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ORIGINAL ARTICLE

High-frequency protocorm-like bodies and shoot regeneration through a combination of thin cell layer and RITA® temporary immersion bioreactor in *Cattleya forbesii* Lindl.

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Abstract

An efficient in vitro mass propagation through protocorm-like bodies (PLBs) was established in *Cattleya forbesii* Lindl., a commercially important orchid. Whole PLBs (W-PLB) and transverse thin cell layers of PLB (tTCL-PLB) explants were cultured in RITA® bioreactors based on temporary immersion system. Explants were transferred in semi-solid or RITA® bioreactor for protocorm production or shoot regeneration. The effect of different immersion frequencies, medium volumes and inoculum densities were studied and optimized. RITA® bioreactor cultures were found to be superior compared with semi-solid cultures regarding PLB production and shoot regeneration. tTCL-PLB explant types cultured in the RITA® bioreactor with immersion for 1 min/4 h, 250 mL of medium and 20 explants showed the highest number of PLBs per RITA® (2237 PLBs) and per explant (111.9 PLBs). The highest number of PLBs per explant was 21 times higher than those from semi-solid culture. The highest number of shoots per RITA® (3998 shoots) and per explant (199.9 shoots) were observed on tTCL-PLB cultured in RITA® bioreactor (1 min/4 h; 150 mL of medium and 20 explants). The highest number of shoots per explant was 95 times higher than those grown on semi-solid culture. Mass propagation of PLBs and shoots of *C. forbesii* Lindl. using combined thin cell layer and RITA® temporary immersion has been adapted in commercial practice.

Keywords *Cattleya forbesii* Lindl. · Protocorm · Thin cell layer · In vitro · Temporary immersion system · Bioreactor · Mass propagation

Introduction

The Orchidaceae family, one of the largest families in the plant kingdom, is represented by 899 genera and 27.801 species (Almeida et al. 2017). Orchids originated 102–120 million years ago in Australia when Neotropics, Antarctica and Australia were close to each other and then they spread to the other two continents. Therefore, orchids are now widespread around the world in every continent except for Antarctica (Schiff 2018). Since orchids developed specialized pollination strategies (food deception, pollinarium bending, pollination by deceit, sexual deceit) (Cozzolino and Widmer 2005) and adaptations to epiphytic habit and have mostly bilaterally symmetric flowers, fused reproductive organs and specialized secondary metabolites (Unruh et al. 2018), the Orchidaceae family is the most evolved flowering family (Schiff 2018). The cultivation of orchids as ornamental plants commenced a long time ago owing to their diversity in size, shape and color of beautiful flowers (Tee et al. 2008) and orchids represents 8% of the world floriculture trade as

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an international business (Chugh et al. 2009; Colombo et al. 2017). Orchids besides their ornamental uses are also evaluated for multipurpose uses including the food and cosmetic sectors and medicinal studies (Schiff 2018). The flowers of *Cattleyas* are long lasting and have beautiful colors ranging from lavender to pink and white, magnificent shapes, and pleasant odor (De et al. 2014; Schiff 2018). It is very popular in the production of hybrids and is crossed with many different genera (Schiff 2018). Most *Cattleya* species are epiphytes and rarely lithophytes. Since *Cattleya* species have popularity and commercial importance among orchid species, they are mostly propagated as ornamental purposes (Caballero-Villalobos et al. 2017).

Orchids are propagated sexually by seeds and asexually by division and cutting depending on their growth habits (sympodial, monopodial), life forms (epiphytic, lithophytic, and terrestrial) and whether they have pseudobulb or not (Lee 2018; Schiff 2018). Due to the heterozygosity of seed, being highly fragile, including no endosperm, the long juvenile period before flowering, requiring fungal infection (symbiotic germination) for germination of some orchids, propagation of orchids through seeds is unsuitable (Chugh et al. 2009). *Cattleyas* are sympodial orchids and contain storage organs called pseudobulb. Sympodial orchids with multiple shoots growing along a rhizome can be multiplied by dividing the rhizomes where there is an axillary bud and these rhizome fragments can produce a new individual. In sympodial orchids with pseudobulbs including a single or several thickened internodes, pseudobulbs can be used for vegetative propagation purpose by division and cutting. *Cattleyas* are vegetatively propagated by dividing the pseudobulb containing rhizome fragment. In some orchids, there are dormand buds along a mature pseudobulb and excision of pseudobulb with one or more live dormand bud can give rise to new plants (Lee 2018). Since the production of orchids with classical propagation methods is not sufficient to meet the demand and not suitable for large-scale production, in vitro propagation methods have been developed for production of these wonderfully exotic plants. Moreover, orchids are the first plants cultured in vitro for virus-free plant production (Adelberg et al. 1998).

Plant tissues such as shoot tips, root tips, floral stalks, stem nodes, apical buds, protocorm-like bodies, leaves, rhizomes, and mature seeds have been used as explants in vitro micropropagation of orchids (Zhao et al. 2008). Since protocorms, which are intermediate structure between embryo and plant, are quite meristematic, they can be produced in a large number in a relatively short period of time. Therefore, protocorms obtained from different plant parts (Protocorm-like bodies; PLBs) are preferred in the micropropagation of orchid species (Roy et al. 2011). The seeds first form the protocorm, which grows into a plant in the classical seed germination process of orchids. The orchids produce PLBs in response to biotic or abiotic stresses in vitro conditions (Teixeira da Silva and Winarto 2016). PLBs, which are well-differentiated tissues, are assumed as embryos of orchids as they have two discrete polar structures called shoot and root meristem and are easily converted to plantlets when cultured in plant growth regulator-free medium (Ng and Saleh 2011). Protocorms and PLBs are often the only explants for the production of orchids, which have low seed formation or germination (Murdad et al. 2006).

In thin cell layer (TCL) technique, a variety of small size explants obtained from different plant organs excised either longitudinally or transversely is used (Vyas et al. 2010). The advantage of this technique is higher frequency regeneration and more conservative of explant tissue compared with conventional in vitro production techniques (Nayak et al. 2002). Therefore, TCL technique has made micropropagation easier and more efficient for many angiosperm species, including orchids (Bose et al. 2017) and this technique has been effectively used for the production of the PLBs in several orchid species such as *Aranda* Deborah (Lakshmanan et al. 1995), *Cymbidium* Sleeping Nymph (Vyas et al. 2010), *Dendrobium aqueum* (Parthibhan et al. 2018), *Dendrobium candidum* Wall Ex Lindl. (Zhao et al. 2007), *Doritaenopsis* (Park et al. 2002), *Rhynchostylis gigantean* (van Le et al. 1999).

Micropropagation can provide a rapid large scale production on a commercial scale of valuable plant species, which may be difficult to produce through traditional methods, in a limited space under the controlled environment without any seasonal constraints all over the year (Bayraktar et al. 2015; Jena et al. 2018). However, the use of semi-solid media in micropropagation increases production costs, requires intensive labor, and makes automation difficult (Etienne and Berthouly 2002). Therefore, bioreactors operating with liquid cultures are prefered for large-scale plant production. Bioreactors have several advantages such as allowing scale-up, easy control of the physical and chemical environment, theoretically low production costs, and uniform environmental conditions (Etienne and Berthouly 2002). Moreover, plant tissues cultured in bioreactors are in better contact with culture medium and can better utilize nutrient sources and growth regulators (Ross and Castillo 2009). Since the explants are continuingly in contact with the liquid nutrient medium, in bioreactors based on continuous immersion system, some disadvantages like hyperhydricity and asphyxia, which adversely affect plant growth and result in morphological abnormalities like shorter internodes and brittle, translucent, and wrinkled leaves etc. can be are arisen. These problems can be reduced with bioreactors functioning on the principle of temporary immersion system (TIS) (Mamun et al. 2015). TIS combines the advantages of liquid and semi-solid culture. In TISs, plant material is periodically immersed with the liquid medium and the atmosphere in the bioreactor is refreshed. Thus, the plant tissue can efficiently

utilize from nutrient and disorders such as asphyxia, hyperhydricity and ethylene and carbon dioxide accumulation in the culture can be eliminated (Escalona et al. 1999; Etienne and Berthouly 2002). The use of TIS bioreactors in micropropagation makes possible the scaling-up of the production and reduces the production cost (Young et al. 2000; McAlister et al. 2005) and the energy demand (Etienne and Berthouly 2002). Bioreactor technology has been successfully applied in some orchid species such as *Bletilla striata* (Zhang et al. 2018), *Cymbidium sinense* (Gao et al. 2014), *Dendrobium* (Winarto et al. 2013), *Oncidium* 'Sugar Sweet' (Yang et al. 2010), *Phalaenopsis* (Young et al. 2000). However, *Cattleya* does not have significant commercial production in bioreactors according to the literature, and there are insufficient studies on production using TCL technique. This study was conducted to compare the semi-solid culture systems and the liquid culture systems in the RITA® bioreactor to develop a convenient process for the commercial production of *Cattleya forbesii* Lindl. Inoculation density, medium volume, and immersion frequency were investigated in bioreactor cultures, and the efficiency of protocorms as an explant source (as a whole and TCL) was examined.

Materials and methods

Plant material and induction of protocorm-like bodies

In vitro propagated 6-month-old *C. forbesii* Lindl. plantlets with well-developed roots were used as the starting plant material to regenerate protocorm-like bodies (PLBs).

The stem segments with nodes were transversely sliced into pieces of about 0.5–1.5 mm thickness, and the slices from the nodes were used as thin cell layer (TCL) explants for PLBs regeneration. To induce PLBs, TCL explants were cultured on 210-cc-capped glass culture jars with 25 mL Knudson C (KC) medium (Knudson 1946) supplemented with 2% (w/v) sucrose, 1.2 mg/L 6-Benzylaminopurine (BAP) and 1.2 mg/L Naphthaleneacetic acid (NAA). The medium was solidified with 0.3% (w/v) Gelrite (Duchefa-Biochemie) (pH 5.8). For multiplication purpose, PLBs obtained from primary culture were subcultured in glass culture jars containing the same medium bimonthly.

PLB production from PLB

Explant types

Whole PLB (2–5 mm) (W-PLB) and transverse thin cell layers of PLB (1-2.5 mm) (tTCL-PLB) were used as explant types for all of the experiments. The PLBs (approximately 2–5 mm) were cross-sectional and transversely sliced into pieces each approximately 1–2.5 mm and these pieces were utilized as tTCL-PLB.

Semi-solid culture

Two different explants (W-PLB and tTCL-PLB) as specified above were cultured in 210-cc-capped glass culture jars containing 25 mL KC medium supplemented with 2% (w/v) sucrose, 15% (v/v) coconut water (CW) (PhytotechLab) and 1 mg/L BAP solidified with 0.3% (w/v) Gelrite (protocorm induced medium: PIM). The pH of medium was adjusted to 5.3 with 1 N HCl or 1 N NaOH before the addition of the gelling agent. The CW was filter-sterilized through a 0.22 µm syringe Millipore filter (Minisart®, Sartorius, Germany), and then added to the autoclaved PIM aseptically at the desired concentrations. The experiment was conducted in three replications; 12 explants were used for each replication. Thirty-six explants per treatment were tested. The data were recorded 60 days after culture initiation.

Liquid RITA® bioreactor culture

The Récipient à Immersion Temporaire Automatique (RITA®) (VITROPIC, Saint-Mathieu-de-Tréviers, France) bioreactor with a 1-L capacity autoclavable feature consists of two compartments. The explants are cultured in the upper compartment of the bioreactor, and the nutrient medium is in the lower compartment. The air pressure is supplied with an air pump (KNF Pump N022AN.18 type; KNF Neuberger GmbH, Freiburg, Germany) created an air flow of 15 L/min. and the air is distributed through silicone hoses. During the immersion period, the solenoid valve opens, and air pressure pushes the culture medium from its compartment to the plant material compartment by forming air bubbles for immersing the explants completely, and thus, both the explants take the nutrients and the headspace atmosphere inside the RITA® is refreshed. A timer is used to control the frequency and duration of the immersion period. Then, the second solenoid valve opens to stop the air pressure provided by the air pump and to escape the excess pressure through an outlet at the upper part of the bioreactor. Finally, the culture medium is returned to the lower compartment by gravity (Teisson and Alvard 1995).

W-PLB and tTCL-PLB explant types were cultured in RITA® bioreactor containing liquid PIM (pH 5.3). Three different medium volumes (150, 200 and 250 mL), two different immersion frequencies (1 min/4 h and 1 min/8 h) and three different inoculum densities (10, 20 and 30 explants) were tested. The experiments were conducted in three replications, and one RITA® bioreactor was used for each replication. Three RITA® bioreactors were tested in total per treatment. The data were recorded 60 days after culture initiation.

Shoot regeneration from PLB

Semi-solid culture

Two different explants (W-PLB and tTCL-PLB) as specified above were cultured in 210-cc-capped glass culture jars containing 25 mL KC medium supplemented with 2% (w/v) sucrose, 15% (v/v) CW and 2.5 mg/L BAP solidified with 0.3% (w/v) Gelrite (shoot regeneration medium: SRM). The experiment was conducted in three replications; 12 explants were used for each replication. Thirty-six explants per treatment were tested. The data were recorded 60 days after culture initiation.

Liquid RITA® bioreactor culture

W-PLB and tTCL-PLB explant types were cultured in RITA® bioreactor containing liquid SRM. Three different medium volumes (150, 200 and 250 mL), two different immersion frequencies (1 min/4 h and 1 min/8 h) and three different inoculum densities (10, 20 and 30 explants) were tested. The experiments were conducted in three replications, and one RITA® bioreactor was used for each replication. Three RITA® bioreactors were tested in total per treatment. The data were recorded 60 days after culture initiation.

Media and culture conditions

The media were autoclaved at 121 °C at 1.04 kg/cm^2 for 15 min. All the cultures were incubated in a growth room under approximately 25 ± 2 °C in a cool white fluorescent light (35 µmol/m^2 s) for a light/dark photoperiod of 16:8.

Statistical analysis

The experiments were set up in a completely randomized design. For semi-solid cultures, a single-factor design for PLB production from PLB and shoot regeneration from PLB was established. The results obtained for semi-solid cultures were evaluated with Mann–Whitney and MINITAB program, which were applied statistically for nonparametric tests. Except for the results of semi-solid culture, all data were analyzed by four-way ANOVA procedures using MINITAB 17.0 Statistical Software (2010). Pairwise comparisons were performed using Tukey's test. The experiment was a 2 × 3 × 2 × 3 factorial design with two immersion frequencies (IF)-(1 min/4 h and 1 min/8 h), three volumes of medium (VM)-(150, 200 and 250 mL), two types of explant types (TE)-(W-PLB and tTCL-PLB), and three inoculum densities (ID)-(10, 20 and 30 explants). All experiments were performed in three replicate. The grouping of the number of PLBs/RITA® and the number of PLBs/Explant means and the grouping of the number of Shoots/RITA®and the number of Shoots/Explant means were made according to Tukey pairwise comparison. The significance of main effects of factors (immersion frequency, volume of medium, type of explant and inoculum density) as well as their interactions were determined. The Homogeneity tests of Group Variances are investigated by Levene Test. And also, the main effects and the comparisons of their interactions that give the most significant results in statistical applications were determined for the number of PLBs and for the number of shoots.

Results

PLBs production from PLB

The number of PLBs per RITA® and the number of PLBs per explant indicated considerable differences among the treatments (Table 1). Regarding these examined parameters, the highest values were obtained as 2237 PLBs per RITA® and 111.9 PLBs per explant from the application no. 8 (1 min/4 h; 250 mL medium; 20 explants) where the tTCL-PLB explant type was used (Fig. 1a–c). These results were followed by application no. 8 where the W-PLB explant type was used (2052 PLBs per RITA® and 102.6 PLBs per explant) (Fig. 1d). They were statistically placed in the same group. The lowest number of PLBs per RITA® (12 PLBs) and the lowest number of PLBs per explant (1.2 PLBs) were observed in application no. 10 (1 min/8 h; 150 mL medium; 10 explants) where the tTCL-PLB explant type was used. According to analysis of variance (Table 2), except for explant types tested and the interaction of immersion frequency (IF) × type of explant (TE) × inoculation density (ID) and TE × ID, the four-way analyses revealed significant interactions ($P < 0.001$) the remaining factors for the number of PLBs per RITA® and per explant.

Effect of the main factors and the interactions among these factors on the number of PLBs per RITA® and per explant is shown in Fig. 2. In addition, the levels that give the most significant results at all levels from these factors and interactions were determined (Online Resource 1). Accordingly, the immersion for 1 min/4 h caused an increase in the number of PLBs per RITA®. Besides, reducing of the volume of medium from 200 mL to 150 mL increased both the number of PLBs per RITA® and explant, while decreasing from 250 mL to 200 mL led to a significant decrease in both the number of PLBs per RITA® and explant (Fig. 2a, b). When the explant types were evaluated, it was observed that the W-PLB explant had more significant effect than tTCL-PLB explant ($P = 0.035 < 0.05$) for both the number of PLBs per RITA® and explant. In the case of different inoculum density, reducing the number of explants from 20 to 10 and from 30 to 10 decreased the number of PLBs per RITA®. Additionally, reducing the number of explants from 20 to 10

Table 1 Effect of immersion frequency (1 min every 4 and 8 h), volume of medium (150, 200 and 250 mL), and inoculum density (10, 20 and 30 explants) on PLBs produced from different explant types (W-PLB and tTCL-PLB) in RITA® culture of *Cattleya forbesii* Lindl.

Immersion frequency (min/h)	Volume of medium (mL)	Inoculation density (number of explant per RITA®)	Number of application	Number of PLBs/RITA® (± SE) Type of explant		Number of PLBs/Explant (± SE) Type of explant	
				W-PLB	tTCL-PLB	W-PLB	tTCL-PLB
1/4	150	10	1	388 ± 2.5hijk	748 ± 5.0de	38.8 ± 2.5de	74.8 ± 5.0b
		20	2	679 ± 3.9defg	713 ± 2.0defg	34.0 ± 1.9defg	35.7 ± 1.0def
		30	3	1220 ± 15.9c	463 ± 1.4fghij	40.7 ± 5.3d	15.4 ± 0.5hijklmn
	200	10	4	224 ± 9.8jklmno	39 ± 5.7no	22.4 ± 1.0ghij	3.9 ± 0.6mno
		20	5	74 ± 6.4mno	75 ± 7.4mno	3.7 ± 0.3mno	3.8 ± 0.4mno
		30	6	219 ± 3.4jklmno	319 ± 2.1ijklm	7.3 ± 1.1lmno	10.6 ± 0.7jklmno
	250	10	7	49 ± 2.1mno	640 ± 2.9defgh	4.9 ± 0.2mno	64.0 ± 2.9bc
		20	8	2052 ± 9.7a	2237 ± 1.8a	102.6 ± 4.8a	111.9 ± 9.1a
		30	9	637 ± 2.7defgh	1702 ± 5.0b	21.2 ± 0.9ghijk	56.7 ± 1.7c
1/8	150	10	10	82 ± 4.0mno	12 ± 1.7o	8.2 ± 0.4klmno	1.2 ± 0.2o
		20	11	373 ± 15.0hijkl	30 ± 6.2no	18.7 ± 0.8hijkl	1.5 ± 0.3o
		30	12	500 ± 2.1efghi	110 ± 8.4lmno	16.7 ± 0.7hijklm	3.7 ± 0.3mno
	200	10	13	31 ± 2.1no	100 ± 3.2mno	3.1 ± 0.2no	10.0 ± 0.3jklmno
		20	14	226 ± 10.7jklmno	446 ± 4.9ghij	11.3 ± 0.5ijklmno	22.3 ± 0.2fghi
		30	15	300 ± 11.5ıjklmn	727 ± 3.2def	10.0 ± 0.4jklmno	24.2 ± 1.1e
	250	10	16	725 ± 10.3def	104 ± 4.9lmno	72.5 ± 1.0b	10.4 ± 0.5jklmno
		20	17	819 ± 14.9d	150 ± 1.5klmno	40.9 ± 0.7d	7.5 ± 0.8lmno
		30	18	793 ± 18.9d	160 ± 1.9klmno	26.4 ± 0.6efgh	5.3 ± 0.7mno

Each value represents the mean ± SE of three replicates

The same *letter* within a *column* denotes statistically equal means with the Tukey test at $P \leq 0.05$

had a decreasing effect on the number of PLBs per explant, while decreasing from 30 to 10 increased the number of PLBs per explant.

In the two-way interactions of main factors, all two-way interactions (IF × VM, IF × TE, IF × ID, VM × TE, VM × ID and TE × ID) were significant (P = 0.00 < 0.05) for the number of PLB per RITA® and explant (Online Resource 2). According to IF × VM interactions, in the immersion for 1 min/4 h, the use of 250 mL medium instead of 200 mL increased the number of PLBs per RITA® and explant. For IF × TE interactions, there was a significant increase in the number of PLBs per RITA® and explant by using tTCL-PLB in the immersion for 1 min/4 h. In the IF × ID interactions, a significant increase in the number of PLBs per RITA® and explant occurred with the use of immersion for 1 min/4 h and 20 explants instead of the immersion for 1 min/8 h and 10 explants. When VM × TE interactions were evaluated, a significant increase in the number of PLBs per RITA® and explant was observed by increasing the volume of medium from 200 mL to 250 mL in the application where W-PLB was used. Considering VM × ID interactions, the use of 250 mL medium instead of 200 mL and 20 explants instead of 10 explants increased the number of PLBs obtained per RITA® and explant (Online Resource 2 and Online Resource 3 a, b) (Fig. 2c, d). When looking at interactions between TE × ID, the using W-PLB explant type and the reducing in the inoculation density from 20 explants to 10 led to a decrease in the number of PLBs per RITA® and explant (Online Resource 2) (Fig. 2c, d).

In the three- and four-way interactions of main factors, all interactions (IF × VM × TE, IF × VM × ID, VM × TE × ID and IF × VM × TE × ID) were significant (P = 0.00 < 0.05) for the number of PLBs per RITA® and the number of PLBs per explant (Online Resource 4). According to IF × VM × TE interactions, in the immersion for 1 min/4 h, the use of 250 mL medium volume and tTCL-PLB explant type increased the number of PLBs per RITA® and explant. In the IF × VM × ID interactions, the number of PLBs per RITA® and explant were higher in the immersion for 1 min/4 h, 250 mL medium and 20 explants compared to immersion for 1 min/8 h, 150 mL medium and 10 explants. Considering VM × TE × ID interactions, the use of 250 mL medium instead of 200 mL, W-PLB explant type instead of tTCL-PLB and 20 explants instead of 10 explants increased the number of PLBs obtained per RITA® and explant. When IF × VM × TE × ID interactions were evaluated, a significant increase in the number of PLBs per RITA® and explant was observed by increasing the immersion frequency from once every 8 h to once every 4 h, volume of medium from 150 mL to 250 mL

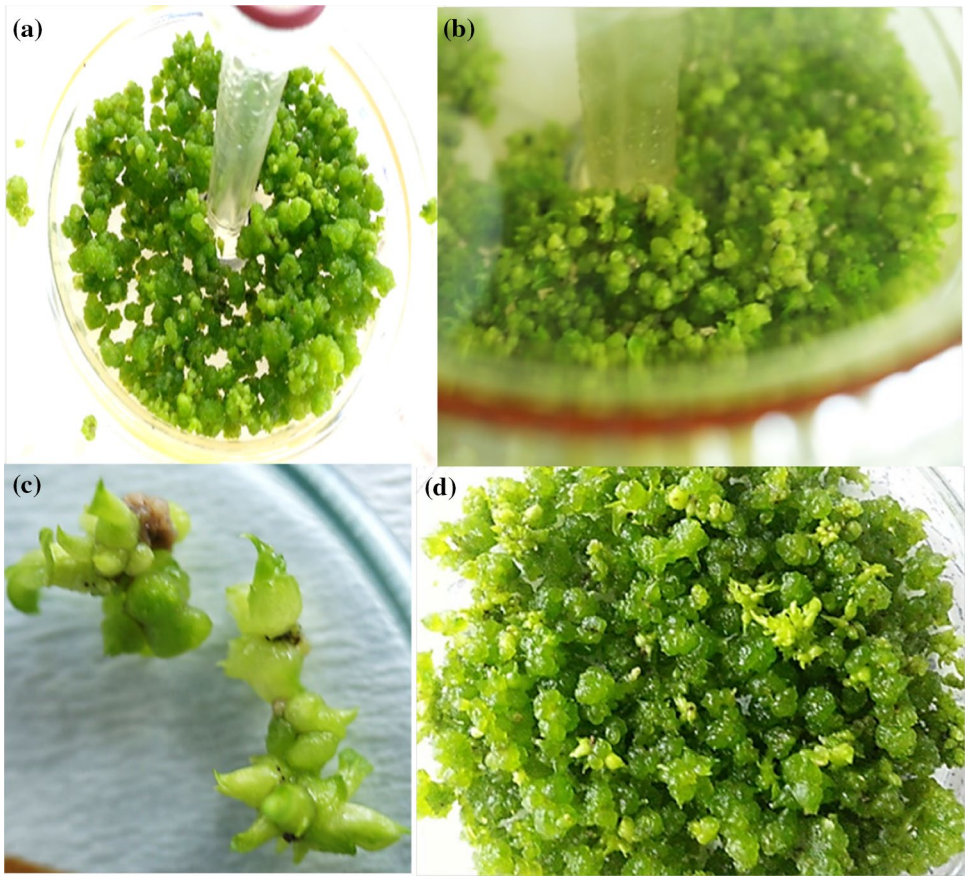

Fig. 1 PLBs produced from tTCL-PLB explants in (**a, b, c**) RITA® bioreactor and from W-PLB explants in (**d**) RITA® bioreactor

Table 2 Analysis of variance for the number of PLBs/RITA® and the number of PLBs/Explant

Source	DF	Adj SS		Adj MS		F-Value		P-Value	
		Number of PLBs/ RITA®	Number of PLBs/ Explant	Number of PLBs/ RITA®	Number of PLBs/ Explant	Number of PLBs/ RITA®	Number of PLBs/ Explant	Number of PLBs/ RITA®	Number of PLBs/ Explant
IF	1	3,842,008	10,702	3,842,008	10,702	559.95	668.02	0.000	0.000
VM	2	6,843,241	19447.7	3,421,620	9723.8	498.68	606.96	0.000	0.000
TE	1	31,553	35	31,553	35	4.60	2.18	0.035	0.144
ID	2	3,248,339	3018	1,624,169	1509	236.71	94.19	0.000	0.000
IF×VM	2	3,972,385	8302	1,986,192	4151	289.48	259.11	0.000	0.000
IF×TE	1	965,223	4139.5	965,223	4139.5	140.68	258.39	0.000	0.000
IF×ID	2	978,955	2007.4	489,477	1003.7	71.34	62.65	0.000	0.000
VM×TE	2	409,846	230.8	204,923	115.4	29.87	7.2	0.000	0.001
VM×ID	4	2,952,695	7310.7	738,174	1827.7	107.59	114.08	0.000	0.000
TE×ID	2	63,962	230.2	31,981	115.1	4.66	7.18	0.012	0.000
IF×VM×TE	2	2,783,846	9190.3	1,391,923	4595.1	202.87	286.83	0.000	0.000
IF×VM×ID	4	3,737,865	13182.6	934,466	3295.7	136.19	205.71	0.000	0.000
IF×TE×ID	2	24,244	1600.3	12,122	800.2	1.77	49.95	0.178	0.000
VM×TE×ID	4	1,187,365	2435.1	296,841	608.8	43.26	38	0.000	0.000
IF×VM×TE×ID	4	547,289	2293.5	136,822	573.4	19.94	35.79	0.000	0.000
Error	72	494,014	1153.5	6861	16				
Total	107	32,082,830	85278.5						

IF immersion frequency, *VM* volume of medium, *TE* type of explant, *ID* inoculation density

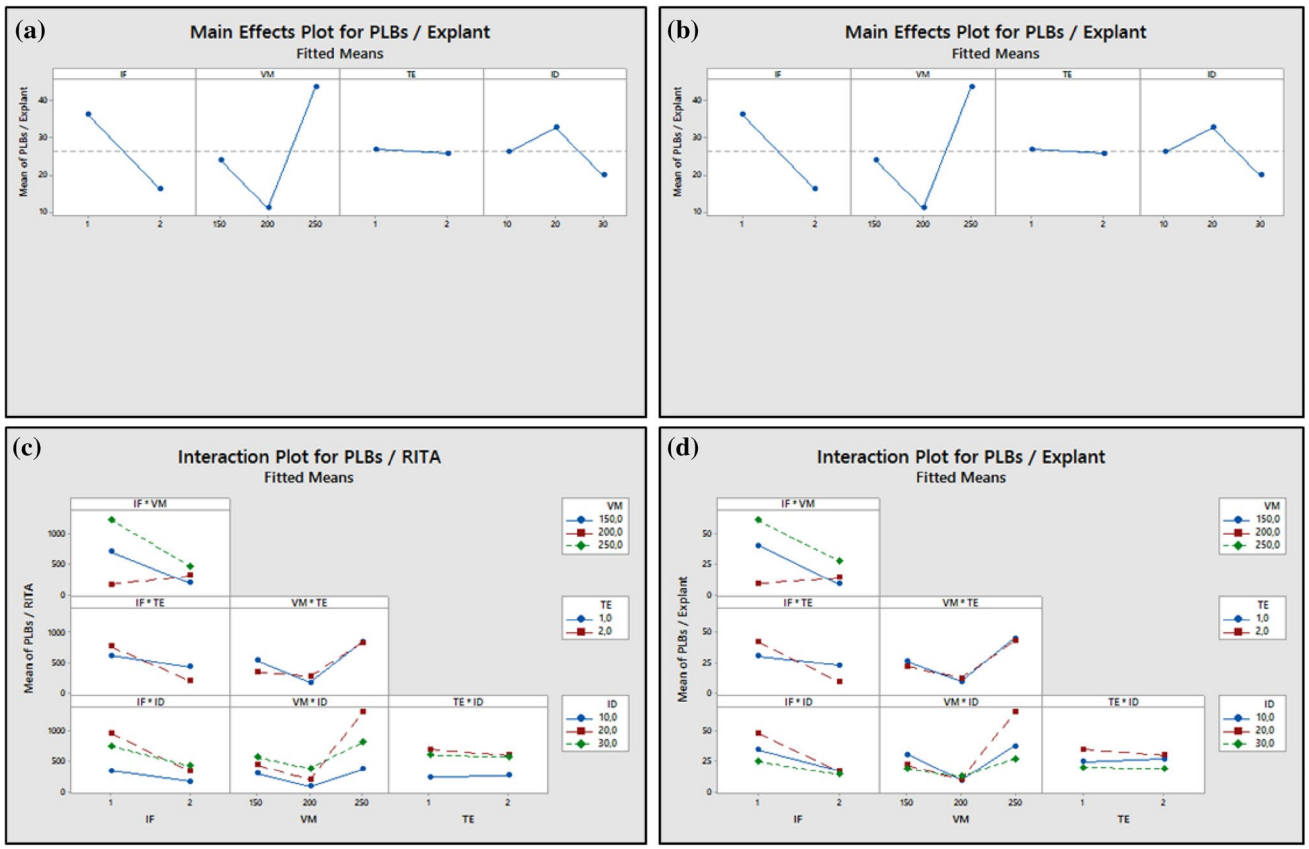

Fig. 2 Main effects and interaction plots for PLBs. **a** Main effects plot for the number of PLBs/RITA® for main groups [(IF-1: 1 min/4 h; 2: 1 min/8 h), (VM-150, 200 and 250 mL), (TE-1: W-PLB; 2: tTCL-PLB), (ID-10, 20 and 30 explants)]. **b** Main effects plot for the number of PLBs/Explant for main groups. **c** Interaction plot for the number of PLBs/RITA® for main groups. **d** Interaction plot for the number of PLBs per explant for main groups

and inoculum density from 10 explants to 20 and using tTCL-PLB explant type.

PLB induction from PLBs of *C. forbesii* Lindl. were also cultured in semi-solid PIM medium. This study were evaluated by Mann–Whitney test. According to this test, there was not significant differences between W-PLB and tTCL-PLB explant types ($P = 0.0809 > 0.00$). The number of PLBs per culture vessel obtained from W-PLB and tTCL-PLB was 90 and 63.3 PLB, respectively, while the number of PLBs per explant observed as 7.5 and 5.3, respectively (Table 3) (Online Resource 5). A comparison of the best results obtained from the semi-solid culture and RITA® bioreactor applications showed that the number of PLBs per explant in RITA® bioreactors increased approximately 14-fold in the W-PLB explant type and nearly 21-fold in the tTCL-PLB explant type compared with the semi-solid cultures (Table 3).

Table 3 Effect of semi-solid and liquid (RITA® bioreactor) cultures on PLBs production from different explant types (W-PLB or tTCL-PLB) of *Cattleya forbesii* Lindl.

Culture types	Number of PLBs/Culture vessel (±SE) Type of explant		Number of PLBs/Explant (±SE) Type of explant	
	W-PLB	tTCL-PLB	W-PLB	tTCL-PLB
Semi-solid	90 ± 4.6	63 ± 3.5	7.5 ± 0.4	5.3 ± 0.3
RITA®	2052 ± 9.7	2237 ± 1.8	102.6 ± 4.8	111.9 ± 9.1

Shoot regeneration from PLB

According to the results of shoot regeneration from PLBs, there are significant differences in the number of shoots obtained per RITA® and the number of shoots obtained per explant (Table 4). The highest shoot regeneration per RITA® was achieved as 3998 shoots regeneration in application no. 2 (1 min/4 h; 150 mL medium; 20 explants) using the tTCL-PLB explant type (Fig. 3a, b). This result was

Table 4 Effect of immersion frequency (1 min every 4 and 8 h), volume of medium (150, 200 and 250 mL), and inoculum density (10, 20 and 30 explants) on shoot regeneration from different explant types (W-PLB and tTCL-PLB) in RITA® culture of *Cattleya forbesii* Lindl.

Immersion frequency (min/h)	Volume of medium (mL)	Inoculation density (number of explant per RITA®)	Number of application	Number of Shoots/RITA® (± SE) Type of explant		Number of Shoots/Explant (± SE) Type of explant	
				W-PLB	tTCL-PLB	W-PLB	tTCL-PLB
1/4	150	10	1	1137 ± 6.4efgh	1314 ± 8.1defg	113.7 ± 6.4bc	131.4 ± 8.1b
		20	2	1404 ± 3.2def	3998 ± 22.4a	70.2 ± 1.6def	199.9 ± 11.2a
		30	3	1188 ± 11.1efgh	2934 ± 6.1b	39.6 ± 0.4ghij	97.8 ± 20.5c
	200	10	4	32 ± 1.7n	40 ± 5.9n	3.2 ± 0.2n	4.0 ± 0.6n
		20	5	110 ± 3.6mn	144 ± 12.7mn	5.5 ± 0.2n	7.2 ± 0.6mn
		30	6	279 ± 8.5lmn	187 ± 4.4mn	9.3 ± 0.3lmn	6.2 ± 0.1mn
	250	10	7	265 ± 3.2lmn	981 ± 17.3fghij	26.5 ± 0.3hijklm	98.1 ± 1.7c
		20	8	714 ± 5.5ghijklmn	1416 ± 17.9def	35.7 ± 0.3ghijk	70.8 ± 0.9de
		30	9	1060 ± 4.0efghi	2117 ± 5.0c	35.3 ± 1.3ghijk	70.6 ± 1.7de
1/8	150	10	10	478 ± 12.6ijklmn	332 ± 13.4klmn	47.8 ± 1.3efgh	33.2 ± 1.3ghijkl
		20	11	728 ± 8.9ghijklmn	425 ± 7.5ijklmn	36.4 ± 4.5ghijk	21.3 ± 3.8ijklmn
		30	12	134 ± 6.9mn	209 ± 5.9mn	4.5 ± 0.2n	6.9 ± 0.2mn
	200	10	13	396 ± 2.5jklmn	945 ± 15.6fghijk	39.6 ± 2.5ghij	94.5 ± 1.6cd
		20	14	445 ± 19.5ijklmn	900 ± 18.0fghijkl	22.3 ± 1.0ghijklm	45.0 ± 0.9fghi
		30	15	579 ± 3.5hijklmn	146 ± 7.0mn	19.3 ± 1.2jklmn	4.9 ± 0.2n
	250	10	16	315 ± 7.8klmn	1918 ± 3.5cd	31.5 ± 0.8ghijklm	191.8 ± 3.6a
		20	17	223 ± 9.8mn	455 ± 8.7ijklmn	11.1 ± 0.5klmn	22.8 ± 0.4hijklm
		30	18	1700 ± 5.3cde	213 ± 1.8mn	56.7 ± 1.8efg	7.1 ± 0.1mn

Each value represents the mean ± SE of three replicates

The same *letter* within a *column* denotes statistically equal means with the Tukey test at $P \leq 0.05$

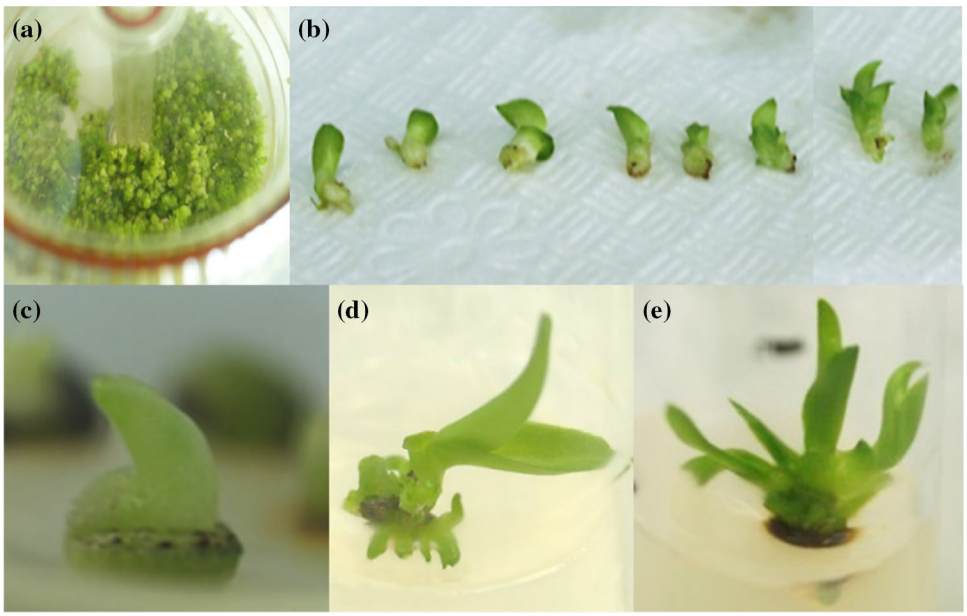

Fig. 3 Shoot regeneration from tTCL-PLB explants in (**a, b**) RITA® bioreactor and (**c, d, e**) semi-solid culture

followed by application no. 3 (1 min/4 h; 150 mL medium; 30 explants) where 2934 shoots regeneration per RITA® was obtained using the same tTCL-PLB explant type. The lowest shoot regeneration per RITA® (32 shoots) was detected in application no. 4 (1 min/4 h; 200 mL medium; 10 explants) where the W-PLB explant type was used. The highest shoot regeneration per explant (199.9 shoots) was obtained from application no. 2 where the tTCL-PLB explant type was used. This result was followed by application no. 16 (1 min/8 h; 250 mL medium; 10 explants) with 191.8 shoots

regeneration per explant. They were statistically placed in the same group. The lowest shoot regeneration per explant (3.2 shoots) was observed in application no. 4 where the W-PLB explant type was used. W-PLB and tTCL-PLB explants formed both single shoots with 2–3 leaves and shoot clusters. According to analysis of variance (Table 5), except for explant types tested, the four-way analyses revealed significant interactions ($P < 0.001$) the remaining factors for the number of PLBs per RITA® and per explant.

Effect of the main factors used in this study on the number of shoots obtained from RITA® bioreactors are shown in Fig. 4a, b and the comparisons of significant interactions were determined (Online Resource 6). Accordingly, the number of shoots obtained from RITA® bioreactors was found to be more important in the immersion for 1 min/4 h. In addition, increasing the volume of medium from 150 mL to 200 mL resulted in a significant decrease in the number of shoots per RITA® and explant, while a significant increase occurred in the number of shoots per RITA® and explant as a result of increasing the volume of medium from 200 mL to 250 mL. When the explant types were evaluated, it was observed that the tTCL-PLB explant had more significant effect than W-PLB explant ($P = 0.00 < 0.05$) for both the number of shoots per RITA® and explant. In the case of different inoculum density, reducing the number of explants from 20 to 10 and from 30 to 10 decreased the number of shoots per RITA®. Additionally, reducing the number of explants from 30 to 20 did not statistically affect the number of shoots per RITA® ($P = 0.920 < 0.05$).

In the two-way interactions of main factors, all two-way interactions (IF × VM, IF × TE, IF × ID, VM × TE, VM × ID and TE × ID) were significant ($P = 0.00 < 0.05$) for the number of shoots per RITA® and explant (Online Resource 7). According to IF × VM interactions According to the results of shoot regeneration from PLBs, there are significant differences in the number of shoots obtained per RITA® and the number of shoots obtained per explant., in the immersion for 1 min/4 h, the use of 150 mL medium instead of 200 mL increased the number of shoots per RITA® and explant. For IF × TE interactions, there was a significant increase in the number of shoots per RITA® and explant by using tTCL-PLB in the immersion for 1 min/4 h. In the IF × ID interactions, a significant increase in the number of shoots per RITA® occurred with the use of immersion for 1 min/4 h and 20 explants instead of the immersion for 1 min/8 h and 30 explants. When VM × TE interactions were evaluated, a significant increase in the number of shoots per RITA® and explant was observed by increasing the volume of medium from 150 mL to 200 mL in the application where tTCL-PLB was used (Fig. 4c, d). Considering VM × ID interactions, the use of 150 mL medium instead of 200 mL and 20 explants instead of 30 explants increased the number of shoots obtained per RITA® and explant (Online Resource 7 and Online Resource 8a, b) (Fig. 4c, d). When looking at interactions between TE × ID, the using W-PLB explant type and the reducing in the inoculation density from 20 explants to 10 led to a decrease in the number of shoots per RITA® and explant (Online Resource 7) (Fig. 4c, d).

Table 5 Analysis of variance for the number of Shoots/RITA® and the number of Shoots/Explant

Source	DF	Adj SS		Adj MS		F-Value		P-Value	
		Shoots/RITA®	Shoots/Explant	Shoots/RITA®	Shoots/Explant	Shoots/RITA®	Shoots/Explant	Shoots/RITA®	Shoots/Explant
IF	1	6,425,009	8994	6,425,009	8993.6	164.94	147.44	0.000	0.000
VM	2	13,455,702	39,334	6,727,851	19,667	172.72	322.42	0.000	0.000
TE	1	4,670,848	21,277	4,670,848	21277.1	119.91	348.82	0.144	0.000
ID	2	1,221,468	26,372	610,734	13185.9	15.6	216.17	0.000	0.000
IF × VM	2	19,410,973	63,236	9,705,487	31,618	249.16	518.35	0.000	0.000
IF × TE	1	3,412,622	2971	3,412,622	2970.6	87.61	48.70	0.000	0.000
IF × ID	2	4,709,036	11,601	2,354,518	5800.7	60.45	95.10	0.000	0.000
VM × TE	2	1,679,875	5127	839,937	2563.6	21.56	42.03	0.001	0.000
VM × ID	4	5,071,046	12,336	1,267,762	3084	32.55	50.56	0.000	0.000
TE × ID	2	1,078,423	8685	539,211	4342.6	13.84	71.19	0.001	0.000
IF × VM × TE	2	3,798,699	11,707	1,899,350	5853.3	48.76	95.96	0.000	0.000
IF × VM × ID	4	1,390,641	9086	347,660	2271.5	8.93	37.24	0.000	0.000
IF × TE × ID	2	4,254,401	11,269	2,127,201	5634.7	54.61	92.38	0.000	0.000
VM × TE × ID	4	4,443,365	22,693	1,110,841	5673.4	28.52	93.01	0.000	0.000
IF × VM × TE × ID	4	3,054,722	7236	763,680	1809.1	19.61	29.66	0.000	0.000
Error	72	2,804,580	4392	38,952	61				
Total	107	80,881,411	266,317						

IF immersion frequency, *VM* volume of medium, *TE* type of explant, *ID* inoculation density

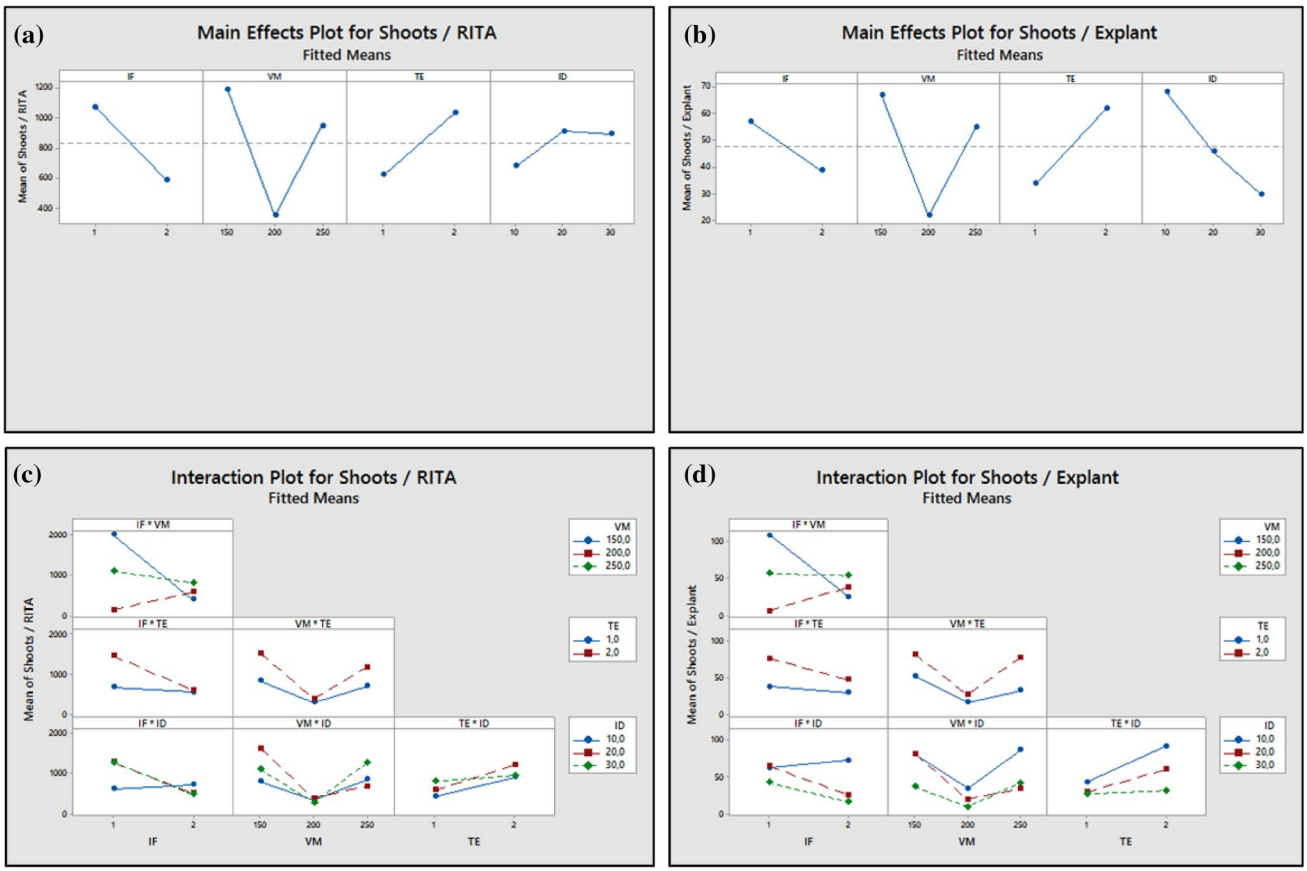

Fig. 4 Main effects and interaction plots for shoots. **a** Main effects plot for the number of Shoots/RITA® for main groups [(IF-1: 1 min/4 h; 2: 1 min/8 h), (VM-150, 200 and 250 mL), (TE-1: W-PLB; 2: tTCL-PLB), (ID-10, 20 and 30 explant)]. **b** Main effects plot for the number of Shoots/Explant for main groups. **c** Interaction plot for the number of Shoots/RITA® for main groups. **d** Interaction plot for the number of shoots per explant for main groups

In the three- and four-way interactions of main factors, all interactions (IF × VM × TE, IF × VM × ID, IF × TE × ID, VM × TE × ID and IF × VM × TE × ID) were significant ($P = 0.00 < 0.05$) for the number of shoots per RITA® and explant (Online Resource 9). According to IF × VM × TE interactions, in the immersion for 1 min/4 h, the use of 150 mL medium volume and tTCL-PLB explant type increased the number of shoots per RITA® and explant. In the IF × VM × ID interactions, the number of shoots per RITA® and explant were higher in the immersion for 1 min/4 h, 150 mL medium and 20 explants compared to immersion for 1 min/4 h, 200 mL medium and 10 explants. For IF × TE × ID interactions, there was a significant increase in the number of shoots per RITA® by using tTCL-PLB and 20 explants in the immersion for 1 min/4 h. Considering VM × TE × ID interactions, the use of 150 mL medium instead of 200 mL, tTCL-PLB and 20 explants instead of 30 explants increased the number of shoots obtained per RITA®. When IF × VM × TE × ID interactions were evaluated, a significant increase in the number of shoots per RITA® and explant was observed in the immersion for 1 min/4 h, using tTCL-PLB explant type, 150 mL medium volume and 20 explant (Online Resource 9).

The number of regenerated shoots per culture vessel and explant obtained from W-PLB and tTCL-PLB explant types cultured in semi-solid SRM medium (Fig. 3c–e) to promote shoot regeneration from PLBs of *C. forbesii* Lindl. were evaluated by Mann–Whitney test. According to this test, there was not significant differences between W-PLB and tTCL-PLB explant types ($P = 0.0809 > 0.00$). The number of shoots per culture vessel obtained from W-PLB and tTCL-PLB was 156 and 25 shoots, respectively, while the number of shoots per explant observed as 13 and 2.1, respectively (Table 6) (Online Resource 10). When the best results obtained from the semi-solid culture and RITA® bioreactor applications were compared, the number of shoots per explant in RITA® bioreactors increased approximately ninefold in the W-PLB explant type and almost 95-fold in the tTCL-PLB explant type compared to the semi-solid cultures (Table 6).

Table 6 Effect of semi-solid and liquid (RITA® bioreactor) cultures on shoot regeneration from different explant types (W-PLB or tTCL-PLB) of *Cattleya forbesii* Lindl.

Culture types	The number of Shoots/ Culture vessel (±SE) Type of explant		The number of Shoots/ Explant (±SE) Type of explant	
	W-PLB	tTCL-PLB	W-PLB	tTCL-PLB
Semi-solid	156 ± 12.6	25 ± 2.4	13.0 ± 1.1	2.1 ± 0.2
RITA®	1700 ± 5.3	3998 ± 22.4	113.7 ± 6.4	199.9 ± 11.2

Discussion

Propagation of orchids by conventional methods such as seeds, division and cutting is slow and not efficient. Therefore, micropropagation is used for large scale propagation of orchid plants with the same genetic makeup. However, current micropropagation methods with semi-solid medium have some disadvantages like time consuming, expensive, requiring more labor, making automation difficult. So, liquid-based culture methods are used to eliminate these disadvantages. Recently, bioreactor systems such as airlift, bubble and temporary immersion bioreactors have been used for large-scale propagation of PLBs and shoots of various orchids and the efficiency of bioreactor systems has been demonstrated (Murthy et al. 2018). Temporary immersion system (TIS) has been particularly designed for plant tissue culture and bioreactors operating with this principle are more advantageous than classical in vitro production techniques using semi-solid cultures in large-scale plant production (Zhang et al. 2018). In the present study, the semi-solid culture and TIS based bioreactor (RITA®) were compared for PLB and shoot production in *C. forbesii* Lindl. According to our results, the number of PLBs and shoots obtained per RITA® bioreactor and explant were much higher than semi-solid culture.

Immersion frequency is an important parameter affecting the efficiency of TIS. As plant tissues are periodically ventilated, and come into contact with liquid medium and the incoming air, the duration and frequency of the immersion affect the nutrient uptake, the atmosphere composition inside the bioreactor, the formation of hyperhydricity, and consequently, plant growth and propagation (Pérez-Alonso et al. 2009). In the present study, a positive relation was observed between the immersion frequency and PLB production and shoot regeneration. By progressively increasing in the immersion frequency from once every 8 h to once every 4 h, in both explant types, immersion for 1 min/4 h was more effective regarding the PLB production and shoot regeneration.

Medium volume (Roels et al. 2005; Ramos-Castellá et al. 2014) and inoculation density (Yang et al. 2010; Gatica-Arias and Weber 2013; Cui et al. 2014; Gao et al. 2014) are the another important parameters affecting the efficiency of temporary immersion bioreactors. In our study, medium volume was found to significantly affect the PLB production and shoot regeneration. A significantly higher PLB production per RITA® bioreactor and explant was achieved when the volume of medium was 250 mL compared to 150 and 200 mL. Unlike PLB production, shoot regeneration were higher in the applications where 150 mL of medium was used. The use of 200 mL medium volume remarkably reduced both the PLB production and shoot regeneration compared to 150 and 250 mL medium volume.

The inoculation density tested in the present study also affected the PLB production and shoot regeneration. Among the explant densities used, 20 explants cultured per RITA® gave the best response regarding PLB production per RITA® and explant and shoot regeneration per RITA®. The relationship between the inoculation density and cultures' growth and propagation in a bioreactor culture has been also reported in other orchid species such as *C. sinense* (Gao et al. 2014), *D. candidum* (Cui et al. 2014) and *Oncidium* 'Sugar Sweet' orchid (Yang et al. 2010).

Different type of explants (W-PLB and tTCL-PLB) was also evaluated in terms of PLB and shoot propagation in RITA® bioreactors. In our study, while both the W-PLB and t-TCL-PLB explants were convenient for PLB production, it was determined that t-TCL-PLB explant was more suitable for shoot production. Since it is easy and rapid to induce and propagate new protocorms, PLBs formed from various explants either directly or through an intermediate callus phase is usually used as explant sources for the in vitro vegetative propagation of orchids (Vyas et al. 2010; Romero et al. 2017). Thin cell layer (TCL) culture system offers a better alternative compared to other conventional in vitro micropropagation methods for rapid propagation of orchids (Zhao et al. 2007). However, this culture system has not been used effectively for the propagation of commercially valuable orchids except a few orchid species such as *Cymbidium* (Nayak et al. 2002; Teixeira da Silva et al. 2006; Vyas et al. 2010; Rittirat et al. 2017), *Dendrobium* (Nayak et al. 2002; Jaiphet and Rangsayatorn 2010), and *Phalaenopsis* (Murdad et al. 2006). After excision of TCL explants from the plant tissue, TCL develops alternate developmental pathways to itself under the effect of in vitro culture conditions and medium components (most likely by removing the influences of maternal tissue) (Park et al. 2002). In general, since cells in the 6–7-week-old PLBs are highly meristematic, more efficient production can be achieved by applying the TCL technique on PLBs (Teixeira da Silva 2013). The wounding caused by cutting the PLB into TCLs may be considered as a trigger for cell division (Vyas et al. 2010). After the wounding, quiescent unwounded cells near the cut surface become active, and cell proliferation starts with some biochemical

changes in the cells. As there are no other tissues in TCL explants, the supply of nutrients and growth promoting substances from the medium is facilitated, and they are transferred to the region where a direct regeneration will occur (Nayak et al. 2002; Park et al. 2002; Murdad et al. 2006; Vyas et al. 2010). This explains the reason for the highest shoot regeneration from tTCL-PLB explant in our study. Nayak et al. (2002) used thin uniform transverse sections excised from PLBs in *Cymbidium aloifolium* (L.) Sw. and *Dendrobium nobile* Lindl and obtained 28.20 PLBs (89.66% regeneration frequency) and 34 PLBs (87.85% regeneration frequency) per explant from *C. aloifolium* and *D. nobile* Lindl, respectively. Teixeira da Silva et al. (2006) used whole PLBs and different types of TCL as explant sources for PLBs production from PLBs of *Cymbidium* hybrids and obtained 8.41 PLBs per explant from the whole PLB explant (96.4% regeneration frequency). Murdad et al. (2006) used trimmed or untrimmed base protocorms in *Phalaenopsis gigantea* and obtained 7.01 and 1.94 protocorms per explant in trimmed and untrimmed explants, respectively. Vyas et al. (2010) obtained 5 PLBs per explant from the tTCLs of the PLB in *Cymbidium* Sleeping Nymph (83% regeneration frequency). Jaiphet and Rangsayatorn (2010) cultured PLBs of *Dendrobium gratiosissimum* orchids by dividing them into two halves (each half was considered as TCL) and obtained 18 PLBs per explant (83% regeneration frequency). Roy et al. (2011) obtained 16 PLBs per whole protocorm in *Vanda coerulea* Griff ex. Lindl. (Blue Vanda). Romero et al. (2017) used whole protocorm and TCL from protocorm as an explant source in a study with *Chloraea gavilu*. They obtained 11 PLBs per explant from whole protocorms, but there was no regeneration from the TCL protocorm. Rittirat et al. (2017) obtained 5.2 PLBs per protocorm from *Cymbidium finlaysonianum* protocorms (85.7% regeneration frequency). In our study, 102.6 and 111.9 PLBs per explant and 113.7 and 199.9 shoots per explant were obtained from W-PLB and tTCL-PLB explants of *C. forbesii* Lindl. respectively. These results are relatively higher than those found in the abovementioned studies. The differences between these studies may be due to the culture type and the content of the medium. Moreover, the use of bioreactors with temporary immersion system may be advantageous for the PLB production in this study.

Conclusions

In conclusion, the use of PLBs transverse thin cell layer technology with RITA® bioreactor technology based on temporary immersion systems provides an effective method for rapid mass propagation of PLBs and shoots in *C. forbesii* Lindl. Immersion frequency, volume of medium, type of explant and inoculum density significantly affected PLB and shoot regeneration in RITA® bioreactor. It was observed that PLB and shoot production were the best in RITA® bioreactor with immersion for 1 min/4 h, 250 mL of medium, 20 tTCL-PLB explants and RITA® bioreactor containing 150 mL of medium, 20 tTCL-PLB explants at an immersion frequency of 1 min every 4 h, respectively. At the end of the 60-day culture period, up to 2237 PLBs and 3998 shoots per bioreactor were obtained from the RITA® experiments. This number generates approximately 13,422 PLBs and 23,988 shoots annually from a single RITA® bioreactor. Therefore, this micropropagation method is more advantageous compared with micropropagation on semi-solid medium regarding explant manipulation, labor costs, and storage area. Moreover, this is the first report on the mass propagation of *Cattleya* sp. using the thin cell layer and RITA® bioreactor technologies. This propagation protocol is currently used for the mass propagation of *C. forbesii* Lindl. by a commercial plant tissue culture laboratory.

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Author contributions ME performed all experiments and wrote the manuscript. MB wrote the manuscript. ÖA calculated all statistical analyses and interpreted the data. AG supervised the research and edited and reviewed this manuscript. All authors designed research, read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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