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ORIGINAL ARTICLE

Improved in vitro germination of *Colobanthus quitensis*: a key step for Antarctic plant conservation

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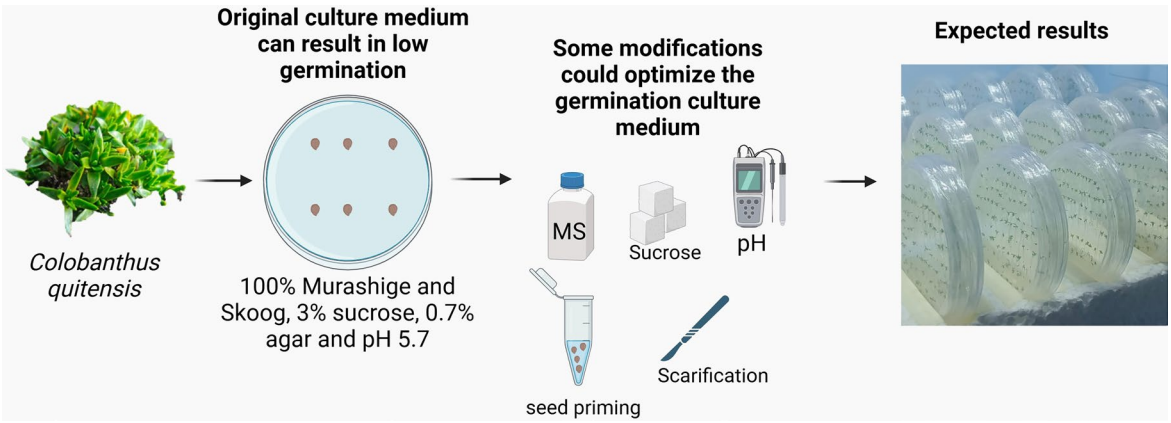
Abstract

In vitro plant tissue culture is an important strategy for plant regeneration, micropropagation, and germplasm conservation. However, its implementation requires efficient and cost-effective protocols. The extremophile species *Colobanthus quitensis* has been proposed as a model plant for stress biology studies. Its distribution in Antarctica faces increasing threats from tourism and scientific activities, making ex situ conservation urgent. However, low in vitro germination rates hinder this approach, highlighting the need for protocol optimization. This study addressed two key questions: (1) What culture medium composition best stimulates *C. quitensis* germination? and (2) Which preconditioning and dormancy-breaking treatments are most effective for Antarctic populations? We tested variations in culture medium composition (Murashige and Skoog [MS] basal salts and sucrose), priming treatments, pH (5.5 and 5.7), and scarification methods. The results demonstrated that omitting MS in a pH 5.7 medium achieved 46.67% germination, increasing to 73.33% with 5% KCl halo-priming. However, complete MS exclusion impaired seedling development. A 25% MS medium at pH 5.5, combined with halo-priming, optimized both germination (81.67%) and seedling growth. For highly dormant Antarctic populations, mechanical scarification was essential to achieve significant germination. In conclusion, minor adjustments to medium composition and concentration provided a low-cost, efficient protocol suitable for *C. quitensis* and other commercially relevant species with similar dormancy challenges.

Key message

The present study successfully optimized a simple, low-cost in vitro germination protocol for *Colobanthus quitensis*, using halo-priming and manual testa scarification.

Graphical abstract



Keywords Dormancy · Seeds · Priming · Scarification · Medium modification

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Introduction

In vitro cultivation has become a cornerstone tool for conservation and biotechnological research, enabling the mass production of plants from seeds or small plant tissue samples, commonly referred to as explants (Cruz-Cruz et al. 2013; Hesami et al. 2021). This technique preserves genetic material without impacting natural populations and contributes to germplasm banking. These tools are invaluable for studying physiological and genetic processes, optimizing growth conditions, and understanding stress adaptation mechanisms (Bhattacharyya et al. 2014; Sané et al. 2021; Ontivero et al. 2024b). An effective in vitro germination protocol facilitates large-scale plant production while providing germinated seedlings for use as young explants in secondary metabolite extraction, genetic transformation, and callus production (Sorokin et al. 2021; Lian et al. 2022). Germination is a complex process influenced by genetic and physical factors; therefore, protocol components must be adapted to each species, considering variables such as culture medium, sterilization, temperature, light, and pH (Roni et al. 2018; dos Santos et al. 2021).

Among germination-enhancing techniques, priming involves preconditioning plants to tolerate impending stress. This may include controlled seed hydration in water (hydropriming), polyethylene glycol (osmopriming), or salt solutions (halopriming: $CaCl_2$, $CaSO_4$, KCl, KNO_3, or NaCl) (Sadeghi and Robati 2015; Wang et al. 2022; Zhou et al. 2024). Controlled hydration at low water potential initiates metabolic activity without radicle emergence, ensuring synchronized germination (Soughir et al. 2013). Priming effectively breaks dormancy, enhances seed vigor, and improves germination/seedling establishment under stress (Pawar and Laware 2018).

For in vitro cultivation, the culture medium type (e.g., Murashige and Skoog, Gamborg B5, Thomale GD, Woody Plant Medium) and concentration (0–2X strength) are critical (Rodríguez et al. 2014; Huh et al. 2016; Li and Zhang 2018; Tinoammini et al. 2024). Other key factors include carbohydrate source/concentration (Stewart et al. 2010; Huh et al. 2016), phytohormone type/concentration (Agha et al. 2022), and medium pH (4.2–5.9) (Zhang et al. 2004; Li and Zhang 2018).

Colobanthus quitensis (Kunth) Bartl. (Caryophyllaceae), one of Antarctica's two native vascular plants, is a model for extreme-environment resilience (Convey and Biersma 2024). In harsh environments, germination is tightly regulated by physiological adaptations (e.g., metabolic dormancy, antioxidant activity), genetic factors (e.g., LEA stress-response genes), and environmental triggers (e.g., temperature fluctuations, light spectra, nitrate availability) (Bewley et al. 2013; de Freitas et al. 2024; Nyasulu et al. 2024). Antarctic populations face exacerbated controls, with seeds exhibiting deep dormancy to avoid unfavorable-season germination (Gielwanowska et al. 2011; Kellmann-Sopyła et al. 2017).

In vitro, challenges include absent natural cues (soil microbiota, diurnal shifts), suboptimal medium composition (growth regulator/osmotic imbalances), and oxidative stress (Bhattacharyya et al. 2014; Tiwari et al. 2024). Despite this, *C. quitensis* has been studied for salinity (Cuba-Díaz et al. 2017a; Ontivero et al. 2024b), freezing (Min et al. 2024), and metal toxicity tolerance (Cuba-Díaz et al. 2017c; Contreras et al. 2018; Farías 2018). However, low in vitro germination rates especially in Antarctic populations limit ex situ conservation (Cuba-Díaz et al. 2017b; Ontivero et al. 2024b), necessitating physiological/technical solutions. While stratification, hormones, and chemical/mechanical scarification have been tested (Cuba-Díaz et al. 2019; Tai et al. 2021; Khuat et al. 2022; Rocha et al. 2022), an optimized, species-specific protocol remains needed (Coelho and Romano 2021).

For *C. quitensis*, full-strength MS medium with 3–4% sucrose (pH 5.7) is standard (Zúñiga et al. 2009; Cuba-Díaz et al. 2017a; Ontivero et al. 2024b). However, other species show higher germination with reduced MS/sucrose (Rodríguez et al. 2014; Huh et al. 2016). Naturally occurring in acidic soils (pH 4.9–6.6; Upson et al. 2008; Beber-Vieira et al. 2011), *C. quitensis* has been propagated in vitro at pH 4.5–5.8 (Zúñiga et al. 2009; Cuba-Díaz et al. 2017a, 2020), suggesting slightly acidic conditions may enhance germination/establishment.

Given Antarctic populations' germination constraints, we hypothesize that in vitro interactions between dormancy-breaking treatments, seed priming, and medium optimization will improve germination. This study addresses: (1) What is the optimal culture medium composition for *C. quitensis* germination? and (2) Which priming/dormancy-breaking treatment is most effective for Antarctic populations? We aim to optimize an in vitro germination protocol and develop targeted strategies for Antarctic specimens.

Materials and methods

Plant material

For experiments 1 to 4, a homogeneous mixture of *C. quitensis* seeds in equal proportions was used, representing different origins: the Antarctic population Arctowski, the

sub-Antarctic coastal populations La Marisma and Laredo, and the sub-Antarctic non-coastal population Vega. This strategy aimed to develop a baseline germination protocol for the species, which can be adapted to the specific requirements of each population (as explored in experiment 5). To achieve this, plants collected from the field were transferred to the common garden of the Active Collection of Antarctic Vascular Plants at the Laboratory of Biotechnology and Environmental Studies (LABEA), University of Concepción, where they were propagated in growth chambers at 13 ± 1 °C, with a photoperiod of 16/8 h light/dark, a light intensity of 120 ± 20 µmol m^2 s^{-1}, and 85–90% relative humidity. *Colobanthus quitensis* seeds were collected from fully open floral capsules containing mature seeds (Still 1999). The capsules were dried at room temperature for 2–3 days, after which the seeds were manually extracted and stored in airtight Eppendorf tubes at 4 °C for 18 months until they were used. The seed storage period corresponded to the maximum vigor period identified by Salgado (2023). The experiments were conducted at one-month intervals.

To improve the germination protocol for Antarctic seeds, corresponding to experiment 5, seeds were collected in the field from the Antarctic population of Byers Peninsula (62°41′07.36"S/60°51′26.75"W, Livingston Island, South Shetland Islands), during the summer of 2023 (PERMIT No. 203/2023 from Instituto Antártico Chileno). These seeds were air-dried for 2 days and stored in Eppendorf tubes at room temperature during their transport from Antarctica to LABEA. Subsequently, they were preserved at 4 °C for 18 months, following the same procedure as the seeds used in the previous experiments.

The culture media evaluated in this study were based on the general protocol for the in vitro germination of *C. quitensis* proposed by Cuba-Díaz et al (2017a). This protocol includes disinfecting the seeds with 70% ethanol for 30 s, followed by 7 min treatment with 5% sodium hypochlorite (NaOCl 35 g L^{-1}, commercial bleach). The seeds are then rinsed three times with sterile distilled water. After disinfection, the seeds are placed in 10 cm diameter Petri dishes containing 20 mL of in vitro culture medium prepared with 100% Murashige and Skoog (MS) medium (Murashige and Skoog 1962), 3% sucrose, 0.7% agar, and a pH adjusted to 5.7. The in vitro seed sowing process is conducted under a laminar flow hood to maintain aseptic conditions. Seeds were germinated at 20 ± 2 °C with a photoperiod of 16 h light/8 h darkness and a light intensity of 45 ± 2 µmol m^2 s^{-1} for 30 days. Germination is recorded daily to calculate several germination indicators (see below), considering a seed germinated when its radicle reaches twice the length of the seed (Sanhueza et al. 2017).

Experiments

Experiment 1: MS modification with pH maintained at 5.7

To evaluate the optimal concentration of MS medium, the original germination protocol was maintained (3% sucrose, 0.7% agar and pH 5.7), with modifications made only to the MS concentrations in the medium. Germination was analyzed using MS concentrations of 0%, 25%, 50%, and 100% (the concentration from the initial protocol proposed by Cuba-Díaz et al. 2017a). Three replicates were performed for each treatment, with 15 seeds per replicate. Daily germination counts were conducted to calculate various germination indicators (see below 2.3).

Experiment 2: Application of priming treatments to seeds

To assess the effect of various priming treatments on the germination of *C. quitensis*, seeds were immersed for 24 h in one of the following solutions: distilled water (W) for hydro-priming, 5% polyethylene glycol (PEG5) or 10% polyethylene glycol (PEG10) for osmo-priming, 5% potassium chloride (KCl5) or 10% potassium chloride (KCl10) for halo-priming. After treatment, the seeds were rinsed three times and disinfected (Cuba-Díaz et al. 2017a). Germination was conducted in vitro using the culture medium from experiment 1 that yielded the best germination results. Four replicates were prepared for each treatment, with 15 seeds per replicate. Daily germination counts were performed to calculate various germination indicators (see below 2.3).

Experiment 3: MS modification by pH reduction (5.5) and optimal priming treatment

Seed germination of *C. quitensis* was evaluated using the priming treatment that achieved the best results in experiment 2 (maintaining a medium with 3% sucrose and 0.7% agar). The MS content in the germination medium was modified (0%, 25%, 50%, and 100%). For in vitro cultivation, plant cells require acidic pH levels between 5.5 and 5.7 (Gamborg and Shyluk 1981). Previous studies have demonstrated that *C. quitensis* can grow in vitro in media at pH 5.5 (Salgado 2023); thus, this pH value was maintained for the present experiment. Four replicates were performed for each treatment, with 21 seeds per replicate. Daily germination counts were conducted to calculate various germination indicators (see Sect. 2.3).

Experiment 4: Sucrose presence/absence analysis at varying pH in the culture medium

For germination to begin, only water is required to activate the enzymatic machinery of seeds (Hasanuzzaman et al. 2013); therefore, MS was not used. Therefore, this experiment evaluated whether sucrose (S + or S−) should be included in the culture medium and how the pH levels previously tested in experiments 1 and 3 influence germination. In this essay, the priming treatment selected in experiment 2 was maintained. Four replicates were performed for each treatment, with 21 seeds per replicate. Daily germination counts were conducted to calculate various germination indicators (see below 2.3).

We consolidated data from the most effective treatments identified in Experiments 1–4 to perform a comparative analysis of their germination efficacy. This systematic approach enabled evidence-based selection of the optimal protocol for enhancing in vitro germination in *C. quitensis*, ensuring the highest germination percentage.

Experiment 5: Scarification on germination in Antarctic *Colobanthus quitensis*

The low germination rate observed in Antarctic populations of *C. quitensis*, even after treatment with sulfuric acid (Cuba-Díaz et al. 2019), highlights the need to explore different treatment combinations to overcome dormancy. In this study, three treatments were evaluated: sulfuric acid (Sa), testa breakage with a scalpel (B), and the combination of both chemical and mechanical scarification methods (Sa + B). Given the hardness of the testa of *C. quitensis* seeds, priming treatments were used to soften the seed coat and promote its rupture. Two treatments from experiment 2 were selected, as they were the most effective. After 24 h of priming, seeds requiring chemical scarification (Sa and Sa + B) were treated with 2% sulfuric acid (Cuba-Díaz et al. 2019) and subsequently disinfected (Cuba-Díaz et al. 2017a). For treatments involving mechanical scarification (B and Sa + B), this procedure was performed after disinfection. These seeds were placed to germinate in media containing 25% MS, 0.7% agar, and pH adjusted to 5.5%. Five replicates were performed per treatment, with 21 seeds per replicate, and daily germination counts were carried out to calculate various germination indicators.

Germination indicators calculated in each experiment

Using daily germination measurements from each experiment were used to calculate the following:

Percentage of germination (Eq. 1).

$$GP = n/N * 100 \tag{1}$$

where *n* is the number of germinated seeds at the end of the experiment and *N* is the total number of seeds used.

From these count data, the accumulated germination rate was obtained as the fraction of germinated seeds per Petri dish recorded each day.

The mean germination time (MGT) (Eq. 2) is calculated as:

$$MGT = \Sigma ni * t/n \tag{2}$$

where *ni* is the number of seeds germinated on day *t* and *n* is the total number of seeds germinated during the experiment.

The time to reach 50% of germination (Eq. 3) is calculated as described by Farooq et al. (2005):

$$T50 = ti + ((n/2 - ni)(tj - ti))/((nj - ni)) \tag{3}$$

where *n* is the total number of germinated seeds by the end of the experiment, *ni* and *nj* are the accumulated numbers of germinated seeds from adjacent counts at times *tj* and *ti*, respectively, such that $ni < \frac{n}{2} < nj$

Germination Synchronization Index (Z) (Eq. 4), as described by Primack (1980), is calculated as:

$$\begin{aligned} &Z = \Sigma Cn_{i,2}/N, \text{siendo } Cn_{i,2} = n_i(n_{i-1})/2y \\ &N = \Sigma n_i(\Sigma n_{i-1})/2 \end{aligned} \tag{4}$$

where $Cn_{i,2}$ represents the combination of the seeds germinated at time *i*, taken two at a time; and n_i is the number of seeds germinated at time *i*. The index Z equals 1 when all seeds germinate simultaneously, and it equal 0 when at least two seeds germinate at different times.

Statistical analysis

To analyze the effect of treatments in Experiments 1, 2, and 3 on the different germination indicators, a one-way ANOVA was performed. For Experiments 4 and 5, a two-factor factorial analysis was conducted. Treatments from Experiments 1 to 4 that exhibited the strongest germination response were compared using one-way ANOVA. In both cases, a post hoc Tukey's Honest Significant Difference (HSD) test was applied using the Tukey HSD function in R, with a 95% confidence interval. Both the one-way and two-way ANOVA were performed using the "aov" function in R (R Core Team 2024). Graphs were subsequently generated using the "ggplot2" package (Wilkinson 2011).

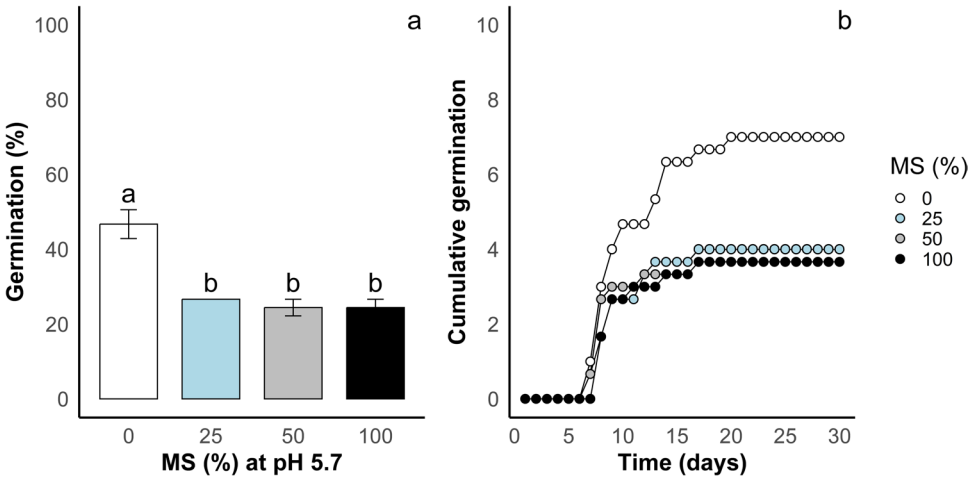

Fig. 1 Effect of modifying the Murashige and Skoog (MS) medium concentration in the in vitro culture medium on (**a**) germination percentage and (**b**) cumulative germination of *Colobanthus quitensis*. Different letters indicate significant differences ($P < 0.05$) according to Tukey's HSD test ($n = 3$)

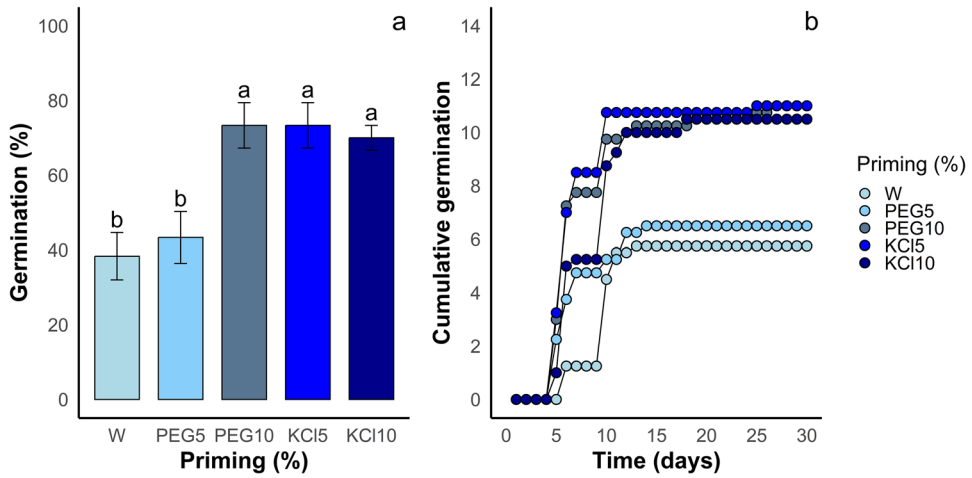

Fig. 2 Effect of different priming treatments on *Colobanthus quitensis* seeds for (**a**) germination percentage and (**b**) cumulative germination. Different letters indicate significant differences ($P < 0.05$) according to Tukey's HSD test ($n = 4$). DW: distilled water, PEG: polyethylene glycol, KCl: potassium chloride

Results

Among the indicators used in this research to evaluate germination, the T50 and Z indices did not show statistically significant differences in any of the experiments conducted. Therefore, they were not relevant indicators for this study on *C. quitensis* and will not be included in the description of the results of the different experiments. However, the results of these indices are available in the supplementary tables for each experiment. Thus, only the germination percentage and MGT indices were considered in the description and discussion of the results.

MS medium modification

The modification of the MS content in the original medium showed that the absence of this medium (0% MS) significantly stimulates germination (46.67%) compared to treatments that contain MS, where the germination percentage did not exceed 26.67% (Fig. 1a, Supplementary Table 1). In the treatments from Experiment 1, germination began on day 7, with no additional seeds germinating after day 20 (Fig. 1b). No significant differences were observed in MGT (Supplementary Table 2).

Application of priming treatments to seeds

The use of different osmo- and halo-priming treatments showed that the incorporation of PEG10 and KCl at concentrations of 5% and 10% into the original medium without MS resulted in germination percentages above 70%, with no significant statistical differences ($P > 0.05$) among these three treatments (Fig. 2a, Supplementary Table 1). The application of any of these priming methods allowed germination to begin as early as the fifth day of the experiment (Fig. 2b). However, PEG is more expensive than KCl in the market (Supplementary Table 3), and among the two halo-priming treatments, the use of 5% KCl requires a lower amount of product, contributing to a reduction in cultivation costs. Although none of the treatments significantly affected MGT, the use of 5% KCl showed a tendency to reduce MGT (Supplementary Table 4).

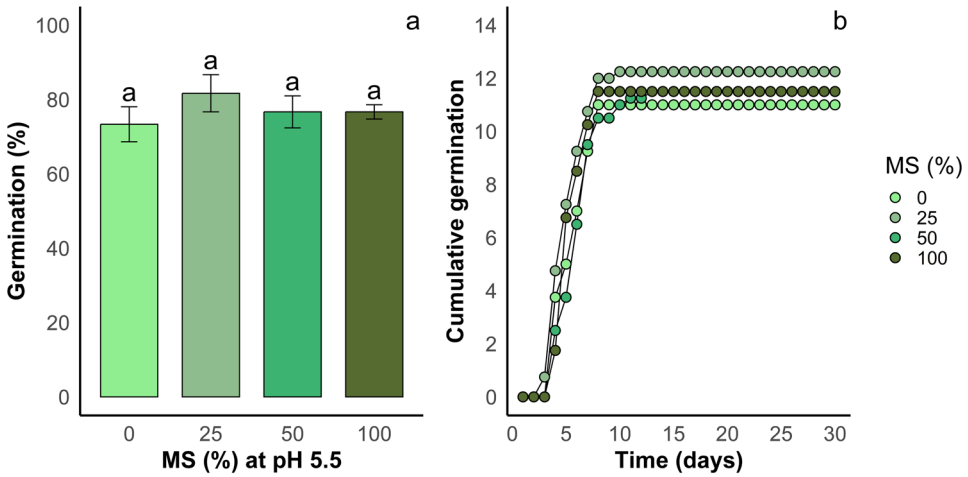

Fig. 3 Effect of modifying the MS medium content in the in vitro culture medium at pH 5.5 when seeds are treated with halopriming (KCl 5%) on (**a**) germination percentage and (**b**) cumulative germination of *Colobanthus quitensis*. Different letters indicate significant differences ($P < 0.05$) according to Tukey's HSD test ($n = 4$)

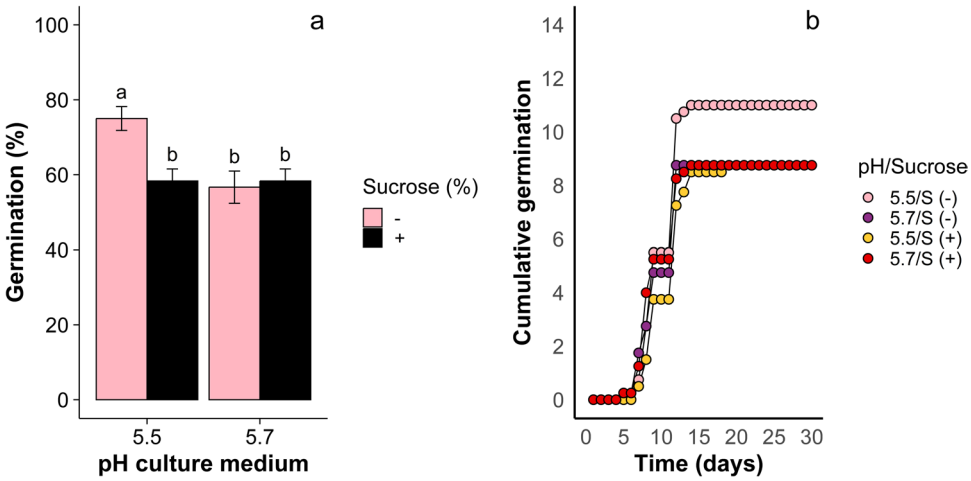

Fig. 4 Effect of medium pH modification and the presence or absence of sucrose in the in vitro culture medium on (**a**) germination percentage and (**b**) cumulative germination of *Colobanthus quitensis*. Different letters indicate significant differences ($P < 0.05$) for Tukey's HSD test ($n = 4$)

MS modification by pH reduction (5.5) and optimal priming treatment

When incorporating halo-priming with 5% KCl (one of the three best results from Experiment 2) into seeds placed in in vitro culture media adjusted to pH 5.5, variations in MS content did not significantly affect the germination percentage ($P > 0.05$) (Supplementary Table 1). However, among these treatments, the medium with 25% MS showed a tendency to increase the germination percentage, which reached 81.67%, 8.34% higher than the medium without MS (Fig. 3a). The new variations in the medium, combined with the halo-priming, allowed germination to be observed starting on the fifth day of the experiment, extending until day 13 for the treatment with 50% MS (Fig. 3b). No significant differences were observed in MGT, the medium with 25% MS tended to reduce MGT (Supplementary Table 5).

Sucrose presence/absence analysis at varying pH in the culture medium

The presence of sucrose in the culture medium at pH 5.5 and 5.7 in the absence of MS did not stimulate germination. However, the removal of sucrose had a stimulating effect on germination when the medium pH was 5.5 (Fig. 4a, Supplementary Table 1). Upon analyzing the cumulative germination, it was evident that germination began in all treatments between days 5 and 7 (Fig. 4b). None of the treatments influenced the germination time indicator (Supplementary Table 6).

Comparison of the best treatments for germination of *Colobanthus quitensis*

To determine the best in vitro germination protocol for *C. quitensis*, the germination percentage and mean germination time of the treatments from experiments 1–4 that most effectively stimulated germination were compared (Table 1). This analysis revealed that T1 (MS (−), 3% sucrose, 0.7% agar, and pH 5.7) had a significantly lower germination

Table 1 Description of the best protocols for in vitro seed germination of *Colobanthus quitensis* from experiments 1 to 4

Nomenclature	Experiment	Description of the protocol
T1	1	MS (–), 3% sucrose, 0.7% agar, and pH 5.7
T2	2	PEG10, MS (–), 3% sucrose, 0.7% agar, and pH 5.7
T3	2	KCl5, MS (–), 3% sucrose, 0.7% agar, and pH 5.7
T4	2	KCl10, MS (–), 3% sucrose, 0.7% agar, and pH 5.7
T5	3	KCl5, MS (–), sucrose (–), 0.7% agar, and pH 5.5
T6	3	KCl5, 25% MS, sucrose (–), 0.7% agar, and pH 5.5
T7	3	KCl5, 50% MS, sucrose (–), 0.7% agar, and pH 5.5
T8	3	KCl5, 100% MS, sucrose (–), 0.7% agar, and pH 5.5
T9	4	KCl5, MS (–), sucrose (–), 0.7% agar, and pH 5.5 (same as T5)

(–): without; PEG10: polyethylene glycol 10%, KCl5: potassium chloride 5%, KCl10: potassium chloride 10%

percentage compared to the other treatments, while T6 (KCl5, 25% MS, sucrose (−), 0.7% agar, and pH 5.5) tended to show the highest germination percentage (Fig. 5a). Regarding mean germination time, the results were much more variable, with the longest germination times recorded for T1 (10.39 days) and T9 (10.3 days), while the shortest germination times were observed for T5 (5.72 days), T6 (5.41 days), and T8 (5.63 days) (Fig. 5b).

Experiment 5: Scarification on germination in Antarctic *Colobanthus quitensis*

The treatments involving mechanical scarification alone or combined with sulfuric acid scarification resulted in germination percentages higher than 55% in the Antarctic population, while the use of sulfuric acid alone did not stimulate more than 2.86% (Fig. 6a, Supplementary Table 1). However, neither of these treatments produced uniform germination, as new seeds continued to germinate throughout the entire experimental period (Fig. 6b). The lack of germination in treatments where hydro-osmotic treatment followed by sulfuric acid scarification, and halo-osmotic treatment with 5% KCl followed by sulfuric acid scarification, did not

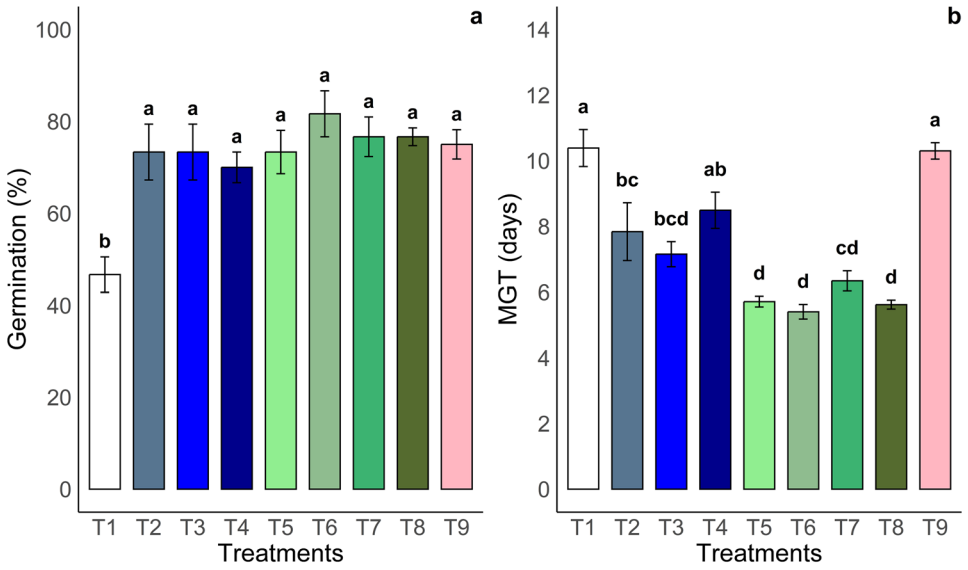

Fig. 5 Comparison of the best in vitro germination protocols of *Colobanthus quitensis* from experiments 1 to 4: (**a**) germination percentage and (**b**) mean germination time. Different letters indicate statistically significant differences according to the Tukey HSD test ($P < 0.05$). The description of each treatment is available in Table 1

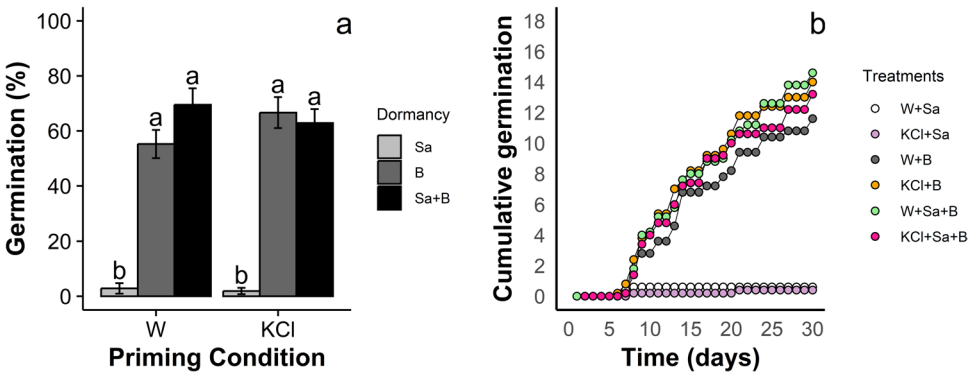

Fig. 6 Effect of combining seed halo-priming (KCl 5%) with scarification methods on (**a**) germination percentage and (**b**) cumulative germination of *Colobanthus quitensis*. Different letters indicate significant differences ($P < 0.05$) according to Tukey's HSD test ($n = 5$). W: distilled water; Sa: sulfuric acid; B: breaking of the testa

allow sufficient germination to determine MGT (Supplementary Table 7).

Discussion

Conservation of germplasm helps maintain the genetic diversity of species, provides necessary material for reforestation processes, or supports research activities (Bhattacharyya et al. 2014; Cuba-Díaz et al. 2020; Hesami et al. 2021). However, conservation efforts can be costly, so alternatives that make this process more economically accessible are continually being explored (Quazi et al. 2021). Although, in vitro cultivation is not considered one of the most expensive conservation techniques, adjusting the concentrations or types of compounds used in media preparation can help reduce costs and facilitate the development of simpler and more efficient protocols (Coelho and Romano 2021; Salgado 2023).

Requirements of in vitro germination in *Colobanthus quitensis*

In natural environments, especially those with extreme conditions, a low germination percentage may suffice to sustain a healthy population (Kildisheva et al. 2020). However, achieving higher germination percentages is critical for managing plant collections or germplasm banks, therefore, seed germination under controlled conditions offers a promising alternative. In vitro germination is a complex process where the relationship between the composition of the medium and carbohydrate sources plays a crucial role in achieving optimal germination (Hesami et al. 2021). Most in vitro germination studies on *C. quitensis* seeds have utilized 100% MS medium which includes various sources of mineral salts and organic compounds (Murashige and Skoog 1962). However, even with this composition, some populations of the species have failed to achieve high germination percentages (Cuba-Díaz et al. 2017b, 2019; Ontivero et al. 2024b). It is known that the osmotic potential of culture media influences the water absorption rate of seeds.

Culture media containing full-strength MS salts and sucrose exhibit more negative osmotic potentials, which can impede water uptake and consequently reduce germination rates (George et al. 2008). Our results confirmed this phenomenon, showing significantly lower germination percentages when using the standard in vitro medium (100% MS, 3% sucrose, pH 5.7) proposed by Cuba-Díaz et al (2017a) or even reduced MS concentrations (25–50%) compared to MS-free controls (Fig. 1a, Supplementary Fig. 1a, b). This inhibitory effect of MS salts on germination has been similarly reported in orchids, where decreased germination was attributed to the osmotic stress caused by medium components (Huh et al. 2016). Notably, ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) are the MS components with most negative osmotic potential, can induce physiological alterations that promote phytotoxic compound production, particularly at higher concentrations (Cárdenas and Villegas 2002; Turkan and Demiral 2009; Rodríguez et al. 2014).

Interestingly, when combining pH adjustment to 5.5 with halo-priming treatment in MS-containing media, germination differences between treatments became non-significant (Fig. 3), highlighting the interactive effects of osmotic regulation and pH optimization. Nevertheless, media lacking either MS salts, sucrose, or both components still achieved high germination percentages (Figs. 1, 2, 3, 4). It should be noted that while diluted MS (1/8 strength) or its complete absence supported germination, these conditions significantly reduced orchid seedling biomass (fresh and dry weight) in previous studies (Huh et al. 2016). Although this study prioritized germination optimization over seedling quality assessment, results showed that while MS-free medium enhanced germination rates (Figs. 2, 3, 4, 5), it impaired subsequent vegetative growth (Supplementary Fig. 1), as plants depended solely on their initial reserves. This lower biomass hinders handling, compromising their preservation in germplasm banks. Therefore, the exclusion of MS is not a viable strategy for the in vitro conservation of this species.

Sometimes, the embryo's own energy reserves and seed imbibition are sufficient to achieve germination (de Carvalho et al. 2024). However, both germination and seedling establishment are energy-intensive processes that require the mobilization of carbohydrates, proteins, and lipids, which constitute the primary reserve sources in seeds (De Mello et al. 2022; Wang et al. 2022). In in vitro culture studies, it has been suggested that each species has specific carbohydrate requirements for optimal germination (Stewart and Kane 2010). In the case of *C. quitensis*, a 3% sucrose concentration has been consistently used (Cuba-Díaz et al. 2017a, b, c, 2019; Ontivero et al. 2024b). However, different studies have identified that sucrose can interfere with both the germination process and seedling establishment (Huh et al. 2016; Bozdemir et al. 2018).

In our study, we remove this sugar from the in vitro culture medium. Thus, higher germination percentages are achieved when the seeds are treated with 5% KCl and germinated in a medium with 25% MS adjusted to pH 5.5, without sucrose (Fig. 5). This result suggests that the soluble carbohydrates reserve available in the seeds of *C. quitensis* are sufficient to support germination. This could be related to the fact that the main reserve substance in *C. quitensis* seeds is starch (Kellmann-Sopyła et al. 2017), a compound

that serves as the primary energy source in seeds, ensuring germination and the initial establishment of seedlings (Shaik et al. 2014).

Many soluble carbohydrates have also been identified, with sucrose, raffinose, D-pinitol, maltose, myo-inositol, glucose, galactinol, D-ononitol, and fructose found in descending order of concentration. These compounds play a key role as antifreeze agents, which is beneficial for the survival of *C. quitensis* in its natural distribution areas (Kellmann-Sopyła et al. 2015). Sucrose and raffinose are compounds that influence seed longevity (Vandecasteele et al. 2011), being quickly mobilized as an energy source during the early stages of the germination process (Bewley et al. 2013; Dadlani and Yadava 2023). It has also been mentioned that glucose is the main respiratory substrate utilized during germination and seedling establishment (Muscolo et al. 2007).

Osmotic and saline priming have an important effect on in vitro germination

In this study, the application of 10% PEG and 5% and 10% KCl stimulated seed germination (Fig. 2). This supports the premise that osmo-priming and halo-priming enhance germination by allowing controlled water uptake, which activates initial metabolic processes without inducing radicle protrusion, thereby preventing damage from excessive hydration (Rahimi 2013; Vasconcelos et al. 2017; Ma et al. 2024). Additionally, priming has been shown to increase ATPase activity in germinating seeds, as well as acid phosphatase and RNA synthesis, ultimately improving the germination rate (Nawaz et al. 2013). Priming treatments help repair cellular structures damaged by seed deterioration, reduce oxidative stress, and minimize variability in germination times, leading to a more synchronized process (Soleimanzadeh 2013; Sadeghi and Robati 2015; Pawar and Laware 2018).

However, in this study, priming did not lead to greater uniformity in germination (Supplementary Table 2). Nonetheless, the application of these three priming treatments significantly reduced the mean germination time compared to the control medium, which lacked MS and contained 3% sucrose, 0.7% agar, and a pH of 5.7 (Fig. 5b). In science, the cost of research is an important factor to consider. Although PEG10, KCl5, and KCl10 produced the same positive effects on the germination of *C. quitensis*, PEG was ruled out due to its higher cost compared to KCl (Supplementary Table 3). Additionally, among the two KCl treatments, the 5% solution was chosen as the best alternative, as it not only required fewer resources to prepare but also tended to reduce the time needed for the germination process (Supplementary Table 2).

Effect of medium pH and MS concentration on seed germination

In in vitro culture, the pH of the medium is a crucial factor due to its impact on nutrient availability, either facilitating or inhibiting it (George et al. 2008), which directly influences vegetative growth (Hakim and Dalimunthe 2022). In this study, lowering the pH from 5.7 to 5.5 promoted seed germination, especially when combined with priming using 5% KCl, without sucrose, and with MS concentrations ranging from 0 to 100% (Fig. 3). While no significant differences in germination percentage occurred among treatments, the 25% MS medium showed a consistent trend: higher germination (Fig. 5a), faster germination (Fig. 5b), and better vegetative development (Supplementary Fig. 1c).

A slightly more acidic pH is likely to enhance nutrient availability in MS, even at low concentrations, contributing to higher germination rates without the negative osmotic effects associated with higher MS concentrations. The presence of MS provides a balanced formula that supports seed and plant organ development, meeting the essential nutritional requirements of most plant species (Zurita-Valencia et al. 2014). Among the treatments evaluated in Experiment 3, despite their good germination results, the MS-free medium was discarded because it did not support seedling vegetative growth. On the contrary, the treatments containing 50% and 100% MS were discarded, as they involve using a larger amount of the product, which increases the cost of the protocol without providing additional benefits for the germination of *C. quitensis* compared to the medium containing 25% MS. Supplementary Fig. 3 shows the cost of MS, a product widely used in tissue culture and in vitro germination, although its use is not strictly necessary if the sole objective is to assess germination.

Previous in vitro germination experiments using the protocol proposed by Cuba-Díaz et al (2017a) have been applied in studies analyzing the germination of various *C. quitensis* populations from Antarctica, the sub-Antarctic region, and central-southern Chile. When considering these data, that is, as a seed pool, germination percentages range from 17.42% to 57.75% (Farías 2018; Cuba-Díaz et al. 2019; Arroyo-Marín 2023; Ontivero et al. 2024a, b). In contrast, our proposed protocol, which includes pre-treatment with 5% KCl, 25% MS, 0.7% agar, and a pH of 5.5, could achieve germination rates above 80% (Figs. 3a, 5a).

Seed dormancy may limit the in vitro germination of Antarctic populations

It has been demonstrated that the germination of *C. quitensis* populations collected directly from the field or obtained from a common garden is very similar (Koc et al. 2018).

For this reason, only field-collected seeds were used in this experiment, without including those from a common garden. Germination rates in a species can be highly variable, as they are influenced by numerous environmental, genetic, and conservation factors (Zhou et al. 2019). However, multiple studies report that in vitro germination rates of Antarctic *C. quitensis* populations reach 50% at best. These low success rates may result from the mismatch between controlled laboratory conditions and the species'natural growth environment (Cuba-Díaz et al. 2017b, 2019; Arroyo-Marín 2023; Ontivero et al. 2024b). In contrast, studies using moistened filter paper or various substrates report germination percentages ranging from 0% to nearly 100%, even with freshly collected seeds (Ruhland and Day 2001; Gielwanowska et al. 2011; Vera 2017; Koc et al. 2018). This highlights not only the high variability in germination rates among Antarctic populations depending on the protocol used but also the limited efforts towards the long-term ex situ conservation of this species. Therefore, establishing an in vitro germination protocol that consistently ensures high germination rates is crucial.

Given that Antarctic populations of *C. quitensis* exhibit difficulties in germination (Ruhland and Day 2001; Gielwanowska et al. 2011; Cuba-Díaz et al. 2019; Arroyo-Marín 2023), previous studies have reported positive results using sulfuric acid scarification (Cuba-Díaz et al. 2019). However, in this study, the combination of seed priming in the Antarctic population, followed by chemical scarification and sowing in a modified in vitro germination medium based on the best result obtained (Fig. 5), resulted in germination rates below 3%. This suggests that chemical scarification is not effective for Antarctic populations.

It is important to note that, although the same amount of material per population was used in experiments 1 to 4, only one Antarctic population and three subantarctic populations were used, which could have influenced the fact that analyzing only the Antarctic population did not result in higher germination percentages. However, by incorporating mechanical scarification or combining chemical scarification with mechanical scarification (via seed coat rupture), this limitation was overcome, achieving germination percentages between 55.24% and 69.52% (Fig. 6). Previous studies have demonstrated that in species such as *Avena sativa*, *Senna italica*, *Sorghum sudanense*, *Tephrosia nubica*, *Senna angustifolia*, *Acacia tortilis*, *Prosopis cineraria*, and *Crotalaria persica*, mechanical scarification of the seed coat is more effective at stimulating germination than sulfuric acid treatment (Rocha et al. 2022; Debouza et al. 2024). The accumulation of electro-dense and osmophilic materials in the epidermal cell walls of the seed coat in Antarctic populations has been reported to form a hard layer that protects the seeds from the extreme Antarctic conditions (Kellmann-Sopyła et al. 2017). Therefore, it is possible that low concentrations of sulfuric acid are not the most effective option for overcoming this physical barrier, and mechanical scarification may be required instead.

In optimizing the germination protocol for Antarctic populations of *C. quitensis*, seeds soaked in water for 24 h and subjected to both chemical and mechanical scarification achieved the highest germination percentage (69.52%), followed by seeds treated with 5% KCl and mechanical scarification (66.67%). Although manual seed coat rupture can be a time-consuming process, it is considered highly effective, safe, simple, and accessible for any laboratory (Debouza et al. 2024). In contrast, sulfuric acid is a costly product (Supplementary Table 3) that requires stricter safety measures due to its corrosive nature. Therefore, we recommend using the second protocol, which yielded the highest germination percentages, for the in vitro germination of Antarctic *C. quitensis* populations.

In vitro germination is a widely used strategy for the conservation and production of plants with ornamental, economic, or ecological value. This research highlights how small modifications to a germination protocol can significantly improve its effectiveness. We believe that the aspects analyzed in this study can be applied to various existing protocols for commercially valuable species with characteristics similar to *C. quitensis* (Ontivero et al. 2024a). Developing step-by-step research strategies that discard protocols based on verifiable results, as well as their economic and technical feasibility, is crucial for achieving better outcomes in propagation, conservation, and research.

Conclusion

The results demonstrate that although *C. quitensis* is capable of germinating in a minimal medium consisting solely of water, 0.7% agar, and pH 5.5, this approach is inadequate for germplasm bank conservation due to the suboptimal vegetative development observed in the seedlings. Protocol optimization through the combination of a 5% KCl priming treatment for 24 h and the use of a culture medium containing only 25% MS salts, without sucrose and adjusted to pH 5.5, achieves significantly higher germination percentages while substantially reducing costs. According to Supplementary Table 3, the use of KCl results in an 81% cost saving compared to PEG, while reducing MS salts to one-quarter of their standard concentration lowers reagent costs by 75%. Furthermore, eliminating sucrose from the medium and favoring mechanical over chemical scarification further reduces operational expenses and enhances protocol safety. This integrated strategy not only ensures high technical efficacy but also improves accessibility for resource-limited

laboratories, thereby supporting conservation efforts for this keystone species in fragile Antarctic ecosystems.

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Declarations

Conflict of interest The authors declare no competing interests.

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