



Techniques *for* **Tasmanian native orchid germination**

for the Department of Primary Industries and Water



Jasmine Janes

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Jasmine K Janes

Resource Management and Conservation Division
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More than a third of Tasmania's native orchid flora and more than half of its endemic orchids are considered threatened. Tasmania has 68 orchid species listed as threatened under the *Threatened Species Protection Act 1995*. Over half of these species are endemic to Tasmania and nationally listed as threatened under the *Environment Protection and Biodiversity Act 1999*. However, in comparison to other Australian states with large numbers of threatened terrestrial orchids, conservation targeted research programs have been rare in Tasmania with funding for active research limited. For example between 2003 and 2006 there was no specific orchid conservation program operating in Tasmania.

In 2006 the Threatened Species Section (Department of Primary Industries and Water) secured two years NRM funding to implement the *Threatened Tasmanian Orchid Flora Recovery Plan 2006-2010*. The aim of the orchid recovery plan is to improve the conservation status of native Tasmanian orchids and its implementation involves wide ranging *in situ* recovery actions. An additional priority of the orchid recovery plan is to establish a threatened orchid ex situ seed bank and investigate the mycorrhizal associations of these species in collaboration with the

Tasmanian Seed Conservation Centre (Royal Tasmanian Botanical Gardens).

The use of skilled volunteers in orchid conservation is becoming increasingly important because of the sheer scope of the effort needed, and a strong community interest in orchid conservation. In particular, The Australian Native Orchid Society (ANOS) has been extremely active in assisting managers and botanic gardens in establishing and maintaining "ex situ" orchid collections. Presently there is no ANOS chapter in Tasmania, however the newly formed Threatened Plants Tasmania (TPT) (Wildcare Inc.), established in conjunction with the Threatened Species Section, has made Tasmanian orchid conservation their priority and recently secured funding (through a Threatened Species Network grant) to make it their flagship project. This manual was developed as a practical training aid for educational purposes and interested TPT volunteers, consultants and scientific researchers. It is hoped that the practical outcomes from this manual will strengthen the ongoing relationship between the numerous collaborators that will assist in the growth and maintenance of the collection after the completion of the *Threatened Tasmanian Orchid flora Recovery Plan 2006-2010*.

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Australian Government



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I Background

Orchids belong to one of the most species rich and floristically diverse families in the world. Orchids are believed to have spread from the Malaysian tropics during the Cretaceous (65-144 mya), the geographical range now extending from Alaska through to subantarctic Macquarie Island (Dressler 1981, Rittershausen 2002). The vast colonising capacity of orchids is attributed to their dust-like seed. A single orchid capsule may contain between 1,000–100,000 seed (Selosse *et al.* 2002), although fruit set in some orchid species is often low due to a unique pollination system, which relies on sexually deceiving male insects (Cozzolino and Widmer 2005). Further complicating the issue, orchid seeds are almost devoid of reserves (Selosse *et al.* 2002). Consequently, the undifferentiated embryo relies on a symbiotic fungal infection for its carbon, water and nutrient supply (Batty *et al.* 2001).

Similar to other plant species, orchid germination begins with the seed imbibing water however, at this point, a fungus penetrates the testa of the seed and enters either through the epidermal hairs or the suspensor of the undifferentiated embryo (Masuhara and Katsuya 1994). After invagination of the plasma membrane

the fungus forms hyphal coils that feed the plant, allowing for a heterotrophic form of growth known as “mycotrophy” or “myco-heterotrophy” (Selosse *et al.* 2002). Initial contact between the fungus and the seed appears to be haphazard with no attractant released by the orchid, although seeds do appear to be able to resist entry from incompatible fungi (Clements 1988). Thus, initial infection can have one of three results: the fungus and embryo form a functional mycorrhiza, the fungus colonizes all plant tissues and parasitizes them, or infection does not occur. Subsequently, some seeds germinate and form a protocorm, some senesce and others fail to germinate, presumably because of a lack of recognition or specificity between the orchid seed and fungus.

It is not clear how long the orchid-fungus relationship is maintained during seedling development. Typically orchids become chlorophyllous and thereby capable of photosynthesizing, although some species are saprophytic (Bayman *et al.* 2002). Regardless of habit, the vast majority of orchid species retain the mycorrhizal fungus or fungi for life, restricting them to the velamen of the root in epiphytic species or the collar cells of the tuber in terrestrial species (Smith and Read 1997). It is thought that this arrangement is particularly

useful for Australian terrestrial species that undergo prolonged periods of dormancy because the orchid can continue to acquire inorganic nutrition whilst supplying the fungal partner with carbon in return (Irwin *et al.* 2007). Furthermore, orchid mycorrhizal associations typically have a degree of specificity. For example, some relationships show absolute specificity or a “one orchid-one fungal species” arrangement (i.e. *Glossodia major*) (Warcup 1971), others exhibit taxon-level specificity, where one orchid species is infected by more than one member of the same fungal genus/family (i.e. *Microtis parviflora*) (Warcup 1981). Alternatively, non-specificity, or the presence of a range of unrelated fungal taxa in a single orchid species, has also been reported (i.e. *Caladenia reticulata*) (Warcup 1971, 1981). Absolute and taxon-level specificity are suspected to be responsible for the low recruitment rates of many orchid species as a result of the patchy distribution of specific mycorrhiza (Brundrett *et al.* 2003).

There are currently 210 formally recognised species in Tasmania from 34 genera (Buchanan 2007). Approximately 30% of these orchids are endemic, occurring only in Tasmania, and 15% of Tasmanian orchids are recognised as threatened on the *Commonwealth Environmental Protection and Biodiversity Conservation Act 1999* and the *Tasmanian Threatened Species Protection Act*

1995. Very little orchid research has been conducted within Tasmania, but research on other terrestrial orchids in the Southern Hemisphere suggests that orchid species have highly variable population sizes and display irregular patterns in flowering, pollination, germination and dormancy (Feuerherdt *et al.* 2005, Coates *et al.* 2006, Gregg and Kéry 2006). Such studies also indicate that habitat loss, human induced changes in fire frequency, introduced and invasive plant species (Jones *et al.* 1999), casual picking of flowers grazing and digging from animals and the use of fungicides are all recognised threats to the current and future management of orchids (Dockrill 1992, Jones *et al.* 1999, Feuerherdt *et al.* 2005, Flora Recovery Plan: Tasmanian Threatened Orchids 2006). With such a high proportion of endemic and endangered orchid species it is important that the collection of orchid seed and its associated mycorrhizal fungi begins in Tasmania. Several other Australian states have made significant progress in the collection of their orchid flora, illustrating that orchid seed and fungal collection requires accurate, detailed knowledge of population locations, anthesis, pollination ecology and fungal isolation techniques. This document has been compiled from several sources and details the techniques required to successfully propagate Tasmanian native orchids from seed to re-introduction into selected native habitat.

2 Legal requirements and field hygiene

The collection of threatened flora (or fauna) is illegal without a permit issued under the provisions of the *Tasmanian Threatened Species Protection Act 1995*, risking prosecution and/or fines if collection is undertaken without a valid permit. Threatened flora includes species that are listed on the schedules of the Act as endangered, vulnerable or rare. The list of threatened species and associated information can be found at <http://www.dpiw.tas.gov.au/threatenedspecieslists>.

In relation to native orchid work, a collection permit is required prior to the collection of threatened orchids and threatened orchid seed, and prior to the disturbance of surrounding soil and leaf litter regardless of tenure (i.e. even if collecting from private property a permit is required). The collection of non-threatened orchid species requires an authority when collecting from Crown Land or land that has been reserved under the *Nature Conservation Act 2002*. However, due to the difficulty associated with orchid identification it is recommended that a precautionary permit to collect is obtained from the Tasmanian Department of Primary Industries and Water. The Department will be able to advise regarding permit requirements. A precautionary permit will protect the collector from

legal action if a threatened species is mistakenly collected, **but it is important to get the identification of species verified and never collect any orchid species extensively**. Permits may include conditions regarding limitations on the amount of material that can be collected and provision of data to be entered into the Natural Values Atlas. Species identifications can be checked by staff at the Tasmanian Herbarium and Threatened Species Section (Dept. Primary Industries and Water). Figure 1 provides an overview of the process for obtaining a collection permit.

When working with threatened orchid species (and all other species) it is important to develop and use good field hygiene techniques. This requires some basic knowledge of fungal infections, diseases and invasive weed species that could be introduced into a new area, or spread further within an existing area. For example, fungal infections such as *Phytophthora cinnamomi* and Chytrid Fungus are easily carried between areas on dirty shoes and can have devastating effects on areas of native flora and frog populations. Invasive weed species often out-compete native species and negatively impact on biodiversity. Weedy species can be introduced and spread into new areas through seeds

that get into socks, packs and shoes. For these reasons it is important to wash down shoes and equipment (a thorough scrubbing, drying and spray with methylated spirits), and check for seeds and other foreign plant material between field trips. In some instances it may be necessary to carry a small spray bottle of methylated spirits to clean equipment and clothing between sites. Further reading on the above topics is recommended to ensure that a basic understanding is gained.

Additional information can be obtained from the following websites:

DPIW weeds, pests and diseases page:
<http://www.dpiw.tas.gov.au/inter.nsf/ThemeNodes/DREN-4VH82R?open>

DPIW native plants and animals page:
<http://www.dpiw.tas.gov.au/inter.nsf/ThemeNodes/SSKA-4X33SG?open>

Australian Government Department of Environment, Water, Heritage and the Arts Environmental Protection and Biodiversity Conservation page:
<http://www.environment.gov.au/epbc/index.html>

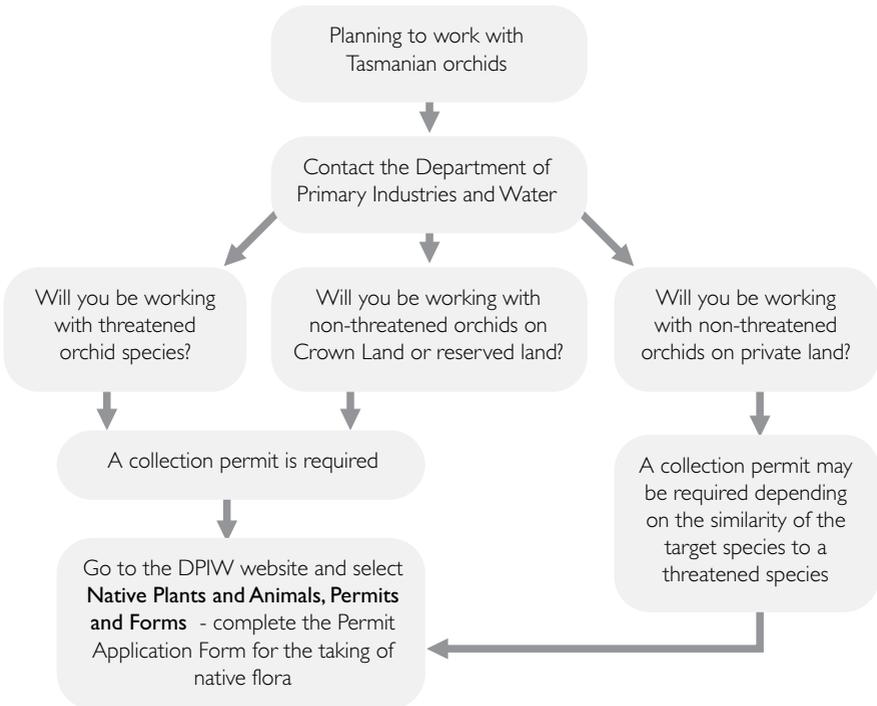


Figure 1. Flow chart outlining the process of obtaining a collection permit.

3 Hand pollination and collection of mycorrhizae from the field

You will need:

- A valid collection permit if required
- Pencil and paper
- Permanent marker
- A hand held GPS
- Camera
- Ruler
- Scalpel/razor blade
- Ethanol/methanol
- Water
- Flagging tape (also called surveying tape)
- Small tubes/bottles
- Toothpick or small stick
- Paper towel
- Plant press if on an extended field trip
- Esky and cooler blocks if on an extended field trip

Methodology

1. Locate orchid populations and confirm the identity of the species.
2. **Collect herbarium specimens:** If the population size is sufficient (i.e. minimum of 30 individuals), three (3) voucher specimens should be collected. If the population size is not sufficient, one (1) voucher specimen should be collected and photographed. Photograph the plant with the ruler next to it to provide a scale prior to removal. Photograph the floral parts and leaf of the orchid with the ruler alongside from various angles. Remember, that the labellum (lip) and column (reproductive structures) can be important in the identification of certain orchid species, so be sure to include representative photos of these structures.
3. **Dig down to collect fungi:** Before removing the individual vouchers, carefully dig around the base of the plant to expose the roots, collar or tuber (see Figure 2). If the orchid is a *Caladenia*, the voucher may be picked just below the collar. The collar is a small nodule just below the leaf and soil level that contains fungal pelotons. All other species require small sections of

root tissue to be cut away. Try to collect from three individuals. To avoid contaminating the orchids, rinse the scalpel or razor blade in ethanol/methanol between uses.

4. **Replace the tuber:** Be very careful to replace the tuber, cover with dirt and pour a little water directly on top to minimise water loss. This will maximise the chance of survival.
5. **Store the fungi:** Brush excess dirt off of root or collar material, wrap in moist paper towel and place inside a small collection tube. Label this tube with the date, location and species. Keep tubes cool if you are on an extended field trip.
6. **Store the vouchers:** Voucher specimens can be stored in a small collection tube with water (like a vase of flowers) or wrapped loosely in damp paper towel and kept cool. Label the tube or paper with the date, location and species. Otherwise, flowers can be pressed immediately and labelled with the date, location and species.
7. **Hand pollinate:** The remaining plants may now be hand pollinated. Using a toothpick or small stick, remove the pollinia from the column (four large yellow or cream packets) of one individual. The pollinia will adhere to the pollinating stick. Move to the next individual and smear the pollinia across the stigma (a small disc

below the pollinia). Once some pollen is visible on the stigma the individual is considered pollinated. Repeat this process for as many individuals as possible, a minimum of 10 and a maximum of 20.

NOTE: hand pollination is not necessary for Prasophyllum species and in some instances human scent appears to attract herbivores. If hand pollinating try to minimise contact with the plants and/or erect small chicken wire enclosures around pollinated individuals.

8. **Mark and record:** Because flowering orchids and developing seed capsules (fruits) can be difficult to locate, it is necessary to mark the location of each pollinated individual. Tie flagging tape to surrounding vegetation, or to each individual. If the orchid is too small to tag, push a stick into the ground next to it and tie flagging tape to that. For small orchid species such as *Corunastylis* the plastic clips from commercially bought bread bags are ideal for tagging individuals, simply place the tag around the base of the plant.

Record the following in a notebook:

- Date
- Location
- Name of collector
- GPS coordinates in WGS84 latitude/longitude and GDA94 easting/northing
- Altitude
- Species name
- Number of individuals within the population
- Area occupied by the population (m²)
- Geology
- Soil texture (e.g. sand, clay, silt, rock, firm, peat)
- Aspect (e.g. north, southwest)
- Drainage (e.g. good, medium, poor)
- Slope (e.g. approximation of slope degrees)
- Associated species (e.g. *Eucalyptus ovata*, *Lomandra longifolia*)
- Vegetation type (e.g. wet sclerophyll, grassland, coastal heath)

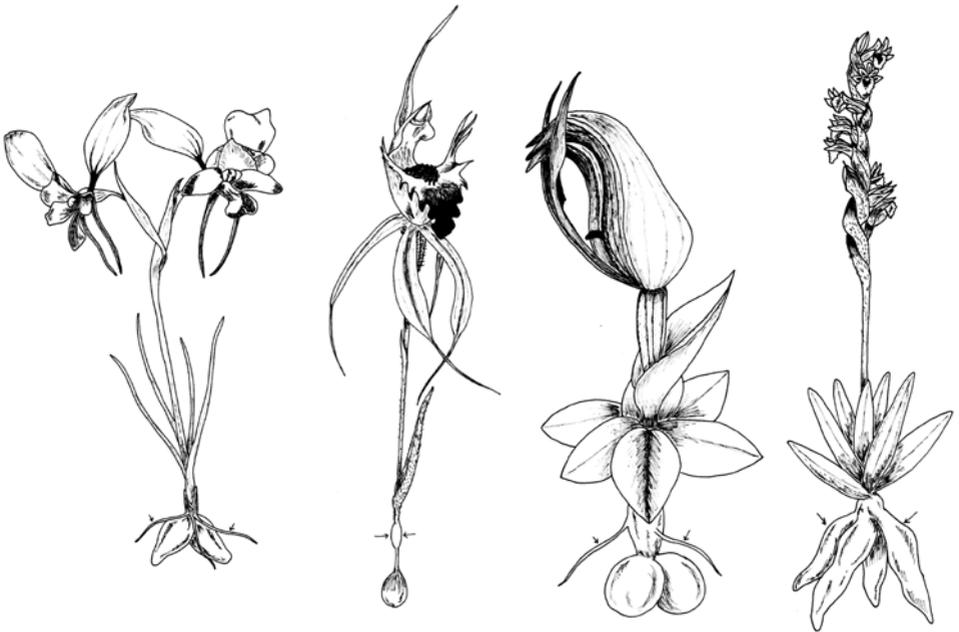


Figure 2. Sites of root, collar or tuber infection for representative orchid genera – *Diuris*, *Caladenia*, *Pterostylis* and *Spiranthes*.

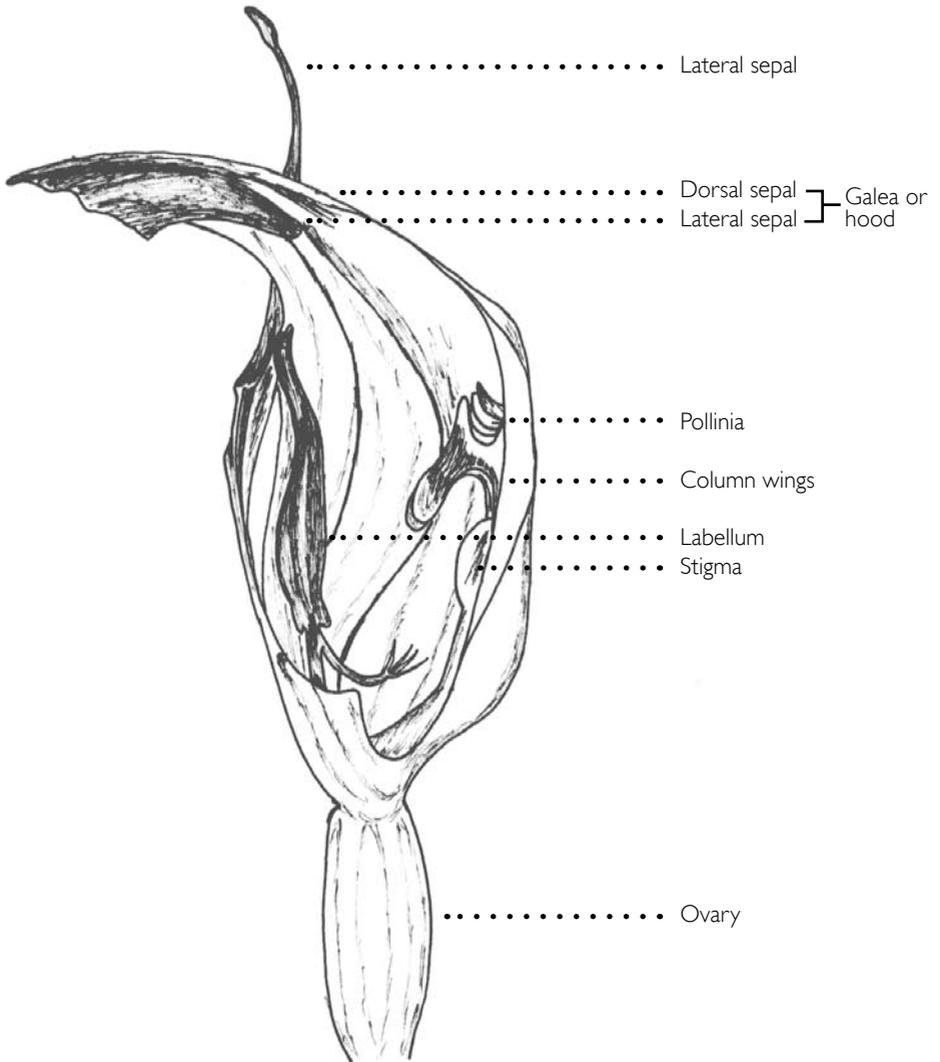


Figure 3. Location of pollinia in relation to the stigma using a dissected specimen of *Pterostylis alata*

4 Collection of seed from the field

You will need:

A valid collection permit if required

Pencil and paper

Permanent marker

Small tubes/bottles

Hand held GPS

Water

Methodology

1. **Re-locate pollinated populations:** Approximately 4-6 weeks after pollination, return to the site to collect developing seed capsules (fruits). Use the GPS to assist in relocation. When close to the coordinates begin looking for the flagging tape that marks individual locations.
2. **Collect developing capsules:** Successfully pollinated individuals will have a swollen green ovary below the dead flower. These individuals should be picked and all flagging tape removed from the location. If ovaries are not very swollen, leave the site intact and return in 2 more weeks to collect the capsules.
3. **Store the capsules:** Capsule stalks should be placed in a small tube or bottle with some water in the bottom (like a vase of flowers). Label the tube with the date, location and species. Refer to Figure 3 for a diagrammatic representation.
4. **Allow the seed to ripen:** Once back in the lab, allow the seed to continue ripening by placing the tube on a sunny windowsill. Check the level of water in the tube daily and do not allow all of the water

to evaporate – the seed will die. Once capsules begin to brown, place them into a labelled paper envelope. The capsule will split and the seeds will be released. Leave the seeds in the paper envelope for 1 month then place seeds into a small vial for 1 month (or indefinitely) at 4°C. Remove the capsule and prepare it for the Herbarium.

- NOTE:** An alternative, although not as popular; collection method using commercial tea bags can be used. Tea bags should be cut open and emptied of tea leaves. These bags can then be placed over the pollinated flowers and tied at the base. This method allows the capsules to develop and dehisce naturally whilst collecting the seed in the tea bag. Seed bags can be collected after 6-8 weeks. This method is not used widely because anecdotal evidence suggests that herbivores are attracted to the tea bags and the seed may become mouldy if weather conditions are bad.

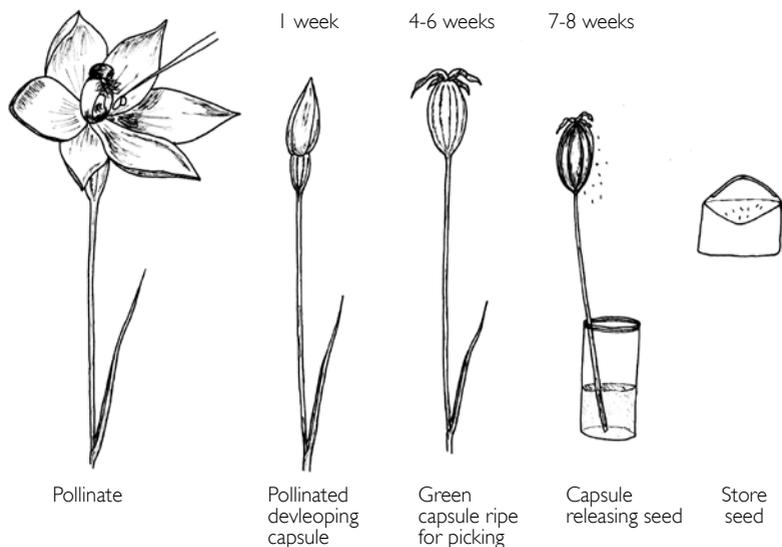


Figure 4. Seed collection schedule (top), example of swelling ovary and developing seeds in *Thelymitra* (above)

5 Isolation of fungal pelotons from orchid tissue

You will need:

Tap water
Autoclave or pressure cooker
Sterile water (deionised and autoclaved for 20 mins at 121°C)
SSE + streptomycin media in petri dishes **OR** FIM + streptomycin media
Permanent marker
Scalpel and forceps
Laminar flow cabinet
A bin
Dissecting microscope and light source
Plastic petri dishes (empty)
Bunsen burner
Ethanol/methanol
20-200µl pipette
Micocapillary gel loading tips
Masking tape
Small screw top tubes/bottles
Latex gloves (optional)

Methodology

1. **Setup:** Spray the laminar flow bench with ethanol/methanol and wipe down with paper towel. Turn on the laminar flow cabinet and Bunsen burner. Place a bin next to the laminar flow. Set up the dissecting microscope inside the laminar flow. Place all other necessary equipment towards the sides of the laminar flow cabinet. Spray your hands with ethanol/methanol and rub together; alternatively you can use latex gloves. A laminar flow cabinet will pump out clean air from the back, the air travels towards you. If anything is in between your working area and the back of the bench, contaminants can be spread by the movement of the air. Always place objects to the sides. **Remember: if you leave the bench and touch other objects, you must put on new gloves or re-spray your hands on return. This method relies on using aseptic techniques – that means being as sterile as possible at all times.**
2. **Rinse tissue:** Remove orchid tissue from tubes, one at a time so that samples are not mixed up. Rinse the tube and gently wash the orchid tissue under running tap water. Place the tissue back into

the appropriately labelled tube. At this point copy the label details onto a strip of masking tape and wrap this around the collection tube. Move all cleaned samples to the side of the laminar flow.

3. **Sterile water tubes:** Prepare sterile water tubes (these should have been autoclaved). For each sample tube, 3 sterile water tubes are required. This results in 3 serial dilutions for the orchid tissue. Place these to the side of the laminar flow, towards the back so that contaminants are not blown onto them.
4. **Sterilise:** Dip the scalpels and forceps into ethanol/methanol and then run through the blue portion of flame of the Bunsen burner. Make sure that all ethanol/methanol has evaporated and place the tools to the side of the laminar flow.
5. **Serial dilutions:** Using the sterile forceps, remove rinsed orchid tissue from collection tubes and place into one of the sterile water tubes, replacing the lid quickly. Remove the masking tape label from the **collection tube** and wrap around the first sterile water tube. Let each sterile rinse tube containing orchid tissue sit for approximately 3 minutes, and then gently shake it. Sterilise the forceps (step 4) and remove the orchid tissue from **rinse tube 1**. Place the tissue into **rinse tube 2** and apply the masking tape label. Let rinse tubes sit for 3 minutes and gently shake. With sterile forceps, remove the orchid tissue from rinse tube 2 and place it into **rinse tube 3**, applying the masking tape label. Let sit for 3 minutes and gently shake. Remember, the collection tubes may contain multiple pieces of orchid tissue; all pieces of tissue from one collection tube may be placed in each sterile rinse. Repeat this process for each collection tube sample, ensuring that each sample has been rinsed in sterile water 3 times. Do not reuse sterile water tubes for different samples and do not leave used tubes in front of where you are working, this will lead to contamination.
6. **Clean the workspace:** Remove all of the used sterile rinse tubes from the laminar flow to avoid clutter and contamination. These will be washed, filled with deionised water and autoclaved later to sterilise them. Spray the bench with ethanol/methanol and wipe down with paper towel to remove any bacteria.
7. **Isolating fungal pelotons:** Place one side of an empty petri dish under the dissecting microscope. Make sure nothing is placed in front of the microscope under the laminar flow. Place a gel loading tip on the pipette and, using a fresh tube of sterile water, pipette some into the middle of the petri dish. The

droplet should be the size of a 20 or 50c piece. Take one of the rinse tubes containing orchid tissue, pick out one piece using sterile forceps and place it into the water droplet. Replace the lid on the rinse tube. With sterile forceps, hold the piece of orchid tissue steady. Take the sterile scalpel in hand, look down the microscope and begin gently scraping the blade along the section of orchid tissue. Fungal pelotons should burst out of the orchid tissue and begin floating in the sterile water droplet. The pelotons will look like small grains of sand, white or cream in colour. Remove the orchid tissue from the water and throw it in the bin.

8. **Serial dilution of pelotons:** Take the pipette and using the same tip, place another sterile water droplet to the side of the petri dish (the size of a 10c piece). Pick up pelotons from the **isolation droplet**, trying not to pick up too much water; and eject them into the fresh **rinse droplet 1**. Try to transfer approximately 30 pelotons to rinse droplet 1. Eject the pipette tip into the bin and replace with a fresh tip. Place another sterile water droplet to the side of the petri dish. Transfer approximately 20 pelotons from rinse droplet 1 to **rinse droplet 2**, trying to minimise the amount of water being transferred. Using a fresh pipette tip, prepare **rinse droplet 3**. Transfer approximately

10 pelotons from rinse droplet 2 to rinse droplet 3.

9. **Plate out pelotons:** Take one of the prepared SSE + streptomycin **OR** FIM + streptomycin plates (petri dish with lid) and check it for contamination (mould, coloured spots). If the plate is contaminated throw it in the bin. If the plate is clean, label the plate lid with the species name, location, plate number and date. With a fresh pipette tip pick up a single peloton and place it in the middle of the plate. Repeat this process (using the same pipette tip) so that 6-7 more pelotons are placed in a circle around the plate. Try to replace the lid of the plate between each peloton placement to minimise contamination.

Note: Each collection tube should contain 3 pieces of orchid tissue. Each piece of tissue will undergo separate isolation, diluting and plating. Thus, there should be three isolation plates for each species collected at a given location. This will provide a representative spread of fungal diversity and will provide enough material to account for the occasional contaminant.

10. **Dry the plate:** Once finished, place the plate directly in front of the microscope with the lid slightly to one side. This will allow the moisture to evaporate and minimise potential bacterial growth.

Provided that no other object is in front of the isolation plate, the air blowing on to it will be clean.

11. **Repeat steps 8-9:** Repeat the dilution and plating process for all collection tubes, ensuring that each collection tube results in 3 plates, one per piece of tissue. Once the second plate is ready for drying, the first plate should be dry. Replace the lid of the dry plate and place it to the side of the laminar flow cabinet making sure that nothing else is in front of it. Plates may be stacked on top of each other. See Figure 4 for a diagrammatic representation.

12. **Store plates:** Put the isolation plates into a plastic sleeve. Label this sleeve with your initials, the date, the type of media (i.e. SSE + Strep) and the temperature they will be stored at.

13. **Monitor plates:** After one week the isolation plates should be checked. Some species of fungi grow more quickly than others. Fungus associated with *Pterostylis* (Greenhoods) grows typically within 3 days, *Caladenia* (finger and spider orchids) fungus grows in about 8 days and other fungal types can take up to 1 month. Fungal growth will appear as a white/cream circle with little branches (hyphae) growing out of it. Bright yellow or pink spots are evidence of bacteria.

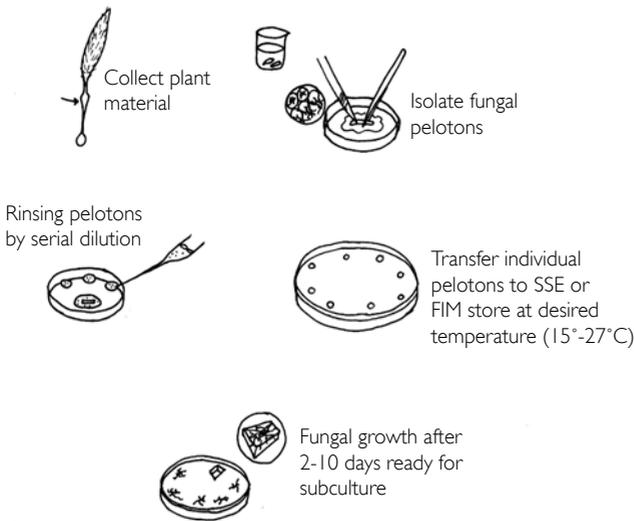


Figure 5. Overview of the fungal isolation process

6 Subculturing fungi to SSE or FIM – first subculture

You will need:

Laminar flow cabinet
Bunsen burner
Matches/lighter
Scalpel and forceps
SSE + streptomycin media OR FIM + streptomycin in petri dishes
Permanent marker
Ethanol/methanol

Methodology

1. **Setup the laminar flow:** Spray the laminar flow bench with ethanol/methanol and wipe down with paper towel. Turn on the laminar flow cabinet and Bunsen burner. Place isolation plates (those with fungi growing) and fresh plates to one side of the bench. Spray your hands with ethanol/methanol or wear latex gloves. Sterilise the scalpel and forceps by dipping them in ethanol/methanol and running them through the Bunsen burner flame. Make sure that all ethanol/methanol has evaporated.
2. **Check fungal growth:** Without removing the petri dish lid, observe the surface of the agar. Select one peloton that has grown sufficiently and cleanly. The fungus will have a fluffy white/cream centre with small branches growing out of it. These branches are the growing edge of the fungal culture.
3. **Subculture:** Remove the lid of the fungal plate. Using the sterile scalpel, cut a small section of the agar that contains the growing edge of the fungus. Remove this section, close the plate lid and transfer the section to a fresh plate of SSE or FIM. Label the plate lid with your initials, the date, species

- name, location and media type. Place the old isolation plate to one side and the subcultured plate to the other side of the laminar flow bench to prevent contamination.
4. **Repeat steps 2-3:** Repeat the process for all fungal isolates. If some plates have failed to grow fungi, but they are uncontaminated, keep them with the old isolation plate pile. If some plates are badly contaminated or failed to grow fungi, throw them in the bin. See Figure 4 for a diagrammatic representation.
 5. **Storage:** Put the old isolation plates back into the plastic sleeve they came out of and return to original growing conditions. Place the subcultured plates into a separate plastic sleeve and label with the type of media, storage temperature, your initials and the date. Put the subcultures into their respective growing conditions.
 6. **Monitor plates:** After one week the subculture plates should be checked. Remember that some orchid mycorrhiza grow faster than others. The size of the growth should be at least the size of a 5c piece before the second subculturing can take place. If subculture plates are heavily contaminated it may be necessary to repeat the first subculturing process or perform Subculturing of contaminated fungi. Record all observations.

7 Subculturing fungi to PDA – second subculture

You will need:

Laminar flow cabinet
Bunsen burner
Matches/lighter
Scalpel and forceps
Ethanol/methanol
Permanent marker
PDA media in petri dishes
Parafilm

Methodology

1. **Setup the laminar flow:** Spray the laminar flow bench with ethanol/methanol and wipe down with paper towel. Turn on the laminar flow cabinet and Bunsen burner. Place first subculture plates (those with fungi growing) and fresh plates to one side of the bench. Spray your hands with ethanol/methanol or wear latex gloves. Sterilise the scalpel and forceps by dipping them in ethanol/methanol and running them through the Bunsen burner flame. Make sure that all ethanol/methanol has evaporated.
2. **Check fungal growth:** Leaving the lids on the subculture plates, check the fungal growth for signs of bacterial contamination. If subculture plates are heavily contaminated you should refer to the Subculturing of contaminated fungi section. Record all observations. Pure fungal subculture plates should be stored to the side of the laminar flow cabinet ready for the second subculture.
3. **Subculture:** Remove the lid of the first subculture plate. Using the sterile scalpel, cut a small section of the agar that contains the growing

edge of the fungus. Remove this section, close the plate lid and transfer the section to a fresh plate of PDA. Label the PDA plate lid with your initials, the date, species name, location and media type. Place the first subculture plate to one side and the second subcultured plate to the other side of the laminar flow bench to prevent contamination.

4. *Repeat steps 2-3:* Repeat the process for all fungal subcultures. If some subcultures have failed to grow fungi, but they are uncontaminated, keep them with the first subculture plate pile.
5. *Storage:* PDA plates should be double wrapped with parafilm around the lid to prevent mites entering the plate. Label the second subculture plates with the date, media type, species, location, plate number etc. Store the plate at the growing temperature used for the first subculture. After 2 weeks place the second subculture plates into a fridge/incubator at 4°C to slow the fungal growth. Plates can be kept for several months in these conditions.

Note: Due to space limitations, the long term storage of second fungal subcultures may be in 20ml tubes on PDA slopes. These tubes will be prepared so that the PDA runs down the tube on an angle. The section of fungi will be placed at the bottom of the slope so that the fungus can grow up the slope. The storage conditions will remain the same. The slopes need to be monitored monthly to ensure that moisture does not build up at the bottom and flood the fungus.

8 Subculturing of contaminated fungi

You will need:

Laminar flow cabinet
Bunsen burner
Matches/lighter
Scalpel and forceps
SSE + streptomycin media in petri dishes
Permanent marker
Ethanol/methanol

Methodology

1. **Window plates:** The purification of contaminated fungal samples requires window plates or partial plates. These plates should be prepared in advance following the **Making SSE or FIM protocol** up until step six. At this point, the petri dishes should be laid out as in step seven. A small amount of SSE should be poured toward one side of each petri dish so that a small spill cools and solidifies at one half of the plate. It may be necessary to tilt each petri dish to ensure that the bottoms are only partially covered. Store the plates according to step eight.
2. **Setup the laminar flow:** Spray the laminar flow bench with ethanol/methanol and wipe down with paper towel. Turn on the laminar flow cabinet and Bunsen burner. Place the contaminated subculture plates (those with fungi growing) and fresh plates to one side of the bench. Spray your hands with ethanol/methanol or wear latex gloves. Sterilise the scalpel and forceps by dipping them in ethanol/methanol and running them through the Bunsen burner flame. Make sure that all ethanol/methanol has evaporated.

3. **Subculture:** Remove the lid of the contaminated subculture plate. Using the sterile scalpel, cut a small section of the agar that contains the growing edge of the fungus. Remove this section, close the plate lid and transfer the section to the uncovered side of a fresh window plate (i.e. the side that is not covered by SSE media). Label the window plate lid with your initials, the date, species name, location and media type. Discard the contaminated subculture plate and move the subcultured window plate to the other side of the laminar flow bench to prevent contamination.
4. **Repeat steps 2-3:** Repeat the process for all contaminated fungal subcultures.
5. **Storage:** Window plates should be double wrapped with parafilm around the lid to prevent mites entering the plate. Store the window plates at the growing temperature used for the first subculture. After 1 week check the subcultured window plates. Fungal hyphae should have grown across the petri dish toward the SSE or FIM media. These hyphae should be free of bacterial contamination because the bacteria have nothing to feed on in the bare section of the petri dish. Bacteria free hyphae can now be subcultured to PDA – see **Subculturing fungi to PDA**.

9 Sterilisation and symbiotic germination of orchid seed

You will need:

Sterile water (deionised and autoclaved for 20 min at 121°C)

TWEEN 80

Bleach (Sodium hypochlorite)

Filter paper (Whatman no. 1, 42 mm) or empty tea bags

Paperclips

Masking tape

Permanent marker

Screw top jars or beakers

Laminar flow cabinet

Forceps

Bunsen burner

OMA media in petri dishes

Alfoil

Orchid seed

Compatible mycorrhizae

Parafilm

Methodology

1. **Setup the laminar flow:** Spray the laminar flow bench with ethanol/methanol and wipe down with paper towel. Turn on the laminar flow cabinet and Bunsen burner. Place the required jars and media plates inside the laminar flow to one side of the bench. Spray your hands with ethanol/methanol or wear latex gloves. Sterilise the scalpel and forceps by dipping them in ethanol/methanol and running them through the Bunsen burner flame. Make sure that all ethanol/methanol has evaporated.
2. **Prepare the sterilisation mix:** In a jar or beaker make a 1% mix of bleach in sterile water (e.g. if making 100 ml total solution, add 1 ml of bleach). Add 1 drop of TWEEN 80 to the solution and mix.
3. **Sterilise seed:** Make a small envelope using the whatman filter paper or empty tea bag. Take a small portion of seed from one (species) seed collection and place it inside the filter paper envelope. Make sure the envelope is not too full. Seal the envelope using a paperclip. Using sterile forceps immerse the envelope in sterile water and squeeze out

any air. Remove the envelope and immerse it in the sterilisation solution. Shake or stir the solution jar for 5 minutes. Remove the envelope and place it into sterile water (**rinse 1**), stirring it for approximately 5 mins. Remove the envelope from rinse 1 and place it into sterile **rinse 2** for another 5 mins. If performing several sterilisations for different species, use the masking tape to label each jar with the species name, collection number and location.

4. **Plate out the seed:** Take the sterile envelope and remove the paperclip. Take a prepared petri dish of OMA and remove the lid. Carefully unfold the envelope and smear the seeds across the filter paper surface. Ensure that the seed is spread evenly across the surface. If necessary use the forceps and/or scalpel to spread the seeds further, but **be sure to sterilise them first** (dip them in ethanol/methanol and pass them through the flame of the Bunsen burner).
5. **Inoculate with compatible fungus:** Sterilise the scalpel and remove 2-3 small sections of compatible mycorrhizae from a PDA plate. Place these sections around the filter paper. Replace the lids of each petri dish. Reseal the PDA subculture plate with parafilm (double layer). Label the germination plate with the date, species, collection number, location,

your initials, the fungus details and the growing temperature being used.

6. **Repeat steps 3-5:** Perform steps 3-5 for each species.
7. **Storage:** Wrap each petri dish in foil and label the foil with the same details from the plate lid. Place each plate in the respective growing conditions.
8. **Monitor:** Check petri dishes weekly for signs of contamination and germination. After 8 weeks a small achlorophyllous (yellow due to lack of light for photosynthesis) leaf should be present. This stage of development is known as the protocorm. Protocorms are now ready for potting out. If no leaf is present and the plate is uncontaminated leave the plate for longer.

Note: if the PDA subculture plate is getting low on fungi or the fungal growth has filled the plate, it will need to be subcultured again. Subculture the fungus to a fresh PDA plate following the instructions from Subculturing fungi to PDA – second subculture. This will ensure a continuous supply of fungus.

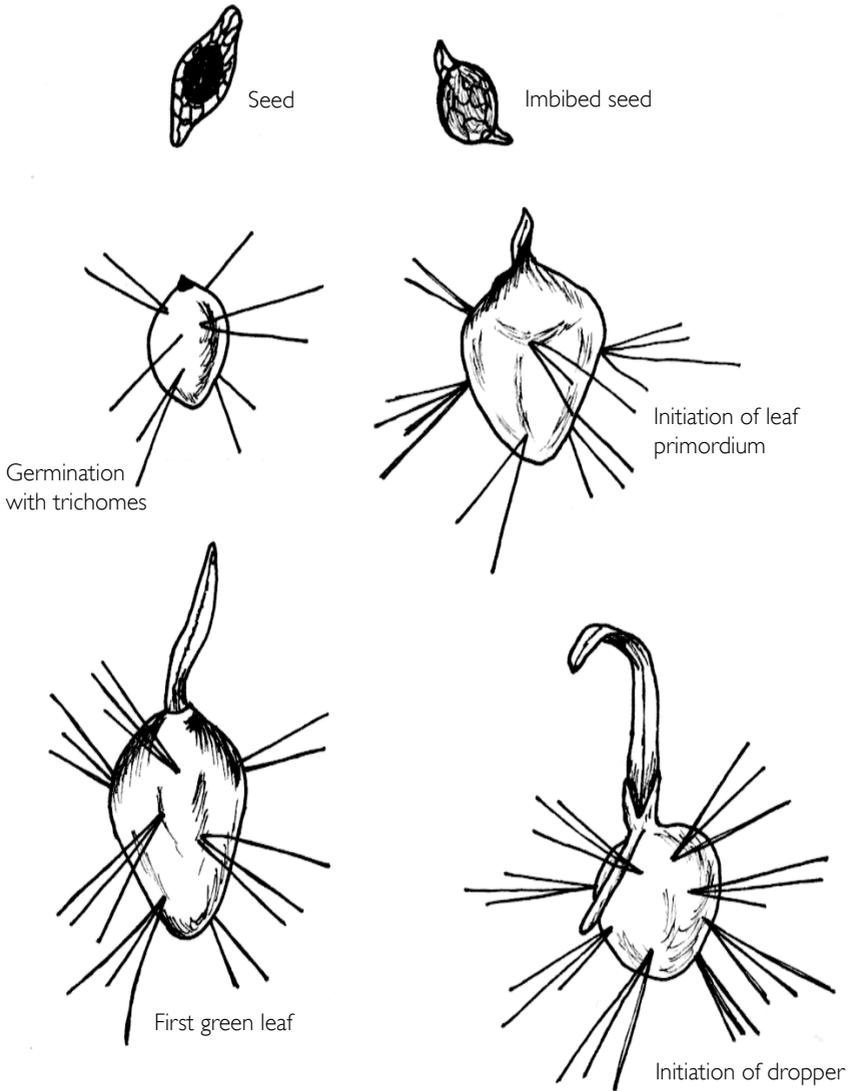


Figure 6. Developmental stages of protocorms

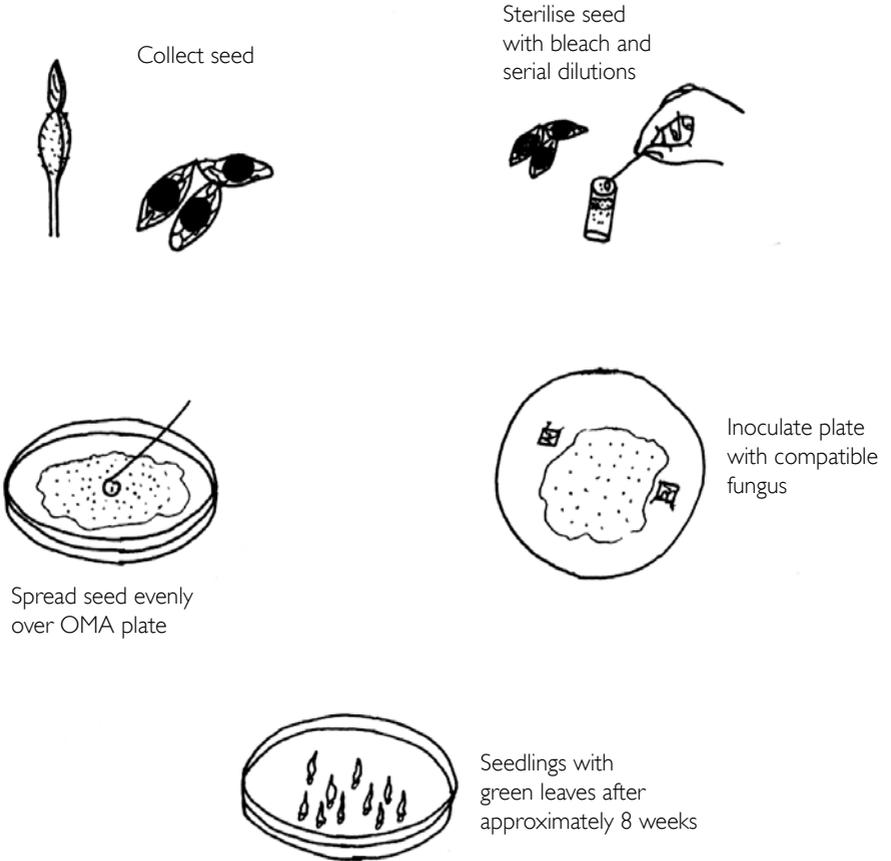


Figure 7. Overview of the seed sterilisation and fungal inoculation process for the symbiotic germination of orchid seed

10 Sterilisation and asymbiotic germination of orchid seed

You will need:

W3 media mixed with banana and coconut water in petri dishes

TWEEN 80

Bleach (Sodium hypochlorite)

Filter paper (Whatman no. 1, 42 mm) or empty tea bags

Paperclips

Masking tape

Permanent marker

Screw top jars or beakers

Laminar flow cabinet

Forceps

Bunsen burner

Alfoil

Orchid seed

Methodology

1. **Setup the laminar flow:** Spray the laminar flow bench with ethanol/methanol and wipe down with paper towel. Turn on the laminar flow cabinet and Bunsen burner. Place the required jars and media plates inside the laminar flow to one side of the bench. Spray your hands with ethanol/methanol or wear latex gloves. Sterilise the scalpel and forceps by dipping them in ethanol/methanol and running them through the Bunsen burner flame. Make sure that all ethanol/methanol has evaporated.
2. **Prepare the sterilisation mix:** In a jar or beaker make a 1% mix of bleach in sterile water (e.g. if making 100 ml total solution, add 1 ml of bleach). Add 1 drop of TWEEN 80 to the solution and mix.
3. **Sterilise seed:** Make a small envelope using the whatman filter paper or empty tea bag. Take a small portion of seed from one (species) seed collection and place it inside the filter paper envelope. Make sure the envelope is not too full. Seal the envelope using a paperclip. Using sterile forceps immerse the envelope in sterile water and squeeze out

- any air. Remove the envelope and immerse it in the sterilisation solution. Shake or stir the solution jar for 5 minutes. Remove the envelope and place it into sterile water (**rinse 1**), stirring it for approximately 5 mins. Remove the envelope from rinse 1 and place it into sterile **rinse 2** for another 5 mins. If performing several sterilisations for different species, use the masking tape to label each jar with the species name, collection number and location.
4. **Plate out the seed:** Take the sterile envelope and remove the paperclip. Take a prepared petri dish of W3 and remove the lid. Carefully unfold the envelope and smear the seeds across the surface of the W3 media, **discard the filter paper**. Ensure that the seed is spread evenly across the surface. If necessary use the forceps and/or scalpel to spread the seeds further; but **be sure to sterilise them first** (dip them in ethanol/methanol and pass them through the flame of the Bunsen burner).
 5. **Repeat steps 3-5:** Perform steps 3-5 for each species.
 6. **Storage:** Wrap each petri dish in foil and label the foil with the same details from the plate lid. Place each plate in the respective growing conditions.
 7. **Monitor:** Check petri dishes weekly for signs of contamination and germination. After 8 weeks a small achlorophyllous (yellow due to lack of light for photosynthesis) leaf should be present. This stage of development is known as the protocorm. Protocorms are now ready for potting out. If no leaf is present and the plate is uncontaminated leave the plate for longer.

*W3 can be obtained from Western
Laboratories, South Australia*

|| Potting out protocorms to OMA and sand

You will need:

Pre-prepared OMA media (0.25% not solid)

Round polycarbonate containers

Silica sand (double sterilised – autoclaved at 121°C for 20 min, rest 24 hrs, 121°C for 20 min)

Forceps and scalpel

Bunsen burner

Laminar flow cabinet

Matches

Ethanol/methanol

Protocorm plates from symbiotic or asymbiotic germination

Autoclave or pressure cooker

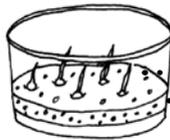
Methodology

1. **Setup the laminar flow:** Spray the laminar flow bench with ethanol/methanol and wipe down with paper towel. Turn on the laminar flow cabinet and Bunsen burner. Place the required jars and media plates inside the laminar flow to one side of the bench. Spray your hands with ethanol/methanol or wear latex gloves. Sterilise the scalpel and forceps by dipping them in ethanol/methanol and running them through the Bunsen burner flame. Make sure that all ethanol/methanol has evaporated.
2. **Prepare OMA containers:** Under the laminar flow, pour OMA into the round containers until approximately 1 cm deep. Put the lids on the containers and autoclave them for 20 minutes at 121°C. Once the autoclave cycle is complete, remove the containers and place them under the laminar flow. Allow the OMA to solidify with the lids on.
3. **Add sand layer:** Once the OMA has solidified, add a 1 cm layer of double sterilised silica sand. Spread the sand evenly. The sand should become moist as it draws moisture from the OMA media. **DO NOT** add more water to the containers.

4. **Transfer protocorms:** Using the sterile forceps and scalpel, remove protocorms from the germination plates one at a time. Place each protocorm into the OMA containers, gently pushing them into the sand. Ensure that the protocorms are evenly distributed throughout the container. Replace the lid and label it with the species name, the date, collection number, your initials, compatible fungi (if used) or asymbiotic status, the location of the collected seed and the growing conditions the container will be placed in. Place protocorm containers to the opposite side of the laminar flow. **Note:** depending on the number of germinated seed, use several OMA containers per germination petri dish to avoid overcrowding and excessive competition between protocorms.
5. **Storage:** Place the OMA growing containers into an appropriate incubator/growth cabinet. Preferably, this should be between 10-20°C and with an alternating light regime of 16 hours light and 8 hours dark. Check the containers weekly for contamination.
6. **Monitor:** Check the containers weekly for signs of contamination and growth. After 4 weeks the seedlings should be ready for hardening.
7. **Hardening:** Containers should be placed inside a glasshouse and left for 5 days with the lid on so that they can adjust to a more natural climate. Seedlings are then hardened for a further 5 days with the lid of the container removed. Seedlings are now ready for transfer to soil.

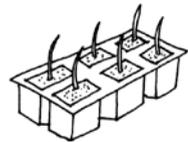


Seedlings ready for potting out after approximately 8 weeks



Seedlings in oma and sand containers for approximately 4 weeks in incubator and 4-8 weeks in glasshouse

••• Silica sand
••• 0.25% OMA



Seedlings in soil after approximately 12 weeks

Figure 8. Overview of the potting out procedure for orchid seedlings

12 Transfer of seedlings to soil

You will need:

OMA containers with orchid seedlings
Autoclave or pressure cooker
Native plant potting mix
Perlite (4 mm diameter)
Seedling punnets/trays
Pot labels
Pencil or permanent marker
Jars or containers

Methodology

1. **Prepare potting mix:** Combine 4 parts of native potting mix with 1 part of perlite in large jars or containers. Autoclave these containers for 40 minutes at 121°C. Once the autoclave cycle has finished, remove the potting mix and take it back to the glass house.
2. **Transfer seedlings:** Fill seedling trays or punnets with sterile orchid potting mix. Transfer one seedling at a time from the OMA growth containers to the seedling trays. If using punnets, plant one seedling per punnet. If using trays, plant each seedling approximately 4 cm apart. Record the number of seedlings of each species that are transferred to soil. Be sure to label seedling trays and punnets so that each species can be readily identified. **Remember:** seedlings will not flower for at least 1 year so it will be impossible to distinguish between species if they accidentally get mixed up.
3. **Storage:** Lightly water the seedling trays and leave them in the glasshouse.

4. **Monitor:** Check the seedlings every few weeks. Record the number of seedlings that have survived. Check that the seedlings are being adequately watered and that common glasshouse pests have not invaded the trays (i.e. slugs, snails, scarid fly). Seedlings may need to be drenched with Mesurool 750 (Methicarb) to deter such pests.
5. **Long term growth:** After 3-4 months of growth in the glasshouse, seedlings can be placed outside if required. At this time, seedlings may also be re-introduced to field sites. It is preferable that the transfer of seedlings to outdoor areas coincides with the onset of the dormant season (Autumn for most species, Spring for most *Pterostylis*).

Appendix I

Preparation of stock solutions for Soil Solution Equivalent (SSE)

You will need:

3 × 1 litre Schott bottles
Scales
Chemicals outlined in Table I
Sterile water
Measuring cylinder/beaker
Masking tape
Permanent marker
Foil
Measuring spoon
Magnetic stirrer
Magnetic stir bar
Forceps (long-handle)

Methodology

1. **Preparation:** Ensure that the 1 L bottles are clean and dry. Collect all of the chemicals required for the solutions and place them near the scales. Tear a strip of masking tape and label the bottles as Stock A etc.
2. **Measurements:** Turn on the scales. Place a small container or section of foil on the scales and re-zero them to account for the weight of the container/foil. Using the spoon, begin weighing the chemicals required for Stock A. Try to make the measurements as accurate as possible. Tip the weighed chemicals into the respective Schott bottle.

Table I. Stock solution chemicals and quantities for SSE media

Chemical	Common name	grams per litre (g/L)
STOCK A		
NH ₄ NO ₃	Ammonium nitrate	0.4
KH ₂ PO ₄	Potassium dihydrogen orthophosphate	0.0136
MgCL ₆ H ₂ O	Magnesium chloride	0.61
NaCl	Sodium chloride	0.058
STOCK B		
CaSO ₄ 2H ₂ O	Calcium sulphate	0.861
STOCK C		
FeEDTA (Na)	Iron	0.073

Once all of the chemicals for Stock A have been weighed and placed in the bottle, all 1 L of sterile water. Continue with Stock B and C.

3. **Mix:** The three stock solutions need to be mixed so that all of the chemicals have dissolved thoroughly. Place the magnetic stir-bar inside the Stock A bottle and place the bottle on the magnetic stirrer. Turn the stirrer on, slowly at first. Once the stir bar has settled into a central swirling motion, increase the spin speed.

4. **Storage:** When all of the chemical powder has dissolved into the solution turn off the magnetic stirrer. Using the long handled forceps, remove the magnetic stir bar. Put the lid on the stock bottle and store in the fridge at 4°C. Rinse the stir bar quickly under sterile water.

5. **Repeat steps 3-4 for Stock B and C.**

Note: Stock solutions will make 10 L of SSE media. Always keep the stock solutions refrigerated between uses. Do not combine the stock solutions unless making SSE – the chemicals can react over time.

Making Soil Solution Equivalent (SSE) media with streptomycin sulphate

You will need:

SSE stock solutions (200 ml of each total)

Sterile water (1400 ml total)

Syringe

Sucrose (4 g total)

Agar (16g total)

Syringe filter – arodisc

4 x 400 ml Schott bottles

2 L bottle

MES buffer (0.4g total)

Streptomycin sulphate

Laminar flow cabinet

Autoclave or pressure cooker

Petri dishes

Permanent marker

Scales

Foil

Measuring cylinder

pH strips

pH buffers

Laminar flow cabinet

Ethanol/methanol

Microwave

Methodology

1. **Make the media:** Make sure that the 2 L bottle and 500 ml Schott bottles are clean and dry. Using the measuring cylinder, put 1400 ml of sterile water into the 2 L bottle. Turn on the scales, place a section of foil on the balance and re-zero. Weigh out 0.4 g of MES buffer and add to the 2 L bottle. Measure out 200 ml each of Stock A, B and C for the 2 L bottle. Weigh out 4 g of sucrose and add to the 2 L mix. Weigh out 16 g of agar and gently swirl once added.
2. **Check the pH:** Using pH strips, dip one into the 2 L bottle. Check the colour of the strip. The media should have a pH of 5.5. Adjust the media accordingly using the pH buffers (hydrochloric acid - HCl or potassium hydroxide - KOH), adding 1-2 drops of the pH buffer at a time. Swirl the bottle gently and check the pH again using a fresh strip. Repeat the process until the pH has reached 5.5.
3. **Melt the agar:** Microwave the bottle for 3 min at a time, watching that it does not boil and overflow. Gently swirl the bottle between each heating. Once the agar has completely dissolved pour the mix into the four 400 ml Schott bottles.

4. **Sterilise the agar:** Loosely screw the lids on and autoclave them for 20 mins at 121°C. Once the autoclave cycle has finished, remove the bottles and place them in a hot water bath until cool enough to handle.
5. **Make the streptomycin solution:** Streptomycin is an antibiotic and should be considered toxic, please be very careful when using this reagent. Using the scales, weigh out 1 g of streptomycin sulphate powder and pour it into a clean, dry bottle (approximately 200 ml in size). Add 70 ml of sterile water to the powder and swirl the bottle until the streptomycin has dissolved. Streptomycin can be made in advance and stored in a bottle covered with foil at 4-5°C.
Note: Streptomycin must always be added to the media after it has been autoclaved – heating streptomycin will inactivate the antibiotic. For this reason, agar media with streptomycin cannot be made in advance and put in the microwave to re-melt it.
6. **Add streptomycin:** Turn on the laminar flow cabinet and wipe down with ethanol/methanol, and place each bottle of media inside. Unwrap an acrodisc syringe filter and place it on the end of a fresh syringe. Add 5 ml of streptomycin to each bottle. Gently swirl each bottle to mix. Place each bottle back into a hot water bath to keep the media in a liquid form.
7. **Plate out the media:** Spray the laminar flow bench with ethanol/ methanol and wipe down. Arrange 3 rows of petri dishes with the lids half off. Take one bottle of media and, starting from the back wall row of the laminar flow, pour media into each dish until the bottom is just covered (about half a cm deep). Once the plates are filled, push the lids forward slightly so that a small gap remains. Leave the plates under the cabinet until the condensation has evaporated and the media has solidified. Starting from the front of the laminar flow, close the lids on the petri dishes and stack them to the side of the cabinet.
Remember: a laminar flow cabinet will pump out clean air from the back, the air travels towards you. If anything is in between your working area and the back of the bench, contaminants can be spread by the movement of the air
8. **Storage:** Place the petri dishes back into a plastic sleeve and seal it. Label the sleeve with the media type (i.e. SSE) and date. Store the bags of petri dishes on a shelf in the lab. Repeat steps 6-7 for the remaining bottles of media.

Making Fungal Isolation Media (FIM)

You will need:

Chemicals outlined in Table 2

Agar 4 g

Streptomycin sulphate 1g

Sterile water 1000 ml/1 L

Sterile water 70 ml

Laminar flow cabinet

1 × 1 L Schott bottle

Petri dishes

Autoclave or pressure cooker

Ethanol/methanol

Scales

Measuring cylinder

Microwave

pH strips

pH buffers

Foil

Syringe filter – acrodisc

Syringe

Methodology

1. **Make the media:** Make sure that the bottles are clean and dry. Turn on the scales, place a section of foil on the balance and re-zero. Weigh out the ingredients from Table 2 and the agar. Combine the ingredients and 1000 ml of sterile water in the 1 L bottle and gently swirl.
2. **Check the pH:** Using pH strips, dip one into the 2 L bottle. Check the colour of the strip. The media should have a pH between 5.0 and 6.0. Adjust the media accordingly using the pH buffers (hydrochloric acid - HCl or potassium hydroxide - KOH), adding 1-2 drops of the pH buffer at a time. Swirl the bottle gently and check the pH again using a fresh strip. Repeat the process until the pH is optimum.

Table 2. Chemicals and quantities for FIM media

Chemical	Common name	grams per litre (g/L)
$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	Calcium nitrate	0.5
KH_2PO_4	Potassium dihydrogen orthorhosphate	0.1
KCl	Potassium chloride	0.05
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	Magnesium sulphate	0.05
$\text{C}_6\text{H}_{12}\text{O}_6$	Sucrose	2.5
	Yeast extract	0.25

3. **Melt the agar:** Microwave the bottle for 3 min at a time, watching that it does not boil and overflow. Gently swirl the bottle between each heating. Once the agar has completely dissolved prepare to sterilise the agar.
4. **Sterilise the agar:** Loosely screw the lid on and autoclave for 20 mins at 121°C. Once the autoclave cycle has finished, remove the bottle and place it in a hot water bath until cool enough to handle.
5. **Make the streptomycin solution:** Streptomycin is an antibiotic and should be considered toxic, please be very careful when using this reagent. Using the scales, weigh out 1 g of streptomycin sulphate powder and pour it into a clean, dry bottle (approximately 200 ml in size). Add 70 ml of sterile water to the powder and swirl the bottle until the streptomycin has dissolved. Streptomycin can be made in advance and stored in a bottle covered with foil at 4-5°C.
Note: Streptomycin must always be added to the media after it has been autoclaved – heating streptomycin will inactivate the antibiotic. For this reason, agar media with streptomycin cannot be made in advance and put in the microwave to re-melt it.
6. **Add streptomycin:** Turn on the laminar flow cabinet and wipe down with ethanol/methanol, and place the FIM media bottle inside.

Unwrap an acrodisc syringe filter and place it on the end of a fresh syringe. Add 10 ml of streptomycin to the 1 L bottle. Gently swirl to mix. Place the bottle back into a hot water bath to keep the media in a liquid form.

7. **Plate out the media:** Spray the laminar flow bench with ethanol/ methanol and wipe down. Arrange 3 rows of petri dishes with the lids half off. Take one bottle of media and, starting from the back wall row of the laminar flow, pour media into each dish until the bottom is just covered (about half a cm deep). Once the plates are filled, push the lids forward slightly so that a small gap remains. Leave the plates under the cabinet until the condensation has evaporated and the media has solidified. Starting from the front of the laminar flow, close the lids on the petri dishes and stack them to the side of the cabinet.
Remember: a laminar flow cabinet will pump out clean air from the back, the air travels towards you. If anything is in between your working area and the back of the bench, contaminants can be spread by the movement of the air
8. **Storage:** Place the petri dishes back into a plastic sleeve and seal it. Label the sleeve with the media type (i.e. FIM) and date. Store the bags of petri dishes on a shelf in the lab. Repeat steps 6-7 for the remaining bottles of media.

Making Potato Dextrose Agar (PDA)

You will need:

PDA powder 13.6 g
Agar 12 g
Sterile water 2000 ml
Laminar flow cabinet
2 L bottle
4 x 400 ml Schott bottles
Petri dishes
Autoclave or pressure cooker
Ethanol/methanol
Scales
Measuring cylinder
Microwave
pH strips
pH buffers
Foil

Methodology

9. **Make the media:** Make sure that the bottles are clean and dry. Turn on the scales, place a section of foil on the balance and re-zero. Weigh out the powder ingredients. Combine the PDA, agar and 2000 ml of sterile water in the 2 L bottle and gently swirl.
10. **Check the pH:** Using pH strips, dip one into the 2 L bottle. Check the colour of the strip. The media should have a pH of 6.8. Adjust the media accordingly using the pH buffers (hydrochloric acid - HCl or potassium hydroxide - KOH), adding 1-2 drops of the pH buffer at a time. Swirl the bottle gently and check the pH again using a fresh strip. Repeat the process until the pH has reached 6.8.
11. **Melt the agar:** Microwave the bottle for 3 min at a time, watching that it does not boil and overflow. Gently swirl the bottle between each heating. Once the agar has completely dissolved pour the mix into the four 400 ml Schott bottles.
12. **Sterilise the agar:** Loosely screw the lids on and autoclave them for 20 mins at 121°C. Once the autoclave cycle has finished, remove the bottles and place them in a hot water bath until cool enough to handle.

13. **Plate out the media:** Spray the laminar flow bench with ethanol/ methanol and wipe down. Arrange 3 rows of petri dishes with the lids half off. Take one bottle of media and, starting from the back wall row of the laminar flow, pour media into each dish until the bottom is just covered (about half a cm deep). Once the plates are filled, push the lids forward slightly so that a small gap remains. Leave the plates under the cabinet until the condensation has evaporated and the media has solidified. Starting from the front of the laminar flow, close the lids on the petri dishes and stack them to the side of the cabinet.

Remember: a laminar flow cabinet will pump out clean air from the back, the air travels towards you. If anything is in between your working area and the back of the bench, contaminants can be spread by the movement of the air

14. **Storage:** Place the petri dishes back into a plastic sleeve and seal it. Label the sleeve with the media type (i.e. PDA) and date. Store the bags of petri dishes on a shelf in the lab. Repeat steps 6-7 for the remaining bottles of media.

Making Oatmeal Agar (OMA)

You will need:

Crushed oats 5 g
Sterile water 2000 ml
Agar 16 g
Laminar flow cabinet
Ethanol/methanol
Petri dishes
Permanent marker
Foil
Autoclave or pressure cooker
2 L bottle
4 × 400 ml Schott bottles
Scales
Measuring cylinder
Microwave
pH strips
pH buffers

Methodology

1. **Make the media:** Make sure that the bottles are clean and dry. Turn on the scales, place a section of foil on the balance and re-zero. Weigh out the oats and agar. Combine the oats, agar and 2000 ml of sterile water in the 2 L bottle and gently swirl.
2. **Check the pH:** Using pH strips, dip one into the 2 L bottle. Check the colour of the strip. The media should have a pH of 5.5. Adjust the media accordingly using the pH buffers (hydrochloric acid - HCl or potassium hydroxide - KOH), adding 1-2 drops of the pH buffer at a time. Swirl the bottle gently and check the pH again using a fresh strip. Repeat the process until the pH has reached 5.5.
3. **Melt the agar:** Microwave the bottle for 3 min at a time, watching that it does not boil and overflow. Gently swirl the bottle between each heating. Once the agar has completely dissolved pour the mix into the four 400 ml Schott bottles.
4. **Sterilise the agar:** Loosely screw the lids on and autoclave them for 20 mins at 121°C. Once the autoclave cycle has finished, remove the bottles and place them in a hot water bath until cool enough to handle.

5. **Plate out the media:** Spray the laminar flow bench with ethanol/ methanol and wipe down. Arrange 3 rows of petri dishes with the lids half off. Take one bottle of media and, starting from the back wall row of the laminar flow, pour media into each dish until the bottom is just covered (about half a cm deep). Once the plates are filled, push the lids forward slightly so that a small gap remains. Leave the plates under the cabinet until the condensation has evaporated and the media has solidified. Starting from the front of the laminar flow, close the lids on the petri dishes and stack them to the side of the cabinet.
Remember: a laminar flow cabinet will pump out clean air from the back, the air travels towards you. If anything is in between your working area and the back of the bench, contaminants can be spread by the movement of the air
6. **Storage:** Place the petri dishes back into a plastic sleeve and seal it. Label the sleeve with the media type (i.e. OMA) and date. Store the bags of petri dishes on a shelf in the lab. Repeat steps 6-7 for the remaining bottles of media.

Achlorophyllous – lacking chlorophyll pigment and unable to photosynthesise.

Agar – a complex polysaccharide obtained from certain types of seaweed. When heated with water and subsequently cooled, agar readily forms a gel.

Androecium – a collection of stamens that form the male reproductive parts of a flower.

Anther – the pollen bearing part of a stamen.

Asymbiotic – the attempted growth of a symbiotic organism without its symbiont or beneficial partner.

Autotroph – an organism that uses carbon dioxide as its main or sole source of carbon i.e. an organism that is able to internally manufacture its own food.

Capsule – a dry fruit that normally dehisces or splits.

Chlorophyllous – containing chlorophyll, the green pigment in plants that functions in photosynthesis by absorbing radiant energy from the sun for conversion to energy.

Collar – a small swollen area below the leaf of some terrestrial orchids that serves as the containment site for mycorrhizal infection. Similar to a pea nodule.

Column – a central fleshy structure in orchid flowers composed of the style and staminal filaments, also called the gynostemium. The column arises from the fusion of the androecium and gynoecium.

Endemic – restricted to a particular country, region or area.

Epidermal – the outermost layer or layers of the plant tissue.

Epiphytic – a plant that uses another plant, typically a tree, for its physical support, but which it does not draw nourishment from.

Family – a taxonomic group of related genera.

Genus – (pl. Genera) a taxonomic group of related species.

Gynoecium – the collective term for the female reproductive parts of a flower comprising one or more carpels.

Heterotrophic – a term referring to an organism's inability to manufacture its own food from simple chemical compounds, it must consume other organisms, living or dead to acquire carbon.

Hypae – a thread-like filament that is the structural unit in many fungi.

Imbibe – the uptake of water and rehydration of the seed tissues.

Mycorrhiza – a close physical association between a fungus and the roots of a plant, from which both fungus and plant appear to benefit.

Mycotrophy – a term referring to the food or energy acquisition of a plant that is associated with a fungus in a mycorrhiza.

Ovary – the part of the gynoecium that encloses the ovules and after fertilisation develops into a fruit.

Peloton – fungal hyphae that coil tightly to form a small ball.

Photosynthesis – the series of metabolic reactions that occur in certain autotrophs, whereby organic compounds are synthesised by the reduction of carbon dioxide using energy absorbed by chlorophyll from sunlight.

Pipette – a narrow, usually calibrated glass or plastic tube into which small amounts of liquid are suctioned for transfer or measurement.

Plasma membrane – the sheet-like membrane that encloses and delimits the contents of a cell. It is a living structure that controls the passage of water and other molecules into and out of the cell. Also known as the cell membrane.

Pollinium – (pl. Pollinia) a coherent mass of pollen grains, the product of a single anther lobe and transported as a single unit in pollination.

Propagate – to cause an organism to produce plants, grow and/or reproduce.

Protocorm – a tuber structure that develops from the embryo of orchids and lycopods.

Saprophytic – the absorption of soluble organic nutrients from inanimate sources (e.g. dead plant or animal matter or dung etc.) by a plant.

Senesce – the deteriorative process that terminates naturally the functional life of an organism or organ.

Species – a closely related group of individuals all of which possess a common set of characters that set them apart from other species.

Stigma – the part of the female reproductive organs on which pollen grains germinate. In orchids it refers to the enlarged sticky area on the column that is receptive to pollen.

Symbiotic – a general term describing the situation in which two dissimilar organisms live together in close association providing mutual benefit for each other.

Taxon – (pl. Taxa) a term used to describe any taxonomic group, e.g. genus or species.

Terrestrial – living on or growing in the ground.

Testa – the seed coat or integument.

Tuber – a swollen stem or root that functions as an underground storage organ.

Velamen – several layers of densely packed, dead cells on the epidermis of the aerial roots of an epiphytic orchid

Voucher – an individual that is collected and preserved for taxonomic reference.

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CONTACT DETAILS

Threatened Species Section
Biodiversity Conservation Branch
Resource Management and
Conservation Division
Department of Primary Industries
and Water
GPO Box 44
Hobart, Tasmania, 7001
Australia