Project Re-introduction of *Spiranthes aestivalis* in The Netherlands
Interim report #1: Seed collection, sowing and germination

Authors: Anneke Wagner, Jos Lammers and Joop ten Dam
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August 30 (2021): In the project plan it is described that seeds of *S. aestivalis* would be collected at two locations: in La Flèche (Pays de la Loire) and in Lessay (Normandie). Unfortunately, not a single specimen of *S. aestivalis* was found that day on the location in La Flèche where they were supposed to be. Therefore, the results described in this and following progress reports relate only to seeds collected in Lessay.

August 31 (2021): Seed pod and soil collection Lessay
Stems with ripe pods from *S. aestivalis* in five different spots spread across the location were cut off with scissors and collected separately in marked dry paper sacks.
In addition, about a kilo of soil was collected from spots close to a few groups of *S. aestivalis.*

Fig. 1. Seeds are released from the pods.

September 1-11 (2021): Drying of the seed pods
The collected stems were laid on paper sheets at room temperature in Zoetermeer (The Netherlands). After two days the first seed pods started to release their seeds (Fig. 1). Seeds from the same stem were combined, packed in a small paper envelope and stored at 4°C.
When all pods were empty, the five portions of seed thus obtained, were, in order to reduce any risk (f.i. contamination) divided over three 'experienced sowers' of the Dutch Society for Propagation of Orchids.
The collected soil was stored in a loosely folded plastic bag at 4°C

September 11 – 30 (2021): Sowing of the seeds and determination of seed viability
Seeds were sown *in vitro*, both asymbiotically (BM1 medium) and symbiotically (fungus strains B1 and A36, obtained from the British Hardy Orchid Society). Depending on the size of the seed portion, 2-4 jars/petri dishes per treatment could be prepared[1]. Jars and petri dishes were stored at room temperature in the dark.

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[1] See for details of the sowing protocol and recipes the Addendum to the project: 'A detailed account of the experimental protocols for asymbiotic and symbiotic seed sowing of Summer Lady's-tresses *(Spiranthes aestivalis*) orchids', July 2021

During the sowing process, small amounts of seed before and after sterilization were used for microscopic examination of the seeds and determination of the viability of the embryos with the tetrazolium method. This method is based on the fact that all living tissues, which respire, are capable of reducing a colourless chemical into a red coloured compound (catalysed by socalled dehydrogenase enzymes).[2]

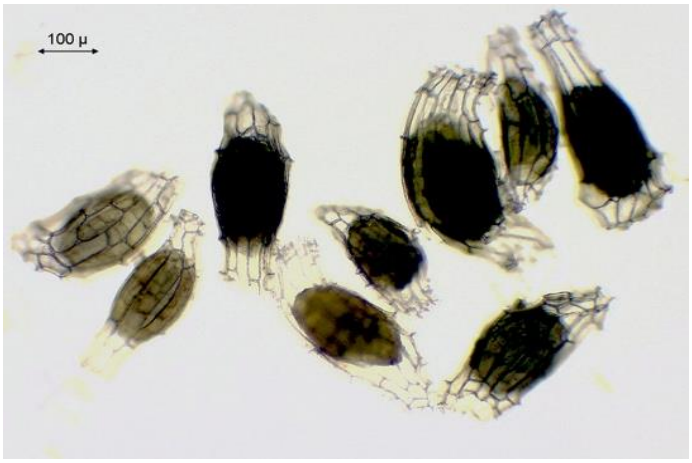

Fig.2. S. aestivalis seeds before sterilization

Fig. 2 shows *S. aestivalis* seeds before sterilization. All seed portions rendered similar images: almost all seeds contain an embryo, the seed coat is yellow-brownish and the embyos are surrounded by a very dark inner seed coat (carapax). Because of the latter characteristic, the tetrazolium test was performed after sterilization with bleach, so that the red colour of the embryo– if present- would become visible.

In Fig 3 it can be seen that after sterilization the seed coats and the carapax have indeed lost their brown colour and that, as a result of the tetrazolium treatment, many, but not all, embryos are coloured brightly red, indicating that they are viable. This is a very normal result for orchid seeds, in our experience usually around 60% of terrestrial orchid seeds, collected in the wild, react with the tetrazolium chemicals.

There was no significant difference in the percentage of coloured embryos between the five portions of seeds.

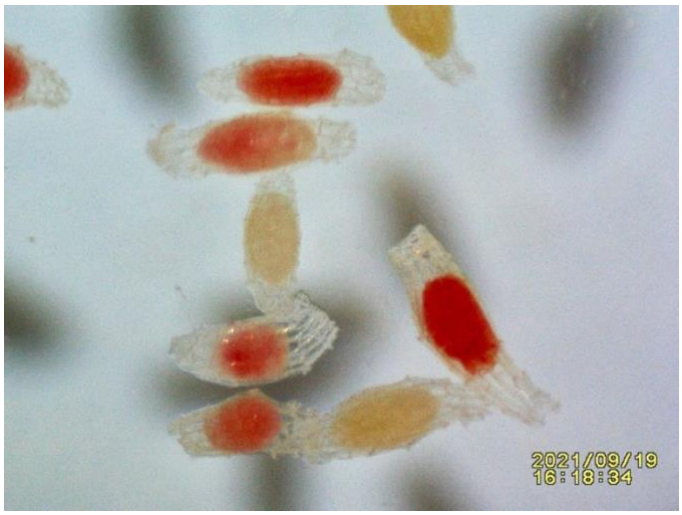

Fig.3. Seeds after sterilization and after treatment with tetrazolium. (dark spots in the background are shadows on the object slide).

Besides sowing *in vitro*, seeds were also sown on soil from the location Urkhovense Zeggen (the intended reintroduction location) and Lessay. Pots with a cardboard inlay were used in order to provide an extra carbon source for the fungi present in the soil. From each seed portion 2 pots were prepared, each with one of the soil samples.
Pots were placed outdoors, covered loosely with aluminium foil and placed in a low plant tray with a 1 cm layer of water in order to keep the soil permanently moist.

[2] See for more detailed description of the method: http://seednet.gov.in/pdffiles/chapter%2014.pdf

September 23 (2021): First germination of the symbiotically sown seeds

In less than two weeks after symbiotically sowing, it became apparent that seeds from two of the five portions (both with B1 as well as with the A36 fungus strain) started to germinate: with a magnifying glass protocorm formation was clearly visible. (Fig. 4)

In the following week(s) this also occurred in the other three portions.

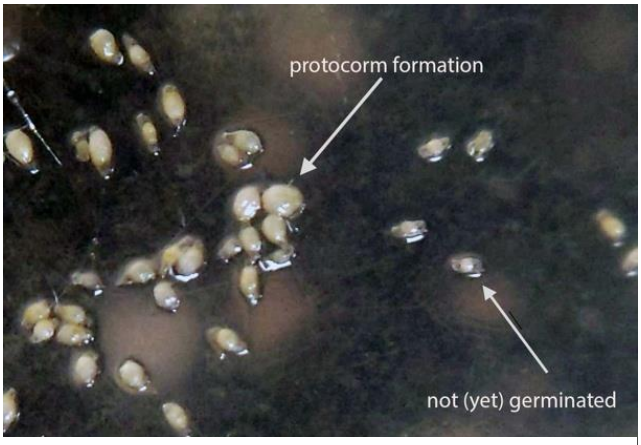

Fig.4. Protocorm formation in vitro after two weeks in the presence of HOS fungus A36

End of October/beginning of November (2021):

In all five seed portions protocorm and root hair formation proceeded, and stems became visible. The first seedlings with a proper stem (like the one in the lower left corner of Fig. 5) are now transferred to a container (with the same culture medium) where they are widely spaced (max 10 per container) and exposed to moderate light conditions. This leads, after a few days, to chlorophyll formation (Fig 6).

Fig.5. Root hair and stem formation (on HOS fungus A36)

In all asymbiotically sown seed portions swelling of the seeds is visible, so it looks as if germination starts there as well, but - as with other orchid species - the process proceeds much slower than in the symbiotic cultures.

No germination is yet observed of seeds sown in soil from the intended location and Lessay, but that is probably not to be expected before spring.

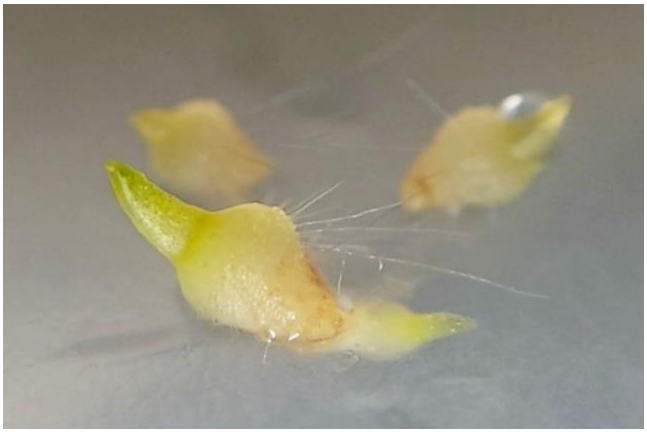

Fig.6. Seedling after replate and exposed to light (on HOS fungus B1)

Conclusion:
Apart from the disappointment of not having been able to collect seeds in the La Flèche area (which would have been good for bringing in more genetic variety), the project started extremely well.
The *S. aestivalis* seeds collected in Lessay germinate quickly in the presence of the B1 and A36 fungi, continue to grow after the first replate and chlorophyll synthesis occurs.
The asymbiotical cultures also germinate according to our expectations.

Inherent to this kind of *in vitro* cultures is that only a very small portion of the germinating seeds will eventually make it to potted mature plants, but if the cultures continue to develop as they do now, we are confident that we will obtain enough plants for our goal: trying to get the Summer Lady's Tresses back in the Netherlands.

Acknowledgement
We are very grateful to Mme. Catherine Zambettakis (Conservatoire botanique national de Brest, Antenne Normandie Caen) for her expert and enthusiastic guidance in the Lessay location, which has enabled this successful start of this project.