GAYANA BOTANICA Gayana Bot. (2024) vol. 81, No. 1, 66-76

ORIGINAL ARTICLE

Symbiotic and asymbiotic germination *in vitro* of *Chloraea longipetala* (Orchidaceae), an endemic orchid from Chile

Germinación simbiótica y asimbiótica *in vitro* de *Chloraea longipetala* (Orchidaceae), una orquídea endémica de Chile

Orlando Jeldes-Cajas[1], Guillermo Pereira[2], Marco A. Molina-Montenegro[3,4] & Cristian Atala[1,*]

[1]Laboratorio de Anatomía y Ecología Funcional de Plantas (AEF), Instituto de Biología, Facultad de Ciencias, Pontificia Universidad Católica de Valparaíso, Campus Curauma, Curauma, Valparaíso, Chile.

[2]Laboratorio de Biotecnología de Hongos, Departamento de Ciencias y Tecnologıa Vegetal, Universidad de Concepción, Campus Los Ángeles, Los Ángeles, Chile

[3]Centre for Integrative Ecology (CIE), Instituto de Ciencias Biológicas, Universidad de Talca, Campus Lircay, Avda. Lircay s/n, Talca, Chile.

[4]Centro de Investigación en Estudios Avanzados del Maule (CIEAM), Universidad Católica del Maule, Talca, Chile.

*Corresponding author: cristian.atala@pucv.cl

ABSTRACT

Chile has about 72 species of terrestrial orchids, most of them endemic. Some are threatened due to habitat loss, fires and herbivores damage and many have potential as ornamental plants or cut flowers. *In vitro* plant germination could be a key strategy for the long-term conservation and propagation of these species. *In vitro* asymbiotic and symbiotic, germination and subsequent embryo development in the orchid *Chloraea longipetala* were evaluated using five different culture media, aiming to provide efficient protocols for its propagation. For asymbiotic germination, we used water agar (AW), banana culture medium (CMB), tomato culture medium (CMT), oatmeal agar (OMA) and Malmgren terrestrial orchid medium (MM). For symbiotic germination, just AW and OMA were used. In addition, seed viability was estimated and reached 86.6 %. After four weeks, in all media except AW, the germination stage (embryo emerges from seed coat) was reached, for asymbiotic germination. However, there were no significant differences in the germination percentage among all five media, not exceeding 10 % in any treatment. After eight weeks, only seeds in MM reached the protocorm stage. On the other hand, symbiotic germination resulted in a significantly higher germination percentage (40 %), and embryos reached the rhizoid stage. For *C. longipetala*, unlike other Chilean species, the presence of orchidiod fungi seems to be necessary for successful and efficient germination and embryo development. This information is key for future propagation initiatives, for conservation, restoration or commercial applications as ornamental plants.

Keywords: *Chloraea longipetala*, in-vitro germination, orchid propagation, terrestrial orchids.

RESUMEN

Chile posee alrededor de 72 especies de orquídeas terrestres, la mayoría de ellas endémicas. Algunas están amenazadas debido a la pérdida de hábitat, incendios y daños por herbívoros y muchas tienen potencial como plantas ornamentales o flores cortadas. La germinación in vitro de plantas podría ser una estrategia clave para la conservación y propagación a largo plazo de estas especies. Se evaluó la germinación in vitro, asimbiótica y simbiótica, y el posterior desarrollo embrionario de la orquídea *Chloraea longipetala* utilizando cinco medios de cultivo diferentes, con el objetivo de proporcionar protocolos eficientes para su propagación. Para la germinación asimbiótica, se utilizó agar agua (AW), medio de cultivo de plátano (CMB), medio de cultivo de tomate (CMT), agar avena (OMA) y medio Malmgren para orquídeas terrestres

(MM). Para la germinación simbiótica, sólo se utilizaron AW y OMA. Además, se estimó la viabilidad de las semillas, que alcanzó el 86,6 %. Después de cuatro semanas, en todos los medios excepto AW, se alcanzó la fase de germinación (el embrión emerge de la cubierta de la semilla), para la germinación asimbiótica. Sin embargo, no hubo diferencias significativas en el porcentaje de germinación entre los cinco medios, no superando el 10 % en ningún tratamiento. Al cabo de ocho semanas, sólo las semillas en MM alcanzaron la fase de protocor. Por otro lado, la germinación simbiótica dio lugar a un porcentaje de germinación significativamente mayor (40 %), y los embriones alcanzaron el estadio de rizoide. Para *C. longipetala*, a diferencia de otras especies chilenas, la presencia de hongos orquidioides parece ser necesaria para una germinación y desarrollo embrionario exitosos y eficientes. Esta información es clave para futuras iniciativas de propagación, para conservación, restauración o aplicaciones comerciales como plantas ornamentales.

Palabras clave: *Chloraea longipetala*, germinación in vitro, propagación de orquídeas, orquídeas terrestres.

INTRODUCTION

The Orchidaceae, the orchid family, is characterized by the complexity of their flowers and their ecological interactions with pollinators and fungi. This family is characterized, especially in terrestrial species, by being mycoheterotrophic during the initial phase of development (Leake 2004), so they need mycorrhizal fungi to allow seed germination and plant growth (Warcup 1973, Mitchell 1989, Rasmussen 1995). Orchids reproduce by sexually produced seeds where a single plant can generate thousands of seeds. These are microscopic and during the maturation process, the endosperm (storage tissues) is aborted (Takao *et al.* 2012). Specific mycorrhizal fungi and seeds form a non-photosynthetic spherical body called protocorm, which is completely dependent on the fungus for organic nutrients needed (Rasmussen 2002, Kuga *et al.* 2014). Meanwhile, fungi receive carbon compounds from orchids at a later stage (Cameron *et al.* 2006, 2007). Some orchid species uphold this mutualistic association in adult stages (Leake 1994, Pereira *et al.* 2014); however, in many partially mycoheterotrophic orchids, the mycobiont supplies minerals and organic nutrients just until the first green leaf emerges (Gebauer & Meyer 2003, Merckx 2013).

Species in the Orchidaceae family are among the most threatened plants, mostly due to land-use changes and the threat of climate change (Batty *et al.* 2002, Backhouse 2007, Seaton *et al.* 2010, Atala *et al.* 2017). At the end of 2022, the International Union for Conservation of Nature (IUCN) World Red List included assessments for 1970 orchid species, of which 36.3 % are considered threatened (IUCN 2022). For example, terrestrial orchid communities in the Argentine and Chilean Andes are particularly affected by the introduction of herbivorous mammals such as hares, wild boars, feral goats and cattle (Vázquez 2002, Novillo & Ojeda 2008), as they lack mechanical and chemical defenses but have highly palatable leaves and fleshy tuberous roots, which are intensely preyed upon by these herbivores (Fracchia *et al.* 2016, Atala *et al.* 2020). Due to the difficulties involved in natural seed germination, resulting from high human pressure on natural populations, alternative propagation methods need to be established (Novoa *et al.* 2015). In this context, the development of *ex situ* conservation approaches, such as *in vitro* germination, are an important complement for the conservation of threatened taxa (Stewart & Kane 2006), as genetic variation is preserved, compared to cloning, *in vitro* somatic cell culture, or similar techniques.

One approach to *ex situ* germination of orchids is symbiotic germination, in which a small portion of the species-specific mycorrhizal fungus grows in a culture medium in contact with orchid seeds and colonizes them giving rise to symbiosis, with the expectation that the outcome of this association will benefit the germinated protocorms or embryos (Mala *et al.* 2017). This method is a powerful tool for both the production of mycobiont-infected seedlings for use in reintroduction initiatives and for the study of fungal specificity within Orchidaceae (Stewart & Kane 2006). However, this process firstly requires the isolation of orchid fungi and their cultivation on a storage medium, and also demands the maintenance of the genetic stability of the mycorrhizal fungi, which can be problematic (Brundrett 2007, Wright *et al.* 2009). Under *in situ* conditions, an orchid may form mycorrhizal associations with more than one fungal species, so that a fungus that promotes germination may not be the same one that leads the plant to an adult stage (Masuhara & Katsuya 1994,

Herrera *et al.* 2022), making it difficult to isolate the fungus required for germination. Additionally, contamination, fungal and bacterial, often occurs in symbiotic methods (Johnson *et al.* 2007, Abraham *et al.* 2012) and the seeds inoculated with mycorrhizal fungi can become completely covered by fungal hyphae, making it difficult to evaluate germination and embryo development, sometimes requiring a change in culture media, increasing the risk of contamination (Pereira *et al.* 2017).

Another option for *ex situ* germination of orchids is asymbiotic germination, which consists of mimicking the nutritional conditions of germination supplied by symbiotic fungi in a natural state (Steinfort *et al.* 2010) using culture media enriched with carbon, nitrogen and phosphorus sources (Knudson 1921, Bustam *et al.* 2014) and the addition of soluble sugar that serves as a source of carbohydrates for orchid embryos (Yam & Arditti 2009). Setting protocols for asymbiotic germination is species-specific and depends on several factors, such as capsule maturity, growing medium components, nutrient requirements of the species, and light and temperature conditions (Paul *et al.* 2011). Asymbiotic seed germination can be a simpler process relative to symbiotic germination (Johnson *et al.* 2007), providing an effective method to generate plants of a wide range of orchid taxa in more controlled and aseptic conditions (Johnson *et al.* 2007, Pereira *et al.* 2017), especially since this do not require prior isolation of the mutualistic fungal species, which reduces the risk of contamination (Castillo-Novales *et al.* 2022). Nevertheless, association with orchidiod fungi could be essential for progressing to higher developmental stages and plant establishment in the field (Bustam *et al.* 2014, Pereira *et al.* 2021), especially considering that native Chilean orchid species associate with fungi in adult stages (Pereira *et al.* 2014, Atala *et al.* 2015). Likewise, because of possible ecological changes in historical orchid sites, mycobionts of a target orchid species may not be present at a site if the orchid itself is not present (Johnson *et al.* 2007).

Yamazaki & Miyoshi (2006) defined the process of how orchid seeds develop *in vitro*, which were modifications of those given by Miyoshi and Mii (1995). These qualitative developmental steps are used to determine the success of germination treatments and further embryo growth. This process is divided into five stages: When no embryonic growth was observed, it was considered that no germination had occurred (Stage 0). In the pre-germination stage (Stage 1), the embryo swells to fill the seed testa. This is clearly evident since the orchid seeds lack other typical structures of monocot seeds and only contain the embryo. Germination (Stage 2) is reached when the embryo emerges from the seed coat. In this stage the testa is broken and the swelled embryo is partially visible. In the protocorm stage (Stage 3), the embryo is completely detached from the seed coat and can be seen as a rounded mass of non-chlorophyllic cells. The rhizoid stage (Stage 4) is achieved when the protocorm produce rhizoids, which are hair-like structures. Finally, the shoot stage (Stage 5) is reached when a shoot differentiates from the protocorm. In this stage the shoot will then produce true leaves and the individual becomes photosynthetic.

In Chile, 72 species have been identified, all of them terrestrial, classified in 7-8 genera distributed from the Tarapacá Region to the Region of Magallanes and Chilean Antarctica, many of which are endemic to the national territory (Novoa *et al.* 2015, Rodríguez *et al.* 2018). In many cases, the conservation status of these species is not even evaluated (Correa 1969, Elórtegui & Novoa 2009, Novoa *et al.* 2015). The number of reports on the success of *in vitro* production of terrestrial orchids has been increasing due to the growing concern of conservationists that many habitats harboring terrestrial orchids are being converted into residential and commercial areas (Stewart & Kane 2006). Even so, currently few native Chilean orchids have been cultivated through *in vitro* germination. Among those few, we can find *Bipinnula fimbriata* (Poepp.) I.M.Johnst. and some species of the genus *Chloraea* (Steinfort *et al.* 2010, Pereira *et al.* 2015, Herrera *et al.* 2017, Pereira *et al.* 2017, Quiroz *et al.* 2017, Romero *et al.* 2017, Mujica *et al.* 2020, Pereira *et al.* 2021, Castillo-Novales *et al.* 2022). In these cases, germination percentages above 60 % have been reported for both symbiotic and asymbiotic approaches, however symbiotic germination methods have been shown to be more effective in progressing to higher developmental stages. Herrera *et al.* (2017) studied the symbiotic germination of *Chloraea longipetala* Lindl. However, no data on asymbiotic germination of this species is, to our knowledge, currently available.

In this study, we seek to study *in vitro* symbiotic and asymbiotic germination of *C. longipetala* seeds to provide reliable germination protocols for this species. This may contribute to future conservation and restoration initiatives for this native and endemic orchid species, and also establish the basis for commercial applications as ornamental plants.

MATERIALS AND METHODS

Plant material and seed harvesting

Chloraea longipetala, a geophyte orchid distributed between the regions of Biobío (Concepción) to Los Lagos (Llanquihue), is not included in the list of evaluated species, despite the fact that urban development throughout its range threatens the species' current habitat. This is a perennial plant between 60

and 80 cm, having inflorescence with 8 to 10 white flowers with green warts and macules, medium to large within the genus, which bloom in summer (Novoa *et al.* 2015).

The plants of *C. longipetala* were collected at the stage of scape development in a population near the Pangue hydroelectric power plant, Alto Biobío commune, Biobío region, Chile (37°54'11" S - 71°37'16" W). The site corresponds to a patch of degraded native forest of the Roble-Raulí-Coigüe type, with a temperate rainy climate. The species was identified by its distinctive floral morphology following Elórtegui & Novoa (2009) and Novoa *et al.* (2015). At the stage of flower development, controlled pollination (xenogamy) was carried out in the greenhouse. At 28 days after pollination, before the capsules opened, they were taken to the laboratory where they were dried at room temperature for seed harvesting.

Seeds from all capsules were mixed and stored in an airtight glass container and kept at 4 °C for 16 months until the start of the experiment. Seed viability was assessed by the tetrazolium test (TTC). For this purpose, 150 approximately randomly selected seeds were immersed in a 1 % TTC solution in an eppendorf tube for 72 hours in the dark (Vujanovic *et al.* 2000, Muñoz & Jiménez 2008). The seeds were then observed under an optical microscope. Viable seeds had robust, ovoid, pinkish-brown embryos. This procedure was repeated three times and the results were expressed as percentage of viable seeds.

Seed disinfection and culture

Seed disinfection was performed following the methods proposed in Batty & Brundett (2001), Pereira *et al.* (2015, 2017). First, seeds were introduced in eppendorf tube to which 70 % ethanol was added and stir for 30 seconds, followed by a 1 % sodium hypochlorite (NaClO) solution with one drop of Tween 20 (polyoxyethylene sorbitan monolaurate) for five minutes under constant vortex agitation. Afterwards, three washes were performed with sterile deionized water, proceeding subsequently, under a horizontal flow chamber, to the corresponding seeding in Petri dishes containing the different culture media described below.

Culture media

Five culture media (Table 1), modified oat agar (OMA), water agar (AW), banana culture medium (CMB; Barbery & Molares 2014), tomato culture medium (CMT; Barbery & Molares 2014) and Malmgren's modified terrestrial orchid culture medium (MM; Malmgren 1996) were used to evaluate the germination of *C. longipetala* seeds. The modified oatmeal agar (OMA) (Steinfort *et al.* 2010), was prepared by combining 10 g/L of crushed oats that was allowed to homogenize at a temperature of 95 °C for 30 minutes (stirring constantly) and then filtered through a gauze, to the obtained mixture was added 0.1 g/L of yeast extract and 10 g/L of agar, adjusting its pH to 5.8 before being autoclaved at 1 atm of pressure (121°C) for 20 minutes. Finally, the agar was allowed to cool to 50-55 °C before adding 0.04 g/L of Streptomycin sulfate, in order to avoid contamination by bacteria (Waksman 1949). The other media were prepared following the instructions of the creator, sterilized in autoclave at 1 atm pressure (121 °C) for 20 minutes, and once all the media were prepared, the pH was adjusted to 5.6. All culture media were poured into 55 mm diameter Petri dishes in a laminar flow chamber.

Seed germination

Once the plates were cooled and solidified, sterilized *C. longipetala* seeds were spread on each plate. Each plate contained at least 100 seeds. A minimum of four plates (replicates) per culture medium were used for asymbiotic germination (four plates × five culture media = 20 plates total).

For symbiotic germination, another four plates with OMA medium and four plates with AW were inoculated with a 5 mm disc of mycorrhizal fungi, placed at equidistant distances in the center of each plate. The fungal material used was the orchid fungus species *Ceratobasidium* sp. accession MN199626 (GeneBank), belonging to the ceparium of the Fungal Biotechnology Laboratory of Los Angeles Campus of the University of Concepción. This fungus was previously isolated from the root system of *Chloraea disoides*, the colony before being used in this study was activated in fresh culture medium.

Petri dishes containing media and seeds were placed in a growth chamber at 24 ± 1 °C with a 16/8 h light/dark photoperiod (light: 70 µmol m-2 s-1). Plates were examined weekly and germinated seeds were evaluated every 2 weeks for up to 14 weeks with an optical microscope. Germination was evaluated based on the developmental stages of terrestrial orchid seeds of Yamazaki & Miyoshi (2006) described earlier, namely; Stage 0: No germination stage, no growth of embryo occurs. Stage 1: Pre-germination stage, embryo swells to fill the seed coat. Stage 2: Germination stage, embryo emerges from the seed coat and is partially visible. Stage 3: Protocorm stage, embryo is completely discharged from the seed coat. Stage 4: Rhizoid stage, rhizoids are formed on the protocorm surface. Stage 5: Shoot stage, a shoot is differentiated from the protocorm, showing the first signs of chlorophyll production. For further details see Pereira *et al.* (2015; 2017).

Statistical analysis

Germination percentages were calculated by dividing the number of germinated seeds by the total number of seeds with

embryo (×100). All percentage data were arcsine transformed to normalize for variation. A one-way ANOVA was used to test significance, followed by Tukey's post-hoc test at P ≤ 0.05. Statistical analyses were performed with the free software package R (Institute for Statistics and Mathematics 2022; https://www.rproject.org).

RESULTS

Seeds of *C. longipetala* had high viability according to the tetrazolium test, reaching an average of 86.6 ± 0.8 %. Seeds on all tested culture media, after two weeks, developed to stage 1 (pre-germination) or more advanced developmental stages in at least one of the replicates, minus the OMA medium under asymbiotic conditions (Fig. 1A). After four weeks, all media reached the germination stage, except AW and OMA medium under asymbiotic conditions, which only reached stage 1. The Malmgren modified terrestrial orchid medium (MM) showed seeds at stage 3 six weeks after the start of the trial, being the only culture medium to reach this stage in the asymbiotic trial (Fig. 1A). Both media inoculated with *Ceratobasidium* sp., AW and OMA, reached stage 3 development at 2 and 6 weeks of incubation, respectively (Fig. 1B). The symbiotic AW treatment was the only one to present protocorms with rhizoid (Stage 4), around week eight. After week 8, MM and both symbiotic media reached stage 3, however, in week 14 only in MM could be distinguished protocorms that continued growing, becoming visible to the naked eye, in the rest of the media the seeds stagnated their development. In the symbiotic AW, a number of protocorms that formed rhizoids were evident, but in a very low percentage.

In the second week, the presence of *Ceratobasidium* sp. hyphae was evident in the symbiotic culture media, although they had a different expression in each medium. In the AW medium, the hyphae grew radially around the inoculum through the culture medium, presenting long arms and few ramifications. On the other hand, in the OMA medium, the hyphae grew on the culture medium without penetrating it, forming dense and branched clusters similar to cotton, only around the seed concentration zones, which made it difficult to calculate the percentage of germinated seeds in this medium. From the sixth week on, there was an evident decline in the density and coverage of the fungus in the OMA medium.

Initially, each treatment consisted of seven replicates. However, contamination, of bacterial and fungal origin, occurred in some experimental units and these were excluded from the data analysis. The number of replicates of each treatment used in the data analysis is shown in Table 2. At the eighth week from the beginning of the germination trial, the symbiotic OMA medium presented the highest germination percentage, followed by the symbiotic AW medium, with statistically significant difference between both ($p<0.05$) (Table 2). The asymbiotic culture media presented germination percentages lower than 8 % in all cases, with CMT being the one with the worst results (Table 2). There is no statistically significant difference between the asymbiotic culture media,

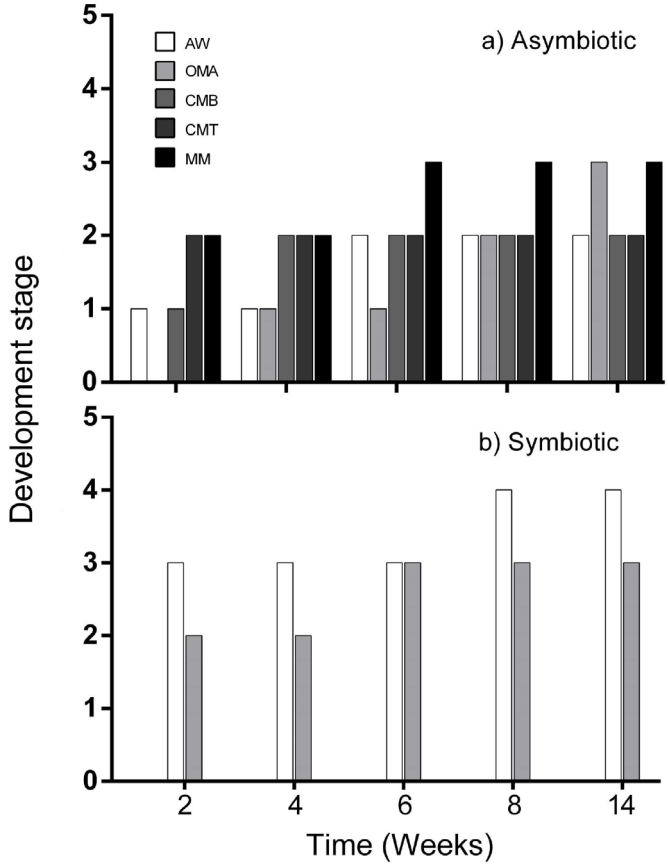

Figure 1. Developmental stages of *C. longipetala* seeds at different times in a germination trial comparing symbiotic and asymbiotic protocols using five culture media. Malmgren's modified terrestrial orchid medium (MM), banana culture medium (MCB), modified tomato culture medium (MCT), water agar (AW) and modified oat agar (OMA). S. AW and S. OMA as abbreviation for symbiotic AW and symbiotic OMA respectively. / Etapas de desarrollo de las semillas de *C. longipetala* en diferentes momentos de un ensayo de germinación en el que se compararon protocolos simbióticos y asimbióticos utilizando cinco medios de cultivo. Medio de orquídea terrestre modificado de Malmgren (MM), medio de cultivo de plátano (MCB), medio de cultivo de tomate modificado (MCT), agar agua (AW) y agar avena modificado (OMA). S. AW y S. OMA como abreviatura de AW simbiótico y OMA simbiótico respectivamente.

which does appear when compared to the symbiotic culture media. At week 14 from the beginning of the experiment, all the media obtained a higher germination percentage, except the symbiotic OMA, which reduced the number of seeds in stage 2 (germination stage) compared to week 8. Finally, none of the asymbiotic media exceeded 12% germination, and the AW medium inoculated with *Ceratobasidium* sp. was the one that obtained the best results, without exceeding 30 %.

Table 1. Comparative composition of culture media used for orchid seed germination. Malmgren's modified terrestrial orchid medium (MM), banana culture medium (CMB), modified tomato culture medium (CMT), water agar (AW) and modified oat agar (OMA). / Composición comparativa de los medios de cultivo utilizados para la germinación de semillas de orquídeas.Medio de cultivo de orquídeas terrestres modificado de Malmgren (MM), medio de cultivo de plátanos (CMB), medio de cultivo de tomates modificado (CMT), agar agua (AW) y agar avena modificado (OMA).

	MM	CMB	CMT	AW	OMA
Macronutrients (mg/L)					
KH_2PO_4	75				
$Ca_3(PO_4)_2$	75				
$MgSO_4$	97,69				
Na_2 EDTA - 2 H_2O	75				
Micronutrients (mg/L)					
$MnSO_4$ - H_2O	1,54				
$FeSO_4$ - 7 H_2O	27,8				
Others (mg/L)					
Ripe banana (pulp)		150000			
Ripe tomato (pulp)			150000		
Oats (crushed)					10000
Yeast extract	1000				100
Pineapple powder	20000				
D-Biotin	0,05				
Casein, enzymatic hydrolyzate	400				
Folic acid	0,5				
Glycine (free base)	2				
Pyridoxine	5				
Myo-Inositol	100				
Nicotinic acid (free acid)	5				
Thiamine	10	1200	1200		
Sucrose	10000	15000	15000	5000	
Sulfate Streptomycin				40	40
Agar	7000	10000	10000	10000	10000
Activated charcoal (plant based)	1000				
Deionized water (mL)	1000	1000	1000	1000	1000

Table 2. Effects of culture medium and fungal inoculation on developmental stages of *Chloraea longipetala* seeds and protocorms at 8 and 14 weeks after seed seeding. Means with the same letter do not differ significantly within stage (Tukey's test, p<0.05). / Efectos del medio de cultivo y de la inoculación fúngica sobre los estadios de desarrollo de las semillas y protocormos de Chloraea longipetala a las 8 y 14 semanas después de la siembra de las semillas.Las medias con la misma letra no difieren significativamente dentro del estadio (prueba de Tukey, p<0,05).

Treatment	*n*	Percentage of seed/protocorms									
		Stage 0		Stage 1		Stage 2		Stage 3		Stage 4	
		8	14	8	14	8	14	8	14	8	14
Asymbiotic											
AW	6	44.3 ± 3.2^{a}	18.4 ± 2.5	44.3 ± 3.2^{a}	62.1 ± 2.8^{a}	3.0 ± 0.9^{a}	11.5 ± 0.9^{a}				
OMA	4	81.5 ± 2.5^{b}	3.3 ± 1.1^{a}	81.5 ± 2.5^{b}	79.8 ± 5.0^{b}	7.8 ± 1.9^{a}	10.5 ± 2.7^{a}	0.2 ± 0.2^{a}	0.3 ± 0.3^{a}		
CMB	4	24.9 ± 2.7^{ac}	42.7 ± 6.8^{b}	24.9 ± 2.7^{c}	40.3 ± 3.6^{c}	4.9 ± 1.3^{a}	7.8 ± 0.6^{a}				
CMT	5	29.8 ± 2.1^{ac}	32.7 ± 4.9^{b}	29.8 ± 2.1^{c}	48.9 ± 5.3^{ac}	1.5 ± 0.4^{a}	9.4 ± 1.7^{a}				
MM	5	25.3 ± 3.8^{c}	57.7 ± 4.1	25.3 ± 3.8^{c}	33.5 ± 2.6	4.8 ± 1.1^{a}	9.7 ± 1.3^{a}	2.2 ± 0.6^{b}	4.5 ± 0.5^{b}		
Symbiotic											
AW	7	67.4 ± 1.5^{b}	2.5 ± 0.4^{a}	67.4 ± 1.5^{b}	61.7 ± 2.3^{a}	25.0 ± 1.4	28.9 ± 2.3^{b}	1.1 ± 0.3^{b}	3.1 ± 0,8^{b}	0.1 ± 0.1	0.1 ± 0.1
OMA	4	49.1 ± 2.5^{b}	2.0 ± 0.5^{a}	49.1 ± 2.5^{a}	73.5 ± 3.1^{ab}	37.5 ± 3.4	21.7 ± 3.1^{b}	0.4 ± 0.3^{a}	0.3 ± 0.3^{a}		

DISCUSSION

We demonstrated that seeds of *C. longipetala* can be germinated asymbiotically *in vitro*, although seed germination rates in all treatments were unusually low (close to 10 %) compared to seed germination rates of other species of the genus *Chloraea* that are within 60-80 % (see Herrera *et al.* 2017, Pereira *et al.* 2017, 2021). Furthermore, none of the media allowed the seeds to form rhizoids on the surface of the protocorm and develop further, at least in the duration of the experiment. To establish a successful *in vitro* asymbiotic germination method, it is necessary to evaluate a number of factors, both individually and in combination. These include: the nutritional composition of the culture media, the effect of growth regulators and hormones, environmental conditions such as temperature and light, time, and general seed management (Pedroza-Manrique *et al.* 2010). It is likely that further studies comparing more germination conditions (i.e. different light intensities and temperatures) could shed light on possible strategies to increase germination and embryo development using asymbiotic methods. There are few reports on *in vitro* seed germination of American terrestrial orchid species, and even fewer on Chilean terrestrial species (but see Herrera *et al.* 2017, Pereira *et al.* 2015, 2017, 2021), especially compared to the total orchid diversity in the region. This is the first study to compare symbiotic and asymbiotic germination methods for seeds of *C. longipetala*. More research is likely required both to improve and optimize current methods for *C. longipetala* and to test the specific requirements of other species present in the country. This could greatly contribute to conservation and propagation initiatives.

Considering the minimal nutritional content of the water agar (AW) culture medium, low germination and stunted embryo development were to be expected. On the other side, culture media supplemented with organic additives, such as banana culture medium (CMB), modified oat agar (OMA) and tomato culture medium (CMT) have been tested in some Chilean terrestrial orchids, obtaining positive results in seed germination (Pereira *et al.* 2015, 2017, 2021, Castillo-Novales *et al.* 2022). This differ from the relatively low germination percentage and embryo development that were achieved here using Malmgren modified terrestrial orchid culture medium (MM). This could be due to the intrinsically slow development of *C. longipetala* embryos, the lack of specific nutritional elements or the essential need for association with the mycobiont (Smith & Read 2008, Herrera *et al.* 2017). Nevertheless, the MM medium allowed the development of seeds up to stage 3, which did not happen in the rest of the asymbiotic culture media. The development of protocorms in this medium can be evaluated in a longer time span in order to test if seedlings can be eventually obtained from this medium. Within the composition of this medium, we can find dehydrated pineapple powder and

activated carbon, which have the capacity to reduce phenols and have a positive effect on root differentiation (Malmgren 1989, Thomas 2008), which could have contributed to the development of seeds in the protocorm stage. Another point to consider is the source of nitrogen and its availability in the media. Certain growing media can be more effective than others in stimulating protocorm development, this partly due to the effect of N sources and their bio-availability (Johnson *et al.* 2007). Inorganic nitrogen can limit germination, due to the low level of nitrate reductase enzyme activity during germination (Van Waes & Debergh, 1986), whereas organic nitrogen can be more readily utilized by young protocorms, since the available amino acids can bypass certain stages of the nitrogen assimilation process (Malmgren 1992, 1996). In addition, media containing glutamine and glycine obtained better results in the development of protocorms (Johnson *et al.* 2007). All the culture media used for asymbiotic germination, except AW, contain organic nitrogen, either in the form of organic matter (oat, tomato, banana) or in the form of a free base (glycine in MM). However, the concentration of N was not explicitly measured, so the role of nitrogen in the obtained results cannot be determined. The physiology and nutritional requirements of Chilean orchids are not well studied, so it is difficult to determine if this species requires particular nutrients or a specific proportion of these for its development. Despite this, Fracchia *et al.* (2014) suggest that some embryos have the capacity to develop without specific nutrients, which is probably related to the absorption of water and dissolved substances directly from the seed (Smith 1967). This agrees with what was observed in this case, where embryo swelling and rupture of the seed coat was evidenced in all plates, regardless of nutrient treatment, without reaching higher developmental stages.

On the other hand, the results obtained through symbiotic germination protocols suggest a certain degree of dependence of *C. longipetala* on the mycobiont to achieve higher germination percentage and higher developmental stages. Herrera *et al.* (2017) obtained a germination percentage of about 60 % for seeds of *C. longipetala* in symbiosis with four different mycobionts, one of them from the genus *Ceratobasidium* and three from the family Tulasnellaceae. Since in this study the seeds of *C. longipetala* were grown in symbiosis with another fungus of the genus *Ceratobasidium*, which was not isolated from individuals of the studied orchid species, it is corroborated that *C. longipetala* presents a low mycorrhizal specificity, at least under conditions of low nutrient availability. Fungal specificity in the Orchidaceae has been considered controversial for many years (Curtis 1939, Hadley 1970). Differences in fungal specificity of orchids under *in vitro* versus *in situ* conditions have been identified (Masuhara & Katsuya 1994, Taylor & Bruns 1999, Taylor *et al.* 2003, Bidartondo & Bruns 2005). However, the results obtained in both *in vitro* symbiotic germination treatments presented lower results than those reported in Herrera *et al.* (2017) and lower than the viability of seeds obtained with the tetrazolium test, which may suggest the need for a specific mycorrhizal fungus to achieve higher germination percentages. The isolation of a mycobiont suitable for germination of *C. longipetala*, likely isolated form individuals of the species, is a promising line of development in the efforts for its successful propagation.

The nutritional supply of culture media also plays a role in mycobiont development. Depending on the availability of nutrients, the orchid-fungus association can vary between mutualism and fungal parasitism (Beyrle *et al.* 1991). In this study, both culture media used have a different nutritional content, which is reflected in the growth form of the mycobiont (data not shown) and in the germination percentages obtained. It should be taken into consideration that regarding the commonly used OMA culture medium, there are no studies on the effect of oat concentration and its relationship to symbiotic germination of orchid seeds (Mala *et al.* 2017). Previous studies of symbiotic germination generally used OMA with oat concentrations between 2.5 and 4 g/l (Zettler *et al.* 2007, Øien *et al.* 2008, Tan *et al.* 2014, Zhou & Gao 2016), but higher oat concentration might allow better performance of OMA medium as higher amount of mycorrhizal fungus results in more germination-promoting nutrients available. In the symbiotic cultures of *C. longipetala* with OMA medium, the growth of the fungus was truncated, with an evident decrease in fungal density after week six of the trial, which may be related to a low concentration of oat, resulting, in turn, in a reduction of embryo growth and development and germination percentage.

One aspect to consider for both symbiotic and asymbiotic germination protocols is light exposure. The idea that terrestrial orchids require light and dark conditions to germinate their seeds, reflecting the natural conditions of their natural habitats, is widely accepted (Quiroz *et al.* 2017). However, some authors have indicated that seed germination of terrestrial orchids may be inhibited by light (Van Waes & Debergh 1986, Yamazaki & Miyoshi 2006). In the same trend, Quiroz *et al.* (2017) report that protocorm formation is promoted by a total darkness regime (0/24 h light/dark) in seeds of *Chloraea crispa* Lindl. In this study, a light/dark regime was used that approximately mirrored the natural spring/summer conditions of the *C. longipetala* habitat (16/8 hours of light/dark). A study of the effects of photoperiod on germination of this species are needed to elucidate whether the difference between viability test results and actual

germination percentage could be the product, at least in part, of inhibition resulting from light exposure.

Although asymbiotic protocols have advantages, such as lower economic cost, are simpler to carry out and allow for more aseptic conditions, in this case they do not represent an adequate option for germination of *C. longipetala* seeds. On the contrary, symbiotic germination methods represent a viable alternative for the reproduction of this species. In addition, symbiotically grown seedlings are likely to be better adapted to *ex vitro* conditions than asymbiotic seedlings, as mycorrhizal fungi in orchids have been described to facilitate water and nutrient uptake in adult plants (Yoder *et al.* 2000, Smith & Read 2008), promote photosynthetic performance, increase radiation use efficiency (Lee *et al.* 2014) and improving growth (Pereira *et al.* 2021).

The rarity of this orchid in the wild and the threatened status of its natural habitat make it necessary to develop efficient seed germination protocols, otherwise the species may not exist as an independent entity in its natural habitat for a long time. In this study, it was established that *C. longipetala* seeds can be germinated symbiotically and asymbiotically under laboratory conditions using different culture media. However, the results obtained are inferior to those reported in previous studies for other species of the genus *Chloraea*. Symbiotic seed germination has been shown to effectively enhance the development of *C. longipetala* protocorms compared to asymbiotic germination. However, it cannot be ruled out that a fully optimized asymbiotic seed germination protocol may begin to parallel the aforementioned in-vitro seed germination. The latter may shed light on possible in-situ plant responses and their environmental requirements. This information will be critical for future efforts to produce and reintroduce plants for the conservation of this species in its natural habitat. However, further studies are needed to determine the specific requirements of this species for germination and development of optimal protocorms.

ACKNOWLEDGMENTS

OJ-C received financial support from the Vicerrectoría Académica of Pontificia Universidad Católica de Valparaíso.

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Received: 04.03.2024
Accepted: 17.05.2024

Editor: Alfredo Saldaña