WOSA 3 Conference

Dullstroom

19-21 January 2018

WILD ORCHIDS SOUTHERN AFRICA

WOSA is a public benefit and non-profit organisation
Registration numbers: PBO 930050871; 141-119 NPO

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Published by: Wild Orchids Southern Africa

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Note: Detailed location data of indigenous orchid and endangered species sightings presented at the Conference have been removed from the papers in these Proceedings as a precautionary measure. Should you require access to this information please contact the respective speakers.

Propagation of Indigenous Orchids

Karsten Wodrich

Introduction

Very few of the indigenous orchids other than a few specific genera like the *Disa uniflora* alliance and *Ansellia* have been propagated from seed on a large scale. Twenty-five years of propagation experience with various terrestrial and epiphytic genera have shown that most of the species can be germinated from seed using asymbiotic methods. The biggest challenge faced by growers is the ability to deflask and acclimatize the plants to life outside the flask. It is here where most plants are lost.

Overview

In-vitro propagation is the method where seed is sown on a gel-like medium containing all the necessary nutrients for seed to germinate. As the medium will also favour the germination of fungal spores and growth of bacteria, the sowing process must take place under sterile conditions. There is also great merit to use a process called symbiotic germination where specific mycorrhizal fungi are introduced into the culture process to improve germination and survival of the seedlings after deflasking. For the purposes of this article only the asymbiotic method of germinating seed will be discussed which does not make use of mycorrhizal fungi.

The first step is to make up a suitable medium with a gelling agent to allow the medium to become semi-solid. This medium is either bought ready mixed or it can be made up from scratch to a specific formulation. This medium is added to water and heated to allow the gelling agent to dissolve and then dispensed into clear flasks which can either be glass or plastic.
Once dispensed, the flasks are sterilised in an autoclave or a pressure cooker. At the same time a sterile area is prepared in which the sowing will take place. This can either be a sterile enclosure or a laminar flow cabinet where sterile air is gently blown over the work area.

The crucial step in the process is then to sterilise the seed (without killing it in the process) before introducing it onto the sterile medium. The easiest is to use the green capsule method where an unopened seed capsule can be sterilized on the outside. The seed inside is in a sterile environment and can be

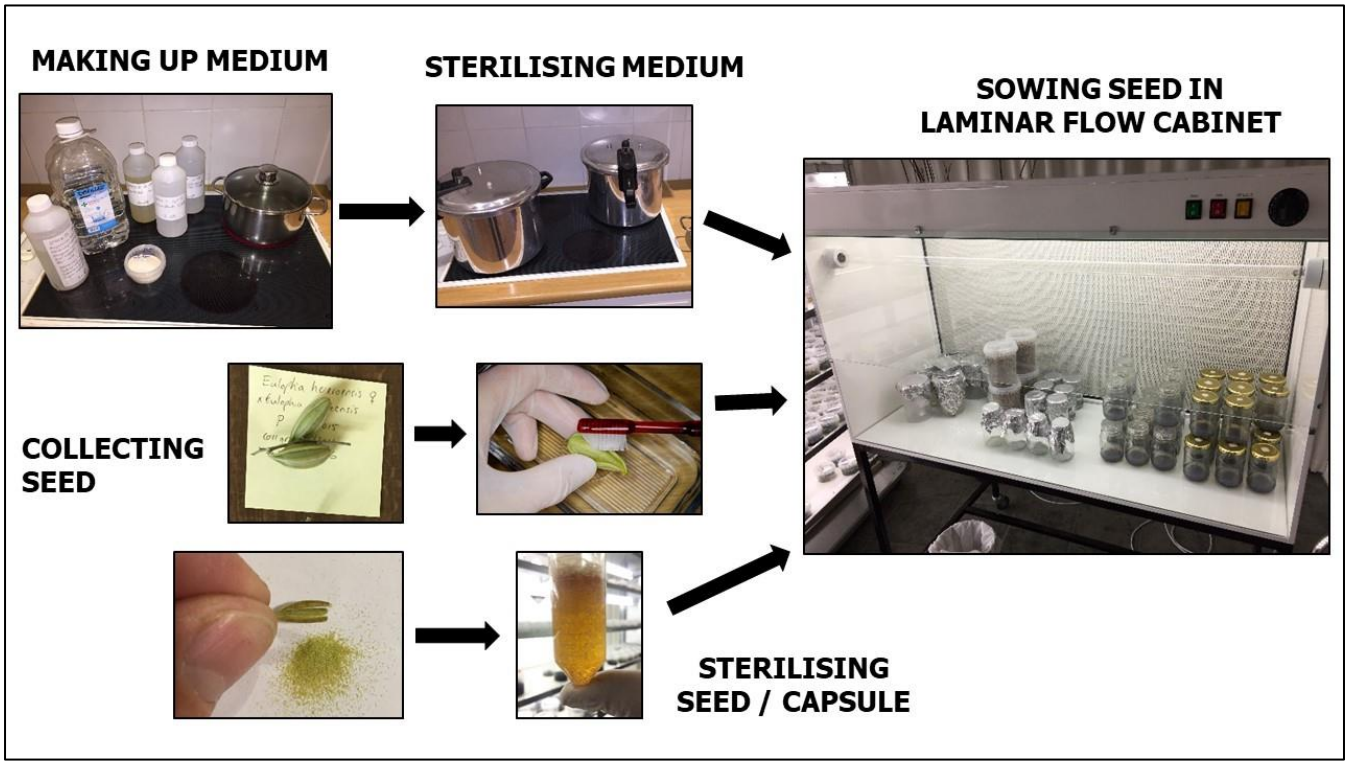

Figure 1.1 Summary of the seed propagation process up to the point where the seed is sown

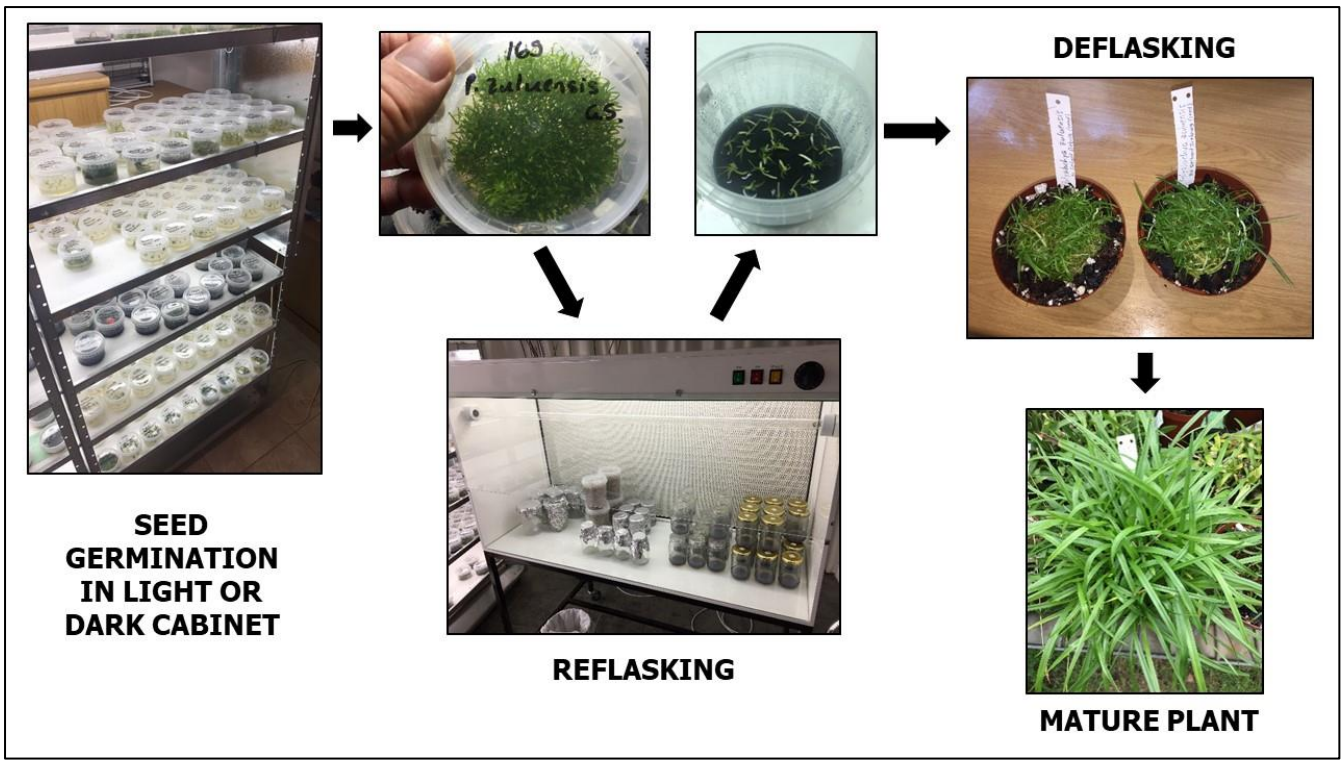

Figure 1.2 Summary of the seed propagation process up to the point where the plants are mature

sown without further sterilising procedures. Where a seed capsule has already split and the seed come into contact with air – and by virtue with fungal spores and bacteria – the seed needs to be surface sterilized with a liquid sterilant, washed with sterile water to remove the sterilant and then sown.

After sowing the flasks are set into a light cabinet or stored in the dark depending on the genus in question. Once germination has taken place the seedlings need to be thinned out onto a fresh medium – again under sterile conditions – to allow the seedling to develop leaves and roots.

Once mature enough the seedlings are deflasked, the remaining medium washed from the roots and the plantlets then planted out in a shady, humid environment until they are sufficiently hardened off.

Specific Challenges

There are a few challenges with our indigenous orchids that need to be taken into account. One of these must no doubt be the fact that the flowers of many species are tiny. Pollination of these is a challenge – especially so when we tend to get on in years and the eye-sight is no longer what is used to be. Typically for the terrestrials, *Stenoglottis*, many of the smaller flowered Disas and in particular the genus *Habenaria*, *Corycium* and *Holothrix* comes to mind. Even with the epiphytes there are some very small flowered species such as for example *Angraecum sacciferum* and species of the genus *Microcoelia*.

Figure 1.3. Tiny flowers of *Angraecum saccifferum*

The only way to pollinate these artificially is to make use of a dissecting microscope. One then needs to be able to manipulate the plant in such a way that the flowers can be viewed under the microscope and pollinated using a sowing needle without damaging the flower or the flowering stem. A *Cattleya* for

example is child's play compared to some of our indigenous species.

And if that is not enough, having successfully pollinated the small flowered species, the next challenge is to collect the seed from tiny seed capsules. A very fine meshed tea sieve is often handy for sifting out capsule debris from the seed once the capsules have split.

Figure 1.4. A tea strainer is useful to sift out debris from dry seed.

When using the green capsule sowing method, the other challenge is to sterilise the outer surface of the capsules without damaging or splitting the capsules. I use a very fine soft paintbrush for this purpose as a tooth brush – often used to scrub the larger capsules – is not a suitable tool for the smaller capsules.

The next challenge is in the flask selection. Traditionally these have been glass jars either with the original metal lids or with rather expensive custom-made polycarbonate screw lids. More recently I have been trying to use clear Polypropylene tubs – typically used for the packaging of Feta cheese and associated foods. There is a definite advantage in using them. Firstly, they are light weight – great for reducing transport costs. They can be stacked when purchased which reduces the size of the boxes one needs to transport home and thirdly they are very competitively priced at between R 2.20 and R 4.00 per tub with lid depending on the size used.

Figure 1.5. A selection of suitable tubs used for flasking.

Having gone down this road in the last four years there are some issues that one needs to be aware of. One of these is that Polypropylene softens slightly at autoclave or pressure cooker steam temperatures which are around 121^{o}C. This means that the lids or the tubs need to be vented in some way. The lids tend to drop onto the sealing surface during sterilisation, even if they are not pushed into place. When the sealed flask cools, the water vapour in the form of steam inside, condenses. Bear in mind that at that point there is also no air left in the flask. This condensing of the steam creates a vacuum that completely collapses the flask if the vacuum is not broken.

Figure 1.6. Successive collapse of a sealed polypropylene tub during cooling once they have been removed from the autoclave.

Quite by accident I noted that it is easy to make a hole on the inside surface of the rim of the tub and then stuff the space between the ribbing of the rim on the outside of the tub with non-absorbent cotton wool.

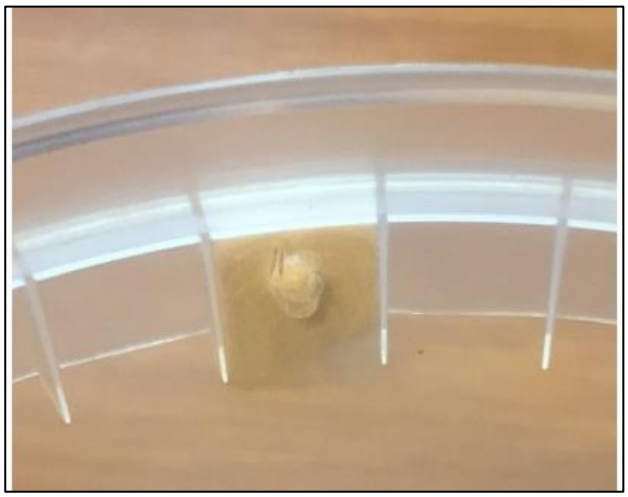

Figure 1.7. A non-absorbent cotton wool plug pushed into the space created by the rib of the tub with a hole pierced to the inside of the tub that allows for venting.

This works well for the smaller flasks up to around 250ml. The larger 500ml or 600ml tubs cannot draw air in through the cotton wool plug fast enough during cooling and still tend to collapse. For these I place a small strip of folded aluminum foil on the rim of the tub so that the lid has no chance of sealing. That foil strip needs to be removed before closing the flask lid once sowing or reflasking has been completed.

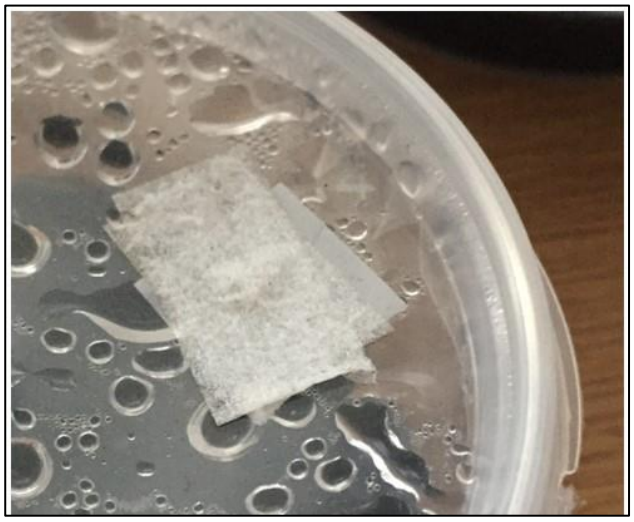

Figure 1.8. Another venting method is to cover a hole drilled in the lid with 3M Micropore tape.

Other methods of venting are by piecing the lid with a small hole and taping two layers of 3M Micropore tape to the top of the hole and one layer underneath. Taping a non-absorbent cotton wool wad to the top of the hole in the lid with the 3M tape also works well. When using plastic tubs, the venting is also essential to prevent the tubs from collapsing slightly during changing atmospheric pressure when the weather changes. This is even more important when the flasks are to be transported between places of differing altitude or transported by air.

The need for venting flask during germination and when the seedlings are reflasked is debatable. With glass jars that are structurally able to withstand slight changes in pressure, I have used both the vented and unvented method and have not seen any noticeable difference is growth rates.

Commercial growers in the USA generally make use of Polycarbonate flasks and not Polypropylene. These withstand higher temperatures and are available at reasonable prices in there when compared to South Africa. Also available overseas are Polypropylene flasks that are irradiated to sterilize them (in the same way many of the medical syringes and plastic devices are sterilized). They are thus not heat sterilised.

I have been struggling with two issues since changing to Polypropylene flasks. One is a phenomenon called phenolic browning where protocorms and plants exude a phenolic compound and, in the process, poison themselves to the point where protocorms and plants are irreparably damaged and die. The trigger for the plant exuding this phenolic compound is not yet fully understood and many growers counteract this by using media containing activated charcoal powder which tends to absorb phenolic substances. But even with charcoal containing medium I was having heavy losses with some species – especially so many of the epiphytic species when growing them in the Polypropylene tubs.

Figure 1.9. Phenolic browning of plantlets in polypropylene flasks.

Secondly many of the species started tissue proliferation without growing roots and/or leaves. A great thing to have happen if you are germinating a very rare species. But this is definitely unwanted when you finally need the plants to grow roots and leaves so that they can be de-flasked.

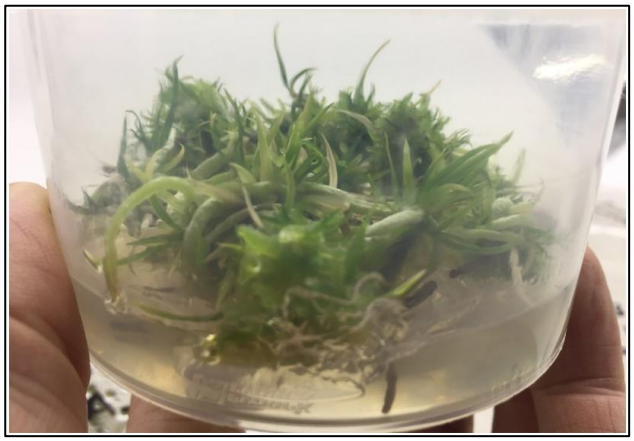

Figure 1.10. Excessive tissue proliferation with plantlets of *Angraecum stella-africae* in polypropylene flasks.

After varying all factors around the flasking process including changing media, leaving out banana as a growth stimulant, changing pH and moving over to using distilled water instead of purified water nothing seems to have improved the situation. At a point of frustration, I discussed the issue with Peter Ashton and he pointed out that the culprit may actually be the flasks themselves. Firstly, Polypropylene is not really designed to be heated to sterilisation temperatures around 121^{o}C. Compounds called Plasticisers are used during the manufacture of Polypropylene and these would possibly leach out into the medium at elevated temperatures. In addition, mold release agents are sprayed into the mold to allow the extruded tub to be released from the mold after forming. Where these are food grade release agents – there is very little information available whether these agents are compatible with live plant material. Mold release agents could be removed by washing each and every tub before use – but hat again defeats the object of an easy to use tub. Certainly, polypropylene can be used for flasking – but indications are that the containers must then be cold sterilised by the process of irradiation if the issue of the leaching of chemicals into the medium is to be minimized.

That has now prompted me to revert back to glass jars and the results seem to indicate that the plants are now growing much better with better root and leaf development. Currently I am busy developing a clear lid for the glass jar with the quick release lids. These jars are mass produced and, in the moment, the most cost-effective ones to use.

One other particular challenge that I have is that the lab is currently housed in a guest cottage on our property. When we receive guests, the whole lab has to be moved to various other parts of the house. That transformation takes place within half an hour thanks to everything being mounted on wheels – but it is still a disruptive process.

Figure 1.11. The current lab is housed in a cottage used as visitor's accommodation. The left shows the room being used as lab, the right the transformation back into a room with a bed.

Media selection and cost

Generally, I use three different media for the various genera. The original Murashige and Skoog medium was published in 1962 (Murashige & Skoog, 1962) and has remained a firm favourite amongst orchid enthusiasts. The three media have proven themselves suitable over the years. The media formulations can be found in the appendix at the end of this article. For an in-depth discussion on media refer to the publication found under the references (Wodrich 1997).

Seed germination

Table 1.1 outlines the different media used for seed germination of various genera.

Figure 1.12. Species germinated using the Murashige and Skoog medium (left to right): *Orthochilus welwitschii*, *Disa uniflora*, *Stenoglottis fimbriata* and *Schizochilos zeheri*.

Figure 1.13. Species germinated using Malmgrens medium (left to right): *Disa versicolor*, *Satyrium carneum*, *Satyrium princeps* and *Satyrium membranaceum*.

Figure 1.14. Species germinated using the Zak medium (left to right): *Bonatea speciosa*, *Brachycorythis conica subsp. transvaalensis*. The picture on the right shows the typical protocorm development of *Corycium* and *Bonatea* with numerous fine rhizoids covering each protocorm.

Medium name	Genus / species
¼ & ½ strength Murashige & Skoog Medium (¼ strength generally used for the terrestrials and the ½ strength for epiphytes)	All epiphytes, *Eulophia*, *Oeceoclades*, *Orthochilus*, *Stenoglottis*, evergreen *Disa* species, *Schizochilus*
Malmgrens Medium	Summer rainfall *Disa* as well as deciduous winter rainfall *Disas*, *Satyrium*, *Holothix*
Zak Medium	*Bonatea*, *Habenaria*, *Corycium*, *Brachycorythis*

Table 1.1. Seed germination media

Reflasking

The reflasking medium is generally a ¼ and ½ strength Murashige and Skoog medium. Addition of banana pulp for example tends to improve root growth which is crucial to allow the plants to be deflasked successfully.

Figure 1.15. *Eulophia horsfallii* showing good root development in a ¼ strength Murashige & Skoog medium augmented with 30g/l of fresh banana pulp.

Media suppliers

Ready made media are available from various suppliers and with online purchasing, even obtaining media from overseas is now easy. The only issue is the cost of transport. It often pays to pool an order with another person in order to reduce the transport cost. The safest way to get the product to South Africa is by courier. That is the more expensive option. The other option is to use the US postal service. They will generally get the media to South Africa in under 5 days. Thereafter you are left at the mercy of the South African Post Office from a delivery time point of view.

The cost per liter of medium (assuming you are using around 40ml of medium per flask) is in the region of R 6.00 to R 8.00 at the time of writing.

The other option is to make up media from scratch. This is generally the cheaper method for lager volumes but requires some very accurate weighing of some of the micronutrient components. The chemicals are made up in higher concentration stock solutions and then portions of that dispensed to make up a volume of medium. There are various chemical supply companies available in South Africa but many of the chemicals can also be procured from overseas labs. Just be aware that some media components are subject to import restrictions. Peptone (made from meat) for example comes to mind. That should rather be obtained from a local supplier. Making media up from scratch is more time consuming but reduces the cost to between R 2.00 to R 3.00 per flask.

Media, equipment and chemical suppliers:

Phytotech Laboratories (USA) – large selection of media, chemicals, flask and equipment
Gallup & Stribling (USA) – ready made Hills germination and replate medium
Western Laboratories (Australia) – custom made media for both terrestrials and epiphytes
Sigma Aldrich (South Africa) – ready made media and chemicals

Sterilising seed

The first point when working with dry seed (i.e. seed from a split capsule) is to remove any fine contaminants that may be present with a fine pair of tweezers. Even the tea strainer used to sift out coarse contaminants will still let a few smaller particles through. And it is these particles that may harbor fungal spores and bacteria.
The next challenge is to sterilise the dry seed without killing the delicate embryo inside the seed coat. If this is not done properly the seed will either be partially sterilised. This will result in contaminated cultures that can then be discarded. Or if overdone, it will kill the seed itself resulting in no germination.
Various solutions are used for this and I tend to use a 0.6% bleach solution (made up by measuring out 17.14 ml of 3.5% bleach and topping that up to 100ml with distilled water) for a period of 10 minutes. This is

Figure 1.16. Any foreign particles found in the batch of seed need to be removed with a fine pair of tweezers.

done in sterilised test tubes or 45-50ml plastic centrifuge tubes. When the seed sterilising time is up the seed is placed in the laminar flow cabinet and immediately decanted into a funnel with filter paper.

Figure 1.17. Once sterilised the sterilant needs to be washed off the seed with sterile water.

The sterilant is allowed to drain off and the seed is then washed two or three times with sterile distilled water using a pipette. Once washed the seed is transferred to the flask using a small spatula or sucked up with the pipette and a little sterile water. This is them squirted into the flasks for distribution.

The green capsule sterilising method is by far the easiest method and reduces the possibility of contamination. The seed is always sterile inside a healthy, undamaged capsule. Only the surface of the capsule is thoroughly disinfected and the seed itself is not exposed to the sterilant. This method is especially useful for sensitive seed. As a rule of thumb green seed capsules can be harvested between ½ to $^{2}/_{3}$ of their normal ripening time. This reduces the time to sowing considerably – especially with some of the species that carry their capsules for up to a year.
To sterilise a capsule, first clean away any dry leaves, bracts and flower parts. A sharp pair of nail scissors is useful for this operation.

Figure 1.18. When sowing green capsule all dry flower parts and bracts are cut off.

Then dip the capsule in pure bleach (i.e. Jik) for 5 minutes, gently scrubbing it with a toothbrush or for the smaller capsules with a small paint brush. This ensures that all bubbles are removed and the sterilant gets to all surfaces. Finally, I transfer the capsule into 70% alcohol solution and also give it a gentle scrub in that before moving them into the sterile are. Allow the alcohol to evaporate from the capsule surface completely before cutting the capsule open and gently tapping some seed into the prepared flasks. It is a good idea to distribute the seed over the medium surface with a small spatula. That allows better surface contact.

Figure 1.19. The capsule is sterilised by gently brushing it with full strength bleach and finally rinsed with a 70% alcohol solution.

Equipment

Over the years I have made much of the equipment required for sowing myself – purely because of the high cost of much of the imported equipment such as for example tweezers and spatulas. Spatulas, for example, can easily be made out of stainless steel welding rods. The rods can be cut to the desired length and then the tip heated and flattened with a hammer. After bending the tip, you are left with a perfect little spatula at a fraction of the price of a purchased one.

Figure 1.20. Spatulas can be made by flattening and bending the tip of a piece of a stainless steel TIG welding rod.

For tweezers I am fortunate to have access to a laser cutter. Each leg of the tweezer is laser cut from stainless steel plate and then spot welded at the handle end to create a pair of tweezers. The issue with tweezers is finding ones that are long enough to be able to reflask seedings into a 500ml glass jars without having to push the fingers into the glass jar opening.

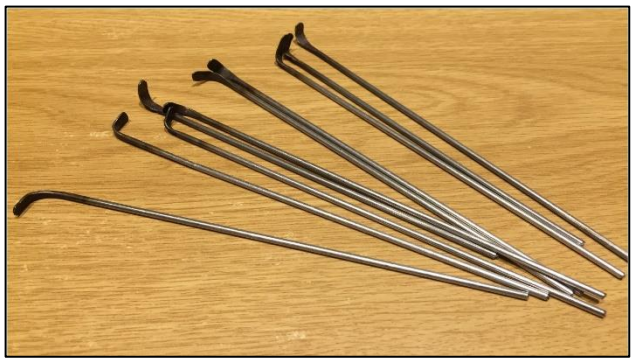

Figure 1.21. A selection of finished spatulas.

Filter paper discs are also available in various diameters from suppliers but I use coffee filter paper and cut two round discs out of each of the coffee filter triangles. Each one is folded in half and then in half again. That then gives you the required funnel shape. And coffee filter paper is available at any of the leading supermarkets.

Figure 1.22. Round discs cut from triangular coffee filters.

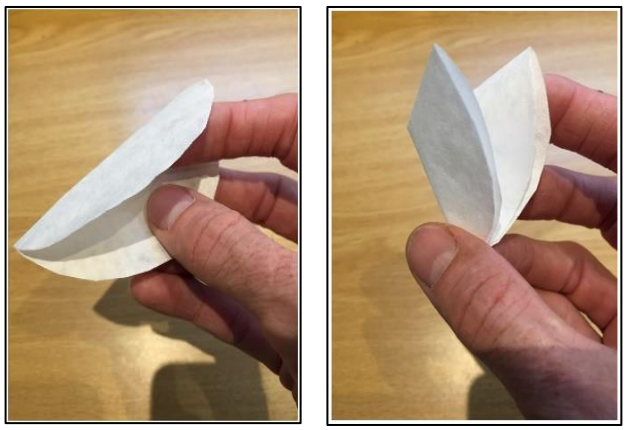

Figure 1.23. Fold these twice to get a funnel shape.

Figure 1.24. Opening the paper up between the folds gives the required filter paper funnel.

A stack of folded filter paper triangles can be wrapped in aluminum foil and sterilised in the pressure cooker or autoclave. This packet can then be placed into the sterile area and filter paper triangles can be removed from the foil wrapper as required.

Deflasking and keeping the plants alive after deflasking

This is the most crucial phase in the life of an orchid seedling and as said before I believe that 95% of the terrestrial orchids are lost during this process.

The timing of deflasking is important. Take some time to determine the natural growth cycle of the species you are wanting to deflask. Terrestrials are ideally deflasked when their natural growth cycle begins. With that one must bear in mind that we have summer rainfall species and winter rainfall species both of which will start their growth cycle 6 months out of sync. Winter rainfall *Satyrium* for example should be deflasked in March or April while those from the summer rainfall areas are ideally deflasked in September or October.

For epiphytes for example one would also deflask these when the temperatures increase in spring. There is an exception and that would for example be the *Mystacidium capense*. Where they can be deflasked in spring, they will normally only show root growth from December onwards (after their normal flowering is complete). So, in order to have the roots rapidly establish themselves I would generally deflask these later in the growing season.

When deflasking it is important to remove as much of the gel medium in which the plants are growing in reflasking so that he medium washed off easily. The reason for this is to remove and much of the gel which can cause fungi and bacteria to grow on them once exposed. Then the plants are tied to a suitable hardwood mount. Place a pad of sphagnum moss on the mount before securely strapping the plant to the mount. The sphagnum allows some moisture retention for the seedling that needs to be hardened off.

The environment that the plantlets are placed in needs to be high in humidity but still allow for a good amount of air movement and fresh air supply. Bear in mind that they come out of an environment where they have been growing in a 100% humidity environment and leaves have not yet developed their resistance to dryer environments. The plants must then be gradually weaned to the typical environment that the mother plants would grow in over a period of 4-6 weeks. For epiphytes one way to do this is to place the mounted plants into a clear plastic container with vent holes. The plants are watered daily and after two weeks the lid can be removed and the plant then finally moved out of the container into the open after 4-6 weeks.

Figure 1.25. Small mounted epiphytes can be placed in clear containers with sufficient vent holes to allow for air movement.

Figure 1.26. After a period of 2 weeks the lids can be removed.

Figure 1.27. Once the roots have established themselves on the mount the plants can be removed from the container.

Some epiphytes like for example *Polystachya zuluensis* will do much better potted up in coarse coconut fiber nuggets which mimic their fibrous natural host the bobbejaanstert (*Xerophyta retinervis*).
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To retain moisture a clear tub or cut off cooldrink bottle with additional vent holes can be placed over the plants for the first few weeks after deflasking.

Figure 1.28. Community pots are ideal for seedlings that are difficult to separate when deflasking.

Figure 1.29. *Polystachya zuluensis* two years after deflasking.

For the terrestrials the best time to deflask is when the plant has produced a tuberoid and is just about to sprout new growth after the natural dormancy or rest period. This would then be the start of the second growing season in flask. They need to be potted up in a well-drained medium and also kept in a slightly shaded position. I often hang trays under the benches and place the newly potted seedling in the tray. This

allows the plantlets to acclimatize better before they are moved onto the benches next to the mother plants. Terrestrials as with epiphytes tend to do very well in community pots and it is often impossible to separate plantlets and tuberoids when they come out of flask without damaging roots and growths. These should be potted into a community pot and only separated once the new tubers have formed and the plants have gone dormant.

Figure 1.30. Seedlings of *Habenaria schimperiana* just sprouting new growths from the new tubers. The plants have gone through one dormancy period in flask and are de-flasked at the start of the second growing period. *Habenaria* seedlings and many other terrestrials typically have rhizoid covered roots that prevent he seedling from being teased apart when potting them up. Rather place these as an unseparated clump in a community pot.

Figure 1.31. Seedlings with pseudobulbs are first washed and as much agar as possible removed from the roots. Seedlings can be left in flask to soak in water for a few hours before attempting to wash off the remaining agar.

Figure 1.32. These *Eulophia* hybrid seedlings are potted up in a community tray using coconut husk. Place a row of seedings against a mound of potting medium, cover the roots with potting medium and then set the next row of seedlings onto that.

Figure 1.33. Finally, a layer of coarse silica sand is places on the medium surface to improve moisture retention, reflect heat and prevent the medium from being washed away when watering.

Figure 1.34. Trays with seedlings are kept under the benches in a shaded position and watered daily until they have acclimatised to dryer conditions. They are then moved to the top of the benches..

Vegetative propagation

Many of the terrestrial species readily propagate vegetatively. Amongst these are *Eulophia*, *Habenaria*, some *Satyriums* and many of the evergreen Disas. I was amazed to what extent *Eulophia* propagates simply by branching out the underground pseudobulbs. This also explains some of the very large colonies of Eulophia that one often finds in nature.

The way to separate these is to repot the plants at the start of their natural growing season before the new roots have developed on new growths. Carefully tip the plants out of their pot and gently separate the plants. Normally the connecting stem between the pseudobulbs will readily separate. Dust any open surfaces or breaks with flowers of Sulphur before repotting the plants. Pots should be prepared with extra holes to improve drainage and also use a layer of coarse gravel or polystyrene chips in the bottom of the pot. Keep the pots small and the plants pot bound to prevent wet spots in the where there are no roots to take up moisture. These wet spots often lead to rot and fungal growth that can impact the plant.

Figure 1.35. A tray of *Eulophia speciosa* at the start of the growing season.

Figure 1.36. The plant is carefully tipped from the old tray.

Figure 1.37. The plants are then separated into sections with two to three pseudobulbs and at least one active growth.

Figure 1.38. Plants are then potted up individually.

Figure 1.39. Vegetative propagation of *Liparis bowkeri*. Separate the individual pseudobulbs and place these on the surface of fresh potting medium. Each pseudobulb may produce up to two new growths per season.

Liparis bowkeri is another prime example of a species that will make two new growths off each of the previous seasons pseudobulbs. Once the new growths are mature the connecting tissue between the old and new pseudobulbs will dry and the old pseudobulb with wither and during dormancy. You are then left with two plants instead of only one. This species makes a beautiful display if allowed to fill a tray over a number of years. What the flowers lack in colour a tray of plants will make up with the sheer number of growths and flowers.

References

Wodrich, K.H.K., 1997. *Growing South African Indigenous Orchids*. A.A.Balkema Publishers, Rotterdam.

Murashige, T. & F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Appendix A – Media Formulations

1/4 STRENGTH MS MEDIUM

Chemical Name	Chemical Formula	Amount per litre of medium (gr)	Stock Solution	Stock Solution Concentration (g/l)	Stock Solution per liter of medium (ml)
Ammonium Nitrate	NH_4NO_3	0.4125	SOLUTION A	20.625	20
Potassium Nitrate	KNO_3	0.475	SOLUTION A	23.75	
13% Iron Chelate (Kompel)		0.043	SOLUTION B	2.15	20
or as alternative:					
Ferrous Sulphate	Fe_2SO_4-$7H_2O$	0.0278	SOLUTION B	1.39	20
Chelate	Na_2 EDTA-$2H_2O$	0.0373	SOLUTION B	1.865	
Calcium Chloride	$CaCl_2$-$2H_2O$	0.11	SOLUTION C	22	5
Magnesium Sulphate	$MgSO_4$-$7H_2O$	0.0925	SOLUTION D	18.5	5
Potassium Phosphate	KH_2PO_4	0.0425	SOLUTION D	8.5	
Manganese Sulphate	$MnSO_4$-H_2O	0.0169	SOLUTION D	3.38	
Zinc Sulphate	$ZnSO_4$-$7H_2O$	0.008625	SOLUTION D	1.725	
Boric Acid	H_3BO_3	0.0062	SOLUTION D	1.24	
Potassium Iodide	KI	0.00083	SOLUTION D	0.166	
Sodium Molybdate	Na_2MoO_3-$2H_2O$	0.00025	SOLUTION D	0.05	
Cobaltous Chloride	$CoCl_2$-$6H_2O$	0.000025	SOLUTION D	0.005	
Cupric Sulphate	$CuSO_4$-$5H_2O$	0.000025	SOLUTION D	0.005	
Nicotinic acid (Vitamin B_3)		0.0005	SOLUTION E	0.05 g/100ml 95% ethyl alcohol	1
Pyridoxine HCl (Vitamin B_6)		0.0005	SOLUTION E	0.05 g/100ml 95% ethyl alcohol	
Thiamine HCl (Vitamin B_1)		0.0001	SOLUTION E	0.01 g/100ml 95% ethyl alcohol	
Agar		8	NO STOCK	weigh out	
Peptone		2	NO STOCK	weigh out	
Myo inositol		0.1	NO STOCK	weigh out	
Sucrose		20	NO STOCK	weigh out	
Banana pulp (optional)		20	NO STOCK	weigh out	
Distilled water		1000	NO STOCK	measure out	

Alternatively use Phytotech Laboratories Medium No: P727 and add 20g/l of Banana Pulp (optional)

Reference: Wodrich, 1997; Phytotech Laboratories Datsheet P727

Appendix A1. ¼ STRENGTH MURASHIGE & SKOOG MEDIUM.

1/2 STRENGTH MS MEDIUM

Chemical Name	Chemical Formula	Amount per litre of medium (gr)	Stock Solution	Stock Solution Concentration (g/l)	Stock Solution per liter of medium (ml)
Ammonium Nitrate	NH_4NO_3	0.825	SOLUTION A	20.625	40
Potassium Nitrate	KNO_3	0.95	SOLUTION A	23.75	
13% Iron Chelate (Kompel)		0.043	SOLUTION B	2.15	20
or as alternative:					
Ferrous Sulphate	Fe_2SO_4-$7H_2O$	0.0139	SOLUTION B	1.39	10
Chelate	Na_2 EDTA-$2H_2O$	0.01865	SOLUTION B	1.865	
Calcium Chloride	$CaCl_2$-$2H_2O$	0.22	SOLUTION C	22	10
Magnesium Sulphate	$MgSO_4$-$7H_2O$	0.04625	SOLUTION D	18.5	2.5
Potassium Phosphate	KH_2PO_4	0.02125	SOLUTION D	8.5	
Manganese Sulphate	$MnSO_4$-H_2O	0.00845	SOLUTION D	3.38	
Zinc Sulphate	$ZnSO_4$-$7H_2O$	0.0043125	SOLUTION D	1.725	
Boric Acid	H_3BO_3	0.0031	SOLUTION D	1.24	
Potassium Iodide	KI	0.000415	SOLUTION D	0.166	
Sodium Molybdate	Na_2MoO_3-$2H_2O$	0.00013	SOLUTION D	0.05	
Cobaltous Chloride	$CoCl_2$-$6H_2O$	0.0000125	SOLUTION D	0.005	
Cupric Sulphate	$CuSO_4$-$5H_2O$	0.0000125	SOLUTION D	0.005	
Agar		5	NO STOCK	weigh out	
Sucrose		20	NO STOCK	weigh out	
Banana pulp (optional)		75	NO STOCK	weigh out	
Distilled water		1000	NO STOCK	measure out	

Alternatively use Phytotech Laboratories Medium No: P748 (also contains 2 g/l Charcoal)

Reference: Wodrich, 1997; Phytotech Laboratories Datsheet P748

Appendix A2. ½ STRENGTH MURASHIGE & SKOOG MEDIUM.

MALMGRENS MEDIUM

Chemical Name	Chemical Formula	Amount per litre of medium (gr)	Stock Solution	Stock Solution Concentration (g/l)	Stock Solution per liter of medium (ml)
Calcium Phophate	$Ca_3(PO4)_2$	0.075	SOLUTION A	7.5	10
Ferrous Sulphate	Fe_2SO_4-$7H_2O$	0.0278	SOLUTION B	2.78	10
Chelate	Na_2 EDTA-$2H_2O$	0.03726	SOLUTION B	3.726	10
Magnesium Sulphate	$MgSO_4$	0.09769	SOLUTION D	9.769	10
Potassium Phosphate	KH_2PO_4	0.075	SOLUTION D	7.5	
Manganese Sulphate	$MnSO_4$-H_2O	0.00154	SOLUTION D	0.154	
Activated Charcoal		1	NO STOCK	weigh out	
Sucrose		10	NO STOCK	weigh out	
Casein, Enzymatic Hydrolised		0.4	NO STOCK	weigh out	
Glycine (amino acid)		0.002	NO STOCK	weigh out	
Myo-Inositol		0.1	NO STOCK	weigh out	
D-Biotin (Vitamin B_7)		0.00005	SOLUTION E	0.05 g/100ml 95% ethyl alcohol	0.1
Folic acid (Vitamin B_9)		0.0005	SOLUTION F	0.05 g/100ml 95% ethyl alcohol	1
Nicotinic acid (Vitamin B_3)		0.005	SOLUTION F	0.5 g/100ml 95% ethyl alcohol	
Pyridoxine HCl (Vitamin B_6)		0.005	SOLUTION F	0.5 g/100ml 95% ethyl alcohol	
Thiamine HCl (Vitamin B_1)		0.01	SOLUTION F	1g/100ml 95% ethyl alcohol	
Agar		5	NO STOCK	weigh out	
Pineapple Pulp (optional)		25 ml	NO STOCK	measure out	
Distilled water		1000	NO STOCK	measure out	

Alternatively use Phytotech Laboratories Medium No: M534 and add 5 g/l Agar and 10 g/l Sucrose

Reference: Wodrich, 1997; Phytotech Laboratories Datsheet M534

Appendix A3. MALMGRENS MEDIUM.

MODIFIED ZAK MEDIUM

Chemical Name	Amount per litre of medium (gr)
Liquid endosperm from Coconut	200
Sucrose	10
Agar	5
Distilled water	800

Reference: Wodrich, 1997

Appendix A4. MODIFIED ZAK MEDIUM.