

Gardenia (Rubiaceae) seed conservation physiology with emphasis on rare Hawaiian species

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Abstract

Background and aims – *Gardenia* species are ecologically, culturally, and economically significant but the three native species of *Gardenia* in Hawai'i are assessed as Critically Endangered. Seed banking is the most cost effective and efficient means of conserving plant material ex situ. To better understand the conservation physiology of Hawaiian and South Pacific *Gardenia* spp. and support their conservation, we asked 1) How do seeds respond to different temperatures and light and dark regimes? 2) What class of dormancy, if any, do seeds exhibit? 3) How does seed germinability respond over time in a seed bank? and 4) What is the conservation status and level of ex situ representation of *Gardenia* globally?

Material and methods – To answer these questions, we used 19 accessions of fresh seeds and seeds stored for varying periods of time in the National Tropical Botanical Garden's Conservation Seed Bank and Laboratory of Hawaiian (*G. brighamii*, *G. remyi*), New Caledonian (*G. aubryi*, *G. oudiepe*), and Tahitian (*G. taitensis*) species. Seeds were incubated at varying temperatures and in light, and in dark.

Key results – We found that (1) seeds of all species tested germinated slowly and only at higher temperatures in the light and dark, (2) seeds have non-deep physiological dormancy, (3) seeds of the Hawaiian species are short lived at conventional seed bank conditions, and (4) only 40% of *Gardenia* spp. are represented in ex situ facilities, and 66% of the species have not been evaluated for the IUCN Red List.

Conclusion – Seeds of Hawaiian *Gardenia* spp. are short lived in storage. Since seeds germinate in darkness, they are unlikely to form a persistent soil seedbank. Although seeds of all species tested are physiologically dormant, they can be easily propagated from seed at warmer temperatures, giving some hope to the conservation and restoration of the Critically Endangered Hawaiian species. Since our dataset was limited by a lack of continuous viability monitoring, we emphasize the need for initial germination testing and ongoing viability tests to better understand seed longevity. Lastly, we discuss the ecological relevance of our results in the context of the Hawaiian archipelago.

Keywords

dormancy, exceptional species, germinability, Hawaii, longevity, recollection intervals, seed banking, threatened species, viability

INTRODUCTION

Plant species are becoming extinct tens to hundreds of times faster than the background extinction rate, making the need for effective plant conservation programs more acute than before (Humphreys et al. 2019). Meanwhile,

the Convention on Biological Diversity developed the Global Strategy for Plant Conservation (GSPC) to help halt the continuing loss of plant diversity (CBD 2011). Within the GSPC is Target 8, which sets the goal of conserving 75% of threatened plant species ex situ. Ex situ conservation strategies include a variety of locations

outside the wild habitat including botanical gardens, seed banks, tissue culture, and cryopreservation. Of these ex situ environments, seed banking is recognized as the most cost-effective and efficient means of ex situ plant conservation (Walsh et al. 2024). Unfortunately, not all species can be conserved using conventional seed-banking methods (i.e. drying seeds within 15–25% relative humidity, hermetically sealing and freezing at -18°C). Wyse et al. (2018) report that 36% of critically endangered (CR) species on the IUCN Red List are considered recalcitrant (i.e. cannot be conserved using conventional methods). In order for seed banking to be successful, seeds must be withdrawn and used before viability falls to an unacceptable level (i.e. 15% (FAO 2014) or 30% (Chau et al. 2019) of initial/maximum viability). Once this threshold is reached, if the continued safeguarding of the maternal genetic line is desirable, seeds must be recollected from the original individual maternal founder/population (preferred) or regenerated (i.e. original seeds propagated and yield deposited into the seed bank) if recollection is not possible (e.g. the maternal founder/population is extinct).

Located over 4,000 km from the nearest continental landmass, the Hawaiian Archipelago is home to 1,367 native vascular plant taxa (Sakai et al. 2002; Wagner et al. 2005), 90% of which are endemic, growing nowhere else on earth. Isolated oceanic islands like the Hawaiian Islands experience high levels of endemism but unfortunately are also subject to high extinction rates (Humphreys et al. 2019). As more oceanic islands like those in Hawai'i are subject to invasive species, habitat degradation, new plant and pollinator diseases, as well as climate change, urgent conservation measures are critical to reverse this trend of species losses (Rønsted et al. 2023).

The Critically Endangered Hawaiian taxa include three native *Gardenia* J.Ellis: *Gardenia brighamii* H.Mann (Fig. 1A–B), endemic to the dry-mesic forests of Hawai'i, Maui, Moloka'i, O'ahu, and Lāna'i Islands; *Gardenia mannii* H.St.John & Kuykendall, endemic to mesic-wet forests on the island of O'ahu; and *Gardenia remyi* H.Mann (Fig. 1C–F) endemic to Kaua'i, Moloka'i, Maui, and Hawai'i Islands. Hawaiian *Gardenia* are famous for their fragrant solitary white flowers and spherical fruits usually filled with orange yellow or cream pulp (Fig. 1).

Globally, *Gardenia* species are native to the tropics and subtropics from Africa through the Indo-Pacific region (Fig. 2). They are described as possessing some of the most attractive and fragrant flowers of tropical flora with subsequently produced seeds protected by 'woody' endocarps (Troup 1921). Healthy mature fruits can possess hundreds of seeds.

Critically Endangered Hawaiian *Gardenia* spp. demonstrate the need for improved understanding of seed biology

Hawaiian *Gardenia* species are at risk of going extinct. The most recent IUCN Red List assessments for the three

Hawaiian endemic *Gardenia* species designate them as Critically Endangered (CR) (World Conservation Monitoring Centre 1998; Bruegman and Caraway 2003; Grave et al. 2020), with less than 100 mature individuals in the wild. This represents three out of eight (38%) *Gardenia* identified as CR on the IUCN Red List. These three Hawaiian species are identified as Exceptional Species (Pence et al. 2022a) and are considered species of focus by the Hawai'i Plant Extinction Prevention Program (<http://www.pepphi.org/>) as having important connections to Hawai'i's natural and cultural heritage. While the 5-year conservation updates of Hawaiian *Gardenia* by the US Fish and Wildlife Service (USFWS) on Endangered Species do not outline germination data, they recommend "continued collection of genetic resources for storage, propagation, and reintroduction. The USFWS further expands this recommendation to include an evaluation of genetic resources currently in storage. This evaluation will determine the need to place additional genetic resources into long-term storage due to the species' vulnerability to climate change" (USFWS 2021).

In a recent study estimating ex situ seed longevity of 295 native Hawaiian species, Chau et al. (2019) recommends a recollection threshold of 70% of initial or maximum viability for the Hawaiian flora. Based on the data available at that time, this threshold was reached in 5–10 years for *G. brighamii*, and was not yet reached after 1 year for *G. mannii* (*G. remyi* was not assessed). "Freeze-sensitivity" (i.e. the phenomenon of seeds being desiccation tolerant yet viability declining faster at -18°C compared to 5°C) was observed at the family level (Chau et al. 2019). Globally, 100% (36 species) of *Gardenia* spp. with available storage behaviour information are listed as having some form of orthodox (i.e. able to be conserved by conventional methods) storage behaviour (SER-INSR and RBGK 2023). Clearly, more work is necessary to fully elucidate the ex situ longevity and storage behaviour of Hawaiian *Gardenia* seeds.

Seed banking and restoration efforts from seeds are often impeded by a lack of a priori knowledge of seed dormancy-breaking and germination requirements (Kildisheva et al. 2020). For Hawaiian *Gardenia*, Baskin et al. (2021) found that seeds of *G. brighamii* had physiological dormancy (i.e. seeds are water permeable, embryos are fully developed, and an environmental signal is needed to elicit germination). In eight other species of *Gardenia* globally, Baskin and Baskin (2014; Supplementary material 2) report that all have or are inferred to have physiological dormancy or be non-dormant. Further, *Gardenia* species with non-dormant seeds were found in either tropical evergreen forest (the vegetation zone of Hawaiian *Gardenia*) or moist warm temperature woodlands, while *Gardenia* spp. with physiological dormancy occurred only in the former vegetation zone/region/habitat (Baskin et al. 2021). Knowing the classification of dormancy and germination conditions for the remaining two Hawaiian *Gardenia*

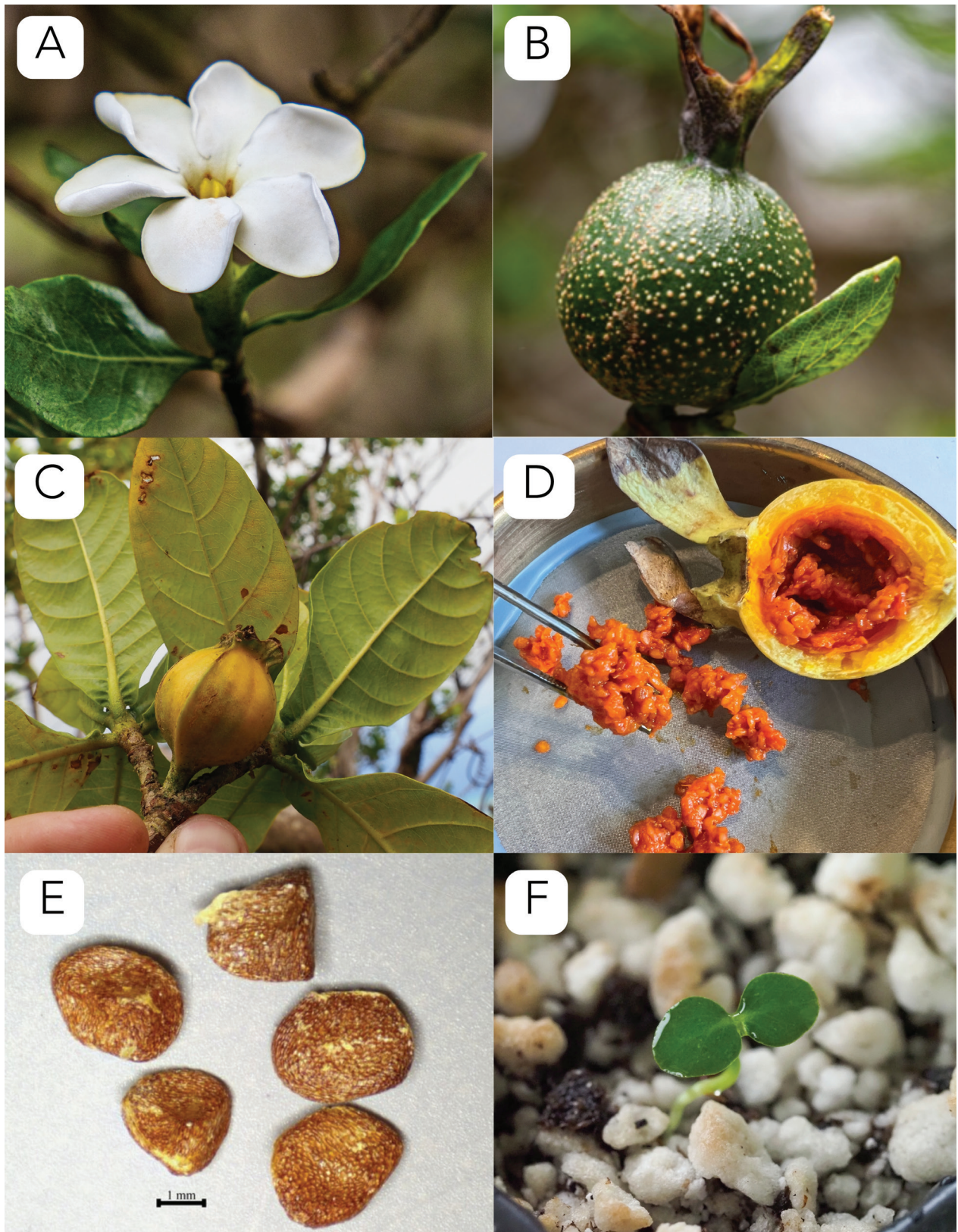


Figure 1. Hawaiian *Gardenia*. A. Flower of *G. brighamii* at Kānepeʻu, Lānaʻi. B. Immature fruit of *G. brighamii* at Kānepeʻu, characterized by hard fruits with green surface and nodular texture. C. Maturing fruit from *G. remyi* at Kaluaʻaha, Molokaʻi, fruit texture is fleshy and compressible by hand. D. Removing seeds from fruit pulp of *G. remyi* to prepare for sowing and/or storage. Mesocarp is amber, with the endocarp displaying deep orange pigment with over 100 seeds inside. E. Cleaned *G. remyi* seed profile when pulled out of storage prior to sowing. F. Healthy seedling of *G. remyi* (#20150377) eight weeks after sowing collected from Hūleʻia, Kauaʻi.

species will help improve conservation and restoration success.

In the present study, with respect to Pacific Island *Gardenia* spp., we ask (1) How do seeds respond to different temperatures and light regimes? (2) What class of dormancy, if any, do seeds exhibit? (3) How does seed germinability respond over time in a seed bank? and (4) What is the conservation status and level of ex situ representation of *Gardenia* globally? By answering these questions, we aim to inform conservation and restoration practitioners, thereby improving the conservation outlook of these rare species.

MATERIAL AND METHODS

In total, 19 accessions of five species collected between 1992 and 2023 were included in the 48 accession experiment combinations in the present study (Table 1). Seeds were collected from New Caledonia and in Hawai'i from Kaua'i, Lāna'i, and Moloka'i Islands.

Seed source

Of the three native Hawaiian *Gardenia* spp., *G. brighamii*, and *G. remyi* seeds were used in the present study (no seeds or living plants of *G. mannii* existed at NTBG Seed Bank (National Tropical Botanical Garden, Hawai'i) and Laboratory or gardens at the time this manuscript was written). *G. brighamii* fruits were collected from Kaua'i and Lāna'i Islands between May 1992 and December 2016. Seeds of *G. remyi* were collected from Kaua'i and

Moloka'i Islands between September 2008 and June 2023. Seeds of *Gardenia aubryi* Vieill and *G. oudiepe* Vieill were collected in New Caledonia in June 1998. *Gardenia taitensis* DC. seeds were collected from a private property on Kaua'i Island in February 2023 (Supplementary material 1). All seeds were manually extracted from the fruit. Prior to 2008, seeds were stored at a target relative humidity (RH) of 20% at ambient indoor temperature in non-hermetic containers (usually paper coin envelopes). After 2008, seeds were hermetically sealed inside trilaminar aluminium foil pouches and stored at 5°C (freeze sensitivity has been observed in other genera of Hawaiian Rubiaceae (Chau et al. 2019), thus a storage temperature of 5°C is recommended). All seeds were withdrawn from the NTBG Seed Bank for this study (see accession numbers and further details in Supplementary material 1).

Effects of temperature and light

To determine the temperature and light/dark requirements for germination, three previously stored *G. remyi* accessions (#080811, #120384, #20150613) and one freshly harvested (26 days since collection) *G. taitensis* accession (#20230068) were used (Supplementary material 1). Three replicates of 15 seeds each per germination condition (except for *G. taitensis* where 2 replicates were used for the 20/10°C dark) were incubated in either light (12 h light/12 h dark) or dark (Petri dishes wrapped in aluminium foil) with alternating daily temperature regimes of 15/5°C, 20/10°C, 25/15°C, and 30/20°C. The light was generated by fluorescent tubes, and the illuminance at the seed level

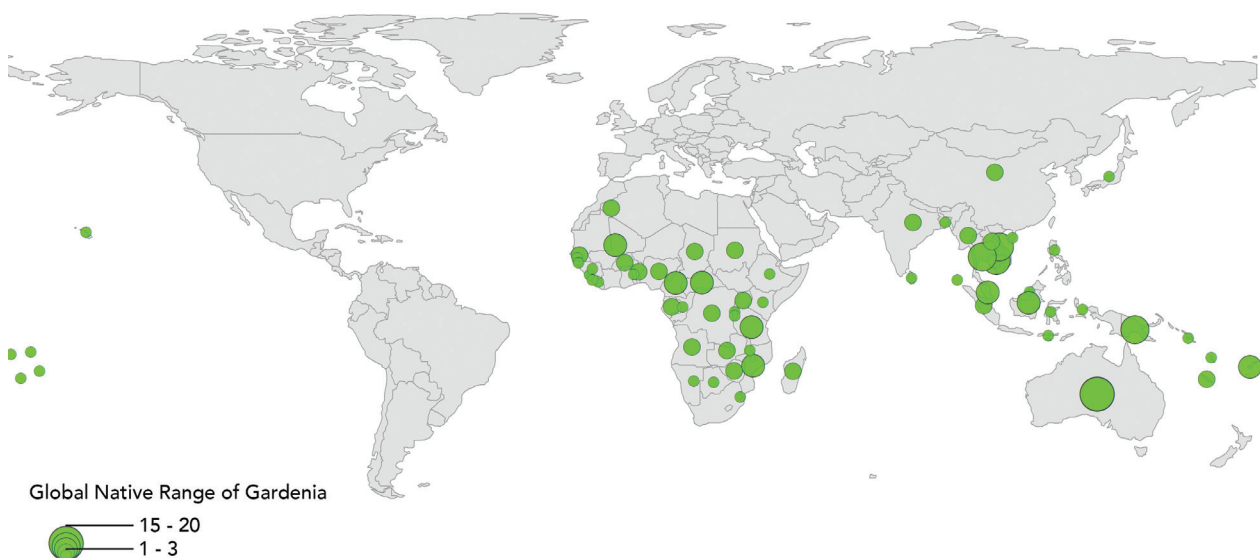


Figure 2. Global native range of *Gardenia*. There are currently 128 recognized species of *Gardenia*, with diversity centres in Australia, Borneo, Cambodia, Central African Republic, Fiji, Papua New Guinea, Thailand, and Vietnam, all of which are home to eight or more native *Gardenia*. Map created with ArcGIS Desktop v.10.4 (ESRI 2024) by Ben Nyberg, National Tropical Botanical Garden, Hawai'i.

Table 1. Summary of mean and standard deviation germinated for each accession and experiment. NTBG: National Tropical Botanical Garden, Hawai'i.

NTBG accession number	Taxon	Experiment	Mean (s.d.) proportion germinated
980618	<i>Gardenia aubryi</i>	25 years old	0(0)
980673	<i>Gardenia aubryi</i>	25 years old	0(0)
010250	<i>Gardenia brighamii</i>	22 years old	0.92(0)
060803	<i>Gardenia brighamii</i>	20 years old	0.16(0.15)
120542	<i>Gardenia brighamii</i>	5 years old	0.22(0.19)
130066	<i>Gardenia brighamii</i>	10 years old	0.6(0.1)
130066	<i>Gardenia brighamii</i>	5 years old	0.71(0.1)
20160848	<i>Gardenia brighamii</i>	5 years old	0.03(0.06)
20160848	<i>Gardenia brighamii</i>	immature - no soak	0(0)
20160848	<i>Gardenia brighamii</i>	immature - soaked in 2% PPM 17 h	0(0)
20160848	<i>Gardenia brighamii</i>	mature - soaked in 2% PPM 17 h	0.79(0.16)
20160848	<i>Gardenia brighamii</i>	mature - no soak	0.91(0.01)
900569	<i>Gardenia brighamii</i>	31 years old	0(0)
920714	<i>Gardenia brighamii</i>	31 years old	0(0)
950116	<i>Gardenia brighamii</i>	28 years old	0(0)
980625	<i>Gardenia oudiepe</i>	25 years old	0(0)
080811	<i>Gardenia remyi</i>	15/5 light	0(0)
080811	<i>Gardenia remyi</i>	20/10 light	0(0)
080811	<i>Gardenia remyi</i>	25/15 dark	0(0)
080811	<i>Gardenia remyi</i>	25/15 light	0(0)
080811	<i>Gardenia remyi</i>	30/20 light	0(0)
080811	<i>Gardenia remyi</i>	8 years old	0.94(0.12)
080811	<i>Gardenia remyi</i>	heat shock	0(0)
080811	<i>Gardenia remyi</i>	sterile agar	0(0)
080811	<i>Gardenia remyi</i>	surgery	0(0)
120384	<i>Gardenia remyi</i>	15/5 light	0(0)
120384	<i>Gardenia remyi</i>	20/10 light	0(0)
120384	<i>Gardenia remyi</i>	25/15 dark	0(0)
120384	<i>Gardenia remyi</i>	25/15 light	0(0)
120384	<i>Gardenia remyi</i>	30/20 light	0(0)
120384	<i>Gardenia remyi</i>	4 years old	0.73(0.06)
120384	<i>Gardenia remyi</i>	5 years old	0.43(0.1)
120384	<i>Gardenia remyi</i>	heat shock	0(0)
120384	<i>Gardenia remyi</i>	sterile agar	0(0)
120384	<i>Gardenia remyi</i>	surgery	0(0)
20150377	<i>Gardenia remyi</i>	8 years old	0.16(0.08)
20150613	<i>Gardenia remyi</i>	25/15 dark	0.8(0.07)
20150613	<i>Gardenia remyi</i>	25/15 light	0.73(0)
20230237	<i>Gardenia remyi</i>	initial	1(NA)
20230238	<i>Gardenia remyi</i>	initial	1(NA)

Table 1 (continued). Summary of mean and standard deviation germinated for each accession and experiment.

NTBG accession number	Taxon	Experiment	Mean (s.d.) proportion germinated
20230257	<i>Gardenia remyi</i>	initial	0.84(0.1)
20230068	<i>Gardenia taitensis</i>	15/5 dark	0(0)
20230068	<i>Gardenia taitensis</i>	15/5 light	0(0)
20230068	<i>Gardenia taitensis</i>	20/10 dark	0(0)
20230068	<i>Gardenia taitensis</i>	20/10 light	0.02(0.04)
20230068	<i>Gardenia taitensis</i>	25/15 light	0.98(0.04)
20230068	<i>Gardenia taitensis</i>	30/20 light	0.98(0.04)

was about 300 lux. See the “Germination” section below for details on how seeds were germinated.

Effects of sterile conditions

Two accessions of *G. remyi* (#080811, #120384) seeds were sterilized (Supplementary material 1) by soaking them in a solution of 1% sodium hypochlorite (NaOCl) in distilled water for 1 min. All seeds were then rinsed by soaking in three consecutive baths of sterilized distilled water for 30 s each. Five replicates of 20 seeds each were sown in 60 mm Petri dishes on 1% agar with the addition of 0.002% Plant Preservative Mixture (PPM; Plant Cell Technology, Washington, D.C., USA) to inhibit fungal growth without affecting germination (Assaf Guri, Plant Cell Technology, pers. comm.) and sealed with plastic paraffin film to exclude external contamination. Seeds were incubated at a 25/15°C thermoperiod and 12/12 h photoperiod since this was the optimal reported temperature for the sister taxon *G. brighamii* (Baskin et al. 2021). Seeds used as the control were those from the same two accessions and incubated in the same conditions (25/15°C; 12/12 h light) as described in the temperature experiment above.

Maturity and pathogen abatement

Mature and immature fruits of one accession (#20160848) of *G. brighamii* collected on 20 Dec. 2016 from NTBG’s McBryde Garden was used to assess the effects of maturity on germination, as well as to assess the effects of PPM on pathogen abatement and viability. Seeds were held at ambient temperature and humidity until experiments started 23–27 Dec. 2016. Three replicates of 30–33 seeds each were used in the following treatments; immature (seed arising from immature fruit), immature + PPM (17-hour soak in a solution of 2% PPM in distilled water), and two replicates of 25 and 30 seeds each of mature (seeds arising from mature fruit), mature + PPM (Supplementary material 1).

Heat shock

For some species, desiccation tolerant seeds lose viability more rapidly than expected at conventional storage temperatures. The incomplete melting of fatty acids upon retrieval from cold storage could be a source of damage during seed imbibition, and brief exposure to high temperatures (“heat shock”) could melt lipids that would otherwise be frozen at incubation temperatures (Crane et al. 2003; Volk et al. 2006). We therefore performed a “heat shock” treatment in an attempt to “resurrect” previously stored seeds that did not germinate to test whether this phenomenon was occurring in *Gardenia* seeds. Three replicates of 15 seeds each per germination condition of two accessions of *G. remyi* seeds (#080811, #120384) were placed in 60 mm Petri dishes on seed germination paper and placed above a saturated potassium sulphate solution in a sealed chamber (~98% RH) overnight to avoid imbibition damage (Table 1). Seeds were then placed in a 45°C oven (Quincy Lab, Inc Model 20 Lab Oven, Illinois, US) for approximately 15 h and then wetted with 0.1% PPM in distilled water, sealed with plastic paraffin film, and finally placed in the 25/15°C germination chamber.

Surgical treatment

In some species with physiological dormancy, low growth potential, or push power, of the embryo plays an important role in delaying germination (Baskin and Baskin 2014). In an attempt to “resurrect” previously stored seeds that did not germinate and to test whether this phenomenon was occurring in *Gardenia* seeds, we performed a surgical treatment to reduce the push power needed for radical emergence. Three replicates of 15 seeds each per germination condition of two accessions of *G. remyi* (#080811, #120384) seeds were withdrawn from long-term cool storage and placed under a dissecting scope. A portion of the seed coat was surgically removed approximately where the radicle was expected to emerge, so as to reduce the push power required for radicle emergence. Seeds were then placed in 60 mm Petri dishes on seed germination paper and placed above a saturated potassium sulphate solution (achieving a RH of ~98%)

in a sealed chamber for approximately 8 h, then wetted with 0.1% PPM in distilled water, and then sealed with parafilm and placed in the 25/15°C germination chamber.

Germination

Seeds removed from storage as well as fresh collections were placed in 60 mm Petri dishes on seed germination paper (Anchor Paper Company, St. Paul, MN, USA) and placed above a saturated potassium sulphate solution in a sealed chamber (achieving an RH of ~98% at 25°C) overnight to avoid imbibition damage (for previously stored seeds unless indicated above). Following this step, seeds and germination paper were wetted with 0.1% PPM. Dishes were then sealed with plastic paraffin film to prevent excessive water loss. Seeds were exposed to daily alternating regimes of 12 h of light (~41 mmol/m²/s cool white [4100 K] fluorescent light) and 12 h of dark at corresponding temperatures of 25/15°C in a germination chamber (GR36L, Percival Scientific, Perry, IA, USA). Germination was defined as radicle emergence, and the proportion germination was calculated by dividing the number of seeds germinated by the number of seeds sown.

Global *Gardenia* summary

In order to place Hawaiian and Pacific *Gardenia* germination data into context of the larger genus, we identify three primary sources that constitute the majority of published *Gardenia* studies to date. First, the Seed Information Database (SER-INSR and RBGK 2023) is a compilation of seed biological trait data, with records derived from measurements and observations on seed collections at RBGK Millennium Seed Bank and from other unpublished and published sources. Second, Baskin and Baskin (2014), which serves as a comprehensive source for seed dormancy data, identified ten different species of *Gardenia* with germination information. Finally, a recent study of 295 Hawaiian plant species (Chau et al. 2019) investigating storage conditions of the seeds is the most comprehensive publication on Hawaiian *Gardenia*.

Some of the global *Gardenia* taxa listed in the Seed Information Database were synonyms of other accepted species names according to the Plants of the World Online (POWO 2024) and the World Flora Online (WFO 2024), which integrates information from regional floras and other online databases. We followed the POWO checklist for *Gardenia* and resolved synonyms with WFO, except for *G. petiolata*, which may require further taxonomic study as it occurs out of the region of the genus. *Gardenia ambanjensis* Humbert ex Rakoton. & A.P.Davis, was resolved as a synonym of *Gardenia brevicalyx* Rakoton. & A.P.Davis and *Gardenia savannensis* Rakoton. & A.P.Davis is an unpublished name, derived from *Gardenia rutenbergiana* (Baill. ex Vatke) J.-F.Leroy, both occurring in Madagascar. In addition, *Gardenia turgida* (Roxb.) Tirveng. was resolved as a synonym of *Ceriscoides turgida*

(Roxb.) Tirveng., occurring on the Indian Subcontinent to Indo-China.

Environmental variables

Mean monthly temperature and mean annual temperature data for each Hawaiian accession that included latitude and longitude were harvested from the Online Climate Atlas of Hawai'i (Giambelluca et al. 2014).

Statistical analysis

The software RStudio (Posit Team 2023) and environment R v.2023.06 (R Core Team 2023) were used for all statistical analyses, and the package ggplot2 v.2023.06 was used for construction of graphs (Wickham 2016). Means (\pm s.d.) are presented. When two different treatments were compared a Welch two sample t-test was performed ($\alpha = 0.05$).

RESULTS

Longevity

For the two New Caledonian *Gardenia* species stored for 25 years, seeds neither germinated from *G. aubryi* (#980618, #980673) nor from *G. oudiepe* (#980625) (Table 1). Since no previous germination tests were performed, it is unclear when germinability fell to 0 or the rate of decline.

For the eight tested accessions of *G. brighamii* stored for 0–31 years, germination ranged from 0–92%. In one accession stored for five years with no other longevity tests (#120542), mean germination was 22% (\pm 19). Only two *G. brighamii* accessions had more than one test. For #130066, germination declined from 71% (\pm 10) at year 5 to 60% (\pm 10) at year 10, but the difference between them was not significant ($p = 0.2488$). In #20160848, the highest initial viability test was 91% (\pm 10), and declined to 3% (\pm 6) at year 5. For the remaining 5 accessions stored for 20–31 years germination occurred in only a single accession (#010250; 22 years old), and was, remarkably, 92% (\pm 0) (Table 1). Again, no previous germination tests were performed, thus it is impossible to determine when germinability fell to 0 or indeed if seeds were ever germinable to begin with.

In one accession of *G. remyi* (#080811), mean germination was quite high after 8 years at 92% (\pm 12). However, at year 14, no seeds germinated (including those used in other experiments, see below) (Table 1). Similarly, for #120384, mean germination was 73% (\pm 6) at year 4 but declined to 43% (\pm 10) at year 5, then at year 10, no seeds germinated (including those used in other experiments, see below). One other accession (#20150377) had one test at year 8 with 16% (\pm 8) mean germination (Table 1). As with the earlier mentioned species, no initial germination tests were performed on these three accessions. However,

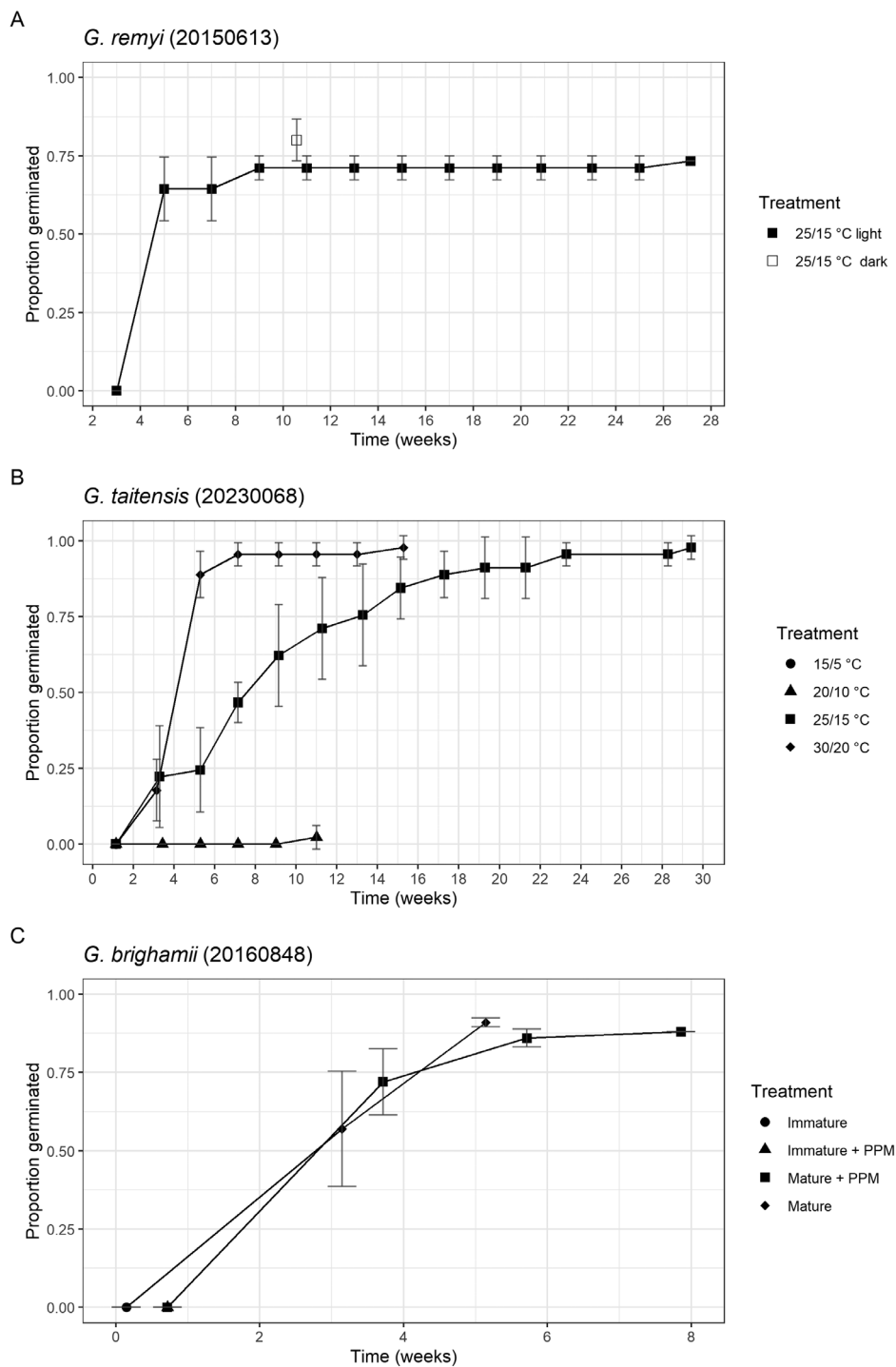


Figure 3. Germination time courses with mean proportion germinated, and \pm standard deviation (error bars). **A.** *Gardenia remyi* (#20150613) used in the light requirement experiment. The dark treatment was incubated at 25/15°C in constant darkness (wrapped in aluminium foil) and unopened until 74 days had elapsed since sowing. The light treatment was incubated at 25/15°C and exposed to a daily alternating photoperiod of 12 h light/ 12 h dark. There was no significant difference in mean maximum germination between the two treatments ($p = 0.2254$). **B.** *Gardenia taitensis* (#20230068) used in the temperature requirements experiment. Mean proportion germinated \pm standard deviation (error bars) is presented. The four treatments shown were incubated in a daily alternating photoperiod of 12 h light/ 12 h dark. No seeds germinated at 15/5°C. Mean maximum germination was equivalent at 25/15 and 30/20°C ($p = 1.0000$). **C.** Effects of maturity and plant preplant preservative mixture (PPM) on germination of *Gardenia brighamii* (#20160848). Mean proportion germinated \pm standard deviation (error bars) is presented. No seeds germinated from immature fruits. There was no significant difference in mean maximum germination between the two mature treatments ($p = 0.3169$).

in three freshly collected accessions, mean germination ranged from 84% (± 10 ; #20230257; three replicates) to 100% (#20230237, #20230238; no replication). One other historic accession of *G. remyi* (#20150613; 8 years old) was tested for a light requirement and germinated to 73–80% (see below section for details) (Supplementary material 1). Although we did not have the data to model longevity specifically, the steepest decline in germinability occurred in one accession of *G. remyi* (#080811) which demonstrated 94% germination at year 8 to 0% at year 14.

Seeds neither germinated from the surgical treatment experiment, nor from the sterile condition experiment, nor from the heat shock experiment. Unbeknown to us, germinability of the seeds used in these experiments had already decreased to zero by the time the experiments were begun.

Effects of temperature and light

For one accession of *G. remyi* (#20150613) stored for 8 years then incubated at 25/15°C, germination in light (12 h light/12 h dark) was observed to have begun between weeks 3 and 5, and maximum germination was observed at week 27 (the seeds exposed to constant darkness was left unopened until after week eleven). Mean maximum germination was 73% (± 0) and 80% (± 7) in light and dark, respectively, with no significant difference between them ($p = 0.2254$; Fig. 3A; Table 1). However, for two accessions of *G. remyi* stored for 10 (#120384) and 14

(#080811) years, viability had already declined to zero and thus no results were obtained for the effects of temperature on germination for those accessions (Table 1).

For *Gardenia taitensis* (#20230068) collected in February 2023, no seeds germinated at 15/5°C regardless of light regime. Similarly, only one seed germinated (in light) for seeds sown in light and dark at 20/10°C. For seeds incubated at 25/15°C and 30/20°C, 50% germination was reached at between weeks 7–9 and 3–5, respectively, and had concluded by weeks 30 and 15, respectively. Mean germination in light at 25/15°C and 30/20°C at week 5 was 24% (± 14) and 89% (± 8), respectively with a significant difference between them ($p = 0.0052$), and mean maximum germination were both 98% (± 4) (Fig. 3B; Table 1), with no significant difference between them ($p = 1.000$). Unfortunately, the dark-treatment Petri-dishes wrapped in aluminium foil at the higher temperatures of 25/15 and 30/20°C were opened during the course of the experiment, and thus omitted from the experiment.

Maturity and pathogen abatement

No seeds germinated from *G. brighamii* immature fruits. Germination from *G. brighamii* seeds arising from mature fruit ranged from 79% (± 16 ; mature + PPM) to 91% (± 1 ; mature), and was not significantly different ($p = 0.3169$) according to a Welch Two Sample t-test (Fig. 3C).

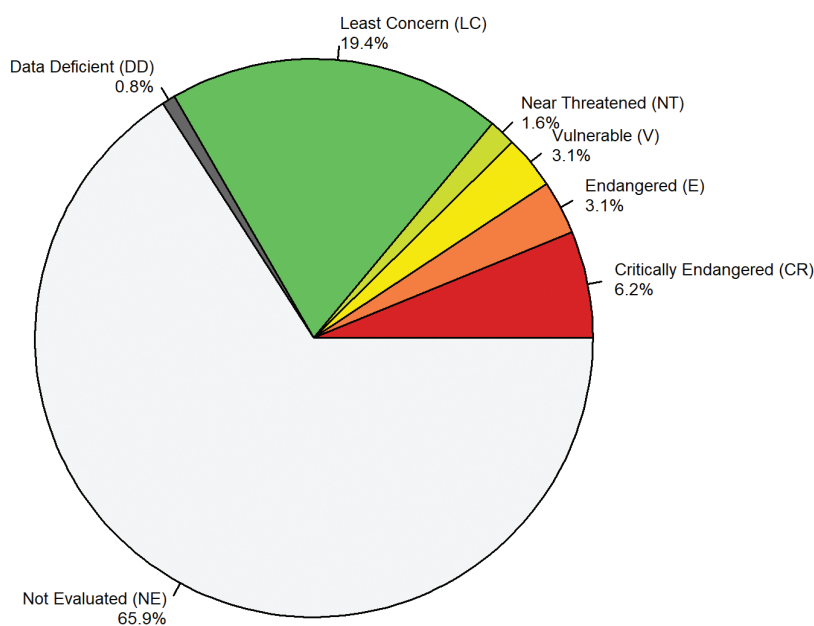


Figure 4. IUCN Conservation status for 128 global *Gardenia* spp. Eight (6.2%) are Critically Endangered, four (3.1%) are Endangered, four (3.1%) are Vulnerable, two (1.6%) are Near Threatened, 25 are of Least Concern, and one is Data Deficient. The remaining 84 taxa are Not Evaluated (NE) (IUCN 2023).

Global synthesis of *Gardenia* seed conservation physiology

The World Flora Online (WFO 2024) recognizes 128 species of *Gardenia* with all species being native to the African, Asian, and Pacific tropics and subtropics (Fig. 2). Of those, 43 species (34%) are represented on the IUCN Red List of Threatened Species. Their respective conservation status includes eight Critically Endangered, four Endangered, four Vulnerable, two Near Threatened, 25 of Least Concern, and one as Data Deficient. The remaining 85 taxa are Not Evaluated (NE) (IUCN 2023) (Fig. 4; Supplementary material 3).

The Botanical Garden Conservation International (BGCI) PlantSearch tool for accessing and sharing information about living collections at ex situ facilities around the globe have records for 51 species (40%) of *Gardenia*, not including hybrids, subspecies, and synonyms. Of these 51 species, 27 (21%) are represented at a single ex situ site (BGCI 2023).

Of the 15 species (12%) of *Gardenia* with reported seed storage behaviour in the Seed Information Database, 100% of them are reported as some form of orthodox (orthodox, orthodox?, or orthodox p), i.e. no recalcitrance or intermediate storage behaviour is reported (SER-INSR and RBGK 2023). The Seed Information Database reports data on germination conditions for 17 species (SER-INSR and RBGK 2023) and Baskin and Baskin (2014) report data for 9 species of *Gardenia*. Together with one additional species (*G. brighamii*) from Baskin et al. (2021), this represents 20% (25 out of 128 species) of the species of *Gardenia*.

DISCUSSION

Longevity

Longevity of the study species and accessions varied widely. This is not surprising, since the rate of decline as well as the theoretical initial viability are collection level traits (Hay et al. 2022). Although no germination was observed from the seeds of the three New Caledonian accessions (*G. aubryi* and *G. oudiepe*), there are NTBG living collection plantouts, indicating that the seeds once had germinability > 0%. Unfortunately, this was the very first time these accessions had been systematically tested and therefore it is impossible to determine the rate of decline. In three *G. brighamii* accessions, the germinability after five years ranged widely (3–71%). In five accessions, stored for 20–31 years, germination occurred in only a single accession that was 22 years old, and was, remarkably, 92%.

With the exception of three accessions having one previous test each, previous germination tests were never performed. Thus, it is impossible to determine when germinability declined to p_{70} (the recollection interval suggested by Chau et al. (2019) for Hawaiian species

defined as a percent of initial/maximum germination), triggering the need for recollection (or regeneration) or indeed if the seeds were ever germinable. Still, one result of high viability after 22 years is remarkable, and this accession represents the oldest known Hawaiian *Gardenia* species that has not yet declined past its recollection threshold. If the maternal founder of this remarkable accession still exists, it could confer high longevity to its offspring making it a high priority for recollection if seeds are harvested at the time of natural dispersal (Pedrini et al. 2020) and kept cool and dry until submitted to a seed bank (FAO 2014; CPC 2019).

The trait of seed longevity can vary with seed maturity (Hay and Probert 1995; Hay et al. 1997), population (Kochanek et al. 2009; Long et al. 2015), and genotype (Pritchard and Dickie 2003; Long et al. 2015), as well as the maternal plant environment (Kochanek et al. 2009). Therefore, it is not possible to determine the causes of decline (or lack thereof) in the present study. More research is needed to understand longevity in *Gardenia* globally and in other wild native Hawaiian plant species.

The pattern of seeds stored cool having higher longevity compared to seeds stored in subfreezing temperatures as observed in Hawaiian *Gardenia* (see Chau et al. 2019) was also observed in a rare species of *Gardenia* from Queensland Australia, *G. actinocarpa* Puttock, where seeds stored at 4°C declined to a 50% loss of viability in 7–8 months compared to seeds stored at < 0°C that had 0% germination after the first check at three months (Osunkoya and Swanborough 2001). Although this pattern was similar, the rate of decline of *G. actinocarpa* appears to be on a wholly different scale-losing viability in a matter of months compared to years in Hawaiian *Gardenia*. However, it is unclear if the Australian *G. actinocarpa* was desiccated before being placed in its respective storage temperatures which could explain why 4°C storage maintained 50% viability for six months and why < 0°C storage resulted in complete seed death upon the first check after three months.

One measure of longevity that is biologically meaningful and intuitive is p_{50} , defined as the time for viability to decline to 50% (Hay et al. 2022). In an Australian continental-scale study of seed longevity in experimental storage, Merritt et al. (2014) found that seeds sourced from regions of higher mean annual temperature (MAT) and rainfall had significantly higher p_{50} than seeds from cooler and drier regions. In the present study, MAT at the collection site ranged from 18.37 (*G. remyi* #20230257) to 23.21°C (*G. brighamii* #20160848), but how these factors affect longevity of the accessions is unknown and further study is warranted. In a study of ca 42,000 species stored real-time in genebanks, Walters et al. (2005) found no correlation with p_{50} and soil persistence. Therefore, in order to understand the longevity of *Gardenia* soil seed banks, burial studies should be performed. While using recollection thresholds such as p_{85} (FAO 2014) or p_{70} (Chau et al. 2019) are inappropriate for comparisons of species, seed lots, or treatments, p_{50} is appropriate for

comparative purposes (Hay et al. 2022). To understand the longevity trait fully, either during storage or in soil seed bank studies, seed survival curves and p_{50} should be investigated.

Effects of temperature and light

Gardenia taitensis seeds experienced little to no germination ($\leq 0.02\%$) in light at the cooler temperatures of 15/5 and 20/10°C, but they germinated to 98% in light at both 25/15 and 30/20°C. Thus, *G. taitensis* seeds appear to require these warmer temperature regimes for germination to occur. In a similar experiment that included *G. brighamii*, the optimal temperature regime for germination to occur was 25/15°C but 30/20°C was not tested (Baskin et al. 2021). While we were not able to gather data on the effects of temperature on germination of *G. remyi* seeds, one accession of the species germinated to 94% at 25/15°C (the only temperature tested). Interestingly, the collection location for the one accession of *G. brighamii* with GPS data was a location with a mean annual temperature (23.21°C) higher than the hottest month (September) at all six *G. remyi* collection locations for which we have GPS data (20.14–21.37°C) as well as the *G. taitensis* collection location (22.59°C). Thus, in addition to testing the effects of temperature on *G. remyi* seed germination, comparing the effects of 30/20 vs 25/15°C for *G. brighamii* is warranted. The initial temperature of seed germination (T_{\min}) was found to be strongly negatively correlated with habitat temperature and that T_{\min} – mean annual temperature (MAT) provided insights into species distribution patterns and thus T_{\min} should be included in species distribution models (Rosbakh and Poschlod 2015). Although T_{\min} was not specifically assessed, *G. brighamii* seeds germinated at 15/6°C (Baskin et al. 2021) and in the present study, T_{\min} was 25/15°C for *G. taitensis*. In a landscape-based assessment of climate change vulnerability for all native Hawaiian plant species, Fortini et al. (2013) used current and future climate envelopes for each species to model response to climate change and assigned each species a vulnerability index score. However, T_{\min} was not a factor in that analysis. Although much is known about T_{\min} in the Hawaiian flora (e.g. Baskin and Baskin 2014, and references therein; Wolkis et al. 2018, 2022, 2023; Baskin et al. 2020, 2021; Hawai'i Seed Bank Partnership (<https://laukahi.org/hawai%CA%BBi-seed-bank-partnership/>) unpublished data), T_{\min} should be continued to be investigated, and used in future models of vulnerability under climate change in Hawai'i.

There was no significant difference in one 8-year-old accession of *G. remyi* seeds incubated at 25/15°C in the light vs dark. Thus, there is no light requirement for the germination of this species. The germination of seeds in continuous darkness suggests that *G. remyi* is unlikely to form a long-lasting soil seed bank; however, more studies are needed to determine whether this is the case in their natural habitat. If the seeds germinate while buried, this

would lead to the loss of a viable soil seed bank. Therefore, the re-establishment of extirpated populations will require reintroduction. After the germination experiment for *G. taitensis* had ended, the Petri-dishes were placed at ambient temperature and light conditions. When we were cleaning up and ready to dispose of the seeds, we noticed that germination had occurred in three Petri-dishes, all of which had been in the cool and dark treatments (two dishes/4 total seeds at 15/5°C and one dish/5 total seeds at 20/10°C). Because there was no difference in light vs dark in *G. remyi* and because few to no seeds of *G. taitensis* germinated at these temperatures, we hypothesize that this germination was due to an increase to ambient temperature (~25°C) rather than the addition of light. However, more research is needed to understand the effects of moving seeds from colder to warmer temperatures (Baskin and Baskin 2003).

Dormancy

Seeds of *Gardenia remyi* stored for 8 years and then incubated at 25/15°C light started to germinate between weeks 3 and 5 and had concluded by week 27. Because seed coats are water permeable (inferred from congener *G. brighamii*; Baskin et al. 2021), embryos are fully developed (inferred from congener *G. brighamii*, Baskin et al. 2021 and from the spatulate embryo in congener *G. jasminoides*, Martin 1946), and seeds take around 4 weeks to start germination, seeds of *G. remyi* have non-deep physiological dormancy (PD). Unfortunately, germinability had already declined to zero in the two accessions of stored seeds we incubated at multiple temperatures (15/5, 20/10, 25/15, and 30/20°C). However, it is important to use freshly collected seeds for such a study since viability can decline as seen in the present study, or seeds stored for even short periods of time can come out of dormancy, thereby confounding results (Baskin et al. 2006). Interestingly, seeds of all other species of Hawaiian Rubiaceae found in the literature also have (or are inferred to have) PD, including *Bobea elatior* Gaudich., *B. sandwicensis* (A.Gray) Hillebr., *Coprosma cymosa* Hillebr., *C. rhynchocarpa* A.Gray, *Kadua acuminata* Cham. & Schltld. (could also be non-dormant), *K. centranthoides* Hook. & Arn. (could also be non-dormant), *Psychotria hathewayi* Fosberg, *P. hawaiiensis* (A.Gray) Fosberg, *P. hobdyi* Sohmer, *P. kaduana* (Cham. & Schltld.) Fosberg, *P. mariniana* (Cham. & Schltld.) Fosberg, *Psydrax odoratus* (G.Forst.) A.C.Sm. & S.P.Darwin (Baskin and Baskin 2014), *Coprosma kauensis* (A.Gray) A.Heller (Wolkis et al. 2023), *C. ernodeoides* A.Gray, *C. montana* Hillebr., *C. ochracea* W.R.B.Oliv., *C. rhynchocarpa*, *Kadua affinis* Cham. & Schltld. (Baskin et al. 2021).

Freshly harvested seeds of *G. taitensis* did not germinate at 15/5°C regardless of light regime, and only one seed germinated for seeds sown in light and dark at 20/10°C. For seeds incubated at 25/15 and 30/20°C, 50% germination was reached between weeks 7–9 and 3–5, respectively, and had concluded by weeks 30 and

15, respectively. Given this information and because seed coats are water permeable and embryos are fully developed (see discussion above), seeds of *G. taitensis* have non-deep PD. Of the *Gardenia* spp. studied by Baskin and Baskin (2014), six have (or are inferred to have) PD: *G. actinocarpa*, *G. brighamii*, *G. latifolia* Aiton, *G. obtusifolia* Roxb. ex Hook.f., *G. ovularis* F.M.Bailey, and *G. turgida* (synonym of *Ceriscoides turgida*) and four have (or are inferred to have) non-dormant seeds: *G. carinata* Wall. ex Roxb., *G. jasminoides* J.Ellis, *G. megasperma* F.Muell., and *G. tubifera* Wall. (Baskin and Baskin 2014, and references therein; Baskin et al. 2021; Supplementary material 2). Interestingly, the non-dormant species are native to tropical evergreen rainforests and moist warm temperature woodlands, while the PD species are native only to tropical evergreen rainforests (Baskin et al. 2021). Our results for *G. taitensis* are consistent with previous findings from other *Gardenia* species.

A global analysis on the effect of macroclimate on seed dormancy found that species with seeds exhibiting PD had a high proportion of species in relatively drier climates, and that increasing annual range of temperature had a significant positive effect, precipitation seasonality had a small negative, yet significant effect, and mean annual temperature had no significant effect (Rosbakh et al. 2023). Much is known about species' dormancy classifications in Hawai'i (e.g. Baskin and Baskin 2014, and references therein; Baskin et al. 2020, 2021), thus a similar study exploring the climatic variables that may predict dormancy patterns at the scale of the Hawaiian archipelago are warranted and timely.

Maturity and pathogen abatement

In the *G. brighamii* maturity experiment, no seeds from immature fruit germinated compared to 91% in seeds arising from mature (control) fruit. This highlights the need for collections to be made when fruits are at the stage of natural dispersal, especially for collections bound for ex situ seed storage. Seeds collected immature will be undeveloped and will lose viability when dried, or even fail to germinate altogether, and seeds collected too late may have reduced viability (de Vitis et al. 2020). A seasoned field botanist with 30 years of experience working with native Hawaiian plants reports that *G. brighamii* seeds are only viable when fruits transition from green to brown, are full size, lose rigidity, can be squeezed open, and the endocarp will be orange, instead of brown and too rigid to open by hand (Hank Oppenheimer, PEPP, pers. comm.). Seeds of *G. brighamii* in the pathogen abatement experiment germinated to a high percent, and although germination was higher in the untreated control (91%), there was no significant difference compared the pathogen abatement treatment (17-hour soak in 2% PPM; 79% germination), indicating that seeds of this species likely need no pathogen pre-treatment.

Heat shock

Hawaiian Rubiaceae are reported to be desiccation tolerant yet short lived at conventional storage temperatures (intermediate “freeze-sensitive”; Chau et al. 2019) as observed in the present study. One possible mechanism for this intermediate “freeze-sensitive” trait is a time-related structural and/or oxidative damage inferred by the transformation (Pritchard and Seaton 1993) or crystallization and melting kinetics of storage lipids (i.e. triacylglycerols (TAGs)) when stored, generally, below 0°C (Crane et al. 2003, 2006; Hamilton et al. 2009; Ballesteros et al. 2020). In addition, incomplete melting of the TAGs upon retrieval from storage, could also be a source of damage during seed imbibition (Crane et al. 2003; Volk et al. 2006). Differential scanning calorimetry (DSC) could be used to determine if *Gardenia* “freeze-sensitivity” could be bypassed if failure to germination was related to imbibitional damage due to the incomplete melting of the seed TAGs (Crane et al. 2003; Volk et al. 2006). Germination neither occurred in the heat shock treatment nor in the control. Thus, in addition to using DSC to investigate TAGs, heat shock treatments could be used on other accessions of *Gardenia* spp. where germinability has declined to 0%.

Surgical treatment

In seeds of species exhibiting physiological dormancy such as the *Gardenia* spp. discussed in the present study, a surgical treatment can reduce the push power needed for the radicle to emerge and therefore to overcome dormancy (Baskin and Baskin 2014). Germination neither occurred from the surgically treated nor from control seeds. Additionally, in vitro plant tissue culture protocols for embryo rescue have been used to assess extreme seed longevity from historic botanical collections (Porteous et al. 2019). Thus, more research is needed to evaluate the efficacy of these methods in Hawaiian *Gardenia* species.

Gardenia conservation assessments require updates

The Global Strategy for Plant Conservation (GSPC) is a program of the United Nations Convention on Biological Diversity with the aim to halt continuing loss of plant diversity. Within the GSPC, Target 8 calls for 75% of plant species to be preserved ex situ, preferably in their country of origin (CBD 2011). However, only 51 (40%) *Gardenia* spp. are currently represented at an ex situ facility (BGCI 2023), which lies well below the goals outlined within Target 8. To reach 75% of the genus *Gardenia*'s 128 recognized species, at least 95 taxa should be conserved at ex situ facilities, 44 more species than at current.

In addition, as only 43 out of 128 (34%) recognized species of *Gardenia* have been assessed under the IUCN Red List, a substantial information gap exists related to the conservation status of a majority of the members of

this important genus. The remaining 66% of species that are Not Evaluated (NE) on the IUCN Red List require conservation assessments to determine which additional *Gardenia* species are threatened, and therefore prioritize which species should benefit from conservation activities in situ and ex situ.

Globally, at least 33% of IUCN Red List assessed threatened species (i.e. the three threatened categories; Critically Endangered, Endangered, Vulnerable) are estimated to produce desiccation intolerant seeds. Further, there is a positive relationship between the proportion of a species list that is likely to produce recalcitrant seeds and the proportion of the list made up by tree species from tropical moist forest and mangrove habitats (Wyse et al. 2018). While *Gardenia* spp. are thought to be desiccation tolerant (Chau et al. 2019; SER-INSR and RBGK 2023), we observed short lifespans in the present study. Many of Hawai'i's microclimates include tropical moist forests, and work continues to assess their ecosystem biodiversity and plant behaviours across the archipelago (Craven et al. 2018). Forests like these which still harbour *Gardenia* spp., and the existence of this pattern of desiccation tolerance in Hawai'i specifically should be investigated.

Exceptionality

Recently, Pence et al. (2022b) updated the definition of an exceptional species to: “plant species that cannot be efficiently and effectively conserved long-term ex situ under the conditions of conventional seed banking, requiring modified approaches,” and outlined a set of conditions as exceptionality factors. Exceptionality factor 1 (EF1) is that seeds are not produced or are extremely limited in quantity, viability, or accessibility. Exceptionality factor 2 (EF2) states that seeds cannot survive drying to ca 15% RH. For exceptionality factor 3 (EF3), seeds may be desiccation tolerant, but their viability will decline to 50% (p_{50}) in < 20 years. For exceptionality factor 4 (EF4), seeds are deeply dormant with very long germination times (> 1 year), and germination has not yet been successful with any conventional dormancy-breaking method (Pence et al. 2022b). As discussed above Hawaiian *Gardenia* spp. are reported to be desiccation tolerant yet short lived at the conventional storage temperature of -18°C (Chau et al. 2019). As a result, all three Hawaiian *Gardenia* spp. were added to the global list of exceptional plant species under EF3 (Pence et al. 2022a). With the exception of the one long lived accession of *G. brighamii* (92% germinability after 22 years, discussed above), the results of the present study confirm that the three Hawaiian *Gardenia* spp. exhibit EF3 (see discussion of longevity above). Interestingly, *G. brighamii* and *G. mannii* were also listed under EF1 following personal communication from Helm Wallace (Pence et al. 2022a). Notably, the three Hawaiian *Gardenia* spp. were the only members of the genus included in the global gap analysis and global list of exceptional plant species (Pence et al. 2022b), thereby highlighting the need for more research into the storage

behaviour and other exceptionality factors of *Gardenia*. Of the 15 species of *Gardenia* reporting seed storage behaviour on the Seed Information Database, 100% of them (including the two Hawaiian species *G. brighamii* and *G. remyi* presented in this study) are reported as some form of orthodox (orthodox, orthodox?, or orthodox p), i.e. no recalcitrance or intermediacy is reported (SER-INSR and RBGK 2023).

Concluding remarks

The results of the present study suggest that *G. remyi* and *G. taitensis* seeds exhibit non-deep physiological dormancy. We identified for the first time germination conditions for Critically Endangered *G. remyi*. Notably, there was no light requirement for germination indicating that the species is unlikely to form a persistent soil seed bank, which could, in part, explain some of the rarity of the species. Our results agree with those from other studies on Hawaiian species of *Gardenia* that generally seeds are “short lived”. We also found that seeds from immature seeds do not germinate, therefore fruits must reach full maturity prior to harvest for seed banking or propagation to be successful. Finally, with only 40% of the species of *Gardenia* represented at ex situ facilities, and 66% of the genus having not been evaluated for the IUCN Red List, we express the need for additional research and conservation assessments of more members of this iconic plant group.

The fact that the majority of the accessions used in the present study were never tested for initial germinability (nor any viability metric) as fresh seed highlights the need for initial germination testing especially, as well as subsequent longevity testing (CPC 2019; FAO 2022). From a wild species perspective any “conservation collections” should have tests of initial germination plus periodic long-term viability testing (CPC 2019). Indeed, the practical guide to Plant Genetic Resources for Food and Agriculture recommends that, “Initial seed germination testing should be conducted as soon as possible after obtaining the accession” and that “A monitoring system is in place to test the viability status of samples at regular intervals during storage” (FAO 2022).

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SUPPLEMENTARY MATERIALS

Supplementary material 1

Summary of *Gardenia* accessions used in the experiment. The table presents accession provenance data, length of experiment, number of replicates, temperature, light, and media.

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Supplementary material 2

Gardenia data from Baskin and Baskin (2014), with presumed or confirmed dormancy class. PD = physiological dormancy; ND = non-dormant. Asterisk * indicates presumed dormancy.

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Supplementary material 3

Global *Gardenia* distribution by region. The 128 *Gardenia* taxa recognized by Plants of the World Online (POWO) organized by taxonomic name, their IUCN status, and native range.

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