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Asymbiotic seed germination and *in vitro* propagation of *Cattleya trianae* Linden & Reichb.f. (Orchidaceae)

Germinación asimbiótica de semillas y propagación *in vitro* de *Cattleya trianae* Linden y Reichb.f. (Orchidaceae)

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Abstract

Cattleya trianae (Linden & Reichb.f., 1860), Colombian national flower, is in danger of extinction due to the destruction of its natural habitats and excessive collection for horticultural purposes. Therefore, *in vitro* culture is a tool for the conservation of endangered species. In this study we determined the most suitable culture medium for asimbytic seed germination and *in vitro* propagation of *C. trianae*. Initially, mature capsules were collected, the seeds were subsequently disinfected and seeded with the syringe method (Vendrame *et al.*, 2007), to evaluate the effect of five media on the development of *C. trianae* after 20 weeks. The seedlings were transplanted and acclimated using different substrates. The best percentage (54.2%) of seedling formation after 20 weeks was found in MS + JP medium with significant differences (P <0.05: *Tukey HSD*). In this research, it is reported that the addition of organic additives to the MS medium improves the efficacy of this, and therefore, allows a greater growth and development of *C. trianae* under *in vitro* conditions.

Key words*:* Organic additives; coconut water; Pineapple juice; *in vitro* propagation.

Resumen

Cattleya trianae (Linden y Reichb.f., 1860) flor nacional de Colombia, se encuentra en peligro de extinción, debido a la destrucción de sus hábitats naturales y a la recolección excesiva con fines comerciales y hortícolas. Por lo tanto, el cultivo *in vitro* es una herramienta para la conservación de especies amenazadas. En este estudio se determinó el medio de cultivo más adecuado para la germinación asimbiótica de semillas y propagación *in vitro* de *C. trianae*. Inicialmente, se colectaron cápsulas maduras, posteriormente, las semillas se desinfectaron y se sembraron con el método de la jeringuilla (Vendrame *et al.,* 2007), para evaluar el efecto de cinco medios en el desarrollo de *C. trianae* después de 20 semanas. Las plántulas se trasplantaron y aclimataron utilizando diferentes sustratos (corteza de pino triturada, arcilla expandida, carbón vegetal y fibra de coco). El mejor porcentaje (54.2%) de formación de plántulas después de 20 semanas se encontró en el medio de cultivo MS+JP con diferencias significativas (*P<0.05: Tukey HSD).* En esta investigación se reporta que la adición de aditivos orgánicos al medio MS mejora la eficacia de éste, y por lo tanto, permite un mayor crecimiento y desarrollo de *C. trianae* bajo condiciones *in vitro.*

Palabras clave : Aditivos orgánicos; Agua de coco; Jugo de piña; Propagación *in vitro.*

Introduction

Orchidaceae family is one of the most diverse groups and with a high risk of extinction of the Plantae kingdom (Salazar & Gélvez, 2015; Zhang, Yan, Tian, Li & He, 2015), comprises about 25000 species integrated in 880 genera. In Colombia, there are approximately 4010 species and 260 genera (Mejía & Pino, 2010). *Cattleya trianae i*s classified as the national flower, according to the concept issued by the Colombian Academy of History in 1936. It is endemic to Colombia, grows epiphyte or litophyte, usually on tall caracolí (*Anacardium excelsum* (Bertero ex Kunth) Skeels) trees, walnut (*Cordia alliodora* (Ruiz & Pav.) Oken), hobo (*Spondias mombin* L.), ceiba (*Ceiba pentandra* (L.) Gaertn.) and purple oak (*Tabebuia rosea* (Bertol.) Bertero ex A.DC.).

It is known that is found in the Colombian departments of Cundinamarca, Tolima and Huila at an altitude of 1000 to 1800 m.a.s.l. (Calderón, 2007). The plant has large flowers with great ornamental and economic value (Galdiano *et al.*, 2012). *C. trianae* is in the global category of the International Union for Conservation of Nature (IUCN) putting this species at risk of becoming endangered according to the red book of Colombian plants (Calderón, 2007). Due mainly to a quality habitat deterioration, therefore, many plants of this species have been illegally collected from wild populations, which can be translated into species damaging for more than 150 years and have allowed a decreasing of some subpopulations in their natural environment. In addition, orchid seeds needs special conditions to have achieved germination in their habitat, which leads to a symbiotic relationship with a mycorrhizal fungus (Chen, Goodale, Fan & Gao, 2015).

In vitro propagation is a technique that results in an important alternative for the conservation of endangered orchid species (Bhattacharyya, 2017; Chen, Goodale, Fan & Gao, 2015; Salazar, 2012). Several authors have achieved asimbiotic germination using different simple means with minerals, vitamins, hormones and sugars (Kauth *et al.*, 2011; Arditti & Ghani, 2000; Knudson, 1946). Likewise, cultures have been improved supplementing the media with organic components (Gallo *et al.*, 2016; Salazar *et al.*, 2013; Salazar & Cancino, 2012). The present research aims to determine the most suitable culture medium for asymbiotic seed germination and *in vitro* propagation of *C. trianae* for the species conservation.

Materials and methods

Plant material

Mature capsules of *C. trianae* were collected 8 months after being manually pollinated under greenhouse conditions in the municipality of Bochamela, Norte de Santander, Colombia (Figures 1A, B, C).

To identify mature capsules, was observed the transition from green to yellow. Subsequently, the mature seeds were extracted from capsules, stored for 5 days in kraft paper sachets, and in glass vials, preserved with a desiccating agent containing silica gel (to avoid excessive moisture) at temperature of 4°C (Salazar, 2012).

Figure 1. *in vitro* propagation procedure of *Cattleya trianae*. (A) *C. trianae* inflorescence. (B) Green capsule of *C. trianae*. (C) Mature capsule of *C. trianae*. (D) Protocorm. (E),(F) Adapted seedlings. (G) Development of *C. trianae*.

Sterilization and seed sowing

The syringe method described by Vendrame *et al.* (2007), was used for seeds disinfection and planting. A small portion of seeds was placed in a 5 ml sterile syringe with a cloth filter. In fact, seeds were immersed for 30 seconds in 70% ethanol, followed by immersion in 0.75% (v/v) in sodium hypochlorite solution (NaOCl) at 1.0 % plus 0.1% Tween 20® (surfactant), for 5 minutes under constant rinsing. Subsequently, five washes were performed with sterile deionized water; the filter was withdrawn from the syringe to perform the seeding in a laminar flow chamber. Therefore, 100 seeds per species were grown in petri dishes, containing 25 mL of culture medium.

Media and culture conditions

The basal culture medium was MS using 100% macro and micronutrient concentrations with 3000 $mg.L^{-1}$ Saccharose, 700 $mg.L^{-1}$ agar, 100 $mg.L^{-1}$ Myo-inositol and 1000 $mg.L^{-1}$ activated carbon. Five basal media were tested in this study: basal MS media as control, MS supplemented with pineapple juice (MS + JP), MS with coconut water (MS + AC), MS plus 0.5 $mg.L^{-1}$ indoleacetic acid (AIA) and MS with 0.5 $mg.L^{-1}$ gibberellic acid (MS + GA_3). Media with organic supplements were prepared by adding 200 $mL.L^{-1}$ (20%) of coconut water and pineapple juice, respectively. With a pH of 6.0, it was sterilized at 15 pounds of pressure (Psi) at 121°C for 20 minutes. The culture media were incubated under controlled environmental conditions (23 ± 2°C, photoperiod 16 hours light and 8 hours darkness, with a light intensity of 25-µmol $m^{-2}s^{-1}$, given by fluorescent light and 60% relative humidity).

Transplanting and acclimatization

Six months later, seedlings of *in vitro* culture were taken, with roots and more than two leaves (seedlings more than 3 cm in length), then the seedlings were transplanted in polystyrene containers with a pine bark substrate (1: 1), bark substrate with carbon (CC; 1: 1), pine bark and coconut fiber (CF; 1: 1) and single expanded clay (AA). Therefore, seedlings were placed in transparent plastic boxes to perform the acclimatization process, gradually decreasing relative humidity for 30 days after transplanting, under greenhouse conditions at a temperature of 25 ± 2°C. The survival percentage in acclimatization for three months after transplanting was recorded.

Experimental design and statistical analysis

A completely randomized 6 x 5 factorial analysis (six developmental stages and five culture media) was performed with 5 replicates (each with 100 seeds). All data were statistically analyzed by analysis of variance (ANOVA). Therefore, Means were compared by Tukey's HSD (Honest Significant Difference) test to determine significant differences at a level of $P<0.05$.

Seed germination process to seedling formation was evaluated at 20 weeks of planting, through the development stages of orchids adapted by Johnson & Kane, 2007 (Table 1). Statgraphic Centurion® software version 16 was used for the statistical analysis.

Table 1. Development phases of orchid seeds

Phase	Description
0	Seeds with embryos did not germinate
1	The embryo expands, capsule rupture (= Germination).
2	Appearance of the proto-corm and rhizoids
3	Emergence and appearance of the first leaf.
4	One leaf and one or more root presence.
5	Presence of two or more leaves, present root (= seedling).

Source: Adapted from: Johnson & Kane (2007).

The survival percentage in acclimatization for three months after transplantation was recorded with a completely randomized experimental design, with five replicates and 10 seedlings per substrate.

Results

The germination process started when the expansion and capsule (15 days) rupture were visualized, with an average of 95% (phase 1; Tables 1, 2). The highest percentage was found in MS + GA_3 culture medium, with no significant differences ($P<0.05$: *Tukey HSD*; Table 2).

Table 2. Effect of the culture medium on the germination percentage and protocorms formation of *C. trianae*

Culture media	Germination percentage	Protocorms formation
MS	94^a	50.6^a
MS+JP	95.6^a	6.4^b
MS+AC	94.2^a	9.6^b
MS+AIA	94.4^a	27.4^c
MS+GA_3	96.4^a	29.4^c

The means values with different letter of each column indicate statistically significant differences, according to Tukey HSD test ($P \leq 0.05$).

In phase two, the highest protocorms percentage was found in the MS culture medium (Tables 2,3; Figure 1D).

Table 3. Effect of the culture medium on germination and seedling formation of *C. trianae* culture at 20 weeks.

	Effect of the culture medium					
Medios	**F0**	**F1**	**F2**	**F3**	**F4**	**F5**
MS	6.0[a]	15.8[a]	50.6 [a]	27.6[a]	0[a]	0[a]
MS+AC	5.8[a]	8.4[a],b	9.6b	23.2[a]	26.0b	27.0b
MS+JP	4.4[a]	5.6b	6.4b	10.4b	19.0b	54.2c
MS+AIA	5.6	41.8d	27.4c	20.8[a]b	4.4[a]	0[a]
MS+GA_3	3.6[a]	15.4[a]c	29.4c	30.0[a]	21.6b	0[a]

The means values with different letter of each column indicate statistically significant differences, according to Tukey HSD test ($P \leq 0.05$).

In the appearance and development of the first leaf (phase 3), the MS + JP culture medium (10.4%; Table 3) was observed with a lower percentage. Therefore, means which had the presence of the initial root (phase 4), were higher to lower percentage MS + AC, MS + GA3, MS + JP, MS + AIA, respectively. In contrast, the MS culture medium, which had no roots present (Table 3). In the seedlings formation (phase 5), pineapple medium was found to be more efficient with significant differences (P <0.05: Tukey HSD; 54.2%) in seedling development and growth of *C. trianae* (Table 3; Figure 1G), followed by the MS + AC culture medium, which generated a percentage of 27% (Table 3). It is important to note that after the final evaluation of *C. trianae* seeds, the culture media MS, MS + AIA and MS + GA3, did not form seedlings at 20 weeks (Table 3).

In transplanting and acclimatization of *C. trianae,* which was *in vitro* seedlings propagated, the most suitable substrate for plant adaptation (Figures 1E, F) was determined, which had achieved 94% of survival percentage in the crushed pine bark substrate and expanded clay (CA, Table 4). Additionally, the AA substrate with 86% efficacy and with less effectiveness in the CF substrate with 68%.

Tabla 4. Supervivencia de *C. trianae* en diferentes sustratos después de 12 semanas de haber sido trasplantadas.

Substrate	Survival rate
CA	94[a]
CC	70[b]
CF	68[b]
AA	86[a,b]

The means values with different letter of each column indicate statistically significant differences, according to Tukey HSD test ($P \leq 0.05$).

Discussion

Orchid species have different needs for mineral nutrients for germination and seedling development. For this reason, the most suitable *in vitro* culture medium for each of them is studied. Arditti & Ghani (2000), consider phase 1 as the first stage in the germination process in orchid seeds. Similar research with *Cattleya mendelli* reported a high degree of germination of 95.7% in the MS + AC culture medium (Salazar, 2012). However, germination varies considerably, and in many species, have a decreasing in storage longevity (Vendrame *et al.*, 2007). In addition, can be inferred that germination is influenced not only by the culture medium, but by seeds maturity and quality. Studies carried out by Salazar & Cancino (2012), found similarities in protocorms formation, where the highest percentage was found in the MS culture medium in this orchid species: *Prosthechea vespa* (Vell.) W.E.Higgins and *Sobralia klotzscheana* Rchb.f. In this research, a beneficial effect of using organic additives to the culture medium is demonstrated for seedlings germination, formation and development, respectively. This is in concordance with other authors, where the effectiveness of the organic components (pineapple juice and coconut water) have allowed a verification in the *in vitro* culture of a great variety of orchid species. (Gallo *et al.*, 2016; Salazar *et al.*, 2013). Nonetheless, Pedroza (2009), found that the addition of AIA (0.5 $mg.L^{-1}$) to the MS culture medium, promoted the development phases of *Epidendrum elongatum.* In the same way, Coello *et al.* (2010), highlights the importance of the GA_3 hormone in orchids germination and growth. However, it should be noted that despite the difficulty in analyzing its composition and the lack of information in the literature about the effects of organic components, such as coconut water and pineapple juice, these are rich in energy, vitamins, amino acids and phytohormones. Recent studies have shown that coconut water in the culture medium significantly stimulates the formation of roots and shoots in *Dendrobium lasianthera* J.J.Sm. orchid species (Wida *et al.,* 2017). However, in this research the most efficient culture medium was that supplemented with pineapple juice.

In transplanting and acclimatization, the seedlings grew vigorously with a high survival degree, which demonstrates the importance of using suitable substrates, which had achieved a good water retention and humidity. Santos *et al.* (2016), obtained a high survival percentage using vermiculite as substrate. Conversely, is convenient to use substrates of easy availability and of low costs to optimize the production.

Conclusions

This study demonstrated that the addition of organic additives such as coconut water and pineapple juice to *in vitro* culture medium optimizes the growth and development of *C. trianae,* orchid species, which could be applied to mass-scale propagation as well as ex situ

species conservation of endangered orchids in their natural habitats. Given these concerns, the use of organic supplements could be an effective alternative for reducing the high costs generated by the use of plant hormones in the culture medium.

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