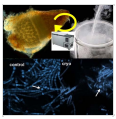

Nov 14, 2019

Azolla filiculoides sporocarp cryo-preservation

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1 Works for me dx.doi.org/10.17504/protocols.io.9bdh2i6

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ABSTRACT

Method extracted from https://doi.org/10.1111/nph.12708

Brouwer P, Bräutigam A, Külahoglu C, Tazelaar AO, Kurz S, Nierop KG, van der Werf A, Weber AP, Schluepmann H. A zolla domestication towards a biobased economy?. New Phytologist. 2014 May;202(3):1069-82.

EXTERNAL LINK

https://doi.org/10.1111/nph.12708

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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nph.12708.pdf

MATERIALS TEXT

Sporulating A. filiculoides Lam. was collected in mid-October 2012 from a ditch in Utrecht, the Netherlands (52°04'24"N; 5°08'53"E) and kept in demineralized water in a glasshouse at 5–15°C and 14 h days with a light intensity of at least 70 micromol/ s.m2 Photosynthetic Photon Flux Density (PPFD).

Mostly fertilized megasporocarps from sediment accumulating at the bottom of the containers in which sporulating cultures were kept was used for the cryo-preservation procedure. To purify these, the method according to Toia et al. (1987;https://doi.org/10.1111/j.1469-8137.1987.tb00142.x) was used except that the sorbitol: water step gradient was layered on top of the residue of the 200-microm mesh size sieve to obtain a clear layer of fertilized megasporocarps.

Unfertilized megasporocarps were also collected according to Toia et al. (1987;https://doi.org/10.1111/j.1469-8137.1987.tb00142.x) with modifications: sporulating plants were placed on top of a stack of sieves with 1000-, 500- and 200- lm mesh sizes and megasporocarps were detached from the stems using a strong water jet. Residue recovered on the 200- microm mesh size sieve was then layered on a 3 M sorbitol: water step gradient and centrifuged at 400 g for 10 min, resulting in a clear layer of pure megasporocarps. Megasporocarps were washed three times by centrifugation with 50 ml water before use for cross fertilization experiments.

For crosses, mature microsporocarps were plucked manually from the plants.

1 **Preliminary knowledge: cryo-preservatives were not effective but drying was**.

The cryopreservation protocols were explored on batches of 20-50 megasporocarps. Each condition was evaluated in duplicate.

For cryopreservation involving cryoprotectant pre-treatment, 1 ml of the cryoprotective solution was added to the megasporocarps immediately before snap-freezing in liquid nitrogen (LN). Batches were kept for 5 min in LN, then thawed on a heating plate set at 30°C for 1.5 min and washed thrice in 1 ml medium.

To test cryopreservation using a drying pre-treatment, megasporocarps were dried for 1, 4 and 8 d in the fume hood at RT and then snap frozen in LN without added fluid. Batches were kept for 5 min in LN then thawed for 1.5 min at 30°C before adding 1 ml medium.

For each cryopreservation protocol, a treatment control was included that was not frozen but still exposed to the cryopreservation pre-treatment; additionally two controls were included that received no treatment.

	No freezing			Freezing		
Pre-treatment	*N*	Viability (%)	Germination (%)	*N*	Viability (%)	Germination (%)
None (control)	32	67	23	42	0	0
DMSO + Glycerol	29	93	7	34	0	0
DMSO + EG + PVP	36	79	23	41	0	0
Sucrose	37	92	29	49	0	0
Trehalose	50	82	57	70	0	0
DMSO + EG + Glucose + Trehalose	35	71	48	71	0	0
1 d drying	31	51	9.40	36	19	0
4 d drying	24	24	21	27	30	0
8 d drying	31	38	10	36	25	6

[1]Dimethyl sulfoxide (DMSO), ethylene glycol (EG) and Polyvinyl pyrrolidone (PVP).
[2]Viability is the percentage of megasporocarps with floats. Freezing was in liquid nitrogen. Each condition was tested in duplicate. *N*, number of megasporocarps tested.

Table 1 Cryo-preservatives were not effective but drying was.

Drying of megasporocarps collected from sediments (with massulae attached) were tested using batches of 247-391megasporocarps.

Pre-treatment	Freezing	*N*	Germination (%)
None (control)	No	281	50.96
RT 1 d	LN	391	0.00
RT 4 d	LN	382	0.79
RT 7 d	LN	247	5.66
RT 16 d	LN	283	1.06
RT 32 d	LN	305	2.62
CT 26°C 1 d	LN	371	4.86
CT 26°C 4 d	LN	340	26.50
CT 26°C 7 d	LN	279	50.62

Megasporocarps collected from sediment were dried 1–32 d at either fluctuating room temperature (RT) of constant temperature (CT) of 26°C. *N*, number of megasporocarp tested. Megasporocarps were either not frozen (No) or frozen in liquid nitrogen (LN), thawed and then germinated as described in the Materials and Methods section.

Table 2 Constant temperature drying at 26 degrees for seven days yielded high viability after the freeze-thaw cycle.

2 **Drying at constant temperature of 26 degrees Celsius**
Use mature megasporocarps with sporocarps attached (we used what was found in the culture sediment), place these in an oven at 26 oC for 7 days, transfer to tubes fit for cryo-preservation.

3 **Snap freeze, then transfer to -80 degree freezer** for indefinite storage- we tried up until 4 years and this yielded the same viability.

4 **Thaw the frozen megasporocarps with massulae attached by transfer into 400 micro liter of Azolla growth medium** at pH 5.5 (Watanabe et al., 1992#) in a growth cabinet set at 25°C day: 15°C nights with 12 h light (40– 70 micromol/s m2 PPFD). This can be up-scaled in small petri-dishes, but small volumes with small batches of megaspores allow for more controlled temperatures.

Spores may be scored for germination over a period of 6 wk. Morphological changes to the megasporocarps can be tracked under a binocular Leica Axioskop light microscope, using either x 10 or x 5 objectives.
We used a Leica SP2 confocal laser scanning fluorescence microscope, equipped with either x 16 or x 40 objectives and a helium–neon laser with excitation wavelength of 543 nm, to visualize N. azollae fluorescence in the range 630–670 nm. We visualized plant tissue fluorescence in the range 560–630 nm and/or 680–750 nm.

This is IRRI medium. AZOLLAIRRI.pdf

5 Good luck!
Please report tests of the method on other Azolla species here.
The aim of such endeavor should be to eventually generate a frozen biodiversity collection of Azolla ferns*.

* We have yet to verify whether the preservation method preserves other bacteria persistently associated with Azolla ferns (Dijkhuizen LW, Brouwer P, Bolhuis H, Reichart GJ, Koppers N, Huettel B, Bolger AM, Li FW, Cheng S, Liu X, Wong GK. Is there foul play in the leaf pocket? The metagenome of floating fern Azolla reveals endophytes that do not fix N2 but may denitrify. New Phytologist. 2018 Jan;217(1):453-66).

6 Note: we found that **megasporocarps and microsporocarps may also be stored wet**- simply in water- in the 4oC fridge in our laboratory for up to 4 years.