In vitro germination and development of “Canelita” (Lycaste aromatica (Graham) Lindl.) in gravity immersion bioreactors

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Abstract

The orchid *Lycaste aromatica* (Graham) Lindl. is known for its outstanding cinnamon aroma, which has caused its illegal extraction from its natural habitat. For this reason, procedures were developed for the asymbiotic seeds germination and seedlings development in a gravity immersion bioreactor (GIB) system. Four culture media were tested for in vitro germination: ½ Murashige and Skoog (MS), MS, ½ Knudson C 15 g L^{-1} sucrose, Knudson C. The efficiency of the in vitro culture protocol using the semi-solid medium was compared and with four immersion frequencies of the GIB system in a liquid medium, using 30 mL of ½ MS medium per seedling. Germination began at eight weeks and lasted for six weeks. The best treatment was ½ MS with 161 germinated seeds. In 90 days, the cultures in bioreactors reached significant differences in the number of leaves (10 leaves), number of roots (4.7 roots), and seedling height (5.4 cm). However, the seedlings developed in the semi-solid medium reported a higher number of shoots (1.6 shoots) and root length (2 cm); furthermore, the bioreactor culture showed the highest photosynthesis rate (0.74 µmol CO_2 g^{-1} s^{-1}). The culture system in bioreactors is an excellent alternative to develop this species because it increases photosynthesis and the size of the seedlings, which favor their acclimatization.

Key Message

Lycaste aromatica begins germination in vitro at 8 weeks, lasts for six weeks, develops functional stomata, and promotes photosynthesis in the gravity immersion bioreactor.

Introduction

Lycaste aromatica (Graham) Lindl. is a wild orchid commercially attractive due to the cinnamon aroma of its flowers, which is why it is known as “Canelita”. It is illegally traded for as low as $ 15.00 Mexican pesos (US $ 0.75) in Chiapas, Mexico market (Jiménez-López et al. 2019). This species population is at risk due to those illegal removals from its habitat, the cloud forest or mountain mesophilic forest (MMF), causing a shortage of individuals. So that, in a 4 km journey in the Chocamán, Veracruz MMF, a single specimen of *L. aromatica* was found (Baltazar-Bernal *et al*. 2014).

Seeds are the best reproductive structure to conserve genetic diversity, yet they are rarely collected, sown, or stored. Despite various attempts to preserve orchid populations in the wild, they are declining at an alarming rate (Diengdoh et al. 2017). *L. aromatica* in their natural habitat 8–11% of the flowers formed capsules, but in 2016 it was only 1%. There are several *in situ* germination studies to help preserve orchids; however, the reported germination is less than 20% without showing later development (Aewsakul *et al*. 2013; Diengdoh et al. 2017). In vitro germination is an alternative for orchids preservation; for example, in other species between 50 and 75% germination has been reported (Hossain et al. 2010) in Murashige and Skoog culture medium (Murashige and Skoog 1962) and 85% in ½ of the MS medium (Ferreira et al. 2017).

The asymbiotic germination of orchid seeds has been used to produce orchids of ornamental interest, such as *Phalaenopsis amboinensis* J.J. Sm. (Utami and Hariyanto 2019), *Myrmecophila grandiflora* (Lindl.) Carnevali, J.L. Tapia & I. Ramírez (Bello-Bello et al. 2020) and *Spathoglottis plicata* Blume (Shekhawat et al. 2021), which have also proven to be a tool to produce orchids for conservation purposes (Jiang et al. 2017).

On other hand, interest in using temporary immersion bioreactors has increased over the last decade (Aragón et al. 2014), since they guarantee better seedling development (Aragón et al. 2014; Ramos-Castellá et al. 2014; Latawa et al. 2015; Ramírez-Mosqueda and Iglesias-Andreu 2016; Silva et al. 2016), compared to a semi-solid medium. The temporary immersion system (TIS) is the culture of cells, tissues, or organs temporarily exposed to a liquid culture medium in semi-automated bioreactors under axenic and controlled conditions (Bello-Bello et al. 2014) and allows better seedlings development (Monja - Mio *et al*. 2015), a greater stomatal cells function, an increase in the net photosynthesis rate (Aragón et al. 2014) and improves the activity of photosynthetic pigments to aid in acclimatization (Shekhawat et al. 2021).

The purpose of this research was to evaluate the culture media for in vitro germination and to evaluate the development of *L. aromatica* seedlings in four frequencies of immersion in a gravity immersion bioreactor (GIB) and semi-solid medium.

Materials And Methods

Vegetal material

The seeds of a mature 6 cm long and 1.5 cm wide capsule of *L. aromatica* were used. The capsule was collected at the community of Tepexilotla, municipality of Chocamán, state of Veracruz. The capsule was stored at 5°C for seven days before sowing.

Experiment 1

in vitro *germination*

Before disinfecting the *L. aromatica* capsule, the remains of the flower and peduncle were removed. The capsule was washed with Axion® lemon detergent and distilled water; then immersed in a solution of distilled water with 20% Cloralex® chlorine plus a drop of Axion® lemon detergent, for 20 min with constant stirring; the solution was removed and rinsed; it was then immersed in a Ridomil Bravo® 81 1 g L^{-1} solution for 5 min and rinsed four times with sterile distilled water, giving one minute for each rinse in a laminar flow hood, and finally sprayed with alcohol and ignited for three seconds. The capsule was subsequently cross-sectioned at the ends and longitudinally with a scalpel. Seeds were then extracted with a scalpel.

Four culture media were used for seed germination: MS (Murashige and Skoog 1962), ½ MS and 15 g L^{-1} sucrose, KC (Knudson 1946) and ½ KC. All media was adjusted to pH 5.7 ± 0.1 before 2.5 g L^{-1} of Phytagel ™ (Sigma-Aldrich, St. Louis, MO) was added, the media was sterilized at 120°C for 15 minutes.

Approximately 200 seeds were spread on culture media in 200 ml flasks, containing 20 ml of culture medium, covered with transparent plastic caps, and sealed with Kleen Pack®. Subsequently, stored an incubation room at 22 ° C ± 1°C and a 16 h of light photoperiod with a 72 µmol m^{-2} s^{-1} light intensity of photosynthetically active radiation, produced by fluorescent cold white light lamps, and 8 h in darkness.

From the eighth week after sowing on, the number of germinated seeds per bottle that reached 1 mm in diameter were evaluated and subcultured, weekly, for six weeks after exhibiting the first germinated seed. A completely randomized experimental design with repeated measures and three repetitions was used. The data were analyzed using an ANTE [1] covariance model structure and means comparison with the Tukey test using the SAS software version 9.4 (SAS, 2012).

Experiment 2: seedling development

For the development phase, which lasted 14 weeks, 2.5 cm long seedlings were used in a semi-solid medium and in a gravity immersion bioreactor (GIB). In both methods, the culture medium was ½ MS supplemented with 15 g L^{-1} sucrose, with a 5.7 pH adjusted before autoclaving at 120°C for 15 minutes. For the semisolid medium, the seedlings were placed in 1 000 mL glass containers containing 100 mL of medium, gelled with 2.5 g L^{-1} of Phytagel™ (Sigma-Aldrich, St. Louis, MO) with 15 seedlings per bottle. The gravity immersion bioreactor was made in pairs of 1 000 mL flasks, one containing 15 seedlings and the other containing 450 mL of liquid culture medium (Georgiev et al. 2014). The GIB system was set to a three-minute immersion period, at frequencies of 4, 6, 8, and 12 h. The cultures were incubated at 22 ± 1°C with a 16 h light photoperiod providing 57 µmol m^{-2} s^{-1} of photosynthetically active radiation with fluorescent cold white light lamps and 8 h darkness.

At the end of the experiment the survival, the number of leaves, the height of the seedling, number and diameter of the shoots, number and length of roots, fresh weight, and the dry weight of the plants were assessed.

Stomata

The stomatal index was also assessed; the length and width of the stoma were measured using a Nikon Axio Zeiss optical microscope with a 40x objective equipped with an AxioCam 1Cc3 digital camera. Fifteen mature leaves, per treatment, were used for this measurement, and transparent nail polish was applied to their adaxial and abaxial central parts (Li et al. 2010).

Once the nail polish had dried and a membrane formed, it was detached and fixed on a slide with a drop of water to allow the sample´s measurement (Silva *et al*. 2000).

Photosynthesis and respiration

The CO_2 exchange in light and dark conditions (µmol CO_2 g^{-1} s^{-1}) was evaluated with a portable system for measuring photosynthesis model LI-6400 (Li-Cor, Inc, Lincoln, Nebraska, USA) using an accessory to measure insect respiration and a setup to measure photosynthesis. Global photosynthesis (µmol CO_2 g^{-1} s^{-1}) was calculated as the difference between the exchange of CO_2 in darkness and light. After measuring CO_2 exchange in light, the chamber was covered for three minutes to measure CO_2 exchange in dark. Measurements were made between 11:00 am and 1:00 pm in randomly selected plants of each treatment and three repetitions. Measurements were made at 30 ± 1°C, 400 ppm of CO_2, and 57 µmol g^{-1} s^{-1} of photosynthetically active radiation. The data were recalculated for analyses as µmol CO_2 g^{-1} s^{-1}.

Chlorophyll

The total, a and b chlorophyll content present in the leaves at six weeks after their development was measured following Harborne (1973) with a spectrophotometer (Genesys 10S UV-Vis, Thermo Scientific) and 80% acetone as blank with three repetitions.

Statistical analysis

A completely randomized experimental design was used and the data were analyzed by an analysis of variance and means comparison with the Tukey test using the SAS software version 9.4 (SAS, 2012).

Results And Discussion

Experiment 1

in vitro *germination*

There were significant differences in germination; the ½ MS medium had the highest number of germinated seeds, 33 seeds, compared to the ½ Knudson C medium, with five germinated seeds. This may be because the KC medium contains a low amount of macro and micronutrients and lacks vitamins, compared to the MS medium. In research on *Alatiglossum fuscopetalum* (Hoehne) Baptista using ½ MS culture medium, and 85% germination was obtained (Ferreira et al. 2017); however, in *Cypripedium lentiginosum* P.J. Cribb & S.C. Chen, it was 52.8% (Jiang et al. 2017) and in *Paphiopedilum insigne* (Wall. ex Lindl.) Pfitzer it was 41.5% (Diengdoh et al. 2017).

The seeds began to germinate (1 mm long protocols) eight weeks after sowing, like on *Cymbidium giganteum* (Thunb.) Sw. (Hossain et al. 2010) and *Paphiopedilum insigne* (Diengdoh et al. 2017).

The seeds germination reached its maximum value in 8 and 9 weeks in the case of the ½ MS medium. At the same time, in the KC treatment maximum germination occurred between weeks 12 and 13. The ½ KC and KC treatments showed less germination and required more time to germinate (Fig. 1). Previous research in *L. aromatica* did not report germination percentages or germination period (Mata-Rosas et al. 2010; Scheffknecht et al. 2010). However, in *L. skinneri* Lindl. in modified KC medium a 100% germination was reported (Mata-Rosas and Salazar-Rojas 2009). This research indicates that *L. aromatica* germination begins by week eight after sowing, extends until week 13, and reaches 80% in the ½ MS medium (Fig. 1).

Experiment 2

The gravity immersion bioreactor system (GIB) and an immersion frequency of 3 min every 8 h obtained 100% survival (Table 1); this is probably due to the fact that a greater exposure of the medium to the explants allows a greater assimilation

of nutrients from the medium (Bello-Bello et al. 2020). The GIB with immersion every 12 h obtained 62.2%. Similarly, to this research, Bello-Bello et al. (2020), reported 66% survival in *Myrmecophila grandiflora* Lindl. in GIB with immersion every 12 h.

Table 1
Effect of the culture system and the immersion frequency on the development of *Lycaste aromatica* grown in vitro for 14 weeks.

In vitro culture system	Immersion frequency (hours)	Survival (%)	Number of leaves	Number of shoots	Seedling length (cm)	Seedling diameter (cm)	Number of roots	Root length (cm)	Fresh weight (g)	Dry weight (g)
BIG	4	80.0 ± 10.18ab	8.5 ± 0.62b	1.2 ± 0.10b	4.3 ± 0.49ab	0.6 ± 0.06a	3.6 ± 0.28b	0.9 ± 0.25b	0.403 ± 0.13a	0.021 ± 0.00a
BIG	6	97.8 ± 10.18a	10.6 ± 0.62a	1.3 ± 0.10ab	3.8 ± 0.49b	0.7 ± 0.06a	3.8 ± 0.28b	1.0 ± 0.25b	0.493 ± 0.13a	0.022 ± 0.00a
BIG	8	100.0 ± 10.18a	8.4 ± 0.62b	1.2 ± 0.10b	5.1 ± 0.49ab	0.65 ± 0.06a	4.3 ± 0.28ab	1.0 ± 0.25b	0.483 ± 0.13a	0.037 ± 0.00a
BIG	12	62.2 ± 10.18b	8.2 ± 0.62b	1.1 ± 0.10b	5.5 ± 0.49a	0.61 ± 0.06a	4.7 ± 0.28a	1.2 ± 0.25b	0.410 ± 0.13a	0.026 ± 0.00a
Semi-solid medium	NA	77.8 ± 10.18ab	8.7 ± 0.62ab	1.6 ± 0.10a	4.1 ± 0.49ab	0.71 ± 0.06a	4.3 ± 0.28ab	2.1 ± 0.25a	0.193 ± 0.13a	0.013 ± 0.00a

Each value represents the mean ± SE of three replicates. A column followed by the same letter in each treatment is not significantly different (Tukey, $p \leq 0.05$). BIG: Gravity immersion bioreactor

A greater number of leaves were developed in GIB with immersion every six hours (10.6), while in the other frequencies, the number of leaves was lower (8.2 to 8.5) (Table 1). Similar results to this study were reported by Moreira et al. (2013) who reported that there is no significant difference in the number of *Cattleya walkeriana* Gardner leaves between the temporary immersion bioreactor and continuous immersion one; they also found no differences between conventional micropropagation and natural ventilation (Silva et al. 2016). However, in *Vanilla planifolia* Jacks. ex Andrews the highest number of leaves was obtained in a temporary immersion bioreactor (BIT®) and automated temporary immersion container (RITA®) compared to GIB (Ramírez-Mosqueda and Iglesias-Andreu 2016). Also, in *Epipactis flava* Seidenf seedlings there are reports of a greater number of leaves in the temporary immersion system (TIS) than with the semi-solid medium and with the continuous immersion system (Kunakhonnuruk et al. 2019).

The highest shoots number was observed in the semi-solid medium, with 1.6 shoots, followed by GIB with 6 h of immersion with 1.3 shoots. In the GIB with 12 h of immersion, 4.7 roots were obtained, which was the best treatment for this variable. In *Cattleya walkeriana* no significant differences were reported in the number of roots between conventional micropropagation and natural ventilation (Silva et al. 2016).

Seedling height was highest in GIB with 12 h of immersion (5.4 cm) and the lowest in GIB with 6 h immersion (3.8 cm). Ramos-Castellá et al. (2014) reported for *V. planifolia* and Aragón et al. (2014) in *Musa* AAB cv. Cemsa ¾ a greater height of the sprout in a temporary immersion system than in a solid medium. Similarly, in this research, the greater plant height is possible because in GIB the culture medium has contact with all parts of the seedling. Also, in GIB the highest shoot height was obtained in *V. planifolia* (Ramírez-Mosqueda and Iglesias-Andreu 2016).

For the root length variable, the best result was obtained in the semi-solid medium (2.08 cm). These results are consistent with that reported by Silva et al. (2016), where they obtained 2.4 cm root length in conventional micropropagation compared to 1.6 cm in the natural ventilation systems in *Cattleya walkeriana* seedlings.

No significant differences were observed in stem diameter (Fig. 2), fresh weight, and dry weight (Table 1). Our results coincide with those obtained for *Cattleya walkeriana*, which did not show significant differences in the fresh weight between the temporary immersion bioreactor and the continuous immersion (Moreira et al. 2013), nor in the fresh weight and dry weight between conventional micropropagation and natural ventilation in *Cattleya walkeriana* (Silva et al. 2016).

Stomata

Lycaste aromatica leaves showed stomata in the abaxial and adaxial parts, for which this species is classified as amphistomatic (Reyes-López et al. 2015). The stomatal index and stomatal length on the underside of the leaf blade were statistically equal between treatments (Table 2). The width of the stoma on the underside was greater in the leaves of explants grown in the semisolid medium (245.79 µm), followed by the GIB system with 6 and 12 h immersions, with 209.72 µm and 204.76 µm each (Table 2). It can be that in the semi-solid medium, the stomata are open and not very functional, and the stomata in the GIB are functional because some stomata are closed (Fig. 3). The stomatal index, length, and width of the stoma in the upper part of the leaf blade were higher in the semi-solid medium compared to the GIB system (Table 2). Monja-Mio et al. (2015) reported that the BioMINT bioreactor allowed better development of stomatal cells and greater epicuticular cells deposition in "Bacanora" *Agave angustifolia* Haw. leaves compared to the semi-solid media, due to the high humidity inside the semi-solid medium containers. The functioning of the stomata and the higher gas regulation exchange in plants grown in BIT can better control the loss of water during the first acclimatization period (Aragón et al. 2014). The *L. aromatica* leaf had stomata on the underside and the upper side, but the stomatal index on the underside was 23 times higher than that in the upper side in GIB, and six times higher than in the semi-solid medium.

Table 2
Effect of the culture system and immersion frequency on stomatal index and stomata size of the under side and upper side of the leaves *of Lycaste aromatica* developed in vitro

In vitro culture system	Immersion frequency (hours)	Abaxial part of the leaves			Adaxial part of the leaves		
		Stomatal index (mm^2)	Length of stomata (µm)	Width of stomata (µm)	Stomatal index (mm^2)	Length of stomata (µm)	Width of stomata (µm)
BIG	6	9.29 ± 0.40a	249.67 ± 15.90a	209.72 ± 8.23b	0.58 ± 0.18b	81.07 ± 23.24b	68.27 ± 21.65b
BIG	12	8.43 ± 0.40a	231.28 ± 15.90a	204.76 ± 8.23b	0.29 ± 0.18b	60.27 ± 23.24b	48.80 ± 21.65b
Semi-solid medium	24	8.28 ± 0.40a	264.95 ± 15.90a	245.79 ± 8.23a	1.32 ± 0.18a	177.44 ± 23.24a	165.56 ± 21.65a

Each value represents the mean ± SE of three replicates. A column followed by the same letter in each treatment is not significantly different (Tukey, $p \leq 0.05$). BIG: Gravity immersion biorreactor

Photosynthesis and respiration

The CO_2 exchange assessment indicates that explants respire less in light than in dark conditions. Respiration in light and dark conditions did not show significant differences between treatments (Table 3). This is possible because no variation was applied in temperature or in the supplied sucrose, which participates in seedlings' respiration (Yang et al. 2015). The global photosynthetic rate was significantly higher in GIB with immersion every 12 hours (0.74 µmol CO_2 g^{-1} s^{-1}) than in the seedlings grown in semisolid media (0.27 µmol CO_2 g^{-1} s^{-1}) because the gas exchange is reduced in semi-solid medium (Oliveira et al. 2019).

Table 3
Effect of the culture system and immersion frequency on respiration and global photosynthesis in *Lycaste aromatica* seedlings developed in vitro

In vitro culture system	Immersion frequency (hours)	Respiration (μmol CO_2 g^{-1} s^{-1})			Global photosynthesis* (μmol CO_2 g^{-1} s^{-1})
		Day			
		Light	Darkness	Night	
BIG	4	0.96 ± 0.19a	1.49 ± 0.16a	1.20 ± 0.20a	0.53 ± 0.09ab
BIG	6	0.85 ± 0.19a	1.16 ± 0.16a	1.39 ± 0.20a	0.31 ± 0.09b
BIG	8	0.95 ± 0.97a	1.54 ± 0.16a	1.45 ± 0.20a	0.59 ± 0.09ab
BIG	12	0.61 ± 0.19a	1.35 ± 0.16a	1.16 ± 0.23a	0.74 ± 0.09a
Semi-solid medium	24	0.88 ± 0.19a	1.15 ± 0.16a	1.43 ± 0.20a	0.27 ± 0.09b

Each value represents the mean ± SE of three replicates. A column followed by the same letter in each treatment are not significantly different (Tukey, $p \leq 0.05$). BIG: Gravity immersion biorreactor. *Global photosynthesis: difference between daytime respiration in dark conditions and in light conditions

No significant differences were observed in respiration in light and dark conditions when comparing culture systems, however, semi-automated bioreactors are considered to promote photosynthesis and respiration, both caused by an increase in chlorophyll synthesis and stomatal functionality (Mancilla-Álvarez et al. 2021).

On average, global photosynthesis was twice as high in the bioreactors with liquid medium (0.54 μmol CO_2 g^{-1} s^{-1}) compared to the semi-solid one (Table 4). These results concur with those reported by Aragón et al. (2014) were the seedlings of *Musa* AAB cv. Cemsa ¾ were photosynthetically active after 14 days of elongation with a net photosynthetic rate 3 to 4 times higher in a temporary immersion bioreactor than in a semi-solid medium, which is attributed to greater efficiency in water use. Also, seedlings under conditions of 150 μmol g^{-1} s^{-1} of photosynthetically active radiation in the temporary immersion system favor the photosynthetic net rate (Aragón et al. 2010). Photosynthetic activity is important to consider for seedling production and successful acclimatization in greenhouse and field conditions (Park et al., 2020).

Table 4
Effect of the culture system on respiration and global photosynthesis in *Lycaste aromatica* seedlings grown in vitro

In vitro culture system	Respiration (μmol CO_2 g^{-1} s^{-1})		Global photosynthesis* (μmol CO_2 m^{-2} s^{-1})
	Light	Darkness	
BIG	0.84 ± 0.091a	1.34 ± 0.077a	0.50 ± 0.055a
Semi-solid medium	0.88 ± 0.183a	1.15 ± 0.090a	0.27 ± 0.110b

Each value represents the mean ± SE of three replicates. A column followed by the same letter in each treatment is not significantly different (Tukey, $p \leq 0.05$). GIB: Gravity Immersion Biorreactor. * Global photosynthesis: difference between daytime respiration in dark conditions and in light conditions

Chlorophyll

The total chlorophyll content ranged between 0.183 to 0.208 mg g^{-1} FW and the chlorophyll a, b, and total contents had no significant effect between the hours of immersion of GIB and the semi-solid medium; however, the GIB system with 6 hours immersions reported the highest total chlorophyll content. The cultivation of seedlings in closed glass jars in semisolid medium produces ethylene, and the movement of the culture medium in temporary immersion reduces it, in such a way that it favours increasing chlorophyll concentration (Silva et al. 2016), such as that observed in *Corylus L*. seedlings (Latawa et al.

2015). Ramírez-Mosqueda and Iglesias-Andreu (2016) reported that in *Vanilla planifolia* the highest chlorophyll concentration was recorded in the BIT® Temporary Immersion Bioreactor followed by GIB system.

Conclusions

In vitro germination of *Lycaste aromatica* (Graham) Lindl seeds starts in week 8 after sowing and continued until week 13. This shows that the ½ MS culture medium favors 80% germination. The seedlings' development in the gravity immersion bioreactor treatment with 3 min immersion every 8 hours obtained 100% survival. The GIB system favored the number of leaves, number of roots, seedling height, stomatal functionality, and global photosynthesis. However, the seedlings developed in the semi-solid medium achieved a greater number of shoots, root length, and stomata size. Overall, the stomatal index of the underside was greater than that of the upper side of the leaves.

Declarations

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Authors Contributions FYSZ: research, completed the experiments and manuscript draft. OBB: Conceptualization, research suggestion, methodology, and finalized the manuscript, NCH: responsible for result interpretation and editing draft manuscript, and. JVHC, JAPS: designed experiments and statistical analysis. All authors have read and approved the final manuscript.

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Figures

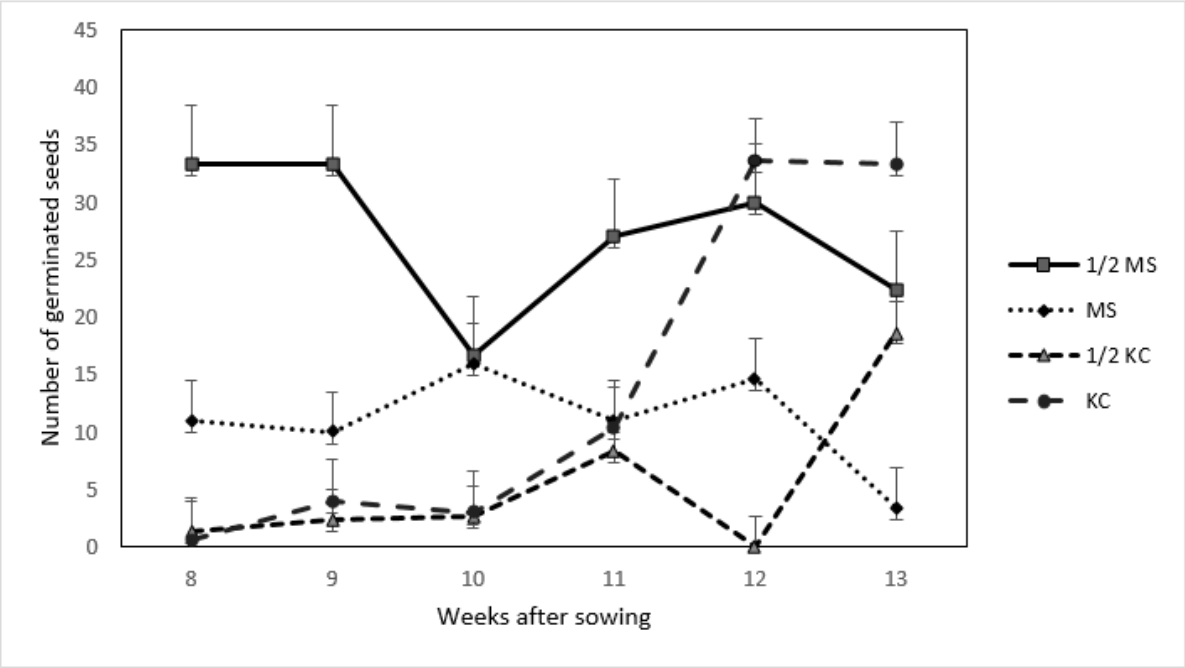

Figure 1

Number of germinated seeds of *Lycaste aromatica* per week in four culture medium. MS = Murashige and Skoog medium, KC = Knudson C medium. Results represent the mean ± SE of three replications.

Figure 2

Lycaste aromatica seedlings grown in MS in a gravity immersion bioreactor for 3 min. A: 4 h. B: 6 h. C: 8 h. D 12 h. E semi-solid medium.

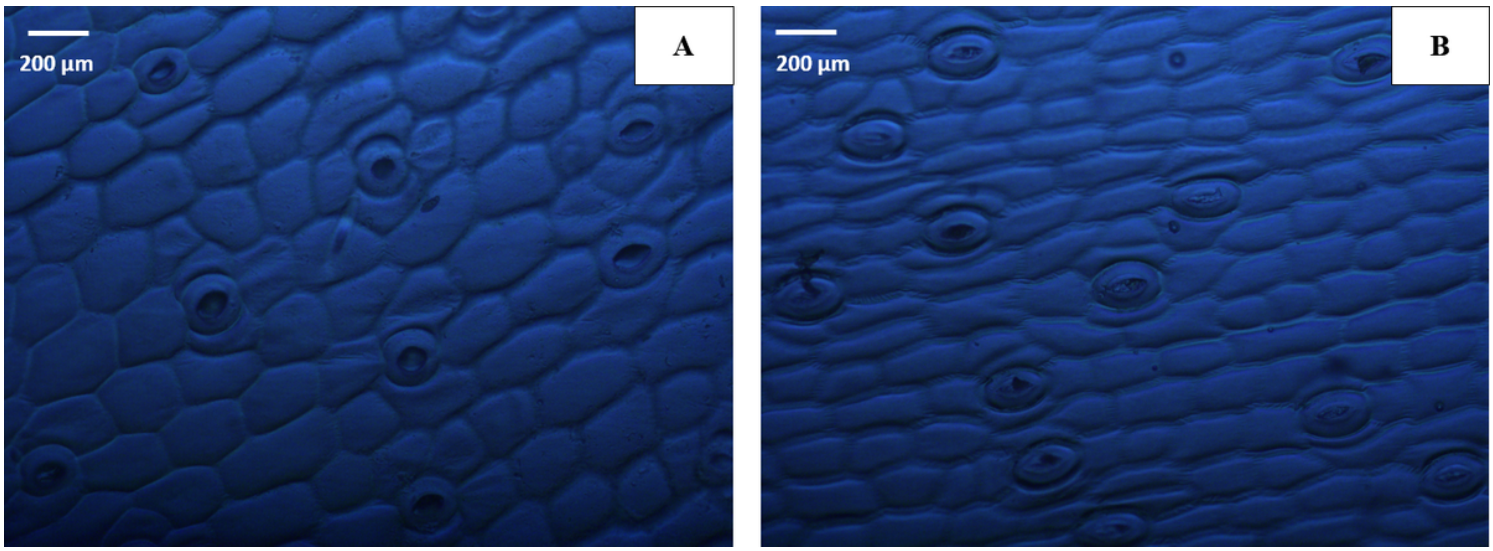

Figure 3

Stomata of the abaxial part of *Lycaste aromatica* leaves developed in MS. A) Semi-solid medium. B) Gravity immersion bioreactor.