

Establishment of a Refined Somatic Embryogenesis protocol and Light-Spectrum-Based Acclimatization in *Caladium bicolor* 'White'

Maryam Dehestani-Ardakani

mdehestani@ardakan.ac.ir

Ardakan University

Heidar Meftahizadeh

Ardakan University

Mohsen Karimi

Ardakan University

Jalal Gholamnezhad

Ardakan University

Research Article

Keywords: 6-benzyladenine, In vitro regeneration, LED light spectra, Somatic embryogenesis

Posted Date: August 27th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7250887/v1>

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Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at BMC Plant Biology on November 13th, 2025. See the published version at <https://doi.org/10.1186/s12870-025-07551-1>.

Abstract

This study aimed to develop an efficient protocol for direct somatic embryogenesis (SE) from leaf explants of *Caladium bicolor* and to assess the impact of different light spectra on *ex vitro* acclimatization of regenerated plantlets. Leaf explants of *C. bicolor* 'White' were cultured on Murashige and Skoog (MS) medium supplemented with varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with 6-benzyladenine (BA). Explants bearing direct somatic embryos were subcultured on MS medium supplemented with four concentrations of BA (0.5, 1.0, 1.5 and 2.0 mg L⁻¹) to shoot regeneration. Regenerated plantlets were then acclimatized under four light spectra: red (R), blue (B), combined blue-red- (BR), and white (W) fluorescent light. Morphological, physiological, and biochemical traits were evaluated. The highest embryogenic callus formation (31.25%) was observed in the treatment with 1.5 mg/L 2,4-D + 1.0 mg/L BA (T6), compared to just 1.25% in the 0.5 mg/L 2,4-D alone treatment (T1). Organogenesis was significantly enhanced at 2.0 mg/L BA, producing up to 6.33 shoots and 55.33 roots per jar, compared to 2.00 shoots and 1.00 root in the lowest BA treatment. During acclimatization, plantlets grown under B LED light showed superior vegetative performance with the highest plant height (5.98 cm), leaf number (28.6), and root weight (1.79 g), whereas white fluorescent light (control) resulted in the poorest outcomes across most traits, including plant height (3.08 cm) and root weight (0.94 g). The study establishes a reproducible SE protocol for *Caladium bicolor* and highlights the critical role of B and R light spectra in enhancing acclimatization. These findings provide a foundational framework for commercial-scale propagation and light optimization strategies in ornamental plant tissue culture.

Introduction

Caladium constitutes a genus of perennial herbaceous flora indigenous to the tropical zones of Central and South America, with a particular prevalence in the Amazon rainforest, where these plants flourish in exposed locales or adjacent to watercourses (Ye et al. 2022; Croat et al. 2019). Leaf colors in *caladium* can be remarkably vibrant and diverse, with varieties featuring bright colors, veins, stripes, and patches in different combinations (Zhou et al. 2024; Maqsood et al. 2015). In vitro plant propagation is conventionally executed through the mechanisms of organogenesis or somatic embryogenesis. Somatic embryogenesis (SE) denotes a developmental phenomenon whereby a somatic (non-reproductive) cell can differentiate into a fully formed plant independent of gamete fusion (Fehér et al. 2003). This phenomenon can arise from a singular cell (unicellular) or a consortium of cells (multicellular) (Maximova et al. 2002). Consequently, augmenting the efficacy of the indirect pathway presents an ongoing challenge. SE is elicited under *in vitro* circumstances utilizing various forms of plant tissue explants and is generally instigated by the incorporation of plant growth regulators (PGRs) into the culture medium (Zavattieri et al. 2010). Among the diverse array of plant growth regulators employed in somatic embryogenesis, 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin, is predominantly utilized. The operational mechanism of 2,4-D encompasses the stimulation of plant cells to proliferate into undifferentiated cells, effectively reprogramming their developmental trajectory (Chin et al. 2018). This phenomenon seemingly entails the cessation of extant gene expression within the explant tissue, subsequently supplanted by an embryogenic gene expression program (Chin et al. 2018). Empirical evidence indicates that this reprogramming may be augmented through DNA methylation modulated by auxins, elucidating their pivotal role in somatic embryogenesis (Chin et al., 2018). At the molecular scale, 2,4-D engages with various signaling pathways at both transcriptional and epigenetic tiers throughout somatic embryogenesis (Wu et al. 2023; Wu et al. 2019; Horstman et al. 2017; Tian et al. 2018). The concentration of 2,4-D is paramount for the successful formation of callus and subsequent embryo development, with the optimal concentration exhibiting variability across distinct species and tissue types (Long et al. 2022). Typically, diminished concentrations facilitate the formation of embryonic callus, whereas elevated concentrations serve to inhibit this process (Long et al. 2022). Significantly, 2,4-D is generally only requisite during the initial induction phase of somatic embryogenesis. Upon the development of embryonic callus into embryoids, the hormone is frequently withdrawn from the medium, implying that 2,4-D fosters the induction of embryogenic callus but impedes the subsequent progression into fully developed plants (Long et al. 2022). Nevertheless, it is important to acknowledge that the application of 2,4-D may also induce anomalies in somatic embryos by disrupting the endogenous auxin equilibrium and polar transport, potentially compromising embryo apical-basal polarity (Garcia et al. 2019).

Somatic embryogenesis protocols for caladium species typically utilize 2,4-D as the primary auxin for embryogenic callus induction. In caladium, different explant sources respond to varying concentrations of 2,4-D. Research has shown that petiole explants respond well to 2,4-D at a concentration of 1.0 mg L^{-1} , producing a maximum of 31 somatic embryos per callus (Abbasi et al. 2016). Leaf explants have also demonstrated success in somatic embryo formation when treated with 2.26 and $4.52 \text{ }\mu\text{M}$ 2,4-D, while stolon tips required higher concentrations of $9.04 \text{ }\mu\text{M}$ 2,4-D to initiate embryogenic response (Joshee et al. 2007). The embryogenic mechanism in *Caladium* adheres to a conventional developmental trajectory when stimulated with 2,4-D. Initial embryogenic aggregates manifest as nodular callus, which subsequently differentiate into distinct somatic embryos displaying characteristic globular, heart-shaped, and cotyledonary phases (Joshee et al. 2007). In temporal analysis, somatic embryos commence emergence on the peripheral cell layer of the callus within a span of 2–3 weeks following the transition to embryo induction medium enriched with 2,4-D (Abbasi et al. 2016). The integration of 2,4-D with additional plant growth regulators has been demonstrated to significantly augment somatic embryogenesis in Caladium. The incorporation of thidiazuron (TDZ) into 2,4-D-infused media has been evidenced to further amplify the quantity of somatic embryos derived from both petiole and leaf explants (Abbasi et al. 2016). Likewise, the synergistic application of 2,4-D (0.8 mg L^{-1}) in conjunction with kinetin (1 mg L^{-1}) has proven effective for callus induction in *Caladium humboldtii* corm and petiole explants (Sakpere et al. 2007).

Suboptimal survival rates during the acclimatization process are frequently correlated with inadequate lighting conditions during micropropagation, which may lead to insufficient morphological and anatomical leaf development, dysfunctional stomata, excessive transpiration, and diminished photosynthetic efficiency (Cioc and Pawłowska 2020). The transition from *in vitro* to *ex vitro* environments represents a critical challenge as plantlets must adapt from heterotrophic to autotrophic growth while coping with dramatically different environmental conditions.

The manipulation of light spectra during *in vitro* culture can significantly enhance acclimatization success by preparing plants for natural environments. Studies have demonstrated that different light treatments during *in vitro* culture create carry-over effects that persist into the acclimatization phase (Vendrame et al. 2022). For example, plantlets previously cultured under specific LED combinations showed better performance during acclimatization than those grown under traditional lighting (Vendrame et al. 2022; Nhut et al. 2005). The amalgamation of red (R) and blue (B) light spectra has demonstrated notably favorable outcomes for enhancing acclimation success. The ratio of 80:20 R to B LEDs markedly enhanced the survival and acclimation of *Spathiphyllum* in comparison to traditional fluorescent lighting (Fan et al. 2022; Nhut et al. 2005). Plants subjected to monochromatic light sources during *in vitro* culture may encounter difficulties during acclimation. Studies indicate that plants cultivated under monochromatic B or R light spectra exhibited reduced growth during the acclimation phase relative to those produced under mixed light spectra (Rodrigues et al. 2022). Interestingly, while B light can benefit *in vitro* development, prolonged exposure to solely B light may have negative effects during acclimatization. In *Heliconia Champneiana* cv. Splash, treatment with 100% B light resulted in the lowest level of development during storage periods but achieved 100% survival during subsequent acclimatization (Rodrigues et al. 2018). Light spectrum manipulation can also influence stomatal characteristics, which are critical for acclimatization success. Since stomatal functionality is a major factor of acclimatization success, particularly in woodland creatures' species, regulating stomatal development via light quality can profoundly influence plant survival during this pivotal phase (Gonzales-Alvarado and Cardoso 2024; Marin-Martinez and Iglesias 2022). The impact of LED lighting on stomata may facilitate a reduction in stomatal density and diameter, hence enhancing the acclimation of plantlets (Gonzales-Alvarado and Cardoso 2024).

Hence, the goal of this study was to develop an efficient and reproducible protocol for direct somatic embryogenesis in *Caladium bicolor* 'White' using leaf explants and to establish the LED light spectra on the acclimatization performance of *in vitro* regenerated plantlet system. This integrated approach aims to improve the propagation efficiency and post-transfer survivability for commercial and conservation purposes.

Material and methods

Plant material and the construction of a culture

In this experiment, young, vigorous leaves of *Caladium bicolor* cultivar 'White' were selected as the primary source of explants. To ensure sterility, a thorough surface disinfection procedure was implemented. To begin, sterile distilled water was used to rinse the leaves three times. After that, they were submerged for three minutes in a sterilized solution that contained 0.1% mercuric chloride and 0.05% citric acid. The sterilization process concluded with an additional three rinses using 0.05% citric acid to eliminate any residual contaminants, as outlined by Khamushi et al. (2019). Following sterilization, three explants were placed in each glass jar (10 cm high × 6 cm diameter). It has 30 mL of Murashige and Skoog (MS) basal medium already inside of it (Murashige and Skoog, 1962). The culture medium was enriched with various combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA) to evaluate the influence of plant growth regulator (PGR) concentrations on somatic embryogenesis from leaf explants. All treatments were incubated under standardized growth chamber conditions, with a photoperiod of 16 hours light and 8 hours darkness. During the light phase, the temperature was maintained at $24 \pm 2^\circ\text{C}$, while during the dark period it was kept at $21 \pm 2^\circ\text{C}$. The light intensity was regulated within a range of 34 to 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density (PPFD).

Somatic embryo induction process

To initiate somatic embryogenesis, leaf explants of *C. bicolor* measuring 1×1 cm were cultured on Murashige and Skoog (MS) medium supplemented with varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyladenine (BA). The experimental treatments were as follows: T1 (0.5 mg L^{-1} 2,4-D), T2 (1.0 mg L^{-1} 2,4-D), T3 (1.5 mg L^{-1} 2,4-D), T4 (0.5 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA), T5 (1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA), and T6 (1.5 mg L^{-1} 2,4-D + 1.0 mg L^{-1} BA). The medium was solidified with 7 g L^{-1} agar and supplemented with 30 g L^{-1} sucrose. Each treatment was replicated five times, totaling 30 culture vessels (6 treatments × 5 replicates), with three explants placed in each jar. To enhance embryogenic induction, cultures were initially incubated in complete darkness for 14 days. Subsequently, the jars were transferred to a growth chamber under the previously described environmental conditions. Embryogenic structures began to form approximately two weeks after culture initiation, and the percentage of explants exhibiting somatic embryogenesis was recorded at this stage. By the eighth week, all jars were evaluated for somatic embryogenesis induction rate and the time required for embryo emergence. Additionally, observations on the extent of explant browning were documented.

Somatic embryo maturation and shoot regeneration

Explants containing direct somatic embryos were sorted according to their origin and transferred to MS medium enriched with standard salts, vitamins, 30 g L^{-1} sucrose, 7.0 g L^{-1} agar, and one of four concentrations of 6-benzyladenine (BA): 0.5, 1.0, 1.5, or 2.0 mg L^{-1} . The cultures were incubated for 10 weeks to support further development. Upon transfer to the BA-supplemented medium, the embryos-initiated organogenesis, producing both roots and shoots (Fig. 3C). Afterward, the regenerated shoots, now 10 weeks old, were placed onto PGR-free MS medium for an additional 2-week period. At 12 weeks, several growth parameters were evaluated, including the rate of organogenesis, number of leaves, roots, and shoots per plantlet.

Ex vitro acclimatization under different LED light spectra

Twelve-week-old *in vitro*-cultured plantlets of *Caladium bicolor* cv. 'White' were carefully selected, excised from their culture containers, and rinsed thoroughly under running tap water to detach adhering agar from the plantlets. These plantlets were then transplanted into a sterilized substrate consisting of a 1:1 (w/w) ratio of cocopeat and perlite, which had been autoclaved prior to use. For the hardening phase, the potted plantlets were placed in growth chambers under Light regimes applied included 100% red light emitting diode (LED) light at 660 nm (R), 100% blue LED light at 450 nm (B), an equal ratio blend of blue and red LEDs (50% B + 50% R (BR)), and 100% white fluorescent light (W) (Fig. 4). These specific light wavelengths were chosen due to their critical role in chlorophyll absorption and photosynthetic activity (Aalifar et al. 2020), with the white fluorescent light providing a full-spectrum control within the photosynthetically active radiation (PAR) range. Light spectra were verified with a Sekonic C7000 SpectroMaster spectrometer (Sekonic Corp., Japan) across a 300–800 nm range. The LED setups were mounted in aluminum chambers ($100 \times 110 \times 50 \text{ cm}^3$) provided by Iranian Grow Light Company, with the light

sources positioned 50 cm above the plantlets. All growth chambers were calibrated to maintain a PPFD of $250 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$. PPFD measurements were conducted with a LI-250A light meter (LI-COR Biosciences, Lincoln, NE, USA). Each treatment included five pots (replicates), totaling 20 pots across the four light regimes. Containers measuring 7 cm in diameter were employed and were uniformly filled with the sterilized cocopeat + perlite (CP + P) mixture. To create a high-humidity microenvironment during the early acclimatization phase, inverted transparent plastic cups were used to cover individual pots. Small ventilation holes were gradually introduced to the covers, enabling gradual exposure to ambient conditions. The inverted covers were removed after two weeks of acclimatization for increasing intervals each day. After four weeks of acclimatization under controlled conditions, the regenerated plantlets were relocated to a greenhouse to continue the hardening process. Ten weeks after transplantation, comprehensive growth and physiological data were collected. Parameters measured included survival rate (%), height of plant (cm), leaf and root count per plant, root length (cm), and fresh weight (g) of both aerial and root tissues. The content of photosynthetic pigments, including chlorophyll a, chlorophyll b, total chlorophyll, and total carotenoids, was also evaluated. Leaf dimensions (length and width, mm) were recorded using a Winarea-UT-11 electronic leaf area meter (manufactured in Iran). Fresh biomass was recorded using a high-precision digital balance with 0.0001 g sensitivity, and the diameter of the leaf stalk was quantified employing a digital caliper.

2.5. Assessment of Chlorophyll and Carotenoid Content

The quantification of chl. a, chl. b, total chl., and total carotenoids was executed in accordance with the methodology delineated by Lichtenthaler (1987). In summary, foliar samples from the micropropagated plantlets were harvested, and 0.1 g aliquots from each experimental group were pulverized with 5 mL of ice-cold acetone and acid-washed sand utilizing a mortar and pestle. Subsequently, the resultant mixture was subjected to filtration, after which the filtrate underwent centrifugation at ambient temperature for 5 minutes at a force of $1957 \times g$. The absorbance of the supernatant was measured at wavelengths of 470 nm, 645 nm, and 662 nm employing a UV-visible spectrophotometer, with acetone utilized as the blank standard. The concentrations of pigments were computed and articulated in $\mu\text{g/mL}$ in accordance with the equations posited by Lichtenthaler (1987).

Chlorophyll a (Chl a) = 11.24 A662 – 2.04 A645	(1)
Chlorophyll b (Chl b) = 20.13 A645 – 4.19 A662	(2)
Total chlorophylls (Total chl) = Chl a + Chl b	(3)
Total carotenoids = (1000 A470 – 1.90 Chl a – 63 .14 Chl b)/214	(4)

2.6. Data analysis

Data were statistically analyzed using a completely randomized design (CRD) that included six treatments during the initial stage of the first experiment (somatic embryogenesis), four treatments in the second stage of the first experiment (organogenesis), and four different light spectra in the ex-vitro acclimatization experiment. Statistical analyses were carried out using IBM SPSS Statistics version 27, with mean differences evaluated by Duncan’s multiple range test (DMRT) at $p < 0.05$ significance. Hierarchical cluster analysis (HCA) was applied to group treatments in both the first and second experiments, and heatmaps were created using the ClustVis web tool for visualization. Principal Component Analysis (PCA) was utilized to explore the relationships among the measured variables using Paleontological Statistics Software (PAST, version 4.03). In addition, the same software was used to conduct cluster analysis based on the UPGMA method.

Results

Somatic embryogenesis

In this study, the somatic embryos appeared light yellow and formed coherent structures exhibiting a rapid growth rate (Fig. 2A). Treatments combining BA with 2,4-D significantly enhanced the induction of embryogenesis compared to treatments with 2,4-D alone (Table 1). The highest percentage of explants undergoing embryogenesis was recorded in treatment T6, with

31.25% of explants showing embryo formation (Table 1). Somatic embryos were also detected in treatments T5, T4, and T1, with induction rates of 25.75%, 11.25%, and 1.25%, respectively (Table 1). Notably, treatments T2 and T3, which included 2,4-D without BA, failed to induce somatic embryo formation (Table 1).

Table 1				
Effect of different PGRs treatments on somatic embryogenic induction, time to somatic embryogenesis, and leaf browning of <i>C. bicolor</i> after four weeks. The bars labeled with distinct letter(s) indicated a significant difference ($p < 0.05$) as determined by DMRT				
Treatments	PGRs (mg L ⁻¹)	Somatic embryogenic induction (%)	Time to somatic embryogenesis (day)	Leaf browning (%)
T1	0.5 mg L ⁻¹ 2,4-D	1.25 ^c ± 1.25	14.25 ^{bc} ± 4.25	82.50 ^a ± 0.25
T2	1 mg L ⁻¹ 2,4-D	0 ^c ± 0.0	0.0 ^c ± 0.0	100.0 ^a ± 0.0
T3	1.5 mg L ⁻¹ 2,4-D	0 ^c ± 0.0	0.0 ^c ± 0.0	100.0 ^a ± 0.0
T4	0.5 mg L ⁻¹ 2,4-D + 1 mg L ⁻¹ BA	11.25 ^{bc} ± 2.39	48.25 ^a ± 1.68	32.75 ^b ± 1.03
T5	1 mg L ⁻¹ 2,4-D + 1 mg L ⁻¹ BA	25.75 ^{ab} ± 4.09	45.50 ^a ± 3.27	68.75 ^a ± 10.68
T6	1.5 mg L ⁻¹ 2,4-D + 1 mg L ⁻¹ BA	31.25 ^a ± 2.79	33.00 ^{ab} ± 2.13	72.00 ^a ± 4.37
*The values presented in this study were expressed as the mean ± standard error ($n = 5$). The values associated with distinct letters exhibited significant differences as determined by ANOVA ($p < 0.05$) followed by Duncan's test ($p < 0.05$). For each trait, only the maximum values were highlighted in bold font				

Somatic embryo induction was observed approximately 14 days after inoculation in the medium containing 0.5 mg L⁻¹ 2,4-D (Table 1). For induction, MS medium was supplemented either with 2,4-D alone or combined with BA. Results indicated that somatic embryo induction occurred in nearly all media supplemented with both 2,4-D and BA. The highest induction rate was achieved in MS medium containing 2 mg L⁻¹ BA, with embryos forming within 33 days post-inoculation. A decrease in 2,4-D concentration corresponded with a reduced induction rate and a prolonged time required for somatic embryo formation (Table 1). The initial indication of browning on the explants manifested as small yellow-brown spots on the leaf blade surfaces after approximately three weeks of culture. This discoloration progressively extended over the entire explant. Media supplemented solely with 2,4-D induced the highest explant browning rate (100%). However, the inclusion of BA in combination with 2,4-D significantly mitigated browning. Treatments T2 and T3 exhibited the greatest browning incidence, reaching 100% (Table 1).

Organogenesis

Organogenesis was observed across all treatments (Fig. 1A). Application of BA stimulated shoot regeneration and development (Fig. 1A). The greatest organogenesis percentages were recorded at BA concentrations of 0.5 mg L⁻¹ (95%), 1.0 mg L⁻¹ (100%), and 2.0 mg L⁻¹ (100%). The emergence of new shoots was first detected directly from the cells of the leaf segments approximately four weeks after transfer to MS basal medium supplemented with different BA concentrations (Figs. 1B and 2C).

Following the transfer of somatic embryos (Fig. 2C) to MS medium supplemented with varying concentrations of BA, significant growth and shoot development were observed (Fig. 2C). These shoots underwent continuous division, resulting in the formation of leaf clusters (Fig. 2C). Adventitious shoots appeared not only at the cut edges but also across the entire leaf surface as small green protrusions within four weeks (Figs. 1B and 2C). The number of shoot regenerations per jar ranged from 2.00 to 6.33 (Fig. 1B). Notably, treatment T4 (2 mg L⁻¹ BA) exhibited the highest shoot regeneration rate, with an average of 6.33 shoots per jar. After eight weeks of *in vitro* culture, somatic embryos began leaf regeneration (Figs. 1, 2). Results

indicated that all treatments containing different BA concentrations successfully induced embryo regeneration and leaf production (Figs. 1C and 2C, D). The greatest number of leaves per jar (48.66) was recorded in treatment T4 (2 mg L⁻¹ BA) (Fig. 1C).

By day 30, root primordia had developed, leading to the formation of adventitious roots (Figs. 2E–F), and fully expanded regenerated plantlets were achieved after 12 weeks (Fig. 2F). The highest average number of roots per jar was recorded in the 2 mg L⁻¹ BA treatment (55.33) (Fig. 1D), while the lowest was observed in the 1.0 mg L⁻¹ BA treatment (1.00) (Fig. 1D).

Multivariate analyses

In order to assess the impact of varying concentrations of BA on organogenesis in *Caladium bicolor* 'White', a principal component analysis (PCA) was conducted using Paleontological Statistics Software (PAST, version 4.03) (Fig. 3). The eigenvalues derived from the covariance matrix indicated that the first two principal components (PC1 and PC2) accounted for 100% of the total variance, with PC1 and PC2 explaining 84.34% and 15.66%, respectively. The PCA revealed clear separation among the BA treatments based on organogenetic parameters, suggesting differential responses across the measured traits (Fig. 3). The traits most strongly associated with PC1 included root number/jar and shoot number/jar, while organogenesis contributed primarily to the variation along PC2. Notably, treatments with higher BA concentrations tended to cluster along the positive axis of PC1, indicating their influence on shoot and root proliferation.

Ex vitro acclimatization

Role of Light Spectral Quality in the Hardening and Acclimatization of Plantlets

Based on the initial results from the first phase of the study, the treatment containing 2.0 mg L⁻¹ BA was identified as the most effective concentration of plant growth regulators (PGRs) for the in vitro propagation of *Caladium*. The second phase of the experiment focused on optimizing four different light spectra during the hardening and acclimatization stages (Fig. 4), evaluating 14 parameters: root number, root length (cm), leaf number, leaf stalk diameter (mm), plant height (cm), fresh weight (FW) of both aerial parts and roots (g), leaf length and width (mm), survival rate, chl. *a* content, chl. *b* content, total chl., and total carotenoid content. Notably, no significant differences were observed in the survival rates of acclimatized plantlets across treatments (Table 2). However, analysis of variance (ANOVA) revealed that light spectra significantly affected the majority of the measured traits ($p < 0.05$). Detailed mean comparisons for each parameter are presented in Table 2.

Leaf Number

The number of leaves per plant ranged from 15.40 in pots exposed to the BR light spectrum to 28.60 under the B light spectrum (Table 2).

Plant Height

The measurements of plant height varied from 3.08 to 5.98 cm. The highest plantlet height was observed in B light spectrum, as well as, plantlet height when grown in R light spectrum. However, the grown of plantlets in W light spectrum resulted in significant decrease in the plant height (Table 2).

Plant height measurements ranged from 3.08 to 5.98 cm. The tallest plantlets were observed under the B light spectrum, closely followed by those grown under the R light spectrum. In contrast, plantlets cultivated under the white (W) light spectrum showed a significant reduction in height (Table 2).

Root Number

The number of roots per plant ranged from 12.20 to 28.20, with the lowest count in the B treatment and the highest in the BR treatment (Table 2). Notably, as shown in Table 2, root formation reached an impressive 28.20 roots per plant under the B light spectrum.

Root Length

The newly emerging roots of all young plants displayed gentle curves and diverse branching patterns. As multiple fibrous roots developed, the plantlets could easily penetrate the substrate, increasing the root surface area and improving nutrient uptake from the environment. In all pots containing CP + P, roots were notably more abundant and demonstrated lateral spreading or penetration prior to growing downward. However, no significant differences in root length were observed among plantlets grown under different light spectra (Table 2).

Leaf stalk diameter

The diameter of leaf stalks ranged between 0.96 mm and 1.28 mm, with the minimum measurement recorded in the R treatment and the maximum in the B treatment, as shown in Table 2.

Fresh Weight of root and aerial parts of plantlets

Among the four spectra analyzed, the B light spectrum exhibited the greatest fresh weight of the aerial parts of plants, measuring 0.83 g, whereas the W light spectrum recorded the lowest value for this parameter at 0.45 g (Table 2). Nonetheless, the differences observed in the data were not statistically significant.

The fresh weight (FW) of roots per plant ranged from 0.94 to 1.79 g, with the minimum value recorded in the W light spectrum treatment and the maximum in the B light spectrum treatment (Table 2). Furthermore, as indicated in Table 2, no significant difference was noted in the FW of roots between the B and BR light spectrum treatments.

Leaf length and width

The leaf width measurements ranged from a minimum of 10.81 mm to a maximum of 14.40 mm for plantlets subjected to the W and BR light spectrum treatments, respectively (Table 2). Additionally, the B light spectrum treatment contributed to an increase in leaf width. However, the leaf length of the plantlets did not demonstrate any significant differences across the four light spectra (Table 2).

Table 2 Duncan's mean comparison test for the effects of four different light spectra treatments on the selected features recorded from the <i>ex vitro</i> acclimatization of the tissue cultured plantlets of <i>Caladium bicolor</i> 'White'										
Light treatment	Leaf number	Plantlets Height (cm)	Length of leaf (mm)	Width of leaf (mm)	Root length (cm)	Root number	Leaf stalk diameter (mm)	FW of aerial part (g)	FW of root (g)	Survival rate (%)
W	20.20 ^{ab} ± 0.8	3.08 ^b ± 0.42	10.05 ^a ± 0.25	10.81 ^b ± 0.48	5.56 ^a ± 0.46	23.20 ^a ± 2.85	1.06 ^{ab} ± 0.06	0.45 ^a ± 0.04	0.94 ^b ± 0.14	60.00 ^a ± 5.00
B	28.60 ^a ± 3.62	5.98 ^a ± 0.52	11.88 ^a ± 0.26	14.32 ^a ± 0.63	6.44 ^a ± 0.49	28.20 ^a ± 2.69	1.28 ^a ± 0.11	0.83 ^a ± 0.15	1.79 ^a ± 0.21	80.00 ^a ± 4.00
R	19.80 ^{ab} ± 3.97	5.34 ^a ± 0.44	10.05 ^a ± 0.65	12.80 ^{ab} ± 0.70	7.14 ^a ± 0.96	13.40 ^b ± 1.88	0.96 ^b ± 0.11	0.52 ^a ± 0.13	1.29 ^{ab} ± 0.15	100.0 ^a ± 0.0
BR	15.40 ^b ± 2.87	4.44 ^{ab} ± 0.80	11.90 ^a ± 2.12	14.40 ^a ± 1.76	7.48 ^a ± 1.93	12.20 ^b ± 0.91	1.10 ^{ab} ± 0.4	0.61 ^a ± 0.20	1.68 ^a ± 0.31	100.00 ^a ± 0.0
*The values presented in this study were expressed as the mean ± standard error (n = 5). The values associated with distinct letters exhibited significant differences as determined by ANOVA (p < 0.05) followed by Duncan's test (p < 0.05). For each trait, only the maximum values were highlighted in bold font										

Chlorophyll and Carotenoid Content

The highest concentration of Chl. a was observed in the B light spectrum, with a value of 5.85 µg/mL (Fig. 5A). In contrast, the greatest amounts of Chl. b were found in both the W and BR light spectra, measuring 3.36 and 3.28 µg/mL, respectively (Fig. 5B). Notably, plants exhibited a lower level of Chl. a when grown under the B light spectrum (Fig. 5A). Additionally, the B and R light spectra resulted in the lowest quantities of Chl. b (Fig. 5B). The highest total Chl. concentration was recorded in the BR light spectrum, with a value of 9.13 µg/mL (Fig. 5C). Conversely, the lowest total Chl. amount was observed in the B light spectrum, measuring 5.61 µg/mL (Fig. 5C). The increased chlorophyll levels noted in the BR light spectrum can be attributed to the activation of the enzymes involved in chlorophyll biosynthesis. Plants grown in the W light spectrum exhibited the highest total carotenoid concentration, recording a value of 3.25 µg/mL (Fig. 5D). In contrast, the lowest carotenoid levels were found in the R, B, and BR light spectra, which measured 1.93, 1.97, and 2.17 µg/mL, respectively (Fig. 5D).

Clustered HeatMap for PigmentBased, MorphoBased, and All 14 Traits

The analysis of the clustered heat-map revealed two primary groups based on the response of the plants to different light spectra (Fig. 6). The first major cluster was characterized by the B LED light spectrum, which exhibited the highest values for various morphological traits. In contrast, this cluster also showed the lowest values for photosynthetic pigments. The second major cluster encompassed the remaining three treatments: W, R, and BR LED light spectra. Within this cluster, two distinct subgroups were identified. The first subcluster included only the W LED light spectrum, while the second contained both the R and BR LED light spectra. This classification highlights the differential impact of the various light treatments on plant morphology and pigment composition.

Discussion

This study developed a micropropagation protocol for *Caladium bicolor* using direct SE from leaf explants, with subsequent regeneration of plants subjected to hardening under various light spectra. The standardization of plant growth regulators was identified as a crucial factor for achieving successful direct embryogenesis. The leaf explants exhibited a high embryogenic frequency, producing a substantial number of direct somatic embryos and successful conversion when cultured on an optimized combination of plant growth regulators. Previous studies have reported that embryogenic and regeneration responses are highly genotype-dependent, with the effectiveness of PGR combinations varying according to the explant source and cultivar type (Meziane et al., 2017; Syeed et al., 2022). Plant cells possess totipotency, allowing them to develop into a complete plantlet through SE or organogenesis. SE can be triggered *in vitro* by subjecting various explant types to appropriate growth conditions (Yang and Zhang 2010; Horstman et al. 2017). In recent years, SE has gained prominence as an alternative to conventional seed-based propagation, especially for species with limited natural reproductive capacity or when the goal is to maintain desirable agronomic characteristics (Ipekci and Gozukirmizi, 2003; Li et al. 2012). In this study, the combination of the plant growth regulators 2,4-D and BA was found to be highly effective in promoting direct SE from leaf explants. A notably high frequency of direct embryo formation was achieved using a medium supplemented with 2,4-D at 1.5 mg L⁻¹ and BA at 1.0 mg L⁻¹. Leaf explants demonstrated strong embryogenic capacity at elevated concentrations of 2,4-D, and the inclusion of BA further enhanced the frequency of embryogenesis. Auxins, particularly 2,4-D, are frequently implemented for the induction of SE and serve as both a stress factor and an auxin source (Ghiorghita 2019). The concentration of 2,4-D found to be most favorable varies by species; for example, in Chinese chestnut (*Castanea mollissima* Blume), embryogenic callus was successfully obtained using 1.8 µM 2,4-D combined with 1.1 µM 6-BA (Liu et al. 2020; Lu et al. 2017). The mechanisms operating at the molecular scale by which 2,4-D induces direct SE involve multiple interconnected pathways that reprogram somatic cells toward an embryogenic fate. At the epigenetic level, 2,4-D exposure leads to significant changes in DNA methylation patterns and chromatin structure. These modifications are crucial for the acquisition of embryogenic competence, as they silence genes associated with the current differentiated state while activating embryogenic gene expression programs. Multiple studies have demonstrated that 2,4-D influences nuclear methylation at the DNA level, which leads to genomic reprogramming in somatic cells (Bajpai et al. 2016; Silveira et al. 2020; De-la-Pena et al. 2015). Additionally, 2,4-D has been reported to affect histone modifications that contribute to chromatin remodeling (Silveira et al. 2020). 2,4-D also functions as a stress inducer, which is a critical aspect of its embryogenic capacity. Mechanisms activated during stress exposure triggered by 2,4-D exposure contributes to cellular reprogramming and is considered a key step in redirecting somatic cells toward embryogenesis (Bajpai et al. 2016; Opabode et al. 2011; Zavattieri et al. 2010). This stress-induced reprogramming involves the

activation of specific stress genes that ultimately contribute to the expression pattern of the embryogenic pathway (Simoes et al. 2010; Mahendran et al. 2015; Stanisic et al. 2015).

The ratio and levels of these plant growth regulators (PGRs) play a pivotal role in maximizing embryogenic efficiency. A concentration of 1 mg L⁻¹ 2,4-D combined with 2 mg L⁻¹ BA was found to be the most effective for inducing somatic embryos, which yielded the highest number of globular-stage embryos (28.6 embryos per explant). Increasing either hormone beyond these levels led to a decline in embryo numbers, indicating an inhibitory effect at higher concentrations (Lizawati et al. 2023). This suggests a finely tuned auxin-to-cytokinin ratio is crucial for triggering embryogenic potential and embryo development. Following a 10-week period in differentiation and maturation media, the majority of the somatic embryos exhibited an elongated morphology and yellow pigmentation, resembling the torpedo stage of development. While some embryos developed individually, most were found clustered together (Fig. 3D–E). The progression of somatic embryo development was asynchronous, a phenomenon commonly observed during somatic embryogenesis in various plant such as palm species, including those belonging to the genus *Euterpe precatoria* Mart (Ferreira et al. 2022). These embryos initiated development at different time points and were exposed to alterations in nutrient conditions during successive subcultures, leading to variability in their developmental stages.

Findings suggested that different levels of BA affected both the maturation and conversion of somatic embryos. A high frequency of embryo conversion was observed at specific BA concentrations, which further enhanced the overall conversion rate. BA facilitates organogenesis through multiple interconnected molecular and physiological mechanisms. At the genetic level, BA activates crucial genes responsible for shoot development, including Wuschel (WUS), Enhancer of Shoot Regeneration (ESR1 and ESR2), and SHOOTMERISTEMLESS (STM) (Eskundari et al. 2021). The collaboration between WUS and STM genes is particularly significant in inducing in vitro organogenesis (Eskundari et al. 2021; Gallois et al. 2004). These genes follow specific expression patterns during organogenesis, with ESR1 being expressed during the initial days of culture on shoot-inducing medium, while ESR2 expression occurs after approximately four days (Eskundari et al. 2021). BA influences cytokinin metabolism at the biochemical level by upregulating genes associated with cytokinin synthesis and activation, thereby increasing endogenous levels of isopentenyladenosine (iPA) (Pan et al. 2024; Deng et al. 2020). The process significantly stimulates adventitious bud regeneration and proliferation. In *Petunia hybrida*, exogenous BA has been reported to elevate levels of isoprenoid cytokinins, such as isopentenyl adenine (iP) and isopentenyl adenosine (iPR), thereby supporting shoot organogenesis (Mercier et al. 2003; Auer et al. 1999).

In this study, light quality had a significant influence on the morphology and growth of *Caladium* plants. Successful acclimatization and sustained post-transfer development of *in vitro* plantlets may be attributed to the re-establishment and functional improvement of the photosynthetic apparatus during axillary shoot proliferation (Cioc et al. 2021; Tokarz et al. 2021). Plants depend on a specific range of light intensity for optimal growth and development; deviations above or below this optimal threshold can impair photosynthetic activity (Shafiq et al. 2021). B and R light wavelengths provide essential energy for photosynthesis and interact with photoreceptors that govern key morphogenetic processes, including tissue elongation, leaf expansion, stomatal regulation, circadian rhythm synchronization, and floral induction (Chen et al. 2004; Su et al. 2017).

The growth parameters of *Caladium* were significantly enhanced under B LED light compared to R, combined blue-red (BR), and white fluorescent light (control). This improvement is primarily attributed to the higher photosynthetic efficiency observed under B light, whose wavelengths closely align with the absorption peaks of chlorophyll (Gao et al. 2021). Among the tested treatments, both R and B LED lights promoted greater plant height relative to other light sources (Table 2). Previous studies suggest a linear relationship between stem elongation and the phytochrome photostationary state (Pfr/Ptotal), wherein an increase in Pfr/Ptotal leads to a reduction in stem elongation rate (Smith, 1982). Supporting our findings, Marin-Martínez (2022) also reported enhanced plant height in *Pinus pseudostrobus* Lindl. under similar light spectra. The spectral components of light serve as key environmental cues regulating numerous life processes in plants (Jiao et al. 2007). Experimental results indicated that the leaf number (28.60) and plant height (5.98 cm), leaf width (14.32 mm), root number (28.20), leafstalk diameter (1.28 mm), and fresh weight of roots (1.79 g) of *caladium* plants subjected to B light exhibited significantly higher values than the control (W light), indicating that this spectrum provides more favorable conditions for growth and morphological traits. Nonetheless, exposure to W light in this study resulted in reduced *Caladium* growth. Light quality has

been shown to be a critical factor influencing various physiological and developmental processes in plants throughout their growth cycle (Aalifar et al. 2020). Actually, among the treatments, the B LED light treatment yielded the highest growth parameters. The B light spectrum is known to enhance vegetative growth, as it promotes chlorophyll synthesis and influences phototropism, leading to healthier and more robust plantlets (Morrow, 2008). The findings confirm the positive correlation between B light exposure and increased leaf production and height, a pattern consistent with previous studies that reported enhanced growth in various plant species under B light conditions (Hogewoning et al., 2010). Unlike the outcomes observed in this study, Dehestani-Ardakani et al. (2025) found that B light had the most negative effects on growth of two *Caladium* cultivars in *in vitro* condition compared to other light spectra.

Conversely, the R light treatment, while showing a commendable survival rate of 100%, exhibited lower values in leaf number (19.80) and leafstalk diameter (0.96 mm) relative to the B LED spectrum. This indicates that while R light may be beneficial for certain physiological processes, it may not equally promote vegetative growth. Despite its role in promoting certain growth parameters, monochromatic R light can cause physiological imbalances in plants. Studies have shown that monochromatic R light can reduce stomatal conductance, which may limit CO₂ uptake for photosynthesis (Hogewoning et al. 2010; Savvides et al. 2011). In consist to our results, in cucumber plants, leaves grown under R light showed lower net photosynthesis, these imbalances were linked to lower internal CO₂ levels in the leaves and diminished photosystem II (PSII) efficiency (Savvides et al. 2011).

Interestingly, the combined B and R light treatment showed reduced leaf number (15.40). This outcome suggests that an excess of R light may inhibit some features of growth. W fluorescent light resulted in significantly reduced plant height relative to other spectral treatments (3.08 cm), leaf width (10.81 mm), and fresh weight of root (0.94 g). W fluorescent light efficacy is contingent upon the species and their distinct growth preferences. For *Caladium bicolor* 'White', while W fluorescent light can support basic growth, it may not provide the targeted benefits of specific spectra, such as those offered by B and R combinations that enhance specific growth parameters significantly (Chung et al. 2010). In a controlled environment, B, R, and FR LED treatments promoted greater leaf development and biomass, along with higher chlorophyll levels in *Oncidium* compared to W fluorescent lamp conditions (Chung et al. 2010). Significantly increased leaf width was observed in plants exposed to B and BR LED lighting, with measurements reaching 14.32 mm and 14.40 mm, respectively. These observations can be attributed to the B light's role in leaf expansion and development, which is essential during the acclimatization phase (Bourget, 2008). The notable increase in root number in the W (florescent) and B LED light treatments is particularly significant, aligning with other plants such as cherry (Iacona and Muleo 2010), wheat (*Triticum aestivum* L.) (Dong et al. 2014) and *Rehmannia glutinosa* (Manivannan et al. 2015). These findings were consistent with those of Dehestani-Ardakani et al. (2025), who reported that the highest root count in the 'Red' *Caladium* cultivar occurred under W light, while the lowest root number (8.33 roots per jar) was observed in the same cultivar under R light. The fresh weight of the aerial part did not show any significant difference in different light spectra, but and the fresh weight of roots, crucial indicators of plant health and vigor, was highest in the B and BR LED light treatments with 1.79 and 1.68 g respectively. Consistent with previous research, these results highlight the significant impact of combined B and R light spectra on plant productivity (Fang et al., 2021; Wang et al., 2016; Hernández and Kubota, 2016). The survival rates were also maximized under B and BR treatments (80 and 100% respectively). These findings highlight the critical role of choosing suitable light spectra during the plant acclimatization process, as they contribute significantly to the establishment and vigor of plantlets. In studies with chrysanthemum cuttings, different light qualities induced distinct growth patterns. B light promoted increased aerial growth and a higher shoot-to-root mass ratio, whereas R light favored biomass accumulation in the underground parts of the plant (Moosavi-Nezhad et al., 2022). Photosynthesis serves as the fundamental process driving biomass accumulation and growth in plants. Among the various light characteristics, both intensity and quality exert the most direct influence on plant morphology and photosynthetic efficiency (Tarakanov et al. 2022). Chlorophyll plays a vital role in photosynthesis as the main pigment responsible for capturing light energy. Chlorophyll molecules exhibit specific absorption spectra: chlorophyll a primarily absorbs light within the B and R wavelengths, whereas chlorophyll b absorbs predominantly in the B and yellow-green regions (Malekzadeh et al. 2024). Significant variations in chl. a, chl. b, and total chl. content were detected among plantlets cultivated under different light spectra (Fig. 5). Higher amount of these photosynthetic pigments was observed in the plantlets grown under the BR LED light (Fig. 5). Photosynthetic pigments respond dynamically to the quality of light they receive, showing remarkable adaptability

across different wavelengths of the spectrum. Plants adjust their photosynthetic pigment composition in response to the ambient light spectrum and intensity, with chlorophyll content and chlorophyll a/b ratios generally rising under B light exposure (Alsanius et al. 2019; Hogewoning et al. 2010). This adaptation allows plants to optimize their light-harvesting capabilities under varying environmental conditions. While individual wavelengths of light can support specific aspects of plant growth, research consistently demonstrates that combining different light spectra produces superior results for overall plant photosynthetic efficiency. The integration of R and B light spectra has been shown to stimulate greater photosynthetic efficiency compared to exposure to monochromatic light alone (Alsanius et al., 2019). Monochromatic light, whether R or B, can be harmful to plants compared to combined wavelengths (Alsanius et al. 2019). The ability of R light to support the accumulation of photosynthetic compounds likely contributes to the synergistic effect observed with R and B light combinations, while B light enhances chloroplast development. Together, these complementary roles make the R-B light combination particularly effective in promoting photosynthetic efficiency in plants (Li et al. 2024). Tomato and citrus plants cultivated under a combination of R and B light generally exhibit increased chlorophyll and carotenoid content, along with enhanced overall photosynthetic efficiency, when compared to those grown under monochromatic light conditions (Naznin et al. 2019; Ma et al. 2012).

Total carotenoids serve as essential pigments that not only assist in photosynthesis but also offer protective functions against photooxidative damage. In this study, the highest carotenoid content was found in the W light treatment (3.25 µg/ml), indicating that W light's broader spectrum may facilitate greater carotenoid accumulation. This aligns with research suggesting that full-spectrum light enhances not only chlorophyll production but also other pigments like carotenoids (Bourget 2008). Although the BR treatment resulted in a slightly lower carotenoid concentration (2.17 µg/mL) compared to the W treatment, it still demonstrated substantial carotenoid accumulation, indicating the plants' capacity to adapt to varying light conditions while preserving the metabolic pathways involved in carotenoid biosynthesis.

Conclusion

This study established an efficient micropropagation protocol for *Caladium bicolor* 'White' through direct somatic embryogenesis from leaf explants, optimized by the synergistic application of 2,4-D and BA. The best embryogenic and regenerative responses were observed with 1.5 mg L⁻¹ 2,4-D and 1.0 mg L⁻¹ BA, demonstrating the importance of balanced auxin–cytokinin interactions in SE induction. Moreover, the light quality during acclimatization markedly affected morphological development, pigment synthesis, and survival rates of regenerated plantlets. B LED light enhanced plantlet growth and root development, while the combined BR spectrum improved photosynthetic pigment accumulation and physiological adaptation. These findings underline the significance of integrating precise PGR regulation with tailored light spectra to maximize micropropagation success. The proposed protocol offers a scalable, genotype-compatible method for commercial propagation and conservation of *Caladium* cultivars.

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid

B: blue

BA: 6-benzyladenine

BR: Blue + Red

Chl: Chlorophyll

iP: isopentenyl adenine

iPR: isopentenyl adenosine

LED: Light Emitting Diode

MS: Murashige and Skoog

R: red

SE: Somatic Embryogenesis

W: White

Declarations

Acknowledgements

Not applicable.

Authors' contributions

Dehestani-Ardakani conceived the original idea, carried out the experiments managed project and all results, and contributed to the final version. Gholamnezhad designed experiments. Karimi carried out the experiments. Meftahizadeh wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Funding

No specific financial credit was used in this experiment.

Conflicts of interest

The author declares they have no financial interests.

Availability of data and material

Data is provided within the manuscript

Authorship contribution statement

The authors conducted the experiments in collaboration and wrote the manuscript.

Clinical trial number

not applicable

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Figures

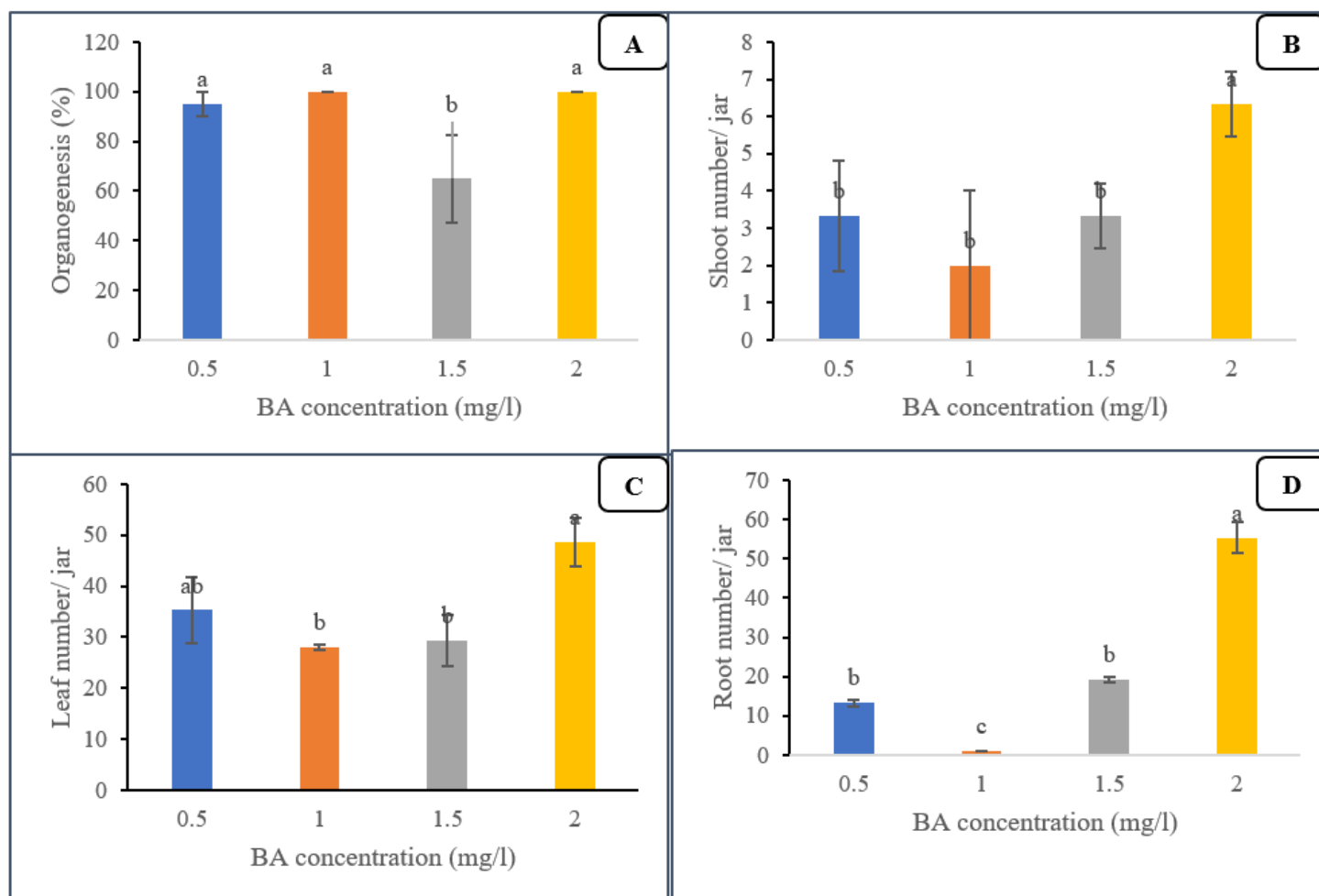


Figure 1

Effect of four concentrations of BA on MS medium on **A** Organogenesis percentage, **B** Shoot number, **C** Leaf number and **D** Root number production after 12 weeks. The bars labeled with distinct letter(s) indicated a significant difference ($p < 0.05$) as determined by DMRT

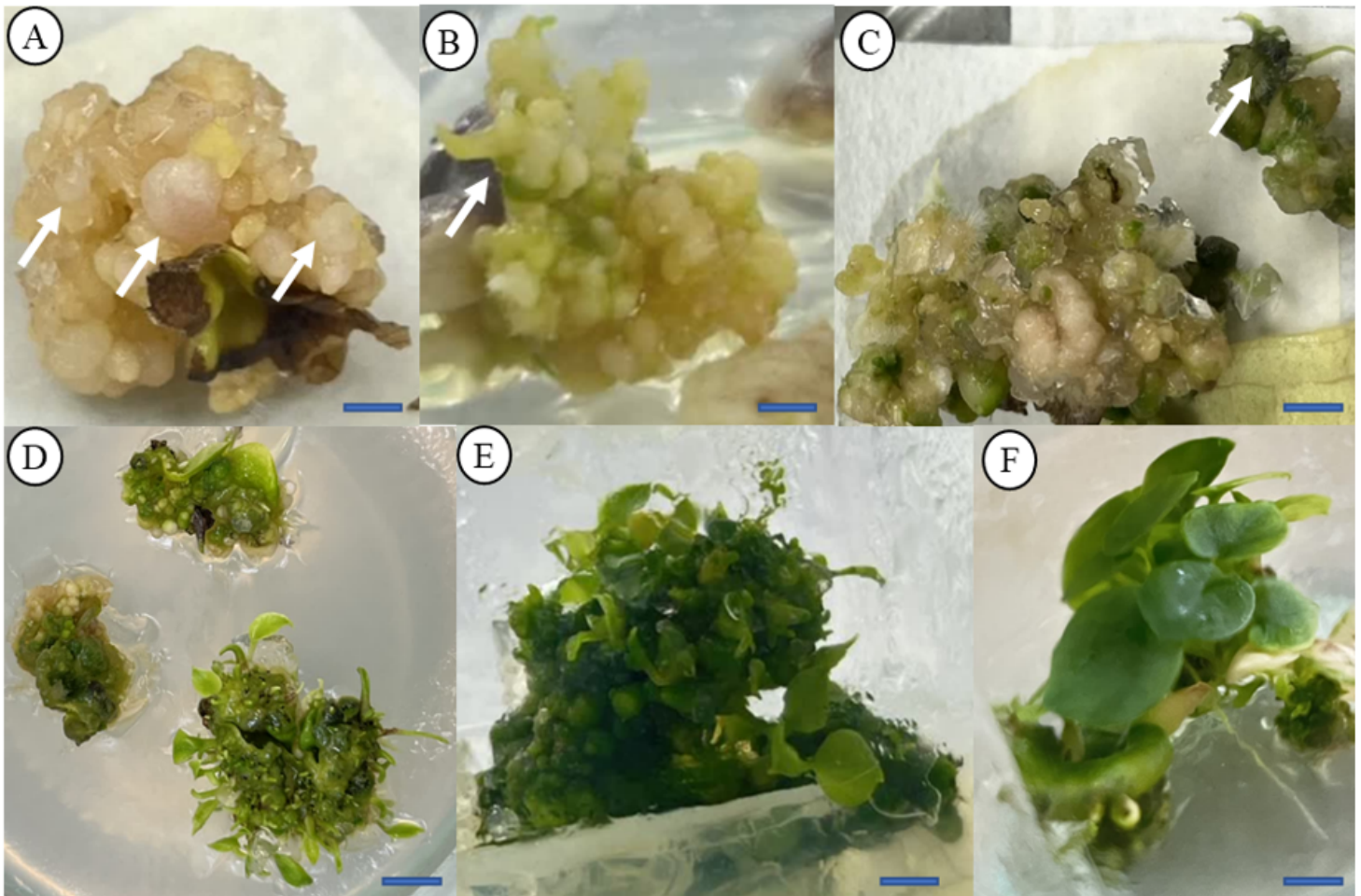


Figure 2

Production steps of plantlet in *C. bicolor* (A) somatic embryos in globular and heart stage (white arrows) regenerated from leaf explant of *C. bicolor* derived from combination of 1.5 mg L^{-1} 2,4-D and 1 mg L^{-1} BA cultured on MS medium. Bars=10 mm, (B) Developmental stages of embryo with distinct globular, and torpedo shape (white arrows), (C, D, E) Cluster of embryos on embryo conversion medium with new emerging shoots (arrow heads). Bars = 4 mm, and (F) bunch of shoots and roots formed after 12 weeks of culture. Bars =4 mm,

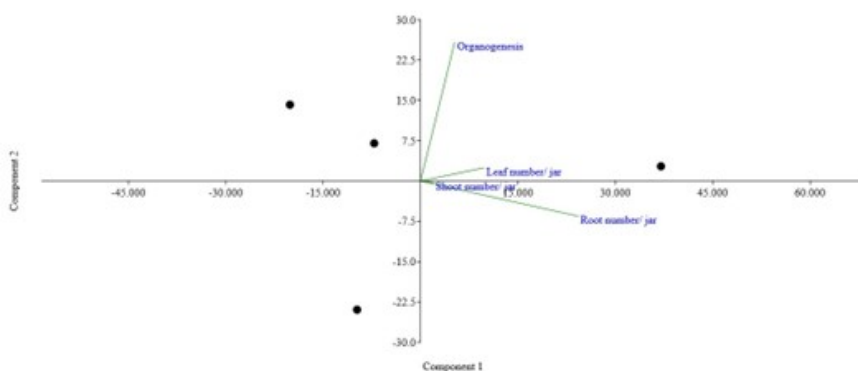


Figure 3

Biplot of the first two principal components (PC1 and PC2) based on the somatic embryogenesis traits of *C. bicolor*, which were significant.

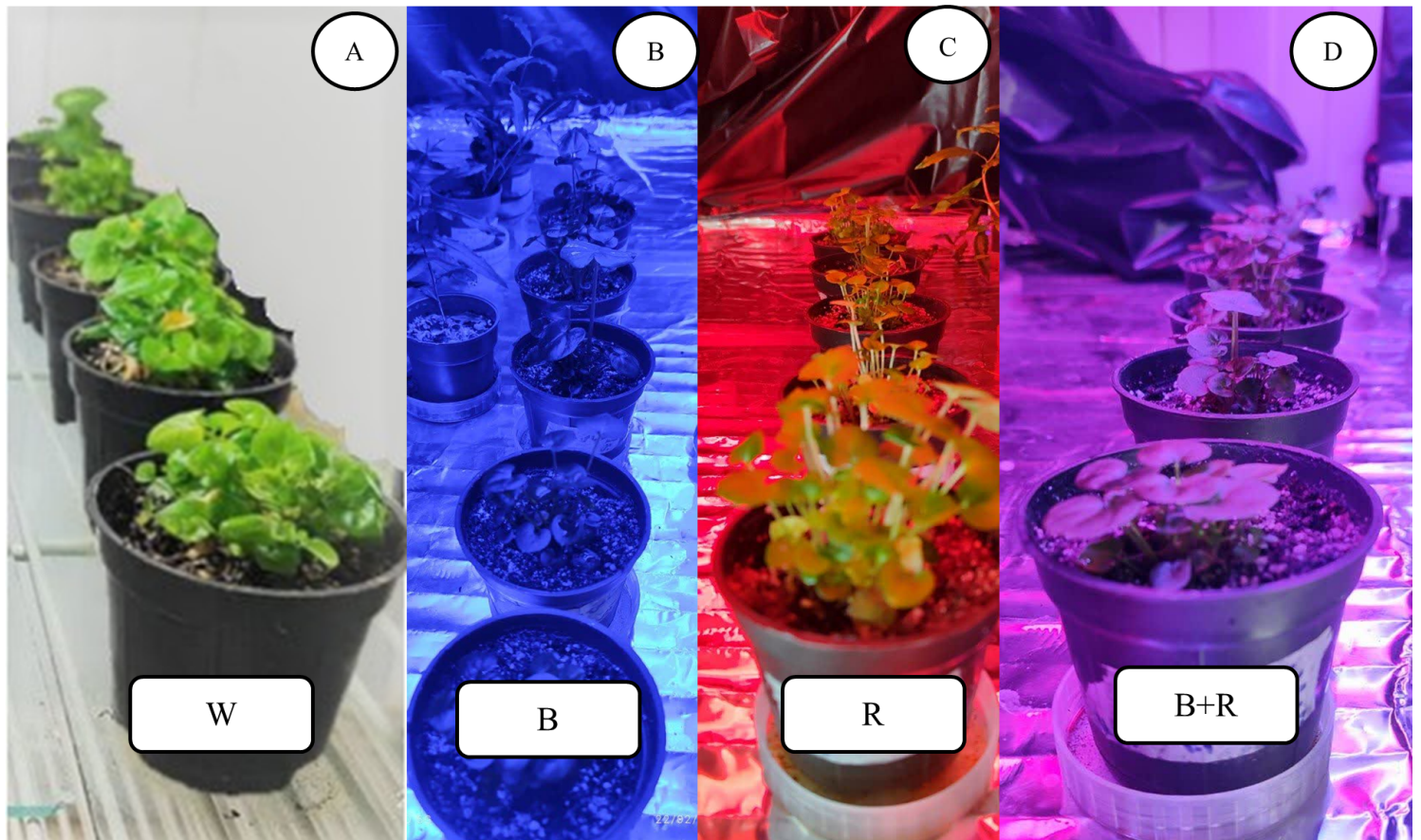


Figure 4

Survival, growth, and development of *in vitro* raised plantlets of *Caladium bicolor* cv. 'White' during *ex vitro* acclimatization under room conditions in (A) 100% White fluorescent lamp, (B) 100% Blue LED spectrum (C) 100% Red LED light spectrum, and (D) 50% Blue+ 50% Red LED spectrum.

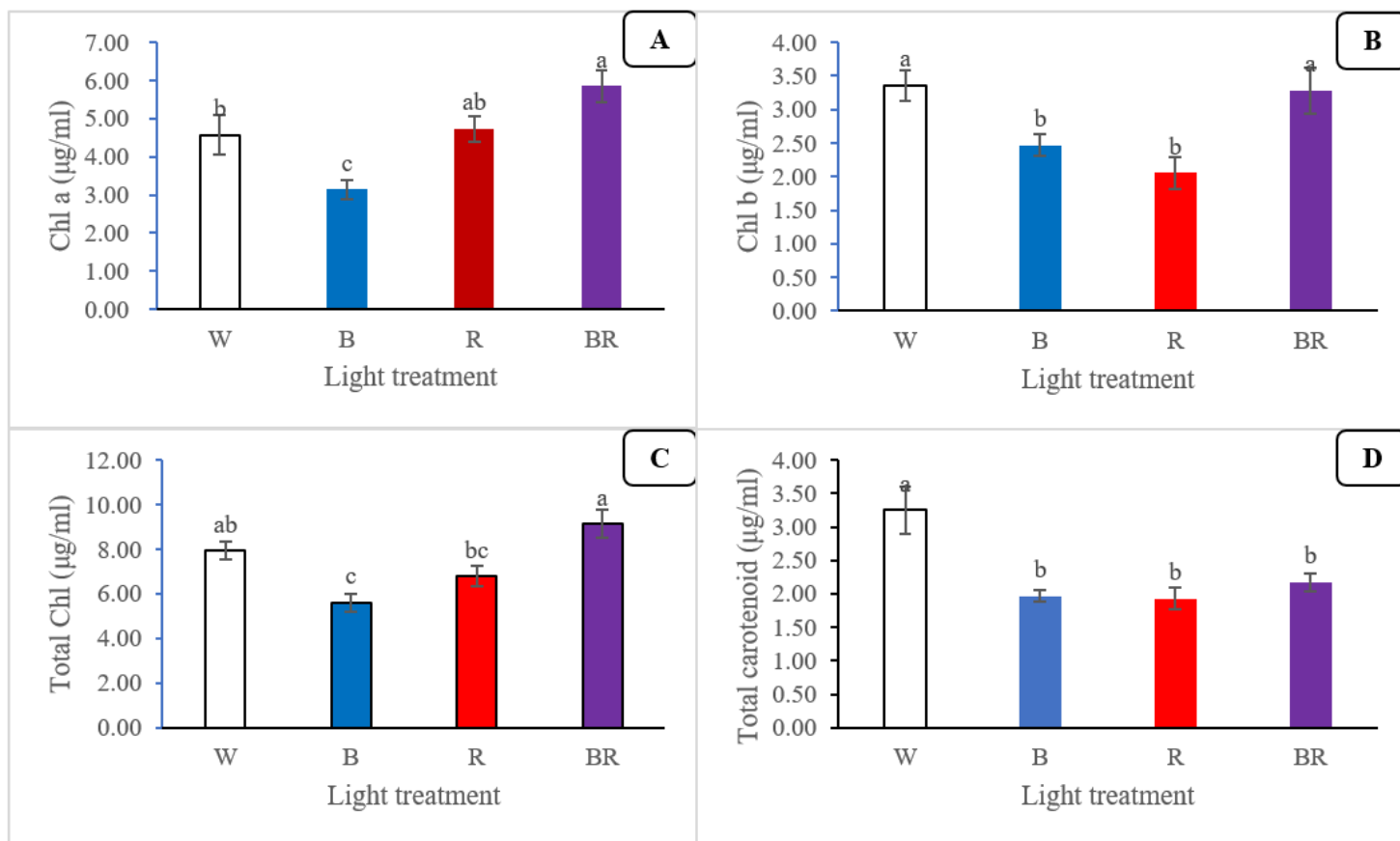


Figure 5

Effect of various light spectra on (A) Chl. a, (B) Chl. b, (C) Total Chl., and (D) total carotenoid on *ex vitro* acclimatization of tissue cultured of 'White' cultivar of *C. bicolor*. The bars labeled with distinct letter(s) indicated a significant difference ($p < 0.05$) as determined by DMRT

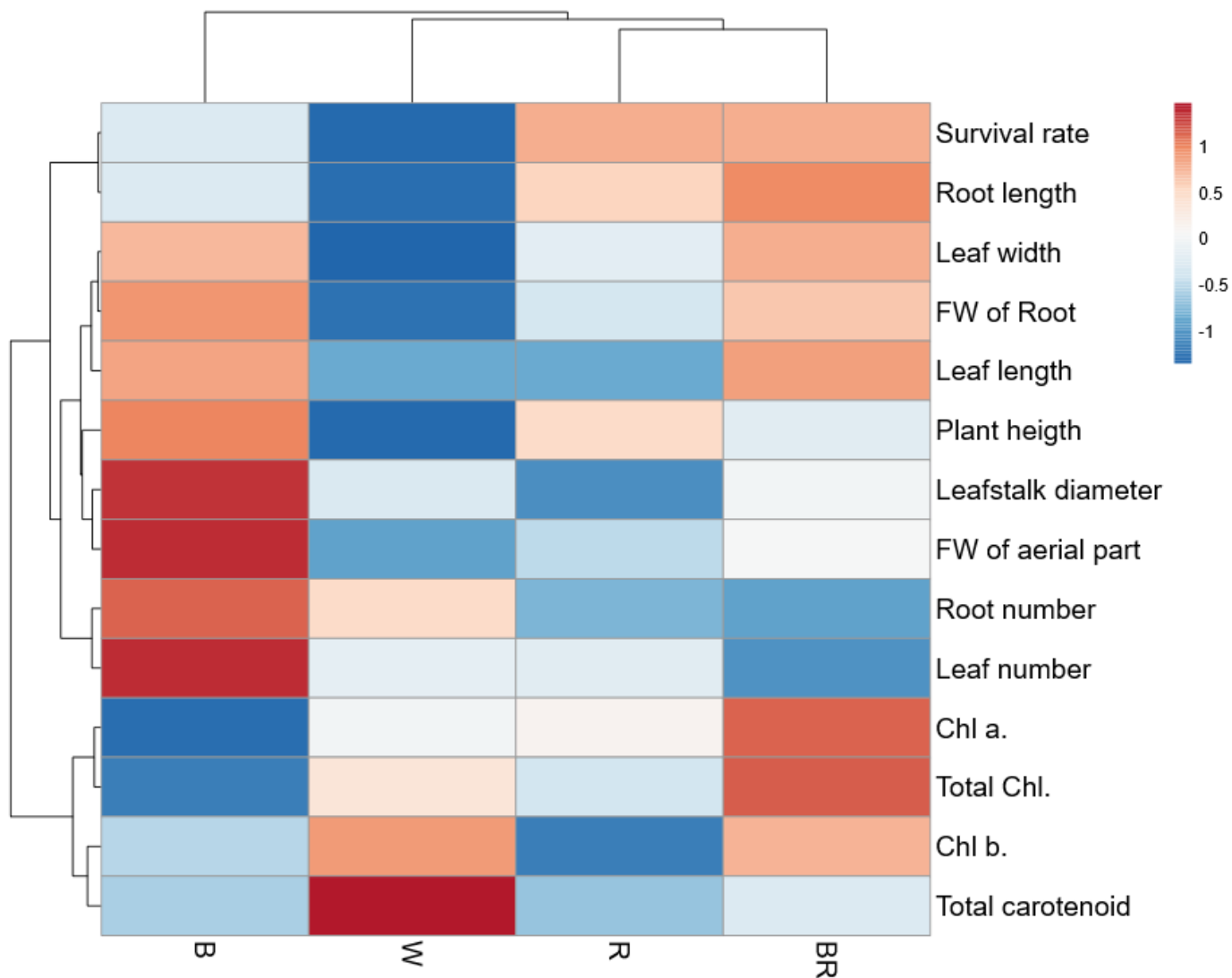


Figure 6

Clustering of the four light spectra using HCA in terms of all 14 traits. For HCA, rows were clustered using Euclidean distance and Ward linkage, while, columns were clustered using Maximum distance and Complete linkage