Addendum to the
Project Re-introduction of *Spiranthes aestivalis* in The Netherlands.

A detailed account of the experimental protocols for asymbiotic and symbiotic seed sowing of Summer Lady's-tresses *(Spiranthes aestivalis*) orchids

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Illustrations by AMW

Methods for seed sowing[2]

In the project proposal 3 different methods of seed sowing are proposed:

1. Sowing directly in pots with soil obtained from the accepting locations Urkhovense Zeggen (NL) and donor locations La Flèche and Lessay (France).
2. Sowing directly in pots with soil obtained from the accepting locations Urkhovense Zeggen (NL) and donor locations La Flèche and Lessay (France), mixed with grinded egg carton moistened with liquid from a preculture of tap water, sucrose and soil from the respective locations (in order to enrich the soil with cellulose thereby providing an extra carbon source for the fungi that are essential for germination to occur).
3. Sowing *in vitro* on a "fungus replacement" nutrient medium (asymbiotic cultivation).

However, during the time between writing the project proposal and obtaining permission from the French authorities to collect seed from *Spiranthes aestivalis*, members of the Dutch Society for Propagation of Orchids (in Dutch: Vereniging Orchideeën Vermeerdering, total number of members currently 91) gained experience with symbiotic orchid cultivation. The fungal isolates HOSB1 and HOSA36, generously made available by the British Hardy Orchid Society, were found to be quite effective in obtaining good and especially rapid germination in a number of species (including some *Spiranthes* species like *S. cernua* but also *S. sinensis* and *S. spiralis.* Thus, we have decided to add a 4th method for sowing:

4. Sowing *in vitro* on pre-cultivated fungal cultures HOSB1 or HOSA36 (symbiotic cultivation).

Methods used for sowing may vary depending on the stage of the project

In all sowing experiments, careful record will be made of which seed pods have been used for what sowing method. Preferably, seed from one seed pod (of which the location and parent plant has

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[2]The methods described here are based on scientific literature, but also on modifications and 'tricks' from practice. See for a comprehensive overview of how the *in vitro* culture of orchids has developed:
Yam TW, Arditti J (2009): History of orchid propagation: a mirror of the history of biotechnology Plant Biotechnol Rep 3: 1–56

been noted) will be used for one treatment. If there is not enough seed in a seed pod for a particular treatment, a second pod of the same parent plant will be used. This allows the results obtained to be followed on the basis of the individual parent plant.

It seems tempting to immediately sow a large portion of the collected seeds in soil from the intended location, enriched or not with cellulose (methods 1,2). However, the amount of seed that we can collect in the French locations is limited and immediate success is not guaranteed: in general the yield of such direct sowing is low.
Therefore, the very first goal in the project will be to use the collected seed for obtaining as many seedlings as quickly as possible and to collect as much seed as possible after flowering of the mature plants developing from these seedlings, thus creating our own substantial source of seed supply. And that is why, at the start of the project, the emphasis will be, at least with the two seed-pods that can be maximally obtained at the donor location La Flèche, on *in vitro* propagation, asymbiotic as well as symbiotic (method 3,4). With respect to the location Lessay: if the permitted 5 stems with ripe pods indeed can be obtained, enough seeds will probably be left, after sowing symbiotically and asymbiotically, for sowing in soil from donor and/or acceptor location.
For one sowing session, one to two seed pods are needed, depending on how large and well-filled they are. Should it come to the point that with the donor La Flèche seed pods a choice must be made, preference will be given to asymbiotic sowing. Development of the seedlings is slower than with symbiotic sowing, but it produces many more seedlings per sowing session.

The seeds, obtained in this way will be used for further propagation under controlled conditions (private greenhouses of VOV members in at east 4 different locations) and for direct sowing outdoor in the intended nature area.
Also a selection of mature plants can be planted in the acceptor location Urkhovense Zeggen (NL). Permission has already been obtained from the landowner.

We will carefully monitor and share the results as well as the details of the sowing and planting protocols with anyone interested (see step 7. Dissemination below for further details). However, because not everyone may be very familiar with the methods used in *in vitro* propagation of orchids, and because this is not described in much detail in the project plan, we have decided to expand the project plan with this addendum, in which the asymbiotic and symbiotic sowing protocols may be explained and illustrated.

Asymbiotic sowing of *S. aestivalis*

Orchid seeds, in nature, germinate only when they get infected with mycorrhizal fungi that provide the developing embryo with water, carbohydrates, minerals, and vitamins. But if orchid seeds are placed on a suitable cultivation medium, they get the necessary nutrition from the medium and they no longer need special fungi to germinate.
Because it is not desirable that all kinds of harmful bacteria and fungi start to grow on such rich culture media, we work under sterile conditions (in a laminar flow cabinet). The seeds are sterilized with bleach (NaClO). In addition to a disinfectant effect, the bleach also has the effect of weakening

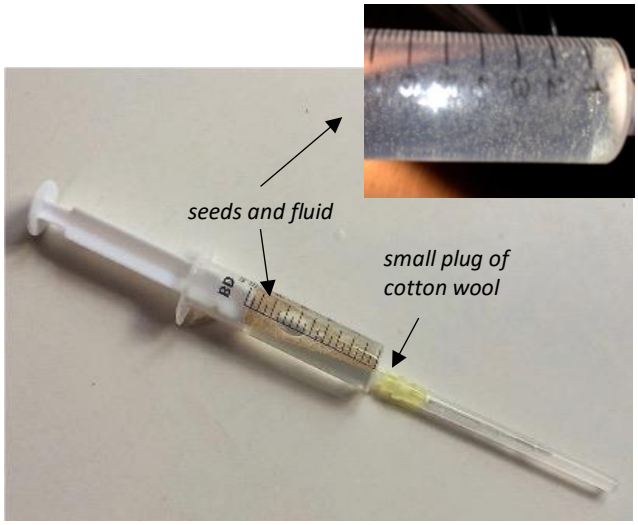
the hard seed coat, allowing water and nutrients to enter more easily (in nature, the fungus breaks down the seed coat).

Step 1. Preparation of the sowing medium
Seeds will be sown on BM1 medium with Nitsch vitamin mixture, sucrose and agar:

Macronutrients		*Vitamins*	
KH_2PO_4	300 mg.L^{-1}	Biotin	0.05 mg.L^{-1}
$MgSO_4$ * 7 H_2O	100 mg.L^{-1}	Folic acid	0.5 mg.L^{-1}
Caseine hydrolysate	500 mg.L^{-1}	L-glycine	2.0 mg.L^{-1}
L-glutamine	100 mg.L^{-1}	Myo-inositol	100 mg.L^{-1}
		Nicotinic acid	5.0 mg.L^{-1}
Micronutrients		Pyridoxine	0.5 mg.L^{-1}
$CuSO_4$ * 5 H_2O	0.025 mg.L^{-1}	Thiamine	0.5 mg.L^{-1}
$FeSO_4$ * 7 H_2O	27.85 mg.L^{-1}		
Na_2EDTA	37.25 mg.L^{-1}	Sucrose	20 g.L^{-1}
Na_2MoO_4 * 2 H_2O	0.25 mg.L^{-1}	Agar	6 g.L^{-1}
H_3BO_3	10 mg.L^{-1}		
$MnSO_4$ * 4 H_2O	25 mg.L^{-1}		
$CoCl_2$ * 6 H_2O	0.025 mg.L^{-1}	pH 5.5	
$ZnSO_4$ * 4 H_2O	10 mg.L^{-1}		

The nutrient medium is sterilized in a pressure cooker and aliquots of 30 ml are poured in sterile plastic containers.

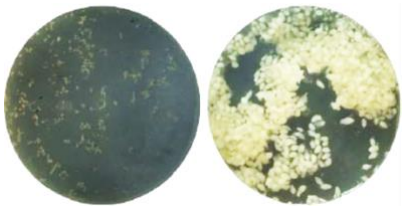
Step 2. Sterilization of seeds
The most widely used method of sterilizing seeds is by means of a (5 or 10 ml) sterilized syringe, with a small plug of cotton wool in the bottom of the needle hub. Fluid can then be drawn into the syringe and the syringe can be emptied again while the seeds remain in the syringe.
The procedure is as follows:



- A 1% solution is prepared of commercially available 'thin bleach' with 2 drops of dish detergent per 250 ml (in order to lower the surface tension so that optimal contact between seed and sterilization fluid is achieved).
- A pinch of seeds is placed in the barrel of the syringe, 8-10 ml of the bleach is drawn and the syringe is shaken vigorously until the seeds are dispersed in the fluid. Note that the seeds stay afloat.
- Syringes are shaken well at least every five minutes. The brownish color of the seeds will slowly disappear and the seeds will start 'whirling' upon shaking. This will usually take some 20 minutes. At that point, the syringe is emptied and 6-7 ml of sterilized and demineralized water is drawn. Shake well and empty immediately. The syringe is refilled with 6-7 ml sterilized and demineralized water and the seeds are rinsed for at least an hour. Shake every 10 minutes.

Step 3. Seed sowing

The syringe is emptied and 2 ml of sterilized and demineralized water is drawn. Seeds are dispersed by vigorous shaking. The needle (including the cotton wool plug) is removed and the syringe content is injected into the sterile nutrient medium container(s). Shake gently to disperse the seeds evenly over the agar. Excess water is removed with a sterile syringe. The container is closed and stored in the dark at room temperature. Protocorm formation usually starts after ± 6 weeks.



immediately after sowing *after 6 weeks*

Orchid seeds on nutrient medium (photographed against a dark background)

Step 4. Replating of seedlings

When roots and shoot formation has started, seedlings will be replanted on 1/5 MS medium with Nitsch vitamin mixture, sucrose, grinded unripe banana, grinded potato and agar and placed under dim light conditions. The nutrient medium is sterilized in a pressure cooker and aliquots of 150 ml are poured in sterile cultivation containers.

Orchid seedlings after 1 year of growth

Macronutrients		*Vitamins*	
$CaCl_2$	66 mg.L^{-1}	Biotin	0.05 mg.L^{-1}
KH_2PO_4	34 mg.L^{-1}	Folic acid	0.5 mg.L^{-1}
KNO_3	380 mg.L^{-1}	L-glycine	2.0 mg.L^{-1}
$MgS0_4$	36mg.L^{-1}	Myo-inositol	100 mg.L^{-1}
NH_4NO_3	330 mg.L^{-1}	Nicotinic acid	5.0 mg.L^{-1}
		Pyridoxine	0.5 mg.L^{-1}
Micronutrients		Thiamine	0.5 mg.L^{-1}
$CuSO_4 * 5 H_2O$	0.025 mg.L^{-1}		
$FeSO_4 * 7 H_2O$	27.85 mg.L^{-1}	Sucrose	20 g.L^{-1}
Na_2EDTA	37.25 mg.L^{-1}	Agar	6 g.L^{-1}
$Na_2MoO_4 * 2 H_2O$	0.25 mg.L^{-1}	Ripe banana 1cm^3.L^{-1}	
H_3BO_3	10 mg.L^{-1}	Potato 1cm^3.L^{-1}	
$MnSO_4 * 4 H_2O$	25 mg.L^{-1}		
$CoCl_2 * 6 H_2O$	0.025 mg.L^{-1}		
$ZnSO_4 * 4 H_2O$	10 mg.L^{-1}	pH 5.5	

A variety of asymbiotically cultured orchids in the private greenhouse of one of the members of the Dutch Society for Propagation of Orchids

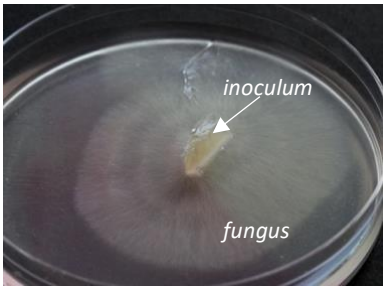
Step 5. Planting

When tuber formation has taken place plantlets will be transferred to pots with soil from the Urkhovense Zeggen location. Pots are then placed in a cold green house in moderate light (regime following outdoor conditions).

Symbiotic sowing

Orchid seeds, in nature, germinate only when they become infected with mycorrhizal fungi that provide the developing embryo with water, carbohydrates, minerals and vitamins. The British Hardy orchid Society generously made two fungal strain available, HOSB1 and HOSA36 respectively, that are proven to germinate a range of terrestrial orchids, including several *Spiranthes* species (see for more details https://www.hardyorchidsociety.org.uk/HOS%201012/Cultivation.html).
Good results have already been obtained by members of the Dutch Society for Propagation of Orchids with *Spiranthes sinensis and S. spiralis.*
With this method, seeds are sown in the presence of one of the fungus cultures.
It has to be avoided that all kinds of harmful bacteria and fungi other than HOSB1 and HOSA36 infect the germinating seeds, so also here, we work under sterile conditions (laminar flow cabinet).

Step 1. Establishing and maintaining the fungal cultures



Symbiotic fungi HOSB1 and HOSA36 are grown in individual sterile Petri dishes, containing 40 ml culture medium consisting of pressure cooker sterilized 6g.L^{-1} agar and 2.5g.L^{-1} finely grinded oatflakes.
The Petri dishes are inoculated with a small section of fungal mycelium. This quickly spreads across the agar plate.
The fungus is replated every 3 weeks on fresh culture medium.

Step 2. Seed sterilization

Seed sterilization is performed as described in step 2 of the asymbiotic sowing procedure.

Step 3. Seed sowing

Seeds are sown as described in step 3 of the asymbiotic sowing procedure on Petri dishes containing a culture medium consisting of pressure cooker sterilized 6g.L^{-1} agar and 2.5g.L^{-1} finely grinded oatflakes. Small sections of fungus culture (mycelium and agar) are then added to the Petri dish containing the sterilized seed. The fungal mycelium quickly spreads and infects the individual orchid seeds enabling seed germination to take place.

Step 4. Replate of seedlings

S. sinensis in a symbiotic (HOSB1) culture

As soon as protocorms are visible they are transferred to similar culture vessels as shown in step 4 of the asymbiotic culture procedure, but then filled with a culture medium consisting of pressure cooker sterilized 6g.L^{-1} agar and 2.5g.L^{-1} finely grinded oatflakes. Seedlings are replated every 4 weeks.

Step 5. Planting

When tuber and root formation has taken place plantlets will be transferred to pots with soil from the Urkhovense Zeggen location. Pots are then placed in a cold green house in moderate light (regime following outdoor conditions).

Step 6. Monitoring survival of seedlings

After planting, survival and growth of individual plantlets over a three year period will be recorded.

Step 7. Dissemination of results

All participating VOV members will sign a Memorandum of Understanding (MOU) stating that they will not distribute any seeds to third parties not involved in this project. Results obtained with the experiments will be shared with publications in relevant journals.