RESEARCH ARTICLE

Testing seed germination from herbaria: Application of seed quality enhancement techniques and implication for plant resurrection and conservation

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Abstract Herbaria are an important source of data and material useful in many fields, including plant conservation. Seeds preserved in herbarium specimens may have the potential to germinate, although few studies focused on this topic. Here, the first systematic assessment of six techniques, including priming techniques and melatonin application, aimed at improving the germination of seeds from herbarium specimens is presented. Seed germination of 26 species common in Europe, some of which congeneric to extinct species, collected in herbaria and in the wild (20,549 seeds in total, including 19,509 from 297 herbarium specimens from 8 different herbaria) was tested with the following treatments: exogenous melatonin addition to the germination medium, priming with melatonin, osmopriming, hydropriming for 24 and 48 hours, standard soil, heat sterilization and gibberellins addition. More than 85% of the fresh seeds and 1% of the seeds collected in herbaria germinated, including seeds older than 50 years. Data show that treatment with exogenous melatonin had a positive effect on the germination of fresh seeds, but a negative effect on the germination of herbarium-derived seeds. Furthermore, osmopriming treatment had a slightly positive effect on the germination of herbarium-derived seeds. Osmopriming and exogenous melatonin addition seem to be promising techniques that need further investigation and improvement and might be useful for the development of an optimal germination protocol for old and herbarium-derived seeds. The germination of seeds from herbaria could be an important tool in plant conservation, with the aim of reversing the extinction trend of many species through de-extinction, safeguarding biodiversity, and genetic variability. This study provides preliminary data for the development of germination protocols, especially for old seeds of species of conservation interest, to maximise the chance of recovering lost genetic diversity and leading to the first de-extinction ever.

Keywords de-extinction; natural history collections; melatonin; priming; seed test; seed viability

Supporting Information may be found online in the Supporting Information section at the end of the article.

■ INTRODUCTION

Anthropocene is a period characterised by important changes in our planet's dynamics due to direct and indirect intensive human exploitation and disturbance (Lewis & Maslin, 2015). Habitat loss, deforestation, climate change, land use change, and pollution are some of the factors that derive from human activities and that result in severe consequences, including species extinction (Corlett, 2015). Current estimates indicate that over 42,000 red-listed species

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are threatened with extinction (IUCN, 2022); other estimates indicate that 39% (Antonelli & al., 2020) to 45% (Bachman & al., 2023) of plant species are threatened with extinction. The importance and urgency of preventing, limiting, and interrupting this trend have led to international and national responses (e.g., the Aichi Biodiversity Targets, and the UN Sustainable Development Goals) in recent decades, but we are still far from achieving these goals (Secretariat of the Convention on Biological Diversity, 2020). The efforts made so far have led to the enrichment of the conservationist toolbox with means and ways to implement actions as effectively as possible to restore, protect and improve the condition of species, habitats, and ecosystems.

Among these instruments, natural history collections like herbaria and carpological collections are useful sources of information about species population trends in time (Willis & al., 2017; Rosche & al., 2022), functional traits (Heberling, 2022) and more (Albani Rocchetti & al., 2021). Herbaria may also be a source of material for recovering lost genetic diversity, including the recovery of extinct cultivars and genotypes, e.g., unique alleles or genes that do not exist in wild populations (Nakahama & al., 2015; Brewer & al., 2019).

In recent years, a few studies investigated the use of herbaria for recovering extirpated populations or even extinct species from seeds preserved in herbarium specimens (Nakahama & al., 2015; Abeli & al., 2020; Albani Rocchetti & al., 2022a), and several studies have shown that old seeds stored under suboptimal conditions in herbarium specimens can germinate (Magrini & al., 2010; Godefroid & al., 2011; Molnár & al., 2015; Cosac & al., 2016; Wolkis & al., 2022). Generally, germination of seed from seed banks should be preferred, as the material is stored under optimal conditions to maintain seed viability over time, so herbaria may be useful in exceptional cases, e.g., if no seeds from seed banks are available (e.g., *Vincetoxicum pycnostelma* Kitag.; Nakahama & al., 2015), if no collections representing genetically different populations exist (e.g., when only one sex of a dioecious species is known: *Symonanthus bancroftii* (F.Muell.) Haegi; Bunn & Turner, 2019), or in the extreme case of recovering extinct species like *Vicia dennesiana* H.C.Watson (Albani Rocchetti & al., 2022a).

The recovery of extinct species ("de-extinction" sensu IUCN SSC, 2016), is defined as the creation of a proxy or a functional equivalent of an extinct taxon. This topic is still only theoretical and vividly debated among conservation biologists and practitioners (Greely, 2017; Seddon, 2017; Slater & Clatterbuck, 2018). Concerning plants, de-extinction could be achieved by germinating or tissue-culturing old diaspores (i.e., seeds/spores) preserved in herbaria (Abeli & al., 2020). In any case, this would result in the recovery of actual species, not proxies, with major implications for plant conservation and the definitions of "extinction" and "de-extinction" themselves (Albani Rocchetti & al., 2022a). Moreover, it is important to note that an extinct species with accessions stored in *ex situ* collections such as germplasm banks is considered Extinct in the Wild (EW), whereas if only accessions exist in natural history collections such as carpological collections and herbaria, the species is considered Extinct (EX), ignoring its germination potential (Abeli & al., 2020). The philosophy behind this distinction is still understudied, although it is of interest not only formally and legislatively, but also practically for taking conservation actions.

Although the abovementioned examples of germination of old seeds and seeds from herbaria are key cases, a systematic attempt to develop germination protocols for old seeds is lacking. Most tests on germination of seeds collected in herbaria have focused mainly on breaking seed dormancy (Molnár & al., 2015; Nakahama & al., 2015).

An overview of tests conducted so far on herbarium seed germination showed a relatively low probability of success. Indeed, only 8 seeds out of more than 2500 germinated in tests (Godefroid & al., 2011). Similarly, the germination rate of spores from two herbarium specimens of the fern *Osmunda regalis* L. was quite low (1% and 2%, respectively; Magrini & al., 2010). Molnár & al. (2015) showed a higher germination percentage (43%, 344 out of 793 sown seeds) in 18 herbarium specimens of *Astragalus contortuplicatus* L. This species belongs to the Fabaceae family, which is well known for its seed longevity (Colville & Pritchard, 2019). These examples show that the successful germination of old seeds from herbaria is affected by several limitations, e.g., the taxonomic, historical, and cultural value of many specimens, where curators may be reluctant to destructive sampling; the use of chemicals and freezing cycles to preserve specimens from pests; the seed longevity, which is extremely variable within genera and species; the storage conditions (temperature and humidity) in herbaria, which are not ideal for preserving the viability of old seeds (Albani Rocchetti & al., 2022b). The maturity stage of the seeds at which the voucher was collected (Hay & al., 1997) and the post-harvest conditions (Hay & Probert, 1995) also play an important role in seed viability and longevity. The initial viability is retained longer in fully ripened seeds than in immature seeds (Stein & al., 1974), and storage potential is poor if the collection was too early (Hay & Smith, 2003). In addition, plants collected for herbarium specimens are often harvested during flowering, which reduces the probability of finding mature, well-developed fruits (e.g., in Godefroid & al. [2011], 1342 specimens out of a total of 1426 were discarded).

Despite these limitations, the abovementioned studies demonstrated the feasibility of conservation actions such as recovering lost genetic variation (Nakahama & al., 2015), or increasing genetic variation in inbred populations (Magrini & al., 2010). However, to date, *ad hoc* germination protocols to overcome the morphological and physiological barriers that prevent the germination of viable seeds are still lacking.

In this study, we provide the results of different seed germination treatments (including priming) here tested for the first time on herbarium seeds. In particular, we tested for the first time the effect of exogenous melatonin in the germination medium, priming with melatonin, and osmopriming

on germination of old seeds. Melatonin is widely recognised as an effective molecule for improving seed germination due to its antioxidant capacity when used both for seed priming (i.e., a pre-sowing seed treatment; Cao & al., 2019; Bahcesular & al., 2020; Yan & al., 2020) and as an exogenous additive (i.e., when the solution is added to culture media; Simlat & al., 2018; Castañares & Bouzo, 2019; Xiao & al., 2019). Osmopriming is the controlled rehydration of seeds by soaking seeds in a solution at low water potential (Ventura & al., 2012). This process allows "the transient activation of the pre-germinative metabolism that includes antioxidant functions and DNA repair processes", reducing cellular damage that can occur with rapid seed imbibition (e.g., with hydropriming; Ventura & al., 2012: 196). While the abovementioned techniques effectively improve germination of fresh seeds (e.g., Sadeghi & al., 2011; Cao & al., 2019; Castañares & Bouzo, 2019), their efficacy in prompting germination of aged seeds is unknown.

The main aim is, therefore, to provide preliminary data for the development of germination protocols, in particular for old seeds of threatened, rare or extinct species, in order to maximise the chance of recovering lost genetic diversity, and eventually leading to the first de-extinction ever.

■ MATERIALS AND METHODS

Target species and seed collecting. — The species were selected according to three criteria: (i) taxonomic relatedness (i.e., within genus or family) of the most common species found in our herbarium network with the species listed as extinct by Humphreys & al. (2019), (ii) known germination requirements, and (iii) availability and abundance of herbarium specimens containing mature seeds.

Based on these criteria, 26 common European species were selected, and seeds were collected from herbarium specimens. See Table 1 for details. The abundance of herbarium specimens of the 26 selected species and their age were preliminarily verified by contacting the curators of the following herbaria, from which specimens were used (suppl. Table S1): Meise Botanic Garden (BR), University of Ferrara (FER), Natural History Museum of Florence (FI), Natural History Museum of Milan (MSNM), Natural History Museum of Piacenza (MSPC), University of Pavia (PAV), La Sapienza University of Rome (RO), Roma Tre University (URT), and the educational herbarium of the University of Florence (not in Index Herbariorum, referred to as "FD" in suppl. Table S1). As the removal of seeds can reduce the taxonomic and diagnostic value of a specimen, we collected a maximum of 25% of the available mature seeds per specimen according to Albani Rocchetti & al. (2022b) and followed the curators' instructions on a case-by-case basis. Moreover, a label with the collector's name and date of collection was added to each specimen to keep track of destructive sampling.

In addition, seed samples of *Silene latifolia* Poir. and *Daucus carota* L. were collected from the wild near Cerveteri (Italy) on 25 May 2020 for comparison with fresh seeds. All seeds were stored in labelled paper envelopes, under controlled conditions of humidity (~15%) and temperature (4°C). SID (SER, INSR, RBGK, Seed Information Database, https://ser-sid.org/; accessed 18 Dec 2023) and ENSCOBASE (http://enscobase.maich.gr/; accessed 27 Apr 2022), two of the most important and complete databases for seed biological trait data, were used to identify the germination requirements of the selected species. Taxonomy follows Global Names Verifier v.1.1.1 (https://verifier.globalnames.org/).

Germination tests. — Germination tests were set as follows: addition of exogenous melatonin to the germination substratum, priming with exogenous melatonin, osmopriming, hydropriming for 48 and 24 hours, germination in soil, and heat disinfection and gibberellic acid addition (Table 1).

In each of the abovementioned tests, seeds from each herbarium specimen were tested separately. Germination tests were performed in different test sessions, as explained in the following sections. Due to the limited number of seeds per specimen, the germination test sessions were optimised by grouping the treatment application and species selection as reported in Table 1.

Within each test, scarification (using a scalpel), cold stratification (4 weeks at 4°C) or warm stratification (4 weeks at 30°C/20°C) was applied to the seeds of those species whose seeds showed a better germination rate according to SID and ENSCOBASE if pre-treated (Table 1). Detailed information on each germination test is reported in the sections below.

Application of exogenous melatonin in the germination medium. — Exogenous melatonin application has recently been successfully used to improve germination of crop seeds (e.g., Simlat & al., 2018; Castañares & Bouzo, 2019; Xiao & al., 2019), but tests have never been carried out on seeds of wild species or on aged seeds. Furthermore, there is insufficient literature data to infer an optimal melatonin concentration that can improve germination performance. We therefore tested three exogenous melatonin concentrations (50, 100 and 200 µM) at the seed bank of the University La Sapienza, Rome. No exogenous melatonin was added in the control group.

Seeds of *Daucus carota* and *Silene latifolia* harvested from the wild and collected from herbarium specimens were used for this test. Since the URT Herbarium holds a substantial number of well-preserved, non-chemically treated, and relatively recently collected specimens (1996 to 2000), several seeds from different seed lots were tested. A total of 2265 seeds were disinfected in bleach (1% sodium hypochlorite for a maximum of 3 minutes, depending on seed volume) and rinsed three times in distilled water. This disinfection method was used in all the tests, except for the heat disinfection test.

Melatonin solutions at different concentrations were prepared by dissolving 23.228 g/mol of melatonin (Alfa Aesar melatonin 99+% powder) in ethanol, then progressively diluting the solution with distilled water to obtain the aforementioned concentrations. Each treatment consisted of sowing three replicates, each with 10–25 seeds per specimen,

depending on seed availability. Seeds were sown in Petri dishes containing double filter paper moistened with the different melatonin solutions or, in the case of the control group, with distilled water, and incubated (Intercontinental incubator; Amcota incubator) for 16 weeks at 20°C with a 12-hour photoperiod (*Daucus carota*), or first incubated for 8 weeks at 25°C–15°C followed by 8 weeks at 15°C–9°C, both with a 12-hour photoperiod (*Silene latifolia*). Germination was checked twice a week for 16 weeks. If necessary, the Petri dishes were rehydrated with distilled water.

Seed priming with melatonin. — Melatonin priming is reported to be one of the most promising priming techniques

Table 1. Species for which the germination was tested with the different methods, as well as germination conditions.

		Germination test								Germination conditions	
Family	Taxon	EM	MP	OP	HP48	HP24	SO*	HE	Pre-Tr	Incubation temperature [°C]	Photoperiod
Caryophyllaceae	*Arenaria serpyllifolia* L.							x		20	12/12
Fabaceae	*Astragalus cicer* L.				x		x		SC	20	12/12
Fabaceae	*Astragalus glycyphyllos* L.				x		x	x	SC	20, 23/9†	12/12
Campanulaceae	*Campanula trachelium* L.			x	x		x	x		20	12/12
Caryophyllaceae	*Cerastium alpinum* L.		x	x	x		x		CS	20	12/12
Caryophyllaceae	*Cerastium arvense* L.		x	x	x		x	x		20	12/12
Caryophyllaceae	*Cerastium tomentosum* L.		x	x			x			20	12/12
Onagraceae	*Chamaenerion angustifolium* L. subsp. *angustifolium*		x	x				x		20	12/12
Lamiaceae	*Clinopodium nepeta* (L.) Kuntze		x	x	x	x	x	x	CS	25/15, 23/9†	12/12
Apiaceae	*Daucus carota* L.‡	x								20	12/12
Brassicaceae	*Drabella muralis* (L.) Fourr.		x	x	x		x	x	WS	20	12/12
Ericaceae	*Erica tetralix* L.							x		23/9	12/12
Asteraceae	*Filago arvensis* L.							x		23/9	12/12
Brassicaceae	*Lepidium sativum* L.			x	x		x	x		20	12/12
Boraginaceae	*Myosotis arvensis* (L.) Hill		x	x	x		x	x		20	12/12
Caryophyllaceae	*Petrorhagia prolifera* (L.) P.W.Ball & Heywood				x		x			20	12/12
Ranunculaceae	*Ranunculus acris* L.				x	x	x	x		30/20, 33/19†	12/12, 16/8†
Lamiaceae	*Salvia pratensis* L.			x	x		x	x		20	12/12
Caryophyllaceae	*Silene flos-cuculi* (L.) Greuter & Burdet§						x			20	12/12
Caryophyllaceae	*Silene latifolia* Poir.‡	x	x	x	x		x	x		20	12/12
Caryophyllaceae	*Silene nutans* L.		x	x				x		23/9	12/12
Caryophyllaceae	*Silene vulgaris* (Moench) Garcke		x		x		x	x		20	12/12
Asteraceae	*Sonchus arvensis* L.		x	x	x	x	x	x		30/20, 33/19†	12/12, 16/8†
Caryophyllaceae	*Stellaria media* (L.) Vill.						x	x		30/20, 23/9†	12/12
Fabaceae	*Trifolium pratense* L.			x				x	SC	20	12/12
Urticaceae	*Urtica dioica* L.		x	x	x		x	x	WS	20/15	12/12

EM, exogenous melatonin addition; MP, melatonin priming; OP, osmopriming; HP48, hydropriming for 48 hours; HP24, hydropriming for 24 hours; SO, use of soil medium; HE, heat sterilization and gibberellic acid addition.
Pre-treatments (Pre-Tr) were: SC, seed scarification using a scalpel; CS, cold stratification (4 weeks at 4°C); WS, warm stratification (4 weeks at 30°C/20°C).
* SO germination test was performed under greenhouse conditions (ca. 18/15°C) and no pre-treatment was applied.
† Additional conditions tested only at Meise Botanic Garden.
‡ Seeds harvested from the wild were also tested.
§ Only seeds harvested from the wild were tested.

for enhancing seed germination in crop seeds (Cao & al., 2019; Bahcesular & al., 2020; Yan & al., 2020). Melatonin priming was performed on 2135 seeds of 12 different species (suppl. Table S1) at the seed bank of the University La Sapienza, Rome. Three melatonin concentrations were tested (50, 100, and 200 µM), while no melatonin priming was used in the control group. For each melatonin treatment, the seeds were placed in sealed tea bags soaked in a 1-litre melatonin solution for 24 hours in the dark at room temperature (ca. 20°C). Seeds of the control group were soaked in 1 litre of distilled water under the same conditions. Aeration was provided by an aquarium air pump (Shego optimal). After melatonin priming, all seeds both from the melatonin priming treatments and the control group were disinfected in bleach (1% sodium hypochlorite for a maximum of 3 minutes, depending on seed volume), rinsed 3 times in distilled water, and placed in Petri dishes containing 1% agar (10 g/l distilled water) supplemented with 250 mg/l gibberellic acid (Merck, Darmstadt, Germany) in three replicates, each with 3–25 seeds per specimen, depending on seed availability. Seeds were incubated at different temperatures (Intercontinental incubator; Amcota incubator) according to their germination requirements (Table 1). Germination was checked twice a week for 16 weeks.

Osmopriming. — Osmopriming has been proven to effectively improve seed germination (Ventura & al., 2012; Mirmazloum & al., 2020; Lei & al., 2021). We tested two osmotic potential treatments (−0.8 MPa and −1.5 MPa) against a control group (0 MPa on 1% agar), on 1239 seeds of 15 species (suppl. Table S1) at the seed bank of the University la Sapienza, Rome. In each osmotic treatment, seeds were primed by placing them in sealed tea bags soaked in the corresponding polyethylene glycol solution (PEG 8000, VWR Chemicals) for 40 hours at room temperature (ca. 20°C). After osmopriming, all seeds, including those from the control group, were disinfected in bleach (1% sodium hypochlorite for a maximum of 3 minutes, depending on seed volume), rinsed 3 times in distilled water, and then placed in Petri dishes containing 1% agar supplemented with 250 mg/l gibberellic acid in three replicates, each with 2–6 seeds per specimen, depending on seed availability. Seeds were incubated at different temperatures (Intercontinental incubator; Amcota incubator) according to their germination requirements (Table 1). Germination was checked twice a week for 16 weeks.

Hydropriming. — Hydropriming is a pre-sowing treatment whose effect on seed germination is still discussed and mostly tested on crop seeds (Paparella & al., 2015; Damalas & al., 2019; Adhikari & al., 2021). Two hydropriming test sessions, for 48 hours and 24 hours, were tested each against its control (seeds sown directly in Petri dishes without hydropriming treatment) at the University of Pavia.

For the 48-hour hydropriming treatment and its control group, 3598 seeds of 16 species were tested (suppl. Table S1). Seeds were sealed in tea bags and soaked in 1 litre of distilled water for 48 hours at room temperature (ca. 20°C) in dark conditions. Aeration was provided by an aquarium air pump (Shego optimal). After hydropriming, all seeds both from the 48-hour hydropriming treatment and the control group were disinfected in bleach (1% sodium hypochlorite for a maximum of 3 minutes, depending on seed volume), rinsed 3 times in distilled water, and placed in Petri dishes containing 1% agar supplemented with 250 mg/l gibberellic acid. Germination tests were performed sowing three replicates, each with 1–16 seeds per specimen, depending on seed availability. Seeds were incubated in LMS 250A incubators (LMS, Sevenoaks, U.K.) at different temperatures and photoperiods according to their germination requirements (Table 1). Germination was checked twice a week for 16 weeks.

For the 24-hour hydropriming treatment and its control group, a total of 190 seeds of *Clinopodium nepeta* (L.) Kuntze, *Ranunculus acris* L., and *Sonchus arvensis* L. were tested using the same procedure as for the 48-hour hydropriming treatment, but over 24 hours. However, in this test, all primed and untreated seeds were placed in Petri dishes in 1/2 Murashige and Skoog (MS) salt mixture medium (pH 5.7) added with 1% (w/v) agar (solid medium), under sterile conditions.

Standard soil. — In this test, seeds were sown in pot trays filled with standard soil (TerraPlant 1 Compo, Cesano Maderno, Italy) to recreate the most natural conditions possible, avoiding the use of agar or filter paper as substrate. A total of 1410 seeds of 19 species from herbarium specimens were sown in three replicates of five seeds per specimen (suppl. Table S1) and placed in the cold greenhouse of the Botanic Garden of the University of Ferrara without disinfection or pre-treatments. Pots were regularly watered with tap water and soil emergence was checked once a week, from mid-February 2021 to September 2022.

Heat disinfection and gibberellic acid addition. — Dry heat exposure has been proposed as a promising seed disinfection technique with a positive effect on infection control without affecting seed viability (Godefroid & al., 2016) or even promoting seed germination if the phenomenon of incomplete lipid melt occurs (Hsu & al., 2003; Baskin & Baskin, 2014). Moreover, a single study reported the use of gibberellic acid to enhance the germination of old seeds from herbaria (Godefroid & al., 2011). Here, we tested the effect of heat disinfection and gibberellic acid addition (250 mg/l) as separate treatments compared to the control (i.e., using 1% agar alone) on the germination of 9712 seeds of 20 species (suppl. Table S1). In each treatment, seeds were sown in three replicates with 3–25 seeds per specimen, depending on seed availability.

These tests were conducted at Meise Botanic Garden, Belgium using material from BR. Heat disinfection was performed by placing seeds in unsealed envelopes in a dry heat chamber at 60°C for 1 hour. During the first three weeks of incubation (LMS Cooled Incubator A280), seeds were checked every 2–3 days. For the next three months, because of lab access restrictions due to the COVID-19 pandemic, germination was checked every 2–4 weeks.

Data analysis. — After germination tests, non-germinated seeds were cut-tested to identify the number of

fresh, empty, and dead (mouldy or infested) seeds. Data analyses were performed in R Studio (v.4.3.2; R Foundation for Statistical Computing, 2021). Due to the fact that different materials from different specimens were used, and given the variability of seed number, specimens, collection year, and herbarium specimens storage conditions, the described experiments are not directly comparable to each other. Therefore, the statistical analyses were applied to identify differences within each experiment.

To this aim, generalized linear models (GLM) with binomial error structure and *logit* link function were performed for each species separately in each experiment. The proportion of germinated seeds on viable sown seeds (intended as the total number of sown seeds at the beginning of the experiments minus empty seeds) was used as the response variable and treatments – according to the experiments described above – as the explanatory continuous variable. The specimen collection year (associated with herbarium ID) was included in the models as a categorical variable in interaction with treatment to test for differences in germination between seed lots of different ages. As germinations were null in most experiments, species with null seed germination in any treatment under all conditions tested were not included in the analysis.

Finally, ANOVA tests were run on the single species models to check for overall differences in "treatment", "year", and the interaction term. Further statistical analyses on the results from standard soil and heat disinfection and gibberellic acid addition tests were impossible due to the null or low germination rates. Generalised linear models were performed with package lme4 v.1.1-27.1 (Bates & al., 2015), and ANOVA with package car v.3.0-12 (Fox & Weisberg, 2019). Model assumptions were tested with the DHARMa package v.0.4.6 (Hartig & Lohse, 2022).

■ RESULTS

A total of 888 out of 1040 fresh seeds (85%), and 195 out of 19,509 seeds from herbarium specimens (1%) germinated (suppl. Table S1). Considering only herbarium seeds, germination was observed in 10 species or 12 herbarium specimens, with the age of the germinated seeds ranging from 1906 to 2015 (median = 2011). Noteworthy germinations include a seed of *Silene flos-cuculi* from 1906, a seed of *Astragalus glycyphyllos* from 1957, a seed of *Silene latifolia* from 1962 and a seed of *Chamaenerion angustifolium* subsp. *angustifolium* from 1984. At the family level, germinations occurred in Fabaceae (*Astragalus glycyphyllos*, *Trifolium pratense*), Brassicaceae (*Drabella muralis*, *Lepidium sativum*), Caryophyllaceae (*Cerastium tomentosum*, *Silene flos-cuculi*, *S. latifolia*, *S. vulgaris*), Onagraceae (*Chamaenerion angustifolium* subsp. *angustifolium*), and Lamiaceae (*Salvia pratensis*).

The list of germinated seeds from herbaria is presented in Table 2. For the complete list of ungerminated and germinated seeds, see suppl. Table S1.

Application of exogenous melatonin in the germination medium. — In this experiment, only freshly collected seeds of *Daucus carota* and *Silene latifolia* germinated, plus three seeds of *S. latifolia* from two control replicates of the 2011 herbarium specimen (suppl. Table S1). In *D. carota*, we observed germination only in fresh seeds, and different concentrations of exogenous melatonin had no significant effect on final seed germination. Fresh seeds of *S. latifolia* showed significantly higher germination than seeds from herbaria, and the effect of melatonin concentration differed significantly among seeds from different ages, as higher melatonin concentrations promoted germination in fresh seeds, while they inhibited germination in seeds from herbaria (Table 3).

Seed priming with melatonin. — In this experiment, only freshly collected seeds of *Silene latifolia* (suppl. Table S1) germinated. Priming with melatonin had no significant effect on seed germination (Table 3).

Osmopriming. — In this experiment, 54 seeds were induced to germinate, most from a 2015 specimen of *Drabella muralis* that had a 100% germination rate in all treatments, and from a 2000 specimen of *Trifolium pratense*, in which the effect of osmopriming was more evident but no significant effect of different osmotic potential on germination was observed (Table 3). A single seed of *Chamaenerion angustifolium* (from 1984) and *Cerastium tomentosum* (from 2001) also germinated under control conditions. However, no significant differences were found between treated and untreated seeds. The effect of osmopriming on seed germination was marginally significant (i.e., for *T. pratense* and *Silene latifolia*; see Table 3).

Hydropriming. — Of the 3598 seeds tested with hydropriming for 48 hours, 65 freshly harvested seeds (100% of fresh seeds tested; suppl. Table S1) and 1 seed of *Silene latifolia* from a 1962 herbarium specimen germinated (Table 2). For *Silene latifolia*, seed germination in fresh seeds was significantly higher than in herbarium seeds; however, both GLM and ANOVA showed no significant effect of treatments on seed germination (Table 3). None of the 190 herbarium seeds tested with hydropriming for 24 hours germinated (suppl. Table S1).

Standard soil. — Of the 1410 seeds tested in the soil medium, 6 freshly harvested seeds (20% of the fresh seeds tested) and 1 seed of *Silene flos-cuculi* from a 115-year-old herbarium specimen germinated (Table 2; suppl. Table S1).

Heat disinfection and gibberellic acid addition. — Concerning this test, 135 of the 9712 seeds from herbarium specimens tested germinated (1.39%). Considering only the specimens in which germination was observed, the percentage of germinated seeds was 37% of seeds in the control group, 47% of seeds treated with heat sterilization, and 50% of seeds treated with gibberellic acid (suppl. Table S1).

■ DISCUSSION

Germinating seeds from natural history collections is a promising tool for recovering lost genetic diversity, yet a

Table 2. Complete list of the germination of seeds collected from herbarium specimens, for which are reported the collection year ("Year"), Treatment (C = control; S = sterilization with heat; GA3 = addition of gibberellic acid), number of seeds sown per replica ("Sown"), number of seeds germinated per replica ("Germ"), and germination in per cent ("Germ%").

Species	Year	Germination test	Sown	Germ	Germ%
Exogenous melatonin - EM					
Silene latifolia	2011	C	12	1	8.3
Silene latifolia	2011	C	12	2	16.7
Osmopriming - OP					
Cerastium tomentosum	2001	C	5	1	20
Chamaenerion angustifolium subsp. *angustifolium*	1984	C	5	1	20
Drabella muralis	2015	C	5	5	100
Drabella muralis	2015	C	5	5	100
Drabella muralis	2015	C	5	5	100
Drabella muralis	2015	–0.8 MPa	5	5	100
Drabella muralis	2015	–0.8 MPa	5	5	100
Drabella muralis	2015	–0.8 MPa	5	5	100
Drabella muralis	2015	–1.5 MPa	5	5	100
Drabella muralis	2015	–1.5 MPa	5	5	100
Drabella muralis	2015	–1.5 MPa	5	5	100
Trifolium pratense	2000	C	5	1	20
Trifolium pratense	2000	–0.8 MPa	5	2	40
Trifolium pratense	2000	–0.8 MPa	5	1	20
Trifolium pratense	2000	–1.5 MPa	5	1	20
Trifolium pratense	2000	–1.5 MPa	5	1	20
Trifolium pratense	2000	–1.5 MPa	5	2	40
Hydropriming (48 hours) - HP48					
Silene latifolia Poir.	1962	HP	5	1	20
Soil - SO					
Silene flos-cuculi	1906	Soil	5	1	20
Heat disinfection and gibberellic acid addition - HE					
Astragalus glycyphyllos	1957	S	8	2	25
Astragalus glycyphyllos	1992	C	16	1	6.3
Astragalus glycyphyllos	1992	GA3	18	2	11.1
Astragalus glycyphyllos	1992	S	14	1	7.1
Drabella muralis	2015	C	25	14	56
Drabella muralis	2015	GA3	25	22	88
Drabella muralis	2015	S	25	18	72
Lepidium sativum	2011	C	14	11	78.6
Lepidium sativum	2011	GA3	14	12	85.7
Lepidium sativum	2011	S	13	12	92.3
Salvia pratensis	2015	C	9	5	55.6
Salvia pratensis	2015	GA3	10	6	60
Salvia pratensis	2015	S	10	5	50
Silene vulgaris	2013	C	25	3	12

(Continues)

Table 2. Continued.

Species	Year	Germination test	Sown	Germ	Germ%
Silene vulgaris	2013	GA3	25	5	20
Silene vulgaris	2013	S	25	4	16
Trifolium pratense	2008	C	6	4	66.7
Trifolium pratense	2008	GA3	6	2	33.3
Trifolium pratense	2008	S	7	6	85.7

challenging task. Despite the large number of herbarium seeds sown (19,509), only a low percentage of seeds germinated (1%). Nevertheless, some of the 195 germinations are remarkable. The germination of a seed of *Silene flos-cuculi* collected in 1906, i.e., 115 years old, is an impressive event that sets a new record for the longevity of *Silene* seeds from herbaria. In fact, there are very few cases where old seeds from the Caryophyllaceae family germinated, and in none of these cases were the tested seeds previously stored under herbarium-like conditions (Steiner & Ruckenbauer, 1995; Yashina & al., 2012).

A specimen of *Astragalus glycyphyllos* from 1957 showed a germination rate of 25% of the seeds tested in the heat disinfection and gibberellic acid addition test. For *A. glycyphyllos* and other representatives of the Fabaceae, this could be favoured by the fact that this family is characterised by physical dormancy (Baskin & Baskin, 2014) and therefore their impermeable seed coat might protect them better than other species from adverse environmental conditions (Mohamed-Yasseen & al., 1994). Interestingly, this impermeability leads to a lower susceptibility of the seeds to humidity fluctuations during storage and therefore to a higher stability of the moisture content of the seeds (Hay & al., 2023). Therefore, in this case, the way in which the herbarium specimens were dried is more relevant than the storage conditions to which they were exposed. In addition, the dynamic process of lipid melting and (re)crystallising must be considered. Lipids could solidify (i.e., freeze) at low and ambient storage temperatures, but then fail to melt at the incubation temperature or require more time to melt, delaying germination. Thus, the application of heat prior to incubation might completely melt the lipids and improve seed germination (Crane & al., 2003, 2006).

Germination of old herbarium seeds can also occur in other families that do not have this type of dormancy. For instance, we achieved germination of a seed of *Chamaenerion angustifolium* subsp. *angustifolium* (Onagraceae) from a specimen from 1984 and a seed of *Silene latifolia* (Caryophyllaceae) from a specimen from 1962. Godefroid & al. (2011) succeeded in germinating eight seeds from three herbarium specimens of *Bupleurum tenuissimum* (Apiaceae) that were 101, 125 and 144 years old. In the present study, the germinated seeds belonged to species from 12 different families, which shows that this kind of event, although rare, is possible within many families. The germination of seeds over 50 years old that have been stored in non-optimal conditions is very encouraging and could be a gleam of hope for extinct species, such as *Centaurea tuntasia* Heldr. ex Halácsy (Asteraceae), *Eryngium sarcophyllum* Hook. & Arn. (Apiaceae), *Schiedea amplexicaulis* Mann (Caryophyllaceae) and other candidates of the same family of germinated seeds here reported, some of which are supposed to produce medium or short-lived seeds (see Albani Rocchetti & al., 2022a and references therein). Concerning seed longevity, it should be noted that it is a complex trait that varies greatly between species, and even between seed lots of the same species (Ellis & Roberts, 1980; Priestley & al., 1985; Mondoni & al., 2011). Moreover, the longevity of seeds can be strongly affected by the conditions under which the seeds have been stored (e.g., Liu & al., 2011). A correlation between seed longevity and post-harvest treatments, seed quality parameters (such as germination, vigour, viability, and seed coat permeability) and specific molecular pathways – to name but a few – has been found in the literature (see Hay & al., 2022; Nadarajan & al., 2023; and references therein). However, other herbarium-specific factors such as the way and speed at which the specimens were dried and pressed and/or the specimen preservation treatments (e.g., use of chemicals, freezing and thawing cycles) may affect the longevity of seeds in herbaria. This aspect is evident from our results (suppl. Table S1), e.g., from the osmopriming test, where eight *Trifolium pratense* seeds from 2000 but no seeds from 2005 germinated; from the heat disinfection and gibberellic acid addition test, a single *Chamaenerion angustifolium* subsp. *angustifolium* seed from 1984 germinated, while no seed from 1998 and 2011 germinated; and from the application of exogenous melatonin test, in which three *Silene latifolia* seeds from 2011 but no seeds from 2017 germinated. In these cases, there does not appear to be an age effect, as the younger seeds do not necessarily germinate better than the older seeds (excluding fresh seeds).

The germination of old seeds of genera for which long-lived seeds are not reported, such as *Cerastium* and *Chamaenerion* (19- and 36-year-old seeds, respectively), is a remarkable result of our study that provides further insight into seed physiology.

Maximum germination percentage (100%) was obtained with the *Drabella muralis* (Brassicaceae) specimen from 2015 (5 years old) in the osmopriming test. A high germination percentage (>75%) was also found for the

Lepidium sativum (Brassicaceae) specimen from 2011 (9 years old) in the heat disinfection and gibberellic acid addition test session. Further in-depth studies on these two genera should be done, as the data reported here may be considered a key reference for the extinct Brassicaceae *Lepidium obtusatum* Kirk, *L. amissum* de Lange & Heenan, *L. bidentatum* var. *remyi* (Drake) Fosberg (formerly *L. remyi* Drake) (Albani Rocchetti & al., 2022a), yet not sufficient to assess the longevity of seeds of these genera. Moreover, samples tested here may not be representative of differences that may occur between accessions from different environmental conditions (e.g., climate, altitude) or different populations. In addition, taxonomic and phylogenetic constraints may influence the germination patterns of different species. Germination can be affected by a variety of factors beyond sample age, seed longevity and dormancy, e.g., genetic traits inherited from ancestors and environmental conditions. Obligatory symbiotic seed germination, e.g., in orchids and mycorrhizal fungi, myrmecophilous plants, and endozoochoric species where intestinal digestion and gut passage affect seed germination, are some intriguing examples of additional aspects to consider (Van Leeuwen & al., 2023).

To narrow the range of variables influencing germination tests and obviate many of the constraints listed above, one strategy is to use seeds stored in *ex situ* collections (e.g., seed banks; Godefroid & al., 2010). This method solves the problem of storage under non-ideal and/or unknown conditions, uncertainty about seed maturity, provenance of samples etc., and seeds undergo natural ageing over time (Crawford & al., 2007). On the contrary, these accessions are limited, easily depleted, and not always available for germination tests, as they are primarily collected for the purpose of safeguarding threatened species and are intended for conservation actions (Godefroid & al., 2010). The use of artificially aged specimens can be a valid alternative. Indeed, it is possible to collect a larger amount of material and induce seed ageing by high temperatures and high relative humidity in a short period of time (Probert & al., 2009; Mondoni & al., 2014; Fenollosa & al., 2020; Delouche & Baskin, 2021). On the other hand, few protocols are available, most of which are species-specific (Gianella & al., 2022) and often require further validation to verify the most effective treatment to achieve controlled and repeatable ageing. However, for both *ex situ* accessions and artificially aged seeds, comparative studies are subject to insufficient material, which also affected our study. In this work, the choice to use many common species and to collect seeds from different herbaria was made in order to mitigate this limitation and at the same time create a study that is both comprehensive and informative.

Table 3. Results from ANOVA performed on the binary logistic models on the application of hydropriming (HP), exogenous melatonin in the germination medium (EM), melatonin priming (MP), and osmopriming (OP).

		HP			EM			MP			OP		
Species	Source	χ^2	df	*p*	χ^2	df	*p*	χ^2	df	*p*	χ^2	df	*p*
Silene latifolia	Treatment	1.487	1	0.222	1.940	1	0.163	0.866	1	0.352	9.599	1	**0.002**
	Year	111.707	1	**<0.001**	752.80	3	**<0.001**	85.501	4	**<0.001**	11.569	3	**0.009**
	Treatment*Year	0.000	1	0.999	8.120	3	**0.043**	0.000	4	0.999	0.000	3	0.999
Daucus carota	Treatment	–	–	–	1.980	1	0.159	–	–	–	–	–	–
	Year	–	–	–	1117.980	5	**<0.001**	–	–	–	–	–	–
	Treatment*Year	–	–	–	0.000	5	0.999	–	–	–	–	–	–
Trifolium pratense	Treatment	–	–	–	–	–	–	–	–	–	2.166	1	0.141
	Year	–	–	–	–	–	–	–	–	–	12.082	1	**<0.001**
	Treatment*Year	–	–	–	–	–	–	–	–	–	0.000	1	0.999
Draba muralis	Treatment	–	–	–	–	–	–	–	–	–	0.000	1	0.999
	Year	–	–	–	–	–	–	–	–	–	124.770	1	**<0.001**
	Treatment*Year	–	–	–	–	–	–	–	–	–	0.000	1	0.999
Cerastium tomentosum	Treatment[†]	–	–	–	–	–	–	–	–	–	2.243	1	0.134
	Year	–	–	–	–	–	–	–	–	–	–	–	–
	Treatment*Year	–	–	–	–	–	–	–	–	–	–	–	–
Epilobium angustifolium	Treatment	–	–	–	–	–	–	–	–	–	2.243	1	0.134
	Year	–	–	–	–	–	–	–	–	–	2.243	2	0.326
	Treatment*Year	–	–	–	–	–	–	–	–	–	0.000	2	0.999

Only species that showed germination are reported. Significant values ($p < 0.05$) are shown in bold.
[†] Only one year available in *Cerastium tomentosum*.

Other propagation techniques (e.g., in vitro culture, embryo rescue) applied to seeds from herbaria should also be further investigated as a means to achieve germination of old seeds. In this context, research on crop species offers a variety of techniques that have been tested specifically to improve seed quality and propagation, including the use of hormones, antioxidants, and priming. The application of exogenous melatonin in the germination medium significantly affected the germination percentage of fresh seeds (positively) and seeds from herbaria (negatively). These opposite effects and the only three herbarium seeds that germinated under the effect of melatonin do not allow to generalise the results on the effectiveness of this technique on herbarium seeds, and further tests are needed. The marginal positive effect of osmopriming on seeds from herbaria might be promising, as it slightly increases the germination of *Trifolium pratense* with increasing osmotic potential. Fast water uptake during imbibition in old and dried seeds can cause cell death and tissue damage (Neya & al., 2004; Baskin & Baskin, 2014; Li & al., 2017), while slow imbibition may reduce the level of oxidative DNA damage through the transient activation of the antioxidant response and DNA repair, thus improving seed germination (Balestrazzi & al., 2011). Therefore, it would be interesting to repeat this test with more seeds per replicate. Several studies have shown that melatonin can promote seed germination by increasing the activity of antioxidant enzymes, thereby regulating plant hormones such as abscisic acid and gibberellins (Xiao & al., 2019). Further tests using higher melatonin concentrations at a finer scale are needed, as they could reveal the concentrations that most effectively promote seed germination, especially for seeds stored under suboptimal conditions. Moreover, the application of antioxidants has been reported to be a promising method to promote germination of old and aged seeds (Draganić & Lekić, 2012; Siadat & al., 2012; Xu & al., 2020), and therefore it would be interesting to further investigate this method to carry out other germination studies.

Fast water imbibition may have occurred in the other germination tests, where seeds were soaked in distilled water (24- and 48-hour hydropriming tests), melatonin solutions (exogenous melatonin addition and melatonin priming tests), or on highly hydrated substrates (agar, filter paper, soil). This hypothesis should be further tested for seeds collected in herbaria, as it may vary considerably depending on the species and age of the samples. For instance, Plaza Arregui & Rodríguez Hiraldo (2009) successfully germinated 4-year-old seeds from a herbarium specimen of the formerly locally extinct *Linaria lamarckii* Rouy (now *L. polygalifolia* subsp. *lamarckii* (Rouy) D.A.Sutton) by 24-hour hydropriming.

Our results also suggest that the germination medium should be carefully evaluated and its role in seed germination further analysed, as some of the most notable germinations occurred on different substrates and some germinations of seeds collected from the same specimen differed depending on the substrate (e.g., *Silene flos-cuculi* from 1906 in soil, *Astragalus glycyphyllos* from 1957 on agar).

The next recommended steps for developing germination protocols for seeds from herbaria would be: (i) further, refined germination tests on seeds from herbaria using exogenous melatonin and osmopriming (e.g., more species, more concentrations, shorter and longer exposure times, etc.); (ii) treatment variations, i.e., the development of further treatments to test different combinations of factors; (iii) comparative germination tests between artificially aged seeds and seeds collected from herbaria; (iv) a larger sample size to ensure sufficient replication within each treatment, which would provide data that can both be statistically sounder and take into account intraspecific natural variations in seed germination.

Although the role of herbaria has been increasingly investigated in recent years, there have been few attempts to germinate seeds from herbarium specimens and test their viability, and – until now – no systematic attempt to test different techniques for the development of germination protocols. According to Thiers (2022), there are 3522 active herbaria in the world housing more than 397 million specimens. In terms of conservation, this is a remarkable heritage and a unique source of data, genetic material, tissues, diaspores and more (Funk, 2003). In case there were no collections in seed/genebanks or botanical gardens, herbarium specimens may represent the last *ex situ* collections preserving lost intraspecific genetic variation, distinct evolutionary features, and also viable seeds of globally extinct taxa (EX, sensu IUCN) (Albani Rocchetti & al., 2021). In recent years, the fascinating yet controversial topic of de-extinction has gained increasing attention in the scientific community and in the media. Unlike the definition of "de-extinction" given by the IUCN, i.e., the process of creating a proxy or functional equivalent for an extinct species (IUCN SSC, 2016), what is meant here is the restoration of a "true" extinct plant species (sensu Albani Rocchetti & al., 2022b). It is at least theoretically possible to use diaspores (i.e., seeds and spores) stored in collections other than seed/genebanks, for conservation purposes (Sallon & al., 2008; Abeli & al., 2020; Albani Rocchetti & al., 2022b; Wolkis & al., 2022) and to reverse the current anthropogenic extinction trend. For instance, extinct taxa could be resurrected by germinating or in vitro culturing (e.g., endosperm culture and embryo rescue) diaspores from archaeological sites and natural history collections.

This study helps to demonstrate the feasibility of germinating seeds from herbarium material, providing consistent data on the germination of seeds of common European species collected decades ago. The useful, albeit incomplete, insight into the germination potential of seeds from herbarium collections provided here also aims to be a first step for the development of optimised germination protocols for old seeds and seeds from herbaria, and we hope this study will be an inspiration for further studies and projects, as suggested above. Finally, we emphasize the importance of raising awareness of natural history collections – herbaria in particular – for plant conservation. Improved storage conditions in herbaria that also take into account the preservation of their seeds could

be seen as a cost-effective conservation strategy and further increase the importance of these already extremely valuable "multi-role" collections (Funk, 2003; Abeli & al., 2020; Albani Rocchetti & al., 2021). The last, and in some cases the only, resort to recover lost genetic diversity of threatened or extinct species is through non-conventional *ex situ* collections.

■ AUTHOR CONTRIBUTIONS

GAR and TA designed the research; GAR, LB, TA and AR performed the seed sampling and tests; TA and FP performed the data analysis; AC, GF, GG, SG, IF, LLa, AMo, SO, FS, and AT contributed to seed sampling and/or testing; GAR wrote the manuscript; all these authors, together with GC, MI, Llo, AMa and AV, contributed to manuscript drafting and revision.

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