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EFFECTS OF DIFFERENT CARBON SOURCES ON IN VITRO SHOOT MULTIPLICATION: SOLID MS MEDIUM OPTIMIZATION AND BIOREACTOR VALIDATION FOR AQUATIC PLANT *BUCEPHALANDRA PYGMAEA* (BECC.) P. C. BOYCE & S.Y. WONG

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ABSTRACT

Bucephalandra pygmaea (Becc.) P. C. Boyce & S.Y. Wong is an aquarium plant that is gaining importance in the ornamental plant sector due to its vibrant leaf colours and high adaptability to underwater conditions.

This study aimed to enhance the in vitro shoot propagation efficiency of *B. pygmaea* 'Bukit Kelam' through a two-stage experimental design. In the first stage (solid MS optimisation), three carbon sources—maltose, sucrose, and glucose—were tested at 10 and 20 g L^{-1} concentrations to determine their effects on key growth parameters, including shoot multiplication rate, plant height, leaf number, leaf area, and plant width. According to the results, 10 g L^{-1} maltose promoted the highest shoot proliferation and leaf expansion, whereas 20 g L^{-1} glucose caused a pronounced reduction in all morphological traits.

In the second stage (bioreactor validation), the optimised 10 g L^{-1} maltose medium was applied in both solid MS and temporary immersion (Plantform TIS) systems. Plants grown on solid MS displayed superior morphological performance (plant height, leaf area, and SPAD values), while bioreactor-cultured plants exhibited colour values, reflecting enhanced pigment intensity. Overall, 10 g L^{-1} maltose was identified as the most effective carbon source for *Bucephalandra* micropropagation. The findings suggest that solid MS medium ensures compact growth and high chlorophyll content, whereas the bioreactor system favours visual colour quality—providing complementary strategies for sustainable large-scale propagation of this ornamental aquatic species.

1. INTRODUCTION

Aquatic plants, as natural components of aquatic ecosystems, have been shown to produce oxygen and organic matter [1]. Furthermore, they play an important role in maintaining ecological balance by providing shelter, nutrition and breeding grounds for other aquatic organisms [2,3]. Furthermore, some species have been employed in the biological purification of aquatic environments, a consequence of their capacity to absorb heavy metals and other pollutants [4]. Beyond their ecological functions, aquatic plants are economically significant in the ornamental industry due to their aesthetic appeal and morphological diversity [5]. Among these, species of *Araceae* family have recently become popular in aquascaping because of their durable foliage, vivid colours, and adaptability to submerged conditions [6]. One noteworthy member of this genus is *Bucephalandra pygmaea* (Becc.) P. C. Boyce & S.Y. Wong an epiphytic species from Borneo. It is distinguished by its metallic leaves and its ability to thrive in low-light conditions. However, the species' restricted natural distribution, in conjunction with habitat degradation and overharvesting, has placed its wild populations at risk [7,8]. Consequently, plant tissue culture techniques are regarded as a vital method for the sustainable cultivation of the species, with regard to both ecological conservation and commercial production.

To ensure the long-term survival of *Bucephalandra* species, sustainable propagation practices should replace direct collection from natural habitats. Effective propagation methods must generate numerous, fast-growing, and pathogen-free plantlets while maintaining the species' genetic integrity. Among these, in vitro micropropagation is particularly advantageous, as it enables mass production under aseptic and space-efficient conditions, independent of climatic or seasonal limitations, while yielding genetically uniform and disease-free plants [9,10].

Plant tissue culture techniques have a wide range of applications in plant-based studies. These include the production of disease-free plants, the conservation of rare genotypes, the synthesis of secondary metabolites, and genetic transformation. The success of these techniques is influenced by a number of factors. These include the genotype, the explant type, the medium composition, plant growth regulators, and environmental conditions. In particular, the carbon source is important in providing energy and carbon skeletons that are essential for cell growth and morphogenesis [11,12]. Sucrose is the most commonly used carbon source; however, the different effects of alternative carbon sources, such as maltose, glucose, mannitol and fructose, on osmotic pressure and metabolic regulation necessitate the development of species-specific protocols. It is therefore vital to determine the most suitable carbon source and concentration for each plant species in order to ensure the success of *in vitro* propagation [13,14].

In recent years, bioreactor systems have provided promising alternatives to solid cultures by enhancing gas exchange and nutrient homogeneity, thereby accelerating in vitro growth [15–17]. However, the physiological response of each species may differ under liquid conditions, sometimes resulting in morphological abnormalities or pigment loss. Therefore, it is crucial to validate bioreactor performance comparatively with conventional solid media. The objective of this study was to determine the most suitable carbon source and concentration for in vitro shoot proliferation of *Bucephalandra pygmaea* 'Bukit Kelam' and to evaluate the effectiveness of the optimised medium under solid MS and temporary immersion bioreactor systems. It was hypothesised that (i) carbon source and concentration would significantly affect shoot multiplication rate, leaf area, and chlorophyll index (SPAD), and (ii) the culture system (solid vs. bioreactor) would differentially influence morphological growth and pigment characteristics. The study aimed to establish a reliable, scalable protocol that supports both propagation efficiency and colour stability for sustainable commercial production and conservation of *Bucephalandra* species.

2. MATERIAL AND METHOD

2.1. Source of Explants and Cultural Conditions

In this study, Murashige and Skoog basal medium [18] was utilised to preserve *in vitro Bucephalandra pygmaea* cv. 'Bukit Kelam'.

B. pygmaea is an obligate rheophyte, which means it grows only in streams or waterfalls under moist or perhumid lowland forest, at elevations between 10-55 metres above sea level. It grows on shale or, very occasionally, on sandstone. The Latin name, pygmaeus, refers to the stature of the whole plant [19]. The taxonomic history of *B. pygmaea* was detailed by Boyce and Wong [20].

The plant materials were obtained from AKVARED Aquarium Plants. Segments of rhizome/node (containing ≥1 axillary bud) were selected as explant material and standardised to an initial length of approximately 1.0–1.5 cm. A total of six replicates were utilised for each application, with three plants evaluated in each replicate. Consequently, a total of 108 plant materials were subjected to rigorous scrutiny. The cultures were maintained for 21 days under conditions of 24 ± 2 °C temperature, 16-hour photoperiod, and 40–60 µmol m^{-2} s^{-1} light intensity. At the conclusion of the 21-day period, measurements were taken.

2.2. Determination of Carbon Type and Concentrations in Solid Media (Stage 1)

Murashige and Skoog basal salt was utilised as the medium for the experiment. Consequently, a total of six treatment groups were created in a factorial design, comprising three different sugar types (maltose, sucrose and glucose) and two concentrations (10 and 20 g L^{-1}). The pH of all culture media was adjusted to 5.7 ± 0.1 using 0.1 N NaOH or 0.1 N HCl prior to autoclaving. The pH meter (Mettler Toledo

SevenCompact S210) was calibrated daily using standard buffer solutions of pH 4.0, 7.0, and 10.0. The media were consolidated through the incorporation of 7 g L^{-1}Bacto Agar prior to undergoing an autoclaving process at 121 °C for a duration of 20 minutes. Following autoclaving heat-labile components (Thidiazuron) were filter-sterilised (0.22 µm) and added to the cooled (≈45–50 °C) sterile medium under laminar airflow to prevent thermal degradation and contamination. As outlined in the study by Zalan et al. [21], Thidiazuron (TDZ) was first dissolved in a minimal volume of 0.1 N NaOH to prepare a 1.0 mg mL^{-1} stock solution. At this stage, the following parameters were measured: plant height, plant width, number of tillers, number of leaves and leaf area.

2.3. Comparison of Solid Medium and Plantform TIS Systems (Stage 2)

In the second stage of the experiment, the solid culture medium was compared with the temporary immersion system (Plantform TIS). In the temporary immersion system (Plantform TIS), explants were cultured in vessels containing 500 mL of liquid MS medium, with the medium being supplemented with 10 g L^{-1} maltose. The immersion cycle was programmed as follows: an initial five-minute immersion period was followed by a six-hour interval, ensuring periodic contact with nutrients and gas exchange. Each vessel was connected to a HEPA-filtered air inlet (0.22 µm pore size) to maintain sterility and aeration during culture. The media used in this study were prepared as MS + 0.5 mg L^{-1} TDZ + 10 g L^{-1} maltose (10M). It should be noted that agar (7 g L^{-1}) was the only additional component utilised in the solid medium.

The experiment was conducted using a randomised design, with the single factor being the culture system. A minimum of four pots (i.e. experimental units) were used for each system, with each pot containing five explants. The culture conditions were maintained as they were in Experiment 1 and the duration was set at 21 days. At the conclusion of the experiment, the following parameters were measured: plant height, plant width, number of tillers, number of leaves, leaf area, colour values (L^*, a^*, b^*, c^*, $h°$) and SPAD chlorophyll index parameters. The estimation of chlorophyll content was conducted utilising a SPAD-502 Plus chlorophyll meter (Konica Minolta, Japan), a device specifically designed for the quantification of photosynthetic pigments. The measurements were obtained from the third fully expanded leaf of each plant, with three readings per plant being averaged for the purpose of analysis. Colour indices were recorded using a CR-400 Chroma Meter (Konica Minolta, Japan) calibrated with a standard white calibration plate (CR-A44) prior to each measurement session.

All morphological measurements were performed under laminar airflow conditions using sterile forceps and a disinfected ruler to ensure aseptic handling of the explants. For SPAD and colour measurements, the specified number of explants was destructively harvested and immediately evaluated to prevent physiological alteration during measurement.

2.4. Experimental Design and Data Analysis

The data obtained from the study were statistically evaluated according to a two-stage experimental design. Data obtained from Stage 1 (solid MS optimisation) were subjected to a two-way analysis of variance (ANOVA) to evaluate the main effects of carbon source (carbon source: maltose, sucrose, glucose) and concentration (10 and 20 g L^{-1}), as well as their interaction (sugar × dose) on all morphological parameters (plant height, number of leaves, shoot multiplication rate, plant width, and leaf area). Mean separations were performed using Tukey's HSD test ($p < 0.05$), and the significance of the main and interaction effects was verified according to F-test results. In Stage 2 (bioreactor validation), each treatment consisted of four culture pots and bioreactor system containing five explants each (4 pots × 5 explants, n = 4 independent replicates).Data were first tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene test).Variables meeting both assumptions ($p > 0.05$) were compared between the solid MS medium and bioreactor system using the independent samples t-test, while those violating normality or variance homogeneity ($p < 0.05$) were analyzed using the Mann–Whitney U test. All results were expressed as mean ± standard deviation (SD), and significance was determined at $p < 0.05$. Furthermore, R package (R 4.3.3) was utilized to perform Heatmap correlation (HCA), Pearson correlation, and Principal Component Analysis (PCA) in order to visualize the relationships between parameters and sources of variation. Prior to conducting principal component analysis (PCA) and correlation mapping, all datasets were subjected to centring and scaling (center = TRUE, scale = TRUE) to ensure the comparability of data across variables.

3. RESULTS AND DISCUSSION

3.1. Effects of Carbon Type and Concentrations in Solid Media (Stage 1)

The present study statistically analysed the effects of applying different carbon sources (sucrose, glucose, and maltose) at two different doses (10 g L^{-1} and 20 g L^{-1}) on the *in vitro* growth parameters of the 'Bukit Kelam' plantlets. The results of the one-way analysis of variance (ANOVA) and Tukey's multiple comparison test revealed significant differences between the treatments for all parameters at the 0.05 level of significance.

According to Table 1, carbon source type and concentration significantly affected all morphological parameters. Low sugar doses, particularly 10 g L^{-1} maltose and glucose, promoted shoot elongation, leaf area, and proliferation, whereas higher concentrations (20 g L^{-1}) of glucose and maltose markedly reduced growth. The greatest plant height (2.81 cm) and leaf number (10.17) were obtained with 20 g L^{-1} sucrose, indicating that sucrose mainly supported elongation and leaf formation. Shoot multiplication reached its maximum (2.0 shoots explant^{-1}) in 10 g L^{-1} maltose, confirming that moderate maltose levels enhance proliferation efficiency. Similarly, 10 g L^{-1} maltose and glucose yielded the highest plant width (≈ 2.3 cm) and leaf area (1.18 cm²), while 20 g L^{-1} glucose caused severe inhibition (0.12 cm² leaf area). Overall, Table 1 clearly demonstrates that low maltose concentration provides the most balanced development, supporting both shoot multiplication and leaf expansion, whereas excessive glucose or maltose levels impose osmotic stress and suppress morphogenesis.

Table 1. Example for table usage in manuscript Effects of different carbon sources on growth parameters of B. pygmaea 'Bukit Kelam'

Treatment	Plant Length	Number of Leaves	Shoot multiplication	Plant Width	Leaf Area
10g L^{-1} Glucose	2.66 ± 0.56ab	9.00 ± 1.10a	1.50 ± 0.55ab	2.33 ± 0.40a	0.83 ± 0.35ab
10g L^{-1} Maltose	2.70 ± 0.51ab	8.17 ± 2.48a	2.00 ± 0.63a	2.26 ± 0.35a	1.18 ± 0.20a
10g L^{-1} Sucrose	2.22 ± 0.20b	8.00 ± 3.16a	1.17 ± 0.75b	2.18 ± 0.28ab	0.79 ± 0.19b
20g L^{-1} Glucose	1.64 ± 0.26b	6.33 ± 1.37b	0.50 ± 0.55c	1.78 ± 0.26bc	0.12 ± 0.03c
20g L^{-1} Maltose	1.54 ± 0.29c	6.83 ± 1.47a	0.67 ± 0.52c	1.55 ± 0.32c	0.48 ± 0.17bc
20g L^{-1} Sucrose	2.81 ± 0.13a	10.17 ± 1.47a	1.33 ± 0.52ab	1.90 ± 0.22b	0.78 ± 0.10b
Two-way ANOVA (p values)					
Carbon Source	p<0.001	p<0.01	p<0.001	p<0.05	p<0.001
Concentration	p<0.001	p<0.05	p<0.01	p<0.01	p<0.001
Interaction	p<0.05	ns	p<0.01	ns	p<0.05

Mean ± SD; different letters within the same column indicate significant differences according to Tukey's HSD test ($p < 0.05$). ns: non-significant.

Sucrose inclusion (10 g L^{-1}) exhibited a green and vibrant appearance, yet exhibited a reduced number of shoots. Conversely, the leaf growth exhibited a marked weakness in glucose-containing media, particularly at a 20 g L^{-1} concentration. The occurrence of osmotic stress symptoms, such as leaf chlorosis and deformation, were observed. The results obtained demonstrate that utilising maltose as a carbon source in plant tissue culture medium at a concentration of 10 g L^{-1}fosters optimal conditions for shoot development, leaf pigmentation, and plant integrity in *B. pygmaea* 'Bukit Kelam' (Figure 1).

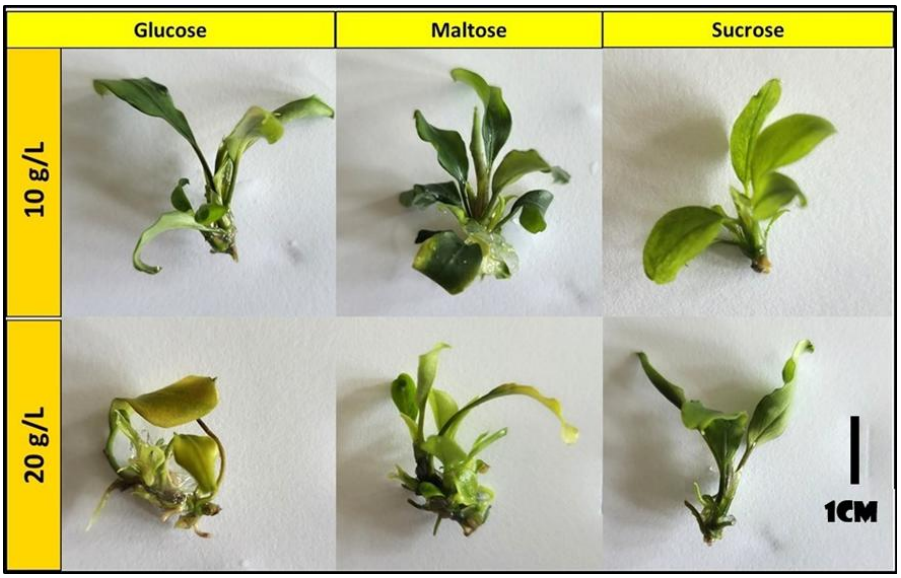

Figure 1. Effects of different carbon sources and concentrations on in vitro shoot development of B. pygmaea 'Bukit Kelam'

Heatmap analysis (Figure 2) visually demonstrated the effects of different carbon sources (sucrose, maltose, glucose) and their doses (10 and 20 g L^{-1}) on the *in vitro* propagation of *B. pygmaea* 'Bukit Kelam'. The presence of row clusters suggests that applications were grouped according to performance differences, while column clusters indicate that plant development parameters (number of leaves, shoot length, plant width, shoot propagation, and leaf area) behaved in related blocks. Specifically, the data demonstrated that leaf number and shoot length exhibited similar trends and were placed in the same cluster, while shoot multiplication and leaf area were clustered together. A thorough examination of the colour scale revealed that red tones were indicative of values that exceeded the mean, while blue tones were indicative of values that fell below the mean. In this context, 10 g L^{-1} maltose was found to have a significant impact on shoot multiplication and leaf area, thereby enhancing multiplication success. The addition of 20 g L^{-1} sucrose resulted in a significant increase in leaf number and shoot length, indicative of its positive impact on these parameters. Conversely, 20 g L^{-1} glucose was represented by blue tones and demonstrated suboptimal performance across all parameters. The heatmap demonstrates that carbon sources elicit divergent effect mechanisms depending on their dose and type, and that these differences can be clearly visualized through the use of clusters and colour scales.

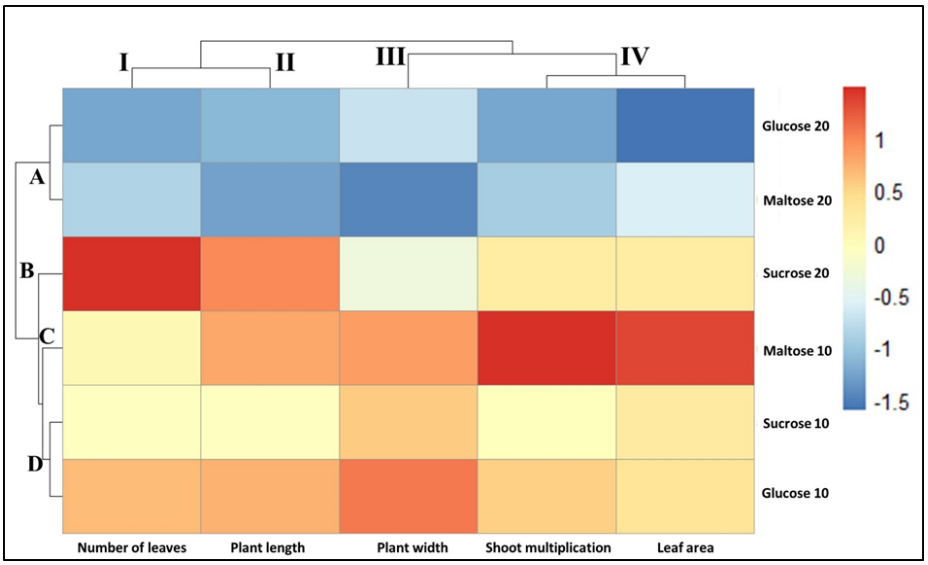

Figure 2. Heatmap showing the clustering of morphological parameters in B. pygmaea 'Bukit Kelam' cultured on MS medium supplemented with different carbon sources (glucose, maltose, and sucrose) at 10 and 20 g L^{-1}concentrations.

Heatmap analysis revealed the effects of different carbon sources on the morphological development of *B. pygmaea* 'Bukit Kelam', and the findings were in complete agreement with the results of the one-way ANOVA analysis. The findings of both analyses indicated that the medium containing 10 g L^{-1} maltose achieved the statistically highest values in terms of plant height, number of leaves, shoot multiplication, and leaf area. In addition, the markedly diminished performance of the 20 g L^{-1} glucose application across all parameters was repeatedly substantiated by both ANOVA and heatmap analyses. This finding indicates that multivariate visualization facilitates statistical evaluations and that the selection of carbon source is a pivotal factor in plant development.

3.2. Validation of the Plantform TIS System with Solid Media (Stage 2)

At this stage, the morphological and physiological parameters obtained by cultivating the *B. pygmaea* 'Bukit Kelam' plant in a solid MS medium supplemented with 10 g L^{-1} maltose and under bioreactor system conditions were compared. The following Table 2 illustrates the mean values, standard deviations, and statistical significance levels obtained from both culture systems. The results obtained from this study indicate that the culture system creates significant differences in plant development and pigment characteristics.

Table 2. Comparison of morphological, physiological, and color parameters of B. pygmaea 'Bukit Kelam' grown in solid medium and Plantform TIS bioreactor

Parameter	Solid Medium	Bioreactor	p-value	Significance
Plant length (cm)	2.75 ± 0.56	2.05 ± 0.27	0.027	*
Number of leaves	9.17 ± 2.48	7.33 ± 1.21	0.193	NS
Shoot multiplication	2.00 ± 0.63	1.67 ± 0.52	0.342	NS
Plant width (cm)	2.32 ± 0.43	2.09 ± 0.21	0.271	NS
Leaf area (cm^2)	1.28 ± 0.14	0.88 ± 0.19	0.005	**
*L** (lightness)	25.19 ± .34	33.71 ± 3.27	0.001	**
*a** (green-red)	-5.59 ± 0.21	-9.77 ± 2.25	0.021	*
*b** (blue-yellow)	4.53 ± 2.08	12.45 ± 3.01	0.001	**
*c** (chroma)	9.96 ± 1.42	14.84 ± 3.77	0.019	*
h^o (hue angle)	141.77 ±1.72	130.97 ± 2.59	<0.001	**
SPAD	7.88 ± 2.10	4.32 ± 3.05	0.049	*

Each value represents mean ± SD. Statistical significance determined by independent samples t-test or Mann–Whitney U test depending on normality (Shapiro–Wilk, Levene).

Plants cultivated in a solid medium exhibited considerably elevated values with respect to plant height and leaf area in comparison to the bioreactor. This phenomenon can be attributed to the more balanced uptake of water and nutrients in the solid medium. Furthermore, the SPAD value was found to be considerably elevated in the solid medium, suggesting that the leaves contained a greater quantity of chlorophyll and that photosynthetic capacity was more effectively supported in this environment. Consequently, the solid medium is advantageous in terms of overall morphological development and physiological quality. Statistical analysis revealed no significant disparities between the two systems with regard to leaf count, branching, and plant width parameters. However, the mean values were higher in favour of the solid medium. This finding suggests that the solid medium may offer a marginal benefit in terms of shoot multiplication and leaf development, although the bioreactor also demonstrates competitiveness in these characteristics. The results of the study demonstrated that the plants cultivated within the bioreactor exhibited a lighter hue in their foliage, characterised by higher values of lightness (*L*), yellowish tones (*b*), and heightened saturation (*c*). When these results are considered collectively, it can be posited that leaves cultivated under bioreactor conditions exhibit a more vivid and luminous appearance. It is evident that the bioreactor offers a distinct advantage in terms of colour intensity and visual aesthetics. The *a* value on the colour scale was more negative in the bioreactor, indicating that the leaves shifted towards a more 'green' tone. However, the hue angle (*h*) was also found to be higher in the solid medium, indicating that the colour tone shifted more towards green in the solid medium and towards yellow in the bioreactor. In summary, TIS leaves exhibit a lighter yellowish-green hue, while leaves in the solid medium display a darker green shade.

Solid medium: The subject is distinguished by its notably elevated stature, extensive leaf area and SPAD levels. This results in the provision of optimal conditions for enhancing biomass and augmenting

photosynthetic capacity. Bioreactor: The application of this technique reveals disparities in colour parameters, including lightness, the yellow component, saturation, and green orientation. Consequently, the leaves exhibit a heightened degree of brightness, vividness, and a yellowish-green hue. Whilst solid media is advantageous in terms of morphological quality and chlorophyll content, bioreactors are noteworthy in terms of the visual colour profile of the leaves. It is imperative that the selection of system is predicated on the intended production objective (i.e. propagation efficiency or visual quality).

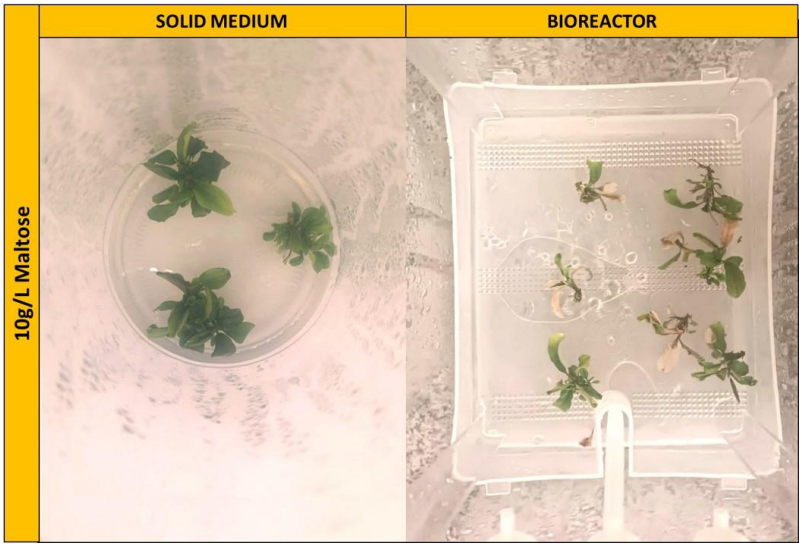

Figure 3. Differences between explants in agar solid medium and Plantform TIS medium

As demonstrated in Figure 3, morphological differences are clearly visible between *B. pygmaea* 'Bukit Kelam' plants cultivated on solid MS medium containing 10 g L^{-1} maltose and those grown in the bioreactor system. In contrast, plants grown in the bioreactor system exhibited leaf yellowing, tissue softening, and weak shoot development. Therefore, the visual findings are entirely consistent with analyses showing that the solid medium affects morphological growth, while the bioreactor system affects pigment structure and colour indices.

The Principal Component Analysis (PCA) performed in this study demonstrates that plants cultivated in a bioreactor and in solid medium can be distinctly distinguished from each other with respect to their morphological and physiological parameters. The two principal components thus obtained account for 100% of the total variance, with the first component (PC1) alone responsible for 89.91% of the variation. This finding suggests that the majority of the observed characteristics can be attributed to a single factor: the difference in growth medium. A thorough examination of the graph reveals that plants cultivated in the bioreactor environment demonstrate a tendency to congregate on the negative side of PC1, while those cultivated in solid medium exhibit a propensity to align on the positive side. This distinction indicates a significant difference between the two culture systems in terms of plant development and physiological responses. Upon examination of the vector directions, parameters such as 'shoot multiplication', 'number of leaves', 'plant width', 'plant length', 'leaf area' and 'SPAD' are observed to align in a consistent direction. This finding suggests a collaborative enhancement of these characteristics, thereby establishing a variation axis that signifies comprehensive plant development.

On the other hand, the colour parameters '*a**', '*b**', '*c**' and 'h' show a different trend, suggesting that morphological growth and colour/pigment characteristics have independent sources of variation. Plants grown in a bioreactor environment likely exhibit better shoot development and leaf width due to higher gas exchange, homogeneous nutrient distribution, and humidity control; conversely, plants grown in solid media display a different profile in terms of colour and pigment components. Overall, the PCA results indicate that the bioreactor system provides a more effective environment for supporting plant growth and physiological development, while the solid medium creates differentiation in characteristics such as leaf colour and pigmentation (Figure 4).

The heatmap of the Pearson correlation analysis comprehensively reveals the direction and strength of the relationships between the morphological, physiological and colour parameters of the plants. The analysis results generally indicate the existence of two distinct relationship groups: the first represents morphological characteristics associated with plant growth, and the second represents colour parameters related to leaf colour and pigmentation. A strong positive correlation (r = 0.70–0.90) was identified between the variables 'plant length', 'number of leaves', 'shoot multiplication', 'plant width' and 'leaf area'. This finding suggests that as plant height increases, the number of leaves, shoot multiplication, and leaf area also tend to increase. Therefore, these parameters appear to exhibit a common biological orientation representing overall plant development. The 'SPAD' value (representing chlorophyll content) also demonstrated a positive relationship, particularly with 'plant width' and 'shoot multiplication'. This finding suggests that chlorophyll content may increase in parallel with leaf expansion and shoot multiplication. A divergent correlation structure was observed among the colour parameters. The '*a**' (green-red axis) parameter exhibited a robust negative correlation with 'SPAD' (r ≈ -0.75), thereby substantiating the notion that an increase in chlorophyll content results in a greener leaf colour (negative *a** value). In a similar manner, the parameters '*b**' (blue-yellow axis) and '*h*' (hue angle) have also been found to be inversely related to SPAD; that is, a shift in leaf tone towards yellow is indicative of a decrease in chlorophyll content. The positive correlations between '*L**' (lightness) and '*a**' and '*b**' indicate that as colour saturation increases, the leaf colour shifts towards lighter tones. The findings indicate a high degree of synchronisation between morphological growth parameters, while pigment parameters demonstrate independence from morphological variables. In other words, as developmental growth increases in plants, there is an observed rise in leaf number, area, and chlorophyll content. However, colour parameters demonstrate an opposing response to this change. Overall, correlation analysis supports the hypothesis that the rapid growth observed in the bioreactor environment provides strong synchronisation among morphological parameters, whereas colour and pigment values differ in solid media.

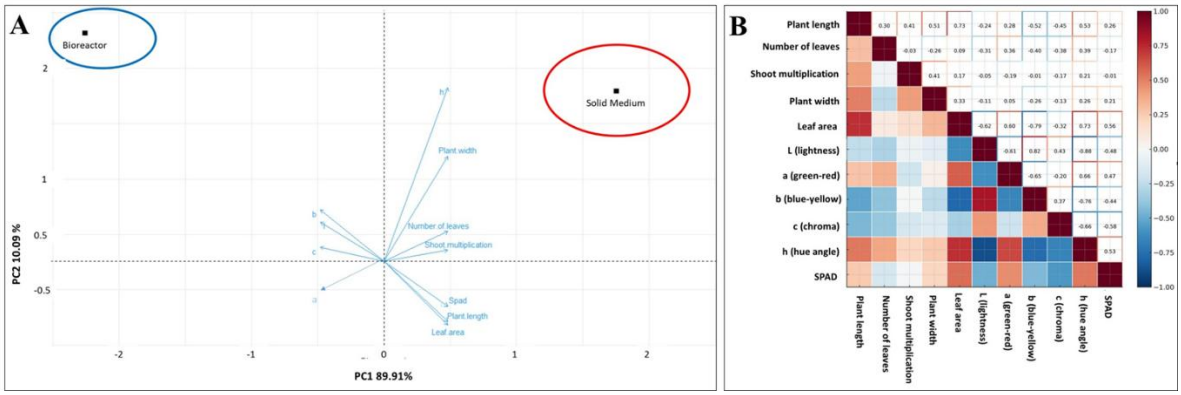

Figure 4. (A) Principal component analysis (PCA) biplot showing the distribution of B. pygmaea 'Bukit Kelam' cultured in solid MS medium and bioreactor systems. (B) Pearson correlation heatmap displaying relationships among morphological and pigment parameters.

The present study evaluated the effects of different carbon sources (glucose, maltose, and sucrose) and two different culture systems (solid MS medium and bioreactor) on shoot development of *B. pygmaea* 'Bukit Kelam' under *in vitro* conditions. The primary carbon sources in *in vitro* tissue cultures are sucrose, fructose, glucose, and maltose. These carbon sources provide the energy necessary for cellular metabolism and growth, thereby supporting the healthy development of explants. Furthermore, they have been demonstrated to play an important role in osmotic regulation, morphogenetic responses, biochemical synthesis, and regeneration processes [22]. However, the presence of elevated carbon concentrations within the medium may increase the risk of microbial contamination. It has been reported that, particularly with an increase in sucrose content, the abundance of easily metabolised sugars leads to increased fungal colony development [13,14].

The findings indicate that the type and concentration of carbon source play a fundamental role in determining the morphological, physiological, and pigment characteristics of the plant in the culture

system. The carbon source has been demonstrated to provide energy and to regulate osmotic balance, cell expansion, and metabolic processes [22]. The findings of Experiment 1 indicated that a low-dose of maltose (10 g/L) was identified as the optimal carbon source for 'Bukit Kelam'. In this medium, the highest values were obtained in terms of proliferation, leaf area, plant width, and shoot number. The slower rate of hydrolysis of maltose in comparison to sucrose and glucose provides a continuous energy source by balancing carbon flow. In addition, it has been documented that maltose promotes more compact shoot development in aquatic species such as *Bacopa monnieri* [23]., *Limnophila aromatica* [24] and *Pogostemon erectus* [25].

Conversely, the application of elevated glucose levels (20 g L^{-1}) resulted in a substantial decline in all measured morphological parameters. This phenomenon can be attributed to the elevated glucose concentrations, which induce osmotic stress and disrupt cellular physiology by increasing the accumulation of reactive oxygen species [26]. The 20 g L^{-1}sucrose application was found to have a significant impact on plant height and leaf number, particularly with regard to supporting shoot elongation and leafing. This is related to sucrose's role as the primary carbon source in most plants, influencing cell elongation and osmotic regulation [27].

In the second experiment, maltose at 10 g L^{-1}, identified as the most suitable carbon source, was tested both in solid MS medium and in a bioreactor system. The results obtained demonstrate that the culture system engenders substantial disparities in plant morphology and physiology. Plants cultivated in a solid medium exhibited superior characteristics, including increased plant height, leaf area, branching ratio, and SPAD values. The outcomes can be attributed to the solid medium providing a more balanced microenvironment in terms of oxygen diffusion, gas exchange, and moisture control.

In the comparison of solid media and bioreactor systems, the results obtained are consistent with findings reported in other aquatic species such as *Ceratophyllum demersum* and *Rotala rotundifolia*. In their study comparing the propagation performance of *C. demersum* in agar-solidified and liquid media, Karataş et al. [28] noted that higher shoot development was achieved in solid media. In a similar vein, a study on *R. rotundifolia* by Doğan [29] reported that although plants formed shoots rapidly in liquid culture media, structural stability decreased. Conversely, in solid media, more compact and healthy development was achieved. The findings of the present study on *B. pygmaea* 'Bukit Kelam' are corroborated by extant literature, which demonstrates that solid culture medium provides a more suitable microenvironment in terms of morphological growth and leaf colour stability.

In the context of the bioreactor system, the cultivation of plants resulted in the development of pale-coloured leaves, accompanied by symptoms of partial chlorosis. This phenomenon may be attributed to a number of factors, including reduced oxygen solubility in the liquid medium, limited light transmission, and imbalanced gas exchange. A plethora of literature supports the hypothesis that this phenomenon may be attributable to a decline in pigment saturation and chlorophyll content within the leaves. As demonstrated in the research by Zalan et al. [21], the colour changes exhibited by aquatic species such as *Bucephalandra* are directly linked to the type of carbon source and the oxygen dynamics of the mediums.

The results obtained from this study are consistent with those reported in the literature for *Bucephalandra* species. Yunita et al. [8] reported that the combination of BAP and TDZ increased shoot proliferation in *Bucephalandra* species. It is well established that carbon sources function not only as an energy source in plant cells but also as an osmotic regulator and trigger for morphogenetic processes. In a similar vein, Silva [30] reported that the carbon source had a direct impact on the organogenesis rate in chrysanthemum tissues, with maltose providing a more balanced energy release compared to sucrose and glucose. Furthermore, Welander & Pawlicki [31] emphasised that different carbon compounds elicit divergent physiological responses on growth and organogenesis in plant tissue cultures, and that the carbon composition of the culture medium varies from genotype to genotype. These findings are consistent with the increase in shoot development observed with low-dose maltose in the present study. In a recent study, Zalan et al. [21] reported that sucrose concentrations in *Bucephalandra* sp. 'Red Mini' cultures have significant effects on shoot tip proliferation, with sucrose at 3–5% providing the highest multiplication rate.

The findings of this study provide significant contributions not only at the morphological and physiological levels but also in terms of aquatic plant biotechnology and sustainable production. It is evident that *Bucephalandra* species are endemic, with limited populations in nature, and are at risk of extinction due to overharvesting [8]. Consequently, *in vitro* propagation protocols developed under laboratory conditions have considerable potential for both commercial and conservation-oriented sustainable production models. This developed protocol facilitates the production of healthy plant material in a controlled environment, thereby ensuring the sustainable utilisation of natural resources and contributing to the preservation of ecological balance. The aim of the study and its significant results should be given briefly in a concrete way. In addition, suggestions and opinions that are requested to be conveyed to the readers regarding the results of the study can be stated.

4. CONCLUSION AND SUGGESTIONS

This study demonstrated a reliable in vitro propagation strategy for *Bucephalandra pygmaea* 'Bukit Kelam', thus contributing to its sustainable production and conservation. The results obtained demonstrate that the carbon source exerts a significant influence on plant development, with MS medium containing 10 g L^{-1} maltose providing optimal conditions. In the present study, the highest shoot multiplication rate (2.0 shoots explant^{-1}) and leaf area (1.18 cm^2) were recorded under these conditions, while SPAD values increased by approximately 40% compared with the bioreactor treatment. Plants cultivated on a solid MS medium demonstrated superior morphological development and increased chlorophyll accumulation. In contrast, bioreactor-cultured plantlets exhibited higher lightness (L^*), chroma (c^*) and yellow hue (b^*) values, indicative of enhanced pigment brightness. These findings demonstrate that the solid medium fosters compact, vigorous growth, while the bioreactor system enhances colour intensity. It is therefore recommended that medium selection be aligned with production objectives, whether these be biomass increase or visual quality. In conclusion, the protocol that has been developed provides a practical and sustainable approach to large-scale propagation of the *Bucephalandra* species. This approach is beneficial in supporting both commercial cultivation and conservation of natural populations.

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Conflict of Interest Statement

There is no conflict of interest between the authors.

Statement of Research and Publication Ethics

The study is complied with research and publication ethics.

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All scientific content, including data analysis, figures, and the manuscript's structural composition, was entirely generated by the authors without any AI assistance.

Contributions of the Authors

İbrahim Halil HATİPOĞLU: Conceptualization, methodology development, modeling, data analysis, manuscript writing.

Meral DOĞAN: Simulations validation, data collection, contribution to the interpretation of results., evaluation of the results, and contribution to manuscript writing.

Yakup Mert KUL: Material supply, Physiological analyses, Investigation, Data curation

Burak AKYÜZ: Literature review, visualization, and manuscript review and editing and final supervision.

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