Polibotánica
ISSN electrónico: 2395-9525
polibotanica@gmail.com
Instituto Politécnico Nacional
México
http://www.polibotanica.mx

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Núm. **53**: 135-149 México. Enero 2022
DOI: 10.18387/polibotanica.53.9

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RESUMEN: La deforestación y recolecta ilegal de las orquídeas ha generado la necesidad de plantear alternativas para conservarlas y multiplicarlas. Una orquídea de interés es *Catasetum integerrimum*. cuyo hábitat está en América Central y México. El objetivo de este estudio fue proporcionar métodos para la conservación y uso sustentable de *C. integerrimum*. Se establecieron las condiciones para la germinación asimbiótica de semillas mediante la evaluación de cuatro formulaciones de medios basales: 1) Murashige y Skoog (MS) con macro y micronutrientes y vitaminas; 2) MS con macro y micronutrientes sin vitaminas; 3) Medio basal para orquídeas con carbón activado y polvo de plátano y 4) Medio basal Knudson. Para la conservación *in vitro* del germoplasma se desarrolló un protocolo de crecimiento lento para lo cual se evaluó el efecto de la concentración del medio basal MS (Fuerza iónica completa y a la mitad de su fuerza iónica) y el efecto del sorbitol, manitol y sacarosa a 1, 2 y 3%. Con el fin de establecer un protocolo para la regeneración *in vitro* para la multiplicación de plantas se evaluó el efecto de tres tipos de explante (raíz, nudo, y hoja) y el efecto individual de N6-furfuriladenina (kinetina) a 4.64, 9.29 y 23.2 µM y 6- Bencil Amino Purina (BAP) a 4.43, 8.87 y 22.2 µM. Los resultados mostraron que la germinación asimbiótica de *C. integerrimum* fue exitosa en el medio Murashige y Skoog con macro y micronutrientes sin vitaminas. La conservación *in vitro* fue posible en el medio basal MS a la mitad de su fuerza iónica suplementado con sorbitol al 2%, ya que en este tratamiento las plantas mostraron menor crecimiento después de 6 meses de cultivo. La organogénesis directa de brotes se observó en el explante de nudo en MS suplementado con BAP y en MS con kinetina en todas las concentraciones evaluadas. La organogénesis indirecta de brotes se observó en explante de raíz en MS suplementado con 4.64 o 9.29 µM de kinetina.
Palabras clave: lento crecimiento; kinetina; manitol; sorbitol, conservación a mediano plazo.

ABSTRACT: The deforestation and illegal over-collection of orchids have generated the need to propose alternatives to conserve and multiply them. One orchid of interest is *Catasetum integerrimum*, whose habitat is in Central America and Mexico. This study aimed to provide methods for the conservation and sustainable use of *C. integerrimum*. Asymbiotic seed germination was established by evaluating four formulations of basal media: 1) Murashige and Skoog (MS) with macro and micronutrients and vitamins; 2) MS with macro and micronutrients without vitamins; 3) Basal medium for orchids with activated carbon and banana powder; 4) Knudson basal medium. We developed the slow-growth protocol for which evaluated MS basal medium concentration (full and half of the ionic strength) and the effect of sorbitol, mannitol, and sucrose at 1, 2, and 3%. The influence of three explant types (root, node,

and leaf) and kinetin at 4.64, 9.29, and 23.2 µM and Benzyl Amino Purine (BAP) at 4.43, 8.87, and 22.2 µM were evaluated to establish *in vitro* regeneration for plant multiplication. Asymbiotic germination of *C. integerrimum* was successful on Murashige and Skoog medium with macro and micronutrients without vitamins. *In vitro* conservation was viable in half-strength basal MS medium supplemented with 2% sorbitol; it was the best treatment for conserving seedlings under slow-growth conditions after six months of *in vitro* culture. Node explant formed direct shoot organogenesis in MS with BAP or kinetin at all concentrations evaluated. Indirect shoot organogenesis was observed in root explant in MS supplemented with 4.64 or 9.29 µM kinetin.
Keywords: slow growth; kinetin, mannitol; sorbitol, medium-term conservation

INTRODUCTION

Orchidaceae is one of the largest families of flowering plants (Pérez Gutiérrez, 2010; Noguera-Savelli & Cetzal-Ix, 2014), which are part of complex biological interactions of ecosystems (Seaton *et al*., 2013; Pereira *et al*., 2015). Orchids are also considered an important crop in the floriculture industry. This plant family is usually cultured due to its medicinal properties and uses in traditional food, as well as the generation of nutraceutical products, perfumes, and cosmetics (Hinsley *et al*., 2018; Kanlayavattanakul *et al*., 2018; Salazar-Mercado & Botello-Delgado, 2020; Singh *et al*., 2021). In recent years, many species of orchids have been threatened in their natural habitats because of the environmental pressure generated by humans (Juras *et al*., 2020; Fonseka, 2020; Puspitaningtyas & Handini, 2020). *C. integerrimum is* an orchid that reaches to adult stage in approximately two years. This development stage is less than 50 percent of the time required by most of the not deciduous taxa orchids (Benzing *et al*., 1982). This species lives in Guatemala, Belize, Honduras, El Salvador, Nicaragua and Mexico (Morales-Báez *et al*., 2016). *C. integerrimum* is an ornamental plant whose flowers are very showy and attractive that produce a fragrance compound by pinene (32.52%) and carvone oxide (46.21%) (del Mazo Cancino & Damon, 2007). It is often collected, resulting in a decrease in the population of their natural habitat (Morales-Báez *et al*., 2016). This over-exploitation generates a loss of their population. Moreover, germination in a natural environment entirely depends on the mycorrhizal fungi association (Pérez Gutiérrez, 2010). This association provides water, carbohydrates, minerals, and vitamins for embryos development; however, mortality rates of embryos developed are high despite this association (Kauth *et al*., 2006). Asymbiotic seed propagation, which is an excellent method for the micropropagation of orchids for conservation purposes (Kim *et al*., 2021). Asymbiotic germination of orchid seeds was established by Lewis Knudson (1884–1958), and it was the first practical procedure for *in vitro* propagation of any plant in pure (i.e., axenic) culture (Yam & Arditti, 2009). Since then, many ornamental species of orchids have been micropropagated using this method (Pereira *et al*., 2017; Vudala & Ribas, 2017; Acemi & Özen, 2019; Franceschi *et al*., 2019; Koene *et al*., 2019; Fonseka, 2020; Kang *et al*., 2020; Salazar-Mercado & Botello-Delgado, 2020; Sorgato *et al*., 2020;. Chin *et al*., 2021;Kim *et al*., 2021; Navarro *et al*., 2021; Yeh *et al*., 2021); The asymbiotic germination process generates protocorm-like-bodies (PLBs) which are embryogenic structures (Chin *et al*., 2021). The process goes from stage 0: No germination to stage 5: the differentiation (Yamazaki & Miyoshi, 2006).

Due to the conservation of the germplasm of this species is needed, *in vitro* culture techniques have been successfully applied for conservation. Also, it facilitates the availability of germplasm for use in genetic improvement programs (Arrigoni-Blank *et al*., 2014).

One of the methods used is slow growth, which reduces plant metabolism and consequently decelerates its development, which allows the maintenance of *in vitro* plant collections without the need for subculture (Chauhan *et al*., 2019; Santos Lima *et al*., 2021; Petrova *et al*., 2021). The slow growth procedure is called medium-term conservation or minimal growth storage (Muñoz *et al*., 2019).

The advantage of the slow growth technique is that enabling *in vitro* maintenance of shoot cultures under aseptic conditions by markedly reducing the frequency of periodic subculturing without affecting the viability and regrowth potential of such shoot cultures (Koeda *et al*., 2018). The slow growth can be achieved by basal culture medium modification, sucrose, and plant growth regulators (PGRs) concentration changes (Ruta *et al*., 2020). Other substances used to slow growth are mannitol and sorbitol (da Costa Santos *et al*., 2011; Tavazza *et al*., 2015; Hammond Hammond *et al*., 2019; Muñoz *et al*., 2019; Petrova *et al*., 2021;). Furthermore, reports of slow-growth techniques include protocols for conserved material multiplication (Hammond-Hammond *et al*., 2019; Ramírez-Mosqueda *et al*., 2019; Fonseka, 2020).

The present study describes the asymbiotic germination of *C. integerrimum*, its conservation in a slow-growth medium and the regeneration by direct and indirect adventitious organogenesis.

MATERIAL AND METHODS

Obtaining plant material

Fully closed of *C. integerrimum* from two capsules with good condition were collected from experimental field El Tormento, Campeche, Mexico at the "Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP)". Afterward, the capsules collected were transferred to the laboratory and put inside a desiccator with 1 kg of silica gel at 25±2ºC for five days. Then they were washed with a solution of Extran® at 5% for 5 minutes and rinsed three times with sterile distillate water.

In vitro establishment of plant material

The capsules were disinfected in a laminar flow cabinet by immersion in a solution of sodium hypochlorite at 1% with 10 µL of Extran® for 10 min, then were rinsed three times with distilled water, afterward were atomized with 70% of ethanol and flamed with a burner. The capsules sterilized were longitudinally cut with a scalpel to obtain the seeds, which were put on a sterile Petri dish. Finally, 300 seeds were cultured in Magenta® boxes with treatments for asymbiotic germination.

Asymbiotic germination

Semi-solid media of MS medium with macro-micro nutrients and vitamins (M5519 SIGMA®), MS medium with macro-micro nutrients (M5524 SIGMA®), Orchid Medium with charcoal and Banana Powder (P1056 SIGMA®), and Knudson C (K4003 SIGMA®) were used to investigate the asymbiotic germination of *C. integerrimum* without PGRs. The pH of the media was adjusted at 5.8 before sterilizing in an autoclave at 121°C. The treatments were dosed at 25 mL per Magenta® box, and seeds were established in three Magenta® boxes per treatment. The evaluation was carried out every 4 weeks for 16 weeks, recording data and digital pictures.

Slow growth for *in vitro* conservation

Plantlets of 2 cm from asymbiotic germination were established in a factorial experiment of 2 x 3 x 3 for conservation. The first factor was the concentration of basal medium (Full and half ionic strength), the second factor was the carbon source (sorbitol, mannitol, and sucrose), and the third factor was the concentration of carbon source (1, 2, and 3%). The experiment was carried out with two MS basal medium control: half-strength MS basal medium (2.2 g L^{-1}) and full-strength MS basal medium (4.4 g L^{-1}) without carbon source. In total were evaluated 18 treatments with two controls and three replicates per treatment. Treatments with half-strength MS basal medium were solidified with 3.1 g L^{-1} of Gelrite® and the treatments with full strength MS basal medium with 2.2 g L^{-1} of Gelrite®. The variables evaluated were the plantlet growth and the shoots number formed per treatment. Plant growth was obtained by subtracting the plantlet length (mm) at six months of culture from plantlet length at the beginning of

culture. The result was the plantlet growing after six months of culture. The shoots number formed per treatment was recorded at 12 months.

Plant regeneration

Induction of regeneration was evaluated in a factorial design of 3 x 2 x 3, which the first factor was the explant source (node, leaf, and root), the second factor was kinetin or BAP, and the third factor PGR concentration, kinetin at 4.64, 9.29 and 23.2 µM, and BAP at 4.43, 8.87 and 22.2 µM. The experiment had 18 treatments and one control with three replicates per treatment. The control was the MS basal medium without PGRs. All treatments were formulated with full strength MS basal medium added with 3% sucrose and 2.2g L^{-1} of Gelrite®. The variable evaluated was shoot number per explant at the 60 days, and shoots were allowed to grow until 120 days of culture. All treatments were supplemented with 2 g L^{-1} of activated carbon.

***In vitro* culture conditions**

All treatments were incubated at 23±2°C and relative humidity of 60% with a photoperiod of 16/8 hours (light/darkness); the light source was provided by LED lamps with a light intensity of 60 $\mu mol^{-2}s^{-1}$.

Statistical analysis

Data were subjected to one-way ANOVA analysis of variance for comparison of means. Significant differences were calculated according to the Tukey test at the 5% significance level using a statistical Statgraphics® Centurion XVI statistical software. Data were presented as means ± standard error.

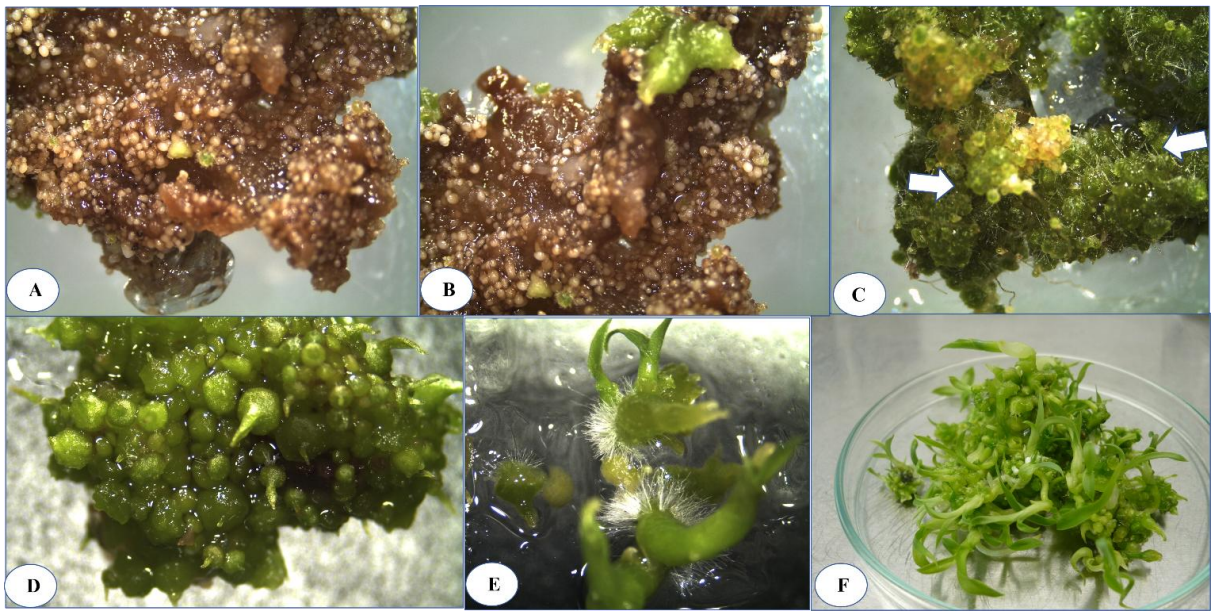
RESULTS

Asymbiotic germination

Asymbiotic germination of seeds of *C. integerrimum* occurred in basal MS medium with macro and micronutrients without vitamins, 250 plantlets were obtained. At four weeks after sowing, the seeds formed a brown friable callous mass jelly-like, from which white embryogenic structures emerged (WES) (fig. 1 A). At six weeks, the WES began to grow into PLBs that turned green. (fig. 1 B). Root formation in PLBs (fig. 1 C) and shoot differentiation into PLBs from protocorms (fig. 1 D) occurred at 8 and 10 weeks, respectively. The plantlets formed rootlets and leaves at 12 weeks (fig. 1 E). The same basal medium where germination occurred completed growth up to 16 weeks and reseeding was unnecessary during this process (fig. 1 F). *C. integerrimum* seeds sown on basal MS media with macro and micronutrients and vitamins, Orchid Medium with Charcoal and Banana Powder and Knudson had no germination response.

Slow growth for *in vitro* conservation

There was significant difference among the 20 treatments selected to evaluate the growth of *C. integerrimum* plantlets (Table 1). T3 allows the highest growth value (76.24±30.5) followed by T1 (54.74±7.6), T5 (52.69±20.1), T4 (48.32±15.8), and T2 (37.39 ±3.7) (Table 1, fig. 1). The treatments with the lowest growth values of cultivated plantlets were T19 (8.54±5.2), T20 (13.10±5.3), T17 (14.18±6.1), T18 (17.70±5.8), T16 (18.61±4.0), T10 (19.71±2.6) and T11 (21.07±8.6), there was no significant difference among these seven treatments (Table 1), the controls T19 and T20 showed the lowest growth values, the leaves of the plantlets showed etiolation and died. Treatment T17 was selected because it presented the lowest value of growth in plantlet length among the other treatments. The plantlets from T17 did not show changes in plant morphology. At six months the shoot formation in the slow growth treatments was evaluated. The treatment T3 (Table 1) formed the highest number of shoots (28.4± 3.2). On the other hand, T17 showed the lowest shoot formation (1±00).



Fig, 1. Asymbiotic seed germination of *C. integerrimum*. Callus with embryogenic structures (A); Embryogenic structures transforming into PLBs (B); Rhizoids formed on the protocorm (white arrows) (C); Shoots differentiated from the protocorm (D); Plantlets formed 3 months after sowing seeds (E); Fully developed plantlets at four months of age (F).

Table 1. Effect of culture medium composition on plantlets growth and shoot proliferation of *C. integerrimum* Hook. to determine the best treatment for *in vitro* conservation

Treatment	MS concentration (%)	Carbon source	Carbon concentration (%)	Average of plantlet length (mm)	No. Shoot per treatment
1	100	Sucrose	1	54.74 ± 7.6^{h}	9.20 ± 2.2^{c}
2			2	37.39 ± 3.7^{fg}	17.60 ± 2.7^{e}
3			3	76.24 ± 30.5^{j}	28.40 ± 3.2^{f}
4	50	Sucrose	1	48.32 ± 15.8^{gh}	12.60 ± 1.9^{cd}
5			2	52.69 ± 20.1^{h}	14.40 ± 2.3^{de}
6			3	26.84 ± 8.8^{cdef}	5.40 ± 1.3^{b}
7	100	Mannitol	1	31.95 ± 13.1^{ef}	3.40 ± 1.1^{ab}
8			2	32.26 ± 6.0^{ef}	2.60 ± 0.8^{ab}
9			3	24.23 ± 4.5^{bcdef}	2.20 ± 0.4^{ab}
10	50	Mannitol	1	19.71 ± 2.6^{abcde}	2.40 ± 0.5^{ab}
11			2	21.07 ± 8.6^{abcde}	3.40 ± 1.1^{ab}
12			3	31.57 ± 7.4^{ef}	3.20 ± 1.7^{ab}
13	100	Sorbitol	1	29.31 ± 5.1^{def}	2.60 ± 0.8^{ab}
14			2	30.14 ± 3.98^{def}	2.80 ± 1.3^{ab}
15			3	27.51 ± 8.7^{cdef}	2.60 ± 0.5^{ab}
16	50	Sorbitol	1	18.61 ± 4.0^{abcde}	2.60 ± 1.8^{ab}
17			2	14.18 ± 6.1^{abc}	1.00 ± 0.0^{ab}
18			3	17.70 ± 5.8^{abcd}	3.20 ± 1.3^{b}
19	100	None	0	8.540 ± 5.2^{a}	0.00 ± 0.0^{a}
20	50	None	0	13.10 ± 5.3^{ab}	0.00 ± 0.0^{a}

Different letters indicate significant statistical differences according Tukey test ($P \leq 0.05$). MS: Murashige and Skoog basal medium.

Plant regeneration

The type of explant influenced shoot formation (Table 2). The leaf explant did not show regeneration. Shoot induction from node explant of *C. integerrimum* via direct organogenesis occurred at 30 days (fig. 3 A) on treatments T2-T7. There was no significant statistical difference among treatments; T5, T16, and T17 showed 3.0 adventitious shoots per explant, followed by T3 with 2.5 shoots (Table 2). Rooting of shoots was observed in the same induction medium; the plantlets morphology at 60 and 120 days was normal (fig. 3 B-C).

The root explant formed callus after 30 days of cultivation in full strength MS basal medium supplemented with kinetin at 4.64 or 9.29 µM (fig. 4 A). Indirect shoot organogenesis at 60 days of culture (fig. 4 B), the rooting of shoots occurred in the same induction medium. Plantlets showed an average height of 8 cm after 120 days of cultivation. (fig. 4 C). BAP did not induce organogenesis in root explant (Table 2).

Table 2. Effect of PGR and type of explant on the induction of adventitious organogenesis of *C. integerrimum* Hook.

Treatment	Explant	PGR	PGR Concentration (μM)	# adventitious shoots/explant
1	Node	None	0.00	0.00±0.00[a]
2		Kinetin	4.64	2.25±1.20[b]
3			9.29	2.50±1.20[b]
4			23.2	2.25±1.50[b]
5		BAP	4.43	3.00±0.81[b]
6			8.87	2.00±1.15[b]
7			22.2	1.75±0.50[b]
8	Leaf	None	0.00	0.00±0.00[a]
9		Kinetin	4.64	0.00±0.00[a]
10			9.29	0.00±0.00[a]
11			23.2	0.00±0.00[a]
12		BAP	4.43	0.00±0.00[a]
13			8.87	0.00±0.00[a]
14			22.2	0.00±0.00[a]
15	Root	None	0.00	0.00±0.00[a]
16		Kinetin	4.64	3.00±0.81[b]
17			9.29	3.00±0.00[b]
18			23.2	0.00±0.00[a]
19		BAP	4.43	0.00±0.00[a]
20			8.87	0.00±0.00[a]
21			22.2	0.00±0.00[a]

Different letters indicate significant statistical differences according Tukey test ($P \leq 0.05$). PGR: Plant Growth Regulator, BAP: Benzyl Amino Purine. MS: Murashige and Skoog basal medium.

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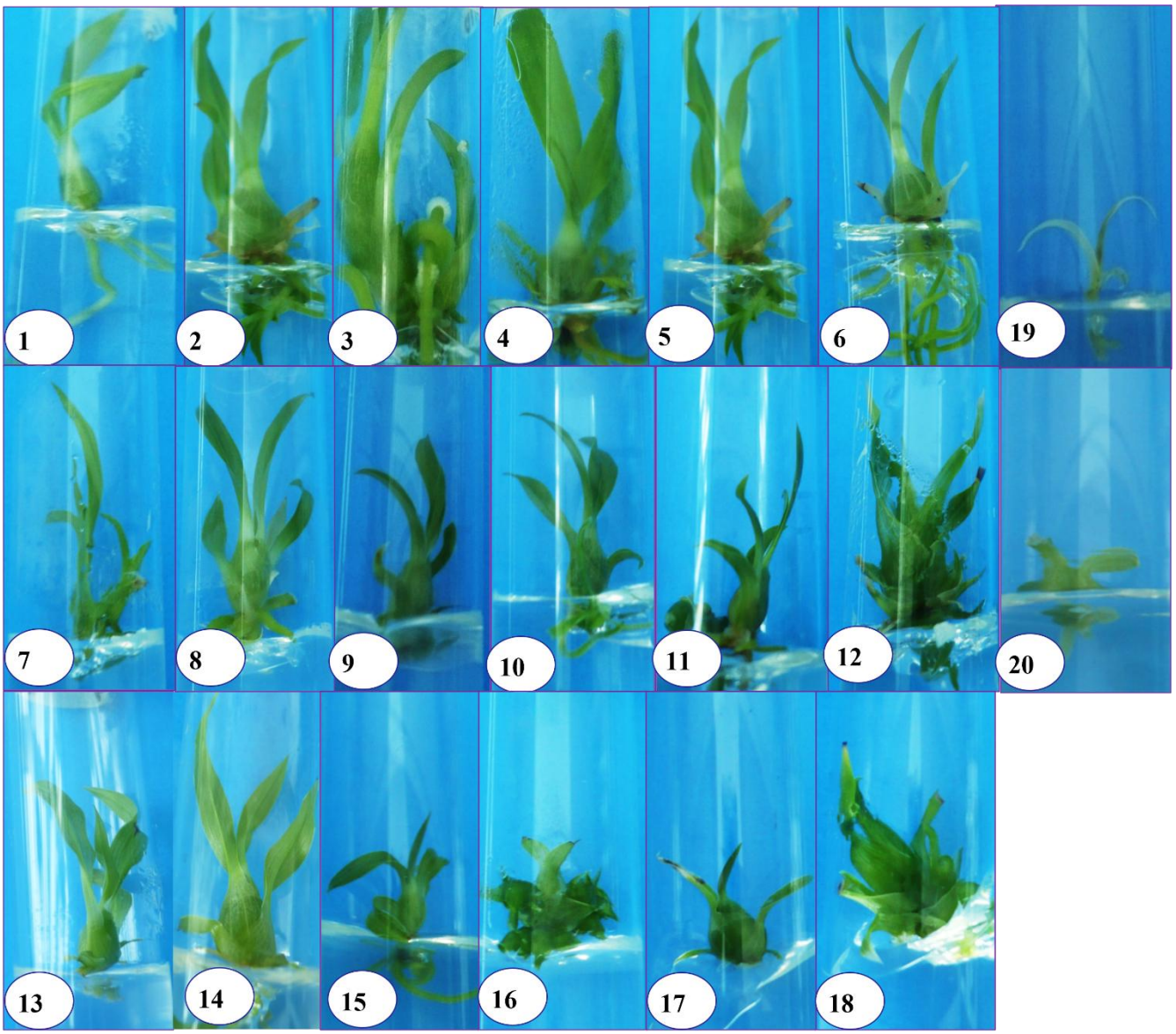

Fig. 2. *In vitro* conservation of *C. integerrimum* growth after 6 months of cultivation in treatments evaluated for its conservation. Full strength MS basal medium supplemented with sucrose 1, 2, and 3%: (1), (2), and (3) respectively. Half strength MS basal medium with sucrose 1, 2, and 3%: (4), (5), and (6) respectively. Full strength MS basal medium supplemented with mannitol 1, 2, and 3%: (7), (8), and (9) respectively. Half strength MS basal medium with mannitol 1, 2, and 3%: (10), (11), and (12) respectively. Full strength MS basal medium supplemented with sorbitol 1, 2, and 3%: (13), (14), and (15) respectively. Half strength MS basal medium supplemented with sorbitol 1, 2, and 3%: (16), (17), and (18) respectively. Controls: half-strength MS basal medium without carbon source (19) and full-strength MS basal medium without carbon source (20).

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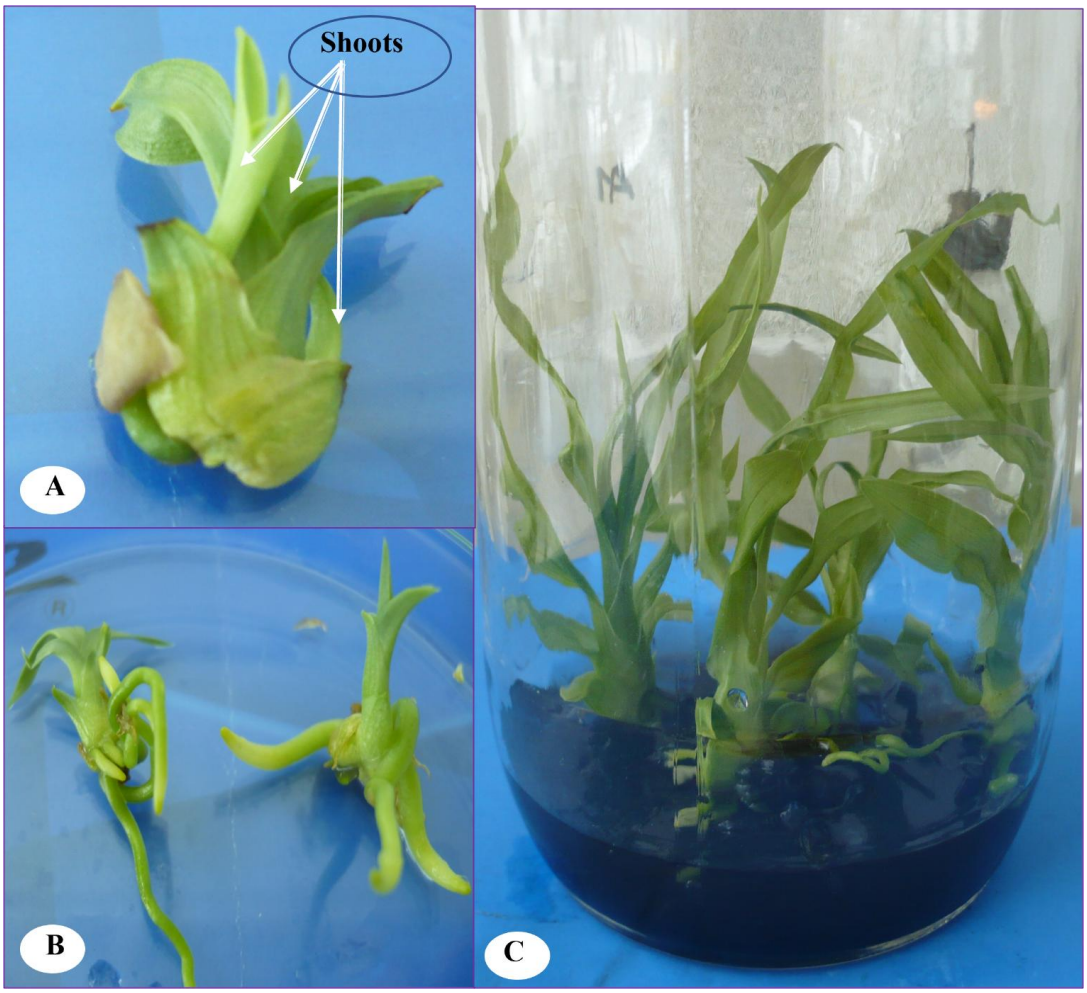

Fig. 3. Direct adventitious organogenesis from node explant of *C. integerrimum.* Adventitious shoots from node explant on full strength MS basal medium+ BAP at 4.43 µM, 30 days culture (A); Plantlets at 60 days of culture (B); Plantlets at 120 days of culture (C).

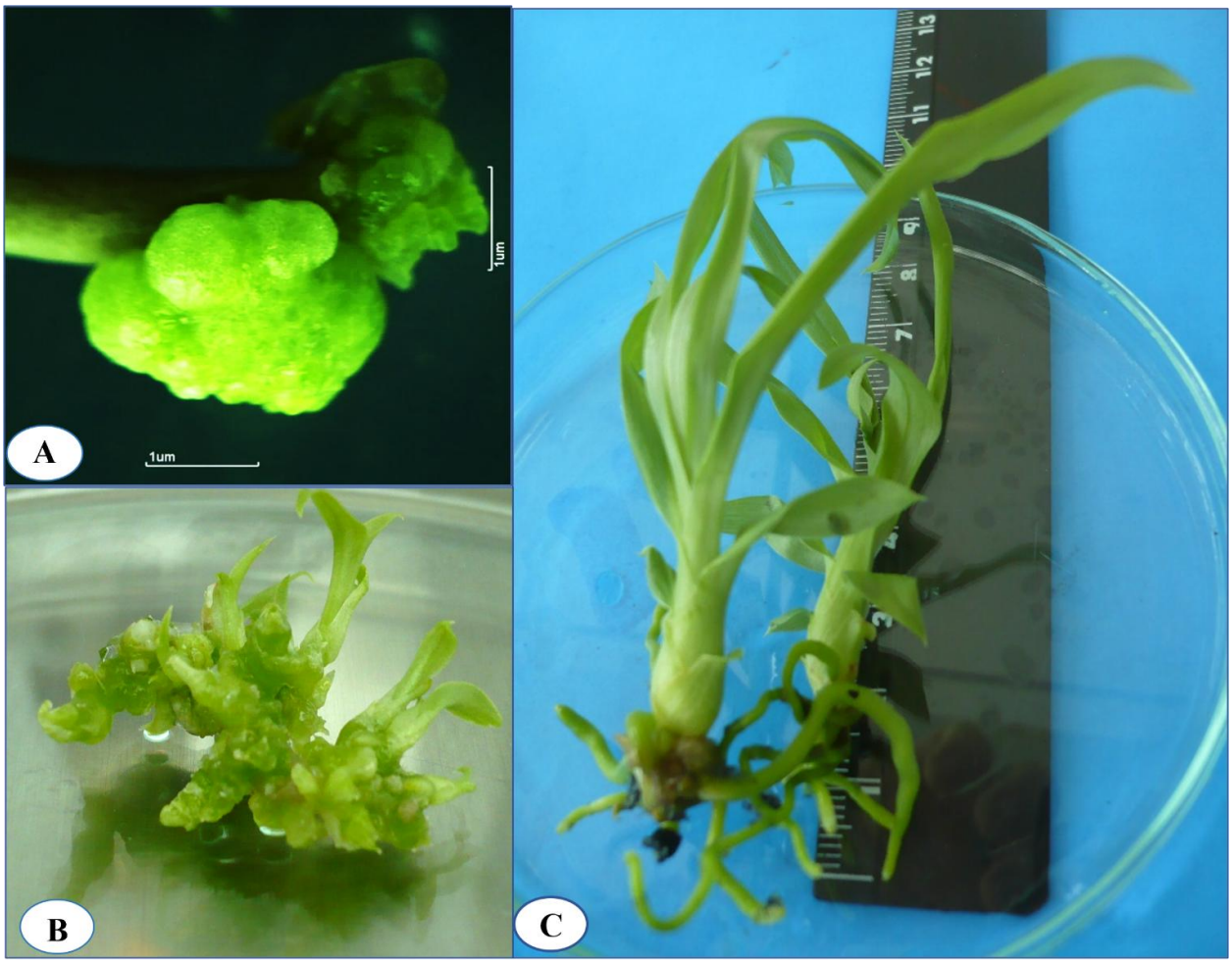

Fig. 4. Indirect adventitious organogenesis from root explant of *C. integerrimum*. Callus formed in root explant at 30 days of culture (A); Adventitious shoots formed on the callus at 60 days of culture (B); Plantlets at 120 days of culture (C).

DISCUSSION

Tissue culture methods are an alternative for help in the rescue of species endangered by deforestation and unmeasured extraction from their natural habitat. This study presents asymbiotic germination, conservation, and *in vitro* regeneration of *C. integerrimum* that have not been previously reported.

The germination of orchid seeds in their natural habitat is complex and inefficient because there must be a symbiotic relationship with a fungus. Association between orchids and fungi was observed in 1824 (Yam & Arditti, 2009). On the other hand, *in vitro* germination is efficient when the composition of the culture medium is known.

According to the classification described by (Yam & Arditti, 2009), the stages of "pre-germination," "germination," and "protocorms formation" corresponding to stages 1-3 were not visible in this study. This observation could be due to the seeds cultivated on MS basal medium with macro and micronutrients without vitamins formed callus. Furthermore, white embryogenic structures formed from the callus, becoming PLBs, the stages 4 and 5 were clearly

visible. Full strength MS basal medium including all macro and micronutrients, without vitamins, was the only one of the four media evaluated that gave a response to asymbiotic germination of *C. integerrimum*. Unlike reports of asymbiotic germination using MS medium with half of the macro and micronutrients (Koene *et al*., 2019; Kang *et al*., 2020; Yeh *et al*., 2021; Navarro *et al*., 2021; Kim *et al*., 2021). *In vitro* culture conditions and the lack of vitamins in the MS basal medium may have generated stress in the *C. integerrimum* seed culture, triggering a signaling cascade for dedifferentiation leading to callus formation. It is going by concepts reported by Fehér (2014) that differentiated plant cells, under certain circumstances, can revert to an earlier developmental state and regain totipotency.

Mineral nutrition, vitamins, and medium composition influence asymbiotic seed germination have been reported (Salazar-Mercado & Botello-Delgado, 2020; Kim *et al*., 2021). Other formulations have been necessary, for instance: New Dogashima medium was used for germination of mature seeds of *Calanthe tricarinata* (Godo *et al*., 2010) or Woody plant medium (WPM) (Vudala & Ribas, 2017; Franceschi *et al*., 2019); Vacin and Went medium (VW) (Pereira *et al*., 2017; Utami & Hariyanto, 2019; Acemi & Özen, 2019). New Dogashima medium has microelements and organic compounds of Nitsch and Morel respectively, NH_4NO_3 and KNO_3 are in minor amount in comparison with MS, this media was developed for micropropagation of orchids (Tokuhara *et al*., 1993). On the other hand, Woody Plant Medium contains the macro-micronutrients and vitamins described by Phillips & Garda (2019).

In this study *C. integerrimum* exogen PGRs were not necessary during the asymbiotic germination and plantlet development contrary to other protocols reported, asymbiotic germination of *Calanthe tricarinata* Lindl occurred using the basal medium New Dogashima with BAP or α-naphthalene acetic acid (NAA) (Godo *et al*., 2010); On the other hand, the germination of *C. integerrimum* was achieved using full-strength MS basal medium added with 2% BAP; in the same study the protocorms development was necessary to use full strength MS basal medium added with 5 mg L^{-1} of activated charcoal and indole acetic acid (IAA) at 5 mg L^{-1} (Velázquez Kú *et al*., 2016).

The conservation protocols for *Dendrobium draconis*, *Bulbophyllum auricomum* Lindl., *Ullucus tuberous* species of the subfamily Epidendroideae, *Arnica montana* L. used MS reduced to half its ionic strength as basal medium (Than, 2013; Hammond Hammond *et al*., 2019; Menezes-Sá *et al*., 2019; Petrova *et al*., 2021).

Other basal media used in preservation protocols include Vacin and Went (Puspitaningtyas & Handini, 2020). The woody plant medium (WPM) (Matos Alvim *et al*., 2020). Culture media for slow growth can use sucrose as a carbon source (Than, 2013; Sanghamitra *et al*., 2019; Menezes-Sá *et al*., 2019; El-Hawaz *et al*., 2019; Matos Alvim *et al*., 2020; Gomes *et al*., 2021). Another modification made to the culture medium is sorbitol and mannitol as carbon sources. The use of mannitol on slow growth protocols has been reported for Globe artichoke, *Ullucus tuberosus* (Tavazza *et al*., 2015; Hammond Hammond *et al*., 2019). On the other hand, the combined effect of mannitol and sucrose allowed the conservation of *Amburana cearensis* plants with better characteristics than using sucrose alone (Matos Alvim *et al*., 2020). The results showed that *C. integerrimum* plantlets have higher growth in treatments T1-T5 supplemented with sucrose, even inducing multiple sprouting, so they are recommended for propagation but not for slow growth. T17 treatment formulated with half-strength MS basal medium and 2% sorbitol was selected for *in vitro* conservation of *C. integerrimum*. The use of sorbitol to slow growth rate has been reported in *Hancornia speciosa* Gomes; *Arnica montana* L., *Solanum tuberosum* (da Costa Santos *et al*., 2011; Muñoz *et al*., 2019; Petrova *et al*., 2021);. The combination of sorbitol with sucrose preserves *in vitro* plants of *Solanum tuberosum* without phenotypic abnormalities (Nasiruddin & Rafiul Islam, 2018). The combination of sorbitol with sucrose and mannitol with sucrose allows slow growth storage of potato germplasm (Muñoz *et al*., 2019).

In this study, kinetin induced the regeneration of adventitious shoots from root explants, and BAP induced a similar response from node explants. Kinetin induces callus (clusters of dedifferentiated plant cells) to differentiate into adventitious buds (Barciszewski *et al*., 2007; Sianipar & Mariska, 2020; Sharma *et al*., 2021).

CONCLUSIONS

Asymbiotic germination, *in vitro* conservation, and adventitious shoots regeneration of *C. integerrimum* are methods effective and efficient. It is also important to highlight that the *in vitro* conservation protocol has been so effective that it has maintained this species *in vitro* conservation for ten years. The asymbiotic germination method and the regeneration method can be used for propagation, helping to reduce the collection of this species in its habitat.

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Recibido:
3/agosto/2021

Aceptado:
12/enero/2022

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