Original Article

Asymbiotic germination in three *Chloraea* species (Orchidaceae) from Chile

Germinación asimbiótica en tres especies de *Chloraea* (Orchidaceae) de Chile

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ABSTRACT

Orchids require symbiotic fungi and/or specific conditions to germinate. Asymbiotic techniques have been shown successful for orchid germination. In Chile, *Chloraea* include many endemic, and potentially ornamental, terrestrial orchid species. In this study, individuals of *Chloraea crispa*, *C. gavilu* and *C. virescens* were manually autopollinated. The resulting capsules were sterilized and seeds were aseptically obtained. We evaluated asymbiotic germination in: Agar Water (AW), Knudson C (KC), Banana Culture Media (CMB), Tomato Culture Media (CMT), Malmgren Modified (MM), Murashige and Skoog (MS), and MS modified (MS1/2). Seeds were incubated in the dark at 24±1 ºC for 2 weeks. Then they were put in 16/8 h light/dark cycles for 14 weeks. We registered germination and embryo development in the different culture media. After 8 weeks, all tested *Chloraea* species germinated in most culture media. After 16 weeks, embryos in MM showed an evident shoot. In AW only the pre-germination stage was achieved. In *C. gavilu* germination was over 90% in MM, CMT, and CMB. In KC, a lower germination was obtained in all tested species compared to the other culture media, except for AW. Asymbiotic germination of endemic Chilean orchids could be a viable strategy for their conservation and propagation as ornamental species.

Keywords: Terrestrial orchids, endemic species, *in vitro* germination, culture media, orchid propagation, asymbiotic germination.

RESUMEN

Las orquídeas requieren hongos simbióticos y/o condiciones específicas para germinar. Las técnicas asimbióticas se han mostrado efectivas para la germinación de orquídeas. En Chile *Chloraea* incluye muchas especies de orquídeas endémicas terrestres y con potencial ornamental. En este estudio, individuos de *Chloraea crispa*, *C. gavilu* y *C. virescens* fueron autopolinizados manualmente. Las cápsulas resultantes fueron esterilizadas y se obtuvieron las semillas asépticamente. Se evaluó la germinación asimbiótica en: Agar Agua (AW), Knudson C (KC), Medio de Cultivo Banana (CMB), Medio de Cultivo Tomate (CMT), Malmgren Modified (MM), Murashige y Skoog (MS), y en MS modificado (MS1/2). Las semillas se incubaron en oscuridad a 24±1 ºC por 2 semanas. Luego fueron puestas en ciclos de 16/8 h luz/oscuridad por 14 semanas. Se registró la germinación y desarrollo del embrión en los diferentes medios de cultivo. Luego de 8 semanas, todas las especies de *Chloraea* estudiadas germinaron en la mayoría de los medios de cultivo. Luego de 16 semanas, los embriones en MM mostraron un brote evidente. En AW sólo se alcanzó la etapa de pregerminación. En *C. gavilu*, la germinación fue sobre el 90% en MM, CMT y CMB. En KC se obtuvo una menor germinación en todas las especies comparado con los otros medios de cultivo, excepto AW. La germinación asimbiótica de orquídeas endémicas de Chile puede ser una estrategia viable para su conservación y propagación como especies ornamentales.

Palabras clave: Orquídeas terrestres, especies endémicas, germinación *in vitro*, medios de cultivo, propagación de orquídeas, germinación asimbiótica.

INTRODUCTION

Orchids are highly valued plant species, mainly due to their applications as ornamental plants, bioresources for perfumes and medicines, and condiments (Dearnaley 2007, Dearnaley *et al.* 2012, Abraham *et al.* 2012). Most of these species are from the tropics, and are cultivated and propagated in controlled conditions (Stenberg & Kane 1998, Kauth *et al.* 2006). In the tropics, orchid species are usually epiphytic, with only 20-30% of the species terrestrial, and are important elements of the epiphytic diversity of tropical forests, forming many complex interactions with insects and fungi (Heywood *et al*. 2007). However, other Orchidaceae, particularly temperate species which are commonly terrestrial, are understudied and many are endangered due to habitat loss and other anthropic effects such as fires and cattle damage (Atala *et al.* 2017). Orchid seeds are very small and lack an endosperm, which is the food reserve of the typical seed (Heywood *et al*. 2007, Paudel *et al.* 2012). In the soil, orchid seeds associate with fungal partners that induce germination and promote embryo growth, supplying the required nutrients to the embryo (Valadares *et al*. 2012, Rasmussen *et al.* 2015). Thus, propagating orchids through seeds may require the presence of specific fungi or specific culture media that can replace the nutrient supply given by such fungi (Otero & Bayman 2009). This method of propagation can be very effective since orchids produce large amount of seeds and genetic variation is preserved, compared to cloning or similar techniques.

Asymbiotic seed germination (*i.e.* without fungal inoculation) has been shown to be a proper tool for the production of plantlets of several orchid species for commercial and conservation purposes (Yamazaki & Miyoshi 2006, Dutra *et al*. 2008, Kauth *et al.* 2006, 2008, Stewart & Kane 2010, Pereira *et al.* 2015). Knudson (1922, 1946) was the first to demonstrate that orchid seed could be germinated *in vitro* on a defined nutrient medium supplemented with a simple carbohydrate source. Other culture media with varied carbon sources have been tried for orchid germination such as tomato culture medium, banana culture medium, MS, among others (Pereira *et al.* 2015). These media can have different results depending on the orchid species (Paul *et al.* 2011, Pereira *et al.* 2015), suggesting intrinsic differences in nutrient requirements for different species. It has also been suggested that particular species may have different limiting factors for germination and seedling early growth (Rasmussen *et al*. 2015). These limitations can include the presence of specific fungi in the soil (Dixon 1991, Otero & Bayman 2009, Thakur & Dongarwar 2013) or abiotic conditions, that can ultimately affect regeneration and can result in local extinction of some populations if conditions change over time, especially considering that orchid plants can be very long-lived (Pedersen *et al.* 2012, Romero 2012, Rasmussen *et al*. 2015). Thus, laboratory technics for orchid propagation can be very helpful for conservation of rare or endangered orchid species.

Asymbiotic germination can be more effective than symbiotic germination for several reasons; 1) it does not require the isolation and identification of the mycobiont of the target orchid species. 2) It allows for more controlled, aseptic conditions, resulting in less overall contamination (Johnson *et al.* 2007, Aggarwal & Zettler 2010, Abraham *et al.* 2012). 3) In symbiotic germination, the seeds inoculated with the mycorrhizic fungi can be completely covered with the fungal hyphae, making difficult the evaluation of germination and embryo development (personal observation), sometimes requiring a change in the culture media, increasing the risk of contamination. However, it is likely that further plant development and/or re-introduction of plants in the field could require fungal partners to enhance survival (Otero & Bayman 2009, Thakur & Dongarwar 2013, Pereira *et al*. 2015), especially considering that adult plants of some Chilean *Chloraea* have been found symbiotically associated with fungal partners in the field (Pereira *et al.* 2014, Atala *et al.* 2015).

In Chile there are 7 terrestrial orchid genera including 49 to 63 species (Lehnebach 2003, Novoa *et al*. 2006, Zuloaga *et al*. 2008), this variation in species number reflecting the poor taxonomic knowledge of the group. *Chloraea* is the most diverse genus in Chile and includes 29 species (Lehnebach 2003), some of which are considered endangered and most are not properly evaluated (Correa 1969, Novoa *et al.* 2006, Elórtegui & Novoa 2009, Romero 2012). Moreover, Chile is considered the center of diversity of the genus (Bernhardt 1995). *Chloraea* species occur in Chile in diverse environments, growing in the understory of native forests to open and highly disturbed sites (Reiche 1910, Correa 1969, Romero 2012). Some *Chloraea* species, such as *Chloraea gavilu* Lindl., are found between Valparaíso (~32 °S) and Araucanía (~39 °S) Regions, while other species such as *Chloraea crispa* Lindl., have very narrow distribution, growing mostly in Bío-Bío Region between 36 to 38 °S (Novoa *et al*. 2006, Elórtegui & Novoa 2009). *Chloraea crispa* and *C. gavilu* are endemic to Chile, and *C. virescens* (Willd.) Lindl. (syn: *C. piquichen* (Lam.) Lindl.) is a native Chilean species that grows in Chile from 36 to 41 °S (Novoa *et al*. 2006, Elórtegui & Novoa 2009, Novoa *et al*. 2015). All three species have high ornamental potential (Fig. 1A, B, C) and, to our knowledge, have not been reproduced asymbiotically (or by other means) before.

The aim of this study is to asymbiotically germinate the seeds of *Chloraea crispa*, *C. gavilu*, and *C. virescens*, and to evaluate embryo development of these species using different culture media. If successful, this technique could be a viable alternative for plant propagation to be used with commercial or conservation purposes. A previous study on a rare terrestrial orchid reported that *in vitro* germination can be a successful tool for orchid conservation (Stewart & Kane 2006).

MATERIALS AND METHODS

Plant collection and seed production

Individuals of three *Chloraea* species (*C. crispa*, *C. gavilu*, and *C. virescens*) were collected from natural populations located in Central-South Chile in late spring 2014-summer 2015 (November 2014-January 2015). *C. crispa* plants were collected close to the town of Quilleco, Bío-Bío Region (37º 24' 46.81'' S, 72º 1' 8.22'' W). *C. gavilu* individuals were collected in a road close to the town of Yumbel, Bío-Bío Region (36º 59' 54.93'' S, 72º 34' 24.10'' W). Lastly, *C. virescens* plants were collected in Pucón (Chilean city) aerodrome, Araucanía Region (39º 17' 40.34'' S, 71º 54' 25.49'' W). The species was identified by its distinctive floral morphology following Novoa *et al.* (2006) and Elórtegui & Novoa (2009). Collected plants were transplanted to 2000 mL plastic pots and put in a greenhouse at Universidad de Concepción, Campus Los Ángeles (37º 28' 20.33'' S, 72º 20' 45.50'' W). In the same season, it was conducted manual autopollination (Fig 1D) in 4 flowers per plant (4 plants per species). Previous to the dehiscence, capsules were taken (Fig. 1E) and the surface immediately sterilized with ethanol 98% and flamed under laminar flow chamber to avoid contamination. Capsules were then open and seeds were collected and used in the experiment described below.

Asymbiotic germination

Seven culture media were used in the asymbiotic seed germination (Table 1): agar water (AW), banana culture medium (CMB; Barbery & Molares 2011), tomato culture medium (CMT; Barbery & Molares 2011), Knudson C (KC; Knudson 1946, Arditti 1982), Malmgren Modified Terrestrial Orchid Medium (MM; Malmgren 1996), Murashige & Skoog medium (MS; Murashige & Skoog 1962) and MS modified (MS1/2). All media were sterilized in an autoclave at 121 °C and 1 atm for 20 min and then poured into 40 mm-diameter Petri dishes in a laminar flow chamber. Once all media were prepared, pH was adjusted to 5.8. Four plates (replicates) per culture media were used (four plates × seven culture media = 28 plates total). Each plate contained at least 200 seeds. Petri dishes containing media and seeds were put in an incubator at 24 ± 1 °C in the dark. After two weeks, seed developmental stage was evaluated (see Table 2, adapted from Yamazaki & Miyochi 2006). Seeds were then put for four weeks in a growth chamber at 20 ± 1 °C with a photoperiod of 16/8-hours light/dark (light: 70 µmol m^{-2}s^{-1}). Germination percentage was determined by counting the number of germinated seeds in all Petri dishes divided by total viable seeds. Seed viability was previously determined by the tetrazolium test (data not shown). Germination was achieved when the embryo emerged from the seed coat (stage 2 in Table 2).

Data analysis

The software Statistica 6.0 (StatSoft Inc., Tulsa, OK, USA) was used for the statistical analyses. Germination percentage was arcsine-transformed to normalize the data. One-way ANOVA with culture media as a fixed factor was used to determine differences in germination percentage between treatments followed by a posteriori Tukey Test. This was done for each orchid species separately.

RESULTS

Floral traits confirmed the identity of the studied species (Fig. 1A-C). After two weeks of the beginning of the experiment, seeds of some of the tested *Chloraea* species germinated in CMT, MM and CMB culture media (Fig. 2A). After eight weeks of incubation, embryos of all tested *Chloraea* species reached stage 3 (protocorm stage) in CMT and MM media (Fig 2B). Also, only *C. gavilu* seeds reached stage 3 in CMB. MS medium resulted in embryo development to stage 3 only in *C. virescens*. At 16 weeks after the beginning of the experiment, embryos of all species in MM medium reached the highest development, evidencing a distinct shoot (Shoot stage, Fig 1I, Fig. 2C), stage that is previous to true leaf initiation. Additionally, stage 4 (Rhizoid stage, Fig. 1H) was reached in all species in CMT and only in *C. gavilu* and *C virescens* in MS medium. Plants in AW showed poor development and only reaches stage 1 (pre-germination stage, Fig. 2C).

The three tested *Chloraea* species showed differences in seed germination in the different culture media tested in our study (one-way ANOVA, *p*<0.001 for each species separately). Maximal germination in *Chloraea crispa* was obtained in CMT (close to 85%), followed by MM and CMB with ~70% germination (Fig. 3A), differing significantly from MS and KC (Tukey test, *p*<0.05). *C. gavilu* was the species with highest seed germination, reaching over 90% in MM, CMT, and CMB (Fig. 3B). In this species germination was significantly higher in MM compared to KC, and in CMT and CMB compared to KC (Tukey test, *p*<0.05). In *C. virescens* the best results were obtained in MM and CMB where germination reached over 85% (Fig. 3C), being significantly higher than in CMB, MS, MS1/2, and KC (Tukey test, *p*<0.05). For all tested species, AW culture media did not allowed seed germination (Fig. 3A-C).
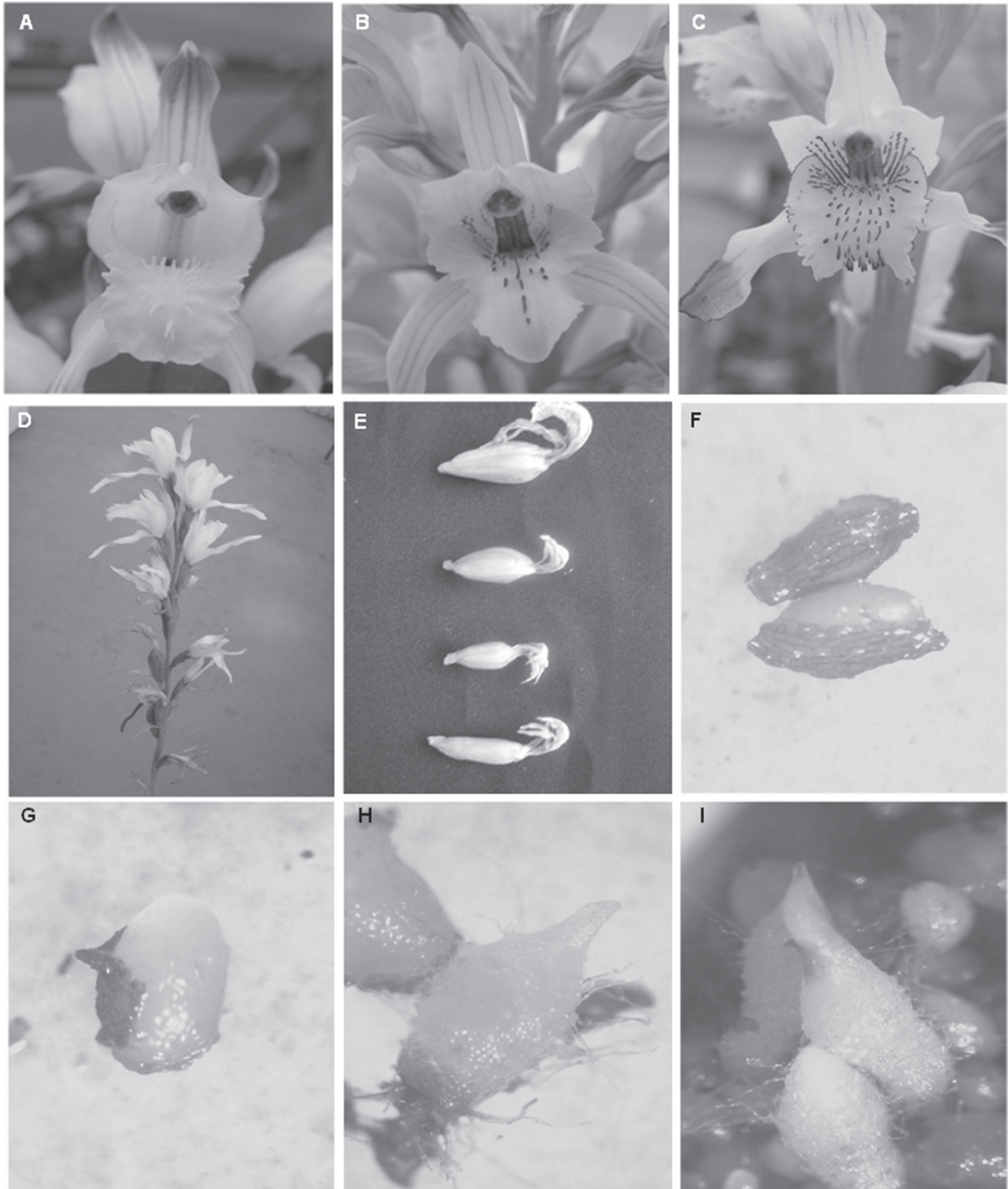
Figure 1. Images of the studied species. A) *C. crispa*. B) *C. gavilu*. C) *C. virescens*. D) Capsules forming after manual pollination. E) Mature capsules. F) Embryos breaking the seed coat. G) Protocorm. H) Protocorm with rhizoids. I) Protocorm with evident apical shoot. / Fotografías de las especies estudiadas. A) *C. crispa*. B) *C. gavilu*. C) *C. virescens*. D) Cápsulas formadas luego de la polinización manual. E) Cápsulas maduras. F) Embriones rompiendo la testa. G) Protocormo. H) Protocormo con rizoides. I) Protocormo evidenciando brote apical.

Table 1. Comparative composition of culture media used for asymbiotic seed germination of three *Chloraea* species. AW: Agar Water. CMB: Banana Culture Medium. CMT: Tomato Culture Medium. KC: Knudson C. MM: Malmgren modified terrestrial orchid medium. MS: Murashige and Skoog, and MS1/2: MS modified. / Composición comparativa de los medios de cultivo usados para la germinación asimbiótica de semillas de tres especies de *Chloraea*. AW: Agar Agua. CMB: Medio de Cultivo Banana. CMT: Medio de Cultivo Tomate. KC: Knudson C. MM: Medio Malmgren modificado para orquídeas terrestres. MS: Murashige y Skoog, y MS1/2: MS modificado.

	KC	MM	CMB	CMT	AW	MS	MS1/2
Macronutrients (mg/L)							
$(NH_4)_2 SO_4$	500						
$(NH_4)(NO_3)$						1650	825
$Ca(NO_3)_2 \cdot 4H_2O$	1000						
$MgSO_4 \cdot 7 H_2O$	250						
KH_2PO_4	250	75				170	85
KNO_3						1900	950
KI						0.83	0.415
$CaCl_2$						332.2	166.1
$Ca_3(PO_4)_2$		75					
$MgSO_4$		97.69				180.7	90.35
$Na_2 EDTA \cdot 2 H_2O$		75				37.26	18.63
Micronutrients (mg/L)							
$MeSO_4 \cdot 7 H_2O$	25						
$MnSO_4 \cdot 4 H_2O$	7.5						
$MnSO_4 \cdot H_2O$		1.54				16.9	8.45
H_3BO_3	0.0560					6.2	3.1
$CuSO_4 \cdot 5 H_2O$	0.0624					0.025	0.0125
MoO_3	0.0160						
$MoO_4Na_2 \cdot 2 H_2O$						0.25	0.125
$ZnSO_4 \cdot 7 H_2O$	0.3310					8.6	4.3
$FeSO_4 \cdot 7 H_2O$		27.8				27.8	13.9
$CoCl_2 \cdot 6 H_2O$						0.025	0.0125
Others (mg/L)							
Ripe banana (pulp)			100000				
Ripe tomato (pulp)				100000			
Yeast extract		1000					
Pineapple powder		20000					
D-Biotin		0.05					
Casein, Enzymatic Hydrolysate		400					
Folic acid		0.5					
Glycine (free base)		2				2.0	1.0
Pyridoxine		5				0.5	0.25
myo-Inositol		100				100	50
Nicotinic acid (free acid)		5				0.5	0.25
Thiamine		10	1200	1200		0.1	0.05
Thidiazuron (TDZ)		0.1					
Sucrose	20000	10000	15000	15000		3000	3000
Agar	10000	7000	10000	10000	10000	7000	7000
Activated charcoal		1000					
Deionized water (mL)	1000	1000	1000	1000	1000	1000	1000

TABLE 2. Developmental stages of terrestrial orchid seeds (modified from Yamazaki & Miyoshi 2006). / Estados de desarrollo de semillas de orquídeas terrestres (modificado de Yamazaki & Miyoshi 2006).

| STAGE	DESCRIPTION
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.
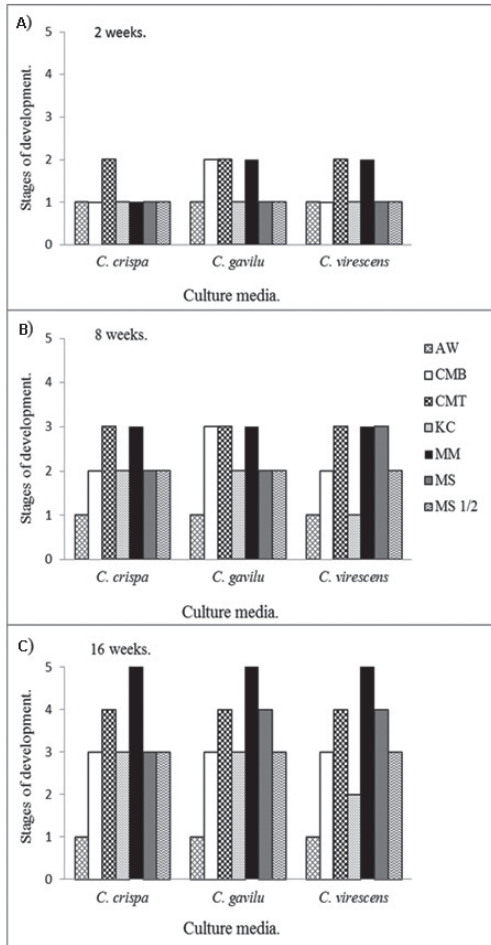
FIGURE 2. Developmental stages of seeds and embryos in three *Chloraea* species after 2 (A), 8 (B) and 16 weeks (C) of incubation in different culture media. AW: Agar Water. CMB: Banana Culture Medium. CMT: Tomato Culture Medium. KC: Knudson C. MM: Malmgren Modified terrestrial orchid medium. MS: Murashige and Skoog, and MS1/2: MS modified. Developmental stages follow a modified version of Yamazaki & Miyochi (2006). / Estados de desarrollo de las semillas y embriones en tres especies de *Chloraea* luego de 2 (A), 8 (B) y 16 (C) semanas de incubación en diferentes medios de cultivo. AW: Agar Agua. CMB: Medio de Cultivo Banana. CMT: Medio de Cultivo Tomate. KC: Knudson C. MM: Medio Malmgren modificado para orquídeas terrestres. MS: Murashige y Skoog, y MS1/2: MS modificado. Los estados de desarrollo siguen una versión modificada de Yamazaki & Miyochi (2006).
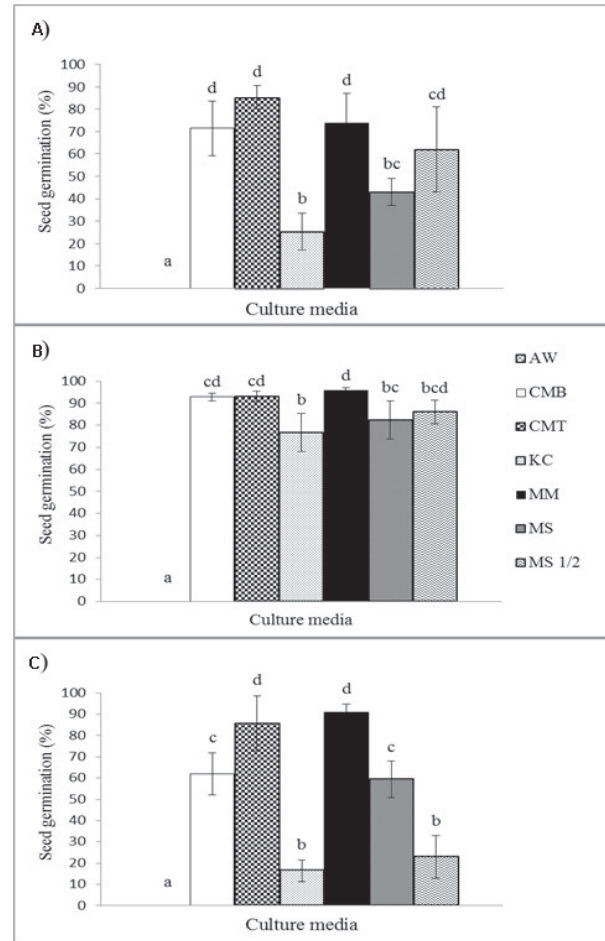
FIGURE 3. Average seed germination in *C. crispa* (A), *C gavilu* (B), and *C. virescens* (C) after 16 weeks of incubation in different culture media. Different letters correspond to statistical differences (t-test, $p < 0.05$). AW: Agar Water. CMB: Banana Culture Medium. CMT: Tomato Culture Medium. KC: Knudson C. MM: Malmgren Modified terrestrial orchid medium. MS: Murashige & Skoog, and MS1/2: MS modified. / Germinación promedio en *C. crispa* (A), *C. gavilu* (B) y *C. virescens* (C) luego de 16 semanas de incubación en diferentes medios de cultivo. Letras diferentes corresponden a diferencias estadísticamente significativas (test de t, p<0.05). AW: Agar Agua. CMB: Medio de Cultivo Banana. CMT: Medio de Cultivo Tomate. KC: Knudson C. MM: Medio Malmgren modificado para orquídeas terrestres. MS: Murashige y Skoog, y MS1/2: MS modificado.

DISCUSSION

Many orchids are economically important (Dearnaley 2007, Dearnaley *et al*. 2012), mainly for their beautiful flowers which made them stand out among ornamental plants (Abraham *et al*. 2012). *Chloraea* and *Gavilea* are the most represented orchid genus in Chile (Elórtegui & Novoa 2009, Novoa *et al*. 2015). The Chilean orchids *Chloraea crispa, C. gavilu*, and *C. virescens* have high ornamental value and potential as cut flowers. Thus, efficient propagation methods are required to propagate these species. In this study it was established that seeds of these orchid species can be asymbiotically germinated in laboratory conditions using different culture media. To our knowledge, asymbiotic (or symbiotic) germination for these species has not been previously reported. Studies on terrestrial temperate orchids are relatively scarce (but see Dixon 1991, Dutra *et al*. 2008, Steinford *et al*. 2010, Fracchia *et al*. 2014, Pereira *et al*. 2014, Atala *et al*. 2015) compared to studies on tropical species. Moreover, studies on orchid biology and asymbiotic reproduction of Chilean species are even rarer and for many species non-existent (but see Lehnebach & Riveros 2003, Valdivia *et al*. 2010, Pereira *et al*. 2015). This is particularly important considering that many of those species are considered critically endangered (Novoa *et al*. 2006, Romero 2012). The shortage of ecological and functional studies on this group could be partially blamed on the lack of taxonomic knowledge in the country and the difficulties for the identification of the species in the field, especially individuals lacking reproductive structures. Currently, the only sources available for the identification of the species are out-of-date papers and divulgation books (Correa 1969, Novoa *et al*. 2006, Elórtegui & Novoa 2009, Romero 2012).

In nature, orchids reproduce by seeds and depend on direct association with specific (to some degree) mycorrhizal fungi to germinate (Murguía & Lee 2007, Pereira *et al*. 2005, Smith & Read 2008, Valadares *et al*. 2012). As stated early, this dependency is due to the minimal amount, or even absence, of nutrients stored in the small seeds of orchids (Elórtequi & Novoa 2009, Smith & Read 2008, Abraham *et al*. 2012). The fungal partner supplies the required nutrients for embryo growth, nutrients that can also be supplied by specific culture media (Knudson 1946, Nadarajan *et al*. 2011, Pereira *et al*. 2015). The appropriate choice of nutrient medium seems to be crucial for seed germination and also for embryo development (Dutra *et al*. 2008, Rasmussen *et al*. 2015, Pereira *et al*. 2015). Seed germination varied among the studied *Chloraea* species depending on the used culture media. In *C. crispa* and *C. virescens* the highest germination (over ~75%) was achieved in CMT and MM. On the other hand, *C. gavilu* germinated over 75% in all tested culture media except AW. Previous studies on *Bipinnula fimbriata* reported a germination of 73 and 65.7% in MM and CMT,

respectively (Pereira *et al*. 2015). Stewart & Kane (2006) registered close to 89% asymbiotic seed germination of the terrestrial orchid *Habenaria macroceratitis* using Lindermann and Knudson C media after 7 weeks. However, in the same study MM medium was the only culture media that induced protocorm development to leaf-bearing after 16 weeks of incubation. More recent studies on the same orchid species reported asymbiotic germination percentages of 83.9% using MM medium, with embryos evidencing elongation of the first leaf after 21 weeks (Stewart & Kane 2010). In our study, Knudson C media resulted in relatively low germination (below 30%) in *C. crispa* and *C. virescens*. However, the same media resulted in over 70% germination in *C. gavilu*. These results reinforce the idea that interspecific differences in nutrient requirements exist, and specific protocols should be developed for each orchid species to be propagated. The ecological and physiological consequences of these differences should be addressed in future studies on temperate terrestrial orchids.

In terms of embryo development MM media showed the best results, evidencing the more advanced stage (stage 5, shoot is differentiated from the protocorm) after 8 weeks compared to the other tested media. As mentioned above, the response of orchid seeds to different nutrient media varies between species (Nadarajan *et al*. 2011, Paul *et al*. 2011, Mohanty *et al*. 2012) and specific nutritional and environmental conditions are likely needed for *in vitro* germination of a determinate species (Arditti *et al*. 1990, Rasmussen 1995). The asymbiotic media used in this study varied greatly in mineral salt, nitrogen organic additives and vitamin compositions. For *C. gavilu,* all tested media (except for AW) supported a high germination, suggesting that this species is able to utilize a broad range of carbon and nitrogen forms and concentration. KC, MS and MS1/2 contain only inorganic sources of nitrogen (ammonium and nitrate). In contrast, MM (and probably CMB and CMT) contains only organic nitrogen sources, coinciding with the highest germination for *C. crispa* and *C. virescens*. Several researchers have reported that inorganic nitrogen may limit orchid germination, possibly due to low nitrate reductase activity during seed germination, and early protocorm development (Malmgren 1992, Van Waes & Debergh 1986, Nadarajan *et al*. 2011). The high germination of *C. gavilu* in media with inorganic nitrogen sources could be explained by an early activation of nitrate reductase during germination and/or because organic nitrogen does not play an important role in seed germination as other nutrients or environmental factors, such as in the terrestrial orchid *Bletia purpurea* (Lam.) DC. (Dutra *et al*. 2008). In the field, *C. gavilu* shows a broad latitudinal distribution and can be found in diverse environments (Elórtegui & Novoa 2009, Romero 2012, Novoa *et al*. 2015). In its wide geographical range it is likely that this species finds different nutrient, soil, and even biotic conditions, possibly explaining why

is less demanding of specific nutrients and culture media in laboratory conditions. On the other hand, *C. crispa* and *C. virescens* have more narrow latitudinal distributions and more specific ecological conditions (Elórtegui & Novoa 2009, Romero 2012, Novoa *et al*. 2015). Following the same argument used above, this could explain the higher selectiveness for Nitrogen sources and culture media.

In vitro germination of *Chloraea* species can shed light on possible *in situ* plant responses and their environmental requirements, and also further the knowledge of orchid development. This information can in turn be used to propagate plants for potential economic purposes and to establish better management policies for their *in situ* and *ex situ* conservation. *In vitro* methods can achieve high germination in these species, but the reintroduction of adult individuals in the field could likely need previous inoculation with species fungal partners to increase plant survival. Further studies are needed to confirm these still open questions.

ACKNOWLEDGMENTS

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