Universidad de Concepción
Dirección de Postgrado
Facultad de Agronomía
Programa de Doctorado en Ciencias de la Agronomía

DETERIORO FISIOLÓGICO Y DESARROLLO DE ESTRATEGIAS DE ALMACENAMIENTO EN *COLOBANTHUS QUITENSIS*: UNA PLANTA MODELO CON SEMILLAS PEQUEÑAS Y DORMANTES

PHYSIOLOGICAL DETERIORATION AND DEVELOPMENT OF STORAGE STRATEGIES IN *COLOBANTHUS QUITENSIS*: A MODEL PLANT WITH SMALL AND DORMANT SEEDS

Tesis para optar al grado de Doctora en Ciencias de la Agronomía

YADIANA ONTIVERO VASALLO

LOS ÁNGELES-CHILE

2025

Profesor Guía: Marely Cuba Díaz
Dpto. de Ciencias y Tecnología Vegetal
Universidad de Concepción
Campus Los Ángeles

Esta tesis ha sido realizada en el Departamento de Ciencias y Tecnología Vegetal de la Escuela de Ciencia y Técnica de la Universidad de Concepción.

Profesor Guía

_____

Dr. Marely Cuba Díaz
Dpto. de Ciencias y Tecnología Vegetal
Universidad de Concepción

Comisión Evaluadora

_____

Dr. León A. Bravo Ramírez
Facultad de Cs. Agropecuarias y Forestales
Universidad de La Frontera

_____

Dr. Miguel Garriga Caraballo
Facultad de Agronomía
Universidad de Concepción

_____

Dr. Daniel Eduardo Chávez Matamala
Dpto. de Ciencias y Tecnología Vegetal
Universidad de Concepción

Director de Programa

_____

Dr. Susana Ursina Fischer Ganzoni
Facultad de Ciencias Biológicas
Universidad de Concepción

Yadiana Ontivero Vasallo, estudiante del Programa Doctorado en Ciencias de la Agronomía, Facultad de Agronomía, Universidad de Concepción, declara ser autor del presente trabajo titulado "**Deterioro fisiológico y desarrollo de estrategias de almacenamiento en *Colobanthus quitensis*: una planta modelo con semillas pequeñas y dormantes**".

AGRADECIMIENTOS

- A mi tutora Marely Cuba Díaz por sus enseñanzas durante estos cuatro años y los que continúan
- A mi comisión de tesis, por transmitir sus conocimientos encausando mi formación.
- Al programa de Doctorado en Ciencias de la Agronomía, por darme las herramientas necesarias para cursar este programa de estudios.
- A la Agencia Nacional de Investigación y Desarrollo, por el fondo Doctorado Nacional 21210100 que me permitió formarme en Chile
- Al Instituto Antártico Chileno, por el proyecto DT_05_22 que me permitió conocer la Antártica y manejar mi primer proyecto como Investigadora Responsable.
- Agradezco a mi familia por siempre estar pendientes y por su apoyo incondicional.
- A mi esposo, que ha sido fundamental estos últimos años para mantener la cordura
- A los amigos con los que comencé este viaje, Camila y Richard, y que se volvieron mi familia en Chile.
- A mi familia chileno-cubana, los Perelli-Cuba, por acogerme desde el primer día.
- A Adrián, mi primer tutor, porque sin él jamás hubiese podido estudiar.
- A mi grupo de trabajo del Laboratorio de Biotecnología y Estudios Ambientales de la Universidad de Concepción.

TABLA DE CONTENIDO

ÍNDICE DE TABLAS

ÍNDICE DE ILUSTRACIONES

RESUMEN

La calidad de las semillas es una característica compleja de evaluar, ya que está influida por factores ambientales, genéticos, estrategias de conservación y condiciones de germinación. *Colobanthus quitensis* es una especie extremófila con un alto potencial como modelo biotecnológico para el estudio del estrés ambiental y el cambio climático. La amplia diversidad de ecotipos de esta especie la convierte en un material valioso para investigar la calidad de semillas, especialmente en semillas pequeñas, con testa dura y dificultades para germinar.

Con este propósito, se desarrollaron distintos ensayos para evaluar la influencia de las características de las semillas en la germinación *in vitro*. Se probaron estrategias de priming y escarificación mecánica para optimizar el protocolo de germinación. Asimismo, se emplearon técnicas comunes de análisis de vigor para determinar las estrategias más adecuadas para el análisis de semillas de *C. quitensis* y otras especies con características similares. Además, se adoptó un enfoque biológico al evaluar el impacto de hongos endófitos y sus extractos sobre la reproducción, calidad de semillas y su conservación.

Esta investigación logró avances importantes en la caracterización y conservación de *C. quitensis*. Se identificó por primera vez la presencia de heteromorfismo en las semillas, demostrando que esta característica no solo influye en la capacidad germinativa, sino que también confiere ventajas frente a la salinidad y posiblemente ante otras condiciones de estrés. Se evidenció que el medio de germinación ideal debe contener una baja concentración de sales Murashige y Skoog (MS), un pH ligeramente ácido, halo-priming con KCl al 5% y escarificación mecánica en semillas de poblaciones antárticas. La inclusión de hongos endófitos tuvo efectos positivos tanto en el desarrollo de las plantas como en las características de las semillas. Además, se comprobó que temperaturas de conservación de 4°C, –20°C y –80°C son adecuadas para mantener la viabilidad de semillas ortodoxas como las de *C. quitensis*, observándose incluso un aumento en los porcentajes de germinación tras seis meses de almacenamiento, en comparación con semillas recién colectadas.

ABSTRACT

The quality of seeds is a complex characteristic to evaluate, as it is influenced by environmental conditions, genetic factors, conservation strategies, and germination conditions. *Colobanthus quitensis* is an extremophile species with high potential as a biotechnological model for studying environmental stress and climate change. Its wide range of ecotypes makes it a valuable resource for studying seed quality, particularly in small, hard-coated seeds with low germination capacity.

To address this, various experiments were conducted to evaluate the influence of seed traits on *in vitro* germination. Priming strategies and mechanical scarification were tested to optimize the germination protocol. Common vigor analysis techniques were used to identify the most appropriate seed evaluation strategies for *C. quitensis* and other species with similar characteristics. A biological approach was also incorporated to assess the impact of endophytic fungi and their extracts on reproduction, seed quality, and conservation.

This research achieved important advances in the characterization and conservation of *C. quitensis*. Seed heteromorphism was identified for the first time in this species, demonstrating that it not only influences germination capacity but may also confer advantages under saline and other stress conditions. It was found that the optimal germination medium for *C. quitensis* should contain a low concentration of Murashige and Skoog medium, a slightly acidic pH, halo-priming with 5% KCl, and mechanical scarification for seeds from Antarctic populations.

The inclusion of endophytic fungi had positive effects on both plant development and seed characteristics. Moreover, conservation at 4°C, –20°C, and –80°C proved effective for maintaining the viability of orthodox seeds like those of *C. quitensis*, with even higher germination rates observed in seeds stored for six months compared to freshly collected ones.

I. INTRODUCCIÓN GENERAL

El comercio internacional de semillas, tanto en diversidad como volumen, es un indicador clave de la economía de un país (Abaidia & Lalmi, 2024). Sin embargo, para su comercialización, las semillas deben cumplir con estrictos estándares de calidad (Milošević et al., 2010; Taylor, 2020). Uno de los principales desafíos es el deterioro fisiológico durante el almacenamiento, el cual afecta tanto la producción vegetal como la viabilidad comercial de las semillas (Taylor, 2020; Wawrzyniak et al., 2020). Los bancos de germoplasma conservan la diversidad genética vegetal, y su eficiencia depende del conocimiento disponible sobre las especies que almacenan, el cual suele ser mayor en plantas de importancia económica (Taylor, 2020; Whitehouse et al., 2020). Los bancos de germoplasma se enfrentan a una creciente necesidad de ampliar el conocimiento sobre las semillas, desarrollar métodos para evaluar su calidad y establecer protocolos de almacenamiento que optimicen su longevidad y minimicen su deterioro (Solberg et al., 2020). La falta de conocimiento sobre las características de las semillas y sus patrones de pérdida de calidad fisiológica genera que los mayores gastos de los bancos de germoplasma estén relacionados con el monitoreo frecuente de la viabilidad (Taylor, 2020; Whitehouse et al., 2020).

La calidad de las semillas es el resultado de la viabilidad, longevidad, germinación y vigor (Marcos-Filho, 2015), lo que, a su vez, depende de factores ambientales, fitosanitarios, morfológicos, fisiológicos y genéticos relacionados con la planta y sus áreas de distribución (Sanhueza et al., 2017; Zhou et al., 2019; Raju-Ahmed et al., 2020). El deterioro de las semillas es un proceso complejo y aún no bien definido, que implica cambios en genes, rutas bioquímicas y la acumulación de compuestos relacionados con el estrés (Bewley et al., 2013; Shibata et al., 2020; López-Hidalgo et al., 2021). En el análisis de calidad de semillas, es recomendable realizar varias pruebas de vigor, ya que una sola prueba no puede explicar todos los factores relacionados con el deterioro de las semillas. Algunas de las pruebas más utilizadas para este propósito son el envejecimiento acelerado, la prueba de frío, la conductividad eléctrica y las pruebas con sales de tetrazolio (Milošević et al., 2010; Marcos-Filho, 2015). Por otro lado, las variaciones en los tipos y la cantidad de reservas nutricionales con el tiempo de almacenamiento ofrecen pistas sobre cómo será la energía germinativa de las semillas según su deterioro (Bewley, 2001; Bewley et al., 2013; Zhao et al., 2018).

La mayoría de las pruebas de vigor han sido estandarizadas para cultivos que producen semillas grandes como maíz y trigo (Nicoleti y Coelho, 2018; Correia et al., 2020; García y Coelho, 2021). En cambio, es más complejo analizar la calidad de semillas de tamaño pequeño (Marcos-Filho, 2015), como la quinua (*Chenopodium quinoa*) y el amaranto (*Amaranthus* spp.), que son cultivos de importancia en la alimentación humana (Souza et al., 2020; Hernández et al., 2020), o las orquídeas (Orchidaceae), que tienen un reconocido valor ornamental (Li et al., 2021).

Colobanthus quitensis (Kunt) Bartl. (Caryophyllaceae) es una especie extremófila que se distribuye desde el sur de México hasta la Península Antártica (Moore, 1970; Convey, 1996). Es considerada un sensor del cambio climático (Bertini et al., 2021) y un referente para analizar la respuesta morfofisiológica y bioquímica de las plantas a condiciones de estrés ambiental (Cuba-Díaz et al., 2014; Koc et al., 2018b; Bertini et al., 2022; Arroyo, 2023; Ontivero et al., 2024b), aportando nuevo conocimiento que puede ser empleado en programas de mejoramiento genético de cultivos de interés agronómico.

Las poblaciones de *C. quitensis* presentan variabilidad en su morfología, características genéticas, fisiológicas e incluso en su germinación (Gianoli et al., 2004; Cuba-Díaz et al., 2017b; Koc et al., 2018a; Cuba-Díaz et al., 2019). Esta variabilidad podría estar influenciada por las marcadas diferencias ambientales entre sus hábitats: mientras que en la Antártica las condiciones son extremas (bajas temperaturas constantes, escases de nutrientes, déficit hídrico, radiación UV elevada y cortos periodos de crecimiento) (Moore, 1970), en regiones subantárticas como Punta Arenas o en Los Andes, los factores abióticos son más benignos existiendo mayor disponibilidad hídrica y estacionalidad térmica menos drástica (Cuba-Díaz et al., 2013; Koc et al., 2018a). Estas presiones selectivas podrían haber favorecido estrategias diferenciadas de dormancia en las semillas, como mecanismo para sincronizar la germinación con ventanas de oportunidad ecológica en cada ambiente (Jurado & Flores, 2005).

Las tasas de germinación varían dentro de una misma especie debido a la influencia de factores ambientales, genéticos y de conservación de las semillas de cada población (Zhou et al., 2019). En estudios de conservación *in vitro* de *C. quitensis*, se ha observado que las poblaciones antárticas y subantárticas presentan diferencias en sus tasas de germinación. En particular, las poblaciones antárticas rara vez alcanzan el 50% de germinación y, en la

mayoría de los casos, menos del 5% de las semillas logran germinar (Cuba-Díaz et al., 2017b, 2019; Arroyo-Marín, 2023; Ontivero et al., 2024b). Estudios donde se emplea papel de filtro humedecido o diversos sustratos han reportado tasas de germinación de poblaciones antárticas entre el 0% y 100%, observándose porcentajes muy bajos incluso cuando se analizan semillas recién colectadas (Ruhland & Day, 2001; Gielwanowska et al., 2011; Vera, 2017; Koc et al., 2018a). Esto pone de manifiesto no solo la alta variabilidad en las tasas de germinación entre las poblaciones antárticas en función del protocolo utilizado y la procedencia de las poblaciones, sino también la escasez de esfuerzos dirigidos a la conservación *ex situ* a largo plazo de esta especie que potencie la investigación científica sin afectar la conservación del ecosistema antártico, el cual es cada vez más afectado por la actividad turística y científica en la región (Tejedo et al., 2022).

La incapacidad de *C. quitensis* de germinar, incluso cuando los embriones de las semillas son viables (Giełwanowska et al., 2011), podría deberse a la necesidad de optimizar los protocolos de germinación existentes (Sanhueza et al., 2017; Whitehouse et al., 2020) o a la presencia de dormancia (Cuba-Díaz et al., 2019). La dormancia es un mecanismo de defensa frente al estrés ambiental que limita la germinación, incluso en condiciones óptimas, al mantener un estado metabólicamente reducido pero reversible (McGraw y Day, 1997; Cuba-Díaz et al., 2019; Nonogaki, 2019) y debido a su relación con la longevidad se ha vuelto un parámetro importante en bancos de germoplasma (Pipatpongpinyo et al., 2020; Solberg et al., 2020). La dormancia puede tener componentes ambientales y/o genéticos (Graeber et al., 2012; Bewley et al., 2013; Pipatpongpinyo et al., 2020), razón por la cual se espera, que este fenómeno varíe entre poblaciones de una misma especie según su tolerancia a entornos con condiciones progresivamente más extremas (Jurado & Flores, 2005). La dormancia de las semillas y la capacidad de germinación se analizan con frecuencia cuantificando el perfil hormonal de las semillas (Ali-Rachedi et al., 2004), sus sustancias de reserva (Zhou et al., 2019; Taylor, 2020), así como la coloración de la testa (Debeaujon et al., 2000; Attri et al., 2021).

Las semillas ortodoxas son aquellas que maduran con un contenido de humedad entre el 5 y el 10%, aumentando su longevidad cuando se almacenan a bajas temperaturas (Kim, 2018). La capacidad de *C. quitensis* para formar bancos de semillas naturales se considera un indicador de que la especie produce semillas ortodoxas (Kellmann-Sopyla y Gielwanowska,

2015) y así están catalogadas en la base de datos del *Royal Botanic Gardens, Kew* (Seed Information Database, 2024).

Observaciones preliminares de laboratorio sugieren que las semillas de *C. quitensis* presentan problemas para germinar después de ser conservadas por un año a 4°C, que es la temperatura estándar de almacenamiento para diferentes cultivos y plantas ornamentales (Singh et al., 2016; Hernández et al., 2020; Kompe et al., 2020, Souza et al., 2020). El deterioro fisiológico de las semillas es un proceso normal; sin embargo, los bancos internacionales de germoplasma abogan por temperaturas extremas de -18°C a -20°C para la conservación de semillas a largo plazo con el fin de mantener su viabilidad. Este procedimiento requiere la deshidratación previa de las semillas a niveles de humedad entre 3 y 7% (FAO, 2014; Singh et al., 2016; Asdal et al., 2019). En trigo y cebada, el almacenamiento a -20°C ha mantenido porcentajes de germinación superiores al 90%, en comparación con el almacenamiento tradicional a 4°C (62%-75%) (van Treuren et al., 2018). A pesar de esto, hay especies que requieren condiciones de almacenamiento más extremas, para garantizar su viabilidad en el tiempo (Ballesteros et al., 2021). Un estudio que evaluó el almacenamiento de semillas de *Populus davidiana* a -80°C durante 48 meses evidenció que esta es una alternativa viable para la conservación de semillas a largo plazo, incluso si el contenido de agua de sus semillas es variable (3%-24%) (Kim, 2018). Las semillas de orquídeas pueden mantener una alta viabilidad cuando se criopreservan a temperaturas entre -20°C y -196°C (Batty et al., 2001; Merritt et al., 2014; Schofield et al., 2018). No obstante, la criopreservación puede aumentar la peroxidación lipídica de las membranas debido al efecto del congelamiento y descongelamiento de las estructuras externas de las semillas (Acosta et al., 2020).

En condiciones naturales, las poblaciones antárticas de *C. quitensis* pueden tolerar temperaturas ambientales por debajo de 0°C durante gran parte del año (Gianoli et al., 2004; Bascuñán-Godoy et al., 2010; Turner et al., 2020). El suelo en la Antártida tiende a estar congelado o cubierto de nieve la mayor parte del tiempo (Potapowicz et al., 2019), y bajo estas condiciones se mantienen bancos de semillas viables en el suelo (Kellmann-Sopyła y Gielwanowska, 2015). Por lo tanto, el almacenamiento *ex situ* de semillas a temperaturas por debajo de 0°C podría ser una alternativa viable de conservación para mantener o prolongar la viabilidad de las semillas de *C. quitensis*.

Factores biológicos como los hongos endófitos influyen positiva y/o negativamente en las características de las semillas, la germinación, la longevidad y la supervivencia de las plántulas, especialmente bajo condiciones de estrés (Gundel et al., 2009; Barrera et al., 2020; Ueno et al., 2020). En condiciones naturales *C. quitensis* se asocia con gran variedad de hongos endófitos (Hereme et al., 2020). En estudios de simulación de cambio climático, en los que se impusieron condiciones de cultivo benignas en términos de disponibilidad de agua y temperatura, durante el crecimiento de *C. quitensis*, disminuyó la presencia y función de estos hongos (Torres-Díaz et al., 2016). Por lo tanto, es posible que las plantas propagadas y cultivadas en condiciones de laboratorio carezcan de estos hongos endófitos o hayan establecido nuevas relaciones simbióticas con otras especies endófitas, lo que podría ser una de las causas de la pérdida de calidad de las semillas con el tiempo y las condiciones de almacenamiento. Por ende, su inoculación con hongos endófitos podría ser una alternativa para mejorar tanto el proceso reproductivo como las características de las semillas de *C. quitensis.*

En agricultura, los hongos endófitos utilizados para estimular la germinación, pueden provenir o no de aislamientos de la misma especie cuyo desempeño se desea mejorar. Por ejemplo, los aislamientos de hongos detectados en *C. quitensis* y *Deschampsia antarctica* se han utilizado para estimular el desempeño de tomates y lechugas bajo estrés salino (Molina-Montenegro et al., 2020). *Phialocephala fortinii*, un hongo endófito oscuro septado detectado en *Araucaria araucana* (Rivas, 2020), ha demostrado estimular el crecimiento de espárragos (*Asparagus officinalis*) (Surono y Narisawa, 2017). En orquídeas, la inoculación de semillas con hongos endófitos se ha utilizado para promover su germinación *in vitro* y la floración (Frericks et al., 2018). En este último caso, se ha demostrado que las semillas de orquídeas inoculadas con hongos micorrícicos endófitos pueden almacenarse en nitrógeno líquido (-196°C) y aun así estimular la germinación de semillas, demostrando que estos microorganismos pueden mejorar la calidad fisiológica de las semillas después de haber sido sometidas a temperaturas extremadamente frías (Batty et al., 2001).

La caracterización de las semillas de *C. quitensis* y la descripción de sus cambios fisiológicos y bioquímicos relacionados con la pérdida de calidad son los principales desafíos de esta investigación. El conocimiento de estos parámetros permitiría perfeccionar las condiciones de manejo y almacenamiento para su conservación, así como definir los mejores indicadores

para analizar el deterioro fisiológico y establecer estrategias que puedan seguirse para ralentizar este proceso.

1. Hipótesis

1.1 Las semillas de poblaciones antárticas de *C. quitensis* tienen una dormancia más prolongada y una mayor expresión de factores protectores contra el deterioro que las poblaciones continentales.

1.2 *Colobanthus quitensis* produce semillas ortodoxas cuya longevidad aumenta cuando se almacenan a temperaturas inferiores a 4°C.

1.3 La inoculación de plantas con hongos endófitos favorece la reproducción de *C. quitensis* y germinación de sus semillas tras el almacenamiento a bajas temperaturas.

1.2 Objetivo

1.2.1 Objetivo general

Analizar variables que determinan la capacidad de dormancia y el deterioro fisiológico de semillas de diferentes poblaciones de *C. quitensis* y sus modificaciones tras someter las semillas a diferentes tiempos de almacenamiento.

1.3 Objetivos específicos

1.3.1 Caracterizar morfológica, fisiológica y bioquímicamente las semillas de poblaciones de *C. quitensis*.

1.3.2 Analizar la presencia de hongos endófitos en *C. quitensis* y el impacto de la inoculación de plantas de diferentes poblaciones con estos microorganismos en la reproducción y germinación.

1.3.3 Evaluar parámetros de la calidad fisiológica de las semillas de *C. quitensis* después de su almacenamiento a bajas temperaturas.

REFERENCIAS

Abaidia, Y., & Lalmi, H. (2024). Investing in the Pioneering Varieties as an Agreement to Attaining Economic Take-off on Smallholder Seed Firms in West Africa. *International Journal of Economic Behavior and Organization,* *12*(1), 34-45.

https://doi.org/10.11648/j.ijebo.20241201.13

Acosta, Y., Perez, L., Linares, C., Hernández, L., Escalante, D., Pérez, A., Zevallos, B. E., Yabor, L., Martinez-Montero, M. E., Cejas, I., Fontes, D., Sershen, & Lorenzo, J. C. (2020). Effects of *Teramnus labialis* (L.f.) Spreng seed cryopreservation on subsequent seed and seedling growth and biochemistry. *Acta Physiologiae Plantarum, 42*(7). https://doi.org/10.1007/s11738-020-3012-9

Ali-Rachedi, S., Bouinot, D., Wagner, M.H., Bonnet, M., Sotta, B., Grappin, P., & Jullien, M. (2004). Changes in endogenous abscisic acid levels during dormancy release and maintenance of mature seeds: Studies with the Cape Verde Islands ecotype, the dormant model of *Arabidopsis thaliana*. *Planta, 219*, 479–488. https://doi.org/10.1007/s00425-004-1251-4

Araujo, J.O., Pinheiro, D.T., Fernandes, D.C. Hilst, P.C., & Medeiros, A.D. (2019). Adequacy of the tetrazolium test to evaluate the viability of Jatropha curcas L. seeds. *Journal of Seed Science, 41*(4), 470–477. https://doi.org/10.1590/2317-1545v41n4221373

Arroyo-Marín, F. B. (2023). Respuestas diferenciales en la tolerancia a la salinidad en poblaciones de *Colobanthus quitensis* Kunth (Bartl.) [Tesis de pregrado, Universidad de Concepción]. Universidad de Concepción.

Asdal, Å., Brodal, G., Solberg, S.Ø., Yndgaard, F., von Bothmer, R., & Meen, E. (2019). Seed longevity and survival of seed borne diseases after 30 years conservation in permafrost: Report from the 100 year storage experiment. Nordic Genetic Resource Center.

Attri, P., Ishikawa, K., Okumura, T., Koga, K., Shiratani, M., & Mildaziene, V. (2021). Impact of seed color and storage time on the radish seed germination and sprout growth in plasma agriculture. *Scientific Reports, 11*, 2539. https://doi.org/10.1038/s41598-021-82080-6

Ballesteros, D., Fanega-Sleziak, N., & Davies, R. M. (2021). Cryopreservation of seeds and seed embryos in orthodox-, intermediate-, and recalcitrant-seeded species. En W. F. Wolkers & H. Oldenhof (Eds.), Cryo-preservation and freeze-drying protocols. Humana 353–371). https://doi.org/10.1007/978-1-0716-0783-1_25

Barrera, A., Hereme, R., Ruiz-Lara, S., & Molina-Montenegro, M. A. (2020). Fungal endophytes enhance the photoprotective mechanisms and photochemical efficiency in the Antarctic *Colobanthus quitensis* (Kunth) Bartl. exposed to UV-B radiation. *Frontiers in Ecology and Evolution, 8*, 122. https://doi.org/10.3389/fevo.2020.00122

Bascuñan-Godoy, L., Garcia-Plazaola, J.I., Bravo, L.A., & Corcuera, L.J. (2010). Leaf functional and micro-morphological photoprotective attributes in two ecotypes of *Colobanthus quitensis* from the Andes and Maritime Antarctic. *Polar Biology, 33*(7), 885–896. https://doi.org/10.1007/s00300-010-0766-6

Batty, A. L., Dixon, K. W., Brundrett, M., & Sivasithamparam, K. (2001). Long-term storage of mycorrhizal fungi and seed as a tool for the conservation of endangered Western Australian terrestrial orchids. *Australian Journal of Botany, 49*(5), 619–628. https://doi.org/10.1071/BT00051

Bertini, L., Cozzolino, F., Proietti, S., Falconieri, G. S., Iacobucci, I., Salvia, R., Falabella, P., Monti, M., & Caruso, C. (2021). What Antarctic plants can tell us about climate changes: Temperature as a driver for metabolic reprogramming. *Biomolecules, 11*(8), 1094. https://doi.org/10.3390/biom11081094

Bertini, L., Proietti, S., Fongaro, B., Holfeld, A., Picotti, P., Falconieri, G. S., Bizzarri, E., Capaldi, G., Polverino de Laureto, P., & Caruso, C. (2022). Environmental signals act as a driving force for metabolic and defense responses in the Antarctic plant *Colobanthus quitensis*. *Plants, 11*(23), 3176. https://doi.org/10.3390/plants11223176

Bewley, J. D., Bradford, K. J., Hilhorst, H. W. M., & Nonogaki, H. (2013). Seeds: Physiology of development, germination and dormancy (3rd ed.). Springer. https://doi.org/10.1007/978-1-4614-4693-4

Bewley, J. D. (2001). Seed germination and reserve mobilization. Encyclopedia of Life Sciences, 1–7. https://doi.org/10.1038/npg.els.0001236

Correia, L.V., Pereira, L.C., Matera, T.C., Pereira, R.C., Suzukawa, A.K., Santos, R.F., Pastorini, L.H., Carvalho, C., Osipi E.A.F., & Braccini A.L. (2020). Accessing the relevance of tests for estimating the physiological quality of wheat grains. *Plant, Soil and Environment, 66*(10), 477–482. https://doi.org/10.17221/405/2020-PSE

Cuba-Díaz, M., Acuña, D., & Cordero, C. M. (2014). Optimización de parámetros para la propagación *in vitro* de *Colobanthus quitensis* (Kunth) Bartl. *Gayana Botánica, 71*(1), 58–67. https://doi.org/10.4067/S0717-66432014000100007

Cuba-Díaz, M., Acuña, D., & Fuentes-Lillo, E. (2019). Antarctic pearlwort (*Colobanthus quitensis*) populations respond differently to pre-germination treatments. *Polar Biology, 42*, 1383–1390. https://doi.org/10.1007/s00300-019-02505-4

Cuba-Díaz, M., Acuña, D., Klagges, M., Dollenz, O., & Cordero, C. (2013). *Colobanthus quitensis* de La Marisma, una nueva población para la colección genética de la especie. En: Libro De Resúmenes VII Congreso Latinoamericano De Ciencia Antártica, 436-439.

Cuba-Díaz, M., Klagges, M., Fuentes-Lillo, E., & Cordero, C. M. (2017). Phenotypic variability and genetic differentiation in continental and island populations of *Colobanthus quitensis* (Caryophyllaceae: Antarctic pearlwort). *Polar Biology, 40*, 2397–2409. https://doi.org/10.1007/s00300-017-2132-4

Debeaujon, I., Leon-Kloosterziel, K. M., & Koornneef, M. (2000). Influence of the testa on seed dormancy, germination, and longevity in Arabidopsis. *Plant Physiology, 122*(2), 403–413. https://doi.org/10.1104/pp.122.2.403

Fantazzini, T.B., Boas, S.V., Oliveira, R.M., Rocha, D.K., Moreira, M.L., & Rodrigues, V.O. (2021). Electrical conductivity test to evaluate the physiological quality of tobacco seeds. *Brazilian Journal of Development, 7*(2), 14903–14916. https://doi.org/10.34117/bjdv7n2-464

FAO. (2014). Genebank standards for plant genetic resources for food and agriculture. Food and Agriculture Organization of the United Nations. https://www.fao.org/3/i3704e/i3704e.pdf

Frericks, J., Munkacsi, A., Ritchie, P., Luo, Y. B., & Lehnebach, C. A. (2018). Phylogenetic affinities and *in vitro* seed germination of the threatened New Zealand orchid *Spiranthes novaezelandiae*. *New Zealand Journal of Botany, 56*(1), 91–108. https://doi.org/10.1080/0028825X.2017.1383451

García, J., & Coelho, C. M. M. (2021). Accelerated aging predicts the emergence of rice seedlings in the field. *Semina: Ciências Agrárias, 42*(3), 1397–1410. https://doi.org/10.5433/1679-0359.2021v42n3p1397

33. Gianoli, E., Inostroza, P., Zúñiga-Feest, A., Reyes-Díaz, M., Cavieres, L.A., Bravo, L.A., & Corcuera, L.J. (2004). Ecotypic differentiation in morphology and cold resistance in populations of *Colobanthus quitensis* (Caryophyllaceae) from the Andes of Central Chile and the Maritime Antarctic. *Arctic, Antarctic, and Alpine Research, 36*(4), 484–489. https://doi.org/10.1657/1523-0430(2004)036[0484:EDIMAC]2.0.CO;2

Graeber, K., Nakabayashi, K., Miatton, E., Leubner-Metzger, G., & Soppe, W.J.J. (2012). Molecular mechanisms of seed dormancy. *Plant, Cell & Environment, 35*(10), 1769–1786. https://doi.org/10.1111/j.1365-3040.2012.02542.x

Gundel, P.E., Martinez-Ghersa, M.A., Garibaldi, L.A., & Ghersa, C.M. (2009). Viability of *Neotyphodium* endophytic fungus and endophyte-infected and non-infected *Lolium multiflorum* seeds. *Botany, 87*(1), 88–96. https://doi.org/10.1139/B08-121

Hereme, R., Morales-Navarro, S., Ballesteros, G., Barrera, A., Ramos, P., Gundel, P.E., & Molina-Montenegro, M.A. (2020). Fungal endophytes exert positive effects on *Colobanthus quitensis* under water stress but neutral under a projected climate change scenario in Antarctica. *Frontiers in Microbiology, 11*, 264. https://doi.org/10.3389/fmicb.2020.00264

Hernández, E. H. M., Coronado, A. C. M., & Ardila, W. L. R. (2020). Seed quality of 22 quinoa materials (Chenopodium quinoa Willd.) from the department of Boyacá. *Revista Ceres, 67*(4), 306–314. https://doi.org/10.1590/0034-737X202067040004

Jurado, E., & Flores, J. (2005). Is seed dormancy under environmental control or bound to plant traits? *Journal of Vegetation Science, 16*(5), 559–564. https://doi.org/10.1111/j.1654-1103.2005.tb02398.x

Kehinde, T. O., Ajala, M. O., Daniel, I. O., & Ojo, D. K. (2013). Physiological and genetic integrity of amaranth (Amaranthus spp.) seeds during storage. *International Journal of Plant Breeding and Genetics, 7*(1), 35–46. https://doi.org/10.3923/ijpbg.2013.35.46

Kellmann-Sopyła, W., & Giełwanowska, I. (2015). Germination capacity of five polar Caryophyllaceae and Poaceae species under different temperature conditions. Polar Biology, 38, 1753–1765. https://doi.org/10.1007/s00300-015-1742-x

Kim, D. H. (2018). Extending Populus seed longevity by controlling seed moisture content and temperature. *PLOS ONE, 13*(8), e0203080. https://doi.org/10.1371/journal.pone.0203080

Koc, J., Androsiuk, P., Chwedorzewska, K.J., Cuba-Díaz, M., Górecki, R., & Giełwanowska, I. (2018a). Range-wide pattern of genetic variation in *Colobanthus quitensis*. *Polar Biology, 41*, 2467–2479. https://doi.org/10.1007/s00300-018-2384-0

Koc, J., Wasilewski, J., Androsiuk, P., Kellmann-Sopyla, W., Chwedorzewska, K. J., & Gielwanowska, I. (2018b). The effects of methanesulfonic acid on seed germination and morphophysiological changes in the seedlings of two *Colobanthus* species. *Acta Societatis Botanicorum Poloniae, 87*(4), 3601. https://doi.org/10.5586/asbp.3601

Kompe, Y. O., Mutlu, V. A., & Ozkoc, I. (2020). The viability of *Anacamptis laxiflora* (Orchidaceae) seeds and the symbiotic germination. *Plant Biology, 22*(6), 800–805. https://doi.org/10.1111/plb.13143

Li, C., Dong, N., Zhao, Y., Wu, S., Liu, Z., & Zhai, J. (2021). A review for the breeding of orchids: Current achievements and prospects. *Horticultural Plant Journal, 7*(5), 380 – 392. https://doi.org/10.1016/j.hpj.2021.02.006

Lopez-Hidalgo, C., Meijon, M., Lamelas, L., & Valledor, L. (2021). The rainbow protocol: A sequential method for quantifying pigments, sugar, free amino acids, phenolics, flavonoids and MDA from a small amount of samples. *Plant Cell and Environment*, 1 – 10. https://doi.org/10.1111/pce.14007

Marcos-Filho, J. (2015). Seed vigor testing: an overview of the past, present and future perspective. *Science Agriculture, 72*(4), 363-374. https://doi.org/10.1590/0103-9016-2015-0007

McGraw, J.B., & Day, T.A. (1997). Size and characteristics of a natural seed bank in Antarctica. *Arctic, Antarctic and Alpine Research, 29*(2), 213-216. https://doi.org/10.1080/00040851.1997.12003234

Merritt, D.J., Hay, F.R., Swarts, N.D., Sommerville, K.D., & Dixon, K.W., (2014). *Ex situ* conservation and cryopreservation of Orchid germplasm. *International Journal of Plant Science, 175*(1), 46–58. https://doi.org/10.1086/673370

Milošević, M., Vujaković, M., & Karagić, D. (2010). Vigour tests as indicators of seed viability. *GENETIKA, 42*(1), 103-118. https://doi.org/10.2298/GENSR1001103M

Molina-Montenegro, M.A., Acuña-Rodríguez, I.S., Torres-Díaz, C., Gundel, P.E., Dreyer, I. (2020). Antarctic root endophytes improve physiological performance and yield in crops under salt stress by enhanced energy production and Na+ sequestration. *Scientific Reports, 10*(5819). https://doi.org/10.1038/s41598-020-62544-4

Moore, D.M. (1970). Studies in *Colobanthus quitensis* (Kunth) Bartl. and *Deschampsia antarctica* Desv. II. Taxonomy, distribution and relationships. *British Antarctic Survey Bulletin*, *23*, 63–80.

Nicoleti, L. F. & Coelho, A. D. L. (2018). Physiological maturity of corn seeds (*Zea mays*) by tetrazolium salt. *Journal of Food Science and Engineering, 8*, 248-256. https://doi.org/10.17265/2159-5828/2018.06.004

Nonogaki, H. (2019). Seed germination and dormancy: The classic history, new puzzles, and evolution. *Journal of Integrative Plant Biology, 61*, 541–563. https://doi.org/10.1111/jipb.12762

Ontivero, Y., Fuentes-Lillo, E., Navarrete-Campos, D., Vázquez-Villa D., Cabrera-Barjas, G., & Cuba-Díaz, M. (2024). Preliminary assessment of seed heteromorphism as an adaptive strategy of *Colobanthus quitensis* under saline conditions. *Scientific Report, 14*(31120), https://doi.org/10.1038/s41598-024-82381-z.

Pipatpongpinyo, W., Korkmaz, U., Wu, H., Kena, A., Ye, H., Feng, J., & Gu, X. Y. 2020 (2020). Assembling seed dormancy genes into a system identified their effects on seedbank longevity in weedy rice. *Heredity, 124*,135–145. https://doi.org/10.1038/s41437-019-0253-8

Potapowicz, J., Szumińska, D., Szopińska, M., & Polkowska, Z. (2019). The influence of global climate change on the environmental fate of anthropogenic pollution released from the permafrost Part I. Case study of Antarctica. *Science of The Total Environment, 651*(1), 1534-1548. https://doi.org/10.1016/j.scitotenv.2018.09.168

Raju-Ahmed, M., Jannat, Y., Collins, W., Perez, M., & Byoung- Kwan, C. (2020). Classification of pepper seed quality based on internal structure using X-ray CT imaging. *Computers and Electronics in Agriculture, 179*(105839), 1-8. https://doi.org/10.1016/j.compag.2020.105839

Rivas, G. F. (2020). Efecto de la inoculación de hongos micorrícicos arbusculares y endófitos en la tolerancia al estrés por sequía en plántulas de Araucaria araucana. Tesis de Ingeniería en Biotecnología Vegetal. Universidad de Concepción, Los Ángeles, Chile, 52 pp.

Sanhueza, C., Vallejos, V., Cavieres, L.A., Sáez, P., Bravo, L.A., & Corcuera, L. J. (2017). Growing temperature affects seed germination of the Antarctic plant *Colobanthus quitensis* (Kunth) Bartl (Caryophyllaceae). *Polar Biology, 40*, 449–455. https://doi.org/10.1007/s00300-016-1972-4

Schofield, E., Jones, E.P., & Sarasan, V. 2018. Cryopreservation without vitrifcation suitable for large scale cryopreservation of orchid seeds. *Botanical Studies, 59*(13), 1-9. https://doi.org/10.1186/s40529-018-0229-7

Seed Information Database: Royal Botanic Gardens, Kew. Available online: https://ser-sid.org/species/603ee8ea-d4ae-47e7-8d84-36d1162ec530 (accessed: 15 October 2024).

Shibata, M., Coelho, C. M. M., Garighan, J. A., Santos, H. P., Araldi, C. G., & Maraschin, M. (2020). Seed development of Araucaria angustifolia: plant hormones and germinability in 2 years of seed production. *New Forests, 52*, 759–775. https://doi.org/10.1186/s40529-018-0229-7

Singh, N., Dash, S., & Khan, Y.J. (2016). Survival of chickpea, sesame, niger, castor and safflower seeds stored at low and ultra-low moisture contents for 16- 18 years. *Seed Science Technology, 44*, 1–14. https://doi.org/10.15258/sst.2016.44.3.09

Solberg, S, Ø., Yndgaard, F., Andreasen, C., Von Bothmer, R., Loskutov, I. G., & Asdal, Å. (2020). Long-term storage and longevity of orthodox seeds: a systematic review. *Frontiers in Plant Science, 11*(1007). https://doi.org/10.3389/fpls.2020.01007

Souza, F.F.S., Devilla, I.A., Souza, R.T.G. Teixeira, I.R., & Spehar, C.R. (2020). Physiological quality of quinoa seeds submitted to different storage conditions. *African Journal of Agricultural Research, 11*(15), 1299-1308. https://doi.org/10.5897/AJAR2016-10870

Surono & Narisawa, K. (2017). The dark septate endophytic fungus *Phialocephala fortinii* is a potential decomposer of soil organic compounds and a promoter of *Asparagus officinalis* growth. *Fungal Ecology, 28*, 1-10. https://doi.org/10.1016/j.funeco.2017.04.001

Taylor, A.G. (2020). Seed storage, germination, quality, and enhancements. En: Wien, H. C. y Stützel, H. (Eds) Physiology of vegetable crops. Second Edition. CAB International. 30 pp.

Tejedo, P., Benayas, J., Cajiao, D., Leung, Y.F., De Filippo, D., & Liggett, D. (2022). What are the real environmental impacts of Antarctic tourism? Unveiling their importance through a comprehensive meta-analysis. *Journal of Environmental Management, 308*(114634). https://doi.org/10.1016/j.jenvman.2022.114634

Torres-Díaz, C., Gallardo-Cerda, J., Lavin, P., Oses, R., Carrasco-Urra, F., Atala, C., Acuña-Rodriguez, I.S., Convey, P., & Molina-Montenegro, M.A. (2016). Biological interactions and simulated climate change modulates the ecophysiological performance of *Colobanthus quitensis* in the Antarctic ecosystem. *PLoS ONE, 11*(10), e0164844. https://doi.org/101371/journal.pone. 0164844

Turner, J., Marshall, G.J., Clem, K., Colwell, S., Phillips, T., & Lu, H. (2020). Antarctic temperature variability and change from station data. *International Journal of Climatology, 40*, 2986–3007. https://doi.org/10.1002/joc.6378

Ueno, A. C., Gundel, P.E., Seal, C.E., Ghersa, C.M., & Martínez-Ghersa, M.A. (2020). The negative effect of a vertically-transmitted fungal endophyte on seed longevity is stronger than that of ozone transgenerational effect. *Environmental and Experimental Botany, 175*(104037). https://doi.org/10.1016/j.envexpbot.2020.104037

van Treuren, R., Bas, N., Kodde, J., Groot, S.P.C., & Kik, C. (2018). Rapid loss of seed viability in *ex situ* conserved wheat and barley at 4°C as compared to –20°C storage. *Conservation Physiology, 6*(1), coy033. https://doi.org/10.1093/conphys/coy033

Vázquez, D., Fuentes-Lillo, E., Farias, D., & Cuba-Díaz, M. (2017). Germination of heteromorphic seeds of *Colobanthus quitensis* (Kunth) Bartl. under salinity conditions. En Guaiquil, I., Leppe, M., Rojas, P y Canales, R. (Eds). Visiones de Ciencia Antártica. Libro Resúmenes, IX Congreso Latinoamericano de Ciencias Antártica. Punta Arenas-Chile, 900-903.

Vera, M.L. (2017). Factors affecting germination of *Deschampsia antartica* and *Colobanthus quitensis* seeds from an altitudinal gradient on Livingston Island (South Shetland Island, Antártica). http://hdl.handle.net/10651/40759.

Wawrzyniak, M. K., Kalemba, E. M., Ratajczak, E., Chmielarz, P. (2020). Oxidation processes related to seed storage and seedling growth of *Malus sylvestris*, *Prunus avium* and *Prunus padus*. *PLoS One, 15* (6): e0234510. https://doi.org/10.1371/journal.pone.0234510

Whitehouse, K.J., Hay, F.R., & Lusty, C. (2020). Why seed physiology is important for genebanking. *Plants, 9*(584). https://doi.org/10.3390/plants9050584

Zhao, M., Zhang, H., Yan, H., Qiu, L., & Baskin, C.C. (2018). Mobilization and role of starch, protein, and fat reserves during seed germination of six wild grassland species. *Frontiers in Plant Science, 9*(234). https://doi.org/10.3389/fpls.2018.00234

Zhou, W., Chen, F., Luo, X., Dai, Y., Yang, Y., Zheng, C., Yang, W., & Shu, K. (2019). A matter of life and death: Molecular, physiological, and environmental regulation of seed longevity. *Plant Cellular Environment, 43*, 293–302. https://doi.org/10.1111/pce.13666

II. CHAPTER I: GERMINATION STRATEGIES AND SEED QUALITY OF *Colobanthus quitensis*: IMPLICATIONS FOR SUSTAINABLE ANTARCTIC ECOSYSTEMS AND *EX SITU* PLANT CONSERVATION

Yadiana Ontivero[1,2], Marely Cuba-Díaz[1,3]*, Eduardo Fuentes-Lillo[4,5] and Peter Convey

[1]Laboratorio de Biotecnología y Estudios Ambientales, Departamento de Ciencias y Tecnología Vegetal, Escuela de Ciencias y Tecnologías, Campus Los Ángeles, Universidad de Concepción, Juan Antonio Coloma 0201, Los Ángeles 4440000, Chile

[2]Facultad de Agronomía, Universidad de Concepción, Campus Chillán, Av. Vicente Méndez 595, Chillán 3812189, Chile

[3]Programa de Ciencia Antártica y Subantártica (PCAS), Universidad de Concepción, Concepción Barrio Universitario s/n, Concepción 4030000, Chile

[4]Laboratorio de Invasiones Biológicas (LIB), Facultad de Ciencias Forestales, Universidad de Concepción, Concepción Barrio Universitario s/n, Concepción 4030000, Chile

[5]Instituto de Ecología y Biodiversidad (IEB), Universidad de Chile, Las Palmeras 3425, Ñuñoa, Santiago 7800020, Chile

[6]British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge CB3 0ET, UK

[7]Department of Zoology, University of Johannesburg, P.O. Box 524, Auckland Park 2006, South Africa

[8]Millennium Institute Biodiversity of Antarctic and Subantarctic Ecosystems (BASE), Pontificia Universidad Católica de Chile, Av. Libertador Bernardo O'Higgins 340, Santiago 8331150, Chile

[9]Cape Horn International Center (CHIC), O'Higgins 310, Cabo de Hornos 6350000, Chile

* Corresponding author (mcuba@udec.cl).

Publicado en Sustainability MDPI, Volumen 16, Número 23, 2024, https://doi.org/10.3390/su162310726.

ABSTRACT

The conservation of the Antarctic ecosystem is linked to scientific and tourism activities. The Colección Activa de Plantas Vasculares Antárticas at the Universidad de Concepción aims to help conserve the region's vascular flora to support scientific research while minimizing anthropogenic pressure on natural ecosystems. *Colobanthus quitensis* is the only native dicotyledonous plant to Antarctica and, as an extremophilic plant, is capable of withstanding extreme abiotic conditions. This species has potentially important use as a biological model to study the physiological deterioration of dormant seeds and other traits, some of which may

contribute to vegetation development in Antarctica. Although studies have addressed the reproduction and germination of *C. quitensis*, there is no recent synthesis of information regarding its reproductive biology and seed traits that could support its conservation. This work synthesizes and critically analyzes the current knowledge of *C. quitensis* reproduction in natural and controlled environments, identifying factors that contribute to the decline in seed quality. We propose that growth conditions, seed morphology, and interactions with endophytic fungi influence germination and seedling establishment. We also highlight critical knowledge gaps that must be addressed to better understand the species' phenology, the impact of biotic and abiotic factors on its development, strategies for seed conservation, and biotechnological applications.

Keywords: Antarctic ecosystem; extremophilic plant; reproduction; seed physiological quality; seed conservation.

1. INTRODUCTION

Few regions of the world are characterized by environmental conditions as extreme for vegetation development as those of Antarctica. Since the Antarctic Treaty came into force in 1961, its ecosystems have received varying degrees of protection, with the entire region declared a natural reserve dedicated to peace and science [1]. However, human activities, including industrial fishing, tourism, and scientific research, have contributed to environmental pollution [2], as well as the introduction and propagation of exotic species [3,4]. These activities threaten the fragile ecosystems of Antarctica, highlighting the importance of conservation efforts and sustainable research practices.

Colobanthus quitensis (Kunth) Bartl. (Caryophyllaceae) is the only native dicotyledonous plant present in Antarctica and has a broad geographic distribution [5,6,7]. Its Antarctic populations are found along the western coast of the Antarctic Peninsula, as well as in the South Shetland and South Orkney Islands (known collectively as the maritime Antarctic) [7,8]. Some of these populations are located within Antarctic Specially Protected Areas (ASPAs), where strict conservation measures aim to prevent disturbances caused by human activities (Figure 1) [9]. However, there is a notable lack of germplasm banks that could provide an alternative to field samples of this species for research purposes. For this reason, in 2009, the Laboratorio de Biotecnología y Estudios Ambientales (LABEA) created the Colección Activa de Plantas Vasculares Antárticas (220.418.012-INV) at the Universidad de Concepción, Campus Los

Ángeles. Here, different populations of *C. quitensis* are grown from seed under controlled common garden conditions or *in vitro* from germinated seeds or vegetative segments (explants) [10]. The common garden conditions maintain plants in growth chambers at 14 ± 1 °C with a light intensity of 100–120 µmol photons m−2 s−1, photoperiod of 16/8 h light/dark, and relative humidity between 75 and 80%, using a leaf–peat–perlite (3:2:1)-based substrate and with fertilization every two weeks [9]. This collection aims to generate scientific knowledge that contributes to Antarctic research while reducing the negative impacts of human presence in the region. Therefore, the collection supports the long-term sustainability of the Antarctic ecosystem.

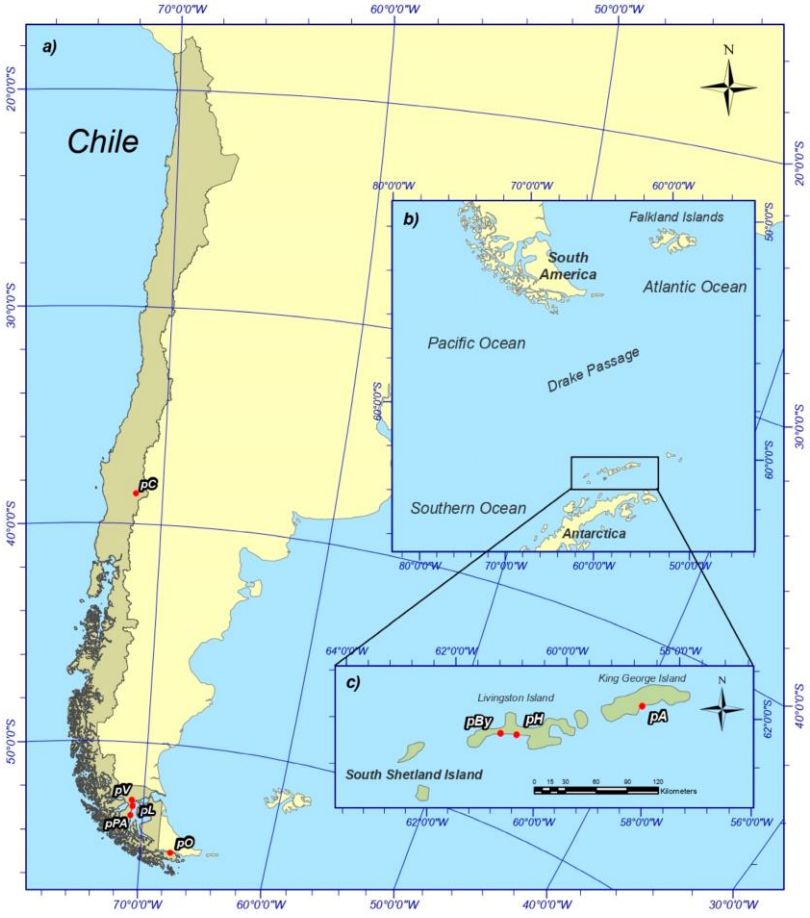


Figure 1. Map showing the geographic locations of populations of *Colobanthus quitensis* analyzed in the articles identified in this review. (a) Geographic location of South American populations. (b) Drake Passage region with (c) enlargement of the South Shetland Islands archipelago indicating King George Island and Livingston Island. The location of the

populations indicated with red dots are Conguillío (pC), La Vega (pV), Sector Laredo (pL), La Marisma (pPA), Omora (pO), Arctowski (pA), Hannah Point (pH), and Byers Peninsula (pBy). Map modified from [11].

The growing interest in understanding plant responses to extreme environmental conditions has led researchers to focus on unconventional biological models like extremophilic plants. Such species can contribute to fields as wide as genetic improvement in crops and ecosystem sustainability [12,13]. *Colobanthus quitensis* provides a promising model for studying the adverse effects of abiotic stress on plant development, particularly in the context of climate change, and for exploring the role of endophytic and rhizospheric microorganisms in these processes [3,14,15,16,17,18]. A model organism for scientific studies should facilitate intensive and extensive investigation of biological phenomena, providing a deeper understanding of these processes in similar organisms as well as in more complex ones [19]. It should also be easy to cultivate and maintain in large numbers under laboratory conditions [20]. Advances in understanding the genetic characteristics of *C. quitensis* [9,11,21] and the development of protocols for *in vitro* cultivation and growth under common garden conditions [10,22,23] further support its use as a biological model species.

Colobanthus quitensis primarily reproduces sexually through seeds [6,24], and its high tolerance to extreme environmental conditions enables it to establish reproductive populations in the <0.4% of ice-free land available in Antarctica [7,8]. However, the harsh conditions in Antarctica affect the species' physiology from the early stages of its development [25,26,27,28]. For collections such as the Colección Activa de Plantas Vasculares Antárticas, it is essential to preserve seeds with optimal physiological quality to ensure their long-term viability and thereby contribute to the conservation of *C. quitensis*. Seed quality is the result of a combination of factors, including viability, longevity, germination success, and vigor, all of which are influenced by environmental, phytosanitary, morphological, physiological, and genetic factors [29,30,31]. Studying seed quality allows the establishment of storage protocols that reduce physiological deterioration [32]. However, most research in this field has prioritized species of agricultural or economic significance [33], often overlooking species with no immediate commercial value that produce small, dormant seeds. These traits present additional challenges for conducting comprehensive physiological seed quality assessments. *Colobanthus quitensis*, as an extremophilic species, produces small seeds ranging from 0.5 to

0.85 mm in size [34], which undergo dormancy [35] and are prone to physiological deterioration when stored at 4 °C [22], the standard temperature used for seed storage across various species [36]. This review aims to highlight how *C. quitensis* can provide new insights into the physiological seed quality of extremophilic species, and what the potential applications of this knowledge are for economically significant crops and ecological studies. Consequently, identifying current knowledge gaps regarding its reproduction and the physiological quality of its seeds, based on the methodologies and conditions used in these studies, is crucial for advancing research in this field.

2. Reproductive Strategies of *Colobanthus quitensis*

In natural ecosystems, *C. quitensis* produces reproductive structures after the second or third year of development, when the diameter of its vegetative cushion ranges between 0.2 and 1 cm [37]. Under controlled conditions, the specific cushion size or plant age at which flowering occurs has not been reported. However, laboratory observations have documented the presence of floral buds in small plants grown both in common garden settings (Figure 2C) and *in vitro* cultures (Figure 3D) (Table 1). Seed propagation is the main form of plant establishment in the natural environment, although asexual reproduction through cushion fragmentation has been recorded [6,24]. Successful sexual reproduction is associated with increased temperature during the spring and summer months [6,38,39]. The average air temperature faced by plants near Arctowski station on King George Island South Shetland Islands (Figure 1) during their growth period can be highly variable, generally above 0.9 °C and below 3.7 °C [40]. However, mature seeds are produced even in the southernmost known population in northern Alexander Island (69 °S) [7] (Table 1).

The number of flowers produced by *C. quitensis* ranges from 1 to 28 per plant and depends on the age of the plant, the size of the plant cushion, and the soil temperature and moisture conditions during the growth stage, as well as its geographic location [21,39,41]. However, even though flowers are produced, they do not always successfully develop seeds [6]. In Chilean Antarctic expeditions conducted during the summer of 2021–2022 in Byers Peninsula (Livingston Island) and Arctowski, as well as during the summer of 2023–2024 in Arctowski (Figure 1), numerous flowers were observed on plant cushions of various sizes (Figure 2A,B, Table 1). The onset of flowering on Signy Island (South Orkney Islands) in 2023/24 was also delayed compared to observations from previous Antarctic expeditions [42]. It is known that

flowering in any given year is influenced by both the climatic conditions of that year and by the climate and resources dedicated to reproduction in the previous year. In species from cold ecosystems, such as Arctic and alpine regions, annual variability in flowering is associated with the timing of snowmelt and seasonal temperatures. Earlier snowmelt can increase exposure to freezing conditions and alter temperatures during the flowering period, thereby influencing flower abundance and overall plant fitness [43,44]. As a result, the balance between flowering in one year and the following year can offset the resources allocated to flowering in the current year [45]. However, increases in temperatures in the maritime Antarctic in recent years have been linked with increased frequency of successful sexual reproduction by *C. quitensis*, along with considerable expansion of some populations [6,17,24,46,47]. This may be due to an increase in the number of flowers per plant, in the number of seeds matured per flower, or in germination success. These observations have received support from studies where the effects of changing environmental conditions on the growth, reproduction, and seed germination of this species have been simulated [29,41,48,49].

In the 'common garden' facility at the Universidad de Concepción, where *C. quitensis* is grown at photosynthetically optimal temperatures (14 ± 1 °C), the species has successfully reproduced for several years. Growing *C. quitensis* in a common garden alters the characteristics of its leaves and flowers, as evidenced by a larger flower peduncle (Figure 2C) compared to plants grown in the field where the flower peduncle and flower capsules do not usually exceed the height of the plant cushion (Figure 2A,B, Table 1).

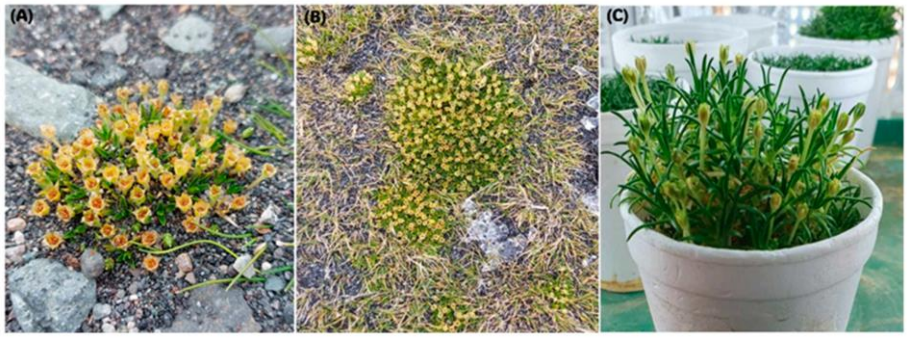


Figure 2. Reproduction by *Colobanthus quitensis* plants. (**A**) A small cushion in Antarctica showing open seed capsules (summer 2023/24, ≈0 °C *). (**B**) Large and small cushions with seed capsules in Antarctica (summer 2023/24, ≈0 °C *). (**C**) Plants flowering under common garden growing conditions (summer 2019/20, 13 ± 2 °C **). * Average temperature in the

vicinity of the plants during the reproductive period [50]; available information at https://www.meteoblue.com/en/weather/historyclimate/weatherarchive/henryk-arctowski-polish-antarctic-station_antarctica_6620757, accessed October 21, 2024. ** Growing temperature under common garden conditions.

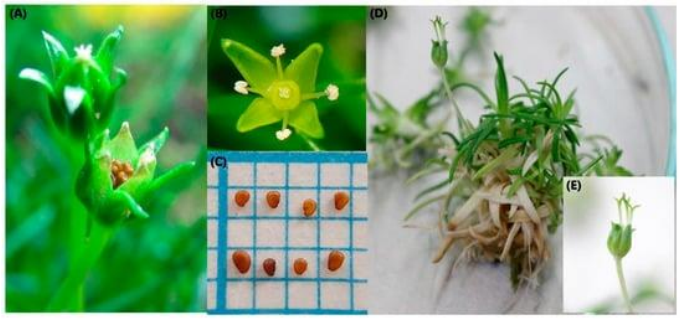


Figure 3. *Colobanthus quitensis* capsule and seeds obtained under laboratory conditions. (**A**) Closed flower bud and open capsule showing seeds. (**B**) Open flower from a common garden showing the stigma and stamens. (**C**) Example seeds from different populations: from left to right, top: Hannah Point, Byers Peninsula, Arctowski, and Omora Park; bottom: La Marisma, Laredo, La Vega, and Conguillío Park (the square measures 1 mm per side). (**D**) Plant from *in vitro* culture showing vivipary. (**E**) Close-up showing the seed attached to the cotyledon of a new seedling generated by vivipary.

Under low-temperature and windy conditions, the flowers are cleistogamous and self-fertilizing, opening only when the seeds are ripe or have almost completed their maturation process (Figure 1a,b) [39,51]. Even in the common garden, flowers tend to open only when the seeds are ripe (Figure 3A). However, in field plants that develop between rocks where the influence of wind is less, flowers can open before generating seeds [52]; in these conditions, fertilization is likely to occur with the help of raindrops. In the common garden, it has been observed that warmer temperatures stimulate flower opening, exposing reproductive structures, which may promote cross-pollination, thus facilitating genetic exchange (Figure 3B, Table 1). The germination success of cleistogamic seeds is expected to be higher compared to chasmogamic seeds due to reduced contamination from pathogens present in air and water [53]. However, no studies are available on the temperature ranges in which *C. quitensis* flowers self-pollinate or cross-pollinate, the role of soil fertility in seed production and quality, or

the frequency of pathogen occurrence. The final stage of reproduction of *C. quitensis* occurs when the capsules open and the mature seeds are exposed [54]. Seed size varies between the different *C. quitensis* populations held in the Colección Activa de Plantas Vasculares Antárticas at the LABEA (Figure 3C). It is also likely that the morphological characteristics of seeds will vary because of their growth conditions, even within the same population. The implications of these factors for the establishment of new individuals are yet to be studied. Under natural conditions, permanent seed banks form in the soil in the immediate vicinity of the parent plants, although some seeds may be dispersed further by wind, rain, or during snowmelt [38,55]. Under *in vitro* culture conditions, the occasional presence of viviparism has been detected (Figure 3D,E). Viviparism in flowering plants refers to the ability of some seeds to generate seedlings while still attached to their mother plant. This phenomenon has been reported in 0.1% of flowering plant families [56] and is a potentially valuable strategy for species in extreme environments because it shortens the period required for the establishment of progeny [57]. In C. apetalus, a species closely related to *C. quitensis*, viviparism has been detected under controlled growth conditions with an air humidity of 75% [52]. Some *C. quitensis* seeds cultivated *in vitro*, where ambient humidity is high, have shown vivipary (Figure 3D,E, Table 1), a phenomenon previously detected in plants with orthodox seeds [58,59]; however, to date, this phenomenon has not been recorded in their natural environment.

Recently, the presence of seed color heteromorphism has been detected in different populations of *C. quitensis*, with light and dark brown seed forms; however, only the influence of seed form on tolerance to salinity has been studied, with some populations identified as being tolerant to this stress [60,61]. The environmental, metabolic, physiological, or genetic causes underlying heteromorphism in *C. quitensis* and its possible influence on other aspects of the phenology of the species require further research (Table 1).

At low temperatures, *C. quitensis* allocates more resources to vegetative growth than to sexual reproduction [6,38], whereas under warmer temperatures and higher water availability, both the growth and reproduction of the species are favored [21,38,62]. However, the detailed relationship between environmental conditions and seed production and quality has not been analyzed. In agricultural crops, nutritional deficiencies can affect pollen fertility as well as seed production and germination success [63]. Under natural conditions, larger and more extensive populations of *C. quitensis* have been recorded in areas where the soil is more fertile and there

is high enzymatic activity of rhizosphere microorganisms [64]. However, research has yet to address whether there is any relationship between soil nutrient availability and the nutritional reserves of seeds produced by *C. quitensis*.

Table 1. Summary of reproductive features of *Colobanthus quitensis* grown under natural or controlled laboratory conditions.

Reproduction Features	Natural Conditions	Controlled Conditions	References
Reproduction type	Sexual reproduction is the main form of establishment. Asexual reproduction is also recorded	Plants can be established either in the common garden or *in vitro* from seeds (sexual reproduction) or explants (asexual reproduction).	[6,24]
Reproduction time	Successful reproduction occurs with increased temperature and water availability during spring and summer.	The environmental conditions remain constant. Flowering is detected throughout most of the year, although it is concentrated in spring and summer months.	[6,38,39]
Plant size for sexual reproduction	Flowering is detected in small plant cushions, whose minimum diameter varies from 0.2 to 1 cm, or in cushions of several centimeters in diameter.	No published reports, although small plants (<2 cm) have been observed at LABEA producing viable flowers and seeds.	[6,24,37] Figure 2A,B
Reproductive strategies	In cold and windy conditions, the flowers remain cleistogamous and self-fertilize.	Cleistogamous (higher frequency) and chasmogamous (lower frequency) flowers have been detected, so there may be self-fertilization or cross-fertilization.	[39,51,52] Figure 3A,B
Flower morphology	The flower stalk does not usually exceed the height of the plant cushion.	The flower stalk tends to exceed the height of the plant cushion.	[39,51] Figure 2
Seed viability	Not all flowers produce viable seeds successfully. The presence of dormant seeds has also been described.	Viable seeds are produced, some of which are dormant.	[6,35,39,41]
Seed heteromorphism	No seed color differences have been noted.	Seed color heteromorphism has been detected, with light and dark brown seeds.	[60,61]
Viviparism	No viviparism has been reported.	Viviparism has been observed in some seeds grown *in vitro* under high relative humidity conditions.	Figure 3D,E

3. Specific Features of *C. quitensis* Seed Production

Colobanthus quitensis produces small, triangular seeds that are wider towards the cotyledon area (Figure 3C), and their inability to germinate in some studies has been associated with the presence of deep primary dormancy [35,39]. Dormancy is a defense mechanism against environmental stress that, in the case of *C. quitensis*, could prevent germination late in the summer season in Antarctica, thereby avoiding seedling mortality [55,65]. It is still unclear whether the trigger for dormancy is an environmental condition or a physiological characteristic of the seeds, although laboratory evidence indicates that there is a genetic component influencing the presence of dormancy in *C. quitensis* populations, which is associated with their origin [66]. It is important to note that physiological dormancy is classified as primary when seeds are dispersed in a dormant state directly from the mother plant or secondary when it develops in response to specific environmental conditions that inhibit germination [67]. Laboratory studies have shown significant differences in *in vitro* germination between seeds

from Antarctic and southern Chilean populations [9,22], which could indicate the presence of dormancy in some populations but not in others. Therefore, we hypothesize that the clear climatic differences between the regions where this species occurs (maritime Antarctic, sub-Antarctic, and throughout the Andes, including tropical latitudes at high altitudes) will exert different levels of stress that influence the expression of dormancy and, consequently, have an additional impact on seed quality. Given the observed differences in germination among populations, we believe that a more thorough study on dormancy is necessary.

Since dormancy may be related to embryo and seed coat characteristics, it affects seed longevity, water absorption rate, water content, resistance to mechanical damage, germination ability, and speed [68,69]. The inability of *C. quitensis* seeds to germinate under controlled conditions, despite embryo viability, as observed with the tetrazolium test [39,51], is related to dormancy [22] or the use of suboptimal protocols to stimulate this process. The germination capacity of seeds under certain temperature conditions will also be influenced by the conditions under which the mother plant developed, thus suggesting that maternal influence is fundamental to the success of germination [29].

Colobanthus quitensis seeds consist of a seed coat, perisperm, sparse endosperm, and an embryo [35]. The endosperm is involved in embryo nutrition, seed longevity, seed protection, and growth control during development and germination [30,70]. Some very small seeds of plant species that accumulate very limited nutritional reserves, such as orchids, have insufficient energy to germinate [71]. In such cases, natural associations with mycorrhizal fungi of genera such as Ceratobasidium, Tulasnella, Rhizoctonia, Epulorhiza, or Ceratorhiza provide the nutrients necessary for germination and initial seedling development [72]. Further, association with non-mycorrhizal endophytic fungi can also increase endogenous production of indoleacetic acid and gibberellins, which encourage germination [73]. It is known that *C. quitensis* establishes symbiotic relationships with endophytic fungi, which benefit growth under environmentally stressful conditions [74,75,76]. Therefore, it is likely that some of these fungi may also influence seed germination.

Colohanthus quitensis plants produce between 12 and 43 seeds per capsule, with the lowest production reported in populations growing under more extreme environmental conditions [5,6,9,29,38,54], along with lower germination success [6,28]. In an ecological context, even when the germination percentage is low, it may still be sufficient for the maintenance of

populations [77]. Harsh environmental conditions generally limit seed production and quality in agricultural settings [78]. The climatic conditions to which *C. quitensis* plants are subjected in Antarctic and high Andean ecosystems are more intense than those faced by agricultural species, albeit the species shows considerable stress adaptation and resistance [25,47,75,79], which may affect not only seed production and germination success but also the plant's ability to develop seed nutritional reserves, testa thickness, and the expression of dormancy.

The coloration of the seed coat varies within *C. quitensis* populations, and the germination percentages differ between light and dark brown seeds when exposed to different NaCl concentrations [60,61]. Seed heteromorphism, not only in testa coloration but also in morphology, has been reported in other Caryophyllaceae species [80,81]. This phenomenon is influenced by genetic factors, the position of the seeds on the plant, and their distance from the plant's vascular tissues [82]. Flower position determines seed filling, its hormonal gradient, seed coat structure, and composition which, combined, influence both the germination process and dormancy [83]. However, studies have not addressed whether *C. quitensis* seed color variation is due to asynchronous seed maturation in flowers, or to biochemical, molecular, or nutritional factors; it is also unknown whether seed coloration is related to other indicators of seed physiological quality. Identifying such correlates of seed quality would help inform conservation decisions.

The long-term storage of seeds under dry air conditions generally depends on their ability to tolerate desiccation and exposure to low temperatures [84]. The ambient moisture content and temperature range employed during seed preservation are critical to maintaining seed viability [32] as some reserve substances such as storage triacylglycerides can crystallize at temperatures of −20 °C [85]. Although *C. quitensis* has shown a rapid loss of germination capacity with storage time at 4 °C, sharing characteristics of recalcitrant seeds [86], the Royal Botanic Gardens informs that it is a species that produces orthodox seeds that can tolerate freezing at −20 °C and show 100% germination when using gibberellic acid (GA3) in *in vitro* culture [87]. This classification is supported by the fact that the species can maintain permanent seed banks in the soil [51], which maintain some degree of viability for periods of between two and four years [39,54]. However, no studies have been conducted evaluating different methods of seed preservation.

4. Characteristics of *C. quitensis* Seed Bank Formation

Colobanthus quitensis populations generate permanent seed banks, providing a mechanism of adaptation to extreme environmental conditions [51,54,55], implicitly assuming an extended length of time that the seeds will remain viable and contributing to the persistence of the population over time [88]. Such a mechanism could be particularly important in the extreme environmental conditions of Antarctica. Three types of seed banks are generally recognized, transient, short-term persistent, and long-term persistent, with the latter having longevity of more than five years [88].

Once seeds enter the soil seed bank, their persistence depends on their ability to remain viable by neutralizing reactive oxygen species, which can cause physiological damage [67]. As a result, each non-lethal stress condition encountered by the seeds in the soil seed bank triggers a phenotypic response that acts as priming, allowing the seeds to respond more effectively to any future event [89]. It is therefore believed that every seedling that emerges carries the memory of the parent plants from the time of seed formation and their subsequent entry into the soil seed bank. In this way, both genetic and epigenetic factors—viewed as memory mechanisms—contribute to the variability found in soil seed banks [67].

The available studies on the seed banks of *C. quitensis* have had a maximum duration of four years [34], so it is not yet possible to confirm which type of seed bank the species generates or if there are any differences between the seed banks of maritime Antarctic, sub-Antarctic, and lower latitude populations. Recently, there has been recognition of the importance of seed dormancy [90] and its relationship with longevity [91], temperature variation [92], and soil characteristics, which together influence the dynamics of soil seed banks [93].

Small, light, round seeds are more likely to penetrate more deeply into the soil profile and generate permanent banks [88]. *Colobanthus quitensis* seeds typically measure between 0.5 and 0.85 mm [34] and weigh approximately 0.05 mg [35], features that could facilitate the formation of a permanent soil seed bank. The ease of burial due to the small size of seeds and positive photoblastism are characteristics that contribute to the formation of seed banks in the soil [94]. Regarding the ability of *C. quitensis* seeds to germinate in the presence of light or darkness, the literature includes studies where seeds are placed to germinate under photoperiods of 24 h light [54] or 24 h darkness [29], as well as in photoperiods that combine 12 h light/12 h darkness [28] or 16 h light/8 h darkness [22]. This indicates that the species can

germinate under all daylength conditions; however, no single study has compared germination success under different lighting conditions to classify *C. quitensis* seeds as positively photoblastic, negatively photoblastic, or neutral, although available evidence suggests that the species is neutral to light conditions for germination.

Plant growth conditions influence the characteristics of the seeds produced and their subsequent persistence in soil, with Antarctic populations producing heavier seeds than those growing in greenhouses [35,51,95]. Longer seeds generally have larger embryos and more endosperms, factors that facilitate germination [70]. Therefore, we hypothesize that differences in seed size among populations of *C. quitensis* may influence various germination indicators, such as germination percentage or speed, and that these differences will be maintained even when parent plants are grown under common garden conditions. Consistent with this hypothesis, differences in morphology, genetic factors, and the expression of mechanisms of tolerance to environmental conditions between *C. quitensis* populations have been reported under common garden conditions [9,11,96].

Local soil characteristics are likely to influence the development of seed banks [6,55,97], not least as the presence of an apparently healthy reproducing population does not automatically lead to the existence of a seed bank [55]. Further studies are required to elucidate the relationship between soil characteristics and the persistence of seeds in the soil. A detailed study of the microbial community is also required since fungi present in the soil can interact with seeds and establish both symbiotic relationships that favor germination [98] and pathogenic relationships that will reduce viability. Recent temperature increases in Antarctica are expected to favor the activity of some soil microorganisms, including in the plant rhizosphere, increasing the availability of nutrients in the soil [64,99,100] and, in turn, the establishment and survival of *C. quitensis*, which could favor the growth, reproduction, and seed production of this species.

A seed bank potentially includes different soil strata related to annual reproductive cycles [88], so seed quality might be expected to vary in relation to position. Applying this concept to *C. quitensis*, the shallowest soil layers have been associated with the youngest seeds [34]. However, the most recently produced seeds do not always show the best germination rates due to the presence of dormancy [54]. To date, studies have focused on the seed density, germination capacity, and survival of samples collected in the first 10 cm of the soil profile

[34,55]. Most of the soils have been influenced by permafrost and extreme cold, with the presence of volcanic and granitic types primarily in the Antarctic Peninsula. They typically lack or have very few of the strata commonly identified elsewhere, resulting in soil depths that rarely exceed a few centimeters, where the organic matter content is often scarce [101,102,103]. At this time, there is no research available on *C. quitensis* that indicates the depth of its seed banks, how many seeds are introduced each year into the soil strata, or the rate at which soil depth increases over time.

5. The Relationship of *C. quitensis* with Endophytic Fungi, with Specific Emphasis on Seeds

Most plants establish symbiotic relationships with endophytic fungi, whose influence promotes plant growth in the presence of biotic and abiotic stresses [74,104,105]. Endophytic fungi are often found in roots, rather than leaves, although they may be present in both [106]. The presence of these fungi leads to increased seed mass and enhances the accumulation of soluble sugars, auxins, and gibberellins, which lead to increased germination success [73,105,106,107,108]. The diversity of endophytic fungi differs among plant species and is also influenced by the characteristics of the environment in which they develop, including soil and climatic factors [53]. These fungi can be vertically transmitted to plant offspring [107]; however, their presence in the parent plant does not necessarily result in transfer to the offspring [98]. Vertical transmission depends on the species involved, genotype, resource availability, environmental stress, and timing of seed initiation [107,109,110].

Endophytic fungi including *Alternaria* sp., *Eupenicillium osmophilum*, *Penicillium chrysogenum*, *P. brevicompactum*, and *Phaeosphaeria* sp. have been identified in the roots of *C. quitensis*, with a frequency of occurrence greater than 1% of the total fungal community [75]. These have beneficial effects on plant growth and flowering [75,76]. On the other hand, endophytic fungi belonging to the genera *Aspergillus*, *Cadophora*, *Davidiella*, *Entrophospora*, *Fusarium*, *Geomyces*, *Gyoerffyella*, *Microdochium*, *Mycocentrospora*, and *Phaeosphaeria* have also been identified in leaves of *C. quitensis*, which are believed to confer resistance against freezing and ultraviolet radiation [111]. *Fusarium* and *Penicillium* species positively influence orchid seed germination by producing hormones and metabolites that stimulate both germination and seedling growth [112,113]. However, studies have yet to address the transmission of

endophytic fungi associated with *C. quitensis* or the effect of these fungi on seed germination under field conditions.

Plant domestication reduces their associated microbial diversity relative to that found under natural conditions [114]. However, no studies comparing the diversity of endophytic fungi associated with *C. quitensis* plants in natural and cultivated conditions have yet been carried out. Plants collected from the field and propagated in the laboratory for more than five years produce seeds that rapidly lose their germination capacity with storage at 4 °C, with even seeds collected recently showing poor germination [115]. Factors underlying this loss of germination capacity potentially include the loss of these endophytic fungi over time or the establishment of new symbiotic relationships that do not favor seed germination. A simulated climate change study showed that in the face of increased temperature and water availability, there was a reduction in the number and functionality of endophytic fungi associated with *C. quitensis* [41]. This suggests that multiple factors, including illumination, temperature, irrigation, soil type, and fertility, used in laboratory studies may lead to important modifications in the microbial community associated with this species.

6. Interpretation of Physiological Quality of Seeds

Seed storage is a frequent practice in the agricultural industry and in germplasm banks [32]. There is a clear need to perfect the process of seed conservation, as suboptimal storage conditions cause and accelerate physiological deterioration in seeds, leading to partial or total loss of germination and resulting in economic losses [36].

Seed quality can be assessed in terms of the integrity of cellular structures, physiological processes, and morphological and phenotypic characteristics [31,116,117] that guarantee high germination, viability, longevity, emergence, and vigor. The study of these parameters allows for the characterization of species, varieties, and ecotypes [32]. In some species, the maximum physiological potential is reached at seed maturation, after which deterioration begins [33], evidenced by the accumulation of free radicals and lipid peroxidation that generate damage to the integrity of membranes, DNA, RNA, protein synthesis, and metabolic processes [30]. Understanding the process of seed quality loss and its underlying causative factors is critical for maximizing the success of *ex situ* seed bank conservation [118,119].

Germination testing allows for discrimination between batches of seeds of the same species tested under optimal conditions, along with assessing the appearance of abnormal growth

structures [120]. This approach does not consider any influence of environmental stress or evaluate other growth parameters, and the optimal conditions will differ between species and populations [33]. For example, in *C. quitensis*, the optimal germination temperature depends on the conditions under which the parent plant grows, as it has been shown that plants grown at 5 °C produce seeds capable of germinating between 5 and 15 °C, whereas plants grown at 11 °C allow for an extended germination temperature range up to 25 °C [29]. Germination tests typically last between 7 and 28 days [120]; however, the time required for germination also depends on the degree of seed deterioration [121] and the presence of dormancy [68].

Longevity refers to the period over which seed germination remains above a critical level under constant storage conditions. Longevity is affected by genotype and pre- and post-harvest environmental conditions, although the integration of these components is poorly understood [30,122]. Mathematical models based on biochemical studies to determine seed longevity [117,123] have not yet been applied in studies of *C. quitensis*.

Seed vigor and longevity are polygenetic traits, the products of interactions between physiological, biochemical, physical, and genetic properties, and contribute to the rapid and uniform emergence of seedlings with normal development [124,125]. From an ecological perspective, considering that the reproductive process in Antarctica is concentrated within the four summer months, flower production and the opening of seed capsules with mature seeds can take up to two months, and the germination and initial seedling establishment process in soil seed bank studies can take up to 45 days [115]. Rapid germination can contribute to successful establishment while conditions remain optimal for plant growth before the onset of winter. From a conservation standpoint in plant collections, achieving uniform and rapid germination reduces study time and resource consumption, facilitating the development of research. Multiple tests of vigor exist based on biochemical parameters, the imposition of stress conditions, and seedling growth [33]. However, since vigor includes multiple parameters, none of these approaches fully explain the properties involved in quality assessment or are applicable to all species. It is therefore recommended that several vigor tests should be performed to try and ensure the inclusion of all possible parameters [126].

6. Studies Related to Seed Quality in *C. quitensis*

Seed germination success relates to the imposed conditions [119], nutritional reserves [70], testa permeability, and the presence of dormancy [22,30]. Understanding each component is necessary to analyze all factors implicated in seed quality [31].

Dormancy extends longevity at the expense of germination [30]. In *C. quitensis*, factors influencing dormancy such as testa structural characteristics or seed hormone content are not completely understood, although it is known that the amounts of calcium, hemicellulose, and lignin in the testa determine water permeability, which limits germination [30]. In some crop plants, a lower lipid content in seeds has been associated with reduced longevity [32] as lipids help protect seeds from oxidative damage caused by reactive oxygen species, which affects the metabolic capacity of the seeds, preventing them from germinating [127,128]. There remain many gaps in knowledge about *C. quitensis* reproduction, seed production, factors causing seed deterioration, and the techniques required to analyze or control this process (Figure 4).

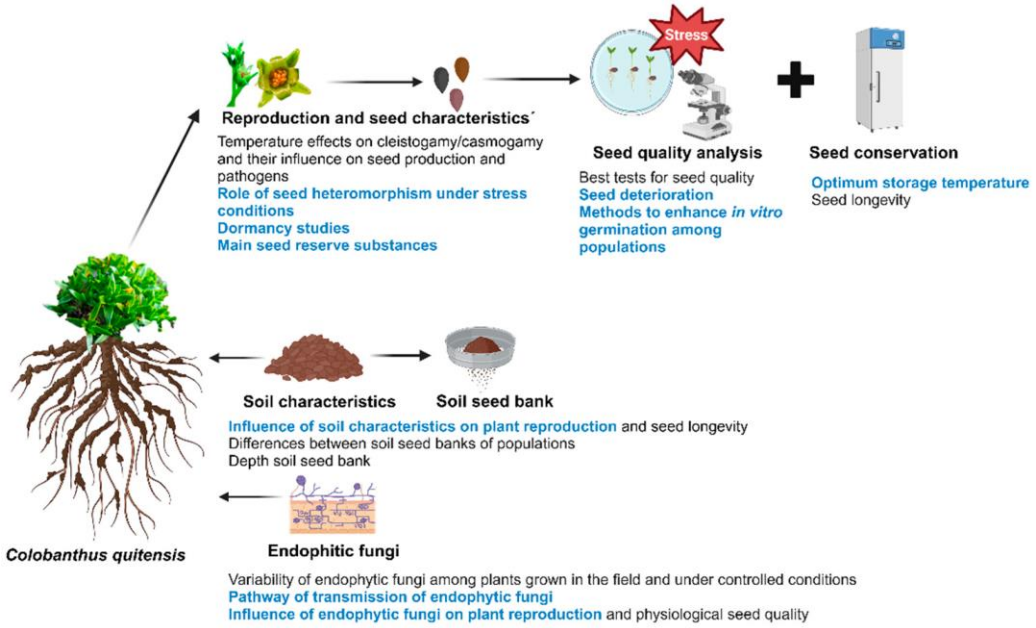


Figure 4. Summary of the main information gaps identified in this review relating to *Colobanthus quitensis* reproduction, seed production, and physiological quality. In blue are the topics that are being investigated at the Laboratorio de Biotecnología y Estudios Ambientales at Universidad de Concepción.

A flotation test is a very simple method of analyzing the physiological quality of seeds that distinguishes between viable and non-viable seeds according to whether they float in water, where seeds that float are assumed to be non-viable [129]. Air cavities that limit viability and affect germination have been detected in pepper (Piper nigrum) seeds [31]. Such cavities could underlie the success of the flotation test. This technique has been used in the analysis of *C. quitensis* seeds [9]. However, it is not 100% effective for *C. quitensis* as germination tests subsequently performed on seeds classified as non-viable showed that some could still germinate [115]. It is possible that *C. quitensis* does not generate these cavities inside its seeds, but this is unknown. Alternativity, because of the dormancy present in the species, the rate of water absorption by the seeds may be too slow for this technique to be effective. However, there is evidence that seeds floating in water have a greater dispersal capacity [130], which could form a dispersal strategy that the species uses under natural conditions.

Seed physiological quality testing based on stress conditions, where seeds are subjected to high humidity and extreme temperature conditions (accelerated and cold aging test), has allowed the evaluation of seed physiological quality, vigor, longevity, storability, and field germination in species such as rice, quinoa, and maize [131,132,133]. The germination capacity of *C. quitensis* seeds has been studied using stress tests based on the effect of low and high temperatures [29], different concentrations of sodium chloride [25,60], sodium fluoride [28], Fe3+ [134], copper [26], methanesulfonic acid [135], and a marine air component involved in the sulfur cycle [136], as well as elevated salt concentrations due to their proximity to the coast [101,102]. However, none of these analyses had the primary objective of evaluating the physiological quality of the seeds, but rather the resistance of the species to extreme environmental conditions.

It is challenging to study *C. quitensis* seeds biochemically due to the large number of samples required for this type of analysis; in addition, some seed quality analyses are also difficult to apply due to the size of the seeds. To some extent, this can be solved by using image processing software such as SVIS®, Vigor-S, ImagJ, or Germinator, which allow more detailed analysis of the different structures resulting from germination [124,131,137]. In the analysis of small seeds, X-ray spectroscopy and attenuated total reflection Fourier transformed infrared spectroscopy can be used to identify changes in seed morphology at the level of the seed coat or deeper layers involving the embryo, allowing for the identification of anomalies in the

embryo or detection of compounds that influence germination [30,133,137,138,139]. The application of these techniques in studies of *C. quitensis* will help to determine more precisely how seed quality loss manifests, without the need for destructive studies. A summary of the factors affecting the seed quality of *C. quitensis* and strategies to evaluate or counteract these is given in Table 2.

Table 2. Factors affecting *Colobanthus quitensis* seed quality and methodologies that can be used to study this phenomenon.

Factors Contributing to the Decline in Seed Quality	Change Origin	Evidence of Seed Quality Loss	Seed Quality or Vigor Evaluation Method	References
Environmental conditions	Environmental origin due to changes in temperature, relative humidity, amount of nutrients, or salinity.	Decreased germination and increased time required for germination. Changes in the structural integrity of the seed coat.	Germination tests under stress conditions. Image processing.	[25,29,60,101,102,124,131,137]
Conservation and storage conditions	Anthropogenic influence due to the imposition of suboptimal conditions of temperature and relative humidity. Possible presence of pathogens.	Decreased germination and increased time required for germination. Embryo death. Accumulation of reactive oxygen species and damage to the genetic material of the seed.	Germination tests under stress conditions. Image processing.	[32,36,120,124,131,137]
Dormancy	Possible physiological origin and/or influence of environmental factors.	Absence of germination until methods to interrupt dormancy are used.	Germination test using techniques to interrupt dormancy (scarification, stentification, mechanical rupture of the testa, or the use of hormones). Viability analysis using tetrazolium salt staining.	[30,68]
Physiological deterioration	Natural process that occurs in any seed once it has matured.	Germination is affected by oxidative stress generated by reactive oxygen species, depletion of nutritional reserves, changes in hormone profiles and/or damage to genetic material.	Germination test. Viability analysis using tetrazolium salt staining, Development of biochemical studies. Image processing.	[30,32,33,115,119,124,131,137]

7. CONCLUSIONS AND FUTURE PERSPECTIVES

This review highlights the relevance of studying the reproduction of *C. quitensis* and the *ex situ* conservation of its seeds as fundamental elements for the proper management of germplasm banks. These efforts are crucial for promoting scientific research on this unconventional model species and contributing to the preservation and sustainability of the protected ecosystems in which it thrives, thereby minimizing the negative impacts of human activity.

We identify several critical areas that require further research, including seed conservation and storage, quality analysis techniques, soil seed banks, and the interaction between microorganisms and the reproduction of *C. quitensis* (Figure 4). To fill these critical knowledge gaps, it is necessary to improve our understanding of the phenology of the species and the impact of biotic and abiotic factors on its development and evaluate strategies for seed

protection and biotechnological applications. The reproductive success of the species is strongly influenced by the environmental conditions of both the previous and the current year. The formation of permanent seed banks represents an adaptive strategy that allows *C. quitensis* seeds to remain viable for extended periods. This is further facilitated by seed dormancy and Antarctic soil conditions, which favor the long-term viability of seeds. These characteristics can inform seed conservation, which serves as a preventive resource to support biodiversity conservation in ecosystems vulnerable to climate change.

New seed analysis tools, such as imaging techniques, can offer innovative solutions for assessing the physiological quality and longevity of species with small seeds like *C. quitensis*. However, the morphological, physiological, biochemical, and genetic factors influencing germination loss under controlled conditions remain largely unknown, complicating the development of efficient storage protocols and the understanding of dormancy in this species. Additionally, uncertainty persists regarding the role that microorganisms present in their natural habitats may play in germination and seedling establishment.

8. Acknowledgments

We would like to express our gratitude for the use and access to the Colección Activa de Plantas Vasculares Antárticas (code 220.418.012-INV—Vicerrectoría de Investigación y Desarrollo of the Universidad de Concepción). We thank Darío Navarrete for support in taking seed images and Diego Echeverria Rivas (Forestry Eng.) for preparing the map showing *C. quitensis* population locations.

9. Funding

This research was funded by Grand ANID (National Agency for Research and Development)/Scholarship Program/Doctorado Beca Nacional/2021-21210100, Instituto Antártico Chileno (INACH) DT_05_22 and Programa de Doctorado en Ciencias de la Agronomía, Facultad de Agronomía, Universidad de Concepción, Campus Chillán/2021300031 (Y.O). EFL was funded by Grand ANID PIA/BASAL FB210006, and PC is supported by NERC core funding to the BAS ‘Biodiversity, Evolution and Adaptation’ team.

REFERENCES

1. Headland, R.K. Territory and Claims in the Antarctic Treaty Region: A Disquisition on Historical and Recent Developments. *Cartogr. J.* **2020**, 57, 160–174.
2. da Silva, J.R.M.C., Bergami, E., Gomes, V. Corsi, I. Occurrence and distribution of legacy and emerging pollutants including plastic debris in Antarctica: Sources, distribution and impact on marine biodiversity. *Mar. Pollut. Bull*. **2023**, 186, 114353.
3. Cuba Díaz, M.; Fuentes Lillo, E.; Navarrete Campos, D.; Chwedorzewska, K.J. Effects of climate change conditions on the individual response and biotic interactions of the native and non-native plants of Antarctica. *Polar Biol*. **2023**, 46, 849–863.
4. Hughes, K. A., Pescott, O. L., Peyton, J., Adriaens, T., Cottier-Cook, E. J., Key, G., ... & Roy, H. E.Invasive non-native species likely to threaten biodiversity and ecosystems in the Antarctic Peninsula region. *Glob. Change Biol*. **2020**, 26, 2702–2716.
5. Moore, D. M. Studies in *Colobanthus quitensis* (Kunth) Bartl. and *Deschampsia antarctica* Desv. II. Taxonomy, distribution and relationships. *Br. Antarct. Surv. Bull.* **1970**, 23, 63–80.
6. Convey, P. Reproduction of Antarctic flowering plants. *Antarct. Sci.* **1996**, 8, 127–134.
7. Convey, P.; Hopkins, D.W; Roberts, S.J.; Tyler, A.N. Global southern limit for flowering plants and moss peat accumulation. *Polar Res.* **2011**, 30, 8929.
8. Brooks, S.T.; Jabour, J.; Van Den Hoff, J.; Bergstrom, D.M. Our footprint on Antarctica competes with nature for rare ice-free land. *Nat. Sustain.* **2019**, 2, 185–190.
9. Cuba-Díaz, M.; Klagges, M.; Fuentes-Lillo, E.; Cordero, C.; Acuña, D.; Opazo, G.; Troncoso-Castro, J.M. Phenotypic variability and genetic differentiation in continental and island populations of *Colobanthus quitensis* (Caryophyllaceae: Antarctic pearlwort). *Polar Biol.* **2017**, 40, 2397–2409.
10. Cuba-Díaz, M.; Acuña, D.; Cordero, C.M.; Klagges, M. Optimización de parámetros para la propagación *in vitro* de *Colobanthus quitensis* (Kunth) Bartl. *Gayana Bot*. **2014**, 71, 58–67.
11. Koc, J.; Androsiuk, P.; Chwedorzewska, K.J.; Cuba-Díaz, M.; Górecki, R.; Giełwanowska, I. Range-wide pattern of genetic variation in *Colobanthus quitensis*. *Polar Biol*. **2018**, 41, 2467–2479.

12. Hmidi, D.; Messedi, D.; Corratgï-Faillie, C.; Marhuenda, T.O.; Fizames, C.C.; Zorrig, W.; Abdelly, C.; Sentenac, H.; Vï Ry, A.N. Investigation of Na+ and K+ transport in halophytes: Functional analysis of the HmHKT2;1 transporter from Hordeum maritimum and expression under saline conditions. *Plant Cell Physiol.* **2019**, 60, 2423–2435.
13. Kouhen, M.; García-Caparrós, P.; Twyman, R.M.; Abdelly, C.; Mahmoudi, H.; Schillberg, S.; Debez, A. Improving environmental stress resilience in crops by genome editing: Insights from extremophile plants. *Critical Rev. Biotech.* **2022**, 43, 559–574.
14. Turner, J.; Bindschadler, R.; Convey, P.; di Prisco, G.; Fahrbach, E.; Gutt, J.; Hodgson, D.; Mayewski, P.; Summerhayes, C. (Eds.) Antarctic Climate Change and the Environment; Scientific Committee on Antarctic Research: Cambridge, UK, 2009; xi+526p.
15. Bertini, L.; Cozzolino, F.; Proietti, S.; Falconieri, G.S.; Iacobucci, I.; Salvia, R.; Falabella, P.; Monti, M.; Caruso, C. What Antarctic plants can tell us about climate changes: Temperature as a driver for metabolic reprogramming. *Biomolecules* **2021**, 11, 1094.
16. Jun, S.Y.; Kim, J.H.; Choi, J.; Kim, S.J.; Kim, B.M.; An, S.I. The internal origin of the west-east asymmetry of Antarctic climate change. *Sci. Adv.* **2020**, 6, eaaz1490.
17. Cannone, N.; Malfasi, F.; Favero-Longo, S.E.; Convey, P.; Guglielmin, M. Acceleration of climate warming and plant dynamics in Antarctica. *Curr. Biol.* **2022**, 32, 1599–1606.
18. Chown, S.L.; Leihy, R.I.; Naish, T.R.; Brooks, C.M.; Convey, P.; Henley, B.J.; Mackintosh, A.N.; Phillips, L.M.; Kennicutt, M.C., II; Grant, S.M. Antarctic Climate Change and the Environment: A Decadal Synopsis and Recommendations for Action; Scientific Committee on Antarctic Research: Cambridge, UK, 2022; 109p.
19. Meinke, D.W.; Cherry, J.M.; Dean, C.; Rounsley, S.D.; Koornneef, M. Arabidopsis thaliana: A model plant for genome analysis. *Science* **1998**, 282, 662–682.
20. Ankeny, R.A.; Leonelli, S. Organisms in Experimental Research. In Handbook of the Historiography of Biology. Historiographies of Science; Dietrich, M.R., Borrello, M.E., Harman, O., Eds.; Springer: Cham, Switzerland; Berlin, Germany, 2021; pp. 265–289.
21. Galleguillos, C.; Acuña-Rodríguez, I.S.; Torres-Díaz, C.; Gundel, P.E.; Molina-Montenegro, M.A. Genetic control underlying the flowering-drought tolerance trade-off in the Antarctic plant *Colobanthus quitensis*. *Plant Cell Environ*. **2023**, 46, 3158–3169.

22. Cuba-Díaz, M.; Acuña, D.; Fuentes-Lillo, E. Antarctic pearlwort (*Colobanthus quitensis*) populations respond differently to pre-germination treatments. *Polar Biol*. **2019**, 42, 1209–1215.
23. Cuba-Díaz, M.; Rivera-Mora, C.; Navarrete, E.; Macarena, K. Advances of native and non-native Antarctic species to *in vitro* conservation: Improvement of disinfection protocols. *Sci. Rep*. **2020**, 10, 3845.
24. Fowbert, J.A.; Lewis-Smith, R.I. Rapid population increases in native vascular plants in the Argentine Islands, Antarctic Peninsula. *Arct. Alp. Res*. **1994**, 26, 290–296.
25. Cuba-Díaz, M.; Castel, K.; Acuña, D.; Machuca, A.; Cid, I. Sodium chloride effect on *Colobanthus quitensis* seedling survival and *in vitro* propagation. *Antarct. Sci*. **2017**, 29, 45–46.
26. Cuba-Díaz, M.; Marín, C.; Castel, K.; Machuca, A.; Rifo, S. Effect of copper (II) ions on morpho-physiological and bio-chemical variables in *Colobanthus quitensis*. *J. Soil Sci. Plant Nutr.* **2017**, 17, 429–440.
27. Contreras, R.A.; Pizarro, M.; Köhler, H.; Sáez, C.A.; Zúñiga, G.E. Copper stress induces antioxidant responses and accumulation of sugars and phytochelatins in Antarctic *Colobanthus quitensis* (Kunth) Bartl. *Biol. Res*. **2018**, 51, 48.
28. Dulska, J.; Wasilewski, J.; Androsiuk, P.; Kellmann-Sopyła, W.; Głowacka, K.; Górecki, R.; Chwedorzewska, K.; Giełwanowska, I. The effect of sodium fluoride on seeds germination and morphophysiological changes in the seedlings of the Antarctic species *Colobanthus quitensis* (Kunth) Bartl. and the Subantarctic species *Colobanthus apetalus* (Labill.) Druce. *Pol. Polar Res*. **2019**, 40, 255–272.
29. Sanhueza, C.; Vallejos, V.; Cavieres, L.A.; Saez, P.; Bravo, L.A.; Corcuera, L.J. Growing temperature affects seed germination of the Antarctic plant *Colobanthus quitensis* (Kunth) Bartl (Caryophyllaceae). *Polar Biol*. **2017**, 40, 449–455.
30. Zhou, W.; Chen, F.; Luo, X.; Dai, Y.; Yang, Y.; Zheng, C.; Yang, W.; Shu, K. A matter of life and death: Molecular, physiological, and environmental regulation of seed longevity. *Plant Cell. Environ*. **2019**, 43, 293–302.
31. Raju-Ahmed, M.; Jannat, Y.; Collins, W.; Perez, M.; Byoung-Kwan, C. Classification of pepper seed quality based on internal structure using X-ray CT imaging. **Comput. Electron. Agric.** *2020*, 179, 1–8.

32. Taylor, A.G. Seed storage, germination, quality, and enhancements. In Physiology of Vegetable Crops, 2nd ed.; Wien, H.C., Stützel, H., Eds.; CAB International: Boston, MA, USA, 2020; pp. 1–30.
33. Marcos-Filho, J. Seed vigor testing: An overview of the past, present and future perspective. *Sci. Agric*. **2015**, 72, 363–374.
34. Vera, M.L. Factors Affecting Germination of *Deschampsia antartica* and *Colobanthus quitensis* Seeds from an Altitudinal Gradient on Livingston Island (South Shetland Island, Antartica). 2017. Available online: http://hdl.handle.net/10651/40759 (accessed on 15 July 2024).
35. Kellmann-Sopyła, W.; Koc, J.; Górecki, R.J.; Domaciuk, M.; Giełwanowska, I. Development of generative structures of polar Caryophyllaceae plants: The Arctic *Cerastium alpinum* and *Silene involucrata*, and the Antarctic *Colobanthus quitensis*. *Pol. Polar Res*. **2017**, 38, 83–104.
36. van Treuren, R.; Bas, N.; Kodde, J.; Groot, S.P.C.; Kik, C. Rapid loss of seed viability in *ex situ* conserved wheat and barley at 4 °C as compared to –20 °C storage. *Conserv. Physiol.* **2018**, 6, coy033.
37. Vera, M.L. Colonization and demographic structure of *Deschampsia antarctica* and *Colobanthus quitensis* along an altitudinal gradient on Livingston Island, South Shetland Islands, Antarctica. *Polar Res*. **2011**, 30, 7146.
38. Edwards, J.A. Studies in *Colobanthus quitensis* (Kunth) Bartl. and *Deschampsia antarctica* Desv. VI. Reproductive performance on Signy Island. *Br. Antarct. Surv. Bull.* **1974**, 39, 67–86.
39. Gielwanowska, I.; Bochenek, A.; Gojlo, E.; Gorecki, R.; Kellmann-Sopyla, W.; Pastorczyk, M.; Szczula, E. Biology of generative reproduction of *Colobanthus quitensis* (Kunth) Bartl. from King George Island, South Shetland Islands. *Pol. Polar Res.* **2011**, 32, 139–155.
40. Sierra-Almeida, A.; Cavieres, L.A.; Bravo, L.A. Warmer Temperatures Affect the in situ Freezing Resistance of the Antarctic Vascular Plants. *Front. Plant Sci.* **2018**, 9, 1456.
41. Torres-Díaz, C.; Gallardo-Cerda, J.; Lavin, P.; Oses, R.; Carrasco-Urra, F.; Atala, C.; Acuña-Rodríguez, I.S.; Convey, P.; Molina-Montenegro, M.A. Biological interactions and simulated climate change modulates the ecophysiological performance of *Colobanthus quitensis* in the Antarctic ecosystem. *PLoS ONE* **2016**, 11, e0164844.

42. Convey, P.; British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge, UK. Unpublished work. 2024.
43. Ernakovich, J.G.; Hopping, K.A.; Berdanier, A.B.; Simpson, R.T.; Kachergis, E.J.; Steltzer, H.; Wallenstein, M.D. Predicted responses of arctic and alpine ecosystems to altered seasonality under climate change. *Glob. Change Biol.* **2014**, 20, 3256–3269.
44. Wheeler, H.C.; Høye, T.T.; Schmidt, N.M.; Svenning, J.C.; Forchhammer, M.C. Phenological mismatch with abiotic conditions—Implications for flowering in Arctic plants. *Ecology* **2015**, 96, 775–787.
45. Obeso, J.R. The costs of reproduction in plants. *New Phytol*. **2002**, 155, 321–348.
46. Cannone, N.; Guglielmin, M.; Convey, P.; Worland, M.; Favero-Longo, S. Vascular plants changes in extreme environments: Effect of multiple drivers. *Clim. Change* **2016**, 134, 651–665.
47. Ramírez, C.F.; Cavieres, L.A.; Sanhueza, C.; Vallejos, V.; Gómez-Espinoza, O.; Bravo, L.A.; Sáez, P.L. Ecophysiology of Antarctic vascular plants: An update on the extreme environment resistance mechanisms and their importance in facing climate change. *Plants* **2024**, 13, 449.
48. Sáez, P.L.; Cavieres, L.A.; Galmés, J.; Gil-Pelegrín, E.; Peguero-Pina, J.J.; Sancho-Knapik, D.; Vivas, M.; Sanhueza, C.; Ramírez, C.F.; Rivera, B.K.; et al. In situ warming in the Antarctic: Effects on growth and photosynthesis in Antarctic vascular plants. *New Phytol.* **2018**, 218, 1406–1418.
49. Penfound, E.; Wellen, C.; Vaz, E. Modelling the impact of temperature under climate change scenarios on native and invasive vascular vegetation on the Antarctic Peninsula and surrounding islands. *Geomatics* **2022**, 2, 390–414.
50. Weather Archive Henryk Arctowski Polish Antarctic Station. Available online: https://www.meteoblue.com/en/weather/historyclimate/weatherarchive/henryk-arctowski-polish-antarctic-station_antarctica_6620757?fcstlength=1y&year=2024&month=10 (accessed on 25 October 2024).
51. Kellmann-Sopyła, W.; Giełwanowska, I. Germination capacity of five polar Caryophyllaceae and Poaceae species under different temperature conditions. *Polar Biol.* **2015**, 38, 1753–1765.

52. Giełwanowska, I. Specyfika rozwoju antarktycznych roślin naczyniowych *Colobanthus quitensis* (Kunth) Bartl. i *Deschampsia antarctica* Desv. Rozprawy i Monografie; Wydawnictwo Uniwersytetu Warmińsko-Mazurskiego: Olsztyn, Poland, 2005.
53. Milarska, S.; Androsiuk, P.; Gielwanowska, I. Anatomy of the generative structures of the Subantarctic flowering plant *Colobanthus apetalus* (Labill.) Druce. *Pol. Polar Res*. **2021**, 42, 45–58.
54. Mackin, H.C.; Shek, K.L.; Thornton, T.E.; Evens, K.C.; Hallett, L.M.; McGuire, K.L.; Peterson, M.L.; Roy, B.A. The “black box” of plant demography: How do seed type, climate and seed fungal communities affect grass seed germination? *New Phytol.* **2021,** 231, 2319–2332.
55. Ruhland, C.T.; Day, T.A. Size and longevity of seed banks in Antarctica and the influence of ultraviolet-B radiation on survivorship, growth and pigment concentrations of *Colobanthus quitensis* seedlings. *Environ. Exp. Bot*. **2001**, 45, 143–154.
56. McGraw, J.B.; Day, T.A. Size and characteristics of a natural seed bank in Antarctica. *Arct. Antarct. Alp. Res*. **1997**, 29, 213–216.
57. Cota-Sánchez, J.H.; Abreu, D.D. Vivipary and offspring survival in the epiphytic cactus Epiphyllum phyllanthus (Cactaceae). *J. Exp. Bot.* **2007**, 58, 3865e3873.
58. Lee, J.A.; Harmer, R. Vivipary, a reproductive strategy in response to environmental stress? *OIKOS* **1980**, 35, 254–265.
59. Farrant, J.; Pammenter, N.W.; Berjak, P. Seed development in relation to desiccation tolerance: A comparison between desiccation-sensitive (recalcitrant) seeds of Avicennia marina and desiccation-tolerant types. *Seed Sci. Res*. **1993**, 3, 1–13.
60. Malovichko, Y.V.; Shikov, A.E.; Nizhnikov, A.A.; Antonets, K.S. Temporal control of seed development in dicots: Molecular bases, ecological impact and possible evolutionary ramifications. *Int. J. Mol. Sci.* **2021**, 22, 9252.
61. Vázquez, D.; Fuentes-Lillo, E.; Farias, D.; Cuba-Díaz, M. Germinación de semillas heteromórficas de *Colobanthus quitensis* (Kunth) Bartl. bajo condiciones de salinidad. In Visiones de Ciencia Antártica Libro Resúmenes, IX Congreso Latinoamericano de Ciencias Antártica; Guaiquil, I., Leppe, M., Rojas, P., Canales, R., Eds.; Publicación del Instituto Antártico Chileno: Punta Arenas, Chile, 2017; pp. 900–903.

62. Ontivero, Y.; Fuentes-Lillo, E.; Navarrete-Campos, D.; Vázquez-Villa, D.; Cabreras-Barjas, G.; Arroyo-Marín, F.B.; Cuba-Díaz, M. Is seed heteromorphism an adaptive strategy of *Colobanthus quitensis* to saline environments? Preliminary assessments. *Sci. Rep.* **2024**; accepted.
63. Fuentes-Lillo, E.; Cuba-Díaz, M.; Rifo, S. Morpho-physiological response of *Colobanthus quitensis* and *Juncus bufonius* under different simulations of climate change. *Polar Sci.* **2017**, 11, 11–18.
64. Zewide, I.; Sherefu, A. Review paper on effect of micronutrients for crop production. *J. Nutr. Food Proc.* **2021**, 4, 1–8.
65. Machuca, A.; Cuba-Díaz, M.; Córdova, C. Enzymes in the rhizosphere of plants growing in the vicinity of the Polish Arctowski Antarctic Station. J. Soil Sci. Plant Nutr. **2015**, 15, 833–838.
66. Klupczyńska, E.A.; Pawłowski, T.A. Regulation of seed dormancy and germination mechanisms in a changing environment. *Int. J. Mol. Sci*. **2021**, 22, 1357.
67. acchello, G.; Bomers, S.; Böhme, C.; Postma, F.; Agren, J. Seed dormancy varies widely among *Arabidopsis thaliana* populations both between and within Fennoscandia and Italy. *Ecol. Evol.* **2022**, 12, e8670.
68. Bicalho, E.M. Soil seed banks, persistence and recruitment: Memories of a partially non-lived life? *Theor. Exp. Plant Physiol*. **2024**, 36, 583–587.
69. Galussi, A.A.; Argüello, J.A.; Moya, M.E.; Zuriaga, F.D.; Zimmermann, L.R. Seed dormancy mechanism as a factor influencing seed physiological quality in alfalfa (*Medicago sativa*) cv. Baralfa 85. *Seed Sci. Tech*. **2013**, 41, 50–59.
70. Kokila, M.A.; Vanitha, C.; Sundareswaran, S.; Marimuthu, S. Evaluation of role of testa, cotyledon and embryonic axis in manifestation of seed dormancy in groundnut varieties. *Pharma Innov. J*. **2021**, 10, 1185–1188.
71. Shen, X.; Cho, M.J. Factors affecting seed germination and establishment of an efficient germination method in sugar pine (*Pinus lambertiana* Dougl.). *Hort. Sci.* **2021**, 56, 299–304.
72. Salazar, J.M.; Pomavilla, M.; Pollard, A.T.; Chica, E.J.; Peña, D.F. Endophytic fungi associated with roots of epiphytic orchids in two Andean forests in southern Ecuador and their role in germination. *Lankesteriana* **2020**, 20, 37–47.

73. Sathiyadash, K.; Muthukumar, T.; Karthikeyan, V.; Rajendran, K. Orchid mycorrhizal fungi: Structure, function, and diversity. In Orchid Biology: Recent Trends and Challenges; Khasim, S.M., Hegde, S.N., González-Arnao, M.T., Thammasiri, K., Eds.; Springer: Singapore, 2020; pp. 239–280.
74. Suwannarach, N.; Kumla, J.; Rachanarin, C.; Srimuang, K.O. *In vitro* symbiotic seed germination of *Epipactis flava* (Orchidaceae) promoted by endophytic fungus, *Tulasnella phuhinrongklaensis*. *Chiang Mai J. Sci*. **2021**, 48, 787–792.
75. Hereme, R.; Morales-Navarro, S.; Ballesteros, G.; Barrera, A.; Ramos, P.; Gundel, P.E.; Molina-Montenegro, M.A. Fungal endophytes exert positive effects on *Colobanthus quitensis* under water stress but neutral under a projected climate change scenario in Antarctica. *Front. Microbiol*. **2020**, 11, 264.
76. Barrera, A.; Hereme, R.; Ruiz-Lara, S.; Larrondo, L.F.; Gundel, P.E.; Pollmann, S.; Molina-Montenegro, M.A.; Ramos, P. Fungal endophytes enhance the photoprotective mechanisms and photochemical efficiency in the Antarctic *Colobanthus quitensis* (Kunth) Bartl. exposed to UV-B radiation. *Front. Ecol. Evol*. **2020**, 8, 122.
77. Ramos, P.; Rivas, N.; Pollmann, S.; Casati, P.; Molina-Montenegro, M.A. Hormonal and physiological changes driven by fungal endophytes increase Antarctic plant performance under UV-B radiation. *Fungal Ecol*. **2018**, 34, 76–82.
78. Kildisheva, O.A.; Dixon, K.W.; Silveira, F.A.; Chapman, T.; Di Sacco, A.; Mondoni, A.; Turner, S.R.; Cross, A.T. Dormancy and germination: Making every seed count in restoration. *Restor. Ecol.* **2020**, 28, 256–265.
79. Sehgal, A.; Sita, K.; Siddique, K.H.M.; Kumar, R.; Bhogireddy, S.; Varshney, R.K.; HanumanthaRao, B.; Nair, R.M.; Prasad, P.V.V.; Nayyar, H. Drought or/and heat-stress effects on seed filling in food crops: Impacts on functional biochemistry, seed yields, and nutritional quality. *Front. Plant Sci*. **2018**, 9, 1705.
80. Bertini, L.; Proietti, S.; Fongaro, B.; Holfeld, A.; Picotti, P.; Salvatore-Falconieri, G.; Bizzarri, E.; Capaldi, G.; Polverino de Laureto, P.; Caruso, C. Environmental signals act as a driving force for metabolic and defense responses in the antarctic plant *Colobanthus quitensis*. *Plants* **2022**, 11, 3176.
81. Abdel-Maksoud, H.S.; Fawzi, N.M. Seed morphology of some taxa of Caryophyllaceae. *Bull. Fac. Agric. Cairo Univ*. **2016**, 67, 213–227.

82. Ullah, F.; Papini, A.; Shah, S.N.; Zaman, W.; Sohail, A.; Iqbal, M. Seed micromorphology and its taxonomic evidence in subfamily Alsinoideae (Caryophyllaceae). *Microsc. Res. Tech*. **2018**, 82, 250–259.
83. Matilla, A.; Gallardo, M.; Puga-Hermida, M.I. Structural, physiological and molecular aspects of heterogeneity in seeds: A review. *Seed Sci. Res*. **2005**, 15, 63–76.
84. Rasheed, A.; Nisar, F.; Gul, B.; Khan, M.A.; Hameed, A. Ecophysiology of seed heteromorfism in halophytes: An over-view. In Halophytes and Climate Change: Adaptative Mechanism and Potential Uses; Hasanuzzaman, M., Shabala, S., Fujita, M., Eds.; CAB International: Boston, MA, USA, 2019; pp. 104–114.
85. Smolikova, G.; Leonova, T.; Vashurina, N.; Frolov, A.; Medvedev, S. Desiccation tolerance as the basis of long-term seed viability. *Int. J. Mol. Sci*. **2021**, 22, 101.
86. Crane, J.; Miller, A.L.; Van Roekel, J.W.; Walters, C. Triacylglycerols determine the unusual storage physiology of Cuphea seed. *Planta* **2003,** 217, 699–708.
87. Seed Information Database: Royal Botanic Gardens, Kew. Available online: https://ser-sid.org/species/603ee8ea-d4ae-47e7-8d84-36d1162ec530 (accessed on 15 October 2024).
88. Walters, C. Orthodoxy, recalcitrance and in-between: Describing variation in seed storage characteristics using threshold responses to water loss. *Planta* **2015**, 242, 397–406.
89. Bakker, J.P.; Poschlod't, P.; Strykstra, R.J.; Bekker, R.M.; Thompson, K. Seed banks and seed dispersal: Important topics in restoration ecology. *Acta Bot. Neerl.* **1996**, 45, 461–490.
90. Freitas, R.S.; Silva, E.C. Unveiling the infuence of stress memory: Enhancing stress tolerance in seedlings through seed stage stress exposure. *Plant Physiol. Rep*. **2024**, 29, 165–175.
91. Shi, Z.; Zhang, J.; Wei, H. Research progress on soil seed bank: A bibliometrics analysis. *Sustainability* **2020**, 12, 4888.
92. Pipatpongpinyo, W.; Korkmaz, U.; Wu, H.; Kena, A.; Ye, H.; Feng, J.; Gu, X.Y. Assembling seed dormancy genes into a system identified their effects on seedbank longevity in weedy rice. *Heredity* **2020**, 124, 135–145.
93. Daibes, L.F.; Zupo, T.; Silveira, F.A.O.; Fidelis, A. A field perspective on effects of fire and temperature fluctuation on Cerrado legume seeds. *Seed Sci. Res*. **2017**, 27, 74–83.

94. Ristau, T.E.; Royo, A.A. Influence of stand age, soil attributes, and cover type on Rubus (Rosaceae) seed bank abundance. *J. Torrey Bot. Soc*. **2020**, 147, 222–231.
95. Rodrigues, E.R.S.; Silveira, F.A.O. Seed germination requirements of Trembleya laniflora (Melastomataceae), an endemic species from neotropical montane rocky savannas. *Plant Species Biol.* **2012**, 28, 165–168.
96. Peng, D.L.; Hu, X.J.; Yang, J.; Sun, H. Seed dormancy, germination and soil seed bank of *Lamiophlomis rotata* and *Marmoritis complanatum* (Labiatae), two endemic species from Himalaya-Hengduan Mountains. *Pl. Biosyst.* **2018**, 152, 642–648.
97. Bascuñán-Godoy, L.; Sanhueza, C.; Cuba-Díaz, M.; Zuñiga, G.E.; Corcuera, L.J.; Bravo, L.A. Cold-acclimation limits low temperature induced photoinhibition by promoting a higher photochemical quantum yield and a more effective PSII restoration in darkness in the Antarctic rather than the Andean ecotype of *Colobanthus quitensis* Kunt Bartl (Cariophyllaceae). *BMC Plant Biol*. **2012**, 12, 114.
98. Cuba-Díaz, M.; Acuña, D.; Klagges, M.; Dollenz, O.; Cordero, C. *Colobanthus quitensis* de la Marisma, una nueva población para la colección genética de la especie. In Avances en Ciencia Antártica Latinoamericana. Libro de Resúmenes VII Congreso Latinoamericano de Ciencia Antártica; Publicación del Instituto Antártico Chileno–Centro de Estudios Avanzados en Zonas Áridas: La Serena, Chile, 2013; pp. 436–439.
99. Wysoczański, W.; Węgrzyn, E.; Lembicz, M.; Jaroszewicz, B. Fungal microbiota in seeds, seedlings and mature plants of raspberry (Rubus ideaus L.). *Eur. J. Plant Pathol*. **2021**, 161, 815–820.
100. Acuña-Rodríguez, I.S.; Newsham, K.K.; Convey, P.; Biersma, E.M.; Ballesteros, G.I.; Torres-Díaz, C.; Goodall-Copestake, W.P.; Molina-Montenegro, M.A. The role of the soil microbiome in the colonization of glacier forefields by Antarctic pearlwort (*Colobanthus quitensis*) under current and future climate change scenarios. *Soil Biol. Biochem*. **2024**, 188, 109249.
101. Molina-Montenegro, M.A.; Ballesteros, G.I.; Acuña-Rodríguez, I.S.; Pertierra, L.R.; Greve, M.; Richardson, D.M.; Convey, P.; Biersma, E.M.; Goodall-Copestake, W.P.; Newsham, K.K. The “Trojan horse” strategy: Seed fungal endophyte symbiosis helps to explain the invasion success of *Poa annua* in Maritime Antarctica. *Divers. Distrib*. **2024**, 29, 1432–1444.

102. Ugolini, F.C.; Bockheim, J.G. Antarctic soils and soil formation in a changing environment: A review. *Geoderma* **2008**, 144, 1–8.
103. Bockheim, J.G.; Lupachev, A.V.; Blume, H.P.; Bölter, M.; Simas, F.N.B.; McLeod, M. Distribution of soil taxa in Antarctica: A preliminary analysis. *Geoderma* **2015**, 245, 104–111.
104. Bockheim, J.G. Antarctic Soil Properties and Soilscapes. In Antarctic Terrestrial Microbiology; Cowan, D., Ed.; Springer: Berlin/Heidelberg, Germany, 2014; pp. 293–315.
105. Gundel, P.E.; Seal, C.E.; Biganzoli, F.; Molina-Montenegro, M.A.; Vázquez-de-Aldana, B.R.; Zabalgogeazcoa, I.; Bush, L.P.; Martínez-Ghersa, M.A.; Ghersa, C.M. Occurrence of alkaloids in grass seeds symbiotic with vertically-transmitted Epichloë fungal endophytes and its relationship with antioxidants. *Front. Ecol. Evol*. **2018**, 6, 211.
106. Wang, Z.; Chunjie, L.; White, J. Effects of Epichloë endophyte infection on growth, physiological properties and seed germination of wild barley under saline conditions. *J. Agron. Crop Sci.* **2020**, 206, 43–51.
107. Coelho, L.C.; Carvalho, C.R.; Rosa, C.A.; Rosa, L.H. Diversity, distribution, and xerophilic tolerance of cultivable fungi associated with the Antarctic angiosperms. *Polar Biol*. **2021**, 44, 379–388.
108. Gundel, P.E.; Garibaldi, L.A.; Martínez-Ghersa, M.A.; Ghersa, C.M. Trade-off between seed number and weight: Influence of a grass–endophyte symbiosis. *Basic Appl. Ecol*. **2012**, 13, 32–39.
109. Chen, Y.; Su, K.; Li, C.; White, J.F. Interactive effects of Epichloë endophyte, dormancy-breaking treatments and geographic origin on seed germination of *Achnatherum inebrians*. *Microorganisms* **2021**, 9, 2183.
110. Ueno, A.C.; Gundel, P.E.; Seal, C.E.; Ghersa, C.M.; Martínez-Ghersa, M.A. The negative effect of a vertically-transmitted fungal endophyte on seed longevity is stronger than that of ozone transgenerational effect. *Environ. Exp. Bot*. **2020**, 175, 104037.
111. Ueno, A.C.; Gundel, P.E.; Ghersa, C.M.; Agathokleousc, E.; Martínez-Ghersa, M.A. Seed-borne fungal endophytes can strain reproductive success of host plants under ozone pollution. *Environ. Res.* **2021**, 202, 111773.

112. Rosa, L.H.; Almeida Vieira, M.L.; Furtado Santiago, I.; Rosa, C.A. Endophytic fungi community associated with the dicotyledonous plant *Colobanthus quitensis* (Kunth) Bartl. (Caryophyllaceae) in Antarctica. *FEMS Microbiol. Ecol*. **2010**, 73, 178–189.
113. Jiang, J.; Zhang, K.; Cheng, S.; Nie, Q.; Zhou, S.X.; Chen, Q.; Zhou, J.; Zhen, X.; Li, X.T.; Zhen, T.W.; et al. *Fusarium oxysporum* KB-3 from Bletilla striata: An orchid mycorrhizal fungus. *Mycorrhiza* **2019**, 29, 531–540.
114. Meng, Y.Y.; Zhang, W.L.; Selosse, M.A.; Gao, J.Y. Are fungi from adult orchid roots the best symbionts at germination? A case study. *Mycorrhiza* **2019**, 29, 541–547.
115. Cuba-Díaz, M.; Laboratorio de Biotecnología y Estudios Ambientales, Universidad de Concepción, Los Ángeles, Chile; Ontivero, Y.; Laboratorio de Biotecnología y Estudios Ambientales, Universidad de Concepción, Los Ángeles, Chile. Unpublish work. 2024.
116. Patil, B.L.; Narayan, K.S.; Gopalkrishna, A.M. Diversity profiling of seed associated endophytic microbiome in important species of Caricaceae family. *Microbiol. Res*. **2021**, 12, 779–792.
117. Zemouri, Z.; Djabeur, A.; Frimehdi, N.; Khelil, O.; Kaid-Harche, M. The seed diversity of Carob (Ceratonia siliqua L.) and the relationship between seed color and coat dormancy. *Sci. Hortic*. **2020**, 274, 109679.
118. Sethi, R.; Kaur, N. Germination ecology of heteromorphic seeds of bur clover (Medicago denticulata Willd.). *Indian J. Weed Sci*. **2021**, 53, 59–66.
119. Fenollosa, E.; Jené, L.; Munné-Bosch, S. A rapid and sensitive method to assess seed longevity through accelerated aging in an invasive plant species. *Plant Methods* **2020**, 16, 64.
120. Whitehouse, K.J.; Hay, F.R.; Lusty, C. Why seed physiology is important for genebanking. *Plants* **2020**, 9, 584.
121. ISTA (International Seed Testing Association). International Rules for Seed Testing; ISTA: Zurich, Switzerland, 2017.
122. Fatokun, K.; Beckett, R.P.; Pammenter, N.W. Germination indices of orthodox seeds as influenced by controlled deterioration and cathodic water seed invigoration. *J. Environ. Biol.* **2020**, 41, 1105–1111.
123. Kijak, H.; Ratajczak, E. What do we know about the genetic basis of seed desiccation tolerance and longevity? *Int. J. Mol. Sci*. **2020**, 21, 3612.

124. Hay, F.R.; Valdez, R.; Lee, J.S.; Cruz, P.C.S. Seed longevity phenotyping: Recommendations on research methodology. *J. Exp. Bot.* **2019**, 70, 425–434.
125. Hampton, J.; TeKrony, D.M. (Eds.) Handbook of Vigour Test Methods; International Seed Testing Association: Zurich, Switzerland, 1995.
126. Navarro, M.; Febles, G.; Torres, V. Bases conceptuales para la estimación del vigor de las semillas a través de indicadores del crecimiento y el desarrollo inicial. *Pastos Forrajes* **2012**, 35, 233–246.
127. Javorski, M.; Carrara, D.O.; da Silva, S.S.; Gomes-Junior, F.G.; Moure, S. Image analysis to evaluate the physiological potential and morphology of pearl millet seeds. *J. Seed Sci.* **2018**, 40, 127–134.
128. Nadarajan, J.; Walters, C.; Pritchard, H.W.; Ballesteros, D.; Colville, L. Seed Longevity—The Evolution of Knowledge and a Conceptual Framework. *Plants* **2023**, 12, 471.
129. Suma, N.; Srimathi, P. Influence of water flotation technique on seed and seedling quality characteristics of *Sesamum indicum*. *IOSR J. Agric. Vet. Sci*. **2014**, 7, 51–53.
130. Najberek, K.; Olejniczak, P.; Berent, K.; Gąsienica Staszeczek, M.; Solarz, W. The ability of seeds to float with water currents contributes to the invasion success of Impatiens balfourii and I. glandulifera. *J. Plant Res.* **2020**, 133, 649–664.
131. García, J.; Medeiros-Coelho, C.M. Accelerated aging predicts the emergence of rice seedlings in the field. *Semin. Ciênc. Agrár*. **2021**, 42, 1397–1410.
132. Jojoa, W.A.; Peixoto, J.R.; Spehar, C.R.; Souza Vilela, M.; Fagioli, M.; Nobrega, D.; Cruz, J.; Oliveira, A. Evaluation of the physiological quality of quinoa seeds. *African J. Agric. Res*. **2021**, 17, 802–808.
133. Milivojević, M.; Srdić, J.; Filipović, M.; Petrović, T.; Branković Radojčić, D.; Marković, K.; Boćanski, J. Application of standard germination and vigour tests for seed quality assessment of maize inbred lines. *Sel. I Semen*. **2021**, 27, 35–45.
134. Farías, D.O. Efectos de los Iones Fe3+ en la Germinación *In Vitro* de *Colobanthus quitensis*; Memoria de título presentada para optar al título de Ingeniero en Biotecnología Vegetal; Universidad de Concepción: Concepción, Chile, 2018; 46p.
135. Koc, J.; Wasilewski, J.; Androsiuk, P.; Kellmann-Sopyła, W.; Chwedorzewska, K.J.; Giełwanowska, I. The effects of methanesulfonic acid on seed germination and

morphophysiological changes in the seedlings of two Colobanthus species. *Acta Soc. Bot. Pol.* **2018**, 87, 3601.

136. Brimblecombe, P. The Global Sulfur Cycle. In Biogeochemistry; Schlesinger, W.H., Ed.; Elsevier: Amsterdam, The Netherlands, 2005; pp. 645–682.
137. Carvalho, S.M.C.; Torres, S.B.; Sousa, E.C.; Sousa, D.M.M.; Pereira, K.T.O.; Paiva, E.P.; Matias, J.R.; dos Santos, B.R.V. Viability of *Carica papaya* L. seeds by the tetrazolium test. *J. Agric. Sci.* **2018**, 10, 335–340.
138. Leite, M.S.; Barros, S.; Gomes, F.G.; Queiroz, C.H.; Pereira, E.; Leite, T.S. Viability of seedling image analysis (Vigor-S) to determine the physiological potential of melon seeds. *J. Seed Sci.* **2020**, 42, e202042043.
139. Waskow, A.; Howling, A.; Furno, I. Advantages and Limitations of Surface Analysis Techniques on Plasma-Treated Arabidopsis thaliana Seeds. *Front. Mater.* **2021**, 8, 642099.

III. CHAPTER II: PRELIMINARY ASSESSMENT OF SEED HETEROMORFISM AS AN ADAPTIVE STRATEGY OF *Colobanthus quitensis* UNDER SALINE CONDITIONS

Yadiana Ontivero[1,2], Eduardo Fuentes-Lillo[3,4], Darío Navarrete-Campos[1], Dante Vázquez-Villa[1], Gustavo Cabreras-Barjas[5], Francisca B. Arroyo-Marín[1] & Marely Cuba-Díaz[1,6]

[1]Laboratorio de Biotecnología y Estudios Ambientales, Departamento de Ciencias y Tecnología Vegetal, Escuela de Ciencias y Tecnologías, Universidad de Concepción, Campus Los Ángeles, 4440000, Concepción, Chile.

[2]Facultad de Agronomía, Universidad de Concepción, Campus Chillán, 3812189, Chillán, Chile.

[3]Laboratorio de Invasiones Biológicas (LIB), Facultad de Ciencias Forestales, Universidad de Concepción, 4030000, Concepción, Chile.

[4]Instituto de Ecología y Biodiversidad (IEB), Ñuñoa, 7800020, Santiago, Chile.

[5]Facultad de Ciencias para el Cuidado de la Salud, Universidad San Sebastián, Campus Las Tres Pascualas, Lientur 1439, 4080871, Concepción, Chile.

[6]Programa de Ciencia Antártica y Subantártica, Universidad de Concepción, 4030000, Concepción, Chile.

Publicado en Scientific Reports Volumen 14, Número 31120, 2024, https://doi.org/10.1038/s41598-024-82381-z

ABSTRACT

Colobanthus quitensis is known for enduring extreme conditions, such as high salinity in Antarctica, making it an excellent model for studying environmental stress. In plant families, variations in seed color heteromorphism have been linked to various germination under stress conditions. Preliminary laboratory observations indicated that dark brown seeds of *C. quitensis* had higher germination rates, suggesting that this phenotypic trait might offer a germination advantage, particularly under saline conditions. To investigate this, germination of heteromorphic seeds from Antarctic, sub-Antarctic, and Andean populations of *C. quitensis* was assessed under *in vitro* saline conditions. Among all populations, dark brown seeds exhibited greater germination and shorter germination time than other seeds in the absence of salinity. In the Antarctic population, dark brown seeds showed better salinity tolerance. In the sub-Antarctic La Marisma population, salt tolerance was not affect by seed color, showing the population was the most salt-tolerant. The other two populations showed very low germination even at low salinity concentration. This study is the first scientific report of seed

heteromorphism in *C. quitensis* populations, offering insights into mechanisms of salinity tolerance and potentially other stress conditions that enhance the species' resilience. In addition, the identification of La Marisma populations as a salinity-tolerant population will holds biotechnological importance for agriculture.

1. INTRODUCTION

Global climate change contributes to increased soil salinity due to rising sea levels and changing rainfall patterns. This fact negatively affects plant development, especially germination and initial seedling growth (1). The osmotic stress caused by salinity decrease germination, plant nutrient uptake, elevate ion toxicity, as well as confer morphological (e.g., reducing root and shoot length, changing root architecture), biochemical (e.g., decreased chlorophyll and carotenoid content, excessive Na+ accumulation, lower K+/Na+ ratio), and physiological changes (e.g., decreasing stomatal conductance, changes in chloroplast structure, lower photosystem II efficiency) (2,3,4,5).

During evolution, plants have developed different stress tolerance mechanisms to survive in different environments, including Antarctic and desertic ones. Among them can be mentioned the accumulation of protective osmolytes, scavenging of reactive oxygen species, selective ion uptake, changing root architecture, protection of the photosynthetic system, and reduction of their metabolism and growth (6). Also, researchers have identified seed heteromorphism as a survival strategy that helps plants grow and endure in harsh conditions (7). In this regard, heteromorphism is evident when a species produces seeds with different morphology, size, weight, and/or color. These characteristics influence seed germination success, dormancy, and vigor (7). Studies on the impact of seed heteromorphism on germination often use scanning electron microscopy (SEM) to analyze testa microstructure, as color and morphological alterations are sometimes linked (8,9). Moreover, heteromorphic seeds in the presence of abiotic stress conditions such as salinity, temperature, and illumination changes show different germination rates (10,11). The ratio of one type of heteromorphic seed to another varies with populations, varieties, climatic, and environmental conditions (11,12). Then, a greater germination percentage of heteromorphic seeds on certain ecosystem conditions suggest that these seeds have higher adaptability to this environment than other

ones. Hence, color heteromorphism may serve as an indicator of the specific stress condition prevailing in each habitat and its impact on the ecological dynamics of plant species (13).

Colobanthus quitensis (Kunth) Bartl. is considered a model plant for biotechnological studies and for understanding adaptive mechanisms to extreme environments, as well as an indicator of the effects of climate change in Antarctica (14,15). It is an extremophile species distributed from southern Mexico to the north of the Antarctic Peninsula (16). Thus, to perform successful scientific research, deep understanding of the physiology and genetics of model plant species (17,18), like *C. quitensis* is crucial. Certain populations of this species are located along the coast (19), which exposes them to marine aerosols directly or enables them to grow in soils drenched with saltwater (20). Recent studies have categorized *C. quitensis* as moderately tolerant to salinity, capable of withstanding from 150 mM NaCl in common garden conditions and up to 400 mM NaCl *in vitro*, showing morphological, physiological, and biochemical adaptations to salinity stress (21,22,23). Therefore, throughout the germination process, seeds do not exhibit the same responses or adaptive strategies against salinity as observed in mature plants (7). For instance, *C. quitensis* seeds show a reduction in their germination and seedling survival rates when exposed to salt concentrations exceeding 100 mM NaCl (21,22). Therefore, further research is required to distinguish the salinity tolerance of *C. quitensis* seeds among its populations. Seed stress can affect plant reproduction and productivity (24). Then, germination tests are a rapid and effective alternative to detect genotypes resistant to environmental stresses (25).

Seed heteromorphism has been previously described in different families such as Fabaceae8, Amaranthaceae (11,13), and Caryophyllaceae26, to which *C. quitensis* belongs. There are several reports that studied the germination process in different populations of *C. quitensis* (27,28,29,30,31), but none have mentioned the presence of heteromorphism or its influence on germination. These studies documented variations in germination rates among *C. quitensis* populations. These variations may relate to dormancy (30), but seed traits likely influenced the results, as dark brown seeds showed higher germination rates.

Whe hypothesized that the color heteromorphism observed in *C. quitensis* confers plasticity in the response to salinity, which could be influenced by local adaptation acquired by the mother plants. This work aims to answer three research questions: (I) Are there differences in germination among different populations of *C. quitensis*? (II) Does color heteromorphism

influence the germination rate of *C. quitensis* populations? (III) Does the presence of seeds color heteromorphism in *C. quitensis* constitute an adaptation mechanism of salinity tolerance? Therefore, the objective of this work was to analyze whether seeds from different populations of *C. quitensis*, grown under controlled conditions, vary in their tolerance to salinity depending on their color heteromorphism. For this purpose, the morphology of light and dark brown seeds was analyzed for structural differences, as well as *in vitro* germination analyses of heteromorphic seeds in the presence and absence of salinity.

2. RESULTS

2.1 Electron microscopy analysis

SEM analysis was conducted to ascertain any morphological disparities among *C. quitensis* seeds of varying colors. According to SEM micrographs, both the light brown and dark brown seeds of *C. quitensis* populations are triangular and kidney shaped. Furthermore, morphological examination of the seeds reveals a uniform, mostly smooth tegument surface, with the presence of small puzzle-like striations near the micropyle area (Fig. 1). Overall, there were no discernible differences in the seed coat with different colors.

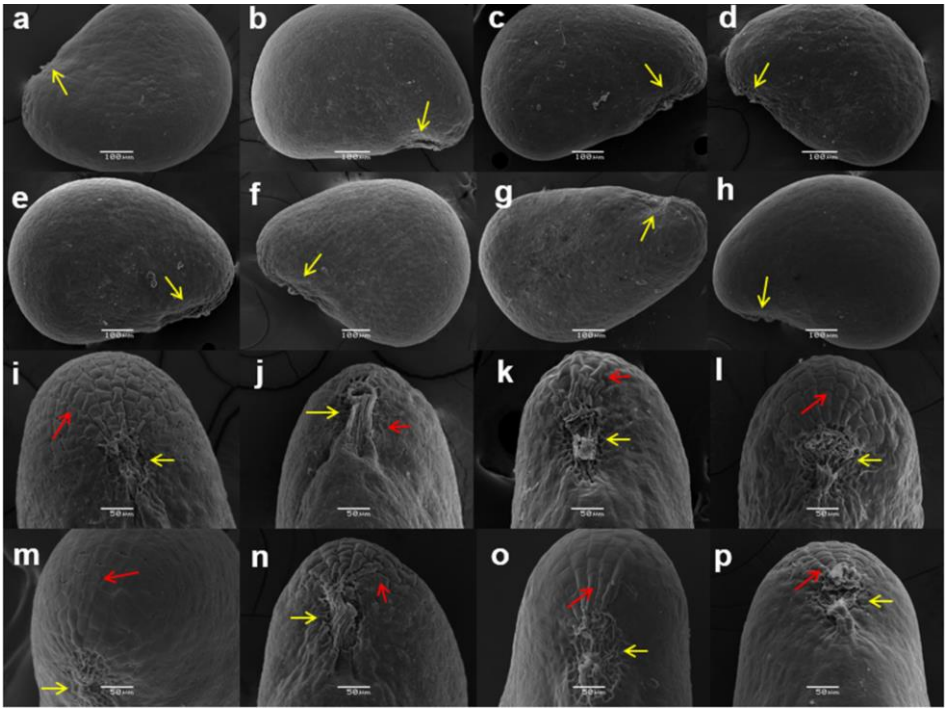


Figure 1. SEM micrographs of heteromorphic *Colobanthus quitensis* seeds side view. Lateral view of light brown seeds from (a) Arctowski, (b) La Marisma, (c) Laredo and (d) Conguillío

populations; and dark brown seeds from (e) Arctowski, (f) La Marisma, (g) Laredo and (h) Conguillío populations. Close-up of the light brown seed micropyle area from (i) Arctowski, (j) La Marisma, (k) Laredo and (l) Conguillío populations; and dark brown seeds from (m) Arctowski, (n) La Marisma, (o) Laredo and (p) Conguillío populations. Yellow arrows indicate the position of the micropile. Red arrow indicates the presence of surface striations.

2.2 Effect of population on seed germination

In each population, the germination percentage of *C. quitensis* seeds was over 40% (Fig. 2a). The pPA and pC populations showed the highest germination percentage, 78% and 66%, respectively, with no statistical difference ($p < 0.05$). Conversely, the germination percentage of pL (41%) and pA (46%) populations were statistically similar ($p < 0.05$), but 1.9-fold and 1.6-fold lower ($p < 0.05$) than pPA population.

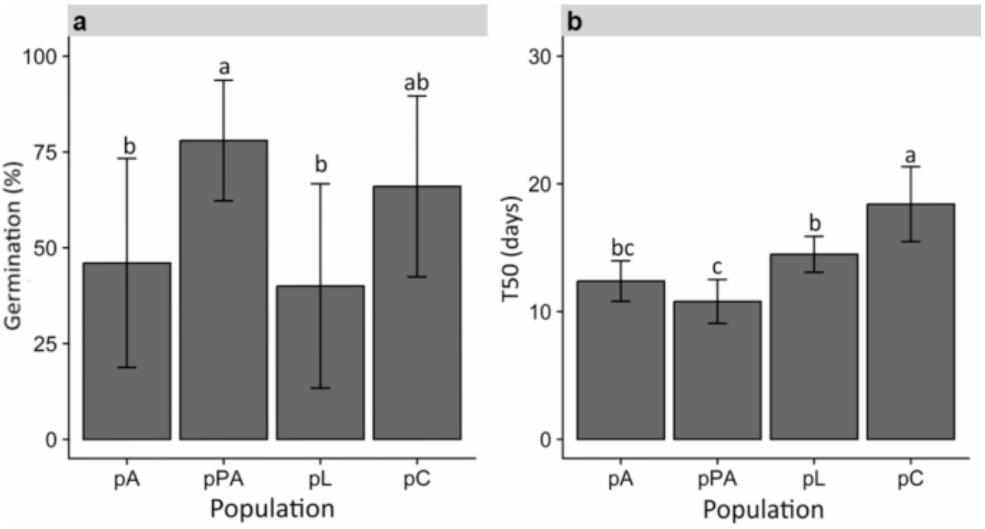


Figure 2. Germination indicators of different populations of *Colobanthus quitensis*. (a) Germination and (b) time to reach 50% germination (T50). Bars represent mean ± SD ($n = 10$). Different letters indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA followed by a Tukey HSD test.

On the other hand, the time at which 50% germination is reached (T50) was higher than 10 days for all populations (Fig. 2b). A significantly lower T50 ($p < 0.05$) was observed in pPA (10.8 days) compared to pL (14.48 days) and pC (18.4 days). While the T50 of pA (12.39 days) does not differ statistically ($p > 0.05$) from the T50 of pPA and pL.

2.3 Effect of color heteromorphism on seed germination

Seed color had a significant effect on germination rates (Supplementary Table 1), which was evidenced by the fact that dark brown seeds in all populations had a significantly ($p < 0.05$) higher germination percentage than light brown seeds (Fig. 3a). The dark seeds of pPA (91%) and pC (84%) achieved the highest germination percentages. It should be noted that light brown seeds of pA and pL barely exceeded 20% germination.

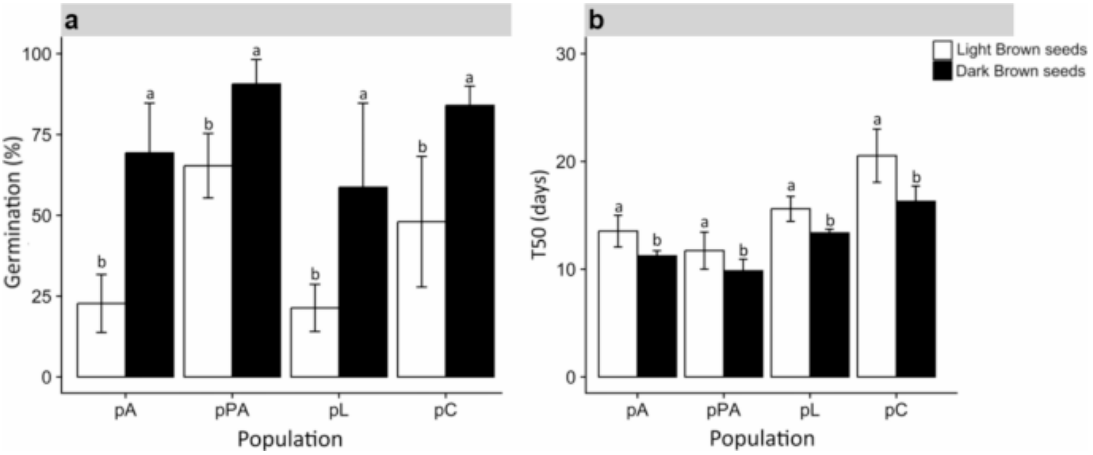


Figure 3. Germination indicators of different populations of *Colobanthus quitensis*. (a) Germination and (b) time to reach 50% germination (T50) of heteromorphic seeds from Arctowski (pA), La Marisma (pPA), Laredo (pL) and Conguillío populations (pC). Bars represent mean ± SD ($n = 5$). Different letters indicate statistically significant differences ($p < 0.05$) according to one-way ANOVA followed by a Tukey HSD test.

Also, the dark brown seeds required significantly ($p < 0.05$) less time to reach 50% germination than the light ones. From all populations, the light (11.7 days) and dark (9.9 days) pPA seeds reached their T50 faster (Fig. 3b).

2.4 Interplay between color heteromorphism and salinity on seed germination

The presence of NaCl salt in the medium affected the germination of all *C. quitensis* populations, while seed color only showed influence on the germination of pA and pC populations. The interaction of both factors had significant effect ($p < 0.05$) in germination percentage of all populations except for pC (Supplementary Table 1).

Both light and dark seeds show a general trend of germination percentage decreasing with increasing salinity. When the results were analyzed, we could observe that dark seeds from the pA and pC populations have a higher germination percentage than light seeds.

Conversely, for the pPA and PL populations, light seeds tend to have a higher germination percentage than dark brown seeds (Fig. 4).

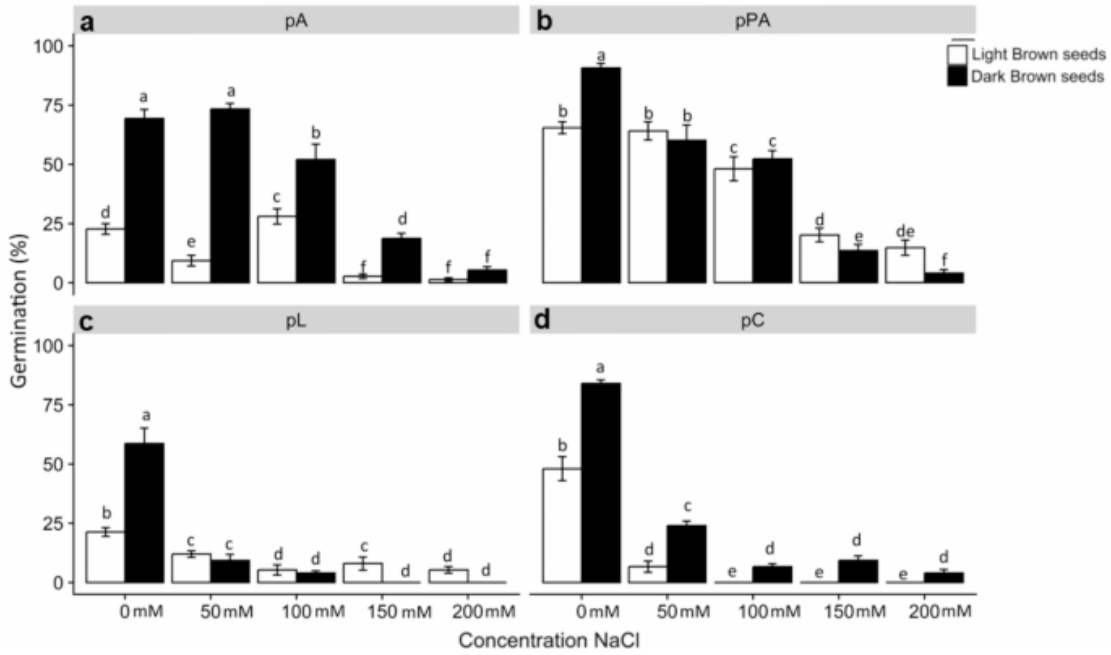


Figure 4. Germination percentage of heteromorphic seeds of *Colobanthus quitensis* treated with different concentrations of sodium chloride (50–200 mM). Light and dark brown seeds from (a) Arctowski (pA), (b) La Marisma (pPA), (c) Laredo (pL) and (d) Conguillío populations (pC). Bars represent mean ± SD (n = 5). Different letters indicate statistically significant differences ($p < 0.05$) according to factorial analysis (2 × 5), followed by a Tukey HSD test.

The germination percentage of dark brown seeds from pA populations in NaCl 50 mM was the highest (73%), then it decreased to 52% at 100 mM (Fig. 4a). The presence of 50 and 100 mM NaCl did not generate differences in germination between light and dark brown seeds in the pPA population (Fig. 4b). On the other hand, the pL and pC populations showed a higher susceptibility to salt concentrations higher than 150 mM NaCl (Fig. 4c, d).

Salinity in the medium affected the T50 of all populations (Fig. 5), while seed color only influenced the T50 of pL and pC ($p < 0.05$). The interaction between these factors significantly affected the T50 of pL and pC ($p < 0.05$) (Supplementary Table 2).

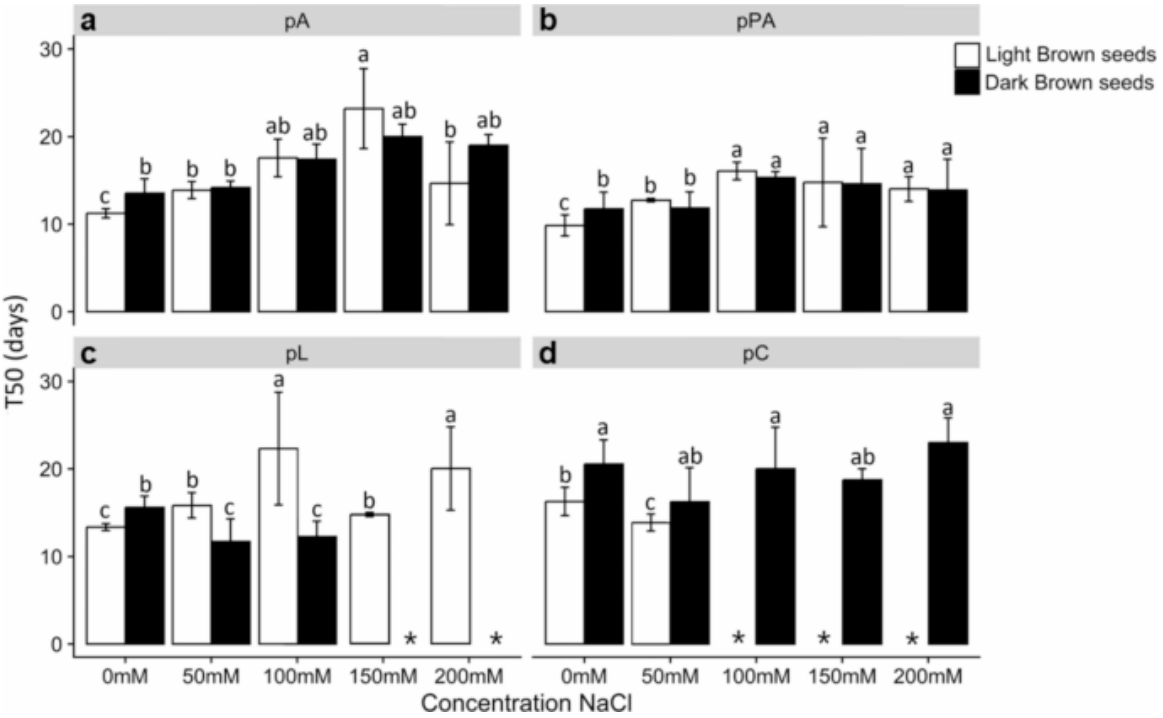


Figure 5. Time to reach 50% germination (T50) of heteromorphic seeds of *Colobanthus quitensis* subjected to different concentrations of sodium chloride. Light and dark brown seeds from (a) Arctowski (pA), (b) La Marisma (pPA), (c) Laredo (pL) and (d) Conguillío populations (pC). The bars represent the mean ± SD (n = 75). Different letters indicate statistically significant differences ($p < 0.05$) according to factorial analysis (2 × 5), followed by a Tukey HSD test.

The pPA population had the lowest T50 in the presence of 200 mM NaCl for both seed types (14 days), compared to the other populations (Fig. 5b). In contrast, the pC population maintained a high T50 regardless of NaCl concentration. For example, dark seeds took 17 days to reach their T50 under control conditions (0 mM NaCl) and 23 days in the presence of 200 mM NaCl (Fig. 5d).

The cumulative germination analysis indicates that the dark brown seeds of pA (Fig. 6b) and pPA (Fig. 6d) began to germinate faster than their light brown counterparts (Fig. 6a, c). Increasing the salt concentration in the *in vitro* medium resulted in a decreased germination percentage and an increased germination time. Salinity has the most severe negative effect on the light brown seeds of pA (Fig. 6a), as well as on both seed colors of the pL (Fig. 6e, f) and pC (Fig. 6g, h) populations. The dark brown pA seeds (Fig. 6b) and both seed colors of pPA

(Fig. 6c, d) showed better tolerance in the presence of 50 mM and 100 mM NaCl in the *in vitro* germination medium.

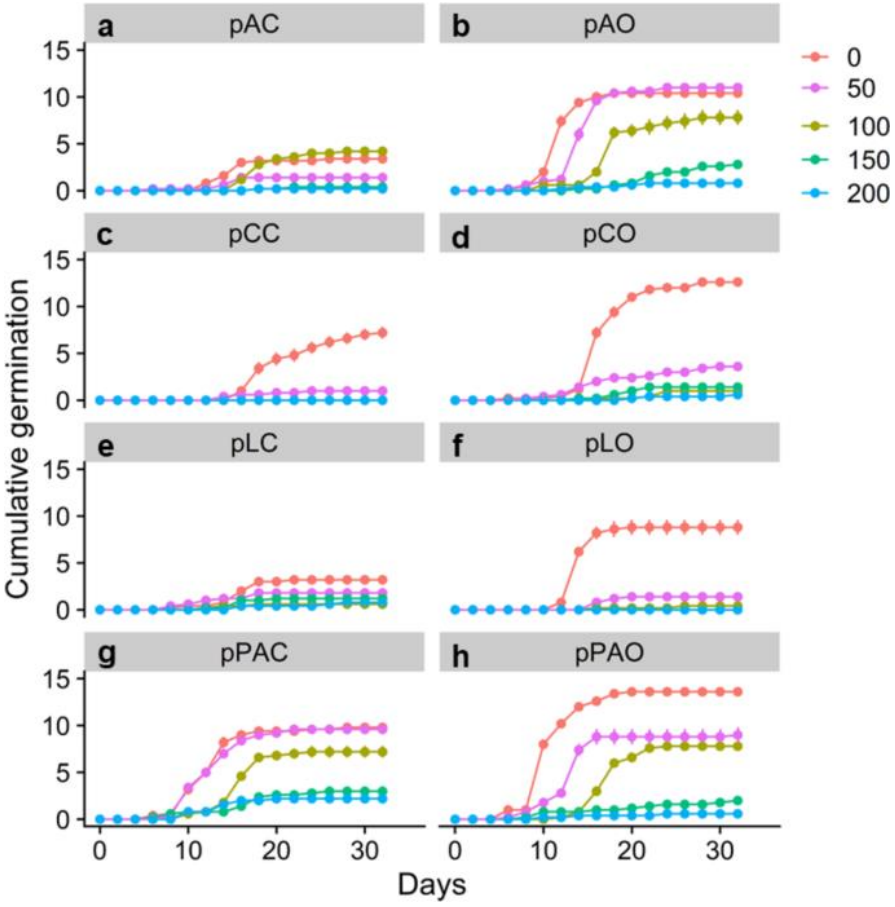


Figure 6. Cumulative germination of *Colobanthus quitensis* under different salinity concentration. Light brown (C) and dark brown (O) seeds of Arctowski population (pA) (a, b), La Marisma (pPA) (c, d), Laredo (pL) (e, f) and Conguillío (pC) (g, h) (n = 75).

3. DISCUSSION

This study, for the first time, provides scientific evidence and describes the presence of color heteromorphism in the seeds of four *C. quitensis* populations grown under controlled conditions, originating from habitats with varying marine influence: pA, a coastal Antarctic population; pPA, a sub-Antarctic coastal population that develops in soils flooded by seawater; pL, a sub-Antarctic coastal population; and pC, an Andean population not exposed to marine influence (Supplementary Fig. 1). This phenotypic characteristic in the seeds contributes to the salinity tolerance of the different *C. quitensis* populations.

The *C. quitensis* triangular and kidney-shaped seed morphology (Fig. 1) agrees with previous descriptions, where it is mentioned that seeds tend to be flattened and wider towards the cotyledon area (32). Conversely, variations in seed testa microstructure, dormancy, and germination are associated with seed coloration (33). In particular, the effect of seed color on germination varies among species (8,9).

SEM analysis performed in lateral view reflected little variation in testa structure, with striations evident only in the dark pPA seeds (Fig. 1d). However, a close-up of the seed micropyle area revealed that heteromorphic seeds from all populations show striations that form small pieces of a puzzle (Fig. 1i-p). Similar shallow marks were previously detected on the surface of the periclinal walls of the pA testa (32). It is possible that light and dark brown seeds differ in their biochemical and structural composition at the deeper layers of the seed coat, so more detailed analyses, including the use of Fourier-transform infrared spectroscopy, may be required (34). Genes regulating the synthesis of flavonols and proanthocyanidin pigments, along with other chemicals, are known to play a crucial role in determining seed coat color and their arrangement is found in the different layers of the seed coat (35,36). However, this study did not cover these aspects, so further research is necessary to understand the factors that lead to heteromorphism in *C. quitensis*. Seed coat characteristics play an ecological role in species dispersal, longevity, water uptake capacity, and germination in ecosystems dominated by changes in temperature, salinity, and water availability (37).

The wide distribution of *C. quitensis* has generated genetic and morphological differentiation among its populations (27,38), which influences their germination (30). Plants growing in cold climates are usually small in size and slower growing than their counterparts. Therefore, they produce small seeds with dormancy, showing low germination speed and high temperature requirements for germination (39). Antarctic populations of *C. quitensis* have been reported to have lower germination relative to sub-Antarctic or Andean populations (27), respectively. However, in this study, the Antarctic population pA showed equal germination percentage and T50 to the sub-Antarctic population pL (Fig. 2a, b).

Seed heteromorphism is a critical adaptive mechanism that enables plants to maintain high germination rates under stress conditions like high salinity and intense UV radiation (40), significantly influencing the ecological resilience of plants to changes in the environment (41). *C. quitensis* frequently encounters these stressors within its natural habitats, making this

mechanism particularly vital. Factors such as seed maturity, flavonoid and pigment concentrations, environmental variations, and genetic influences all play a role in testa pigmentation (42). Consequently, under increasing salt concentrations, seed heteromorphism is expected to differentially affect the germination of *C. quitensis* populations (Figs. 4, 5 and 6). However, this character only contributed to the higher salinity tolerance of dark brown pA seeds, inferring, therefore, that seed color heteromorphism might be fundamental for this population to tolerate increased salinity conditions to a greater extent in Antarctic.

The testa thickness of heteromorphic seeds is determined by the amount of suberin, cutin, and lignin in the cell walls, as well as the presence of fatty acids in the intercellular spaces. This thickness influences seed permeability, impacting the rate of water uptake by the embryo, dormancy, and germination (43,44). Additionally, the levels of phytohormones such as abscisic acid, indole-3-acetic acid, and zeatin riboside vary with seed color, potentially affecting the germination process45. Understanding these complex interactions provides valuable insights into the resilience mechanisms of *C. quitensis*, offering potential strategies for enhancing crop tolerance to abiotic stressors.

Varieties or genotypes among species exhibit different levels of tolerance to the same stress (46), a phenomenon observable from the early stages of plant development (47). For instance, dark brown pA seeds demonstrated a higher germination percentage than light brown seeds up to 150 mM NaCl (Fig. 4a). However, in the presence of 150 and 200 mM NaCl, light brown pPA seeds showed a higher germination percentage than dark brown seeds (Fig. 5b). This suggests that within a population, the higher germination percentage of one seed type over another indicates the possible presence of biochemical compounds that enable tolerance to NaCl. Both pA and pPA populations thrive in areas exposed to saline conditions (Supplementary Fig. 1a, b) (19,20), suggesting they possess genetically determined mechanisms that confer salinity tolerance. Previous research has shown that the addition of 50 mM NaCl to *in vitro* culture media did not affect the germination percentage of pPA compared to seeds germinated under non-saline (control) conditions (21,22).

Nevertheless, in this investigation, pPA seeds with a dark brown color exhibited a greater germination percentage when the *in vitro* germination media did not contain NaCl (Figs. 3a and 4b). The pPA has a higher tolerance to salt than pL, even though both species originate in similar locations and are associated with coastal ecosystems (Supplementary Fig. 1b, c). A

possible reason could be that the pPA ecosystem undergoes frequent flooding of the soil with seawater, exposing the roots to continuous and direct contact with saltwater (20). This may have led to the development of stronger genetic mechanisms in this population that can tolerate salinity. Because the habitats and microclimates that comprise a natural landscape are ever-changing. Therefore, the populations that grow there tend to show differences in response to environmental pressures (48). However, pL is subject to strong anthropic pressure that has influenced its habitat and therefore the ecology of this population (M. Cuba-Díaz, pers. comm.). This could also explain why, although they grow in Punta Arenas associated with coastal environments, pL and pPA have different levels of salt tolerance.

Both pL and pC showed low tolerance to salinity (Fig. 4c, d). However, at concentrations up to 100 mM NaCl, pPA seeds and only the dark brown seeds of pA had a germination percentage higher than 50% (Fig. 4a, b). The observed difference in salinity tolerance between pPA and pA could be attributed to the different ways these populations are exposed to salinity: pPA is found in areas that are flooded with seawater, while pA receives marine spray. This distinction should be analyzed in depth. For this experiment, seeds were obtained from plants grown under controlled conditions and temperatures, without exposure to salinity for at least 8 to 10 months (49). Nonetheless, seeds from pPA and the dark brown seeds from pA exhibited greater tolerance to salinity, suggesting the presence of genetically inherited mechanisms that confer tolerance, even in the absence of direct exposure to saline conditions during their production.

For each population, it is evident that when the concentration of NaCl increases, seeds of all colors are impacted. In fact, there was a delay in seed germination, starting with an increase in salt concentration (Fig. 4). In numerous species, studies have documented the negative influence of salinity (46,50,51) because the increase of Na+ ions in the medium decreases the water uptake rate of seeds, generating drought stress, thereby delaying and decreasing germination percentage (52). Furthermore, it generates toxicity that changes and disrupts the activity of enzymes involved in nutrient mobilization, such as α-amylase, and decreases the content of gibberellins required for germination (50,53).

Researchers have observed that *C. quitensis* does not germinate quickly under laboratory conditions. The development of long germination periods is considered an adaptive trait in species that occur in ecosystems with a high degree of stress or disturbance (54). However, in

this study, pA and pPA required less time to achieve 50% germination (Fig. 5a, b) than pL and pC (Fig. 5c, d). This is advantageous because faster-germinating plants are less susceptible to disease and experience less competition for environmental resources, both of which are critical for healthy growth and subsequent seed production (55).

This research shows that there is a close relationship between seed color and the germination of *C. quitensis* in response to salinity. Although this phenomenon was not observed in the other three populations, it is possible, according to the literature, that seed heteromorphism may be a useful mechanism for tolerating other types of environmental stress, in the remaining populations of *C. quitensis*. Considering that the thickness of the testa, the composition and abundance of chemical compounds in the seed coat, and the presence of secondary metabolites in seeds may provide relevant information about the ecology of species in extreme environments (7). Finally, there is a need for further investigation focusing on seed heteromorphism to clarify the causes of its origin and its implications for the species' ecology (Fig. 7).

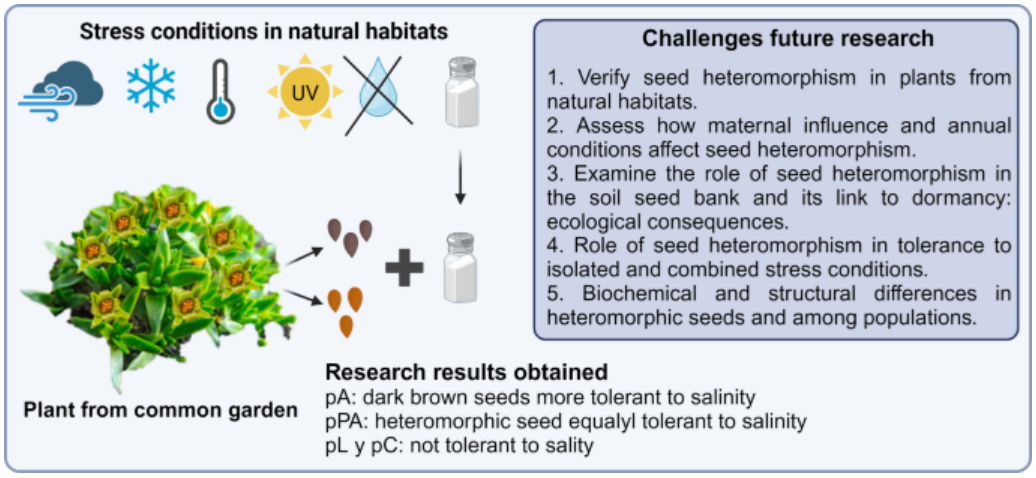


Figure 7. Diagram of the main research results and proposal for future research focused on the heteromorphism of *Colobanthus quitensis* seeds.

While heteromorphism has been documented in the Caryophyllaceae family (26), this is the first report of such a phenomenon in *C. quitensis*, highlighting an additional adaptive strategy this species uses to endure the harsh conditions of its native habitats. Heteromorphism in seeds has been reported across numerous plant families, including Amaranthaceae (41),

Poaceae (56), Fabaceae (57), and Asteraceae (58), many of which contain crops of economic importance. The discovery of heteromorphism in *C. quitensis* opens the possibility of leveraging this trait to understand seed survival mechanisms under variable environmental conditions. This knowledge could be instrumental in selecting populations, varieties, or genotypes of commercially significant species that exhibit enhanced tolerance to abiotic stresses, such as increased soil salinity.

4. CONCLUSION

This work represents the first effort to identify the existence of color heteromorphism in *C. quitensis* seeds from different habitats. The studied populations vary in their germination capacity, with dark brown seeds showing greater success than light brown ones. The most salt-tolerant population is pPA, where the heteromorphic seeds respond equally to the presence of NaCl. This opens the possibility of using pPA as a biotechnological model to investigate the mechanisms of salinity tolerance in extremophilic species. In pA, dark brown seeds showed a higher germination percentage than light brown seeds in the presence of NaCl, showing that heteromorphism is one of the mechanisms used by this population to cope with saline conditions. Heteromorphism in pL and pC did not influence their ability to withstand salt in the medium, making them the most susceptible populations. Finally, seed heteromorphism related color of seed coat in *C. quitensis* may be one of the strategies used by this species to tolerate the harsh conditions within its natural habitat, although the advantages of this phenomenon for the species need further study.

5. MATERIALS AND METHODS

5.1 Plant material

For this study, seeds from four populations of *C. quitensis* plants were analyzed 8 to 10 months after their collection in the field. These plants were kept in a common garden belonging to the Antarctic plant collection of the Laboratorio de Biotecnología y Estudios Ambientales of Universidad de Concepción, Chile. Seeds were coded with the name of the location where the plants were collected: Arctowski (pA) (King George Island, South Shetland Islands, Antarctica; 62°09’S; 58°28’W), La Marisma (pPA) (Santa María Point, South of Punta Arenas, Chile; 53°22’S; 70°58’W), Laredo (pL) (Laredo sector, North of Punta Arenas, Chile; 52°58’S; 70°49’W) and Conguillío (pC) (Conguillío National Park, Araucanía Region, Chile; 38°36’S;

71°36'W) (Supplementary Fig. 1). The populations pA, pPA, and pL are coastal populations, so they are constantly exposed to marine spray. Notably, pPA inhabits areas that are continuously flooded with seawater, while pC is located in the mountain range and does not receive marine influence.

C. quitensis plants were grown in growth chambers at 13 ± 1 °C, with a 16/8 h light/dark photoperiod, a light intensity of 120 ± 20 µmol m2 s− 1 and 85–90% relative humidity. Seeds from each population were collected when the flower capsules were fully opened, and the seeds were matured (Fig. 8a). Mature seeds were considered as those able to tolerate desiccation (59) and whose color varied from immature seeds. The capsules were dried at room temperature for 2–3 days. Subsequently, seeds were manually extracted, sorted by color into light and dark brown seeds (Fig. 8b, c), and stored in hermetically sealed Eppendorf tubes at 4 °C until use.

Figure 8. Open floral capsule of *Colobanthus quitensis* showing mature seeds ready to be collected (a), light brown seeds (b) and dark brown seeds (c).

5.2 Electron microscopy analysis

The light and dark brown seed testa of the different populations was analyzed by scanning electron microscopy (SEM) (model JSM-6380) to detect differences in their morphology. Seeds from different colors were fixed to a holding plate and sputtered with an Au layer using a Leica EM ACE600 high vacuum coater. SEM images were taken using an acceleration voltage of 30 kV modified from Kellman-Sopyla et al. (32).

5.3 Seed selection and conditioning

Seeds were submerged for 24 h in distilled water, and seeds that floated were considered non-viable and those that did not were considered viable (60). All viable seeds were scarified with

1% (v/v) H2SO4 during 30s30 and subsequently disinfected with 70% (v/v) ethanol for 30 s in vortex and 5% (v/v) NaClO for 7 min in vortex, followed by three times washes with sterile distilled water (21).

5.4 Effect of population, seed color heteromorphism and salinity on seed germination

For the experiments, 10 cm diameter Petri dishes were used containing 20 mL of MS culture medium (61), 3% sucrose, 0.7% agar were prepared. Seeds were placed on dishes for *in vitro* germination at 20 ± 2 °C, with a 16 h light/ 8 h dark photoperiod and a light intensity of 45 ± 2 µmol m-2 s-1.

To evaluate the effect of populations (n = 4) on germination without making distinctions in seed color, an experimental design was carried out with 10 replicates per treatment (n = 40). Seeds were randomly selected, maintaining the color proportions for each population of *C. quitensis* and 15 seeds per replicate were placed to germinate in the mentioned medium.

In addition, to evaluate the effect of seed color2 within the population, five replicates of 15 seeds of the same color were used for each replicate and placed to germinate on the same medium.

Finally, to explore the combined effect of seed color and salt concentration, five concentrations of NaCl (0, 50, 100, 150 and 200 mM) were added to the medium. Five replicates of 15 seeds per color and NaCl concentration, respectively, were used.

For the three tests, the number of germinated seeds on each plate was recorded every 48 h for 32 days. Germinated seeds were considered to be those whose radicle was at least twice the size of the seed (28). Data was calculated for germination percentage (GP) according to the Eq. 1.

$$GP = \frac{n}{N} * 100 \quad (1)$$

Where n is the number of germinated seeds at the end of the experiment and N is the total number of seeds. In addition, it was determined the time at which 50% germination is reached (T50) according to the Eq. 2 (62).

$$T50 = ti + \frac{(\frac{n}{2} - ni)(tj - ti)}{(nj - ni)} \quad (2)$$

Where n is the number of germinated seeds at the end of the experiment; ni and nj are the cumulative number of germinated seeds per adjacent count at tj and ti times, respectively, where

$$ni < \frac{N}{2} < nj$$

Based on these count data, it was obtained cumulative germination rate as the fraction of the number of germinating seeds per Petri dish every two days.

5.5 Statistical analysis

To analyze the effect of populations and seed heteromorphism on germination percentage and T50 of each population, a one-way ANOVA was performed. Germination percentage and T50 were considered as dependent variables, and population and seed color were used as independent variables. To evaluate whether color heteromorphism affects germination response to salt stress, a factorial analysis was performed. Germination percentage and T50 were again the dependent variables, while seed color and different salinity treatments acted as independent variables. In both analyses, a post hoc Tukey's Honestly Significant Difference (HSD) analysis was employed with a 95% confidence interval. These analyses were executed using the "aov" function within the R Studio program (R Core Team, 2023). Subsequently, the graphs were generated utilizing the "ggplot2" package63.

6. REFERENCES

1. Uçarly, C. Intech Open, UK. Effects of salinity on seed germination and early seedling stage in Abiotic stress in plants (ed Fahad, S., Saud, S., Chen, Y., Wu, C., Wang, D.) 211–232 (2021).
2. Shahid, M. A. *et al*. Insights into the physiological and biochemical impacts of salt stress on plant growth and development. Agron 10, 938. https://doi.org/10.3390/agronomy10070938 (2020).
3. Hameed, A. *et al*. Effects of salinity stress on chloroplast structure and function. Cells 10 https://doi.org/10.3390/cells10082023 (2021).
4. Li, H. *et al*. Effective root responses to salinity stress include maintained cell expansion and carbon allocation. New. Phytol. 238, 1942–1956 (2023).

5. Irik, H. A. & Bikmaz, G. Effect of different salinity on seed germination, growth parameters and biochemical contents of pumpkin (Cucurbita pepo L.) seeds cultivars. Sci. Rep. 14, 6929. https://doi.org/10.1038/s41598-024-55325-w (2024).
6. Liang, W., Ma, X., Wan, P. & Liu, L. Plant salt-tolerance mechanism: a review. Biochem. Biophys. Res. Commun. 495, 286–291 (2018).
7. Liu, R. R., Wang, L., Tanveer, M. & Song, J. Seed heteromorphism: an important adaptation of halophytes for habitat heterogeneity. Front. Plant. Sci. 9, 1515. https://doi.org/10.3389/fpls.2018.01515 (2018b).
8. Mandizvo, T. & Odindo, A. O. Seed coat structural and imbibitional characteristics of dark and light-colored Bambara groundnut (Vigna subterranean L.) landraces. Heliyon 5, e01249. https://doi.org/10.1016/j.heliyon.2019.e01249 (2019).
9. Sethi, R., Kaur, N. & Singh, M. Morphological and physiological characterization of seed heteromorphism in Medicago denticulata Willd. Plant. Physiol. Rep. 25, 107–119 (2020).
10. Nisar, F., Gul, B., Ajmal Khan, M. & Hameed, A. Germination and recovery responses of heteromorphic seeds of two co-occurring Arthrocnemum species to salinity, temperature and light. S. Afr. J. Bot. 121, 143–151 (2019).
11. Toderich, K. *et al*. Seed heteromorphism and germination in *Chenopodium quinoa* Willd. Related to crop introduction in marginalized environments. Pak. J. Bot. 55, 1–17 (2023).
12. Gul, B., Ansari, R., Flowers, T. J. & Khan, M. A. Germination strategies of halophyte seeds under salinity. Environ. Exp. Bot. 92, 4–18 (2013).
13. Ma, Y. *et al*. Seed heteromorphism and effects of light and abiotic stress on germination of a typical annual halophyte Salsola Ferganica in cold desert. Front. Plant. Sci. 8, 2257. https://doi.org/10.3389/fpls.2017.02257 (2018).
14. Bertini, L. *et al*. What Antarctic plants can tell us about climate changes: temperature as a driver for metabolic reprogramming. Biomolecules 11, 1094. https://doi.org/10.3390/biom11081094 (2021).
15. Cuba-Díaz, M., Rivera-Mora, C., Navarrete, E. & Klagges, M. Advances of native and non-native Antarctic species to *in vitro* conservation: improvement of disinfection protocols. Sci. Rep. 10, 3845. https://doi.org/10.1038/s41598-020-60533-1 (2020).

16. Moore, D. M. Studies in *Colobanthus quitensis* (Kunth) Bartl. And *Deschampsia antarctica* Desv. II. Taxonomy, distribution and relationships. Br. Antarct. Surv. Bull. 23, 63–80 (1970).
17. Meinke, D. W., Cherry, J. M., Dean, C., Rounsley, S. D. & Koornneef, M. Arabidopsis thalaana: a model plant for genome analysis. Sci 282, 662–682 (1998).
18. Niedbała, G., Niazian, M. & Sabbatini, P. Modeling Agrobacterium mediated gene transformation of Tobacco (Nicotiana tabacum)—a model plant for gene transformation studies. Front. Plant. Sci. 12, 695110. https://doi.org/10.3389/fpls.2021.695110 (2021).
19. Convey, P. *et al*. The spatial structure of Antarctic biodiversity. Ecol. Monogr. 84, 203–244 (2014).
20. Cuba-Díaz, M., Acuña, D., Klagges, M., Dollenz, O. & Cordero, C. *Colobanthus quitensis* de la Marisma, una nueva población para la colección genética de la especie in Avances en Ciencia Antártica Latinoamericana. Libro de Resúmenes VII Congreso Latinoamericano de Ciencia Antártica, Chile, 436–439 (2013).
21. Cuba-Díaz, M., Castel, K., Acuña, D., Machuca, A. & Cid, I. Sodium chloride effect on *Colobanthus quitensis* seedling survival and *in vitro* propagation. Antarct. Sci. 29, 45–46 (2017a).
22. Castel, K. Evaluación de tolerancia a la salinidad (NaCl) de tres poblaciones de *Colobanthus quitensis in vitro*. Tesis de Pregrado de Ingeniería en Biotecnología Vegetal. Universidad de Concepción, Chile (2015).
23. Arroyo-Marín, F. B. Respuestas diferenciales en la tolerancia a la salinidad en poblaciones de *Colobanthus quitensis* Kunth (Bartl.). Tesis de Pregrado de Ingeniería en Biotecnología Vegetal. Universidad de Concepción, Chile (2023).
24. Kranner, I., Minibayeva, F. V., Beckett, R. P. & Seal, C. E. What is stress? Concepts, definitions and applications in seed science. New. Phytol. 188, 655–673 (2010).
25. Mbinda, W. & Kimtai, M. Evaluation of morphological and biochemical characteristics of sorghum (Sorghum bicolor (L.) Moench) varieties in response salinity stress. Annu. Res. Rev. Biol. 33, 1–9 (2019).
26. Ullah, F. *et al*. Seed micromorphology and its taxonomic evidence in subfamily Alsinoideae (Caryophyllaceae). Microsc. Res. Tech. 82, 250–259 (2018).

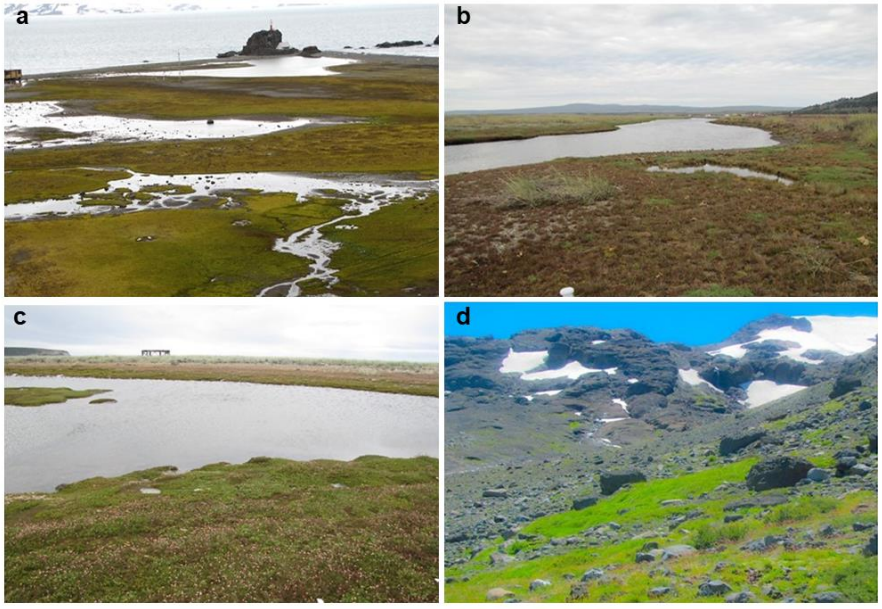
27. Cuba-Díaz, M. *et al*. Phenotypic variability and genetic differentiation in continental and island populations of *Colobanthus quitensis* (Caryophyllaceae: Antarctic pearlwort). Polar Biol. 40, 2397–2409 (2017b).
28. Sanhueza, C. *et al*. Growing temperature affects seed germination of the Antarctic plant *Colobanthus quitensis* (Kunth) Bartl (Caryophyllaceae). Polar Biol. 40, 449–455 (2017).
29. Koc, J. *et al*. The effects of methanesulfonic acid on seed germination and morphophysiological changes in the seedlings of two Colobanthus species. Acta Soc. Bot. Pol. 87, 3601. https://doi.org/10.5586/asbp.3601 (2018b).
30. Cuba-Díaz, M., Acuña, D. & Fuentes-Lillo, E. Antarctic pearlwort (*Colobanthus quitensis*) populations respond differently to pre-germination treatments. Polar Biol. 42, 1209–1215 (2019).
31. Dulska, J. *et al*. The effect of sodium fluoride on seeds germination and morphophysiological changes in the seedlings of the Antarctic species *Colobanthus quitensis* (Kunth) Bartl. And the subantarctic species *Colobanthus apetalus* (Labill.) Druce. Pol. Polar Res. 40, 255–272 (2019).
32. Kellmann-Sopyla, W., Koc, J., Górecki, R. J., Domaciuk, M. & Gielwanowska, I. Development of generative structures of polar Caryophyllaceae plants: the Arctic *Cerastium alpinum* and *Silene involucrata*, and the Antarctic *Colobanthus quitensis*. Pol. Polar Res. 38, 83–104 (2017).
33. Loades, E. *et al*. Distinct hormonal and morphological control of dormancy and germination in *Chenopodium album* dimorphic seeds. Front. Plant. Sci. 14, 1156794. https://doi.org/10.3389/fpls.2023.1156794 (2023).
34. Waskow, A., Howling, A. & Furno, I. Advantages and limitations of surface analysis techniques on plasma-treated Arabidopsis thaliana seeds. Front. Mater. 8, 642099. https://doi.org/10.3389/fmats.2021.642099 (2021).
35. Wang, L. *et al*. Transcriptome analysis of a new peanut seed coat mutant for the physiological regulatory mechanism involved in seed coat cracking and pigmentation. Front. Plant. Sci. 7, 01491. https://doi.org/10.3389/fpls.2016.01491 (2016).

36. Cao, J. *et al*. Effects of genetic and environmental factors on variations of seed heteromorphism in Suaeda Aralocaspica. AoB Plants. 12, plaa044. https://doi.org/10.1093/aobpla/plaa044 (2020).
37. Baskin, C. C. & Baskin, J. M. Seeds: Ecology, Biogeography, and Evolution of Dormancy and Germination 2nd edn (Academic Press - Elsevier, 2014).
38. Koc, J. *et al*. Range-wide pattern of genetic variation in *Colobanthus quitensis*. Polar Biol. 41, 2467–2479 (2018a).
39. Rosbakh, S., Chalmandrier, L., Phartyal, S. & Poschlod, P. Inferring community assembly processes from functional seed trait variation along elevation gradient. J. Ecol. 110, 2374–2387 (2022).
40. Xie, J. *et al*. Seed color represents salt resistance of alfalfa seeds (Medicago sativa L.): based on the analysis of germination characteristics, seedling growth and seed traits. Front. Plant. Sci. 14, 1104948. https://doi.org/10.3389/fpls.2023.1104948 (2023).
41. Žerdoner-Čalasan, A. & Kadereit, G. Evolutionary seed ecology of heteromorphic Amaranthaceae. Perspectives in plant ecology. Evol. Syst. 61, 125759. https://doi.org/10.1016/j.ppees.2023.125759 (2023).
42. Debeaujon, I., Lepiniec, L., Pourcel, L. & Routaboul, J. M. Seed development, dormancy and germination, in: (eds Bradford, K. J. & Nonogaki, H.) Seed coat Development and Dormancy. Blackwell Publishing Ltd, USA, 25–49 (2007).
43. Kelly, K. M., Staden, J. V. & Bell, W. E. Seed coat structure and dormancy. Plant. Growth Regul. 11, 201–209 (1992).
44. Marles, M. A. S. & Gruber, M. Y. Histochemical characterization of unextractable seed coat pigments and quantification of extractable lignin in the Brassicaceae. J. Agric. Food Chem. 84, 251–262 (2004).
45. Wang, F. X. *et al*. Salinity affects production and salt tolerance of dimorphic seeds of Suaeda salsa. Plant. Physiol. Biochem. 95, 41–48 (2015).
46. Dehnavi, A. R., Zahedi, M., Ludwiczak, A., Cardenas, S. & Piernik, A. Effect of salinity on seed germination and seedling development of sorghum (Sorghum bicolor (L.) Moench) genotypes. Agronomy 10, 859. https://doi.org/10.3390/agronomy10060859 (2020).

47. Pinheiro, C. *et al*. Salinity effect on germination, seedling growth and cotyledon membrane complexes of a Portuguese salt marsh wild beet ecotype. Theor. Exp. Plant. Physiol. 30, 113–127 (2018).
48. Lum, T. D. & Barton, K. E. Ontogenetic variation in salinity tolerance and ecophysiology of coastal dune plants. Ann. Bot. 125, 301–314 (2020).
49. Xiong, F. S., Mueller, E. C. & Day, T. A. Photosynthetic and respiratory acclimation and growth response of Antarctic vascular plants to contrasting temperature regimes. Am. J. Bot. 87, 700–710 (2000).
50. Liu, L. *et al*. Salinity inhibits rice seed germination by reducing α-amylase activity via decreased bioactive gibberellin content. Front. Plant. Sci. 9, 275. https://doi.org/10.3389/fpls.2018.00275 (2018a).
51. Javaid, M. M. *et al*. Influence of environmental factors on seed germination and seedling characteristics of perennial ryegrass (*Lolium perenne* L). Sci. Rep. 12, 9522. https://doi.org/10.1038/s41598-022-13416-6 (2022).
52. Misra, N. & Gupta, A. K. Effect of salt stress metabolism in two high yielding genotypes of green gram. Plant. Sci. 169, 331–339 (2005).
53. Ryu, H. & Cho, Y. G. Plant hormones in salt stress tolerance. J. Plant Biol. 58, 147–155 (2015).
54. Brits, G. J., Brown, N. A. C., Calitz, F. J. & Van Staden, J. Effects of storage under low temperature, room temperature and in the soil on viability and vigor of Leucospermum cordifolium (Proteaceae) seeds. S. Afr. J. Bot. 97, 1–8 (2015).
55. Bueno, M. Adaptation of halophytes to different habits. In: Jiménez-López JC ed. Seed dormancy and germination. IntechOpen 97–120 (2020).
56. Wang, A. B., Baskin, C. C., Baskin, J. M. & Ding, J. Environmental and seed-position effects on viability and germination of buried seeds of an invasive diaspore-heteromorphic annual grass. Physiol. Plant. 176, e14353. https://doi.org/10.1111/ppl.14353 (2024).
57. Wango, M., Medaglia-Mata, A., Villalta-Romero, F., Alvarado-Marchena, L. & Rocha, O. J. Relationship between flooding regimes, time of year, and seed coat characteristics and coloration within fruits of the annual monocarpic plant Sesbania emerus (Fabaceae). Int. J. Plant. Sci. 182, 295–308 (2021).

58. Junting, L. *et al*. Seed and fruiting phenology plasticity and offspring seed germination rate in two Asteraceae herbs growing in karst soils with varying thickness and water availability. J. Resour. Ecol. 13, 319–327 (2022).
59. Still, D. W. Development of seed quality in Brassicas. HortTechnology 9, 335–340 (1999).
60. Suma, N. & Srimathi, P. Influence of water flotation technique on seed and seedling quality characteristics of Sesamum indicum. IOSR J. Agric. Vet. Sci. 7, 51–53 (2014).
61. Murashige, T. & Skoog, F. A revised medium for the rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15, 473–497 (1962).
62. Farooq, M., Basra, S. M. A. & Hafeez, K. Thermal hardening: a new seed vigor enhancement tool in rice. J. Integr. Plant. Biol. 47, 187–193 (2005).
63. Wilkinson, L. ggplot2: elegant graphics for data analysis by Wickham. H Biometrics. 67, 678–679 (2011).

7. ELECTRONIC SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Natural ecosystems and collecting sites of different populations of *Colobanthus quitensis*. a) Arctowski, b) La Marisma, c) Laredo, d) Conguillío.

Supplementary Table 1. Factorial analysis of the effect of seed color (light or dark brown) and sodium chloride content (0,50,100,150 or 200mM) on the germination percentage of *Colobanthus quitensis* seeds.

***C. quitensis* populations**	**Abbreviation**	**Variables effects on Germination (%)**	**Statistical parameters**			
			df	**SS**	**F**	**p-value**
Arctowski	pA	Seed color (SC)	1	2.006	70.409	**<0.001***
		Salinity (S)	4	3.085	26.288	**<0.001***
		SCxS	4	0.652	5.560	**0.0011***
		Error	40	1.173		
La Marisma	pPA	Seed color (SC)	1	0.00021	0.0056	0.940
		Salinity (S)	4	5.426	36.577	**<0.001***
		SCxS	4	0.473	3.192	**0.0229***
		Error	40	1.483		
Laredo	pL	Seed color (SC)	1	0.000039	0.001	0.974
		Salinity (S)	4	2.656	17.919	**<0.001***
		SCxS	4	0.709	4.785	**0.0029***
		Error	40	1.482		
Conguillío	pC	Seed color (SC)	1	0.947	47.749	**<0.001***
		Salinity (S)	4	5.514	69.465	**<0.001***
		SCxS	4	0.113	1.425	0.243
		Error	40	0.793		

df degree of freedom; SS sum of squares; F test F; *p-value with significant differences ($p<0.05$).

Supplementary Table 2. Factorial analysis of the effect of seed color (light or dark brown) and sodium chloride content (0, 50, 100, 150 or 200 mM) on the time taken to reach 50 % germination of *Colobanthus quitensis*.

***C. quitensis* populations**	**Abbreviation**	**Variables effects on T_{50} (days)**	**Statistical parameters**			
			df	**SS**	**F**	**p-value**
Arctowski	pA	Seed color (SC)	1	3.702	0.604	0.443
		Salinity (S)	4	362.877	14.812	**<0.001***
		SCxS	4	39.862	1.627	0.194
		Error	29	177.620		
La Marisma	pPA	Seed color (SC)	1	0.001	0.000	0.992
		Salinity (S)	4	148.812	5.888	**<0.001***

		SCxS	4	12.304	0.487	0.745
		Error	35	221.155		
Laredo	pL	Seed color (SC)	1	181.852	29.134	**<0.001***
		Salinity (S)	4	344.035	13.779	**<0.001***
		SCxS	4	1032.051	41.336	**<0.001***
		Error	28	174.769		
Conguillío	pC	Seed color (SC)	1	1191.791	193.590	**<0.001***
		Salinity (S)	4	553.905	22.493	**<0.001***
		SCxS	4	1269.276	51.544	**<0.001***
		Error	31	190.845		

df degree of freedom; SS sum of squares; F test F; *p-value with significant differences ($p<0.05$).

IV. CHAPTER III: IMPROVED *IN VITRO* GERMINATION OF C*olobanthus quitensis*: A KEY STEP FOR ANTARCTIC PLANT CONSERVATION

Yadiana Ontivero[1,2], Constanza Salgado[1], Eduardo Fuentes-Lillo[3,4], Carolina Sanhueza[1]; Darío Navarrete Campos[1], Solange Alveal[1,2], Marely Cuba-Díaz[1,5*]

[1]Universidad de Concepción, Laboratorio de Biotecnología y Estudios Ambientales, Departamento de Ciencias y Tecnología Vegetal, Escuela de Ciencias y Tecnologías, Campus Los Ángeles 4440000, Chile.

[2]Universidad de Concepción, Programa de Doctorado en Ciencias de la Agronomía, Facultad de Agronomía, Chillán 381 2189, Chile.

[3]Laboratorio de Invasiones Biológicas (LIB), Facultad de Ciencias Forestales, Universidad de Concepción, Concepción 4030000, Chile.

[4]Instituto de Ecología y Biodiversidad (IEB), Ñuñoa, Santiago 7800020, Chile.

[5]Programa de Ciencia Antártica y Subantártica, Universidad de Concepción, Concepción 4030000, Chile.

*MCD: mcuba@udec.cl (correspondence author)

Manuscript submitted for peer review to Plant Cell, Tissue and Organ Culture on April 7, 2025, and accepted for publication on May 4, 2025.

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.

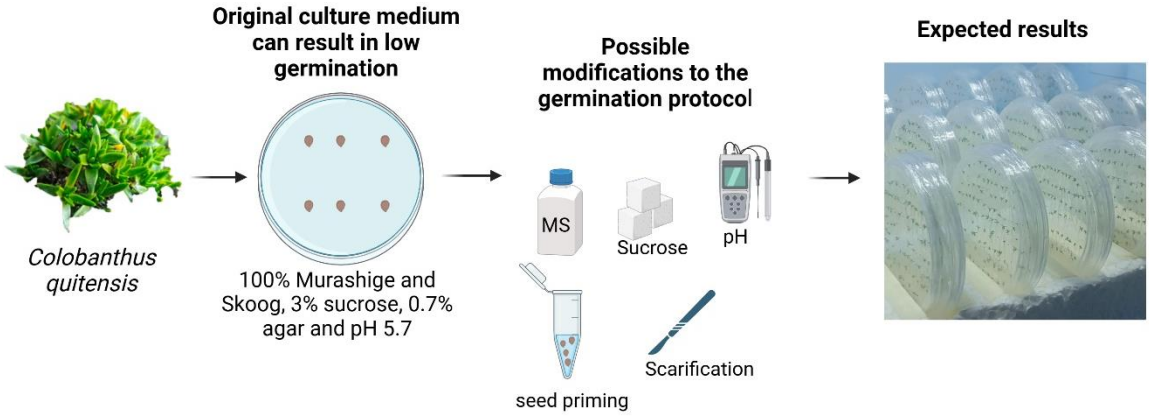
The extremophile species *Colobanthus quitensis* has been proposed as a model plant for stress biology studies. Its distribution in Antarctica faces increasing threats from tourism and scientific activities, making *ex situ* conservation urgent. However, low *in vitro* germination rates hinder this approach, 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.

Keywords: dormancy; seeds; priming, scarification, medium modification.

Highlights

- Halo-priming with 5% potassium chloride increases the germination rates
- *Colobanthus quitensis* can germinate with only 25% Murashige and Skoog medium.
- This species germinates better in slightly acidic media.
- Sucrose is not an essential component for *in vitro* germination.
- Testa breaking of seeds from Antarctic populations is effective in germination protocols.

Graphical abstract



1. 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.

2. MATERIALS AND METHODS

2.1 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 seconds, followed by 7 minutes 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 hours light / 8 hours 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).

2.2 Experiments

2.2.1 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).

2.2.2 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 hours 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).

2.2.3 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 section 2.3).

2.2.4 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.

2.2.5 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 hours 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.

2.3 Germination indicators calculated in each experiment

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

Percentage of germination (Equation 1).

$$GP = n/N * 100 \quad (1)$$

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) (Equation 2) is calculated as:

$$MGT = \Sigma\, ni * t/n \quad (2)$$

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 (Equation 3) is calculated as described by Farooq *et al*. (2005):

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

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) (Equation 4), as described by Primack (1980), is calculated as:

$Z = \Sigma\, Cn_{i,2}/N$, siendo $Cn_{i,2} = n_i(n_i - 1)/2$ y $N = \Sigma\, n_i(\Sigma n_i - 1)/2$

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.

2.4 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, 2023). Graphs were subsequently generated using the "ggplot2" package (Wilkinson, 2011).

3. 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.

3.1 Experiment 1: MS 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).

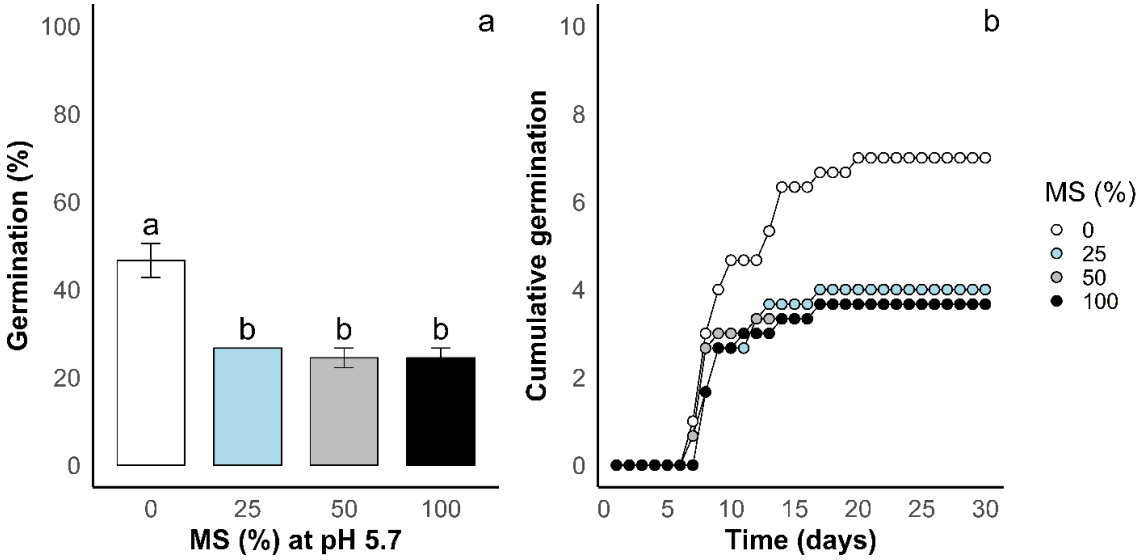


Figure 1. Effect of modifying the Murashige and Skoog (MS) medium concentration in the *in vitro* culture medium on (a) germination percentage and (b) cumulative germination of *Colobanthus quitensis*. Different letters indicate significant differences (P≤ 0.05) according to Tukey's HSD test (n=3).

3.2 Experiment 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).

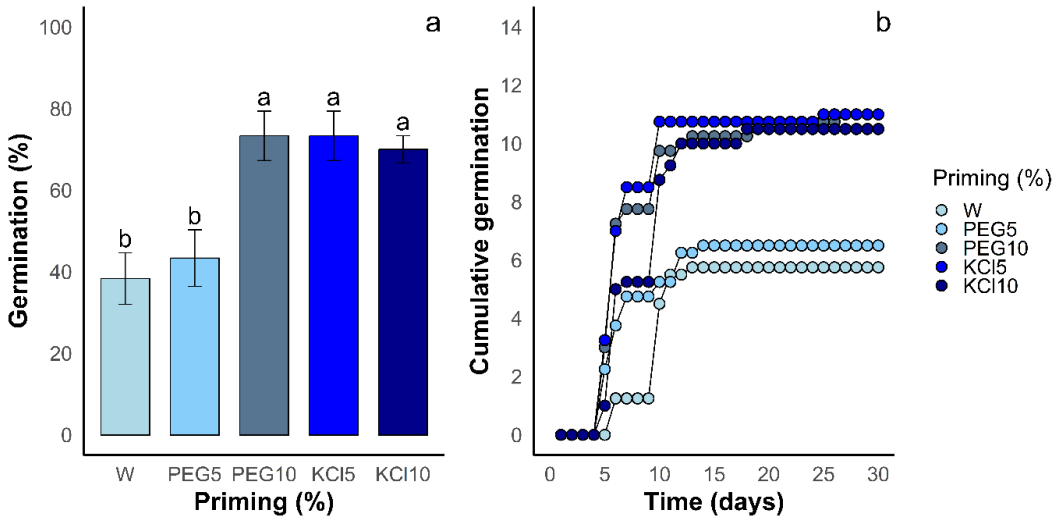


Figure 2. Effect of different priming treatments on *Colobanthus quitensis* seeds for (a) germination percentage and (b) cumulative germination. Different letters indicate significant differences (P ≤ 0.05) according to Tukey's HSD test (n = 4). DW: distilled water, PEG: polyethylene glycol, KCl: potassium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

3.3 Experiment 3: 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).

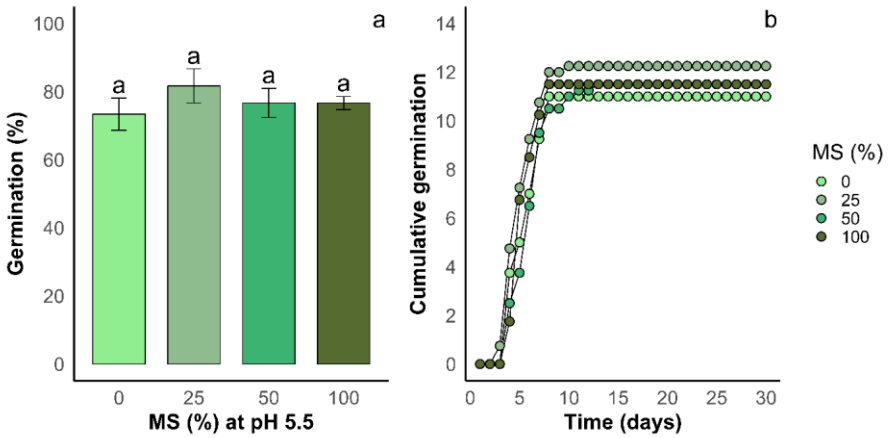


Figure 3. Effect of modifying the MS medium content in the *in vitro* culture medium at pH 5.5 when seeds are treated with halopriming (KCl 5%) on (a) germination percentage and (b) cumulative germination of *Colobanthus quitensis*. Different letters indicate significant differences (P ≤ 0.05) according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

3.4 Experiment 4: 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).

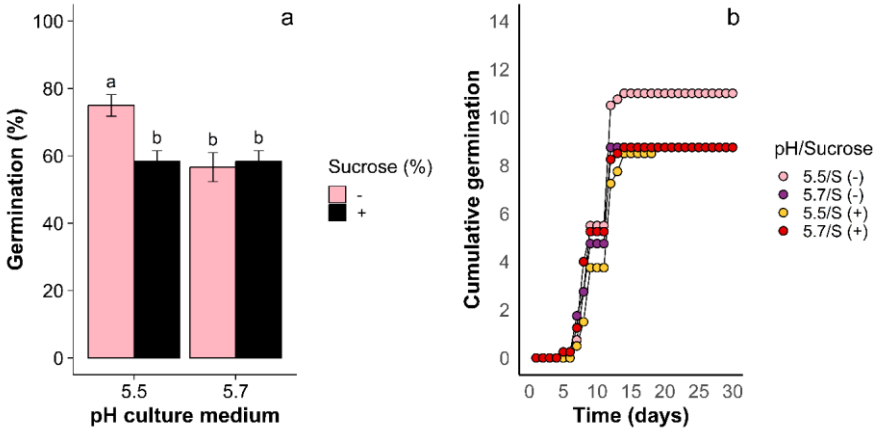


Figure 4. Effect of medium pH modification and the presence or absence of sucrose in the *in vitro* culture medium on a) germination percentage and b) cumulative germination of

Colobanthus quitensis. Different letters indicate significant differences (P ≤ 0.05) for Tukey's HSD test (n=4).

3.5 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 was compared (Table 1). To determine the best treatment combination for stimulating the germination of *C. quitensis*, the germination percentage and mean germination time of the best treatments from each experiment were compared (Table 1). This analysis revealed that T1 (MS (-), 3% sucrose, 0.7% agar, and pH 5.7) had a significantly lower germination 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).

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%.

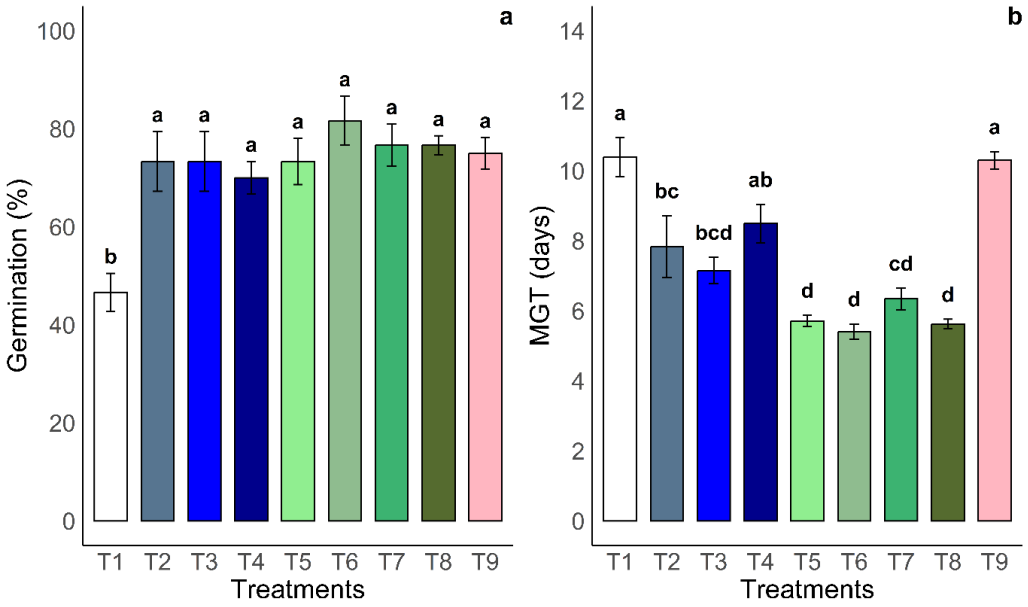


Figure 5. Comparison of the best *in vitro* germination protocols of *Colobanthus quitensis* from experiments 1 to 4: a) germination percentage and b) mean germination time. Different letters indicate statistically significant differences according to the Tukey HSD test ($P \leq 0.05$). The description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

3.6 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 of Byers Peninsula, 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 allow sufficient germination to determine MGT (Supplementary Table 7).

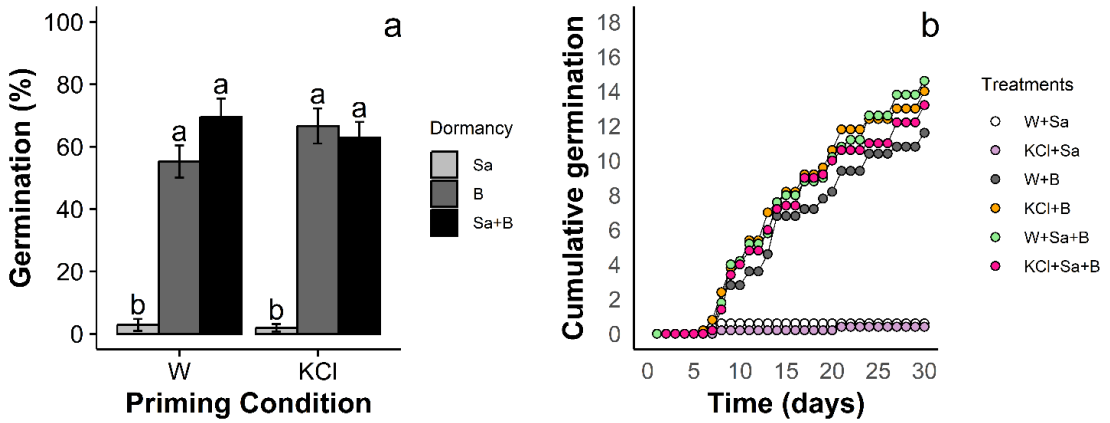


Figure 6. Effect of combining seed halo-priming (KCl 5%) with scarification methods on a) germination percentage and b) cumulative germination of *Colobanthus quitensis*. Different letters indicate significant differences (P ≤ 0.05) according to Tukey's HSD test (n = 5). W: distilled water; Sa: sulfuric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

4. 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 & Romano, 2021; Salgado, 2023).

4.1 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. 1-4). 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. 2-5), 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).

4.2 Osmotic and saline priming have an important effect on in vitro germination of *Colobanthus quitensis*.

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).

4.3 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 Figure 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. 2024). 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% (Fig. 3a, 5a).

4.4 Seed dormancy may limit the *in vitro* germination of Antarctic populations of *Colobanthus quitensis*

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 hours 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.

5. 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 hours 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.

6. REFERENCES

Agha H, Sidik N, Radzun K, Jawad A, Mohammed A (2022) The influence of different concentrations of plant hormones in vitro on seeds germination of fenugreek (*Trigonella foenum-graecum*). J Asian Sci Res 12:104-113. https://doi.org/10.55493/5003.v12i2.4528

Arroyo-Marín F (2023) Respuestas diferenciales en la tolerancia a la salinidad en poblaciones de *Colobanthus quitensis* Kunth (Bartl.). (Tesis de Pregrado de Ingeniería en Biotecnología Vegetal). Universidad de Concepción, Chile

Beber-Vieira F, Batista-Pereira A, Schünemann A, Albuquerque F, Pereira de Albuquerque M, Putzke J, Strassburger de Oliveira C (2011) Soil chemical attributes as affected by vegetal cover and seabirds in Punta Hennequin, Antarctica. Annu Act Rep 57-61. https://doi.org/10.4322/apa.2014.062

Bewley J, Bradford K, Hilhorst H, Nonogaki H (2013) Seeds: Physiology of Development, Germination and Dormancy. Springer, New York

Bhattacharyya P, Kumaria S, Diengdoh R, Tandon P (2014) Genetic stability and phytochemical analysis of the *in vitro* regenerated plants of Dendrobium nobile Lindl. an endangered medicinal orchid. Meta Gene 2:489-504. https://doi.org/10.1016/j.mgene.2014.06.003

Bozdemir H, Çığ A, Türkoğlu N (2018) Effects of different concentrations of carbohydrate forms on *Orchis sancta* L. propagation *in vitro*. Appl Ecol Environ Res 16:4849-4864. https://doi.org/10.15666/aeer/1604_48494864

Cárdenas M, Villegas A (2002) Potencial osmótico del medio de cultivo con diferentes componentes para la propagación *in vitro*. Rev Fitotec Mex 25:213-217. https://doi.org/10.35196/rfm.2002.2.213

Coelho N, Romano A (2021) Impact of chitosan on plant tissue culture: recent applications. PCTOC. 148:1–13. https://doi.org/10.1007/s11240-021-02156-6

Contreras R, Pizarro M, Köhler H, Sáez C, Zúñiga G (2018) Copper stress induces antioxidant responses and accumulation of sugars and phytochelatins in Antarctic *Colobanthus quitensis* (Kunth) Bartl. Biol Res 51. https://doi.org/10.1186/s40659-018-0197-0

Convey P, Biersma E (2024) Antarctic Ecosystems. In: Levin SA (ed) Encyclopedia of Biodiversity, 3rd edn. Elsevier, Oxford, pp. 133–148

Cruz-Cruz C, González-Arnao M, Engelmann F (2013) Biotechnology and Conservation of Plant Biodiversity. Resources 2:73-95. https://doi.org/10.3390/resources2020073

Cuba-Díaz M, Acuña D, Cordero CM, Klagges M (2014) Optimización de parámetros para la propagación *in vitro* de *Colobanthus quitensis* (Kunth) Bartl. Gayana Bot 71:10-17. https://doi.org/10.4067/S0717-66432014000100009

Cuba-Díaz M, Acuña D, Fuentes-Lillo E (2019) Antarctic pearlwort (*Colobanthus quitensis*) populations respond differently to pre-germination treatments. Polar Biol 42:1209-1215. https://doi.org/10.1007/s00300-019-02505-4

Cuba-Díaz M, Castel K, Acuña D, Machuca A, Cid I (2017a) Sodium chloride effect on *Colobanthus quitensis* seedling survival and *in vitro* propagation. Antarct Sci 29:45-46. https://doi.org/10.1017/S0954102016000432

Cuba-Díaz M, Klagges M, Fuentes-Lillo E, Cordero C, Acuña D, Opazo G, Troncoso-Castro JM (2017b) Phenotypic variability and genetic differentiation in continental and island populations of *Colobanthus quitensis* (Caryophyllaceae: Antarctic pearlwort). Polar Biol 40:2397-2409. https://doi.org/10.1007/s00300-017-2152-x

Cuba-Díaz M, Marín C, Castel K, Machuca A, Rifo S (2017c) Effect of copper (II) ions on morpho-physiological and biochemical variables in *Colobanthus quitensis*. J Soil Sci Plant Nutr 17. https://doi.org/10.4067/S0718-95162017005000031

Cuba-Díaz M, Rivera-Mora C, Navarrete E, Klagges M (2020) Advances of native and non-native Antarctic species to *in vitro* conservation: improvement of disinfection protocols. Sci Rep 10. https://doi.org/10.1038/s41598-020-60533-1

Dadlani M, Yadava D (eds) (2023) Seed Science and Technology: Biology, Production, Quality. Springer Nature, Singapore

de Carvalho J, Nascimento G, Fernandes A, Gonçalves E, dos Santos P, Santos AC, Gonçalves J (2024) Understanding the role of storage reserve mobilization during seed germination and initial seedling growth in species of the genus *Carapa*. Botany 102:268-281. https://doi.org/10.1139/cjb-2023-0120

de Freitas EM, Gomes CN, da Silva LJ, da Cunha FF (2024) Germination Performance of *Physalis peruviana* L. Seeds under Thermal and Water Stress Conditions. Agronomy 14. https://doi.org/10.3390/agronomy14061213

De Mello T, Rosa T et al (2022) Reserve mobilization and in vitro germination of *Euterpe edulis* (Martius) seeds at different maturation stages. Trees 36:415–426. https://doi.org/10.1007/s00468-021-02216-6

Debouza N, Mundra S, Shah I, Ksiksi T (2024) Mechanical scarification: The key to optimal germination parameters in nine flowering species of the United Arab Emirates. Innov Agric 7:1-10. https://doi.org/10.3897/ia.2024.124153

dos Santos M, Soares T, Rodrigues E, Leite R, Nunes O, Góes T, Duarte F, (2021) Optimization of culture medium for the *in vitro* germination and histochemical analysis of *Passiflora* spp. pollen grains. Sci Hortic 288. https://doi.org/10.1016/j.scienta.2021.110298

Farías D (2018) Efectos de los iones Fe^{3+} en la germinación *in vitro* de *Colobanthus quitensis*. (Tesis de Ingeniería en Biotecnología Vegetal). Universidad de Concepción, Chile

Farooq M, Basra S, Hafeez K (2005) Thermal hardening: a new seed vigor enhancement tool in rice. J Integr Plant Biol 47:187-193. https://doi.org/10.1111/j.1744-7909.2005.00031.x

Gamborg OL, Shyluk JP (1981) Nutrition, media and characteristics of plant cell and tissues culture. In Thorpe TA (ed) Plant Tissue Culture: Methods and Applications in Agriculture. Academic Press, New York, pp 21–44

George E, Hall M, De Klerk G (2008) The Components of Plant Tissue Culture Media II: Organic Additions, Osmotic and pH Effects, and Support Systems. In: George EF, Hall MA, Klerk GJD (eds) Plant Propagation by Tissue Culture, Springer, Dordrecht, pp 115-173

Gielwanowska I, Bochenek A, Gojlo E, Górecki R, Kellmann-Sopyla W, Pastorczyk M, Szczula E (2011) Biology of generative reproduction of *Colobanthus quitensis* (Kunth) Bartl. from King George Island, South Shetland Islands. Pol Polar Res 32:139-155. https://doi.org/10.2478/v10183-011-0008-6

Hakim J, Dalimunthe A (2022) Season, basal media and plant growth regulators effect in wood plant *in vitro* propagation: a comprehensive review. IOP Conf Ser Earth Environ Sci 1115. https://doi.org/10.1088/1755-1315/1115/1/012051

Hasanuzzaman M, Nahar K, Alam M, Roychowdhury R, Fujita M (2013) Physiological, biochemical, and molecular mechanisms of heat stress tolerance in plants. Int J Mol Sci 14:9643-9684. https://doi.org/10.3390/ijms14059643

Hesami M, Pepe M, Monthony AS, Baiton A, Phineas-Jones AM (2021) Modeling and optimizing in vitro seed germination of industrial hemp (*Cannabis sativa* L.). Ind Crops Prod 170. https://doi.org/10.1016/j.indcrop.2021.113753

Huh Y, Lee J, Nam S, Hong E, Paek K, Son S (2016) Effects of altering medium strength and sucrose concentration on *in vitro* germination and seedling growth of *Cypripedium macranthos* Sw. J Plant Biotechnol 43:132-137. https://doi.org/10.5010/JPB.2016.43.1.132

Kellmann-Sopyła W, Koc J, Górecki R, Domaciuk M, Giełwanowska I (2017) Development of generative structures of polar Caryophyllaceae plants: the Arctic *Cerastium alpinum* and *Silene involucrata*, and the Antarctic *Colobanthus quitensis*. Pol Polar Res 38:83-104. https://doi.org/10.1515/popore-2017-0001

Kellmann-Sopyła W, Lahuta L, Giełwanowska I, Górecki R (2015) Soluble carbohydrates in developing and mature diaspores of polar Caryophyllaceae and Poaceae. Acta Physiol Plant 37. https://doi.org/10.1007/s11738-015-1866-z

Khuat Q, Kalashnikova E, Kirakosyan R, Nguyen H, Baranova E, Khaliluev M, (2022) Improvement of *In Vitro* Seed Germination and Micropropagation of *Amomum tsao-ko* (*Zingiberaceae Lindl*.). Horticulturae 8. https://doi.org/10.3390/horticulturae8070640

Kildisheva O, Dixon K, Silveira F, Chapman T, Di Sacco A, Mondoni A, Turner S, Cross A (2020) Dormancy and germination: making every seed count in restoration. Restor Ecol 28:256-265. https://doi.org/10.1111/rec.13140

Koc J, Wasilewski J, Androsiuk P, Kellmann-Sopyła W, Chwedorzewska KJ, Giełwanowska I (2018) The effects of methanesulfonic acid on seed germination and morphophysiological changes in the seedlings of two *Colobanthus* species. Acta Soc Bot Pol 87. https://doi.org/10.5586/asbp.3601

Li H, Zhang D (2018) *In Vitro* Seed Germination of *Kalmia latifolia* L. Hybrids: A Means for Improving Germination and Speeding Up Breeding Cycle. HortScience 53:535-540. https://doi.org/10.21273/HORTSCI12829-17

Lian Z, Nguyen C, et al (2022) Application of developmental regulators to improve in planta or *in vitro* transformation in plants. Plant Biotechnol J 20:2857-2876. https://doi.org/10.1111/pbi.13837

Rocha R L, Khalil Y, Maity A, Beckie H, Ashworth M (2022) Mechanical scarification technique breaks seed coat-mediated dormancy in wild oat (*Avena fatua*). Weed Technol 36:152-159. https://doi.org/10.1017/wet.2021.94

Ma L, Wei J, Han G, Sun X, Yang X (2024) Seed osmopriming with polyethylene glycol (PEG) enhances seed germination and seedling physiological traits of *Coronilla varia* L. under water stress. PLoS ONE 19. https://doi.org/10.1371/journal.pone.0303145

Min K, Sulaiman S, Jeong J, Lee H, Lee J, Lee J, Lee H (2024) Exploring the mechanisms underlying recovery from freeze–thaw injury in *Colobanthus quitensis*: mechanistic insights via transcriptome profiling. Physiol Plant 176. https://doi.org/10.1111/ppl.14642

Murashige T, Skoog F (1962) A revised medium for the rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473-497

Muscolo A, Sidari M, Mallamaci C, Attinà E (2007) Changes in germination and glyoxylate and respiratory enzymes of *Pinus pinea* seeds under various abiotic stresses. J Plant Interact 2:273-279. https://doi.org/10.1080/17429140701713795

Nawaz J, Hussain M, Jabbar A, Nadeem GA, Sajid M, Subtain M, Shabbir I (2013) Seed priming a technique. Int J Agric Crop Sci 6:1373-1381

Nyasulu M, Zhong Q, Li X, Liu X, Wang Z, Chen L, He H, Bian J (2024) Uncovering novel genes for drought stress in rice at germination stage using genome wide association study. Front. Plant Sci 15. https://doi.org/10.3389/fpls.2024.1421267

Ontivero Y, Cuba-Díaz M, Fuentes-Lillo E, Convey P (2024a) Germination strategies and seed quality of *Colobanthus quitensis*: implications for sustainable Antarctic ecosystems and ex situ plant conservation. Sustainability 16. https://doi.org/10.3390/su162310726

Ontivero Y, Fuentes-Lillo E, Navarrete-Campos D, Vázquez-Villa D, Cabreras-Barjas G, Arroyo-Marín FB, Cuba-Díaz M (2024b) Preliminary assessment of seed heteromorphism as an adaptive strategy of *Colobanthus quitensis* under saline conditions. Sci Rep 14. https://doi.org/10.1038/s41598-024-82381-z

Primack R (1980) Variation in the phenology of natural populations of montane shrubs in New Zealand. J Ecol 68:849-862. https://doi.org/10.2307/2259460

Quazi S, Golani T, Capuzzo A (2021) Germplasm conservation. In: Kumar S (ed) Endangered Plants. IntechOpen, London, 180 pp.

Rahimi A (2013) Seed priming improves the germination performance of cumin (*Cuminum cyminum* L.) under temperature and water stress. Ind Crops Prod 42:454-460. https://doi.org/10.1016/j.indcrop.2012.06.026

Rodríguez M, Chacón M, Carrillo R (2014) Efecto de la concentración y de los componentes del medio de cultivo MS sobre la germinación *in vitro* de *Ugni molinae*. Bosque 35:119-122. https://doi.org/10.4067/S0717-92002014000100012

Roni M, Islam M, Shimasaki K (2018) In vitro seed germination and tracking the seedling growth of Eustoma. N. Z. J Crop Hortic Sci 46:224-242. https://doi.org/10.1080/01140671.2017.1391300

Ruhland C, Day T (2001) Size and longevity of seed banks in Antarctica and the influence of ultraviolet-B radiation on survivorship, growth and pigment concentrations of *Colobanthus quitensis* seedlings. Environ Exp Bot 45:143-154. https://doi.org/10.1016/S0098-8472(00)00089-7

Sadeghi H, Robati Z (2015) Response of *Cichorium intybus* L. to eight seed priming methods under osmotic stress conditions. Biocatal Agric Biotechnol 4:443-448. https://doi.org/10.1016/j.bcab.2015.08.003

Salgado C (2023) Estrategias para promover la germinación *in vitro* de *Colobanthus quitensis* (Kunth.) Bartl. (Caryophyllaceae). (Tesis de Ingeniería en Biotecnología Vegetal). Universidad de Concepción, Chile

Sané A, Diallo B, Kane A, Sagna M, Sané D, Sy MO (2021) In vitro germination and early vegetative growth of five tomato (*Solanum lycopersicum* L.) varieties under salt stress conditions. Am J Plant Sci 12:796-817. https://doi.org/10.4236/ajps.2021.125055

Sanhueza C, Vallejos V, Cavieres L, Sáez P, Bravo L, Corcuera L (2017) Growing temperature affects seed germination of the Antarctic plant *Colobanthus quitensis* (Kunth) Bartl (Caryophyllaceae). Polar Biol 40:449-455. https://doi.org/10.1007/s00300-016-1972-4

Shaik S, Carciofi M, Martens H, Hebelstrup K, Blennow A (2014) Starch bioengineering affects cereal grain germination and seedling establishment. J Exp Bot 65:2257-2270. https://doi.org/10.1093/jxb/eru107

Soleimanzadeh H (2013) Effect of seed priming on germination and yield of corn. Int J Agric Crop Sci 5:366-369

Sorokin A, Yadav N, Gaudet D, Kovalchuk I (2021) Development and standardization of rapid and efficient seed germination protocol for *Cannabis sativa*. Bioprotocol 11. https://doi.org/10.21769/BioProtoc.3875

Soughir M, Elouaer M, Hannachi C (2013) The effect of NaCl priming on emergence, growth and yield of fenugreek under saline conditions. Cercet Agron Mold 154:73-83. https://doi.org/10.2478/v10298-012-0085-7

Stewart S, Kane M (2010) Effects of carbohydrate source on the in vitro asymbiotic seed germination of the terrestrial orchid Habenaria macroceratitis. J Plant Nutr 33:1155-1165. https://doi.org/10.1080/01904161003763757

Still D (1999) Development of seed quality in Brassicas. HortTechnology 9:335-340

Tai L, Wang H, Xu X, Sun W, Ju L, Liu W, Li W, Sun J, Chen K (2021) Pre-harvest sprouting in cereals: genetic and biochemical mechanisms. J Exp Bot 72:2857-2876. https://doi.org/10.1093/jxb/erab024

Tinoammini N, Aazhivaendhan G, Kumar TS (2024) In vitro germination and optimization of basal media for protocorm-like bodies proliferation in *Dienia ophrydis* (J. Koenig) Seidenf. Rhizosphere 29. https://doi.org/10.1016/j.rhisph.2024.100854

Tiwari P, Bose SK, Park KI, Dufossé L, Fouillaud M (2024) Plant-Microbe Interactions under the Extreme Habitats and Their Potential Applications. Microorganisms 12. https://doi.org/10.3390/microorganisms12030448

Turkan I, Demiral T (2009) Recent developments in understanding salinity tolerance. Environ Exp Bot 67:2-9. https://doi.org/10.1016/j.envexpbot.2009.05.008

Upson R, Newsham K, Read D (2008) Root-fungal associations of *Colobanthus quitensis* and *Deschampsia antarctica* in the maritime and sub-Antarctic. Arct Antarct Alp Res 40:592-599. https://doi.org/10.1657/1523-0430(07-057)[UPSON]2.0.CO;2

Vandecasteele C, Teulat-Merah B et al (2011) Quantitative trait loci analysis reveals a correlation between the ratio of sucrose/raffinose family oligosaccharides and seed vigour in Medicago truncatula. Plant Cell Environ 34:1473-1487. https://doi.org/10.1111/j.1365-3040.2011.02346.x

Vasconcelos P, Loureiro M, Lima A, Ribeiro P, Bernal D, Moreno M, de Castro R (2017) New insights into the mechanism underlying *Ricinus communis* L. tolerance to drought stress during germination. Ind Crops Prod 103:99-106. https://doi.org/10.1016/j.indcrop.2017.03.038

Vera M (2017) Factors affecting germination of *Deschampsia antarctica* and *Colobanthus quitensis* seeds from an altitudinal gradient on Livingston Island (South Shetland Island, Antarctica). http://hdl.handle.net/10651/40759. Accessed 15 July 2024

Wang W, Zhang C, Zheng W, Lv H, Li J, Liang B, Zhou W (2022) Seed priming with protein hydrolysate promotes seed germination via reserve mobilization, osmolyte accumulation and antioxidant systems under PEG-induced drought stress. Plant Cell Rep 41:2173-2186. https://doi.org/10.1007/s00299-022-02914-6

Zhang M, Han M, Wang X, Tian Y, Wang S, Wang A, Zhu X (2004) Factors affecting embryo rescue of early ripening nectarines. J Fruit Sci 21:382–384

Zhou H, Shi H, Yang Y, Feng X, Chen X, Xiao F, Lin H, Guo Y (2024) Insights into plant salt stress signaling and tolerance. J Genet Genomics 51:16-34. https://doi.org/10.1016/j.jgg.2023.08.007

Zhou W, Chen F, Luo X, Dai Y, Yang Y, Zheng C, Yang W, Shu K (2019) A matter of life and death: molecular, physiological, and environmental regulation of seed longevity. Plant Cell Environ 43:293-302. https://doi.org/10.1111/pce.13666

Zúñiga G, Zamora P, Ortega M, Obrecht A (2009) Short Note: Micropropagation of Antarctic *Colobanthus quitensis*. Antarct Sci 21:149-150. https://doi.org/10.1017/S0954102008001570

Zurita-Valencia W, Gómez-Cruz J, Atrián-Mendoza E, Hernández-García A, Granados-García M, García-Magaña J, Salgado-Garciglia R, Sánchez-Vargas N (2014) Establishment of an efficient method of *in vitro* germination and micropropagation of the cirimo tree (*Tilia mexicana* Schlecht.) (Tiliaceae). Polibotánica 38, 129-144

7. Competing interests

The authors declare no competing interests.

8. Funding

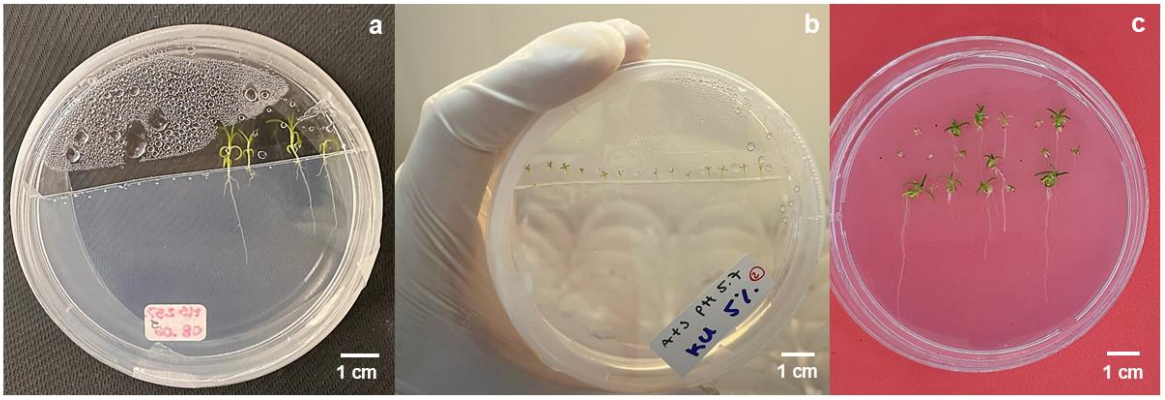
Yadiana Ontivero was supported by ANID/Scholarship Program/Doctorado Beca Nacional/2021-21210100; INACH DT_05_22 Project (Instituto Antártico Chileno) and Programa de Doctorado en Ciencias de la Agronomía 2021300031, Facultad de Agronomía, Universidad de Concepción, Campus Chillán. Eduardo Fuentes-Lillo was funded by ANID BASAL 210006. Marely Cuba-Díaz was supported by VRID 220.418.012 Project

(Vicerrectoría de Investigación de Desarrollo, Universidad de Concepción) and 2023000418ESP-VRID.

9. Authors contributions

Yadiana Ontivero: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review and editing. **Constanza Salgado**: Data curation, Investigation, Methodology, Writing – original draft. **Eduardo Fuentes-Lillo**: Formal analysis, Funding acquisition, Writing – review and editing. **Carolina Sanhueza**: Formal analysis, Writing – original draft, Writing – review and editing. **Darío Navarrete-Campos**: Methodology, Resources, Writing – review and editing. **Solange Alveal**: Methodology, Writing – original draft. **Marely Cuba-Díaz**: Conceptualization, Formal analysis, Funding acquisition, Supervision, Investigation, Methodology, Project administration, Resources, Writing – original draft, Writing – review and editing.

Supplementary materials



Supplementary Figure 1. Effect of the germination protocol used on the vegetative development of *Colobanthus quitensis* seedlings at the end of the experiment. Seeds were germinated in: a) Murashige and Skoog (MS) 25% medium + sucrose + pH 5.7 (experiment 1); b) application of 5% potassium chloride (KCl) in medium without MS + sucrose + pH 5.7; and c) application of 5% KCl + a combination of 2% sulfuric acid followed by testa rupture with a scalpel in MS 25% medium without sucrose + pH 5.5.

Supplementary Table 1. Analysis of variance of the effect of the variables evaluated in the different experiments on the *in vitro* germination of *Colobanthus quitensis*.

Experiment	Variables effects on germination	Statistical parameters

	(%)	df	MS	F	p-value
1	MS at pH 5.7	3	0.0390	18.04	**0.000641*** **
	Error	8	0.0022		
2	Priming treatments	4	0.1350	8.024	**0.00115** **
	Error	15	0.0168		
3	MS at pH 5.5	3	0.0082	0.771	0.532
	Error	12	0.0107		
4	pH	1	0.03858	7.183	**0.02***
	Sucrose	1	0.02658	4.948	**0.0461***
	pHxSucrose	1	0.03858	7.183	**0.02***
	Error	12	0.00537		
5	Priming	1	0.0007	0.117	0.850
	Scarification	2	2.3107	121.190	**1.48e^{-13} *** **
	PrimingxScarification	2	0.0261	2.066	0.149
	Error	24	0.0180		0.253

df degree of freedom; MS mean of squares; F test F; *p-value with significant differences (p<0.05); ** p-value with significant differences (p<0.01); *** p-value with significant differences (p<0.001)

Suplementary Table 2. Effect of the modification of Murashige and Skoog (MS) medium content in the *in vitro* culture medium at pH 5.7 on the mean germination time (MGT), the time required to reach 50% germination (T50), and the germination uniformity index (Z) of *Colobanthus quitensis* (n=3). No significant differences were detected (p>0.05) according to Tukey HSD (n=3).

MS (%) at pH 5.7	MGT (days) ± SD	T_{50} (days) ± SD	Z ± SD
0	10.39 ± 0.98	8.83 ± 1.09	0.094 ± 0.04
25	9.92 ± 1.18	8.78 ± 1.07	0.056 ± 0.10
50	9.11 ± 1.02	7.58 ± 0.14	0.222 ± 0.10
100	10.03 ± 1.43	8.72 ± 0.86	0.167 ± 0.17

Supplementary Table 3. Costs of different chemical products in Chile.

Product	Price (clp)	Quantity	Company	Product link
Murashige and Skoog	19400	1L	Sigma Aldrich	https://www.sigmaaldrich.com/CL/es/product/sigma/m5519?utm_source=google&utm_medium=cpc&utm_campaign=22179178721&utm_content=177793365521&gad_source=1&gad_campaignid=22179178721&gbraid=0AAAAD8kLQTx4nzzd_F6VaDomLQJSjSp7&gclid=Cj0KCQjw8cHABhC-ARIsAJnY12zi9gIonk4mRyS5RprJvAwfRTUtAe_w0nX-WWYROXn6TthEU-wUzjIaAmZ-EALw_wcB

Polyethylene glycol	55620	1kg	Productos químicos	https://productosquimicos.cl/producto/polietilenglicol-1-lt/?srsltid=AfmBOooMzvK0xge0q8EwJnZnYuZKZzhbsUiKwF-TdLaxgcoUHQ6zG7RZ
Potassium chloride	10591	1 kg	Bioquímica.cl	https://www.bioquimica.cl/cloruro-de-potasio-1kg?srsltid=AfmBOoqKibzVmmOlS8VDCOOJMsY-80MIu7KmEff09fkX9votTnZ2y99d
Sufuric acid	34986	500 mL	Hanna	https://hannachile.com/producto/reactivo-de-acido-sulfurico-al-16-500-ml-hi70425/?gad_source=1&gclid=Cj0KCQiA2oW-BhC2ARIsADSIAWpxPWapQut5Uw1HTWjk40CQJW2ppPdtj85JlyYks_TUhDmDJ1sVEqIaAufiEALw_wcB

Suplementary Table 4. Effect of osmotic pre-treatments on the mean germination time (MGT), the time required to reach 50% germination (T50), and the germination uniformity index (Z) in *Colobanthus quitensis*. No significant differences were detected (p>0.05) according to Tukey HSD (n = 4).

Priming treatment	MGT (days) ± SD	T_{50} (days) ± SD	Z ± SD
Distilled water	9.55 ± 1.16	8.72 ± 1.91	0.55 ± 0.30
Polyethylene glycol 5%	7.08 ± 1.16	5.63 ± 0.83	0.41 ± 0.42
Polyethylene glycol 10%	7.84 ± 1.76	5.73 ± 0.36	0.32 ± 0.22
Potassium chloride 5%	7.15 ± 0.76	5.59 ± 0.22	0.23 ± 0.03
Potassium chloride 10%	8.49 ± 1.10	7.56 ± 2.12	0.29 ± 0.11

Suplementary Table 5. Effect of modifying the Murashige and Skoog (MS) medium content in *in vitro* culture medium at pH 5.5 on the mean germination time (MGT), the time required to reach 50% germination (T50), and the germination uniformity index (Z) of *Colobanthus quitensis.* No significant differences were detected (p>0.05) according to Tukey HSD (n = 4).

MS at pH 5.5	MGT (days) ± SD	T_{50} (days) ± SD	Z ± SD
0	5.72 ± 0.33	5.01 ± 0.63	0.18 ± 0.03
25	5.41 ± 0.45	4.81 ± 0.63	0.17 ± 0.02
50	6.34 ± 0.61	5.85 ± 0.48	0.15 ± 0.06
100	5.63 ± 0.27	4.90 ± 0.27	0.26 ± 0.07

Suplementary Table 6. Effect of pH modification and the presence or absence of sucrose in the *in vitro* culture medium on the mean germination time (MGT), time to reach 50% germination (T50), and germination uniformity index (Z) of *Colobanthus quitensis.* No significant differences were detected (p>0.05) according to Tukey HSD (n=4).

pH /Sucrose (S)	MGT (days) ± SD	T_{50} (days) ± SD	Z ± SD

5.5/S (-)	10.30 ± 0.50	9.06 ± 0.56	0.30 ± 0.16
5.7/S (-)	9.79 ± 0.27	8.83 ± 0.39	0.26 ± 0.06
5.5/S (+)	10.92 ± 1.07	9.60 ± 0.76	0.22 ± 0.04
5.7/S (+)	9.66 ± 0.44	8.76 ± 0.74	0.20 ± 0.09

+: presence, -: absence

Suplementary Table 7. Effect of different dormancy breaking methods on the mean germination time (MGT), time to reach 50% germination (T50), and germination uniformity index (Z) of *Colobanthus quitensis* (n=5).

Priming	**Scarification**	**MGT (days) ± SD**	**T_{50} (days) ± SD**	**Z ± SD**
Distilled water	Sulfuric acid	-	-	-
	Testa breaking	15.81 ± 1.81	13.97 ± 3.59	0.095 ± 0.042
	Sulfuric acid + Testa breaking	16.28 ± 1.19	13.7 ± 1.60	0.059 ± 0.016
Potassium chloride	Sulfuric acid	-	-	-
	Testa breaking	15.45 ± 1.41	12.43 ± 2.31	0.061± 0.032
	Sulfuric acid + Testa breaking	15.8 ± 1.66	13.68 ± 1.93	0.068 ± 0.028

- Measurements are absent due to lack of data

V. Chapter VI. ACCELERATED AGING IN *Colobanthus quitensis* SEEDS: UNDERSTANDING STRESS RESPONSES IN AN EXTREMOPHILE SPECIES

Yadiana Ontivero[1,2], Darío Navarrete-Campos[1], Vicente Carrillo[1]; Marely Cuba Díaz[1,3]

[1]Laboratorio de Biotecnología y Estudios Ambientales, Departamento de Ciencias y Tecnología Vegetal, Escuela de Ciencias y Tecnologías, Universidad de Concepción, Los Ángeles, Chile.

[2]Facultad de Agronomía, Universidad de Concepción, Chillán, Chile.

[3]Programa de Ciencia Antártica y Subantártica, Universidad de Concepción, Concepción.

Submitted for review to the journal Planta on May 16, 2025

ABSTRACT

Seed deterioration affects all species and presents a major challenge for the management of germplasm banks. *Colobanthus quitensis* is one of the two extremophile vascular species native to Antarctica and is recognized for its biotechnological potential in environmental stress studies and as a bioindicator of climate change. However, the manifestation of physiological deterioration in the seeds of this species remains unknown. This study aimed to analyze the physiological changes occurring in *C. quitensis* seeds during the deterioration process. To achieve this, the accelerated aging method was applied to evaluate variations in germination and various biochemical indicators between aged and non-aged seeds. The results showed that the germination percentage, mean germination time, time required to reach 50% germination, and germination speed coefficient significantly decreased with seed deterioration. However, once the seed coat is broken, germination may be enhanced. Additionally, total sugar content decreased significantly with aging, while indole-3-acetic acid content increased. In conclusion, accelerated aging leads to a reduction in germination indices, and both total sugar and indole-3-acetic acid contents may serve as markers of physiological deterioration in *C. quitensis* and other species characterized by small seeds, seed dormancy, and hard seed coats.

1. INTRODUCTION

Seed deterioration is a phenomenon that affects all plant species and complicates the work of germplasm banks, as it necessitates continuous quality assessments that demand time and

resources (Taylor, 2020). The accelerated aging technique allows for seed quality evaluation without requiring long waiting periods between monitoring sessions (Fenollosa *et al*., 2020).

Colobanthus quitensis (Kunth) Bartl. (Caryophyllaceae) is an extremophile species with a broad distribution, ranging from Mexico to Antarctica (Moore, 1970). In recent years, it has garnered growing interest in biotechnological research on environmental stress (Clemente-Moreno *et al*., 2020; Min *et al*., 2024). The inaccessibility of its natural habitats and the need to protect Antarctic ecosystems from human influence have driven efforts toward its *ex situ* conservation as a research resource (Ontivero *et al*., 2024aa).

Ex situ conservation of this species through *in vitro* cultivation has revealed germination issues in seeds from several populations (Cuba-Díaz *et al*., 2019). This problem has been linked to dormancy (Gielwanowska *et al*., 2011). However, the early indicators affected by seed deterioration remain unknown. This knowledge gap has implications not only for the management of its collections but also for advancing the understanding of extremophilic plant biology. This study aims to analyze the physiological changes that occur in *C. quitensis* seeds during deterioration.

2. MATERIALS AND METHODS

2.1 Plant Material and Accelerated Aging

A seed pool was generated from the Antarctic populations of Arctowski (62°09′S; 58°28′W) and Byers Peninsula (62°40′S; 60°55′W), the Parque Omora population from Puerto Williams in Patagonia (54°56′S; 67°39′W), and the Punta Arenas populations of La Marisma (53°22′S; 70°58′W), Laredo (52°58′S; 70°49′W), and La Vega (52°41′S; 70°56′W) of *C. quitensis.* The seeds were obtained from plants cultivated under controlled common garden conditions (Cuba-Díaz *et al*., 2017a) and had been stored for 12 to 18 months at 4°C. These seeds, referred to as *non-aged seeds* (NAS), corresponded to the period of optimal physiological quality (Salgado, 2023). A portion of the seeds was subjected to accelerated aging (AS) with modifications to the protocol of Fenollosa *et al*. (2020). The seeds were placed in airtight containers with 200 mL of water and incubated in ovens at 45°C and 90% relative humidity for 3 days. Subsequently, both NAS and AS seeds were divided into two groups to assess their quality.

2.2 *In vitro* germination test

For the *in vitro* germination test, 5 replicates of 21 seeds were disinfected with ethanol and sodium hypochlorite and placed in Petri dishes with 100% Murashige and Skoog medium, 3% sucrose, 0.7% agar, and pH 5.7 (Cuba-Díaz *et al*., 2017a). They were maintained at 20 ± 2 °C under a 16-hour light/8-hour dark photoperiod with a light intensity of 45 ± 2 µmol m^{-2} s^{-1} for 30 days. A seed was considered germinated when its primary root was at least twice the size of the seed. Germination data were collected daily to determine:

1. Germination percentage: $G\,(\%) = \frac{\mathrm{N}}{\mathrm{A}}\ x\ 100$; where N: number of germinated seeds at the end of the test. A: total number of seeds set to germinate.
2. Mean Germination Time: MGT = $\frac{\sum \mathrm{ni} * \mathrm{t}}{\sum n}$ (days); where ni: number of seeds germinated on day t. n: number of seeds germinated in the trial.
3. Time to reach 50% of germination: $T50 = \frac{ti + (n/2 - ni)(tj - ti)}{(nj - ni)}$ (days); where *n:* total number of germinated seeds; *ni* and *nj* are the accumulated numbers of germinated seeds from adjacent counts at times *tj* and *ti*, respectively, such that $ni < \frac{n}{2} < nj$.
4. Coefficient of velocity of germination: $CVG = \frac{G1 + G2 + \cdots + Gn}{(1xG1) + (2xG2) + \cdots + (nxGn)}$; where G: number of germinated seeds.

Dormancy in *C. quitensis* has been considered one of the possible factors affecting its germination (Gielwanowska *et al*., 2011). To determine whether this phenomenon was influencing the results, 60 ungerminated seeds from each group were randomly selected after 30 days of experimentation, following the completion of the initial *in vitro* germination test. These seeds had their seed coat punctured with a scalpel and were then placed back under the same previously described germination conditions to assess their germination percentage (Ontivero *et al*., 2025).

2.3 Determination of Chemical Compounds

A total of 2 g of seeds for each experimental condition was sent to the Laboratory of Bioactives in Plants and Plant-Based Ingredients at the Universidad de Concepción, where the chemical profile of the samples was determined. The total sugar and polyphenol content was determined by spectrophotometry using a Synergy™ H1 microplate reader. For the analysis of gibberellic acid, indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) profiles, a high-

performance liquid chromatograph (HPLC) coupled to a diode array detector Hitachi Primaide, equipped with a Kromasil® C18 column, was used. Finally, the protein content was determined using the Kjeldahl nitrogen method. A total of three replicates were performed for each variable.

2.4 Statistical Analysis

To analyze the effect of accelerated seed aging on the germination of *C. quitensis* and its chemical compounds, a Student's t-test was performed using the t.test() function in R, with a 95% confidence interval (R Core Team, 2023). Subsequently, the graphs were generated using the "ggplot2" package.

3. RESULTS AND DISCUSSION

Accelerated aging in *C. quitensis* seeds significantly affected the germination percentage, although it did not cause total seed mortality. The germination percentage of NAS was 34.29%, whereas AS only reached 5.71% (Fig. 1A). These results highlight the difficulties this species faces in germination, which have previously been associated with dormancy (Gielwanowska *et al*., 2011) or the need to optimize its germination protocols (Ontivero *et al*., 2024a). The germination time of the AS was significantly longer (Fig. 1B, C), while their CVG was lower (Fig. 1D), indicating physiological deterioration, likely caused by the accumulation of reactive oxygen species and lipid peroxidation (Dadlani and Yadava, 2023). This effect occurs because high temperatures can cause microfractures in the seed coat, disrupting its impermeability to oxygen and promoting the formation of peroxide radicals in the endosperm, leading to seed deterioration (Huang *et al*., 2021). The significant reduction in germination corresponds to the impact of high temperature and humidity (Fenollosa *et al*., 2020), demonstrating that accelerated aging studies allow the assessment of *C. quitensis* behavior in response to deterioration.

The germination test conducted after the initial germination analysis showed that 20% of the NAS seeds germinated, while 23.3% of the AS seeds germinated, with no significant differences ($p > 0.05$) observed between these groups (data not shown). These results indicate the presence of dormancy in both seed groups, a process that was alleviated by mechanical scarification (Rocha *et al*., 2022).

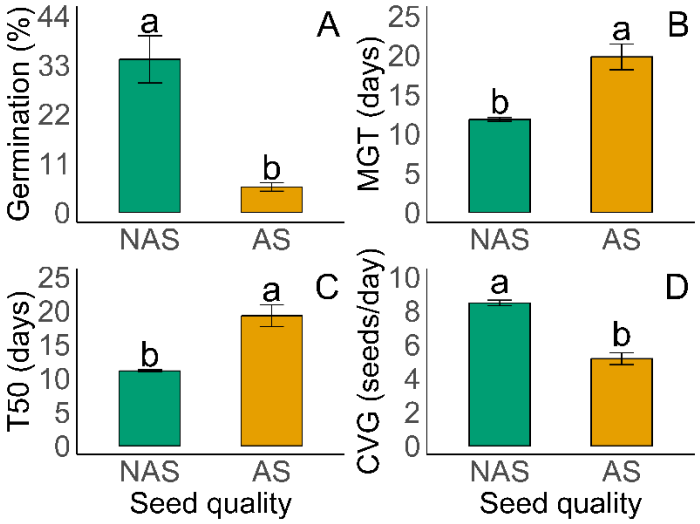


Figure 1. Germination indices of non-aged (NAS) and aged seeds (AS) of *Colobanthus quitensis*. a) Germination percentage; b) Mean germination time; c) Time to reach 50% germination and d) Coefficient of velocity of germination. Different letters indicate statistical differences according to Student's t-test ($p<0.05$, $n=5$).

Accelerated seed aging induces an uncontrolled accumulation of reactive oxygen species (ROS), whose cytotoxicity compromises the structural and functional integrity of cells. Lipid peroxidation, protein carbonylation, and oxidative damage to nucleic acids disrupt key metabolic processes, leading to a progressive loss of viability and germination capacity (Gill *et al*., 2010). The deterioration of seeds causes complex and multifactorial metabolic alterations. Since physiological responses to stress conditions are not uniform, a detailed analysis of the specific chemical profile of each species under defined conditions is a key tool for assessing seed vigor and their *ex situ* conservation potential (Chen *et al*., 2022). This would be feasible in *C. quitensis*, where the geographical distribution of its populations has led to the formation of ecotypes (Gianoli *et al*., 2004) that differ in their genetic and morphological characteristics (Cuba-Díaz *et al*., 2017; Koc *et al*., 2018).

In *C. quitensis*, the total sugar content and indole-3-acetic acid levels were the only two compounds significantly influenced by seed quality (Fig. 2A, D). Sugar is crucial in the germination process, as the first step in the mobilization of nutrient reserves involves the hydrolysis of sugars, which are then supplied to the seed embryo during germination (Dadlani and Yadava, 2023). Sugars could mediate the signaling pathways of hormones and reactive

oxygen species, cell division, and programmed cell death (Liu *et al*., 2013), while also acting as osmoregulators to protect proteins and membranes from oxidative damage (Sachdev *et al*., 2023). Therefore, the decrease in sugars in the AS (Fig. 1A) could be directed towards responding to stress rather than being used as an energy source. The increase in IAA helps respond to oxidative stress, making it a stress signal (Lecube *et al*., 2014). Its high production has been linked to lower germination rates, as it can induce dormancy (Gupta *et al*., 2024). However, although the content of polyphenols, gibberellic acid, and IBA were not significantly different between NAS and AS, a trend was observed indicating that AS had higher contents of these compounds (Fig. 2B, C, E), while the protein content remained unchanged (Fig. 1F). In plants, polyphenols have been identified as playing a significant protective role against oxidative damage (Šamec *et al*., 2021). For this reason, this indicator tended to be higher in aged seeds (Fig. 2B), although it may not have been sufficient to counteract the effects of deterioration, which resulted in lower germination percentages in the aged seeds (AS). It is important to highlight that, initially, NAS contains the necessary sugars to obtain the energy that allows them to germinate at a higher rate than AS. The latter must conserve their energy to protect themselves and counteract stress, mainly oxidative stress. However, when the physical barrier of the seed coat is removed, AS can germinate at a rate like NAS. In this study, the chemical composition of a set of seeds was analyzed. However, a previous study using electron microscopy showed that the structure of the seed coat in *C. quitensis* does not vary among seeds, although there are differences in germination capacity between populations. This suggests that the composition of the seed coat plays a fundamental role in this process (Ontivero *et al*., 2024b).

Gibberellins are essential for germination, but their effectiveness depends on the balance with abscisic acid (ABA), a phytohormone that regulates dormancy and germination (Gong *et al*., 2022). In accelerated aging studies, a negative correlation has been observed between gibberellin content and seed vigor, and the impact of this hormone on seed quality has been linked to ABA levels (Wang *et al*., 2022). Although ABA content was not determined in this study, research on wheat has shown that indole-3-acetic acid (IAA) can complement the role of ABA in germination (Ramaih *et al*., 2003), which may be contributing to the lower germination rates in accelerated aged seeds (AS) compared to non-aged seeds (NAS).

Indole-3-butyric acid was long considered a synthetic auxin. However, it is now known that its biosynthesis is closely related to its precursor, IAA, as well as to the endogenous production of ABA and stress conditions such as drought and osmotic imbalance (Ludwig-Müller, 2000). In this sense, the results obtained in this study are consistent with the higher production of IAA and IBA in AS, which, along with their possible association with the ABA produced due to the stress from accelerated aging, could explain the lower germination percentages observed in AS compared to NAS.

The effects of accelerated aging on seed protein content have yielded contradictory results in the literature. In some cases, this process does not alter total protein levels, whereas in others, it leads to a significant reduction. These discrepancies are closely related to the specific stress conditions imposed and the intrinsic ability of each species to tolerate deterioration (Rajjou *et al*., 2008; Wang *et al*., 2022). In this study, protein content remained unchanged between the evaluated seed groups (Fig. 2F). It is possible that the temperature and humidity parameters used were intense enough to induce changes in germination (Fig. 1) and in more sensitive compounds, such as sugars (Fig. 2A), without affecting total protein abundance. The antioxidant activity of polyphenols may have been sufficient to prevent protein degradation. Although the low germination rate in AS suggests possible embryo mortality, the ability to germinate after seed coat rupture indicates that not all embryos are dead. Therefore, in future studies, it would be advisable to use tetrazolium analysis to more accurately assess their viability. In *C. quitensis*, various heat shock proteins (HSPs) have been identified (Bertini *et al*., 2022), and their synthesis may have increased in response to the thermal stress induced by accelerated aging. These proteins could have played a crucial role in stabilizing and protecting other proteins, thereby mitigating structural and functional damage during the deterioration process.

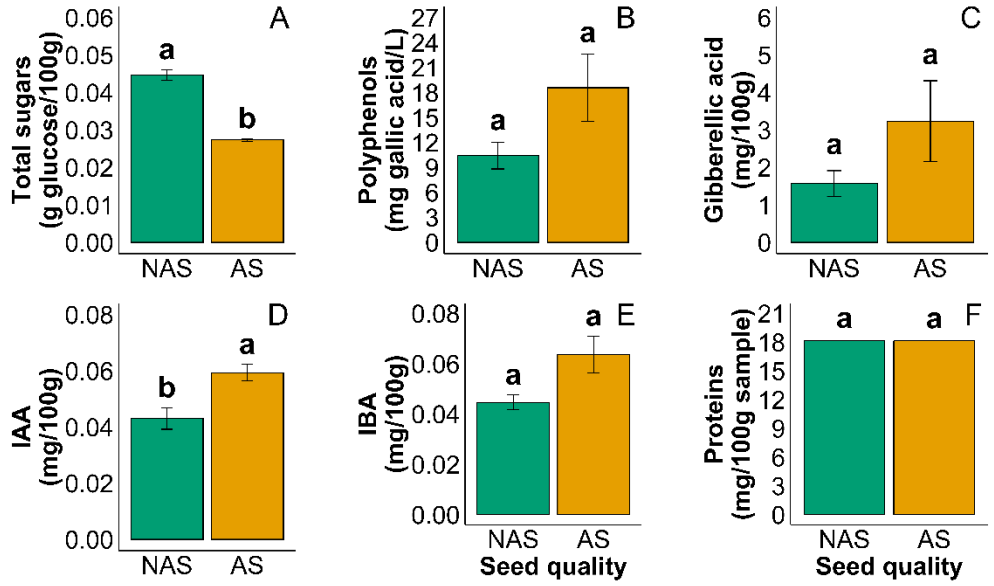


Figure 2. Seed germination indices of *Colobanthus quitensis* with different physiological qualities. A) Germination percentage; B) Mean germination time; C) Time to reach 50% germination; and D) Germination speed coefficient. Different letters indicate statistical differences according to the Student's t-test ($p<0.05$, $n=5$). NAS: non-aged seeds; AS: aged seeds.

This study demonstrates that accelerated aging negatively impacts the physiological quality of *C. quitensis* seeds, as evidenced by a significant reduction in germination indices. However, a subsequent germination test following mechanical scarification revealed that the species exhibits dormancy-related germination constraints. This finding underscores the need for refining its *in vitro* germination protocol to optimize seedling establishment and maximize viability. Based on this study, it can be considered that the combination of soluble sugar analysis and indoleacetic acid are potential metabolic markers that can indicate seed vigor and its potential aging degree. These findings are of great importance for the rapid evaluation of seeds, which can be used in agronomic studies and seed quality control processes for commercialization and conservation. This is also the first report on the indole-3-acetic acid content in *C. quitensis* seeds, highlighting the need for further studies to determine whether the concentration of this hormone, along with others such as ABA, is related to dormancy in this species.

4. REFERENCES

Bertini L, Proietti S, Fongaro B, Holfeld A, Picotti P, Falconieri GS, Bizzarri E, Capaldi G, Polverino de Laureto P, Caruso C (2022) Environmental Signals Act as a Driving Force for Metabolic and Defense Responses in the Antarctic Plant Colobanthus quitensis. Plants, 11(22):3176. https://doi.org/10.3390/plants11223176

Clemente-Moreno MJ, Omranian N, Sáez P, et al (2020) Cytochrome respiration pathway and sulphur metabolism sustain stress tolerance to low temperature in the Antarctic species Colobanthus quitensis. New Phytol 225:754-768. https://doi.org/10.1111/nph.16167

Cuba-Díaz M, Castel K, Acuña D, Machuca A, Cid I (2017a) Sodium chloride effect on Colobanthus quitensis seedling survival and in vitro propagation. Antarct Sci 29:45–46. https://doi.org/10.1017/S0954102016000432.

Cuba-Díaz M, Klagges M, Fuentes-Lillo E, Cordero C, Acuña D, Opazo G, Troncoso-Castro JM (2017b) Phenotypic variability and genetic differentiation in continental and island populations of Colobanthus quitensis (Caryophyllaceae: Antarctic pearlwort). Polar Biol 40:2397–2409. https://doi.org/10.1007/s00300-017-2152-x

Cuba-Díaz M, Acuña D, Fuentes-Lillo E (2019) Antarctic pearlwort (Colobanthus quitensis) populations respond differently to pre-germination treatments. Polar Biol 42:1209–1215. https://doi.org/10.1007/s00300-019-02505-4

Dadlani M, Yadava D (eds) (2023) Seed Science and Technology: Biology, Production, Quality. Springer Nature, Singapore.

Fenollosa E, Jené L, Munné-Bosch S (2020) A rapid and sensitive method to assess seed longevity through accelerated aging in an invasive plant species. Plant Methods 16:64. https://doi.org/10.1186/s13007-020-00607-3

Gianoli E, Inostroza P, Zúñiga-Feest A, Reyes-Díaz M, Cavieres LA, Bravo LA, Corcuera LJ (2004) Ecotypic differentiation in morphology and cold resistance in populations of Colobanthus quitensis (Caryophyllaceae) from the Andes of Central Chile and the Maritime Antarctic. Arct Antarct Alp Res 36(4):484–489. https://doi.org/10.1657/1523-0430(2004)036[0484:EDIMAC]2.0.CO;2

Gielwanowska I, Bochenek A, Gojlo E, Górecki R, Kellmann W, Pastorczyk M, Szczuka E (2011) Biology of generative reproduction of Colobanthus quitensis (Kunth) Bartl. from King

George Island, South Shetland Islands. Pol Polar Res 32:139–155. https://doi.org/10.2478/v10183-011-0008-6

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Biochem 48:909–930. https://doi.org/10.1016/j.plaphy.2010.08.016

Gong D, He F, Liu, J, Zhang C, Wang Y, Tian S, Sun C, Zhang X (2022) Understanding of Hormonal Regulation in Rice Seed Germination. Life 12(7):1021. https://doi.org/10.3390/life12071021

Gupta S, Hrdlička J, Kulkarni M, Doležalova I, Pěnčík A, Van Staden J, Novák O, Doležal K (2024) Karrikinolide1 (KAR1), a Bioactive Compound from Smoke, Improves the Germination of Morphologically Dormant Apium graveolens L. Seeds by Reducing Indole-3-Acetic Acid (IAA) Levels. Plants 13:2096. https://doi.org/10.3390/plants13152096

Huang Y, Lu M, Wu H, Zhao T, Wu P, Cao D (2021) High Drying Temperature Accelerates Sunflower Seed Deterioration by Regulating the Fatty Acid Metabolism, Glycometabolism, and Abscisic Acid/Gibberellin Balance. Front Plant Sci 12:628251. https://doi.org/10.3389/fpls.2021.628251

Koc J, Androsiuk P, Chwedorzewska KJ, Cuba-Díaz M, Górecki R, Giełwanowska I (2018) Rangewide pattern of genetic variation in Colobanthus quitensis. Polar Biol 41:2467–2479. https://doi.org/10.1007/s00300-018-2383-5

Lecube ML, Noriega GO, Santa Cruz DM, Tomaro ML, Batlle A, Balestrasse KB (2014) Indole acetic acid is responsible for protection against oxidative stress caused by drought in soybean plants: The role of heme oxygenase induction. Redox Rep 19(6):242–250. https://doi.org/10.1179/1351000214Y.0000000095

Liu YH, Offler CE, Ruan YL (2013) Regulation of fruit and seed response to heat and drought by sugars as nutrients and signals. Front Plant Sci 4:282. https://doi.org/10.3389/fpls.2013.00282

Ludwig-Müller J (2000) Indole-3-butyric acid in plant growth and development. Plant Growth Regul 32:219–230. https://doi.org/10.1023/A:1010746806891

Rocha R, Khalil Y, Maity A, Beckie HJ, Ashworth MB (2022) Mechanical scarification technique breaks seed coat-mediated dormancy in wild oat (Avena fatua). Weed Technol 36:152–159. https://doi.org/10.1017/wet.2021.94

Min K, Sulaiman S, Jeong J, Lee H, Lee J, Lee JH, Lee H (2024) Exploring the mechanisms underlying recovery from freeze–thaw injury in Colobanthus quitensis: mechanistic insights via transcriptome profiling. Physiol Plant 176(6):e14642. https://doi.org/10.1111/ppl.14642

Moore DM (1970) Studies in Colobanthus quitensis (Kunth) Bartl. and Deschampsia antarctica Desv. II. Taxonomy, distribution and relationships. Brit Antarct Surv Bull 23:63–80.

Ontivero Y, Cuba-Díaz M, Fuentes-Lillo E, Convey P (2024a) Germination Strategies and Seed Quality of Colobanthus quitensis: Implications for Sustainable Antarctic Ecosystems and Ex Situ Plant Conservation. Sustainability 16, 10726. https://doi.org/10.3390/su162310726

Ontivero Y, Fuentes-Lillo E, Navarrete-Campos D, Vázquez-Villa D, Cabreras-Barjas G, Arroyo-Marín FB, Cuba-Díaz M (2024b) Preliminary assessment of seed heteromorphism as an adaptive strategy of Colobanthus quitensis under saline conditions. Sci Rep 14:31120. https://doi.org/10.1038/s41598-024-82381-z

Ontivero Y, Salgado C, Fuentes-Lillo E, Sanhueza C, Navarrete-Campos D, Alveal S, Cuba-Díaz M (2025) Improved in vitro germination of Colobanthus quitensis: a key step for Antarctic plant conservation. PCTOC (in press).

Rajjou L, Lovigny Y, Groot SP, M Belghazi, Job C, Job D (2008) Proteome-wide characterization of seed aging in Arabidopsis: A comparison between artificial and natural aging protocols. Plant Physiol 148:620–641. https://doi.org/10.1104/pp.108.123141

Ramaih S, Guedira M, Paulsen GM (2003) Relationship of indoleacetic acid and tryptophan to dormancy and preharvest sprouting of wheat. Funct Plant Biol 30(9):939 – 945. https://doi.org/10.1071/FP03113

Sachdev S, Ansari SA, Ansari MI (2023) Role of Osmolytes in Alleviation of Oxidative Stress. In Reactive Oxygen Species in Plants. Springer, 173–202.

Salgado C (2023) Estrategias para promover la germinación in vitro de Colobanthus quitensis (Kunth.) Bartl. (Caryophyllaceae). (Tesis de Ingeniería en Biotecnología Vegetal). Universidad de Concepción, Chile.

Taylor AG (2020) Seed storage, germination, quality, and enhancements. In: Wien, HC, Stützel H (eds) Physiology of vegetable crops. 2nd edition. CAB International.

Wang B, Yang R, Ji Z, Zhang H, Zheng W, Zhang H, Feng F (2022) Evaluation of Biochemical and Physiological Changes in Sweet Corn Seeds under Natural Aging and Artificial Accelerated Aging. Agronomy 12(5):1028. https://doi.org/10.3390/agronomy12051028

Declaration Section

Authors contributions

YO, VC, and DN participated in data collection. YO and MCD carried out the analysis and interpretation of data. YO and MCD wrote the first draft of the manuscript. YO, VC, DN, and MCD revised the final manuscript.

Funding

This research was funded by the National Agency for Research and Development (ANID, Chile) through the National Doctorate Scholarship Program (2021–21210100), the Chilean Antarctic Institute (INACH) project DT_05_22, and the Doctoral Program in Agricultural Sciences, Faculty of Agronomy, University of Concepción, Campus Chillán (2021300031, YO). MCD was supported by project 2023000418ESP from VRID-UdeC.

Competing interests

The authors declare no competing interests.

Data Availability

The data of this study are available from the corresponding author upon request.

VI. CHAPTER V: ENDOPHYTIC FUNGI INOCULATION AND REPRODUCTION OF *Colobanthus quitensis*: IMPLICATIONS FOR SEED PRODUCTION AND CONSERVATION

Yadiana Ontivero[1,2], Daniel Chávez[3], Cristian Deramond[3], Patricio Ramos[4,5], Darío Navarrete-Campos[1], Marely Cuba-Díaz[1,6*]

[1]Laboratorio de Biotecnología y Estudios Ambientales, Departamento de Ciencias y Tecnología Vegetal, Escuela de Ciencias y Tecnologías, Universidad de Concepción, Campus Los Ángeles, 4440000, Concepción, Chile

[2]Facultad de Agronomía, Universidad de Concepción, Campus Chillán, 3812189, Chillán, Chile

[3]Laboratorio de Investigación de Micorrizas y Aplicaciones Biotecnológicas de Hongos, Departamento de Ciencias y Tecnología Vegetal, Universidad de Concepción, Campus Los Ángeles, Juan Antonio Coloma 0201, Los Ángeles 4440000, Chile

[4]Plant Microorganism Interaction Laboratory, Instituto de Ciencias Biológicas, Campus Talca, Universidad de Talca, Talca, Chile

[5]Vicerrectoría de Investigación y Postgrado, Universidad Católica del Maule, Talca, Chile

[6]Programa de Ciencia Antártica y Subantártica, Universidad de Concepción, 4030000, Concepción, Chile

* Correspondence author: mcuba@udec.cl

ABSTRACT

Microorganisms play a fundamental role in plant development. Under natural conditions, *Colobanthus quitensis* establishes symbiotic relationships with various fungi that contribute to its survival under extreme environments. However, when conserved *ex situ*, these associations may be altered, potentially affecting its reproduction and seed quality. This study aimed to answer two key questions: (1) What is the effect of endophytic fungal inoculation on the reproduction of *C. quitensis*? and (2) What benefits does inoculation with endophytic fungi provide for seed germination and long-term conservation? To address these questions, *C. quitensis* plants were inoculated with *Trichoderma harzianum* and *Hypocrea lixii*, two fungal strains previously isolated from the roots of this species. The effects on plant phenology, seed quality, and seed conservation over six months were subsequently evaluated. The results showed that the fungi influenced each population differently, affecting flowering time, peduncle development, and the number of seeds per flower. The fungal treatments also altered certain seed traits, although they did not significantly affect germination rates. Regarding germination, the seeds from inoculated plants exhibited varied responses. This research reveals that

Antarctic populations display extremely low germination rates, likely due to seed dormancy. However, seeds from the Byers Peninsula and Conguillío populations showed improved germination after storage at temperatures between 4°C and –80°C. Seeds from the La Marisma population tolerated these storage conditions, although germination percentages did not improve. Identifying the metabolites and hormones produced by these fungi, along with understanding their role in nutrient solubilization, is essential for the development of a biotechnological product with potential agronomic applications.
Key words: seed characteristics, germination, conservation temperature, endophytic fungi

1. INTRODUCTION

Colobanthus quitensis has a broad distribution, extending from southern Mexico to the northern Antarctic Peninsula, where it is consistently associated with extreme environmental conditions (Moore, 1970; Smith, 2003). Its ability to tolerate habitats with adverse climates has driven the development of various survival strategies, including high phenotypic plasticity (Gianoli *et al*., 2004; Cuba-Díaz *et al*., 2017; Fuentes-Lillo *et al*., 2017), photoprotective mechanisms that mitigate the impact of intense ultraviolet radiation (Pérez-Torres *et al*., 2007; Contreras *et al*., 2019), and the accumulation of osmoprotective compounds that enhance tolerance to low temperatures (Clemente-Moreno *et al*., 2020; Ramírez *et al*., 2024). Additionally, modifications in the species' epigenetic regulation have been documented, specifically through variations in the methylation of genes involved in the cold response (Hereme *et al*., 2025).

The association between plants and endophytic fungi plays a fundamental ecological and adaptive role, promoting symbiosis rather than leaving plants as isolated entities within the ecosystem (Hassani *et al*., 2018). These symbiotic relationships enhance plant fitness by improving nutrient solubilization, stimulating growth through metabolic compound production, and providing protection against stress conditions (Bai *et al*., 2017; Chagas *et al*., 2018). Although the effect of endophytic fungi on a given species depends on multiple factors, they can influence seed traits, germination, longevity, and seedling survival (Gundel *et al*., 2009; Barrera *et al*., 2020; Ueno *et al*., 2020).

In field-collected *C. quitensis* plants, a wide diversity of endophytic fungi has been identified, belonging to the genera *Aspergillus, Alternaria, Cadophora, Cladosporium, Davidiella,*

Entrophospora, Eupenicillium, Fusarium, Geomyces, Gyoerffyella, Lenzites, Leucosporidium, Microdochium, Mollisia, Mycocentrospora, Penicillium, Peniophora, Phaeosphaeria, Phlebia, Sistotrema, Trametes, and *Ypsilina* (Rosa *et al*., 2010; Hereme *et al*., 2020; Bertini *et al*., 2022). These associations contribute to the activation of molecular mechanisms that enhance plant adaptation and performance (Barrera *et al*., 2020; Acuña-Rodríguez *et al*., 2024), enabling them to cope with water stress (Hereme *et al*., 2020; Morales-Quintana *et al*., 2021, 2024), rising temperatures (Ballesteros *et al*., 2020), ultraviolet radiation (Barrera *et al*., 2020), and contamination by organic compounds (Egas *et al*., 2025). These interactions may represent a key factor in the survival of this species under the extreme conditions of Antarctica. The fungal community within plants of the same species is not random and tends to vary significantly depending on habitat, soil type, the presence of environmental microbes, plant developmental stage, plant tissues, and climatic conditions, both within and across years (Collado *et al*., 1999; Bonito *et al*., 2014; Jiawen *et al*., 2022; Tang *et al*., 2022). In climate change simulation studies, where benign growth conditions in terms of water availability and temperature were imposed compared to the extreme conditions of Antarctica, the presence and function of these fungi in *C. quitensis* decreased (Torres-Díaz *et al*., 2016). Since 2014, an active collection of Antarctic vascular plants has been maintained, where different populations of *C. quitensis* are preserved *in vitro* and under controlled common garden conditions (Ontivero *et al*., 2024a). In *ex situ* populations of *C. quitensis*, low germination rates have been observed, which have been linked to physiological deterioration due to prolonged storage or seed dormancy (Gielwanowska *et al*., 2011; Cuba-Díaz *et al*., 2019; Ontivero *et al*., 2024b). However, until now, the impact of endophytic microorganisms on seed reproduction and quality over time and under different storage temperatures has not been considered. The marked differences between field and laboratory growth conditions may have led to the disappearance of original symbiotic relationships or the establishment of new interactions that do not support the reproductive process or seed quality.

Preliminary laboratory observations suggest that *C. quitensis* seeds have difficulty germinating after being stored for one year at 4°C, a standard storage temperature for various crops and ornamental plants (Singh *et al*., 2016; Hernández *et al*., 2020; Kompe *et al*., 2020; Souza *et al*., 2020). The use of colder temperatures has contributed to maintaining and prolonging the viability of orthodox seeds (Chmielarz, 2010). For this reason, international germplasm banks

recommend temperatures around -20°C for long-term seed conservation (FAO, 2014; Singh *et al*., 2016; Asdal *et al*., 2019). However, some species may require even lower temperatures, between -80°C and -196°C, also known as cryopreservation, to ensure their long-term viability (Ballesteros *et al*., 2021).

Under natural conditions, Antarctic populations of *C. quitensis* tolerate ambient temperatures below 0°C for most of the year (Gianoli *et al*., 2004; Bascuñán-Godoy *et al*., 2010; Turner *et al*., 2020). As a result, Antarctic soils tend to remain frozen or covered with snow for most of the time (Potapowicz *et al*., 2019), and under these conditions, viable seed banks persist in the soil (Kellmann-Sopyła & Gielwanowska, 2015). Therefore, *ex situ* seed storage at temperatures below 0°C could be a viable conservation strategy to maintain or extend the viability of *C. quitensis* seeds, while also potentially helping to break dormancy. This is possible because, as orthodox seeds (Kellmann-Sopyła & Gielwanowska, 2015), they may benefit from preservation at lower temperatures.

In agriculture, the inoculation of plants with endophytic fungi is a practice that can enhance plant growth, flowering, productivity, and germination (Frericks *et al*., 2018). This strategy could serve as a viable alternative to optimize the *ex situ* reproductive process of *C. quitensis*, contributing to its conservation. To evaluate the effect of endophytic fungal inoculation on the reproductive phenology and physiological seed quality of *C. quitensis*. Based on the presented background, this study hypothesizes that inoculating *C. quitensis* populations with endophytic fungi isolated from plants in their natural habitat improves the species' reproductive parameters and seed quality. This study aims to answer two key questions: (1) What is the effect of endophytic fungal inoculation on the reproduction of *C. quitensis*? and (2) What benefits does inoculation with endophytic fungi provide for seed germination and long-term conservation?

2. MATERIALS AND METHODS

2.1 Plant Material

For the search of endophytic fungi, plants of *C. quitensis* from both *in vitro* cultivation and common garden were analyzed. The studied populations corresponded to the Byers Peninsula (pBy) (Livingston Island, South Shetland Islands; 62°40'S; 60°55'W), La Marisma (pPA) (Punta Santa María, south of Punta Arenas, Chile; 53°22'S; 70°58'W), and Conguillío (pC) (Conguillío National Park, La Araucanía Region, Chile; 38°36'S; 71°36'W) (Cuba-Díaz *et al*., 2017b).

The *in vitro* plants were maintained in growth chambers at 20 ± 2 °C, with light intensity ranging from 28 to 45 µmol m^{-2} s^{-1} and a photoperiod of 16 hours of light and 8 hours of darkness. The culture medium contained Murashige & Skoog (MS) salts and vitamins at 100%, 3% sucrose, 0.5 mg L^{-1} of 6-benzylaminopurine, 0.25 mg L^{-1} of indoleacetic acid, 7 g L^{-1} of agar, 10 µM of silver thiosulfate, and a pH adjusted to 5.7 (Cuba-Díaz *et al*., 2014, 2020). On the other hand, the common garden plants were grown in growth chambers with a substrate composed of peat, leaf soil, and vermiculite in a 2:3:1 ratio. They were kept at a temperature of 13 ± 2 °C, with a photoperiod of 16/8 hours light/dark, light intensity of 100-120 µmol photons m^{-2} s^{-1}, and relative humidity of 75% (Cuba-Díaz *et al*., 2017).

For inoculation with endophytic fungi, the plants from the mentioned populations, with one year of *in vitro* cultivation, were propagated again and maintained under these conditions for 3 months to standardize the material to be used. These plants were acclimated to the common garden for three weeks under the same conditions previously described, using a substrate sterilized twice at 120 atm for 20 minutes and fertilized every two weeks with Phostrogen (NPK 13:10:27). For the experiment, a total of six pots with five plants per population were arranged. The seeds from the experimental plants 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 for varying times and temperatures until analysis according to the experimental conditions explained below.

2.2 Search for endophytic fungi

To select plant material for inoculation assays, it was essential to use endophyte-free plants. Therefore, root samples were taken from ten plants per population, cultivated either *in vitro* or in a common garden (positive control), and stained with 0.05% trypan blue (Phillips and Hayman, 1970). The roots were washed three times with distilled water to remove any remaining *in vitro* culture medium or substrate, depending on the case, and then preserved in 70% ethanol. Before staining, the ethanol was discarded, and the roots were thoroughly rinsed with distilled water. Bleaching was not required for *in vitro*-grown plants since their root systems are thinner and lighter in color than those of common garden plants (Figure 1 a, b). In contrast, roots from common garden plants were bleached using 20 mL of 10% potassium hydroxide (KOH) for five minutes in a temperature-controlled water bath at 95°C.

Subsequently, both types of roots were acidified with 20 mL of 2% hydrochloric acid (HCl) for one minute at room temperature. After removing the HCl, the samples were stained with 15 mL of 0.1% trypan blue in lactic acid for ten minutes in a 90°C water bath. The stain was then removed, and the roots were preserved in lactoglycerol, in a 1:1:1 ratio of lactic acid, glycerol, and distilled water, until analysis. To search for fungal structures, ten root fragments measuring 0.5 to 1 cm in length were selected (Rothen, 2015).

2.3 Plant Inoculation with Endophytic Fungi

The endophytic fungi *Trichoderma harzianum* and *Hypocrea lixii*, isolated previously from the roots of *C. quitensis* plants randomly collected from various locations near the Henryk Arctowski Polish Station in Admiralty Bay, King George Island (Rabert *et al*., 2020), were used for the inoculation experiment. These fungi were cultured in 250 mL of liquid medium with 1% malt extract for one month. For plant inoculation, the mycelium of each fungus was filtered, and the mycelial biomass was mixed with sterile distilled water and blended with a homogenyzer for 5 seconds to obtain the inoculum (Rivas, 2020).

Plants measuring 2 to 4 cm in size, with a maximum of two developed shoots, were selected for the experiment. Three experimental groups were established: Control group, receiving only 10 ml of distilled water, Treatment with *T. harzianum* or with *H. lixii*, Inoculated with 10 mL of homogenized mycelium along with the culture medium. Two inoculations were performed 15 days apart, with the inoculum applied at three points along the root system using a syringe to facilitate root colonization.

To assess the effect of the inoculum on reproduction, 20 randomly selected plants were evaluated for the number of flowers per plant. From this group, 50 flowers were selected, and the peduncle length, time to floral capsule opening, and number of seeds produced per Flower were measured.

To analyze the impact of the treatments on seed morphology and physiology, 50 seeds per population were photographed to determine area and perimeter using ImageJ software (NIH, Bethesda, MD; https://imagej.nih.gov/ij/). The weight of 100 seeds was measured using a RADWAG AS 220/C/2 analytical balance, with an error margin of ±0.1 mg, performing five replicates per population. To determine the water content (WC) at seed maturity, 10 mg of fresh seeds were dried in an oven at 105°C until a constant weight was reached (Brasil, 2009), with five replicates per population. The WC was calculated using the following equation:

$$WC = \frac{\text{FW}-\text{DW}}{\text{FW}}\ x\ 100$$

where FW represents the fresh weight of the seeds, and DW represents the dry weight.

2.4 Germination of seeds from inoculated plants and short-term conservation

A group of freshly produced seeds (<1 month since collection and stored at 4 ºC) from each inoculation treatment was used to evaluate their initial physiological quality (control) through an *in vitro* germination test. The remaining seeds were divided into three groups and stored for six months at 4, -20, and -80 ºC to later assess their germination. Before storage, the seeds designated for -20 and -80 ºC were gradually dried with silica gel for 3 to 4 days until reaching approximately 12% moisture to prevent freezing damage during the germination test. To achieve this moisture percentage, the seeds were dried in silica gel until they reached the desired moisture content (Sultana et al., 2021). This was verified using the following equation:

$$\%\ Moisture = \frac{Wi - Wf}{Wi} x100$$

Where *Wi* is the initial weight of the seeds, and *Wf* is the final weight of the seeds after drying.

For the *in vitro* germination test, the seeds from each experimental condition were disinfected with 70% (v/v) ethanol for 30 seconds in a vortex, followed by 5% (v/v) sodium hypochlorite for 7 minutes in a vortex. They were then rinsed three times with sterile distilled water (Cuba-Díaz *et al*., 2017a). Subsequently, 15 randomly selected seeds were placed in 10 cm diameter Petri dishes containing 20 mL of full-strength MS medium (Murashige & Skoog, 1962) supplemented with 3% sucrose, 0.7% agar, and adjusted to a pH of 5.7 for *in vitro* germination. The seeds were maintained at 20 ± 2 °C under a 16 h light/8 h dark photoperiod with a light intensity of 45 ± 2 µmol m^{-2} s^{-1} for 30 days. A seed was considered germinated when its radicle was at least twice the size of the seed (Sanhueza *et al*., 2017).

Based on the collected data, the following parameters were calculated:

1. Germination percentage:

$$G(\%)=(N/A)\times 100$$

Where: *N* = number of germinated seeds at the end of the test; *A* = total number of seeds subjected to germination.

2. Mean Germination Time (MGT):

$$MGT=\sum(ni\times t)/n$$

Where: n_i = number of seeds germinated on day t; n = total number of germinated seeds in the experiment.

2.5 Statistical analysis

To evaluate the effect of fungal inoculation on the reproductive parameters of different populations, as well as on the morphological and physiological characteristics of their seeds, one-way analysis of variance (ANOVA) was performed whenever the assumptions of normality and homogeneity of variances were met. When these assumptions were not satisfied, the non-parametric Kruskal–Wallis test was used to detect differences between groups.

To analyze the combined effect of fungal inoculation and storage temperature on germination percentage and mean germination time, a two-factor experimental design was used. For statistical analysis, data expressed as percentages were transformed using the arcsin(√%/100) function to meet normality assumptions. When the assumptions of normality and homogeneity of variances were verified, a two-way ANOVA was applied; otherwise, the Kruskal–Wallis test was used. Subsequently, post hoc tests were conducted to identify significant differences between groups. For parametric analyses, Tukey's Honestly Significant Difference (HSD) test was applied, while for non-normally distributed data, Dunn's test with Bonferroni correction was used. In all cases, a 95% confidence level was considered.

Statistical analyses were performed in RStudio (R Core Team, 2023), using the *aov*() function for ANOVA and kruskal.test() for non-parametric analyses. Finally, graphs were generated using the ggplot2 package (Wilkinson, 2011).

3. RESULTS

The search for endophytic fungi in the roots of *C. quitensis* plants grown *in vitro* (Fig. 1a) and in a common garden (Fig. 1b) revealed that the association of this species with endophytic fungi is strongly influenced by its cultivation method. In plants grown *in vitro*, no fungal structures were identified (Fig. c-e), making them suitable for the endophytic fungal inoculation process. In contrast, a great variety of endophytic fungi were found in the roots of plants from the common garden. Hyphae and spores at different developmental stages were detected in the roots (Fig. 1 f-m), and specimens probably of *Chytridium* spp. (Fig. 1k-l) and *Fusarium* spp. (Fig. 1m) were identified based on their morphology.

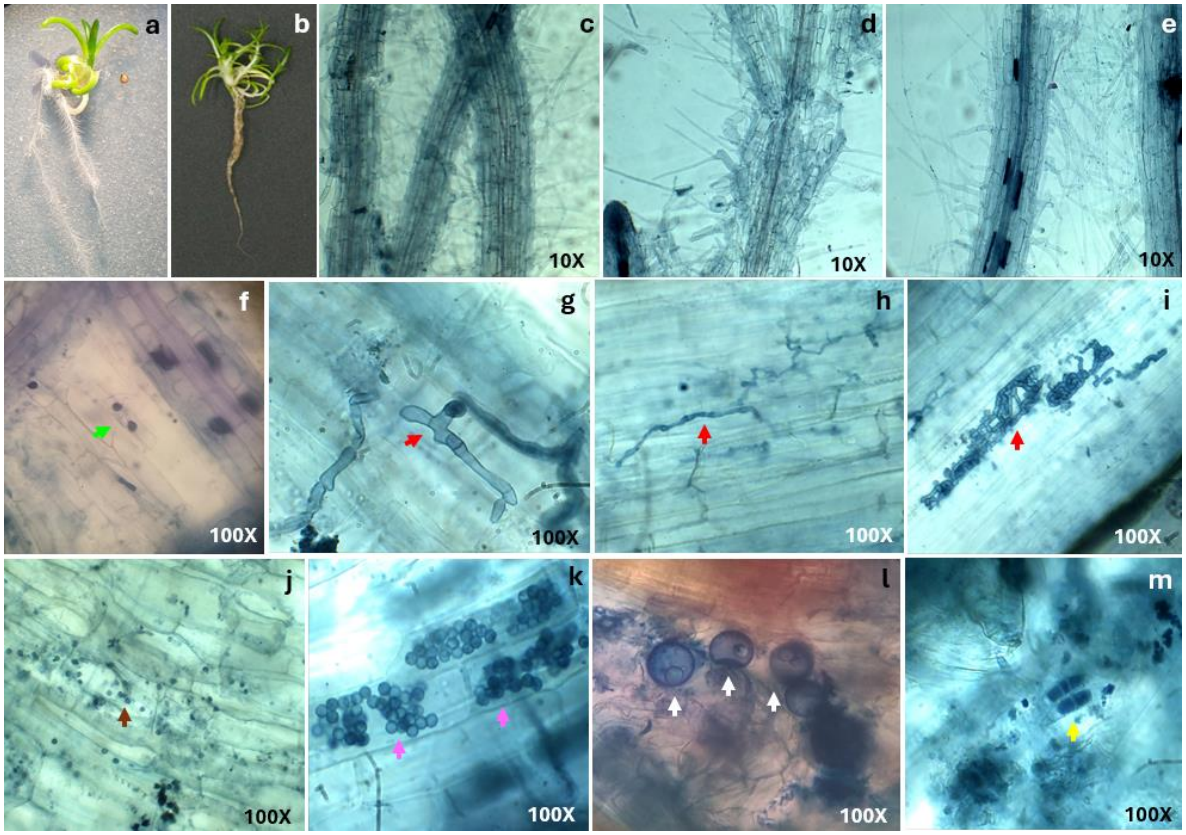


Figure 1. Identification of endophytic fungi in the roots of *Colobanthus quitensis* plants grown *in vitro* and in a common garden. Color difference in the roots between a) a plant grown *in vitro* and b) another in a common garden. c-e) roots of *in vitro*-grown plants free of endophytic fungi. f-m) presence of endophytic fungi in the roots of common garden plants. f) colonization of adjacent cells (green arrow); g-i) hyphae colonizing different cells (red arrow); j) fungal spores; k) sclerotium (pink arrow); l) *Chytridium* spp. spores; m) *Fusarium* spp. All populations exhibited the same fungi; therefore, the figure presents a representation of this pattern.

The effect of endophytic fungal inoculation on the reproductive parameters of the plants varies depending on the population (Table 1). In the Antarctic population of Byers Peninsula, no significant changes were observed in floral opening, the number of floral capsules, or seed production with fungal inoculation, although *Trichoderma* increased peduncle length. However, when analyzing the experiment as a global phenomenon, it is evident that plants in the control treatment produced a total of 115 open floral capsules, while *Trichoderma* and *Hypocrea* produced only 93 and 86, respectively, which has a significant biological impact on the conservation of the population.

In the Subantarctic population pPA, *Trichoderma* reduced the time required for floral opening ($p<0.001$) and lengthened the peduncle without affecting other reproductive parameters. In this population, the control treatment generated 154 open floral capsules, whereas *Trichoderma* and *Hypocrea* led to a higher production of 238 and 228 capsules, respectively.

In the Andean population Conguillío, *Hypocrea* delayed floral opening compared to *Trichoderma* and promoted peduncle growth ($p<0.05$). Both fungi reduced the number of flowers per plant, but *Trichoderma* significantly increased seed number per floral capsule ($p<0.001$) (Table 1). In this population, the control treatment produced 175 open floral capsules, while both *Trichoderma* and *Hypocrea* resulted in a lower number, with 102 capsules each.

Table 1: Influence of endophytic fungal inoculation on reproductive parameters in *Colobanthus quitensis* plants from different populations. Different letters indicate significant differences between treatments ($p<0.05$) according to Tukey-HSD post-hoc test or Dunn-Bonferroni post-hoc test (ǂ).

Population	**Inoculation**	**Time floral capsule opening (days)***	**Number floral capsule/plant****	**Peduncle size (mm)***	**No. seeds/floral capsule***
Byers Peninsula	Control	38 ± 5.53 ns	5.75 ± 3.27 ns	7.37 ± 2.74 b	31.06 ± 8.11 A
	Trichoderma	38.22 ± 5.21 ns	4.64 ± 2.06 ns	9.12 ± 2.66 a	27.18 ± 12.4 B
	Hypocrea	37.36 ±4.43 ns	4.3 ± 2.87 ns	7.8 ± 2.46 b	23.66 ± 10.87 B
La Marisma	Control	45.66 ± 5.33 a ǂ	7.7 ± 4.76 ns	19.8 ± 5.83 ab	26.32 ± 7.48 ns
	Trichoderma	38.32 ± 11.06 b ǂ	11.9 ± 7.15 ns	21.57 ± 5.06 a	25.68 ± 7.67 ns
	Hypocrea	46.62 ± 5.32 b ǂ	11.4 ± 6.82 ns	17.71 ± 5.98 b	27.02 ± 9.67 ns
Conguillío	Control	44.88 ± 4.42 ab	8.75 ± 2.57 a	24.91 ± 4.89 b ǂ	33.62 ± 9.66 b ǂ
	Trichoderma	43.6 ± 3.53 b	5.1 ± 2.27 b	24.48 ± 4.48 b ǂ	40.54 ± 7.74 a ǂ
	Hypocrea	46.82 ± 5.39 a	5.1 ± 2.31 b	27.23 ± 4.59 a ǂ	37.4 ± 8.59 a ǂ

ns: no statistically significant differences between treatments; *: n=50; **: n=20.

The fungal treatment significantly affected seed area and perimeter, the weight of 100 seeds, and the relative water content in Byers Peninsula and La Marisma. In Conguillío, fungi influenced all the analyzed seed traits except for the weight of 100 seeds (Table 2).

Table 2. Parametric and non-parametric analyses used to evaluate the effect of fungal inoculation with *Trichoderma harzianum* and *Hypocrea lixii* in three populations of *Colobanthus quitensis* on seed morphological and physiological characteristics.

Population	Statistical analysis type	Dependent variable	Independent variable	Statistical parameters			
				df	SS	F	p-value
Byers Peninsula	Parametric test	Seed area	Fungal	2	0.0125	3.46	0.034*
			Error	147	0.2664		
	Non-parametric test	Seed Perimeter	Fungal	2	-	-	0.033*
	Parametric test	100 seeds weight	Fungal	2	0.0011	14.94	0.00055***
			Error	12	0.0005		
	Parametric test	Water content	Fungal	2	20.861	19.77	0.00016***
			Error	12	6.332		
La Marisma	Parametric test	Seed area	Fungal	2	0.1595	20.74	0.00000***
			Error	147	0.5654		
	Non-parametric test	Seed Perimeter	Fungal	2	-	-	0.00000***
	Parametric test	100 seeds weight	Fungal	2	11.89	5.42	0.021*
			Error	12	13.16		
	Parametric test	Water content	Fungal	2	71.68	41.66	0.00000***
			Error	12	10.32		
Conguillío	Parametric test	Seed area	Fungal	2	0.1043	19.34	0.00000***
			Error	147	0.3966		
	Parametric test	Seed Perimeter	Fungal	2	0.0895	27.86	0.00000***
			Error	147	0.2362		
	Parametric test	100 seeds weight	Fungal	2	0.0173	0.063	0.939
			Error	12	1.6520		
	Parametric test	Water content	Fungal	2	74.34	12.38	0.00121**
			Error	12	36.03		

Parametric test: ANOVA; Non-parametric test: Kruskal-Wallis; df: degree of freedom; SS: sum of squares; F: test F; significant differences were determined at $p < 0.05$*; $p < 0.01$** and $p < 0.001$***.

Fungal inoculation had a significant effect on seed area (Fig. 2a) and perimeter (Fig. 2b), with variations depending on the population. In the Antarctic Byers Peninsula population, *Hypocrea* reduced the seed area compared to the control and perimeter compared to *Trichoderma*. In the Subantarctic La Marisma population, *Trichoderma* promoted an increase in seed area and perimeter compared to the other treatments. In contrast, in the Andean population pC, both fungi contributed to a larger seed area, while *Hypocrea* resulted in the greatest perimeter, followed by *Trichoderma*.

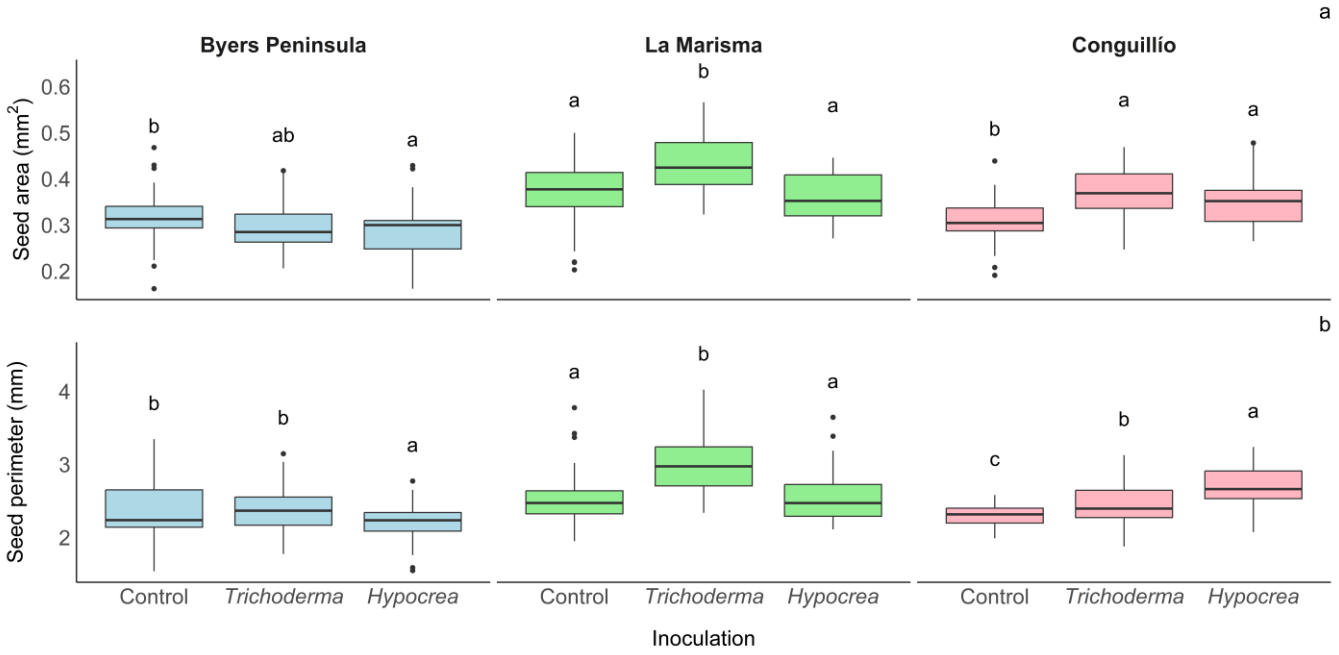


Figure 2. Effect of fungal inoculation on seed area (a) and perimeter (b) in different populations of *Colobanthus quitensis*. Different letters indicate significant differences (p<0.05) between treatments according to Tukey's test (n=50).

In the Byers Peninsula population, inoculation with endophytic fungi significantly increased both seed weight and WC at maturity. In contrast, in La Marisma, only *Trichoderma* led to a significant increase in seed weight and WC compared to the control. In the Conguillío population, inoculation with endophytic fungi did not affect seed weight, although *Hypocrea* contributed to a higher seed WC.

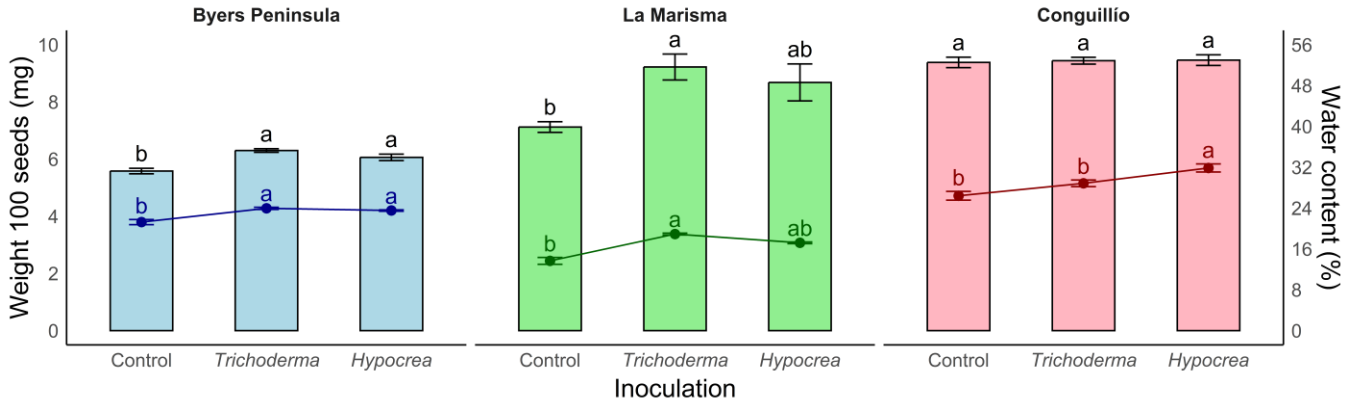


Figure 3. Physiological characteristics of seeds from three populations of *Colobanthus quitensis* obtained from non-inoculated plants (control) and plants inoculated with *Trichoderma harzianum* and *Hypocrea lixii*. The bar graph represents the weight of 100 seeds, while the line graph shows the seed water content. Different letters indicate significant differences ($p < 0.05$, $n = 5$) according to Tukey's HSD post-hoc test.

When analyzing the combined effect of fungal inoculation and storage temperature on germination and mean germination time (MGT), the results varied among populations (Table 3). For the *Byers* Peninsula population, only the temperature factor had a significant effect on both germination indicators (Table 3, Supplementary Figure 1A, B). In the La Marisma population, the control and *Trichoderma* treatments produced the highest germination percentages, although there were no statistically significant differences between these two treatments (Table 3). However, only the control treatment reduced the MGT (Supplementary Figure 1C, D). For *Conguillío* population, temperature was the factor that significantly influenced both germination percentage (Supplementary Figure 1E) and mean germination time (Table 3). The seeds stored for six months at 4°C had a lower MGT, followed by those stored at -80°C (Supplementary Figure 1F).

Table 3. Factorial analysis of the effect of plant inoculation with *Trichoderma harzianum* and *Hypocrea lixii* on the germination of seeds from three *Colobanthus quitensis* populations after storage at different temperatures (Control, 4, -20, and -80°C).

***Colobanthus quitensis* population**	**Statistical analysis**	**Dependent variable**	**Independent variable**	**Statistical parameters**			
				df	**SS**	**F**	**p-value**

Byers Peninsula	Parametric test	Germination	Fungal (F)	2	0.0114	1.363	0.269
			Temperature (T)	3	0.5505	43.966	**0.0000*** **
			FxT	6	0.0239	0.952	0.471
			Error	36	0.1503		
	Parametric test	MGT	Fungal (F)	2	0.502	0.161	0.8519
			Temperature (T)	2	12.797	4.106	**0.0282***
			FxT	4	4.991	1.601	0.2037
			Error	26	3.117		
La Marisma	Non-parametric test	Germination	Fungal (F)	2	-	-	**0.01033***
			Temperature (T)	3	-	-	0.7749
			FxT	6	-	-	0.3918
	Non-parametric test	MGT	Fungal (F)	2	-	-	**0.0003*** **
			Temperature (T)	3	-	-	**0.0130***
			FxT	6	-	-	**0.0002*** **
Conguillío	Parametric test	Germination	Fungal (F)	2	0.246	2.595	0.0886
			Temperature (T)	3	10.131	71.247	**0.0000*** **
			FxT	6	0.186	0.656	0.6855
			Error	36	1.706		
	Non-parametric test	MGT	Fungal (F)	2	-	-	0.5927
			Temperature (T)	3	-	-	**0.0000*** **
			FxT	6	-	-	**0.0006*** **

Parametric test: ANOVA; Non-parametric test: Kruskal-Wallis; df: degree of freedom; SS: sum of squares; F: test F; significant differences were determined at $p < 0.05$*; $p < 0.01$** and $p < 0.001$***.

The germination percentage of freshly collected seeds (stored for less than one month at 4ºC) from the Byers Peninsula population was significantly lower than that of seeds from the other treatments, with a trend observed in which seeds stored at -80ºC had slightly higher germination percentages (Fig. 4A). In the Antarctic population, a significant effect on MGT was detected only in seeds from plants inoculated with *Trichoderma*, specifically between those stored at 4ºC and -20ºC (Fig. 4B).

For La Marisma population, none of the combined treatments affected germination percentage, which remained high in all cases, exceeding 88% (Fig. 4C). The presence of these fungi did not contribute to a reduction in MGT compared to control treatment (Fig. 4D).

Finally, in Conguillío population, germination percentages after six months of storage were higher than in the control condition for all three inoculation treatments (control, *Trichoderma*, and *Hypocrea*) (Fig. 4E). Additionally, storage contributed to a reduction in MGT, with no significant differences between temperatures or inoculation conditions (Fig. 4F).

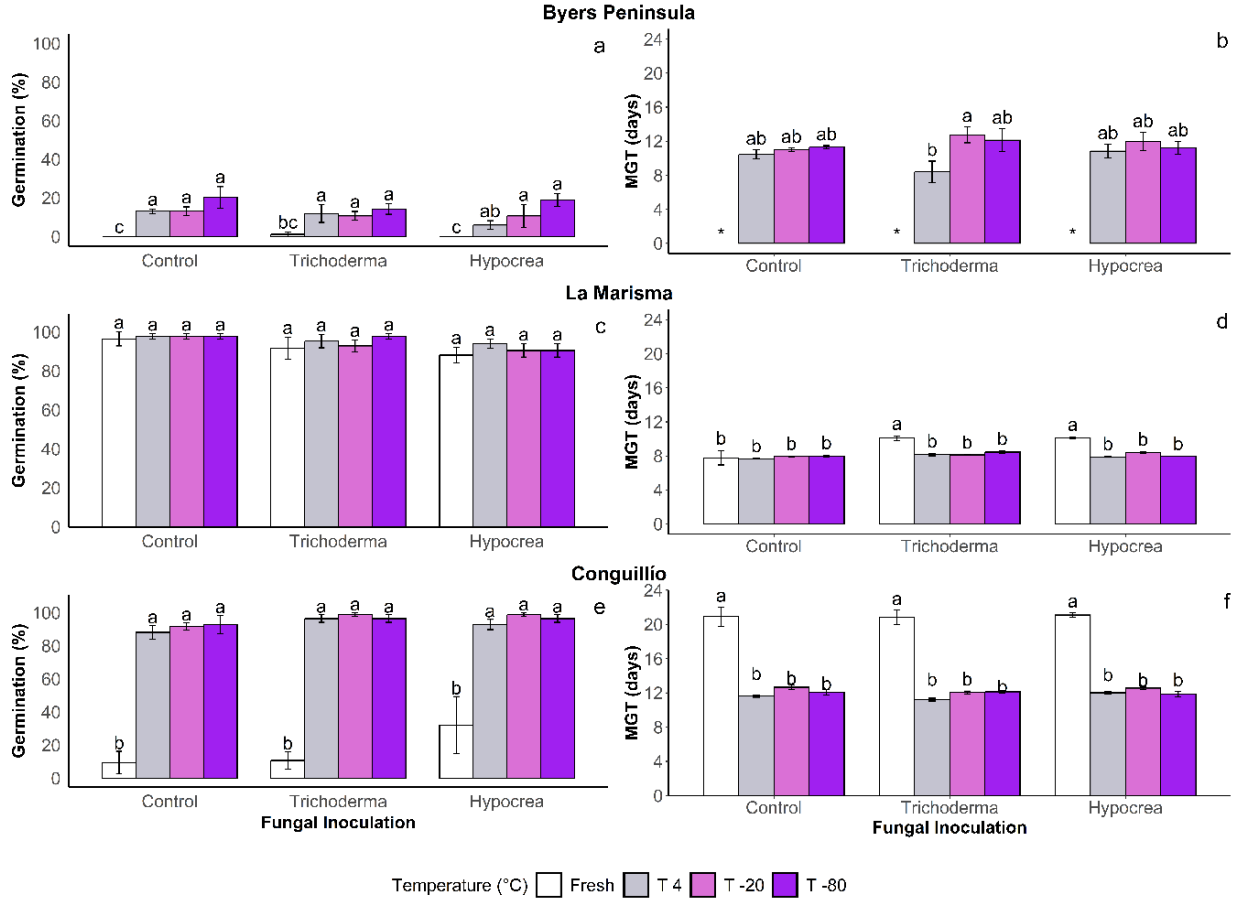


Figure 4. Combined effect of seed germination and storage temperature on three populations of *Colobanthus quitensis* from non-inoculated plants (Control) and plants inoculated with *Trichoderma harzianum* and *Hypocrea lixii*. Seeds were stored for either <1 month at 4ºC (Fresh) or for six months at 4, -20, and -80ºC. The effects were evaluated on (A) germination percentage and (B) mean germination time. Different letters indicate significant differences ($p < 0.05$) between treatments according to Tukey's HSD post-hoc test (Byers Peninsula, germination in Conguillío) or Dunn-Bonferroni test (La Marisma, MGT in Conguillío). * Indicates unavailable results due to missing data.

4. DISCUSSION

The use of endophytic fungi in agriculture is a beneficial practice aimed at promoting plant growth and productivity (Khan *et al*., 2021; Das et al., 2025) due to their role as phytohormone producers, their ability to solubilize nutrients, and their contribution to tolerance against biotic and abiotic stresses through the production of secondary metabolic compounds (Sánchez-Fernández *et al*., 2013; Baro and Rigobelo, 2022; Acuña-Rodríguez *et al*., 2024; Díaz-Valenzuela *et al*., 2024). Despite the proven effectiveness of these microorganisms, their role can vary considerably depending on the fungal species and strain used, the plant species and its genotypes, as well as the environmental conditions under which the inoculation is applied (Madawala, 2021; Díaz-Valenzuela *et al*., 2024; González-Ortega-Villaizán *et al*., 2024).

4.1 Fungal effects on plant reproduction and seed characteristics

The effects of inoculation on reproduction and seed characteristics varied among populations depending on the treatment used. The Byers Peninsula population benefited from the absence of inoculation, as the fungi only had a positive effect on the size of the floral peduncle. In contrast, the use of *T. harzianum* reduced the time required for the floral capsule to open in La Marisma and Conguillío populations (Table 1). In species such *Cucumis melo, Zea mays* and *Helianthus annuus,* it has been observed that inoculation with endophytic fungi accelerates the phenological process of flowering (Raya-Díaz *et al*., 2017; Kuzhuppillymyal-Prabhakarankutty *et al*., 2020; González-Mas *et al*., 2023). Since the floral capsules of *C. quitensis* open once the seeds are mature and ready to germinate (Ontivero *et al*., 2024a), the use of *T. harzianum* accelerates, at least in the La Marisma and Conguillío populations, the production of mature seeds. This contributes to the *ex situ* conservation of the species and reduces the time required for research. In crops of agricultural interest, earlier flowering has been correlated with increased fruit production, reducing the need for crop inputs (González-Mas *et al*., 2023).

Factors such as plant age, the size of the plant cushion, and the soil temperature and moisture conditions during the growth stage influence the number of flowers that *C. quitensis* plants can produce, with up to 28 flowers recorded per plant (Galleguillos *et al*., 2023; Ontivero *et al*., 2024a). In this study, the number of flowers ranged from 4 to 12 flowers (Table 1). A comparison between *C. quitensis* plants from seed disinfected to be free of endophytic fungi (E-) and non-disinfected seeds (E+) showed that the presence of endophytic fungi contributes to higher production of reproductive structures (Ballesteros *et al*., 2022). In this research, none

of the populations showed a significant benefit in their flowers per plant production. However, when considering the total number of flowers produced throughout the experiment, plants from La Marisma inoculated with endophytic fungi produced between 228 and 238 flowers, while the control only produced 154. In contrast, plants from the Byers Peninsula and Conguillío populations inoculated with endophytic fungi tended to decrease the number of flowers per plant (Table 3). In fungal inoculation studies, the use of endophytic fungus is not always aimed at increasing the number of flowers per plant (González-Mas *et al*., 2023). However, it is expected that this increased total flower production will lead to a greater number of seeds, which is an important aspect to explore further in future research on inoculation tests of these fungi in plants of agricultural interest.

Studies on the reproduction of *C. quitensis* show that it can produce between 12 and 43 seeds per floral capsule, which ensures the stability of its populations even in one of the most extreme conditions in the world, such as Antarctica (Moore, 1970; Edwards, 1974; Convey, 1996; Cuba-Díaz *et al*., 2017b). In this study, the number of seeds per floral capsule, regardless of the population, ranged from 23 to 40 seeds, which aligns with the reported ranges. However, in the Byers Peninsula population, the number of seeds decreased when endophytic fungi were used. In contrast, in the Conguillío population, the number of seeds increased when inoculated with *T. harzianum* (Table 1). Genetic studies on *C. quitensis* have shown that there is considerable genetic variability between different populations of the species, including those from Byers Peninsula and Conguillío (Koc *et al*., 2018). Therefore, the different responses in seed production among populations under experimental conditions could be influenced by the genetic characteristics of each population, as it has been reported that host plant–fungal interactions are primarily determined by their genotypes (Rai and Agarkar, 2016; Wu *et al*., 2020).

The genus *Trichoderma* has been classified as capable of producing indole acetic acid (IAA) (Ortuño et al., 2013; Kubiak et a., 2023), a phytohormone that regulates growth processes such as cell elongation, vascular tissue development, apical dominance, and fruit development and maturation (Kou et al., 2022). Among its species, *T. harzianum* has specifically been identified as an IAA producer (Bader et al., 2020; Mazrou et al., 2020). Therefore, it is logical to observe certain effects of this treatment on the reproductive phenology of the species. However, for *Hypocrea lixii*, the teleomorph of *T. harzianum*, little information is available, as

this fungus is primarily used as a biological control agent (Kiarie *et al*., 2020; Chebet *et al*., 2021; Muhorakeye et al. 2024). Nevertheless, a study of its genome revealed that it contains genes encoding nitrilases, enzymes capable of converting indole-3-acetonitrile into indole-3-acetic acid in plants (Piotrowski & Volmer, 2006).

For Byers Peninsula, the use of *T. harzianum* had the same effect as the control on seed size. In contrast, in La Marisma, the presence of this fungus resulted in larger seeds, while in Conguillío, the largest seeds were obtained with *Hypocrea*, followed by *Trichoderma* (Fig. 2). Achieving larger seeds generally promotes the development of larger embryos and more endosperm, factors that facilitate germination (Kokila *et al*., 2021). The increase in seed weight in inoculated plants has been associated in various crops with the biological nitrogen-fixing capability of endophytic fungi (Adeleke and Babalola, 2021). In this study, the metabolic profile or the effects of these fungi on soil nutrient availability were not determined. However, different strains of *T. harzianum* have been shown to enhance nutrient availability in the soil and modify the composition of beneficial bacterial communities in the rhizosphere (Silletti *et al*., 2021; Wei *et al*., 2023), which could have contributed to the increase in seed size. Further studies are needed on the role of these fungi in soil fertility, as this would help determine whether their use is sufficient to ensure optimal nutrient levels and eliminate chemical fertilization in this species or in its future agronomic application in the field. The seeds from the Byers Peninsula and La Marisma exhibited the same behavior regarding seed weight and moisture content, with the latter being higher when endophytic fungi were used. In Conguillío, although none of the treatments affected seed weight, those from plants inoculated with *H. lixii* matured with a higher WC.

In *Oryza sativa* plants inoculated with species of the genus *Trichoderma*, including *T. harzianum*, the production of heavier seeds has been reported (Abdel-Fattah *et al*., 2007; Gateta *et al*., 2023). From an agronomic perspective, the use of endophytic fungi from this genus could benefit crops intended for grain production for food purposes. The literature indicates that when a seed has a moisture content between 5 and 20%, it is most likely orthodox and, therefore, capable of resuming its metabolism when rehydrated (Kim, 2018; Silva *et al*., 2022; Trusiak *et al*., 2023). *Colobanthus quitensis* has been classified as such by the Seed Information Database: Royal Botanic Gardens, Kew (https://ser-sid.org/species/603ee8ea-d4ae-47e7-8d84-36d1162ec530). In this study, the moisture content

of the seeds from the three populations ranged between 16 and 32%, significantly higher than expected (Fig. 3). A high moisture content in seeds leads to greater long-term viability losses, so it is recommended to reduce the water percentage to between 5 and 12%, although this may vary slightly depending on whether the seed is oil-bearing or not (Roberts & Ellis, 1989). In this regard, there is no scientific evidence to classify this species into either of these groups.

4.2 Role of endophytic fungi and storage conditions in short-term seed quality

In this study, the inoculation of plants with endophytic fungi did not have a positive effect on germination. Previously, Ballesteros *et al*. (2022) demonstrated that in *C. quitensis*, the presence of seeds with endophytic fungi (E+) does not increase the germination percentage compared to fungus-free seeds (E-). However, it was observed that freshly collected seeds from Byers Peninsula and Conguillío had significantly lower percentages compared to seeds stored for six months at different temperatures (Fig. 4A, E). This behavior aligns with the low germination percentages reported in Antarctic populations of this species (Cuba-Díaz *et al*., 2017, 2019; Ontivero *et al*., 2024b), which have been associated with dormancy (Ruhland & Day, 2000; Gielwanowska *et al*., 2011). It is possible that cold period acted as a stratification treatment that facilitated the germination process in Byers Peninsula and Conguillío populations (Fig. 4). In *Deschampsia antarctica*, the second native plant of Antarctica that shows serious germination issues, it was found that stratification at 5°C for 21 months successfully stimulated up to 55% germination (Kunakh *et al*., 2023). However, on Byers Peninsula, germination did not exceed 20.23% after six months of storage at cold temperatures. *Colobanthus quitensis* is characterized by hard testas due to the deposition of electro-dense and osmophilic material in their epidermal cell walls (Kellmann-Sopyla *et al*., 2017). Therefore, it would be advisable to apply other scarification treatments to enhance germination percentages, such as mechanical seed coat rupture or scarification with sulfuric acid (Cuba-Díaz *et al*., 2019; Rocha *et al*., 2022; Ontivero *et al*., 2025).

Short-term cryopreservation of *C. quitensis* seeds did not have a negative effect on seed quality, but rather maintained it, like the effect of 4ºC storage, making it a viable conservation alternative for this species. The use of temperatures between -20ºC and -196ºC has been applied in the conservation of seeds from various species, including orchids (Merritt *et al*., 2014; Schofield *et al*., 2018), *Solanum lycopersicum* (Zevallos *et al*., 2013), *Allium cepa*, *Oryza*

sativa, and *Zea mays* (Walters & Wheeler, 2004), ensuring high seed viability rates. However, several studies suggest that cryopreserved seeds with a water content lower than 5% may result in poor seedling emergence (Chmielarz, 2010). In this study, no abnormalities were identified in the germination of any population, nor were there any effects on germination percentages, except for the Peninsula Byers population, which initially showed a low germination percentage. Therefore, it can be considered that drying the seeds at room temperature for 3 - 4 days with silica gel until they reached a constant weight corresponding to 12% moisture content was sufficient to avoid oxidative damage due to the cooling and rewarming of the seeds' external structures (Acosta *et al*., 2020).

In recent years, scientific research on the role of endophytic fungi isolated from Antarctic plants or soil in agriculture has increased, as these microorganisms are adapted to extreme climatic conditions and have potential applications in agriculture under current climate change conditions. For example, fungal isolates detected in *C. quitensis* and *D. antarctica* have been used to enhance tomato and lettuce performance under saline stress (Molina-Montenegro *et al*., 2020). Furthermore, recent studies have used fungi isolated from *C. quitensis* to enhance strawberry resilience to drought and heat stress, as well as in blueberry plants exposed to drought conditions (Balbontin et al., 2025; Yañez et al., 2025). Despite this, there is still much information to be gathered to understand how the characteristics and metabolites of Antarctic endophytic fungi influence agricultural crop productivity

5. CONCLUSIONS

This study provides the first evidence that inoculating ex situ conserved *C. quitensis* with Antarctic endophytic fungi can enhance reproductive outcomes, while also demonstrating the feasibility of seed cryopreservation. Our findings partially support the hypothesis that fungal inoculation improves reproductive parameters, with *T. harzianum* accelerating reproductive timing and increasing seed size/weight in two of three populations. However, the anticipated improvement in seed germination was not observed, revealing a population-specific response where Antarctic accessions showed particularly low germination rates likely due to dormancy mechanisms. The cryopreservation results proved promising, with Byers Peninsula and Conguillío seeds maintaining viability at 4°C to -80°C, though La Marisma accessions only tolerated these conditions without germination enhancement. These outcomes suggest that while fungal inoculation shows potential for ex situ conservation protocols, its effects are

parameter-specific rather than universally beneficial across all reproductive traits. The superior performance of *T. harzianum* in modifying phenology and seed morphology indicates its prime candidacy for applied use, though potential synergies with *H. lixii* warrant further investigation given the known benefits of fungal consortia in stress conditions. Moving forward, research should focus on characterizing the underlying mechanisms through metabolite profiling and nutrient solubilization assays to develop targeted biotechnological applications. This work establishes a foundation for using Antarctic fungal symbionts in conservation strategies while highlighting the need for population-specific approaches given the variable responses observed.

6. REFERENCES

Abdel-Fattah, G.M., Shabana, Y.M., Ismail, A.E., & Rashad, Y.M. (2007). *Trichoderma harzianum*: a biocontrol agent against *Bipolaris oryzae*. *Mycopathologia 164*, 81–89, https://doi.org/ 10.1007/s11046-007-9032-9

Acosta, Y., Perez, L., Linares, C., Hernández, L., Escalante, D., Pérez, A., Zevallos, B. E., Yabor, L., Martinez-Montero, M. E., Cejas, I., Fontes, D., Sershen, & Lorenzo, J. C. (2020). Effects of *Teramnus labialis* (L.f.) Spreng seed cryopreservation on subsequent seed and seedling growth and biochemistry. *Acta Physiologiae Plantarum, 42*(7). https://doi.org/10.1007/s11738-020-3012-9

Acuña-Rodríguez I. S., Ballesteros, G. I., Gundel, P. E., Castro-Nallar, E., Barrera, A., Carrasco-Urra, F., & Molina-Montenegro, M.A. (2024). Fungal endophyte symbionts enhance plant adaptation in Antarctic habitats. *Physiologia Plantarum, 176*(6), e14589. https://doi.org/10.1111/ppl.14589.

Adeleke, B.S. & Babalola O.O. (2021). Biotechnological overview of agriculturally important endophytic fungi. *Horticulture Environment and Biotechnology*, *62*, 507-520. https://doi.org/10.1007/s13580-021-00334-1

Bader, A.N., Salerno, G.L., Covacevich, F. & Consolo, V.F. (2020). Native *Trichoderma harzianum* strains from Argentina produce indole-3 acetic acid and phosphorus solubilization, promote growth and control wilt disease on tomato (*Solanum lycopersicum* L.). *Journal of King Saud University Science, 32*, 867–873. https://doi.org/10.1016/j.jksus.2019.04.002

Bai, Y., Kissoudis, C., Yan, Z., Visser, R.G.F., & van der Linden, G. (2017). Plant behaviour under combined stress: tomato responses to combined salinity and pathogen stress. *Plant Journal, 93*(4), 781–793. https://doi.org/10.1111/tpj.13800

Balbontín, C., Flores, S., Reyes, M., Urrutia, V., Parra Palma, C., Morales Quintana, L., Ramos, P. (2025). Antarctic fungal inoculation enhances drought tolerance and modulates fruit physiology in blueberry plants. *Current Plant Biology 42*, 100462. https://doi.org/10.1016/j.cpb.2025.100462

Ballesteros G.I., Acuña-Rodríguez, I.S., Barrera A., Gundel, P.E., Newsham, K.K., & Molina-Montenegro, M.A. (2022). Seed fungal endophytes promote the establishment of invasive Poa annua in maritime Antarctica. *Plant Ecology & Diversity, 15*, 199-212. https://doi.org/10.1080/17550874.2022.2145579.

Ballesteros, G.I., Torres-Díaz C., Bravo, L.A., Balboa, K., Caruso, C., Bertini, L., Proietti, S., & Molina-Montenegro, M.A. (2020). *In silico* analysis of metatranscriptomic data from the Antarctic vascular plant *Colobanthus quitensis*: Responses to a global warming scenario through changes in fungal gene expression levels. *Fungal Ecology, 43*, 100873. https://doi.org/10.1016/j.funeco.2019.100873

Baron, N. C. & Rigobelo, E. C. (2022). Endophytic fungi: A tool for plant growth promotion and sustainable agriculture. *Mycology. 13*, 39–55. https://doi.org/10.1080/21501203.2021.1945699

9. Barrera, A., Hereme, R., Ruiz-Lara, S., Larrondo, L.F., Gundel, P.E., Pollmann, S., Molina-Montenegro, M.A., Ramos, P. (2020). Fungal endophytes enhance the photoprotective mechanisms and photochemical efficiency in the Antarctic *Colobanthus quitensis* (Kunth) Bartl. exposed to UV-B radiation. *Frontiers in Ecology and Evolution, 8*(122). https://doi.org/10.3389/fevo.2020.00122

Bertini, L., Perazzolli, M., Proietti, S., Capaldi, G., Savatin, D. V., Bigini, V., Longa, C. M. O., Basaglia, M., Favaro, L., Casella, S., Fongaro, B., Polverino de Laureto, P., & Caruso, C. (2022). Biodiversity and Bioprospecting of Fungal Endophytes from the Antarctic Plant *Colobanthus quitensis*. *Journal of Fungi, 8*(9), 979. https://doi.org/10.3390/jof8090979

Bonito, G., Reynolds, H., Robeson, M. S., Nelson, J., Hodkinson, B.P., Tuskan, G., Schadt, C.W., & Vilgalys, R. (2014). Plant host and soil origin influence fungal and bacterial assemblages in the roots of woody plants. *Molecular Ecology, 23*(13), 3356–3370. https://doi.org/10.1111/mec.12821

Clemente-Moreno, M.J.; Omranian, N.; Sáez, P. *et al*. (2020). Cytochrome respiration pathway and sulphur metabolism sustain stress tolerance to low temperature in the Antarctic species *Colobanthus quitensis*. *New Phytologist. 225*, 754–768. https://doi.org/10.1111/nph.16167

Collado, J., Platas, G., Gonzalez, I. & Pelaez, F. (1999). Geographical and seasonal influences on the distribution of fungal endophytes in *Quercus ilex*. *New Phytologist, 144*, 525–532. https://doi.org/10.1046/j.1469-8137.1999.00533.x

Contreras, R. A., Pizarro, M., Köhler, H., Zamora, P., & Zúñiga, G.E. (2019). UV-B shock induces photoprotective flavonoids but not antioxidant activity in Antarctic *Colobanthus quitensis* (Kunth) Bartl. *Environmental and Experimental Botany, 159*, 179-190. https://doi.org/doi:10.1016/j.envexpbot

Convey, P. 1996. Reproduction of Antarctic flowering plants. *Antarctic Science, 8*, 127–134.

Cuba-Díaz, M., Acuña, D., Cordero, C.M. & Klagges, M. (2014). Optimización de parámetros para la propagación *in vitro* de *Colobanthus quitensis* (Kunth) Bartl. *Gayana Botánica. 71*(1). http://dx.doi.org/10.4067/S0717-66432014000100009

Cuba-Díaz, M., Castel, K., Acuña, D., Machuca, A., & Cid, I. (2017a). Sodium chloride effect on *Colobanthus quitensis* seedling survival and *in vitro* propagation. *Antarctic Science, 29*, 45–46. https://doi.org/10.1017/S0954102016000432

Cuba-Díaz, M., Klagges, M., Fuentes-Lillo, E., Cordero, C., Acuña, D., Opazo, G., & Troncoso-Castro, J.M. (2017b). Phenotypic variability and genetic differentiation in continental and island populations of *Colobanthus quitensis* (Caryophyllaceae: Antarctic pearlwort). *Polar Biology, 40*, 2397–2409. https://doi.org/10.1007/s00300-017-2152-x

Chagas, F. O., Pessotti, R. D., Caraballo-Rodriguez, A. M. & Pupo, M.T. (2018). Chemical signaling involved in plant-microbe interactions. *Chemical Society Reviews, 47*(5), 1652–1704. https://doi.org/10.1039/c7cs00343a

Chebet, O. N., Omosa, L. K., Subramanian, S., Nchiozem-Ngnitedem, V.-A., Mmari, J. O., & Akutse, K. S. (2021). Mechanism of Action of Endophytic Fungi *Hypocrea lixii* and *Beauveria bassiana* in *Phaseolus vulgaris* as Biopesticides against Pea Leafminer and Fall Armyworm. *Molecules, 26*(18), 5694. https://doi.org/10.3390/molecules26185694

Chmielarz, P. (2010). Cryopreservation of Dormant Orthodox Seeds of European Hornbeam (*Carpinus betulus*). *Seed Science Technology*, 38, 146–157. https://doi.org/10.15258/sst.2010.38.1.15

Das, D., Sharma, P.L., Paul, P., Baruah, N.R., Choudhury, J., Begum, T., Karmakar, R., Khan, T., & Kalita, J. (2025). Harnessing endophytes: innovative strategies for sustainable agricultural practices. *Discover Bacteria, 2*, 1. https://doi.org/10.1007/s44351-025-00011-z

Díaz-Valenzuela, C., Deramond-Gálvez, C., Machuca, A. & Chávez, D. (2024). Actividad promotora del crecimiento vegetal de cuatro hongos endófitos aislados desde Araucaria araucana. *Siembra 11* (2) (2024): e6802. https://doi.org/10.29166/siembra.v11i2.6802

Edwards, J. A. (1974). Studies in *Colobanthus quitensis* (Kunth) Bartl. and *Deschampsia antarctica* Desv. VI. Reproductive performance on Signy Island. *British Antarctic Survey Bulletin, 39*, 67–86.

Egas, C., Ballesteros, G., Galbán-Malagón, C., Luarte, T., Guajardo-Leiva, S., Castro-Nallar, E., & Molina-Montenegro, M.A. (2025). Fungal endophytes modulate the negative effects induced by Persistent Organic Pollutants in the antarctic plant *Colobanthus quitensis*. *Physiologia Plantarum, 177* (1), e70079. https://doi.org/10.1111/ppl.70079

Frericks, J., Munkacsi, A., Ritchie, P., Luo, Y. B., Lehnebach, C. A. (2018). Phylogenetic affinities and *in vitro* seed germination of the threatened New Zealand orchid *Spiranthes novae-zelandiae. New Zealand Journal of Botany, 56*(1), 91-108. https://doi.org/10.1080/0028825X.2017.1418398

Fuentes-Lillo, E., Cuba-Díaz, M. & Rifo, S. 2017. Morpho-physiological response of *Colobanthus quitensis* and Juncus bufonius under different simulations of climate change. *Polar Science, 11*, 11–18. https://doi.org/doi:10.1016/j.polar.2016.11.003

Galleguillos, C., Acuña-Rodríguez, I. S., Torres-Díaz, C., Gundel, P.E. & Molina-Montenegro, M.A. (2023). Genetic control underlying the flowering-drought tolerance trade-off in the Antarctic plant *Colobanthus quitensis*. *Plant Cell & Environment 46*, 3158–3169.

Gateta, T., Nacoon, S., Seemakram, W., Ekprasert, J., Theerakulpisut, P., Sanitchon, J., Suwannarach, N., & Boonlue, S. (2023). The Potential of Endophytic Fungi for Enhancing the Growth and Accumulation of Phenolic Compounds and Anthocyanin in Maled Phai Rice (*Oryza sativa* L.). *Journal of Fungi, 9*(9), 937. https://doi.org/10.3390/jof9090937

Gianoli, E., Inostroza, P., Zúñiga-Feest, A., Reyes-Díaz, M., Cavieres, L.A., Bravo, L.A., & Corcuera, L.J. (2004). Ecotypic differentiation in morphology and cold resistance in populations of *Colobanthus quitensis* (Caryophyllaceae) from the Andes of Central Chile and the Maritime

Antarctic. *Arctic, Antarctic, and Alpine Research, 36*(4), 484–489. https://doi.org/10.1657/1523-0430(2004)036[0484:EDIMAC]2.0.CO;2

Gielwanowska, I., Bochenek, A., Gojlo, E., Górecki, R., Kellmann, W., Pastorczyk, M., & Szczuka E. (2011). Biology of generative reproduction of *Colobanthus quitensis* (Kunth) Bartl. from King George Island, South Shetland Islands. *Polish Polar Research*, *32*, 139–155. https://doi.org/10.2478/v10183−011−0008−6

González-Mas, N., Cuenca-Medina, M., García-Mozo, H., Muñoz-Redondo, J.M., Moreno-Rojas, J.M., Padilla-Álvarez, F., Rodríguez-Gómez, I.M., & Quesada-Moraga, E. (2023). Endophytic *Beauveria bassiana* modifies flowering phenology, floral volatile profile and pollinator behaviour in melon. *Entomologia Generalis*, 1-9, https://doi.org/10.1127/entomologia/2023/1991

González-Ortega-Villaizán, A., King, E., Patel, M. K. *et al*. (2024). The endophytic fungus *Serendipita indica* affects auxin distribution in *Arabidopsis thaliana* roots through alteration of auxin transport and conjugation to promote plant growth. *Plant, Cell & Environment*, 1–21. https://doi.org/10.1111/pce.14989

Gundel, P. E., Martinez-Ghersa, M. A., Garibaldi, L. A. & Ghersa, C. M. (2009). Viability of Neotyphodium endophytic fungus and endophyte-infected and noninfected *Lolium multiflorum* seeds. *Botany, 87*, 88–96. https://doi.org/10.1139/B08-119

Hassani, M. A., Duran, P. & Hacquard, S. (2018). Microbial interactions within the plant holobiont. *Microbiome, 6*(1),58. https://doi.org/10.1186/s40168-018-0445-0

Hereme, R., Galleguillos, C. & Molina-Montenegro, M. A. (2025). Climate change and epigenetics: Unraveling the role of methylation in response to thermal instability in the Antarctic plant *Colobanthus quitensis. Physiologia Plantarum, 177*(1), e70043. https://doi.org/10.1111/ppl.70043

Hereme, R., Morales-Navarro, S., Ballesteros, G., Barrera, A., Ramos, P., Gundel, P.E. Molina-Montenegro, M.A. (2020). Fungal endophytes exert positive effects on *Colobanthus quitensis* under water stress but neutral under a projected climate change scenario in Antarctica. *Frontiers in Microbiology, 11*(264). https://doi.org/10.3389/fmicb.2020.00264

Jiawen, C., Yuan, W., Xin, Z., Junjie, G., Xing, H., & Jinglei, X. (2022). Diversity analysis of leaf endophytic fungi and rhizosphere soil fungi of Korean Epimedium at different growth stages. *Environmental Microbiome, 17*(52). https://doi.org/10.1186/s40793-022-00446-w.

Kellmann-Sopyła, W. & Giełwanowska, I. (2015). Germination capacity of five polar *Caryophyllaceae* and *Poaceae* species under different temperature conditions. *Polar Biology, 38*, 1753–1765. https://doi.org/10.1007/s00300-015-1740-x

Kellmann-Sopyła, W., Koc, J., Górecki, R.J., & Domaciuk, M. (2017). Development of generative structures of polar Caryophyllaceae plants: the Arctic *Cerastium alpinum* and *Silene involucrata*, and the Antarctic *Colobanthus quitensis*. *Polish Polar Research, 38*, 83–104. https://doi.org/10.1515/popore-2017-0001

Khan, M. S., Gao, J., Munir, I., Zhang, M., Liu, Y., Moe, T.S., Xue, J., & Zhang, X. (2021). Characterization of endophytic fungi, *Acremonium* sp., from *Lilium davidii* and analysis of its antifungal and plant growth-promoting effects. *BioMed Research International,* 9930210. https://doi.org/10.1155/2021/9930210

Kiarie, S., Nyasani, J. O., Gohole, L. S., Maniania, N.K., & Subramanian, S. (2020). Impact of Fungal Endophyte Colonization of Maize (*Zea mays* L.) on Induced Resistance to Thrips- and Aphid-Transmitted Viruses. *Plants, 9*(4), 416. https://doi.org/10.3390/plants9040416

Koc, J., Androsiuk, P., Chwedorzewska, K.J., Cuba-Díaz, M., Górecki, R., & Giełwanowska, I. (2018). Range-wide pattern of genetic variation in *Colobanthus quitensis*. *Polar Biology, 41*, 2467–2479. https://doi.org/10.1007/s00300-018-2383-5

Kokila, M. A., Vanitha, C., Sundareswaran, S. & Marimuthu, S. (2021). Evaluation of role of testa, cotyledon and embryonic axis in manifestation of seed dormancy in groundnut varieties. *Pharma Innovation Journal, 10*, 1185–1188.

Kou, X., Zhao, X., Wu, B., Wang, C., Wu, C., Yang, S., Zhou, J., & Xue, Z. (2022). Auxin Response Factors Are Ubiquitous in Plant Growth and Development and Involved in Crosstalk between Plant Hormones: A Review. *Applied Science* 12, 1360. https://doi.org/10.3390/app12031360.

Kubiak, A., Wolna-Maruwka, A., Pilarska, A. A., Niewiadomska, A., & Piotrowska-Cyplik, A. (2023). Fungi of the *Trichoderma* Genus: Future Perspectives of Benefits in Sustainable Agriculture*. Applied Sciences, 13*(11), 6434. https://doi.org/10.3390/app13116434

Kunakh, V., Twardovska, M., Andreev, I., Drobyk, N., Navrotska, D., Nuzhyna, N., Poronnik, O., Konvalyuk, I., Myryuta, G., Ivannikov, R., Parnikoza, I. (2023). Development, integrative study and research prospects of *Deschampsia antarctica* collection. *Polish Polar Research, 44*, 254-261 https://doi.org/10.24425/ppr.2023.144537

Kuzhuppillymyal-Prabhakarankutty, L., Tamez-Guerra, P., Gomez-Flores, R., Rodriguez-Padilla, M.C. & Ek-Ramos, M.J. (2020). Endophytic *Beauveria bassiana* promotes drought tolerance and early flowering in corn. *World Journal of Microbiology & Biotechnology, 36*(3), 47. https://doi.org/10.1007/s11274- 020-02823-4.

Madawala, M. S. P. (2021). Chapter 7 - Arbuscular mycorrhizal fungi as biofertilizers: Current trends, challenges, and future prospects. In: Rakshit, A., Meena, V.S., Parihar, M., Singh, H.B., Singh, A.K. (Eds.) Biofertilizers. Woodhead Publishing, pp 83-93, https://doi.org/10.1016/B978-0-12-821667-5.00029-4.

Mazrou, Y. S. A.; Makhlouf, A. H.; Elseehy, M. M., Awad, M.F., & Hassan, M.M. (2020). Antagonistic activity and molecular characterization of biological control agent *Trichoderma harzianum* from Saudi Arabia. *Egyptian Journal of Biological Pest Control*, *30*, 120. https://doi.org/10.1186/s41938-020-0207-8

Merritt, D. J., Hay, F. R., Swarts, N. D., Sommerville, K.D., & Dixon, K.W. (2014). *Ex situ* conservation and cryopreservation of Orchid germplasm. *International Journal of Plant Science, 175* (1), 46–58. https://doi.org/10.1086/673370

Moore, D. M. (1970). Studies in *Colobanthus quitensis* (Kunth) Bartl. and *Deschampsia antarctica* Desv. II. Taxonomy, distribution and relationships. *British Antarctic Survey Bulletin 23*, 63–80.

Morales-Quintana, L., Barrera A., Hereme, R., Jara, K., Rivera-Mora, C., Valenzuela-Riffo, F., Gundel, P.E., Pollmann, S., Ramos, P. (2021). Molecular and structural characterization of expansins modulated by fungal endophytes in the Antarctic *Colobanthus quitensis* (Kunth) Bartl. Exposed to drought stress. *Plant Physiology and Biochemistry, 168,* 465-476. https://doi.org/10.1016/j.plaphy.2021.10.036.

Morales-Quintana, L., Rabert, C., Méndez-Yáñez, A., Ramos, P. (2024). Transcriptional and structural analysis of non-specific lipid transfer proteins modulated by fungal endophytes in Antarctic plants under drought. *Physiologia Plantarum, 176*(3), e14359. https://doi.org/10.1111/ppl.14359

Muhorakeye, M. C., Namikoye, E. S., Khamis, F. M., Wanjohi, W., & Akutse, K.S. (2024). Biostimulant and antagonistic potential of endophytic fungi against fusarium wilt pathogen of tomato *Fusarium oxysporum* f. sp. *lycopersici*. *Scientific Reports, 14*, 15365. https://doi.org/10.1038/s41598-024-66101-1

Murashige, T. & Skoog, F. (1962). A revised medium for the rapid growth and bioassay with tobacco tissue cultures. *Physiology Plantarum, 15*, 473–497.

Niu, Y., Xu, Y., Liu, X.F., Yang, S.X., Wei, S.P., Xie, F.T., & Zhang, Y.M. (2013). Association mapping for seed size and shape traits in soybean cultivars. *Molecular Breeding, 31*, 785-794. https://doi.org/10.1007/s11032-012-9833-5

Ontivero, Y., Cuba-Díaz, M., Fuentes-Lillo, E. & Convey, P. (2024a). Germination Strategies and Seed Quality of *Colobanthus quitensis*: Implications for Sustainable Antarctic Ecosystems and *Ex Situ* Plant Conservation. *Sustainability, 16*, 10726. https://doi.org/10.3390/su162310726

Ontivero, Y., Fuentes-Lillo, E., Navarrete-Campos, D., Vázquez-Villan D., Cabrera-Barjas, G. Arroyo-Marín, F.B., & Cuba-Díaz, M. (2024b). Preliminary assessment of seed heteromorphism as an adaptive strategy of *Colobanthus quitensis* under saline conditions. *Scientific Report, 14* (31120). https://doi.org/10.1038/s41598-024-82381-z.

Ontivero, Y., Salgado, C., Fuentes-Lillo, E., Sanhueza, C., Navarrete-Campos, D., Alveal, S. & Cuba-Díaz, M. (2025). Improved *in vitro* germination of *Colobanthus quitensis*: a key step for Antarctic plant conservation. *Plant Cell, Tissue and Organ Culture*. (in press)

Ortuño, N, Miranda, C. & Claros, M. (2013). Selección de cepas de *Trichoderma* spp. generadoras de metabolitos secundarios de interés para su uso como promotor de crecimiento en plantas cultivadas. *Journal of the Selva Andina Biosphere, 1*(1), 16-24. https://sars.org.bo/index.php/jsab/article/view/134/173

Pérez-Torres, E., Bravo, L. A., Corcuera, L. J. & Johnson, G.N. (2007). Is electron transport to oxygen an important mechanism in photoprotection? Contrasting responses from Antarctic vascular plants. *Physiology Plantarum, 130*, 185–194. https://doi.org/10.1111/j.1399-3054.2007.00899.x

Phillips, J.M. & Hayman, D.S. (1970). Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society, 55*, 158–160

Piotrowski, M. & Volmer, J. J. (2006). Cyanide metabolism in higher plants: cyanoalanine hydratase is a NIT4 homolog. *Plant Molecular Biology, 61*, 111–122. https://doi.org/10.1007/s11103-005-6217-9

Rabert, C., Larama, G., Fuentes, A. *et al*. (2020). *In situ* rhizosphere microbiome in Antarctic vascular plants, modulated by soil condition. Microbiology Resource Announcements, 9 (e1125–20). https://doi.org/10.1128/MRA.01125-20

Rai, M. & Agarkar, G. (2016). Plant–fungal interactions: what triggers the fungi to switch among lifestyles? *Critical Reviews in Microbiology, 42*, 428-438. https://doi.org/10.3109/1040841X.2014.958052

Ramírez, C.F., Cavieres, L.A., Sanhueza, C., Vallejos, V., Gómez-Espinoza, O., Bravo, L.A., & Sáez, P.L. (2024). Ecophysiology of Antarctic Vascular Plants: An Update on the Extreme Environment Resistance Mechanisms and Their Importance in Facing Climate Change. *Plants, 13*(3), 449. https://doi.org/10.3390/plants13030449.

Raya-Díaz, S., Sánchez-Rodríguez, A.R., Segura-Fernández, J.M., del Campillo, M.C., & Quesada-Moraga, E. (2017). Entomopathogenic fungi-based mechanisms for improved Fe nutrition in sorghum plants grown on calcareous substrates. *PLoS ONE, 12*(10), e0185903. https://doi.org/10.1371/journal.pone.0185903

Rivas, G. F. (2020). Efecto de la inoculación de hongos micorrícicos arbusculares y endófitos en la tolerancia al estrés por sequía en plántulas de Araucaria araucana. Tesis de Ingeniería en Biotecnología Vegetal. Universidad de Concepción, Los Ángeles, Chile, 52 pp.

Roberts, E.H. & Ellis, R.H. (1989). *Water and Seed Survival. Annals of Botany, 63*(1), 39–39. https://doi.org/10.1093/oxfordjournals.aob.a087727.

Rocha, R.L., Khalil, Y., Maity, A., Beckie, H.J., & Ashworth, M.B. (2022). Mechanical scarification technique breaks seed coat-mediated dormancy in wild oat (*Avena fatua*). *Weed Technology, 36*(1), 152-159. 10.1017/wet.2021.94.

Rosa, L.H., Almeida M.L., Furtado I. & Rosa, C. (2010). Endophytic fungi community associated with the dicotyledonous plant *Colobanthus quitensis* (Kunth) Bartl. (*Caryophyllaceae*) in Antarctica. *FEMS Microbiology Ecology, 73* (1) 178–189. https://doi.org/10.1111/j.1574-6941.2010.00872.x.

Ruhland, C. T. & Day, T. A. (2001). Size and longevity of seed banks in Antarctica and the influence of ultraviolet-B radiation on survivorship, growth and pigment concentrations of *Colobanthus quitensis* seedlings. *Environmental and Experimental Botany, 45*, 143–154. https://doi.org/10.1016/S0098-8472(00)00089-7

Schofeld, E., Jones, E.P. & Sarasan, V. (2018). Cryopreservation without vitrifcation suitable

for large scale cryopreservation of orchid seeds. *Botanical Studies, 59* (13): 1-9. https://doi.org/10.1186/s40529-018-0229-7

Silva, J. P. N., Salatino, M. L. F., Barbedo, C., Figueiredo-Ribeiro, R.C.L., & Centeno, D.C. (2022). Active Metabolism during Desiccation of the Desiccation Tolerant Short-Lived Seeds of *Poincianella pluviosa* (DC.) L. P. Queiroz. *Journal of Seed Science, 44*, e202244028. https://doi.org/10.1590/2317-1545v44261911

Silletti, S., Di Stasio, E., Van Oosten, M. J., Ventorino, V., Pepe, O., Napolitano, M., Marra, R., Woo, S. L., Cirillo, V., & Maggio, A. (2021). Biostimulant Activity of *Azotobacter chroococcum* and *Trichoderma harzianum* in Durum Wheat under Water and Nitrogen Deficiency. *Agronomy 11*, 380. https://doi.org/10.3390/agronomy11020380.

Smith, R. (2003). The enigma of *Colobanthus quitensis* and *Deschampsia antarctica* in Antarctica. In: Huiskes, A., Gieskes, W., Rozema, J. et al. (Eds.), Antarctic Biology in a Global Context. Backhuys, Leiden, pp. 234–239.

Sultana, R., Kunusoth, K., Amineni, L., Dahal, P., & Bradford, K. J. (2021). Desiccant drying prior to hermetic storage extends viability and reduces bruchid (*Callosobruchus chinensis* L.) infestation of mung bean (*Vigna radiata* (L.) R. Wilczek) seeds. *Journal of Stored Products Research, 94*, 101888. https://doi.org/10.1016/j.jspr.2021.101888

Tang, M., Liu, J., Hou, W., Stubbendieck, R.M., Xiong, H., Jin, J., Gong, J., Cheng, C., Tang, X., Liu, Y., Li, Z., Wang, J., & Yi, Y. (2022). Structural variability in the bulk soil, rhizosphere, and root endophyte fungal communities of *Themeda japonica* plants under different grades of karst rocky desertification. *Plant Soil, 475*, 105–122. https://doi.org/10.1007/s11104-021-04969-y

Torres-Díaz, C., Gallardo-Cerda, J., Lavin, P., Oses, R., Carrasco-Urra, F., Atala, C., Acuña-Rodriguez, I.S., Convey, P., & Molina-Montenegro, M.A. (2016). Biological interactions and simulated climate change modulates the ecophysiological performance of *Colobanthus quitensis* in the Antarctic ecosystem. *PLoS ONE, 11*(10): e0164844. https://doi.org/101371/journal.pone. 0164844.

Trusiak, M., Plitta-Michalak, B. P. & Michalak, M. (2023). Choosing the Right Path for the Successful Storage of Seeds. *Plants, 12*, 72. https://doi.org/10.3390/plants12010072

Ueno, A. C., Gundel, P.E., Seal, C.E., Ghersa, C.M., & Martínez-Ghersa, M.A. (2020). The negative effect of a vertically-transmitted fungal endophyte on seed longevity is stronger than

that of ozone transgenerational effect. Environmental and Experimental Botany, 175(104037). https://doi.org/10.1016/j.envexpbot.2020.104037

Walters, C., Wheeler, L. & Stanwood, P. C. (2004). Longevity of Cryogenically Stored Seeds. *Cryobiology, 48*, 229–244. https://doi.org/10.1016/j.cryobiol.2004.01.007

Wei, Y., Yang, H., Hu, J., Li, H., Zhao, Z., Wu, Y., Li, J., Zhou, Y., Yang, K., & Yang, H. (2023). *Trichoderma harzianum* inoculation promotes sweet sorghum growth in the saline soil by modulating rhizosphere available nutrients and bacterial community. *Frontiers in Plant Science, 14*,1258131. https://doi.org/10.3389/fpls.2023.1258131.

Wilkinson, L. 2011. ggplot2: elegant graphics for data analysis by Wickham. H. *Biometrics, 67*, 678–679.

Wu, L. S., Dong, W. G., Si, J. P., Liu, J.J., & Zhu, Y.Q. (2020). Endophytic fungi, host genotype, and their interaction influence the growth and production of key chemical components of *Dendrobium catenatum. Fungal Biology, 124*(10), 864-876. https://doi.org/10.1016/j.funbio.2020.07.002

Yáñez, M.A., Flores, S., Hormazábal-Abarza, F., Pollmann, S., Gundel, P.E., Cabrera-Ariza, C., Santelices-Moya, R., Morales-Quintana, L., & Ramos, P. (2025). Antarctic endophytic fungi enhance strawberry resilience to drought and heat stress by modulating aquaporins and dehydrins. *Plant Stress, 16*, 100805. https://doi.org/10.1016/j.stress.2025.100805.

Zevallos, B., Cejas, I., Escriba, R. C., Yabor, L., Aragón, C., González, J., Engelmann, F., Martínez, M. E., & Lorenzo, J. C. (2013). Biochemical Characterization of Ecuadorian Wild *Solanum lycopersicum* Mill. Plants Produced from Non-Cryopreserved and Cryopreserved Seeds. *CryoLetters, 34*, 413–421.

VII. CHAPTER VI: CAN FUNGAL EXTRACTS ENHANCE THE GERMINATION AND EARLY DEVELOPMENT OF *Colobanthus quitensis*?

Yadiana Ontivero[1,2], Christian Deramond[3], Daniel Chávez[3], Patricio Ramos[4,5], Marely Cuba-Díaz[1,6*]

[1]Laboratorio de Biotecnología y Estudios Ambientales, Departamento de Ciencias y Tecnología Vegetal, Escuela de Ciencias y Tecnologías, Universidad de Concepción, Los Ángeles, Chile.

[2]Facultad de Agronomía, Universidad de Concepción, Chillán, Chile.

[3]Laboratorio de Investigación de Micorrizas y Aplicaciones Biotecnológicas de Hongos, Departamento de Ciencias y Tecnología Vegetal, Universidad de Concepción, Campus Los Ángeles, Juan Antonio Coloma 0201, Los Ángeles 4440000, Chile

[4]Plant Microorganism Interaction Laboratory, Instituto de Ciencias Biológicas, Campus Talca, Universidad de Talca, Talca, Chile

[5]Vicerrectoría de Investigación y Postgrado, Universidad Católica del Maule, Talca, Chile

[6]Programa de Ciencia Antártica y Subantártica, Universidad de Concepción, Concepción.

* Correspondence author: mcuba@udec.cl

ORCID

YO: 0000-0002-3558-2552; DC: 0000-0003-4130-1339; PR: 0000-0001-8341-314X y MC: 0000-0002-2981-0328

Submitted to the Journal of Soil and Plant Nutrition on May 9, 2025.

ABSTRACT

Aim: Germination is a key step in plant establishment and can be enhanced by using endophytic fungi. However, in aseptic environments such as in vitro cultivation, fungal metabolites may be preferable to live fungi. *Colobanthus quitensis*, an extremophile plant native to Antarctica with biotechnological potential as a climate change indicator, often shows low germination rates under *in vitro* conditions. This study tested the hypothesis that fungal extracts from *Umbelopsis dimorpha*, *Trichoderma harzianum*, and *Hypocrea lixii* improve seed germination and early growth in *C. quitensis*.

Methods: The effect of fungal extracts from *U. dimorpha*, *T. harzianum*, and *H. lixii* on *in vitro* germination and early growth of *C. quitensis* was evaluated.

Results: While the extracts had a modest effect on germination, they significantly enhanced early seedling growth. Treated seedlings developed larger leaves, a higher number of leaves,

and more extensive root systems. Notably, the response varied among *C. quitensis* populations, highlighting genetic or ecotypic influences.

Conclusions: This is the first study to report the use of fungal extracts isolated from Araucaria and *C. quitensis* as biostimulants in this species. These results contribute to improving in vitro propagation protocols for *C. quitensis*, with potential applications in the conservation of other species with similar challenges, such as small seeds, physical dormancy, and hard seed coats.
Keywords: conservation, root development, Antarctic science, endophytic fungi

1. INTRODUCTION

The challenges of modern agriculture highlight the urgent need to explore ecological alternatives that enhance agricultural production while reducing environmental impact. The indiscriminate and prolonged use of chemical fertilizers has led to adverse effects on natural biota, soil contamination, and risks to human health (El-Nagar *et al*., 2024). In this context, endophytic fungi are emerging as key allies in agriculture due to their ability to improve the availability of essential nutrients such as nitrogen, phosphorus, and potassium, in addition to promoting plant hormone synthesis (Poveda *et al*., 2021; Díaz-Valenzuela *et al*., 2024). These biological properties not only enhance germination and plant growth but also stimulate flowering and increase crop yields, even under abiotic stress conditions (Frericks *et al*., 2018; Yáñez *et al*., 2025).

Although some fungi establish species-specific relationships with plants (Gange *et al*., 2007), it is possible to inoculate plants with fungi isolated from different species (Surono & Narisawa, 2017; Molina-Montenegro *et al*., 2020). Species from the genera *Trichoderma*, *Hypocrea*, and *Umbelopsis* have been used for their ability to produce metabolically active compounds of interest that stimulate plant growth and/or provide protection against diseases (Ortuño *et al*., 2013; Díaz-Valenzuela *et al*., 2024; Muhorakeye *et al*., 2024; Zhang *et al*., 2025). However, there is currently a growing interest in identifying natural bioactive compounds that can replace the use of chemical substances in agriculture without the need for inoculation (Karthikeyan *et al*., 2022). Fungal extracts, unlike live inoculants, offer scalable and standardized applications while mitigating the risks of microbial proliferation in sensitive ecosystems (Hart *et al*., 2018). These products are often rich in plant hormones such as indole-3-acetic acid, antioxidants, and antimicrobial compounds, making them highly promising for enhancing crop resistance to pathogens and optimizing plant growth (Khan *et al*., 2021; Shaker *et al*., 2022; El-Nagar *et al*.,

2024). However, their effects on extremophiles species remain largely unexplored, despite their potential to unveil novel stress-tolerance mechanisms.

Colobanthus quitensis, one of the two native plant species of Antarctica, has gained increasing interest as a biotechnological model for studying adaptation mechanisms to environmental stress (Clemente-Moreno *et al*., 2020; Alveal, 2023; Min *et al*., 2024). The vulnerability of the Antarctic ecosystem to anthropogenic pressure has driven efforts to conserve this species *ex situ* to provide material for scientific research (Ontivero *et al*., 2024). The *ex situ* conservation of *Colobanthus quitensis* faces the limitation that some populations exhibit low *in vitro* germination rates (Cuba-Díaz *et al*., 2017b; 2019; Ontivero *et al*., 2024b), a phenomenon associated with the presence of dormancy (Gielwanowska *et al*., 2011; Ontivero *et al*., 2024a). *In vitro* cultivation is a technique that ensures the asepsis of the propagated material (Tegen & Mohammed, 2016). However, under natural conditions, endophytic microorganisms play a crucial role in the development of this species (Hereme *et al*., 2020). Since the primary goal of tissue culture is to obtain microorganism-free material, the effect of fungal extracts on the germination and early growth of *C. quitensis* was evaluated without exposing the seeds to live microorganisms, only to the beneficial compounds they produce.

This study hypothesizes that fungal extracts derived from *Umbelopsis dimorpha*, *Trichoderma harzianum*, or *Hypocrea lixii* enhance seed germination and early growth performance in *C. quitensis*. To test this hypothesis, we evaluated the effects of fungal extracts from these species on key germination parameters and vegetative growth indicators in seedlings under *in vitro* controlled conditions. The central research question was: Do fungal extracts from *U. dimorpha*, *T. harzianum*, or *H. lixii* significantly improve germination efficiency and early-stage development in *C. quitensis* compared to untreated controls?

2. MATERIAL AND METHODS

2.1 Plant Material

For this research, populations of *C. quitensis* representing the Antarctic (Byers Peninsula, Livingston Island, South Shetland Islands; 62°40'S; 60°55'W), Subantarctic (La Marisma; Punta Santa María, south of Punta Arenas, Chile; 53°22'S; 70°58'W), and Andean (Conguillío, Conguillío National Park, Araucanía Region, Chile; 38°36'S; 71°36'W) regions were used (Cuba-Díaz *et al*., 2017b; Koc *et al*., 2018). These plants were grown under common garden conditions in a substrate composed of peat, leaf soil, and vermiculite in a 2:3:1 ratio, at a

temperature of 13 ± 2°C, with a photoperiod of 16/8 h light/dark, a light intensity of 100–120 µmol photons m^{-2} s^{-1}, and 75% relative humidity. Seeds obtained from plant propagation were collected at maturity (Still, 1999), dried at room temperature for 2 to 3 days, and then stored at 4°C for one year until they were treated with fungal extracts.

2.2 Seed fungi extract treatment

For the preparation of fungal extracts, *Umbelopsis dimorpha* (*Umbelopsis*), isolated from *Araucaria araucana* in Nahuelbuta National Park (Rivas, 2020), and *Trichoderma harzianum* (*Trichoderma*) and *Hypocrea lixii* (*Hypocrea*), isolated from the roots of *C. quitensis* plants collected from various locations near the Henryk Arctowski Polish Station in Admiralty Bay, King George Island (Rabert *et al*., 2020), were used. These fungi were cultivated in 250 mL of 1% (w/v) malt extract liquid medium for 7 days at 24 ± 1 °C without agitation and in complete darkness. They were kept in dark conditions for one month until use. For seed treatment, the fungal extract produced during fungal growth was filtered using a sterile 0.22-micrometer filter (biocomma).

2.3 *In vitro* germination and plant analysis

The seeds from each previously stored population were disinfected with 70% ethanol and 5% sodium hypochlorite following the protocol of Cuba-Díaz *et al*. (2017a). They were then divided into four groups and immersed for 24 hours in 1 mL of sterile distilled water (Control) or filtered fungal extract from *U. dimorpha, T. harzianum* and *H. lixii*. After this period, the excess liquid was removed using a sterile paper towel, and the seeds were placed for *in vitro* germination. For the *in vitro* germination test, 4 replicates of 15 seeds were placed in Petri dishes with 100% Murashige and Skoog medium, 3% sucrose, 0.7% agar, and pH 5.7 (Cuba-Díaz *et al*., 2017a). They were maintained at 20 ± 2 °C under a 16-hour light/8-hour dark photoperiod with a light intensity of 45 ± 2 µmol m^{-2} s^{-1} for 30 days. A seed was considered germinated when its primary root was at least twice the size of the seed. Germination data were collected daily to determine:

5. Germination percentage: $G\,(\%) = \frac{N}{A}\ x\ 100$; where N: number of germinated seeds at the end of the test. A: total number of seeds set to germinate.
6. Mean Germination Time: MGT = $\frac{\sum ni*t}{\sum n}$ (days); where ni: number of seeds germinated on day t. n: number of seeds germinated in the trial.

To assess the effect of fungal extracts on initial growth, on day 45 after the experiment began, 15 plants from each treatment were randomly selected, whenever possible, that were between 40 ± 1 days old. The aerial and root length, number of leaves, and leaf length of these plants were measured. A Kamasa KM-477 digital caliper with an accuracy of 0.01 mm was used for the measurements.

2.4 Statistical analysis

To evaluate the effect of fungal extract on seed germination and seedling growth independently for each *C. quitensis* population, separate statistical analyses were performed. A one-way analysis of variance (ANOVA) or Student's t-test was applied when the assumptions of normality and homogeneity of variances were met. In cases where these assumptions were not satisfied for a given variable in a particular population, the non-parametric Kruskal–Wallis test was used to detect differences between groups.

For multiple comparisons between treatments, Tukey's or Duncan's test was used for parametric data, while Dunn's test was applied when the data did not meet normality assumptions. A 95% confidence interval was used for all analysis. Subsequently, graphs were generated using the "ggplot2" package in RStudio (R Core Team, 2023).

3. RESULTS

Fungal extracts had no statistically significant effect on the germination indicators analyzed (Table 1), although some stimulatory responses were observed depending on the species. In the Byers Peninsula and Conguillío populations, seeds treated with extract of *T. harzianum* showed higher germination, meanwhile in La Marisma, *U. dimorpha* promoted higher germination.

Table 1. Germination indicators of *Colobanthus quitensis* populations treated with extracts obtained from endophytic fungi of *Araucaria araucana* (*Umbelopsis dimorpha*) and *C. quitensis* (*Trichoderma harzianum* and *Hypocrea lixii*) (n = 4).

***Colobanthus quitensis* population**	**Fungal extract**	**Germination (%) ± SD**	**p value**	**Mean Germination Time ± SD (days)**	**p value**
Byers Peninsula	Control	5 ± 3.3	0.060	7.67 ± 2.08	0.591
	Umbelopsis dimorpha	0		-	

	Trichoderma harzianum	5 ± 6.48		8.75 ± 1.77	
	Hypocrea lixii	0		-	
La Marisma	Control	53.33 ± 9.43	0.516	7.65 ± 0.65	0.721
	Umbelopsis dimorpha	63.33 ± 11.55		7.18 ± 1.92	
	Trichoderma harzianum	55 ± 3.33		7.99 ± 2.87	
	Hypocrea lixii	55 ± 13.74		9.12 ± 3.44	
Conguillío	Control	71.67 ± 14.78	0.052	9.06 ± 1.47	0.205
	Umbelopsis dimorpha	88.33 ± 15.75		11.34 ± 2.26	
	Trichoderma harzianum	96.67 ± 3.85		9.73 ± 0.95	
	Hypocrea lixii	76.67 ± 15.67		11.02 ± 1.57	

-: Results unavailable due to the absence of germination data.

Fungal extracts had a significant effect on plant growth, with effects varying for each population (Fig. 1). In Byers Peninsula, seeds treated with *T. harzianum* produced larger plants compared to the control (Fig. 1 A, B blue). In La Marisma, significant differences in aerial length were observed only between *H. lixii* and *T. harzianum*. However, the presence of all fungal extracts increased root system length compared to the control (Fig. 1A, B). For Conguillío, *T. harzianum* produced plants with the greatest aerial and root lengths, followed by *Hypocrea*, while control plants consistently showed the lowest growth (Fig. 1A, B, pink).

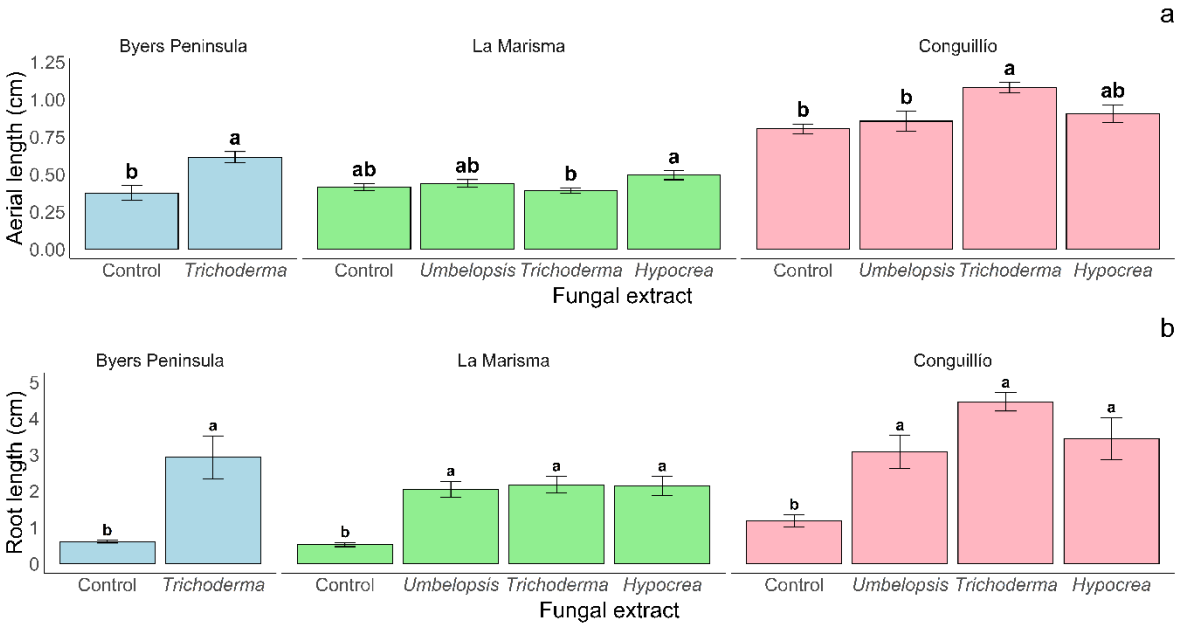


Figure 1. Growth of *Colobanthus quitensis* populations treated with fungal extracts from endophytes of *Araucaria araucana* (*Umbelopsis dimorpha*) and *C. quitensis* (*Trichoderma harzianum* and *Hypocrea lixii*), showing a) shoot length and b) root length. Different letters indicate significant differences ($p < 0.05$): Byers Peninsula was analyzed using Student's t-test ($n = 3$); La Marisma with one-way ANOVA followed by Tukey's test ($n = 15$) for multiple comparisons; and Conguillío with Kruskal-Wallis test (non-parametric ANOVA) followed by Dunn's test ($n = 15$) for non-normal data.

In Byers Peninsula, seeds treated with *Trichoderma* extracts produced a greater number of leaves with longer length compared to the control seeds (Fig. 2 A, B blue). In La Marisma, there were no significant differences in the number of leaves among the three fungal extracts; however, *Hypocrea* produced more leaves than the control, while no differences in length were observed between these two treatments (Fig. 2 A, B green). In the Andean population of Conguillío, the number of leaves was unaffected by any treatment, with Umbelopsis producing seedlings with fewer leaves compared to the other treatments, including the control (Fig. 2 A, B pink).

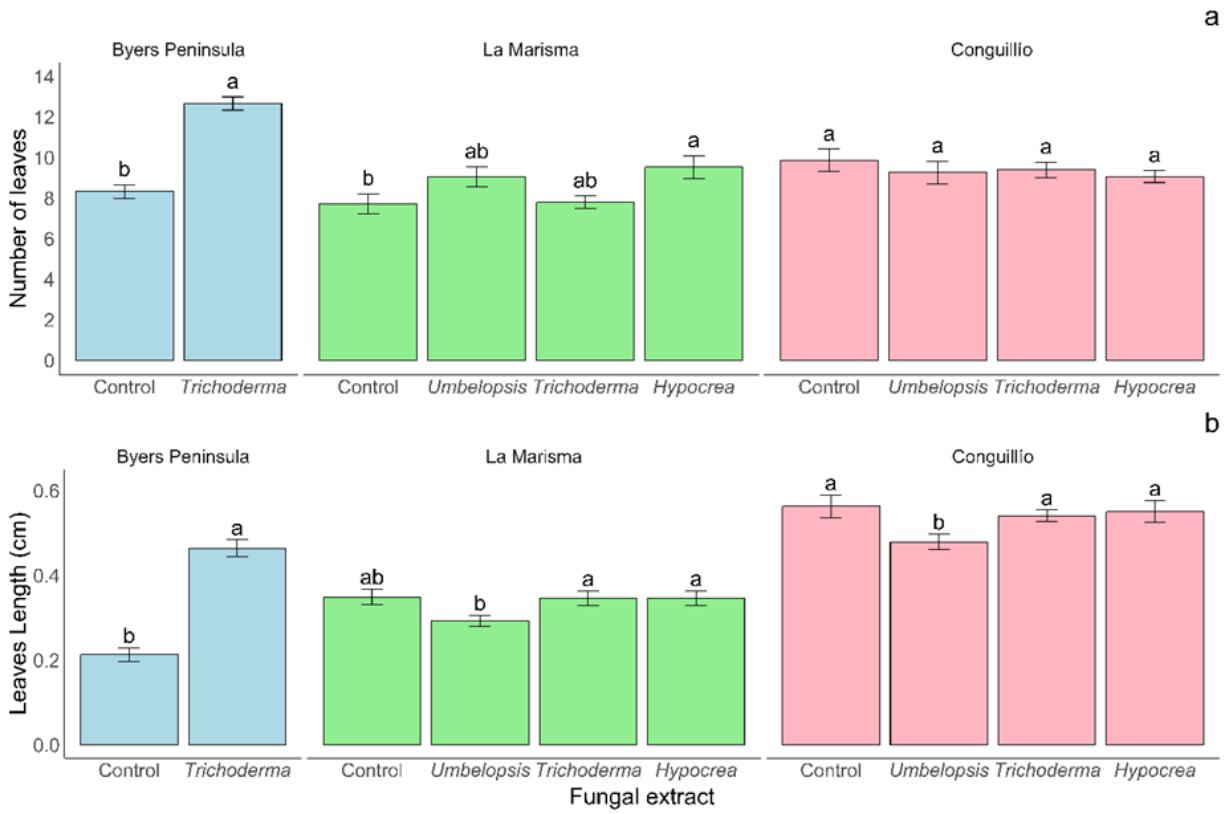


Figure 2: Number of leaves (a) and leaf length (b) produced in seedlings of three *Colobanthus quitensis* populations treated with fungal extracts from endophytic fungi of Araucaria araucana (*Umbelopsis dimorpha*) and C. *quitensis* (*Trichoderma harzianum* and *Hypocrea lixii*). In Byers Peninsula, significant differences ($p < 0.05$) were determined using Student's t-test (leaves: n = 3; leaf length: n = 6). For La Marisma and Conguillío, one-way ANOVA ($p < 0.05$) followed by Tukey's test was applied (leaves: n = 15; leaf length: n = 30). Different letters indicate statistically significant differences between treatments.

4. DISCUSSION

There are various reports on the importance of inoculating seeds and plants with endophytic fungi to stimulate germination, growth, and resistance to pathogens (Díaz-Valenzuela *et al*., 2024; Muhorakeye *et al*., 2024; Zhang *et al*., 2025). Therefore, it is not surprising that the biofertilizer market is projected to reach a value of $3.72 trillion in 2025, with expectations of surpassing $9 trillion by 2030 (Mordor Intelligence, 2025). The most common application method is through microorganism inoculation. However, their survival and effectiveness in the field, as well as their actual association with plants, are still under evaluation, along with their impact on soil microbiota (O'Callaghan *et al*., 2022). In contrast, the use of fungal extracts rich

in metabolic products generated by fungi emerges as a promising alternative, as it does not interfere with the native soil microbiology while simultaneously enhancing species productivity (Kramski *et al*., 2022).

Fungi can produce metabolites that can break dormancy and induce germination by weakening the seed coat and cotyledons, degrading germination-inhibiting compounds, or generating plant growth-promoting substances such as phytohormones (Kagithoju *et al*., 2013). In *C. quitensis*, low germination rates have been attributed to dormancy (Gielwanowska *et al*., 2011), sometimes requiring the use of priming, sulfuric acid scarification, and/or mechanical scarification to stimulate germination (Cuba-Díaz *et al*., 2019; Ontivero *et al*., 2025). Therefore, treating its seeds with fungal extracts was expected to enhance germination, particularly in the Antarctic population, where the most significant germination issues have been reported (Gielwanowska *et al*., 2011; Cuba-Díaz *et al*., 2019).

Umbelopsis dimorpha and *T. harzianum* are known for their ability to produce indole-3-acetic acid (IAA) (Bader *et al*., 2020; Díaz-Valenzuela *et al*., 2024), while *H. lixii* has shown a more prominent role as a biological control agent (Chebet *et al*., 2021). Indole-3-acetic acid is a phytohormone that regulates various growth processes, including cell elongation, vascular tissue development, apical dominance, fruit formation, and maturation (Kou *et al*., 2022). However, it has also been reported to induce dormancy (Gupta *et al*., 2024).

In this study, we do not consider that the use of these fungi increased dormancy, as none of the fungal extracts resulted in lower germination rates compared to the control. Nevertheless, these extracts alone were not sufficient to achieve high germination percentages, except for *Trichoderma* in Byers Peninsula, although it only stimulated 5% more germination compared to the control (Table 1). Therefore, it would be more advisable to incorporate them as part of a technological package that includes strategies to break dormancy, such as seed priming, sulfuric acid scarification, or mechanical scarification (Cuba-Díaz *et al*., 2019; Rocha *et al*., 2022; Ontivero *et al*., 2025).

Our findings demonstrate that fungal extracts from *U. dimorpha*, *T. harzianum*, and *H. lixii* differentially modulate early growth traits in *C. quitensis* across its geographic distribution (Fig. 1, 2), despite showing limited effects on germination parameters (Table 1). The role of *U. dimorpha* as a plant growth promoter was recently confirmed and linked to its ability to produce indole-3-acetic acid (IAA), solubilize phosphate (Díaz-Valenzuela *et al*., 2024), and enhance

metabolite production in plants (Qin *et al*., 2018). Likewise, *T. harzianum* is also capable of producing IAA and is considered an excellent alternative for promoting plant growth (Abdenaceur *et al*., 2022). In contrast, there are no reports on the use of *H. lixii* as a plant growth promoter. However, its genome is believed to contain genes encoding nitrilases, enzymes capable of converting indole-3-acetonitrile into indole-3-acetic acid in plants (Piotrowski & Volmer, 2006). Although this study did not determine the metabolic compound profile produced by these fungi, the observed effects of their extracts on plant growth could indicate their ability to produce indole-3-acetic acid.

This study demonstrated that in Byers Peninsula, only *Trichoderma* had beneficial effects on all evaluated parameters, except for MGT. In La Marisma, all fungal extracts improved germination to some extent, with no significant effects on the aerial part but noticeable effects on the roots. In contrast, in pC, there was a clear trend toward improved germination (p = 0.052) and a positive impact on vegetative growth with the use of fungal extracts, with *Trichoderma* showing the most pronounced effect. From an ecological perspective, root growth stimulated by metabolites produced by endophytic fungi provides plants with an additional advantage in accessing nutrients and water under extreme environmental conditions (Qin *et al*., 2024).

The fact that not all populations responded similarly to the same fungal extract may be related to the host plant–fungal interactions being primarily determined by their genotypes (Rai and Agarkar, 2016; Wu *et al*., 2020). In *C. quitensis*, different ecotypes have been identified based on their geographical distribution (Gianoli *et al*., 2004). Moreover, studies have shown that genetic and morphological variability is preserved in populations even after cultivation and propagation under common garden conditions, suggesting a certain inherent stability in each population (Cuba-Díaz *et al*., 2017b). Additionally, each microorganism produces a specific set of metabolic compounds that allow it to chemically interact with its potential host plant. This chemical variability may lead certain populations to benefit more from one fungal extract than another, depending on their chemical affinity (Schmidt *et al*., 2019).

This study provides the first evidence of the use of fungal extracts isolated from *Araucaria* and *C. quitensis* as biostimulants in the early stages of plant development in this species. The results demonstrate that these extracts promote a more extensive root system, a greater number of leaves, and an increase in leaf size. This not only optimizes the *in vitro*

establishment of *C. quitensis* but also represents a significant advancement for the *in vitro* cultivation and conservation of related species.

The production of larger plants increases the availability of viable plant material for use as explants, thereby facilitating the propagation and conservation of this species. Future research should focus on characterizing the metabolic and hormonal profiles of these fungi, both individually and in consortium, to develop a biostimulant enriched with the most effective compounds for promoting plant growth. Also, these extracts should be evaluated in future technological packages aimed at stimulating germination, in combination with *priming* strategies, modifications in the composition of the medium, scarification, and stratification.

5. REFERENCES

Abdenaceur R, Farida B, Mourad, D, Rima H, Zahia O, Fatma SH (2022) Effective biofertilizer *Trichoderma* spp. isolates with enzymatic activity and metabolites enhancing plant growth. Int Microbiol 25:817–829. https://doi.org/10.1007/s10123-022-00263-8

Alveal S (2023) Optimización de protocolo para la conservación *in vitro* en poblaciones de *Colobanthus quitensis* (Kunth) Bartl. (Tesis de Ingeniería en Biotecnología Vegetal). Universidad de Concepción, Chile.

Barrera A, Hereme R, Ruiz-Lara S, Larrondo LF, Gundel PE, Pollmann S, Molina-Montenegro MA, Ramos P (2020) Fungal endophytes enhance the photoprotective mechanisms and photochemical efficiency in the Antarctic *Colobanthus quitensis* (Kunth) Bartl. exposed to UV-B radiation. Front Ecol Evol 8 (122). https://doi.org/10.3389/fevo.2020.00122

Chebet ON, Omosa LK, Subramanian S, Nchiozem-Ngnitedem VA, Mmari JO, Akutse KS (2021) Mechanism of Action of Endophytic Fungi *Hypocrea lixii* and *Beauveria bassiana* in *Phaseolus vulgaris* as Biopesticides against Pea Leafminer and Fall Armyworm. Molecules, 26(18):5694. https://doi.org/10.3390/molecules26185694

Clemente-Moreno MJ, Omranian N, Sáez P, et al. (2020) Cytochrome respiration pathway and sulphur metabolism sustain stress tolerance to low temperature in the Antarctic species *Colobanthus quitensis*. New Phytol 225:754-768. https://doi.org/10.1111/nph.16167.

Cuba-Díaz M, Acuña D, Fuentes-Lillo E (2019) Antarctic pearlwort (*Colobanthus quitensis*) populations respond differently to pre-germination treatments. Polar Biol 42:1209–1215. https://doi.org/10.1007/s00300-019-02505-4

Cuba-Díaz M, Klagges M, Fuentes-Lillo E, Cordero C, Acuña D, Opazo G, Troncoso-Castro JM (2017b) Phenotypic variability and genetic differentiation in continental and island populations of *Colobanthus quitensis* (Caryophyllaceae: Antarctic pearlwort). Polar Biol 40:2397–2409. https://doi.org/10.1007/s00300-017-2152-x

Cuba-Díaz M, Castel K, Acuña D, Machuca A, Cid I (2017a) Sodium chloride effect on *Colobanthus quitensis* seedling survival and *in vitro* propagation. Antarct Sci 29:45–46. https://doi.org/10.1017/S0954102016000432

Díaz-Valenzuela C, Deramond-Gálvez C, Machuca A, Chávez D (2024) Actividad promotora del crecimiento vegetal de cuatro hongos endófitos aislados desde *Araucaria araucana*. Siembra 11(2):e6802. https://doi.org/10.29166/siembra.v11i2.6802

Frericks J, Munkacsi A, Ritchie P, Luo YB, Lehnebach CA (2018) Phylogenetic affinities and *in vitro* seed germination of the threatened New Zealand orchid *Spiranthes novae-zelandiae*. New Zealand J Bot, 56(1):91–108. https://doi.org/10.1080/0028825X.2017.1418398

Gange AC, Dey S, Currie AF, Sutton BC (2007) Site- and species-specific differences in endophyte occurrence in two herbaceous plants. J Ecol 95(4):614-622. https://doi.org/10.1111/j.1365-2745.2007.01245.x.

Gianoli, E., Inostroza, P., Zúñiga-Feest, A, Reyes-Díaz M, Cavieres LA, Bravo LA, Corcuera LJ (2004) Ecotypic differentiation in morphology and cold resistance in populations of *Colobanthus quitensis* (Caryophyllaceae) from the Andes of Central Chile and the Maritime Antarctic. Arct Antarct Alp Res, 36(4):484–489. https://doi.org/10.1657/1523-0430(2004)036[0484:EDIMAC]2.0.CO;2

Gielwanowska, I, Bochenek, A., Gojlo, E, Górecki R, Kellmann W, Pastorczyk M, Szczuka E (2011) Biology of generative reproduction of *Colobanthus quitensis* (Kunth) Bartl. from King George Island, South Shetland Islands. Pol Polar Res 32:139–155. https://doi.org/10.2478/v10183-011-0008-6

Gupta S, Hrdlička J, Kulkarni M, Doležalova I, Pěnčík A, Van Staden J, Novák O, Doležal K (2024) Karrikinolide1 (KAR_1), a Bioactive Compound from Smoke, Improves the

Germination of Morphologically Dormant *Apium graveolens* L. Seeds by Reducing Indole-3-Acetic Acid (IAA) Levels. Plants 13:2096. https://doi.org/10.3390/plants13152096.

Hart MM, Antunes PM, Chaudhary VB, Abbott LK (2018) Fungal inoculants in the field: Is the reward greater than the risk? Funct Ecol, 32:126–135. https://doi.org/10.1111/1365-2435.12976.

Hereme R, Morales-Navarro S, Ballesteros G, Barrera A, Ramos P, Gundel PE, Molina-Montenegro MA (2020) Fungal endophytes exert positive effects on *Colobanthus quitensis* under water stress but neutral under a projected climate change scenario in Antarctica. Front Microbiol 11, 264. https://doi.org/10.3389/fmicb.2020.00264

Kagithoju S, Hussain A, Godishala V, Nanna RS (2013) Role of Fungi and Fungal Extract on Strychnos Potatorum Seed Germination. Res J Biotechnol 8(11):32-36.

Karthikeyan A, Joseph A, Nair BG (2022) Promising bioactive compounds from the marine environment and their potential effects on various diseases. J Genet Eng Biotechnol 20(14). https://doi.org/10.1186/s43141-021-00290-4

Khan MS, Gao J, Munir I, Zhang M, Liu Y, Moe TS, Xue J, Zhang X (2021) Characterization of Endophytic Fungi, *Acremonium* sp., from *Lilium davidii* and Analysis of Its Antifungal and Plant Growth-Promoting Effects. BioMed Res Int 9930210:1-14. https://doi.org/10.1155/2021/9930210

Kou, X., Zhao, X., Wu, B, Wang C, Wu C, Yang S, Zhou J, Xue Z (2022) Auxin Response Factors Are Ubiquitous in Plant Growth and Development and Involved in Crosstalk between Plant Hormones: A Review. Appl Sci 12,1360. https://doi.org/10.3390/app12031360

Kramski DJ, Nowinski D, Kowalczuk K, Kruszyński P, Radzimska J, Greb-Markiewicz B (2023) *Beauveria bassiana* Water Extracts' Effect on the Growth of Wheat. Plants, 12(2), 326. https://doi.org/10.3390/plants12020326

Rocha R, Khalil Y, Maity A, Beckie HJ, Ashworth MB (2022) Mechanical scarification technique breaks seed coat-mediated dormancy in wild oat (*Avena fatua*). Weed Technology 36:152–159. https://doi.org/10.1017/wet.2021.94

Mastouri F, Bjorkman T, Harman GE (2010) Seed treatment with *Trichoderma harzianum* alleviates biotic, abiotic, and physiological stresses in germinating seeds and seedlings. Phytopathol 100:1213-1221. https://doi.org/10.1094/PHYTO-03-10-0091

Min K, Sulaiman S, Jeong J, Lee H, Lee J, Lee JH, Lee H (2024) Exploring the mechanisms underlying recovery from freeze–thaw injury in *Colobanthus quitensis*: mechanistic insights via transcriptome profiling. Physiol Plant 176(6):e14642. https://doi.org/10.1111/ppl.14642

Molina-Montenegro MA, Acuña-Rodríguez IS, Torres-Díaz C, Gundel PE, Dreyer I (2020) Antarctic root endophytes improve physiological performance and yield in crops under salt stress by enhanced energy production and Na^+ sequestration. Sci Rep 10:5819. https://doi.org/10.1038/s41598-020-62544-4

Mordor Intelligence 2025. Biofertilizer Market SIZE & SHARE ANALYSIS - GROWTH TRENDS & FORECASTS UP TO 2030 Source: https://www.mordorintelligence.com/industry-reports/global-biofertilizers-market-industry/

O'Callaghan M, Ballard RA, Wright D (2022) Soil microbial inoculants for sustainable agriculture: Limitations and opportunities. Soil Use Manage 38(3):1340-1369. https://doi.org/10.1111/sum.12811

Ontivero Y, Cuba-Díaz M, Fuentes-Lillo E, Convey P (2024) Germination Strategies and Seed Quality of *Colobanthus quitensis*: Implications for Sustainable Antarctic Ecosystems and *Ex Situ* Plant Conservation. Sustainability 16(10726). https://doi.org/10.3390/su162310726

Ontivero Y, Salgado C, Fuentes-Lillo E, Sanhueza C, Navarrete-Campos D, Alveal S, Cuba-Díaz M (2025) Improved *in vitro* germination of *Colobanthus quitensis*: a key step for Antarctic plant conservation. PCTOC (in press).

Ortuño N, Miranda C, Claros M (2013) Selección de cepas de *Trichoderma* spp. generadoras de metabolitos secundarios de interés para su uso como promotor de crecimiento en plantas cultivadas. J Selva Andina Biosph 1(1):16-24. https://sars.org.bo/index.php/jsab/article/view/134/173

Piotrowski M, Volmer JJ (2006) Cyanide metabolism in higher plants: cyanoalanine hydratase is a NIT4 homolog. Plant Mol Biol 61:111–122. https://doi.org/10.1007/s11103-005-6217-9

Poveda J, Eugui D, Abril-Urías P, Velasco P (2021) Endophytic fungi as direct plant growth promoters for sustainable agricultural production. Symbiosis 85:1–19. https://doi.org/10.1007/s13199-021-00789-x

Qin D, Wang L, Han M, Wang J, Song H, Yan X, Duan X, Dong J (2018) Effects of an Endophytic Fungus *Umbelopsis dimorpha* on the Secondary Metabolites of Host–Plant *Kadsura angustifolia*. Front Microbiol 9(2845). https://doi.org/10.3389/fmicb.2018.02845

Qin X, Xu J, An X, Yang J, Wang Y, Dou M, Wang M, Huang J, Fu Y (2023) Insight of endophytic fungi promoting the growth and development of woody plants. Crit Rev Biotechnol 44(1):78–99. https://doi.org/10.1080/07388551.2022.2129579

Rabert C, Larama G, Fuentes A, Gutiérrez-Moraga A, Tapia-Valdebenito D (2020) *In situ* rhizosphere microbiome in Antarctic vascular plants, modulated by soil condition. Microbiol Resour Announc 9:e1125–20. https://doi.org/10.1128/MRA.01125-20

Rivas GF (2020) Efecto de la inoculación de hongos micorrícicos arbusculares y endófitos en la tolerancia al estrés por sequía en plántulas de *Araucaria araucana.* Tesis de Ingeniería en Biotecnología Vegetal. Universidad de Concepción, Los Ángeles, Chile, 52 pp.

Schmidt R, Ulanova D, Wick LY, Bode HB, Garbeva P (2019) Microbe-driven chemical ecology: past, present and future. ISME J 13(11):2656–2663. https://doi.org/10.1038/s41396-019-0469-x

Shaker KH, Zohair MM, Hassan AZ, Sweelam HM, Ashour WE (2022) LC–MS/MS and GC–MS based phytochemical perspectives and antimicrobial effects of endophytic fungus *Chaetomium ovatoascomatis* isolated from *Euphorbia milii*. Arch Microbiol 204:661. https://doi.org/10.1007/s00203-022-03262-5

Still D (1999) Development of seed quality in *Brassicas*. HortTechnology 9, 335–340.

Surono, Narisawa K (2017) The dark septate endophytic fungus *Phialocephala fortinii* is a potential decomposer of soil organic compounds and a promoter of *Asparagus officinalis* growth. Fungal Ecol 28:1-10. https://doi.org/10.1016/j.funeco.2017.04.001

Tegen H, Mohammed W (2016) The Role of Plant Tissue Culture to Supply Disease Free Planting Materials of Major Horticultural Crops in Ethiopia. JBAH 6(1):122-129.

Yáñez MA, Flores S, Hormazábal-Abarza F, Pollmann S, Gundel PE, Cabrera-Ariza C, Santelices-Moya R, Morales-Quintana L, Ramos P (2025) Antarctic endophytic fungi enhance strawberry resilience to drought and heat stress by modulating aquaporins and dehydrins. Plant Stress 16(100805). https://doi.org/10.1016/j.stress.2025.100805

Zhang ZY, Fu X, M, Shu YC, Qin XJ, Zhang YJ, Zhao P, Yang XQ, Zou LH, Wang WH, Zhu GL (2025) Three new compounds of Umbelopsis dimorpha VDG10, a dominant endophytic fungus from *Vaccinium dunalianum* Wight. Fitoterapia 181(106358). https://doi.org/10.1016/j.fitote.2024.106358

Competing interests

The authors declare no competing interests.

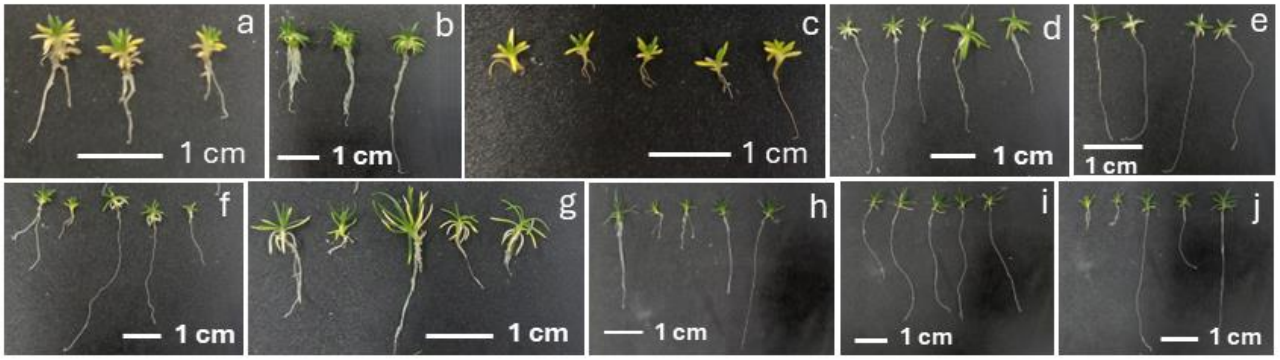
Funding

This research was funded by the National Agency for Research and Development (ANID, Chile) through the National Doctorate Scholarship Program (2021–21210100), the Chilean Antarctic Institute (INACH) project DT_05_22, and the Doctoral Program in Agricultural Sciences, Faculty of Agronomy, University of Concepción, Campus Chillán (2021300031, YO). Patricio Ramos was supported by ANID FONDECYT 1240771 and ANILLO ATE220014. MCD was supported by INACH projects RT_17_22 and 2023000418ESP, and by VRID UdeC. Additional funding was provided by ANID (Chile) through FONDECYT project 1240771 and ANILLO project ATE220014.

Contributions

YO, DC, and MCD designed scientific research. YO, CD, DC y PR carried out the technical work. YO, PR, and MC wrote the first draft of the manuscript. YO, CD, DC, PR, and MC revised the final version of the manuscript.

Supplementary material



Supplementary Figure 1: Vegetative growth of *Colobanthus quitensis* seedlings after treatment with fungal extracts from endophytic fungi associated with *Araucaria araucana* and *C. quitensis*. Seeds from the Antarctic population Arctowski: (a) untreated (control), (b) treated with *Trichoderma harzianum*. Sub-Antarctic population La Marisma: (c) control, (d) treated with *Umbelopsis dimorpha*, (e) with *T. harzianum*, (f) with *Hypocrea lixii*. Andean population Conguillío: (g) control, (h) with *U. dimorpha*, (i) with *T. harzianum*, (j) with *H. lixii*.

Chapter VII: IMPACT OF STORAGE TIME ON THE VIABILITY AND VIGOR OF *Colobanthus quitensis* SEEDS

Yadiana Ontivero[1,2]; Constanza Salgado[1]; Darío Navarrete-Campos[1], Marely Cuba-Díaz[1,3*]

[1]Universidad de Concepción, Laboratorio de Biotecnología y Estudios Ambientales, Departamento de Ciencias y Tecnología Vegetal, Escuela de Ciencias y Tecnología, Campus Los Ángeles 4440000, Chile.

[2]Universidad de Concepción, Programa de Doctorado en Ciencias de la Agronomía, Facultad de Agronomía, Chillán 381 2189, Chile.

[3]Programa de Ciencia Antártica y Subantártica, Universidad de Concepción, Concepción 4030000, Chile.

ABSTRACT

Seed physiological quality is a key indicator for commercialization, but storage time can negatively impact seed viability and vigor. While several tests exist to evaluate seed quality, not all are suitable for every species. *Colobanthus quitensis*, an extremophile species with biotechnological potential, lacks information regarding its seed quality and appropriate evaluation methods, posing challenges for *ex situ* conservation. This study aimed to assess the physiological quality of seeds from various *C. quitensis* populations stored between 2015 and 2022 using flotation, electrical conductivity, *in vitro* germination, and tetrazolium tests. The flotation test successfully separated seeds into viable and non-viable groups, although both groups later showed germination potential. The electrical conductivity test did not correlate with germination performance. Seeds from Antarctic populations had germination rates below 1% in most storage periods. Higher germination percentages were observed in seeds stored for 12 to 24 months, while those stored for over four years lacked vigor. Storage time also affected seedling fresh weight. This study underscores the need to apply multiple vigor tests to accurately determine seed quality. Buoyancy and electrical conductivity are not recommended for evaluating deterioration in *C. quitensis* seeds. The most reliable methods were *in vitro* germination and tetrazolium testing. The fact that some viable seeds failed to germinate suggests either the need to optimize germination protocols or the presence of dormancy mechanisms.

Keywords: conservation, vigor analysis, germination, tetrazolium salts, electrical conductivity

1. INTRODUCTION

The international seed trade is a key indicator of a country's agricultural economy. However, this trade is subject to strict quality standards to ensure commercial viability (Milošević *et al*., 2010; Taylor, 2020). One of the main challenges is the physiological deterioration of seeds during storage, a problem that differentially affects all agricultural crops and impacts both their production and market value (Gang *et al*., 2019; Wawrzyniak *et al*., 2020).

Germplasm banks play a critical role in conserving plant genetic diversity (Whitehouse *et al*., 2020), though their efficiency is directly tied to the available knowledge about the species they store. This knowledge is particularly limited for species without established agricultural use (Taylor, 2020). Currently, there is a growing demand to develop techniques for accurately assessing seed quality and optimizing storage protocols to prolong longevity and minimize deterioration (Solberg *et al*., 2020). Notably, the lack of data on seed viability loss patterns forces germplasm banks to allocate significant resources to frequent collection monitoring (Taylor, 2020; Whitehouse *et al*., 2020).

Seed physiological quality is a complex trait influenced by morphological, physiological, genetic, and environmental factors (Zhou *et al*., 2019). Therefore, it is recommended to perform multiple vigor tests simultaneously when assessing the deterioration of a seed lot (Milivojević *et al*., 2021). Although germination testing is the most commonly used method for evaluating seed quality, several alternative vigor tests such as flotation, electrical conductivity, and tetrazolium, offer quicker, simpler, and less resource-intensive options. These tests assess cell membrane integrity and provide insights into the biochemical status of the seeds (Marcos-Filho, 2015).

Regarding evaluation methodologies, most vigor tests have been developed for large-seeded crops such as maize, wheat, and rice (Nicoleti & Coelho, 2018; Correia *et al*., 2020; García & Coelho, 2021). In contrast, evaluating small-seeded species like quinoa (*Chenopodium quinoa*), amaranth (*Amaranthus* spp.), which are crucial for food security (Kehinde *et al*., 2013; Souza *et al*., 2020), or orchids (Orchidaceae), known for their high ornamental value (Li *et al*., 2021), presents greater technical challenges.

Colobanthus quitensis (Kunt) Bartl. (Caryophyllaceae) is a species of special interest due to its broad geographic distribution, from southern Mexico to the Antarctic Peninsula, where it faces extreme environmental conditions (Moore, 1970; Convey, 1996; Smith, 2003). This uniqueness

has led to remarkable variability among populations in traits such as germination capacity, morphology, and genetic adaptation (Gianoli *et al*., 2004; Cuba-Díaz *et al*., 2017; Koc *et al*., 2018), making it a promising model for biotechnological studies on environmental stress (Clemente-Moreno *et al*., 2020; Min *et al*., 2024). Despite its potential, Antarctic vascular plant collections, where *C. quitensis* has been included since 2014 have shown declines in germination viability during storage (preliminary observations). However, no systematic studies have yet evaluated the physiological quality of its stored seeds. Understanding deterioration patterns in this species would not only improve seed bank management but also contribute to protocol development for small-seeded species with low germination (Ontivero *et al*., 2024a). Thus, this study aimed to assess the physiological quality of seeds from different *C. quitensis* populations stored between 2015 and 2022, using multiple vigor analysis methodologies.

2. MATERIALS AND METHODS

2.1 Plant material

Plants from the Hannah Point (pH) (Livingston Island, South Shetland Islands; 62°39'S, 60°37'W), Arctowski, (pA) (King George Island, South Shetland Islands; 62°09'S, 58°28'W), La Marisma (pPA) (Punta Santa María, southern Punta Arenas, Chile; 53°22'S, 70°58'W), Laredo (pL) (Laredo sector, northern Punta Arenas, Chile; 52°58'S, 70°49'W), and Conguillío (pC) (Conguillío National Park, Araucanía Region, Chile; 38°36'S, 71°36'W) populations (Cuba-Díaz *et al*., 2017) were grown under common garden conditions. The plants were cultivated in a substrate composed of peat, leaf soil, and vermiculite in a 2:3:1 ratio. The growth environment was maintained at 13 ± 2 °C, with a 16/8 h light/dark photoperiod, light intensity of 100–120 µmol photons m^{-2} s^{-1}, and 75% relative humidity (Cuba-Díaz *et al*., 2019). Seeds produced by these plants were collected from fully open floral capsules containing mature seeds. The seeds were cleaned and dried at room temperature for 2 to 3 days, then stored in tightly sealed Eppendorf tubes at 4 °C.

2.2Loss of germination capacity over storage time

2.2.1 Buoyancy test and electrical conductivity

Seeds from each population stored between 2015 and 2022 were grouped by quarters to assess viability using the buoyancy test (Suma and Srimathi, 2014) and membrane integrity using the electrical conductivity test (Fantazzini *et al*., 2021). For all populations, the available material was insufficient, so the analysis periods vary between populations

For each storage period, three replicates of 10 mg of seeds were used and placed in 25 mL of deionized water for 24 h at 25 °C. After this period, seeds were then separated into viable (non-floating) and non-viable (floating) groups, which were dried at room temperature for four days. Viability percentage was calculated using the following equations:

$$Viable\ seeds(\%) = \frac{Weight\ of\ non-floating\ seeds}{Weight\ of\ total\ seeds}\ x\ 100$$

$$Non-viable\ seeds(\%) = \frac{Weight\ of\ floating\ seeds}{Weight\ of\ total\ seeds}\ x\ 100$$

In addition, the percentage of material loss was determined using the buoyancy test, calculated with the following equation:

$$Lost = 100 - (Viable\ seeds + Non-viable\ seeds)$$

The water used in the buoyancy test was analyzed with a PL-700PC device (Santiago, Chile) to measure its conductivity, which was expressed in $\mu S \cdot cm^{-1} \cdot g^{-1}$.

2.2.2 Germination test and initial growth of seedlings

Viable seeds obtained from the buoyancy test were disinfected with 70% ethanol and 5% sodium hypochlorite (Cuba-Diaz *et al*., 2017). Subsequently, 12 seeds randomly selected for each population and storage time were placed for *in vitro* germination in Petri dishes containing 100% MS medium (Murashige and Skoog, 1962), supplemented with 3% sucrose and 0.7% agar, and adjusted to pH 5.7. The plates were incubated for 30 days under a 16 h light / 8 h dark photoperiod at 20 ± 2 °C. A seed was considered germinated when its radicle reached at least twice the seed length (Sanhueza *et al*., 2017). Three replicates were conducted in total. The following indicators were evaluated:

Germination: G(%) = *N*/*A* x 100; where *N* is the number of germinated seeds at the end of the test; *A* is the total number of seeds set to germinate.
Mean Germination Time: MGT (days) =∑*ni* x *t*/*n*; where ni is the number of seeds germinated on day t; n is the number of seeds germinated in the trial.
Germination Index: GI =∑ *Gt*/*Dt*; where *Gt* is the number of seeds germinated each day *t*; *Dt* is the number of germination days.
Vigor Index: VI = *GI* x *S*; where *S* is the height of plants 20 days post-germination

An additional germination analysis was performed with a new group of viable and non-viable seeds to verify if the seeds classified as non-viable by the buoyancy test were incapable of germinating. The same germination protocol outlined previously was followed, but only the germination percentage was determined.

2.2.3 Tetrazolium salt test

A tetrazolium staining test was performed to assess the viability of both the germplasm bank seeds and those that did not germinate in the germination test. The seeds from the germplasm bank were placed in distilled water for 24 hours at 25°C to soften the seed coat. An incision was then made with a scalpel to open the seed coat and expose the embryo to a 1% 2,3,5-triphenyl tetrazolium chloride solution, where the seeds were immersed for 24 hours at 25°C (ISTA, 2017). After incubation, the tetrazolium solution was discarded, and the seeds were examined under a microscope to verify the staining. Seeds with a bright pink embryo color were considered viable, while those with a uniform white color were deemed non-viable. The viability percentage was calculated by dividing the number of viable seeds by the total number of seeds analyzed.

2.2.4 Fresh weight

To determine the fresh weight of the seedlings for each conservation time and population, between 3 and 10 seedlings were selected at 30 days post-germination, with each plant considered as a replicate. The amount of material weighed varied according to the availability

of seedlings for each treatment, depending on seed germination. An analytical balance, RADWAG AS 220/C/2, with a precision of ± 0.1 mg was used.

2.3 Statistical Analysis

To evaluate the effect of storage time on seed quality in different populations of *Colobanthus quitensis*, a one-way analysis of variance (ANOVA) was applied when the assumptions of normality and homogeneity of variances were met. When these assumptions were not satisfied for a given variable within a specific population, the non-parametric Kruskal–Wallis test was used to detect differences between groups.

To analyze the relationship between the germination test and the electrical conductivity analysis, a Pearson correlation was performed. This correlation aimed to assess the degree of linear association between seed germination percentages and membrane integrity, as indicated by electrolyte leakage.

For multiple comparisons between treatments, Tukey's or Duncan's test was used for parametric data, while Dunn's test was applied for non-parametric data when normality assumptions were not met. A 95% confidence interval was used for all statistical analyses. Graphs were generated using the "ggplot2" package in RStudio (R Core Team, 2023).

3. RESULTS

Material loss was detected in all populations through the buoyancy test. In the populations pH ($p=0.154$), pA ($p=0.159$), and pPA ($p=0.791$), no significant differences were observed in the proportion of viable and non-viable seeds according to the buoyancy test (Fig. 1a, b, c), although the trend in pH for the 4_6 and 16_18 month periods indicates that viable seeds are present in much lower proportions. In the case of pL, the test revealed a higher proportion of non-viable seeds compared to viable ones, although this effect was only significant for the conservation periods of 4–6, 7–9, 22–24, and 25–27 months (Fig. 1d). Similarly, in pC, the buoyancy test indicated a greater number of non-viable seeds, with this difference being significant only for the 16–18 and 82–84 month periods (Fig. 1e).

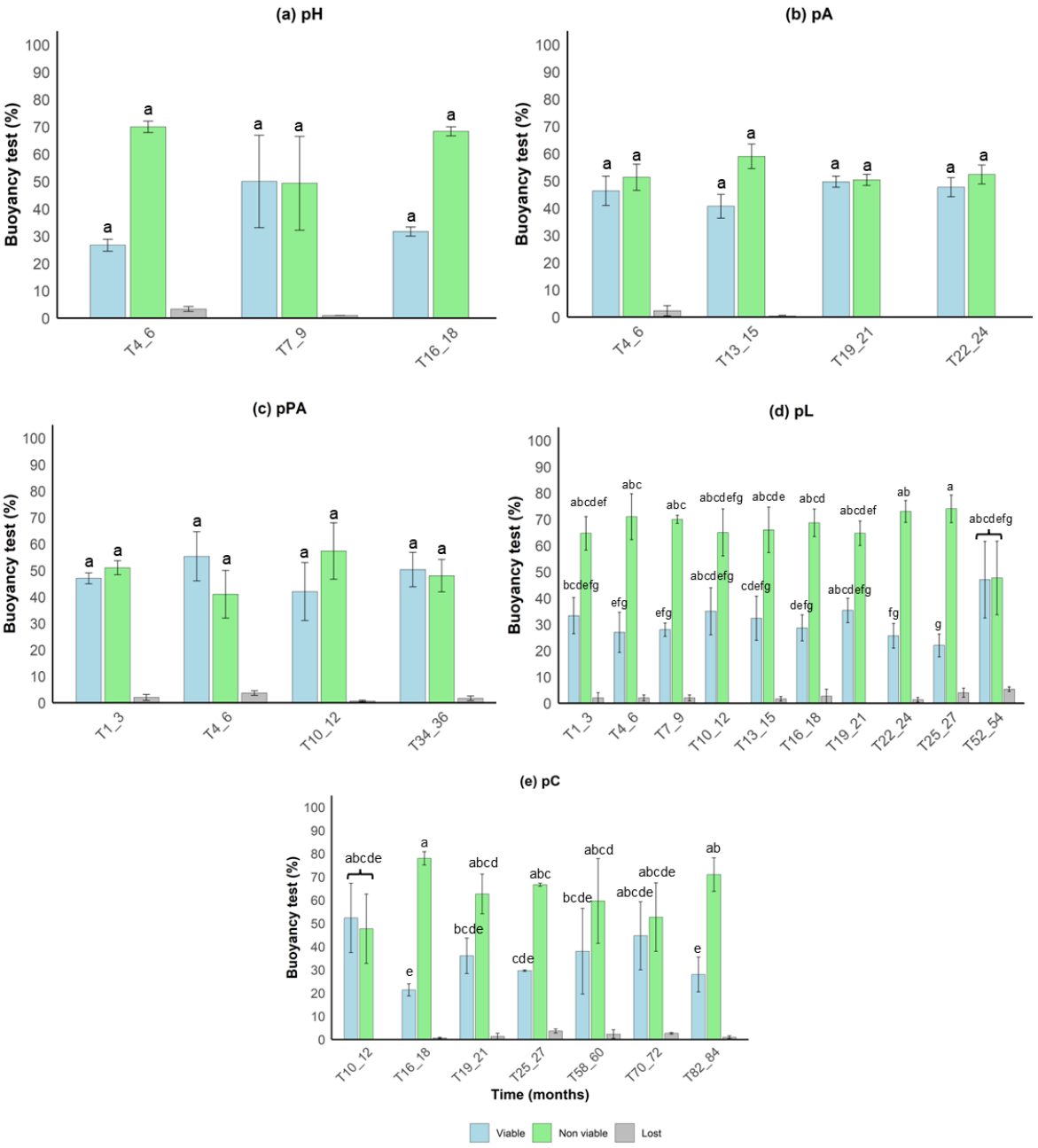


Figure 1. Identification of viable, non-viable seeds, and material loss based on the buoyancy test in seeds stored for different periods from various populations of *Colobanthus quitensis*. Different letters indicate significant differences ($p < 0.05$) according to Dunn's post-hoc test following Kruskal-Wallis (pH), Tukey HSD following ANOVA (pA, pPA, pL), and Duncan's test following ANOVA (pC) (n = 3). pH: Hannah Point; pA: Arctowski; pPA: La Marisma; pL: Laredo; and pC: Conguillío.

For populations pH, pA, pPA, and pC, no significant changes in electrical conductivity were observed across different seed storage periods (Fig. 2 a, b, c, and d). In the pL population, a significant effect of storage on electrical conductivity was detected; however, no clear pattern was observed among the different storage durations (Fig. 2 d).

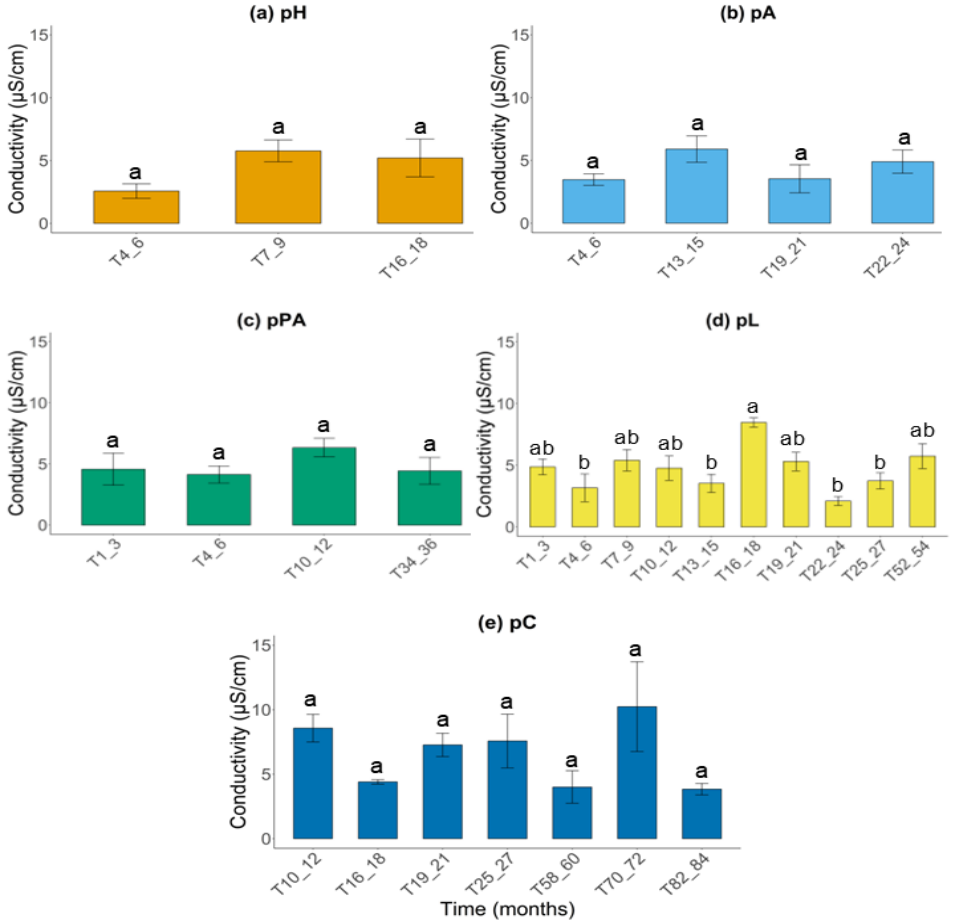


Figure 2. Electrical conductivity analysis of seeds from different populations of *Colobanthus quitensis* subjected to various storage periods. pH: Hannah Point; pA: Arctowski; pPA: La Marisma; pL: Laredo; and pC: Conguillío. Different letters indicate significant differences ($p < 0.05$) according to Tukey's HSD test following ANOVA (n = 3).

In pH population, germination never exceeded 5%, regardless of the storage duration (Fig. 3 a). In pA, freshly harvested seeds exhibited lower germination percentages compared to those stored for longer periods (Fig. 3 b). In pPA, seeds showed high overall germination rates; however, those stored for up to 3 months had lower germination percentages than the rest (Fig. 3 c). In pL, a bell-shaped distribution was observed: seeds with shorter and longer

storage times displayed lower germination, while the highest values were recorded between 7 and 24 months of storage (Fig. 3 d). Finally, in pC, seeds stored for more than 25 months showed a significant decrease in germination compared to the more recently stored seeds (Fig. 3 e).

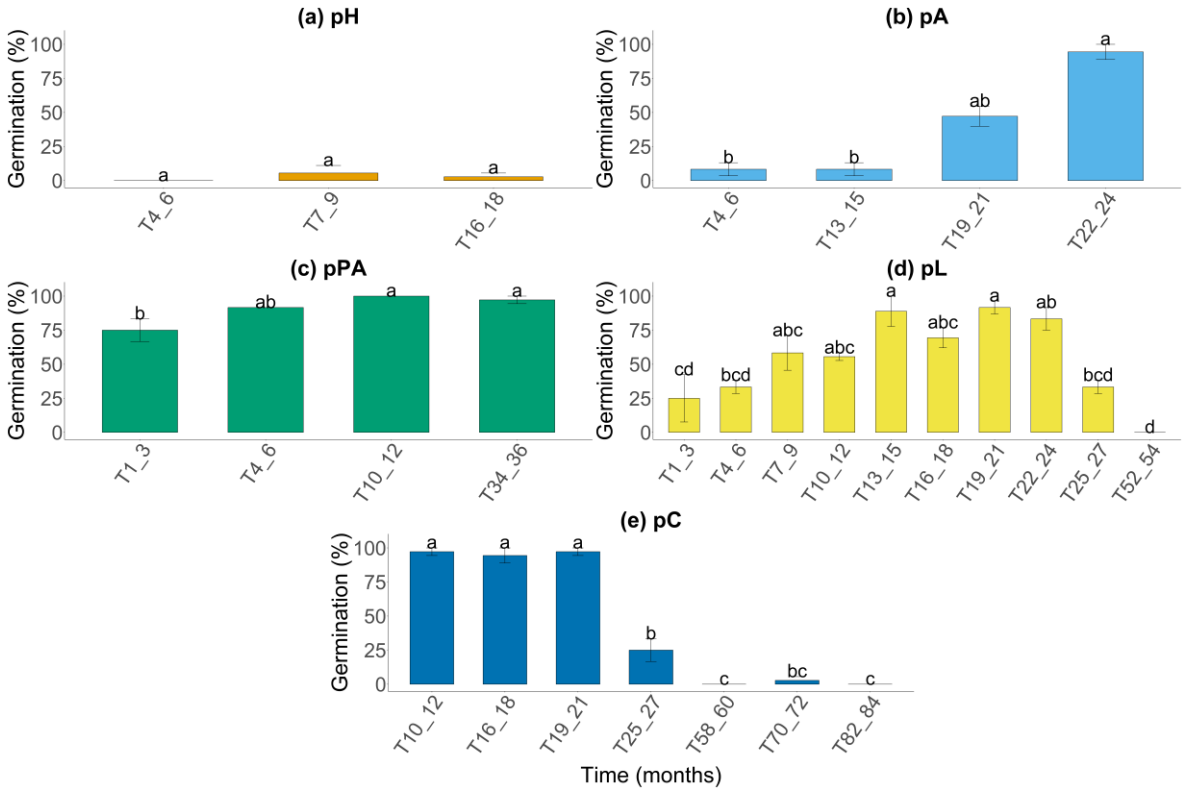


Figure 3. Germination percentage of different *Colobanthus quitensis* populations subjected to various storage durations. Different letters indicate significant differences ($p < 0.05$) according to Tukey's HSD test ($n = 3$). pH: Hannah Point; pA: Arctowski; pPA: La Marisma; pL: Laredo; and pC: Conguillío.

Pearson's correlation analysis revealed no significant relationship between electrical conductivity and germination percentage in any of the analyzed *Colobanthus quitensis* populations (Fig. 4).

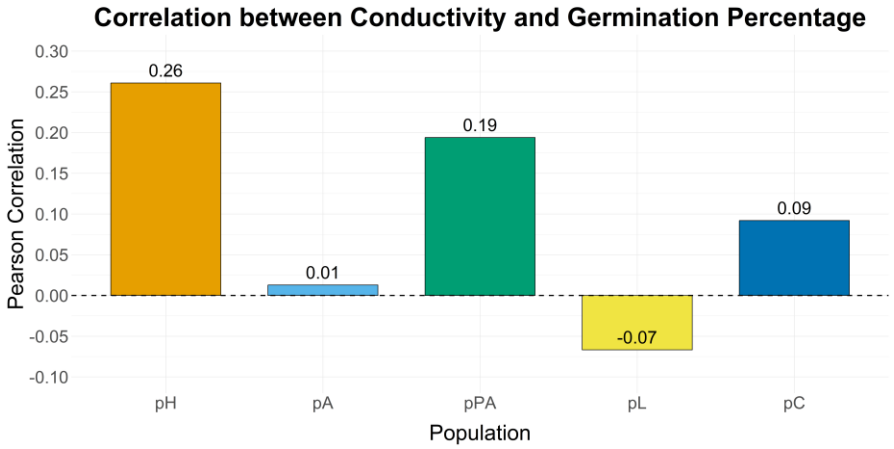


Figure 4. Correlation between electrical conductivity and germination percentage in different *Colobanthus quitensis* populations. pH: Hannah Point; pA: Arctowski; pPA: La Marisma; pL: Laredo; and pC: Conguillío.

The absence of germination in the pH population prevented the evaluation of the remaining germination indicators. For the other populations, storage time significantly affected at least one germination parameter: mean germination time (MGT), germination index (GI), or vigor index (VI). In the pA population, seeds stored for 19_21 and 22_24 months had a lower MGT and higher GI compared to those stored for 4_6 and 13_15 months. Regarding VI, seeds stored for 22_24 months showed the highest values, although they did not differ significantly from those stored for 19_21 months (Table 1).

In the pPA and pL population, storage time only affected the germination index (GI). For pPA, seeds stored for more than 3 months show the highest values. No significant differences were observed between seeds stored for 1_3 months and those stored for 4_6 months. In pL, the seeds stored for 19_21 months showed the highest GI, although this was only significantly different from the seeds stored for a maximum of 6 months and those stored for 25_27 months (Table 1).

In the pC population, storage time did not affect the VI. For MGT, differences were observed only between seeds stored for 16_18 and 25_27 months, with faster germination in the former. The GI was significantly lower in seeds stored for 25_27 months (Table 1).

Table 1. Germination indices in *Colobanthus quitensis* populations subjected to different storage periods. Different letters indicate significant differences ($p < 0.05$) according to Tukey-

HSD test (n = 3). MGT: mean germination time; GI: germination index; VI: vigor index. pA: Arctowski; pPA: La Marisma; pL: Laredo; and pC: Conguillío.

Population	Time (months)	Mean Germination Time (days)	p value	Germination Index	p value	Vigor Index	p value
pA	4_6	10.75 ± 1.77ab	0.0243	0.15 ±0.10 c	0.0003	0.52 ± 0.36 b	0.00653
	13_15	12 ± 0.00 a		0.13 ± 0.06 c		0.45 ± 0.25 b	
	19_21	8.94 ± 0.25 b		0.68 ± 0.17 b		3.64 ± 0.74 ab	
	22_24	8.82 ± 0.25 b		1.32 ± 0.17 a		6.83 ± 2.31 a	
pPA	1_3	9.10 ± 0.41 a	0.544	1.04 ± 0.17 b	0.00493	5.56 ± 0.46 a	0.386
	4_6	8.85 ± 0.26 a		1.29 ± 0.03 ab		5.54 ± 1.06 a	
	10_12	8.89 ± 0.80 a		1.42 ± 0.09 a		6.93 ± 1.63 a	
	34_36	8.41 ± 0.67 a		1.41 ± 0.05 a		6.71 ± 1.30 a	
pL	1_3	12.18 ± 0.32 a	0.272	0.37 ± 0.27 bc	0.0002	1.74 ± 1.59 a	0.143
	4_6	11.1± 1.39 a		0.42 ± 0.05 bc		1.46 ± 0.35 a	
	7_9	11.1± 0.49 a		0.65 ± 0.26 abc		2.43 ± 1.84 a	
	10_12	12.1 ± 1.4 a		0.57 ± 0.10 abc		1.79 ± 0.71 a	
	13_15	13.64 ± 3.71 a		0.86 ± 0.22 ab		3.09 ± 1.75 a	
	16_18	11.77 ± 0.99 a		0.72 ± 0.13 abc		2.87 ± 0.24 a	
	19_21	11.41 ± 0.69 a		0.98 ± 0.13 a		3.84 ± 1.25 a	
	22_24	13.89 ± 0.78 a		0.76 ± 0.17 abc		2.33 ± 1.01 a	
	25_27	13.76 ± 1.87 a		0.31 ± 0.11 c		0.95 ± 0.38 a	
pC	10_12	11.89 ± 0.63 ab	0.0326	1.01 ± 0.06 a	0.0002	6.56 ± 1.21 a	0.0677
	16_18	10.04 ± 1.19 b		1.17 ± 0.21 a		6.46 ± 4.57 a	
	19_21	11.49 ± 1.29 ab		1.07 ± 0.15 a		6.61 ± 1.29 a	

	25_27	15.7 ± 3.30 a		0.21 ± 0.15 b		1.12 ± 0.91 a	

In population pH, no differences were observed in germination percentage between seeds classified as viable and non-viable based on the float test, with both groups showing germination rates below 5% (Fig. 5a). In the remaining populations, germination was recorded in both viable and non-viable seed groups. In some cases, seeds previously classified as non-viable exhibited, or tended to exhibit, higher germination percentages than their viable counterparts (Fig. 5b, c, d, e).

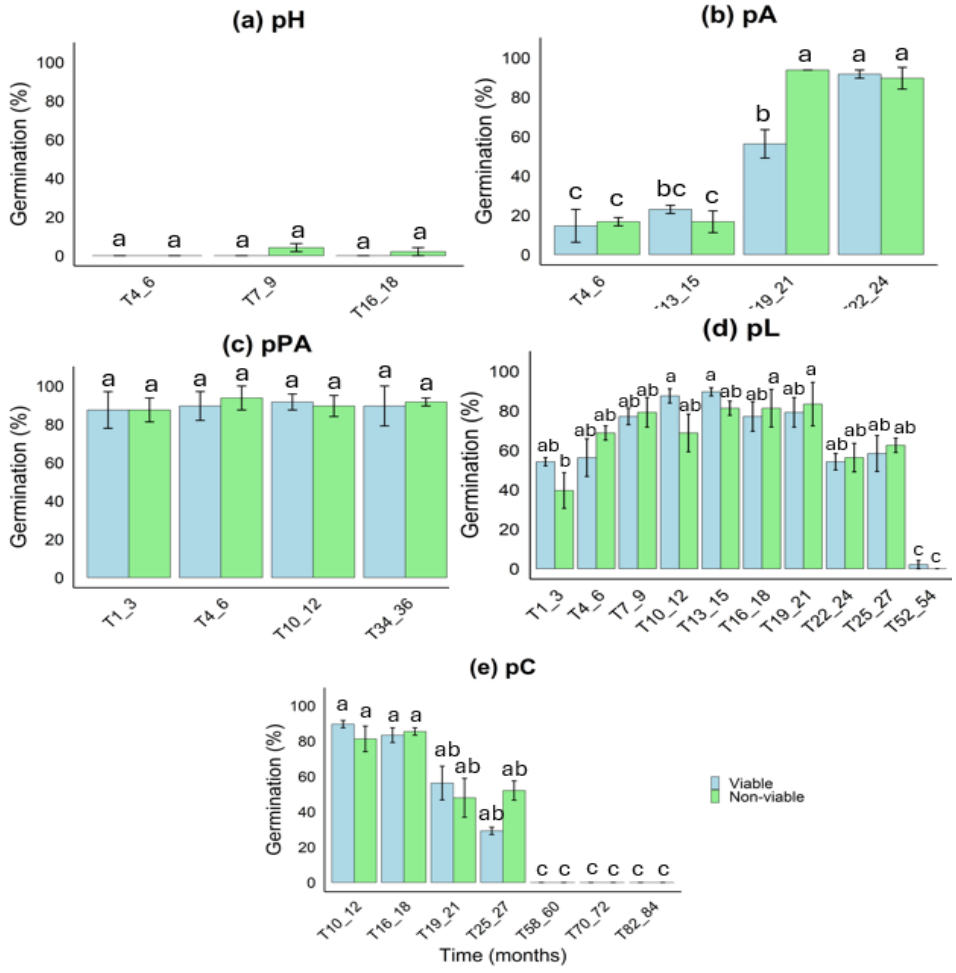


Figure 5. Germination of viable and non-viable seeds identified using the buoyancy test in different *Colobanthus quitensis* populations subjected to various storage periods. Different letters indicate significant differences ($p < 0.05$) according to Tukey HSD test (pH, pA, pPA,

and pL) and Dunn's test (pC). pH: Hannah Point; pA: Arctowski; pPA: La Marisma; pL: Laredo; and pC: Conguillío.

Tetrazolium staining revealed that, under several of the tested conditions, seeds that failed to germinate during the germination test still contained viable embryos. This was confirmed by the viability assessment of seed bank samples used as controls. No germination was recorded for pL seeds stored for 52–54 months, nor for pC seeds stored for 58–60, 70–72, and 82–84 months (Table 2).

Table 2. Viability of seeds from different populations of *Colobanthus quitensis* based on tetrazolium salt staining. pH: Hannah point population; pA: Arctowski population; pL: Laredo; and pC: Conguillío.

Populations	Time (months)	Viability (%) of non-germinated seeds	Viability (%) of seed bank
pH	4_6	83,33	75
pH	7_9	41,66	58,33
pH	16_19	50	58.33
pA	4_6	25	50
pA	13_15	33,33	41,66
pA	19_21	16.66	50
pL	1_3	25	58,33
pL	4_6	25	41,6
pL	10_12	16,66	41,6
pL	25_27	0	25
pL	52_54	0	0
pC	25_27	0	25
pC	58_60	0	0
pC	72_70	0	0
pC	82_84	0	0

The lack of germination in the pH population prevented the assessment of seedling fresh weight. In all other populations, seed storage time influenced seedling biomass. In pA, seeds stored for longer periods produced seedlings with lower fresh weight (Fig. 6a). In contrast, in pPA, seeds stored for up to 6 months resulted in seedlings with lower weight (Fig. 6b). For the

pL population, a decreasing trend in seedling weight was observed as storage time increased, although significant differences were only found between seeds stored for 1–3 months and those stored for 22–24 months (Fig. 6c). In pC, only seeds stored for 16–18 months had significantly lower seedling weight compared to the other treatments (Fig. 6d).

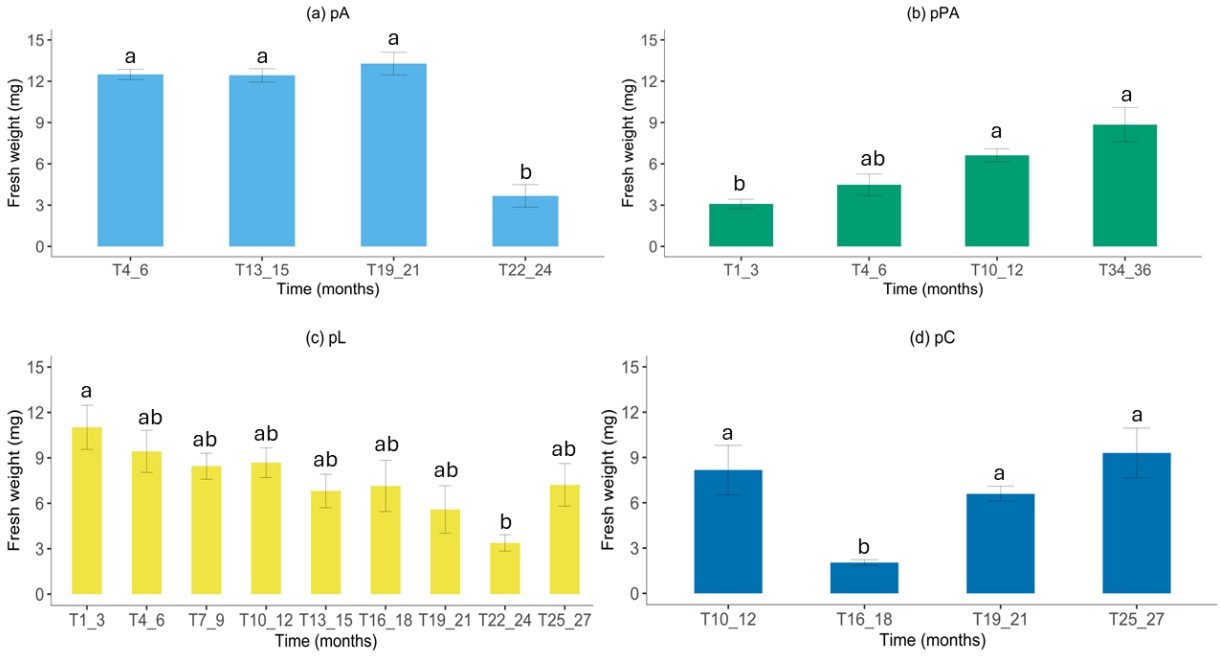


Figure 6. Fresh weight of seedlings derived from seeds stored for different durations in populations of *Colobanthus quitensis*. Different letters indicate significant differences ($p < 0.05$) according to Tukey's HSD test (pA) and Duncan's test (pPA, pL, and pC). pA: Arctowski; pPA: La Marisma; pL: Laredo; and pC: Conguillío.

4. DISCUSSION

4.1 Buoyancy Test as a Method to Assess Viability

The buoyancy test has been used as a strategy to identify damaged or dead seeds with reduced physiological quality (Suma and Srimathi, 2014; Vijay *et al*., 2025). In *Colobanthus quitensis*, this technique has previously been applied as a method to select viable seeds (Cuba-Díaz *et al*., 2017b, 2019). In the present study, the buoyancy test revealed significant differences between the proportion of viable and non-viable seeds only in the pL and pC populations (Fig. 1d, e). However, for most storage times, a higher proportion of seeds were classified as non-viable (floating) across all populations. When both seed groups (classified as viable or non-viable by the flotation test) were subjected to germination testing, germination

was observed in both, with no major differences in germination percentages (Fig. 5), indicating that the flotation test is not a reliable method for assessing the seed quality of *C. quitensis*.

Seed dispersal studies in *C. quitensis* are limited, although bird migration (Parnikoza *et al*., 2007), wind (Vera *et al*., 2013), and water (Kellmann-Sopyła *et al*., 2017) have been identified as influencing factors. Some seeds can float from one day to over a week, enabling water-based dispersal through a mechanism known as hydrochory (Fryirs and Carthey, 2022). A recent study on the phylogenetic relationships among different populations of *C. quitensis* suggests that the species reached Antarctica via maritime routes, indicating its tolerance to salinity (Biersma *et al*., 2020). The hard seed coat of *C. quitensis* (Kellmann-Sopyła *et al*., 2017) may render the seeds impermeable to water, thereby enhancing their buoyancy. Consequently, the flotation test is not a reliable method for assessing seed viability in this species, as germination was observed even in seeds classified as non-viable (Fig. 5).

4.2 Electrical Conductivity for Estimating Seed Quality

The electrical conductivity (EC) test is a biochemical technique used to assess cell membrane integrity by measuring the release of solutes during seed hydration. Seeds with lower vigor or greater deterioration tend to release higher amounts of ions and organic compounds into the medium, which is often associated with reduced germination capacity (Marcos-Filho, 2015). The EC test has proven effective for identifying seed lot vigor and is widely used due to its non-destructive nature, simplicity, speed, and low cost (Demir *et al*., 2012; Pereira & Martins Filho, 2012; Nugraheni *et al*., 2023). However, this method does not always correlate well with actual seed quality (Panobianco & Vieira, 2007; dos Santos *et al*., 2019).

In this study, the EC test showed significant differences ($p < 0.05$) only in the pL population, yet no clear pattern emerged between storage duration and conductivity values. In some cases, seeds stored for longer periods exhibited lower conductivity values than those stored for shorter periods (Fig. 2d). The low correlations observed between the EC test and germination performance suggest that electrical conductivity was not a reliable predictor under the evaluated conditions (Fig. 4). It is likely that the hard seed coat of *C. quitensis* (Kellmann-Sopyła *et al*., 2017) interferes with solute release into the medium, affecting the accuracy of conductivity measurements. Although this study followed a standardized protocol developed for tobacco seeds (Fantazzini *et al*., 2021), it is recommended to optimize the seed quantity and water volume used in the test (Nugraheni *et al*., 2023). This was not feasible here due to

the limited availability of seed material.

4.3 Germination indices and viability using tetrazolium salt staining

The germination test performed on seeds previously classified as viable through the flotation method revealed that the Antarctic population pH exhibited nearly zero germination percentages across all three storage periods analyzed (Fig. 3). A similar pattern was observed for population pA in seeds stored for shorter periods (4–6 and 13–15 months); however, germination percentages increased to over 47% when the storage time was extended (Fig. 3b). Germination rates in Antarctic populations are known to be highly variable, often ranging from 0 to 50%, a pattern commonly attributed to the presence of seed dormancy (Gielwanowska *et al*., 2011; Cuba-Díaz *et al*., 2017b; Kellmann-Sopyła *et al*., 2017; Ontivero *et al*., 2024b).

The increase in germination observed in seeds from the pA population with longer storage periods may be the result of a cold stratification effect due to storage at 4 °C. Although previous studies testing cold stratification for periods ranging from 7 days to 4 months reported no stimulation in germination percentage under these conditions (Day *et al*., 2001; Cuba-Díaz *et al*., 2019), germination can increase when stratification is extended to 4 years (Ruhland and Day, 2001). It is likely that prolonged stratification periods serve as a germination strategy for Antarctic seeds, but not for the other populations analyzed in this study (pL and pC), whose germination was virtually absent after 52 months (4.3 years) (Fig. 3 d, e; 5 d, e).

Population pPA consistently exhibited high germination percentages; however, seeds stored for 1_3 months showed significantly lower germination, with a clear increasing trend as storage time progressed, peaking at 36 months (Fig. 3c). The pPA population has historically shown high germination rates, even under stress conditions such as salinity (Cuba-Díaz *et al*., 2017b, 2019; Ontivero *et al*., 2024b). In population pL, storage time had a significant effect on germination, displaying a bell-shaped trend: both the freshest seeds (1_6 months) and the most aged ones (25_54 months) had lower germination percentages compared to seeds stored between 7 and 24 months (Fig. 3d). In the pC population, a significant decrease in germination was observed after 25 months of storage (Fig. 3e).

Although it was not possible to evaluate the same storage periods across the five populations analyzed, certain patterns were observed. In populations pA, pPA, and pL, freshly harvested seeds showed lower germination percentages. On the other hand, in populations pL and pC,

seeds stored for longer periods exhibited a notable decline in germination capacity. The reduced germination in fresher seeds may be related to the presence of dormancy in the species (Gielwanowska *et al*., 2011). The fact that tetrazolium staining revealed viable embryos in this group of seeds (Table 2) supports the potential role of dormancy in limiting germination. In contrast, in seeds stored for extended periods, physiological deterioration likely contributed to the reduced germination (Zhou *et al*., 2019), which was reflected in their lower viability as indicated by the tetrazolium test (Table 2).

Although the tetrazolium test is an effective tool for assessing seed viability (Dadlani & Yadava, 2023; Ursulino *et al*., 2025) and has previously been used in studies of *C. quitensis* (Gielwanowska *et al*., 2011; Kellmann-Sopyła & Giełwanowska, 2015; Sanhueza *et al*., 2017; Koc *et al*., 2018), it is important to consider that this method may overestimate the actual viability of seed lots. Therefore, it is advisable to complement it with other quality assessment tests, such as germination tests (Marcos-Filho, 2015).

Seed deterioration tends to affect not only the germination percentage but also other indicators such as mean germination time (MGT), germination index (GI), and vigor index (VI) (Yi *et al*., 2025). In this study, it was expected that MGT would increase with storage time, while GI and VI would decrease. However, this pattern was not consistent across all populations. For example, seeds from the pA population stored for 19 to 24 months showed lower MGT and higher GI and VI values, indicating seeds of higher physiological quality. Similarly, in the pPA population, seeds stored for longer periods exhibited higher GI compared to fresher seeds (Table 1). It has been shown that seed coat hardness contributes to protection against deterioration (Dai *et al*., 2023). It is likely that this characteristic in *C. quitensis* contributed to the preservation of seed quality over time.

4.4 Seedlings fresh weight

Seed deterioration can lead to damage in DNA integrity, protein degradation, accumulation of reactive oxygen species (ROS), and depletion of storage reserves. These factors affect not only germination capacity but also seedling vigor (Zhou *et al*., 2019; Das & Biswas, 2021; Dai *et al*., 2023). In this study, not all populations followed the same pattern regarding seedling fresh weight in relation to seed storage duration. For instance, in population pA, seeds stored for longer periods exhibited lower fresh weight (Fig. 6a), even though they also showed the highest germination percentages (Fig. 3b). Conversely, in pPA, it was the freshest seeds that

produced seedlings with the lowest fresh weight (Fig. 6b). In population pL—where the longest storage periods were analyzed—there was a clear trend of decreasing seedling fresh weight with increasing storage time, which may be associated with seed deterioration. This trend could not be clearly identified in other populations due to the limited number of storage time points assessed.

5. CONCLUSIONS

This study reinforces the importance of using multiple vigor tests to accurately assess the physiological quality of seeds. Results demonstrate that the float test is not a reliable method to identify viable seeds in *C. quitensis*, as it may lead to the unnecessary disposal of viable material. Under the experimental conditions used, the electrical conductivity test did not adequately reflect the physiological quality of seeds, although it may be worth optimizing the protocol for this species. Germination testing was the most effective method for evaluating seed quality. However, tetrazolium staining revealed that some viable seeds were unable to germinate, highlighting the need to implement strategies to overcome dormancy, possibly by adjusting parameters of the current *in vitro* germination protocol. A kinetic study of seed quality loss over a greater number of storage periods would be beneficial. However, the findings of this study show that seeds stored for more than 52 months at 4ºC exhibit almost no germination capacity and are therefore not recommended for long-term germplasm conservation or propagation. Nevertheless, lower storage temperatures might help extend the viability of the material.

6. REFERENCES

Biersma, E. M., Torres-Díaz, C., Molina-Montenegro, M. A. *et al*. (2020). Multiple late-Pleistocene colonisation events of the Antarctic pearlwort *Colobanthus quitensis* (Caryophyllaceae) reveal the recent arrival of native Antarctic vascular flora. *Journal of Biogeography, 47* (8), 1663-1673. https://doi.org/ 10.1111/jbi.13843

Clemente-Moreno, M. J., Omranian, N., Sáez, P., *et al*. (2020). Cytochrome respiration pathway and sulphur metabolism sustain stress tolerance to low temperature in the Antarctic species *Colobanthus quitensis*. *New Phytologist, 225*, 754-768. https://doi.org/10.1111/nph.16167.

Convey, P. (1996). Reproduction of Antarctic flowering plants. *Antarctic Science, 8* (2), 127-134.

Correia, L.V., Pereira, L.C., Matera, T.C., Pereira, R.C., Suzukawa, A.K., Santos, R.F., Pastorini, L.H., Carvalho, C., Osipi E.A.F., & Braccini A.L. (2020). Accessing the relevance of tests for estimating the physiological quality of wheat grains. *Plant Soil Environment, 66*, 477–482. https://doi.org/10.17221/213/2020-PSE

Cuba-Díaz, M., Acuña, D., & Fuentes-Lillo, E. (2019). Antarctic pearlwort (*Colobanthus quitensis*) populations respond differently to pre-germination treatments. *Polar Biology, 42*, 1209–1215. https://doi.org/10.1007/s00300-019-02505-4

Cuba-Díaz, M., Klagges, M., Fuentes-Lillo, E., & Cordero, C. M. (2017b). Phenotypic variability and genetic differentiation in continental and island populations of *Colobanthus quitensis* (*Caryophyllaceae*: Antarctic pearlwort). *Polar Biology, 40*, 2397–2409. https://doi.org/10.1007/s00300-017-2152-x

Dadlani, M., & Yadava, D. (Eds.) (2023). *Seed Science and Technology: Biology, Production, Quality*. Springer Nature, Singapore.

Dai, L., Chen, Y., & Wei, X. (2023). Hard Seed Characteristics and Seed Vigor of *Ormosia hosiei*. *Agriculture*, *13*(5), 1077. https://doi.org/10.3390/agriculture13051077

Das, R., & Biswas, S. (2021). Invigoration of various sized sunflower seeds: an evaluation of germination and seedling quality parameters under different aging conditions. *Journal of Oilseeds Research, 38*(2), 137-144.

Day, T. A., Ruhland, C. T., & Xiong, F. S. (2001). Influence of solar ultraviolet-B radiation on Antarctic terrestrial plants: results from a 4-year field study. *Journal of Photochemistry and Photobiology Biology, 62*, 78–87. https://doi.org/10.1016/s1011-1344(01)00161-0

Demir, I., Cebeci, C., & Guloksuz, T. (2012). Electrical conductivity measurement to predict germination of commercially available radish seed lots. *Seed Science and Technology, 40* (2), 229-237. https://doi.org/10.15258/sst.2012.40.2.08

dos Santos, L. A., Carvalho, I. R., Pinto, C. C. *et al*. (2019). Electrical conductivity test for measurement of white clover seeds vigor. *Journal of Agricultural Science, 11* (12), 40-49. https://doi.org/10.5539/jas.v11n12p40

Fantazzini, T.B., Boas, S.V., Oliveira, R.M., Rocha, D.K., Moreira, M.L., & Rodrigues, V.O. (2021). Electrical conductivity test to evaluate the physiological quality of tobacco seeds.

Brazilian Journal of Development, 7 (2), 14903 – 14916. https://doi.org/10.34117/bjdv7n2-219

Fryirs, K. & Carthey, A. (2022). How long do seeds float? The potential role of hydrochory in passive revegetation management. *River Research and Applications, 38*(6), 1139-1153. https://doi.org/10.1002/rra.3989

Gang, G. D., McDonald, T., Walder, B. et al. (2019). International principles and standards for the practice of ecological restoration. *Restoration Ecology, 27*(1), 1–46. https://doi.org/10.1111/rec.13035

García, J., & Coelho, C. M. M. (2021). Accelerated aging predicts the emergence of rice seedlings in the field. *Semina Ciencias Agrarias Londrina, 42*(3), 1397-1410. https://doi.org/10.5433/1679-0359.2021v42n3SupI1p1397

Gianoli, E., Inostroza, P., Zúñiga-Feest, A., Reyes-Díaz, M., Cavieres, L.A., Bravo, L.A., & Corcuera, L.J. (2004). Ecotypic differentiation in morphology and cold resistance in populations of *Colobanthus quitensis* (*Caryophyllaceae*) from the Andes of Central Chile and the Maritime Antarctic. *Arctic, Antarctic, and Alpine Research, 36*(4), 484–489. https://doi.org/10.1657/1523-0430(2004)036[0484:EDIMAC]2.0.CO;2

Gielwanowska, I., Bochenek, A., Gojlo, E., Górecki, R., Kellmann, W., Pastorczyk, M., & Szczuka, E. (2011). Biology of generative reproduction of *Colobanthus quitensis* (Kunth) Bartl. from King George Island, South Shetland Islands. *Polish Polar Research, 32*, 139–155. https://doi.org/10.2478/v10183−011−0008−6

ISTA (International Seed Testing Association) (2017). International rules for seed testing. ISTA, Zurich, Switzerland.

Kehinde, T. O., Ajala, M. O., Daniel, I. O., & Oyelakin, O. O. (2013). Physiological and genetic integrity of amaranth (*Amaranthus* spp.) seeds during storage. *International Journal of Plant Breading and Genetics, 7*(1), 35-46. https://doi.org/10.3923/ijpbg.2013.35.46

Kellmann-Sopyła, W., & Giełwanowska, I. (2015). Germination capacity of five polar Caryophyllaceae and Poaceae species under different temperature conditions. *Polar Biology 38*, 1753–1765. https://doi.org/10.1007/s00300-015-1740-x

Kellmann-Sopyla, W., Koc, J., Górecki, R. J., Domaciuk, M., & Gielwanowska, I. (2017). Development of generative structures of polar Caryophyllaceae plants: the Arctic *Cerastium alpinum* and *Silene involucrata*, and the Antarctic *Colobanthus quitensis*. *Polish Polar Research, 38*, 83–104. https://doi.org/10.1515/popore-2017-0001

Koc, J., Wasilewski, J., Androsiuk, P., Kellmann-Sopyla, W., Chwedorzewska, K. J., & Gielwanowska, I. (2018). The effects of methanesulfonic acid on seed germination and morphophysiological changes in the seedlings of two *Colobanthus* species. *Acta Societatis Botanicorum Poloniae, 87*, 3601. https://doi.org/10.5586/asbp.3601

Li, C., Dong, N., Zhao, Y., Wu, S., Liu, Z., & Zhai, J. (2021). A review for the breeding of orchids: Current achievements and prospects. *Horticultural Plant Journal*, *7*(5), 380 – 392. https://doi.org/10.1016/j.hpj.2021.02.006

Marcos-Filho, J. (2015). Seed vigor testing: an overview of the past, present and future perspective. *Scientia Agricola, 72*, 363-374. https://doi.org/10.1590/0103-9016-2015-0007

Milivojević, M., Srdić, J., Filipović, M., Petrović, T., Branković Radojčić, D., Marković, K., & Boćanski, J. (2021). Application of standard germination and vigour tests for seed quality assessment of maize inbred lines. *Selekcija I Semenarstvo, 27*, 35-45. https://doi.org/10.5937/SelSem2102035M

Milošević, M., Vujaković, M., & Karagić, D. (2010). Vigour tests as indicators of seed viability. *GENETIKA, 42*(1): 103-118. https://doi.org/10.2298/GENSR1001103M

Min, K., Sulaiman, S., Jeong, J., Lee, H., Lee, J., Lee, J.H., & Lee, H. (2024). Exploring the mechanisms underlying recovery from freeze–thaw injury in *Colobanthus quitensis*: mechanistic insights via transcriptome profiling. *Physiologia Plantarum, 176*(6), e14642. https://doi.org/10.1111/ppl.14642.

Moore, D. M. (1970). Studies in *Colobanthus quitensis* (Kunth) Bartl. and *Deschampsia antarctica* Desv. II. Taxonomy, distribution and relationships. *British Antarctic Survey Bulletin, 23*, 63–80.

Nicoleti, L. F., & Coelho, A. D. L. (2018). Physiological maturity of corn seeds (*Zea mays*) by tetrazolium salt. *Journal of Food Science and Engineering, 8*, 248-256. https://doi.org/10.17265/2159-5828/2018.06.004

Nugraheni, Pujiasmanto, B., & Samanhudi, A. T. S. (2023). Comparison between the electrical conductivity method and radicle emergence test as a rapid test of sorghum seed vigor. *Journal Kultivasi, 22*(2), 200-209. https://doi.org/10.24198/kultivasi.v22i2.46547.

Ontivero, Y., Cuba-Díaz, M., Fuentes-Lillo, E. & Convey, P. (2024a). Germination Strategies and Seed Quality of *Colobanthus quitensis*: Implications for Sustainable Antarctic Ecosystems

and *Ex Situ* Plant Conservation. *Sustainability, 16*, 10726. https://doi.org/10.3390/su162310726

Ontivero, Y., Fuentes-Lillo, E., Navarrete-Campos, D., Vázquez-Villa D., Cabrera-Barjas, G., & Cuba-Díaz, M. (2024b). Preliminary assessment of seed heteromorphism as an adaptive strategy of *Colobanthus quitensis* under saline conditions. *Scientific Report, 14*(31120). https://doi.org/10.1038/s41598-024-82381-z.

Panobianco, M., & Vieira, R. D. (2007). Eletrical conductivity and deterioration of soybean seeds exposed to different storage conditions. *Revista Brasileira de Sementes, 29*(2). https://doi.org/10.1590/S0101-31222007000200013

Parnikoza, I. Y., Maidanuk, D. N., & Kozeretska, I. A. (2007). Are *Deschampsia antarctica Desv.* and *Colobanthus quitensis (Kunth) Bartl.* Migratory relicts? *Cytology and Genetics, 41*(4), 226–229. https://doi.org/10.3103/s0095452707040068

Pereira, M. D., & Martins Filho, S. (2012). Adequação da metodologia do teste de condutividade elétrica para sementes de cubiu (*Solanum sessiliflorum* DUNAL). *Revista Agrarian, 5*, 93-98. https://ojs.ufgd.edu.br/agrarian/article/view/707

Ruhland, C., & Day, T. (2001). Size and longevity of seed banks in Antarctica and the influence of ultraviolet-B radiation on survivorship, growth and pigment concentrations of *Colobanthus quitensis* seedlings. *Environmental and Experimental Botany, 45*, 143–154. https://doi.org/10.1016/S0098-8472(00)00089-7

Sanhueza, C., Vallejos, V., Cavieres, L.A., Sáez, P., Bravo, L.A., & Corcuera, L. J. (2017). Growing temperature affects seed germination of the Antarctic plant *Colobanthus quitensis* (Kunth) Bartl (*Caryophyllaceae*). Polar Biology, 40, 449–455. https://doi.org/10.1007/s00300-016-1972-4

Smith, R. (2003). The enigma of *Colobanthus quitensis* and *Deschampsia antarctica* in Antarctica. In: Huiskes, A., Gieskes, W., Rozema, J. *et al*. (Eds.), Antarctic Biology in a Global Context. Backhuys, Leiden, pp. 234–239.

Solberg, S, Ø., Yndgaard, F., Andreasen, C., Von Bothmer, R., Loskutov, I. G., & Asdal, Å. (2020). Long-term storage and longevity of orthodox seeds: a systematic review. *Frontiers in Plant Science, 11*(1007). https://doi.org/10.3389/fpls.2020.01007

Souza, F.F.S., Devilla, I.A., Souza, R.T.G. Teixeira, I.R., & Spehar, C.R. (2020). Physiological quality of quinoa seeds submitted to different storage conditions. *African Journal of Agricultural*

Research, 11(15), 1299-1308. https://doi.org/10.5897/AJAR2016-10870

Suma, N., & Srimathi, P. (2014). Influence of water flotation technique on seed and seedling quality characteristics of *Sesamum indicum. Journal of Agriculture and Veterinary Science, 7*, 51-53.

Taylor, A. G. (2020). Seed storage, germination, quality, and enhancements. In: Wien, H. C. y Stützel, H. (Eds) Physiology of vegetable crops. Second Edition. CAB International. 30 pp.

Ursulino, M. M., Alves, E. U., de Melo P. A. F. R. et al. (2025). Seed selection of *Dimorphandra gardneriana* using the tetrazolium test. *Brazilian Journal of Agricultural and Environmental Engineering, 29*(8), e288877. https://doi.org/10.1590/1807-1929/agriambi.v29n8exxxxxx

Vera, M.L., Fernández-Teruel, T., & Quesada, A. (2013). Distribution and reproductive capacity of *Deschampsia antarctica* and *Colobanthus quitensis* on Byers Peninsula, Livingston Island, South Shetland Islands, Antarctica. *Antarctic Science, 25*(2), 292-302. https://doi.org/10.1017/S0954102012000995.

Vijay, D., Manjunath-Prasad, C.T., & Vijayakumar, H. P. (2025). Seed Quality Control and Assurance. In: Dadlani, M., Das Gupta, P.R., Dadlani, N.K. (eds) Indian Seed Sector. Springer, Singapore. https://doi.org/10.1007/978-981-96-0714-3_11.

Wawrzyniak, M. K., Kalemba, E. M., Ratajczak, E., & Chmielarz, P. (2020). Oxidation processes related to seed storage and seedling growth of *Malus sylvestris*, *Prunus avium* and *Prunus padus*. *PLoS One, 15*(6), e0234510. https://doi.org/10.1371/journal.pone.0234510

Whitehouse, K. J., Hay, F. R., & Lusty, C. (2020). Why seed physiology is important for genebanking. *Plants, 9*, 584. https://doi.org/10.3390/plants9050584

Yi, K., Gao, l., Xu, Y., Yang, J., Mao, P., Dou, L., & Li, M. (2015). Lipid remodeling and response mechanisms during the germination of aged oat seeds. *BMC Plant Biology, 25*(186). https://doi.org/10.1186/s12870-025-06191-9

Zhou, W., Chen, F., Luo, X., Dai, Y., Yang, Y., Zheng, C., Yang, W., & Shu, K. (2019). A matter of life and death: Molecular, physiological, and environmental regulation of seed longevity. *Plant, Cell & Environment, 43*, 293–302. https://doi.org/10.1111/pce.13666

VIII. CONCLUSIONES GENERALES

Esta investigación integra aspectos clave del ciclo de vida de *Colobanthus quitensis*, desde la producción y calidad de semillas, hasta estrategias para potenciar la germinación de la especie, optimizando su conservación y consolidando a esta especie como un modelo para estudios de fisiología y tecnología de semillas.

Esta investigación constituyó la primera evidencia de la presencia de heteromorfismo de color en cuatro poblaciones de *C. quitensis* y su impacto sobre el porcentaje de germinación funcionando como un mecanismo de adaptación a condiciones extremas, donde la población subantártica pPA, al ser la población más tolerante a salinidad, constituye un modelo para estudiar la tolerancia al estrés salino. Identificamos que las modificaciones en los azúcares solubles y ácido indol 3-acético constituyen marcadores bioquímicos para estimar de forma rápida el vigor de las semillas. Los diferentes experimentos permitieron descartar pruebas de análisis de la calidad (Anexo 1) como la prueba de flotación para semillas que tienen testa dura y capacidad para flotar, así como la prueba de conductividad.

Confirmamos que el tiempo de conservación influye diferencialmente sobre la calidad de las semillas (Anexo 2) de las diferentes poblaciones de *C. quitensis*, a pesar de lo cual, la tendencia es que semillas frescas (1_12 meses) y semillas envejecidas (más de 52 meses) tienen menores porcentajes de germinación comparado con semillas que tienen entre 12_24 meses de conservación. Siendo la prueba de germinación y de tetrazolio, las más efectivas para conocer la viabilidad real de las semillas.

Para la germinación *in vitro*, se demostró que *C. quitensis* no requiere medios con altas concentraciones de nutrientes, siendo suficiente un sustrato básico de agar y agua. Sin embargo, un tratamiento previo de halo-priming con KCl (5%) o sus alternativas un poco más costosas con KCl al 10% y osmo-priming con polietilenglicol al 10% mejoran significativamente la germinación, mientras que la escarificación mecánica es esencial para superar la dormancia en poblaciones antárticas.

Además, se identificó que los hongos endófitos (*Umbelopsis dimorpha*, *Trichoderma harzianum* y *Hypocrea lixii*) y extractos fúngicos de (*T. harzianum*, *H. lixii*) son capaces de acelerar la apertura floral, modificar el área, perímetro, peso y contenido de agua de las semillas de *C. quitensis* y el crecimiento de las plántulas. Pero a la vez evidencia que la relación microorganismo-planta depende en gran medida del nivel de afinidad entre la planta y cada hongo, ya que no todas las poblaciones de *C. quitensis* respondieron de igual forma a la inoculación fúngica o a sus extractos.

Se confirmó que las semillas de *C. quitensis* son ortodoxas, manteniendo su viabilidad incluso en criopreservación (-20°C y -80°C), lo que valida su potencial para ser almacenadas a largo plazo en bancos de germoplasma.

Las hipótesis planteadas se cumplieron parcialmente. Se corroboró que las semillas antárticas presentan dormancia y que por ende requieren de un tratamiento adicional de escarificación mecánica para lograr altos porcentajes de germinación. Sin embargo, no se encontraron evidencias sobre expresión de factores protectores contra el deterioro en comparación con poblaciones continentales, sugiriendo que otros mecanismos fisiológicos y ecológicos podrían estar involucrados. La capacidad de tolerar temperaturas de -20 y -80ºC confirma la clasificación de las semillas de esta especie como ortodoxas, aunque se requieren tiempos de evaluación superiores a 6 meses para identificar si estas alternativas de conservación son mejores que la conservación a 4ºC para prolongar la viabilidad por más de 4 años.

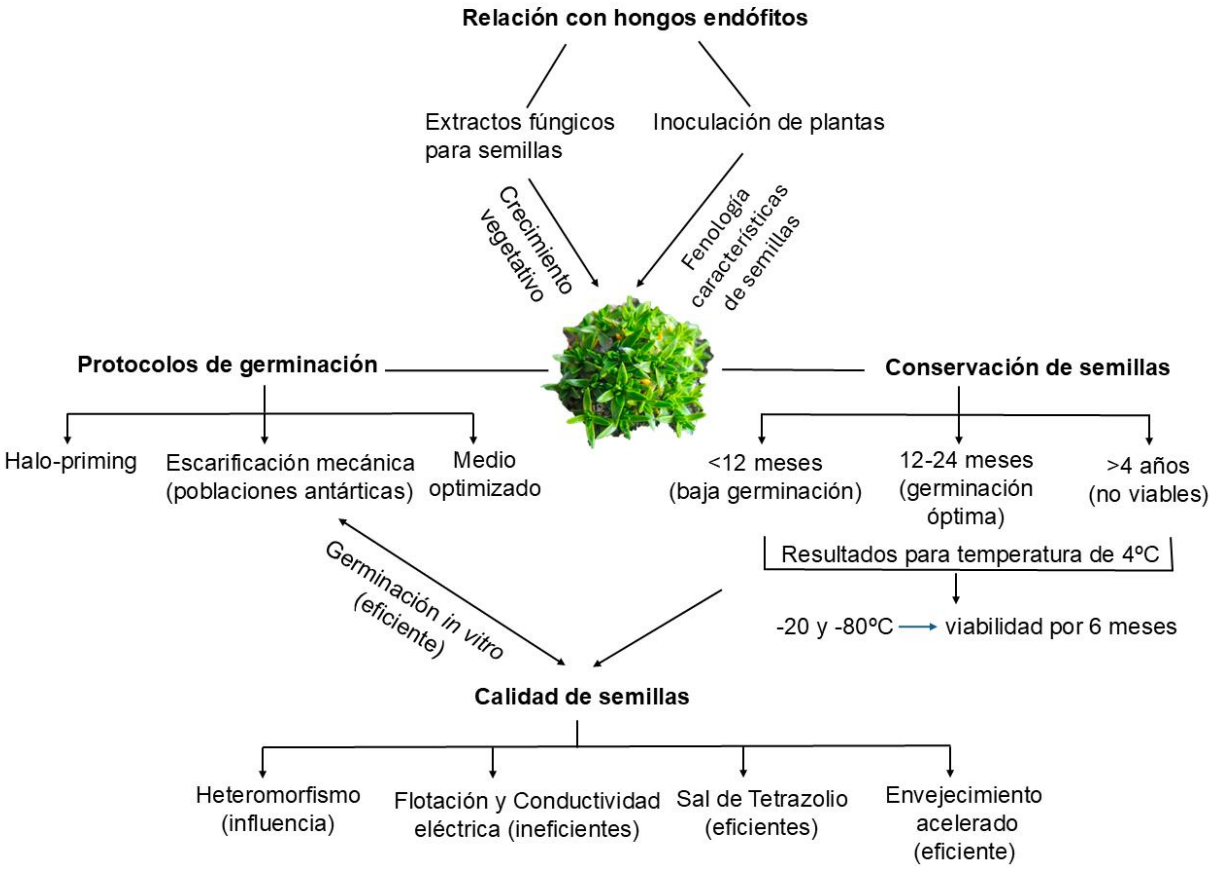
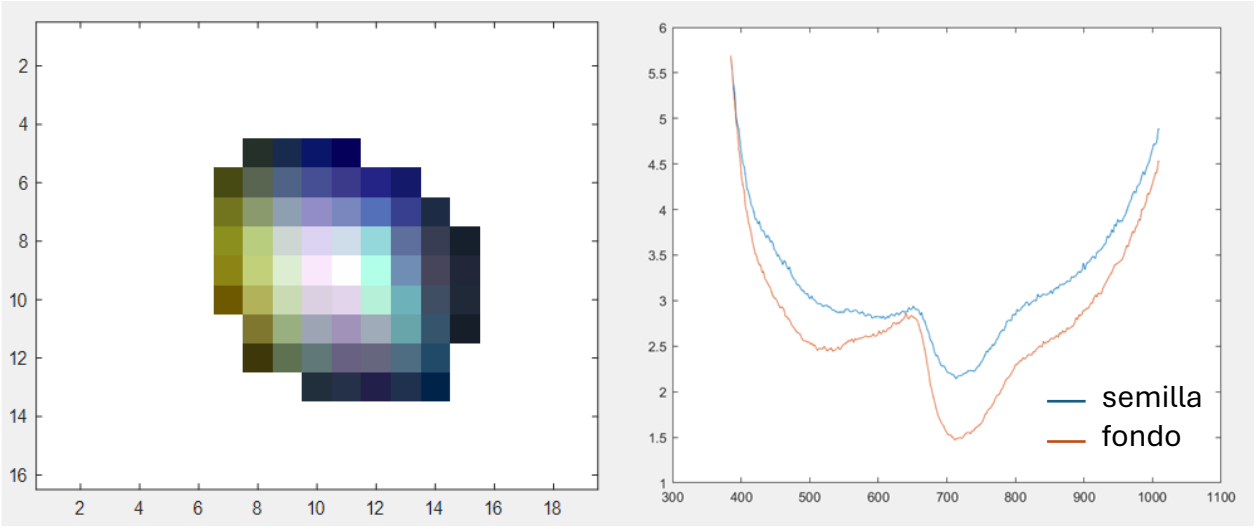


Figura 1. Áreas de investigación y avances generales en el estudio de *Colobanthus quitensis.*

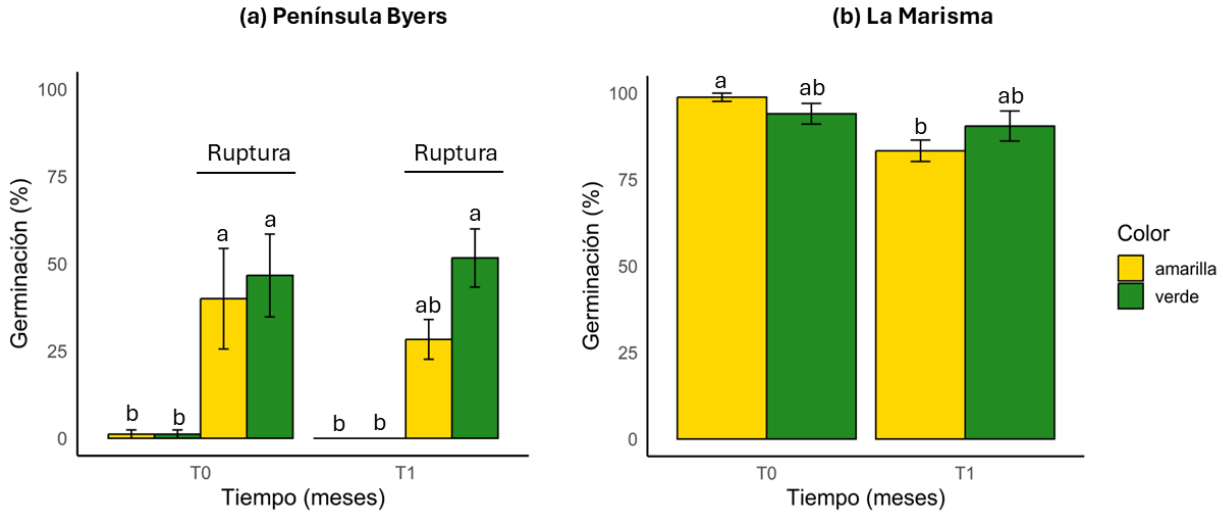
ANEXOS

1. Imágenes hiperespectrales



Anexo 1. Las imágenes hiperespectrales no permiten identificar los espectros de las semillas de *Colobanthus quitensis* y el fondo, por lo que no es adecuada para conocer la calidad de estas semillas cuyo tamaño es aproximadamente de 0,5 a 0,8 mm.

2. Ensayo de germinación de semillas (estudio dormancia)



Anexo 2. Porcentaje de germinación *in vitro* de semillas de *Colobanthus quitensis* de (a) Península Byers y (b) La Marisma, provenientes de cápsulas florales abiertas de color verde o amarillo (secas), recolectadas recientemente (T0) y tras un mes de conservación a 4 °C (T1). Las semillas de Península Byers que no germinaron fueron sometidas a escarificación mecánica (ruptura de la testa) como método para superar la dormancia. Letras diferentes

indican diferencias significativas ($p < 0,05$) según la prueba de Dunn posterior a Kruskal-Wallis (Península Byers) y Tukey HSD posterior a ANOVA (La Marisma) ($n = 4$).

3. Publicaciones

1. Ontivero, Y., Carrillo, V., Navarrete-Campos, D., & Cuba-Díaz, M. (2025). Accelerated aging in *Colobanthus quitensis* seeds: understanding stress responses in an extremophile species. Planta (enviado 16 de mayo de 2025).
2. Ontivero, Y., Deramond, C., Chávez, D., Ramos, P., & Cuba-Díaz, M. (2025). Can fungal extracts enhance the germination and early development of *Colobanthus quitensis*? Journal of Soil Science and Plant Nutrition (enviado 9 de mayo de 2025). Ontivero, Y., Salgado, C., Fuentes-Lillo, E., Sanhueza, C., Navarrete-Campos, C., Alveal, S., & Cuba-Díaz, M. (2025). Improved *in vitro* germination of *Colobanthus quitensis*: a key step for Antarctic plant conservation (aceptado).
3. Ontivero, Y., & Cuba-Díaz, M. (2025). El misterio alrededor del incremento poblacional de *Colobanthus quitensis*: la importancia de los bancos de semillas en el suelo antártico. INACH. https://www.inach.cl/el-misterio-alrededor-del-incremento-poblacional-de-colobanthus-quitensis/#:~:text=Avances%20de%20la%20ciencia%20Ant%C3%A1rtica.%20El%20misterio,siendo%20un%20enigma%20debido%20a%20sus%20extremas
4. Carrasco, M., Ontivero, Y., Navarrete-Campos, D., Convey, P., Cuba-Díaz, M. (2025). First report of vivipary in *Deschampsia antarctica*: a new insight into Antarctic plant reproductive strategies. Antarctic Science. https://doi.org/10.1017/S0954102025000069
5. Ontivero, Y., Cuba-Díaz, M., Fuentes-Lillo, E., & Convey, P. (2024). Germination Strategies and Seed Quality of Colobanthus quitensis: Implications for Sustainable Antarctic Ecosystems and Ex Situ Plant Conservation. Sustainability. https://doi.org/10.3390/su162310726
6. Ontivero, Y., Fuentes-Lillo, E., Navarrete-Campos, D., Vázquez-Villa, D., Cabreras-Barjas, G., Arroyo-Marín, F., & Cuba-Díaz, M. (2024). Preliminary assessment of seed heteromorphism as an adaptive strategy of *Colobanthus quitensis* under saline conditions. Scientific Reports. https://doi.org/10.1038/s41598-024-82381-z