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## Cryopreservation of three *Saprolegnia* species (Oomycota): Preliminary evidence for the long-term archiving of water mould species

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### ABSTRACT

*Saprolegnia* spp. water moulds are opportunistic pathogens that can cause economic losses to aquaculture. The diseases caused by them are difficult to control since use of the effective drug, malachite green oxalate, is no longer permitted in several regions (including the European Union and USA). To develop an effective control strategy, *Saprolegnia* isolates must be maintained in the laboratory. Cryopreservation is a useful solution for long-term maintenance; however, at present, there is no developed protocol for the cryopreservation of *Saprolegnia* spp. Here, we isolated and identified three *Saprolegnia* species, *S. parasitica*, *S. australis* and *S. ferax*, and developed a deep-freezing protocol that enables the long-term archiving of these species. The survival and growth rates of isolates kept at  $-80\text{ }^{\circ}\text{C}$  for 3, 6, 9 and 12 months, were tested and compared among the species examined. Although the growth rates of frozen isolates were significantly lower than those of the control (i.e. non-frozen) isolates, the overall survival rate (>90%) indicated the effectiveness of the technique developed. Thus, the protocol developed appears to be a promising method for the long-term preservation of *Saprolegnia* isolates and may facilitate the creation of stock collections.

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### 1. Introduction

The *Saprolegnia* spp. (Heterokontophyta: Oomycota), also known as water moulds, are endemic in most freshwater habitats worldwide (van West, 2006). *Saprolegnia* spp. are generally a secondary pathogens, although under appropriate conditions, they can also act as primary pathogens, causing substantial economic losses to aquaculture (Wuensch et al., 2018); this is of particular concern for fish farms where fish eggs, fish fry and adult fish are affected by infection with water mould (Eiras et al., 2008; Woo and Bruno, 2011).

**Abbreviations:** MGO, malachite green oxalate; ITS, internal transcribed spacer; GY (P + S), glucose–yeast medium with 500 µg/mL penicillin and 500 µg/mL streptomycin; PGA (P + S), Potato–glucose medium with 500 µg/mL penicillin and 500 µg/mL streptomycin; LB, lysogeny broth; ddH<sub>2</sub>O, double-distilled, sterile water; DFT H<sub>2</sub>O, dechlorinated, sterile-filtered tap water; n.a., not applied; TSA, tryptic soy agar.

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Growing moulds are visible to the naked eye and form cotton swabs-like white patches on fish and eggs. On eggs, the disease is manifested by abundant mycelial growth on the surface and inside of the cells, which often induces the death of affected eggs. *Saprolegnia* invades epidermal tissues in fish, with the infection often starting on the head or fins and spreading over the entire body surface (van West, 2006). Disease control is especially important, since the European Union increasingly restricts the use of medicines and water treatment agents in food-producing fish farms. Malachite green oxalate (MGO) was previously used for many decades to effectively treat fish eggs against saprolegniasis. However, MGO is no longer permitted for use on food-producing fish because of its suspected carcinogenic side effects (EU Commission Regulation No 37/2010). Therefore, recent studies have aimed at finding alternative treatment agents or therapeutic options that are as effective and economical as MGO, but without its harmful side effects (Ali et al., 2014; Eszterbauer et al., 2018; Tedesco et al., 2019). For treatment trials, it is essential to be able to keep *Saprolegnia* isolates continuously available under laboratory conditions. Generally, oomycetes can be maintained in the laboratory by their

serial subculture (Juarros et al., 1993), or by storage in liquid nitrogen, as reported for *Phytophthora* spp. (Houseknecht et al., 2012).

There are several reported methods for culturing *Saprolegnia*. Farkas and Oláh (1979) investigated different culture media, of which Sabouraud dextrose agar proved to be the best. For example, El-Nagdy and Abdel-Hafez (1990) and Khallil et al. (1991) used hemp seed as bait, and glucose or cellulose Czapek Dox agar as culture medium. Hussein et al. (2001) cultured *Saprolegnia* spp. on glucose-yeast (GY) medium containing ampicillin and streptomycin. Carbajal-González et al. (2011) used tryptic soy agar (TSA) and sheep blood agar for culturing, whereas Shin et al. (2017) applied potato dextrose agar containing ampicillin (100 µg/mL) for the same purpose.

Although the cryopreservation of *Saprolegnia* spp. has not yet been resolved, freezing methods have been described for other oomycetes and fungi. Juarros et al. (1993) developed a simple but efficient method for the long-term preservation of *Phytophthora*, *Pythium*, *Sclerotinia*, and *Rhizoctonia* species. This involved placing pieces of mycelium within an agar block with 10% glycerol as cryoprotectant, pre-cooling the samples at +4 °C for 1 h, and then cooling them to –80 °C. Kitamoto et al. (2002) reported the successful cryopreservation of 66 species of Oomycota, Zygomycota, Ascomycota, and Basidiomycota using a special sawdust-based solid medium with 10% glycerol, which was inoculated with mycelium. The inoculated media were incubated for several weeks and then kept at –80 °C. Ito and Akira (1996) studied the survival rates of several Basidiomycota species stored at –80 °C for up to 15 years, and reported an overall survival rate above 88%.

In the present study, we developed an efficient method for the cryopreservation of parasitic *Saprolegnia* spp. based on literature data and our own experience in *Saprolegnia* culture. The efficiency of the optimised method was tested by measuring the survival rates of oomycete isolates and comparing the growth rates of frozen and control isolates.

## 2. Materials and methods

### 2.1. Isolation and culturing of *Saprolegnia* spp.

Water moulds were isolated from water and infected fish eggs from the fish brood houses of the Lillafüred trout hatchery and the Akasztó carp hatchery in Hungary. Samples were taken with sterile cotton swabs and then, the heads of the cotton swabs were used to inoculate GY agar supplemented with penicillin and streptomycin (P + S), which contained 10 g/L glucose, 2.5 g/L yeast extract, 15 g/L agar, 500 µg/mL penicillin G (P), and 500 µg/mL streptomycin-sulphate (S); and the plates were incubated at room temperature (23–26 °C) until *Saprolegnia* growth was clearly visible (usually up to 1 week). Subsequently, pure *Saprolegnia* cultures were prepared by subculturing according to the protocol outlined by Eszterbauer et al. (2018).

### 2.2. Identification of *Saprolegnia* spp.

We collected the hyphae in double-distilled water (ddH<sub>2</sub>O), and homogenised them using TissueLyser LT (Qiagen, Netherlands). The homogenate was digested in lysis buffer (containing 0.2% SDS, 20 mg/mL proteinase K, 100 mM NaCl, 10 mM Tris and 10 mM EDTA) at 55 °C overnight. Genomic DNA (gDNA) was extracted from the lysate using Miniprep Express Matrix following the manufacturer's manual (MP Biomedicals, USA). The amount of extracted gDNA was quantified using NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA). PCR amplification of the internal transcribed spacer (ITS) region was carried out by the use of universal primers (forward primer ITS-1 5'-TCC GTA GGT GAA CCT GCC

G-3', reverse primer ITS-4 5'-TCC TCC GCT TAT TGA TAT GC-3') as recommended by White et al. (1990), with a modified PCR protocol. The obtained, approximately 710-bp-long PCR product included 18S ribosomal RNA gene (rDNA) partial sequence, ITS 1, 5.8S rDNA, ITS 2, and 28S rDNA partial sequence. The PCR conditions comprised an initial denaturation at 94 °C for 5 min, followed by 6 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and elongation (60 s at 72 °C), followed by 34 cycles of denaturation (30 s at 94 °C), annealing (30 s at 58 °C), and elongation (60 s at 72 °C), and a final elongation step at 72 °C for 10 min. The total volume of the PCR mixture was 25 µL and contained 1 × Taq buffer with KCl (ThermoFisher Scientific, USA), 25 µM of both forward and reverse primers (IDT, Belgium), 10 mM dNTPs (Sigma, Germany), 25 mM MgCl<sub>2</sub> (Thermo Scientific, USA), 1.25 U recombinant Taq DNA polymerase (ThermoFisher Scientific, USA), and approximately 10–30 ng template DNA. The PCR fragment was purified using MEGAquick-spin Total Fragment DNA Purification kit (Intron Biotechnology, Korea), and an ABI BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, USA) was applied for Sanger sequencing.

### 2.3. Freezing protocol

For the freezing experiments, the three *Saprolegnia* species most commonly found in the sampled fish farms were selected; these included the following: *Saprolegnia parasitica* Coker (isolated from the water of the brood house in Lillafüred), *Saprolegnia australis* Elliott (isolated from common carp eggs in the Akasztó fish hatchery) and *Saprolegnia ferax* Gruith (isolated from the water of the brood house in Akasztó). Cultures were kept in a sterile, disposable, plastic Petri dish containing GY (P + S) media. Using a sterile biopsy punch of 4 mm in diameter, agar plugs colonised by mycelia were removed, and placed one by one onto fresh GY (P + S) media. Several different protocols were applied for sample preparation, freezing and thawing (Table 1). Protocols varied depending on the culture media, as well as pre-growing and pre-cooling conditions and the use of hemp seed. The most effective freezing protocol (Trial 6) is described in the Results section.

### 2.4. Thawing

After deep-freezing at –80 °C for various durations (for up to 12 months), three parallel samples (i.e. three cryovials) of each of the three species were thawed at room temperature (altogether nine vials per time point). Thereafter, the agar plug and hemp seeds from the same cryovial were removed and placed onto fresh GY (P + S) media (one Petri dish per cryovial), and were incubated at room temperature (Fig. 1). To evaluate the growth of *Saprolegnia* isolates, the size of the colony was determined by measuring the outer margin of the hyphae in the culture media (in diameter), once a day (at the same time of day) until the colony occupied the entire Petri dish.

Additionally, after the 6-, 9- and 12-month-long deep-freezing periods, we conducted a comparative investigation in order to determine whether the deep-freezing itself changed the growth potential of oomycetes. Thus, the growth of the frozen and the non-frozen (i.e. control) isolates was compared for the three *Saprolegnia* species. The controls applied were from the same strains as the frozen ones, but had been maintained by subculturing, and had never been frozen. The growth potential of frozen and control samples was examined in parallel (in three replicates per species).

## 2.5. Statistical analysis

Fisher's exact test was used to determine the differences in survival among the species or the freezing durations. To compare the size of the frozen and control isolates, Welch's t-test was applied. The impact of freezing duration on the growth rate was studied by ANOVA and Tukey's post-hoc test. The level of significance was determined at  $p < 0.05$ , and 'borderline' significance was identified in cases when the  $p$  value was between 0.05 and 0.1. The statistical analyses were performed by the use of the R program, version 3.5.1.

## 3. Results

The most efficient deep-freezing protocol (Trial 6; Table 1) was as follows (for one cryovial): three pieces of autoclaved hemp seed and an agar-mycelium plug 4 mm in diameter were put in 1 mL dechlorinated, sterile-filtered tap water (DFT H<sub>2</sub>O) in a 2-mL centrifuge tube (Greiner, Austria). The tubes were incubated at room temperature for 1 day and in a refrigerator (at +6–7 °C) for another 3 days (pre-growing time was then 4 days altogether). Thereafter, the hemp seeds and the agar plugs were transferred into one 2-mL cryovial (Nunc, Denmark) containing 100 µL sterile glycerol (Sigma–Aldrich, USA) and 900 µL DFT H<sub>2</sub>O. The cryovial was pre-cooled in a refrigerator for 1 h and then it was placed into a –80 °C freezer (Forma Scientific, USA).

The duration of deep freezing did not significantly affect the survival rate of *Saprolegnia* isolates. After 3 months of deep-freezing, eight out of the nine samples started growing within 24 h after thawing, while for the remaining sample, the growing hyphae could be observed 48 h after thawing (Fig. 1). All nine isolates survived the freezing procedure, their growth potentials showed a clear linearity (Table 2; Fig. 2A), and all samples colonised the entire medium within 4 days after thawing. The survival rate of the strains *S. parasitica* and *S. australis* strains was two out of three after 6 months of deep-freezing, while that of the strain *S. ferax* was three of three; all surviving strains colonised the entire medium by day 4 (Fig. 2B). After the 9-month-long freezing, the survival rate of *S. parasitica* isolates was only two out of three, but it was three out of three for the other two strains (Table 2), and all the surviving strains colonised the entire medium by day 4 (Fig. 2C). The survival rate further decreased over time, and only two out of the three isolates survived the 12-month-long freezing for all species (Table 2). Although the majority of the *Saprolegnia* isolates survived the above conditions, deep-freezing decreased the viability of the

strains. The isolates deep frozen for 6, 9 and 12 months had a significantly ( $p < 0.05$ ) lower growth potential than non-frozen isolates of the same *Saprolegnia* species (Figs. 3 and 4). The growth potentials were slightly higher for the 9-month-long deep-freezing, than for the 6-month-long trial period. The lowest growth potential (i.e. the slowest growth) was detected in the samples frozen for 12 months. The differences in the colony size among the frozen isolates were not significant, regardless of the duration of freezing. The divergence among *Saprolegnia* species in terms of how many strains survived the freezing process compared to the controls was also not significant ( $p > 0.05$ ).

## 4. Discussion

Currently, the most effective method for the long-term maintenance of *Saprolegnia* spp. has been subculturing, which is a labour-intensive procedure as the maintenance of isolates requires constant attention. Furthermore, previous studies have reported that, due to the continuous transfer, certain physiological or even genetic properties of the strains could change, such as the reproductive capacity or the deterioration of fruit bodies (Kirsop and Doyle, 1991; Ko, 2003; Marx and Daniel, 1976). Thus, it is very important to have a reliable method for the long-term archiving of *Saprolegnia* isolates, and cryopreservation (deep freezing) is an optimal solution for this. A number of promising cryopreservation methods have been described, but none of these methods has been tested on *Saprolegnia* spp. Juarros et al. (1993) used a method similar to that developed here, for the culture of *Phytophthora*, *Pythium*, *Sclerotinia* and *Rhizoctonia* species, with the differences being that they used PDA agar as the medium, 37 °C as the thawing temperature, and that they did not use hemp seed. The overall survival rate was nearly 100% in their study after a freezing period of up to 12 months, which is consistent with the survival rate obtained in our study. Ito and Akira (1996) also used a similar method, and they reported a survival rate of 88.4% after 15 years of freezing at –80 °C. The method of Kitamoto et al. (2002) was unique to a certain extent, as they used a solid medium for deep-freezing instead of a liquid cryoprotectant.

In the present study, we elaborated and tested several freezing protocols. We optimised an appropriate protocol, in which the use of hemp seed was the key element. Hemp seed is known to be an ideal 'bait' for sampling *Saprolegnia* spp. in fish brood houses, as the oomycetes in environmental samples prefers to colonise these seeds (Hoitsy, 2015; Eszterbauer et al., 2018). Hemp seed is also an optimal tool for trapping *Saprolegnia* in freshwater sites (El-Nagdy

**Table 1**  
Summary of the various trials/protocols used for the cryopreservation of *Saprolegnia* spp. Trial 6 was found to be the most efficient protocol.

	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Trial 6
Medium	GY (P + S) <sup>a</sup>	GY (P + S)	GY (P + S)	GY (P + S)	PGA (P + S) <sup>b</sup>	GY (P + S)
Broth	LB <sup>c</sup>	LB	LB/ddH <sub>2</sub> O <sup>d</sup>	LB/ddH <sub>2</sub> O	ddH <sub>2</sub> O	DFT H <sub>2</sub> O <sup>e</sup>
Glycerol	+	+	+	+	+	+
Broth: glycerol ratio	4:1	4:1	4:1/9:1	4:1/9:1	9:1	9:1
Pre-growing time (days)	n.a. <sup>f</sup>	n.a.	3	n.a.	n.a.	4 <sup>g</sup>
Pre-growing medium	–	–	ddH <sub>2</sub> O	–	–	DFT H <sub>2</sub> O
Pre-cooling (hours)	–	–	1	1	1	1
Thawing	room T	room T	room T	room T	room T	room T
Hemp seed	–	–	+	+	–	+
Freezing duration	1 month	1 week	1 week	1 week	1 week	up to 12 months

<sup>a</sup> GY (P + S): glucose–yeast medium with 500 µg/mL penicillin and 500 µg/mL streptomycin.

<sup>b</sup> PGA (P + S): Potato-glucose medium with 500 µg/mL penicillin and 500 µg/mL streptomycin.

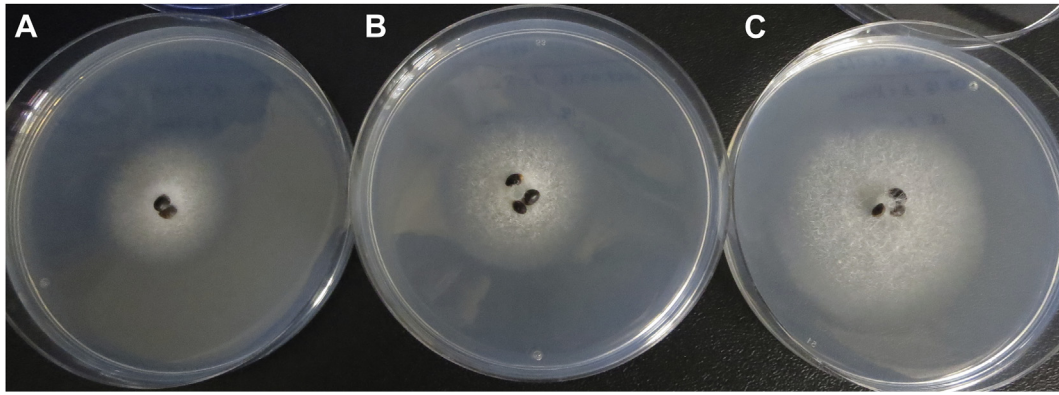
<sup>c</sup> LB: lysogeny broth.

<sup>d</sup> ddH<sub>2</sub>O: double-distilled, sterile water.

<sup>e</sup> DFT H<sub>2</sub>O: dechlorinated, sterile-filtered tap water.

<sup>f</sup> n.a. not applied.

<sup>g</sup> 1 day at RT and further 3 days at +6–7 °C.



**Fig. 1.** *Saprolegnia* cultures growing on GY (P + S) medium 48 h after thawing. A) *S. parasitica*; B) *S. australis*; C) *S. ferax*. The content of a cryovial (i.e. three hemp seeds and a 4 mm agar-mycelium plug) were placed to the centre of the agar medium.

**Table 2**

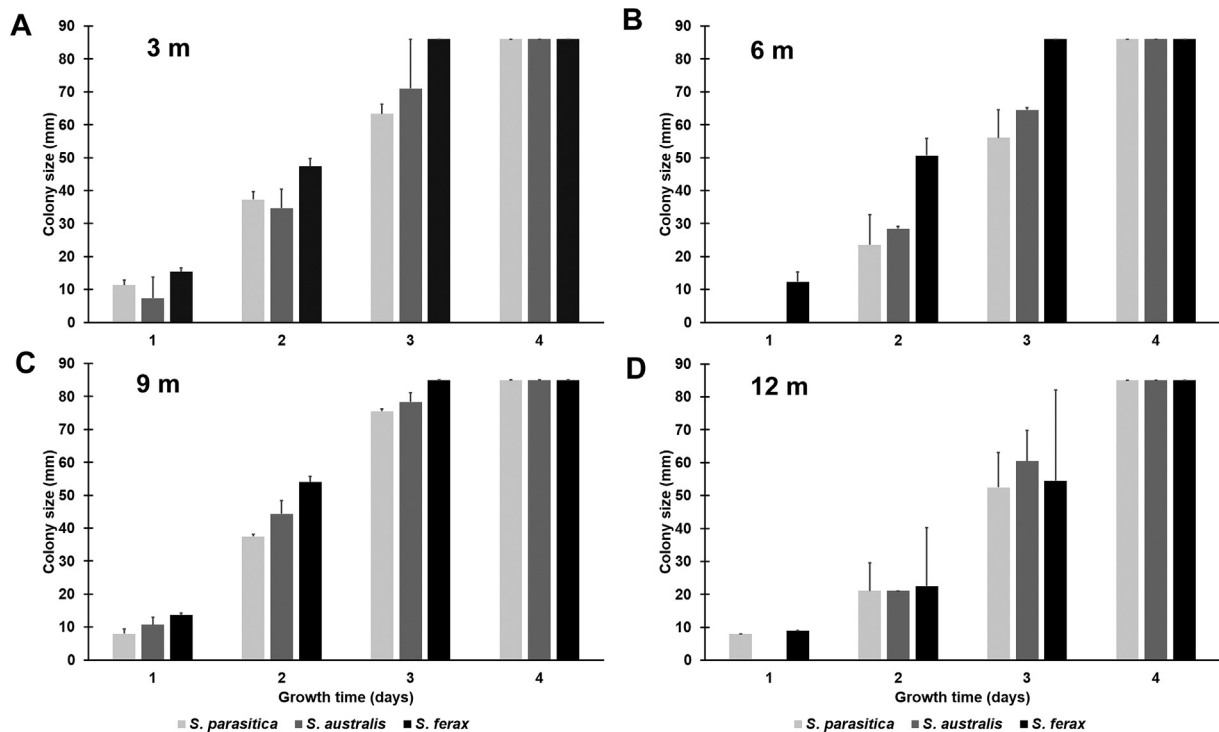
Survival rate of the three tested *Saprolegnia* species frozen at  $-80^{\circ}\text{C}$  for 3-, 6-, 9- and 12 months. Total survival was evaluated by the growth in culture medium over four days of examination (data given in the table show the number of survived samples out of all).

Duration of deep freezing (months)	<i>S. parasitica</i>	<i>S. australis</i>	<i>S. ferax</i>
3	3 of 3	3 of 3	3 of 3
6	2 of 3	2 of 3	3 of 3
9	2 of 3	2 of 3	3 of 3
12	2 of 3	2 of 3	2 of 3

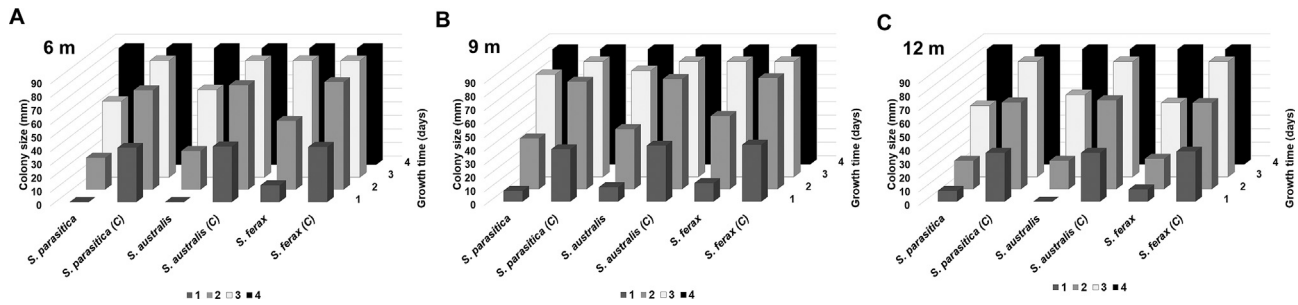
and Abdel-Hafez, 1990; Khallil et al., 1991; Kiziewicz et al., 2011), while Hussein et al. (2001) and Carbajal-González et al. (2011) used hemp seeds to grow *Saprolegnia* spp. *in vitro*. In our study, the use of a solution supplemented with hemp seeds proved to be suitable for

cryopreservation, as mycelia colonising the seeds are likely to be well protected from ice crystallisation inside the seeds. However, deep-freezing had a significantly negative effect on the interrupted growth of *Saprolegnia* spp. Our findings showed that after deep-freezing and thawing, the growth potential of the examined *Saprolegnia* species was significantly lower than that of the controls. While non-frozen (control) isolates managed to colonise the entire Petri dish by day 3, it took a further day for the frozen isolates to do the same. Presumably this period of time (i.e. 1 day) was required for the oomycetes to regenerate their physiological functions.

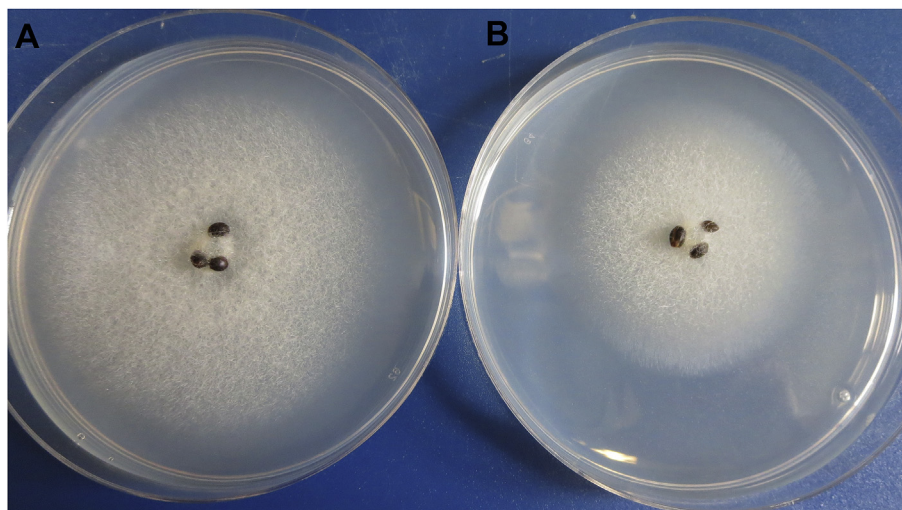
Due to the low number of replicates (i.e. three per species), the survival rate of isolates can only be roughly estimated in our study. Juarros et al. (1993) reported a 91.67% overall survival rate after 3 months of cryopreservation, 73.33% after 6 months and 71.67% after one year. Ito and Akira (1996) observed 93.50% after one year, 92.10% after 5 years and 88.40% after 15 years, whereas Kitamoto



**Fig. 2.** Mean growth of *Saprolegnia* strains that survived: A) 3 months, B) 6 months, C) 9 months, and D) 12 months of deep freezing. Colony size in diameter was measured on four consecutive days. Standard deviation (SD) bars are shown above the columns.



**Fig. 3.** Mean growth of *Saprolegnia* isolates after A) 6 months, B) 9 months, and C) 12 months of deep-freezing as compared to the control (c; i.e. non-frozen) isolates. Colony size in diameter was measured on four consecutive days.



**Fig. 4.** Comparison of the growth rate of A) untreated control and B) deep-frozen isolate of *Saprolegnia ferax* 48 h after thawing and placement onto a GY (P + S) agar medium. The content of a cryovial (i.e. three hemp seeds and a 4 mm agar-mycelium plug) were placed onto the agar medium, and colony sizes in diameter were compared.

et al. (2002) reported at least 92.40% after 21 months. Using the protocol developed in our study, we observed similarly high survival rates compared to previous studies on other taxa of oomycetes. In our case, at least two thirds of the samples of every species survived freezing. The growth potential of the surviving isolates differed depending on the duration of deep-freezing. Surprisingly, a decrease in growth rate was observed after 6 months of freezing, while the samples frozen for 9 months grew quicker, and another decrease in survival was noted after 12 months of freezing. We believe that the room temperature (RT) over 26 °C might be sub-optimal (too high) for thawing the samples frozen for 6 months, while an RT of 23–24 °C provided satisfactory conditions in all other cases. On the basis of the low number of replicates examined, it seems that *S. ferax* was the most viable species (all samples survived after 3, 6, and 9 months, and two thirds of them could be revived after 12 months), and *S. parasitica* had the lowest survival rate (all samples survived after 3 months, whereas only two thirds of them grew after 6, 9 and 12 months of freezing); however the difference was not statistically significant. A more comprehensive study involving further species and isolates is required to determine the difference between *Saprolegnia* species in their tolerance to freezing.

In conclusion, our findings suggest that *Saprolegnia* isolates can be stably archived in the long run with good efficiency using our deep-freezing protocol. The long-term storage of frozen, isolated *Saprolegnia* strains would allow water mould samples to be

continually available for further laboratory use and enable the establishment of strain collections.

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