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Bioreactor Systems to Mass-Produce the Undervalued Crop, *Celosia argentea*, With High Nutrient Impact

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ABSTRACT

Celosia argentea is an undervalued crop that shows potential for production enhancement due to elevated leaf nutrient accumulative ability. By investigating propagation using various in vitro culture systems, thidiazuron (TDZ)-supplemented nutrient media enhanced yield from 10 plants per explant in semi-solid medium, to 27 under continuous immersion in liquid media in recipient for automated temporary immersion (RITA) bioreactors, to 63 under temporary immersion in liquid media in a balloon-type bubble bioreactor (BTBB). TDZ in the BTBB system also increased shoot biomass and subsequent nutrient content relative to TDZ-free media in ex vitro plants. Ex vitro plants originating from both continuous and temporary media immersion in BTBBs outperformed those in all other culture systems in accumulating leaf Mg, Fe, Ca and Zn to meet the recommended dietary allowance for males and females. The genotypic variance and genetic advance of the mean at 5% selection intensity varied for each nutrient per culture system, with and without TDZ. Selective breeding at 5% selection intensity would improve leaf nutrient content but is specific to the culture system and the presence of TDZ. This is the first study to use liquid-based bioreactor systems for *C. argentea* propagation thereby providing new opportunities to upscale plant production for high nutrient-accumulating genotypes. *Practical application*: This study establishes a commercially viable protocol for the large-scale clonal propagation of *Celosia argentea*, a nutrient-rich, fast growing leafy vegetable with untapped agronomic value. Using temporary immersion bioreactors and thidiazuron-supplemented media, the system delivers up to 63 plants per explant, more than 6-fold the yield of conventional methods, while significantly boosting leaf biomass and nutrient content (Mg, Ca, Fe, Zn). These results position *C. argentea* as a functional crop for health-focused markets and ready-to-cook vegetable lines. The low-input cultivation needs and rapid production cycle (8 weeks in vitro, 8 weeks ex vitro) make it ideal for high-turnover commercial nurseries, contract growers, and vertical farming operations. The systems reproducibility and high heritability of nutritional traits further support selective breeding programs for premium-value cultivars. This propagation platform offers agribusinesses a scalable entry point into the expanding market for nutrient-dense indigenous vegetables with health and wellness appeal.

Abbreviations: ANOVA, analysis of variance; BTBB, balloon-type bubble bioreactor; Ca, calcium; Fe, iron; GA, genetic advance; GA_3, gibberellic acid; GAM, genetic advance as a percentage of the mean; GCV, genotypic coefficient of variation; H_2, broad-sense heritability; IAA, indole-3-acetic acid; Mg, magnesium; MS, Murashige and Skoog; PCV, phenotypic coefficient of variation; PGR, plant growth regulator; RDA, recommended dietary allowance; RITA, recipient for automated temporary immersion; TDZ, thidiazuron; Zn, zinc.

1 | Introduction

Globally, the triple-burden of malnutrition—undernourishment, nutrient deficiency and over-nutrition, needs to be addressed, so that equitable food supplies are environmentally and socially sustainable [1]. In Africa, 281.6 million people are undernourished and are at risk for developing chronic illnesses because of inadequate intake, or impaired absorption, of essential vitamins and nutrients [2]. Reduced intake of magnesium (Mg), calcium (Ca), iron (Fe) and zinc (Zn) poses serious health threats that can lead to hypomagnesemia [3], hypocalcaemia [4], iron-deficiency anaemia [5] and impaired immunity [6], which result in metabolic and electrolytic disorders, impaired cognitive function, reduced productivity and increased healthcare costs [2–6]. Biofortification of staple crops or the application of soil/foliar fertilisers can improve the overall nutrient status of seed-based crops (e.g., wheat, maize, beans or rice), but can take many months before a once-off harvest is completed to derive the nutritional benefit [7, 8]. On the other hand, undervalued crops, in particular *Celosia argentea*, has the potential to address nutrient deficiencies, since this species can naturally accumulate elevated nutrient levels in their leaves, thereby providing food security. Moreover, *C. argentea* can be harvested multiple times from 4–6 weeks of age and requires very little agricultural input [9, 10].

Leaves of *C. argentea* are cooked and consumed as part of soups or relish in Western African countries, such as Nigeria but have not been utilised in other African countries, such as South Africa [11]. Adegbaju et al. [12] found that 100 g of pre-flowering dried leaves of *C. argentea* contained 189% magnesium (Mg), 83% zinc (Zn) and 266% iron (Fe) for men, while in females, the leaf Mg, Fe and Zn accounted for 241, 118 and 115%, respectively, and 143% calcium (Ca) for males and females, based on their recommended dietary allowances (RDAs) per day. The daily RDAs considered for males and females aged 19–50 in this study were, respectively, 1000 mg Ca, 420 and 320 mg Mg, 11 and 8 mg Zn, and 8 and 18 mg Fe [13, 14]. Comparative nutrient analyses based on quantitative genetic trait analyses can be useful in determining the % improvement of breeding selected genotypes, such as those with high nutrient accumulating-ability, over time, and the extent to which the environment would affect a particular trait [15]. Although there is no formal breeding programme for *C. argentea* to-date, particularly for high nutrient-accumulating genotypes, there is an increase in research focus on utilising biotechnology tools in other undervalued crops, to screen, select and upscale the production of genotypes of interest, with an emphasis on quantitative genetic trait analyses of genotypic and phenotypic variation in relation to heritability estimates [16–20].

Some micropropagation protocols have been developed for *C. argentea* and related species. Bakar et al. [21] used indirect organogenesis to produce 1.5 and 0.9 shoots per explant of *C. argentea* in semi-solid Murashige and Skoog (MS; [22]) medium supplemented with 1.0 mg L^{-1} benzylaminopurine (BAP) + 0.5 mg L^{-1} naphthaleneacetic acid (NAA) or 1.0 mg L^{-1} BAP + 1.0 mg L^{-1} NAA, respectively. Indirect organogenesis increases the culture period and chances of somaclonal variation, which occur due to an intervening callus phase, and results in potentially undesirable genetic changes that may affect the trait of interest [23]. Using young shoot tips as explants presents the most suitable route for producing clones of interest, increasing genetic stability and negating age-related mutations [24]. Warhade and Badere [25] showed that in vitro propagation of 12-day-old shoot tip explants of *C. cristata* inoculated on semi-solid MS medium containing 0.1 mg L^{-1} NAA + 1.5 mg L^{-1} BAP resulted in 3.6 shoots per explant forming after 4 weeks. Recurrent regeneration of the original explants over 10 culture cycles resulted in 1160 shoots in total, but this was achieved in 40 weeks. Literature related to in vitro propagation of *Celosia* sp. is limited to semi-solid media, which is labour-intensive due to manual manipulation under aseptic conditions, further periodic subcultures every 4–6 weeks due to nutrient depletion are required, and the cost of gelling agents garner an additional expense (reviewed by [26]). The use of liquid media in bioreactors addresses the limitations of semi-solid media by easing subculture procedures, reducing media cost, while also reducing the number of subcultures [26]. Asphyxia and hyperhydricity, typical of continuously immersed explants, can be overcome by using temporary immersion bioreactor (TIB) systems which allow for gas exchange, oxygenation of the culture environment and periods of drying when the medium flows out of the bioreactor chamber [26]. Importantly, TIBs also allow for automation by controlling the intervals of media immersion and periods of rest using a timer and a solenoid valve [26]. Furthermore, different-sized bioreactors offer advantages over other types. Larger bioreactors, such as balloon-type bubble bioreactors (BTBB) have more airspace and hold more medium and explants, which supports improved plant growth. Smaller bioreactors, such as recipient for automated temporary immersion (RITA) systems, prevent large-scale losses due to contamination, use less medium and are better suited for pilot trials [26].

There are several designs and modifications of bioreactors [26, 27] that have been applied to a variety of explant types. These include commercially important species, such as banana [28], sugarcane [29], eucalyptus [30], medicinally important species such as cannabis [31], cancer bush [32], and ginseng [33], in pharmaceutics to produce the anti-malarial drug, artemisinin [34] and the anti-cancer drug, paclitaxel [35], as well as secondary metabolite production [36]. Despite the vast applications of bioreactor culture systems, there are no reports on their use in upscaling production of *C. argentea*, particularly of nutrient-superior genotypes. By screening and selecting genotypes with this desired trait, bioreactors can be used to mass produce their clones for improved nutrient accumulative-ability. The aim of this study, therefore, was to establish an efficient direct shoot organogenesis protocol for clonal propagation of *C. argentea* shoot tips in different-sized bioreactors. The effect of immersion type and the use of TDZ on in vitro shoot proliferation and root formation, as well as on biomass and leaf nutrient content in ex vitro plants were evaluated. Estimates of genetic variability, heritability and genetic advance for leaf nutrient content were also determined. These goals form part of a larger research programme which focuses on the establishment of naturally breeding populations of high-nutrient accumulating undervalued crops. As such, large-scale bioreactor production of superior nutrient-accumulating clones is needed to establish a breeding population of *C. argentea* for cultivar development and research purposes.

2 | Materials and methods

2.1 | Plant Material

Mature inflorescences of *C. argentea* (Voucher specimen: 18283, Ward Herbarium, University of KwaZulu-Natal) were collected from random plants in a naturally occurring population in Durban, South Africa (29°48'19.3"S, 30°58'20.2"E) from 2020–2023. The inflorescences were placed between 5–8 layers of newspaper and sun-dried for 3 d on stainless steel trays. Seeds were then manually removed from the dried inflorescences and stored in hermetically sealed, clear plastic packets overnight, then stored at 4°C in the dark until experimental use.

2.2 | Seed Decontamination

Seeds were surface-decontaminated using serial immersion in different sterilants followed by hand agitation in sterile 2 mL Eppendorf tubes in a laminar air-flow cabinet. Seeds were rinsed in sterile distilled water (dH_2O) for 5 min (5×1 min each), followed by two immersions in 70% (v/v) ethanol (Illovo, South Africa) for 2 min (2×1 min each), five immersions in 2% (w/v) sodium dichloroisocyanurate (Medihealth Distributors, South Africa) containing a drop of Tween 20/80 (Sigma-Aldrich, Malaysia) for 5 min (5×1 min each) and five immersions in 0.3% (v/v) hydrogen peroxide (Sigma, United States of America) containing a drop of Tween 20/80 for 10 min (5×2 min each). Each change of sterilant was preceded by a single rinse using sterile dH_2O for 1 min and at the end for 10 min (10×1 min each).

2.3 | Media Preparation and Growth Conditions

The pH of different plant growth media was adjusted to 5.6–5.8 (before the addition of agar, where relevant). Basal medium consisted of full-strength MS (Sigma, United States of America) containing 3% (w/v) sucrose and supplemented with various plant growth regulators and/or 0.75% (w/v) agar (Sigma, Spain) in all in vitro stages. The media were then autoclaved (vertical type steam Steriliser HL34L, Prestige Laboratory Supplies, South Africa) at 121°C and 1.2 kg cm^{-2} for 20 min. Following inoculation at each in vitro step, culture tubes or bioreactors were sealed with parafilm and maintained in a growth room under fluorescent light (Phillips TLD90 De Lux 58 W, Poland) at 200 µmol m^{-2} s^{-1} and a photoperiod of 16 h light (26 ± 2°C):8 h dark (20 ± 2°C).

2.4 | Seed Germination

Following decontamination, seeds were allowed to air-dry for 15 min in a laminar air-flow cabinet and then individually inoculated onto 10 mL 1% agar. Seeds were germinated in the light for 21 d under growth conditions described in Section 2.3.

2.5 | Seedling Contamination Screening

Healthy seedlings containing no visible signs of contamination were used to screen explants for the subsequent in vitro stages. Seedlings were aseptically excised at the root collar and the aerial portions were individually incubated onto 10 mL nutrient agar (NA) (Neogen, United Kingdom). Cultures were incubated for 18–26 h in the growth room (section 2.3) in the dark. Thereafter, explants without observable contamination were used in the shoot multiplication stage.

2.6 | Shoot Multiplication

Screened axenic seedlings were inoculated onto MS basal medium for three weeks. Thereafter, single shoot tip explants containing two or three leaves were aseptically excised and inoculated in MS basal medium supplemented with 2 mg L^{-1} TDZ (Sigma, Switzerland) for 14 d in a growth room (see section 2.3; n = 20). Thidiazuron-free medium served as the control (n = 20). Axillary shoot development following each culture stage was counted as the number of new shoots produced.

2.6.1 | Semi-Solid Culture

Twenty single shoot tip explants were individually inoculated onto 10 mL semi-solid MS basal medium supplemented with 2 mg L^{-1} TDZ and 0.75% (w/v) agar in 100×20 mm (L x W) culture tubes. The number of explant:volume of medium ratio was 1:10.

2.6.2 | Bioreactor Culture

All bioreactors were supplied with medical-grade air using a 0.2 µm PTFE Midisart 2000 filter (Sartorius, Germany) and the temporary immersion cycle was set at 20 min every 2.5 h (modified after [32]). For continuous immersion, shoot tips were constantly aerated with the same working volume and the number of explant:volume of medium ratio. Four single shoot tip explants were transferred into five separate 0.9 L plastic RITA vessels (Vitropic, France) containing a working volume of 320 mL liquid medium. The number of explant:volume of medium was 1:80. Twenty single shoot tip explants were transferred into a 5 L glass balloon-type bubble bioreactor containing a working volume of 3200 mL liquid medium. The number of explant:volume of medium ratio was 1:160.

2.7 | Elongation

Multiplied axillary shoots from each culture system were aseptically excised and inoculated onto semi-solid MS basal medium supplemented with 2 mg L^{-1} gibberellic acid (GA_3) (Sigma, United States of America) for 21 d in 100×20 mm (L x W) culture tubes (n = 20–80).

2.8 | Rooting

Elongated explants (0.5 cm) from each culture system were inoculated onto semi-solid MS basal medium supplemented with 1 mg L^{-1} indole-3-acetic acid (IAA) (Sigma, China) [21] and 0.75% (w/v) agar for 21 d in 100×20 mm (L x W) culture tubes (n = 20–80).

2.9 | In vitro Growth Measures

Following each in vitro culture stage, the following parameters were recorded: number of newly developed axillary shoots (i.e., visible bud break), leaf number and plantlet height (base of shoot tip to the root collar). During multiplication, not all the initial explants multiplied, however, they were transferred onto elongation medium if they exceeded 0.5 cm, and further to the rooting stage. At the rooting stage, the number of explants forming roots was recorded and expressed as %.

2.10 | Acclimatisation and Growth

In vitro plantlets measuring 2–3 cm in height with 4–5 leaves and maximum root production were rinsed in dH_2O to remove excess agar. They were transferred into 5×5 cm pots containing autoclaved (121°C and 1.2 kg cm^{-2} for 20 min) and cooled seedling mix (Grovida, South Africa). Acclimatisation was performed over three weeks under decreasing humidity (from 100% to ambient) in a growth room and plants were watered bi-weekly with a nutrient solution of ¼-strength MS basal salts. Thereafter, the plants were transferred into 10×8.5 cm pots containing seedling and potting mix (1:1, v/v) (Grovida, South Africa), placed under a mist tent and sprayed automatically with municipal water for 1 min twice a day for one week. The plants were then watered twice a day until medium saturation and maintained in a greenhouse (uncontrolled environment; 25–38°C, 60–80% RH) for 4 weeks. They were also treated with commercial fungicides to prevent the onset of disease. The soil was sprayed weekly with 1 mL L^{-1} Odeon (chlorothalonil) (Grovida, South Africa), 1 g L^{-1} Sporgon 50 WP (BASF, South Africa) and 1.25 mL L^{-1} Folicur 250 EW (triazole) (Bayer, South Africa). Thereafter (at 8-week physiological age and pre-flowering), the entire plant was harvested for growth analysis (see section 2.11).

2.11 | Harvest Parameters

On the day of harvest, roots were thoroughly rinsed in tap water to remove excess soil. The roots were blotted dry with paper towel and each plant was partitioned into shoots and roots. Biomass assessments included plant height (cm; root collar to the base of the apical bud), root length (cm; root collar to the end of the longest root), number of leaves and nodes, and fresh and dry weights of leaves, stems and roots (g). Dry weight was recorded after incubating the material at 50°C in an oven (Labcon, South Africa) for 3 d until constant weight. Leaf area was measured using an Android leaf area app (Easy Leaf Area free v1.02; Developer: Heaslon). Following growth data collection, various calculations were performed: (i) specific leaf area (SLA), i.e., the ratio of the leaf area to the leaf dry weight (cm^2 g^{-1} DW, [37]); (ii) leaf area ratio (LAR), i.e. the ratio of leaf area to the total dry weight (cm^2 g^{-1} DW, [38]), and (iii) an adapted harvest index (HI) for leaves, i.e. the ratio of the leaf fresh weight to the total fresh weight of the plant (g^{-1} FW, [39]).

2.12 | Leaf nutrient Content

Dried leaves from each genotype were stored over activated silica gel until analysis. Leaves were ground to a fine powder using mortar and pestle. Thereafter, 0.07 g dry leaf material was heat-digested in 3 mL nitric acid (Sigma, Germany) for 3 min on a hotplate (Scientific, Laboratory Consumables and Chemical Supplies, South Africa) under a fume hood [40]. Each sample was then diluted to 10 mL with Millipore water, filtered into a polypropylene vial using a 0.45 µm nylon filter (Anatech, South Africa) and analysed for Ca, Mg, Zn and Fe content using inductively coupled plasma optical emission spectroscopy (ICP-OES, Perkin Elmer 5300, Germany). Nutrient content was expressed as mg per 100 g dry leaf weight and as mg per system biomass where relevant. Leaf nutrients were quantified through various calibration curves using standards (Sigma, Switzerland) of Mg (3.125–200 mg L^{-1}), Ca (1.56–100 mg L^{-1}), Zn (0.078–5.0 mg L^{-1}) and Fe (0.039–2.5 mg L^{-1}). All analyses were performed in triplicate on randomly selected leaves per genotype. The yield of nutrients per system was calculated and expressed based on the average leaf biomass per population (n = 20).

2.13 | Comparative Nutrient Analysis in Different Genotypes

The genotypic, environmental and phenotypic variances were estimated by using the following equations with amendments [41–43].

a. Genotypic variance (GV):

$$\sigma^2 g = \frac{MSt - MSe}{r}$$

where MSt = mean sum of squares for the trait of genotype, MSe = mean sum of squares for error of the genotype and r = number of replications.

b. Environmental variance:

$$\sigma^2 e = \frac{MSe}{r}$$

Where MSe = mean sum of squares for error of the genotype and r = number of replications.

c. Phenotypic variance (PV):

$$\sigma^2 p = \sigma^2 g + \sigma^2 e$$

Where $\sigma^2 p$ = phenotypic variance for each trait of the genotype, $\sigma^2 g$ = genotypic variance for each trait of the genotype and $\sigma^2 e$ = environmental variation among the tested traits of the genotype.

d. Phenotypic coefficient of variance (PCV) and genotypic coefficient of variance (GCV) [44]:

The PCV and GCV were classified into three classes: < 10% (Low), 10–20% (Moderate) and > 20% (High).

$$\text{Phenotypic coefficient of variance (PCV)} = \frac{\sqrt{\text{Phenotypic variance}}}{\text{Mean}} \times 100$$

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e. Heritability in broad sense [45]:

These values were classified into three classes based on Comstock et al. [46]: 0.0–30% (Low), 31 - 60% (Moderate) and > 60% (High).

$$\text{Heritability (broad sense)} = \frac{\text{Genotypic variation}}{\text{Phenotypic variation}} \text{x } 100$$

f. Genetic advance (GA) and genetic advance as a percentage of the mean (GAM) [47]:

$$GA = K \text{ x } H^2 \text{ x } P$$

The GAM was classified into three classes: < 10% (Low), 10–20% (Moderate) and > 20% (High).

$$GAM\ \% = \frac{K \text{ x } H^2 \text{ x } P}{\text{Mean}} \text{x } 100$$

where K = 2.06 at 5% selection intensity, H^2 = heritability and P = phenotypic standard deviation.

2.14 | Statistical Analyses

Data were analysed using the Statistical Package for the Social Sciences (SPSS) (version 28 IBM Inc., Chicago, Illinois, United States of America). The data were tested for a normal distribution using a Shapiro Wilk's test and homoscedasticity using a Levene's test. A one-way analysis of variance (ANOVA) followed by mean separation via a Tukey's post-hoc test was performed on in vitro cultures at different stages (1) number of shoots per explant, (2) number of leaves, (3) % elongation > 0.5 cm, (4) % rooting and (5) yield per system. Biomass and leaf nutrient content per culture system were assessed via a one-way ANOVA followed by a Tukey's post-hoc test. Where data did not meet assumptions, even after log transformation, a non-parametric alternative (Kruskal-Wallis H test) was applied on the untransformed ranked data. All differences were considered significant at the 0.05 level.

3 | Results

3.1 | In vitro Propagation

Irrespective of the culture system, addition of TDZ to the medium resulted in significantly more ($p < 0.05$) shoots being produced relative to TDZ-free medium (Table 1, Figure 4) following multiplication and elongation. Shoot multiplication was highest in the bioreactor systems containing TDZ-media. Temporary and continuous immersion resulted in 56 ± 18 and 27 ± 11 shoots, respectively, being produced after five weeks (Table 1). A few explants exhibited hyperhydricity after two weeks of being continuously immersed in TDZ-free medium (Figure 4B). When subcultured onto gelled medium, the symptoms of hyperhydricity decreased. Explants appeared the largest in the BTBBs (Figure 4A), followed by a slight reduction in size in the RITA vessels (Figure 4C), while the semi-solid cultures had the smallest and shortest explants (Figure 4F), although most explants were larger than 0.5 cm (Table 1). Continuous immersion in TDZ-media significantly reduced elongation after five weeks, relative to temporary immersion in the same system and medium ($p < 0.05$).

During the rooting phase, explants exposed to TDZ continued to multiply shoots if they were previously continuously or temporarily immersed in BTBBs ($p < 0.05$) (25 ± 24 and 20 ± 19 shoots, respectively) than those that were continuously immersed in TDZ-supplemented medium in the RITA vessels or semi-solid medium (5 ± 8 and 3 ± 5 shoots, respectively). Continuous explant multiplication at the rooting phase resulted in very small shoot tips that did not readily root when subcultured in the same rooting medium (Figure 5A–J). All *C. argentea* explants that were elongated in the previous stage were > 0.5 cm at the end of the rooting phase. Exposure to TDZ significantly reduced rooting capacity ($p < 0.05$), relative to TDZ-free medium, except for *C. argentea* shoots from temporary immersion in RITA vessels and from the semi-solid medium. The extent of this reduction was specific to the culture system (Table 1). The explants were acclimatised in a growth room for four weeks (Figure 5K) and then placed in the greenhouse for a further four weeks until harvest. Clones that were not harvested were maintained until the flowering stage upon which they were placed outside the greenhouse for pollination (Figure 5L); these clones produced viable seeds after 2–3 months.

The TDZ-supplementation of media resulted in significantly ($p < 0.05$) improved yields (plant per explant) per system, relative to TDZ-free media (Figure 1). Overall, temporary and continuous immersion BTBBs containing TDZ-enriched media resulted in the highest, 63 ± 35 and 30 ± 22, plants per explant, respectively, followed by continuous immersion in RITA vessels containing TDZ-enriched medium (12 ± 5 plants per explant) and semi-solid TDZ medium (9 ± 7 plants per explant) (Figure 1). Semi-solid culture and RITA vessels containing TDZ-supplemented media resulted in similar yields of *C. argentea* plantlets (10–12 plants per explant). Large variations in yield are indicative of genotypic responses within the different populations in the different culture systems.

Following in vitro propagation, acclimatisation and ex vitro growth, the biomass partitioning of *C. argentea* plants from TDZ-supplemented medium showed significantly higher leaf, stem, root and total biomasses relative to TDZ-free medium (Figure 2). Plants that were derived from BTBB temporary immersion with TDZ-enriched medium showed significantly higher ($p < 0.05$) total (184.5 ± 26.8 g), leaf (43.6 ± 6.0 g), stem (66.5 ± 18.5 g) and root (74.4 ± 16.8 g) biomasses when compared with continuous immersion in the same culture system and medium, (61.4 ± 15 g, 19.2 ± 3.3 g, 27.9 ± 8.8 g and 14.3 ± 4.3 g biomasses, respectively) (Figure 2). Biomasses significantly declined in the RITA and semi-solid culture systems, with or without TDZ (Figure 2). The lowest total and stem biomasses were observed in semi-solid culture without TDZ (1.2 ± 0.2 g and 0.5 ± 0.2 g, respectively), and the lowest leaf biomasses were statistically similar between explants from TDZ-free media in RITA continuous and temporary immersion, and semi-solid culture (0.7 ± 0.3 g, 0.5 ± 0.2 g and 0.4 ± 0.1 g, respectively). Root biomasses were significantly the lowest in explants from continuous immersion in RITA vessels and semi-solid culture (0.5 ± 0.2 g and 0.4 ± 0.1 g, respectively), devoid of TDZ (Figure 2).

TABLE 1 | Shoot multiplication, elongation and rooting of *Celosia argentea* using shoot tips in different culture systems containing 2 mg L^{-1} TDZ, 2 mg L^{-1} GA_3 and 1 mg L^{-1} IAA, respectively. Data were recorded after 5 weeks (the number of shoots per explant and % elongation) and 3 weeks (% elongation and % root formation). Data were analysed using a Kruskal-Wallis H test and a post-hoc Tukey test (mean ± SD, $p < 0.05$, n = 20–80). Different lowercase letters denote statistical differences amongst culture systems and immersion times within the culture stage. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, TDZ = thidiazuron, Con = control.

Stage	Culture system	Immersion	PGR	# Shoots /explant	% Elongation > 0.5 cm	% Rooting
Multiplication and Elongation	**BTBB**	**Continuous**	TDZ	27 ± 11^{ab}	80 ± 12^{c}	
			Con	4 ± 2^{d}	100 ± 0^{a}	
		Temporary	TDZ	56 ± 18^{a}	100 ± 0^{a}	
			Con	3 ± 2^{d}	100 ± 0^{a}	
	RITA	**Continuous**	TDZ	12 ± 5^{b}	92 ± 10^{b}	
			Con	1 ± 1^{e}	100 ± 0^{a}	
		Temporary	TDZ	7 ± 1^{c}	100 ± 0^{a}	
			Con	1 ± 1^{e}	100 ± 0^{a}	
	SS	**Continuous**	TDZ	9 ± 7^{c}	98 ± 8^{a}	
			Con	0 ± 0^{e}	100 ± 0^{a}*	
Rooting	**BTBB**	**Continuous**	TDZ	20 ± 19^{a}	100 ± 0^{a}	72 ± 25^{bc}
			Con	0 ± 0^{cd}	100 ± 0^{a}*	100 ± 0^{a}
		Temporary	TDZ	25 ± 24^{a}	100 ± 0^{a}	73 ± 28^{bc}
			Con	1 ± 1^{bcd}	100 ± 0^{a}	100 ± 0^{a}
	RITA	**Continuous**	TDZ	5 ± 8^{b}	100 ± 0^{a}	73 ± 12^{c}
			Con	0 ± 0^{d}	100 ± 0^{a}*	100 ± 0^{a}
		Temporary	TDZ	1 ± 1^{cd}	100 ± 0^{a}	97 ± 9^{a}
			Con	0 ± 0^{cd}	100 ± 0^{a}*	100 ± 0^{a}
	SS	**Continuous**	TDZ	3 ± 5^{bc}	100 ± 0^{a}	81 ± 20^{b}
			Con	0 ± 0^{d}	100 ± 0^{a}*	100 ± 0^{a}

*Based on initial explant inoculated onto TDZ-free media.

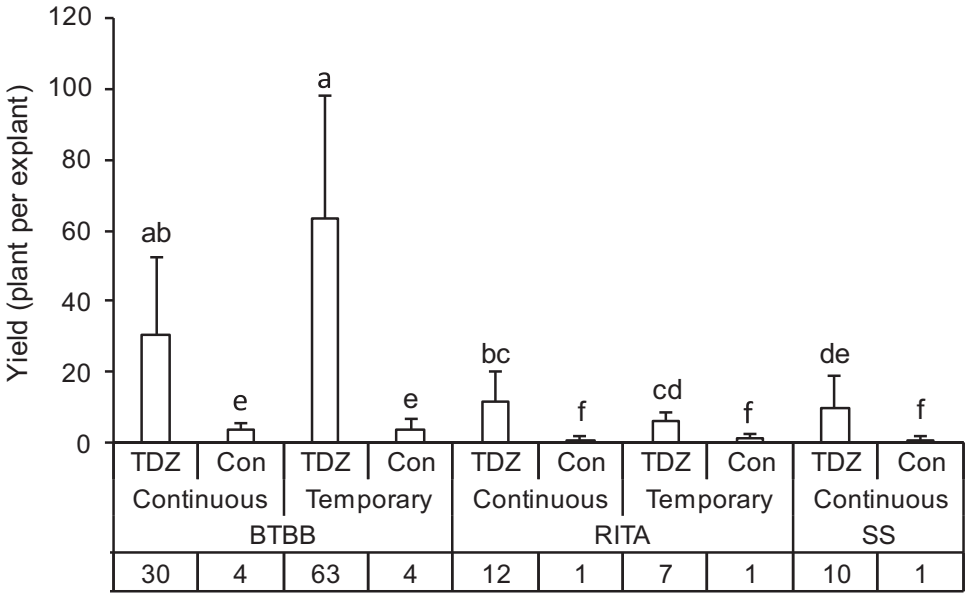
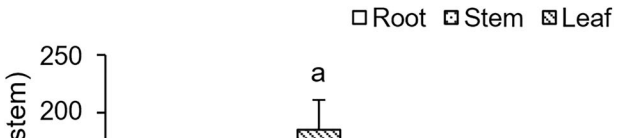


FIGURE 1 | Yield (plants per explant) of micropropagated *Celosia argentea* plants. Data were analysed using a Kruskal-Wallis H test and a post-hoc Tukey test (mean ± SD, $p < 0.05$, n = 20–80). Columns labelled with different letters are significantly different. Values beneath the graph are the mean yield values. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, TDZ = thidiazuron, Con = control.



	TDZ	Con	TDZ	Con	TDZ	Con	TDZ	Con	TDZ	Con
	Continuous		Temporary		Continuous		Temporary		Continuous	
	BTBB				RITA				SS	
Leaf	19.2	2.8	43.6	2.9	7.8	0.7	4.0	0.5	4.6	0.4
Stem	27.9	3.8	66.5	2.3	9.6	1.0	5.9	0.9	4.3	0.5
Root	14.3	1.9	74.4	5.6	4.9	0.5	6.7	1.1	3.4	0.4

FIGURE 2 | Total dry biomass and biomass partitioning (g per yield in each culture system) of 8-week-old ex vitro *Celosia argentea* plants. Data were analysed using a Kruskal-Wallis H test and a post-hoc Tukey test (mean ± SD, $p < 0.05$, $n = 20$). Columns labelled with different letters are significantly different. Values beneath the graph are the mean biomass values. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, TDZ = thidiazuron, Con = control.

3.2 | Leaf Nutrient Content Per System

Leaf nutrient contents of acclimatised *C. argentea* plants were significantly higher with the addition of TDZ to the multiplication medium in all culture systems (Figure 3). Leaf Mg, Ca and Zn contents were significantly higher ($p < 0.05$) in *C. argentea* plants from the BTBB that temporarily immersed shoot tips in TDZ-containing medium, relative to other culture systems (Figure 3A,B and D). In this regard, an in vitro culture period of 8 weeks and ex vitro acclimatisation and growth period of a further 8 weeks would yield 7659 ± 859, 9671 ± 1564 and 93 ± 17 mg DW per system for Mg, Ca and Zn, respectively. Leaf Fe content was significantly elevated in the BTBB system in temporary and continuous immersion culture, resulting in 49 ± 10 and 36 ± 11 mg DW per system, respectively (Figure 3C).

3.3 | Comparative Nutrient Analysis in Different Genotypes

Leaf Mg, Ca, Fe and Zn contents were affected by the presence of TDZ in the multiplication medium which also affected the genetic parameters and resulted in nutrient-specific genotypic and phenotypic variation (Tables 2–5). When compared in a standardised manner (mg per 100 g^{-1} DW), leaf Mg content ranged from 874–1013 mg per 100 g^{-1} DW across all culture systems. Leaf Mg content was only significantly reduced in *C. argentea* plants from TDZ-free medium in the temporary immersion BTBB and in semi-solid medium (570 ± 140 and 625 ± 123 mg per 100 g^{-1} DW, respectively). For the remaining culture systems, leaf Mg contents were comparable (Table 2). For *C. argentea* plants from the BTBB and semi-solid culture system containing TDZ, the GCV % and PCV % for leaf Mg contents were lower than those obtained from TDZ-free media. The opposite was true for the RITA system. For all culture systems, the estimates for PCV % were higher than GCV %, however, both were considered to be low (< 10). The results of the broad-sense heritability revealed that the leaf Mg contents were lower in all culture systems containing TDZ (59–89%), relative to TDZ-free medium (76–88%). The plants from continuous immersion in TDZ-enriched medium in the RITA system exhibited the highest GA at 5% of 141 mg 100 g^{-1} DW, while the plants from temporary immersion in TDZ-enriched medium exhibited the lowest GA at 5% of 49 mg 100 g^{-1} DW. The GAMs for plants from the BTBB and semi-solid systems were 2x higher in TDZ-free medium, relative to the control. For the RITA system, TDZ-supplemented medium in both continuous and temporary immersion, resulted in plants accumulating marginally higher leaf Mg (14 and 11%, respectively), relative to TDZ-free plants (11 and 7%, respectively).

When compared in a standardised manner, leaf Ca content ranged from 643–1850 mg per 100 g^{-1} DW across all culture systems. Leaf Ca content was significantly reduced in *C. argentea* plants from the semi-solid medium containing TDZ only (897 ± 189 mg per 100 g^{-1} DW), relative to the control (1611 ± 349 mg per 100 g^{-1} DW). For the RITA system and temporary immersion in the BTBB, TDZ exposure resulted in significantly higher Ca accumulation in these plants (Table 3). For *C. argentea* plants exposed to TDZ in the BTBB, semi-solid culture and RITA temporary immersion, the GCV % and PCV % for leaf Ca contents were lower than those obtained from TDZ-free media. The GCV % and PCV % were considered low (< 10) for these systems and moderate (> 10.1–20) for plants in RITA systems under continuous immersion in TDZ-enriched medium and temporary immersion in TDZ-free medium. The results of the broad-sense heritability revealed that the leaf Ca contents were high in all culture systems (> 60%). The plants temporarily immersed in TDZ-enriched medium in the RITA system exhibited the highest GA at 5% of 284 mg 100 g^{-1} DW, while their counterparts in the BTBB exhibited the lowest GA at 5% of 51 mg 100 g^{-1} DW. The GAMs for plants continuously immersed in TDZ-medium in the RITA system were 2x higher (29%) than in the same culture

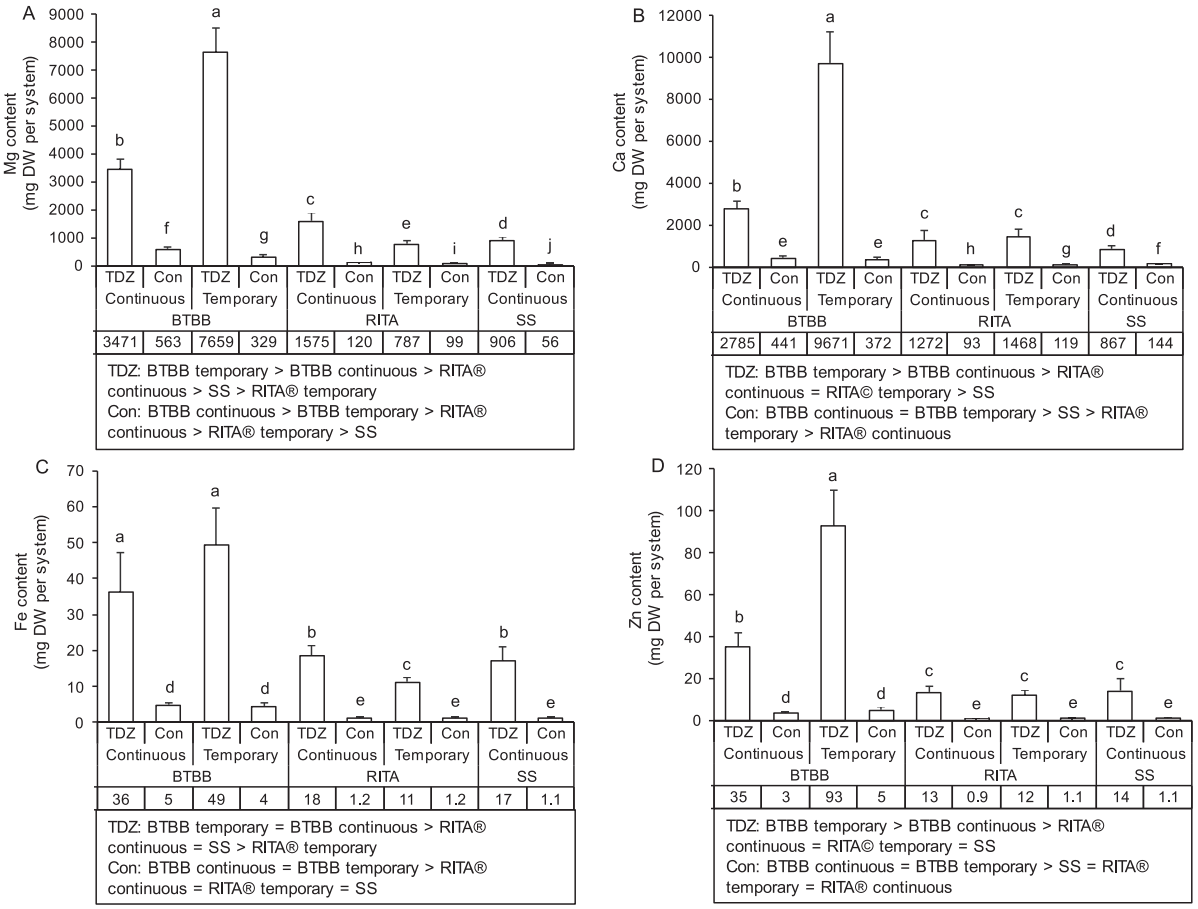


FIGURE 3 | Leaf magnesium (A), calcium (B), iron (C) and zinc (D) content (mg DW in each culture system) in ex vitro *Celosia argentea* plants. Data were analysed using a Kruskal-Wallis H test and a post-hoc Tukey test (mean ± SD, $p < 0.05$, $n = 20$). Columns labelled with different letters are significantly different. Values beneath each graph are the mean nutrient values and the text summarises the statistical trends. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, TDZ = thidiazuron, Con = control.

TABLE 2 | Average (± SD) leaf magnesium content (mg 100 g^{-1} DW), genotypic variance (GV), phenotypic variance (PV), genotypic coefficient of variance (GCV %), phenotypic coefficient of variance (PCV %), heritability (H^2 %), genetic advance at 5% selection intensity (GA, mg 100 g^{-1} DW) and genetic advance as percentage of mean (GAM %) of ex vitro *Celosia argentea* plants from different culture systems (n = 20). Different lowercase letters denote statistical differences amongst culture systems and immersion times. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, TDZ = thidiazuron, Con = control.

Culture system	Immersion	PGR	Mg content (mg 100 g^{-1} DW)	GV	PV	GCV %	PCV %	H^2 %	GA	GAM %
BTBB	Continuous	TDZ	903 ± 91^{ab}	931	1232	3.4	3.9	76	55	6
		Con	1013 ± 217^{ab}	6240	7090	7.8	8.3	88	153	15
	Temporary	TDZ	879 ± 99^{ab}	908	1458	3.4	4.3	62	49	6
		Con	570 ± 140^{c}	2297	2952	8.4	9.5	78	87	15
RITA	Continuous	TDZ	1010 ± 198^{ab}	5238	5863	7.2	7.6	89	141	14
		Con	874 ± 132^{ab}	2000	2621	5.1	5.9	76	81	9
	Temporary	TDZ	992 ± 156^{a}	3061	3650	5.6	6.1	84	104	11
		Con	974 ± 112^{ab}	1422	1888	3.9	4.5	75	67	7
SS	Continuous	TDZ	937 ± 132^{ab}	1542	2604	4.2	5.4	59	62	7
		Con	625 ± 123^{c}	1971	2283	7.1	7.6	86	85	14

TABLE 3 | Average (± SD) leaf calcium content (mg 100 g^{-1} DW), genotypic variance (GV), phenotypic variance (PV), genotypic coefficient of variance (GCV %), phenotypic coefficient of variance (PCV %), heritability (H^2 %), genetic advance at 5% selection intensity (GA, mg 100 g^{-1} DW) and genetic advance as percentage of mean (GAM %) of ex vitro *Celosia argentea* plants from different culture systems (n = 20). Different lowercase letters denote statistical differences amongst culture systems and immersion times. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, TDZ = thidiazuron, Con = control.

Culture system	Immersion	PGR	Ca content (mg 100 g^{-1} DW)	GV	PV	GCV %	PCV %	H^2 %	GA	GAM %
BTBB	Continuous	TDZ	725 ± 91^{de}	868	1229	4.1	4.8	71	51	7
		Con	794 ± 200^{cd}	5502	6011	9.3	9.8	92	146	18
	Temporary	TDZ	1110 ± 179^{b}	3718	4833	5.5	6.3	77	110	10
		Con	643 ± 138^{e}	1891	2730	6.8	8.1	69	75	12
RITA	Continuous	TDZ	815 ± 310^{cde}	13963	14412	14.5	14.7	97	240	29
		Con	679 ± 120^{de}	1854	2151	6.3	6.8	86	82	12
	Temporary	TDZ	1850 ± 441^{a}	23544	29110	8.3	9.2	81	284	15
		Con	1174 ± 355^{b}	16390	18908	10.9	11.7	87	246	21
SS	Continuous	TDZ	897 ± 189^{c}	4269	5348	7.3	8.2	80	120	13
		Con	1611 ± 349^{a}	15142	18310	7.6	8.4	83	231	14

TABLE 4 | Average (± SD) leaf iron content (mg 100 g^{-1} DW), genotypic variance (GV), phenotypic variance (PV), genotypic coefficient of variance (GCV %), phenotypic coefficient of variance (PCV %), heritability (H^2 %), genetic advance at 5% selection intensity (GA, mg 100 g^{-1} DW) and genetic advance as percentage of mean (GAM %) of ex vitro *Celosia argentea* plants from different culture systems (n = 20). Different lowercase letters denote statistical differences amongst culture systems and immersion times. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, TDZ = thidiazuron, Con = control.

Culture system	Immersion	PGR	Fe content (mg 100 g^{-1} DW)	GV	PV	GCV %	PCV %	H^2 %	GA	GAM %
BTBB	Continuous	TDZ	9.2 ± 2.8^{c}	0.99	1.21	10.6	11.7	82	1.9	20
		Con	8.4 ± 1.7^{c}	0.20	0.41	5.4	7.7	49	0.7	8
	Temporary	TDZ	5.7 ± 1.2^{d}	0.11	0.21	5.7	8.1	51	0.5	8
		Con	7.3 ± 2.3^{cd}	0.47	0.79	9.3	12.2	59	1.2	15
RITA	Continuous	TDZ	11.8 ± 2.0^{b}	0.39	0.61	5.3	6.6	64	1.0	9
		Con	8.8 ± 1.8^{c}	0.33	0.48	6.5	7.8	68	1.0	11
	Temporary	TDZ	14.0 ± 2.0^{ab}	0.37	0.62	4.4	5.7	59	1.0	7
		Con	11.7 ± 2.4^{b}	0.69	0.87	7.1	7.9	79	1.5	13
SS	Continuous	TDZ	17.7 ± 4.2^{a}	1.95	2.66	7.9	9.2	73	2.5	14
		Con	12.8 ± 4.5^{b}	2.70	3.05	12.8	13.6	89	3.2	25

system without TDZ (12%). All other culture systems showed lower GAM % in TDZ-enriched media, than their respective controls.

Leaf Fe content ranged from 5.7–17.7 mg per 100 g^{-1} DW across all culture systems. Leaf Fe content was significantly improved in *C. argentea* plants growing in TDZ-enriched semi-solid medium (mean difference 4.9 mg per 100 g^{-1} DW) and in RITA continuous immersion (mean difference 3 mg per 100 g^{-1} DW). For the remaining systems, TDZ exposure resulted in comparable leaf Fe accumulation in these plants (Table 3). For *C. argentea* plants under temporary immersion in both bioreactor types and in semi-solid cultures containing TDZ-enriched media, the GCV % and PCV % for leaf Fe contents were reduced, whereas continuous immersion in BTBB increased the GCV % and PCV % relative to the respective controls. The GCV % and PCV % were considered low (< 10) for all systems except for moderate responses (> 10.1-20) in continuous immersion in TDZ-supplemented media in the BTBB and the semi-solid media without TDZ. For all culture systems, the estimates for PCV % were higher than the GCV %. The results of the broad-sense heritability varied between systems with or without TDZ. The leaf Fe contents were high (> 60%) in TDZ-exposed plants under continuous immersion in BTBB and RITA systems, and in plants under continuous and temporary immersion without TDZ in the RITA system, and in the semi-solid culture system. In plants from continuous immersion in

TABLE 5 | Average (± SD) of zinc content (mg 100 g^{-1} DW), genotypic variance (GV), phenotypic variance (PV), genotypic coefficient of variance (GCV %), phenotypic coefficient of variance (PCV %), heritability (H^2 %), genetic advance at 5% selection intensity (GA, mg 100 g^{-1} DW) and genetic advance as percentage of mean (GAM %) of ex vitro *Celosia argentea* plants from different culture systems (n = 20). Different lowercase letters denote statistical differences amongst culture systems and immersion times. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, TDZ = thidiazuron, Con = control.

Culture system	Immersion	PGR	Zn content (mg 100 g^{-1} DW)	GV	PV	GCV %	PCV %	H^2 %	GA	GAM %
BTBB	Continuous	TDZ	9.1 ± 1.9^{cd}	0.48	0.53	7.6	8.0	90	1.4	15
		Con	6.2 ± 1.8^{e}	0.43	0.47	10.5	10.9	93	1.3	21
	Temporary	TDZ	10.6 ± 2.0^{bc}	0.46	0.59	6.3	7.3	77	1.2	11
		Con	8.0 ± 3.2^{de}	1.31	1.53	14.3	15.4	86	2.2	27
RITA	Continuous	TDZ	8.6 ± 2.0^{cd}	0.53	0.58	8.5	8.8	92	1.5	17
		Con	6.9 ± 2.3^{de}	0.77	0.80	12.7	12.9	96	1.8	26
	Temporary	TDZ	15.3 ± 3.1^{a}	1.15	1.44	7.0	7.8	80	2.0	13
		Con	10.5 ± 3.5^{bc}	1.66	1.80	12.3	12.8	93	2.6	24
SS	Continuous	TDZ	14.4 ± 6.0^{ab}	5.14	5.46	15.7	16.2	94	4.5	31
		Con	12.8 ± 4.4^{ab}	2.51	2.85	12.4	13.2	88	3.1	24

TDZ-free medium in the BTBB, H^2 was lowest at 49%. The plants from semi-solid media exhibited the highest GA at 5%, 3.19 and 2.46 mg 100 g^{-1} DW for TDZ-free and TDZ-supplemented media, respectively. The lowest GA at 5% was 0.48 mg 100 g^{-1} DW in plants from temporary immersion in medium containing TDZ in the BTBB followed by continuous immersion in TDZ-free medium in the same system which was 0.65 mg 100 g^{-1} DW. The GAM for plants from semi-solid TDZ-free medium was the highest (25%), followed by continuous immersion in TDZ-supplemented medium in the BTBB (20%). For the remaining culture systems, the GAM was low to medium (6–18%).

Leaf Zn contents were significantly improved in TDZ-exposed *C. argentea* plants in the BTBB systems and RITA temporary immersion system. For the remaining systems, TDZ exposure resulted in comparable leaf Zn accumulation in these plants (Table 5). For leaf Zn contents, *C. argentea* plants from TDZ-enriched media in all systems but semi-solid culture, reduced the GCV % and PCV %. The GCV % and PCV % were considered low (< 10) for all BTBB and RITA systems containing media with TDZ. Plants from the respective TDZ-free systems and semi-solid culture were considered to be moderate (> 10.1–20) for GCV % and PCV %. For all culture systems, the estimates for PCV % were higher than the GCV %. The broad-sense heritability was high (> 60%) for all systems irrespective of the presence of TDZ. The GA at 5% showed that an improvement of at least 1.0 mg 100 g^{-1} DW in all culture systems can be achieved by selective breeding, and the highest improvement was 4.5 mg 100 g^{-1} DW in high-accumulating genotypes from semi-solid media containing TDZ, followed by the respective TDZ-free medium in the same culture system (3.1 mg 100 g^{-1} DW).

Ex vitro plants that were derived from each culture system were assessed for their ability to meet or exceed the RDA (Table 6) of leaf nutrients (mg 100 g^{-1} DW). Leaf Mg contents were markedly high in all culture systems, resulting in plants accumulating 136–241% of the RDA for males and 178–317% for females. For leaf Ca, plants from four culture systems met and exceeded the RDA, but this was not specific to the culture system nor the presence of TDZ. The remaining six culture systems produced plants that did not accumulate enough leaf Ca to meet the RDA, but contributed 63–91% depending on the system (Table 6). Leaf Fe content met and surpassed the RDA in most culture systems for males, however, for females, none of the culture systems produced plants capable of meeting the RDA. Continuous immersion in the BTBB and RITA systems without TDZ, produced plants that did not meet the Zn RDA for women, while the RDA for men was not met in plants derived from temporary immersion in the RITA system and both semi-solid treatments.

In order to visualise the variability exhibited by acclimatised *C. argentea* plants, a clustergram was constructed using all the variables that were measured in this study. Overall, the micro-propagation data showed higher values for plants grown with TDZ and under temporary immersion in the BTBB (Figure 6). This was also similar to the harvest parameters, although explant elongation was relatively consistent across systems. Nutrient content varied, along with the ability to meet the RDA, per system, although TDZ-exposed plants that were temporarily immersed, and in semi-solid medium, showed higher values than TDZ-free culture systems (Figure 6).

4 | Discussion

Axillary shoot proliferation is the most common method of clonal propagation that maintains genetic fidelity through natural ontogenetic development of existing meristems [48]. In this study, bud proliferation of *C. argentea* from shoot tip explants was assessed following two weeks in liquid medium, in either temporary or continuous immersion bioreactors, and three weeks each in semi-solid media for elongation and rooting (Table 1). Temporary and continuous immersion in MS media containing 2 mg L^{-1} TDZ in the BTBBs promoted rapid and prolific axillary

TABLE 6 | Assessment of nutrient content (mg 100 g^{-1} DW) in plants following culture in different bioreactors and semi-solid medium. Values represent the nutrient % relative to the RDA. Symbols indicate if % RDA per nutrient met (✓) or did not meet the RDA (X). RDA = Recommended Dietary Allowance, BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, SS = semi-solid, PGR = plant growth regulator, TDZ = thidiazuron.

			Mg (%)				Ca (%)		Fe (%)				Zn (%)			
RDA (mg per day)			420		320		1000		8		18		11		8	
Culture system	Immersion	PGR	Male		Female		Male and female		Male		Female		Male		Female	
BTBB	Continuous	TDZ	✓	215	✓	283	✗	72	✓	115	✗	52	✗	83	✓	109
		Con	✓	241	✓	317	✗	79	✓	104	✗	46	✗	57	✗	78
	Temporary	TDZ	✓	209	✓	275	✓	111	✗	71	✗	32	✗	97	✓	133
		Con	✓	136	✓	178	✗	63	✗	91	✗	41	✗	73	✓	100
RITA	Continuous	TDZ	✓	240	✓	316	✗	82	✓	147	✗	66	✗	78	✓	108
		Con	✓	208	✓	273	✗	68	✓	110	✗	49	✗	63	✗	87
	Temporary	TDZ	✓	236	✓	310	✓	185	✓	174	✗	78	✓	139	✓	192
		Con	✓	232	✓	304	✓	117	✓	147	✗	65	✗	95	✓	131
SS	Continuous	TDZ	✓	223	✓	298	✗	91	✓	238	✗	98	✓	131	✓	188
		Con	✓	149	✓	195	✓	161	✓	160	✗	71	✓	117	✓	160

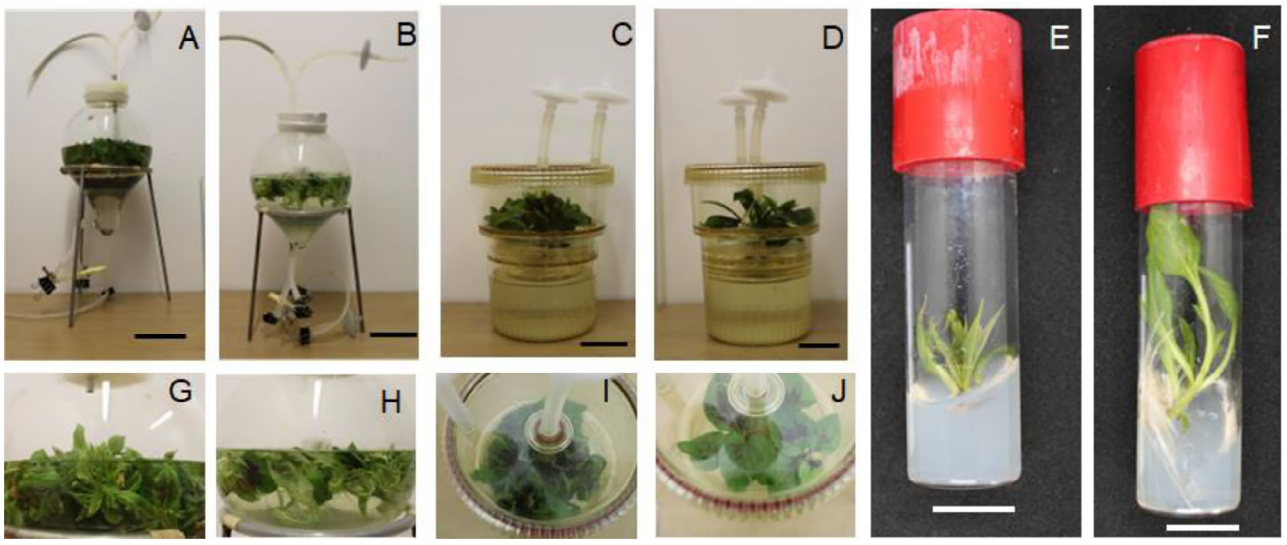


FIGURE 4 | Shoot tips of *Celosia argentea* following continuous immersion multiplication in balloon-type bubble bioreactors in TDZ-supplemented media (A and G), in TDZ-free media (B and H), in continuous immersion in RITA vessels in TDZ-supplemented media (C and I), in TDZ-free media (D and J), semi-solid medium containing TDZ (E) and TDZ-free medium (F). Scalebars: A–B: 15 cm, C–D: 5 cm and E–F: 2 cm.

shoot development (56 ± 18 and 27 ± 11 shoots, respectively) and buds continuously proliferated during the elongation and rooting phases (Table 1, Figures 4 and 5). Thidiazuron is known to induce axillary shoot development [49], as was observed in the present study, while explants in TDZ-free media each produced only 1–3 shoots (Table 1, Figure 5C,D,G,H,J). Thidiazuron can also illicit diverse physiological, morphological and altered endogenous hormonal control responses in plant tissues [50]. Most explants exposed to TDZ exhibited slightly reduced leaf area in culture (Figure 5), when compared to the broad leaves in the respective controls. However, once these plants were acclimatised and placed in a greenhouse, there were no

FIGURE 5 | Range of *Celosia argentea* explant sizes at the rooting phase from continuous immersion in TDZ-supplemented and TDZ-free media in the BTBB (A and C, respectively), and RITA system (E and G, respectively), temporary immersion in TDZ-supplemented and TDZ-free media in the BTBB (B and D, respectively), and RITA system (F and H, respectively), and semi-solid medium containing TDZ (I) and TDZ-free medium (J). Acclimatised plantlets in a growth room (K). Flowering ex vitro plants (L). Scalebars: A–J: 2 cm.

observed differences in their leaf morphology and overall biomass (Figures 5K,L and 6). Continuous shoot proliferation occurred in subsequent in vitro culture stages indicating that there were hormonal interactions between endogenous and exogenous PGRs (Table 1, Figure 5A,B,E,F,I). As explained by Erland et al. [51], exposure to media containing TDZ results in the accumulation of TDZ-like oligomers in the explant tissue and later released as the active TDZ monomer over time. This explains the long-lasting effect on rates of multiplication during the different in vitro stages observed in the present study. Furthermore, TDZ is known to activate all cytokinin receptors and their signalling pathways, and therefore, prevents cytokinin degradation by inhibiting cytokinin oxidase-dehydrogenase. The resulting increase in endogenous cytokinin levels would alter the auxin:cytokinin ratio [52]. This is also why rooting capacity was significantly lower in TDZ-exposed explants relative to explants in TDZ-free medium which exhibited 100% rooting (Table 1). Despite the continuous multiplication, even at the rooting phase, some explants did not produce shoots while others continued to multiply (Table 1). This was attributed to a genotypic effect. In a study by Warhade and Badere [25] on *C. cristata* shoot tips, a combination of NAA and BAP produced 3.6 ± 0.6 shoots per explant in 4 weeks and recurrent regeneration of the original/mother explant in semi-solid medium using NAA and BAP yielded 1160 shoots in 40 weeks. In this study, 1185 shoots were produced in a shorter period (8 weeks) following temporary immersion in liquid MS containing TDZ in the BTBB, indicating that a rapid and efficient protocol for shoot proliferation was developed for production enhancement of *C. argentea*.

Given the ratio of medium to explant, the semi-solid medium (1:10 mL) had limited airspace and medium when compared with the RITA system (1:80 mL) and the BTBB (1:160 mL) (Figure 4). The ratio of the number of explant:volume of medium and frequency of immersions are determined by species-specific responses to changes in the culture environment. In this study, a larger airspace and the number of explant:volume of medium ratio favoured proliferation in *C. argentea* explants (Figure 4). Since there is more medium and therefore, more nutrients available for absorption and accumulation, this explains the larger leaves and explants observed after two weeks in TDZ-containing media (Figure 4). In this study, the temporary immersion bioreactors were programmed to one immersion every 2.5 h, which is more frequent than reported in other studies. Uma et al. [28] found that the optimum explant:medium ratio for *Musa* sp. was 1:42 mL in temporary immersion bioreactors using one immersion every 6 h, which produced 1496 shoots in 12 weeks. For *Alnus glutinosa* shoots in RITA vessels, decreasing the explant:medium ratio from 1:18 mL to 1:9 mL resulted in a slight reduction (6.6 to 6.1 shoots per explant) in shoot proliferation but did not affect % elongation when immersed once or thrice every 24 h [53]. Shaik et al. [32] showed that temporary immersion of *Lessertia frutescens* nodal explants (1:38 mL) produced significantly higher numbers of shoots (12.8 shoots) in BTBBs than in continuous immersion (9.0 shoots) and semi-solid culture (7.8 shoots). Those authors also reported that shoots were 50 and 80% hyperhydric, respectively, in temporary and continuous immersion after 6 weeks. Debnath [54] showed that symptoms of hyperhydric shoots of *Rhodiola rosea* from RITA vessels could be improved when transferred to semi-solid medium. In the present study, hyperhydricity was observed in a few explants after 2 weeks in TDZ-free medium in the continuous immersion BTBB, and then ameliorated by subculturing onto a semi-solid medium. For example, after 2 weeks of continuous immersion in TDZ-free medium in the BTBB, the explants were pale green in colour and were brittle (Figure 4B), but after six weeks of elongation and rooting on semi-solid medium, these symptoms were ameliorated (Figure 5C).

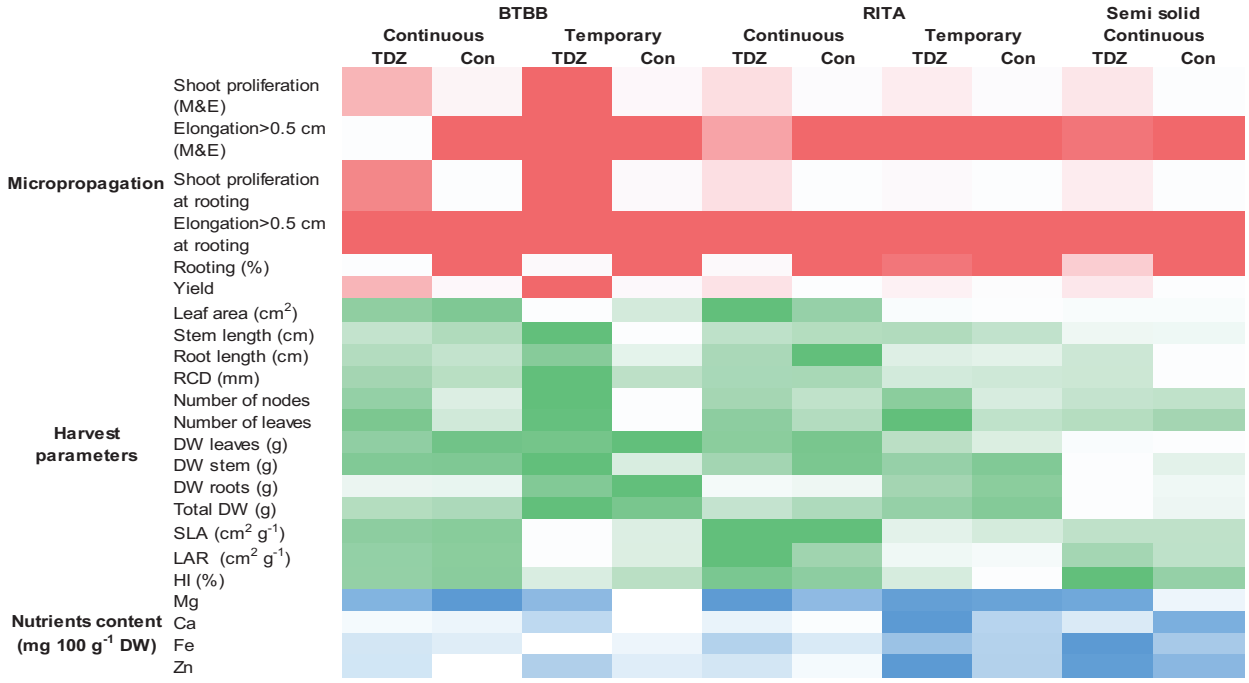


FIGURE 6 | Summary clustergram of micropropagation evaluations, and various ex vitro data viz. harvest measures, nutrient content (mg 100 g^{-1} DW) of *Celosia argentea* plants. Darker shaded cells represent high values. BTBB = balloon-type bubble bioreactor, RITA = recipient for automated temporary immersion, RCD = root collar diameter, DW = dry weight, SLA = specific leaf area, LAR = leaf area ratio, HI = harvest index, M&E = multiplication and elongation.

Other methods to reduce hyperhydricity include the addition of osmotic agents, such as sorbitol or mannitol, in the medium, increasing the gel strength of semi-solid medium in subsequent subcultures or increasing oxygenation via improved aeration to the cultures, which was successful in salvaging *Citrullus lanatus* shoots [55].

Overall, culture in the BTBBs resulted in the highest yield (63 plants per explant), while the yields were lower and comparable between the RITA and the semi-solid culture system containing TDZ-supplemented medium (Figure 1). In the latter systems, the number of shoots produced were also similar (Table 1). The increased plant yields in the bioreactor systems containing TDZ-enriched media led to significantly higher total biomasses than those of semi-solid culture (Figures 1 and 2). Total biomass was also significantly lower in plants from TDZ-free medium relative to the culture system containing TDZ (Figure 2). Since more plants, 63 and 30 plants per explant were produced in temporary and continuous immersion BTBBs, respectively (Figure 1), the resulting leaf, stem, root and total biomasses were proportionately higher in these culture systems (Figure 2). Consequently, since these plants produced more leaves, the nutrient accumulation was significantly higher for leaf Mg, Ca, Fe and Zn, relative to other culture systems (Figure 3). Based on the combination of a large airspace, adequate nutrients in the medium and an effective PGR at the optimum concentration, rapid and efficient shoot production were achieved. Since PGRs control physiological processes related to growth, improving the growth rate, biomass accumulation and root development, the plant's ability to absorb and accumulate soil minerals would improve [56]. *Celosia argentea* is also a known hyperaccumulator of metals [57], and under in vitro conditions, the medium is slightly acidic (pH 5.6–5.8) which promotes further bioavailability and uptake of a range of nutrients contained in the nutrient formulation. The BTBB had a higher number of explant:volume of medium ratio, the enhanced uptake and partitioning of nutrients during the in vitro culture phase, in combination with higher yields, explain why explants from this system exhibited higher total, leaf, stem and root biomass, relative to the RITA and semi-solid culture systems. Leaf biomass almost doubled in plants from BTBB in TDZ-containing medium relative to continuous immersion in the same medium, but when compared with semi-solid medium, leaf biomass was 10x lower (Figure 2). Low concentrations (0.88 mg L^{-1}) of TDZ have recently been shown to reduce biomass in a cadmium hyperaccumulator, *Sedum alfredii* [58]. However, in the present study, higher concentrations of TDZ (2 mg L^{-1}) did not exhibit negative effects on plant biomass (Figure 2). Furthermore, when clones flowered and set seed, these processes did not appear to be hindered by earlier exposure to TDZ, and the seeds exhibited similar rates of germination as seeds collected from the field (70%) (Figure 5L).

Plants absorb and partition nutrients for growth and reproduction [59]. When grown in soil, selective uptake and accumulation of various nutrients are dependent on the cation exchange capacity of the soil and availability of ion-binding sites for transportation [60]. Therefore, under in vitro conditions, it is thought that the uptake of nutrients is less constrained in liquid medium since the explants absorb nutrients directly from all exposed surfaces, while those in semi-solid culture are in contact with semi-solid medium at only the excised surfaces [61, 62]. Furthermore, since agar contains inorganic impurities that vary based on the location of harvest, some nutrients may bind to these and reduce the rate of diffusion into the tissue in semi-solid medium [63, 64]. As

antagonistic or synergistic nutrient interactions occur in the soil, it is possible that they also occur in an in vitro environment as nutrients are being absorbed by the explants. The exhaustion of specific nutrients or sucrose in the media also impacts nutrient availability and accumulation of other nutrients [65, 66]. When leaf Mg, Ca, Fe and Zn contents were expressed per system, these nutrients accumulated in significantly higher quantities in plants from BTBB temporary immersion in TDZ-supplemented medium relative to other culture systems (Figure 3). Leaf Fe content was significantly higher in plants from temporary and continuous immersion in TDZ-supplemented media, relative to other culture systems. Overall, the BTBB system, in combination with the TDZ-supplemented medium, produced higher yields and plant biomass (Figures 1 and 2, respectively), which resulted in greater nutrient accumulation. However, when leaf nutrient content was compared on a mg per 100 g^{-1} DW basis, nutrient accumulation was specific to the culture system and the genotypes in those culture populations (Tables 2–5), with no observable influence of TDZ. The combined effect of TDZ and culture system did not appear to have a consistent effect on the harvest parameters, micropropagation stages or leaf nutrient evaluations either (Table 6, Figure 6). This variability is attributed to the unique genotypes in each culture population. In terms of meeting the RDA, leaf Mg contents could exceed the RDA for males and females (Table 6) in all culture systems, indicating that *C. argentea* is a good source of Mg. Leaf Fe (only for males) and Zn (only for females) contents were able to meet and surpass the respective RDAs in most culture systems, but would benefit from screening and selection for improved, consistent nutrient-accumulating ability. Four of the culture systems generated plants that would accumulate a sufficient amount of leaf Ca to meet the RDA, but for the remaining systems, these would provide 63–91% of the RDA (Table 6). In this regard, a larger screening pool, followed by selective breeding and then mass propagation of high Ca accumulating F1 or F2 generations may improve leaf Ca content following bioreactor culture.

Genotypic heterogeneity within populations can modulate the overall response observed in nutrient accumulative ability. Therefore, an assessment of the genotypes can be useful in identifying specific nutrient-accumulative traits of interest that can be selectively bred followed by mass production [67]. The present study showed that the GV and PV for nutrients were specific to the system, and the large variabilities are indicative of genetic diversity, since each system contained unique seed-derived genotypes in the form of shoot tip explants (Tables 2–5). However, for leaf Mg, Fe and Zn, GV and PV were lower in BTBB continuous immersion in TDZ-containing medium, relative to the corresponding TDZ-free bioreactor. This suggests that either the genotypes in these systems were not as diverse as expected or that TDZ can potentially constrain variability associated with in vitro culture and, therefore, could contribute to reducing incidences of somaclonal variation. Novikova et al. [68] assessed the clonal fidelity of the woody ornamental, *Rhododendron mucronulatum*, using DNA-based markers and flow cytometry. The nodal explants were cultured for 8 weeks in 0.02–0.5 mg L^{-1} TDZ followed by a 6-week culture on hormone-free medium. The results showed that the genetic similarity of all regenerants did not differ between themselves and the mother seedlings, and there were no effects on the ploidy. In the present study, using genetic evaluation through molecular markers would be necessary to determine if TDZ had an effect on the clonal fidelity.

The nutrient GCV %s and PCV %s per system ranged from low to moderate and for all nutrients. The PCV %s were higher than GCV %s for all nutrients, which indicated that the culture systems influenced the accumulation of nutrients with or without TDZ in the medium. The heritabilities of leaf Ca and Zn were high for all culture systems and were moderate to high for leaf Fe and Mg. The GA at 5% varied by culture system for each nutrient with or without TDZ. Since selection at 5% would result in only one genotype being selected from each culture system, in order to prevent compromising other heritable traits due to inbreeding, the culture systems that have a high GAM % and H^2 could be used to establish an interbreeding population of high nutrient-accumulating ability since this will result in high genetic gain [16]. In this regard, the selected genotypes should be from RITA systems with continuous immersion using TDZ-containing medium and temporary immersion using TDZ-free medium for leaf Ca content. Selection of genotypes for high Fe content could be sourced from continuous immersion in TDZ-containing medium in the BTBB or from semi-solid medium devoid of TDZ. Leaf Zn content could be improved by selecting genotypes from TDZ-free media in BTBBs and RITA vessels with temporary and continuous immersion, as well as from either TDZ-containing or TDZ-free semi-solid media. The selection for leaf Mg should be re-evaluated in other genotypes as none of these exhibited the required high H^2 and GAM %. However, since the leaf Mg content was 1.5–2x higher than the RDA required for males and females, there may not be a need to select and breed superior genotypes since these values exceed the RDA.

5 | Concluding Remarks

This study demonstrated the efficacy of a 2-week exposure of *C. argentea* shoot tips to TDZ in promoting shoot multiplication and plant yield, particularly for upscaling production in BTBBs. Since this study only evaluated the effect of multiplication in liquid medium, other in vitro stages could be carried out in a similar manner with osmotic agents to prevent potential hyperhydricity. Overall, the BTBB system, in combination with the TDZ-supplemented medium, produced higher yields, which resulted in more plants being produced and hence, higher nutrient accumulation on a culture system basis. Comparison of leaf nutrient content on a mg 100 g^{-1} DW basis, showed that the medium with or without TDZ did not significantly reduce nutrient accumulation, but showed that nutrient accumulation was specific to the culture system or the genotypes that were cultured in those systems. Due to the increased biomass and the resulting increase in leaf Mg, Ca, Fe and Zn, future studies could use selected genotypes for enhanced nutrient accumulation traits, taken from diverse populations of plants. Furthermore, for the genotypes used in these culture systems, leaf Mg content exceeded the RDA requirements, indicating that a smaller quantity of leaves can be consumed which, in turn, maximises agricultural productivity. Leaf Fe and Zn contents were also high in most culture systems, which indicates the suitability of this propagation system on a large scale. Once high-accumulating genotypes are identified, clonal propagation via temporary immersion in a medium containing 2 mg L^{-1}

TDZ in the BTBB could be used to increase the number of clones, resulting in significantly higher leaf nutrient content on a system basis. High heritability and GAM for specific culture systems for Ca, Fe and Zn indicate that selection can be used to develop breeding populations with improved nutrient accumulation. Although this strategy provides a promising approach for developing nutritionally superior *C. argentea* cultivars, selective breeding may inadvertently result in the loss of other beneficial traits or genetic dilution, such as the ability to grow under restricted water or nutrient conditions. Furthermore, to negate any potential health implications of consuming plants containing high levels of nutrients, consumption in lower amounts, which reduces toxicity and maximises agricultural output could be considered, particularly for resource-poor rural communities.

Author Contributions

CR, NS and SS designed the experiments. CR performed laboratory experiments, data analyses and wrote the manuscript. NS and SS supervised the study and edited the manuscript. All the authors read and approved the final version of the manuscript submitted for publication.

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Conflicts of Interest

The authors declare no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This article does not contain any studies involving animals or human participants as objects of research. The authors declare no conflict of interest.

Data Availability Statement

The datasets generated during and/or analysed during the current study are available at https://doi.org/10.25410/ukzn.29424668.v1.

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