Research Article

Micropropagation of *Cattleya maxima* J. Lindley in Culture Medium with Banana Flour and Coconut Water

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Abstract

Cattleya maxima J. Lindley is one species of the large-flowered, epiphyte and occasional lithophyte of the Orchidaceae family, seriously threatened by indiscriminate extraction and destruction of their natural habitat, climate change and pollution. Therefore, the aim of the present study was to establish a reproducible *in vitro* propagation and mass multiplication protocol for germplasm conservation of this species in natural conditions. Seeds of *C. maxima* were provided by the "Orquídeas Moyobamba" greenhouse, in Moyobamba, San Martín, Peru, of peruvian low jungle. Process began with the culture of viable seeds by hypodermic syringe method. Viability, seeds germination, the morphogenic processes of induction and multiplication of protocorm-like bodies (PLBs), elongation and differentiation of seedlings were determined. The Murashige and Skoog (MS) medium supplemented with two complex organic substances, coconut water and banana flour, was used, as well as the NAA-BAP treatment. The highest germination rate of seeds was 97.12% in treatment with 40 g/l BF and 20% CW, and in the treatment with NAA-BAP, rates of 100% formation and survival of PLBs

were reached. Rooted plantlets were successfully acclimatized and subsequently established under greenhouse conditions, with a survival rate of 70%. In this study, a relevant *in vitro* propagation protocol, using banana flour and coconut water, was established. This protocol can contribute to the *ex situ* conservation of germplasm of *C. maxima* and other endangered orchid species of the Peruvian jungle.

Keywords: Complex organic substances; Orchidaceae; PLBs multiplication; Seed viability and seed germination; Seedlings elongation

Abbreviations: BAP: 6-benzylaminopurine; BF: Banana flour; CW: Coconut water; GA_3: Giberellic acid, MS: Murashige and Skoog; NAA: α-naphtaleneacetic; PLBs: Protocorm-like bodies

1. Introduction

The Orchidaceae family belongs to the Asparagales order, which has 736 genera and 28,000 species approximately. Orchidaceae is the largest family in importance surpassing the Asparagaceae, Iridaceae and Amaryllidaceae families [1-3]. The largest genera are *Bulbophyllum* (2,000 species), *Epidendrum* (1,500 species and *Dendrobium* (1,400 species), and it is possible there are currently more than 100,000 hybrids and cultivars. However, it has been indicated that the high number of invalid, infraspecific and hybrid names, defined as "taxonomic exaggeration", is seriously affecting biodiversity conservation plans [4]. The *Cattleya* genus, native to the Amazonian region of Ecuador and distributed from southern Costa Rica to Argentina, contains 42 species and a large array of hybrids, predominantly epiphytes with floral stems growing on top of fully developed pseudobulbs [5, 6].

The most orchids are propagated by sexually and asexually in their natural habitat. Sexual reproduction via seeds is commonly reported, in contrast to apomixis, whereby seeds are clones of the mother; however, natural sexual propagation is rare as they lack endosperm, poorly organized morphologically, have undifferentiated embryos and only germinated if there is a fungal association/infection occurred in natural conditions [7, 8]. On the other hand, there are orchid species with serious difficulties in seed germination or they produce very few seeds [9]. That is why, as an alternative to *in vitro* seed germination, other techniques have been developed such as *in vitro* propagation of nodal segments from the mother plant or multiplication via protocorm like bodies (PLBs) [10].

The literature reports very few studies on micropropagation of *C. maxima* with the supplement of complex organic substances or inorganic compounds; nonetheless since Arditti [11] suggested complex organic substances can influence in seed germination, seedling growth and PLBs proliferation of orchids, numerous studies have been published using such substances in several species [12]. In a recent study, the effect concentration and interaction of chitosan and coconut water in MS culture medium [25] of nodal segments and PLBs was tested, and the combination of both chemical compounds at higher concentration (150 mg and 200 mL, respectively) resulted in a better growth of PLBs [13]. In another *Cattleya species*, *Cattleya bicolor* and a orchid double-hybrid 'BLC Pastoral Innocence', effect of different concentrations of activated charcoal (AC) or graphite, supplemented to the KC culture medium [14] was evaluated, observing that the higher induction rates of buds and roots, in *C. bicolor* seedlings, occur in the highest concentrations of graphite and AC, respectively [15]. In *Cattleya maxima* x *nobilior* hybrids, the acclimatization of seedlings and multiplied from small and medium size protocorms in KC and MS culture medium, respectively, was evaluated, observing that the highest survival rate (80%)

was better in the AP substrate (mesquite wood shaving and perlite) and the plant height was significantly higher in the PM (moss and perlite) substrate [16].

Although studies using *in vitro* tissue culture techniques are scarce in *C. maxima*, the literature reports numerous studies in other genera of orchids. In *Phalaenopsis* 'Silky Moon', one-leaf shoots from protocorms were used for PLBs induction in culture media with organic supplements as potato juice and peptone, and local fertilisers as Viking Ship (10N-20P-30K) and Saturn fert [17]. In *Cymbodium pendulum*, an epiphytic species of highly floriferous orchids, organic supplements such as banana homogenate (BH), CW and peptone were tested on protocorms multiplication, observing the highest regeneration frequency with 50 g/l BH [12]. In a new *Dendrobium* hybrid known as *D.* Alya Prink, the effect of different organic additives as homogenates of banana, tomato and CW on PLBs proliferation were evaluated, observing that only coconut water was the best organic additive tested [18]. In another *Cymbidium* species, *Cymbidium giganteum*, commonly known as 'Iris-like cymbidium', *in vitro* seedlings were used as explants for PLBs production and mass propagation, highlighting the importance of pre-treatment with 1.0 µM thidiazuron (TDZ) for 7 days in the PBLs proliferation from the base of seedlings [19]. In *Phalaenopsis amboinensis*, a rare Indonesian native orchid, Vacin and Went (VW) medium [20] was suitable for seedling development from protocorm, when coconut water was added together BH [21].

However, orchids have not always been propagated using complex organic supplements, since only growth regulator supplementation has been sufficient in several studies. In the case of *in vitro* propagation of *Guarianthe skinneri* (= *Cattleya skinneri*), a threatened orchid native of Chiapas, Mexico, plantlets regeneration from seed-derived protocorms on a MS medium supplemented with BA, IAA, NAA and GA_3 was observed [22]. Likewise in *in vitro* propagation of *Prosthechea citrina*, other threatened orchid native of Durango State, Mexico, mass propagation through protocorms cultured in MS medium with plant growth regulators was also observed [23]. Recently, it has been suggested that other factors and new technologies such the use of temporary immersion biorreactores and lighting-emitting diodes increasing PLB production efficiency [24].

The aim of this study was to evaluate two complex organic substances, coconut water (CW) and banana flour (BE), of easy access and low cost, in seed germination, induction and multiplication of protocorm-like bodies (PLBs) and seedlings rooting of *Cattleya maxima*, a very representative orchid from the northern jungle of Peru.

2. Methods

2.1 Plant material

A nine-month-old mature capsule of *Cattleya maxima* was provided by the "Orquídeas Moyobamba" greenhouse, in Moyobamba city (San Martín, Peru) of peruvian low jungle (Figure 1a and b). This study consisted three separate experiments: Seed germination, PLBs multiplication and rooting and plantlet development.

2.2 First experiment: Seed germination and sterilization, media and culture conditions

In ten samples of seeds chosen at random, the seed viability was evaluated through presence or absence of the embryo. Used the "Olympus" stereoscopic microscope (100x) and next formula was applied: V (%) = (A/B) x 100, where V is a seed viability (%), A is the total number of seed viability and B is the total number of seed tested [25, 26]. Due to fact the mature capsule was already open, for seed disinfection method of the hypodermic syringe previously sterilized with 70%

ethanol was used. The plunger was removed from the syringe and cotton was placed at the inlet and outlet of the nozzle. Using a thin spatula, seeds were placed inside the syringe and then the plunger was introduced. Disinfection process began with the hydration of seeds with sterile distilled water for 10 min. Then 70% ethanol was used for 30 sec and finally 1:100 (v/v) commercial sodium hypochlorite (Clorox® 5.25% active chlorine) containing 0.02% (v/v) Tween-20 for 7 min.

The medium used for seed germination and seedling growth was MS [27] containing 20% (v/v) coconut water and 20, 30 and 40 g/l banana flour, as the main components. The ripe banana (cv. Cavendishi of AAA genome) was peeled and cut into 2.0 cm in size and 1000 g of the diced fresh material, was oven dried at 40°C for 48 h and then ground to a very fine powder. Fruits of banana and coconut were collected from Sullana, Piura, Peru.

This phase, seed germination was also evaluated using the following formula: G (%) = (A/B) x 100, where G is seed germination (%), A is the total number of seed germination and B is the total number seed tested in 2x2 cm sample [28], estimating the total surface of culture medium in 28.3 cm^2 glass jars with approximately 536 seeds/glass jars. Germinated seed was considered with the radicle emergence.

2.3 Second experiment: Protocorm-like bodies (PLBs) multiplication

To second stage, the survival and differentiation of PLBs in MS culture medium supplemented with 20% CW and 20, 30 and 40 g/l BF were evaluated, as well as a treatment with NAA 0.1 mg/l and 1.0 mg/l BAP, without organic supplements.

2.4 Third experiment: Rooting and seedling development

For third stage, rooting and seedling development were evaluated in MS culture medium supplemented with various combinations of BF (20, 30 and 40 g/l) and CW (20 and 40%) and additional treatment with 0.5 mg/l NAA and 0.1 mg/l BAP. In all treatments 3.0 g/l (w/v) AC was added. The evaluated characteristics were plantlet height, number of shoots and number and length of leaves. Evaluations were made at 30, 60 and 90 days after culture.

Other components of the all culture medium were 2.0% sucrose and vitamins 1.0 mg/l thiamine.HCl and 100 mg/l myo-inositol. The pH all media was adjusted to 5.8 ± 0.1 prior adding 0.7% agar-agar and autoclaving. Explants were cultured in 80 ml glass jars containing 20 ml of culture medium. All cultures were incubated under 24 ± 2°C with a 16 h photoperiod at 35 µmol m^{-2} s^{-2} provided by cool-white fluorescent lights and 80% relative humidity. Each treatment comprised 15 glass jars and was performed twice.

Seedlings rooted with 1.5 to 2.0 cm high were acclimatized in sphagnus moss substrate under natural light conditions, temperature of 24-26°C and photoperiod of 11-13 h.

2.5 Statistical analysis

The data were subjected to analysis of variance (ANOVA), for each of treatments tested, using Tukey parametric test at a significance level of 95%. Statistical software SPSS version 15.0 and Microsoft Office Word, Excel version 2013 and Megastat programs were used.

3. Results

3.1 Seeds viability and germination

Seeds viability of *C. maxima*, determined by the presence or absence of zygotic embryo, was 55.87% (Table 1) (Figure 1c). The highest germination rate of

seeds (Figure 1d), determined by the radicle emergence, was 97.12% in treatment with 40 g/l BF and 20% CW, 33.67 days after the *in vitro* culture was established, although the rate of average germination, among all the treatments evaluated, was 81.89% (Table 2).

3.2 Protocorm-like bodies (PLBs) multiplication: Survival and differentiation

In all the treatments, with exception of treatment supplemented with only 20% CW, the survival rate of PLBs was 100%, including the treatment with growth regulators (0.1 NAA mg/l and 1.0 mg/l BAP), observed from first to fourth week of evaluation (Figure 1e and f). Regarding the seedlings differentiation only in the treatments with 20 and 0.0 g/l BF, the lowest rates were observed, 60.0 and 33.33%, respectively, since in the other treatments differentiation rate was 100%, reached in the fourth week of evaluation (Table 3).

3.3 Seedling development and rooting

Results obtained in the seedling height, number of shoots, number and length of leaves in *C. maxima*, after 90 days of culture, are shown in Tables 4 and 5. The highest seedling height (1.84 cm) was reached in culture medium supplemented only with growth regulators 0.5 mg/l NAA and 0.1 mg/l BAP (Figure 1g). Likewise, in the treatments with 30 g/l BE and 20% CW, those with the best response with organic supplements, the seedling height was 1.17 cm and 1.09 cm, respectively. The highest number of shoots formed (2.37 and 2.10) was reached in the treatments supplemented with 30 g/l BF and 20% CW, respectively. In relation the number of leaves formed, although the highest number (5.28) was reached in the treatment with growth regulators (NAA-BAP), in all the treatments tested the number of leaves formed was greater than 3. In all treatments tested, the length of the leaves was not greater than 1 cm.

In Table 6, Tukey test for the relationship between culture medium - culture duration - morphological responses, only showed significant differences for the treatment 0.5 mg/l NAA - 0.1 mg/L BAP - 90 days of culture - seedling height (1.84 cm). However, for the characteristics number of shoots, number and length of leaves, the differences were not significant between 60 and 90 days of culture. In treatments supplemented with CW, although values similar to those obtained with NAA-BAP were not reached, the seedlings showed optimal growth and development characteristics. On the other hand, in Table 7 the Tukey test, for the relationship between culture medium - culture duration - morphological response, no statistical differences were observed in the characteristics of seedlings height and leaf length (not shown). In the characteristic number of shoots and leaves, the treatments with 30 and 40 mg/l BF greatly surpassed the other treatments tested with 2.90 and 5.57, respectively, in 60 and 90 days of culture. As in CW trials, seedlings in media supplemented with 20, 30, and 40 mg/l BF showed optimal growth and development characteristics.

In all the culture media, with complex organic supplements or with growth regulators (NAA-BAP), a rooting rate of 100% was achieved (Figure 1h), with 1 to 3 roots formed and of length greater than one cm. The survival rate during acclimatization was around 70%, after 45 days of culture (Figure 1i).

Sample number (No)	Total seeds (No)	Viability (%)	No viability (%)
1	120	58.33	41.67
2	158	49.46	50.54
3	170	63.52	36.48
4	162	64.19	35.81
5	134	38.80	61.20
6	120	51.66	48.34
7	174	67.81	32.19
8	120	46.66	53.34
9	180	61.11	38.89
10	168	57.14	42.86
Average	150.60	55.87	44.13

Table 1: Seed viability of *Cattleya maxima* using a stereoscopic microscope.

Complex organic substances		Germination (days)	Germination (%)
Banana flour (g/l)	Coconut water (%)		
0.0	20.0	28.73 ± 8.10^{a}	55.25 ± 3.26^{a}
20.0	20.0	30.33 ± 1.84^{b}	82.99 ± 3.00^{b}
30.0	20.0	32.60 ± 2.87^{ab}	92.20 ± 1.43^{c}
40.0	20.0	33.67 ± 2.26^{ab}	97.12 ± 1.51^{d}
Average		31.37	81.89

Table 2: Seed germination of *Cattleya maxima*, after one month of culture.

Organic compounds				Responses	
Complex organic substances		Growth regulators (mg/l)			
BF (g/l)	CW (%)	NAA	BAP	Survival (%)	Seedlings differentiation (%)
0.0	20.0	0.0	0.0	40.0	33.33
20.0	20.0	0.0	0.0	100.0	60.0
30.0	20.0	0.0	0.0	100.0	100.0
40.0	20.0	0.0	0.0	100.0	100.0
0.0	0.0	0.1	1.0	100.0	100.0

MS-medium supplemented with BF-banana flour and CW-coconut wáter

Table 3: Protocorms survival and differentiation of *Cattleya maxima* seedlings, after four weeks of culture.

Complex organic substances/ Growth regulators (mg/l)			Shoot length (cm)	Shoot number (No)	Leaves number (No)
BF (g/l)	**NAA**	**BAP**			
0.0	0.0	0.0	0.69 ± 0.02^{a}	1.38 ± 0.10^{a}	3.24 ± 0.06^{b}
20.0	0.0	0.0	0.65 ± 0.02^{a}	1.09 ± 0.10^{a}	2.78 ± 0.06^{a}
30.0	0.0	0.0	1.17 ± 0.02^{c}	2.37 ± 0.10^{c}	4.98 ± 0.06^{d}
40.0	0.0	0.0	0.95 ± 0.03^{b}	2.08 ± 0.12^{c}	4.72 ± 0.07^{c}
0.0	0.5	0.1	1.69 ± 0.02^{d}	1.73 ± 0.05^{b}	5.38 ± 0.03^{e}

MS medium supplemented with activated charcoal 3.0 g/l; BF-banana flour; Mean ± standard deviation within a column followed by the same letter are not significantly different according LSD multiple range test at $p < 0.05$

Table 4: Regeneration responses of protocorms of *Cattleya maxima* on MS medium supplemented with banana extract or NAA-BAP combination, after 90 days culture.

Complex organic substances/ Growth regulators (mg/l)			Shoot length (cm)	Shoot number (No)	Leaves number (No)
CW (%)	**NAA**	**BAP**			
0.0	0.0	0.0	0.69 ± 0.02^{a}	1.38 ± 0.10^{a}	3.24 ± 0.06^{a}
20.0	0.0	0.0	1.09 ± 0.04^{b}	2.10 ± 0.12^{c}	3.81 ± 0.07^{b}
40.0	0.0	0.0	0.98 ± 0.04^{b}	1.93 ± 0.14^{b}	4.05 ± 0.06^{c}
0.0	0.5	0.1	1.84 ± 0.03^{c}	1.87 ± 0.09^{b}	5.38 ± 0.05^{d}

MS medium supplemented with activated charcoal 3.0 g/l; CW-coconut water; Mean ± standard deviation within a column followed by the same letter are not significantly different according LSD multiple range test at $p < 0.05$

Table 5: Regeneration responses of protocorms of *Cattleya maxima* on MS medium supplemented with coconut water or NAA-BAP combination, after 90 days culture.

Complex organic substances/ Growth regulators (mg/l)			Duration of culture (days)	Shoot length (cm)	Shoot number (No)	Leaves number (No)
CW (%)	**NAA**	**BAP**				
20	-	-	30	0.71 ± 0.03^{a}	1.22 ± 0.07^{c}	3.43 ± 0.11^{a}
40	-	-	60	0.76 ± 0.04^{a}	1.58 ± 0.11^{abc}	3.56 ± 0.13^{ab}
40	-	-	30	0.83 ± 0.03^{ab}	1.82 ± 0.14^{ab}	3.76 ± 0.09^{abc}
20	-	-	60	0.84 ± 0.03^{ab}	1.68 ± 0.11^{abc}	3.97 ± 0.11^{bc}
40	-	-	90	0.98 ± 0.04^{bc}	1.93 ± 0.14^{ab}	4.18 ± 0.13^{cd}
20	-	-	90	1.09 ± 0.04^{c}	2.10 ± 0.13^{b}	4.75 ± 0.11^{e}
-	0.5	0.1	30	1.52 ± 0.04^{d}	1.60 ± 0.09^{ac}	4.40 ± 0.08^{de}

-	0.5	0.1	60	1.71 ± 0.03^{e}	1.73 ± 0.09^{ab}	5.33 ± 0.08^{f}
-	0.5	0.1	90	1.84 ± 0.03^{f}	1.87 ± 0.09^{ab}	5.38 ± 0.05^{f}

MS medium supplemented with activated charcoal 3.0 g/L; CW-cocunut water; Means ± standard deviation within a column followed by the same letter are not significantly different according LSD multiple range test at $p < 0.05$

Table 6: Tukey's test in regeneration responses of protocorms of *Cattelya maxima* on MS medium supplemented with coconut water or NAA-BAP combination, after 30, 60 and 90 days of culture.

Complex organic substances/ Growth regulators (mg/l)			**Duration of culture (days)**	**Shoot number (No)**	**Leaves number (No)**
BF (g/l)	**NAA**	**BAP**			
20	-	-	30	1.00 ± 0.17^{a}	2.30 ± 0.10^{a}
0	-	-	30	1.00 ± 0.17^{a}	2.50 ± 0.10^{a}
20	-	-	60	1.10 ± 0.17^{ab}	2.63 ± 0.10^{a}
20	-	-	90	1.17 ± 0.17^{ab}	3.40 ± 0.10^{b}
0	-	-	60	1.33 ± 0.17^{abc}	3.23 ± 0.10^{b}
-	0.5	0.1	30	1.60 ± 0.08^{abcd}	4.40 ± 0.05^{d}
40	-	-	60	1.73 ± 0.24^{abcdef}	4.87 ± 0.14^{de}
-	0.5	0.1	60	1.73 ± 0.08^{abcdef}	5.33 ± 0.05^{ef}
30	-	-	30	1.77 ± 0.17^{abcdef}	4.40 ± 0.10^{cd}
0	-	-	90	1.80 ± 0.17^{abcdef}	4.00 ± 0.10^{c}
-	0.5	0.1	90	1.87 ± 0.08^{cdef}	6.40 ± 0.05^{g}
40	-	-	30	2.07 ± 0.12^{def}	4.47 ± 0.07^{d}
30	-	-	60	2.43 ± 0.17^{efg}	4.97 ± 0.10^{e}
40	-	-	90	2.47 ± 0.24^{efg}	5.60 ± 0.14^{f}
30	-	-	90	2.90 ± 0.17^{g}	5.57 ± 0.10^{f}

MS medium supplemented with activated charcoal 3.0 g/l; BF-banana flour; Means ± standard deviation within a column followed by the same letter are not significantly different according LSD multiple range test at $p < 0.05$

Table 7: Tukey's test in regeneration responses of protocorms of *Cattleya maxima* on MS medium supplemented with banana flour or NAA-BAP combination, after 30, 60 and 90 days culture.

Figure 1: In vitro propagation of Cattleya maxima. (a) flower; (b) dehiscent capsule with sedes; (c) sedes; (d) seeds germination; (e) plant regeneration by PLBs; (f) protocorms proliferation; (g) plantlets elongation; (h) roots formation and (i) acclimatization.

4. Discussion

4.1 Seed viability

In asymbiotic germination of orchid seeds, especially when capsule formation occurs under greenhouse conditions, even with manual crosspollination, it is important to carry out previous viability tests. Among the most frequent tests are staining with triphenyl tetrazolium chloride (TTC) [29] and acid fuchsin. For instance, in orchid terrestrial species, *Cypripedium reginae*, *C. parviflorum* and *Platanthera grandiflora*, the efficiency of the bioassay was similar to that of TTC and acid fuchsin for testing seed viability; however, the biassay with *Fusarium* was more appropriate for estimating embryo viability after seed pretreatment in

10% NaClO [30]. To *Cephalanthera falcata*, a terrestrial orchid species native of East Asia, *in vitro* seed viability and asymbiotic germination of immature seed was evaluated within TTC test solution and examined under a stereoscopic microscope [31]. In the case of *Aerides ringens*, an epiphytic and endemic orchid of Western Ghats, India, seeds viability was determined by TTC and fluorescein diacetate (FDA) staining methods [32]. Likewise, In Serapias vomeraceae, in addition to the TTC test, low-g forced centrifugation and stereomicroscopic examination were used as additional methods to separate the seeds without embryos from viable seeds [33]. However, observations made with a steroscopic microscope are also useful and even more so when embryo is easily observed through the seed teguments, suchs as case of the present study carried out in *C. maxima*, which allowed determining a viability rate of 55.87%. This relatively moderate percentage of viability of the *C. maxima* seed can be attributed to fact pollination of the plants was natural, although in greenhouse conditions. In general, the seed viability test is an important phase complements the later phases of germination and propagation, as has been observed in the present study in *C. maxima* and which is even useful in *in vitro* conservation of orchids [34, 35].

4.2 Seed germination

Otherwise, concerning seed germination, with 55.87% of seed viability *C. maximua*, the germination rate was 97.12%, after 33.67 days of evaluation, in culture medium supplemented with 40 mg/l BF and 20% CW. In *C. maxima*, no studies on *in vitro* asymbiotic germination of seeds are presented. In *Cattleya maxima* x *nobilior* hybrid, plant regeneration was from flowering stalkes [17] but in *C. maxima* was from nodal segments and PLBs [14], without reporting seed germination percentages.

On the other hand, the scientific literature reports similar percentages in other orchid species, as well as reported in the present study. In *Phalaenopsis* Taipei Pearl, a hybrid resulting from *Phalaenopsis* crossing (Carmela's Wild Thing x Taipei Peral), seed germination rate was 95.1% and the difference between viability (TTC test) and germination was 2.9% [34]. The *in vitro* mass propagation of *Cymbidium giganteum*, in M medium [36] 98% seeds germinated within 15 days of inoculation, significantly overcoming seed germination in MS and KC media [20]. In *A. ringens*, *in vitro* seed germination reached the highest rate (89%) in Knudson C (KC) medium supplemented with BAP and peptone [32]. Likewise, *in vitro* seeds of *Gomesa divaricatum*, *G. forbessi*, *G. praetexta* and *G. recurva*, native Brazilian orchids from Atlantic Forest, indicated orthodox behavior, with high viability rates (78 to 94%), after 12 monts of storage, using capsules obtained from manual croospollination in greenhouse conditions [37]. In *Phalaenopsis amboinensis* optimum seed germination (90.7%) was achieved on VW medium, very suitable for seedling formation, although only 51.4% of seedling development from protocorm [22]. However, in *Cephalanthera falcata* the highest frequency (39.8%) of *in vitro* asymbiotic germination of immature seed was obtained with seeds harvested 70 d after hand-pollination [31]. In most of these studies, germination rate was higher than 50%, which is in accordance with was established by Arditti [38] for the asymbiotic germination rate of tropical epiphytic orchids.

Coconut water (CW) is one of the most widely used organic substances in *in vitro* tissue culture, especially in germination of orchid seeds. Seeds of *Cattelya bicolor* and double hybrid from a cross of *Brassavola*, *Cattleya* and *Laelia* species ('BCL Pastoral Innocence'), were germinated on KC-medium containing 15% CW; however, the percentage of viability and germination of

seeds was not reported [16]. Likewise, most efficient culture medium in seeds germination (96.7%) of *Phalaenopsis* hybrids was MS supplemented with 20% CW [34]. Recently, in *Gastrochilus matsuran*, an endangered epiphytic orchid, maximal seed germination (93.3%) was achieved on ½ MS medium without 5% CW [39]. None of these studies was BF used as an organic supplement; But, in the present study carried out in *C. maxima*, only 55.25% of germination was reached with 20% CW instead with 20% CW plus 40.0 mg/L BE the germination rate was 97.12%.

4.3 Protocorm-like bodies (PLBs) multiplication: Survival and differentiation

In *C. maxima* the highest survival rate, multiplication of PLBs and seedlings differentiation, which was 100%, were achieved in culture medium supplemented with 30 or 40 g/l BF and 20% CW, and even in the treatment with 0.1 mg/l NAA - 1.0 mg/l BAP, without complex organic supplements. In a similar study by Paris et al. [14] in *C. maxima*, the best growth of PLBs was observed on MS medium with a mixture of 20% CW and 125 or 150 mg chitosan, also resulting in the lowest values of explant necrosis.

In other species of orchids as in *Aerides ringens* the highest rate of seed germination (89.28%) and protocorms formation was achieved in the medium with 4.4 µM BAP and 500 mg/L peptone, obtaining vigorous and compact green protocorms [32]. In *Cymbidium pendulum* the formation of PLBs was better in medium supplemented with 50 g/l BH, 10% CW and 2.0 g/l peptone, while 75 g/L BH was detrimental for the survival of cultures [12]. In *Dendrobium* hybrid (D. Alya Pink) the growth and proliferation of PLBs was better in medium supplemented with 10 or 20% CW than with BH or tomato homogenate [19]. To *Phalaenopsis amboinensis*, when 15% CW and 10 g/l BH was added to VW medium the highest multiplication of PLBs and seedling growth was achieved [22]. In general, in all these studies the supplement of CW, in concentrations of 10 and 20%, promoted the multiplication of PLBs, reaching high levels of survival and plantlet differentiation. The additional supplement of BH, in concentrations less than 50 g/L, substantially improved the growth and development of PLBs, like NAA-BAP treatment. All these results are in broad agreement with results shown in work presented in *C. maxima*.

4.4 Seedling development and rooting

Seedling development and rooting of *C. maxima* are essential to ensure success in the acclimatization phase. To treatment 0.5 mg/l NAA - 0.1 mg/l BAP, highest shoot elongation (1.69 cm) and the leaves number (5.38) formed were achieved, although in treatment 30 g/L BP the shoots number (2.37) formed was slightly higher. A similar result was achieved with 20% CW. All these results, to a greater or lesser degree, are related to those obtained in other species where the culture medium was supplemented with BP, CW and other complex organic substances; likewise, some cytokinin, especially BAP. As in *C. maxima*, in treatments with 125 and 250 mg chitosan supplemented with 200 mL CW, highest shoot number, size, central width leaf, and root length was reached [14]. Mass propagation of *Cymbidium giganteum* when KN was replaced by BAP [20] and in *Guarianthe skinneri* when the MS medium was supplemented with BA [23]. For *Cymbidium pendulum*, a high number of shoots/explant, 7.75 and 9.0, reached when medium was supplemented with 50 g/l BH and 10% CW, respectively [12]. In other species of orchids as in *Dendrobium* sp. the highest number of shoots (40.3) was achieved in ½ MS culture medium supplemented with 10% Sabri banana pulp and with 10% CW (22.3) [40]. In *Phalaenopsis amboinensis*, with 15% CW and 10 g/L BH highest seedling height (62.1 mm) and leaf length (36.7 mm) were reached [22].

The supplement of 3.0 g/l AC, in all treatments tested, contributed to modulate morphogenic processes during seedling elongation and rooting, evidenced in growth and development in treatment without complex organic substances and growth regulators. There are few studies on the micropropagation of orchids where culture medium was supplemented with AC. In *Cattleya bicolor* seedlings largest number of roots occurred on media with 6.0 g/l AC and in the hybrid 'BLC Pastoral Innocence', the largest number of shoots and roots occurred in medium with 4.5 g/l AC [16]. The beneficial effects of AC in *in vitro* plant tissue culture may be due to darkening of the culture medium or adsorption of growth regulators and other organic compounds, which favors roots induction [41].

Regarding the relationship composition of the culture medium - growth and development of the plant material - evaluation days, although in the present research carried out in *C. maxima* the evaluation was after 90 days of culture, in other studies the evaluation depended on the species and composition of culture medium. As long evaluation periods as 6 months have been reported in *C. bicolor* [16], a 12-week half period as in *C. maxima* [14] and *Cymbidium pendulum* [12] and 8-week short period as in *Aerides ringens* [32] and *Dendrobium* sp. [40].

Coconut water or liquid coconut endosperm is one of the most widely used organic compounds in tissue culture due to its high content of sugars, amino acids, antioxidants, minerals, organic acids and growth regulators, especially cytokinins [42, 43]. A review of pharmacological activities and their beneficial effects in plant tissue culture has recently been published by Kamaruzzaman et al. [44]. On the other hand, the chemical composition of banana powder, banana flour, banana extract or banana homogenate shifted according to the variety with a range of 61-76.5% starch, 19-23% amylose, 2.5-3.3% protein, and 0.3-0.8% lipids [45]. As both organic compounds vary significantly in quality and quantity, in its chemical composition, the culture medium is considered "undefined medium, basal or compex médium" [46] and in some way, to minimize the effect of such variation, in this study, the fruits were collected from specific plantations located in Sullana (Piura, Peru). So too, the cytokinins BAP, KIN o TDZ, in low concentrations have been reported to be decisive for shoot proliferation and elongation in several orchids species as *Aerides ringens* [32], and supplemented with auxins, in the rooting of *Vanda spatulata* [47] and *Oncidium* sp. [48]. In general, complex organic supplements such as CW, BF, and NAA-BAP combination stimulated seedling elongation and rooting.

5. Conclusion

This study presents a protocol of seed germination, multiplication of protocoms-like bodies (PLBs), elongation of plantlets and rooting of *Cattleya maxima*, using complex organic substances such as banana flour (BF) and coconut water (CW). These compounds are easily accessible and inexpensive for tissue culture laboratories initiate orchid propagation activities. In this way, there is a useful tool enables the large-scale propagation, not only of *C. maxima* but also of other orchid species threatened by climate change and the destruction of their natural habitat.

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Conflict of Interest

The authors declare no competing financial interest.

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