AG-O, AG-P and AG-Q, induced the symbiotic germination. However, no germination occurred when the seeds were inoculated with Sebacina vermifera (R. globularis) and Waitea circinata (R. zeae and R. oryzae) in vitro

43. Yamashita, T. and K. Nishikawa, *Development and anatomy of the fungus-free, cultivated seedling and of underground organs of Habenaria radiata (Orchidaceae).* Beitrage zur Biologie der Pflanzen, 1991. **66**(3): p. 351-370.

Seeds of Habenaria radiata [Pecteilis radiata] were germinated in sterilized nutrient medium and the seedlings were cultivated for 1 year in the same medium. Protocorms, shoot axes, adventitious roots and tuberous roots of the seedlings were investigated morphologically and anatomically. The tuberous root is initiated just below the shoot apex of the main axis, and the broadened proximal part of the tuberous root primordium envelopes the shoot apex. The apical part of the tuberous root primordium dissolves the surrounding stem cortex tissue and makes a hole through the stem epidermis. Finally the tuberous root, together with the enclosed shoot apex of the main axis, moves out of the plant body through the hole made in the stem epidermis. Thus, the seedlings grow monopodially, despite their peculiar appearance. Comparison with tuberous roots of adult, flowering plants in Ophrydeae is discussed

44. Rasmussen, H. and F.N. Rasmussen, *Climatic and seasonal regulation of seed plant establishment in Dactylorhiza majalis inferred from symbiotic experiments in vitro.* Lindleyana, 1991. **6**(4): p. 221-227.

Seeds of D. majalis (collected from a population in North Zealand, Denmark) were germinated in vitro with a compatible fungus, Tulasnella calospora. Germination itself was almost completely inhibited in long and short day photoperiods and at a very low light intensity, but initial illumination (particularly with red light) raised the eventual germination percentage. These responses would tend to enhance germination in open vegetation but below ground, where there are chances of immediate symbiotic infection and little risk of desiccation. Germination in vitro was highest at about 20 deg C with little diurnal fluctuation. Above 25 deg , seeds remained imbibed but did not germinate and rejected fungal infection. Symbiotic seedlings

grew well at fairly low temperatures and responded to chilling (5 deg for 15 weeks) by developing an aerial shoot and later a tuberoid. These findings suggest that the seeds normally germinate in autumn when saprophytic nutrition of the mycelium is optimal. The requirement of a cold season for further seedling differentiation may be restrictive for the distribution of this species

45. Anderson, A.B., *Symbiotic and asymbiotic germination and growth of Spiranthes magnicamporum (Orchidaceae).* Lindleyana, 1991. **6**(4): p. 183-186.

Seeds of S. magnicamporum, a species which is becoming increasingly rare in Canada and which has commercial potential, were germinated in vitro on (1) water agar, (2) modified Knudson's medium, or (3) oat medium containing the fungal symbiont Epulorhiza repens isolated from a naturally occurring plant of the same species. Symbiotic germination in medium (3) was variable, the fungus parasitizing many of the embryos and developing protocorms, but surviving seedlings grew well. Forty plants were potted at 4 months and kept at 10 deg C in 8-h days until they were 8 months old, when they were planted in a raised bed of calcareous sand in June. All survived to 23 months, when most of them flowered. With medium (2), containing 250 mg calcium nitrate, Burgeff's phosphate buffer, 5% potato extract but no potassium chloride, the germination was 45% with good development until 2 months when signs of N deficiency became evident; this was corrected by increasing the level of calcium nitrate to 1 g/litre. After potting at 4 months, only 2 of the 40 seedlings survived to 23 months. With medium (1) germination was excellent (99%) but there was no development beyond the protocorm stage. However, when protocorms were transferred to medium (3) and became infected with the fungus development was satisfactory; all 40 seedlings survived and most flowered in the following Sep

46. Arditti, J., *et al., The contribution of orchid mycorrhizal fungi to seed germination: a speculative review.* Lindleyana, 1990. **5**(4): p. 249-255.

Topics discussed in this review include physiology of orchid seeds, contributions by the fungus, orchid-fungus specificity and nucleic acid metabolism

47. Rasmussen, H.N., B. Johansen, and T.F. Andersen, *Symbiotic in vitro culture of immature embryos and seeds from Listera ovata.* Lindleyana, 1991. **6**(3): p. 134-139.

Immature embryos as well as seeds of L. ovata were successfully inoculated with a symbiotic fungus (Epulorhiza sp.). Symbiotic protocorms developed from embryos in all stages, whereas asymbiotic culture of embryos only succeeded if the capsules were almost mature. Germination percentage in mature seeds was higher in symbiotic than asymbiotic culture, and symbiotic protocorms developed faster. Inoculation of immature embryos could be a generally applicable method to propagate 'hard-to-germinate' orchid species and to test compatibility of fungi

48. Rasmussen, H.N., *Cell differentiation and mycorrhizal infection in Dactylorhiza majalis (Rchb. f.) Hunt & Summerh. (Orchidaceae) during germination in vitro.* New Phytologist, 1990. **116**(1): p. 137-147.

Seeds of D. majalis were sown in vitro with a compatible fungus (Epulorhiza sp.). Germination, and establishment of mycorrhiza, took c. 14 d in vitro. From day 9 the protein reserves in the embryo were hydrolysed, the protein vacuoles coalesced and starch accumulated in plastids. Certain epidermal cells developed nuclei about 8 times orginal volume and produced rhizoids which emerged from day 11. The mycorrhiza was established after infection through the rhizoids: hyphae formed pelotons in central cells with enlarged nuclei (16-64 times original volume). Intracellular hyphae developed close contacts with the hypertrophied host nuclei. Collapsed pelotons were observed in cells from day 12, one day after infection. Meristematic activity in the uninfected chalazal end of the seedling began on day 12. On day 28 the first vascular tissue started to develop and on day 35 the beginning of a leafy shoot could be detected

49. Rasmussen, H., B. Johansen, and T.F. Andersen, *Density-dependent interactions between seedlings of Dactylorhiza majalis (Orchidaceae) in symbiotic in vitro culture.* Physiologia Plantarum, 1989. **77**(3): p. 473-478.

In vitro growth of the heterotrophic seedlings of D. majalis with a symbiotic fungus (Rhizoctonia sp.) was density-dependent, even at densities that are not high enough to exploit the growth medium fully. Competition was two-sided; increasing density among seedlings did not increase size inequality between them. The slowly growing and smaller seedlings are normally more likely to die than the bigger ones, but mortality was not increased by higher density within the studied range (2-128 seedlings/dish). Growth depression by neighbouring seedlings was independent of their physical distance but varied with the total number of seedlings sharing the same culture dish and the same mycelium. Therefore, if the growth-promoting effects of different fungi or treatments are to be compared in future work, the density of planting in the culture dishes should be comparable

50. Xu, J.T. and S.X. Guo, *Fungus associated with nutrition of seed germination of Gastrodia elata - Mycena osmundicola Lange.* Acta Mycologica Sinica, 1989. **8**(3): p. 221-226.

M. osmundicola was isolated and identified from the protocorms of germinated seeds of G. elata orchids for the first time in China. Seeds infected by str. 01 of M. osmundicola could germinate. Band numbers and mobility of esterase isoenzymes of str. 01 were shown to be the same by PAGE

51. Tsutsui, K. and M. Tomita, *Suitability of several carbohydrates as the carbon sources for symbiotic seedling growth of two orchid species.* Lindleyana, 1990. **5**(2): p. 134-139.

The suitability of mono-, di- and polysaccharides in symbiotic cultures was investigated with three orchid-fungus combinations: Spiranthes sinensis with binucleate Rhizoctonia isolate No. 706, S. sinensis with R. repens isolate No. 624, and Liparis nervosa with isolate No. 624. The orchid seeds were sown on inoculated oat-agar medium. Cellulose and inulin were good carbon sources for all combinations. In general, mono- and disaccharides were inferior to these polysaccharides. This may be due to their early depletion in the media as a result of rapid uptake and utilization by the fungi. Pectin was also a good carbon source for S. sinensis when associated with isolate No. 706, but it was harmful to the orchid when associated with isolate No. 624. Mannitol was utilized very slowly by the fungi and was a good carbon source for symbiotic seedlings of S. sinensis but not so for those of L. nervosa. Galactose and galacturonic acid were detrimental to the orchids but were as suitable for the fungi as other soluble sugars. Galactose was as appropriate as other soluble sugars as a carbon source provided that orchids did not come into direct into contact with it

52. Rasmussen, H., T.F. Anderson, and B. Johansen, *Temperature sensitivity of in vitro germination and seedling development of Dactylorhiza majalis (Orchidaceae) with and without a mycorrhizal fungus.* Plant, Cell & Environment, 1990. **13**(2): p. 171-177.

In vitro germination and development of D. majalis seeds in the presence of a Rhizoctonia strain was very temperature dependent. There was a marked decline in germination percentage at temperatures more than 23-25 deg C. Seeds that germinated at higher temperatures exhibited only slight or no mycorrhizal development and developed few or no rhizoids compared with seedlings raised at optimal or lower temperatures. When 6-week-old seedlings were grown for an additional 4 weeks at temperatures ranging from 10 deg to 28 deg the rate of increase of seedling length was highest at 23-24.5 deg ,. When grown at 26 deg , seedlings had smaller starch reserves than those grown at lower temperatures and increased in length as much as those kept at 13 deg . At 23-24.5 deg , seedlings grew to a larger size before shoot initiation than those kept at higher or lower temperatures. At 23-24.5 deg seed germination in the presence of Rhizoctonia was about double that in the absence of the fungus, and seedling length increased at 45% per week in the presence of the fungus compared with 30% in its absence

53. Tsutsui, K. and M. Tomita, *Effect of plant density on the growth of seedlings of Spiranthes sinensis Ames and Liparis nervosa Lindl. in symbiotic culture.* Journal of the Japanese Society for Horticultural Science, 1989. **57**(4): p. 668-673.

Seeds of each species were cultured with Rhizoctonia isolates on a medium containing a decoction of 25 g oats/litre and 1% agar, and the seedlings were transplanted to fresh medium at densities ranging from 1 to 10 per 60 ml medium. At high densities, the weights of individual seedlings after 10-16 weeks were

proportional to the volume of medium per plant. At densities more than 1 plant/60 ml for S. sinensis and more than 2 plants/60 ml for L. nervosa, the rate of FW increase decreased with increasing seedling density. Mycelium DW per culture flask also decreased with increasing seedlings density. The density effect was thought to be the result of competition for nutrients in the medium, rather than competition for space between the aerial parts

54. Mitchell, R.B., *Growing hardy orchids from seeds at Kew.* Plantsman, 1989. **11**(3): p. 152-169.

The symbiotic and asymbiotic techniques used at the Royal Botanic Gardens for raising hardy terrestrial orchids are described. Details are given of the isolation and storage of mycorrhizal fungi; seed collection, sterilization, sowing and germination media; transferring and growing-on protocorms; natural growth cycles of seedlings; weaning seedlings in the greenhouse; and establishing seedlings outdoors

55. Wilkinson, K.G., K.W. Dixon, and K. Sivasithamparam, *Interaction of soil bacteria, mycorrhizal fungi and orchid seed in relation to germination of Australian orchids.* New Phytologist, 1989. **112**(3): p. 429-435.

Endotrophic bacteria were isolated from the mycorrhizal tissues of 12 of 13 species of Western Australian terrestrial orchids tested. The bacteria were placed into 8 groups based on UV light fluorescence, Gram staining and colony characteristics. The most commonly isolated bacteria from 9 of the 12 orchid species sampled were strs within the Pseudomonas fluorescens-putida group. The abundance of bacteria followed a seasonal pattern that differed between orchid genera especially on the basis of the morphology of fungus infected tissue. There was little evidence of specificity of bacterial groups to orchid taxa or part of the plant infected by the fungus. Symbiotic germination of Pterostylis vittata seed in association with 7 bacterial isolates showed a significant promotion of germination and seedling development with 3 bacterial strs. The influence of a fourth str. was no different to the control while the remaining 3 strs significantly suppressed seedling development

56. Masuhara, G. and K. Katsuya, *Effects of mycorrhizal fungi on seed germination and early growth of three Japanese terrestrial orchids.* Scientia Horticulturae, 1989. **37**(4): p. 331-337.

Effects of mycorrhizal fungi on germination and the early growth stage of Spiranthes sinensis var. amoena, Ponerorchis graminifolia [Gymnadenia sp.] and Bletilla striata were investigated in vitro. Fungi isolated from protocorms or roots of 2 wild and 4 cultivated orchids were used in the germination test with mature seeds of the 3 species on oatmeal agar. Promotion of germination and stimulation of protocorm growth were observed in S. sinensis var. amoena and protocorm growth was also observed in P. graminifolia. However, the symbiotic method was not effective for protocorm growth in B. striata