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Overcoming Recruitment Bottlenecks in an Extinct-in-the-Wild Plant: Evidence for Adaptive Ex Situ Conservation

Denisse Mariana Escarza *, Cristina Sanhueza and Sergio Martin Zalba

GEKKO—Grupo de Estudios en Conservación y Manejo, Departamento de Biología, Bioquímica y Farmacia, Universidad Nacional Del Sur, Bahía Blanca 8000, Buenos Aires, Argentina; ccsanhueza@yahoo.com.ar (C.S.); szalba@criba.edu.ar (S.M.Z.)
* Correspondence: denissemarianaescarza@gmail.com

Abstract

Species classified as Extinct in the Wild depend entirely on ex situ collections, making the optimization of sexual reproduction a key challenge for maintaining genetic diversity and supporting future recovery. *Senecio leucopeplus* (Asteraceae), a perennial shrub endemic to southwestern Buenos Aires Province (Argentina), survives exclusively under cultivation, offering a unique opportunity to investigate factors limiting recruitment in extremely threatened plants. We evaluated the effects of pollination method (natural vs. hand pollination), seed storage time, plant growth site (greenhouse vs. outdoor cultivation), and germination substrate on seed germination, seedling establishment and early survival. Seeds collected in 2015 and between 2020 and 2025 were subjected to controlled germination experiments, and analysed using generalized linear mixed models. Sexual recruitment was severely constrained. Germination of morphologically selected seeds ranged from 0% in older seed lots to 79.55% in the most recent greenhouse-derived lot tested in Petri dishes, whereas seedling establishment remained a major limitation. Nevertheless, germination was generally higher in more recently collected seed lots, in seeds originating from a greenhouse cultivation site, and following natural rather than supplementary hand pollination. No significant pairwise differences were detected between the two substrates tested. This study provides practical evidence-based recommendations for the ex situ management of *S. leucopeplus* and illustrates how adaptive experimental approaches can inform propagation decisions, strengthening the conservation of extremely small plant populations, and supporting the long-term recovery of other critically threatened plant species.

Keywords: sexual recruitment; evidence-based conservation; plant growth site; plant propagation; extremely small populations

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1. Introduction

Biodiversity loss is accelerating worldwide, and vascular plants are among the most affected groups, despite widespread recognition of their fundamental role in ecosystem functioning and the provision of ecosystem services [1–3]. Land-use change, the intensification of human activities, biological invasions, and alterations in disturbance regimes continue to reduce plant diversity across virtually all biomes, while conservation efforts implemented to date have failed to halt the increasing number of threatened species [4,5]. In this context, there is a growing consensus that preventing further plant extinctions will require integrating in situ conservation with effective ex situ strategies capable not only of maintaining living collections, but also of preserving their evolutionary and demographic viability until reintroduction becomes feasible [6].

Ex situ conservation is particularly important for endemic species with restricted distributions and for species whose natural populations have undergone severe declines. Its role becomes indispensable for species classified as Extinct in the Wild, whose persistence depends entirely on cultivated collections that constitute the last remaining reservoir of genetic diversity [7,8]. In these systems, conservation success depends not only on preventing mortality of existing individuals but also on maintaining effective sexual reproduction, thereby allowing genetic recombination and reducing the long-term consequences of clonality, genetic drift and inbreeding [9–11].

However, maintaining viable populations under cultivation involves much more than preserving living individuals. Conservation collections should retain as much of the original genetic diversity as possible, while minimizing the effects of genetic drift, inbreeding, artificial selection, disease, and unintended hybridization, all of which may compromise their long-term conservation value [12,13]. These challenges are especially acute in extremely small populations founded by few individuals, a common situation in species on the brink of extinction, for which every successful reproductive event contributes disproportionately to the conservation of the remaining genetic diversity.

Within this framework, understanding the reproductive biology of threatened species is a fundamental component of ex situ conservation strategies. Although vegetative propagation allows the rapid expansion of cultivated populations, it contributes little to maintaining or increasing genetic diversity [14]. Sexual reproduction, in contrast, promotes genetic recombination and preserves the adaptive potential of populations; although, its success is often constrained by low seed viability, inbreeding, pollinator limitation, self-incompatibility mechanisms, unsuitable germination conditions, or difficulties during seedling establishment [15–17]. Additional limitations may arise from interactions with soil microorganisms, especially mycorrhizal fungi, which play a crucial role during the early developmental stages of most terrestrial plants [18].

These constraints act sequentially throughout the recruitment process, from pollination and seed production to germination and seedling establishment, creating multiple potential bottlenecks that may ultimately determine population persistence. Identifying which of these stages most strongly limits recruitment is, therefore, essential for designing effective conservation interventions.

Despite the importance of these processes, appropriate propagation protocols remain unknown for most threatened plant species. Consequently, ex situ management frequently relies on adaptive approaches, in which cultivation techniques are progressively optimized through the experimental evaluation of alternative treatments. This evidence-based approach transforms ex situ collections from passive repositories into experimental systems capable of generating knowledge directly applicable to conservation management. Beyond their immediate value for the recovery of a particular species, such studies generate transferable knowledge that can benefit other conservation programs facing similar constraints, notably those working with extremely small populations, in which every reproductive event and every seed represents genetic resources of exceptional conservation value.

This issue is particularly relevant in Argentina, where agricultural expansion, urbanization, and biological invasions have profoundly transformed natural ecosystems [19]. The situation is especially critical in the Pampas region, where native grasslands have been almost entirely replaced by productive systems and protected remnants represent only a small fraction of their original extent [20]. As a consequence, numerous endemic plant species now persist in small, fragmented populations, whose survival depends on urgent conservation actions [21].

Senecio leucopeplus Cabrera (Asteraceae) represents one of the most extreme examples of this situation. This perennial shrub, endemic to southwestern Buenos Aires Province,

Argentina, was declared extinct in the wild in 2007 [22,23] and currently survives exclusively in cultivation. A recent study demonstrated that propagation through stem cuttings is an effective tool for rapidly increasing the size of the cultivated population [24]. However, long-term conservation cannot rely exclusively on vegetative propagation because maintaining the evolutionary potential of the species requires successful sexual recruitment. The low germination, previously reported by Pini [25], highlights the need to identify cultivation conditions that maximize reproductive success.

In this study, we evaluated the effects of pollination method, seed storage time, plant growth site, and germination substrate on seed germination, seedling establishment, and early seedling survival in *S. leucopeplus*. By experimentally evaluating these successive stages of the recruitment process, our objective was to develop evidence-based recommendations for ex situ management, while generating transferable knowledge applicable to the conservation of other critically endangered plant species maintained in living collections.

2. Materials and Methods

2.1. Species Description

Senecio leucopeplus is a perennial shrub endemic to southwestern Buenos Aires Province, Argentina, formerly occurring in plains and foothill environments [26,27]. For decades, the species was known only from herbarium specimens until 1996, when—after extensive field surveys guided by herbarium label information—two wild individuals were found growing along a stream bank, and ex situ propagation was initiated at the Pillahuincó Botanical Garden (JBP) using seeds and stem cuttings. After the death of the last known wild individuals in 2007 [22], the species became entirely dependent on cultivated collections. By 2017, only four plants remained at the JBP. Seeds collected from these plants were used in a germination trial [25], which yielded 15 seed-derived individuals that became the founders of the current collection. At present, ca. 230 individuals derived from these founder plants are maintained across three cultivation sites. Morphological variation observed in some cultivated plants has raised the possibility of hybridization or other genetic processes affecting the collection [28].

2.2. Seed Collection

Seeds were collected during the flowering seasons from 2020 to 2025, from plants cultivated both outdoors and under greenhouse conditions, growing in pots with the same fertilized soil substrate. Each year, as fruits approached maturity, capitula were enclosed in tulle bags to prevent seed dispersal (Figure 1). Seeds were harvested once the drying process was complete, as indicated by the grey colour of the receptacle, and stored in kraft paper envelopes placed inside boxes under dry conditions and at room temperature (20 to 25 °C) until germination trials were conducted. Except in the experiment evaluating the effect of seed storage time, all germination assays were conducted approximately six to eight months after seed collection.

To evaluate the effect of long-term storage on germination, an additional seed lot collected in 2015 from outdoor-grown plants at the Pillahuincó Botanical Garden was included. These seeds were stored in paper envelopes under room temperature until the beginning of the experiment.

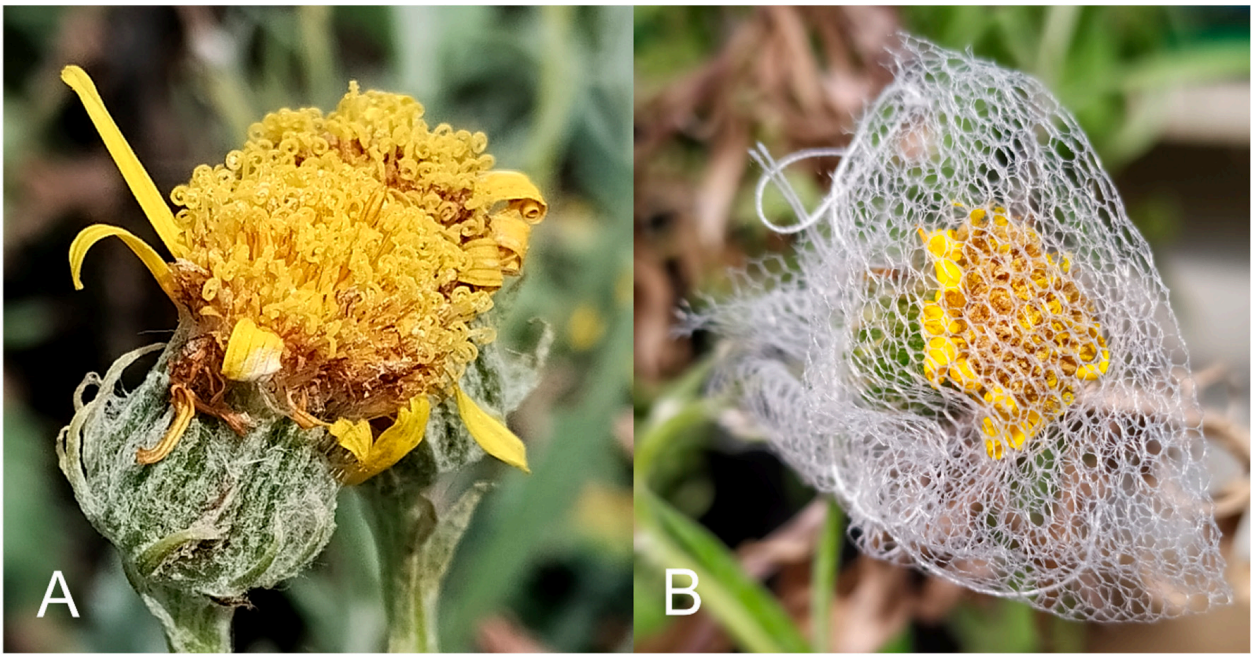


Figure 1. Capitula of *Senecio leucopeplus* and seed collection method. (**A**) Mature capitulum at the time of seed maturation, before being enclosed. (**B**) Capitulum enclosed in a tulle bag to retain the dispersing seeds until harvest.

2.3. Experimental Overview

To identify the principal factors limiting sexual recruitment under ex situ conditions, we conducted four complementary investigations addressing successive stages of the recruitment process. First, explored whether a simple morphological classification could help prioritize seeds for germination trials, without treating appearance as a validated measure of viability. Second, we assessed the effects of pollination method (natural vs. hand pollination) and plant growth site (greenhouse vs. outdoors) on the production of filled seeds and on subsequent germination success. Third, we examined the influence of seed storage time on germination. Finally, we evaluated the effects of plant growth site and germination substrate on seed germination. Together, these experiments allowed us to identify potential bottlenecks affecting sexual recruitment and to develop evidence-based recommendations for the ex situ conservation of *S. leucopeplus*.

2.4. Germination Protocol

Unless otherwise indicated, all germination experiments followed the protocol developed for *S. leucopeplus* by Pini [25]. Seeds were first cold-stratified at 4 °C for 15 days in trays containing moist river sand and were subsequently transferred to a germination chamber under a 12 h light/12 h dark photoperiod, with temperatures of 20 °C during the light period and 15 °C during the dark period, reflecting spring temperatures at the species' native site [25]. Depending on the experiment, seeds were sown either in seedling trays or in Petri dishes. Before each experiment, the germination chamber and all experimental containers were disinfected with 0.5% sodium hypochlorite. The substrate used in seedling trays consisted of an equal-parts mixture of vermiculite, peat, and commercial fertilized soil, previously sterilized in an oven at 90 °C for 30 min. In Petri dish experiments, seeds were placed on moistened paper towels. Seeds were watered every other day with tap water, and germination was monitored three times per week.

For seeds germinated in Petri dishes, germination was defined as radicle emergence of at least 1 mm. In seedling trays, germination was recorded when the hypocotyl emerged

above the substrate surface. Germination trials were terminated one week after the last germination event had been recorded. Following the emergence of the first true leaf, seedlings were transplanted into 1L pots containing a mixture of commercial fertilized soil and perlite, and maintained under controlled environmental conditions (20–25 °C, indirect light, and watering every other day). Seedling survival was recorded as the time elapsed from the first observed germination or emergence until death or the final observation, distinguishing pre- and post-transplant periods. Seedlings that remained alive at the end of the observation period were recorded as surviving individuals. Survival results were summarized descriptively for each experiment and stage (before and after transplantation) and are reported as mean ± standard deviation (SD), together with the corresponding sample size (N).

2.5. Experiment 1—Exploratory Assessment of Seed Morphology

Preliminary observations revealed a marked seed dimorphism, which was used as a provisional screening criterion rather than a validated indicator of viability. Seeds were, therefore, classified into two morphological categories (Figure 2): filled seeds, characterized by a width ≥ 0.7 mm, dark coloration, cylindrical shape and consistency, and empty seeds, characterized by a width ≤ 0.2 mm, pale coloration, flattened shape, and flexible consistency. Seed width was measured using a digital caliper. Because neither seed viability nor embryo development was independently assessed, these categories should be regarded as putatively filled and putatively empty seeds. To evaluate the usefulness of this morphological classification, 1389 seeds (332 putatively full and 1057 putatively empty) obtained during the 2020–2021 flowering season from 11 capitula produced by greenhouse-grown mother plants were sown in five seedling trays maintained under the germination conditions described above.

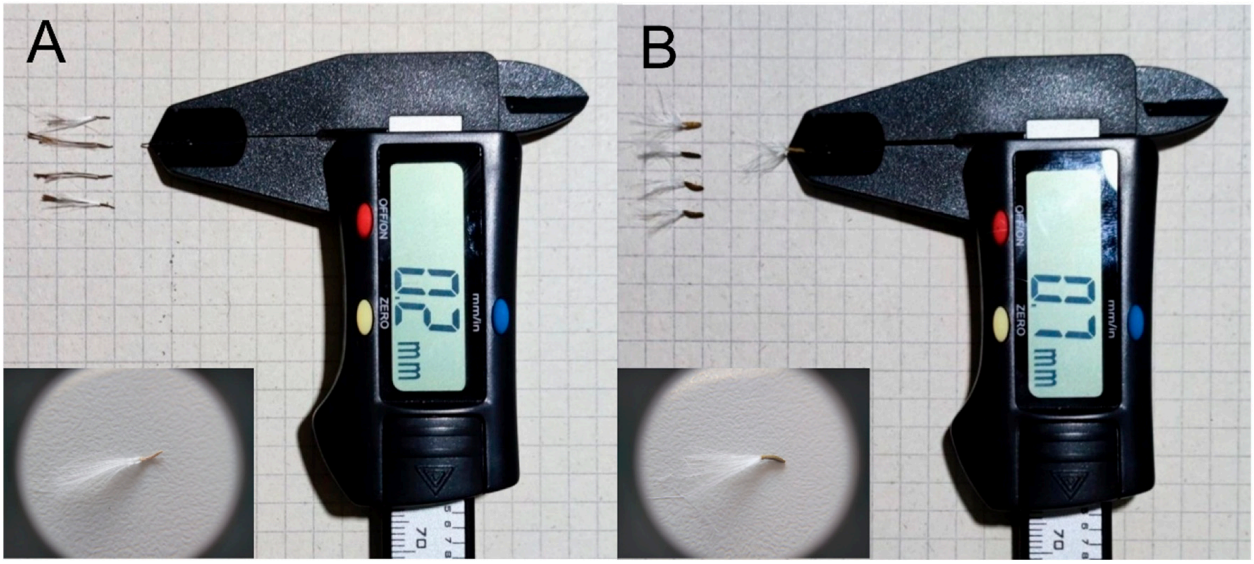


Figure 2. Morphological classification of *Senecio leucopeplus* seeds: (**A**) putatively empty seeds: pale, flattened, flexible, ≤0.2 mm wide; (**B**) putatively filled seeds: dark, cylindrical, firm, ≥0.7 mm wide.

2.6. Experiment 2—Effects of Pollination Method

To evaluate the effect of hand pollination on reproductive success, 18 plants were randomly selected at each of the three cultivation sites (one greenhouse and two outdoor collections) during the 2022–2023 flowering season. The greenhouse treatment was represented by a single cultivation site, whereas outdoor plants were sampled at two sites; thus, cultivation environment and site identity were not independently replicated.

At each site, nine plants were subjected to supplementary hand pollination, whereas, the remaining nine were left under natural pollination. Hand pollination was performed using a fine paintbrush to transfer pollen between capitula from plants with different genetic linage and was repeated three times per week until floral senescence. Seeds were collected from ten randomly selected capitula per plant, and the proportion of filled seeds was estimated for each treatment. The number of filled seeds produced per capitulum is reported as mean ± standard deviation (SD). Subsequently, 959 seeds, classified as filled, were sown in eight seedling trays, ensuring balanced representation of the different plant growth site and pollination treatments.

2.7. Experiment 3—Effects of Seed Storage

To evaluate the influence of storage time, 670 filled seeds (134 per collection year) collected during the 2015, 2020–2021, 2021–2022, 2022–2023, and 2023–2024 flowering seasons were used. All seeds were harvested between December and February of the corresponding season, and tested simultaneously in August 2024, so that seed age at sowing was 104–106 months, 42–44 months, 30–32 months, 18–20 months and 6–8 months respectively. Two germination methods were compared. In seedling trays ($n = 5$), 451 seeds produced by greenhouse-grown and outdoor-grown plants were sown together and distributed evenly among trays. In Petri dishes ($n = 9$), 221 seeds, only produced by greenhouse-grown plants, were used. In both methods, seeds from the different storage time were distributed evenly among trays or dishes.

2.8. Experiment 4—Plant Growth Site and Germination Substrate

To evaluate whether germination success depends on the plant growth site and germination substrate, 513 filled seeds collected during the 2024–2025 flowering season were sown in four seedling trays. Two trays contained the same substrate used in the previous experiments (a sterilized mixture of vermiculite, peat, and commercial fertilized soil, 1:1:1) and two contained soil collected from Cerro Ventana, Ernesto Tornquist Provincial Park. Seeds from each plant growth site (greenhouse and outdoor) were distributed evenly among all four trays.

2.9. Statistical Analyses

For all experiments, treatment effects were analyzed using generalized linear mixed models (GLMMs) with binomial error distributions, fitted with the glmmTMB package in R version 4.1.2 [29]. The response variables were the probability of germination and, in the pollination experiment, the probability of producing filled seeds. Depending on the experiment, genetic lineage (derived from the 15 founder plants), maternal plant, capitulum, and experimental unit (seedling tray or Petri dish) were included as random effects (Table 1). Model selection was based on the Akaike Information Criterion (AIC).

Table 1. Final generalized linear mixed models (GLMMs) fitted to evaluate the effects of pollination method, seed storage, plant grown site, and germination substrate on filled seed production and germination in *Senecio leucopeplus*. All models were fitted using a binomial error distribution with a logit link function (glmmTMB). The symbol * indicates the inclusion of both main effects and their interaction, whereas + separates additive terms; (1|x) denotes a random intercept for variable x. Experimental unit refers to a seedling tray or Petri dish, and genetic lineage refers to a lineage derived from one of the 15 seed-derived founder plants. Germination was recorded as a binary response (germinated = 1; not germinated = 0).

Experiment	Final Model
Pollination: production of filled seeds	cbind(filled_seeds, empty_seeds) ~ pollination * site + (1|maternal_plant) + (1|capitulum)
Pollination: germination	germination ~ pollination * site + (1|experimental_unit) + (1|maternal_plant) + (1|capitulum)
Seed storage: seedling trays	germination ~ season + site + (1|experimental_unit) + (1|genetic_lineage)
Seed storage: Petri dishes	germination ~ season + (1|experimental_unit) + (1|genetic_lineage)
Plant growth site and substrate	germination ~ substrate * site + (1|experimental_unit) + (1|genetic_lineage)

3. Results

Seed morphology was explored as a provisional screening criterion for distinguishing putatively filled from putatively empty seeds. Among the 1389 seeds evaluated in the validation experiment, only four seeds germinated, and all had previously been classified as filled. No germination was recorded among seeds classified as empty. Although the low overall germination precluded a formal validation of the classification, observations did not establish the diagnostic accuracy of the morphological classification; the subsequent experiments nevertheless focused on the putatively filled category.

Pollination treatment influenced both the production of filled seeds and their subsequent germination success, although its effect depended on the plant growth site. The mean total number of seeds produced per capitulum ranged from 124.2 ± 27.75 to 140.9 ± 32.59, with no consistent differences among cultivation sites or pollination treatments. In contrast, the proportion of filled seeds varied according to the interaction between pollination treatment and plant growth site. At both outdoor cultivation sites, naturally pollinated plants produced significantly higher proportions of filled seeds than hand-pollinated plants ($p < 0.05$), whereas the opposite pattern was observed in the greenhouse, where hand pollination resulted in significantly higher proportions of filled seeds ($p < 0.05$, Figure 3).

Despite this result, natural pollination consistently produced seeds with higher germination success than hand pollination. Germination percentages of seeds classified as filled ranged from 11.26% to 29.41%, and the generalized linear mixed model detected a significant interaction between pollination treatment and plant growth site. Under all cultivation conditions, seeds produced through natural pollination showed higher germination than those obtained through supplementary hand pollination ($p < 0.05$, Figure 4). This pattern contrasted with that observed for the production of filled seeds in the greenhouse, indicating that a higher proportion of morphologically filled seeds did not necessarily translate into higher germination success.

Older seed lots generally showed lower germination, and this association was apparent with both germination methods. Germination declined progressively with increasing storage time, and no germination was recorded for seeds stored for 104–106 months and 42–44 months, consistent with reduced germination capacity in older lots, although storage duration cannot be separated fully from collection season and seed-origin differences.

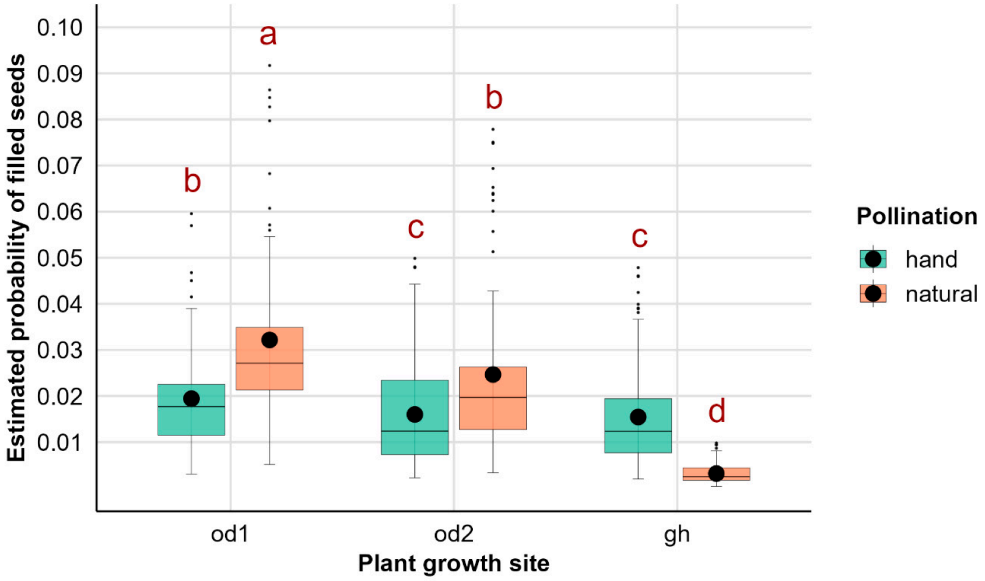


Figure 3. Estimated probability of the proportion of filled seeds produced by *Senecio leucopeplus* plants grown in greenhouse and outdoors, under hand and natural pollination. Values correspond to estimated marginal means (response scale) obtained from a generalized linear mixed model with a binomial distribution. od1: outdoor site 1; od2: outdoor site 2; gh: greenhouse. Boxes show the interquartile range, horizontal lines the median, whiskers the data dispersion (error bars extending up to 1.5 × IQR), small dots the outliers, and central large dots the estimated marginal mean for each group. Letters indicate statistically significant differences among specific combinations of plant growth site and pollination treatments (Tukey-adjusted pairwise comparisons, $p < 0.05$), based on the significant interaction found in the generalized linear mixed model.

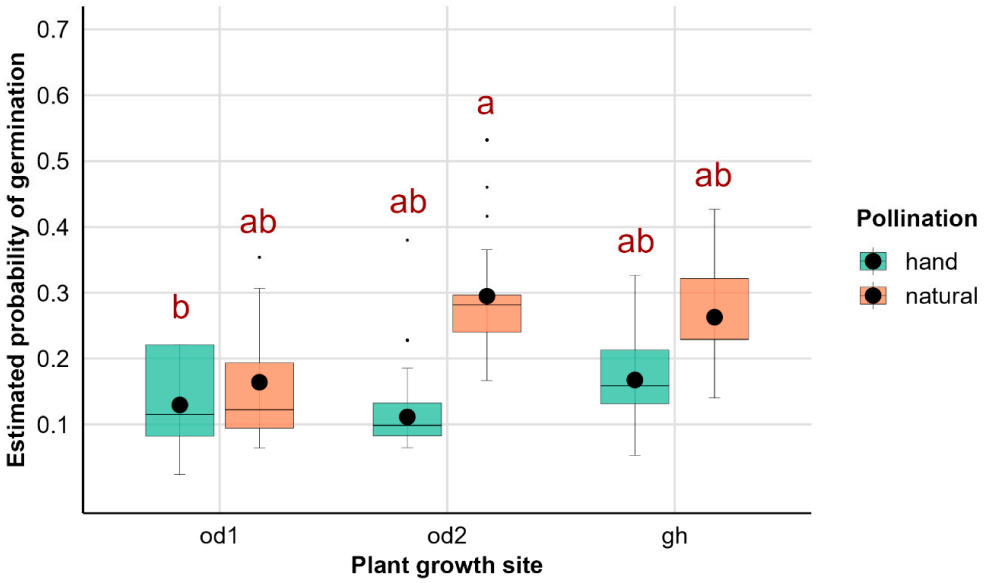


Figure 4. Estimated probability of germination of *Senecio leucopeplus* seeds obtained from plants grown in greenhouse and outdoors, under hand and natural pollination. Values correspond to estimated marginal means (response scale) obtained from a generalized linear mixed model with a binomial distribution. al1: outdoor site #1; al2: outdoor site #2; gh: greenhouse. Boxes show the interquartile range, horizontal lines the median, whiskers the data dispersion (error bars extending up to 1.5 × IQR), small dots the outliers, and central large dots the estimated marginal mean for each group. Letters indicate statistically significant differences among specific combinations of plant growth site and pollination treatments (Tukey-adjusted pairwise comparisons, $p < 0.05$), based on the significant interaction found in the generalized linear mixed model.

For seeds sown in seedling trays, germination success was significantly associated with decreasing storage time ($p < 0.01$, Figure 5). Germination percentages for seeds produced by greenhouse-grown plants increased from 4.44% for seeds stored for 30–32 months to 26.67% for those stored for 18 to 20 months, and 53.33% for the most recent lot stored for 6 to 8 months. Seeds produced outdoors showed an even steeper decline, with germination recorded only for the seeds stored for the shortest period (6 to 8 months), which reached 33.33% germination. No interaction was detected between storage time and plant growth site, suggesting a broadly similar age-associated pattern across the sampled cultivation sites. Nevertheless, seeds obtained from greenhouse consistently exhibited higher germination success than those obtained from the outdoor sites in this comparison ($p < 0.01$, Figure 5).

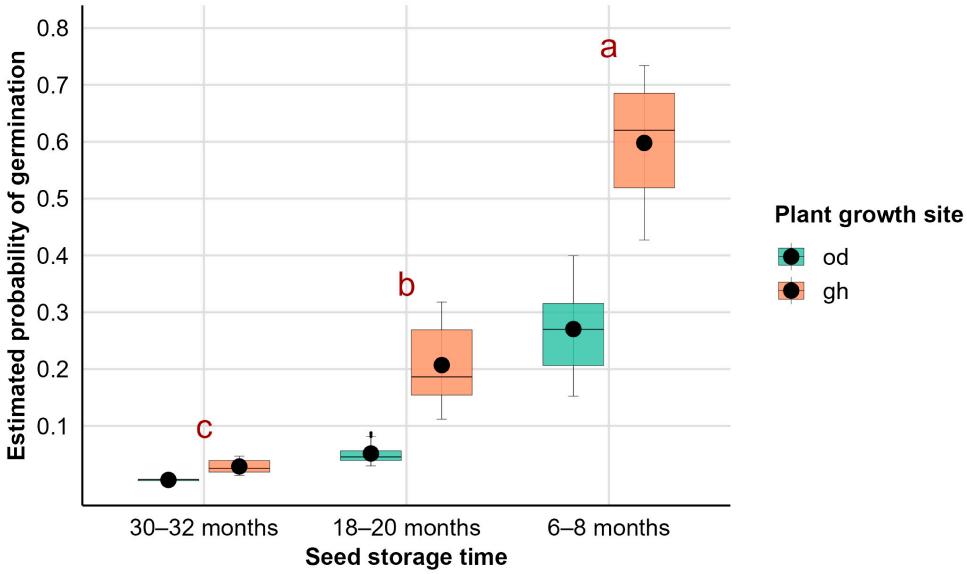


Figure 5. Estimated probability of germination of *Senecio leucopeplus* seeds with different storage ages collected from greenhouse- and outdoor-grown plants and sown in seedling trays. Values correspond to estimated marginal means (response scale) obtained from a generalized linear mixed model with a binomial distribution. gh: greenhouse; od: outdoors. Boxes show the interquartile range, horizontal lines the median, whiskers the data dispersion (error bars extending up to 1.5 × IQR), small dots the outliers, and central large dots the estimated marginal mean for each group. Letters indicate statistically significant differences among storage times (Tukey-adjusted pairwise comparisons, $p < 0.01$). Plant growth site also had a significant effect (greenhouse > outdoors, $p < 0.01$). No significant interaction between factors was detected.

A similar pattern was observed for seeds sown in Petri dishes. Germination increased from 2.38% for seeds stored for 30–32 months to 41.30% and 79.55% for those stored for 18 to 20 months and 6 to 8 months, respectively ($p < 0.01$, Figure 6). As in seedling trays, seeds stored for 104–106 months and 42–44 months failed to germinate.

Although absolute germination percentages were generally higher in Petri dishes than in seedling trays, both methods revealed the same biological pattern: germination capacity declined rapidly with storage time. During the Petri dish experiment, however, fungal growth developed on several seeds, suggesting that this method may be less suitable for routine propagation despite the relatively high germination percentages obtained.

Germination varied across plant growth sites and substrate combinations. Seeds collected from greenhouse-grown plants reached germination percentages of 39.06% in Cerro Ventana soil, and 26.56% in the vermiculite, peat and fertilized soil mixture. Corresponding values for seeds produced outdoors were 21.88% and 30.21%, respectively. Although the generalized linear mixed model detected an interaction between plant growth site and

substrate type, pairwise comparison revealed no significant differences among treatments (Figure 7). These results provide no evidence of a consistent difference between the two substrates under the conditions evaluated; they do not establish a causal effect of plant grown site.

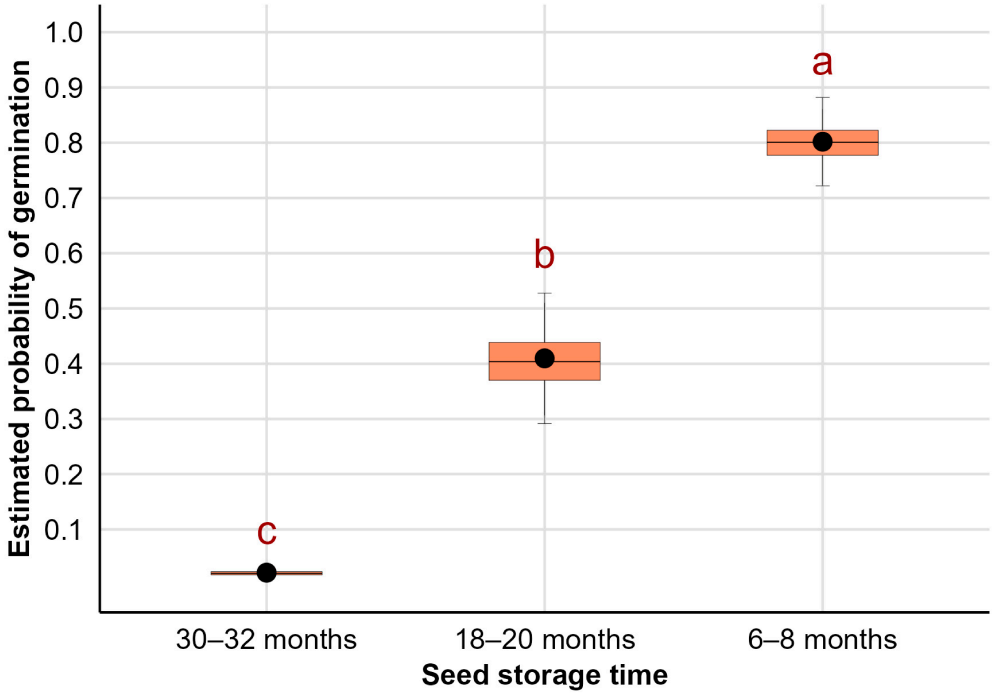


Figure 6. Estimated probability of germination of *Senecio leucopeplus* seeds with different storage ages collected from greenhouse-grown plants and sown in Petri dishes. Values correspond to estimated marginal means (response scale) obtained from a generalized linear mixed model with a binomial distribution. Boxes show the interquartile range, horizontal lines the median, whiskers the data dispersion (error bars extending up to 1.5 × IQR), and central large dots the estimated marginal mean for each group. Seed storage time had significant main effects on germination probability. Letters indicate statistically significant differences among storage times (Tukey-adjusted pairwise comparisons, $p < 0.01$).

Although several treatments increased germination success, post-germination survival remained an important limitation to sexual recruitment in most trials. Survival after germination was consistently low in nearly all experiments, regardless of the treatment applied. In the seed classification experiment, the four seedlings that emerged survived for a maximum of 34 days in the germination chamber. After transplantation into pots, one seedling died immediately, two survived for an additional seven days, and only one individual reached a total survival time of 70 days. Seedlings obtained in the pollination experiment ($N = 178$) survived for 5.8 ± 13.4 days before transplantation and 12.2 ± 10.9 days thereafter ($N = 44$). Similarly, seedlings obtained during the seed storage experiment showed very limited survival. Individuals germinated in seedling trays ($N = 53$) survived for 2.0 ± 7.2 days and were not transplanted because of their poor condition. Seedlings germinated in Petri dishes ($N = 55$) survived for 2.4 ± 6.6 days before transplantation and 9.3 ± 6.4 days after being transferred to seedling trays ($N = 32$). The highest survival was recorded in the final experiment evaluating plant growth site and substrate. Seedlings ($N = 142$) survived for 2.8 ± 5.6 days before transplantation and especially 150.5 ± 82.9 days thereafter ($N = 18$). Unlike the previous experiments, where all seedlings eventually died within the reported survival periods, five individuals were still alive when the manuscript was prepared (early July 2026), showing that some seedlings persisted for substantially longer

in this trial. Because observation durations and transplantation procedures differed among experiments, these survival summaries cannot by themselves establish which conditions improved establishment.

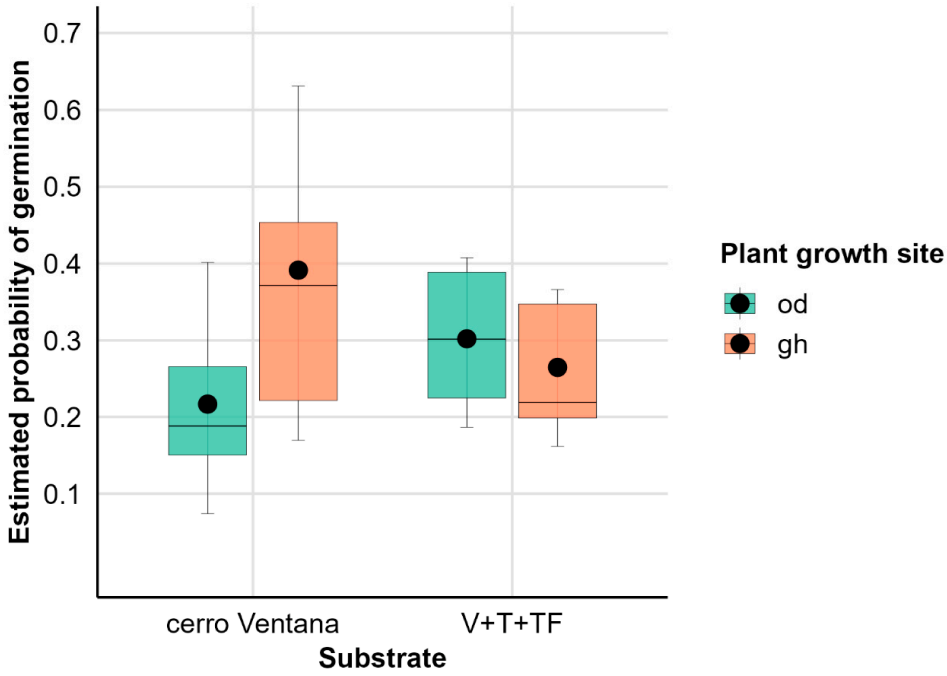


Figure 7. Estimated probability of germination of *Senecio leucopeplus* seeds collected from greenhouse- and outdoor-grown plants and sown in different substrates. Values correspond to estimated marginal means (response scale) obtained from a generalized linear mixed model with a binomial distribution. gh: greenhouse; out: outdoors; Cerro Ventana: substrate collected from Cerro Ventana; V + P + FS: substrate consisting of a mixture of vermiculite, peat, and fertilized soil. Boxes show the interquartile range, horizontal lines the median, whiskers the data dispersion (error bars extending up to 1.5 × IQR), and central large dots the estimated marginal mean for each group. A significant interaction was detected between substrate and plant growth site. Tukey-adjusted pairwise comparisons among specific combination of substrate and plant growth site detected no significant differences.

4. Discussion

This study highlights the severe limitations affecting the sexual propagation of *S. leucopeplus*. The low overall production of morphologically filled seeds, variable germination of the selected seeds, and poor survival of seedlings in most trials indicate that recruitment is constrained by multiple processes acting sequentially from seed production to early establishment. Understanding these limitations is essential for maintaining sexual reproduction and, consequently, preserving the genetic diversity and adaptive potential of the only remaining population of the species. Nevertheless, the results obtained identify factors that significantly influence reproductive success under cultivation conditions. In particular, natural pollination produced higher germination percentages under the different cultivation conditions evaluated. Likewise, higher germination success was observed in seeds collected during more recent flowering seasons and, in general, in those obtained from greenhouse-grown plants, suggesting associations with plant growth site and seed storage time, without independently demonstrating changes in seed viability or causal effects of cultivation site. More importantly, the results illustrate how systematic experimental evaluation can generate evidence to optimize propagation protocols for plant species maintained exclusively under cultivation.

Due to the extremely low germination percentage obtained in the first experiment, it was not possible to confirm the accuracy of the seed classification method used in this study. However, considering that none of the seeds classified as empty germinated, excluding them from the subsequent experiments appeared to be the most appropriate strategy to optimize the experimental effort. Although this morphological classification cannot be considered a definitive assessment of seed viability, it was used as a pragmatic screening rule for allocating scarce material, but its sensitivity and specificity remain unknown.

The establishment stage represents the main bottleneck in the life cycle of many plant species and is also among the most variable phases of the plant life cycle [30]. It comprises two sequential processes, namely seed germination and seedling survival, which, although commonly evaluated together, may be subject to different selective pressures [31]. The persistence of rare and endangered plant species largely depends on the success achieved during these early stages [32], with germination representing a key step for successful establishment, growth, and population expansion [33]. Our results indicate that *S. leucopeplus* experiences limitations at both stages, suggesting that improving germination alone will probably be insufficient to ensure successful recruitment unless seedling establishment is also enhanced.

The germination percentages obtained in all experiments were considerably lower, regardless of the treatments applied, than those reported for other Asteraceae, including species of the genus *Senecio* [34–38], and remained low even when compared with other threatened species [39,40]. This finding is particularly concerning because all experiments were conducted exclusively with the subset of seeds classified as filled, which represented only a small fraction of the total seed production (only 1.51–3.24% in the pollination experiment). Consequently, the effective production of potentially recruitable offspring is dramatically lower than would be expected from total seed output alone.

A possible explanation for the low germination success could be the presence of a physiological constraint preventing germination, as a consequence of either environmental influences or the induction of secondary dormancy [35,41]. However, an important limitation of our study is the absence of an independent assessment of seed viability. Without this information, it is not possible to determine whether the low germination percentages primarily reflect a high proportion of non-viable seeds or the presence of viable but dormant seeds that failed to germinate under the experimental conditions. This distinction is critical because it implies fundamentally different management strategies. Viability tests, such as the tetrazolium test or the assessment of embryo development through dissection or imaging techniques, would provide an estimate of the proportion of potentially germinable seeds and help explain the high variability observed among seed lots and treatments. Such analyses would also allow the relative importance of seed mortality and dormancy to be quantified, thereby clarifying whether management efforts should focus primarily on improving seed quality or on overcoming physiological constraints to germination. Incorporating these analyses should, therefore, be considered a priority for future studies, as they would help explain variation among seed lots, while providing a stronger basis for optimizing propagation protocols.

The combination of extremely low germination, the high proportion of empty seeds and poor seedling survival, the latter possibly exacerbated by transplant shock, suggests that the limitations are unlikely to arise from a single mechanism. Instead, they probably reflect the combined effects of physiological, genetic, and demographic processes that have accumulated during the recent conservation history of the species. In particular, the founding population, established from just two wild individuals, may have experienced genetic drift and inbreeding, although neither mechanism was measured here, processes that are common in threatened species [13]. Several studies have shown that reduced genetic diver-

sity can negatively affect seed germination and seedling recruitment, ultimately decreasing offspring fitness [33,42,43]. These effects are especially concerning in small or declining populations, where genetic bottlenecks and increased demographic stochasticity may further accelerate the loss of genetic diversity and reduce adaptive potential, with important implications for the long-term viability of the species [44,45]. Although these mechanisms were not directly evaluated in our study, they provide a biologically plausible explanation for the consistently poor reproductive performance observed across all experiments.

Added to these factors is the possibility that the cultivated population originated through hybridization with a closely related species [28]. This situation has been identified by Maunder et al. [12] as one of the major limitations of conservation collections maintained in botanical gardens, which frequently face genetic and demographic management challenges and are, therefore, susceptible to genetic drift, artificial selection, hybridization, and the accumulation of deleterious mutations [46]. Because all existing individuals descend from a very small number of founders and have remained under cultivation for nearly three decades, these processes may have acted simultaneously, making it difficult to disentangle their relative contributions to the observed reproductive limitations. In this context, genetic studies are urgently needed to confirm the taxonomic identity of the cultivated individuals, characterize their genetic diversity, and evaluate the possible contribution of these factors to the low and highly variable germination and seedling establishment observed in this species. Clarifying these questions is important not only for understanding the reproductive biology of *S. leucopeplus*, but also for defining appropriate conservation strategies. If reduced genetic diversity proves to be the principal cause of poor reproductive performance, management actions should prioritize maximizing recombination among the existing lineages and minimizing further genetic erosion. Conversely, if hybridization has substantially altered the genetic identity of the cultivated population, conservation efforts may require reassessing the long-term objectives of the ex situ programme and the feasibility of future reintroduction initiatives. Consequently, integrating experimental propagation studies with molecular genetic analyses should be regarded as a priority for the next stage of the conservation programme.

Beyond identifying the factors responsible for the low recruitment success of *S. leucopeplus*, our results also provide practical guidance for improving its ex situ management. Although none of the treatments evaluated completely overcame the reproductive limitations affecting the species, several significantly increased recruitment success and can, therefore, be incorporated into future propagation protocols.

The higher germination success observed in seeds produced under natural pollination suggests that the supplementary hand pollination protocol used did not improve germination. Although brush pollination is a simple and operationally efficient technique [47], its effectiveness depends on appropriate timing and careful manipulation of the reproductive structures. When performed improperly, it may cause mechanical damage to the reproductive structures of the flowers [48], and repeated application may even remove previously deposited pollen, thereby reducing reproductive success. Another possibility is that successful fertilization may occur through additional reproductive mechanisms, such as autogamy, which has been reported in other species of the genus [49]. Alternatively, natural pollinators may transfer pollen more efficiently than manual pollination by ensuring greater pollen loads or more effective deposition on receptive stigmas. Evaluating the contribution of these mechanisms in future studies would be of particular interest, as has been done for other Asteraceae [50], in order to determine which reproductive strategies are employed by *S. leucopeplus* and their relative importance. Taken together, these results suggest that maintaining conditions that favour natural pollinator activity is likely to be a more effective management strategy than implementing routine supplementary hand pollination.

The association between older seed lots and lower germination could partly reflect a progressive loss of viability during storage, a fairly common phenomenon, especially in perennial species [35,51]. Although the seeds used in this study were stored under dry conditions at room temperature, storage at low temperatures has been reported as an effective measure to improve germination success, as it tends to slow physiological changes, preventing both the loss of viability and the induction of secondary dormancy [41]. For example, *Senecio morisii* J. Calvo & Bacch. has been reported to maintain a high germination capacity after ten years of storage under seed bank conditions at −25 °C, following international standards for long-term seed storage [52]. Our results indicate that seed collections should be renewed frequently and that propagation efforts should prioritize recently collected seeds until more effective storage protocols become available. In parallel, evaluating long-term storage under conventional seed-bank conditions represents an important research priority for improving the long-term conservation of the species. However, because seed age was confounded with collection season and potentially with seed-origin or storage history, a controlled storage experiment is needed to establish the effect of duration itself.

In the seedling trays, germination success was lower for seeds obtained from plants cultivated outdoors, in this particular comparison. Moreover, although the model predicts a certain level of germination success for seeds with longer storage time, some older outdoor-derived lots failed to germinate, but this does not establish a more rapid loss of viability in outdoor-derived seeds. One possible explanation is variation in seed provisioning across plant growth sites, but temperature, humidity and other potential causes were not measured sufficiently to test this mechanism. This influence of the maternal ecological environment on the phenotype of the offspring, known as the environmental maternal effect, occurs after embryo formation but before seed dispersal. The reduction in offspring fitness decreases the probability of seedling recruitment and the competitive ability of young individuals [53]. These observations justify further replicated comparisons of cultivation conditions and seed quality. Greenhouse cultivation may be a useful provisional management option, but its benefits should be tested independently of site effects before being generalized.

Regarding substrate type, the differences observed in germination percentages were not significant, but the experiment did not directly assess nutrient availability or mycorrhizal colonization. Although many species of Asteraceae, including species of the genus *Senecio*, establish arbuscular mycorrhizal associations, and these may influence nutrient uptake and plant growth [54–56], the intensity and effects of these associations may be variable or even moderate depending on the ecological context, the fungal species, or the soil type [57,58]. The absence of significant pairwise differences between these two substrates does not exclude substrate effects under other conditions or effects on later seedling establishment. Accordingly, further work should prioritize seed quality and seedling establishment while retaining substrate optimization as an open question.

Overall, our results indicate that the limitations affecting sexual recruitment in *S. leucopeplus* arise from the interaction of multiple processes acting sequentially from seed production to early seedling establishment. Rather than identifying a single limiting factor, our study suggests that successful propagation will require attention to several stages of the recruitment process simultaneously, including the production of filled seeds, seed germination, and seedling survival. Accordingly, the optimization of ex situ conservation should be viewed as an adaptive process in which management actions are progressively refined as new evidence becomes available. The principal recruitment limitations identified in this study, together with the corresponding management implications and research priorities, are summarized in Table 2.

Table 2. Sequential constraints affecting sexual recruitment in *Senecio leucopeplus*, together with the principal management actions and research priorities identified to support adaptive, evidence-based ex situ conservation.

Recruitment Stage	Evidence from This Study	Immediate Management	Research Priorities
Filled seed production	Very low proportion of filled seeds	Promote natural pollination	Assess genetic diversity, hybridization and reproductive biology
Germination	Low and highly variable	Use fresh seeds, maintain mother plants under greenhouse conditions	Assess seed viability and dormancy mechanisms
Seedling establishment	High mortality	Reduce transplant stress, optimize nursery conditions	Identify causes of seedling mortality and evaluate beneficial soil microorganisms

Our study provides an evidence-based framework for improving the ex situ management of *S. leucopeplus*, showing that even in species classified as Extinct in the Wild, some components of propagation success can be improved by systematically identifying and addressing the main constraints affecting recruitment. Our results indicate that reproductive limitations arise from the combined effects of low production of filled seeds, reduced germination capacity, and poor seedling establishment, highlighting the need to consider the entire recruitment process rather than focusing on a single developmental stage. From a management perspective, our findings support the use of recently collected seeds, further evaluation of greenhouse cultivation for mother plants, and the promotion of natural pollination as practical measures capable of increasing recruitment success under cultivation. At the same time, they identify clear priorities for future research, including the evaluation of seed viability and dormancy, the characterization of the genetic diversity of the cultivated population, and the identification of the factors responsible for the high mortality observed during seedling establishment.

Although developed for a single species, the adaptive approach adopted here may be informative for other ex situ conservation programmes involving extremely small or genetically impoverished plant populations, where propagation protocols must be optimized despite limited biological information and scarce propagules. By combining experimental evaluation with adaptive management, this framework can help transform routine cultivation into a source of evidence that supports both immediate conservation actions and the long-term recovery of threatened plant species.

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