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Shoot Induction, Multiplication, Rooting and Acclimatization of Black Turmeric (*Curcuma caesia* Roxb.): An Important and Endangered *Curcuma* Species

Zainol Haida, Uma Rani Sinniah, Jaafar Juju Nakasha and Mansor Hakiman *

Department of Crop Science, Faculty of Agriculture, Universiti Putra Malaysia, Serdang 43400, Selangor, Malaysia
* Correspondence: mhakiman@upm.edu.my; Tel.: +60-3-9786-4903

Abstract: *Curcuma caesia* Roxb., commonly known as Kali Haldi or black turmeric, is one of the important species in the genus *Curcuma*. This species has been classified as one of the endangered *Curcuma* species due to the drastic decrement of this plant in its natural habitat. *C. caesia* has been overharvested for various purposes, including bioactive compound extraction to fulfill the pharmaceutical industry demand. Hence, this study was conducted to establish a protocol for the propagation of *C. caesia* via plant tissue culture techniques. In the shoot induction stage, three basal medium formulations, including Murashige and Skoog (MS medium), the combination of Murashige and Skoog macronutrients and B5 micronutrients (MSB5 medium) and woody plant medium (WPM medium) supplemented with 15 µM of 6-benzylaminopurine (BAP), were used. The results found that the MSB5 medium was the most suitable basal medium formulation for shoot induction of *C. caesia*. In the subsequent experiment, different types of cytokinin, including BAP, kinetin and 2-iP at concentrations of 5, 10, 15 and 20 µM, were fortified in the MSB5 medium for shoot multiplication. The shoot multiplication was further enhanced by supplementing the MSB5 medium with indole-3-butyric acid (IBA) or 1-napthaleneacetic acid (NAA) at the concentrations of 2, 4, 6 and 8 µM. The results showed that a combination of 15 µM of BAP and 6 µM of IBA significantly increased the shoot multiplication with 100% shoot induction, 3.53 shoots/explant, 10.81 cm of shoot length, 9.57 leaves, 0.486 g of leaves fresh weight and 0.039 g of leaves dry weight. After the multiplication, the rooting stage was carried out by altering the basal medium strength into half and full strength and supplementing with 2.5, 5, 7.5 and 10 µM of indole-3-acetic acid (IAA). The full strength of MSB5 medium supplemented with 5 µM of IAA exhibited the highest number of roots and length of roots, with 6.13 roots and 5.37 cm, respectively. After the rooting stage, the plantlets were successfully acclimatized in the potting medium with the combination of cocopeat and peatmoss, and the ratio of 1:1 was found to produce the highest survival rate with 77.78%. In conclusion, the protocol established in this study could be useful for large-scale raw material production, either for conservation or bioactive compound extraction.

Keywords: *Curcuma caesia*; basal medium formulation; cytokinin; auxin; acclimatization

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1. Introduction

Curcuma caesia Roxb. belongs to the family Zingiberaceae and genus *Curcuma*. This species is well known in India as Kali Haldi and is commonly called Indian Black Turmeric in other countries. *C. caesia* has been used since ancient times for the treatment of various ailments and diseases in the Indian community. The *C. caesia* extract is used for the treatment of asthma, cancer, inflammation, epilepsy, fever and allergies [1,2]. In addition, the rhizomes and leaves of *C. caesia* were found to contain essential oils such as camphor, eucalyptol, tropolone, ledol and camphene, which are responsible for the aromatic odor [3–5]. In the numerous pharmacological studies on *C. caesia*, the extracts of *C. caesia* possess anticancer, anti-asthmatic, anti-acne, anti-inflammatory and anti-microbial properties [6–9].

As the pharmaceutical industry keeps growing, the demand for plant raw materials has increased for the extraction of various bioactive compounds. Therefore, there is an urgent need to look for an alternative method for the large-scale production of plant raw materials to fulfill the growing demand from the industry. To date, micropropagation or in vitro propagation is a plant tissue culture technique that has many advantages in producing high amounts of plantlets in a short time, and this technique can also be used for the production of bioactive compounds [10,11]. In addition, the plant tissue culture technique is very useful for the conservation of endangered plant species, including *C. caesia,* which has a drastically reduced population due to overharvesting activity [3]. Previously, several studies related to *C. caesia* micropropagation, including regenerating the plant via direct and indirect organogenesis, have been conducted [12–18]. The studies involved the alteration of basal medium formulation, cytokinin and auxin [12–18].

Hence, the aim of this study was to develop an improved protocol for micropropagation of *C. caesia* as affected by basal medium formulation, plant growth regulators, basal medium strength and potting medium for acclimatization.

2. Materials and Methods

2.1. Plant Materials

Curcuma caesia Roxb. fresh rhizomes were obtained from the nursery located in Muar, Johor, Malaysia (Coordinates: 2.06° N, 102.58° E). The sprouted shoots (approximately 5 cm) were cut and brought to the laboratory for sterilization processes. The sterilization process was conducted by placing the shoots under running tap water for 30 min to eliminate the soils, debris and contaminants. Then, the shoots were transferred to the bottle jar containing 70% commercial bleach (Clorox®, Oakland, CA, USA) and shaken for 20 min. The shoots were rinsed with autoclaved distilled water three times and excised into approximately 1 ± 0.5 cm.

2.2. Shoot Induction of C. caesia as Affected by Basal Medium Formulations

For the shoot induction experiment, shoots were inoculated onto the three formulations of basal medium, namely MS medium [19], MSB5 medium (combination of macronutrients and micronutrients formulated by Murashige and Skoog [15] and vitamins formulated by Gamborg et al. [20]) and Woody Plant Medium (WPM medium) formulated by Lloyd and McCown [21]. All the chemicals used were analytical grade (R&M Chemical, Malaysia; Sigma-Aldrich, USA). All the basal medium formulations were supplemented with 30 g/L of sucrose, 3 g/L of gelrite and 5 µM of BAP. The pH of the basal mediums was adjusted to 5.75 prior to autoclave (Hirayama, Japan). The basal medium was poured into the 250 mL conical flasks, with each flask containing 50 mL of basal medium. All basal mediums were autoclaved at the temperature of 121°C for 20 min at a pressure of 1.06 kg cm^{-2}. For each conical flask, one aseptic microshoot was inoculated onto the basal medium and data were collected after six weeks of inoculation. All cultures were kept in the culture room under 16 h of light and 8 h of dark using LED white light with an irradiation of 45 µmol m^{-2} s^{-1} at a temperature of 25 ± 2 °C.

2.3. Effect of Different Concentrations of Cytokinins and Auxins on Shoot Multiplication of C. caesia

After shoot induction, the multiplication experiment was conducted by supplementing the basal medium with cytokinin, including BAP, kinetin and 2-iP, at the concentrations of 5, 10, 15 and 20 µM. After eight weeks of incubation, the data were recorded. In the subsequent experiment, the shoot multiplication was further enhanced by combining 15 µM of BAP with auxin (IBA and NAA) at the concentrations of 2, 4, 6 and 8 µM. The data were collected after eight weeks of inoculation.

2.4. Effect of Basal Medium Strength and Different Concentrations of Indole-3-Acetic Acid (IAA) on Root Induction of C. caesia Plantlets

For root induction, healthy plantlets at eight weeks old, which were maintained on the MSB5 medium supplemented with 15 µM of BAP and 6 µM of IBA, were used. The rooting medium used was full and half strength of MSB5 medium supplemented with IAA at the concentrations of 2.5, 5, 7.5 and 10 µM. The plantlets were exposed to rooting medium for four weeks, and the data were collected after four weeks of inoculation.

2.5. Acclimatization of C. caesia Plantlets as Affected by Different Potting Mediums

The acclimatization was conducted by transferring the healthy plantlets with at least two leaves and well rooted (more than 1.5 cm long) into the potting medium. The potting medium used was a combination of cocopeat and peatmoss, perlite and peatmoss and vermiculite and peatmoss at the ratio of 1:1 for all potting medium combinations. The data were recorded after two weeks of transplantation.

2.6. Statistical Analysis

All the experiments were conducted in a completely randomized design with three replications and ten explants for each replication (n = 30). The Analysis of Variance (ANOVA) was used for data analysis, and Duncan's Multiple Test (DMRT) was used for means separation. The analysis was performed using Statistical Analysis System (SAS ver. 9.4, Cary, NC, USA).

3. Results and Discussion

3.1. Shoot Induction of C. caesia as Affected by Basal Medium Formulations

In this experiment, the shoot induction was significantly affected by different formulations of the basal medium. The MSB5 medium formulation significantly produced the highest percentage of shoot induction with 100%, followed by MS medium and WPM medium with 93.33 and 83.33%, respectively (Table 1). The MSB5 medium also significantly produced the highest number of shoots, length of shoot and number of leaves, with 2.70 shoots, 7.88 cm and 5.33 leaves (Figure 1). Meanwhile, there was no significant difference between MS medium and WPM medium formulations on number of shoots, length of shoot and number of leaves. For the biomass of leaves, the highest fresh and dry weight of leaves were accumulated from the MSB5 medium formulation with 0.179 and 0.018 g, respectively.

Table 1. Shoot induction of *C. caesia* as affected by different formulations of basal medium.

Basal Medium	Shoot Induction (%)	No. of Shoot	Shoot Length (cm)	No. of Leaves	Leaves Fresh Weight (g)	Leaves Dry Weight (g)	Root Induction (%)	No. of Root	Root Length (cm)
MSB5	100 a	2.70 a	7.88 a	5.33 a	0.179 a	0.018 a	100 a	8.40 a	3.34 a
MS	93.33 a	1.50 b	5.53 b	3.00 b	0.105 b	0.008 b	86.67 b	3.85 b	2.19 a
WPM	83.33 b	1.37 b	4.86 b	2.97 b	0.085 c	0.006 b	80 b	3.67 b	2.02 a

Data were collected after six weeks of incubation. Means (n = 30) followed by the same letter within the columns were not significantly different at $p < 0.05$ using Duncan's Multiple Range Test.

By conducting the plant tissue culture technique, finding the most suitable basal medium formulation is a crucial step for producing healthy plantlets. Since a long time ago, numerous basal medium formulations have been established for propagating various species. In micropropagation of *Curcuma* species, MS medium is the most frequently used for propagating *C. longa*, *C. zedoaria*, *C. aromatica* and also *C. caesia* [22–25], in contrast with this study, which found that MSB5 medium formulation was more efficient than MS medium formulation. The high efficiency of MSB5 medium formulation as compared to MS medium might be due to higher vitamin concentrations in the MSB5 formulation that lead to increased plant growth. Based on the MSB5 medium composition, the pyridoxine HCl and nicotinic acid concentrations were two-fold higher, and thiamine HCl concentration was 100-fold higher than the MS medium vitamin formulations. According to Saad and

Elshahed [26], vitamins such as thiamine HCl, nicotinic acid and pyridoxine HCL at low concentrations are required by plants as a catalyst for various metabolic processes. In addition, the amount needed varies by plant species as some plants are able to synthesize their own vitamins [27].

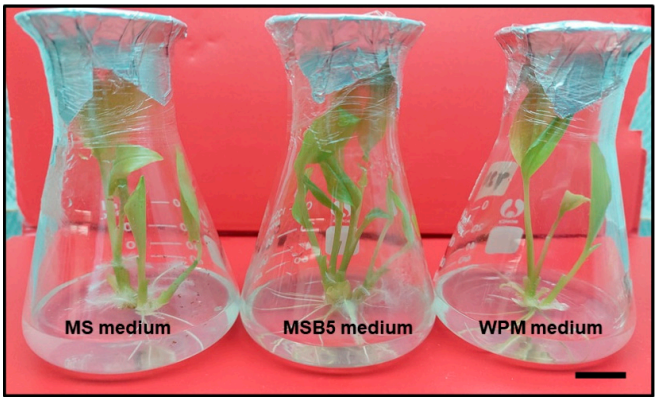

Figure 1. Establishment of *C. caesia* microshoots in MS medium, MSB5 medium and WPM medium after six weeks of inoculation. Scale bar represents 1 cm of actual size.

3.2. Effect of Different Concentrations of Cytokinins on Shoot Multiplication of C. caesia

Based on the previous experiment, the MSB5 medium formulation was found to be the most suitable basal medium for *C. caesia.* In this experiment, the shoots were multiplied by using different concentrations of cytokinin (Table 2). After eight weeks of inoculations, the treatments of 10 and 15 µM BAP and 10, 15 and 20 µM kinetin were recorded at 100% of shoot induction. The lowest percentage of shoot induction was significantly produced by the treatment of 20 µM of 2-iP with 30% of induction. The treatment of 15 µM of BAP significantly produced the highest number of shoots and number of leaves, with 2.77 shoots and 7.00 leaves, respectively. Meanwhile, the highest length of shoots was exhibited by the treatments of 15 µM of BAP, 15 and 20 µM of kinetin with 10.86, 11.10 and 10.14 cm, respectively (Figure 2). As the highest number of shoots and number of leaves were produced from the treatment of 15 µM of BAP, the leaves biomass accumulation was also recorded from the same treatment with 0.343 and 0.029 g of fresh and dry leaves. In this experiment, callus formation was observed in all treatments of 2-iP. The treatment of 5 µM of 2-iP significantly produced the highest callus induction percentage and callus fresh weight with 23.22% and 0.425 g, respectively (Figure 3). In contrast, no callus formation was observed in other treatments.

Figure 2. Shoot multiplication of *C. caesia* in MSB5 medium supplemented with 15 µM BAP at eight weeks after inoculation. Scale bar represents 1 cm of actual size.

Table 2. Effect of different concentrations of cytokinins in MSB5 medium on shoot multiplication of *C. caesia*.

Cytokinin (µM)		Shoot Induction (%)	No. of Shoot	Length of Shoot (cm)	No. of Leaves	Leaves Fresh Weight (g)	Leaves Dry Weight (g)	Callus Induction (%)
Control		93.33 a	1.20 d	8.96 d	4.10 e	0.109 f	0.017 f	0 c
BAP	5	96.67 a	1.97 b	9.54 c	5.73 b	0.248 cd	0.025 b	0 c
	10	100 a	2.07 b	10.10 b	5.73 b	0.277 bc	0.025 b	0 c
	15	100 a	2.77 a	10.86 a	7.00 a	0.343 a	0.029 a	0 c
	20	96.67 a	1.97 b	7.94 e	4.30 de	0.195 e	0.018 ef	0 c
Kin	5	96.67 a	1.37 cd	9.22 cd	4.63 d	0.242 d	0.020 de	0 c
	10	100 a	1.27 cd	9.63 c	4.13 d	0.240 d	0.022 cd	0 c
	15	100 a	1.47 c	11.10 a	5.03 c	0.285 b	0.022 cd	0 c
	20	100 a	1.47 c	10.14 a	5.10 c	0.246 cd	0.024 bc	0 c
2-iP	5	80 b	1.20 d	4.76 f	3.03 f	0.109 f	0.008 g	23.22 a
	10	60 c	0.70 e	3.84 g	1.53 g	0.089 f	0.009 g	16.67 b
	15	36.67 d	0.47 f	2.20 h	1.01 h	0.037 g	0.004 h	16.67 b
	20	30 e	0.43 f	1.84 h	1.17 gh	0.040 g	0.005 h	3.33 c

Data were collected after eight weeks of incubation. Means (n = 30) followed by the same letter within the columns were not significantly different at $p < 0.05$ using Duncan's Multiple Range Test.

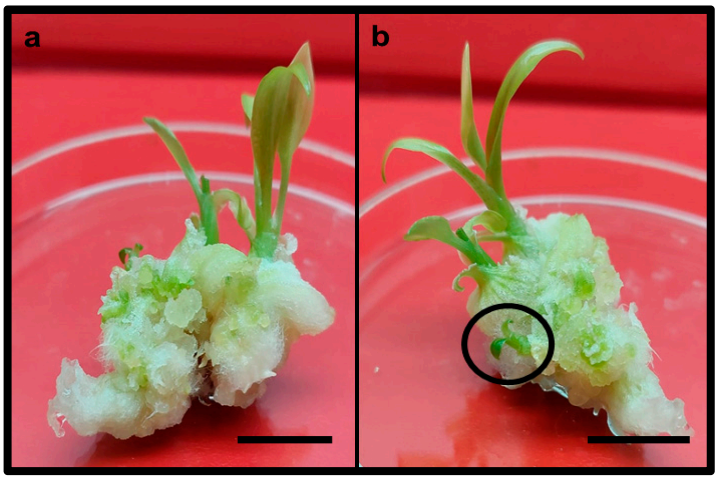

Figure 3. (**a**) Callus produced from the treatment MSB5 medium supplemented with 15 µM BAP and 5 µM 2-iP at eight weeks after inoculation. (**b**) Regenerated shoot (in circle) from the treatment MSB5 medium supplemented with 15 µM BAP and 5 µM 2-iP at eight weeks after inoculation. Scale bars represent 1 cm of actual size.

Based on the results of this experiment, 15 µM of BAP was more prominent than the other treatments. Among the BAP, kinetin and 2-iP treatment, BAP was the most superior, followed by kinetin and 2-iP. The finding was in agreement with Fong and Sani [28], which found that BAP was more effective for shoot production of *C. caesia* compared to kinetin and TDZ. In addition, the efficiency of BAP was also reported on other species, including *Calotropis procera* [29], *Artemisia arborescens* [30], *Boerhaavia diffusa* [31] and *Andrographis alata* [32]. The suitability of BAP over other types of cytokinin in shoot multiplication might be due to the ability of the plant to metabolize BAP more readily as nucleotides, and ribosides stability is present naturally in BAP [33].

3.3. Enhancement of Shoot Multiplication by Supplementation of 15 µM of BAP with Different Concentrations of Auxins

The supplementation of cytokinins in the previous experiment found that 15 µM of BAP was the most prominent in the multiplication of *C. caesia*. Hence, the shoot multiplication was further enhanced by supplementing 15 µM of BAP with different concentrations of auxins. By supplementing the MSB5 medium with auxins, 100% of shoot induction was obtained in all the treatments (Table 3). In terms of the number of shoots, the treatment of 6 and 8 µM of IBA produced the highest number of shoots, with 3.53 shoots. However, the number of shoots recorded was statistically non-significant with all NAA treatments.

However, the highest length of shoots was significantly exhibited by the treatment of 6 µM of IBA with 10.81 cm. In addition, the same treatment also produced the highest number of leaves and leaves biomass accumulation with 9.57 leaves, 0.486 g of leaves fresh weight and 0.039 g of leaves dry weight, respectively. This experiment showed that the application of cytokinin and auxin produced a positive effect on *C. caesia* growth.

Table 3. Effect of 15 µM of BAP with auxins in the MSB5 medium on enhancement of shoot multiplication of *C. caesia*.

PGR	Conc (µM)	Shoot Induction (%)	No. of Shoot	Shoot Length (cm)	No. of Leaves	Leaves Fresh Weight (g)	Leaves Dry Weight (g)
Control	0	100 a	2.57 c	10.00 b	5.90 f	0.294 e	0.022 ef
IBA	2	100 a	2.97 b	9.19 c	7.13 de	0.311 d	0.023 de
	4	100 a	3.00 b	9.74 bc	7.37 cd	0.362 b	0.028 b
	6	100 a	3.53 a	10.81 a	9.57 a	0.486 a	0.039 a
	8	100 a	3.53 a	9.58 bc	8.10 b	0.320 d	0.026 bc
NAA	2	100 a	3.27 ab	8.00 de	7.00 e	0.254 g	0.020 f
	4	100 a	3.40 a	8.57 d	7.57 c	0.333 c	0.025 cd
	6	100 a	3.20 ab	7.68 e	6.83 e	0.295 e	0.021 ef
	8	100 a	3.30 ab	8.13 de	7.00 e	0.278 f	0.022 def

Data were collected after eight weeks of incubation. Means (n = 30) followed by the same letter within the columns were not significantly different at $p < 0.05$ using Duncan's Multiple Range Test.

In plant tissue culture, supplementation of auxin in the basal medium is frequently used for the rooting stage. However, the addition of auxin can also be conducted for the enhancement of shoot multiplication as the synergistic effect between the auxin and cytokinin will enhance the cell division and elongation of the shoot [34,35]. Several studies have reported that the combination of auxin and cytokinin significantly increased plant growth. A study by Hailu et al. [36] found that the combination of BAP and IBA significantly increased the shoot induction percentage, number of leaves and number of shoots of *Aframomum corrorima*. Besides that, the synergistic effect between auxin and cytokinin on shoot growth was also reported on *Petunia hybrida* [37]. The integration of auxin and cytokinin is important to support plant growth and development [38]. The increased plant growth might be due to the different nutrient uptakes, translocation rates, metabolic processes and ability of plants to regulate the level of plant hormones [39].

3.4. Effect of Basal Medium Strength and Different Concentrations of Indole-3-Acetic Acid (IAA) on Root Induction of C. caesia

In micropropagation of Zingiberaceae plant species, including those from the genus *Curcuma*, the roots are commonly produced simultaneously with the shoots. However, the number of roots produced per shoot is not enough to support the plantlets during acclimatization. Hence, the rooting stage was conducted to produce adequate amounts of roots for high survival rates during acclimatization. In this study, the shoots were initially cultured on the MSB5 medium supplemented with 15 µM of BAP and 6 µM of IBA for the first eight weeks. After that, the healthy plantlets with more than two leaves and growth of more than 5 cm were separated from the clumps and inoculated individually on rooting medium. The results in Table 4 showed that full-strength MSB5 medium supplemented with 5 µM of IAA significantly produced the highest number of roots and length of roots, with 6.13 roots and 5.37 cm, respectively. Meanwhile, the treatment of full-strength MSB5 medium supplemented with 10 µM of IAA significantly produced the lowest number of roots and length of roots, with 3.20 roots and 1.79 cm, respectively. In this study, full-strength MSB5 fortified with 5 µM of IAA gave the best results for adventitious root formation of *C. caesia* (Figure 4). The suitability of full-strength basal medium over half-strength medium might be due to a sufficient amount of nutrients to stimulate the root formation. Meanwhile, IAA, which is a frequently used auxin for root induction, alongside IBA, was reported to produce a good rooting response in *Nardostachys jatamansi* and *Alkanna tinctoria* [40,41].

Table 4. Effect of MSB5 medium strength and IAA on root induction of *C. caesia* plantlets.

Strength	IAA (μM)	Number of Roots	Length of Roots (cm)
Full	0	4.40 de	3.85 e
	2.5	5.87 b	4.39 c
	5	6.13 a	5.37 a
	7.5	4.20 ef	3.38 f
	10	3.20 h	1.79 h
Half	0	3.60 g	2.81 g
	2.5	4.27 def	2.59 g
	5	5.13 c	4.88 b
	7.5	4.53 d	4.15 cd
	10	4 f	4.01 de

Data were collected after twelve weeks of incubation. Means (n = 24) followed by the same letter within the columns were not significantly different at $p < 0.05$ using Duncan's Multiple Range Test.

Figure 4. Regeneration of adventitious roots of *C. caesia* in the full strength of MSB5 medium supplemented with 5 μM of IAA.

3.5. Acclimatization of C. caesia as Affected by Different Potting Mediums

Acclimatization is an important step in the micropropagation of commercially important plants. After the rooting stage, the healthy plantlets with at least two leaves, more than 5 cm in plant height, more than 1.5 cm in root length and without any morphology abnormalities were acclimatized on different potting mediums. Among the combination of cocopeat, perlite and vermiculite with peatmoss, cocopeat significantly produced the highest percentage of plantlet survival, with 77.78%, followed by perlite (61.11%) and vermiculite (44.43%) after two weeks of acclimatization (Table 5) (Figure 5). The higher survival percentage of plantlets in the combination of cocopeat and peatmoss could be due to the higher porosity and air space between the cocopeat and peatmoss, which provided better aeration for the roots to grow and, consequently, produced a higher survival percentage compared to the other treatments. The efficiency of cocopeat as a potting medium for acclimatization was reported on *Bacopa monnieri* and *Ficus carica* [42,43].

Table 5. Effect of potting medium on acclimatization of *C. caesia plantlets*.

Medium	Survival (%)
	Day 14
Cocopeat + peatmoss	77.78 a
Perlite + peatmoss	61.11 b
Vermiculite + peatmoss	44.43 c

Data were collected after two weeks of acclimatization. Means (n = 18) followed by the same letter within the columns were not significantly different at $p < 0.05$ using Duncan's Multiple Range Test.

Figure 5. Plantlet during acclimatization in the potting medium of cocopeat and peatmoss.

4. Conclusions

An efficient protocol for micropropagation of *C. caesia* was established in this study with the aim to mass produce the raw materials of *C. caesia* in a short time for the conservation of wild populations of this species. The basal medium formulation and plant growth regulators were tested, and results showed that the MSB5 medium supplemented with 15 µM BAP and 6 µM of IBA was the optimum formulation for shoot induction and multiplication of *C. caesia.* At the rooting stage, full-strength MSB5 medium supplemented with 5 µM IAA was the best concentration for the root formation, and acclimatization was successfully carried out using the potting medium of cocopeat with peatmoss (1:1). Hence, the protocol developed in this study could be used for the large-scale production of *C. caesia.*

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