



Tipping the ex situ orchid seed conservation scale: successful medium-term seed storage of 30 terrestrial orchids

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Abstract

Seeds from several orchid taxa are reported as orthodox; nevertheless, the belief persists that they are short-lived under conventional storage (-20 °C, 15% RH). Previous reports on orchid seed viability and storage behaviour occasionally fail to report initial viability percentages at the time of collection, frequently assuming high initial viability. This study investigates the storage behaviour of 30 terrestrial orchid taxa from Greece, represented by 37 seed lots (2019–2022). Initial viability, tested via TTC/TB double-staining, showed significant variability (1.5% to 94.4%). Crucially, profound intraspecific variation was recorded. Inter-annual collections from the same individuals yielded significantly different viabilities for *Neotinea maculata* (11.8% vs. 92.5%) and *N. lactea* (74.8% vs. 60.6%). Capsule maturity also strongly impacted initial quality; immature seeds of *Ophrys sphegodes* subsp. *aesculapii* and *O. ferrum-equinum* subsp. *ferrum-equinum* demonstrated much lower viability (28.5% and 32.8%) compared to fully mature seeds from the same populations (66.7% and 65.5%). Most collections (64.8%) exhibited initial viability > 50%, while only two recorded viabilities < 10% (*Limodorum abortivum*, *Himantoglossum robertianum*). After 2.7–6 years of storage at 4 °C, follow-up testing showed minor drops in absolute viability (0.5–9.7%) for the majority (75.6%) and moderate loss (10.6–21.1%) for eight seed lots. Only *Cephalanthera damasonium* experienced high viability loss (71.1%). Based on our results, 97.3% of the tested taxa can be stored for at least 3–6 years without significant viability loss.

Keywords Initial viability · Orchidaceae · Seed bank · Storage behaviour · Terrestrial orchids

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Introduction

Orchidaceae arguably represents the largest and most diverse family of flowering plants (Christenhusz et al. 2017). Despite this diversity, it is also proportionally the most threatened plant family based on current IUCN assessments. Compared to the overall percentage of assessed taxa, nearly twice the proportion of assessed orchids falls into a threat category, with six taxa already recorded as extinct (IUCN 2025). This precarious conservation status underscores the urgent need for effective conservation strategies.

The family's complex life history presents significant challenges to conservation. A fundamental peculiarity is the obligate mycorrhizal symbiosis required by all taxa for germination and, for many, throughout their life cycle (Bernard 1899, 1909; Rasmussen and Rasmussen 2014; Rasmussen et al. 2015). This dependence arises because their microscopic (Rasmussen and Rasmussen 2009), dust-like seeds, ranging from less than 0.1 mm to 6 mm in length (Arditti and Ghani 2000), lack endosperm and thus cannot support seedling development independently. However, due to their small size, orchid seeds have long been a focus for *ex situ* conservation in seed banks (Pritchard and Seaton 1993; Seaton et al. 2013). Furthermore, the evolution of deceptive and often intricate pollination mechanisms (Jersáková et al. 2006) makes many taxa particularly vulnerable to ecosystem disruptions, especially when combined with the absence of suitable fungal associates (McCormick and Jacquemyn 2014). These biological constraints, compounded by accelerating habitat loss, fragmentation, and climate change, have led to a dramatic increase in threatened orchid taxa (Wraith and Pickering 2018, 2019), making the development of robust *in situ* and *ex situ* conservation protocols imperative.

Historically, seeds were classified into three main categories (Roberts 1973): recalcitrant seeds, which rapidly lose viability if dried; intermediate seeds, with higher tolerance of dry to a relatively low moisture content but with low tolerance of freezing temperatures; and orthodox seeds, which tolerate drying to low moisture contents (~3–7%) and storage at freezing temperatures (−20°C or below). Orchid seeds have broadly been classified as 'orthodox' (Pritchard and Seaton 1993). However, this classification has been challenged by numerous reports documenting rapid viability loss under low relative humidity conditions, freezing temperatures, or a combination of both (Pritchard and Seaton 1993; Rasmussen 1995; Machado-Neto and Custodio 2005). This inconsistent storage response has led some researchers to propose cryopreservation in liquid nitrogen as a more reliable long-term solution for these exceptional taxa (Merritt et al. 2014; Schofield et al. 2018). Conversely, recent studies have identified many orchid taxa with genuinely orthodox seed behaviour, capable of maintaining high viability for several years under conventional cold storage (Magrini et al. 2019a, b; Francisqueti et al. 2024; Oikonomidis et al. 2024a, b). The existing body of research highlights a critical gap: many early studies failed to report the initial viability of the seed collections tested. This omission makes it difficult to determine whether low post-storage viability was a consequence of storage conditions or simply poor initial seed quality (Pritchard and Seaton 1993). The present work aims to address this gap by investigating the storage behaviour of 37 seed collections from 30 Greek terrestrial orchid taxa, stored at 4 °C and ~30% RH for two to six years. Our primary hypothesis is that while initial orchid seed viability varies considerably across species, localities, and temporal collection conditions (e.g., year of collection and maturity of seed pods), seed viability will not significantly change following medium-term cold storage. To address this hypothesis, we seek to: (1)

quantify the inter- and intraspecific variation in seed viability immediately following collection, to determine if all collections possess high initial viability; (2) determine if the tested seeds rapidly lose viability when stored at cold, non-freezing temperatures, or if they exhibit orthodox-like storage behaviour, as supported by recent studies (Magrini et al. 2019a, b; Francisqueti et al. 2024; Oikonomidis et al. 2024a, b); and (3) assess potential fluctuations in both initial viability and subsequent storage behaviour across different collection years (within the same populations) and varying stages of capsule maturity for a selected subset of taxa to highlight potential differences due to temporal factors.

Materials and methods

This study investigates the viability and storage behaviour of 37 seed collections, representing 30 orchid taxa from 10 genera native to Greece and the Euro-Mediterranean region (Table 1). Collections were conducted between 2019 and 2022. The sampling encompassed 10 of the 18 orchid genera occurring in Greece: *Anacamptis* (4 taxa, 4 collections), *Cephalanthera* (1 taxon, 1 collection), *Dactylorhiza* (1 taxon, 1 collection), *Himantoglossum* (2 taxa, 4 collections), *Limodorum* (1 taxon, 1 collection), *Neotinea* (2 taxa, 4 collections), *Ophrys* (11 taxa, 14 collections), *Orchis* (5 taxa, 5 collections), *Serapias* (2 taxa, 2 collections), and *Spiranthes* (1 taxon, 1 collection).

From each population, 10–80 mature capsules were collected from 10 to 40 individuals, with the sample size adjusted based on population density and the fecundity of each taxon. To ensure representative sampling, an equal number of capsules was collected from each individual where possible (see Supplementary Table 1 for collection details). The capsules for each taxon, population, and collection year were mixed to form seed lots. The experimental design included specific comparisons: for populations of *Neotinea maculata* and *Neotinea lactea*, seeds were collected from the same marked individuals in two consecutive years (2020/2021 and 2019/2020, respectively). Similarly, for one population of *Ophrys ferrum-equinum* subsp. *ferrum-equinum* and one of *Ophrys sphegodes* subsp. *aesculapii*, capsules were collected at two distinct maturity stages: full maturity (approximately one-month post-capsule formation, when capsules were yellowish and seeds were fully formed, brown, and easily detached) and an immature stage (approximately two weeks post-capsule formation, when capsules were still green and seeds were fully formed but white and not easily detached). The full maturity stage was evaluated based on two factors: (1) capsule development, assessed via in situ observation of shape (fully formed) and colouration (green turning to yellowish towards maturity); and (2) seed development, assessed in situ by opening three randomly selected, visually mature capsules to inspect for proper seed colouration (brownish, as opposed to the white colour of immature seeds). Finally, for populations located near the NKUA Seed Bank (26 of the 38 collections), visits were conducted every two weeks after flowering to closely monitor the maturation process. Following collection, capsules were transported to the NKUA Seed Bank, where they were maintained under ambient conditions and kept unopened until the first viability test. Initial viability was assessed immediately after seed extraction, no later than eight days post-collection. For viability testing, we employed the double-staining technique proposed by Magrini et al. (2019a b). This method provides an accurate evaluation of seed viability by accounting for both the viable seeds stained by 2,3,5-triphenyltetrazolium chloride (TTC) and the non-viable seeds that are

Table 1 Viability of the tested collection after the initial test (< 10 days of storage) and after more than 1000 days of storage at 4 °C

Taxon	Col- lection Year	Stor- age Days	Pre-treat- ment min	Ca(OCI) ₂	V ₀ % ± S.E.	V ₁ % ± S.E.	p-value	ES	CI95	Location
<i>Anacamptis collina</i>	2021	1411	15		76.5±2.7	69.3±4.1	0.435	0.42	[-3.2, 17.5]	Atica, Poikilo
<i>Anacamptis coriophora</i> subsp. <i>coriophora</i>	2021	1399	6		88.9±2.5	79.8±5.6	0.500	0.32	[-6.7, 24.3]	Drama, Falakro
<i>Anacamptis morio</i> subsp. <i>caucasica</i>	2021	1399	15		94.4±1.0	88.8±2.0	0.407	0.56	[-0.3, 10.5]	Drama, Falakro
<i>Anacamptis sancta</i>	2019	2132	15		65.5±7.3	62.4±8.2	0.870	0.09	[-28.0, 29.8]	Paros, Paroikia
<i>Cephalanthera damasonium</i>	2021	1317	540		80.3±8.9	9.2±1.2	0.037	0.83	[45.1, 87.8]	Parnitha, Flabouri
<i>Dactylorhiza saccifera</i> subsp. <i>saccifera</i>	2020	1638	30		23.0±1.4	22.5±2.8	0.825	0.14	[-6.5, 8.6]	Parnitha, Haradra Mola
<i>Himantoglossum jankae</i>	2021	1317	30		83.2±4.2	71±4.2	0.407	0.46	[-2.8, 24.8]	Parnitha, Flabouri
<i>Himantoglossum robertianum</i>	2019	2105	45		6.2±1.4	4.2±1.8	0.407	0.35	[-7.22, 3.51]	Spata, Venizelos Airport
<i>Himantoglossum robertianum</i>	2019	2105	30		10.1±1.3	6.6±1.6	0.407	0.601	[-8.0, 0.3]	Spata, Venizelos Airport
<i>Himantoglossum robertianum</i>	2020	1768	45		69.7±3	62.8±3.5	0.435	0.35	[-5.8, 16.6]	Hymettus, Panepistimiopolis
<i>Limodorum abortivum</i>	2019	2054	160		1.5±0.8	0.9±0.5	0.825	0.12	[-2.2, 3.4]	Parnitha, Mpafi
<i>Neotinea lactea</i>	2020	1775	60		60.6±3.8	54.8±5.3	0.444	0.32	[-12.7, 19.4]	Hymettus, Panepistimiopolis
<i>Neotinea lactea</i>	2019	2194	60		74.8±2.5	69±2.5	0.750	0.28	[-2.0, 12.8]	Hymettus, Panepistimiopolis
<i>Neotinea maculata</i>	2020	1725	6		11.8±3.7	10.9±2.8	0.974	0.01	[-10.4, 10.7]	Hymettus, Taxiarches
<i>Neotinea maculata</i>	2021	1402	0		92.5±1.5	83.4±3.6	0.750	0.58	[-0.0, 14.5]	Hymettus, Taxiarches
<i>Ophrys apifera</i>	2021	1399	6		87.0±2.2	66.0±3.9	0.037	0.83	[8.0, 31.4]	Drama, Falakro
<i>Ophrys argolica</i> subsp. <i>argolica</i>	2022	1010	6		53.7±1.8	48.8±4.6	0.500	0.32	[-7.4, 14.4]	Megalopolis, Elenitsa
<i>Ophrys ferrum-equinum</i> subsp. <i>ferrum-equinum</i>	2021	1429	15		32.8±4.7	30.5±3.3	0.825	0.16	[-9.3, 16.3]	(Immature)Hymettus, Panepistimiopolis
<i>Ophrys ferrum-equinum</i> subsp. <i>ferrum-equinum</i>	2021	1405	15		60.0±3.5	51.3±4.5	0.435	0.46	[-3.19, 20.6]	Hymettus, Kaisariani
<i>Ophrys ferrum-equinum</i> subsp. <i>ferrum-equinum</i>	2021	1415	6		65.5±3.5	55.8±4.2	0.435	0.42	[-3.6, 21.1]	(Mature)Hymettus, Panepistimiopolis
<i>Ophrys ferrum-equinum</i> subsp. <i>gotfriediana</i>	2021	1437	15		33.4±7.3	28.9±5.3	0.435	0.14	[-17.1, 32.8]	Hymettus, Kaisariani
<i>Ophrys fusca</i> subsp. <i>fusca</i>	2021	1437	15		87.2±4.8	71.1±4.6	0.407	0.51	[-1.8, 31.1]	Hymettus, Kaisariani
<i>Ophrys lutea</i> subsp. <i>galilea</i>	2021	1437	15		67.9±7.3	66.5±10.1	0.870	0.09	[-24.3, 35.2]	Hymettus, Kaisariani
<i>Ophrys lutea</i> subsp. <i>lutea</i>	2021	1437	15		78.5±8.5	72.9±8.4	0.825	0.16	[-16.7, 36.8]	Hymettus, Kaisariani

Table 1 (continued)

Taxon	Col- lection Year	Stor- age Days	Ca(OCl) ₂ Pre-treat- ment min	V ₀ % ± S.E.	V ₁ % ± S.E.	p-value	ES	CI95	Location
<i>Ophrys sphegodes</i> subsp. <i>aesculapii</i>	2021	1429	6	28.5±2.7	27.6±2.6	0.407	0.01	[-8.5, 8.3]	(Immature)Hymettus, Cemetery
<i>Ophrys sphegodes</i> subsp. <i>aesculapii</i>	2021	1415	6	66.7±1.9	54.8±1.9	0.956	0.01	[5.6, 16.6]	(Mature)Hymettus, Cemetery
<i>Ophrys sphegodes</i> subsp. <i>spruneri</i>	2022	1010	15	50.8±3.7	42.5±3.0	0.500	0.32	[-3.6, 19.4]	Megalopoli, Elenitsa
<i>Ophrys tenthredinifera</i>	2021	1405	6	51.8±4.5	42.5±3.7	0.407	0.46	[-3.3, 26.1]	Hymettus, Kaisariani
<i>Ophrys umbilicata</i> subsp. <i>umbilicata</i>	2019	2158	15	10.7±2.6	8.5±3.4	0.825	0.14	[-6.7, 11.1]	Poikilo, Theatro Foinika
<i>Orchis mascula</i> subsp. <i>mascula</i>	2021	1361	30	79.6±1.2	69.0±4.6	0.407	0.56	[-0.9, 24.0]	Drama, Falakro
<i>Orchis militaris</i> subsp. <i>militaris</i>	2021	1399	15	22.4±6.4	16.7±6.3	0.825	0.19	[-16.3, 28.9]	Drama, Falakro
<i>Orchis pauciflora</i>	2021	1348	15	67.8±7.8	53.3±4.7	0.444	0.37	[-11.2, 35.2]	Parnon, Malevi
<i>Orchis purpurea</i>	2021	1392	60	46.4±3.0	38.8±2.6	0.407	0.56	[-2.2, 18.4]	Parnassus, Elatos
<i>Orchis quadripunctata</i>	2021	1396	15	94.2±1.8	82.2±4.8	0.407	0.49	[-2.04, 22.9]	Hymettus, Taxiarches
<i>Serapias cordigera</i> subsp. <i>cordigera</i>	2022	1066	6	32.0±2.5	28.5±4.1	0.838	0.12	[-8.8, 16.3]	Laconia, Elafonisos
<i>Serapias orientalis</i> subsp. <i>orientalis</i>	2019	2168	0	17.6±2.2	16.6±1.7	0.825	0.14	[-5.9, 7.8]	Spata, Venizelos Airport
<i>Spiranthes spiralis</i>	2020	1587	0	87.2±3.9	72.2±8.6	0.444	0.37	[-6.9, 33.9]	Hymettus, Kaisariani

Notes*: (1) V₀: mean value of initial viability before storage (2) V₁: mean value of viability after seeds storage (3) ES: The effect size ($r = Z/\sqrt{N}$) CI95: the 95% confidence interval for the Hodges–Lehmann estimate of the median difference in initial seed viability and seed viability after storage

either impermeable to water (not stained by trypan blue) or destroyed by the chemical pre-treatment (embryos stained blue by trypan blue). For each collection, six random samples, each containing approximately 100 seeds with a well-formed embryo, were processed. The protocol involved a pre-treatment with 5% $\text{Ca}(\text{OCl})_2$ (see Table 1 for durations), followed by a one-hour imbibition in distilled water. The pre-treatment duration for each taxon was optimized to achieve 100% seed coat permeability while minimizing potential embryo mortality caused by the chemical pre-treatment, following the methodology suggested by Oikonomidis et al. (2024a, b a). Seeds were then stained with a 1% TTC solution ($\text{pH } 6.8 \pm 0.2$) for 48 h in darkness at 25 °C. A 48-hour staining duration was selected to compensate for the relatively low incubation temperature of 25 °C (Magrini et al. 2019a, b a), which has been shown to offer high accuracy in previous studies, equivalent to that of germination tests (Oikonomidis et al. 2024a, b). Subsequently, seeds were stained with a 0.4% trypan blue solution for 5 min. All imbibition steps were performed using a syringe apparatus based on the design by Ponert et al. (2011). For the older collections from 2019, viability staining was initially performed using only the TTC solution, as the double-staining method had not yet been published. These tests were repeated using the double-staining protocol at the beginning of 2020; because no significant differences were observed between the two methods, the double-staining values are reported in this work.

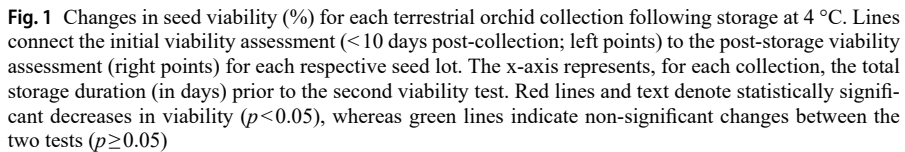
After the initial viability assessment, the seeds were equilibrated for two weeks to $30\% \pm 3\%$ relative humidity (RH) inside dry cabinets containing silica gel. The RH inside the cabinets was monitored using a temperature and humidity sensor (Xiaomi Mijia BT 4.2). The bulk seed collections were then placed in glass vials sealed with rubber caps, and these vials were stored inside larger containers containing silica gel as moisture indicators. These containers were stored at 4 ± 1 °C for a period of two to six years, depending on the initial collection date. Viability was re-tested for all 37 collections between February and April 2025, utilizing the identical double-staining protocol described above.

The adjusted percentage reduction in viability, relative to the initial viability, was calculated as follows: $R = 100 \frac{V_0 - V_1}{V_0}$ where R is the percentage viability reduction, V_0 is the initial viability of the seed lot and V_1 is the viability of the seed lot after storage (see also supplementary Table 1).

Due to the non-normal distribution of the viability data (Shapiro-Wilk test, $p < 0.001$), the non-parametric Mann-Whitney U test was used to compare initial viability against post-storage viability, under the hypothesis that the tested seeds do not undergo rapid viability loss during storage. Given the destructive nature of the TTC+TB test, the samples used for the initial and post-storage viability assessments were independent. To account for multiple comparisons, p -values were adjusted using the Benjamini-Hochberg false discovery rate (FDR) procedure. All statistical and graphical analyses were performed in R (R Development Core Team, 2005). The results of the statistical tests are presented in Table 1.

Results

Initial assessments revealed a wide range of viability across the 37 collections, from a low of $1.5 \pm 0.8\%$ for *Limodorum abortivum* to a high of $94.4 \pm 1.0\%$ for *Anacamptis morio* subsp. *caucasica*. A significant proportion of the collections (13 of 37, representing 12 taxa)



Following a storage period of at least 2.7 years (1,010 days) at 4 °C, most collections (28 of 37) demonstrated excellent preservation (Fig. 1), with absolute viability loss ranging from just 0.5% to 9.7%. A moderate decline in absolute viability (10.6–21.0%) was observed in eight seed lots. In contrast, one collection of *Cephalanthera damasonium* experienced a substantial viability reduction of 71.1%. When adjusting for the initial viability of each taxon (Fig. 1), 15 collections (15 taxa) showed less than a 10% proportional viability reduction during the storage period, while 21 collections (19 taxa) experienced a proportional viability reduction ranging between 10% and 40%. As mentioned above, the *C. damasonium* collection was an extreme exception, exhibiting a proportional reduction of 88.5%.

Significant variability in initial viability was also observed within individual taxa under different conditions. High inter-annual variation was recorded, with collections from the same marked individuals in consecutive years yielding significantly different results. For *Neotinea maculata*, initial viability was $11.8 \pm 3.7\%$ for seeds collected in 2020, compared to $92.5 \pm 1.5\%$ for seeds collected in 2021 (significant at the $\alpha=0.05$ level). Similarly, for *Neotinea lactea*, viability was $74.8 \pm 2.5\%$ for seeds collected in 2019 versus $60.6 \pm 3.8\%$ for those collected in 2020 (significant at the $\alpha=0.05$ level). Seed maturity at the time of collection (from the same populations and individuals) also had a significant impact on viability. For *Ophrys sphegodes* subsp. *aesculapii*, immature seeds showed a viability of $28.5 \pm 2.7\%$, whereas mature seeds had a significantly higher viability of $66.7 \pm 1.9\%$ (significant at the $\alpha=0.05$ level). Likewise, for *Ophrys ferrum-equinum* subsp. *ferrum-equinum*, immature seeds ($32.8 \pm 4.7\%$) were significantly less viable than mature seeds ($65.5 \pm 3.5\%$) (significant at the $\alpha=0.05$ level).

Finally, approximately one-third of the studied collections (13 of 37) exhibited low initial viability (<50%), with two collections—one of *L. abortivum* (1.5%) and one of *Himantoglossum robertianum* (6.2%)—demonstrating less than 10% seed viability immediately following collection. In all of these cases, no significant change in viability was observed after storage.

Discussion

The long-term success of *ex situ* conservation programs for Orchidaceae, a family facing unprecedented threats (Wraith and Pickering 2018; Wraith et al. 2020; IUCN 2025), hinges on a robust understanding of their seed storage physiology. Historically, orchid seeds were often characterised as delicate and challenging to maintain in storage, a perception that cast doubt on the efficacy of conventional seed banking for their conservation (Pritchard and Seaton 1993; Rasmussen 1995). Nevertheless, recent studies (Magrini et al. 2019a, b; Francisqueti et al. 2024) suggest that the seeds of several orchid taxa can be maintained under conventional seed bank conditions for several years without a significant decline in viability.

Greece is a recognized hotspot for Euro-Mediterranean orchid biodiversity, hosting more than half of the taxa found across the broader region (POWO, 2025). By assessing the viability of 37 seed lots from 30 terrestrial orchid taxa—representing approximately 10% of all orchid taxa in Europe and the Mediterranean—our study challenges traditional perceptions of orchid seed fragility, demonstrating that 28 of our 37 tested collections maintained excellent viability after up to six years at 4 °C and 30% RH. These results align with a growing body of evidence indicating that many orchid taxa produce orthodox seeds capable of extended storage under conventional seed bank conditions (Magrini et al. 2019a, b; Francisqueti et al. 2024).

A pivotal finding of our research is the remarkable range of initial seed viability (1.5% to 94.4%) observed across taxa, as well as within individual taxa when collected from the same population in different years or at different maturation stages. This confirms that assuming high initial viability for any given orchid seed collection is inherently flawed. Factors such as pollination success, resource allocation by the parent plant, maturation level, and environmental conditions during seed development can profoundly impact the final quality of a seed lot (Rao et al. 2017). Although literature concerning orchid seed development and quality is relatively sparse, several worldwide case studies examining the impact of pollen origin on seed development suggest that deceptive orchid species are highly susceptible to inbreeding depression. This often manifests as lower seed quality, including high proportions of empty seeds or significantly reduced viability (Light and MacConaill 1998; Metsares et al. 2015; Tałaj et al. 2017; Lara et al. 2018; Andronova et al. 2020; Chen et al., 2009; Oikonomidis et al. 2024a, b). These studies consistently highlight the detrimental effect of autogamy on seed production in deceptive orchids, with some research (e.g., Tałaj et al. 2017) further indicating clear differences in seed quality among species with varying pollination strategies. Based on these insights, we postulate that the low initial viabilities recorded in our study may frequently result from self-pollination events and subsequent inbreeding depression. This is highly probable given that all the studied taxa—with the exception of *Limodorum abortivum* and *Neotinea maculata*, which frequently exhibit

cleistogamy (Balogh et al., 1987; Duffy et al. 2009)—employ deceptive pollination strategies. Regarding the exceptionally low initial viability observed in the saprophytic and cleistogamous orchid *Limodorum abortivum*, other factors—such as severe resource limitation or environmental stress—likely override pollination strategy in dictating seed quality. A similar phenomenon is observed in the cleistogamous *Neotinea maculata*, as discussed in the next paragraph.

The stark contrast in initial viability for *Neotinea maculata*, a predominantly cleistogamous species (Duffy et al. 2009), between 2020 (11.8%) and 2021 (92.5%) from the exact same individuals underscores the dramatic influence of inter-annual environmental fluctuations on seed quality. Such variations are likely linked to specific differences in seasonal weather patterns (Maity et al. 2023). This finding reinforces the critical importance—as originally highlighted by Pritchard and Seaton (1993)—of assessing initial viability immediately upon collection to establish a reliable baseline. Without this baseline, low post-storage viability could be erroneously attributed to deterioration within the seed bank rather than to inherently poor initial seed quality (Pritchard and Seaton 1993; Rasmussen 1995; Machado-Neto and Custódio, 2005).

A central finding of our study is the excellent retention of viability for most orchid seeds under dry, cold storage. After storage periods ranging from 2.7 to 6 years at 4 °C, the majority of the collections (28 of 37) exhibited only a minimal loss of viability (0.5–9.7%). This demonstrates that for these taxa, storage at 4 °C following intermediate drying (~30% relative humidity) is a highly effective medium-term conservation strategy. Based on our results and previous work on similar taxa (e.g., Magrini et al. 2019a, b), comparable orthodox storage behaviour is theoretically expected under standard long-term seed banking conditions (-20 °C and 15% RH). However, this requires further testing to account for specific physiological risks, including the danger of over-drying (below 30% RH), potential cellular damage from ice crystal formation if 30% RH proves insufficiently dry for sub-zero temperatures (Chin et al. 1989), and the risk of lipid melting and crystallization recently investigated for orchid seeds (Magrini et al. 2022). Overall, our results for genera such as *Anacamptis*, *Ophrys*, and *Orchis* are highly consistent with recent findings pointing toward orthodox storage behaviour in European terrestrial orchids (Magrini et al. 2019a, b; Oikonomidis et al. 2024a, b). This demonstrated resilience significantly bolsters the case for mid- to long-term seed banking as a primary pillar of orchid conservation (Francisqueti et al. 2024; Oikonomidis et al. 2024a, b), offering a cost-effective and space-efficient method to safeguard genetic diversity against habitat loss and other mounting threats (Seaton et al. 2010). Finally, given these complexities associated with sub-zero storage, our demonstrated success at 4 °C presents a low risk alternative for medium term storage in seed banks that may lack the precise moisture-control or cryopreservation infrastructure required to safely freeze delicate orchid seeds.

However, our results were not uniform across all tested taxa. Two taxa, *Ophrys apifera* and particularly *Cephalanthera damasonium*, showed statistically significant decreases in viability. While the viability loss in *O. apifera* was moderate (~21%), the dramatic 71.1% drop observed in *C. damasonium* is alarming and warrants closer examination. The moderate decline in *O. apifera* may reflect natural intraspecific variation or a slightly faster aging rate compared to other *Ophrys* spp. tested in our work, though it still retains enough viability to be useful for conservation purposes. For *C. damasonium*, it is possible that this species possesses an atypical storage physiology, perhaps leaning toward an intermediate or recalcitrant

trant behaviour, or it may have specific storage requirements not met by our protocol. While our current results suggest non-orthodox behaviour for this species, its exact desiccation tolerance and sub-zero storage capacity remain to be tested. For instance, some orchid seeds are highly sensitive to low desiccation levels (<30% RH; Pritchard and Seaton 1993; Machado-Neto and Custódio, 2005), and conditions that are optimal for orthodox seeds might be detrimental to these sensitive taxa (Hay and Probert 2013; Merritt et al. 2014; Popova et al. 2016). Furthermore, the genus *Cephalanthera* is well known for its germination challenges, which may be linked to specific types of physiological dormancy or unique seed coat properties (Rasmussen 1995; Batty et al. 2001). From an ecological perspective, this rapid viability loss aligns with early observations of closely related terrestrial Epidendroideae taxa (e.g., *Cephalanthera* spp., *Epipactis* spp., and *Neottia* spp.), which also exhibited rapid deterioration during storage (Rasmussen 1995). Because these taxa typically inhabit deep woodlands and rely heavily on specific mycorrhizal fungi for germination and development, they likely exhibit an opportunistic “hit-or-miss” ecological strategy; seed viability declines rapidly if a suitable symbiont is not immediately present in the soil vicinity (Oikonomidis and Thanos 2024). The significant viability loss in *C. damasonium*, combined with the extremely low initial viability observed in *Limodorum abortivum* (1.5%), highlights that a “one-size-fits-all” approach to orchid seed conservation is inadequate. These outlier taxa require targeted research to elucidate their specific storage sensitivities and to develop conservation protocols, which may ultimately require cryopreservation, as suggested by Merritt et al. (2014) for highly sensitive taxa.

Furthermore, our investigation into the viability of seeds from immature capsules of *Ophrys sphegodes* subsp. *aesculapii* and *Ophrys ferrum-equinum* subsp. *ferrum-equinum* provides valuable practical insights for seed collectors. The significantly lower viability of seeds from immature capsules, compared to mature ones from the exact same populations, underscores that harvesting time is a critical variable. Although seeds may appear morphologically developed, physiological maturity is essential for maximizing initial viability and ensuring subsequent longevity in storage (Hay and Probert 2013; Maity et al. 2023). Consequently, collecting capsules prematurely—a practice often employed to avoid herbivory or seed loss through natural dehiscence—can severely compromise the quality and long-term conservation potential of a seed lot. In many cases, near-mature orchid capsules are preferentially collected to provide a simpler means of surface sterilization for in vitro germination experiments. Disinfecting the intact, closed capsule rather than the fragile seeds themselves helps avoid potential chemical damage from the sterilant, and these near-mature seeds often exhibit higher initial germination rates for immediate propagation (Oliva and Arditti 1984; Kitsaki et al. 2004). However, our findings clearly demonstrate that while this practice is advantageous for short-term cultivation, harvesting seeds before they reach full physiological maturity is highly detrimental to their long-term storage potential in seed banks.

Finally, although approximately one-third of the collections (13 of 37) exhibited low initial viability (<50%), our results still indicate that these seed lots can be successfully stored for the medium-to-long term under moderate desiccation (30% RH) at non-freezing temperatures (4 °C). While these findings should be interpreted with caution—particularly for collections with extremely low initial viability (<10%)—our dataset provides compelling internal validation. For example, in species such as *Neotinea maculata* and *Himantoglossum robertianum* (see Table 1), independent collections from different years or localities that possessed *high* initial viability similarly exhibited no significant reduction in viability

following storage. Nevertheless, given the prolific seed production characteristic of Orchidaceae—where a single capsule may contain tens of thousands of seeds—banking even low-viability collections remains a highly valuable conservation action for propagation purposes, provided the seeds viability do not further decline, taking also into account studies suggesting collection of orchid seeds with less than 60–70% of viability still maintain high germinability (Oikonomidis et al. 2024a, b).

The double staining test (Magrini et al. 2019a, b) utilized in the present study has been shown to yield viability results comparable to actual *in vitro* germination rates in related species (Oikonomidis et al. 2024a, b). Nevertheless, we must acknowledge that discrepancies can arise when assessing viability via different methodological approaches. Historically, the accuracy of vital dyes has varied; they sometimes indicate higher viability compared to actual germination rates in certain orchids (e.g., Francisqueti et al. 2024), while showing no significant differences in other plant groups (e.g. Soares et al. 2016). These potential disparities must be taken into account. However, in the case of orchids, lower germination rates relative to stain-based viability often stem from an insufficient understanding of species-specific germination requirements and complex dormancy-breaking cues, rather than a failure or overestimation by the vital dye itself (Francisqueti et al. 2024).

In conclusion, our study strongly supports the growing consensus that many terrestrial orchids produce orthodox seeds well-suited for long-term *ex situ* conservation. We demonstrated that seeds of most tested orchids from Greece can be stored for several years at 4 °C and 30% relative humidity (RH) with negligible loss of viability. Supported by previous research (Magrini et al. 2019a, b; Oikonomidis et al. 2024a, b), these findings suggest that such conditions are sufficient for the mid- to long-term preservation of numerous European orchid taxa. This resilience provides a clear mandate for integrating these taxa into national and international seed banking initiatives. Nonetheless, the high variability in initial seed quality, alongside the existence of sensitive taxa like *Cephalanthera damasonium*, serves as a crucial reminder that conservation protocols must be evidence-based and species-specific. Future research must prioritize these outlier taxa to elucidate the physiological mechanisms driving their rapid viability loss and to determine how specific moisture and temperature regimes govern this deterioration. Furthermore, prompted by the dramatic inter-annual fluctuations observed in our *Neotinea maculata* collections, investigating the spatiotemporal impacts of biotic and abiotic environmental factors on initial seed development represents a critical area for future study. Finally, while our current results provide a highly successful and accessible mid-term strategy for *ex situ* orchid conservation, the efficacy and safety of standard long-term gene bank conditions (−20 °C and 15% RH) must be also explored to secure the future of these threatened plants.

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Author contributions SO and CAT planned and designed the research, SO conducted fieldwork, performed the experiments analysed the data and wrote the initial version of the manuscript, CAT revised the manuscript before initial submission.

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Data availability The summarized data are available in the Table 1 of the manuscript, and the supplementary Table 1 of the supplementary material. The raw data for the species are available upon request from the authors.

Declarations

Competing interests The authors declare no competing interests.

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