

***In vitro* germination of orchids : a manual**

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This manual outlines the basic biology of orchid seed germination, some techniques used to germinate orchids *in vitro* germination and an appendix of media and species already tested as part of the El Pahuma *in vitro* germination program. It has been specifically designed for the El Pahuma orchid program, but can be adapted to suit other programs.

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1. INTRODUCTION

1.1 Aims of the El Pahuma *in vitro* orchid germination program

In vitro germination of orchid seeds is an important part of the conservation program of the Ceiba Foundation for Tropical Conservation at the El Pahuma reserve, Pichincha Province, Ecuador. The aims of the *in vitro* propagation program are four-fold:

1. To raise orchids in order to stock the Orchid Gallery Trail.
2. To propagate rare species of orchid in order to increase their numbers both in the wild and in places other than El Pahuma (e.g. botanical gardens, other conservation programs), and to provide seedlings for restoration programs in regenerating forests.
3. To provide seedlings for sale at the reserve in order to deter wild collection, stimulate public interest and provide an income for the reserve.
4. To raise seedlings of unusual or ornamental species for export in jars in order to support the conservation work being carried out at El Pahuma.

All profits derived from the sale of orchids both at El Pahuma and through exportation will go to the management of the El Pahuma Orchid Reserve and other orchid conservation projects.

1.2 The biology of orchid seed germination

Orchid seeds are often referred to as "dust seeds", as they are tiny and contain few food reserves. In nature they may germinate but will not grow unless infected by a mycorrhizal fungus, which supplies the young plants with all the sugars and nutrients they need until the plants are large enough to produce food on their own. Once the seed has germinated it produces a fairly undifferentiated mass of cells called a protocorm (see Fig. 1). All being well this protocorm will continue to grow for many weeks, months or even years depending on species, until large enough to produce leaves and roots. In terrestrial orchids it is vitally important that the orchid/fungus relationship is maintained during the early stages of the plants life, as the protocorm is subterranean and cannot produce any food of its own. In epiphytic orchids the protocorms are often green, and thus can produce some food of their own. The orchid/fungus relationship has not been investigated in most tropical orchids.

1.3 Basics of *in vitro* germination

1.3.1 Symbiotic and asymbiotic seed germination

In *in vitro* germination the seeds are germinated in glass or plastic jars or dishes on an agar-based medium which contains all the sugars and minerals the seeds need to germinate and grow. There are two basic types of *in vitro* germination: symbiotic and asymbiotic.

In symbiotic seed germination the seeds are sown with a small piece of an appropriate mycorrhizal fungus. This fungus then grows over the media, colonizes the germinating seeds and a symbiotic relationship is formed which presumably will sustain the protocorm until it produces leaves and becomes autotrophic. This technique is widely used for the propagation of temperate terrestrial orchids. It has the advantage that the media used is very simple (one of the most popular consisting of only powdered oats with a little yeast extract), and the resultant mycorrhizal plants are often stronger and more resistant to fungal infection than some of their asymbiotically propagated counterparts. It has the disadvantage that you need the correct strain of mycorrhizal fungus, or the symbiosis will not develop or might become parasitic and the seedlings die. Very little research has been carried out on the mycorrhizal fungi of tropical orchids, and so the appropriate mycorrhizal fungi are not at this time readily available.

Asymbiotic germination is commonly used in the propagation of tropical orchids, which tend to be easier to grow than their temperate relatives. The media used for asymbiotic germination is more complex than that for symbiotic germination, as all organic and inorganic nutrients and sugars must be in a form readily available to the orchid without the fungus intermediary. Asymbiotic germination is the method employed in the El Pahuma *in vitro* orchid propagation program at this time. Should fungi become available from *in situ* sowings at a later date, then it might also be possible to use symbiotic techniques.

1.3.2 Basics of maintaining sterile conditions

In both symbiotic and asymbiotic germination, it is vital that all media, jars, instruments and seeds are kept sterile at every stage of the germination procedure. Any bacteria or fungi to get into the jars will grow much quicker than the seedlings and will soon outgrow and kill the germinating seeds.

Sterile conditions are created in the preparation of the media by autoclaving all media and jars to be used for 15 minutes at 15 atm. This temperature and pressure is sufficient to kill all bacteria and fungal spores in the media.

The seeds must be sterilized and transferred to the jars without introducing extraneous bacteria or fungi. This is usually done by sowing from surface-sterilized green capsules, or by sterilizing mature seeds with sodium or calcium hypochlorite, or hydrogen peroxide, and ensuring that all instruments used in the transfer are sterile. With a little care and practice sterile conditions can be created and maintained from germination to the planting out of the seedlings.

Sterile conditions for pouring the media and sowing the seeds are obtained in the El Pahuma germination program using a laminar air-flow bench. Other techniques for maintaining sterile conditions are possible (Thompson, 1980), but technically more difficult, and should not be attempted in humid or less than clean conditions.

1.3.3 Use of the laminar air-flow bench

There are some basic rules to be observed in using the laminar air-flow bench.

1. Always sterilize thoroughly before use with 70 - 90 % alcohol (ethanol preferably: be very careful if using "*alcohol antiseptico*" which also contains methanol. Methanol is toxic and can cause blindness if swallowed. Use the spray and swab down all surfaces in the cabinet with cotton wool soaked in alcohol (walls and ceiling too). Sterilize both before and after switching on the cabinet. Be careful not to breathe in ethanol when using spray when the cabinet is switched on.

2. Everything which goes into the cabinet should be sterile or sterilized. Wear gloves, and sterilize them thoroughly before use by putting them on, spraying with alcohol and holding them in the cabinet until the alcohol has dried. It is possible with practice, instead of wearing gloves, to wash the hands and scrub the nails thoroughly using anti/bacterial soap, dry and sterilize with alcohol as before, but should there be any problems with bacterial contamination, revert to gloves. Wear a lab-coat, and clean it regularly.

Make sure glassware is clean. Glassware etc. can be sprayed with alcohol on entering the cabinet but beware! Any marks made with marker pen on the glass will come off if sprayed with ethanol. If the glassware is clean, spraying with ethanol may not be necessary. Instruments can be autoclaved before use either wrapped in aluminum foil or in brown paper sealed with masking tape. Once in the cabinet sterility is ensured by dipping in 100% ethanol and flaming three times before use. The ethanol is best kept in a tall glass jar to allow maximum exposure of the instruments to the ethanol. After flaming the instruments are quickly placed on the top of a sterilized glass jar to continue to burn. Allow to cool before use.

3. Remember that any bacteria or fungal spores in the cabinet will float down and towards you in the flow. Never place hands, sleeves or anything else above or beyond anything sterile (e.g. media). Keep movements smooth and avoid creating air turbulence which could bring in contamination. Do not talk, cough or sneeze in the cabinet. Work towards the back of the cabinet as much as possible, and minimize exposure time of media where possible.

4. Maintain sterile conditions by swabbing bench regularly with alcohol, re-sterilizing instruments after each use and re-sterilizing hands after they have been in contact with anything outside the cabinet. Especially, do not touch face or hair with gloves on!

SAFETY NOTES:

1. BE CAREFUL NOT TO FLAME YOUR HANDS - particularly when wearing gloves! It is easy to do as you can not feel if the alcohol on your gloves has not totally evaporated. Luckily ethanol burns at a low temperature and can be easily put out by flapping like mad!

2. BE CAREFUL NOT TO BURN YOURSELF or any seedlings you might be transferring - the instruments are very hot after only two flames.

3. ALWAYS REMEMBER TO TURN OFF THE GAS, both at the Bunsen burner and at the tap on the top of the cylinder.

2 PREPARATION OF THE MEDIA

2.1 Introduction to media preparation

Media can be prepared either from the basic ingredients (Thompson, 1977, Appendix 1) or bought in powder form from a supplier. There are many different media available commercially and many others devised by amateurs and professionals alike to grow certain species. When attempting to germinate a new species if possible it is good to try with a few different media at both full and half strength, in order to determine what is best for that species. *Masdevallias* for instance are known to be “picky” about which media they will germinate on (Richard Warren, personal communication). pH is also important. Most orchids will germinate on media of pH 5.5, but Andean species may favor slightly higher pHs e.g. 5.6 to 5.9 (Monica de Navaho, pers. comm.). Remember when experimenting with pH that the pH of the media after the addition of agar will be higher than before the addition of agar.

Commercial preparations of Phytamax (Sigma Chemicals, Aldrich, England) and Murashige and Skoog have been the main media used in the EI Pahuma project to date (March 2000). Either of these media can be prepared either half strength or full strength (half strength recommended for Murashige and Skoog).

The general method preparation of these media is as follows.

2.2 General method for preparation of media

1. Measure out the correct quantity of media powder into 1l bottle, taking care to avoid the dust.
2. MS only: add correct quantity of sugar and 2g/l activated charcoal
3. Add magnetic stirrer and a small quantity of distilled water and stir until dissolved
4. Make up to 1l using distilled water, stirring continuously
5. Measure the pH and adjust to 5.6 using HCl or NaOH, stirring throughout with the magnetic stirrer. *Caution - if a pH meter is used then do not stir using the element: the membrane is very delicate and expensive and could easily break! If a pH meter is not available then pH papers are sufficient.*
6. Pour half of the liquid into a flask, add 4g agar to each vessel (8g/l agar) and stir to disperse
7. Screw the lid loosely on the bottle, cover the tops of flasks with aluminum foil and autoclave.

In ideal conditions the media should be made in one bottle and the magnetic stirrer kept in the bottle throughout autoclaving so that the media can be stirred before pouring , thus making a more even distribution of charcoal etc. throughout the jars. However, for safety reasons the University prefers that two 500ml bottles or flasks are used so keep the magnetic stirrer in the bottle and swirl the flask gently before pouring to disperse the charcoal.

SAFETY NOTES:

1. DO NOT BREATHE DUST OF PHYTAMAX AND AVOID CONTACT WITH SKIN AND EYES. If you should get any of the dust of either phytamax, MS or charcoal

on your skin, wash it off immediately with plenty of cold water. If in contact with eyes, wash immediately with cold water and seek medical attention.

2. NEVER SCREW A JAR OR BOTTLE LID TIGHTLY ON BEFORE PUTTING IN THE AUTOCLAVE. The build up of pressure in the autoclave will cause the glass to break. Always make sure that lids are loose when put in for autoclaving: they can be tightened again once the jars have cooled.

3. BE CAREFUL WHEN RETRIEVING JARS AND BOTTLES FROM THE AUTOCLAVE. They will be very hot and could burn.

2.3 Pouring of media

The media must be poured into sterile jars or petri dishes, using the laminar air flow bench (see 1.2.3). Jars must be thoroughly washed and rinsed four times before use, and sterilized for 15 minutes at 15 p.s.i. in an autoclave. If lids are used on the jars, remember to screw them on loosely before autoclaving. If not lids are available then coverings can be made by wrapping placing a piece of aluminum foil over the mouth of the jar and sealing tightly with a twist of the wrist. Heavy-duty brown paper tied with string can also be used, but is more cumbersome. Re-sterilize any jars which come out of the autoclave with a hole in the foil, or the foil lid coming off.

1. Wait for media to cool enough to handle comfortably. When the agar appears less fluid and the bottle or flask can be handled easily, it is ready to pour. Agar poured while still very hot will create a lot of condensation.

2. Line the jars or petri dishes up at the back of the laminar air flow bench. Loosen lids of jars.

3. Open jar lids or petri dish lid and pour, moving left to right (if right handed) to avoid any part of the hand or lab coat hovering over an open jar or dish (see 2.xx)

4. Leave to set, then replace lids. Lids can be closed immediately after the media is poured, but this leads to condensation in the jars. Some free water in the jars may or may not be desirable, depending on whether seed is being sown from green capsules or mature seed.

The preparation of media, from the mixing of the ingredients to the setting of the poured media, takes roughly 4 hours. If 2 l of medium has been made at the same time then a hot plate can be used to keep one bottle of media warm while the other is setting. A hot plate should only be used as a short term emergency measure, however, as longer term use (e.g. half an hour) can cause the charcoal to precipitate out.

3 SOWING THE SEED

3.1 Seed collection and storage

Seeds can be collected either in green capsules or as mature seed. A green capsule is judged to be mature and ready for sowing when it looks full and 'gives' a little

when gently pressed. Capsules can be stored for a few weeks wrapped in kitchen paper, in a well aerated part of a domestic refrigerator (e.g. cheese compartment). Do not store in plastic bags or the capsules will sweat and rot.

If at all possible, try to collect capsules which have already dehisced on a dry day. Ideally seed should be used fresh or dried over a saturated solution of calcium hexahydrate (Seaton & Pritchard, 19xx). If that is not available seeds can be dried over calcium chloride, silica gel (harsher than calcium chloride and really only recommended for short-term use) or simply left to dry at room temperature (dry climates only). Once dried the seeds can be stored for many months in air-tight vials in a refrigerator (4-5 °C). Again, seed should never be stored or transported in plastic bags or air-tight containers.

Times from flowering to seed maturity vary greatly between species and sites, for example it takes roughly 3.5 months for seeds of *Epidendrum* or *Masdevallia* to ripen (ref) and roughly 18 months for *Odontoglossum* (Monica de Navarro, pers. comm.) . Conventional estimates do not take account of different climatic conditions; obviously a capsule grown in a warm glasshouse will ripen much quicker than one growing in a cloud forest.

At Pahuma, mark all plants collected from with a tape and code number, and remember to include this code number in the sowing details.

3.2 Seed sowing

3.2.1 Green capsules vs. dry seed

Seeds can be sown from capsules either before dehiscence ('green capsules') or after dehiscence ('dry seed'). The advantages and disadvantages of using green capsules or dry seeds are as follows.

1. Sowing from green capsules. The inside of an orchid capsule, if intact, is naturally sterile. Therefore if you sterilize the outside of the capsule, where bacteria and fungi can collect, and cut open the capsule under sterile conditions then the seeds should be sterile. This method has the advantage that the seeds themselves do not need to be sterilized (which can sometimes lead to damage). In

addition, some seeds, if taken from capsules which are almost ripe, germinate quicker than those taken from mature capsules as the dormancy mechanisms are not yet in place. It has the disadvantage that once opened all the seeds from an immature capsule must be sown or discarded (some mature seeds may be dried and saved). In addition, seeds sown from capsules which are not sufficiently mature may germinate very slowly or not at all .

2. Sowing from dry seeds. Once a seed capsule has opened the seeds are no longer sterile. The seeds thus need to be sterilized, usually using a solution of sodium hypochlorite (bleach), calcium hypochlorite or hydrogen peroxide. The seeds are shaken in a solution of sterilant containing a drop of detergent to 'wet' the seeds, then rinsed in sterile water and planted on to the medium. This method has the advantage that seeds can be collected, air-dried, stored for many months in the fridge and used when needed.

It must be remembered that at times availability of seed in either form will govern what is sown.

3.2.2 Sowing from green capsules

The general method from sowing from green capsules is as follows

1. Using a scalpel, carefully trim the dead flower off the capsule
2. Using a soft toothbrush gently scrub the capsule with soap solution
3. Rinse the capsule in water
4. Immerse for 10 minutes in 1 % sodium hypochlorite (bleach solution) to which a drop of detergent has been added.
5. Transfer capsule in bleach solution to the laminar air-flow cabinet.
6. Pick capsule out of bleach solution using forceps, preferably holding on to what is left of the stalk. Dip in 100 % alcohol and pass *briefly* through the flame. Allow alcohol to burn off and capsule and forceps to cool. Repeat twice for larger capsules. Use your own discretion as to how many times to flame for small or delicate capsules (perhaps only once). If not possible to hold by the stalk, change the position of the forceps before flaming again.
7. Transfer to a sterile cutting surface (e.g. sterilized petri dish). Using a sterile scalpel slice the capsule in half longitudinally. Use a fresh blade for each new capsule to prevent the spread of any viruses.

8. Lift one half of the capsule using forceps and gently tap over the media to sow seeds
9. Repeat until all loose seed is used up. Less mature seed can be teased out of the capsule using forceps
10. A few drops of water are normally added to each jar, and any clumps of seeds broken up and spread out a little over the agar.

3.2.3 Sowing from dehisced capsules (dry seed)

There are several different methods of sowing from dry seed. All rely on sterilization of the seed, and rinsing in sterile water before sowing, but techniques vary. Sterilization times also vary depending on the species, time after dehiscence when the seed was collected, climatic conditions at the time of collection and methods of drying and storage. A range of sterilization times is therefore recommended for new seeds.

In the El Pahuma conservation program two main techniques are used for sowing mature seeds: the syringe technique and the packet technique. Other techniques are possible (e.g. filtration technique using a Buchner funnel), but are not covered in this manual.

In both the syringe and the packet techniques all instruments and water needed for the sowing is best sterilized the day before needed as it takes a long time for the water to cool down after autoclaving.

In general, the syringe technique is used for most sowings and the more cumbersome packet technique used for very small or rare seeds.

3.2.3.1 Syringe technique

1. Take a 5 ml syringe, plug the tip of the syringe with cotton wool wrapped in a piece of cloth cut from a pair of tights (see diagram), replace the plunger and autoclave. At the same time autoclave a supply of distilled water (in jars or bottles) and forceps.
2. Pull out plunger and pour a *small* quantity of seed into syringe. (Large numbers of seeds will form a crust-like mass if technique 5b is used to sow, making it difficult to sow thinly). Replace plunger.
3. Draw c. 4ml of 1 % bleach solution (to which one drop of detergent has been added) into the syringe. Agitate for 5 minutes (or other sterilization time), making

sure that the seeds are bathed in the solution and not trapped in air bubbles. Eject solution and draw in fresh solution. Agitate for another 5 minutes, then eject bleach.

4. Rinse seeds 3 - 4 times by drawing in sterile distilled water, agitating briefly then ejecting.

5. Sow seeds either by a) sterilizing the neck of syringe and pouring the seeds onto the medium. Remove any excess water or b) expelling all excess water, removing the cotton wool ball using forceps and dabbing seed on to medium.

3.2.3.2 Packet technique

1. Create a packet out of a piece of paper (see diagram)

2. Sow a *small* quantity of seed into the packets. Again, large numbers of seed will clump and hinder penetration of the bleach.

3. Fold and seal using a stapler. Immerse in distilled water for 5 - 10 minutes, squeezing gently to dispel any air bubbles.

4. Transfer packet using forceps to 1 % bleach solution to which one drop of detergent has been added. Leave for 10 minutes (or other sterilization time), stirring or agitating frequently.

5. Transfer packet plus bleach to laminar flow cabinet. Using forceps transfer packet to sterile distilled water (in a jam jar). Agitate. Repeat 3 - 4 times to rinse seeds.

6. Gently squeeze out excess water and transfer to sterile surface. Using sterile scissors cut open packet, and sow seeds by dabbing onto medium.

Normally using this technique the packets are put in a container of bleach over a magnetic stirrer. The theory is that the staples will act as magnetic fleas and stir the packets automatically. I have never found this to work! Merely stirring or agitating frequently does the same job, and several packets can be immersed in bleach at the same time to speed up the sowing process.

3.3 Capping the jars

Once sown the jars can be capped either using cling-film or cellophane covering cut from bags use to cook chickens in the oven. With cling film the film must be unwrapped in the flow cabinet, doubled over and the side closest to the back of the cabinet placed over the jars. The cling film is then secured with an elastic band and surplus film trimmed off.

The cellophane is cut into squares, and autoclaved in jars before being used. A square of cellophane is removed from the jars using forceps, placed over a jar of medium and secured using an elastic band. From the *KLAR 2000 Bolsas transparentes para hornear* it is possible to get 20 tops per bag and 160 tops per packet of bags (8 bags per packet).

The cellophane is easier to handle than the cling-film and is reusable. It is therefore recommended as the preferred capping material.

3.4 Record-keeping

Records are kept both in a laboratory note-book, a card system and on the computer. Details of all media made, sowings and observational notes are recorded in the note-book. The card system records all sowings, recording a code for each sowing consisting of the species code (e.g. TF = *Trichopilia fragrans*) and the sowing code (e.g. TF12 if the twelfth sowing of *Trichopilia*). The date of sowing is in the top left hand corner, and beneath that the number of jars, type of media (and date of manufacture of media if known), the seed code and source and any observations made on sowing. Jars are examined weekly and notes on contamination and germination etc. made under the appropriate date. If a jar is discarded, this is shown by an X on either side of the observation for that jar, and the reason noted (e.g. fungal contamination or bacterial contamination). If all jars of a particular sowing have been discarded an x is written beside the species code and a large x drawn through the card. Do not throw out the card - it may be useful later to investigate what went wrong.

Each jar is labeled with the sowing code and any other relevant information which may prove useful to have without referring to the cards (e.g. time of sterilization). Each jar is also labeled with the medium code at the time of pouring, to prevent any mix-up occurring during storage or sowing.

4 CARE OF SEEDLINGS

4.1 Seedlings in flasks

The seedlings at USFQ are grown 40 cm under 20 Watt fluorescent tubes in a growth room set at 18 °C with 16 hours light and 8 hours dark.

Newly sown flasks should be checked for contamination as often as possible after sowing. If contamination is caught early enough, it can often be cut out before it has had the chance to spread. Once a fungus has formed spores however, or if there is spare liquid in the bottom of the flask, cutting out the visible contamination will do no good. In most cases such as this, the flask should be discarded. Occasionally a fungal contaminant will actually aid germination (Monica de Navarro, pers. comm). In those cases the seedlings can be left to grow, but must be transplanted before the fungus takes over and kills the seedlings (see Monica for advice).

Sterile water can be added if the jars look dry, using a sterilized syringe.

Seedlings are first transferred when the leaves have formed and the jars begin to look crowded. The exception to this rule is if it is more efficacious to remove protocorms from a contaminated jar or petri dish, rather than cut out the infection. Seedlings are transferred using sterile forceps to fresh jars of media. A crochet hook can be useful at this stage to remove delicate seedlings (Monica de Navarro, pers. comm.). The next medium can be the same as the first, but it is often better to add complex substances to the media to encourage root development (e.g. mashed banana, coconut milk, pineapple juice or vitamin B. A small hole can be put in caps (if metal or plastic, possibly if cellophane, not if cling-film!) and sealed two or three times with the micropore to provide a spore-proof breathable surface in the cap. The holes made should not be more than 2 mm in diameter (Monica de Navarro, pers. comm.)

Several transfers will have to take place before the seedlings are ready for potting up.

4.1 Planting out

Seedlings grown in jars have been in a very cosseted environment and must gradually accustom to their new environment before planted into pots. In the growth room light is low and both light and temperature fairly constant. The humidity in the jars is high and the seedlings are protected from the attacks of fungi, bacteria and herbivores. In the field both light and temperature will vary greatly. In particular the new seedlings need to be protected from strong sunlight: even a small amount will burn the leaves of what are essentially shade-grown plants. The new seedlings, having been raised in conditions of high humidity, will have poor cuticles, and thus the seedlings need to be gradually accustomed to a drier atmosphere before planting. Finally, both in the acclimatization stage and the newly-planted stage all seedlings must have daily attention to check for problems, more than daily if the weather is dry and sunny.

The following are guidelines to planting out. The timing of each stage may vary from that given: constant monitoring should give an idea of what is best for each species under the given conditions. Amend these notes where necessary!

1. Start the planting out process at the beginning of the wet season if possible. the lower amounts of light and high humidity at this time should make it easier for the seeds to acclimatize and establish (is this right Monica? Or when planted will the high humidity encourage fungal attack?)
2. Put the jars in a shaded position, sheltered from rain, to acclimatize to the new light and temperature conditions. Leave for 2 - 4 weeks with the lids on.

3. Loosen the jars lids to let in a small amount of air. Leave for about a week. Check daily for any leaf shriveling and make sure media is moist but not wet.
4. Open a little more. Leave for a few days, checking daily.
5. Half open the lids. Leave for a few days, checking daily
6. Open the lids to three-quarters. Leave for a few days, checking daily
7. Fully open the jars. If possible leave for about a week before transporting the seedlings to the nursery for transplanting.
8. Choose the correct potting medium for the seedlings (see Appendix)
9. Carefully remove the seedlings from the jar, tease apart gently and wash off all traces of agar
10. Fill a pot with potting compost and put it in a basin of water. Dip the roots of the seedling in fungicide and plant the seedling into the floating medium
11. When the pot is removed from the water the medium should stick to the roots of the orchids without damaging them. Place the newly planted seedlings into a propagator or small table 'tent' made from clear plastic to ensure high humidity during the establishment phase. Water regularly from the top avoiding the leaves and never leave standing in water. Some spraying with water could be helpful in the early stages to maintain high humidity.
12. Again, gradually acclimatize the seedlings to their new environment (e.g. fully sealed tent, tent door partially opened, tent door fully opened, no tent but protected from rain, no protection from rain, or should all plants be kept sheltered under a plastic cover?.) When fully acclimatized and large enough to be potted up, they can either be transplanted into larger pots or simply tied onto a tree trunk, branch or piece of bark (with or without a pad of moss- experiment!) Spray with water in the early stages of development- develop judgement on how much and when as water addition needs to be enough to keep the plant from drying up but not so much that it encourages fungal rot.

5 CONCLUSION

With *in vitro* germination large numbers of seedlings can be raised in a relatively short time (several months for the quickest growing species). Some of these seedlings can be exported as orchids raised and kept in sterile jars are exempt from the usual CITES and phyto-sanitary regulations. Others can be taken to the orchid nursery at El Pahuma, planted into pots and raised either for sale or for introduction

to the forest at El Pahuma or elsewhere. Each orchid capsule contains many thousands of seeds, so there will be plenty of plants to go round!

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APPENDIX 1

Equipment and materials used for seed germination at USFQ

Laminar air-flow cabinet

Autoclave

Growth room (18 °C, 16 hours light, 8 hours dark)

Shelving with 20 Watt fluorescent lamps (uncovered)

1l Pyrex bottle

1l Pyrex flask

Magnetic stirrer + flea

pH meter

NaOH and HCl for pH adjustment

Distilled water

Phytamax orchid maintenance medium (P6668, Sigma chemicals)

Murashige & Skoog salts

Agar

Sugar

Activated carbon

Vitamin B complex

forceps

scalpel with blades

glass for ethanol

glass jam-jars

Plastic 250 ml beakers

Petri dishes

Small glass vials for seeds

Jam jar lids

Aluminum foil

Thick brown paper

Masking tape

Cling-film

Bolsas de hornear (cellophane)

Elastic bands

Micropore

Lab coat

Hand-towel

Kitchen gloves

Disposable plastic gloves

Cloths

Paper towels

Paper

Alcohol (70% ethanol)

100 % ethanol

Spray bottle for ethanol

Indelible marker pens (black)

Bleach

Syringe

Cotton wool

Tights

Stapler and staples