GAYANA BOTANICA Gayana Bot. (2022) vol. 79, No. 1, 44-50

ORIGINAL ARTICLE

Asymbiotic germination of *Chloraea disoides* Lindl. (Orchidaceae), a critically endangered orchid endemic to Chile

Germinación asimbiótica de *Chloraea disoides* Lindl. (Orchidaceae), una orquídea endémica de Chile en peligro crítico de extinción

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ABSTRACT

Degradation of ecosystems is one of the main causes of the global decline in biodiversity. Many species are threatened with extinction and urgent actions are needed. *Chloraea disoides* is a critically endangered terrestrial orchid endemic to Chile. Our main goal was to evaluate the effect of three culture media in the asymbiotic germination of this species, aiming at its propagation in the long term. We tested the effect of Malmgren modified terrestrial orchid medium (MM), modified Murashige & Skoog medium (MS1/2), and agar-water (AW). After one week of incubation, seeds in all three media reached pre-germination stage. After four weeks, germination was achieved in MM and MS1/2. Protocorm stage was reached after 6 weeks only in MM. After 13-14 weeks, embryos reached the rhizoid stage in MM and MS1/2 and did not further develop until the end of the experiment (week 16). Overall, asymbiotic germination was statistically higher in MM and MS1/2 compared to AW, but very low nevertheless, compared to other Chilean *Chloraea* species, being lower than 8%. The laboratory propagation of *C. disoides* could be a key strategy to avoid its extinction in the short term but further studies are needed to fully understand its low germination capacity.

Keywords: terrestrial orchid, Chilean orchid species, orchid conservation, *in vitro* germination, culture media.

RESUMEN

La degradación de los ecosistemas es una de las principales causas del declive global de la biodiversidad. Muchas especies están amenazadas con la extinción y se requieren acciones urgentes. *Chloraea disoides* es una orquídea terrestre endémica de Chile que se encuentra en peligro crítico de extinción. El objetivo de este trabajo es evaluar el efecto de tres medios de cultivos en la germinación asimbiótica de esa especie, apuntando a su propagación en el largo plazo. Se probó el efecto de los medios Malmgren modificado (MM), Murashige & Skoog modificado (MS1/2) y agar agua (AW). Luego de una semana de incubación, las semillas de los tres medios alcanzaron el estado de pre-germinación. Luego de cuatro semanas, se alcanzó germinación en MM y MS1/2. El estado de protocormo se alcanzó a las 6 semanas, pero solo en MM. Luego de 13-14 semanas, los embriones alcanzaron el estado de rizoides en MM y MS1/2 y no se observó subsecuente desarrollo hasta el fin del experimento (semana 16). En general, la germinación asimbiótica

fue estadísticamente mayor en MM y MS1/2 comparada con AW, pero baja de todas formas comparada con otras especies chilenas de *Chloraea*, siendo inferior al 8%. La propagación de *C. disoides* en laboratorio podría ser una estrategia clave para evitar la extinción de esta especie en el corto plazo, pero más estudios se requieren para entender completamente su baja capacidad germinativa.

Palabras clave: orquídea terrestre, especies de orquídeas chilenas, conservación de orquídeas, germinación *in vitro*, medios de cultivo.

INTRODUCTION

Destruction and degradation of natural ecosystems are among the main causes of decline in global biodiversity (Saunders *et al.* 1991, Rands *et al.* 2010). Anthropic effects had led to the severe reduction of natural populations of many species. For example, it is estimated that over 1/5 of all plants species could be threatened according to UICN criteria (Brummitt *et al.* 2015). Moreover, the extinction rates of seed plants has doubled since 1900 (Humphreys *et al.* 2019).

Orchids belong to the Orchidaceae, one of the largest plant families of the world (Dressler 1993, Heywood *et al.* 2007, Govaerts *et al.* 2016). In Chile, there are 72 terrestrial species distributed in most of the country (Novoa *et al.* 2015). *Chloraea disoides* Lindl. is an endemic orchid that is found in Central Chile (Rodríguez *et al.* 2018) and is currently considered critically endangered (MMA 2011, Novoa *et al.* 2015). Natural populations are severely threatened and subjected to high anthropic pressure (i.e. cattle damage, wildfires, etc.) and some could disappear in the near future due to climate change (Atala *et al.* 2017). Additionally, most registered populations in Chile are not inside protected areas (Atala *et al.* 2017). Thus, urgent actions are needed to avoid the extinction of this species. For several Chilean orchids, including other *Chloraea* species, asymbiotic germination in enriched culture media has shown to be a successful strategy (Pereira *et al.* 2015, 2017). Moreover, plants germinated asymbiotically can be eventually re-inoculated with orchid fungi to form functioning mycorrhizae (Pereira *et al.* 2021). However, there is currently no data available on the germination of *C. disoides*.

The Malmgren modified terrestrial orchid medium (MM) and Murashige & Skoog medium (MS), among other enriched culture media, have been used in asymbiotic germination of terrestrial Chilean orchid with relative high success (Pereira *et al.* 2015, 2017, 2021). On the other hand, culture media with low nutrients, such as agar water (AW), usually result in low germination and in a stalled embryo development, likely due to the seed characteristics and their dependency on mycorrhizal fungi (Smith & Read 2008; Herrera *et al.* 2017). Other studies on symbiotic germination of tropical orchids have obtained the highest germination, and also greater embryo developments, using MS supplemented with pineapple juice, coconut water, and hormones (Salazar & Vega 2017, Salazar & Botello 2020). In some Chilean terrestrial orchids, culture medium supplemented with organic additives such as banana and tomato have been tested with somewhat positive results in seed germination (Pereira *et al.* 2015). However, in that study the greatest germination and developments was reached using MM.

This study aims to address the effect of three different culture media on the asymbiotic germination of mature seed of *C. disoides*. This may contribute to future conservation and ecological restoration initiatives for this critically endangered orchid species.

MATERIALS AND METHODS

Seed collection and viability test

Seeds of *Chloraea disoides* (Fig. 1a) were collected from a natural population located in the Valparaíso region, central Chile (33° 03' 33.1" S, 71° 31' 12.9" W). We took mature capsules before dehiscence from 3 adult plants. These capsules were formed by natural pollination. Since this species is critically endangered (Novoa *et al.* 2015), is very rare to find more than a couple of individuals in a given population (see Atala *et al.* 2017). Capsules were transported to the laboratory and dried at 24°C for two days. Then, capsules were superficially disinfected with a chloride solution at 0.1% v/v and seeds were collected from capsules and stored in a hermetic plastic container at 4°C for 5 months until later use in the experiments described below. We checked seed viability using the tetrazolium (TZ) test. For this, we put 100 *C. disoides* seeds in a 1% TZ solution for 24 h in the dark (see Muñoz & Jiménez 2008). Seeds were then observed with a light microscope (Olympus CX30). Viable seeds contained ovoid embryos that were stained pinkish-brown. We repeated this test for 3 independent 100 seeds sample (n=3).

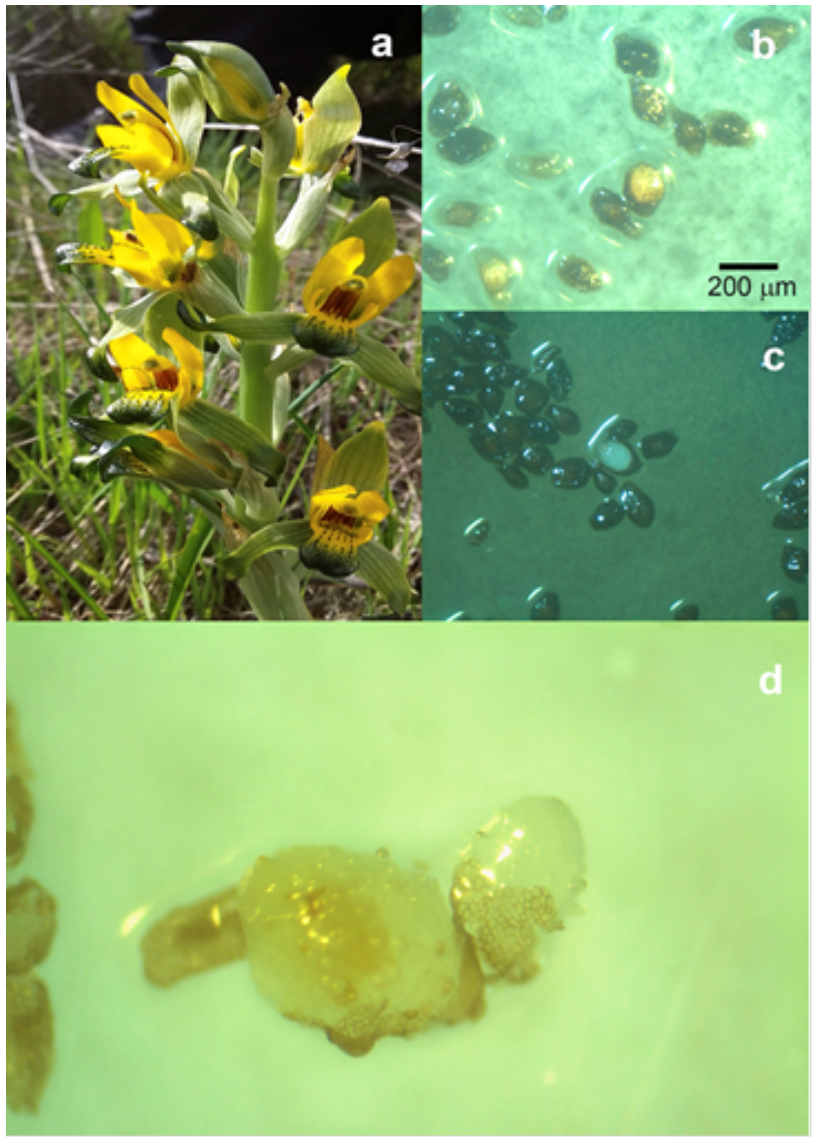

Figure 1. a = Flowering individual of *Chloraea disoides* growing in a natural population. b = Embryo braking the seed coat. c = Protocorm. d = Protocorm with initiating rhizoids. / a = Individuo de *Chloraea disoides* con flores creciendo en una población natural. b = Embrión rompiendo la testa. c = Protocormo. d = Protocormo con rizoides iniciales.

Germination experiment

Seeds to be used in the germination assay were sterilized using an ethanol 70% solution for 30 seconds and then a 1% NaOCl solution with 2 drops of Tween 20 for 5 minutes under constant shacking using a vortex mixer at 1200 rpm (Zx3 vortex mixer, Velp Scientifica, Italy). Immediately afterwards, seeds were washed 5 times with sterile deionized water (see Pereira *et al.* 2015, 2017, 2021). Then, seeds were sown in Petri dishes filled with three different culture media using a sterilized dropper in a laminar flux chamber. We used Malmgren modified terrestrial orchid medium (MM) (Malmgren 1996), 1/2 Murashige & Skoog medium (MS1/2) (Murashige & Skoog 1962), and agar-water (AW). AW was used as a control. All media were autoclaved at 121°C and 1 atm for 20 min and put in 4 cm-diameter Petri dishes in a laminar flux chamber. We used four plates per treatment (n=4) each containing approximately 200 seeds. All plates were put in a stove in the dark at 24 ±1 °C for two weeks as indicated by previous successful protocols with other *Chloraea* species (Pereira *et al.* 2015, 2017). Seeds were then moved to a growth chamber (Archiclima, Chile) set at the same temperature, and at a photoperiod of 16/8 h light/

dark cycles and a PPDF of 70 $mmolm^{-2}\,s^{-1}$. We evaluated seed germination (embryo emergence from seed coat) and embryo development after two weeks and then weekly until the end of the experiment after 16 weeks (see Pereira *et al.* 2015, 2017 for further details on orchid seed germination and embryo developmental stages). At the end of the experiment, we determined germination percentage in each culture media as the proportion of germinated seed relative to possibly viable seeds in the plate (100 seeds per plate) x 100. This was done due to the high percentage of non-viable seeds in this species and to better show the effects of the different culture media on seed germination. Viability was determined earlier (see above) and the average seed viability was used for the calculation of germination percentage. When at least 5 seeds in a plate showed an advancement in embryo developmental stage (i.e. from 1 to 2 or from 2 to 3, etc.), we considered that treatment as advanced to the next stage.

Statistical analyses

The software Statistica 6.0 was used for the analyses. The percentage data was arcsin transformed before they were subjected to analysis of variance (one-way ANOVA). Data are expressed as means ± SD. We used a Tukey Test as the *post-hoc* analysis.

RESULTS

The seeds of *C. disoides* had a very low viability according to the tetrazolium test, reaching in average a 26.20 ± 0.98%. After one week of incubation, seeds of *C. disoides* reach the pre-germination stage (swelling of the embryo inside the seed) in the three testes culture media. After 4 weeks, seed germination was reached in MM and MS1/2 (Fig. 1b) but not in AW, where it took another week to evidence germination in some seeds. Overall, asymbiotic seed germination at the end of the experiment differed between culture media (ANOVA, $p<0.05$). It was higher in MS1/2 and MM compared to AW (Fig. 2, Tukey test, $p<0.05$). No differences were found between MS1/2 and MM in seed germination (Tukey test, $p>0.05$). Considering the most advanced seed/embryo development in the plates, we observed seed germination in all culture media after 5 weeks (Fig. 3). After 10 weeks, all media reached the stage 3 or protocorm stage (embryo totally emerged from seed, Fig. 1c, Fig. 3). After 15 weeks, some embryos reached the rhizoid stage (Fig. 1d, Fig. 3) only in MS1/2 and MM with no further development of embryos in any media until the end of the experiment (Fig. 3).

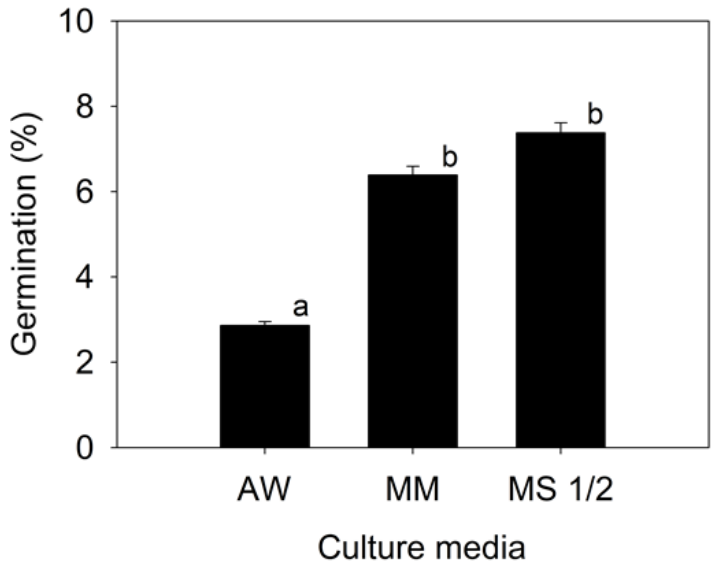

Figure 2. Asymbiotic seed germination of *C. disoides* in three different culture media. Different letters represent statistically significant differences (Tukey test, $p<0.05$). AW = Agar-water. MM = Malmgren modified culture media. MS½ = Modified Murashige & Skoog culture media. / Germinación asimbiótica de *C. disoides* en tres medios de cultivo diferentes. Letras diferentes representan diferencias estadísticamente significativas (Test de Tukey, $p<0,05$). AW = agar agua. MM = Medio de cutivo Malmgren modificado. MS ½ = Medio de cultivo Murashige & Skoog modificado.

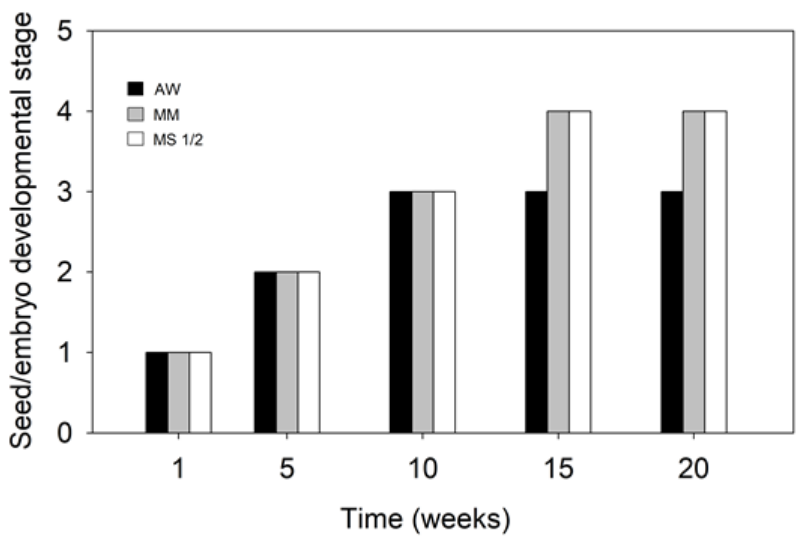

Figure 3. Developmental stages of *C. disoides* seeds at different times in an asymbiotic germination assay using three culture media. AW = Agar-water. MM = Malmgren modified culture media. MS½ = Modified Murashige & Skoog culture media. Developmental stages (modified from Yamazaki & Miyoshi 2006): 0 = No germination stage. No growth of embryo occurs; 1 = Pre germination stage. Embryo swells to fill the seed coat; 2 = Germination stage. Embryo emerges from the seed coat; 3 = Protocorm stage. Embryo is completely discharged from the seed coat; 4 = Rhizoid stage. Rhizoids are formed on the protocorm surface; 5 = Shoot stage. Shoot is differentiated from the protocorm (not observed in this study). We considered a new stage when at least five seeds in a plate evidenced a new developmental stage. / Estados de desarrollo de semillas de *C. disoides* a diferentes tiempos en un ensayo de germinación asimbiótica usando tres medios de cultivo. AW = agar agua. MM = Medio de cutivo Malmgren modificado. MS ½ = Medio de cultivo Murashige & Skoog modificado. Estados de desarrollo (modificados de Yamazaki & Miyoshi 2006): 0 = estado sin germinación; 1 = Estado de pre-germinación. El embrión se hincha hasta llenar la testa; 2 = Estado de germinción. El embrión emerge de la testa; 3 = Estado de protocormo. El embrión se encuentra completamente fuera de la testa; 4 = Estado de rizoides. Rizoides se forman en la superficie del protocormo; 5 = Estado de brote. Brotes se diferencian del protocormo (no observado en este estudio). Se considera el avance de estado de desarrollo cuando al menos cinco semillas de una placa se observan en el estado de desarrollo siguiente.

DISCUSSION

We achieved asymbiotic germination of the critically endangered orchid *Chloraea disoides* which could contribute to the conservation of the species in the short term. However, germination was very low compared to congeneric species using similar asymbiotic methods (Pereira *et al.* 2017, Herrera *et al.* 2017) and had very low seed viability. Previous studies have obtained between 60 to 80% germination in other *Chloraea* species (Pereira *et al.* 2017, Herrera *et al.* 2017) contrasting with our results that reached in the best culture media close to 8%. Additionally, embryo development did not advance to protocorm stage, independent on culture media, unlike previous studies on other Chilean species (Pereira *et al.* 2015, 2017, 2021). Low germination and stalled embryo development could be due to inbreeding depression, since populations usually are composed by only a few individuals (Atala *et al.* 2017). This has been known to reduce plant fitness, particularly seed quality and/or quantity, over time (Angeloni *et al.* 2011). Additionally, it can be also due to some degree of seed dormancy. There seems to be a positive correlation between a progressive decrease in germination and seed maturity, where biochemical changes in the seed induce dormancy (Vasudevan *et al.* 2010, Kaur *et al.* 2016). This can be particularly true in seeds lacking endosperm, such as orchid´s seeds (Heywood *et al.* 2007). Yi *et al.* (2007) and Kaur *et al.* (2016) suggest that while the embryo develops, it dehydrates due to the synthesis of hydrophobic molecules and the lignification of the seed coat. Additionally, these authors show that endogenous ABA levels also increase over time, further reducing germination. On the other hand, is possible that this species is more dependent on their fungal partners for seed germination and embryo development, for example if it is highly specific, as seen in some orchid species (Claro *et al.* 2019, Herrera *et al.* 2020). If this is the case, it may be possible that changes in the availability of the orchid fungi in the soil due to anthropic effects could have negatively affected this species, contributing to its endangered condition.

The main goal with this species is to increase seed germination and to eventually produce more individuals

for reintroduction to avoid its extinction. This species in particular seems to have, as mentioned above, very low seed viability which result in few resulting germinated seeds (in terms of absolute numbers). Previous studies have shown that pretreatment of orchid seeds can increase seed viability and/or increase detection of viable seeds with the tetrazolium test (Salazar *et al.* 2019, 2020, Mercado *et al.* 2020, Salazar & Botello 2020). These studies used immersion of seeds in a sucrose solutions or distilled water to obtain a higher measured viability which also increased with time of immersion. It is possible that using pretreatment of *C. disoides* seeds could yield better results in the TZ test, but further studies are required.

Using MM and MS1/2 culture media embryos developed up to the rhizoid stage, and no further development was observed. This could be due to an intrinsic slow development of *C. disoides* embryos or to the lack of an essential element or association. Other *Chloraea* species develop faster in these culture media and can reach shoot stage in 16 weeks (Pereira *et al.* 2015, 2017, 2021). The physiology and nutritional requirements of Chilean orchids are poorly studied (but see Pereira *et al.* 2017, Romero *et al.* 2018), and it is possible that this species require specific nutrients or proportion of nutrients for its developments. The other possibility is that is more dependent on symbiotic association, following the same argument stated earlier.

Symbiotic seed germination in orchids is a complex process, particularly in terrestrial orchids, where different factors affect germination success (Herrera *et al.* 2020). Asymbiotic germination of orchid seeds can be a good strategy for orchid conservation, propagation, and restoration (Pereira *et al.* 2015, 2017, 2021, Romero *et al.* 2018). This is mainly because this method can produce many individuals, do not require previous isolation of fungal species, reduce contamination risk, and, because uses sexually produced seeds, preserve genetic variability (Johnson *et al.* 2007, Aggarwal & Zettler 2010, Abraham *et al.* 2012). However, there is evidence that inoculation with mutualistic fungi can increase plant growth and survival (Pereira *et al.* 2021). One solution could be the mass production of plants through asymbiotic means, and the later artificial inoculation, as it has already proven effective for another Chilean *Chloraea* species (Pereira *et al.* 2021). Immediate actions are require to avoid the extinction of *C. disoides* in the relatively short term, threatened by human actions and climate change (Atala *et al.* 2017). Thus, further studies are required to establish the most efficient method for obtaining adult individuals, and, eventually, make possible the re-introduction of plants in the field, where with the aid of native shrubs it may be possible to ensure survival in the long term (Baldelomar *et al.* 2019, Atala *et al.* 2020).

Overall, asymbiotic germination of this endangered species is possible, but further research is required to understand why embryo developments stalls and to test other methods such as pretreatments of the seeds, different culture media, or symbiotic methods.

ACKNOWLEDGMENTS

The present work was funded by project VRID-MULTIDISCIPLINARIA UdeC N° 220.418.011-M.

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Received: 18.08.2021
Accepted: 17.05.2022