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Growth and genetic stimulation of Cattleya-91 plantlets enriched with oxygenated ultrafine bubbles *in vitro* under aseptic conditions

Crescimento *in vitro* e estimulação genética de plântulas de Cattleya-91 em diferentes nutrições líquidas enriquecidas com bolhas ultrafinas oxigenadas em condição asséptica

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ABSTRACT

Effective Cattleya micropropagation enables achieving enhanced seedling quality and production. Ultrafine bubbles (UFBs) represent a recent technological breakthrough that is expected to improve the growth and development of orchid plantlets. The objective of this research was to reveal the effects of oxygenated UFB (O-UFB) at different liquid nutrition strengths on the morpho-physiology and gene expression of Cattleya plantlets grown *in vitro* under septic conditions. Uniform plantlets were planted on granular vermiculite-perlite (VP) (1:1) with full-Murashige Skoog (MS), ¾-MS, ½-MS, and full-Hougland (HG) liquid media enriched with oxygenated UFB (O-UFB), and full-MS-0 was used as control. A lower ionic strength (¾-MS) medium with the presence of O-UFB improved Cattleya plantlet growth both morphologically and physiologically. Nutrition strength also induced the overexpression of SUT2 and PEPCK genes during the *in vitro* septic culture, leading to a physiological transition from heterotrophic to autotrophic micropropagation. Therefore, O-UFB supplementation during Cattleya plantlet growth and development can improve its quality and productivity.

Index terms: Gene expression; micropropagation; nanobubbles; orchid; ionic strength.

RESUMO

A micropropagação eficaz de Cattleya melhora a qualidade e a produção de mudas. Espera-se que a aplicação de bolhas ultrafinas (UFB) como um avanço tecnológico recente melhore o crescimento e desenvolvimento das plântulas de orquídeas. O objetivo da pesquisa foi revelar o efeito da UFB oxigenada (O-UFB) em diferentes dosagens de nutrição líquida sobre a morfofisiologia e a expressão gênica de plântulas de Cattleya cultivadas em cultura séptica *in vitro*. Plântulas uniformes foram cultivadas em vermiculita-perlita granular (VP) (1:1) com meio líquido Murashige Skoog (MS) e Hougland (HG) enriquecido com UFB oxigenado (O-UFB) e MS-0 completo como controle. A menor força iônica (3/4-MS) na presença de O-UFB melhorou o crescimento morfológico e fisiológico das plântulas de Cattleya. A força nutricional também induziu a superexpressão dos genes SUT2 e PEPCK durante o cultivo séptico *in vitro*, levando a uma transição fisiológica da micropropagação heterotrófica para a autotrófica. A suplementação de O-UFB no crescimento e desenvolvimento de plântulas de Cattleya pode melhorar a qualidade e a produtividade.

Termos para indexação: Expressão gênica; micropropagação; nanobolhas; orquídea; força iônica.

Introduction

Cattleya is one of the most attractive types of orchids for use as cut flowers due to its high genetic variability, photoperiodically controllable varieties, attractive colors, durability, shape, and profitability. Micropropagation of Cattleya includes the following steps: preparation and sterilization of the plant material, seeds and initial germination, shoot and protocorm-like body induction, and finally, proliferation, plantlet formation, and acclimatization (Dewir et al., 2015; Lando et al., 2016; Menezes-Sá et al., 2021; Oliveira et al., 2021). Several well-known factors influence plant growth and development *in vitro*, such as basal medium and strength (An et al., 2021; Zahara et al., 2017), culture method (solid or liquid) (Winarto et al., 2013), light intensity and quality (Bantis et al., 2018), dissolved oxygen (DO) levels (Bhatia & Sharma, 2015; Winarto et al., 2013), sugar content (Zahara et al., 2017), and plant growth regulators (Xu, Beleski, & Vendrame, 2022).

The basal medium and strength significantly affect the morphogenesis of plant cells, tissues, and organs *in vitro.* The macro- and micronutrients needed to regulate plant growth and development are included in the composition of the basal media

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(An et al., 2021; Zahara et al., 2017). According to Liebig's law of the minimum, the micropropagation medium has the lowest possible quantity of nutrients and is regulated by low nutrient concentrations and balances (An et al., 2021). The basal medium of MS is used widely for the plant cell culture of Cattleya (Oliveira et al., 2021) and Dendrobium (Winarto et al., 2013) because this medium contains high concentrations of nitrate and ammonium ions along with potassium (Oliveira et al., 2021), similar to Hoagland (HG) (Kittiwongwattana & Vuttipongchaikij, 2013). Unoptimal basal medium, which is also referred to as negative effects medium, generally affects the growth stage of explants during *in vitro* culture (Bhowmik & Rahman, 2017; Maissour et al., 2023; Hwang et al., 2024). The number of protocorms that develop on the Knudson medium is lower than that on the MS medium, but the size of the protocorms is greater when the Knudson medium is used. A low amount of macro- and micronutrients in the Knudson medium could be the reason for this greater size of protocorms, which is probably to counter the nutritional stress, but not for the increased number of protocorms. The production of larger protocorms indicates that the cultured seeds require nutrients in sufficient amounts. Thus, it is understood that the nutrient regime for orchid culture is species-specific, and no single culture medium is universally applicable to all orchid species (Bhowmik & Rahman, 2017). In a study, compared to those grown on MS or OMM, the fresh weight of the seedlings of *Dendrobium moniliforme* grown on Hyponex medium was substantially greater, nearly 20 times, and the number of roots and root length were also higher (Hwang et al., 2024). Therefore, optimizing mineral nutrients in the culture medium is regarded as a key strategy for improving the plant micropropagation protocol.

An ultrafine bubble (UFB) is a floating bubble with a volume equivalent diameter of less than 200 nm (Agarwal, Ng, & Liu, 2011). The UFB interface consists of strong hydrogen bonds that allow UFB to maintain its adequate kinetic balance, lowering the UFB's diffusivity against high internal pressure (Takahashi et al., 2007) and high thermal and mechanical strength and enabling high gas solubility (Tao, 2022). Incorporating UFB into a liquid medium for micropropagation would, therefore, be significantly advantageous, as the bubbles could remain for weeks or even months (Tanaka et al., 2021). In contrast, micro and macro bubbles, which are commonly found in bioreactor cultures, directly rise to the surface of the water (Agarwal, Ng, & Liu, 2011).

In fact, UFBs have been successfully used in the field of hydroponics (Kobayashi & Yamaji, 2022) and for the germination of lettuce, carrot, fava bean (Ahmed et al., 2018), and barley (Oshita et al., 2023), and fermented lettuce (Pujiwati et al., 2024). In previous studies, physiologically, the application of UFB increased the germination rate of oil palm seeds (Arif et al., 2023), improved seed invigoration and germination for shallot seeds (Raga, Widajati, & Purwanto, 2023), increased the water and/or nutrient-dissolved absorption and prevented leaf senescence under stress conditions in *Coffea arabica* seedlings (Hirooka, Motomura, & Iijima, 2024), and increased the germination rate and radicle emergence to >80% for shallot seed germination (Raga, et al., 2024). However, there is currently no report on the application of UFB in cattle micropropagation. Therefore, this study aimed to reveal the effects of oxygenated UFB at different liquid nutrition strengths on the growth and genetic stimulation of Cattleya plantlets grown *in vitro* under septic conditions.

Material and Methods

Plant material, plantlet preparation, and culture incubation

Cattleya-91 protocorm-like bodies (plbs) were collected from the Taman Arjuno Research Center (7°51'34.0"S 112°38'51.0"E). The plants were maintained in a semi-solid MS medium supplemented with 3% sucrose, 15% banana extract, 10% coconut water, and 1% agar until young plantlets were produced. Uniform young plantlets with 1–2 young immature leaves, plant length of 0.8–1.2 cm, 1–2 roots, and root length of 0.5–0.7 mm were harvested and selected for plant germination after 90 days of culture.

The cultures were incubated at a temperature of 25 ±2 °C under a 16-h photoperiod provided by cool daylight fluorescent lamps inside a culture room.

Effects of different liquid nutrition strengths on Cattleya 91 plantlet growth performance

The culture system used in the experiment was liquid culture UFB supplemented with a combination of granular porous media, i.e., vermiculite (V) and perlite (P) (< 3 mm in diameter), at a 1:1 ratio and ±1 cm in thickness. The different liquid nutrition strengths tested in the experiment were (1) full-MS-UFB, (2) ¾-MS-UFB, (3) ½-MS-UFB, and (4) full-HG-UFB supplemented with MS vitamins and 100 mg/L myoinositol, while (5) full-MS-0 was used as control. A completely randomized design with six replications was adopted. Each treatment consisted of six culture vessels (jam bottles, 9 cm in height and 6 cm in diameter). Each culture vessel contained 4 uniform plantlets. All media were sugar-free. The pH of each medium was adjusted to 5.6 ±0.2 prior to autoclaving for 20 min at 121 °C. A UFB generator (Qwater, Kanchun Technology Co. Ltd., Nanjun, Taichung City, China) and an oxygen concentrator (Yuwell 8F-3AW, Jiangsu Yuyue Medical Equipment and Supply Co. Ltd., Nanjing, Jiangsu, China) were used in tandem to create oxygenated UFBs for liquid nutrition. Freshly prepared nutrient-oxygenated UFBs were added biweekly to the culture vessel in sufficient amounts (2 mL/vessel) to ensure nutrient availability. The cultures were incubated for 3 months inside a culture room under similar conditions as those described previously.

Morphophysiological aspects and gene expression analysis

The morphological data collected in the experiment included plant length (PH), leaf number (LN), root length (RL) and number (RN), total fresh weight (TFW), shoot fresh weight (SFW), root fresh weight (RFW), and the root-shoot ratio (RSR). The physiological data measured included chlorophyll a and b contents, carotenoid contents, and gene expression data, including catalase (CAT), phosphoenolpyruvate carboxykinase (PEPCK), sucrose transporter 2 (SUT2), ribulose-1,5-bisphosphate carboxylase/oxygenase (RBC), and cyclin D1 (D1) expression. All data were recorded for 90 days after culture.

The chlorophyll a, b, and carotenoid contents were determined using 95% ethanol extraction of young leaf blades. The leaf and ethanol mixtures were homogenized and centrifuged at 10000 rpm for 15 min. The supernatant was diluted 10 times and analyzed using a UV-Vis spectrophotometer at 664, 649, and 470 nm. The concentration (µg/mL) was calculated as described by Lichtenthaler (1987).

A RiboSpin™ isolation kit (GeneAll Biotechnology, South Korea) was used to isolate RNA from 100 mg of leaf tissue from each sample. The RNA quantity and quality were determined using agarose gel electrophoresis. Double-strand cDNA samples were synthesized using the technical manual protocol of the Bio-Rad iScript™ cDNA Synthesis Kit (Applied Biosystems, USA). Bio-Rad's MiniOpticon™ Real-Time PCR System (Applied Biosystems, USA) was used for gene expression analysis.

The PCR mixtures were prepared in a strip-tube and contained individual samples, 1x SYBR Green (Taq polymerase, dNTPs, SYBRGreen dye), and 200 nM forward and reverse primers (SUT2-F 5'-TACTCAACATTTCCATCGTCATCCC-3' SUT2-R 5'-AGTTAGAGCGAGA GAGCCTTGGAA-3'; CAT-F 5'-GGATGATGAAGCTGTGATTGTTGG-3' CAT-R 5'-CA GGCTGAAGAGGCAGGATGTC-3'; D1-F 5'-TATCATTGCCTTCATTGTTGCC C-3' D1-R 5'-AAGTTCATAAGGACCGCCATTGTAC -3'; RBCS-F 5'-TGATGATCTCATCCGC TACCGC'-3' RBCS-R 5'-CAGGGAGGTATGACAGTGTCTCAAAC-3'; PEPC K-F 5'-GC TTCCTACCCTATCGAGTACATTCC-3' PEPCK-R 5'-TGGCTTGCGGCTC CTTGAT-3'). Amplification was performed using the following PCR program: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min, and finally, the melting curve was obtained at 65 to 95 °C, with a 5 °C increase in each step, using fluorescence measurement. The expression levels were calculated based on primer efficiency and normalized according to the housekeeping gene Ubiquitin (Ubi) (F/R 5'-TGAAC TCCATCGCCTTCCTCTTC-3' / 5'-TGAAGC ATGGCATCAATTTC-3').

Data analysis

The data collected from the experiments were analyzed through an analysis of variance (ANOVA) with Genstatf 19th. Differences in means were further evaluated using Tukey's test, with $P = 0.01$ indicating significance. Bio-Rad CFX Maestro software was used to quantify the expressions of RBCs, PEPCK, CAT, D1, and SUT2.

Results and Discussion

Effect of medium condition on plant morpho-physiology characteristics

Plant micropropagation medium usually consists of macro- and microelements, organic supplements, carbon sources, growth regulators, and agar, and is placed in closed vessels to maintain aseptic and humid conditions for plantlet growth and development. In the present study, the application of sugar-free thin-film liquid nutrition media, namely, full-MS, ¾-MS, ½-MS, and full-HG enriched with O-UFG provided an ideal environment for roots to grow and absorb nutrients from a porous VP surface and significantly stimulated growth and development of Cattleya 91 plantlets (**; $p < 0.01$) (Table 1). The MS and HG basal media enriched with O-UFB influenced plantlet parameters. In contrast to full-MS-0, O-UFB supplementation significantly increased the pH, LN, RL, and RN of full-MS, ¾-MS, ½-MS, and full-HG media (Figure 1A-1E).

Table 1: The means value of plant length (PH), leaves number (LN); root length (RL), root number (RN), total fresh weight (TFW), shoot and root fresh weight (FWS, FWR), and root-shoot ratio (RSR) root fresh of Cattleya-91 plantlet on different medium condition.

Medium condition	PH** (cm)	LN**	RL** (cm)	RN**	TFW** (mg)	FWS** (mg)	FWR** (mg)	RSR**
Full-MS-UFB	2.46±0.09^{c}	2.50±0.03^{ab}	2.44±0.09^{b}	2.38±0.14^{a}	414.38±11.22^{bc}	315.38±23.0^{b}	99.00±16.96^{a}	0.32±0.09^{a}
¾-MS-UFB	2.52±0.08^{c}	3.00±0.20^{c}	2.59±0.14^{b}	2.56±0.13^{ab}	425.31±16.95^{c}	314.56±19.71^{b}	110.75±4.25^{ab}	0.35±0.03^{a}
½-MS-UFB	2.32±0.08^{b}	2.38±0.14^{ab}	2.43±0.10^{b}	3.06±0.13^{c}	359.44±7.86^{a}	244.00±3.51^{a}	115.44±6.1 ^{b}	0.47±0.02^{b}
Full-HG-UFB	2.51±0.01^{c}	2.56±0.13^{b}	2.44±0.11^{b}	2.69±0.13^{b}	406.13±12.84^{b}	308.50±10.58^{b}	97.63±6.14^{a}	0.32±0.02^{a}
Full-MS-0	1.99±0.10^{a}	2.25±0.20^{a}	1.91±0.10^{a}	2.63±0.14^{b}	358.00±10.16^{a}	253.19±13.14^{a}	104.81±8.18^{ab}	0.42±0.05^{ab}

Means followed by the same latter in the same column are not significantly different based on Tukey test, *p<0.01* (**).

Figure 1: Cattleya-91 plantlets morphogenesis on different medium conditions of full-MS-UFB (A), ¾-MS-UFB (B), ½-MS-UFB (C), full-HG-UFB (D), and full-MS-0 (E). All figures in similar size with a 1 cm bar.

In many cases, higher nutrient concentrations are superior to lower ionic strengths in terms of plant growth regulation. The results of this study revealed that nutrition-enriched O-UFB promoted growth, as indicated by the PH, LN, RN, and RL parameters, as well as the quality of physiological characteristics. Interestingly, some growth parameters, such as PH, RL, RN, and FWS, were affected similarly by ¾-MS-UFB and full-HG-UFB media, whereas LN was affected by ½-MS-UFB and full-HG-UFB media. The concentration of full-HG-UFB was lower than that of ¾-MS-UFB (34.15 mM) and ½-MS-UFB (22.77 mM), but the presence of UFBs effectively supported plant growth. UFBs influenced several key physiological aspects of plants, primarily through interactions with the growth processes, improved the dissolution of gases in water, and increased oxygen and nutrient availability in the root zone. Enhanced gas exchange promotes better nutrient absorption by plants (Uchida et al., 2018). UFB treatment also upregulated the genes associated with nutrient absorption and stimulated the synthesis of the hormone gibberellin, which plays a role in promoting nutrient absorption and overall plant development (Oshita et al., 2023). The application of UFB has been reported to increase the germination rate and the vigor index of oil palm seeds (Arif et al., 2023) and shallot seeds (Raga, Widajati, & Purwanto, 2023), enhance the absorption of dissolved water and/or nutrients, prevent the leaf senescence of *Coffea arabica* seedlings under stress conditions (Hirooka, Motomura, & Iijima, 2024), and increase gas solubility and the bubble surface area and promote the production of reactive oxygen species (Raga et al., 2024), all of which are essential seed signaling pathways related to the gibberellic and abscisic acid (GA) ratio, cell synthesis, and respiration during the germination of shallot seeds (Raga, Widajati, & Purwanto, 2023), Interestingly, the full MS-UFB medium, which had the highest concentration of nutrients (45.54 mM), did not affect the maximum mean values of the parameters. In fact, RN peaked when the mean values of ½-MS-UFB were used, whereas PH, LN, and RL peaked when the mean values of ¾-MS-UFB were used; the minimum values of PH, LN and RL were noted in full-MS-0. The pH (2.52 cm) of the plantlets grown in ¾×-MS-UFB was 1.27 times greater than that of those grown in full-MS-0 (1.99 cm). The ¾-MS-UFB mediumimproved the NL and RL values by 1.33 and 1.36 times, respectively, compared to those noted in full-MS-0. However, there was no noticeable distinction in the pH between full-HG-UFB and ¾-MS-UFB.

In contrast, the RN of ½-MS-UFB was 1.17 times greater than that of full-MS-0, with 3.06 roots/plantlet noted in the former. According to the present study, O-UFB in the liquid nutrition with varying ionic strengths had a greater effect on plantlet development than full MS-0, potentially because it increased the dissolved oxygen (DO) and reduced the critical amounts of nutrients by increasing nutrient absorption. O-UFB significantly influenced TFW and RSR in liquid full-MS, ¾-MS, ½-MS, and full-HG (**; $P < 0.01$). The average TFW values in the ¾-MS-UFB medium increased rapidly and peaked at 425.31 mg/plantlet, which was 1.05 and 1.19 times greater than those in the full-HG-UFB and full-MS-0 media, respectively. In full-MS-UFB medium, the mean value of FWS peaked at 3.15 mg/plantlet; however, it did not differ significantly from the value noted for ¾-MS-UFB (3.14 mg/plantlet) or full-HG-UFB (406 mg/plantlet). The FWR increased significantly and peaked at 115 mg/plantlet in ½-MS-UFB but differed from that noted for ¾-MS-UFB and full-MS-0. The RSR parameter was influenced considerably by full-MS-UFB (1.14-fold) and full-HG-UFB (1.49-fold) but was not significantly influenced by full-MS-0. According to these results, O-UFB clearly regulated morphogenesis, fulfilled sufficient nutritional requirements similar to HG, and modified plant requirements.

In this study, the presence of O-UFB in the medium with relatively low ionic strength improved plantlet growth, while plant growth in the medium with the highest ionic strength was reduced. The addition of UFB to the medium improved nutrient utilization and plantlet growth (Wang et al., 2021). The author explained that UFB in the rhizosphere can attract positively charged nutrients such as K^+ and Ca^{2+} from the rhizosphere, leading to increased productivity and bioavailability of N and P.

Plant growth in this study also varied with the ionic strength of the basal medium in the modified culture medium. In terms of plantlet growth, the MS basal medium was superior to HG.

Balance and proper composition are two key factors to successfully achieve plant development in micropropagation, as these factors are critical for the proper morphogenesis and multiplication rate of a given genotype. The total concentration of full-MS in this study was greater than that of full-HG-UFB. The minerals provided by MS are suitable for the universal tissue culture of most herbaceous plants and involve high ion concentrations, especially those of nitrogen, potassium, zinc, and chlorine (Leifert, Murphy, & Lumsden, 1995). HG was the first to emerges as a proper medium for hydroponics and tissue culture, after which, MS became the most frequently used medium composition in micropropagation among the numerous compositions developed over several years. In the presence of O-UFB, lowering MS concentrations did not affect nutritional balance.

The critical amount of nutrients required by Cattleya to grow appeared to be a secondary issue in this study. Previous micropropagation studies have reported that nutrients in the culture medium play an essential role in orchid seedling development (Yam & Arditti, 2009), but no consensus has been reached on nutritional requirements (Vudala, Padial, & Ribas, 2019). Micropropagation studies using MS or Vacint and Went (VW) have revealed that cultures having the most diverse nutrient concentrations result in differences in plant growth responses. Previous studies on Cattleya micropropagation reported that the shoot, root, and leaf number parameters of *Cattleya elongata* Barb. were better in the VW medium than in the ½-MS medium. The fresh biomass in the VW medium increased more during early growth (180 days) but decreased at the end of the observation period (270 days), indicating that MS was the superior medium (Oliveira et al., 2021). According to An et al. (2021), ½-MS and full-Hyponex promote the growth of *Sedirea japonica* plantlets more remarkably, in terms of fresh weight, root length, and leaf area, than full-MS-0. Zahara et al. (2017) reported that both ½-MS and full-VW promoted plantlet growth in the *Phalaenopsis* hybrid 'Pink'.

The effects of medium conditions on plantlet physiological characteristics observed in this study are described in Table 2. Compared to the full-MS-0 medium, the full-HG-UFB medium significantly increased the chlorophyll a and b contents (**; $P < 0.01$), the values of which reached 1.61 and 0.66 µg/mL, respectively, for each plantlet; these values were 1.08-fold and 1.26-fold greater than those noted for the full-MS-0 medium. A ¾-MS-UFB medium produced 0.84 µg of carotenoids/mL per plantlet, which was a 1.77-fold increase over that of full-MS-0. The presence of O-UFB in a liquid medium with varying ionic strengths resulted in equal contents of chlorophyll a, b, and carotenoids in the plantlets. However, the medium conditions did not affect the chlorophyll b content while the average chlorophyll b content was greater in the full HG-UFB. Nutrient-enriched UFB promoted increases in the leaf parameters and total chlorophyll content after four weeks of culture.

The nitrogen concentration and the ammonium-nitrate ratio of MS and HG are important factors in micropropagation media. HG basal medium lowered the ammonium-nitrate ratio and promoted the chlorophyll content, which could be attributed to the better ionic conditions provided for plant development. The ammonium-to-nitrate ratio can influence the chlorophyll content in plants. Sotiropoulos et al. (2005) reported that using only ammonium nutrition in the medium reduced the chlorophyll content in apple plants. High ammonium application in the medium was reportedly related to a relatively low pH (Ma et al., 2018). However, Cattleya plantlets grow better in the MS medium, which has a higher ammonium-nitrate ratio (0.52), whereas HG has a lower ratio (0.023). In terms of specific parameters, Cattleya's growth is better in a medium with a low ammonium-nitrate ratio. Plant growth responses to different nitrogen forms or ammonium-nitrate ratios differ across species (Zhu et al., 2021).

Effect of medium conditions on gene expression levels

The gene expression data for plant growth and metabolism during plantlet incubation are shown in Figure 2. Gene expression was regulated by the medium conditions. The ¾-MS-UFB medium resulted in increased expressions of PEPCK (13.25 ΔΔCq), SUT2 (2.61 ΔΔCq), and CAT (10.31 ΔΔCq). The medium ¾-MS UFB induced high expression levels of D1 and PEPCK genes, with a value of 3.94 ΔΔCq for each. Full-HG-UFB also resulted in relatively high expression levels of RBCS and SUT2 genes, with a value of 1.00 for each. The full-MS-0 medium increased the expressions of CAT (23.01 ΔΔCq), D1 (4.79 ΔΔCq), and RBCs (1.37 ΔΔCq) but decreased the expressions of PEPCK and SUT2 genes. In contrast, full-MS-UFB produced the lowest expression levels of all target genes.

Table 2: The chlorophyll and carotenoid characters of Cattleya-91 plantlets on different medium condition.

Medium condition	Chlorophyll-a ** (µg/mL)	Chlorophyll-b ** (µg/mL)	Carotenoid ** (µg/mL)
Full-MS-UFB	1.32±0.01 a	0.59±0.05 a	0.70±0.44 b
¾-MS-UFB	1.56±0.04 bc	0.53±0,11 a	0.84±0.09 c
½-MS-UFB	1.33±0.07 a	0.60±0.06 a	0.51±0.07 a
Full-HG-UFB	1.61±0.03 c	0.66±0.15 a	0.69±0.12 b
Full-MS-0	1.49±0.06 b	0.52±0.03 a	0.48±0.02 a

Means followed by the same letter in the same column are not significantly different based on the Tukey test, p<0.01 (**).

Gene expression could be clustered into two groups: the SUT2 and PEPCK genes in one group and the CAT, RBC, and D1 genes in the other. The overexpression of the SUT2 and PEPCK genes could affect the growth of the plantlets. The greatest shoot and root growth, as well as fresh weight, were observed in the plantlets that were grown on the ¾-MS-UFB medium when the gene was overexpressed. All aerobic organisms use molecular oxygen to generate ATP, which is a chemically valuable energy source for life, and this oxygen may become toxic and mutagenic through the production of reactive oxygen species (ROS), such as H_2O_2 (Buonocore, Perrone, & Tataranno, 2010). In this study, the CAT gene was overexpressed in both full-MS-0 and ¾-MS-UFB media. The catalase (CAT) enzyme is found in many organisms and helps decompose H_2O_2 into non-toxic water and O_2 (Ali, Hahn, & Paek, 2005). The overexpression of CAT can improve plant resistance to abiotic and biotic stresses, and it is, therefore, considered an essential enzyme for reducing oxidative stress (Zeng et al., 2019).

According to a recent study, SUT2 and PEPCK genes also play crucial roles in this process, but in different ways. SUT2 is a gene that transports sucrose in most plant species. It is responsible for transferring sucrose from the source to the sink tissues in plants. Moreover, the SUT2 gene helps regulate sucrose uptake and distribution in plants (Barker et al., 2000). PEPCK is an enzyme that drives gluconeogenesis, a process of the conversion of non-carbohydrate precursors into glucose. This enzyme also participates in photosynthesis, which regulates carbon fixation. PEPCK gene expression is influenced by light, temperature, and water availability (Huang et al., 2015).

This study revealed that the use of O-UFB in liquid nutrition and VP provided an ideal environment for roots to absorb nutrients and oxygen from a porous medium. Vermiculite ($(Mg, Fe^{2+}, Al)_3(Al, Si)_4O_{10}(OH)_2 \bullet 4(H_2O)$) is a hydrous phyllosilicate mineral soil conditioner with a 2:1 layer composed of one octahedral sheet between two tetrahedral sheets. This structure provides a highly negative charge to vermiculite, with a cation exchange capacity of 100–150 meq/100 g (Kumari & Mohan, 2021). Vermiculite improves plant growth because of its high water-holding capacity, inert chemical nature, moderate level of aeration, absence of substrate for microbial growth, effective cation exchange capacity (Indrasumunar & Gresshoff, 2013), and ability to manage nutrient and moisture contents. Vermiculite microsurfaces produce interfacial oxygen UFBs, which are loaded into and delivered to the root zone of rice, stimulating root activities and DW from 4.4 to 6.5 g of root DW and increasing rice shoot biomass from 29.5 when vermiculite free to 37.1 g for adsorption, which regulates water and nutrient management (Sha et al., 2020). While perlite ($Al_2CaFe_2K_2MgNa_2O_{12}Si$) is a hydrated, glassy volcanic rock with a water content of 2%–5% (Khoshraftar, Masoumi, & Ghaemi, 2023). Highly porous growing media are essential because their physical, chemical, and biological properties allow for adsorption

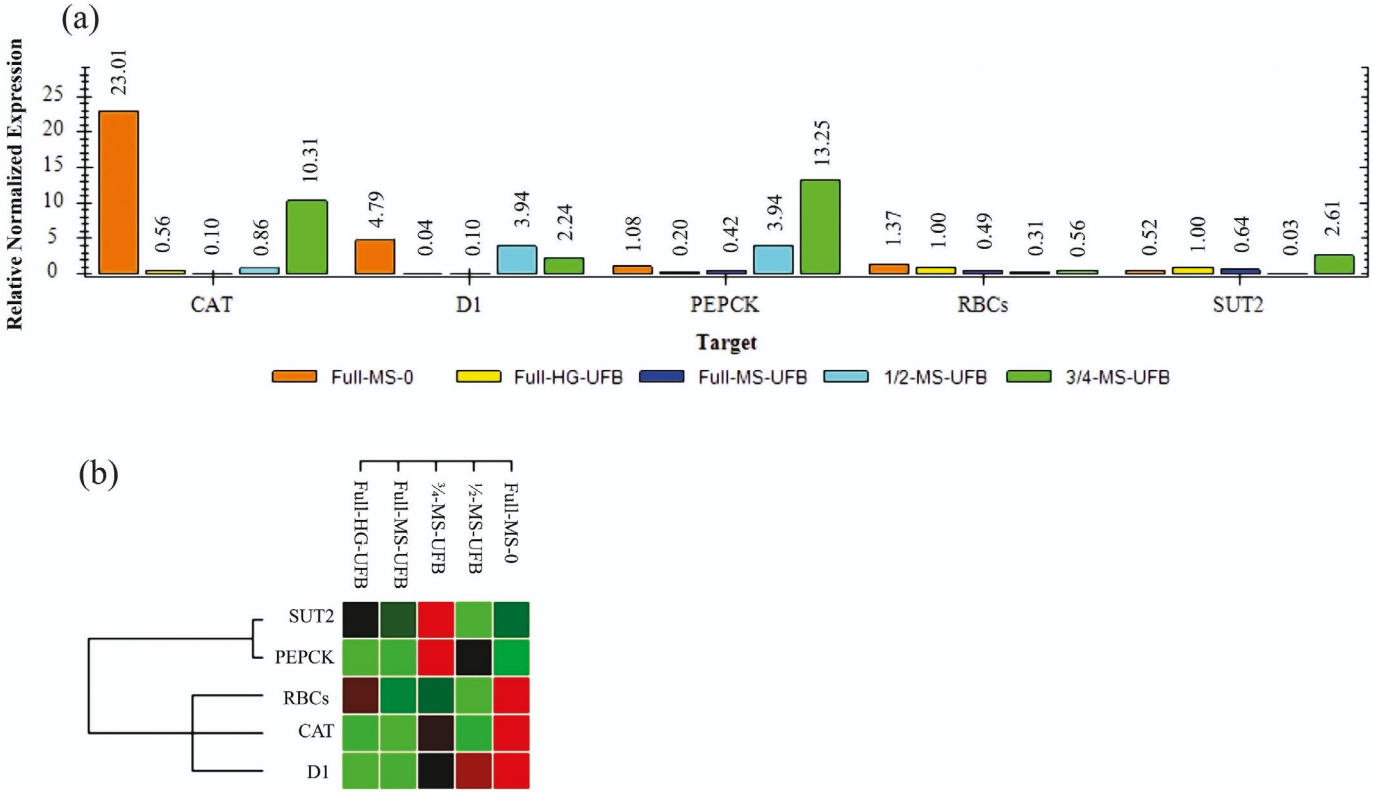

Figure 2: The gene expression pattern (a) and cluster (b) of Cattleya-91 plantlets as a response to different medium condition.

and regulate water and nutrient management (Savvas & Gruda, 2018). UFBs have long retention times in porous media, indicating that they remain available and, therefore, have long-term effects (Hamamoto et al., 2019). As the oxygen diffusion capacity in water is insufficient, a VP may facilitate liquid and gas exchange in the medium during incubation. In fact, VP application reportedly improved the growth of lemon verbena (Aliniaeifard et al., 2010), *Arachnis labrosa* (Deb & Imchen, 2010), soybean (Indrasumunar & Gresshoff, 2013), and grape (Dev et al., 2019).

Using a small volume of liquid nutrients for developing plantlets allows for porosity and continuous nutrient transfer to the plantlets through the attachment of the nutrition-enriched O-UFB to the VP surfaces. A thin film of liquid medium serves multiple purposes, such as supplying elements for plant growth, balancing transpiration, and preventing hyperhydricity (Afreen, 2008). Additionally, this film provides gases (O_2 and CO_2) and humidity to balance transpiration and avoid hyperhydricity. Optimal growth conditions alter metabolism and electron transport, leading to regulatory changes and bioproduct synthesis (Somerville & Proctor, 2009, 2013)

An increase in the oxygen concentration in liquid media is commonly observed in bioreactor cultures. Compared to conventional cultures, continuous air supply in the bioreactor gradually increased the DO levels from 8 to 12 mg/L (Kujawiak et al., 2017) and significantly improved cell growth in Citrus (Agisimanto et al., 2019) and Dendrobium mass propagation (Winarto et al., 2013). In contrast to micropropagation, the application of UFB has advanced to\ *ex vitro* experiments, and increasing evidence suggests that UFBs and microbubbles enhance growth processes (Oshita et al., 2023). The presence and density of UFBs in nutrition media increased the DO levels (Nirmalkar, Pacek, & Barigou, 2018).

Using microbubbles in a hydroponic nutrient solution resulted in about twice as much lettuce growth as the control solution in a previous study (Park & Kurata, 2009). When tap water with UFBs was added, the fresh and dry weights of lettuce shoots produced in the hydroponic system were greater than those produced when normal tap water was used (Kobayashi & Yamaji, 2022). *Brassica campestris* demonstrated a similar effect of growth promotion after four weeks of hydroponic culture in air nanobubble water (Ebina et al., 2013). Laboratory and field studies on rice production revealed that UFBs stimulated gibberellin synthesis and upregulated plant nutrient absorption genes in rice seedlings, increasing rice yield by nearly 8% while reducing fertilizer usage by 25% (Wang et al., 2021). UFB has unique physical-chemical properties and many potential applications in the biological process of plant growth and development, as reported previously for hydroponic and water-based cultures.

The novel combination of nutrition-enriched O-UFB with VP tested in this study provided a positive environment, such as ion absorption and transfer facilitation and gas exchange in liquid, for Cattleya plantlet growth and development, resulting in morphophysiological changes, increased concentrations of photosynthetic pigments, and gene activation, all of which led to a faster physiological transition from heterotrophic to autotrophic micropropagation. Eliminating sugar from the medium promotes photosynthetic activity, leading to healthier plantlets and faster adaptation to greenhouse conditions (Xiao, Niu, & Kozai, 2011). The presence of O-UFB in a sugar-free, septic culture may shorten the micropropagation process for orchids, particularly Cattleya, by combining plant development, hardening, and acclimatization in a single stage. However, the physiological and molecular mechanisms of O-UFB-regulated growth promotion require thorough investigation.

Conclusions

A novel, nutrient-oxygenated UFB-vermiculite-perlite medium enhanced Cattleya plantlet growth and development. A lower ionic strength (¾-MS) in the presence of UFB improved Cattleya morphology and induced the overexpression of SUT2 and PEPCK genes during *in vitro* septic culture, leading to a physiological transition from heterotrophic to autotrophic micropropagation. Exploring and investigating the effects of nutrient-enriched UFB-VP medium in addition to other media for Cattleya on plant proliferation and initial growth of Cattleya are suggested as suitable directions for further research in this field.

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Author contribution

Conceptual idea: Agisimanto, D.; Yulianti, F.; Winarto, B.; Methodology design: Agisimanto, D.; Yulianti, F.; Hardiyanto; Winarto, B.; Data collection: Agisimanto, D.; Yulianti, F.; Mitalarasati.; Fanani, A.K.; Devy, N.F.; Data analysis and interpretation: Agisimanto, D.; Yulianti, F.; Sugiarto. A.T.; Winarto, B., and Writing and editing: Agisimanto, D.; Yulianti, F.; Mitalarasati.; Fanani, A.K.; Hardiyanto.; Devy, N.F.; Sugiarto, A.T.; Winarto, B.

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