Seeds in the lab

I collect the seeds just before the seed pods split open because at this stage the seeds have not yet been exposed to spores from bacteria and fungi. The right time is when the pods start to turn brown.

I sterilise all seeds 10-15 min in 1% sodium hypochlorite. Dissolve one drop of dishwash detergent in 100 ml of demineralized water. Add commercial bleach to a final sodium hypochlorite concentration of 1% (1:5 dilution of Chlorox, in DK Klorin), wear gloves to protect your hands. Sterilise the seeds in small reagent tubes or Eppendorf caps. Shake the tubes now and then.

The dry seeds of hardy orchids float on top of the solution so there is no need for filtering. The seeds are simply transferred to the flasks with a straightened paper clip with the end curled to a small loop. Tropical orchid seeds normally sink, and the hypochlorite solution must be removed using a syringe with a sterile single-use needle.

1.5 ml eppendorf caps with seeds and hypochlorite, and an inoculation loop made from a paper clip.

Most amateur orchidists do not have a sterile cabinet for handling of seeds and flasks. Therefore, to avoid contamination with spores from bacteria or fungi, the sterile medium flasks should only be opened in the steam over a boiling water bath. Simply fill a saucepan with water and put it on the stove.

The sterilized seeds can be transferred to the flasks using a straightened paper clip with one end curled into a small loop. Sterilize the loop by flaming it over a burning candle or by using a pocket lighter, not too long or you will burn your fingers. Hold the flask over the boiling water bath, and loosen the aluminium foil. Lift the alufoil slightly and transfer a drop of hypochlorite/seeds to the flasks using the paper clip. Be careful that only the flamed (=sterile) part of the paper clip touches the glas. The small amount of hypochlorite transferred together with the seeds is diluted in the medium and does not reach harmful concentrations. After sowing the seeds, the aluminium foil is exchanged with two layers of kitchen film (cling film) over a boiling water bath. Then seal the flasks with sticky tape. The kitchen film is semi-sterile when you buy it in the supermarket because it is manufactured at high temperature. To increase the sterility, place the cling film roll in a microwave oven together with a glass of water and radiate for 3 minutes. When applying the cling film to the flasks, be careful not to touch the side of the film that faces the inside of the flask.

The risk of contamination is reduced if the flasks are handled on a boiling water bath when the seeds are sown.

It is important, that there is no air-movement in the room when you sow the seeds and replace the aluminium foil. Air flow increases the risk of contamination, so close all doors and windows and hold your breath.

Inside the flasks there is often a few drops of condensed water; gently distribute the seeds on the gel-surface with these drops. If the gel is dry, you may get a little water by placing the flasks in a tilted position for a few days in the refrigerator.

With the above procedure, you will get contamination with bacteria or fungi in 10 to 70 % of the flasks. Practise increases the success rate. The risk of contamination is much lower if you have access to a sterile cabinet instead of using a boiling water bath.

Sometimes, the only seed you can get of a much wanted species come from old, brown and partly decaying seed pods. In that case, you will get 100% contamination with the above procedure, so more elaborate procedures are needed. Spores of bacteria and fungi are very resistant to chemical attack, so 1% hypochlorite will not kill all spores of heavily contaminated seeds.

Calanthe tricarinata seedlings.

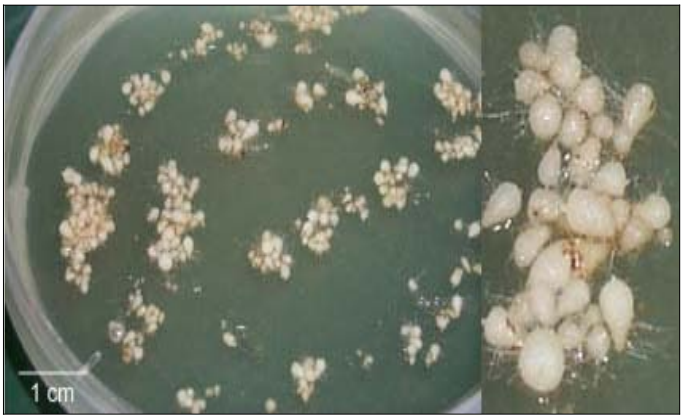
Platanthera hyperborea. Each protocorm produce several shoots that can be divided and transferred to fresh medium where they again produce more shoots etc.

The trick is to make the spores germinate before killing them, and this is done by offering them sugar. Make a sugar solution (app. 1 tea spoon per litre). Mix the seeds with 1 ml sugar solution in a reagent tube or an Eppendorf cap. Incubate over night at room temperature. Remove the sugar solution using a syringe with a needle. Then sterilize the seeds by adding some 1% sodium hypochlorite solution and let it react for 20-30 minutes, shake now and then. Sow the seeds as described above.

Sometimes, the sugar trick is not enough either, and a really harsh treatment is needed, for example silver nitrate ($AgNO_3$). Silver nitrate is a toxic heavy metal and the following procedure

A single spore inside the flask and you have bacteria or fungi all over.

should not be tried in the kitchen, only in professional labs. Make a fresh 1% $AgNO_3$ solution (100 mg in 10 ml water). Mix the seeds with 1 ml $AgNO_3$ solution and incubate 15-20 minutes. Wash the seeds once with sterile demineralized water. Then wash once with sterile 1% NaCl (salt) solution. Wash twice with sterile water and sow the seeds. The salt solution precipitates small silver nitrate grains on the seeds and these grains will have a sterilizing effect for several days, killing the germinating spores. Silver nitrate reacts with light, so the bottle should be wrapped in alu-foil and stored in the dark.



Protocorms of *Dactylorhiza majalis* ready for replate. They have been grown 3.5 months at 15 degrees Celsius. Notice the small shoot initials.

When the seeds have been successfully sown, the flasks should be incubated in the dark. Germination time varies from a few days to more than two years but fresh seeds of easy species like *Dactylorhiza, Bletilla, Calopogon* and some *Platanthera* usually germinate within 2-4 weeks. First, the seed makes a clump of undifferentiated cells, a protocorm (=pre-body). When the

protocorm reaches a certain size, it makes a shoot and a root at one end. Sometimes it makes more protocorms and then you get several plants from each seed.

Dactylorhiza purpurella (14 months) ready for deflasking.

Temperature

Temperature is important for both germination and development. I get the best results by incubating the flasks at 10-15 °C. Growth of the small orchids is actually faster at 15 °C than at higher temperatures. If the temperature reaches 25-30 °C many temperate seedlings simply give up and die.

I give all species a cold period (winter) at 1-5 °C in December, January and February, both ungerminated seeds, protocorms and fully developed seedlings. For northern species, this cold treatment improves germination, some dormant seeds may actually need two "winters" before they germinate almost two years after they were sown. For seedlings of cold-temperate genera like *Dactylorhiza*, *Platanthera*, *Gymnadenia* and *Calopogon*, the small plants need a cold period to break dormancy before they can be transferred to soil in spring.

If you have access to a sterile air cabinet, you may replate the protocorms on fresh medium when they are 2-3 mm, make sure that there is good contact between the protocorms and the medium to avoid dessication. If you do not have a sterile air cabinet, you can just hope that you did not sow too many seed per flask because then they will be crowded and the plants will compete for nutrients. Place the seedlings in dim light when they have made shoots, but no direct sunlight.

Breaking the seedcoat

The cells inside the orchid seeds swell and grow when they get water and nutrients, but some species have thick or waxy seed coats that are non-permeable. This is for example the case for seeds from some of the Mediterranean orchids like *Orchis purpurea and Orchis militaris* where thick seed coats protect the seeds during the warm and dry Mediterranean summer. In nature, the seed coat is probably broken down by enzymes excreted by fungi, but in the lab, we have to break it chemically by etching the seeds with sulfuric acid (H_2SO_4) (4).

Mix the seeds with 1% sulfuric acid in a small reagent tube or an eppendorf cap and incubate for approximately 10 minutes. Some seeds with very thick seed coats may need higher concentrations of sulfuric acid or longer etch-time. Now, make a little funnel by folding twice a round piece of coffee filter paper. Place the funnel in a small glass, add the seed/sulfuric acid mix and let the acid pass through the filter. Wash the seeds several times with tap water by adding a few ml of water with a syringe, let it pass through the filter, add more water, etc. When the seeds are free of sulfuric acid, they may be transferred to a new reagent tube with the tip of a knife and sterilized with hypochlorite as described above.

If hypochlorite is mixed with acid, the result will be toxic chlorine fumes, so be very careful to remove all the sulfuric acid before sterilizing the seeds.

For some *Cypripedium* the permeation of the seed coat can also be achieved by increasing the time in sodium hypochlorite. Optimum bleach-time for *C. macranthos* was reported to be 30 min in a 1% solution which led to 40,2% germination of mature seeds. Permeation of the seed coat was documented by scanning electron microscopy (5). I have not tested this method myself.

*Calanthe tricarinata a*nd other calanthes are easy to propagate from mature seeds or green pods.


A home-made filter for removing sulfuric acid.


A plate of *Dactylorhiza* seedlings ready for transfer to soil 19 months after the seeds were sown.

Green pods
Some *Platanthera*, some *Orchis*, and most *Cypripedium* are more easily grown from immature seeds. I harvest the green pods from most most European orchids about 40 days after pollination, for *Cypripedium* it is variable from 60-80 days. The draw-back of this method is that you need a sterile air cabinet for handling the seeds.

Small pods may be sterilized by dipping in ethanol, then in 2% hypochlorite for 30 min, followed by washing in two changes of sterile water. Large seed pods may be sterilized by dipping in ethanol and the setting them on fire while the pods are moved slowly back and forth to avoid heating of the seed.

Place the sterile seed pod on a piece of tin foil that has been sterilized in the oven at 200 °C for 1 h. Sterilize scissors and forceps by flaming them on all surfaces with an ordinary pocket lighter. Dissect out the immature seeds, and transfer them to the medium flasks.

Easy orchids
Dactylorhiza, Liparis, Calanthe, Bletilla, Pleione, Spiranthes, Calopogon, Goodyera, Serapias, Thelymitra, some *Ophrys*, many *Anacamptis (A. sancta,, A. coriophora, A. fragrans, A. palustris, A. laxiflora, A. papilionacea),* some *Orchis* with thin seed coats (*O. Anatolica, O. pauciflora, O. italica*) some *Platanthera* (*P. dilitata, P. hyperborea, P. blephariglottis*).

These orchids are all straight forward to germinate from fresh mature seeds. Orchids with handshaped tubers (*Dactylorhiza, Gymnadenia*) can be transferred to soil after one year. Orchids with round tubers (*Orchis, Ophrys, Anacamptis, Serapias*) have low survival if the tuber is too small, so they need an additional year in the flasks.


Liparis kumukiri is easy and the mother plants always make a lot of seeds, probably due to autogami.


Platanthera dilitata (23 months) ready for deflasking.

Difficult orchids
Anacamptis pyramidalis, Orchis with thick seed coats (*O. militaris, O. purpurea, O. mascula*), P*latanthera* with thick seed coats (*P. chlorantha, P. bifolia*). *Epipactis, Cephalanthera*, most *Cypripedium*. The mature seeds from these orchids all have rather impermeable seeds. Treatment with sulfuric acid increases germination

of most of the species, but germination may still take more than a year and germination percentages are often low. Germination can however be quite successful for some of the genera if the green pod method is used.

"Winter" in the fridge. Three months at 4º C breaks the dormancy in seeds and seedlings from cold habitats.

References:

The above information is inspired by articles written by Svante Malmgren and others.

(1) Malmgren, S. 1988. Fröförökning av *Dactylorhiza* i stor skala; en kort manual. Svensk Botanisk Tidskrift 82: 161-166.

(3) Malmgren, S. 1992. Hybridisering blandt svenska orkideer; korsnings-, och odlingsförsök. Svensk Botanisk Tidskrift. 86: 337-

(4) Malmgren, S. 1993.Asymbiotisk fröförökning i stor skala av *Anacamptis, Ophrys, Orchis* och andra orkideer med runda rodknölar. Svensk Botanisk Tidskrift v87p221-234.

(5) Bae K H. 2010. Structural changes of seed coats and stimulation of in vitro germination of fully mature seeds of C*ypripedium macranthos* Swartz (Orchidaceae) by NaOCl pretreatment. Propagation of ornamental plants 10: 107-113.

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