

Article

A Comparison of the Horizontal and Vertical Positions of the PET Bioreactor for In Vitro Commercial Propagation of Vanilla

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Abstract

Temporary immersion systems represent an efficient, biotechnology-based alternative for micropropagation. This study compares the in vitro propagation of vanilla shoots in semisolid and liquid media using a polyethylene terephthalate (PET) bioreactor in both horizontal and vertical positions. During the multiplication stage, these PET bioreactors were evaluated by measuring the following development variables: chlorophyll content, stomatal density, percentage of closed stomata, and dry matter percentage. Additionally, survival percentage was evaluated during the acclimatization stage. Our findings indicate that the different culture systems exert differential effects on the evaluated variables during in vitro multiplication. Both horizontal and vertical PET bioreactors yielded a higher number of shoots per explant, as well as increased dry matter percentage, stomatal density, and closed stomata. The longest shoots, featuring more leaves and higher chlorophyll content, were achieved in the horizontal PET bioreactor. Furthermore, plantlets cultured in the PET bioreactors exhibited the highest survival rates during greenhouse acclimatization. In conclusion, the in vitro multiplication of vanilla using PET containers as temporary immersion bioreactors represents an economical and efficient alternative for the micropropagation of this species on a commercial scale.

Keywords: polyethylene terephthalate; photosynthetic pigments; stomata; photomixotrophism

1. Introduction

Vanilla (*Vanilla planifolia* Andrews) is the only orchid species cultivated for the vanillin contained in its fruits. This compound is used for its aroma and flavor by the agri-food, pharmaceutical, and fragrance industries [1,2]. Unfortunately, generative propagation of vanilla using seeds is challenging due to its low germination rate of 5% [3]. Consequently, this species is commercially propagated vegetatively using cuttings. However, this method can promote pests and diseases that compromise commercial production, and it fails to rejuvenate new plantations [4]. Compounding these challenges, vanilla is currently listed as “Endangered” on the Red List of Threatened Species [5] and is under special protection in Mexico [6]. Plant tissue culture techniques, such as micropropagation, can increase in vitro regeneration capacity. In vitro propagation is a useful tool for producing plants of high phytosanitary quality while ensuring the rejuvenation of new plantlets via a process called in vitro multiplication [7–9].



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However, despite the advantages of in vitro propagation, its commercial application remains limited by the reliance on semisolid medium and sealed vessels, which incur high labor costs, require expensive gelling agents, and lack automation. The use of liquid culture media in temporary immersion systems (TISs) presents a cost-effective alternative by (1) eliminating gelling agents, (2) semi-automating key micropropagation stages, and (3) significantly reducing labor requirements [10].

The success of TISs largely depends on their design to cultivate explants exposed to liquid culture for a specific time and frequency. These systems use bioreactors to semi-automate commercial-scale micropropagation under controlled conditions [10]. Certain TIS designs allow for the control of compressor airflow, facilitating the exchange of atmospheric air [8] and improving processes such as photosynthesis, respiration, and stomatal function [11]. These processes can indirectly indicate in vitro photomixotrophism, which refers to the ability to simultaneously use organic carbon from the culture medium and light for photosynthesis [12,13]. Photomixotrophism induced in bioreactors can increase the content of photosynthetic pigments, as well as photorespiration and the opening and closing of stomata. These processes contribute to increased survival during the acclimatization phase [14].

Different TIS models with airflow have been used for the in vitro propagation of *V. planifolia*. These include the following: the Recipient for Automated Temporary Immersion (RITA[®]) [15], manufactured from sterilizable polypropylene; the SETIS[™] bioreactor [16], also made of polypropylene; the Temporary Immersion Modular System (SMIT[®]) [17], likewise made of polypropylene; and the temporary immersion bioreactor (TIB) [18], which typically utilizes glass containers, though plastic can also be used. Despite their efficacy, the widespread adoption of these technologies is limited by high initial investment costs and the fragility of glass components during handling. Thus, efforts have been made to implement TIBs with polyethylene terephthalate (PET) containers, also known as TIB-PET [19]. PET bioreactors are low-cost, lightweight, easy to handle, impact-resistant, recyclable, and do not require an autoclave for sterilization, as they can be sterilized with sodium hypochlorite or UV light. Thus, PET bioreactors can reduce costs and increase the yield capacity of regenerating vanilla shoots. Moreover, using the PET bioreactor in a horizontal rather than a vertical configuration could increase light irradiance, space per explant, and the in vitro multiplication rate [19]. Thus, we hypothesized that in vitro propagation using PET-based bioreactors in either a horizontal or vertical position would increase the multiplication rate, promote shoot physiological development, and improve survival rates during the acclimatization phase, thereby establishing a viable alternative for commercial scale-up. Therefore, this study aimed to compare the in vitro multiplication of vanilla shoots using horizontal and vertical PET systems with both semisolid and liquid culture media.

2. Materials and Methods

2.1. Explant Collection and Surface Disinfection

The initial biological material consisted of cuttings from a single commercial vanilla clone cultivated in Veracruz, Mexico. These cuttings were obtained from mother plants selected for high fruit productivity and vigor, and certified by the National Agro-Alimentary Health, Safety and Quality Service (SENASICA, certificate No. LAB 30044001/2018). These cuttings were cultivated in greenhouses at the Postgraduate College, Cordoba Campus (18°53'56.5" N 96°56'58.9" W) under 78% shade at 30 ± 2 °C, 60–70% relative humidity (RH), and natural light with an irradiance of 180 ± 5 $\mu\text{mol m}^{-2} \text{s}^{-1}$. One year after planting, 60 cm long young stems containing five axillary buds were collected. Nodal segments were trimmed to 1.5 cm and washed for 15 min in a solution of 20 μL Tween 20[®] (Merck KGaA[®], Darmstadt, Germany) per 100 mL of water. In a laminar flow hood,

nodes were submerged in a 0.10% (*w/v*) sodium hypochlorite (NaClO) solution for 15 min. Afterwards, explants were submerged in a 0.08% (*w/v*) mercury chloride (HgCl₂) solution for 12 min and washed three times with sterile distilled water. Explants were trimmed to a length of 1 cm and cultivated in test tubes (22 × 150 mm) containing 15 mL of MS culture medium [20], supplemented with 8.8 µM of benzyladenine (BA, PhytoTech Labs, St Lenexa, KS, USA), 4.9 µM of indole-3-butyric acid (IBA, Merck KGaA®), and 3% sucrose. The culture medium pH was adjusted to 5.7 ± 0.2, and 0.28% (*w/v*) Gelrite® (Merck KGaA®, Darmstadt, Germany) was added as a gelling agent. The culture medium was sterilized using an autoclave (CVQ-B75L, Ecoshel, Pharr, TX, USA) at 1.5 kg cm⁻² and 121 °C for 15 min. The explants were kept under white LED light in an incubation room with a 16 h light/8 h dark photoperiod, 24 ± 2 °C, 78% relative humidity, and a photosynthetic photon flux density (PPFD) of 40 to 50 µmol m⁻² s⁻¹. After three 60 d subcultures in the multiplication phase on semisolid medium, shoots were taken as explant sources for all treatments.

2.2. In Vitro Multiplication in a PET Bioreactor

The TIS used in this study was a PET bioreactor consisting of two transparent containers in both horizontal and vertical designs: one for culturing the explants and the other for holding the culture medium as described by Fuentes-Torres et al. [19]. Both vessels were recycled from 4000 mL purified water bottles, connected by silicone tubing, and fitted with 0.22 µm hydrophobic filters. Each PET bioreactor contained 1000 mL of liquid MS medium and 20 explants (2 shoots each, 2 cm in length), maintaining a ratio of 50 mL of medium per explant. In the TIB-bioreactors, the immersion frequency was 2 min every 4 h for 60 d, following the protocol described by Spinoso-Castillo and Bello-Bello [17]. The control treatment consisted of using the same PET containers with semisolid culture medium under the same conditions and adding 0.28% (*w/v*) Gelrite® (Merck KGaA®, Darmstadt, Germany) as a gelling agent. All recipients were sterilized in a laminar airflow cabinet by rinsing them with a 0.25% (*w/v*) NaClO solution for 5 min, followed by three rinses with sterile deionized water. Finally, the containers were exposed to UV light from the laminar flow hood (CV-2A, Ecoshel, Pharr, TX, USA) for 10 min. The culture medium used in this study was previously described by Ramírez-Mosqueda and Bello-Bello [16]. Briefly, MS medium was supplemented with 3% sucrose and 8.8 µM BA (PhytoTech Labs, St Lenexa, KS, USA), and the pH was adjusted to 5.7 ± 0.2.

The number of leaves and shoots was manually counted, and shoot length was measured using a Vernier caliper. Shoot dry weight was determined after oven-drying at 75 °C for 72 h. Dry matter content was calculated using the following equation: Dry matter (DM) = dry weight/fresh weight × 100. Stomatal index, percentage of closed stomata, and chlorophyll (a, b, and total) content were also evaluated. Light irradiance inside the PET container was also measured using a ceptometer (AccuPAR LP-80, Decagon Devices, Pullman, WA, USA), recording a PPFD of 37 ± 2 µmol m⁻² s⁻¹.

2.3. Analysis of Stomatal Content and Total Chlorophyll

Stomatal content. Stomatal index (SI) and the percentage of closed stomata (%CS) were estimated according to the methodology described by Xu and Zhou [21]. Samples were collected from the third leaf below the plant apex in the culture system. The undersides of fully developed leaves were observed under a compound microscope (Olympus, BX51, Tokyo, Japan). SI was calculated using the following formula:

$$\text{Stomatal index} = \frac{\text{Number of stomata}}{\text{Number of epidermal cells} + \text{Number of stomata}} \times 100$$

The percentage of closed stomata was calculated as follows:

$$\text{Percentage of closed stomata} = \frac{\text{Number of closed stomata}}{\text{Total number of stomata}} \times 100$$

Chlorophyll determination. Chlorophyll a (Chl a) and chlorophyll b (Chl b) were calculated according to Lichtenthaler and Wellburn [22]. Total chlorophyll (Chl t) content is given as Chl a + b. Samples were collected from the foliar tissue of the third leaf below the apex. Samples of 250 mg FW (fresh weight) were crushed in 2.5 mL of a 70% cold acetone and absolute ethanol mixture. The homogenate was incubated for 48 h in a cool, dark environment, and the resulting extract was transferred into a 3 mL vial. Subsequently, extracts were centrifuged at $15,000 \times g$ for 5 min. The supernatant obtained after centrifugation was analyzed using a UV-Vis spectrophotometer (Genesys 10S, Thermo Scientific, Waltham, MA, USA) at wavelengths of 663 and 645 nm for Chl a and b, respectively.

$$(\text{Chl } a) (\text{mg} \cdot \text{g}^{-1}) = \frac{(12.72 \times \text{OD}_{663} - 2.59 \times \text{OD}_{645}) \times V}{1000 \times W}$$

$$(\text{Chl } b) (\text{mg} \cdot \text{g}^{-1}) = \frac{(22.88 \times \text{OD}_{645} - 2.59 \times \text{OD}_{663}) \times V}{1000 \times W}$$

$$\text{Total chlorophyll content (mg} \cdot \text{g}^{-1}) = \text{Chl } a + \text{Chl } b$$

where V is the total volume of acetone–ethanol extract (mL), and W is the FW (g) of the sample.

2.4. Acclimatization

The survival percentage of the different culture systems was evaluated during the acclimatization phase at 90 d of culture. From each culture system, 384 rootless individual shoots (3–5 cm long with at least two leaves) were collected. After that, shoots were transferred to 32-cell polypropylene trays (190 cm³ per cavity) containing a sterile 1:1:1 v/v/v compost + tezontle + sawdust substrate. Shoots were maintained under ex vitro conditions in a greenhouse with 78% shade at 30 ± 2 °C, 60 to 70% RH, and natural light with an irradiance of 180 ± 5 μmol m² s^{−1}. For 90 d, the survivability and growth of the plantlets were observed.

2.5. Statistical Data Analysis

All experiments followed a completely randomized design with three replicates. The experimental design included four containers per treatment (four for the semisolid medium and four bioreactors for each TIB). The explant proportion was as follows: Four containers $\times$ 20 explants $\times$ (replicated three times) = 240 explants in total for each treatment. The acclimatization phase included four polypropylene trays per treatment. This phase consisted of four polypropylene trays $\times$ 32 shoots $\times$ (replicated three times) = 384 shoots in total. After 60 d of culture, a one-way analysis of variance was performed on the plant growth variables and biochemical parameters for a probability of error of 5% ($p \leq 0.05$). Normality and homogeneity of variance were verified using the Kolmogorov–Smirnov and Levene’s tests, respectively. Data were analyzed with ANOVA (analysis of variance) and Tukey’s range test ($p \leq 0.05$). All statistical analyses were conducted using SPSS version 20 software (SPSS Inc., Chicago, IL, USA). Percentage values were transformed using the formula: $Y = \arcsin(\sqrt{(x/100)})$, where “x” is the percentage value.

3. Results

3.1. Evaluation of the Different Culture Systems on Shoot Proliferation

Our findings revealed significant differences in the development variables measured during the in vitro multiplication stage of vanilla across the different culture systems. The highest shoot number per explant was found in the TIB-horizontal and -vertical systems, yielding 18.86 and 20.26 shoots per explant, respectively. Conversely, the lowest number of shoots per explant was found in the horizontal semisolid (SS-horizontal) and vertical semisolid (SS-vertical) systems, with 8.7 and 10.50 shoots per explant, respectively (Figure 1a). Regarding shoot length, the longest shoots were found in the TIB-horizontal system with 3.04 cm, whereas the shortest length was obtained in the SS-vertical system with 1.5 cm (Figure 1b). Regarding the number of leaves per shoot, the highest number was found in the TIB-horizontal system, with 2.6 leaves per shoot. The shoots with the lowest number of leaves were found in the SS-vertical containers with 1.8 leaves per shoot (Figure 1c). As for dry matter, the greatest percentage of dry matter was found in the TIB-horizontal and -vertical systems, with 6.25% and 6.35% dry matter percentage, respectively. Conversely, the lowest percentages were obtained in the SS-horizontal and -vertical systems, with 5.34% and 5.55% dry matter percentage, respectively (Figure 1d). Images of the in vitro vanilla cultures and shoot clumps under these varying conditions are presented in Figure 2.

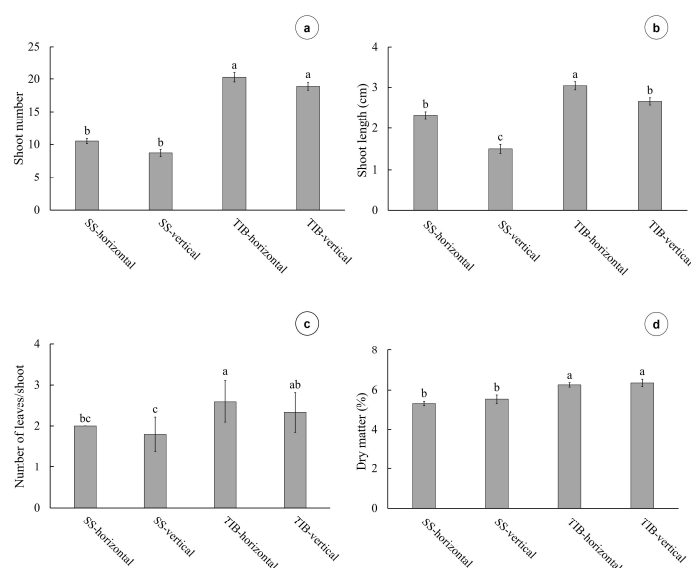


Figure 1. The effect of different culture systems of polyethylene terephthalate on in vitro multiplication of vanilla (*Vanilla planifolia* Andrews) after 60 d. (a) Shoot number, (b) shoot length, (c) leaves per shoot, and (d) dry matter percentage. Values represent the mean $\pm$ standard error. Means with a different letter indicate statistical significance (Tukey, $p \leq 0.05$). SS: semisolid; TIB: temporary immersion bioreactor.

3.2. Stomatal Index (SI), Percentage of Closed Stomata (%CS), and Chlorophyll Content

Significant differences in SI and %CS were observed among the evaluated systems at the end of the in vitro multiplication phase of vanilla (Figure 3a). The TIB-horizontal and TIB-vertical systems showed the highest SI, both with 24.5%. In contrast, the SS-horizontal and SS-vertical systems showed the lowest SI at 18.22% and 20.00%, respectively. Regarding %CS, the TIB-horizontal and TIB-vertical systems showed the highest percentage, both at 0.73%. Conversely, the SS-horizontal and SS-vertical systems showed the lowest %CS, at 0.53% each (Figure 4).

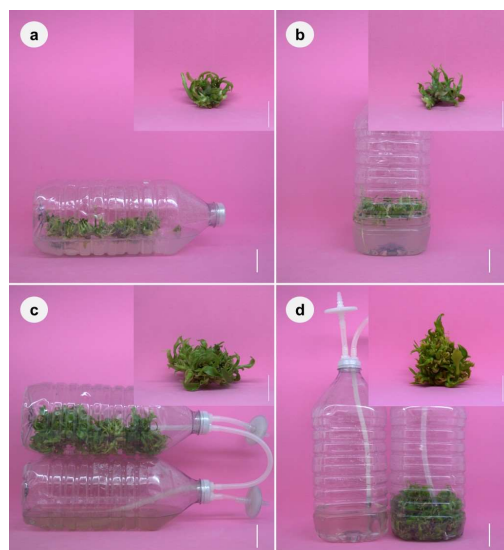


Figure 2. Effect of different culture systems of polyethylene terephthalate on in vitro multiplication of vanilla (*Vanilla planifolia* Andrews) shoots after 60 d. (a) SS-horizontal, (b) SS-vertical, (c) TIB-horizontal, and (d) TIB-vertical. SS: semisolid; TIB: temporary immersion bioreactor. White bars = 5 cm.

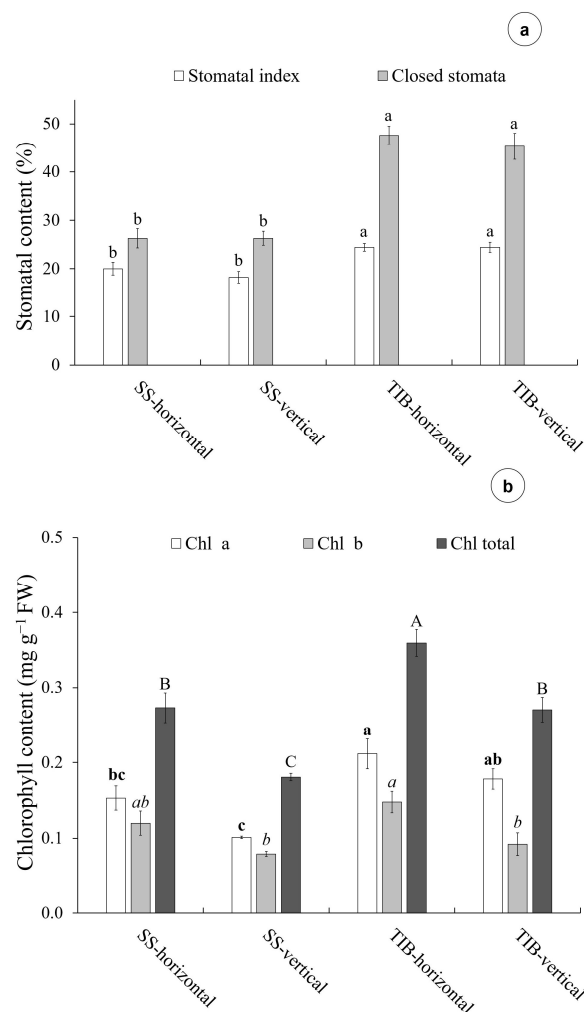


Figure 3. The effect of different culture systems of polyethylene terephthalate on in vitro multiplication of vanilla (*Vanilla planifolia* Andrews) shoots after 60 d. (a) Stomatal content, (b) chlorophyll content. Values represent the mean $\pm$ standard error. Means with a different letter indicate statistical significance (Tukey, $p \leq 0.05$). Chl: chlorophyll; SS: semisolid; TIB: temporary immersion bioreactor; FW: fresh weight.

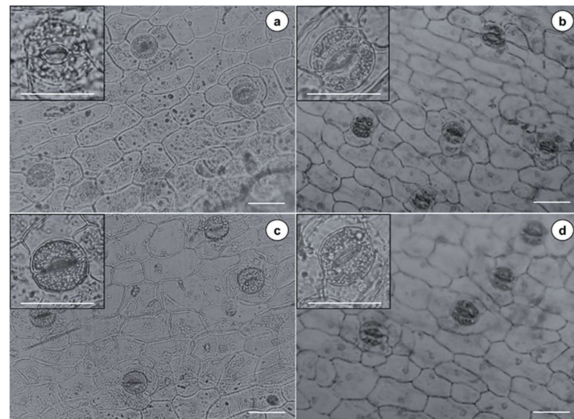


Figure 4. The effect of different culture systems of polyethylene terephthalate on the stomatal index and closed stomata during in vitro multiplication of vanilla (*Vanilla planifolia* Andrews) shoots after 60 d. (a) SS-horizontal, (b) SS-vertical, (c) TIB-horizontal, and (d) TIB-vertical. SS: semisolid; TIB: temporary immersion bioreactor. White bars = 100 µm.

Likewise, Chl a, Chl b, and Chl t contents differed significantly among the evaluated systems during the in vitro multiplication phase of vanilla (Figure 3b). The highest Chl a, b, and t contents were observed in the TIB-horizontal system, yielding 0.21, 0.14, and 0.35 mg g⁻¹ FW, respectively. Conversely, the lowest Chl a, b, and t contents were observed in the SS-vertical containers (0.10, 0.07, and 0.18 mg g⁻¹ FW, respectively) and the TIB-vertical system with 0.09 (Chl b) mg g⁻¹ FW.

3.3. Transfer to Ex Vitro Conditions: Acclimatization of Axenic Plants

The highest survival percentages were found in plantlets grown in the TIB-horizontal and -vertical systems, at 98.6% and 98.3%, respectively. Conversely, the lowest percentages were found in the SS-horizontal and -vertical systems, with 91.6% and 91.3% survival, respectively (Figure 5a). Plantlets showed a healthy appearance after 90 d under greenhouse conditions in trays containing compost, cedar sawdust, and tezontle substrate (Figure 5b). Subsequently, they were transferred to cultivation beds with bamboo stakes for mother plant production (Figure 5c). Finally, plants were cultivated for the commercial production of vanilla under greenhouse conditions (Figure 5d).

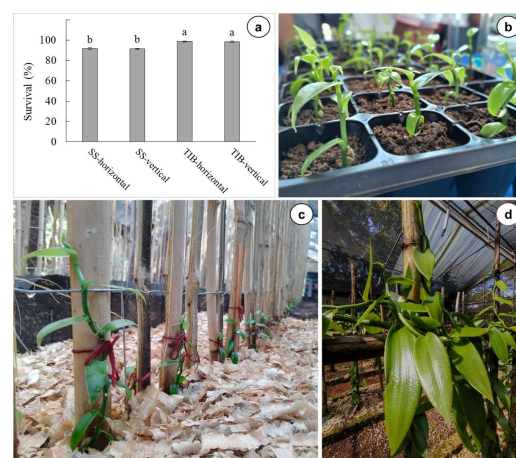


Figure 5. Acclimatization and cultivation of vanilla (*Vanilla planifolia* Andrews) plants under greenhouse conditions. (a) Plantlets survival percentage, (b) acclimatized plantlets after 90 d of cultivation, (c) plantlets after 120 d of cultivation, (d) plants in commercial culture conditions. Values represent the mean $\pm$ standard error. Means with a different letter indicate statistical significance (Tukey, $p \leq 0.05$). SS: semisolid; TIB: temporary immersion bioreactor.

4. Discussion

4.1. Plant Growth in Different Culture Systems

This study evaluated the efficiency of PET-based TIB-horizontal and -vertical culture systems during in vitro multiplication, comparing them against SS-horizontal and -vertical systems. Previous studies have evaluated the efficiency of using vertical TIBs with glass containers to propagate vanilla (*V. planifolia*) [23], taro (*Colocasia esculenta* L.) [24], sugarcane (*Saccharum* spp.) [25], agave (*Agave potatorum* Zucc.) [26], and hybrid rhyncattleanthe (*Rhyncholaeliocattleya* Hwa Yuan Grace × *Cattlianthe* Chocolate Drop) [27]. Furthermore, de Souza et al. [28] evaluated the efficiency of using a vertical glass-container Biotilt™ system for vanilla propagation. In this study, we obtained higher in vitro regeneration rates with the TIB-horizontal and TIB-vertical systems than with semisolid systems. This could be attributed to the increased space available for explant growth and differentiation. Previously, Ramírez-Mosqueda and Iglesias-Andreu [23] compared the propagation of vanilla in TIB (18.06 shoots per explant), BIG (6.83 shoots per explant), and RITA® (12.77 shoots per explant) systems, with an immersion frequency of 2 min every 4 h, demonstrating higher shoot quality in the TIB after 30 d of culture. Fuentes-Torres et al. [19] in sugarcane (*Saccharum* spp.) reported a greater number of shoots per explant using a TIB-horizontal system (29.30 shoots per explant), with an immersion frequency of 2 min every 9 h, compared to the SS-vertical and -horizontal systems, which yielded 10.20 and 12.00 shoots per explant, respectively, after 45 d of culture. Additionally, de Souza et al. [28] reported that the Biotilt™ system enhanced explant length (8.85 cm), shoot number (5.25 shoots per explant), and leaf number (3.30 leaves per shoot), with an immersion frequency of 5 min every 2 h, compared to the semisolid system, which only achieved values of 3.50 cm, 2.82 shoots per explant, and 1.00 leaves per shoot.

The higher number of shoots per explant in the liquid culture media within the TIB-bioreactor could be attributed to more space and better use of the culture medium components (nutrients and organic compounds). In this system, nutrients reach growth areas more readily because temporary immersion in a liquid medium enhances the availability of nutrients, growth regulators, and other organic components. This is facilitated by the larger contact surface area between the explant and the medium compared to semisolid cultures [29]. Future studies should quantify the consumption of sugars, nitrogen, and mineral elements in the culture medium, as well as the nutrient content within plant tissues obtained from PET systems. Additionally, explant movement within the bioreactor can cause loss of apical dominance, leading to greater explant regeneration [10]. Conversely, the lower number of shoots per explant in the semisolid horizontal and vertical container systems likely occurs because explants in semisolid medium can only absorb the components of the medium through the surface area. While explants maintain a fixed position inside the TIB container, the increase in shoot length and leaf number in the horizontal TIB configuration may be due to its specific geometry, which optimizes light availability. Additionally, the horizontal position allows greater leaf growth, likely due to the increased space within the culture system. In vanilla, using the SETIS™ bioreactor, Ramírez-Mosqueda and Bello-Bello [16] reported increased shoot length and fresh and dry weight, attributed to the system's design, which provided greater light availability. In sugarcane (*Saccharum* spp.), Fuentes-Torres et al. [19] found that the TIB-horizontal system yielded an increase in shoot length and number of leaves per shoot compared to the semisolid system.

The increase in dry matter percentage in the TIB-horizontal and TIB-vertical systems could be explained by improved vanilla multiplication in these systems compared to the SS-horizontal and -vertical configurations. In vanilla, using the SETIS™ bioreactor, Ramírez-Mosqueda and Bello-Bello [16] reported increased dry matter in the semisolid system, with 8.7% compared to the SETIS™ bioreactor, which yielded 6.65% dry matter.

4.2. Stomatal and Chlorophyll Content

Stomatal content. Stomata are typically located on the leaf epidermis and open or close in response to environmental and physiological signals. Their function helps the plant maintain an equilibrium between water loss and its oxygen and carbon dioxide requirements [30]. In this study, we observed higher SI and a greater %CS in the TIB-horizontal and -vertical systems than in the SS-horizontal and -vertical ones. This finding suggests enhanced stomatal function in the TIB, which could promote in vitro photosynthesis. Similarly, in vanilla, Ramírez-Mosqueda and Bello-Bello [16] observed a higher percentage of closed stomata in the SETISTM bioreactor than in the semisolid system. In agave (*Agave potatorum* Zucc.), Mancilla-Álvarez et al. [26] observed a higher percentage of closed stomata in the SETISTM bioreactor and TIB in comparison with the semisolid system. The increase in SI in the TIB-horizontal and -vertical systems could be attributed to the continuous ventilation provided by the systems compared to the closed system. In this study, the TIBs likely improved explant conditions by reducing relative humidity and transpiration and by enhancing the assimilation of CO₂ from an air compressor to overcome the limitations of the solid culture medium. Yi et al. [31] note that the gas exchange via stomata is mainly determined by their number, size, and function. Moreover, semisolid systems maintain explants at elevated relative humidity, which reduces stomatal function by lowering transpiration and CO₂ assimilation [32], unlike TISs, which enhance stomatal function by promoting the exchange of O₂, CO₂, and N₂.

Chlorophyll a, b, and total. Chlorophyll is an essential photosynthetic pigment for plants that absorbs photons and participates in photochemical reactions [33]. In this study, we observed significant changes in the content of photosynthetic pigments, including Chl a, Chl b, and Chl t. The highest contents of Chl a, b, and t were obtained in the TIB-horizontal system. This increase in photosynthetic pigments can be interpreted as an indirect measure of photosynthetic efficiency due to the gas exchange in the PET bioreactor. Mancilla-Álvarez et al. [26] describe the in vitro process by which plants transition from a heterotrophic state to an autotrophic state as in vitro photomixotrophism. During the temporary immersion process with airflow, a higher gas exchange rate is achieved. This improves the availability of nutrients that play important roles in biochemical and physiological processes in plants. Nitrogen (N) and magnesium (Mg) are largely involved in chlorophyll synthesis.

The increase in photosynthetic pigments observed in TIBs has been evaluated in vertically positioned glass containers in eucalypt hybrids (*Eucalyptus grandis* × *Eucalyptus urophylla*) [34], banana (*Musa* sp. cultivar Rasthali) [35], agave (*Agave potatorum* Zucc.) [26], and raspberry (*Rubus idaeus* L.) [13]. In this study, the greatest Chl content was found in the TIB-horizontal system. This finding is similar to that reported by Fuentes-Torres et al. [19] in sugarcane (*Saccharum* spp.). These authors observed higher contents of Chl a, Chl b, and t in the TIB-horizontal than in the semisolid system. Yang et al. [36] note that Chl a is a key indicator of photosynthetic efficiency and biomass accumulation potential, which may be related to the high dry matter percentage in this system. Additionally, Quintana-Zaez et al. [37] state that Chl b is essential for capturing light during photosynthesis. Under stress, Chl b levels can decrease, thereby reducing photosynthetic efficiency and thus limiting energy production for growth and repair. Simkin et al. [38] indicate that an increase in chlorophyll a/b could enhance survival during acclimatization under high light intensities and improve photosynthetic electron transport by optimizing the balance between pigment composition and the plant's light-capturing capacity, thus improving photosynthetic efficiency and vegetative performance.

4.3. Acclimatization Stage

The high survival percentage (98%) of plantlets cultured in the TIB-horizontal and -vertical systems during the acclimatization stage guarantees the efficacy of in vitro multiplication of vanilla using these systems. This finding may be related to the increase in chlorophyll content, stomatal density, and the percentage of closed stomata, which help prevent dehydration. According to Quintana-Zaez et al. [37], TIBs enhance specific physiological processes—such as photosynthesis, respiration, chlorophyll synthesis, and stomatal function—that allow plants to adapt to ex vitro conditions during acclimatization. Similarly, Aragón et al. [39] and Martínez-Estrada et al. [40] state that improved plant performance under ex vitro conditions could be due to physiological changes induced by the TIS that favor acclimatization. Survival is a crucial factor for successful micropropagation. Using a TIB, Martínez-Estrada et al. [40] reported a survival percentage of 90% in anthurium (*Anthurium andraeanum* L.). This survival was higher than that observed using a semisolid system (80.4%) due to stomatal function. Vendrame et al. [41] reported a survival percentage of 93.3% for the orchid *Brassavola nodosa* L. in a SETISTM bioreactor, which was higher than the 6.7% survival in the semisolid system. Similar results were reported in raspberry (*Rubus idaeus* L.) by Reyes-Beristain et al. [13]. In this study, the survival percentage was higher in the PET bioreactor (98.6%) than in the semisolid system (91.6%). Moreover, Fuentes-Torres et al. [19] reported that the survival percentage of sugarcane (*Saccharum* spp.) in TIB-horizontal and -vertical systems was 99.5% and 99.0%, respectively. In comparison, the survival percentage in horizontal and vertical semisolid systems was 95.50% and 90%, respectively. In our experience, successful micropropagation depends not only on the number of shoots per explant but also on their morphological quality, survival rate, and the vigor of the plantlets produced under ex vitro conditions.

TIBs have undergone several modifications in their design, position, materials, and accessories, which influence explant physiology. The use of PET containers in TIBs offers several advantages due to their specific characteristics, including transparency, light weight, shatter resistance, thermal stability, and mechanical strength. However, PET material is not autoclavable. According to Doğan [42], UV light can be particularly damaging to polymers, causing degradation, color changes (yellowing), cracking, surface chalking, and reduced mechanical properties. As the UV energy dose accumulates over time, degradation effects such as fragmentation and biodegradation could be accelerated. However, González et al. [43] reported that the efficiency of UV disinfection depends on UV fluence, which is defined as the product of the incident irradiance (fluence rate) and exposure time, corrected for water absorption and the reflection factor. In our study, no visible UV damage was observed in the PET containers or the explants. Future studies should investigate the release of microplastics and their effects. The use of PET optimizes costs and ease of use, as it is a primary recyclable material that can be used to produce new bottles from the same material [44]. The PET container is single-use because dissection is required to extract the explants; however, its associated components—including filters, caps, and silicone tubing—are reusable and autoclavable. The 4L PET bioreactor has a total production cost of approximately US\$63.57 per unit (including PET containers, air filters, lid, and silicone hose), making it more economical than other commercial systems. This represents an efficient alternative for reducing the environmental impact associated with its use, without contributing to plastic waste [45]. Recycling PET is crucial for reducing plastic pollution, supporting sustainability, and advancing the circular economy by lowering the carbon footprint and creating new products like food-grade packaging and textile fibers. Bottle-to-bottle recycling provides a significant reduction in environmental burdens. Post-consumer recycled material can thus be used as packaging material for temporary immersion bioreactors [46].

5. Conclusions

In conclusion, our results demonstrated that the use of temporary immersion systems with bioreactors made of polyethylene terephthalate offers a viable alternative for the commercial micropropagation of *Vanilla planifolia*. Compared to semisolid systems, this system increased multiplication rates and improved in vitro shoot quality without requiring a rooting stage, thereby promoting greater survival during ex vitro acclimatization.

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