

Orchid seeds are not always short lived in a conventional seed bank!

Ana Maria Francisqueti¹, Rafael Rubio Marin¹, Mariane Marangoni Hengling¹, Silvério Takao Hosomi¹,
Hugh W. Pritchard^{2,3}, Ceci Castilho Custódio¹ and Nelson Barbosa Machado-Neto^{1,*}

¹Unoeste Campus II, Agronomy College, Building 2, room 201, Rodovia Raposo Tavares, km 572. Limoeiro, Presidente Prudente SP, 19067-175, Brazil, ²Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650201, PR China and

³Royal Botanic Gardens, Kew, Wakehurst, Ardingly, West Sussex RH17 6TN, UK

*For correspondence. E-mail nbmneto@unoeste.br

Received: 9 October 2023 Returned for revision: 22 January 2024 Editorial decision: 6 February 2024 Accepted: 9 February 2024

- **Background and Aims:** Orchid seeds are reputed to be short lived in dry, cold storage conditions, potentially limiting the use of conventional seed banks for long-term *ex situ* conservation. This work explores whether *Cattleya* seeds are long lived or not during conventional storage (predried to ~12 % relative humidity, then stored at –18 °C).
- **Methods:** We explored the possible interaction of factors influencing seed lifespan in eight species of the genus *Cattleya* using physiological (germination and vigour), biochemical (gas chromatography), biophysical (differential scanning calorimetry) and morphometric methods. Seeds were desiccated to ~3 % moisture content and stored at –18 °C for more than a decade, and seed quality was measured via three *in vitro* germination techniques. Tetrazolium staining was also used to monitor seed viability during storage. The morphometric and germination data were subjected to ANOVA and cluster analysis, and seed lifespan was subjected to probit analysis.
- **Key Results:** Seeds of all *Cattleya* species were found to be desiccation tolerant, with predicted storage lifespans (P_{50y}) of ~30 years for six species and much longer for two species. Cluster analysis showed that the three species with the longest-lived seeds had smaller (9–11 %) airspaces around the embryo. The post-storage germination method impacted the quality assessment; seeds equilibrated at room temperature for 24 h or in 10 % sucrose solution had improved germination, particularly for the seeds with the smallest embryos. Chromatography revealed that the seeds of all eight species were rich in linoleic acid, and differential scanning calorimetry identified a peak that might be auxiliary to selecting long-lived seeds.
- **Conclusions:** These findings show that not all orchids produce seeds that are short lived, and our trait analyses might help to strengthen prediction of seed longevity in diverse orchid species.

Key words: Cryobiotechnology, differential scanning calorimetry, fatty acid composition, orchid conservation, seed lifespan, seed longevity.

INTRODUCTION

Seed banking is a very effective methodology for storing and conserving plant genetic resources (Walters and Pence, 2021). Commonly used for crop species and their relatives, the techniques are well documented and established for many species, including wild species (Nagel and Börner, 2010; Castañeda-Álvarez *et al.*, 2016; Colville and Pritchard, 2019). The suitability of orchid species for *ex situ* seed storage and conservation has been debated for many years, because storability is known to vary with species (Shoushtari *et al.*, 1994). Contrasting findings during storage span refrigerator conditions (Machado-Neto and Custódio, 2005), –10 °C for *Cattleya loddigessi* and *C. coccinea* (syn. *Sophranitis coccinea*) (Bowling and Thompson, 1972), –18 °C or lower temperatures for *Disa uniflora* (Thornhill and Koopowitz, 1992), and down to liquid nitrogen at –196 °C (Pritchard, 1984; Popova *et al.*, 2016; Fileti *et al.*, 2021). (Note that abbreviations of scientific names follow the rules of the Royal Horticultural Society for orchid genera and hybrids.) Moreover, seeds have been predried by different

methods and to varying moisture contents, e.g. over calcium chloride, silica gel and lithium chloride, making the development of reliable predictive tools for orchid seed storage difficult. The problem is compounded by the use of various *in vitro* germination tests, necessary for most orchids (Prasongsom *et al.*, 2022), that can impact the assessment of seed quality. Because the small seeds of orchids have a large surface area-to-volume ratio and must be disinfected before sowing on an *in vitro* germination medium, there is a risk of imbibition damage to the dry seeds (Pritchard *et al.*, 1999). Additionally, the medium used for germination might be suboptimal for the stored seed, which might be evident only over a relatively long period of storage as seed vigour and viability fall and within the individual germination test that can vary from <1 month (Hosomi *et al.*, 2011, 2012) to >1 year (Rasmussen *et al.*, 2015), depending on the species and culture conditions (Diantina *et al.*, 2020). Media must also be devised to support the conversion of seeds into seedlings post-storage. Alternatively, post-storage seed survival can be assessed in ~1 day using the vital stain triphenyl tetrazolium chloride (Hosomi *et al.*, 2011, 2012).

Different sensitivities to these potential confounding factors could have contributed to the regular perception that orchids produce short-lived seeds.

Seeds tend to fit three broad classes of storage responses: orthodox seeds tolerate drying to $<0.12 \text{ g H}_2\text{O g}^{-1}$ dry matter (DM) and storage at -20°C ; intermediate seeds tolerate partial drying to $0.1\text{--}0.15 \text{ g H}_2\text{O g}^{-1}$ DM and tend to be short lived, particularly at -20°C ; and recalcitrant seeds are sensitive to drying in the region $1\text{--}0.25 \text{ g H}_2\text{O g}^{-1}$ DM, requiring tissue cryopreservation for long-term storage (Walters, 2015; Nadarajan et al., 2023). Furthermore, fully desiccation-tolerant, apparently orthodox seeds have vastly different lifespans (Walters et al., 2005; Colville and Pritchard, 2019; Mira et al., 2019). Longevity can be compromised by a negative interaction between lipid transformation, particularly crystallization, and sub-zero storage temperature. This risk has been inferred from differential scanning calorimetry (DSC) thermograms, for orchids (Pritchard and Seaton, 1993), *Cuphea* sp. (Crane et al., 2003, 2006) and brassicas (Mira et al., 2019), supporting the notion that there might be species-specific sub-zero storage temperature optima for oilseeds (Pritchard, 2004). A determinant of such behaviour could be the seed fatty acid (FA) composition, which drives lipid thermal transformation (Crane et al., 2003, 2006; Mira et al., 2019). Variability in lipid composition is evident amongst seeds of *Cattleya* species; for instance, *C. kautskyana*, *C. tigrina* and *C. amethystoglossa* contain four to seven main FAs (Fileti et al., 2021) and *C. crispata*, *C. rupestris*, *C. tenebrosa*, *C. loddigesii*, *C. intermedia*, *C. schilleriana* and *C. warneri* contain five to seven main FAs (Hengling et al., 2020). Similar variation has been observed in seeds of five other species: *Dendrobium strebloceras*, *Den. cunninghamii*, *Gastrodia cunninghamii*, *Pterostylis banksii* and *Thelymitra nervosa* (Diantina et al., 2022). Although ageing can trigger decreases in orchid seed saturated, polyunsaturated, mono-unsaturated and total FA content, composition alone appears not to be an adequate marker for seed longevity in orchids (Diantina et al., 2022).

The known variability in dry seed storage lifespan in orchids remains a concern for conservationists. The seeds of a wide range of species tend to be relatively short lived in various ageing conditions [*Guarianthe aurantiaca* (Seaton and Pritchard, 1999; Pritchard and Dickie, 2003); *Caladenia arenicola*, *Calda. flava*, *Calda. huegelii*, *Diuris laxiflora*, *Microtis media* ssp. *media*, *Pterostylis recurva*, *Ptst. sanguinea*, *Thelymitra crinita*, *Thel. macrophylla* and *Diuris fragrantissima*; (Hay et al., 2010); *Calda. flava*, *Diuris laxiflora*, *Microtis media*, *Ptst. recurva* and *Thel. crinita* (Merritt et al., 2014a); *Cattleya schilleriana*, *C. tigrina*, *C. crispata* and *C. labiata* (Fileti et al., 2015); *Cattleya amethystoglossa*, *C. brevicaulis*, *C. briegei*, *C. crispata*, *C. duvenii*, *C. endsfeldzii*, *C. milleri*, *C. nobilior*, *C. purpurata*, *C. rupestris*, *C. sanguiloba*, *C. tenebrosa*, *C. tigrina*, *C. forbesii*, *Constatia cristinae*, *Oncidium isthmi*, *Onc. hastatum*, *Phalaenopsis* sp., *Trichocentrum pumilum*, *Vanda curvifolia*, *V. tessellata* and *V. testacea* (Machado-Neto and Custódio, 2005); *Bifrenaria inodora*, *Cattleya bicolor*, *C. intermedia*, *C. purpurata*, *Encyclia pygmaea*, *Enc. odorantissima*, *Epidendrum fulgens*, *Grobya* sp., *Maxillaria picta*, *Gomesa enderianum*, *Gom. flexuosum*, *Trt. Pumilum* and *Acianthera glumacea* (Alvarez-Pardo and Ferreira, 2006); *Coelogyne asperata*, *Coel.*

foerstermannii, *Coel. pandurata*, *Coel. rumphii*, *Dendrobium macrophyllum*, *Den. mirbelianum*, *Den. Stratiotes* and *Xylobium undulatum* (Seaton et al., 2013)]. Should all orchids be assigned to exceptionality factor 3 of Exceptional Species *sensu* Pence et al. (2022)? That is, the seeds may be desiccation tolerant but cannot survive ‘long term’ at -20°C . Moreover, how do orchids fit into a new conceptual framework for longevity that has evolved over the last few decades (Nadarajan et al., 2023)? For example, seeds of *Cattleya trianaei* have been described as orthodox (Pritchard and Seaton, 1993) or intermediate (Shoushtari et al., 1994). *Cattleya crispata*, formerly classified as *Laelia flava*, is reputed not to survive in storage (Hengling et al., 2020) or to be orthodox (Machado-Neto and Custódio, 2005). *Cattleya brevicaulis*, *C. forbesii*, *C. tigrina*, *C. rupestris* and *C. sanguiloba* (syn. *C. cinnabarina*) were all considered to be short lived in the same work (Machado-Neto and Custódio, 2005).

Multi-species comparisons in seed longevity are relatively rare in orchids. Seed storage lifespan in *Cattleya* species has previously been assessed for *C. tenebrosa*, *C. warneri*, *C. intermedia*, *C. schilleriana*, *C. loddigesii*, *C. crispata* and *C. rupestris* by Hengling et al. (2020) and *C. amethystoglossa*, *C. tigrina* and *C. brevicaulis* by Fileti et al. (2021). Moreover, we have recently observed that interspecies variability in *Cattleya* species seed survival can be associated with differences in seed morphology, even in such small seeds (Fileti et al., 2021). Thus, *Cattleya* as a genus appears to be a good model to explore both the mechanistic drivers for seed ageing in orchids and to consider practical conservation interventions. In this study, we determined the long-term (i.e. >10 years) dry seed storage potential at -18°C for eight species of *Cattleya* and explored the potential interactions with various seed traits (as determined through morphometry, biochemistry and biophysics). Our main hypothesis was that orchid seeds are always short lived.

MATERIALS AND METHODS

Seed storage

Seeds of eight *Cattleya* species (*C. alvaroana*, *C. briegei*, *C. crispata*, *C. harrisoniana*, *C. hegeriana*, *C. granulosa*, *C. rupestris* and *C. sanguiloba*; Table 1) were obtained from the Aurora Orchid House in Taciba, São Paulo estate, from controlled cross-pollination with pollen from plants of different origins. Fruits were harvested at the first signs of maturity and stored in fine paper envelopes at 45 % relative humidity (RH) and $22 \pm 3^\circ\text{C}$ until complete dehiscence. The dispersed seeds were cleaned, and the seeds from different capsules of the same species were pooled and split into subsamples, placed in open 2 mL cryogenic microtubes and equilibrated over a saturated lithium chloride solution at 20°C at $\sim 12\%$ RH (Hay et al., 2008). To check the moisture content (MC), three samples from each seed lot were weighed in aluminium pans and placed in an oven at $105 \pm 3^\circ\text{C}$ for 24 h. Subsequently, the pans were placed over silica gel, left to cool, and reweighed (BRASIL, 2009). The MC was calculated on a wet basis (MCwb). Seeds reached 3–4 % MCwb, except for *C. hegeriana* (7 %), and were placed into separate 2 mL cryovials, sealed with a silicon ringed cap and stored at

TABLE 1. *Species, origin, growth habit and conservation status of the used Cattleya species.*

Species	Biome	Places of origin	Growth habit	Conservation status
<i>C. alvaroana</i>	Atlantic Rainforest over rocks	RJ	Rupicolous	NE
<i>C. briergeri</i>	Savannah, Atlantic Rainforest	MG	Rupicolous	EN
<i>C. crispata</i>	Savannah	MG	Rupicolous	NT
<i>C. granulosa</i>	Seasonal semi-deciduous forest, Restinga	AL, BA, PB, PE, RN, ES	Epiphytic, terrestrial	VU
<i>C. harrisoniana</i>	Riparian Forest, Atlantic Rainforest	ES, MG, RJ and SP	Epiphytic	VU
<i>C. hegeriana</i>	Atlantic Rainforest	MG, RJ	Rupicolous	LC
<i>C. rupestris</i>	Savannah	MG	Rupicolous	NE
<i>C. sanguiloba</i>	Savannah	MG	Rupicolous	LC

Brazilian estates: AL, Alagoas; BA, Bahia; ES, Espírito Santo; MG, Minas Gerais; PB, Paraíba; PE, Pernambuco RJ, Rio de Janeiro; RN, Rio Grande do Norte; SP, São Paulo. Conservation status: EN, Endangered; LC, Least concern; NE, not evaluated; NT, Near Threatened; VU, vulnerable (CNCFlora2020).

–18 °C. Samples were maintained in tight vials inside large vials containing silica gel orange sachets equilibrated to the same eRH (equilibrium Relative Humidity). These vials were placed in a box containing white silica gel mixed with orange indicator silica gel to enable monitoring of hermetic sealing over the storage evaluation period. Seed longevity was determined as in the studies by Newton *et al.* (2014) and Hengling *et al.* (2020), applying probit analysis to the seed survival curves based on at least four sampling times (i.e. at the establishment of the UNOESTE Orchid Seed Bank more than a decade ago and in 2017, 2020 and 2022).

Germination and viability testing

Seed disinfection was performed according to Hosomi *et al.* (2011), and sowing in Petri dishes was on a half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 30 g L⁻¹ sucrose and 6 g L⁻¹ agar. Before autoclaving, the pH was adjusted to 5.6 (with 0.1 M NaOH). Seed germination dishes were kept in a growth room at 25 ± 3 °C with a 16 h light photoperiod (511 µE m⁻² s⁻¹).

Over the storage experiment, seeds were quality assessed using three different methods: (1) at the beginning, the original technique (OT) of germination was to remove the seed container from storage and keep it at room temperature for ≥1 h; after we made two modifications, seed containers were removed from storage, left for 24 h at room temperature (25 ± 2 °C) and then either handled (2) without sucrose pretreatment (WoS) or (3) given a 24 h sucrose pretreatment (coded as WS), plus washing twice with distilled water as part of the preparation for the germination test. After these handling treatments (1, 2 and 3), the seeds were disinfected and sown *in vitro*.

The seeds were considered to have germinated when the embryonic mass was green and expanded to the point at which the testa was clearly breached, i.e. stage 1, spherical protocorm (Seaton and Ramsay, 2005; Prasongsom *et al.*, 2022). The germinated seeds were counted weekly until germination was stable. The seeds were evaluated in three marked fields to include ≥100 full seeds (embryo present and excluding empty testae) per field. Images were captured with a Sony DSC-P10 (Sony Electronics Inc., Japan) digital camera manually coupled to a stereomicroscope eyepiece lens. Germination (G) was

expressed as a percentage of the number of filled seeds per marked field, with three determinations for each of the three methods at each sampling time.

Viability was evaluated by a tetrazolium (Tz; 2,3,5-triphenyl tetrazolium chloride) test as described by Hosomi *et al.* (2011, 2012). Three repetitions were made for each species and at each sampling time.

Transesterification of fatty acids and gas chromatography

Fatty acid extraction and transesterification followed the method of Colville *et al.* (2016). The FA methyl esters were analysed by gas chromatography (Shimadzu GC QP2010, Shimadzu Corporation Global, Kyoto, Japan) with a flame ionization detector in an NST23 capillary column (Nano Separation Technologies, São Carlos, Brazil) with a length of 60 m, an internal diameter of 0.25 µm and film thickness of 0.20 µm. Nitrogen was used as the carrier gas at a flow rate of 0.6 mL min⁻¹. The temperature programme followed that of Colville *et al.* (2016). Two samples of each species were made. Fatty acids were separated into saturated FA (SFA), mono-unsaturated FA (MUFA) and polyunsaturated FA (PUFA). The MUFAs and PUFAs were used to calculate the double bond index (DBI) and peroxidation index (PI) according to Pamplona *et al.* (1998).

Thermal analysis

Seeds were placed in 34 µL aluminium pans, crimp-sealed and weighed on a UMX2 Ultra-Microbalance (Mettler Toledo; ±0.1 µg) to determine the precise quantity of the sample and submitted to thermal analysis using a differential scanning calorimeter (Shimadzu DSC-60 analyser). The cooling and warming cycle sequence was as follows: cooling to –80 °C at 10 °C min⁻¹; holding for 1 min at –80 °C; warming from –80 °C to 75 °C at 10 °C min⁻¹; holding for 1 min; then cooling down to room temperature. Warming thermograms and melting peaks were measured in ORIGINPro® 2019b software (OriginLab Corporation, Northampton, MA, USA). Representative thermograms are presented based on three independent samples.

Morphometric analysis

Morphometric analysis was performed according to Fileti *et al.* (2021), measuring 40 seeds in each seed lot.

Statistical analysis

The percentages of germination (G) and viability (Tz) were transformed with $y = \arcsin(x/100)^{1/2}$ for normalization. The data were expressed as the mean values, and significant differences in means were compared with Tukey's test ($P \leq 0.05$). The experiment was a factorial 8×3 (eight species $\times$ three conditions of sowing) and conducted as completely randomized with three repetitions each, with ≥ 200 seeds. These data were used to calculate seed conservation parameters for each species and for ANOVA. Probit regressions of the seed-ageing data (σ and P_{50y}) were calculated with GLM (General Linear Model) and fitted with ORIGINPro® 2019b software (Origin LAB Corporation, Northampton, MA, USA). All analyses were performed with SISVAR software (Ferreira, 2011).

Morphometric, principal FAs volumetric relationships between seed and embryo and P_{50y} data were submitted to correlation analysis and cluster analysis using PAST software v.4 (Hammer *et al.*, 2001).

RESULTS

Morphometric analyses

Based on their dimensions and volumes, all *Cattleya* species studied produced micro-seeds (Table 2). In comparison to the six rupicolous species, *C. granulosa* and *C. harrisoniana* produced smaller seeds ($\sim 350 \mu\text{m} \times 170 \mu\text{m}$) and embryos ($\sim 76 \mu\text{m}$ in diameter) (Table 2). Overall, *C. rupestris* had the significantly longest and widest seed. Embryo diameters separated the species into two groups of four, with diameters of 76–97 and 108–120 μm . Embryo volumes ranged 17-fold, from 0.17 to $2.97 \times 10^{-3} \text{ mm}^3$ for *C. harrisoniana* and *C. briergeri*, respectively (Table 2). All seeds had relatively large embryos in comparison to the whole seed, with air spaces

varying from only 9 % (*C. granulosa*) to 27 % (*C. crispata*) of seed volume.

Germination, viability and seed longevity parameters

Seed storage moisture contents were ~ 0.03 – $0.04 \text{ g H}_2\text{O g}^{-1} \text{ DW}$, with only *C. hegeriana* seed being higher ($0.06 \text{ g H}_2\text{O g}^{-1} \text{ DW}$). The germination level was influenced, in most cases, by the post-storage treatment, e.g. being kept for 24 h at room temperature or this first step followed by sucrose imbibition for another 24 h (Table 3). For three of the species, the resulting germination levels were statistically different. Although for the other five species the results of such treatments were not different, there was a tendency for increment. For *C. briergeri*, *C. granulosa*, *C. harrisoniana* and *C. sanguiloba*, the treatment with sucrose was the most effective at increasing germination post-storage. Similar letters, in the columns, are not statistically different by the Tukey's test ($P < 0.05$).

Amongst the species, germination after various storage times was strongly correlated ($R^2 = 0.829$) with Tz viability (Fig. 1), meaning that the Tz test can be a reliable predictor of germination for the studied species.

Thermal and fatty acid analyses

Thermal fingerprints for the seeds of the eight species were similar based on the DSC warming thermograms, with pronounced melting events in seven of the eight species (Fig. 2). In contrast, *C. granulosa* seeds generated a weaker signal in the DSC (Fig. 2A). For the seven species, four main peaks were evident: centred on -45 , -30 and -18 °C and finally at 5 – 15 °C. For *C. granulosa*, no peak was evident at -45 °C, but peaks were observed at -25 , -10 and 25 °C. These complex melting transitions suggest a variability in FA acid composition in the seeds (Fig. 2; Supplementary Data Table S1).

The gas chromatography analysis of the seeds revealed large differences in FAs amongst the species (Supplementary Data Table S2). The major FA components were palmitic acid (16C:0) and linoleic acid (18C:2; Supplementary Data Table S2), which varied among species from 14 to 32 % and from

TABLE 2. Seed morphovolumetric characteristics of eight *Cattleya* species. Non-rupicolous species are shown in bold text.

Species	Dimensions (μm ; mean $\pm$ s.e.m.)			Volume ($\times 10^{-3} \text{ mm}^3$; mean $\pm$ s.e.m.)			Air space (% of seed)
	Seed length	Seed width	Embryo diameter	Embryo (prolapsed spheroid)	Seed	Air	
<i>C. alvaroana</i>	602 $\pm$ 2.0 ^c	248 $\pm$ 0.9 ^c	108 $\pm$ 0.4 ^{ab}	1.54 $\pm$ 0.14 ^{bc}	1.90 $\pm$ 0.15 ^{bc}	0.32 $\pm$ 0.05 ^{bc}	17.7 $\pm$ 2.2 ^{bc}
<i>C. briergeri</i>	779 $\pm$ 2.8 ^b	326 $\pm$ 1.6 ^a	120 $\pm$ 0.5 ^a	2.52 $\pm$ 0.5 ^a	2.97 $\pm$ 0.25 ^a	0.45 $\pm$ 0.07 ^{ab}	15.7 $\pm$ 0.1 ^{bc}
<i>C. crispata</i>	637 $\pm$ 2.2 ^c	244 $\pm$ 1.1 ^c	90 $\pm$ 0.3 ^{cd}	0.99 $\pm$ 0.08 ^{cd}	1.39 $\pm$ 0.11 ^c	0.39 $\pm$ 0.06 ^{ab}	27.3 $\pm$ 2.4 ^a
<i>C. granulosa</i>	357 $\pm$ 1.1 ^d	172 $\pm$ 0.5 ^d	76 $\pm$ 0.3 ^d	0.53 $\pm$ 0.05 ^d	0.58 $\pm$ 0.06 ^d	0.06 $\pm$ 0.01 ^d	9.4 $\pm$ 1.5 ^c
<i>C. harrisoniana</i>	348 $\pm$ 0.9 ^d	164 $\pm$ 0.5 ^d	77 $\pm$ 0.3 ^d	0.17 $\pm$ 0.04 ^d	0.49 $\pm$ 0.04 ^d	0.07 $\pm$ 0.01 ^d	10.4 $\pm$ 2.0 ^c
<i>C. hegeriana</i>	642 $\pm$ 2.8 ^c	264 $\pm$ 0.9 ^{bc}	118 $\pm$ 0.4 ^a	1.94 $\pm$ 0.15 ^{ab}	2.38 $\pm$ 0.21 ^{ab}	0.44 $\pm$ 0.09 ^{ab}	18.9 $\pm$ 2.6 ^{abc}
<i>C. rupestris</i>	874 $\pm$ 2.9 ^a	340 $\pm$ 1.3 ^a	110 $\pm$ 0.4 ^{ab}	2.18 $\pm$ 0.22 ^{ab}	2.82 $\pm$ 0.23 ^a	0.61 $\pm$ 0.01 ^a	21.5 $\pm$ 2.9 ^{ab}
<i>C. sanguiloba</i>	667 $\pm$ 1.9 ^c	297 $\pm$ 0.9 ^{ab}	97 $\pm$ 0.5 ^{bc}	1.59 $\pm$ 0.14 ^{bc}	1.75 $\pm$ 0.16 ^{bc}	0.22 $\pm$ 0.06 ^{bc}	10.9 $\pm$ 2.5 ^c
MSD (minimum significant difference) (5 %)	9.28	4.19	0.389	0.063	0.016	0.006	2.22

TABLE 3. Longevity parameters (σ and P_{50y}) of seeds from eight *Cattleya* species stored for ≥ 13 years at -18°C and low moisture content (MC). Viability (as a percentage) is for the tetrazolium (Tz) test. Initial germination is shown as determined (as a percentage) and estimated (Ki in probits). Final germinations (as a percentage and probit value) obtained with three germination methods are also shown. Total storage times varied from 4963 days (13.6 years) to 5222 days (14.3 years). Four time points were included in the probit analysis of the longevity parameters. Non-rupicolous species are shown in bold text.

Species (days in storage)	Germination treatment ^a	MC (g H ₂ O) g ⁻¹ FW)	Initial germination (%)	Estimated Ki	Final Tz (%)	Final germination (%)	Final probit	σ (years)	P_{50y} (years)
<i>C. alvaroana</i> (5187)	WS	0.026 $\pm$ 0.005	100	8.09	82.6 $\pm$ 0.97	92.2 aA	6.43 aCD	9.6 aBC	38.9 aBC
	WoS					91.1 aAB	6.47 aC	9.0 aA	36.5 aA
	OT					88.0 aAB	6.20 aBC	8.5 aA	34.4 aA
<i>C. briergeri</i> (5187)	WS	0.033 $\pm$ 0.005	100	8.09	80.1 $\pm$ 2.39	93.3 aA	6.57 aBC	6.4 aD	25.7 aD
	WoS					87.1 aB	6.15 aCD	6.8 aA	27.3 aA
	OT					65.0 bD	5.40 bDE	6.5 aA	26.3 aA
<i>C. crispata</i> (4963)	WS	0.027 $\pm$ 0.001	99	7.33	85.0 $\pm$ 0.98	91.0 aA	6.43 aCD	8.1 aD	29.5 aD
	WoS					94.0 aAB	6.47 aBC	7.1 aA	26.0 aA
	OT					87.0 aAB	6.20 aC	7.1 aA	26.1 aA
<i>C. granulosa</i> (5199)	WS	0.041 $\pm$ 0.002	99	7.33	90.6 $\pm$ 0.61	96.3 aA	6.83 aABC	22.4 aAB	82.1 aAB
	WoS					70.2 bC	5.53 bE	8.4 bA	30.9 bA
	OT					47.8 cC	4.94 cE	5.9 bA	21.7 bA
<i>C. harrisoniana</i> (5185)	WS	0.035 $\pm$ 0.001	99	7.33	93.8 $\pm$ 0.25	98.2 aA	7.05 aAB	30.1 aA	110.3 aA
	WoS					89.8 bAB	6.29 bC	16.6 bA	60.9 bA
	OT					80.0 cBC	6.03 bC	9.8 bA	36.0 bA
<i>C. hegeriana</i> (5222)	WS	0.068 $\pm$ 0.002	100	8.09	90.0 $\pm$ 0.44	99.2 aA	7.33 aA	7.4 aD	29.8 aD
	WoS					97.6 aA	7.09 abAB	7.3 aA	29.6 aA
	OT					96.0 aA	6.85 bA	6.3 aA	25.3 aA
<i>C. rupestris</i> (4963)	WS	0.030 $\pm$ 0.003	100	8.09	93.7 $\pm$ 0.13	98.6 aA	7.14 aAB	6.5 aD	26.3 aD
	WoS					99.0 aA	7.33 abA	5.5 aA	22.3 aA
	OT					95.0 aA	6.73 bAB	4.8 aA	19.3 aA
<i>C. sanguiloba</i> (5222)	WS	0.028 $\pm$ 0.002	99	7.33	73.2 $\pm$ 0.45	79.7 aB	5.87 aD	13.2 aBC	48.4 aBC
	WoS					71.9 bC	5.57 aDE	9.2 aA	33.8 aA
	OT					75.7 abC	5.70 aCD	8.9 aA	32.7 aA

^aGermination method: OT, original technique; WoS, without sucrose pretreatment; WS, with 24 h sucrose pretreatment.

Significant differences between species are indicated by: uppercase italics in the columns for WS, uppercase bold in the columns for WoS, and normal uppercase in the columns for OW. Significant differences amongst treatments for each species are denoted by lowercase letters.

51 to 79 %, respectively, of the total amount of FAs. Two species, *C. crispata* and *C. rupestris*, had a similar amount of stearic acid (18C:0). Linolenic acid (18C:3) in high amounts (>3 %) was found only in *C. granulosa*. Oleic acid (18C:1) was 4–8 % in all species, being highest in *C. crispata* and lowest in *C. hegeriana*. The seed UFA:SFA ratio among species varied from 1.7 (*C. granulosa*) to 5.7 (*C. harrisoniana*). Consequently, PI, DBI and PUFA parameters were the lowest in *C. granulosa* and the highest in *C. harrisoniana* (Supplementary Data Table S2).

The amounts of FA also varied among species from 12 to 34 % of the seed weight. When these values were normalized to 100 % filled seed, the values increased to 28–51 % (Table 4). *Cattleya granulosa* exhibited the lowest level of filled seeds (41 %), which might be responsible for the weak signal in the DSC thermograms (Fig. 2).

Co-correlants of seed biochemistry, morphology and storage physiology

The grouping analysis of the morphovolumetric variables, some longevity parameters and principal FAs (Fig. 3) showed that the species were grouped according to taxonomic affiliation by the seed dimensions, linoleic acid content, which also influences PI, or lifespan (P_{50y} , Table 3). Palmitic acid or moisture content also influenced the grouping of species, but with less strength. The bifoliate *Cattleya* of the subgenus *Intermediae* (Berg, 2014), i.e. *C. harrisoniana* and *C. granulosa*, are together in one cluster and the other six species, from series *Parviflorae* (Berg, 2014), in another cluster.

Seed length and width have many correlations with the other variables and a significant strong positive correlation between them and embryo diameter. This means that longer seeds are wider seeds and have bigger embryos. These three variables

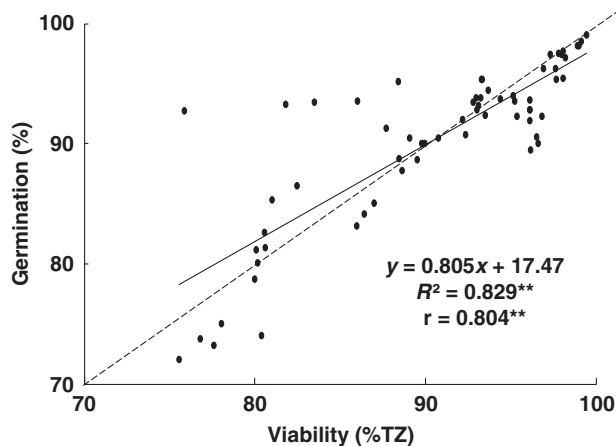


FIG. 1. Correlation between germination with sucrose pretreatment and tetrazolium test of eight stored *Cattleya* seeds. The null line is dashed. ** $P < 0.01$.

had a significant strong negative correlation with the conservation parameter P_{50y} , e.g. the bigger the seed, the worse their conservation for these species. Palmitic acid (C16:0) content had significant negative correlations with linoleic acid (C18:2) content and PI and DBI (Table 5). Oleic acid (C18:1) content exhibited a significant negative correlation with P_{50y} , meaning that seeds with higher contents of oleic acid potentially preserve comparatively less well. The linoleic acid (C18:2) content was strongly correlated significantly with PI and DBI. The PI and DBI had a significant strong negative correlation with palmitic acid (C16:0) and a significant stronger one with linoleic acid (C18:2).

DISCUSSION

A stochastic model for the loss of cell viability over time means that small orchid seeds with relatively undifferentiated and few cells in the embryo are potentially at a disadvantage for lifespan. Amongst *Dendrobium* and other orchid species, seed and embryo dimensions are known to vary, but this might not translate into differences in the comparative rate of decay of the seeds, with all being fairly short lived (Diantina et al., 2020, 2022). In contrast, across seeds of three *Cattleya* species, the one with the biggest seeds, *C. kautskyana*, another species belonging to the series *Parviflorae* (Berg, 2014), had the greater longevity (Fileti et al., 2021). Also, amongst orchid species there was a weak correlation between relative air space and storability (Diantina et al., 2022). These earlier studies used ageing in natural or accelerated conditions, whereas our study compared longevity over the long term (>10 years). We found a significant negative correlation ($r = -0.745$, $P < 0.01$) between relative air space (as a percentage) around the embryo and longevity, such that seeds of *C. granulosa* and *C. harrisoniana* with less air space stored relatively well. However, *C. rupestris* and *C. hegeriana* with higher air space also had good preservation potential, indicating that air space alone is not the determinant of orchid seed longevity. One possibility is that differences in gas permeability into the embryo could also be a modulator of the seed longevity response (Groot et al., 2015).

The seed longevity parameters determined here for eight species of *Cattleya* indicate that the seeds are not short lived,

and these species are not likely to be exceptional, as defined as having a seed P_{50y} of <20 years in conventional seed bank conditions (Walters, 2015; Pence et al., 2022). Such longevity achieved here for *Cattleya* seeds might reflect the optimization of steps in post-harvest handling. Nonetheless, it is difficult to predict the full longevity potential of the stored seeds because 70 % of the germination estimates (i.e. 17 of 24) yielded values of >85 % after 13 years. In seed population ageing studies, this level of viability is considered to be a critical node (Zhou et al., 2022), below which the loss of viability is relatively faster (as a percentage per year). Also, when most of the data analysed in longevity studies are above this value, it is not easy to derive reliable estimates of longevity, or to forecast regeneration requirements (Hay et al., 2021). Thus, regular monitoring of orchid seed longevity is recommended at intervals shorter than the 10-year assessment that is often used in seed bank accession monitoring (Hay et al., 2021).

Both temperature and moisture content are recognized as the main drivers of seed longevity (Roberts and Ellis, 1977; Ellis and Hong, 2007), yet our understanding of what is dry for orchid seeds is incomplete. The relationship between seed moisture content and relative humidity is such that the isotherm shifts to lower equilibrium moisture content (eMC) as oil content increases (Hay et al., 2022). Orthodox (desiccation-tolerant) seed storage guidelines recommend drying in conditions that equilibrate the seeds to 3–7 % moisture content (FAO, 2013). For seven of the species studied here, eMC values were ~3–4 %, which could be expected because the determined seed oil contents are in the range of 28–51 % once empty seeds are accounted for (Tables 3 and 4). The exception is *C. hegeriana* with ~7 % eMC, although the seed oil content is similar to the other species studied. Such a high eMC suggests that the oil content in this seed should be much lower, and we do not yet know the reason for this apparent anomaly. The presence of oil in these seeds is also confirmed by the main melting peaks at –30 and –15 °C in the thermal fingerprint (Fig. 3).

For any oil-rich seed there is a risk of falling germination/viability over time from lipid peroxidation as the first step in a cascade of events that can lead to the formation of several by-products (Zinsmeister et al., 2020). Oxidation occurs preferentially from PUFA to MUFA to SFA. Although some Brassicaceae could be stored successfully in the dry, cold state for >40 years, *Sinapis arvensis* with high oleic acid (19.9 %) stored less well (Mira et al., 2019). In the case of orchids seeds, linoleic acid is often the most common FA present, with low concentrations of linolenic acid and oleic acid (Hengling et al., 2020; Fileti et al., 2021; Diantina et al., 2022). Although seeds with higher levels of unsaturated lipids might be expected to undergo peroxidation the fastest, we found that a relatively high oleic acid content was associated with shorter seed longevity (P_{50y} , $r = -0.837$, $P < 0.05$) and positively correlated with seed air space ($r = 0.837$, $P < 0.01$), which was also negatively correlated with P_{50y} ($R^2 = -0.745$, $P < 0.01$). This is not to suggest that peroxidation of unsaturated fats is unimportant for orchid seed survival. Although the specific mix of FAs is not a reliable marker for the relative rate of ageing, peroxidation is a common feature of decreasing seed viability in a range of orchid species (Diantina et al., 2022).

The accumulation of FAs in seeds is a highly efficient means of energy storage, especially in small seeds. Species that have

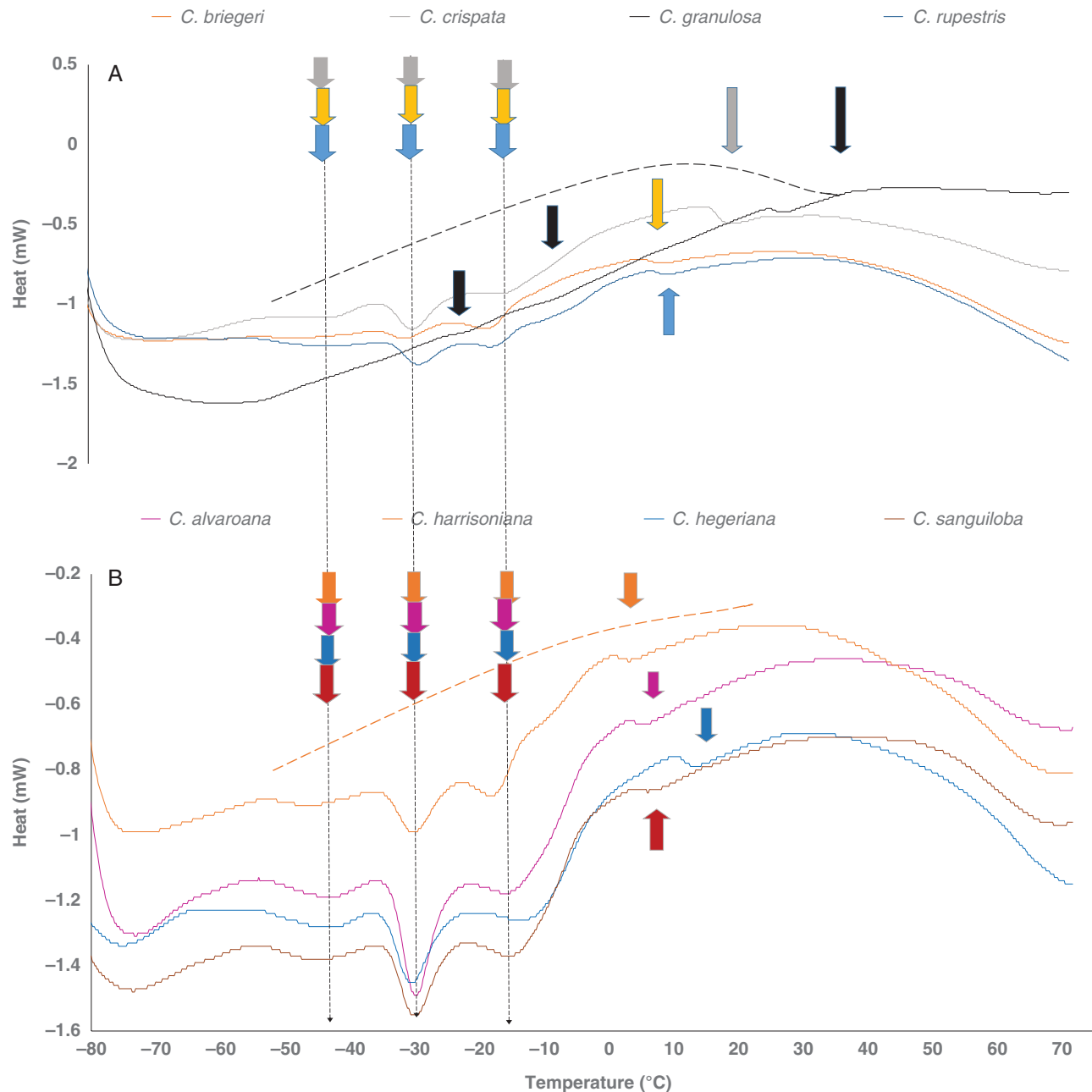


FIG. 2. Warming differential scanning calorimetry curves of stored seeds from eight *Cattleya* species. (A) *Cattleya briegei*, *C. crispata*, *C. granulosa* and *C. rupestris*. (B) *Cattleya alvaroana*, *C. harrisoniana*, *C. hegeriana* and *C. sanguiloba*. Each coloured band is a signal of lipid melting. The scanning baseline is shown as a dashed line for *C. crispata* in A and *C. harrisoniana* in B.

evolved in hot environments in the tropics tend to store relatively less unsaturated FAs than temperate species. Unsaturated fats have lower freezing (crystallization) temperature, and the plants and seeds potentially reduce the risk of freezing stress at any time of their life cycle. In contrast, seeds with more saturated lipids will have higher melting temperatures. This applies to the cell membranes, including the half-unit membrane around the oil bodies, and the storage lipid, mainly triacylglycerol (TAG; Crane *et al.*, 2006; Schwallier *et al.*, 2011). Linoleic acid, which we and others have found to be present in orchid seeds (Hengling *et al.*, 2020; Fileti *et al.*, 2021; Diantina *et al.*,

2022), potentially melts around -12.7°C as free acid and -50°C as a TAG (Knothe and Dunn, 2009). Peaks coinciding with these temperatures are evident for all seven species with clear thermal fingerprints (Fig. 2; Supplementary Data Table S1). Lipid transitions at -80°C , between -40 and -30°C and around -10°C have also been noted in seeds of three other orchid species (*Calda. flava*, *Microtis media* and *Ptst. recurva*; Merritt *et al.*, 2014a). For some species of *Cuphea*, the main storage lipids in the seeds are medium-chain lauric (12C:0) and myristic (14C:0) acids (Crane *et al.*, 2003, 2006; Volk *et al.*, 2007). When present in large quantities, these latter lipids are

thought to compromise seed viability during storage in cold conditions and recovery, requiring melting through the application of a dry thermal shock (45 °C) to facilitate germination. Such a thermal shock was ~20 °C beyond the peak temperature for the final lipid melt in *Cuphea* species, which is ~25 °C. Only for seeds of three of the seven *Cattleya* species for which

TABLE 4. Total of filled seeds in each sample and their total fatty acid content of eight *Cattleya* species. Non-rupicolous species are shown in bold text.

Species	Full-filled seeds (% ± s.d.)	Fatty acid content (% ± s.d.)	Adjusted lipid content (100% filled seeds) ^a
<i>C. alvaroana</i>	76.36 ± 0.20	33.85 ± 2.11	44.33
<i>C. briegei</i>	83.63 ± 0.49	26.205 ± 1.56	31.33
<i>C. crispata</i>	83.47 ± 0.86	33.96 ± 2.91	40.68
<i>C. granulosa</i>	41.00 ± 0.08	11.65 ± 0.60	28.41
<i>C. harrisoniana</i>	69.36 ± 1.32	33.2 ± 0.92	47.87
<i>C. hegeriana</i>	54.85 ± 4.15	27.92 ± 0.01	50.90
<i>C. rupestris</i>	80.34 ± 0.24	31.80 ± 5.99	39.58
<i>C. sanguiloba</i>	77.97 ± 1.48	34.0 ± 2.25	43.61

^aAdjusted to account for the proportionate presence of the embryo by weight and then for fatty acids.

Most species had 70–80 % filled seeds, with *C. granulosa* and *C. hegeriana* in the range ~41–55 %. Seed oil contents were 28 to ~51 %, except for *C. granulosa* at 28 %, contributing to low DSC signal trace of this species (Fig. 2).

we had clear thermal fingerprints is the 25 °C germination treatment used here 20 °C above the final melting peak, viz. *C. alvaroana*, *C. harrisoniana* and *C. sanguiloba* (Fig. 2). Although we did not take specific measures to reduce this potential risk to germination efficiency, the seeds of none of the *Cattleya* species appeared to show the type of compromised storability and germinability noted for *Cuphea* species (Crane et al., 2003, 2006). In the case of *Cuphea* species, it was recommended that heating was applied before the start of the germination test, suggesting a negative interaction with the imbibition process. For orchid seeds, water is likely to enter the embryo in the vapour phase across the air space, suggesting that seed morphology also plays a role in the realization of germination after storage.

But we also found that the method of germination was crucial to our estimate of seed survival after storage, with seeds having the smallest embryos (*C. granulosa* and *C. harrisoniana*) and potentially the fewest cells benefitting the most from both sucrose priming after 1 day of equilibration at 25 °C and from 25 °C equilibration alone after removal from storage (Table 3). Germination of *C. briegei* seeds post-storage also benefitted from these treatments without influencing estimates of longevity (σ and P_{50y}). Given that these orchid seeds do not possess any thick protective structure, such as a lignified coat, to buffer changes in the environment around the seeds, some time might be needed to stabilize cell structures before the start of germination (De Bont et al., 2019; Nadarajan et al., 2023). Given that we are not sure that lipid crystallization was a feature of the post-storage handling of the *Cattleya* seeds, we feel that the impact of a 1-day equilibration period at room temperature

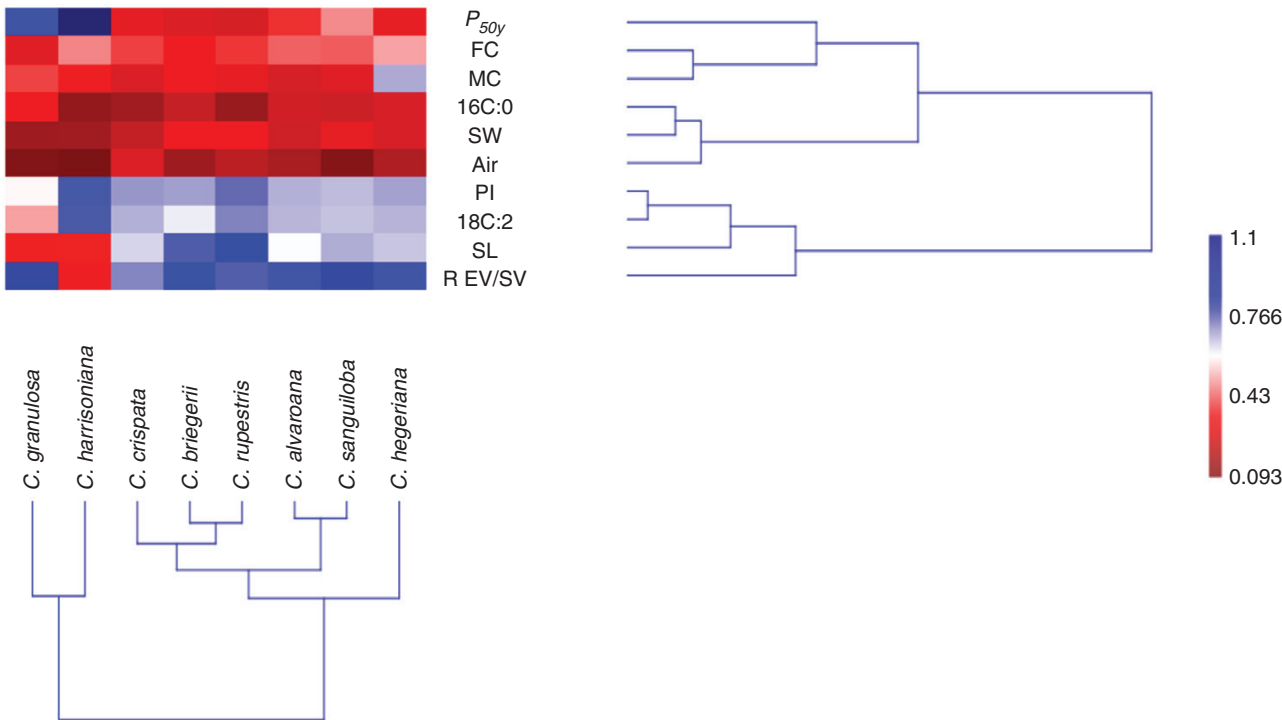


FIG. 3. Species grouping according to morphometric, best longevity parameters and principal fatty acids. Abbreviations: P_{50y} , half live of the seeds in years; FC, fatty acid content; MC, moisture content; 16C:0, palmitic acid; SW, seed width; Air, air content; PI, peroxidation index; 18C:2, linoleic acid; SL, seed length; R EV/SC, relation embryo volume/seed volume.

TABLE 5. Correlation analysis between moisture content, morphovolumetric variables, principal fatty acids and conservation parameter (P_{50y}).

	SL	SW	16C:0	18C:1	18C:2	P_{50y}	PI [#]	DBI ^{##}
SL		–	–	–	–	–0.880**	–	–
SW	0.972***		–	–	–	–0.805*	–	–
ED	0.827*	0.852**	–	–	–	–0.832*	–	–
EV/SV	–	–	0.733*	–	–0.782*	–	–0.837*	–0.871**
Air	–	–	–	0.837**	–	–0.745**	–	–
16C:0	–	–		–	–	–	–	–0.908***
18C:1	–	–	–		–	–0.736*	–	–
18C:2	–	–	–0.848**	–		–	–	0.967***
PI**	–	–	–0.900**	–	0.946***	–		0.991***

Abbreviations: Air, percentage of air inside the testae; 16C:0, palmitic acid; 18C:1 oleic acid; 18C:2, linoleic acid; ED, embryo diameter; EV/SV, ratio of embryo volume/seed volume; P_{50y} , half-life of a seed lot; PI[#], peroxidation index; DBI^{##}, double bond index; SL, seed length; SW, seed width.

Significance: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

(≥ 25 °C) post-storage on the revival of long-term stored orchid seeds should be assessed further. In the case of two of the species studied here (*C. sanguiloba* and *C. rupestris*), we have previously considered the seeds to be intermediate, and two (*C. briegei* and *C. crispata*) were considered short-lived orthodox (Machado-Neto and Custódio, 2005). However, with improved handling throughout the storage and germination processes, we have found that medium-term lifespans ($P_{50} > 30$ years) in gene bank conditions appear to be attainable. The discrepancy that Hengling et al. (2020) documented between germination and Tz tests was related to how the seeds were treated after being removed from the freezer. In the case of Hosomi et al. (2012), some seeds showed an increment during storage. Furthermore, the differences between germination and Tz, when the seeds were manipulated in a similar manner, were not so discrepant.

In our study, we found several interesting correlations between seed biochemistry, morphology and storage physiology, emphasizing the importance of determining a range of seed traits. For example, the strong negative correlation between oleic acid and potential storage (P_{50y} estimates) in *Cattleya* species supports the finding of relative poor storage in another orchid, *Ptst. banksi*, with seeds having a high oleic acid content (~23 %) (Diantina et al., 2022). Given that oleic acid is less dense than linoleic and linolenic acids (Knothe and Steidley, 2014), it might facilitate the production of relatively larger seeds and embryos. Such seeds also tended to contain relatively more air (i.e. larger air space as a proportion of seed volume), which, in turn, affects the storability of the polyunsaturated FAs.

Finally, the long-lived seeds in our long-term conventional seed bank study come from species (*C. harrisoniana* and *C. granulosa*) that are either epiphytic or terrestrial (growing in dunes) and grow in environments that are usually hotter and drier than the other six species investigated. This finding is slightly at odds with the finding of Merritt et al. (2014b), using accelerated seed-ageing conditions on 172 species from many families, who observed that plants from warmer, moist environments have seeds that are longer lived than seeds from colder, drier environments. We also found that conventional seed banking longevity in orchid species with smaller seeds appears to be greater than in congeneric species with bigger

seeds. Together with the findings that larger seeds of Australian alpine species (Satyanti et al., 2018) and *Aegilops* seed morphs (Guzzon et al., 2018) are shorter lived, our results on orchids emphasize the importance of exploring further the association between the seed morph and seed longevity phenotypes.

In conclusion, not all orchids produce seeds that are relatively short lived in conventional seed bank conditions, as demonstrated here for several species. The longevity findings combined with our multi-trait analyses might help to strengthen *ex situ* seed conservation strategies for species in this diverse and highly threatened plant family.

SUPPLEMENTARY DATA

Supplementary data are available at *Annals of Botany* online and consist of the following.

Table S1: differential scanning calorimetry peak measurements of seeds of eight *Cattleya* species. Table S2: lipid composition of eight *Cattleya* species.

FUNDING

This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) scholarships: PIBIC to R.R.M., a PhD Scholarship to M.M.H. (401047/2019-9) and a DT2 fellowship of N.B.M.-N. (07924/2020-3). H.W.P. acknowledges funding from the Chinese Academy of Sciences, Kunming Institute of Botany project on ‘Seed biology on important species of Magnoliaceae, Euphorbiaceae, Acanthopanceae, Orchidaceae and Lauraceae’, and the Government of China, Ministry of Science and Technology (MOST) for ‘MOST’s senior foreign experts introduction plan project’.

ACKNOWLEDGEMENTS

We thank Philip Seaton [orchid seed stores for sustainable use (OSSSU); Darwin Initiative Project 16-012] for the initial

support in the installation of the UNOESTE-Orchid Seed Bank and all collaborators who, during the existence of the UOSB, helped and worked with us to maintain that collection. We also thank the Department of Chemistry and Biochemistry and Chemistry College (DCB—UNESP-Presidente Prudente) for access to facilities.

CONFLICT OF INTEREST

None declared.

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