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Application Article

In vitro asymbiotic germination for micropropagation of the recalcitrant terrestrial orchid *Chloraea crispa* (Orchidaceae)[1]

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- *Premise of the study: Chloraea crispa* is a terrestrial Orchidaceae species native to Chile, characterized by a beautiful and showy inflorescence. The species has a great potential for commercial exploitation in the cut flower industry, but it is essential to improve propagation methods to avoid endangering its natural populations. Because this species is hard to propagate using traditional greenhouse techniques, in vitro techniques offer an effective tool for its large-scale production in terms of germination, growth, and propagation.
- *Methods:* The current study evaluated the effect of the culture medium on the asymbiotic germination of *C. crispa* seeds, as well as the effects of the plant growth regulators 6-benzylaminopurine and indole-3-butyric acid. Different light regimes were also studied.
- *Results:* A significant effect was observed for the interaction between culture media and light regime on the morphogenic response of the seeds. The highest rate of embryonic germination was obtained in Van Waes medium supplemented with 0.1 mg·L^{-1} of 6-benzylaminopurine.
- *Discussion:* For the first time, asymbiotic culture of this species using biotechnology tools has been developed. Plantlets developed very well under in vitro conditions, allowing the possibility to propagate and store genetic material for conservation and domestication purposes.

Key words: asymbiotic in vitro germination; *Chloraea crispa*; Orchidaceae; tissue culture.

Orchids (Orchidaceae) represent one of the most commercially important plants in the world. In Chile, orchids are represented by seven genera, which comprise 52 species. Most of the species are rare, relatively unknown, and endangered because of human activities (Novoa et al., 2006). Among the species known in Chile is *Chloraea crispa* Lindl., a terrestrial orchid endemic to Chile and distributed in coastal zones, in the Central Valley, and in low-elevation areas of the Coastal Cordillera (Humaña et al., 2008).

Flowering in *C. crispa* occurs from October to January (Novoa et al., 2006), when it produces beautiful flowers with long stems and long vase life, thus giving this species great potential as a novel alternative for the cut flower industry (Steinfort et al., 2012). As with most of the orchids (Orchidaceae), this species is naturally propagated using seeds and/or mericlones; however, because of the difficulties involved in natural germination of the seeds along with the high human pressure on the natural populations through use of the flowers, it is necessary to develop alternative propagation methods (Novoa et al., 2006). The species is incapable of apomixis or autogamy, a fact that should be considered for conservation purposes when a propagation method is selected (Humaña et al., 2008).

Asymbiotic methods of germination under in vitro conditions are an alternative tool for the efficient propagation of orchids (Arditti and Ernst, 1993; Pedroza-Manrique and Mican-Gutiérrez, 2006; Zeng et al., 2012). Knudson (1922) first successfully propagated orchids (*Cattleya* Lindl. and *Laelia* Lindl.) using in vitro germination. Since that time, the reports in the literature indicate that in vitro germination is now a significant option for propagating terrestrial orchid species, although marked differences among species are expected (Vogel and Macedo, 2011; Zeng et al., 2012). In addition, in vitro germination percentages can be very low for several recalcitrant species (Kauth et al., 2006; Stewart and Kane, 2006; Thompson et al., 2006; Johnson et al., 2007; Dutra et al., 2008; Vogel and Macedo, 2011), making it necessary for protocols to be developed on a case-by-case basis.

To establish a successful in vitro plant propagation method, it is necessary to assess a number of factors, both individually and

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in combination. These include: the nutritional composition of the culture media, the effect of growth regulators, the environmental conditions, the type of explants, and the general management of the plants (Pedroza-Manrique et al., 2010). The use of artificial supplements with different nutrients can increase the rate of asymbiotic germination, as well as increase flowering and productive yields (Shin et al., 2011). Rasmussen (1995) reported 40 culture media that allow in vitro germination of terrestrial orchids. In parallel with these studies, a number of strategies have been developed, such as seed hydration, modification of the nutritional composition of the culture medium, and managing concentrations and interactions of plant hormones (Stewart and Kane, 2006; Johnson et al., 2007; Dutra et al., 2008; Kauth et al., 2008; Pedroza-Manrique et al., 2010; Zeng et al., 2012).

Research on the role of different hormones, such as auxins and cytokinins, for in vitro orchid cultures has shown that hormones can both favor and inhibit germination, while in other cases they appear not to have any effect on germination. However, Pedroza-Manrique et al. (2010) noted that not all orchids require exogenous hormones, especially for germination, as evidenced in their research in which protocorm development was achieved without any type of growth regulator. In contrast, Godo et al. (2010) reported that auxins such as naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and indole-3-acetic acid (IAA) were less effective in promoting orchid seed germination than cytokinins.

Addition of 6-benzylaminopurine (BAP) has shown an improvement in seed germination and protocorm proliferation in orchids (David et al., 2010; Nongdam and Tikendra, 2014), as well as in protocorm recovery and plant development (Cardoso and Ono, 2011). In addition, application of IBA as an auxin source has been found to promote rooting and plant development in orchids (Aktar et al., 2007; Parthibhan et al., 2015). Furthermore, a combination of IBA and NAA improved protocorm recovery from a rare and endangered orchid from the genus *Orchis* L. (Baker et al., 2014). In any case, according to Arditti and Ernst (1993), the response of orchids to plant hormones can be inconsistent and can depend on both the plant genus and the type of plant growth regulator. Thus, particular studies should be performed for each species to find the best protocols.

Environmental conditions can also influence orchid development, including under in vitro conditions (Vogel and Macedo, 2011). The results for the influence of light on in vitro germination of orchids are contradictory and seem to be closely related to the species; indeed, some species have been found in which germination occurs in both dark and light (Dutra et al., 2008), while other studies have found in vitro germination to be inhibited by light (Yamazaki and Miyoshi, 2006).

The objective of the current study was to develop an effective in vitro asymbiotic germination protocol for the terrestrial orchid *C. crispa*, to propagate this species for conservation as well as for commercial use.

MATERIALS AND METHODS

***Plant material and disinfection of seeds*—**All the experiments were carried out in the Plant Tissue Culture Laboratories of the Universidad de Talca and the Universidad Católica del Maule. Fruits were collected from the farm of Enrique Matthei Jensen, from plants growing under a pine plantation near Yumbel, Región del Bío Bío, Chile. Randomly selected *C. crispa* seeds (*C. crispa* × *C. crispa*) collected from the open pollinated plants were disinfected, and 5 mg of seeds were placed in sterile Eppendorf tubes (Eppendorf, Hamburg, Germany) under a laminar flow hood. A disinfectant solution of 1.3 mL of sodium hypochlorite at 1% and Tween 80 that had been sterilized with a 0.22-µm Millipore filter (EMD Millipore, Billerica, Massachusetts, USA) was placed in each tube. The seeds were sterilized with constant agitation for 10 min. The disinfectant solution was then removed, and the seeds were washed with five changes of sterile water. The seeds were dried under the laminar flow hood and sown in the appropriate culture medium.

***Experimental design and statistical analysis*—**The basal media for all assays and treatments were supplemented with 20 $g \cdot L^{-1}$ of sucrose and 7 $g \cdot L^{-1}$ of agar. The pH level was adjusted to 5.8 before the media were sterilized in an autoclave for 30 min at 121°C and 117.7 kPa of pressure.

Cultivation was carried out at a temperature of 20°C through all the culture stages. In all cases, the first 15 d of culture were in darkness and subsequently the treatments were cultivated under their respective light regimes. The light intensity employed was 100 µmol/m²/s supplied by fluorescent tubes. Seeds under dark conditions were cultivated inside dark boxes in the same light room. Dark boxes were prepared using paperboard boxes and covered with a dark cloth. Glass flasks of 100 mL were used for all the experiments.

All experiments were conducted as described above, according to a factorial design. The first group of experiments (culture medium and light regime) comprised 10 treatments (I-1–5 for each light regime), with four replications for each treatment. For the experiments assessing the effect of plant growth regulators and light regime on the germination efficiency, eight treatments (II-1–4 for each light regime), with four replications per treatment, were set as detailed below. As described previously, the experimental unit was a flask with 5 mg of seeds. Normality of the data was assessed by the Kolmogorov–Smirnov test. Average calli formation and protocorm germination per treatment were recorded in each experiment as dependent factors to select the best treatments. Data were statistically analyzed by analysis of variance (ANOVA) and Tukey's honest significant difference test with 95% confidence level, using the SPSS program (IBM, Armonk, New York, USA). A two-way ANOVA analysis was performed to detect the effect of the interaction of independent factors and the analyzed dependent factors.

***Effects of light regime and media composition on the asymbiotic germination of* C. crispa—**The effectiveness of different light regimes on the asymbiotic germination of *C. crispa* cultivated in five different basal media was evaluated. All treatments were subjected to one of two light regimes: 16 h of light and 8 h of darkness (16 h light : 8 h dark) or continuous darkness (0 h light : 24 h dark). Media labeling and composition are described in Table 1. In contrast to the Murashige and Skoog (MS) medium, BM-2 has no inorganic nitrogen, but it is supplied by the following compounds: hydrolyzed casein (500 $mg \cdot L^{-1}$), L-glutamine (100 $mg \cdot L^{-1}$), D-biotin (0.05 $mg \cdot L^{-1}$), folic acid (0.5 $mg \cdot L^{-1}$), glycine (2 $mg \cdot L^{-1}$), *myo*-inositol (100 $mg \cdot L^{-1}$), nicotinic acid (5 $mg \cdot L^{-1}$), pyridoxine-HCl (0.5 $mg \cdot L^{-1}$), and thiamine-HCl (0.5 $mg \cdot L^{-1}$).

***Effect of plant growth regulators BAP and IBA on asymbiotic germination of* C. crispa *under different light regimes*—**After analyzing the results of the experiment described above, the basal BM-2 medium (Van Waes and Debergh, 1986) was used to induce calli formation and protocorm development. In this experiment, the effect of reducing the concentration of BAP and IBA was tested because, in the first group of experiments, the addition of plant growth regulators reduced the germination efficiency. Also, Van Waes (1984) previously reported that BAP improved germination of some orchid species in a range of 0.1 to 0.2 $mg \cdot L^{-1}$. All treatments were submitted to one of the two previously described light regimes: 16 h light : 8 h dark or 0 h light : 24 h dark. The amounts of growth regulators added to the basal medium for each light regime are detailed in Table 2.

Table 1. Evaluation of the culture medium on asymbiotic germination of *Chloraea crispa*.

Treatment	Basal medium	Salt supplement ($g \cdot L^{-1}$)	Plant growth regulator ($mg \cdot L^{-1}$)
I-1	BM-2[a]	—	—
I-2	50% MS[b]	—	—
I-3	BM-2	$CaNO_3$ (1.0), $(NH_4)_2SO_4$ (0.5)	—
I-4	BM-2	—	BAP (0.2)
I-5	BM-2	—	IBA (0.2)

[a] Van Waes basal medium (Van Waes and Debergh, 1986).

[b] MS basal salts and vitamins (Murashige and Skoog, 1962) diluted at 50% of their original concentration.

Table 2. BAP and IBA treatments under different photoperiod regimes, assayed for inducing asymbiotic germination in *Chloraea crispa*. BM-2 medium (Van Waes and Debergh, 1986) was used for all treatments.

	Plant growth regulator (mg·L^{-1})	
Treatment	BAP	IBA
16 h light : 8 h dark		
II-1	—	—
II-2	0.1	—
II-3	—	0.1
II-4	0.2	0.1
Continuous darkness		
II-1	—	—
II-2	0.1	—
II-3	—	0.1
II-4	0.2	0.1

Note: BAP = 6-benzylaminopurine; IBA = indole-3-butyric acid.

RESULTS

Effects of light regime and media composition on the asymbiotic germination of C. crispa

Embryogenic calli formation—The tested factors of light regime and culture medium had a significant effect, both as individual factors and in combination, depending on the evaluation time *($P \leq 0.05$)*. For light regime, the single effect of this factor on calli formation was significant only at 30 d after germination. For protocorm germination, the significant effect of this factor lasted until 60 d after seed culture was initiated.

After 60 d of culture, calli formation was obtained in all light and media treatments. The rate of new callus induction was decreased in treatment I-1 (for both culture conditions) and in treatment I-2 under the photoperiod throughout the evaluation period. At 60 d, the I-2 treatment showed a significant difference in induction of embryogenic calli (17.3 ± 1.52, $P \leq 0.05$) compared with the other treatments. After 120 d, this treatment continued to have a higher number of embryogenic calli (10.80 ± 3.80, $P \leq 0.05$) than the other treatments, with the exception of I-2 and I-3 cultivated in continuous darkness, which produced 8.00 ± 0.00 and 7.50 ± 0.58 embryogenic calli, respectively. This situation was repeated after 180 d of culture, because the formation of embryogenic calli observed in treatment I-2 under the photoperiod (11.0 ± 2.0 of embryogenic calli) was statistically similar to the effects of treatments I-2 and I-3 under continuous darkness ($P \leq 0.05$). At the end of the evaluation period (180 d), these three treatments generated the highest number of embryogenic calli compared with the rest of the treatments ($P \leq 0.05$).

Protocorm germination—Protocorm formation was influenced by the effect of each individual factor or the interaction between them ($P \leq 0.05$). However, after 90 d of culture, the individual effect of the environmental conditions and culture medium was not significant (Tables 3, 4).

The formation of protocorms began in five of the 10 treatments by 60 d, with treatment I-1 under continuous darkness, resulting in a significantly higher number of protocorms ($P \leq 0.05$), as can also be seen in Tables 3 and 4. Protocorm formation began in the rest of the treatments between days 60 and 120, with the exception of the seedling in treatments I-3 and I-5.

As with embryogenic calli, and independent of the light regime and growth medium, most of the treatments increased protocorm formation after 120 and 180 d of culture. At 60 d, treatment I-1 under continuous darkness induced significantly higher protocorm formation than the other treatments, with a mean of 16.50 ± 1.00 germinated protocorms ($P \leq 0.05$). At the end of the evaluation (180 d), treatment I-1 both under photoperiod and under darkness (24.00 ± 0.86 and 21.0 ± 3.00, respectively) induced higher efficiency for protocorm formation ($P \leq 0.05$). However, after the three evaluations, the total sum of germinated protocorms in treatment I-1 was higher than the rest of the treatments ($P \leq 0.05$).

Effect of plant growth regulators BAP and IBA on asymbiotic germination of C. crispa *under different light regimes*

Embryogenic calli formation—Both the individual factors and their interaction (light regimen × plant growth regulators) had a significant effect on calli formation ($P \leq 0.05$). As can be seen in Table 5, except for treatments II-3 and II-4 under photoperiod, all treatments generated embryogenic calli by 60 d after

Table 3. Effect of media composition and photoperiod on asymbiotic germination of *Chloraea crispa* seeds 60, 120, and 180 d after sowing. Germination efficiency was estimated considering calli formation and germination of protocorms.[a]

	60 d		120 d		180 d	
Treatment[b]	Embryogenic calli	Germinated protocorms	Embryogenic calli	Germinated protocorms	Embryogenic calli	Germinated protocorms
16 h light : 8 h dark						
I-1	5.50 ± 0.60 b	0.00 d	5.50 ± 1.00 c	13.00 ± 2.00 a	3.00 ± 2.00 de	24.00 ± 0.86 a
I-2	17.30 ± 1.52 a	2.80 ± 1.60 c	10.80 ± 3.80 a	7.00 ± 0.00 b	11.00 ± 2.00 a	7.50 ± 1.00 cd
I-3	4.00 ± 1.80 b	0.00 d	5.50 ± 1.00 c	0.00 d	6.70 ± 1.50 bc	1.75 ± 1.50 e
I-4	4.80 ± 0.60 b	0.00 d	6.00 ± 0.00 c	11.00 ± 3.70 ab	7.50 ± 1.00 b	13.30 ± 2.50 b
I-5	0.00 b	0.00 d	0.00 d	0.00 d	0.00 e	0.00 f
Continuous darkness						
I-1	6.80 ± 3.50 b	16.50 ± 1.00 a	3.70 ± 2.90 c	17.00 ± 3.50 a	3.70 ± 2.90 c	21.00 ± 3.00 a
I-2	0.00 c	0.00 d	8.00 ± 0.00 ab	6.50 ± 1.00 bc	10.30 ± 2.20 ab	8.00 ± 0.00 c
I-3	6.70 ± 3.20 b	5.00 ± 1.00 b	7.50 ± 0.58 ab	5.70 ± 0.50 c	9.20 ± 1.70 ab	6.50 ± 1.00 d
I-4	3.70 ± 2.90 b	4.60 ± 2.90 bc	3.70 ± 2.80 c	14.00 ± 2.60 a	3.70 ± 0.60 d	16.00 ± 0.80 b
I-5	4.00 ± 0.80 b	4.00 ± 3.30 bc	4.70 ± 0.50 c	4.00 ± 1.10 c	5.00 ± 0.00 c	4.00 ± 1.00 d

[a] Different letters in the same column signify significant differences between treatments (Tukey's honest significant difference test, $P \leq 0.05$).

[b] I-1 = BM-2 (Van Waes basal medium [Van Waes and Debergh, 1986]); I-2 = 50% MS; I-3 = BM-2 + 1.0 mg·L^{-1} $CaNO_3$ + 0.5 g·L^{-1} $(NH_4)_2SO_4$; I-4 = BM-2 + 0.2 mg·L^{-1} BAP; I-5 = BM-2 + 0.2 mg·L^{-1} IBA.

TABLE 4. Statistical analysis of the treatments summarized in Table 3, showing the effect of media composition and photoperiod on asymbiotic germination of *Chloraea crispa* seeds 60, 120, and 180 d after sowing.

	60 d					120 d					180 d				
Variation source	Sum of squares	Degrees of freedom	Medium squares	*F* ratio	*P* value	Sum of squares	Degrees of freedom	Medium squares	*F* ratio	*P* value	Sum of squares	Degrees of freedom	Medium squares	*F* ratio	*P* value
Calli formation															
Basal medium	190	4	47	13	<0.0001	215	4	54	17	<0.0001	437	4	109	39	<0.0001
Photoperiod regime	42	1	42	11	0.0022	0	1	0	0	>0.9999	1	1	1	0	0.5152
Interaction basal medium × photoperiod regime	605	4	151	40	<0.0001	85	4	21	7	0.0005	106	4	26	9	<0.0001
Standard error	113	30	4			94	30	3			85	30	3		
Protocorm germination															
Basal medium	253	4	63	27	<0.0001	1063	4	266	68	<0.0001	2550	4	637	469	<0.0001
Photoperiod regime	297	1	297	126	<0.0001	106	1	106	27	<0.0001	60	1	60	44	<0.0001
Interaction basal medium × photoperiod regime	385	4	96	41	<0.0001	43	4	11	3	0.047	36	4	9	7	0.0006
Standard error	71	30	2			118	30	4			41	30	1		

plating, both under photoperiod or continuous darkness. The higher number of embryogenic calli were observed after 120 and 180 d of culture ($P \leq 0.05$). With treatments II-2 and II-4 (both under continuous darkness), there was an ongoing decrease in the mean number of embryogenic calli formed throughout the evaluation. At 120 d, treatment II-1 under continuous darkness produced the most embryogenic calli (15.50 ± 3.51). The same situation was observed at 180 d, where this treatment induced the highest number of embryogenic calli (19.00 ± 1.41, $P \leq 0.05$), followed by treatment II-3 under continuous darkness (10.50 ± 1.00) (Tables 5, 6).

Protocorm germination—There was a significant effect of the interaction between light regime and plant growth regulators on the efficiency of protocorm germination ($P \leq 0.05$). Each individual factor also significantly influenced this variable ($P \leq 0.05$). In those treatments cultivated under continuous darkness, protocorm formation began 60 d after sowing, while seeds germinated under 16 h of light only began forming protocorms after 60 d but before 120 d, as is shown in Table 5. As with embryogenic calli, and across light regime and growth medium, the largest number of protocorms was formed between 120 and 180 d (Fig. 1). At 120 d, treatment II-2 under continuous darkness (27.50 ± 2.08) induced a higher number of germinated protocorms than all other treatments ($P \leq 0.05$).

At the end of the evaluation (180 d), treatment II-2 under continuous darkness (30.5 ± 1.73) showed the highest efficiency for protocorm formation ($P \leq 0.05$), followed by treatment II-1 under photoperiod, which generated 26.00 ± 1.63 new protocorms. Furthermore, after the three evaluations, the total sum of germinated protocorms in treatment II-2 under continuous darkness was higher than the rest of the treatments, reaching an average of 66.5 protocorms (Fig. 2).

DISCUSSION

Effect of the basal medium and light regime—The idea that epiphytic and terrestrial orchids require different light and dark conditions for their seeds to germinate, reflecting the natural conditions of their original habitats, is widely accepted. Nevertheless, germination responses to different light regimes vary by the particular species and genotypes, regardless of growth habit (Kauth et al., 2008; Vogel and Macedo, 2011).

The role of light regime in orchid seed germination is often not given the importance it deserves, nor is it fully understood in terrestrial orchids, particularly those belonging to the genus *Chloraea* Lindl. Independent of the light regime, the growth medium and growth regulators used in almost all of the treatments reported here induced germination of the hybrid seeds under study.

At 60 d, treatment I-2 under photoperiod (light regime 16 h light : 8 h dark) showed a significant difference in induction of embryogenic calli compared to the rest of the treatments. The formation of protocorms is favored by a regime of full darkness (0 h light : 24 h dark), the best treatment under this regime being I-1 (complete darkness without any growth regulators) (Table 3). Other authors have also suggested that germination occurs in both dark and light (Stewart and Kane, 2006; Dutra et al., 2008).

In contrast, some authors have indicated that seed germination of terrestrial orchids can be inhibited by light (Van Waes

TABLE 5. Effect of plant growth regulators and photoperiod on calli induction and asymbiotic germination of *Chloraea crispa* seeds 60, 120, and 180 d after sowing. Germination efficiency was estimated considering calli formation and germination of protocorms.[a]

	60 d		120 d		180 d	
Treatment[b]	Embryogenic calli	Germinated protocorms	Embryogenic calli	Germinated protocorms	Embryogenic calli	Germinated protocorms
16 h light : 8 h dark						
II-1	5.00 ± 0.82 ab[3]	0.00 c	2.50 ± 0.58 d	14.00 ± 0.82 b	3.50 ± 0.58 c	26.00 ± 1.63 b
II-2	1.50 ± 1.29 c	0.00 c	5.50 ± 1.91 bc	1.00 ± 0.00 f	8.00 ± 1.41 b	3.00 ± 1.63 f
II-3	0.00 d	0.00 c	3.00 ± 0.82 cd	3.00 ± 0.82 e	2.50 ± 0.58 cd	3.00 ± 0.82 f
II-4	0.00 d	0.00 c	2.50 ± 0.58 d	2.00 ± 0.82 e	1.50 ± 1.00 d	4.50 ± 1.00 f
Continuous darkness						
II-1	4.50 ± 1.92 ab	4.50 ± 1.91 b	15.50 ± 3.51 a	15.50 ± 1.00 b	19.00 ± 1.41 a	21.00 ± 1.63 c
II-2	3.50 ± 0.58 b	8.50 ± 1.00 a	1.50 ± 0.58 d	27.50 ± 2.08 a	1.50 ± 0.58 d	30.50 ± 1.73 a
II-3	5.00 ± 2.31 ab	6.50 ± 0.58 ab	7.00 ± 0.00 b	6.50 ± 0.58 d	10.50 ± 1.00 b	6.00 ± 0.00 e
II-4	7.00 ± 1.41 a	4.00 ± 0.00 b	3.50 ± 1.30 cd	10.00 ± 0.00 c	3.50 ± 1.30 c	15.00 ± 0.58 d

[a] Different letters in the same column signify significant differences between treatments (Tukey's honest significant difference test, $P \leq 0.05$).
[b] II-1 = BM-2 (Van Waes basal medium [Van Waes and Debergh, 1986]); II-2 = BM-2 + 0.1 mg·L^{-1} BAP; II-3 = BM-2 + 0.1 mg·L^{-1} IBA; II-4 = BM-2 + 0.2 mg·L^{-1} BAP + 0.1 mg·L^{-1} IBA.

and Debergh, 1986; Yamazaki and Miyoshi, 2006). *Chloraea crispa* is generally found shaded under forest canopies in central southern Chile, because of which its seeds are not normally exposed to high intensities of light. Orchid seeds in tree-shaded soils are exposed to low intensities of red light (660–770 nm) and higher intensities of far red light (770–800 nm). Exposure to this type of light transforms the phytochrome from its phytochrome far-red (P_{fr}) form, which stimulates germination, to its P_r form, which inhibits germination (Maciel and Bautista, 1997). The fact that fluorescent tubes like those used in the current study emit high intensities of red light (Frankland, 1981) explains why protocorm formation was favored by a light regime of continuous darkness.

Although embryogenic calli and protocorms were formed in the two basal media evaluated, treatment I-2 (MS media prepared at half of its original concentration [50% MS medium] under a light regime of 16 h light : 8 h dark) produced the highest number of embryogenic calli. These results are similar to those reported by Zeng et al. (2012), who found that the best morphogenic responses with orchids of the genus *Paphiopedilum* Pfitzer were obtained in low-salt basal media.

Media composition proved to have a significant effect on calli formation and protocorm germination. The highest protocorm germination and calli formation rates were obtained in 50% MS medium and Van Waes (VW) medium. In a study of the terrestrial orchid *Habenaria macroceratitis* Willd., Stewart and Kane (2006) determined that any form and concentration of nitrogen is beneficial for germination and the early development of protocorms. The current study supports this conclusion, as the 50% MS medium favored the germination and early development of embryogenic calli. However, the 50% MS medium did not favor later development in the protocorm stage. Kauth et al. (2006) obtained positive results in terms of germination and the early development of protocorms with media containing inorganic nitrogen sources like nitrates and ammonium (nitrate : ammonium ratio of 0.5). This could explain the effect of the 50% MS medium in this study, in which the nitrate : ammonium ratio was 0.52. Curtis and Spoerl (1948) determined that a nitrate : ammonium ratio of 0.23 is beneficial for the germination of orchids of the genus *Cattleya*. On the contrary, we have demonstrated that the addition of $CaNO_3$ (1.0 g·L^{-1}) or $(NH_4)_2SO_4$ (0.5 g·L^{-1}) as a supplementary source of inorganic nitrogen reduced both protocorm germination and calli formation.

The type and concentration of nitrogen in the growth medium can play a significant role during the asymbiotic germination of orchid seeds (Kauth et al., 2006; Stewart and Kane, 2006; Johnson et al., 2007; Dutra et al., 2008). Dutra et al. (2008) and Van Waes and Debergh (1986) reported that under natural conditions mycorrhizal fungi contribute the organic nitrogen necessary for germination. Consequently, these authors suggested that adding a source of organic nitrogen to the culture medium (e.g., amino acids) favors germination and the development of terrestrial orchids, among them *Bletia purpurea* (Lam.) DC. and *Platanthera ciliaris* (L.) Lindl., because these compounds are more available to seeds than inorganic sources.

According to Johnson et al. (2007), the fact that certain culture media are more effective than others in stimulating the development of protocorms is due partly to the nitrogen sources and their availability as well as to enzymatic synthesis and/or activation. Van Waes and Debergh (1986) found that inorganic nitrogen can limit germination, possibly because of the low level of activity of the nitrate reductase enzyme during germination. The VW medium used in this research was supplemented with organic nitrogen sources like amino acids and vitamins (see Materials and Methods). Johnson et al. (2007) obtained better results in developing protocorms with media containing glutamine and glycine than with media containing inorganic nitrogen sources like potassium nitrate and magnesium nitrate.

Effect of the plant growth regulators BAP and IBA on asymbiotic germination under photoperiod and continuous darkness—The formation of protocorms was enhanced in darkness with some effect of BAP treatments in doses of 0.1 mg·L^{-1} (treatment II-2 in contrast to II-3 with IBA under continuous darkness; Table 5). Stewart and Kane (2006) obtained similar results, determining that germination and protocorm formation are favored by the use of kinetin, zeatin, and BAP in doses of 0.2 mg·L^{-1}. Our results are also supported by the findings of Godo et al. (2010), who reported that the application of BAP and NAA to the medium stimulates the germination of *Calanthe tricarinata* Lindl. Nevertheless, they suggested that the auxins NAA, IBA, and IAA in the culture medium have a

TABLE 6. Statistical analysis of the treatments summarized in Table 5, showing the effect of plant growth regulators and photoperiod on calli induction and asymbiotic germination of *Chloraea crispa* seeds 60, 120, and 180 d after sowing.

	60 d					120 d					180 d				
Variation source	Sum of squares	Degrees of freedom	Medium squares	*F* ratio	*P* value	Sum of squares	Degrees of freedom	Medium squares	*F* ratio	*P* value	Sum of squares	Degrees of freedom	Medium squares	*F* ratio	*P* value
Calli formation															
Photoperiod regime	91	1	91	53	<0.0001	98	1	98	41	<0.0001	181	1	181	167	<0.0001
Plant growth regulator	27	3	9	5	0.0058	178	3	59	24	<0.0001	331	3	110	102	<0.0001
Interaction photoperiod regime × plant growth regulator	65	3	22	13	<0.0001	306	3	102	42	<0.0001	521	3	174	160	<0.0001
Standard error	41	24	2			58	24	2			26	24	1		
Protocorm germination															
Photoperiod regime	276	1	276	442	<0.0001	780	1	780	814	<0.0001	666	1	666	410	<0.0001
Plant growth regulator	25	3	8	14	<0.0001	673	3	224	234	<0.0001	1629	3	543	334	<0.0001
Interaction photoperiod regime × plant growth regulator	25	3	8	14	<0.0001	781	3	260	272	<0.0001	1156	3	385	237	<0.0001
Standard error	15	24	1			23	24	1			39	24	2		

less pronounced effect in promoting the germination of terrestrial orchid seeds, which corresponds with the negative effect of adding 0.1 mg·L^{-1} of IBA to germinate seeds of *C. crispa* found in the current study. However, it must be stressed that the results from the treatment with no growth regulators and dark conditions (II-1, under continuous darkness) was superior up to the final assessment at 180 d, at which point the treatment with BAP (II-2, under continuous darkness) was higher.

Growth regulators are essential to induce orchid germination, but it is a question of whether additional exogenous applications are beneficial. The response of orchid seeds to exogenous hormones varies from genus to genus and even from one species to another (Stewart and Kane, 2006). The promoter effects attributed to exogenous phytohormones in culture media have not been reported before for the genus *Chloraea*. However, studies have been conducted on the effect of hormones such as the cytokinin N6-(2-isopentenyl) adenine (2-iP), BAP, kinetin, and zeatin on the germination of *Habenaria macroceratitis* (Stewart and Kane, 2006) and *Calanthe* sp. (Shin et al., 2011), and on the effect of auxins on the germination of *Masdevallia coccinea* Linden ex Lindl. (Pedroza-Manrique et al., 2010), *Calanthe tricarinata* (Godo et al., 2010), *Calanthe* sp. (Shin et al., 2011), and *Paphiopedilum wardii* Summerh. (Zeng et al., 2012). Although *C. crispa* germinated in light, more embryogenic calli were formed by 60 d in media without any type of growth regulator. This is congruent with García-Gonzáles et al. (2010), who affirmed that some plants can develop under in vitro conditions without any growth regulators. Similarly, Pedroza-Manrique et al. (2010) reported that not all orchid seeds require exogenous growth regulators to germinate, as evidenced in studies that obtained protocorm development in the species *Masdevallia coccinea* in treatments without growth regulators. Araújo et al. (1999) reported longer germination times and higher protocorm yields in media without growth regulators for the species *Cyrtopodium palmifrons* Rchb. f. & Warm. The fact that embryos contain endogenous levels of hormones that permit them to initiate germination could explain why some species do not require the exogenous application of these components to the culture media.

This is the first report of in vitro asymbiotic germination of *C. crispa*, and some considerations should be noted. First, asymbiotic germination of *C. crispa* can be significantly affected by the interaction between the culture medium and the light regime for morphogenic formation of embryogenic calli and embryonic germination. Second, protocorm formation can be achieved from pre-induced calli or directly from the seeds as demonstrated in those treatments with a high number of protocorm-like bodies but low calli formation.

Terrestrial orchids belonging to the *Chloraea* genus in South America are difficult to propagate and are under great pressure in their natural habitats; thus, these first findings will be useful not only for *C. crispa* but also for other species from this genus, without depending on the symbiotic effect of mycorrhizal fungi. Subsequent to this study, it will be necessary to develop micropropagation protocols for in vitro mass production of plants from this orchid species, as well as efficient ex vitro rooting protocols and methods for plant development under nursery conditions. Taken as a whole, these new protocols will help conservation efforts for the species, and will also enable the production of plants for commercial purposes.

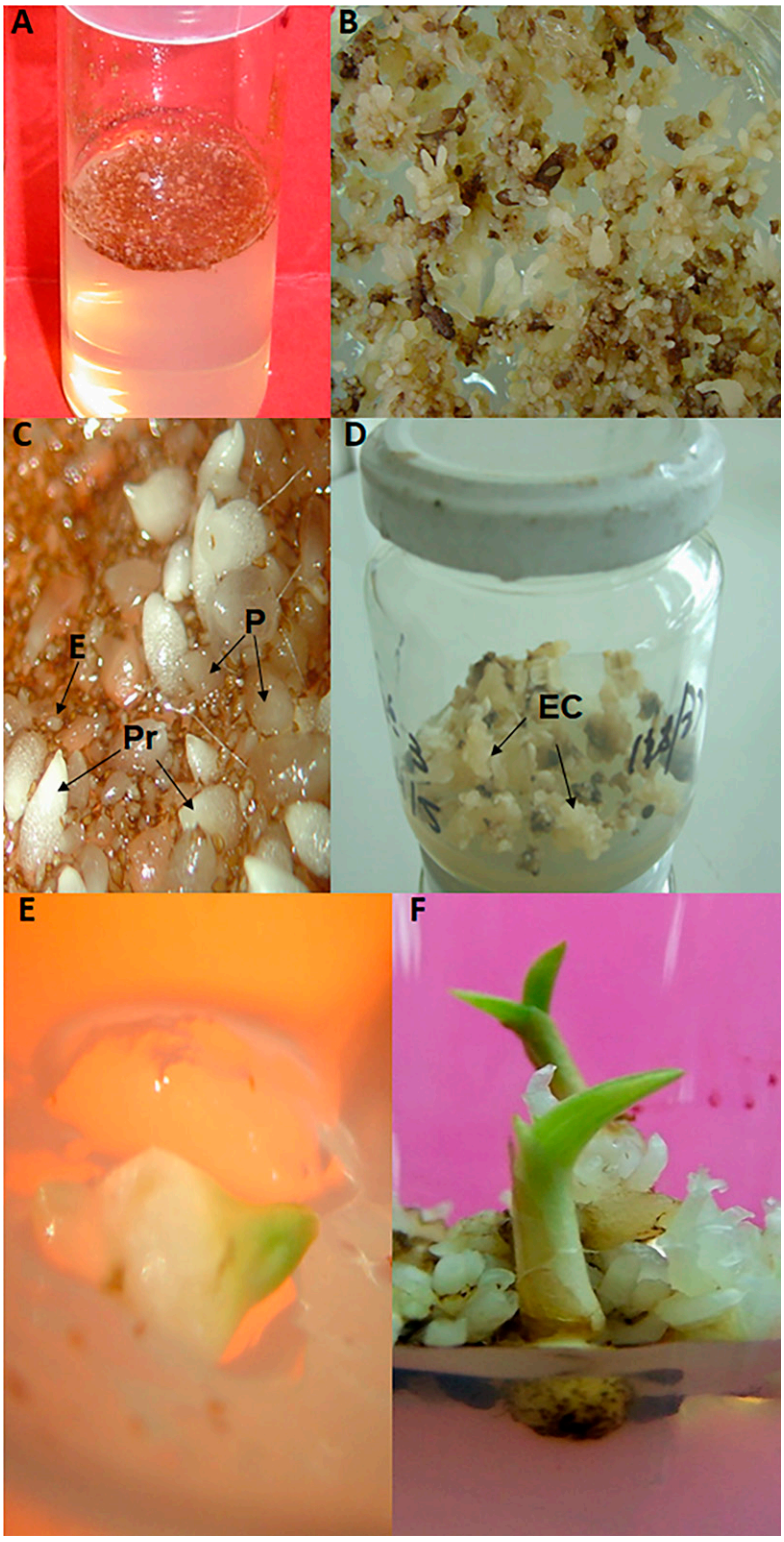

Fig. 1. Developmental stages of *Chloraea crispa* from seeds according the classification of Stewart and Zettler (2002), Stewart et al. (2003), and Johnson and Kane (2007). (A) Stage 0: nongerminated seeds. (B) Stage 1: seed coat rupture and early germination. (C) Stage 2 and Stage 3: embryo growth (E), protomeristem differentiation (P), and protocorm germination (Pr). (D) Stage 3: multiplication of embryogenic calli (EC). (E) Stage 4: differentiation and emergence of the first leaf. (F) Stage 5 and Stage 6: elongation of the first leaf, plant growth, and emergence of the second leaf.

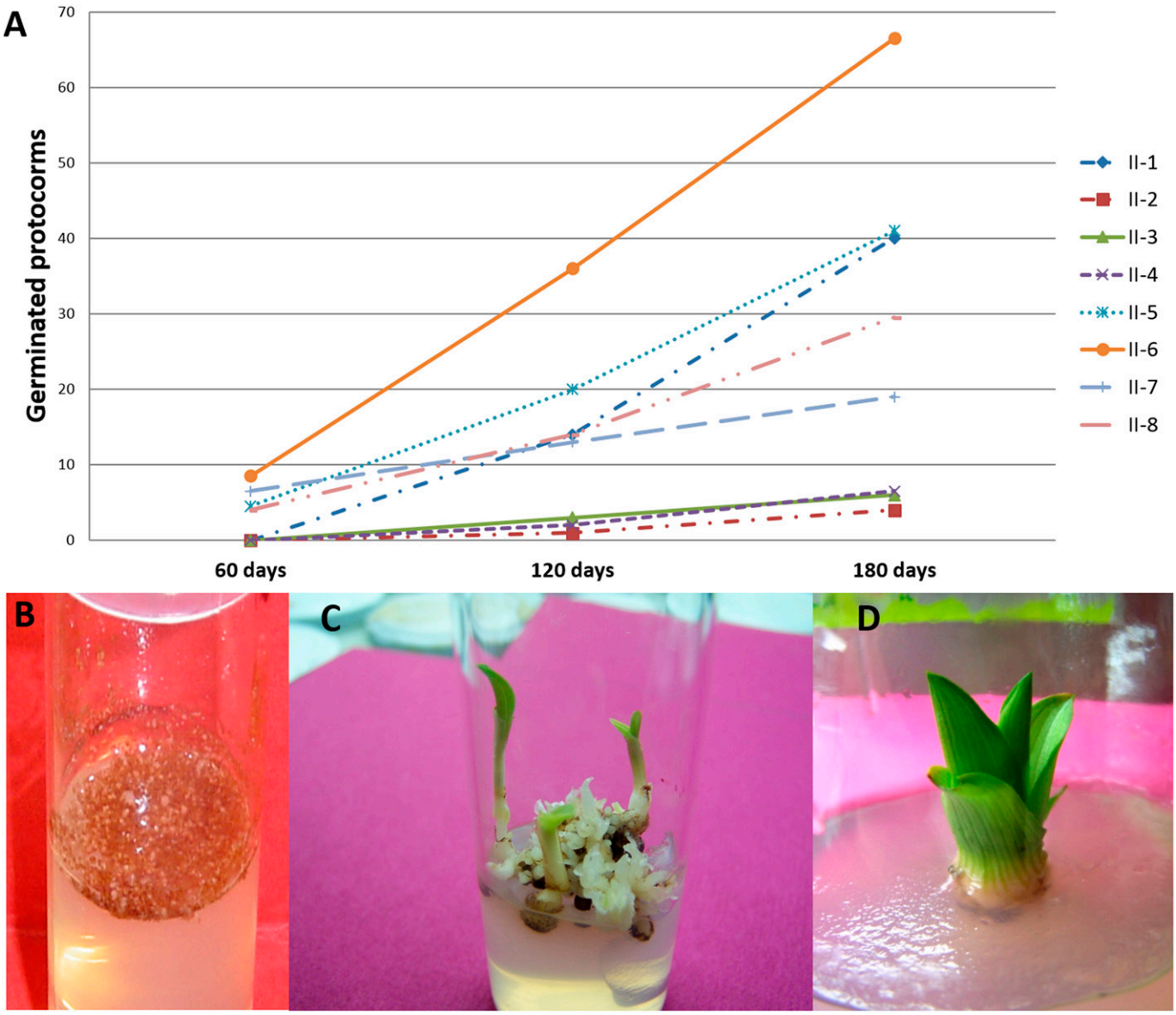

Fig. 2. Effect of plant growth regulators on the asymbiotic germination of *Chloraea crispa*. (A) Cumulative effect of different treatments on protocorm germination at three different evaluation periods. Treatments II-1–4 under photoperiod conditions are represented as II-1, II-2, II-3, and II-4. Treatments II-1–4 under dark conditions are presented as II-5, II-6, II-7, and II-8. (B) Disinfected *C. crispa* seeds three to four weeks after sowing. (C) Protocorm formation 180 d after plating on treatment II-2 (0.1 mg·L^{-1} BAP + 24 h of continuous dark). (D) Individualized from II-2 (0.1 mg·L^{-1} BAP + 24 h of continuous dark) ready for micropropagation.

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