Effect of microalga Desmodesmus subspicatus, polyamines and plant growth regulators on the in vitro propagation of Cattleya warneri

Quezia Rocha Navarro
Universidade Federal do Parana
Diego de Oliveira Corrêa
Universidade Federal do Parana
Alexandre Behling
Universidade Federal do Parana
Miguel Daniel Noseda
Universidade Federal do Parana
Luciana Lopes Fortes Ribas (llfribas@ufpr.br)
Universidade Federal do Parana Setor de Ciencias Biologicas https://orcid.org/0000-0003-1099-1090

Research Article

Keywords: Microalgal biomass, Microalgal extract, Micropropagation, Orchidaceae, Protocorm, Thin cell layer

Posted Date: August 9th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1894253/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on January 18th, 2023. See the published version at https://doi.org/10.1007/s11240-022-02442-x.

Abstract

Cattleya warneri, an orchid with high ornamental potential, suffers indiscriminate harvest and is classified as a vulnerable species, requiring propagation studies. The aim of this study was to evaluate the effect of biomass or aqueous extract of *Desmodesmus subspicatus* microalga, polyamines (PAs) and plant growth regulators (PGRs): 6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA) on the *in vitro* propagation of *C. warneri* using the thin cell layer (TCL) technique. Entire protocorms and transversal sections (tTCLs) or longitudinal sections (lTCLs) were grown in MS culture medium, with the concentration of macronutrients reduced by half (MSM/2) containing *D. subspicatus* biomass or extract Pas, and BA and/or NAA. The results indicated that the apical tTCLs were the best explants and the entire protocorms formed seedlings. The regeneration of protocorm-like bodies (PLBs) was more efficient with the addition of 1.5 g L^{-1} of microalgae biomass (92% and 4.7 PLBs) or 1.0 g L^{-1} of extract (100% and 3.3 PLBs) and 8 µM BA (89% and 5.2 PLBs). The biomass (1.0 g L^{-1}) or extract (2.0 g L^{-1}) also enabled PLBs regeneration from entire protocorms (70% and 4.0 PLBs, 50% and 3.2 PLBs, respectively). The plant acclimatization was recommended using coconut fiber and vermiculite (1:1, v/v) as substrate (survival rate 84%% and greater fresh mass 0.895 g). In conclusion, a rapid and efficient protocol for the mass propagation of *C. warn*eri was achieved using the TCL technique. In addition, MSM/2 medium supplementation with biomass and extract of *D. subspicatus* is an effective alternative to replace conventional PGRs.

Key Message

Complete protocol for micropropagation of *Cattleya warneri* using TCL technique was established. Apical tTCL of protocorm was the most responsive explant and *Desmodesmus subspicatus* microalga is alternative to replace PGRs.

Introduction

Cattleya is one of the most popular orchid genera. The beauty of its flowers accounts for its high demand in the global floriculture trade and is highly priced in national and international markets (Pant et al. 2020). *Cattleya warneri* T. Moore is an epiphytic species, which has one of the largest flowers of its genus, with high ornamental potential and a high market value (CNCFlora 2012). For a long time is suffering great extraction from nature for commercial purposes and destruction of its habitat. Taking into account its restricted distribution and fragmented habitat, this species was classified as vulnerable (CNCFlora 2012), requiring propagation studies. One of the factors that contributes to this vulnerability of orchids is that after pollination, they produce capsules with numerous microscopic seeds, which do not have endosperm as a reserve tissue and depend on specific mycorrhizal fungi to germinate. Thus, the germination rate in nature is very low, preventing species from spreading efficiently (Gupta 2016). As a result of these reproductive characteristics, asymbiotic germination and micropropagation of orchids have been efficient alternatives for seedling production (Cardoso et al. 2020).

Plant tissue culture technique has been extensively used in multiplication of orchids (Lal and Singh 2020) and thin cell layer (TCL) has advantages when compared to conventional micropropagation, as it allows a greater contact surface of the tissues with the culture medium and reduces the time to produce plants (Teixeira da Silva and Dobránszki 2014). Their higher productivity compared to conventional explants has made TCLs continually relevant and useful in plant research and applied plant biotechnology (Teixeira da Silva and Dobránszki 2019). Another benefit of this technique is that the stress response caused by the injury can induce the formation and differentiation of protocorm-like bodies (PLBs) allowing the achievement of high multiplication rates (Teixeira da Silva and Dobránszki 2019). According to Cardoso et al. (2020), the process of induction, multiplication and regeneration of PLBs is one of the most advantageous methods for the mass propagation of orchids. Thus, the TCL technique is a promising alternative for endangered species, such as *C. warneri.*

Orchid embryos, when placed in an appropriate culture medium, will initially form protocorms, and the ability of apical protocorm cells to divide makes them ideal explants for micropropagation studies (Yeung 2017). In addition to selecting the best explant, the TCL technique depends on choosing a suitable culture medium and adding plant growth regulators (PGRs). Various formulations of culture media have been used with the TCL technique, such as: MS (Murashige and Skoog 1962) and their modifications. The most used PGRs for inducing and multiplying PLBs are: cytokinins, alone or combined with auxins, such as: benzyladenine (BA), thidiazuron (TDZ), indole-3-butyric acid (IBA), α-naphthalene acetic acid (NAA) and concentrations vary according to the species (Cardoso et al. 2020; Lal and Singh 2020). An alternative, to reduce costs and improve the efficiency of micropropagation protocols, is to test the addition of biostimulant compounds, as partial or total substitutes for PGRs. Biostimulants are products that, applied in small quantities, stimulate the growth and development of many cultures, both under optimal conditions and under stress (Ronga et al. 2019). Studies using microalgae for *in vitro* cultivation are scarce. Corbellini et al. (2020) found that the addition of microalgae from the Chlorophyceae family to the culture medium was efficient for induction and PLBs regeneration of *Cattleya labiata* and that these biostimulants can replace the use of conventional PGRs. Previous results with biomass and extract from the microalga *Desmodesmus subspicatus* (Chodat) E. Hegewald & A. W. F. Schmidt (Scenedesmaceae) showed a positive effect on the asymbiotic germination of *C. warneri* (Navarro et al. 2021), nonetheless more studies are needed to verify its efficiency with the TCL technique.

Other additives that can be used on *in vitro* culture are polyamines (PAs), which are organic polycationic compounds, consisting of two or more amino groups, used to improve the growth and development of plants, due to their effects on cell division and differentiation (Abbasi et al. 2017). The main PAs present in the superior plants are: spermidine (Spd), spermine (Spm) and putrescine (Put) (Abbasi et al. 2017). According to Rakesh et al (2021), Spd, Spm and Put can also be used to study the effects of PAs in various fields of tissue culture. They have been tested in organogenesis, as they have effects on cell division and elongation, rooting and the adventitious formation of shoots (Chen et al. 2019; Rakhesh et al. 2021). The addition of PAs in the culture media, associated or not with cytokinins and / or other PGRs has also demonstrated a positive effect in several stages of orchids micropropagation, including the TCL technique (Bhattacharyya et al. 2016; Mandal et al. 2020).

The regenerated PLBs can be grown in culture medium, without PGRs and with the addition of activated charcoal to stimulate elongation and root development. This strategy has been effective for some orchids, such as *Hadrolaelia grandis* (Vudala et al. 2019)d *labiata* (Corbellini et al. 2020).

The last step of micropropagation is transplanting and acclimatization of seedlings, requiring special care to obtain high percentages of survival. One of the factors that interfere in this stage is the choice of a suitable substrate for transplanting. Porosity in the growing substratum is essential for *Cattleya* epiphytic roots. Sphagnum moss, coconut fiber, and pine bark were also used for *Cattleya* propagation (Mercado and Contreras 2017; Corbellini et al. 2020; Pant et al. 2020).

There are no studies testing the efficiency of adding extract or biomass of microalgae *D. subspicatus* or PAs as substitutes for the addition of PGRs in the micropropagation of *C. warneri*. Because of the commercial importance of this species and it is threatened with extinction, the objective of this study was to establish a protocol for the mass propagation of *C. warneri* seedlings using the TCL technique, from protocorms. The effects of section type, addition, and concentration of PGRs, PAs and aqueous extract or biomass of microalgae *D. subspicatus* were tested to obtain higher regeneration rates of PLBs to reduce costs and accelerate the large-scale production of *C. warneri* seedlings.

Materials And Methods

Microalgae biomass and aqueous extract

The microalgae *D. subspicatus* was cultivated in a 100 L flat plate photobioreactor for 14 days, with BBM culture medium (Nichols and Bold 1965), continuous illumination (155 µmol m^{-2} s^{-1}), and controlled temperature (23 ± 1 °C). The microalgal biomass was recovered by flocculation (Corrêa et al. 2019), centrifuged (2600 x g, 20 min, 4 °C) and freeze-dried. The extract was obtained from dried biomass by aqueous extraction as described by Mazepa et al. (2021).

The composition of biomass and aqueous extract was presented by Navarro et al. (2021) and consisted of the determination of carbohydrates (Dubois et al. 1956), proteins (Bradford 1976), lipids (Bligh and Dyer 1959), and phytohormones by HPLC-MS.

Plant material, disinfestation and *in vitro* seed germination

Seeds of four mature capsules from manual cross-pollination of three *C. warneri* plants (Fig. 1A) were collected from the Botanical Institute of São Paulo (São Paulo, Brazil) and stored in a refrigerator (4 °C) for 14 months. To verify the viability of the seeds, they were submitted to the tetrazolium test according to the methodology described by Hosomi et al. (2011, 2017).

The seeds (40 mg) were immersed in a 1% (v/v) sodium hypochlorite solution (NaOCl) plus 0.1% (v/v) Tween 20®, for 10 min with shaking. The seeds with the disinfestant solution were transferred to a glass funnel covered with sterile filter paper and rinsed six times with sterilized distilled water. After drying the seeds on the filter paper, they were sown in Petri dishes, containing MS culture medium, with the concentration of macronutrients reduced by half (MSM /2). Each Petri dish contained approximately 400 seeds and 16 repetitions. *In vitro* germination was performed to obtain 90 and 120-days-old protocorms for the TCL experiments with PGRs and PAs. In parallel, germination was performed in MSM/2 medium, supplemented with: 0, 0.25, 0.5, 1.0, and 2.0 g L^{-1} of biomass or aqueous extract of microalga *D. subspicatus.* Each treatment consisted of eight repetitions. Germination with the addition of the microalgae was carried out to obtain protocorms of 90-days-old that were used in a TCL experiment.

Thin cell layer (TCL)

Protocorms with apex and rhizoids from 90 and 120 days of germination in MSM/2 culture medium were used as explants (Fig 1B). Entire protocorms (2 mm thick) and sectioned into two-1 mm-thick transverse sections (apical tTCL and basal tTCL) and two longitudinal sections (lTCLs) were tested. The TCL sections were inoculated with the cut region in contact with the culture medium.

The experimental design was completely randomized, and in each Petri dish: eight entire protocorms, eight tTCLs from each section (apical and basal) and eight lTCLs were cultivated, with eight replications, totaling 32 explants per plate.

Effect of PGRs and PAs on the PLBs regeneration

The 90-days-old explants were inoculated in Petri dishes with MSM/2 culture medium, supplemented with 0, 4.0, 8.0, and 16.0 µM BA and 0, 2.0, and 4.0 µM NAA and with combinations of BA and NAA (Sigma, Aldrich, St. Louis, Mo, USA), totaling 12 treatments.

The 120-days-old explants were inoculated in MSM/2 culture medium, supplemented with the following concentrations of the PAs: Put, Spm and Spd (Sigma, Aldrich): 0.25, 0.5, 1.0, and 1.5 mM and a control (without addition of PA). The PAs were sterilized using a 0.22 µm filter and added to the autoclaved culture media.

Effect of biomass or aqueous microalgae extract on the PLBs regeneration

The 90-days-old explants from *in vitro* germination: entire protocorms (2 mm thick), apical tTCLs, basal tTCLs and lTCLs (1 mm thick) were inoculated in MSM/2 culture medium, supplemented with: 0, 0.25, 0.5, 1.0, and 2.0 g L^{-1} of biomass or

aqueous extract of microalga *D. subspicatus*.

The explants of the experiments with PGRs, PAs and biomass or extracts of microalgae were subcultured twice for media containing the same treatments, every 90 days. The evaluations were carried out after the two subcultures, considering the following variables: the percentage of PLBs regeneration, the average number of PLBs per explant and percentage of seedling formation for the entire protocorms.

Elongation and root development

The explants from the TCLs experiments with PGRs, PAs and microalgae, approximately 0.5 cm in length, were inoculated in flasks containing MSM/2 culture medium, supplemented with 0, 1.0, 2.0 and 4.0 g L^{-1} activated charcoal. Six shoots were inoculated per flask and 36 replicates, totaling 216 explants per treatment. After 120 days of cultivation, rooting percentage, average number of roots, average length of the largest root, average length of the aerial part and fresh mass were evaluated.

Transplanting and acclimatization in greenhouse

After 120 days of cultivation in MSM /2 medium, containing activated charcoal, the plants, at 2 cm long, were individualized, their roots washed and planted in sowing trays containing 128 cells (52 cm x 26 cm), each cell with 4.5 x 4.5 cm^2, containing different substrates (0.5 g per cell). The plants from the TCL experiment with PGRs, PAs and microalgae biomass and extract were planted on the following substrates: 1- sphagnum, 2- fine-grained vermiculite Eucatex®, 3- sphagnum and pine bark in the ratio 1: 1 (v/v) and 4- pine bark, charcoal and coconut fiber in a 1: 1: 1 (v/v) ratio, 5- vermiculite and pine bark in a 1: 1 (v / v) ratio and 6- vermiculite and coconut fiber in a 1: 1 (v/v), 7- Maxfertil® commercial substrate (pine bark, rice husk, vermiculite and NPK) and 8- Forth® commercial substrate containing decomposed pine bark and ash. Each treatment consisted of eight plants and 10 repetitions. Plants were manually irrigated every two days. The survival percentage was evaluated monthly. After nine months, in addition to the percentage of survival, other variables were evaluated: fresh mass, average length of the aerial part and of the largest root and average number of roots.

Culture medium and culture conditions

The culture medium used was MSM/2, supplemented with 5.4 g L^{-1} of Vetec® agar and 3% sucrose (w/v). The pH of the media was adjusted to 5.8 with 1 N NaOH or 1 N HCl, before adding the agar. The culture media was sterilized at 121 °C at 1.06 Kg cm^{-2} pressure for 20 min.

For *in vitro* germination and TCL experiments (with the addition of PGRs, PAs and biomass or aqueous extract of microalga *D. subspicatus*) 30 mL of culture medium was placed in each Petri dish (200 x 150 mm). For shoot elongation and root development, the explants were placed in 100 mL glass flasks (65 x 80 mm) containing 40 mL of culture medium and closed with polypropylene caps.

The disinfestation, *in vitro* germination, and experiments using the TCL technique were performed in a laminar flow hood.

In vitro cultures were maintained in a growth room, with a temperature of 22 ± 2 °C, a photoperiod of 16 h and photosynthetically active radiation of 73 µmol m^{-2} s^{-1} (*in vitro* germination and TCL experiments) and 40 µmol m^{-2} s^{-1} (elongation and root development) provided by Empalux LED tube lamps (20 W, white light 6500 K). The transplanted seedlings were kept in a greenhouse, with an average temperature of 19 ± 3 °C, a minimum of 14 ± 3 °C and a maximum of 22 ± 3 °C, a photoperiod of 12 h and photosynthetically active radiation of 50 µmol m^{-2} s^{-1}, supplied by a mercury vapor lamp (250 W).

Statistical analysis

The experimental design was completely randomized. The data from the TCL, elongation and acclimatization experiments were subjected to analysis of variance (ANOVA) and the means were compared using the Tukey`s test at the 5% level of significance. Regression analysis was used for data from TCL experiments with microalgae and also in the elongation and rooting step. The data was transformed when necessary to achieve the F test assumptions. The analyzes were performed using the Julia 1.1.0 (julialang.org/), programming language (Julia, 2021) in which functions for data analysis and graphic representation were developed by the authors.

Results

TZ viability test and *in vitro* germination

The result of the TZ test indicated 94.3% of viable seeds, 1.9% of non-viable seeds and 3.8% without embryo, after 14 months of storage at 4 ^{o}C. *In vitro* germination was efficient in MSM/2 culture medium, and after 90 days protocorms with apex and rhizoids (Fig. 1B) were obtained for experiments with PGRs and biomass or extract of microalga *D. subspicatus* and 120 days for that of PAs.

TCL experiments

Effect of PGRs (BA and / or NAA) on the PLBs regeneration

The analysis of variance indicated that the interaction between the type of explant and PGR was significant for the variables: percentage of explants that regenerated PLBs and average number of PLBs. For the entire protocorms that formed seedlings, there was a significant difference between treatments, after 90 days of the second subculture.

The entire protocorms showed low percentages of explants that regenerated PLBs, with no difference between the PGRs tested (Table 1). The best responses occurred with explants grown on media containing 8 mM BA (on average 45% of PLBs and 2PLBs per explant). Apical tTCLs were the most responsive explants and the addition of PGRs also did not influence the regeneration responses of PLBs. However, for basal and lTCLs, the highest percentages of PLBs regeneration occurred with the addition of 8 µM of BA in the MSM/2 culture medium (68.75 and 87.50%, respectively), differing significantly from the other treatments with PGRs (Table 1). In general, the percentage responses of PLBs regeneration were higher with apical tTCLs, followed by longitudinal, entire protocorms, and basal tTCLs (Table 1).

The average number of PLBs was not influenced by the addition of PGR, both for the entire protocorms, as well as for the tested TCLs (Table 1). With the addition of BA and NAA isolated and combined, the TCLs showed higher numbers of PLBs than with the entire protocorms. The best responses were obtained with apical tTCLs, lTCLs and basal tTCLs, respectively, in most treatments with the addition of isolated or combined PGRs (Table 1).

The entire protocorms grown in medium containing 4 µM NAA showed the highest percentage of plantlet formation (95.83%), being significantly higher than that obtained with 8 and 16 µM BA and with the combinations of BA and NAA (4, 8, 16 µM BA and 4 µM NAA or 4, 8, 16 µM BA and 2 µM NAA) (Table 1).

The combined analysis of the percentage and average number of PLBs indicated that apical tTCLs (89.58% and 5.2 PLBs) (Fig. 1C) or lTCLs (87.50% and 4.8 PLBs) should be used in MSM/2 culture medium, containing 8 µM BA. The entire protocorms showed the highest percentages of seedling formation and it was not necessary to add PGRs.

Table 1 Effect of the explant type of *Cattleya warneri* on the regeneration of protocorm-like bodies (PLBs) after second subculture (120 days) cultivated in MSM/2 (MS, with the half concentration of macronutrients) containing different concentrations of 6-benzyladenine (BA) and α-naphthaleneacetic acid (NAA).

Treatments	PLBs regeneration (%)				Average number of PLBs per responsive explant				Plantlets formation (%)
BA and NAA (µM)	Entire Protocorms	Apical tTCL	Basal tTCL	lTCL	Entire Protocorms	Apical tTCL	Basal tTCL	lTCL	Entire protocorms
Control	4.16 a C	68.75 a A	4.17 b C	45.83 b B	2.0 a A	2.4 a A	1.0 a A	2.0 a A	87.50 a b
4 + 0	14.58 a B	66.67 a A	4.17 b B	29.17 b B	1.4 a A	3.4 a A	5.5 a A	3.5 a A	81.25 a b c
8 + 0	47.92 a C	89.58 a A	68.75 a B	87.50 a A	1.9 a B	5.2 a A	6.0 a A	4.8 a A	41.67 d
16 + 0	45.83 a B	66.67 a A	16.67 b C	41.67 b B	1.5 a B	6.9 a A	5.6 a A	4.9 a A	50.00 c d
0 + 2	10.42 a C	70.83 a A	4.17 b C	45.83 b B	3.4 a A	3.2 a A	3.5 a A	3.1 a A	87.50 a b
0 + 4	4.17 a C	72.92 a A	4.17 b C	50.00 b B	4.5 a A	4.6 a A	3.5 a A	3.2 a A	95.83 a
4 + 2	20.83 a B	52.08 a A	8.33 b B	27.08 b B	2.6 a B	3.9 a B	6.7 a A	3.3 a B	68.75 a b c d
4 + 4	25.00 a B	58.33 a A	6.25 b C	35.42 b B	2.1 a B	5.2 a A	5.7 a A	4.4 a A	64.58 b c d
8 + 2	43.75 a B	81.25 a A	4.17 b C	43.75 b B	2.2 a B	6.5 a A	2.0 a B	3.9 a B	47.92 d
8 + 4	43.75 a B	66.67 a A	0.00 b D	20.83 b C	2.3 a B	6.7 a A	0.0 a C	6.9 a A	52.08 c d
16 + 2	45.83 a A	56.25 a A	4.17 b B	60.42 b A	1.9 a B	5.3 a A	5.0 a A	4.9 a A	45.83 c d
16 + 4	29.17 a B	70.83 a A	6.25 b C	39.58 b B	1.7 a B	6.2 a A	4.3 a A	5.9 a A	64.58 c d

tTCL = transverse Thin Cell Layer; lTCL = longitudinal Thin Cell Layer

Different lower-case letters in the columns and different uppercase letters in the lines differ statistically by Tukey's test $P \leq 0.05$.

Effects of PAs on PLBs regeneration

The evaluation of the analysis of variance indicated that the type of explant and the concentration of Put had a significant influence on the percentage of explants that regenerated PLBs. The interaction of the type of explant and the concentration of Put was significant for the average number of PLBs. For the entire protocorms there was no difference between treatments for the percentage of plantlet formation, after 90 days of the second subculture.

Apical tTCLs showed better PLBs regeneration responses (85.42%), followed by lTCLs, basal tTCLs and entire protocorms, with only 5% (Table 2). The addition of 0.25 mM of Put in the MSM/2 medium provided the lowest percentage of PLBs regeneration (30.73%), differing significantly from the other treatments (Table 2).

Table 2 Percentage of protocorm-like bodies (PLBs) regeneration of *Cattleya warneri* after second subculture (120 days) cultivated in MSM/2 (MS, with the half concentration of macronutrients) containing putrescine.

Treatments			
Putrescine (mM)	**PLBs regeneration (%)**	**PLBs regeneration (%)**	
0	40.10 a	**Explant type**	
0.25	30.73 b	entire protocorms	5.00 d
0.5	42.19 a	tTCL apical	85.42 a
1.0	42.19 a	tTCL basal	12.08 c
1.5	40.10 a	lTCL	53.75 b

tTCL = “thin cell layer” transverse; lTCL = “thin cell layer” longitudinal

Different lower-case letters in the columns differ statistically by Tukey’s test P≤0.05.

The average number of PLBs did not differ between the types of explants and the Put concentrations had influence only for the basal tTCLs, with a better response (4.3) obtained with the addition of 0.5 mM to the MSM/2 medium, when compared with the other concentrations (Table 3, Fig. 1D). Entire protocorms also formed high percentages of seedling formation, regardless of the Put concentration (Table 3).

The general analysis of the variables indicated the use of apical tTCLs, not requiring the addition of Put for the PLBs regeneration. The entire protocorms formed seedlings, regardless of the addition of Put.

Table 3 Average number of protocorm-like bodies (PLBs) per responsive explant and percentage of plant formation from entire protocorms of *Cattleya warneri* after second subculture (120 days) cultivated in MSM/2 (MS,with the half concentration of macronutrients) containing putrescine.

Treatments	**Average number of PLBs per responsive explant**				**Plantlets formation (%)**
Putrescine (mM)	Entire protocorms	Apical tTCL	Basal tTCL	lTCL	Entire protocorms
0	3.0 a A	1.9 a A	1.7 b A	2.2 a A	95.83 a
0.25	2.0 a A	3.0 a A	2.0 b A	2.7 a A	97.92 a
0.5	4.0 a A	2.3 a A	4.3 a A	2.7 a A	95.83 a
1.0	3.8 a A	2.6 a A	1.7 b A	3.5 a A	87.50 a
1.5	3.0 a A	2.8 a A	2.5 b A	3.3 a A	93.75 a

tTCL = transverse Thin Cell Layer; lTCL = longitudinal Thin Cell Layer

Different lower-case letters in the columns and different uppercase letters in the lines differ statistically by Tukey’s test P≤0.05.

The variance analysis of the experiment with the addition of Spm indicated that the type of explant had a significant effect on the percentage of explants that regenerated PLBs and for the average number of PLBs. For the entire protocorms there was no significant difference between the treatments for seedling formation, after 90 days of the second subculture.

The explants that showed high percentages of PLBs regeneration were the apical tTCLs (88.33%), followed by the lTCLs (57.50%), with the basal tTCLs and the entire protocorms obtaining the lowest percentages (12.08% and 5.83%, respectively) (Table 4). The lowest average number of PLBs occurred with basal tTCLs, when compared with other types of explants (Table 4). The entire protocorms showed high percentage of seedling formation (above 83.33%), regardless of the addition of Spm (Table 4).

The general analysis of the variables indicated that for greater PLBs regeneration, it is recommended to use apical tTCLs, without adding Spm (Fig. 1E). As with Put, the entire protocorms formed the highest percentage of seedlings.

Table 4 Effect of the explant type *of Cattleya warneri* on the regeneration of protocorm-like bodies (PLBs) and percentage of plant formation from entire protocorms after second subculture (120 days) cultivated in MSM/2 (MS, with the half concentration of macronutrients) containing spermine.

Explant type	**PLBs regeneration (%)**	**Average number of PLBs per responsive explant**
entire protocorms	5.83 c	2.6 a
tTCL apical	88.33 a	2.4 a
tTCL basal	12.08 c	1.7 b
lTCL	57.50 b	2.6 a
Plantlets formation (%) (Entire protocorms)		
Spermine (mM)		
0	95.83 a	
0.25	95.83 a	
0.5	83.33 a	
1.0	97.92 a	
1.5	97.92 a	

tTCL = transverse Thin Cell Layer; lTCL = longitudinal Thin Cell Layer

Different lower-case letters in the columns differ statistically by Tukey's test $P \leq 0.05$

The analysis of variance indicated that the interaction between the type of explant and the Spd concentration was significant for the percentage of explant that regenerated PLBs. For the average number of PLBs, only the type of explant had a significant influence. For the entire protocorms, there was no significant difference between the treatments for seedling formation, after 90 days of the second subculture.

The apical tTCLs showed high percentages of PLBs regeneration in all treatments with Spd, followed by lTCLs, with the entire protocorms and basal tTCLs showing low percentages of regeneration (Table 5, Fig. 1F).

The entire protocorms showed high percentages of seedling formation, regardless of the addition and concentration of Spd (Table 6). Although the percentage of PLBs regeneration of the entire protocorms was low, it was these explants that showed better responses to the average number of PLBs (3.5) (Table 5).

The general analysis of the variables indicated that for the maximum regeneration of PLBs it is recommended to use apical tTCLs, grown in MSM/2 medium, without the addition of Spd (Table 5).

Table 5 Effect of the explant type of *Cattleya warneri* on the regeneration of protocorm-like bodies (PLBs) and percentage of plant formation from entire protocorms after second subculture (120 days) cultivated in MSM/2 (MS, with the half concentration of macronutrients) containing different concentrations of spermidine.

Treatments	**PLBs regeneration (%)**				**Plantlets formation (%)**
Spermidine (mM)	Entire protocorms	Apical tTCL	Basal tTCL	lTCL	Entire protocorms
0	4.17 a D	83.33 a A	18.75 a C	54.17 a B	95.83 a
0.25	8.33 a C	97.92 a A	10.42 a C	64.58 a B	91.67 a
0.5	10.42 a C	100.00 a A	14.58 a C	68.75 a B	89.58 a
1.0	18.75 a C	100.00 a A	4.17 a D	60.42 a B	81.25 a
1.5	10.42 a C	91.67 a A	2.08 a C	58.33 a B	89.58 a
Average number of PLBs per responsive explant - Explant type					
entire protocorms			3.5 a		
tTCL apical			2.3 b		
tTCL basal			1.8 b c		
lTCL			2.6 b		

tTCL = transverse Thin Cell Layer; lTCL = longitudinal Thin Cell Layer

Different lower-case letters in the columns and different uppercase letters in the lines differ statistically by Tukey's test $P \leq 0.05$.

Effect of *D. subspicatus* biomass or extract on PLBs regeneration

The apical tTCLs were the explants with the highest regeneration rate of PLBs (above 85%), followed by the entire protocorms and lTCLs and with the lowest percentages obtained for basal tTCLs (Fig. 2A). The high percentages of PLBs were obtained in apical tTCLs, regardless of the addition and concentration of microalgal biomass. Likewise, basal tTCLs were not influenced by the addition of biomass, and they achieved the lowest percentages of PLBs. It is not necessary to add biomass for lTCLs and for entire protocorms the best responses would occur if 1.5 g L^{-1} of biomass were added (Fig. 2A). The biomass concentration only influenced the percentage of PLBs regeneration on entire protocorms, with higher values obtained with the highest concentrations (1.0 and 2.0 g L^{-1}), with 71.88 and 73.44%, respectively, while without addition of biomass, only 7.81% occurred (Fig. 2A).

The highest mean numbers of PLBs occurred with apical tTCLs and lTCLs, and were superior to the control (without addition of biomass) (Fig. 2B). The results in Figure 2b showed that the highest mean numbers of PLBs would occur with the addition of 1.5 g L^{-1} of biomass for apical tTCLs, lTCLs and entire protocorms, and with 0.5 g L^{-1} for basal tTCLs.

With entire protocorms, the formation of plantlets was highest on the medium without added biomass (92.19%) and decreased as the concentration increased (Fig. 2C).

The general analysis of the results indicated that for higher regeneration of PLBs, apical tTCLs should be used and MSM/2 medium supplemented with 1.5 g L^{-1} of *D. subspicatus* biomass (Fig. 1G). Entire protocorms were efficient explants for PLBs formation, and the addition of this same concentration (1.5 g L^{-1}) of biomass was recommended (Fig. 1H). The entire protocorms also regenerated seedlings without adding biomass.

Regarding the addition of aqueous extract, the apical tTCLs were the most responsive explants for the regeneration of PLBs (above 86%), with no differences between the extract concentrations tested, followed by lTCLs, and the entire protocorms and basal tTCLs showed low percentages of PLBs regeneration (Fig. 3A). The analysis of Figure 3A, showed that it is not necessary to add extract to induce regeneration of PLBs in basal tTCLs and lTCLs, while for apical tTCLs can be recommended 1.0 g L^{-1} and for entire protocorms 2.0 g L^{-1} (Fig. 3A).

The type of explant did not influence the average number of PLBs and analyzing fig 3B, the best response occurred with the addition of 1.0 g L^{-1} of the extract. The percentage of seedling formation of entire protocorms was higher in treatments without extract than with addition of 0.25, 1.0, and 2.0 g L^{-1} (Fig 3C).

The combined analysis of the variables indicated that the apical tTCLs were the most responsive explants in MSM/2 culture medium, containing 1.0 g L^{-1} of microalgae extract (Fig. 1I). The entire protocorms induced 50% regeneration of PLBs, with the addition of 2.0 g L^{-1} of extract. It is not necessary to add extract in the culture medium, when entire protocorms are used for seedling formation.

3.4 Effect of activated charcoal on elongation and rooting

The rooting percentage was 100%, regardless of the addition of activated charcoal. The average number of roots and the average length of the largest root was higher in all treatments containing activated charcoal (Figs. 4A, 4B and 5B).

The length of the aerial part of the explants cultivated in medium with 2 and 4 g L^{-1} of activated charcoal was higher than that of medium without charcoal (Fig. 5A) and with 1 g L^{-1} (Fig. 4C). The fresh mass was higher in the activated charcoal treatments (Fig. 4D).

The general analysis of the variables: average number of roots, average length of the largest root, length of the aerial part and fresh mass, indicated that the best response would occur with the addition of 3 g L^{-1} of charcoal activated to the MSM/2 medium (Fig. 4).

Transplanting and acclimatization in a greenhouse

The percentage of survival after 120 days in the greenhouse did not show any significant difference between the tested substrates. The survival percentages obtained were higher than 90% (Fig. 5C). After nine months in the greenhouse, the percentages of plant survival were high for several tested substrates, with the exception of the commercial substrate Maxfértil® (45% survival) (Table 6). The fresh mass of the plants on the substrate containing vermiculite and coconut fiber mixture (1:1, v/v) was significantly higher than that of all tested substrates (Table 6, Tukey's Test, $P< 0.05$, Fig. 5D). The average length of the aerial part and the largest root was lower in treatments using commercial substrates (Maxfértil® and Forth®). The lowest average number of roots occurred when the Maxfértil® substrate was used (Table 6).

Table 6 Effects of substrates used for transplanting and acclimatization on *Cattleya warneri* seedlings after nine months in a greenhouse.

Substrates	Survival (%)	Fresh mass (g)	Average length of the aerial part (cm)	Average length of the largest root (cm)	Average number of roots
Sphagnum	86.25 a	0.511 b	2.26 a	3.57 a	4.0 a
Vermiculite	85.00 a	0.579 b	2.30 a	3.47 a	4.4 a
Sphagnum + pine bark	85.00 a	0.480 b c	2.34 a	3.33 a	3.9 a
Pine bark + activated charcoal + coconut fiber	58.75 a b	0.666 b	2.59 a	3.33 a	4.3 a
Vermiculite + pine bark	75.00 a	0.684 b	2.41 a	3.51 a	4.6 a
Vermiculite + coconut fiber	83.75 a	0.895 a	2.56 a	3.91 a	4.4 a
Maxfértil®	45.00 b	0.279 c	1.57 b	1.62 b	2.4 b
Forth®	58.75 a b	0.273 c	1.64 b	2.13 b	3.3 a b

Different lower-case letters in the columns differ statistically by Tukey's test $P \leq 0.05$.

Discussion

The protocorm showed to be an efficient and responsive explant for the *in vitro* propagation of *C. warneri*, showing positive responses, even in the absence of PGRs. The protocorms, resulting from the germination of orchids, are an intermediate phase between the embryo and the plantlet, being an excellent source of explant for the PLBs regeneration (Yeung 2017). The main response obtained by the entire protocorms of *C. warneri* was seedling regeneration (greater than 90%), in the absence of PGRs, PAs or microalgae. As reported by Yeung et al. (2018) the apical meristem is the first structure formed by the protocorm, followed by the leaf primordia and later the root formation, resulting in a functional plant, as observed in the present study. A similar response was obtained with entire protocorms of *C. labiata*, which with the addition of biomass or aqueous extract of the microalga *Messastrum gracile* showed high percentages of seedling formation (76 and 60%, respectively) (Corbellini et al. 2020).

Entire protocorms were less efficient than TCLs for PLBs regeneration. In the experiment with PGRs, the best result was achieved in the medium containing 8 µM BA (47.92% and 1.9 PLBs), with the addition of PAs, the maximum percentage did not reach 10% and with extract from *D. subspicatus* (2.0 g L^{-1}) 50% and 3.3 PLBs. Of all the treatments tested, the best response was achieved with 1.0 and 2.0 g L^{-1} of biomass (71.88 and 73.44% and 4.7 PLBs), being indicated to test 1.5 g L^{-1} of biomass by regression analysis. Lower results than those achieved in our study occurred with the addition of *M. gracile* biomass in the MSM/2 medium, with the highest regeneration rate of PLBs from *C. labiata* (59%), regardless of the concentration (0.5 to 4.0 g L^{-1}) (Corbellini et al. 2020). In the composition analysis of the dry biomass of microalga *D. subspicatus*, Navarro et al. (2021) found the presence of carbohydrates, proteins, lipids and zeatin, and these organic compounds were detected in higher concentrations than in the aqueous extract. As expected, lipids were not detected in the extract. The concentration of zeatin in the aqueous extract (45.8 ± 1.8 µg g^{-1}) was more than six times higher when compared to that of biomass (7.2 ± 0.8 µg g^{-1}), then, probably, the organic compounds stimulated a greater production of

PLBs in the entire protocorms in medium containing biomass. Stirk et al. (2013) after quantifying chemical compounds in 24 microalgae strains, among them, the same genus in this study *Desmodesmus armatus* (R. Chodat) verified the presence of cytokinins and also auxins, which were not detected in the present study. This response of microalgae in the regeneration of PLBs indicated that they have great potential as a biostimulant in tissue culture.

The TCL technique applied to 90- and 120-days-old protocorms from *in vitro* seed germination was an efficient alternative for propagation of *C. warneri* plants. According to Yeung (2017), rapid divisions occur naturally in the meristematic zone of protocorms and allow better responses from exogenously applied PGRs. Another advantage of this technique is that explants have greater contact with the culture medium and, thus, increase the capacity to absorb nutrients, enabling greater mass propagation (Teixeira da Silva and Dobránszki 2014). Another explanation is that when thin sections are made in explants, the stress response can induce the formation and differentiation of PLBs (Teixeira da Silva and Dobránszki 2019).

The comparison of explant type showed that apical tTCLs were the most responsive explants, with the highest percentages of PLBs regeneration of *C. warneri*, followed by lTCLs, while basal tTCLs showed little regeneration response. According to Yeung et al. (2018), the cells of the apical extremity of the protocorms are meristematic and smaller when compared to those in the basal region, and this may have favored the best response that occurred with the apical tTCLs of *C. warneri.* A similar result was achieved with TCL from *Hadrolaelia grandis*, in which apical tTCLs sections of two-month protocorms also produced a higher PLBs regeneration rate than basal tTCLs during the initial cultivation (Vudala et al. 2019). Corbellini et al. (2020) also found that the apical tTCLs of *C. labiata* showed higher percentages of regeneration than the basal tTCLs and lTCLs. The basal tTCLs of *C. warneri* darkened in the first weeks of cultivation, in experiments with PGRs, PAs and microalgae and this may have occurred because of the damage caused to the tissues when cutting. Basal tTCLs only showed better responses on medium containing 8 M BA (68.75%), while in the other treatments with BA, ANA alone or the two PGRs combined ranged from 0 to 18.75% of regeneration of PLBs. A similar result of little regeneration of PLBs with basal tTCLs occurred with the addition of extract and biomass of microalgae (10.94 and 18.75%, respectively) or PAs (12.08 to 14.58%). In contrast to the present study, Mata-Rosas and Baltazar-García (2011) found more tissue damage in lTCLs of *Oncidium tigrinum* protocorms due to stress caused during sectioning, with only 19.8% of PLBs regeneration in MS medium.

Apical tTCLs and lTCLs from 90-days-old protocorms of *C. warneri*, grown on medium supplemented with 8 µM BA, showed better response in percentage of PLBs regeneration (89 and 87%, respectively) and average number (5.2 and 4.8 PLBs). Although cytokinins and auxins are the main groups of PGRs that promote regeneration and proliferation of PLBs (Cardoso et al., 2020), in our study, the best response occurred with addition of BA alone. Similar result was reported by Vudala et al. (2019), in which the lTCL technique was more efficient with sections from 90-days-old protocorms of *H. grandis* grown on WPM medium containing 8.8 µM BA (83.3% of PLBs). However, the number of regenerated PLBs per explant was higher (34.4) and this may have occurred because the subcultures for the same media as the lTCLs of *H. grandis* were performed every 60 days, while for *C. warneri* it was every 90 days. Another difference was the culture medium used, which was WPM for *H. grandis* and MSM/2 for *C. warneri.* Different responses were obtained by Gomes et al. (2015) with lTCLs from 180-days-old protocorms of *Brasilidium forbesii* grown in WPM medium containing 2.0 µM BA (77% of PLBs regeneration and 22.7 PLBs per explant), indicating that the best BA concentration may vary depending on the species, age of the protocorm, and culture medium.

The regeneration responses of PLBs from explants grown on medium containing different concentrations of PAs (Put, Spd, and Spm) were similar to those of the control treatment (without PAs), so this supplementation to MSM/2 medium is not recommended. PAs can improve plant growth and development because of their effects on cell division and differentiation (Abbasi et al. 2017) and these results were observed in this study, with high percentages of PLBs regeneration in apical tTCLs (83.33 to 100%). In our study, PAs were tested with protocorms obtained after 120 days of *in vitro* germination, while in the experiments performed with microalgae and PAs, the protocorms were 90 days old. Perhaps

the age of the protocorm may have influenced the inferior regeneration responses of PLBs. Superior results from the addition of PAs were achieved for other orchid species, which were tested for the multiplication step or associated with PGRs. An example of this was reported by Kumari and George (2011), who found a positive effect of Spm and Spd (1.0 mM) supplemented to MSM/2, on shoot morphogenesis of *Dendrobium* "Rungnappa Red" and "Miss Snow", when they were combined with kinetin and NAA. On the other hand, Mandal et al. (2020) obtained a positive effect of PAs, with much lower concentrations than those tested in the present study. The addition of PAs (1.0 µM of Put and Spd) to liquid MS medium induced direct PLB formation with a highest frequency of 80%, and associated with 8 µM BA enhanced the direct PLB formation in shoot tip culture of *Dendrobium* hybrid Sonia. While the control with only 8 µM BA showed no direct PLB formation of *Dendrobium* (Mandal et al. 2020), in the present study, using the same concentration of BA, 89% of PLBs regeneration with apical tTCLs was obtained. We only evaluated the responses of PGRs and isolated PAs, requiring experiments of the combination to verify the effect on the PLBs regeneration of *C. warneri*. According to Rakesh et al. (2021), in some cases, PAs supplemented with PGRs or other nutrient compounds or elicitors like sucrose, silver nitrate have a much more noticeable effect rather than individual PA treatments.

The supplementation of biomass and aqueous extract of *D. subspicatus* demonstrated a positive effect on the PLBs regeneration of *C. warneri*. The best responses of apical tTCLs were achieved with the addition of 1.5 g L^{-1} of biomass to the MSM/2 culture medium (92% regeneration and 4.7 PLBs per explant), and 1.0 g L^{-1} of extract (100% regeneration and 3.3 PLBs). The biochemical composition of biomass and aqueous extract of *D. subspicatus* indicated the carbohydrate and protein concentrations of the biomass (312.4 ± 6.8 and 213.6 ± 3.5 mg g^{-1}, respectively) were higher than those of the extract (227.1 ± 4.3 and 164.5 ± 3.8 mg g^{-1}), while the opposite occurred for zeatin. This result also occurred with the chemical compositions of the biomasses and microalgae extracts tested for *C. labiata* (Corbellini et al.,2020). Zeatin is a costly cytokinin and its presence, in conjunction with organic compounds present in the biomass and extract of *D. subspicatus* contributed to the responses of PLBs regeneration. These results indicated that the addition of biomass or extract may replace classic PGRs in the induction and PLBs regeneration of *C. warneri*. Promising results were also observed with TCL from *C. labiata*, with the addition of 0.5 g L^{-1} of biomass from the microalgae *Messastrum gracile* (59% of PLBs regeneration and 4 PLBs) and 0.5 g L^{-1} of *Chlorella vulgaris* extract (35% and 8 PLBs per explant) (Corbellini et al. 2020). Contrary to what was observed in our study, the addition of microalga *Chlorella sorokiniana* was not efficient to replace the addition of BA to the culture medium used to induce shoots of *Schomburgkia crispa* (Pereira et al. 2018).

In the elongation step, explants of *C. warneri* rooted independently of the addition of activated charcoal. However, the concentration of 3.0 g L^{-1} activated charcoal was recommended by the regression analysis to be added to the MSM/2 medium. The explants cultivated in semi-solid culture medium, containing charcoal showed better responses of the aerial part elongation, fresh mass and roots, when compared to the medium without addition. According to Thomas (2008), one of the effects of activated charcoal is to adsorb inhibitory substances released by the media or explants, promoting greater elongation and rooting and this was verified in this study. The optimal concentration of activated charcoal has varied according to the studied species. Similar results were obtained in the PLBs elongation of *Brasilidium forbesii* with the addition of 3.0 g L^{-1} activated charcoal (Gomes et al. 2015), or with 1.0 g L^{-1} for *H. grandis* (Vudala et al. 2019) and 2.0 g L^{-1} for *C. labiata* (Corbellini et al. 2020).

The *C. warneri* plants showed high percentages of survival in a greenhouse in all tested substrates (above 85%), after two months in the greenhouse. The evaluation carried out for a longer period of permanence of the plants in the greenhouse (nine months) and analyzing other variables besides the percentage of survival (fresh mass, average length of shoots and roots) indicated that the substrates sphagnum, vermiculite and combinations: sphagnum + pine bark (1: 1, v/v), vermiculite + pine bark (1: 1, v/v) and vermiculite + coconut fiber (1:1) can be used for transplanting seedlings. However, the combination of vermiculite and coconut fiber is recommended as it provides greater fresh mass (0.895 g) and high

survival rate of *C. warneri* plants (83.75%). Vermiculite is a light substrate that showed good aeration and water retention and coconut fiber is inert, has also a high porosity, low cost and a long life without altering its physical characteristics (Faria et al. 2018). Similar substrates were indicated for other orchid species such as vermiculite for *Brasilidium forbesii* (Gomes et al. 2015)d *picta* (Santos et al. 2016), pine bark and coconut fiber for *Cattleya forbesii* and *C. bowringiana* (Colombo et al. 2017), pine bark and sphagnum for *C. xanthina* (Juras et al. 2019) and sphagnum for *C. labiata* (Corbellini et al. 2020).

Conclusions

An efficient micropropagation protocol for orchid *C. warneri* was established using the TCL technique. The type of section, the concentrations of PGRs and aqueous extract or biomass of microalga *D. subspicatus* influenced the PLBs regeneration responses. Apical tTCLs of 90 days protocorms from *in vitro* germination are recommended for this technique because they were the most responsive explants.

The regeneration of PLBs was more efficient with the addition of 1.0 g L^{-1} of biomass or 1.5 g L^{-1} of *D. subspicatus* extract and 8 µM BA in the MSM/2 medium. PAs were not efficient for PLBs regeneration, and it is suggested that they could be tested in association with cytokinins.

The entire protocorms showed high percentages of seedling formation, except for those grown in the MSM/2 medium, supplemented with 1.0 g L^{-1} of biomass, which also induced high percentages of PLBs. Microalgae are effective alternatives to replace conventional PGRs added to the culture medium.

To promote the elongation of the aerial part and the development of roots, it is recommended addition of 3.0 g L^{-1} of activated charcoal to the culture medium. Plants showed high percentages of survival in the greenhouse on several tested substrates, but we recommend a combination of coconut fiber and vermiculite as a substrate for acclimatization of *C. warneri* plants.

Declarations

CRediT authorship contribution statement

Quezia Rocha Navarro: Methodology, Validation, Formal Analysis, Investigation, Resources, Data curation, Writing - original draft, Visualization. **Diego de Oliveira Corrêa:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Writing – Review & Editing, Supervision. **Alexandre Behling:** Methodology, Software, Formal Analysis, Investigation, Data Curation, Visualization. **Miguel Daniel Noseda:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Visualization. **Luciana Lopes Fortes Ribas:** Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Writing - original draft, Writing – Review & Editing, Visualization, Supervision, Project Administration.

Funding

The authors thank the “Coordenação de Aperfeiçoamento de Pessoal de Nível Superior” (CAPES, Brazil) (Finance Code 001) and “Conselho Nacional de Desenvolvimento Científico e Tecnológico” (CNPq, Brazil) for the Master´s scholarship to Quezia Rocha Navarro. Diego de Oliveira Corrêa acknowledges to CNPq for the postdoctoral scholarship (PDJ) (159590/2018-3). Miguel Daniel Noseda is Research Member of CNPq (306568/2018-7) and acknowledges CNPq for a Universal Call grant (462414/2014-0).

Declaration of competent interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We grateful acknowledge to Dr. Rogerio Mamoru Suzuki for providing the seeds and photo of the *Cattleya warneri* flower used in this study.

References

1. Abbasi NA, Ali I, Hafiz IA, Khan AS (2017) Application of polyamines in horticulture: a review. Int J Biosci 10:319–342. http://dx.doi.org/10.12692/ijb/10.5.319-342
2. APHA (2005) Standard methods for the examination of water and wastewater, 21st edn. American Public Health Association, Washington
3. Battacharyya P, Kumaria S, Job N, Tandon P (2016) En-masse production of elite clones of *Dendrobium crepidatum*: a threatened, medicinal orchid used in Traditional Chinese Medicine (TCM). J Appl Res Med Aromat Plants 3:168–176. https://doi.org/10.1016/j.jarmap.2016.04.001
4. Bligh EG, Dyer WJ (1959) A Rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917. https://doi:10.1139/o59-099
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254. https://doi:10.1016/j.cj.2017.04.003
6. Cardoso JC, Zanello CA, Chen J-T (2020) An overview of orchid protocorm-like bodies: Mass propagation, biotechnology, molecular aspects, and breeding. Int J Mol Sci 21. doi: 10.3390/ijms21030985
7. Chen D, Shao Q, Yin L, Younis A, Zheng B (2019) Polyamine function in plants: metabolism, regulation on development, and roles in abiotic stress responses. Front Plant Sci 9:1–13. https://doi.org/10.3389/fpls.2018.01945
8. CNCFlora (2012) *Cattleya warneri* in Lista Vermelha da flora brasileira versão 2012.2 Centro Nacional de Conservação da Flora. Disponível em < http://cncflora.jbrj.gov.br/portal/pt-br/profile/Cattleya warneri. Acesso em 31 maio 2020
9. Colombo RC, Hoshino RT, Ferrari EAP, Alves GAC, de Faria RT (2017) *Cattleya forbesii* x *Cattleya bowringiana*: A new hybrid of *Cattleya* orchid. Crop Breed Appl Biotechnol 17:184–186. https://doi.org/10.1590/1984-70332017v17n2c28
10. Corbellini JR, Ribas LLF, de Maia FR, Corrêa DO, Noseda MD, Suzuki RM, Amano E (2020) Effect of microalgae *Messastrum gracile* and *Chlorella vulgaris* on the in vitro propagation of orchid *Cattleya labiata*. J Appl Phycol https://. doi:10.1007/s10811-020-02251-9
11. Corrêa DO, Duarte MER, Noseda MD (2019) Biomass production and harvesting of *Desmodesmus subspicatus* cultivated in flat plate photobioreactor using chitosan as flocculant agent. J Appl Phycol 31:857–866. https://doi:10.1007/s10811-018-1586-z
12. Dubois M, Gilles KA, Hamilton JK et al (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356. https://doi:10.1021/ac60111a017
13. Faria RT, Stegani V, Bertoncelli D, Alves GAC, de Assis AM (2018) Substrates for the cultivation of epiphytic orchids. Semin Agrar 39:2851–2866. doi: 10.5433/1679-0359.2018v39n6p2851
14. Gomes LRP, Franceschi CRB, Ribas LLF (2015) Micropropagation of *Brasilidium forbesii* (Orchidaceae) through transverse and longitudinal thin cell layer culture. Acta Sci Biol Sci 37:143–149. https://doi.org/10.4025/actascibiolsci.v37i2.27276

15. Gupta A (2016) Asymbiotic seed germination in orchids: role of organic additives. Int Adv Res J Sci Eng Technol 3:143–147. doi: 10.17148/IARJSET.2016.3530
16. Hosomi ST, Santos RB, Custodio CC, Seaton PT, Marks TR, Machado-Neto NB (2011) Preconditioning *Cattleya* seeds to improve the efficacy of the tetrazolium test for viability. Seed Sci Technol 39:178–189. https://doi:10.15258/sst.2011.39.1.15
17. Hosomi ST, Souza TB, Custódio CC, Machado-Neto NB (2017) Refining the tetrazolium test for evaluation of *Cattleya labiata* and *C. tigrina*. seeds viability Aust J Crop Sci 10:1320–1326. https://doi.org/10.21475/ajcs.17.11.10.pne606
18. Julia (2021) https://docs.julialang.org/en/v1/
19. Juras MCR, Jorge J, Pescador R, Ferreira WM, Tamaki V, Suzuki RM (2019) In vitro culture and acclimatization of *Cattleya xanthina* (Orchidaceae), an endangered orchid of the Brazilian Atlantic Rainforest. Rodriguésia 70:1–11. https://doi:10.1590/2175-7860201970014
20. Kumari IP, George TS (2011) In vitro clonal shoot morphogenesis of commercial *Dendrobium* orchid cultivars in polyamines supplemented medium. J Tropic Agric 49:118–120
21. Lal N, Singh M (2020) Prospects of plant tissue culture in orchid propagation: a review. Indian J Biol 7:103–110. https://doi.org/10.21088/ijb.2394.1391.7220.15
22. Ma X, Liu J, Liu B, Chen T, Yang B, Chen F (2016) Physiological and biochemical changes reveal stress-associated photosynthetic carbon partitioning into triacylglycerol in the oleaginous marine alga *Nannochloropsis oculata*. Algal Res 16:28–35. https://doi:10.1016/j.algal.2016.03.005
23. Mandal S, Pal N, Mondal T, Banerjee N (2020) Comparative efficiency analysis of different explants and contribution of polyamines on *in vitro* propagation of *Dendrobium* hybrid Sonia. Plant Tissue Cult Biotechnol 30:77–86. https://doi.org/10.3329/ptcb.v30i1.47793
24. Mata-Rosas M, Baltazar-García J (2011) In vitro regeneration through direct organogenesis from protocorms of *Oncidium tigrinum* Llave & Lex. (Orchidaceae), an endemic and threatened Mexican species. HortScience 46:1132–1135
25. Mercado SAS, Contreras NAV (2017) Asymbiotic seed germination and *in vitro* propagation of *Cattleya trianae* Linden. & Reichb Acta Agron 66:544–548. https://doi.org/10.15446/acag.v66n4.63597
26. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:474–497. https://doi:10.1111/j.1399-3054.1962.tb08052.x
27. Navarro QR, Corrêa DO, Behling A, Noseda MD, Amano E, Suzuki RM, Ribas LLF (2021) Efficient use of biomass and extract of the microalga *Desmodesmus subspicatus* (Scenedesmaceae) in asymbiotic seed germination and seedling development of the orchid *Cattleya warneri*. J Appl Phycol 33:2189–2207. https://doi.org/10.1007/s10811-021-02442-y
28. Pant M, Negi A, Singh A, Gautam A, Rawat M (2020) *Cattleya* orchids: a mini review. J Crit Rev 7:4592–4598. https://doi.org/10.31838/jcr.07.12
29. Pereira NS, Ferreira BRR, de Carvalho EM, Damiani CR (2018) Application of *Chlorella sorokiniana* (Chlorophyceae) as supplement and/or an alternative medium for the in vitro cultivation of *Schomburgkia crispa* (Orchidaceae). J Appl Phycol 30:2347–2358. https://doi:10.1007/s10811-018-1441-2
30. Rakesh B, Sudheer WN, Nagella P (2021) Role of polyamines in plant tissue culture: an overview. Plant Cell Tiss Organ Cult 145:487–506. https://doi.org/10.1007/s11240-021-02029-y
31. Ronga D, Biazzi E, Parati K, Carminati D, Carminati E, Tava A (2019) Microalgal biostimulants and biofertilisers in crop productions. Agronomy 9:1–22. https://doi:10.3390/agronomy9040192
32. Santos SA, Smidt EC, Padial AA, Ribas LLF (2016) Asymbiotic seed germination and *in vitro* propagation of *Brasiliorchis picta*.. Afr J Biotechnol 15:134–144. https://doi.org/10.5897/AJB2015.15043

33. Stirk WA, Ördög V, Novák O, Rolčík J, Strnad M, Bálint P, van Staden J (2013) Auxin and cytokinin relationships in 24 microalgal strains. J Phycol 49:459–467. https://doi:10.1111/jpy.12061
34. Teixeira da Silva JA, Dobránszki J (2014) Dissecting the concept of the thin cell layer: theoretical basis and practical application of the plant growth correction factor to apple, *Cymbidium* and *Chrysanthemum*. J Plant Growth Regul 33:881–895. https://doi.org/10.1007/s00344-014-9437-x
35. Teixeira da Silva JA, Dobránszki J (2019) Recent advances and novelties in the thin cell layer-based plant biotechnology – a mini-review. J Biotechnol Comput Biol Bionanotechnol 100:89–96. http://doi.org/10.5114/bta.2019.83215
36. Thomas TD (2008) The role of activated charcoal in plant tissue culture. Biotechnol Adv 26:618–631. https://doi:10.1016/j.biotechadv.2008.08.003
37. Vudala SM, Padial AA, Ribas LLF (2019) Micropropagation of *Hadrolaelia grandis* through transverse and longitudinal thin cell layer culture. S Afr J Bot 121:76–82. https://doi.org/10.1016/j.sajb.2018.07.017
38. Yeung EC (2017) A perspective on orchid seed and protocorm development. Bot Stud 58:1–14. https://doi:10.1186/s40529-017-0188-4
39. Yeung EC, Li YY, Lee YI (2018) Understanding seed and protocorm development in orchids. In: Lee YI, Yeung ECT (eds) Orchid propagation: From laboratories to greenhouses—methods and protocols. Springer Protocols Handbooks, New York, pp 3–26

Figures

Figure 1

Cattleya warneri: (A) mature plant, (B) protocorm with apex and rhizoids used in the thin cell layer (TCL) technique, (C-I) regeneration of protocorm-like bodies (PLBs) after 120 days of culture in MSM/2 medium (MS, with the concentration of macronutrients reduced by half). (C) apical tTCL cultivated on medium with 8μM of 6-benzyladenine, (D) basal tTCL cultivated on medium with 0.5 mM putrescine, (E) apical tTCL cultivated on medium without added spermine, (F) apical tTCL cultivated on medium with 0.5 mM spermidine, (G) apical tTCL cultivated on medium with 1.0 g L^{-1} of *Desmodesmus subspicatus* biomass, (H) entire protocorm cultivated on medium with 1.0 g L^{-1} of biomass, (I) apical tTCL cultivated on medium with 1.0 g L^{-1} of *D. subspicatus* aqueous extract.

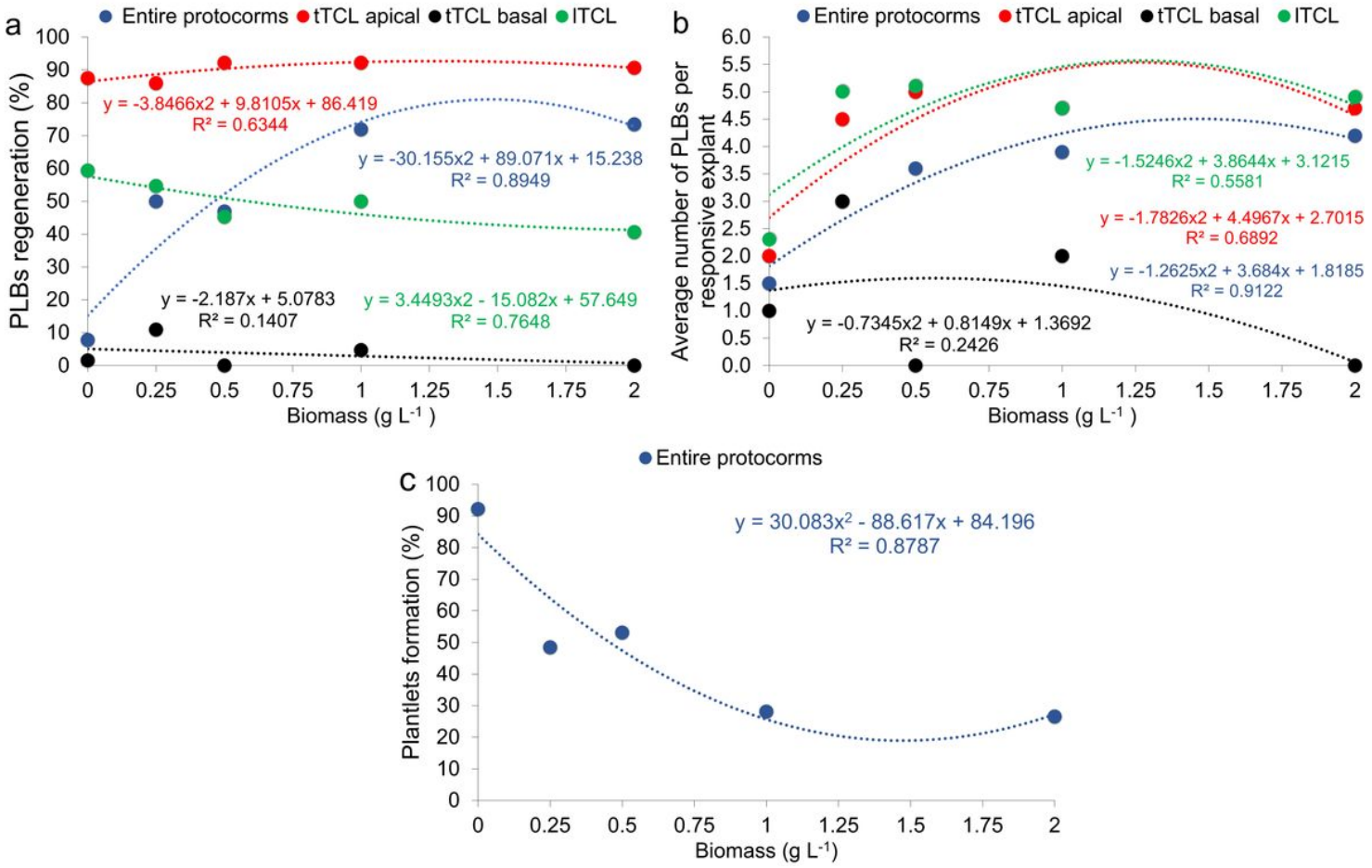

Figure 2

Regeneration responses of protocorm-like-bodies (PLBs) from *Cattleya warneri* explants after 120 days of the second subculture in MSM/2 medium (MS with half the macronutrient concentration), supplemented with *Desmodesmus subspicatus* biomass.

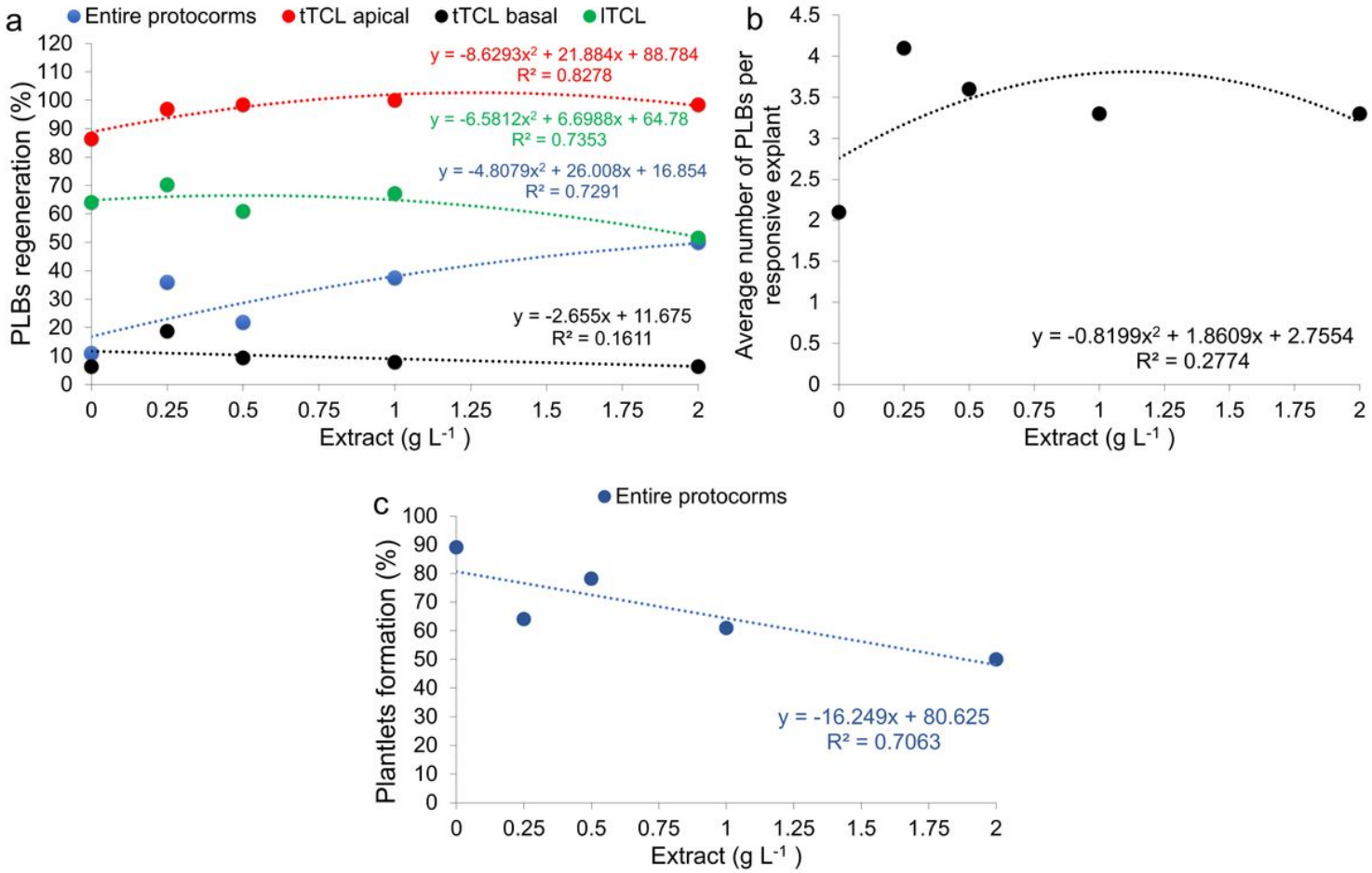

Figure 3

Regeneration responses of protocorm-like-bodies (PLBs) in *Cattleya warneri* explants after 120 days of the second subculture in MSM/2 medium (MS with half concentration of macronutrients) containing *Desmodesmus subspicatus* extract.

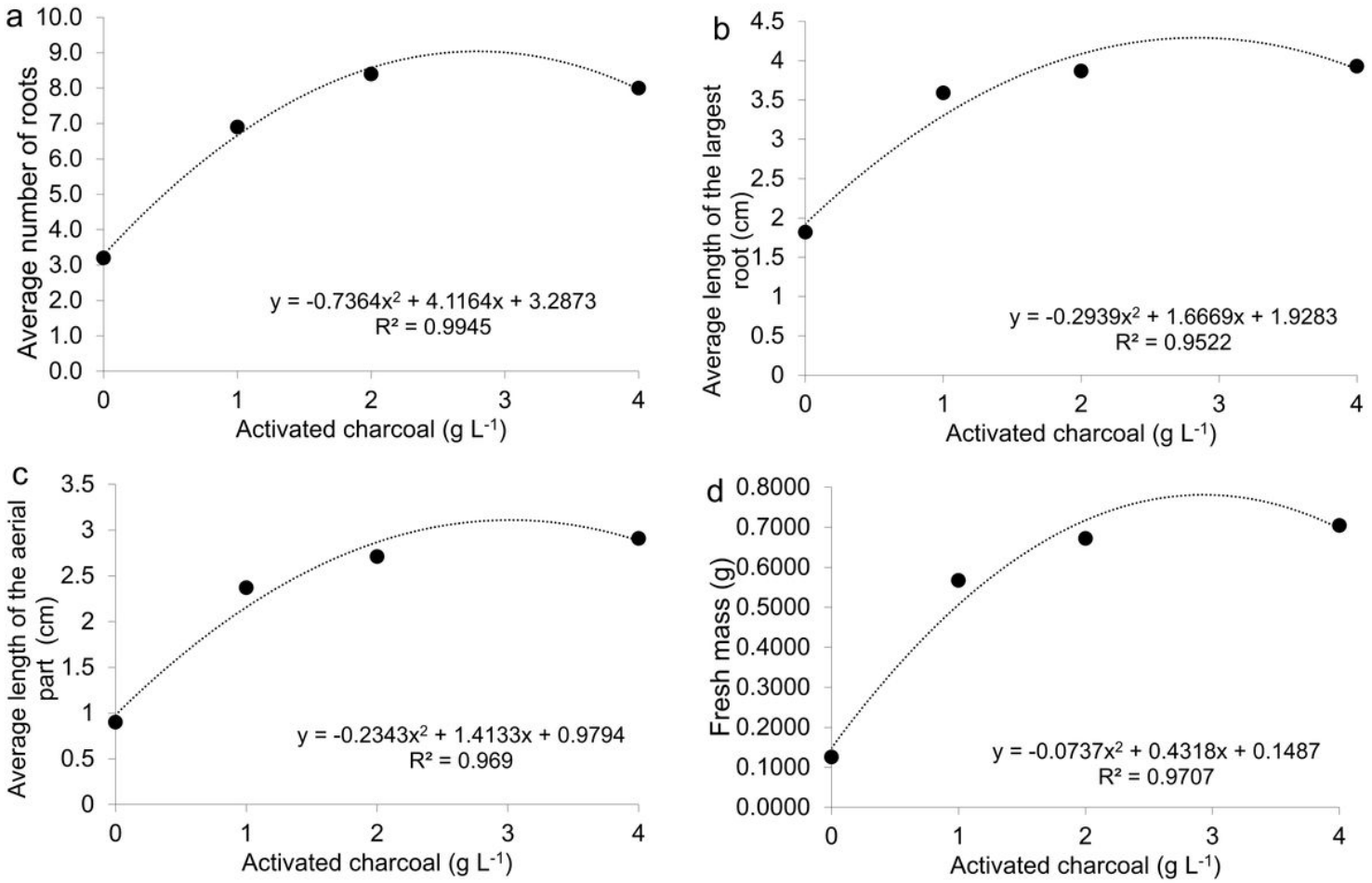

Figure 4

Effects of activated charcoal on the elongation and rooting of *Cattleya warneri* explants after 120 days of culture in MSM/2 medium (MS with half concentration of macronutrients).

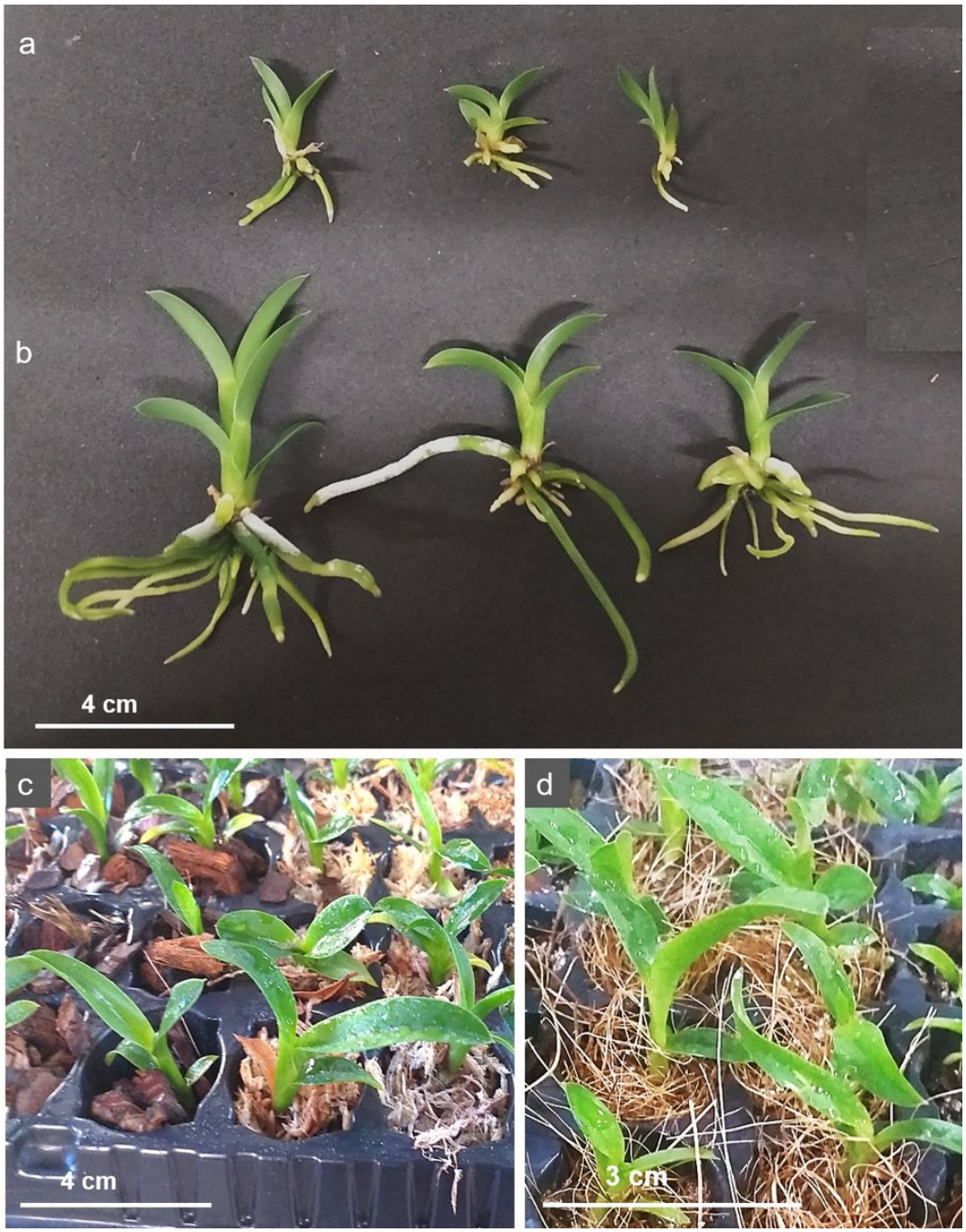

Figure 5

Acclimatization of *Cattleya warneri* plants from thin cell layer experiments: (A) individualized plants grown in MSM/2 medium, without addition of activated carbon, before transplanting, (B) plants after elongation, grown in MSM/2 medium, with activated charcoal, (C) acclimatization of plants on different substrates, (D) acclimatized plants using vermiculite and coconut fiber (1:1, v/v) as substrate, after 9 months in the greenhouse.