Longevity, Cryopreservation, and Propagation of Carnivorous Plants Seeds: Insights From 13 Species in Long-Term Ex situ Collections.

Daniel Ballesteros[2,3,*], Rachael M. Davies[3], Jennifer L. Peach[3], and Valerie C. Pence[1]

[1]Center for Conservation and Research of Endangered Wildlife (CREW). Cincinnati Zoo and Botanical Garden. 3400 Vine Street. Cincinnati, OH45220, USA; [2]Universitat de València. Departamento de botánica y geología. Av. Vicent Andrés Estellés s/n. 46100-Burjassot, Valencia, Spain; [3]Royal Botanic Gardens Kew. Wakehurst, Ardingly, West Sussex, RH17 6TN, UK.

**For correspondence. E-mail: daniel.ballesteros@uv.es*

Abstract

- Background and Aims: A quarter of the assessed carnivorous plants (CPs) are threatened with extinction, and the effectiveness of ex situ conservation initiatives must be evaluated to complement in situ conservation efforts. Conventional seed storage (e.g., 15% relative humidity, -20°C) is the most common and efficient strategy for plant ex situ conservation, but seeds of diverse CPs might be short-lived at these conditions. Thus, there is a need to empirically and comparatively assess their longevity and evaluate the success of alternative storage options.
- Methods: Diverse seed collections were used to assess longevity of seeds of thirteen CP species stored (some up to 30 years) at various storage conditions: ambient/uncontrolled, conventional, and cryogenic.
- Key Results: The relative short longevity of CP seeds stored in ambient/uncontrolled and conventional conditions was confirmed for some taxa (e.g., Drosera rotundifolia L.). Nevertheless, despite this potential short longevity, seeds of 64% of accessions tested retained their initial viability for up to two decades when stored at conventional seed bank conditions. Only some accessions stored for longer times (> 25 years), showed significant signs of deterioration, with D. rotundifolia and D. anglica Huds. showing P50 values in the region of other taxa considered medium/short or short-lived at comparable cold/dry storage conditions. As an alternative (or complement) to conventional seed banking, cryogenic storage of dry seeds was able to preserve a high germination percentage of seeds of CPs stored up to two decades. Furthermore, seedlings obtained developed normally into healthy plants when monitored up to 1.5 years after germination.

• Conclusions: Despite the potential short lifespan of seeds of some CP taxa, this paper highlights and supports their routine dry storage in ex situ conservation programs to support in situ conservation initiatives.

Keywords: seed lifespan, cryopreservation, seed banking, germination, plant growth, Drosera L., Sarracenia L., Pinguicula L., Utricularia L.

INTRODUCTION

Carnivorous plants (CPs) are a polyphyletic group that has evolved multiple times among the angiosperms (Ellison and Gotelli, 2009). These iconic plants have attracted considerable attention from many biologists for several hundred years, among them, Charles Darwin, who studied them in detail and used them in his evolutionary studies (Darwin, 1875). CPs are found on all continents except Antarctica, with some families (e.g., Cephalotaceae and Byblidaceae) being endemics to single continents and families having cosmopolitan distributions (e.g., Droseraceae and Lentibulariaceae) (Ellison et al., 2012). A cosmopolite example can be the genus Pinguicula (Lentibulariaceae), which presents an extensive geospatial distribution range throughout Eurasia, North to South America, the Caribbean, and Morocco, and spans from tropical low-land to chasmophyte and alpine environments (Shimai et al., 2021). Despite this global distribution, CPs are an ecologically defined group, that typically inhabit sunny, moist and nutrient-poor terrestrial habitats, such as fens, bogs, stream sides, and rock faces with dripping, splashing water in the northern hemisphere (e.g., Sarracenia sp., Pinguicula sp., Drosera sp.and Dionaea muscipula J.Ellis) or tropical rainforests throughout East and South-East Asia (e.g., Nepenthes spp.) (Ellison and Adamec, 2018; Skates et al., 2019; Shimai et al., 2021). However, some can also be found living in aquatic habitats (e.g., Utricularia vulgaris L. and Aldrovanda vesiculosa L.), or in less mesic, seasonally dry and fire-prone infertile habitats in Western Europe (Drosophyllum lusitanicum L. (Link)), Brazil (Philcoxia spp.), or South-West Australia (Drosera sp. and Byblis sp.) (Ellison and Adamec, 2018; Skates et al., 2019). In many of these habitats, CPs have important ecological roles and are essential for the subsistence of diverse plant, animal and microbial communities (Jennings and Rohr, 2011; Ellison and Adamec, 2018). CPs are also important for humans as several species provide medicinal compounds, some may

be useful in pest control, and most have important horticultural value (Šamaj et al., 1999; Jennings and Rohr, 2011; Northcutt et al., 2012). However, over a quarter of the CP species assessed are listed as threatened (Cross et al., 2020), mainly due to pressure on their habitats, as well as to over-collection, invasive species, and pollution (Jennings and Rohr, 2011; Northcutt et al., 2012; Khanna et al., 2014; Clarke et al., 2018; Cross et al., 2020). While in situ protection of the threatened species is urgently required, it is vital to perform research that assesses the effectiveness of ex situ CP conservation initiatives and to explore how these activities can be used to complement in situ efforts to conserve and restore remnant and degraded natural habitats (Clarke et al., 2018; Cross et al., 2016, 2018, 2020).

Seed banking is the most common and efficient strategy for plant ex situ conservation that also supports plant production for restoration programs in situ (Guerrant et al., 2004; Cochrane et al., 2007; Li and Pritchard, 2009; Abeli et al., 2020; Di Sacco et al., 2021). Seeds tolerant to desiccation and low temperatures (i.e., orthodox seeds) can be routinely dried to low moisture contents and stored at sub-zero temperatures for long-term preservation under the standard conditions of conventional seed banks (FAO, 2014). Seeds of several CPs have been determined to be orthodox (Cross et al., 2018; Royal Botanic Gardens Kew, 2021), and thus, they may be good candidates for storage in conventional seed banks (Cross et al., 2018). Indeed, seeds of some Australian CPs have been stored successfully for years or decades at such conditions (Cross et al., 2018). However, the possibility that seeds of some CPs may present difficulties during their long-term storage in conventional seed banks cannot be entirely ruled out (Godefroid et al., 2010; Cross et al., 2016, 2018; Royal Botanic Gardens Kew, 2021). For example, diverse references state the ephemeral nature of seeds of many CPs in the soil bank or during storage at (uncontrolled) ambient conditions (Groves, 1917; Bourke, 2003; Baskin and

Baskin, 2014; Cross et al., 2018) and it is known that seeds with a short-lived nature may also age relatively quickly during storage in the controlled conditions of conventional seed banks (e.g., Walters et al., 2005a; Probert et al., 2009; Ballesteros and Pence, 2017). An example of a short-lived CP has been observed with seeds of Aldrovanda vesiculosa, a species in the Droseraceae family. Seeds of A. vesiculosa tolerated the initial desiccation needed for storage in the conventional conditions of a seed bank (e.g., drying at 15% relative humidity; Cross et al., 2016), but were reported to deteriorate within three months when stored at 15°C and within just 24 hours when they were exposed to -18°C (Cross et al., 2016). Similarly, seeds of Cephalotus follicularis Labill. have also been reported to lose viability rapidly when desiccated and stored at -18°C (Cross et al., 2018), although this observation could have been incidental due to the desiccation and storage of underdeveloped seeds (North et al., 2021). As reports on longevity of seeds of CPs are scarce, mostly anecdotal, and sometimes controversial, particularly under the conditions of conventional seed banks (Godefroid et al., 2010; Cross et al., 2018), more information is needed to evaluate the potential of their long-term storage.

Given the case that seeds of CPs might be freezing sensitive (to temperatures near -18 or -20°C) or short-lived during standard storage in seed banks, alternative methods to conventional seed banking should be considered for the long-term conservation of these species (Northcutt et al., 2012; Khanna et al., 2014; Cross et al., 2016). Cryogenic storage is suggested for the long-term preservation of seeds that tolerate sufficient drying to survive freezing but have short lifespans regardless of how they are dried or cooled, and in general, for seeds of unknown longevity from wild species (Li and Pritchard, 2009; Hay and Probert, 2013; FAO, 2014; Pritchard et al., 2016; Pence et al., 2020). Liquid nitrogen (LN) exposure has been demonstrated to be safe for dry seeds of a large number of species (e.g., Stanwood and Roos, 1979; Stanwood,

1980; Styles et al., 1982; Pence, 1991), including some CPs (e.g., Sarracenia sp., Northcutt et al., 2012; Khanna et al., 2014), and has increased survival of seeds of some species when compared with 5°C or -18°C storage (Pritchard, 1995; Walters et al., 2004; Pritchard and Nadarajan, 2008; Ballesteros and Pence, 2017). However, seed cryostorage is a relatively new method in plant conservation (Pritchard et al. 2016) and still requires empirical evaluation of its effectiveness through the use of long-term storage data sets (Pritchard, 2004; Pence et al., 2020). This is important, because it has been shown that some short-lived seeds also age and die during LN storage, particularly if they are of initial low quality (Pritchard and Seaton, 1993; Walters et al., 2004; Ballesteros and Pence, 2017). This could be of particular importance for some CPs, as the cryopreservation procedures themselves may damage the seeds (e.g., Sarracenia sp., Khanna et al., 2014), decreasing their initial quality and thereby affecting longevity.

In addition, the long-term storage of seeds from wild species poses unique challenges that have not been well studied (Pritchard and Nadarajan, 2008; Hay and Probert, 2013; Merrett-Wade et al., 2016; Ballesteros and Pence, 2017). Unlike crop species, collections from the wild may have only a small number of seeds per accession and the initial quality may be highly variable (Hay and Probert, 2013; Ballesteros and Pence, 2017), factors that can influence the recovery of plants from the collection after long-term storage (Pritchard and Seaton, 1993; Walters et al., 2004; Ballesteros and Pence, 2017). Furthermore, the seed biology of many wild and rare species may be unknown at the time of banking or retrieval from storage (Merrett-Wade et al., 2016) and may be difficult to germinate for a proper evaluation of seed viability or for plant production (Godefroid et al., 2010). Finally, it is also important to analyse the ability of seeds recovered from storage to produce vigorous plants, since the ultimate purpose of most collections of wild seeds is to ensure that sufficient viable seeds are available for the production

of healthy, usable plants for habitat restoration and species reintroduction (Merritt and Dixon, 2011; Hay and Probert, 2013; FAO, 2014; Abeli et al., 2020; Di Sacco et al., 2021).

The study reported here focuses on three important aspects for a successful implementation of the ex situ conservation of seeds of CPs: (1) the analysis of the potential longevity of seeds of CPs, (2) the evaluation of the feasibility of different long-term seed storage methods for the conservation of CPs genetic resources, and (3) the development of the optimal propagation protocols for some CPs. The seeds evaluated in this work belong mostly to U.K. and U.S. native species, were collected in the wild and stored ex situ due to their conservation interest locally. In addition, we also evaluated seeds purchased from commercial sources, to have sufficient material to test the effects of liquid nitrogen exposure and storage on seed survival in diverse species within the main genera harvested in the wild. In the British Isles, habitat loss is the most common pressure on CP, which have led to local extinctions over the last centuries (Walker and Preston, 2006). In the United States, Sarracenia populations have been reduced by 90%, and many of these pitcher plants have become rare and endangered, being protected at State or Federal levels (Jennings and Rohr, 2011; Northcutt et al., 2012; Khanna et al., 2014). Several Drosera species are also of conservation concern in the U.S. (Jennings and Rohr, 2011). Long-term stored seeds used in this study were banked in the 1980s and 1990s in the Millennium Seed Bank (MSB) of the Royal Botanic Gardens Kew and the CryoBioBank® of the Center for Conservation and Research of Endangered Wildlife (CREW) using conventional seed bank conditions (i.e., dry storage at -20°C) or dry cryogenic storage in LN, respectively. These seed banks were designed to support the long-term preservation of plant genetic resources from native and endangered species in the U.K and the U.S. In addition, their collections can be useful to illustrate the challenges of dealing with wild plant species during seed ex situ conservation, and

to contribute to the understanding of longevity during long-term storage of biological samples at low temperatures, including those reached in LN (Pritchard et al., 2016; Ballesteros and Pence, 2017). We also tested seeds stored at room temperature in the seed bank of the International Carnivorous Plant Society (ICPS) for comparison purposes. We have tested the hypotheses that (a) longevity of seeds of CPs is short and is comparable to that of other species with known short-lived seeds when stored in comparable storage conditions, (b) seed germination and viability have been preserved over the storage time in collections designed for the ex situ conservation of plant genetic resources, but some viability decrease may be observed in those seeds stored long-term in the conventional conditions of seed banks, and (c) cryopreserved seeds have preserved a high germination rate and have maintained the ability to develop into healthy, viable plants that could be potentially used in restoration initiatives. The results of this work have been used to discuss and evaluate the success of 30 years of ex situ conservation of seeds of carnivorous plants and determine if seed banking can provide efficient conservation tools to ensure the future of these iconic plants.

MATERIAL AND METHODS

Plant material

Thirteen taxa (32 accessions) of CPs from the Lentibulariaceae, Sarraceniaceae and Droseraceae were studied. Seeds were collected from the wild in the U.K. and the U.S., purchased from Thompson & Morgan Inc., or donated to the Seed Bank of the ICPS by different private CP growers in the U.S. Seed origin, accession number, age and storage conditions are indicated in Table 1. Taxa used in this study were selected based on diverse criteria. Firstly, their availability and conservation interests in the U.K. (all species stored at the MSB) or the U.S.

(e.g., all Drosera rotundifolia accessions or the wild harvested Sarracenia purpurea from Triangle Bog). Secondly, the availability of seeds to purchase for increasing the number of species studied within a genera of conservation interest (e.g., all Sarracenia sp. accessions and the Drosera capensis purchased from Thompson & Morgan Inc.). Finally, the availability of sufficient seeds to be donated from diverse taxa and different storage dates (e.g., all seeds donated by the ICPS).

Curation, germination and data analysis of seeds stored at room temperature

Seeds stored at room temperature were donated by the Seed Bank of the ICPS. Here, seeds had been stored at ambient (room) conditions of about 20 ± 5°C and 40-60% RH (J. Brittnacher, pers. comm.).

Seeds were germinated in our lab at CREW using agar 0.8% as substrate. Drosesa finlaysoniana Wall. ex Arn. seeds were germinated without any pretreatment at 26 ± 1°C and 16 h fluorescent light/8 h dark. Sarracenia sp. and Darlingtonia sp., as well as Dionaea muscipula, were cold-moist stratified at 4°C for during 8 weeks, followed by germination at 26 ± 1°C and 16 h fluorescent light/8 h dark.

Seed germination was measured as radicle emergence of at least 2mm. Data were taken daily for 30 days or after steady germination values were accounted (i.e., total germination, usually within 25 days after sowing (DAS)). Germination was considered normal if the seedling developed cotyledons and a root by that time (e.g., Fig. 1A, D).

Curation, germination and data analysis of seeds stored at conventional seed banking conditions in the MSB

Collections at the MSB were dried to equilibrium with 15% RH and 18°C to a moisture content of 3-7% (wet weight basis) and then stored at -20°C under international gene bank standards (ENSCONET, 2009; FAO, 2014). Seeds were removed from cold storage and allowed to equilibrate in their container in a dry room (15% RH and 18°C) for 24 hours before opening for the germination tests.

Germination and viability tests of seeds stored in the MSB came from routine germination tests. For some species, such as Drosera rotuundifolia, germination tests were performed several times during the first years of storage until an optimal germination protocol was developed (Supplementary Table 1), and then after every 10 years of storage using the optimal germination protocol developed for each species. Seed germination was measured as radicle emergence of at least 2mm. Data were taken daily weekly for 30 days or after steady germination values were accounted. At the end of each germination test, a cut test was performed on all non-germinated seeds and internal structures were examined under a microscope. Each seed was determined as either fresh (full and potentially viable), moldy (full and non-viable), empty (non-full) or insect infested (non-full). Generally, seeds were germinated in 15 x 150 mm plastic petri plates half filled with 1% agar placed in controlled temperature and light chambers using the conditions indicated in Supplementary Table 1. Seeds germinated on agar were not sterilized (Davies et al., 2015), except for two germination tests in Drosera accessions where seeds were immersed in 10% commercial bleach (composed of 4.5% Sodium hypochlorite) for 5 minutes (Supplementary Table 1).

Germination assays were performed in one plate containing 50 seeds, but for small seed collections 10-25 seeds in one plate were used. Probit analysis of the seed ageing data from MSB was carried out for individual accessions or species using GenStat for Windows, Version 14.2 (VSN International Ltd., Hemel Hempstead, UK), following Roberts (1973) and using the improved viability equation (Ellis and Roberts, 1980):

$$\nu = Ki - p / \sigma$$

where ν is the viability (normal equivalent deviates, NED) of seeds after p days in storage; Ki is a measure of the initial viability (NED) and σ is the time for viability to decline by 1 NED. In addition to generating estimates for Ki and σ, the probit analysis output can also provide an estimate of the time for viability to fall to 50% (p50) (Hay et al., 2014). Only one replica was used per germination assay over storage time as data generated from this kind of storage experiments are from independent samples collected at different times (Hay et al., 2014). In other words, it is considered that each germination test is an independent replica of the seed population stored for each accession. Then, the probit analysis is carried out using all germination tests generated over time, needing at least three germination tests (i.e., three independent replicas) to be performed.

Significant decline in germination/viability was monitored in conventional storage by comparing the initial (highest) result with the most recent retest. The value of normal deviate Z was calculated using a two-tailed test (Ellis and Roberts, 1985), where a correction factor is applied to enable the normal distribution to be used in the analysis:

$$Z = \frac{(p_1 - p_2)}{\sqrt{\bar{p}\,(100 - \bar{p})\,(1/n_1 + 1/n_2)}}$$

where p_1 is $\frac{100 \text{ x } g_1}{n_1 - 0.5}$, p_2 is $\frac{100 \text{ x } 2}{n_2 + 0.5}$, $\bar{p}$ is the mean value of p_1 and p_2, g_1 and g_2 are the numbers of germinated seeds, and n_1 and n_2 are the numbers of full seeds sown (where n > 19) for the initial and most recent retest respectively. Decline was considered significant ($p < 0.05$) where $Z > 1.64$ and lowest result < 95%.

Curation, germination, plant growth, and data analysis of cryopreserved seeds

Seeds cryopreserved at CREW were either air dried on the bench top or dried over silica gel for up to 1 week to a moisture content of 5-10 % (wet weight basis). After desiccation, seeds were transferred to 2 ml polypropylene cryovials, which were plunged into LN (-196°C). They were stored submerged in LN and were handled over the time using best practice procedures (ISBER, 2012). Seeds removed from LN were warmed slowly on the bench top at room temperature for at least 20 minutes and then, rehydrated at 100% relative humidity for 24 hours before germination.

Germination tests were performed in CREW before and after LN storage. Pre-storage germination tests were performed during the different steps in the cryopreservation process on freshly harvested seeds, dried seeds, and dried seeds exposed to LN for 1 hour (Supplementary Table 2). For each group, half of the seeds were cold-moist stratified at 4°C for 12 weeks, and half were not stratified. Pre-storage germination tests were done by planting the seeds in Metro Mix 250 soilless potting mix (Sun Gro® Horticulture) at two moisture conditions: moist and wet (soil perpetually over-watered). Some Drosera rotundifolia L. seeds (accession from Gallagher Fen) were also germinated on agar (Supplementary Table 2). Germination was done in the greenhouse (26 ± 3°C) except for seeds sowed on agar that germination was done in an incubator set at 30/15°C and 16 h fluorescent light/8 h dark. Due to the limited number of seeds available

for initial tests and storage, only one replica of 50 seeds per test was generally used (Supplementary Table 2).

After cryostorage, most seeds were germinated in 15 x 150 mm plastic petri plates of 0.8% agar in controlled temperature and light. Because the initial germination conditions produced poor results for D. rotundifolia (Supplementary Table 2), we made a pilot study to obtain an optimal germination protocol with seeds of three accessions. In this pilot study seeds (4 plates with 25 seeds per accession) were germinated without stratification at 26 ± 1°C and 16 h fluorescent light/8 h dark. After 7 weeks, 2 plates per accession were moved to 30/15°C (16 h fluorescent light/8 h dark), and two plates were moved to 4°C for 8 weeks of cold/moist stratification. Then the stratified seeds were germinated at 30/15°C (16 h fluorescent light/8 h dark). A fourth accession was used to compare germination after stratification at 30/15°C vs. 26°C. After we determined that D. rotundifolia needed an improved germination protocol based on cold-moist stratification (see in the RESULTS section the results of the pilot experiment indicated above), seeds of this species were cold-moist stratified in wet paper towels at 4°C for 8 weeks to break dormancy.

Seeds of Sarracenia purpurea L., S. minor Walt., and S. flava L. removed from cryostorage, were also cold-moist stratified in wet paper towels at 4°C for 8 weeks to break dormancy based on the results of pre-storage germination tests and conclusion from Baskin and Baskin (1998) and Ellison (2001) germination works for Sarracenia sp. Seeds were incubated at 26 ± 1°C and 16 h fluorescent light/8 h dark, except for D. rotundifolia that were also incubated in a chamber with alternating temperatures: 30/15°C (16 h fluorescent light/8 h dark). Seeds germinated on agar were not sterilized.

Seeds from Drosera capensis L. and two purchased accessions of Sarracenia purpurea (S. purpurea subsp. purpurea and S. purpurea subsp. venosa) were not germinated as explained above and were germinated in vitro. Sterile in vitro germination was selected due to the lack of initial germination tests or the low germination percentage of the initial germination tests (Supplementary Table 2) and because these two accessions were removed from LN before the pilot tests explained above. Seeds were surface sterilized with a 1:9 solution of commercial bleach after recovery from LN and sown on half-strength Murashige and Skoog (1962) medium with 1.5% sucrose in 25 x 150 mm borosilicate culture tubes with Magenta closures, approximately 15 ml of medium per tube. Cultures were incubated at 26 ± 1°C and 16 h fluorescent light/8 h dark up to 200 days, checking germination and development at days 21 and 194.

Seed germination was measured as radicle emergence of at least 2mm. Data were taken daily or weekly (pilot studies) for 30 days or after steady germination values were accounted. Germination was considered normal if the seedling developed cotyledons and a root by that time (e.g., Fig. 1A, D).

After total and normal germination was counted, seedlings were transferred to a substrate composed of 5 parts sphagnum, 3 parts milled peat, and 1 part sand in order to follow further development of the seedlings and young plants. Seedlings were moved at 25 DAS, except for D. rotundifolia (accession from Mantua Bog, stored 15.8 yrs), which was moved to soil 84 DAS. For Drosera species, a thin layer of sphagnum was placed on top of the soil and the plants grew directly on the sphagnum. This substrate, with a large amount of sphagnum, was used to avoid fungal growth observed in seedlings of D. rotundifolia (accessions from Gallagher Fen and Mantua Bog stored 14.7 yrs) and S. purpurea (accession from Triangle Lake Bog), which grew

for 85 days in another substrate, which was used initially (Promix® BX Mycorrhizae TM, Premier Tech Horticulture). All plants grew in the greenhouse inside a terrarium (26 ± 3°C, >75% relative humidity, Fig. 1F).

Growth of the seedlings and young plants for some accessions was measured monthly after seedlings were moved to soil (short-term development), corresponding to 46, 71, and 106 DAS. For other accessions, growth of the seedlings and young plants was measured 106 DAS and one more time ≥ 251 DAS (251, 360 or 529 DAS) (long-term development). A normal young plant was defined as bright green and healthy, with at least 2 non-cotyledonary leaves (e.g., Fig. 1A, B, E). The number of normal leaves per plant (L) was also measured, normal leaves being those with glandular hairs in Drosera (Fig. 1A, B), and those forming a tube for Sarracenia (Fig. 1E, F). Also, for some accessions, we also monitored when Drosera plants went into the hibernaculum stage (Fig. 1C) and if all plants recovered after this dormancy stage.

Pre-storage germination was usually measured in one plate containing 50 seeds. If more than one plate was used the SD of the averaged value is indicated in Annex 2. Post-storage germination was typically measured in four plates of 25 seeds each, when enough seeds were available in the cryovial. If the number of seeds stored was small, at least two plates with usually 10 to 25 seeds per plate were used. In order to analyze significant differences between germination percentages of diverse germination conditions, differences between total and normal germination percentages, or differences between the percentage of normal seedlings at different times after sowing, germination and normal young plant proportions (pulled for each accession and storage time from the different plates showed) were considered to show a binomial response and were analyzed using a two-way binomial test using GenStat® 14th edition. Growth of the plants was considered significant if the average number of leaves produced per plant within each

replicate followed a positive correlation over time (Pearson correlation, alpha = 0.05) using GraphPad Prism software, version 6.

Differential Scanning Calorimetry (DSC)

Differential scanning calorimetry (DSC) was used in this study to determine the thermal fingerprint of seeds of some of the CPs stored in the MSB. One to ten mg of seeds from Drosera rotundifolia (Surrey, Norfolk and East Sussex accessions), D. anglica Huds. (Dorset accession), Pinguicula vulgaris L. (Cumbria1 and Cumbria3 accessions) and Utricularia vulgaris (Norfolk2 accession) were sealed into aluminium DSC pans, weighted and scanned. After DSC scans, pans containing the seeds were punctured and placed at 103°C ± 2°C for 17 ± 1 hours (ISTA, 2007). Water contents were calculated from the difference in fresh and dry weights and are expressed on a dry weight basis as g H_2O/g dry weight (DW). All seeds were scanned at the moisture conditions at which they were stored, which was consistent among samples and averaged 0.039 ± 0.006 g H_2O/g DW, ranging mostly between and 0.033 and 0.046 g H_2O/g DW (Supplementary Tables 3 and 4). This experiment was conducted to determine if seeds of CPs presented thermal fingerprints related to the storage lipids [i.e., triacylglycerols (TAGs)] that could potentially interact with their longevity during storage in the MSB collection (e.g., Crane et al., 2003, 2006) and to investigate changes in the melting properties of these storage lipids over storage time. Seed deterioration has been shown to affect the physical properties of lipids by decreasing the energy of the melting transition of TAG (Vertucci, 1992; Walters et al., 2005b), and this measurement enables us to further characterize the physicochemical properties of the seeds of some of the CPs used in this study.

Phase transitions in embryos were determined using a Mettler-Toledo DSC-1 (Greifensee, Switzerland), calibrated for temperature with indium (156.6°C) standards and for energy with indium (28.54 J g−1). The presence of phase transitions was determined from heating thermograms recorded between −150°C and +90°C while scanning at a rate of 10°C min^{-1}. The onset temperature of the melting transitions was determined from the intersection between the baseline and a line drawn from the steepest portion of the transition peak. The enthalpy (ΔH) of the transition was determined from the area encompassed by the peak and the baseline. All analyses were performed using Mettler-Toledo STARe software version 16.0, Mettler-Toledo, Switzerland. Enthalpies of exothermic and endothermic events were normalized to the dry weight (DW) of the sample used and expressed as mJ/DW.

RESULTS

Viability of CP seeds stored at room temperature

Viability of CP seeds stored at room temperature by the ICPS seed bank was determined by their germination potential. Table 2 shows germination data for several species and accessions. A high rate of germination (77%) was observed in the accession of Darlingtonia californica Torr. seeds stored for less than 5 months. However, germination of this species was much lower (<15%) in accessions stored for more than 1 year. Pre-storage germination rates for this species were not available, but seeds from ‘Parks Creek Rd’ and ‘The Ponds’ were used in a study of seedling development when freshly harvested (Brittnacher, 2014) and the initial germination was “excellent”, likely above 80% (J. Brittnacher, pers. comm.). S. purpurea seeds showed very low (about 1%) or no germination when stored over 13 months (Table 2), and Sarracenia flava seeds stored for 14 months showed germination values <30% (Table 2). Very

low (<2%) or no germination occurred in Dionaea muscipula and Drosera finlaysoniana accessions, which were stored for >1.5 years (Table 2). Unfortunately, pre-storage germination rates for these species were not available.

Germination and seed longevity during conventional seed banking at the MSB

Germination conditions for most of the accessions tested at the MSB have changed over the storage time, particularly during the first germination tests (Supplementary Table 1). This readjustment was done to improve the germination results when the difference between the germination percentage and the percentage of viable seeds (by cut test) was significantly different. As a result, the initial germination percentage of a particular accession did not correspond to the maximum germination percentage obtained in most cases except for D. rotundifolia Surrey (Table 3). On the other hand, initial and maximum viability was the same in most accessions. When comparing maximum and final germination/viability proportions, significant viability decline was detected for five accessions (46% of all accessions tested, table 3), two stored about 17 yrs (P. vulgaris Cumbria 2, U. vulgaris) and three over 25 yrs (D. anglica Dorset, D. rotundifolia Surrey and Hampshire). Significant germination decline over time was detected for three accessions, all three also showing significant viability decline (D. rotundifolia Surrey, P. vulgaris Cumbria2, U. vulgaris, table 3), which represented 25% of the accessions tested.

Seed germination and viability were also represented against the storage time using data for all test intervals when sufficient data points (i.e., >3) were available (Fig. 2). We could observe the typical seed deterioration courses where both germination and viability decreased over storage time following a sigmoidal trend (Fig. 2A, C). However, in some accessions seed

deterioration was only indicated by a viability decrease over storage time as germination proportion increased over time (Fig. 2B, D). Germination/viability time courses were not constructed due to the lack of sufficient data points for some accessions (D. rotundifolia Norfolk, D. rotundifolia East Sussex, P. vulgaris Cumbria 1, and U. vulgaris). For other accessions (e.g., Pinguicula lusitanica L., Wakehurst) the germination/viability time courses followed a straight line due to the absence of germination or viability loss over storage time (Table 3).

Longevity was estimated by the time for germination/viability to fall to 50% (P50) in a probit analysis when three or more storage data points were available (Table 3). We were able to perform this calculation in the viability and germination time courses of only two D. rotundifolia accessions. P50s using viability data ranged between 19.3 and 35.5 years and 10.6 to 26 years from germination data (Table 3).

In addition to the analyses performed for each individual accession, we also plotted viability over time and analyzed P50s at the species level (by merging the viability data of the diverse accessions available per species). Results at the species level reflect the findings made at the accession level (Table 3) and show that viability over time was sharply lost only in D. rotundifolia (Supplementary Fig. 1A; Z (slope)=-9.444; sig.= <0.001) with a P50 calculated in 22.4±5.3 years. Viability seems to slightly decrease over time in D. anglica and P. lusitanica, but in a smooth way (D. anglica, Supplementary Fig. 1B) or only due to the influence of the last germination assay after 33.7 years of storage (P. lusitanica, Supplementary Fig. 1D). P50 projected for D. anglica was estimated at 49.5 years, although the slope was not significant at the 0.05 level (P=0.06).

Germination, seedling development and plant growth of seeds cryopreserved at CREW

Before long-term storage, seeds of Drosera rotundifolia showed low or no germination in most treatments (Supple. Table 2). The highest germination, 38-56%, occurred in one accession with seeds sown in wet soil or on agar. Sarracenia purpurea seeds only germinated after stratification (Supple. Table 2), and dried/LN-treated seeds had a higher germination percentage than freshly harvested or dried seeds (binomial test, $P<0.05$). Unlike D. rotundifolia, there was no germination in wet soil. For some of the accessions of both Drosera and Sarracenia, the data on pre-storage viability had been lost.

Because of the low pre-storage germination rates, for this study pilot germination tests were done using four accessions of D. rotundifolia seeds removed from long-term LN storage to determine if germination could be improved. When, in the first test, seeds of three of the accessions were incubated on agar plates for 7 weeks at 26°C without stratification, no germination was observed (Fig. 3A). However, when half of these seeds were given 8 weeks of stratification and then moved to a 30/15°C temperature regime, >90% germination was obtained within 2 weeks in all three accessions (binomial test, $P<0.05$). The other half of the seeds, which was moved directly to the same alternating temperature regime without stratification, showed germination in only one of the three accessions and at a much lower rate than the stratified seeds. In a second test, using the fourth accession, stratification followed by the alternating temperature regime produced slightly higher germination than stratification followed by incubation at 26°C (binomial tests performed at 31, 35 and 42 DAS, all $P<0.05$) (Fig. 3B). Based on these results, in the following experiments, D. rotundifolia seeds were germinated on agar at the alternating temperatures of 30/15°C after cold/moist stratification for 8 weeks.

Maximum germination of D. rotundifolia seeds removed after 14-20 years of storage in LN was reached about 15-30 DAS (Fig. 2B) and ranged from 80 to 98 % for the four accessions

studied (Fig. 4). Because of the improved germination conditions used post-storage, these germination rates were higher than those observed at the time of banking (Supple. Table 2) but indicated that declines in viability were either absent or low (e.g., maximum 20% loss if an initial 100% germination is considered) over the time in LN storage.

With all four accessions of D. rotundifolia, by 25 DAS, seedlings had developed the normal phenotype (normal seedling) with green cotyledons and a root (Fig. 1A), and the percentage of normal seedlings was not significantly different from the percent total germination (Fig. 4; binomial test, $P>0.05$). The growth of two of the accessions was monitored 106 days (Figs. 4A, B). In the Gallagher Fen accession, there was no significant loss of D. rotundifolia plants over time (Fig. 4A, binomial test, $P> 0.05$), and seedling leaf production increased significantly (Positive Pearson correlation, $P<0.05$). In the Mantua Bog1 accession, there was a significant loss of plants between 71 and 106 DAS (36 % of normal germinated seeds, Fig. 4B), due to large fungal growth in the soil used for this accession. Fungal growth also affected the Gallagher Fen accession, which was grown in the same substrate, and resulted in a smaller increase in the number of leaves between 71 and 106 DAS. Similar losses did not occur with the Prairie Road Fen and Mantua Bog2 accessions (Figs. 4C, D), which were grown in an improved soil mix, which was rich in sphagnum (see Methods). When D. rotundifolia plant viability and growth was monitored longer in the improved soil mixture (250-360 DAS, Figs. 4C, D), plants grown from seed stored for 15.8 or 18.4 years in LN continued growth with a significant increase in leaf production (Figs. 4C, D, Positive Pearson correlation, $P<0.05$). Only about 10% of the normal seedlings died over this time period for the two accessions monitored for a long time (Figs. 4C, D), primarily because plants did not resume growth after they went into the hibernaculum stage, the typical overwintering stage of D. rotundifolia (Fig. 1C).

For Sarracenia sp., post-storage germination ranged from 25-75%, and the growth of seedlings was similar to that observed with Drosera (Fig. 5). There was no significant loss of plants, either during the first 3 months of growth (up to 106 DAS), or after 1 to 1.5 years of growth (360 DAS for S. flava and S. minor or 529 DAS for S. purpurea) (Fig. 5, binomial test, $p>0.05$). In addition, the seedlings of all three species significantly increased leaf production during that time (Fig. 5; Positive Pearson correlation, $P<0.05$). Pre-storage viability data was available for S. purpurea, and there was no significant difference between germination of S. purpurea seeds exposed to LN before storage and of seeds recovered after storage (Fig. 5A, binomial test, $p>0.05$), indicating that the initial viability of the seeds was not lost over the time in LN storage.

Seeds of one accession of D. capensis and two purchased accessions of S. purpurea (subsp. purpurea and subsp. venosa) were germinated in vitro. There was no germination of S. purpurea subsp. venosa, but 37% and 20% germination in D. capensis and S. purpurea subsp. purpurea, respectively (Fig. 6). Most of the seeds germinated in vitro produced normal seedlings, and there was no significant loss of plants when observed at 194 DAS. While the pre-storage data for these S. purpurea accessions was not available, D. capensis viability had not declined after 20 years of storage in LN (binomial test, $p>0.05$).

TAG thermal fingerprints of seeds of some CPs

Distinct endothermic and exothermic events were detected as peaks above and below the baseline in the warming and cooling scans of the seeds of the four CP species scanned (Fig. 7). Based on the low amount of water in the seed tissues (about 0.04 g H_2O g DW^{-1}), these events

were identified as TAG melting and crystallization events (after Vertucci 1992 and Walters et al. 2005b).

Seeds of both Drosera sp. scanned showed very similar TAG thermal fingerprints in the warming scans (Fig. 7A), with a small endothermic transition peaking at about -75°C (ΔH = 0,24 ± 0,07 and 0,25 ± 0,03 for D. rotundifolia and D. anglica, respectively; Supplementary Table 3), followed by a small exothermic event peaking at about -65°C (ΔH = 0,50 ± 0,11 and 0,75 ± 0,08 for D. rotundifolia and D. anglica, respectively; Supplementary Table 3), and a broad endothermic event peaking at about -34°C (ΔH = 1,85 ± 0,24 and 1,97 ± 0,21 for D. rotundifolia and D. anglica, respectively; Supplementary Table 3). These three events were related to the melting and recrystallization of different crystal forms of the same seed TAG. TAG crystallization is a dynamic and polymorphic process that produces different crystal forms based on the cooling history. In our DSC warming scans (Fig. 7A), the lowest temperature peak is related to the melting of the low energetic and transitory α crystals of the seed TAG that were formed after the relatively fast cooling rate used (10°C min^{-1}), followed by a recrystallization of the melted α crystals into more stable β' crystal forms, and ending in the melting of all the β' crystals of the seed TAG (Supplementary Fig 2). These assumptions were made based on Walters et al (2005b) interpretations of the DSC scans of sunflower seeds and some complementary experiments in which the seeds of the CPs studied were hold (i.e., annealed) at -60°C (i.e., near the β' recrystallization temperature) for 60 min to allow the complete crystallization of the seed TAG as β' crystals (Supplementary Fig 2A). When the seeds were annealed at -20°C for 60 min, no changes in the TAG thermal fingerprint of the warming scans were detected (Supplementary Fig 2A).

Seeds of P. vulgaris and U. vulgaris also showed very similar TAG thermal fingerprints in the warming scans, which were different from those observed for Drosera sp. (Fig. 7A). In these scans two main peaks were detected near -90°C and near -20°C (labelled as L2 and L1, respectively; Supplementary Fig 2B). Based on the annealing experiments performed, these peaks could not be clearly related to α and β' crystal forms of the same seed TAG (although L1 tended to increase after 60 min annealing at -60°C). They were considered as part of a large lipid melting event with an average enthalpy of melt of 9,4 ±1,5 and 8,6 ± 1,4 mJ mg DW^{-1} for P. vulgaris and U. vulgaris seeds, respectively (Supplementary Table 4).

When seeds of D. rotundifolia stored for different periods of time were scanned in the DSC, we detected diverse changes in the TAG thermal fingerprints related to storage time and germination percentage of the accession. Seeds stored for shorter times (6.6 and 6.9 years with germination of 94 and 100% respectively) showed comparable fingerprints, with an enthalpy of melting of the β' crystals of 1,85 ± 0,24 and 1,39 ± 0,25 mJ/DW, respectively. On the other hand, seeds stored for a longer time (26,5 yrs with germination of 18%) showed a significant decrease in the enthalpy of melting of the β' crystals ($P<0.05$, ANOVA) which was calculated in 0,40 ± 0,20 mJ/DW (Supplementary Fig 3, Supplementary Table 3). In addition, the seeds stored for a longer time showed very small or nil changes in enthalpy in the area of the melting of the α crystals or the area of the β' recrystallization (Supplementary Fig 3, Supplementary Table 3) in comparison with the clear transitions detected in seeds stored for 6.6 or 6.9 years (Supplementary Fig 2A, Supplementary Fig 3, Supplementary Table 3).

DISCUSSION

In this paper, we aimed to test three hypotheses about longevity and long-term ex situ conservation of seeds of CPs. Firstly, and based on the considerations of their ephemeral nature due to their short life span when stored at (uncontrolled) room conditions (Groves, 1917; Bourke, 2003; Baskin and Baskin, 2014), we hypothesized that longevity of seeds of CPs was going to be short and comparable to that of other taxa with known short-lived seeds when stored in comparable storage conditions. We have confirmed this hypothesis for some taxa stored at diverse storage conditions, such as Darlingtonia californica stored at room conditions at the ICPS and Drosera sp. stored at standard seed bank conditions at the MSB. For example, when enough viability/time point data were available for longevity calculations in the MSB stored seeds, only D. rotundifolia and D. anglica seeds showed significant P50 values, which were in the region of other taxa considered medium/short or short-lived at comparable cold/dry storage conditions (Walters et al., 2005a; Colville and Pritchard, 2019). A detailed discussion about the "comparative longevity of seeds of CPs" and the "determinants for the potential short longevity of seeds of CPs" follows this introductory paragraph. Nevertheless, despite their potential ephemeral nature, seeds of 64% of accessions tested in this work retained up to two decades their initial viability when they were stored in the controlled conditions of a conventional seed bank, where moisture and temperature are adjusted to maximize longevity (ENSCONET, 2009; FAO, 2014). Only some accessions stored for longer times, generally over 25 years, showed significant signs of deterioration (mostly viability decrease). These results, also discussed in depth below, support our second hypothesis that supposed that seed germination and viability would have been preserved over the storage time in most cases in ex situ seed storage collections. Finally, we hypothesized that cryopreserved seeds would have preserved a high germination rate and ability

to develop into healthy and viable plants with potential use in restoration programs. We have found in the seeds stored at CREW that cryogenic storage of dry seeds can be considered as an alternative (or complement) to conventional seed banking, as it was able to preserve a high germination percentage of seeds of CPs stored up to two decades. Furthermore, seedlings obtained developed normally into healthy plants when monitored up to 1.5 years after germination.

The results of this paper were obtained from long-term storage surveys (up to 30 years) performed on thirteen species representing the three main plant orders (and families) with CPs: Ericales (Sarracenaceae), Lamiales (Lentibulariaceae) and Caryophyllales (Droseraceae). Despite the potential short lifespan of seeds of diverse CP taxa, this paper highlights and supports the routine storage of seeds of CP (either in conventional or cryogenic storage) in ex situ conservation programs to support present and future in situ conservation initiatives, such as ecosystem restoration, population reinforcements or reintroductions (Guerrant et al., 2004; Clarke et al., 2018; Cross et al., 2016, 2020). However, more research is needed in this area to fully confirm and support our conclusions across all CPs, considering that over 750 CP species are currently recognised and they are polyphyletic and remarkably diverse in morphology, physiology, ecology, and distribution (Ellison and Adamec, 2018).

Comparative longevity of seeds of CPs

Seeds inevitably deteriorate and age during storage until their tissues succumb to a programmed cell death upon rehydration and seeds are not able to germinate (Ballesteros et al., 2020; Zinsmeister et al., 2020). The period after harvest in which seeds show signs of viability or germinability is considered the longevity and can be indicated or measured by different

parameters. For example, the absolute longevity defines the storage period until the last year of seed germination and the P50 is the time for viability to fall to 50% (Nagel and Börner, 2010). In seed science research, P50 values have been considered an objective measure of longevity and have allowed longevity comparisons across species (e.g., Walters et al., 2005a; Probert et al., 2009; Nagel and Börner, 2010; Hay et al., 2010; Mondoni et al., 2011; Merritt et al., 2014; Colville and Pritchard, 2019; Davies et al., 2020).

Reports on longevity of seeds of CPs are scarce in the scientific literature and often are related to anecdotical information of absolute longevity obtained from the personal experience of some plant growers (Groves, 1917; Bourke, 2003; Baskin and Baskin, 2014). While this information is highly valuable and provides an important starting point, it may not be sufficient to determine if storage of seeds of CPs at the standard conditions of seed banks will be challenging due to the apparent ephemeral nature of these seeds when stored in uncontrolled ambient conditions (Groves, 1917; Bourke, 2003; Godefroid et al., 2010; Baskin and Baskin, 2014; Cross et al., 2016; Royal Botanic Gardens Kew, 2021). To categorize seed longevity, empirical determinations need to be made at specific storage conditions and compared with other species aged in the same or comparable conditions (e.g., Walters et al., 2005a; Probert et al., 2009; Hay et al., 2010; Nagel and Börner, 2010; Mondoni et al., 2011; Merritt et al., 2014; Colville and Pritchard, 2019; Davies et al., 2020). In this sense and based on the absolute longevity determined for seeds stored under ambient storage conditions, seeds of the CPs studied in this paper seem to be short-lived, as they aged as fast or faster than the shorter-lived seeds measured in other studies using ambient storage conditions (e.g., 20.3 ± 2.3°C and 50.5 ± 6.3% RH, Nagel and Börner, 2010). For example, Nagel and Börner (2010) found that the shorter-lived species they tested (chive and lettuce) showed absolute longevities of 5 and 7 years,

respectively, while the longer-lived species had absolute longevities over 20 years. The data presented from seeds stored under ambient conditions in the ICPS seed bank (Table 2) suggest that the absolute longevity for most of the seeds tested was under 5 years, as most seeds stored between 1 and 2 years showed germination between 0 and 2%. As we did not have enough data to calculate P50s in the seeds of this collection, it is difficult to use this parameter for longevity comparisons using, e.g., Nagel and Börner (2010) data. However, considering that seeds of Darlingtonia californica seeds showed germination <15% in less than 2 years of storage and their initial viability was likely above 80%, we can speculate that P50 of this species was around 1 year, which is also significantly lower than the shorter P50 found in Nagel and Börner (2010) report (2 and 4.6 years for chive and lettuce, respectively).

P50s calculated from seeds stored in the MSB, also provide a good estimate to determine the comparative longevity of seeds of some CP species. Whether we take the mean value of the P50s estimated from actual germination (18.3 years) or estimated viability data calculated from individual D. rotundifolia accessions (30.5 years) or for the whole species (22.4 years) (Table 3), these values suggest that seeds of this CP species are relatively short-lived when compared with P50s obtained for species stored dry and cold in other seed banks (e.g., Walters et al., 2005a; Colville and Pritchard, 2019). In these reports, P50 < 36 years were related to the species or families with seeds showing the shorter lifespans. On the other hand, and using calculations at the species level, there is no significant decline over time for Pinguicula sp., and the P50 estimated for D. anglica falls within the range of medium-lived species (medium = 36 years < P50 < 72 years, long = P50 > 72 years; Walters et al., 2005a; Colville and Pritchard, 2019). In addition, we can also infer the relative short lifespan of seeds of some CP species stored in the MSB based on the significant differences between maximum and last germination tests (Table

3). For example, Probert et al. (2009) found that seeds considered short-lived also showed a significant decrease in viability after 20 years of storage in the conditions of the MSB. In our study, we found significant viability decrease in seeds of D. anglica (Dorset), D. rotundifolia (Surrey, Hampshire), P. vulgaris (Cumbria2), and Utricularia vulgaris stored for 25, 26.5, 25, 16.9, and 17.7 years, respectively (Table 3), suggesting that these species could also be relatively short-lived at seed bank conditions.

Determinants for the potential short longevity of seeds of CPs

Seed longevity has been suggested to be influenced by taxonomy, geographical origin, ecology, and seed morphology (Walters et al., 2005a; Probert et al., 2009). For example, on a global scale, seeds of plant species that occur in moist or/and cool habitats, like temperate Europe, tend to be short-lived compared with species growing in dry or/and hot environments (Walters et al., 2005a; Probert et al., 2009). When looking in specific regions such as Australia, seeds tend to be shorter-lived from dry, cool regions compared to those with higher rainfall and temperature (Merritt et al., 2014; Davies et al. 2016). In addition, endospermic seeds with small embryos tend to be short-lived compared with non-endospermic seeds (Probert et al., 2009; Merritt et al., 2014). These two are characteristics of most of the taxa studied in this paper [species were mostly harvested in temperate regions in the UK and the USA (Table 1) and all Droseraceae and Sarraceniaceae studied present endospermic seeds (Martin, 1946; Dwyer, 1983; Ellison, 2001; Baskin and Baskin, 2014)] but particularly of D. rotundifolia. In addition, these ecological and seed morphological characteristics are typical in many CPs reported to present short seed longevity generally at uncontrolled storage conditions (e.g., Aldovandra, Darlingtonia, Drosera, Genlisea, Heliamphora, Nepenthes, Pinguicula, and Sarracenia; Groves, 1917; Bourke,

2003; Baskin and Baskin, 2014; Cross et al., 2016). These species typically grow in wet habitats and have limited tolerance to dry soils (Brewer and Schlauer, 2018) and also tend to present endospermic seeds with small embryos (Smith, 1929; Martin, 1946; Dwyer, 1983; Ellison, 2001; Baskin and Baskin, 2014). On the other hand, the literature indicates that seeds of a few CP species may not have the relative short lifespans found in seeds of most CPs and suggests that this trait may also be related to the ecology or distribution of the species. For example, seeds of species such as Drosophyllum lusitanicum and Byblis sp. have been reported to be stored successfully out of the fridge for many years with no problems of germination at all (Bourke, 2003). Interestingly, D. lusitanicum is one of the few carnivorous plants to grow in dry soil and present distinctly xerophytic features (Adamec, 2009) and Byblis is an endemic genus to Australia and South Asia, both places showing the longest seed lifespans in the species originated there (Walters et al., 2005a; Merritt et al., 2014). In terms of the taxonomy, and despite the polyphyletic nature of CPs (Ellison and Gotelli, 2009), none of the five orders or eleven families holding CPs are considered to present a high proportion of short-lived seeds (Ellison and Gotelli, 2009; Walters et al., 2005a; Probert et al., 2009).

Additionally, seed longevity has been determined to be shaped by certain physicochemical attributes of the seeds, with two "dry architectures" that have been related to promote short-life spans: seeds that present chloroplasts at maturity and oily seeds with specific melting/crystallization behaviors (Ballesteros et al., 2020). While information about chloroplasts presence and oil composition and thermal properties in seeds of CPs is scarce (e.g., SID database does not contain oil contents for any seed of CP; Royal Botanic Gardens Kew, 2021), there are some clues in the literature that support the presence of these two dry architectures in some CP. For example, it has been described that Utricularia species from the Iperua section produce green

seeds with extremely short lifespans (Bourke, 2003). In addition, the seed storage behavior described for Aldovandra vesiculosa (desiccation tolerance but extremely fast ageing when exposed to -18°C, Cross et al., 2016) seem to be analogous to that observed for seeds in the Cuphea genus (Crane et al., 2003, 2006) where the lipid crystallization and melting properties of the seed triacylglycerols determined the anomalous seed storage of these and other oily seeds when stored dry at low sub-zero temperatures (Ballesteros et al., 2020). In our experiments, we detected clear TAG thermal events at temperatures ranging between -15 and -120°C, with the main crystallization and melting events occurring between -15 and -50°C. While the influence of these TAG thermal events on the interspecies differences of CP seed longevity is difficult to establish based on the results presented in this paper (Fig 7, Supplementary Fig 2, Supplementary Fig 3, Supplementary Table 3 and 4), it seems clear that seed ageing alters the physical-chemical properties of the storage lipids which show a decrease in the energy of their melting transitions (Supplementary Fig 3, Supplementary Table 3) as observed for other oily seeds (Vertucci, 1992; Walters et al., 2005b). Nevertheless, more research is needed in this area to characterize seed traits in CPs and their relation to longevity.

Germination and propagation of seeds of CPs

One of the main constrains when analyzing the dataset of this work, was the change of the germination protocols over time to reach acceptable germination percentages in tune with the viability percentages obtained by cut test (e.g., MSB), or the lack of optimal germination tests of the seeds before long-term cryogenic storage, so when freshly harvested, dried, or exposed to LN (e.g., CREW). This constraint, for example, showed higher germination in seeds after LN storage (e.g., Fig. 4), or brought us to use viability percentages instead of germination percentages for

some of the statistical analyses from MSB stored seeds (Table 3), as germination showed in some cases an increase over time due to the improvement of the germination conditions (Fig. 2B, D) and germination decrease due to ageing could not be monitored. This issue has illustrated one of them main issues seed banks may encounter when working with wild species: the lack of knowledge of the seed biology of many wild and rare species at the time of banking or retrieval from storage (e.g., Hay and Probert, 2013; Cross et al., 2016; Merrett-Wade et al., 2016). This was particularly relevant for the CP species studied in this work, as most of them were collected in the late 1980s and 1990s, when not many studies in germination and dormancy breaking of seeds of CP were available (Baskin and Baskin, 1998). However, some lessons are learnt from this work that support previous suggestions for the management of wild species seed banks (including CPs). When germination tests are not optimized for a species due to knowledge gaps on their dormancy and the way to break it, viability tests (e.g., cut test, tetrazolium tests) can provide reliable estimates to monitor seed ageing over time (Godefroid et al., 2010; Hay and Probert, 2013). In addition, low germination percentages initially, should not discourage us to bank seeds of rare or threatened plants if identified as viable somehow, as seed biology of these species may be revealed over storage time and efficient and optimal germination protocols may be available at the time of seed retrieval (compare references for seeds of CPs between Baskin and Baskin 1998 and 2014).

We have also shown that seed germination developments should be complemented by horticultural research on optimal seedling development and plant growth, as the ultimate purpose of most seed collections is to ensure the production of healthy, usable plants for habitat restoration and species reintroduction (Merritt and Dixon, 2011; Hay and Probert, 2013; FAO, 2014; Cross et al., 2016; Abeli et al., 2020; Di Sacco et al., 2021). Popular knowledge from CP

growers is important and the information provided in websites and manuals could be vital to inform about germination and plant growth developments to conservation institutions holding CP collections. However, fundamental research in botanical gardens or specialized nurseries is needed to understand the biological principles that govern seed germination and seedling establishment. This knowledge is essential to foster connections between ex situ and in situ conservation efforts, and properly plan plant reintroductions or reinforcements in nature when they need to be implemented (Godefroid et al., 2011; Cross et al., 2016, 2020). Moreover, other strategies could be needed to ensure more diverse collections for conservation purposes and to provide us with research models to study germination, propagation or longevity issues. These strategies could include gap analyses of the preserved collections in diverse ex situ platforms, collection of species lacking representation in current ex situ collections, and prioritization of genetic resources in function of their threatened status.

CONCLUSIONS

Despite the potential and relative short longevity of seeds of some CP species when compared to other plant species stored in comparable conditions, viability and germination of seeds can be extended in many cases when they are stored dry at low temperatures (Bourke, 2003; Connor and Gibbs, 2012; Baskin and Baskin, 2014). In this sense, the use of the standard storage conditions of seeds banks (ENSCONET, 2009; FAO, 2014) has been proven to preserve high levels of viability and germination up to 2 decades and potentially up to 3 or more decades in most species studied, except for D. rontundifolia (Table 3). In addition, cryogenic storage of seeds of CPs up to 2 decades has been proven to preserve high germination percentages (with no

significant decrease over time) and subsequent seedling development and plant growth. The data presented in this paper support the routine storage of seeds of CPs (both conventional and cryogenic) as an efficient ex situ conservation measure to support in situ restoration programs (Guerrant et al., 2004; Clarke et al., 2018; Cross et al., 2016, 2020). However, due to the large number of CP species with high diversity on phylogenetic origin, morphology, physiology, ecology, and distribution, more research is needed on fundamental aspects of germination, propagation, and longevity of seeds of CP to maximize their successful ex situ storage and conservation in germplasm banks. This will be important to determine whether some seeds may present short longevity due to specific physicochemical properties of their seeds, how to efficiently break the dormancy of seeds of CPs after long-term storage, and how to propagate them using the most appropriate horticultural techniques for restoration ecology projects. This research is necessary to aid CP conservationists and researchers in their fight against CP biodiversity loss.

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FIGURE LEGENDS

Figure 1. The different developmental stages of seedling growth for Drosera rotundifolia (A, B, C) and Sarracenia sp. (D, E, F). C: cotyledon, L: normal leaf, H: hibernaculum.

Figure 2. Germination (black squares) and viability (determined after cut test, grey circles) of seeds of a selection of accessions of Drosera rotundifolia, Pinguicula lusitanica and P. vulgaris stored up to 30 years in the MSB. Black lines represent the germination (solid) and viability (dotted) curves fitted by probit analysis. Grey dashed lines indicate the points where germination is 50% of initial, and arrows indicate the P50 (time at which germination decreases 50% of initial) calculated for germination (black) and viability (grey) data.

Figure 3. Germination of Drosera rotundifolia seeds in the pilot study. A: Seeds from 3 accessions (Gallagher Fen, ○; 240225, Δ; and Mantua Bog1, □), 4 replicates with 25 seeds each per accession, were germinated without stratification at 26 ± 1°C. After 7 weeks, 2 replicates per accession (Group A) were moved to 30/15°C, and two plates were moved to 4°C for 8 weeks for cold/moist stratification, followed by germination at 30/15°C (Group B). B: Seeds of the Prairie Road accession were cold-moist stratified for 8 weeks at 4°C and then germinated at either 25ºC or 30/15ºC, 4 replicates of 25 seeds each per temperature.

Figure 4. Germination, seedling survival, and growth during the first 3 months (up to 106 DAS) for seeds of four accessions of Drosera rotundifolia: Gallagher Fen (A), Mantua Bog1 (B), Prairie Road Fen (C) and Mantua Bog2 (D). Germination and seedling survival was measured as

the percent of seeds that germinated or seedlings that grew normally, respectively. Growth was measured as the number of normal leaves produced. The asterisk indicates that the number of normal seedlings was significantly different at the indicated DAS with respect to the other DAS.

Figure 5. Germination, seedling survival, and growth of Sarracenia sp. A: Seeds of Sarracenia purpurea (Triangle Lake Bog) were stored in LN 15.7 years. Seedling growth was tracked up to 1.5 years (526 DAS). Seeds of Sarracenia minor (A) and S. flava (B) were stored in LN 20.7 years. Seedling growth was tracked up to 1 year (106 and 360 DAS, respectively). Germination and seedling survival was measured as the percent of seeds that germinated or seedlings that grew normally, respectively. Growth was measured as the number of normal leaves produced. The arrow indicates the germination of the accession measured before LN storage (Supplementary. Table 2).

Figure 6. Germination and seedling survival up to 194 DAS of seeds of Drosera capensis and Sarracenia purpurea subsp. purpurea germinated and cultured in vitro. Germination and seedling survival was measured as the percent of seeds that germinated or seedlings that grew normally, respectively. The arrow indicates the germination of the accession measured before LN storage (Supplementary. Table 2).

Figure 7. DSC thermograms of dry seeds of CPs. Warming scans (A) show endothermic and exothermic events arising above and below the baseline (dashed line), respectively. Cooling scans (B) show exothermic events arising below the baseline (dashed line). Endothermic events correspond to the melting of the storage lipids of the seeds (mainly TAG) while exothermic

events correspond to crystallization of the storage lipids of the seeds. Both warming and cooling scans were performed at 10 °C/min.

Table 1. Taxa of carnivorous plants used in this study. Seed origin, accession number (if available), age and storage conditions are indicated in the table.

Species	Source or Collection Site[a] (Accession number)	Collection date	Date of storage	Storage time (yrs)
Seeds stored in LN at CREW (collected or purchased in the USA)				
Drosera capensis L.	Purchased (220217)	Received April 1993	30 July 1993	19.8
Drosera rotundifolia L.	Gallagher Fen (230254)	22 August 1997	01 July 1998	15.5
	Prairie Road Fen (220255)	29 September 1995	22 November 1995	18.4
	Mantua Bog1 (230443)	03 August 1998	24 September 1998	14.7
	Mantua Bog2 (230444)	03 August 1998	24 September 1998	15.8
	no data (240225)	Aug./Sept. 2000	20 February 2001	12.3
Sarracenia purpurea subsp. purpurea L.	Triangle Lake Bog (230257)	20 November 1997	07 January 1998	15.7
	Purchased (220215)	Received April 1993	30 July 1993	19.8
Sarracenia purpurea subsp. venosa (Raf.) Wherry	Purchased (220216)	Received April 1993	30 July 1993	19.8
Sarracenia minor Walt.	Purchased (220218)	Received April 1993	30 July 1993	20.7
Sarracenia flava L.	Purchased (220219)	Received April 1993	30 July 1993	20.7

Seeds stored at -20°C in MSB (collected in the UK)				
Drosera anglica Huds.	Hampshire (86262)	03 September 1990	06 February 1991	25.0
	Dorset (86413)	10 September 1990	06 February 1991	25.0
Drosera rotundifolia L.	Surrey (86192)	28 August 1990	26 November 1991	26.5
	Hampshire (86240)	03 September 1990	06 February 1991	25.0
	Norfolk (Norfolk)	04 September 2008	09 March 2009	6.9
	East Sussex (East Sussex)	16 October 2008	29 July 2009	6.6
Pinguicula lusitanica L.	Isle of Wight (46187)	09 August 1982	13 July 1983	33.7
	I. Wight (Wakehurst)[b] (102270)	01 July 1994[b]	05 December 1994[b]	21.2
Pinguicula vulgaris L.	Cumbria1 (130280)	08 September 1998	17 March 1999	17.7
	Cumbria2 (130327)	09 September 1998	17 March 1999	19.9
	Cumbria3* (995126)	31 July 2018	12 February 2019	2.3
Utricularia vulgaris L.	Norfolk (128148)	12 August 1998	26 June 1999	17.7
	Norfolk2* (1010385)	06 August 2018	06 June 2019	2.4
Seeds stored at room conditions by the ICPS (collected in the USA, except for D. finlaysoniana)				

Darlingtonia californica Torr.	The Ponds	01 September 2012	01 September 2012	1.5
	Parks Creek	01 October 2012	01 October 2012	1.4
	Gasquet	01 October 2013	01 October 2013	0.4
Dionaea muscipula Ellis	From grower "KS"	01 August 2012	01 August 2012	1.6
	From grower "RL"	01 August 2012	01 August 2012	1.6
Drosera finlaysoniana Wall. ex Arn.	Australia	01 October 2006	01 October 2006	7.4
Sarracenia flava var. flava L.	North Carolina	06 November 2012	06 November 2012	1.3
Sarracenia purpurea subsp. purpurea L.	New York	10 November 2011	10 November 2011	2.3
	Massachusetts	07 January 2013	07 January 2013	1.1

[a]Purchased = from Thompson & Morgan Inc.; From grower = seeds donated to ICPS from different private CP growers in the USA; Collection sites in the USA: Gallagher Fen State Nature Preserve, Clark Co, OH; Prairie Road Fen, Clark County, OH; Mantua Bog, Portage Co., OH; Triangle Lake Bog State Nature Preserve, Portage Co., OH; The Ponds, Del Norte Co., California; Parks Creek Rd, Siskiyou Co., California; Gasquet, California; Onslow Co, North Carolina; Easton, Massachusetts. Collection sites in the UK: Seeds collected from wild populations in England located in diverse counties.

[b]Seeds from this accession were regenerated in the early 90s from the other accession of P. lusitanica collected in 1982 in a wild population in Isle of Wight. Regenerated seeds were stored in the MSB in 1994.

*accessions used for DSC scans only

Table 2. Germination percentage of seeds of diverse carnivorous plants stored at room temperature for different lengths of time. Seeds were obtained from the International Carnivorous Plant Society seed bank (http://www.carnivorousplants.org/seedbank/seedmain.html). There are no accession numbers and accession name refer to the collection site or the initials of the grower (Table 1).

species	Accession	Storage time (yrs)	Germination percentage (Average ± SD)
Drosera finlaysoniana		7.4	1 ± 2
Darlingtonia californica	Gasquet	0.4	77 ± 10
	Parks Creek Rd	1.4	1 ± 2
	The Ponds	1.5	14 ± 3
Sarracenia flava var. flava		1.3	29 ± 5
Sarracenia purpurea subsp. purpurea	Massachusetts	1.1	1 ± 2
	New York	2.3	0 ± 0
Dionaea muscipula	KS	1.6	2 ± 2
	RL	1.6	0 ± 0

Table 3. Germination, viability and longevity stats for the CP species stored and germinated in the MSB since 1983. Initial, highest, and last germination and viability percentages (in brackets) are shown for the intervals tested in each accession. When a Z test indicated a significant decline in viability between the highest and the last value measured, the probability values are presented, otherwise the non-significant (NS) decline is indicated. Estimates of time for viability to fall to 50% (p50) from probit seed longevity where significant ($P < 0.05$) and parameters K_i and slope ($1/\sigma$) are shown for each species.

Species	Collection site	Age	Initial % germination (viability)	Max % germination (viability)	Last % germination (viability)	Number of valid test intervals for probit analysis	Z Significant germination (viability) decline between max and last % [b]	p_{50} ± s.e. Years	K_i	slope ($1/\sigma$)
Drosera anglica	Hampshire	25,0	83[c] (100)[c]	90 (100)[c]	90 (96)	1 (3)	-	-	-	-
	Dorset	25,0	55 (100)	74 (100)	74 (86)	1 (3)	- (2.94)	-	-	-
Drosera rotundifolia	Surrey	26,5	76 (100)	76 (100)	10 (18)	5 (5)	6.39 (8.56)	10.6 ± 1.56 (19.3 ± 0.99)	0.84 (3.13)	-0.08 (-0.16)
	Hampshire	25,0	55 (100)	84[c] (100)	62 (95)	3 (7)	- (2.03)	26.0 ± 2.71 (35.5 ± 7.78)	1.69 (2.47)	-0.07 (-0.07)
	Norfolk	6,9	100 (100)	100 (100)	100 (100)	1 (1)	-	-	-	-
	East Sussex	6,6	0 (88)	94 (96)	94 (96)	1 (1)	-	-	-	-
Pinguicula lusitanica	Isle of Wight	33,7	77[d] (100)[d]	83 (100)	83 (88)	1 (1)	-	-	-	-
	Wakehurst Place	21,2	95 (100)	100 (100)	100 (100)	3 (4)	NS (NS)	-	-	-
Pinguicula vulgaris	Cumbria1	17,7	10 (100)	100 (100)	100 (100)	1 (1)	NS (NS)	-	-	-
	Cumbria2	16,9	5 (100)	100 (100)	86 (90)	2 (2)	2.58 (2.33)	-	-	-
Utricularia vulgaris	Norfolk	17,7	33 (100)	82 (100)	82 (89)	2 (3)	2.63 (2.55)	-	-	-

[a] Germination / viability data prior to the highest (Max) result were excluded for probit analysis. Minimum sample size was 10 seeds for probit analysis.

[b] Z test between highest (Max) and last germination/viability percentages were only run when sample size was at least 20 seeds. Significant germination/viability decline is indicated by $Z > 1.64$ ($P < 0.05$).
[c] Less than 20 seeds in the germination/viability test
[d] Less than 10 seeds in the germination/viability test

Figure 1

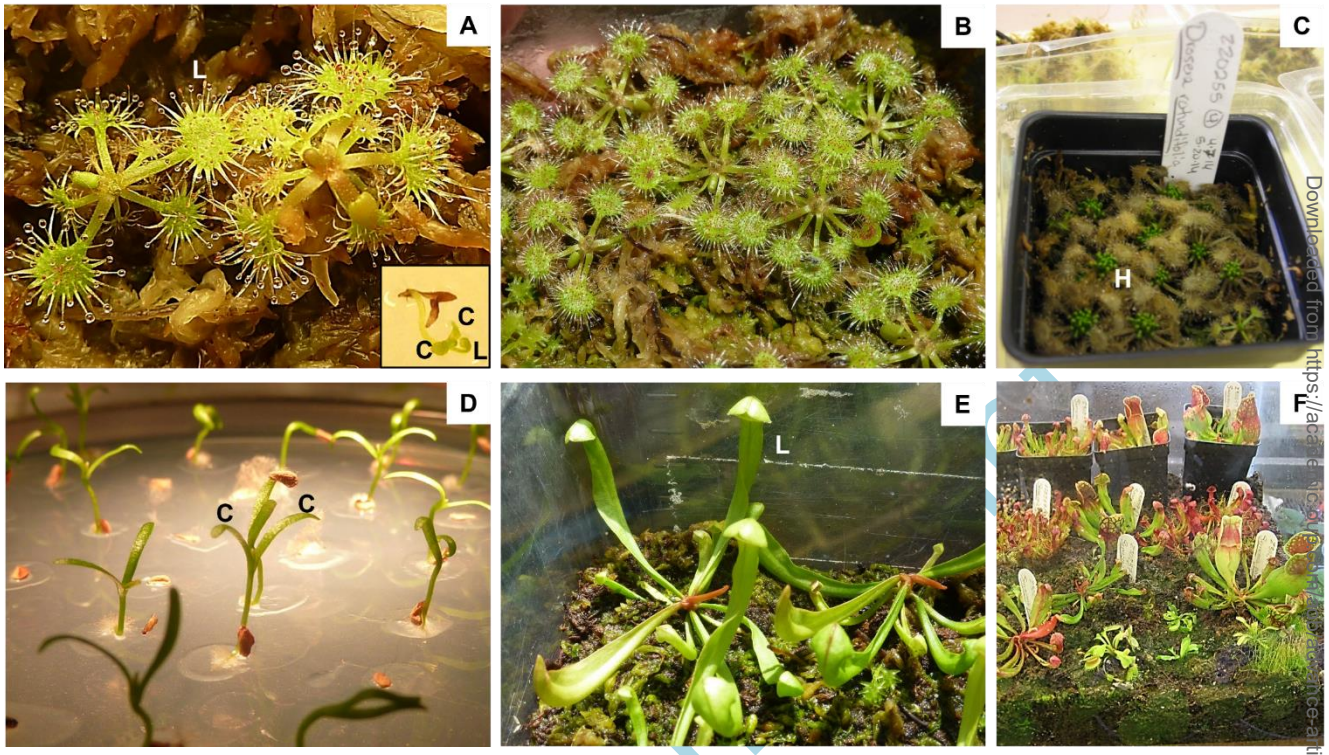

Figure 2

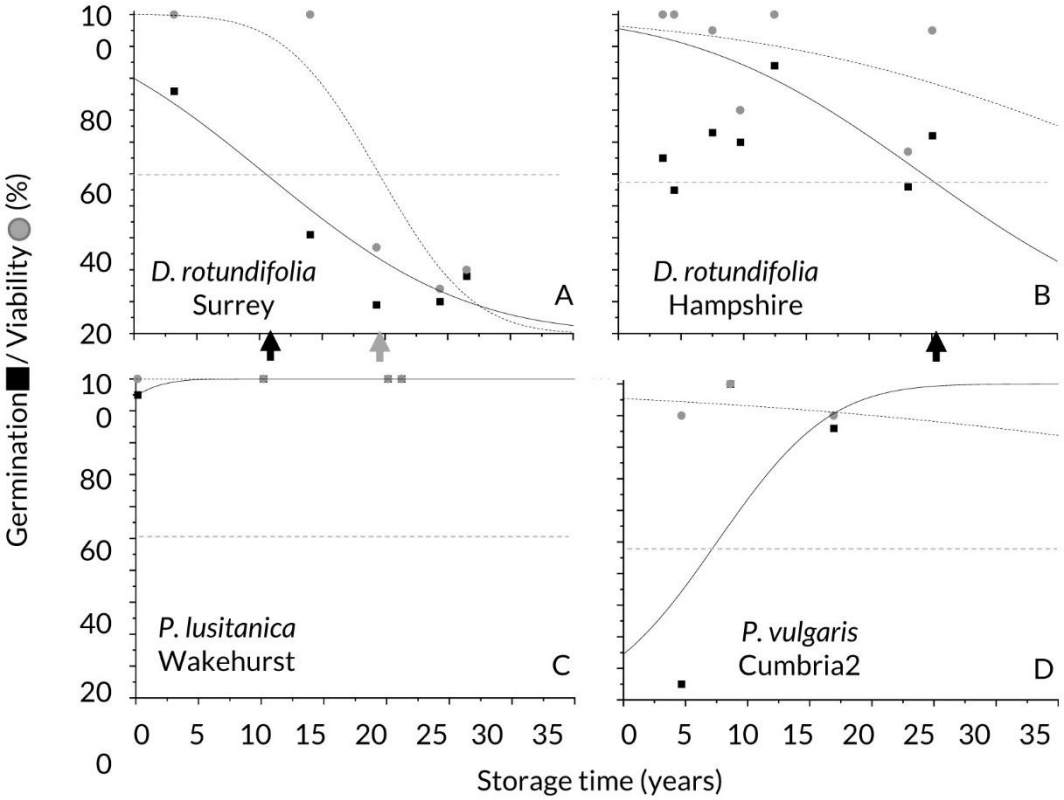

Figure 3

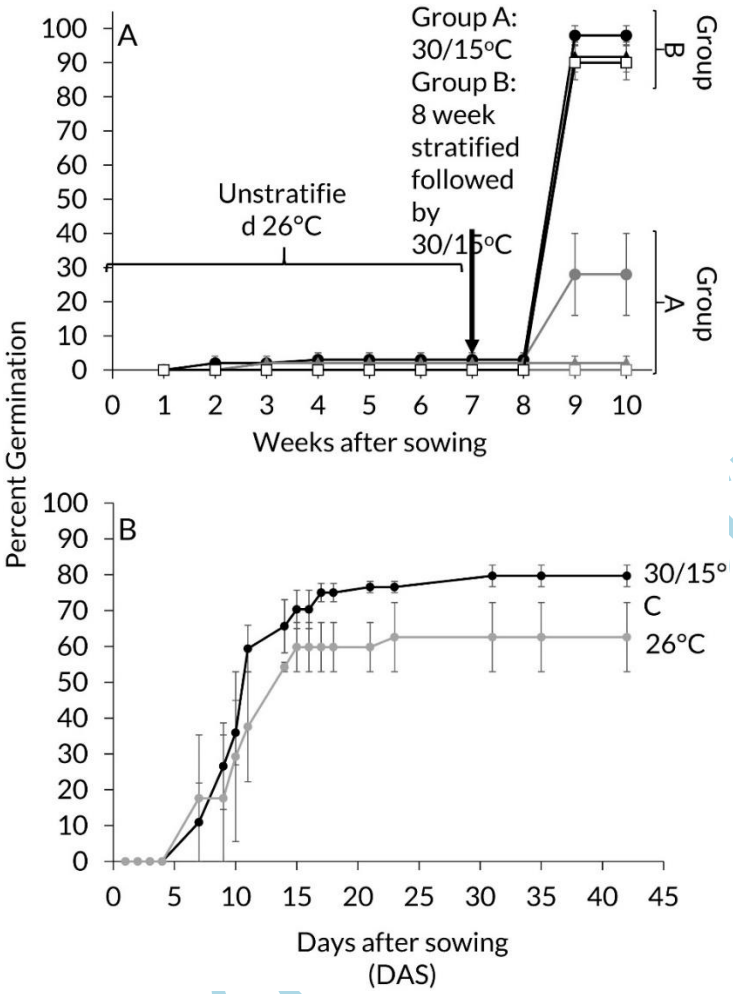

Figure 4

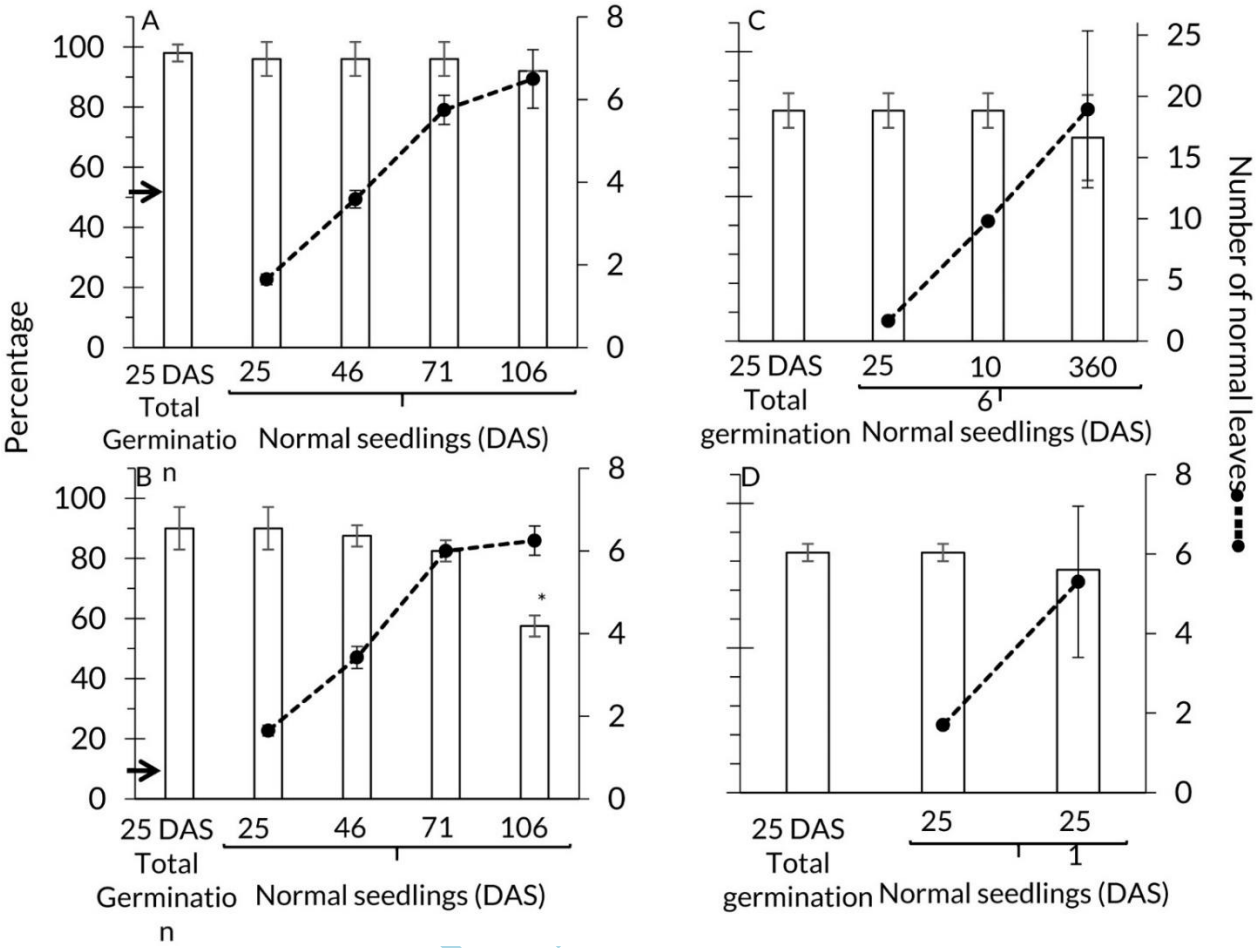

Figure 5

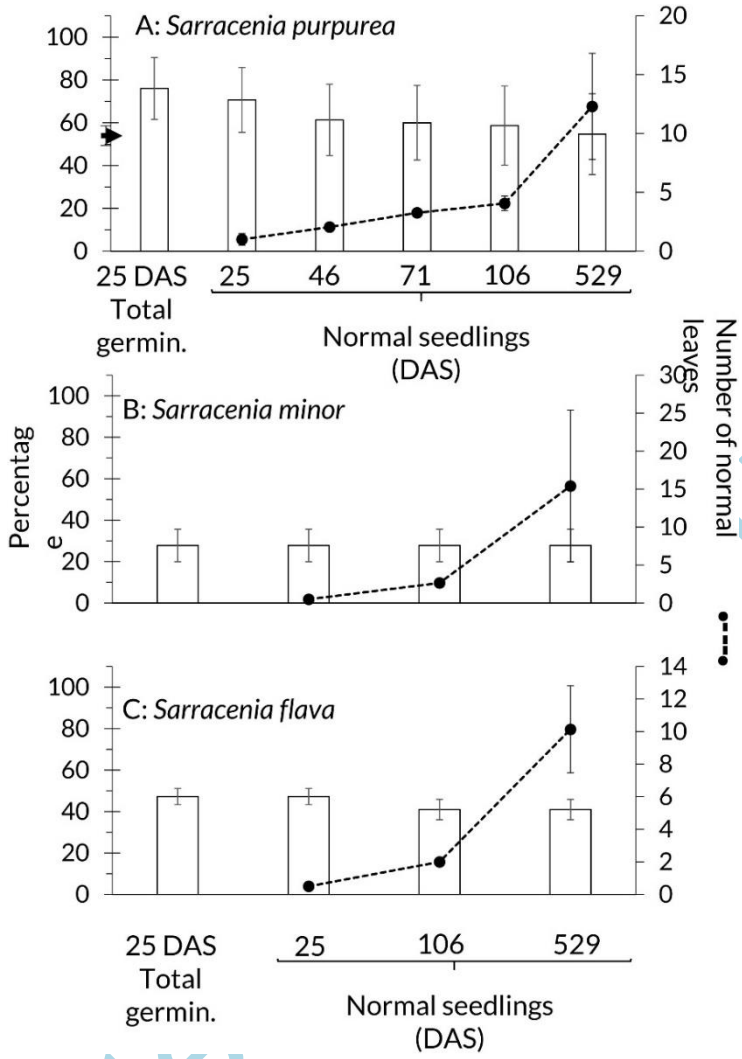

Figure 6

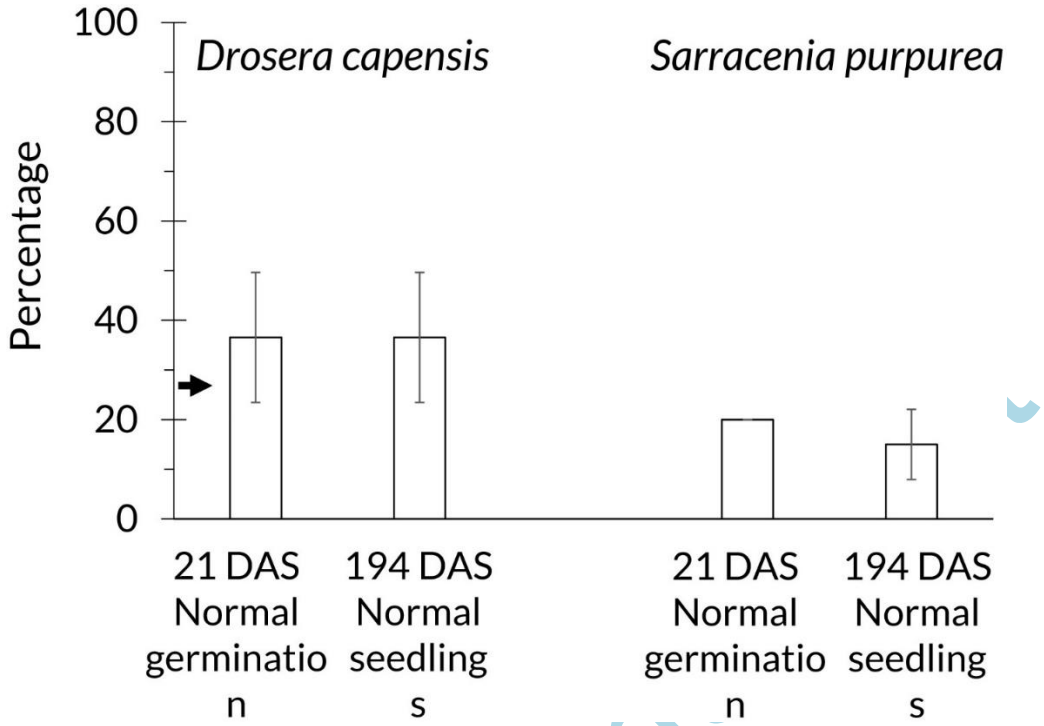

Figure 7