Asymbiotic seed germination and *in vitro* seedling formation of *Cattleya mendelii* Dombrain (Orchidaceae)

Germinación asimbiótica de semillas y formación *in vitro* de plántulas de *Cattleya mendelii* Dombrain (Orchidaceae)

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Abstract

Cattleya mendelii is an endemic orchid from Colombia, which has a great ornamental value that is in danger of extinction due to massive collection and their natural habitat destruction by human activities. *In vitro* culture was an alternative to preserve this specie and carry out its marketing. In this study was evaluated the asymbiotic germination and the orchid seedling formation from seeds of *C. mendelii*, in several *in vitro* cultures. Initially, mature capsules were collected and seed viability with tetrazolium test was done; seeds were disinfected at the same time and planted by the syringe method to evaluate the effect of five growth culture media on the development of *C. mendelii* after 16 weeks of cultivation. It was found that seed viability was 93%, the highest percentage of germination was found in the culture medium Murashige-Skoog plus coconut water (MS+AC) with significant differences compared to other culture media ($P<0.05$, Tukey). This study showed that MS medium supplemented with coconut water and pineapple juice, were more efficient in asymbiotic germination and seedling formation of *C. mendelii* orchids compared with other culture media. This could be an option to reduce the costs generated by using plant hormones.

Key words: *Cattleya mendelii*, culture medium, embryonic development, germination, *in vitro* culture, tetrazolium.

Resumen

Cattleya mendelii es una orquídea endémica de Colombia con alto valor ornamental que se encuentra en peligro de extinción, debido a la recolección masiva y destrucción de sus hábitats naturales por la acción antrópica. El cultivo *in vitro* es una alternativa para la conservación de esta especie y/o su comercialización. En esta investigación se evaluaron la germinación asimbiótica y la formación de plántulas de las semillas de orquídeas de la especie *C. mendelii*, en diferentes medios de cultivos *in vitro*. Inicialmente se recolectaron cápsulas maduras; posteriormente, se determino la viabilidad de las semillas con la prueba de Tetrazolio; en forma paralela las semillas se desinfectaron y sembraron con el método de jeringuilla, para evaluar el efecto de cinco medios de cultivo en el desarrollo de *C. mendelii* después de 16 semanas de cultivo. Se encontró que la viabilidad de las semillas fue de 93%. El mejor porcentaje de germinación se encontró en el medio de cultivo Murashige-Skoog más agua de coco (MS + AC) con diferencias significativas ($P < 0.05$, Tukey) con respecto a los demás medios de cultivo. En este estudio se

demostró que los medios de cultivos MS suplementados con agua de coco y jugo de piña fueron más eficientes en la germinación asimbiótica y formación de plántulas en la orquídea *C. mendelii* con respecto a los otros medios de cultivo, siendo una opción en la reducción de los costos generados por la utilización de las fitohormonas.

Palabras clave: *Cattleya mendelii*, cultivo *in vitro*, desarrollo embrionario, germinación, medio de cultivo, tetrazolio.

Introduction

Orchidaceae family has a wide diversity in the Plantae kingdom with 750 genera and between 25,000 and 30,000 species around the world (Thorpe and Yeung, 2011). Thanks to its evolutionary capacity it shows variability in flower size, shape, texture and color (Nagaraju and Mani, 2005). According to the most recent data, Colombia registry 4010 species distributed in 260 genera (Mejía and Pino, 2010). In Norte de Santander there are descriptions of 37 genera and 105 species, highlighting *Catleya mendelii* (Díaz *et al.*, 2004) which is native of Colombia growing in rocks and in the top of large trees in the Eastern Range of the Andes in Santander and Norte de Santander (1200 – 1800 MASL) (Santos *et al.*, 2010). Plants have large and colorful flowers that give them an ornamental and economic value. It flowers once per year between April and May. This specie is endangered according to the red book of plants of Colombia (Calderón, 2007), meaning that, it is in danger of extinction or population decline in the wild. This threat is caused by its excessive and illegal recollection for marketing and, because of the destruction of host trees and its natural habitat (Salazar *et al.,* 2010; Santos *et al.,* 2010). Additionally, orchid germination in the wild has some limitations, like little nutrient reserves in the seeds which limit germination and survival in vivo. Therefore, it is necessary to establish symbiotic associations, mainly with mycorrizae, to achieve germination in natural conditions (Chen and Chen, 2007.

The first method for orchid seed germination (Moore, 1849) with horticultural interest meant an important and radical change in the way that other seeds of orchids were germinated 160 years ago (Arditti, 1984; Yam *et al.*, 2002; Yam and Arditti, 2009). Half a century after Moore´s discovery, Noël Bernard formulated a method for symbiotic germination of orchid seeds *in vitro* (Rasmussen, 1995; Yam *et al.*, 2002).

Lewis Knudson developed a method for asymbiotic germination of orchid seeds *in vitro* (Knudson, 1921; Yam *et al.*, 2002), which was the first practical procedure for *in vitro* propagation of any plant in axenic conditions, showing that germination was possible on a simple medium supplemented with minerals and sugar. The research done by Knudson shocked the orchids world demonstrating the *Cattleya, Vanilla* and other orchids were able to germinate asymbiotically *in vitro* (Knudson, 1946). Other authors like Arditti (1982) and Fast (1980), have contributed in the formulation of cultivation media for different genera and species.

The MS cultivation media (Murashige and Skoog, 1962) have been tested for germination and growth of several orchid species, getting optimal results because of its content of inorganic salts, carbohydrates, vitamins and amino acids, giving high nitrogen and potassium levels required for nutrition. It is relevant to mention that the *in vitro* culture of orchid seeds is favored by the addition of organic supplements and hormones to the growth medium (Malgren, 2006; Arditti, 2008; Pedroza, 2009; Cancino and Salazar, 2011).

In vitro methods for growth have been successfully used for conservation and propagation of species of endangered and medicinal orchids (Deb and Pongener, 2011; Stewart and Kane, 2006; Lo *et al.*, 2004). When germination conditions are being evaluated is important to take into account the viability of the studied seeds, which will estimate the rate of seeds that will not germinate as consequence of conditions that prevent germination due to low viability (Muñoz and Jiménez, 2008). In orchids the most used method for seed viability determination is the biochemical test with tetrazolium (Johnson *et al.*, 2007;

Lauzer *et al.*, 2007; Vujanovic *et al.*, 2000) which detects signs of life or metabolic activity. Viable seeds are easily identifiable due to the characteristic red coloring (Mweetwa *et al.*, 2008). The present study evaluated seed asymbiotic germination and in vitro formation of *C. mendelii* seedlings. Moreover, seed viability of this specie was determined and compared to the germination percentage.

Materials and methods

Plant material

Mature dehiscent capsules of *C. mendelii* were collected in Bochamela, Norte de Santander, Colombia on March 30, 2011. These capsules had been manually pollinized under greenhouse conditions by transferring two pollen grains to the stigma of another flower using fine forceps. A total of 4 plants, 2 donors and 2 receptors, were used. Afterwards, mature seeds were taken out of the capsules for storage on Kraft paper at 4 °C in glass containers with silica gel to avoid seed deterioration by humidity (Dutra *et al.*, 2008; Vogel and Macedo, 2011).

Seed viability

Seeds were subjected to different sodium hypochlorite concentrations 0.5%, 1%, 1.5%, 2.0%, 2.6%, 3.0%. Next, their viability was evaluated with the tetrazolium test [2,3,5-triphenyltetrazolium chloride (TTC: $C_{19}H_{15}ClN_4$)]. 100 orchid seeds were immersed in a TTC solution (1%) (1 g in 100 ml phosphate buffer: 0.9 % Na_2HP0_4 $2H_20$ + 1.1 % KH_2P0_4, pH 6-7) for 24 hours in the dark (Muñoz and Jiménez, 2008; Ossenbach *et al.*, 2007), before they were examined under the microscope stereoscope (Leica EZ4). The process to determine viability was replicated five times. Viable seeds were dyed red due to the tetrazolium reduction by cell respiration. This test is approved by the International Seed Testing Association (ISTA, 1985).

Seed disinfection and sowing

For seed disinfection and sowing it was used the syringe method (Vendrame *et al.*, 2007), which consists on putting some seeds on a sterile 5 ml syringe with a cloth filter; seeds were submerged on a 70% ethanol solution for 30 seconds; then they were placed on 1% sodium hypochlorite (NaOCl) solution with two drops of Tween-20® (surfactant) for 5 minutes in constant shaking; then they were washed five times with deionized water and, the filter was removed from the syringe to proceed with the sowing under sterile conditions in the flow chamber. 100 seeds were sown on petri dishes containing 25 ml of culture medium.

Asymbiotic medium and culture conditions

Basic culture medium was MS with 100% of macro and micronutrients concentration (Murashige and Sloog, 1962) with 3000 mg/lt sucrose, 700 mg/lt agar, 100 mg/lt myo-inositol and 1000 mg/lt activated carbon. Five culture media were used: MS as control, MS supplemented with pineapple juice (MS+JP), MS with coconut water (MS+AC), MS with 0.5 mg/lt indolacetic acid (MS+AIA) and MS with 0.5 mg/lt giberellic acid (MS+GA_3). Media with organic supplements were prepared adding 200ml/lt (20%) of coconut water or pineapple juice. Pineapple juice was prepared with a blender, then it was filter through gauze, boiled for 10 min and stored in glass flasks 8200 ml) at -80 °C. Medium was adjusted to ph 6.0 with NaOH or 0.5 M HCl. It was sterilized at 15 psi, 121 °C for 20 minutes. Culture media were incubated under controlled conditions (23 ± 2 ºC, 16/8h light/dark, light intensity 25 µmol/m per second using fluorescent light and 60% relative humidity).

Experimental design and statistical analysis

Experimental designed was a completely randomized factorial 6 x 5 (six developmental phases and five culture media), with five replicates and three petri dishes in average (each ne with 100 seeds) for a total of 75 experimental units. Data were subjected to a variance analysis (Anova). Afterwards, means were compared using the HSD (Honestly Significant Difference) multiple range test of Tukey to determine significant differences at $P < 0.05$ (Tukey, 1994).

The process from seed germination to seedling formation was evaluated every 2 weeks for 16 weeks after sowing using the orchid developmental phases adapted by Vasudevan and Staden (2010) (Table 1). Moreover, the total percentage of *in vitro* germination was measured to be compared with the viability test, this was done by summing the orchid developmental phases 1, 2,3,4 and 5 of each culture media used (MS, MS+JP, MS+AC, MS+AIA y MS+GA3). Later on, germination average value was obtained (germination percentage). The correlation coefficient between sodium hypochlorite concentration and viability percentage was determined. For the statistical analysis the software Statgraphic Centurión XV version 15.2.05 (2006) was used.

Results and discussion

Viability percentage vs. sodium hypochlorite concentration

The higher viability percentage of *C. mendelii* was found in seeds treated with 1% sodium hypochlorite, and the lower with the highest concentration of this compound (3%) (Figure 1). According to these results, it is possible to declare with 95% probability that sodium hypochlorite has a moderately strong effect on seed viability and, that there is a correlation coefficient R^2 = 0.7841 (Figure 1, Picture 1). Alvarez-Pardo *et al.* (2006) when seed viability and sodium chloride concentrations are associated finding higher viability percentage in *C. bicolor.* Sodium hypochlorite scarifies seed coat therefore the tetrazolium solution penetrates easily, and in the same way, germination is stimulated (Rännbäck, 2007; Vasudevan and Staden, 2010).

Tetrazolium test is a recomended method due to its efficiency and easy use; viable seeds are easily identified with a red color characteristic of the reaction on living cells, because they release hydrogen via dehydrogenase activity during respiration (Vujanovic *et al.*, 2000; Ossenbach *et al.*, 2007; Muñoz and Jiménez, 2008).

Table 1. Developmental phases on orchid seeds.

Phase	Description
0	Seeds and embryo did nt germinate.
1	Embryo increases its size.
2	Testa ruptura.
3	Protocorm and rhizoids formation.
4	First leaf emergence.
5	First leaf elongation and progressive development.

Source**:** Modified from Vasudevan and Staden, 2010.

Seed viability and germination

In average, the viability of *C. mendelii* was high (93%). Lauzer *et al.* (2007) and Vujanovic *et al.* (2000) demonstrate that viability test is not always a good indicator of germination, since it does not evaluate cell division and development capacities (Padilla, 2011). For this reason, the validity of this test was confirmed with germination test on the nursery (Johnson *et al.*, 2007; Muñoz and Jiménez, 2008).

In vitro asymbiotic germination of *C. mendelii* seeds in the five media tested was, in average, 94.1% (Table 2). The difference between the germination test and the viability test was 1.1% indicating that there are no differences (P > 0.05) between both tests. This demonstrates that the tetrazolium test could be used to predict the germination capability of *C. mendelii.* Hosomi *et al.* (2011) used the tetrazolium test to estimate seed viability of *Catleya* sp. for propagation and preservation. Also, it has been used to determine the germination capacity and seedling

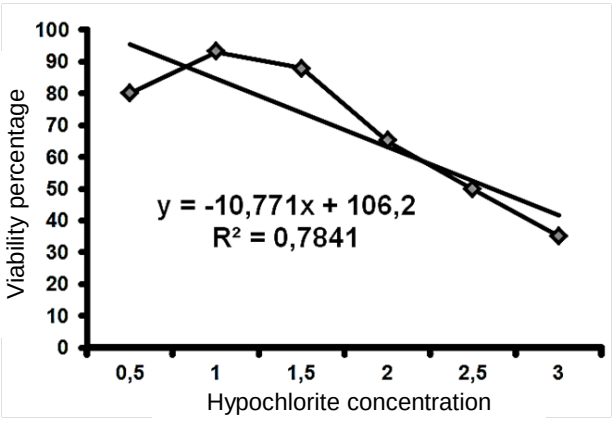
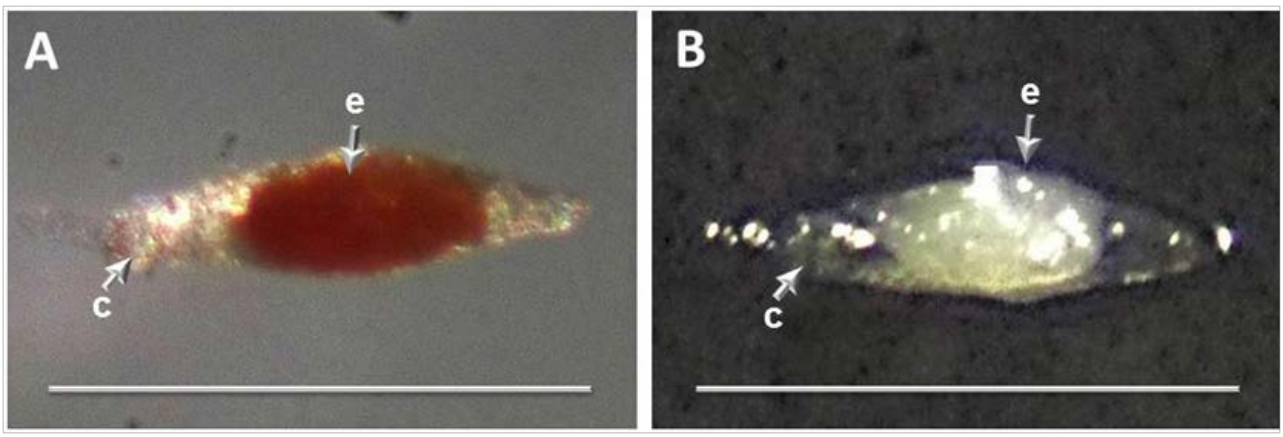


Figure 1. Correlation between sodium hypochlorite concentration and viability percentaje.



Picture 1. Viability evaluation of *Cattleya mendelii*, using the tetrazolium test . (**A**) viable seed. (**B**) unviable seed. Tetrazolium salt is a transparent, soluble and oxidated solution. In presence of respiratory activity it is reduced, producing an isoluble red precipitate called Formazan. Bar scale 1 mm. **c**: seed coat; **e**: embryo.

growth of a vast number of orchids species (Stewart and Kane, 2006; Yamazaki and Miyoshi, 2006; Mweetwa *et al.*, 2008; Cancino and Salazar, 2011). Nonetheless, the test is based only on the internal conditions of seeds and does not revealed the combined behavior of their quality with certain growth characteristics (Ossenbach *et al.*, 2007). Besides, seed viability of different capsules changes considerability and in several orchid species it decreases during storage (Vendrame *et al.*, 2007; Hirano *et al.*, 2011).

Culture media, asymbiotic germination and seedling formation

Germination percentage of *C. mendelii* was constant from week 4 on (Figure 2). The highest percentage was found in the MS+AC medium ($P < 0.05$) (Table 2). The highest per

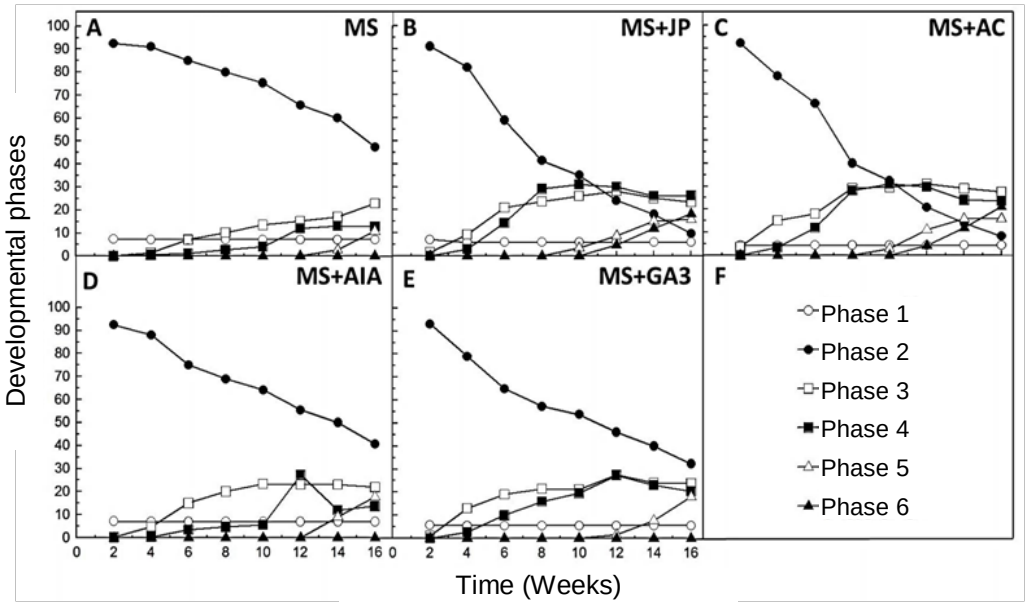


Figure 2. Developmental phases of *Cattleya mendelii* culture don five media. (**A**) MS culture medium (Murashige and Skoog). (**B**) MS + Pineaple juice. (**C**) MS + Coconut water. (**D**) MS + Indolacetic acid. (E) MS + Giberellic acid. (**F**) Phases symbols.

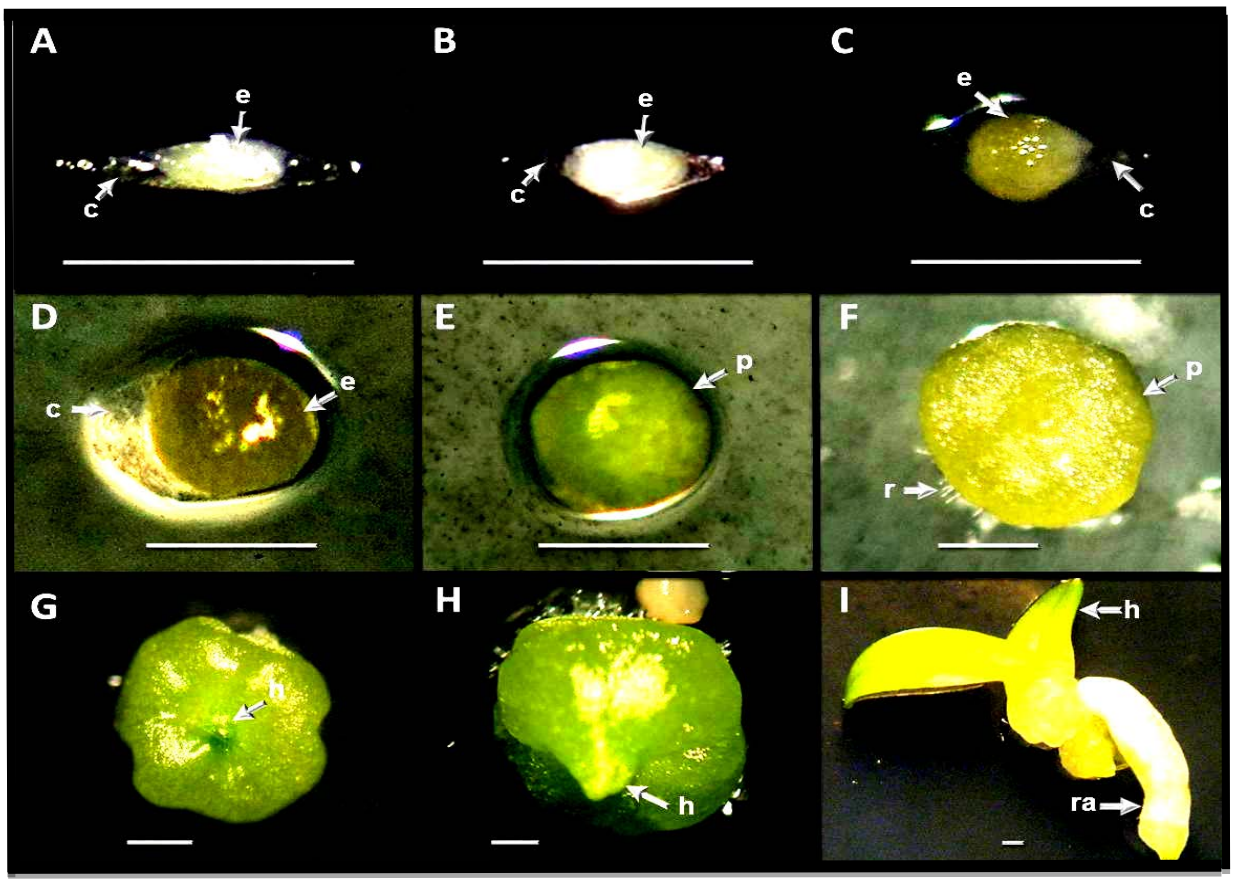
Table 2. Culture media effect on germination percentage and protocorm formation of *Cattleya mendelii*. Evaluation done at 120 days of culture.

Culture medium	Germination percentage	Protocorm formation
MS	92.86 a*	12.798 a
MS + JP	94.06 bc	26.198 b
MS + AC	95.7 d	23.464 bc
MS + AIA	93.34 ab	13.73 a
MS + GA3	94.5 c	20.398 c

*Values in the same column with different letters are significantly different (P < 0.05), according to Tukey´s test.

centage of protocorm formation happened in MS+JP medium that was not different from the MS+AC medium (P > 0.05) (Table 2). Similar results were found by Stewart and Kane (2006) in *Habenaria macroceratitis* (Orchidaceae). Kitsaki *et al.* (2004) found that culture media enriched with coconut water was more suitable for germination and protocorm formation in *Ophys* than the one with pineapple juice. Independently from the growth habit and taxonomy, several species can have different requirements for germination (Vogel and Macedo, 2011). Additionally, it is possible to infer that germination is affected not only for the culture media but also for the seed quality and maturity (Lo *et al.*, 2004; Yam and Arditti, 2009).

C. mendelii developmental phases (Picture 2 and Figure 2) were highly variable in the media treatments during the 16 weeks of evaluation, with the exception of the Phase 0 which did not vary from the week 4 on in all



Picture 2. Developmental phases on *Cattleya mendelii* from seed germination till seedling formation in *in vitro* asymbiotic culture medium. (**A**) Phase 0: Seed with embryo without germination. (**B** and **C**) Phase 1: Embryo increases its size. (**D**) Phase 2: Testa rupture. (**E** and **F**) Phase 3: Protocorm and rhizoids formation. (**G**) Phase 4: First leaf emergence. (**H** and **I**) Phase 5: First leaf elongation and progressive development. Bar scale = 1mm. **c**: seed coat; **e**: embryo; **h**: leaf; **p**: protocorm; **r**: rhizoid; **ra**: root.

the evaluated media (Picture 2A). In the other developmental phases it was found that the highest percentage in Phase 1 (Picture 2B and C) happened in the MS medium (47.33%; $P < 0.05$) (Figure 3). In Phase 2 (Picture 2D) development was not equilibrated in the culture media (MS: 22.798; MS+JP: 23.33; MS+AC: 27.57; MS+AIA: 21.864 and MS+GA3: 23.796; Figure 3). In Phase 3 (Picture 2E and F) the highest percentage was on the MS+JP medium (26.198) (Figure 3), however it was not different ($P > 0.05$) with the development on MS+AC medium (23.464). In Phase 4 (Picture 2G) seedlings on MS medium showed less development (10.794) in relation to other culture media and there were no differences between them ($P > 0.05$; Figure 3). Development on Phase 5 (Picture 2H and I) was achieved only in MS+JP and MS+AC media, but differences were not significant ($P > 0.05$).

Kitsaki *et al.* (2004) found that culture media supplemented with coconut water is more efficient in the first developmental phases (1, 2 and 3), contrasting with the media supplemented with pineapple juice which is better on Phases 4 and 5. Contrasting, the present research showed that the MS+AC and MS+JP media were the best in all the developmental phases of *C. mendelii*. However, the other culture media were efficient until Phase 4. Pedroza (2009) showed that the addition of IAA (0.5 mg/lt) to the MS medium promotes development on *Epidendrum elongatum* (Orchidaceae). In other studies, it was demonstrated, equally, the beneficial effect of IAA when added to MS medium (Farias and Arciga, 2011). Manrique *et al.* (2005) and Coello *et al.* (2010) found that GA3 can accelerate orchid germination and growth, which is not what was found in this study, where the effect of the plant hormones IAA and GA3 was not optimal for the developmental phases of the plant. It should be noted that both, pineapple juice and coconut water, are good sources of energy, vitamins, amino acids and plant hormones (Kitsaki *et al.*, 2004; Yam and Arditti, 2009; Yong *et al.*, 2009). In this research was found that the addition of these organic compounds to the MS medium improved its efficiency, and, favored a better growth and development of *C. mendelii*.

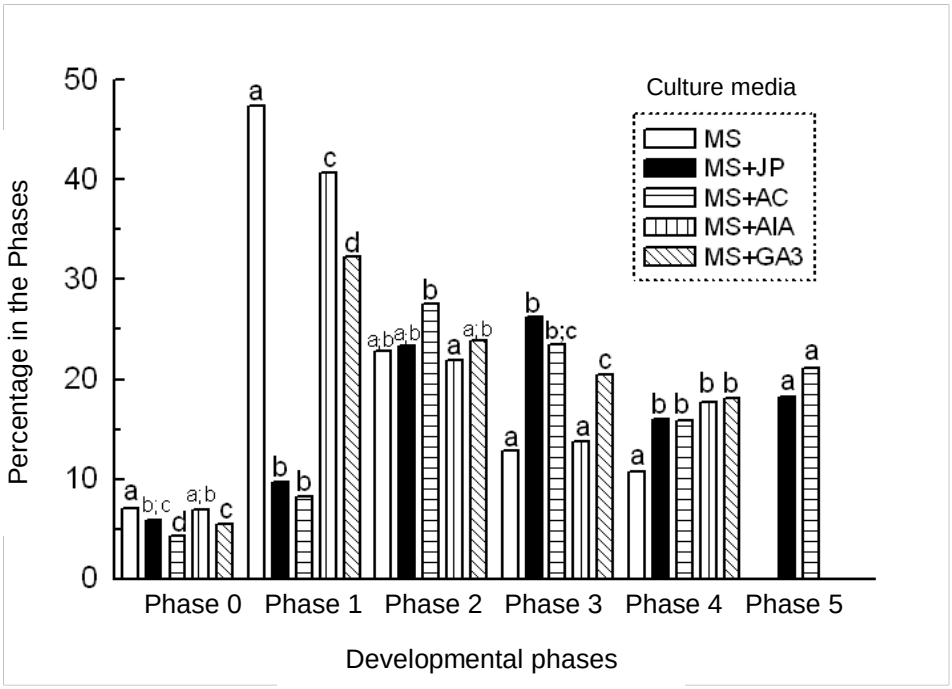


Figure 3. Effect of culture media on the developmental phases of *Cattleya mendelii*. Bars with different letters between each phase show statistically significant differences according to *HSD* Tukey´s test ($P<0.05$). Evaluation done at week 16 of culture.

Conclusions

- The present study demonstrated that the MS media supplemented with organic compound like coconut water and pineapple juice, favor *in vitro* asymbiotic germination and seedling formation of *C. mendelii*, being a suitable combination to produce multiple plants in order to reintroduce endangered species in their natural habitats.
- The use of organic supplements in the culture media could be a possible alternative to reduce high costs of the use of plant hormones in the media. Likewise, the tetrazolium test results on an efficient methodology to predict the germination capacity of *C. mendelii* seeds.
- There is a need of studies associated to multiplication and hardening of endangered or ornamental orchid species, to help their conservation and/or commercialization. Additionally, it is recommended to keep evaluating other organic components with different orchid species to prove their effectiveness.

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