APPLICATION ARTICLE

Seed dormancy and germination of the endangered exceptional Hawaiian lobelioid *Brighamia rockii*

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Abstract

Premise: The Campanulaceae (Lobelioideae) is the Hawaiian plant family with the most endangered and extinct species. Although seeds of Hawaiian lobelioids are desiccation tolerant, the species are exceptional (i.e., they present challenges at various stages of the conventional ex situ conservation chain) due to their generally poor seed survival at the conventional seed-banking temperature (−18°C). Both morphological dormancy (MD) and morphophysiological dormancy (MPD) have been identified in the seeds of other Hawaiian lobelioids; however, the class of dormancy and germination requirements of the Critically Endangered genus *Brighamia* have not yet been determined.

Methods: We measured the embryonic growth in 12-week-old seeds of *B. rockii* and tested their germination at three temperature regimes (15/5°C, 20/10°C, and 25/15°C) in light and at 25/15°C in darkness.

Results: The embryos grew prior to radicle emergence, and the seeds germinated rapidly to high percentages in all tested conditions.

Discussion: Whether fresh *B. rockii* seeds have MD or MPD still needs to be determined; nevertheless, 12-week-old seeds germinated well in light and darkness, and thus the seeds can be used for conservation purposes. Germination in the dark suggests that the species may not form a long-lived soil seed bank in its native habitat.

KEYWORDS
*Brighamia*, critically endangered plants, embryo growth, ex situ conservation, exceptional species, Hawaiian lobelioids

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 urgent than ever before (Humphreys et al., 2019; Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services, 2019). The Global Strategy for Plant Conservation (GSPC) provides a framework for practicing plant conservation. Target 8 of the GSPC sets the goal of conserving 75% of threatened plant species ex situ (Convention on Biological Diversity, 2011), with seed banking being the most efficient and cost-effective means of ex situ plant conservation (Phartyal et al., 2002; Guerrant et al., 2004; Cochrane et al., 2007; Liu et al., 2018, 2020). However, not all species can be conserved using conventional seed-banking methods (i.e., retaining long-term viability when desiccated to a relative humidity [RH] in equilibrium with 10–25% and stored at −18°C), which is the case for endemic Hawaiian Lobelioideae (Chau et al., 2019). For seed banking to be a successful conservation strategy, seeds eventually need to be withdrawn from the seed bank and used for plant propagation; however, when the viability of seeds in the seed bank falls to an unacceptable level, additional seeds must be collected from wild plants and/or regenerated from banked seeds. Regardless of the seed source, species-specific knowledge of dormancy-breaking and germination requirements is needed to successfully grow plants from seeds.

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The endemic Hawaiian Lobelioideae (Campanulaceae; six genera, 126 species) exemplify adaptive radiation in plants, with the Campanulaceae representing the largest family in the flora of the most isolated archipelago on Earth (Wagner et al., 2005). The lobelioid ancestor arrived in the Hawaiian Islands ca. 13 mya (Givnish et al., 2009). Eventually, species of the Lobelioideae became distributed on seven of the eight main Hawaiian Islands at elevations from <100 to >4000 m a.s.l. (Wagner et al., 1999). These plants have remarkably diverse life forms (shrubs, trees, rosettes, succulents, vines, and epiphytes) and occupy a variety of habitats (including high-elevation bogs, cliff faces, and forests; Wagner et al., 1999). The Campanulaceae family also contains the most Hawaiian species listed under the Endangered Species Act and the highest number of recent extinctions in the state (United States Fish and Wildlife Service, 2016; Wood et al., 2019). The reason behind this high endangerment and extinction rate is uncertain; however, these plants are susceptible to destruction by rats, slugs, and diseases, and may have been impacted by the loss of specific pollinators and seed dispersers (Walsh et al., 2019; Wood et al., 2019; Ronsted et al., 2022).

While the majority of species in the Campanulaceae globally produce orthodox seeds (i.e., seeds that are desiccation and freeze tolerant and long lived; Royal Botanic Gardens Kew, 2020), this is not the case with Hawaiian-endemic Campanulaceae, which appear to be desiccation tolerant yet lose viability faster at the conventional seed storage temperature of −18°C than at 5°C. This type of response has been termed “intermediate freeze-sensitive storage behavior” (Chau et al., 2019).

Efforts to restore plants from seeds are often challenged by a lack of a priori knowledge of seed dormancy and germination requirements. Seeds of many species of Hawaiian Campanulaceae have morphological dormancy (MD) or morphophysiological dormancy (MPD) (Baskin et al., 2005, 2020; Baskin and Baskin, 2014). Freshly matured seeds with MD have a water-permeable seed coat, and the embryo is small and either undifferentiated (i.e., lacking a radicle and cotyledon[s], such as members of the Orchidaceae) or differentiated (radicle and cotyledon[s] present) but underdeveloped, and must therefore grow inside the seed before radicle emergence (germination) occurs. Seeds with MD do not require dormancy-breaking treatments per se; they merely need time for the embryo to grow and become fully developed, and can germinate in ≤1 month at favorable conditions. On the other hand, in addition to being underdeveloped, the embryo in seeds with MPD also has a physiological mechanism for germination inhibition (Baskin and Baskin, 2014). The germination of seeds with MPD is therefore delayed >1 month, and treatments such as warm and/or cold stratification are needed to break the physiological component of the embryo dormancy.

_Brighamia_ A. Gray (Campanulaceae) is an endemic Hawaiian genus comprising two species, _B. insignis_ A. Gray and _B. rockii_ H. St. John (Wagner et al., 2005). _Brighamia rockii_ (ʻolulu, alula, or puaʻalain in Hawaiian) is listed as Critically Endangered on the IUCN Red List of Threatened Species and only 11 individuals are known in the wild, all of which are located in a single subpopulation on the cliffs of Molokaʻi (Walsh et al., 2022). Seed dormancy-break and germination have been investigated for a few species of Hawaiian Campanulaceae, and both MD and MPD have been identified (Baskin et al., 2005, 2020; Baskin and Baskin, 2014); however, the dormancy-breaking and germination requirements have not been determined for the endemic and exceptional (i.e., presenting challenges to the conventional ex situ conservation chain) Hawaiian lobelioid genus _Brighamia_. Therefore, our objective in this study was to improve our understanding of the seed dormancy and germination requirements of _Brighamia_ to inform conservation collection management. Because other lobelioids have MD or MPD, we hypothesized that the embryos in the seeds of _B. rockii_ are underdeveloped (small) and must grow inside the seed prior to radicle emergence (germination), and therefore that the seeds have either MD or MPD.

To test our hypothesis, we asked the following questions: (i) Does the embryo length : seed length (E : S) ratio increase prior to seed germination? (ii) How quickly do seeds germinate? (iii) What are the temperature and light/dark requirements for germination? Here, we report the results of our studies, although we note that the outcome was complicated by a 12-week storage period before our experiments began. During this time, any fully developed embryos may have transitioned from dormancy to nondormancy or, in the case of underdeveloped embryos, from MPD to MD. Despite this complication, we have gained important information about the seed germination of an endangered species.

**METHODS**

**Seed source**

_Brighamia rockii_ occurs on the Wailau cliffs of Molokaʻi, Hawaiʻi, USA. The mean hourly temperature for this location ranges from 16.17°C (February, 07:00) to 25.02°C (September, 14:00) (Giambelluca et al., 2014). Freshly matured seeds of _B. rockii_ were collected at the Oliina Rare Plant Facility on Maui on 30 October 2020 by Anna Palomino (Hawaiʻi Plant Extinction Prevention Program; University of Hawaiʻi Center for Conservation Research and Training; Hawaiʻi State Department of Land and Natural Resources Department of Forestry and Wildlife) from three cultivated plants (numbers 02, 03, and 09). The three plants are the progeny of a wild single maternal founder (GeoRef no. BRI-ROC-MO-EHU-A), and the seeds were pooled before shipping. Ane Bakutis (Hawaiʻi Plant Extinction Prevention Program; University of Hawaiʻi Center for Conservation Research and Training; Hawaiʻi State Department of Land and Natural Resources Department of Forestry and Wildlife) coordinated the shipment of seeds.
to the National Tropical Botanical Garden (NTBG), Kalāheo, Hawai‘i. The seeds were received on 3 December 2020 and kept in paper coin envelopes in ambient lab conditions (~46% RH and 23°C) until the experiments (embryo growth and germination) were carried out, beginning on 22 January 2021.

**Embryo growth**

To determine whether the embryos grow inside the seeds prior to germination, the E : S ratio was determined for seeds imbibed for 24 h and for seeds when germination was imminent, i.e., at the time the seed coat had split but before the protrusion of the radicle (germination) had occurred (i.e., the maximum [critical] E : S ratio for germination). To determine the initial E : S ratio, seeds were imbibed for 24 h in 12 h light/12 h dark (hereafter, light) at 25/15°C. The seeds were then cut open lengthwise with a single-edge razor blade and the E : S ratio was determined for 16 seeds using a micrometer in the eyepiece of a dissecting microscope. To determine the critical E : S ratio, the seeds were placed in 60-mm Petri dishes on seed-germination paper (Anchor Paper Company, St. Paul, Minnesota, USA) moistened with a 0.1% solution (in distilled water) of a plant preservative mixture (PPM; Plant Cell Technology, Washington, D.C., USA) to inhibit fungal growth without affecting germination (Assaf Guri, Plant Cell Technology, personal communication). The seeds were incubated in light at 25/15°C and examined every 2–3 days to identify individuals for which germination was imminent. The critical E : S ratio of five seeds was determined.

**Germination**

To determine the temperature and light/dark requirements for germination, three replicates of 25 seeds per germination condition were placed in 60-mm Petri dishes on seed-germination paper moistened with a solution of 0.1% PPM. The Petri dishes were sealed with plastic paraffin film to reduce water loss. The seeds were incubated in 12 h light/12 h dark with alternating temperature regimes of 15/5°C, 20/10°C, and 25/15°C, or in continuous darkness (Petri dishes wrapped in aluminum foil) at 25/15°C. The light was generated by fluorescent tubes, and the illuminance at the seed level was about 300 lux. Germination was monitored at two-week (occasionally three-week) intervals for a maximum period of 292 days. The seeds incubated in darkness were checked using a safe green light, provided by an LED with a peak wavelength at 515 nm (Feit Electric, Los Angeles, California, USA).

“Safe green lights” are commonly used to monitor the germination of dark-incubated seeds, but green light may actually promote germination in some species, depending on the stage of dormancy break between full dormancy and nondormancy (Baskin et al., 2006). Baskin et al. (2006) suggested comparing the germination of seeds exposed to green light to that of seeds not receiving any light; therefore, we tested whether the green light promoted germination of *B. rockii* seeds. To achieve this, 50 seeds were placed in a 60-mm Petri dish on seed-germination paper moistened with a solution of 0.1% PPM. These dishes were sealed with plastic paraffin and wrapped in aluminum foil to exclude light, then incubated at 25/15°C. The dishes were not opened until the termination of the test on day 61.

We monitored the seeds (except those in continuous darkness at 25/15°C) until every seed either germinated, was conspicuously contaminated by fungi, or had been incubated for 292 days. If seeds did not resist gentle pressure from probing, they were considered to have lost viability. Germination was defined as radicle emergence, and the proportion germinated was calculated by dividing the number of seeds germinated by the number of seeds sown. Only one seed died (at 25/15°C in dark) before the experiments were terminated.

**Statistical analysis**

The software RStudio (RStudio Team, 2019) and environment R (R Core Team, 2021) were used for all statistical analyses, and the package ‘ggplot2’ was used for the construction of graphs (Wickham, 2016). The germination data were analyzed using a time-to-event model (Ritz et al., 2013) and the two-step meta-analytic approach described by Jensen et al. (2017, 2020), performed using the ‘drc’ package (Ritz et al., 2019). A non-linear log-logistic three-parameter model was used:

\[
F(t) = \frac{d}{1 + \exp(b \log(t) - \log(t_50))}
\]

where \(d\) is maximum germination, \(t_50\) is time at which 50% of the seeds that germinated during the experiment have done so, and the absolute value \(b\) is proportional to the slope of \(F\) at time \(t\). There were two replicates (Petri dishes) to which the model could not be fitted. In one dish at 20/10°C, the only seeds that germinated were observed at the same time, thus no parameters were estimated. In the other case, at 15/5°C, all the seeds germinated, so \(d\) (maximum germination) did not need to be estimated and was set to 1 and the model was run manually for estimates \(b\) and \(t_50\). Jensen et al. (2017) proposed a meta-analytic approach that allows the incorporation of the experimental design into the statistical analysis when using appropriate models at the level of the individual Petri dishes, which are treated as sub-experiments. We used a linear mixed model in which the treatment-by-model parameter interaction was the only fixed effect, and the replicated Petri dishes were the random effects.

A Welch’s *t*-test (\(\alpha = 0.05\)) was used to determine significant differences between the initial and critical (germination imminent) E : S ratios.
RESULTS

Embryo growth

The mean (±SE) initial embryo and seed lengths were 0.5 ± 0.02 mm and 1.1 ± 0.02 mm, respectively. At imminent germination, the embryo and seed lengths were 1.0 ± 0.04 mm and 1.1 ± 0.05 mm, respectively. The initial E : S ratio was therefore 0.49, while at imminent germination it was 0.92, representing a significant (*P* < 0.0001) increase of 87.8% in 12 days (Figure 1).

Germination

For germination under the different conditions (except for the dark control seeds sown in darkness and not checked for 61 days), mean (± SE) parameter estimates ranged as follows: *d* (the maximum proportion germinated) from 0.78% ± 0.07 (at 25/15°C in light) to 0.94% ± 0.07 (at 20/10°C in light), *t*$_{50}$ (time to 50% germination) from 19.86 days ± 2.50 (at 20/10°C in light) to 25.53 days ± 2.97 (at 25/15°C in darkness), and *b* (absolute value proportional to slope) from −7.76 ± 2.17 (at 20/10°C in light) to −3.07 ± 0.97 (at 15/5°C in light; Table 1, Figure 2). There were no significant differences between any germination conditions for *d* (*P* > 0.10) or *t*$_{50}$ (*P* > 0.10). The parameter estimate *b* for 20/10°C in light was significantly higher (steeper) to that of parameter estimate *b* of 25/15°C in darkness (*P* = 0.04; Table 2, Figure 2, Appendix 1).

A set of dark control seeds were sown in complete darkness, incubated at 25/15°C, and not checked for 61 days to compare their germination against the dark-grown seeds checked under the safe green light. Of the 50 dark control seeds sown, 24 (48%) germinated. By contrast, the seeds incubated in the dark at 25/15°C and exposed to green light during germination monitoring had a germination rate of 87% ± 0.07.

DISCUSSION

We showed that the embryos grew inside the seeds of *B. rockii* prior to their germination; thus, they have MD or MPD. Both MD and MPD have been identified in other members of Hawaiian Campanulaceae; for example, MD and/or MPD was found in *Delissea* Gaudich. (two species), *Clermontia* Gaudich. (10 species), and *Lobelia* L. (seven
species), but only MPD was found in *Cyanea* Gaudich. (four species) and *Trematolobelia* Zahlbr. ex Rock (three species) (Baskin et al., 2005, 2020; Baskin and Baskin, 2014). We did not observe any significant difference in the final germination or $t_{50}$ between any of the temperature and light regimes, and $t_{50}$ was reached in 19–26 days, which suggests that seeds of *B. rockii* have MD. It is important to note, however, that 84 days elapsed between seed collecting and testing, which for some species is ample time for physiological dormancy to be broken via after-ripening (Baskin and Baskin, 2020). A Hawaiian gardening handbook states that seeds of the genus *Brighamia* will germinate within 10 days to three months in nursery conditions (Lilleeng-Rosenberger, 2005); however, for one accession of *B. insignis* (20180161) from NTBG, seeds sown 13 days after collection and incubated in a 25/15°C 12 h light/12 h dark regime germinated between 33–67 days later, reaching 70% germination. In another *B. insignis* accession (20180199) from NTBG, only one of the seeds sown eight days after collection at 25/15°C 12 h light/12 h dark regime germinated within 44 days after sowing, and by day 98, germination had ended at just 22%. By contrast, we found that seeds of this same accession stored for one year at the recommended storage conditions for desiccation-tolerant freeze-sensitive species (e.g., a target equilibrium relative humidity of 20% at the storage temperature of 5°C; Chau et al., 2019) sown under the same conditions had a 70% germination rate in a 34–89-day period, except for one seed that germinated on day 117 (Wolkis, unpublished data); thus, our study has not eliminated the possibility that at least some *B. rockii* seeds have MPD. The time period over which fresh *Brighamia* seeds are reported to germinate (e.g., 10 days to three months) seems to suggest that a seed lot consists of a mixture of seeds with MD and MPD, and that those with MPD undergo after-ripening to break the physiological component of MPD. Clearly, more research is needed to determine whether seeds of *B. rockii* have MD, MPD, or a mixture of both.

For studies of most Hawaiian Campanulaceae species in which multiple temperature regimes were used, high germination percentages occurred at 25/15°C (e.g., for *Clermontia fauriei* H. Lev., *C. hawaiensis* Rock, *C. kakeana* Meyen, *Cyanea angustifolia* (Cham.) Hillebr., *C. kunthiana* Hillebr., *Delisea rhytidosperma* H. Mann, *Lobelia grayana* E. Wimm., *L. hypoleuca* Hillebr., and *Trematolobelia macrostachys* (Hook. & Arn.) Zahlbr., ex Rock). An exception was *Clermontia pyrularia* Hillebr., which germinated only at 15/6°C (Baskin et al., 2005, 2020). Here, we showed that a high germination percentage also occurred for *B. rockii* at 25/15°C, but this was not significantly different from the other two temperatures at which seeds were tested (15/5°C and 20/10°C).

The seeds incubated in continuous darkness at 25/15°C for 61 days had a 48% germination rate, while those incubated in darkness but exposed to green safe light at this temperature regime had an 87% germination rate after 96 days, suggesting that the safe green light did promote germination of some *B. rockii* seeds. The germination of seeds in continuous darkness (without exposure to the safe green light) suggests that this species 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. In a recent case study using the only other species in the genus, *B. insignis*, the authors recommend implementing a pedigree-based approach to management of reproduction of plants ex situ to slow the inevitable loss of genetic diversity and, in turn, result in healthier collections (Foster et al., 2022). As *B. rockii* is held in ex situ collections in at least four locations in Hawai’i (Walsh et al., 2022), it is expected that *B. rockii* should be managed with the same approach.

Recently, Pence et al. (2022a) 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. Under exceptionality factor 3 (EF3), seeds may be desiccation tolerant, but their viability will decline to 50% ($P_{50}$) in <20 years. In 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., 2022a).

| Pairwise germination condition comparison | $P$ value | $d$ | $b$ | $t_{50}$ |
|------------------------------------------|----------|----|----|--------|
| 15/5°C light/dark vs. 20/10°C light/dark | 0.9093   | 0.0543 | 0.2725 |
| 15/5°C light/dark vs. 25/15°C dark*        | 0.6340   | 0.9212 | 0.5508 |
| 15/5°C light/dark vs. 25/15°C light/dark  | 0.2212   | 0.3585 | 0.2620 |
| 20/10°C light/dark vs. 25/15°C dark*        | 0.4603   | 0.0375 | 0.1439 |
| 20/10°C light/dark vs. 25/15°C light/dark  | 0.0962   | 0.1388 | 0.9624 |
| 25/15°C dark* vs. 25/15°C light/dark       | 0.3405   | 0.2058 | 0.1370 |

*Used safe green light.
reached 50% germination in 20–26 days. These results provide the empirical data needed to verify the findings of a recent exceptional species gap analysis, which indicated that Brighamia seeds do not fit under EF4 (but are correctly classified as EF3 as described above; Pence et al., 2022b; list available at http://cincinnatizoo.org/global-list-of-threatened-exceptional-plants [accessed 4 August 2022]).

For many exceptional species, cryobiotechnologies (and, to some extent, in vitro propagation) have been the main focus of preservation research (Pritchard, 2018; Pence et al., 2020; Philpott et al., 2022), but the storage of dry seeds at –18°C would be less expensive than cryobiotechnology. However, seeds of various species, including those of some EF3 species with intermediate storage behavior such as Brighamia, do not survive the long-term combined stresses of desiccation and freezing at –18°C. Triacylglycerol (TAG) has been identified in seeds of the majority of species with intermediate storage behavior, and its crystallization at conventional storage temperatures (–18°C) in diverse species may be an important cause of damage that results in the loss of seed viability (Ballesteros et al., 2020). One possible technological innovation, especially for EF3 species, might be to store the desiccated seeds at –80°C, but this method has not been tested. Lipid thermal fingerprinting could be used to identify biophysical markers in relation to storage stability at different temperatures (e.g., Mira et al., 2019) including –80°C; this would give insight into whether EF3 species could be stored safely at –80°C, making this temperature a viable alternative to cryobiotechnology. Until more is known about lipid thermal properties or viability after storage at –80°C, it is recommended that B. rockii seeds are desiccated to 20% RH at a storage temperature of +5°C (Chau et al., 2019).

Brighamia rockii seeds stored for 12 weeks have high germination over a range of temperature regimes in light and at 25/15°C in darkness (the only dark temperature tested). Thus, this exceptional species can be propagated from seeds for conservation purposes. The ability of many seeds of this species to germinate in darkness suggests that it is unlikely to form a long-lived soil seed bank; therefore, the re-establishment of extirpated populations will require reintroduction. Brighamia rockii seeds have either MD or MPD or a mixture thereof, and more research is needed to determine the class(es) of dormancy in this species.

**AUTHOR CONTRIBUTIONS**
D.W., C.C.B., J.M.B., and N.R. conceptualized the study. D.W. designed and conducted the germination studies, statistical analyses, and drafted the manuscript. All authors read and approved the final version of the manuscript.

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**DATA AVAILABILITY STATEMENT**
The data that support the findings of this study are included as Appendix S1.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

**Appendix S1. Germination data for Brighamia rockii.**