Industrial Crops & Products 223 (2025) 120101

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Micropropagation of white turmeric (*Curcuma zedoaria* (Christm.) Roscoe) and establishment of adventitious root culture for the production of phytochemicals

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ARTICLE INFO

Keywords:
Acclimatization
Elicitation
Flow cytometry
Genetic fidelity
Molecular markers
Multiple shoots

ABSTRACT

Curcuma zedoaria (Christm.) Roscoe (popular as "white turmeric"), a medicinal herb (Zingiberaceae family), is an integral part of traditional medicine and is also used as condiment and dye. Cultivation through conventional rhizome propagation is hindered by slow germination, long dormancy period, and susceptibility to diseases. The roots of *C. zedoaria* contain curcumin and curcumenol, but limited production of roots restricts sufficient production of these phytochemicals. Therefore, this study developed an innovative protocol for micropropagation and establishment of adventitious root culture in *C. zedoaria* for phytochemical production. For high-frequency multiple shoot production, Murashige and Skoog (MS) medium fortified with varying concentrations of 6-benzylaminopurine and *meta*-Topolin (*m*T) were tested. The maximum number of shoots were observed in MS medium + 1.5 mg/L *m*T that yielded an average of 620 shoots from a single explant after 36 weeks following 6 subcultures. The maximum number of roots were produced in MS medium + 1.5 mg/L indole-3-butyric acid. An 86 % survival was obtained by acclimatizing the plants in soil and rice husk (3:1) media. The genetic stability of the plants was substantiated with the aid of four different types of molecular markers coupled with flow cytometry. The highest adventitious root induction frequency (100 %) was recorded in 1.5 mg/L α-naphthalene acetic acid. MS medium at ¼ strength was ideal for the proliferation of roots as well as the accumulation of phytochemicals. Eliciting the adventitious root culture with 400 μM methyl jasmonate further improved the phytochemical content. Therefore, the protocol described in this study can be utilized to produce genetically identical *C. zedoaria* plants at a larger quantity and to generate adventitious roots that can be used as an alternate source of phytochemicals.

1. Introduction

Curcuma zedoaria (Christm.) Roscoe (zedoary) (popular as "White turmeric") is a perennial herb belonging to the Zingiberaceae family. They are indigenous to South and Southeast Asia, mainly Sri Lanka, Bangladesh, and India. The rhizomes of *C. zedoaria* play significant role in the traditional system of medicine in many countries, where they are used to cure an array of ailments such as damaged skin, leukoderma, stomach sickness, blood stagnation, and spleen swelling (Syahid and Heryanto, 2017). In addition, the plant finds extensive application as condiment, dye, and in the perfume sector (Banisalam et al., 2011).

The hepatoprotective, antidiabetic, anticancer, and anti-

Abbreviation: BAP, 6-benzylaminopurine; CDDP, conserved DNA-derived polymorphism; DAMD, directed amplification of minisatellite-region DNA; DW, dry weight; FCM, flow cytometry; FW, fresh weight; IBA, indole-3-butyric acid; ISSR, inter simple sequence repeats; MeJ, methyl jasmonate; MS, Murashige and Skoog; *m*T, *meta*-Topolin; NAA, α-naphthalene acetic acid; PGRs, plant growth regulators; SA, salicylic acid; SCoT, start codon targeted; TFC, total flavonoid content; TPC, total phenolic content.

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https://doi.org/10.1016/j.indcrop.2024.120101
Received 2 June 2024; Received in revised form 14 July 2024; Accepted 17 November 2024
Available online 2 December 2024

inflammatory effects of *C. zedoaria* are ascribed to the presence of terpenoids, flavonoids, phenylpropanoids, and essential oils in its rhizomes (Jadhao and Bhuktar, 2019). Among the bioactive components of this plant species curcumin, curcumenol, curcumenon, and curcuzedoalide are vital ones (Zeeshan et al., 2018). As per the reports of Lobo et al. (2009) and Mishra et al. (2018), curcuzedoalide and curcumin in particular showed chemopreventive properties against gastric and human ovarian cell lines, respectively. Due to its increasing demand and potential benefits, *C. zedoaria* is becoming increasingly valuable for industrial phytochemical extraction (Jena et al., 2020). Nonetheless, the constraints linked to the traditional propagation of *C. zedoaria* resulted in the market supply not being able to keep up with this growing demand (Chong et al., 2012). The crop is conventionally propagated using rhizomes. The prolonged dormancy of rhizome (before germination), slow growth rate (that lasts up to 9–12 months), few propagules per rhizome, soil-borne pathogens causing rhizome rot and leaf spot diseases are the disadvantages of this method, which make it difficult to produce *C. zedoaria* on a large scale for commercial use. The amount of rhizomes that can be used for phytochemical extraction is also decreased by the requirement to put aside 10–20 % of the rhizomes for propagation during the following season (Bharalee et al., 2005; Sarma et al., 2011).

Conventional propagation is being replaced by a biotechnological approach known as *in vitro* propagation, which produces healthy, genetically identical plants quickly and in large quantities from a single explant in an aseptic environment. In addition, the plants developed via *in vitro* propagation are available all year round because this technique remains unaffected by seasonal or climatic variations (Lan et al., 2019).

Plant growth regulators (PGRs), specifically auxin and cytokinin, substantially influence the *in vitro* growth of plants. Cytokinin, which promotes cell division and proliferation, accelerates the induction and multiplication of *in vitro* shoots (Zahid et al., 2021). 6-benzylaminopurine (BAP) is the most widely utilized cytokinin for *in vitro* multiplication of most crops, including *Curcuma* species. While BAP has been widely utilized to facilitate shoot multiplication, new research has revealed the presence of a more potent cytokinin called *meta*-Topolin (*m*T).

The natural aromatic cytokinin *m*T has been proven to be a superior alternative to BAP (Krishna Vrundha et al., 2021; Gantait and Mitra, 2021). It has shown to be remarkably successful in promoting the multiple shoot development in crops such as *Allamanda cathartica* (Khanam et al., 2018) and *Sesamum indicum* (Elayaraja et al., 2019), and in Zingiberaceae members such as *Hedychium coronarium* (Behera et al., 2019), *C. mangga* (Waman et al., 2021), and *C. amada* (Behera et al., 2022). There have been several attempts at *C. zedoaria in vitro* propagation, however the most successful protocol to date has been developed using 3.0 mg/L BAP, which produces 7.60 shoots/explant (Jena et al., 2020). On the other hand, *m*T at 2.0 mg/L resulted in an average of 13 shoots/explant in *C. mangga* (Waman et al., 2021). Therefore, *m*T was tested as a potential substitute to BAP in boosting production of multiple shoots in *C. zedoaria*.

Moreover, the increased number of shoots must result in plants that are true-to-type. However, somaclonal variation is common in plants grown *in vitro*. According to Ranghoo-Sanmukhiya (2021) somaclonal variation is the variation of genetic components in plants produced *in vitro* and is driven by multiple components including PGRs, duration of the culture period, along with the number of subculture cycles. Thus, evaluating genetic fidelity is an essential phase in the micropropagation process and can assist in determining how many subculture cycles a plant could undergo before genetic variation appears. Flow cytometry (FCM) is used alongside molecular markers, which measures variations in ploidy level and genome size, to evaluate the genetic integrity of *in vitro*-regenerated plants (Jena et al., 2018).

The need for a creative approach for phytochemical production arises from the fact that the demand for phytochemicals extracted from *C. zedoaria* rhizomes exceeds the supply. According to Kayum et al. (2021), the roots of *C. zedoaria* contain compounds like curcumin and curcumenol. Mishra et al. (2018) described the anticancerous characteristics of these chemicals, showing that roots have the potential to replace rhizomes as the source of these secondary metabolites. Leveraging *in vitro* adventitious roots is one of the novel strategies for producing phytochemicals from *C. zedoaria* roots. This has the ability to produce phytochemicals of consistent quality and quantity, while also addressing the existing shortage of rhizomes and providing scalable solutions to fulfil the increasing demands in the field of phytochemical research and applications.

Secondary metabolites that build up in roots can be produced by a technique called adventitious root culture. It is perceived to be advantageous since it has the potential to scale up root-based secondary metabolite production to a commercial level (Silja and Satheeshkumar, 2015). Adventitious root cultures were utilized to synthesize secondary metabolites in Zingiberaceae members such as *C. mangga* (Soundar Raju et al., 2015), *Boesenbergia rotunda* (Yusuf et al., 2018), and *Zingiber zerumbet* (Alwakil et al., 2022).

In adventitious root culture, PGR concentration and salt strength (of basal medium) are the two key parameters influencing root growth. Salt strength in the media is an important determinant for root proliferation. Low salt strength in the media promotes faster root growth by increasing ion availability, while PGRs aid in speeding up root development and production of secondary metabolite (Silja and Satheeshkumar, 2015). The auxins α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA) were particularly successful in boosting root growth and proliferation because of their stability and persistency, which allow them to be promptly absorbed by plant cells (Hao et al., 2021).

Stress levels have been shown to affect plant cells' ability to produce secondary metabolites, and these stress levels can be elevated by the addition of elicitors (Rahmat and Kang, 2019). Rodríguez-Sánchez et al. (2020) defined elicitors as substances that activate defence genes, ensuing in the enhanced accumulation of secondary metabolites. Salicylic acid (SA) and methyl jasmonate (MeJ) are the two highly sought-after elicitors utilized to ameliorate the phytochemical accumulation in the root cultures. In the cases of *Thevetia peruviana* (Mendoza et al., 2018), *Rauvolfia serpentina* (Dey et al., 2020), and *Z. zerumbet* (Alwakil et al., 2022), phytochemical enhancement has been achieved with the addition of MeJ and SA to root culture. There are no reports of adventitious root culture being established in *C. zedoaria*, despite the fact that it is a perfect and practical method for producing phytochemicals for commercial use.

Hence, the purpose of this study was to establish a superior protocol for *in vitro* propagation of *C. zedoaria* utilizing the cytokinin *m*T, as well as to establish a methodology for adventitious root culture of *C. zedoaria* as a phytochemical source.

2. Methodology

2.1. Plant material

C. zedoaria rhizomes were obtained from Kaseh Herb Nursery in Kampung Panggau, Kangar, Perlis, Malaysia (coordinates: 6°25'37.8"N, 100°10'15.9"E). A voucher specimen bearing the voucher number KM 0098/23 was submitted to the Institute of Bioscience (IBS) UPM herbarium. The rhizomes were germinated in organic garden soil (Nurfarm Agro, Malaysia) to aid in their sprouting. Subsequently, the rhizome-derived sprouts were employed as explants to initiate *in vitro* cultures.

2.2. Culture media preparation and growth room condition

As the basal culture medium, MS (Murashige and Skoog) (Murashige and Skoog, 1962) medium was employed, which was enhanced with 3 % (w/v) sucrose and 0.3 % (w/v) Gelrite®. Following a pH adjustment (to 5.7) using NaOH or HCl, the medium was autoclaved for 20 min at 121°C and 104 kPa of pressure. A temperature of 22±2°C and a photoperiod of 16 h were maintained for the cultures during their incubation.

2.3. Sterilization of explants and establishment of cultures

C. zedoaria cultures were established by selecting rhizome sprouts with sizes of 1, 2, and 3 cm (Fig. 1A). The sprouts were washed in water for 30 min. Next, the sprouts were immersed in 250 mL of tap water with 3–4 drops of Tween 20® to remove any dirt, debris, or contaminants that might have been attached to the surface of sprouts. The buds were surface sterilized by soaking them in 0.2 % (w/v) Carbendazim for 1 h while being continuously shaken at 70 rpm on an orbital shaker. This was followed by a 15-min soak in 10 % (v/v) sodium hypochlorite. Rinsing with sterile distilled water was done after each stage. Following a 60 sec soak in 70 % ethanol, the buds were washed thrice with sterile water. They were dried with sterile tissue paper after being rinsed in distilled water. Before being inoculated in the culture media, the buds' basal surface and 2–3 outer layers were removed. The cultures were maintained in the media for 6 weeks, with a subculture interval of 3 weeks. The percentage of shoots that survived after 6 weeks, days to shoot induction, number of shoots/explant, and overall number of shoots produced by various bud sizes at the end of establishment were recorded.

2.4. Shoot induction and proliferation

In vitro-induced shoots from established *C. zedoaria* cultures were employed as explants. They were inoculated in media fortified with different concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mg/L) of *m*T after being cut to a size of 1 cm from the base (Fig. 1E). To evaluate the effectiveness of both cytokinins, *in vitro* shoots were inoculated in media supplemented with BAP within the same concentration range as *m*T. As the control, MS medium without PGR was utilized. The cultures were kept in the media for a duration of 6 weeks, following that period, data were recorded on the percentage of induced shoots, number of days for induction, number of shoots per explant, length of the induced shoots, and total number of leaves.

Additionally, tests were conducted on shoot regeneration potential of *C. zedoaria* for up to 6 subculture cycles, each lasting 6 weeks. The optimal *m*T and BAP concentrations were found in the preceding experiment and applied in the ensuing subculture cycles. After being trimmed and removed from the base, each shoot that had been induced in the induction media was subcultured individually in MS medium + 1.5 mg/L *m*T and MS medium + 1.0 mg/L BAP.

2.5. Rooting of shoots

Shoots of 4–5 cm length with 2–3 leaves (Fig. 1I) were removed from multiplication media and inoculated in semi-solid and liquid MS media augmented with variable levels of IBA (1.0, 1.5, and 2.0 mg/L) in order to induce *in vitro* roots. IBA-free medium were used as control. The cultures were incubated in the rooting media for 4 weeks. Using the WinRhizo STD4800 root scanner (Regent Instruments Inc., Canada), the parameters such as root number, length, volume, and diameter were recorded after 4 weeks.

2.6. Acclimatization

Acclimatization was accomplished by selecting plantlets rooted in semi-solid media. In order to get rid of any remaining media from the roots, plantlets with well-developed roots were carefully taken out of rooting media and washed with distilled water. After being soaked with 0.1 % fungicide Carbendazim (w/v) to prevent contamination, the

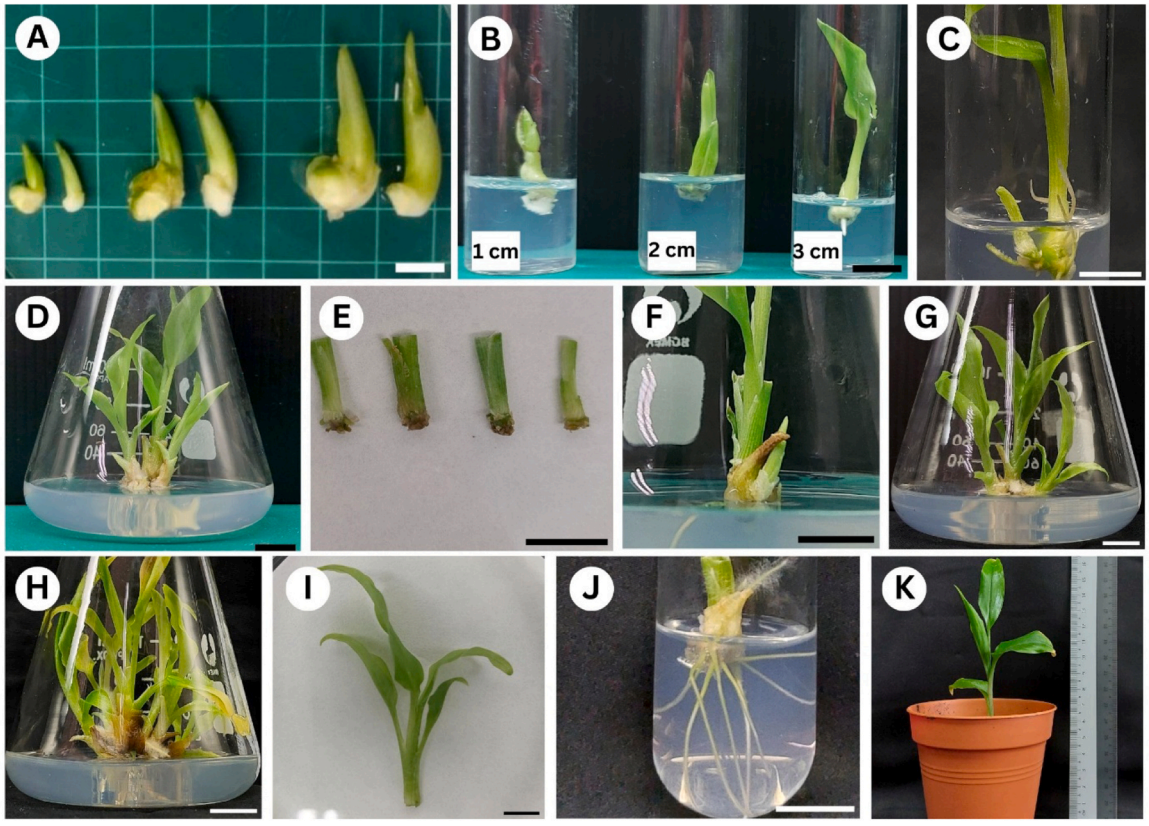

Fig. 1. *In vitro* regeneration and acclimatization of *C. zedoaria* plantlets. (A) Buds of varying size (1, 2, and 3 cm) for selection of ideal explant size; (B) rhizome sprouts at 1 week after inoculation; (C) initiation of shoot; (D) multiple shoot formation in establishment media; (E) *in vitro* shoots cut to a size of 1 cm for induction of multiple shoots; (F) initiation of shoot; (G) multiple shoot formation in MS medium + 1.0 mg/L BAP; (H) multiple shoot formation in MS medium + 1.5 mg/L *m*T; (I) shoots with 2–3 leaves were used for *in vitro* rooting experiment; (J) *in vitro* root formation in MS medium + 1.5 mg/L IBA; (K) *in vitro*-developed plantlet acclimatized in soil: rice husk (3:1) after 4 weeks. (Scale bar = 1 cm).

plantlets were then shifted to pots containing diverse potting medium, such as soil, soil: sand (1:2), and soil: rice husk (3:1). The plants were incubated at 25°C. To maintain humidity, translucent polythene sheets were laid over the plantlets. To progressively lower the humidity, holes were punched in the polythene sheets, and the sheets were taken off from time to time to allow the plantlets to become acclimated. Watering was carried out on alternate days. The percentage of surviving plants was documented after 4 weeks.

2.7. PCR-based molecular analysis

After 6 cycles of subcultures, *in vitro*-regenerated plantlets were assessed for genetic fidelity. Ten plant samples (each from *in vitro*-propagated and conventionally propagated) were randomly selected and from those plants, 2–3 leaves of similar developmental stages were collected for genomic DNA extraction. Four distinct systems of molecular markers i.e. start codon targeted (SCoT) polymorphism markers, inter simple sequence repeats (ISSR), directed amplification of minisatellite-region DNA (DAMD), and conserved DNA-derived polymorphism (CDDP) were employed; using five primers for every marker. The Plant Mini (DNA extraction) Kit from GSure® was used to extract the DNA from the plant samples. Polymerase Chain Reaction (PCR) was run to amplify the isolated DNA utilizing thermocycler (Proflex™, Applied Biosystems® by Life Technologies™, USA). Each 25 µL of the PCR mixture comprised 5 µL 1X Taq polymerase assay buffer, 0.1 mM of each primer, 2 mM of dNTP, 0.5 µL Taq polymerase, and 40 ng of template DNA, in sterile water. Denaturation is the initial stage of PCR, and it involves setting the temperature to 94°C for 5 min. This procedure was followed by 94°C denaturation for 1 min, annealing at 40–58°C for 1 min, and 72°C extension for 1 min. Simultaneously, the final was performed at 72°C for 5 min. The end product of PCR was kept at −20°C. The PCR product was then resolved in 1.5 % agarose gel electrophoresis alongside a 100 bp ladder. With the use of a UVP® Gel Doc (Cambridge, UK), the gel was examined under UV light. The data was recorded as '1' when a band was present and as '0' when none was visible.

2.8. Flow cytometry

In order to further evaluate the genetic fidelity, leaf samples from both conventionally and *in vitro*-developed plants were cut to a size of 1.5–2 cm and sliced with a blade in 5 mL of modified Galbraith's extraction buffer (pH = 7.0), which contained 0.5 % (v/v) Triton X-100, 200 mM Tris, and 45 mM $MgCl_2.6H_2O$, in order to obtain a nuclei suspension. The nuclei suspension was filtered using a double-layered, 30-µm nylon filter. Next, 50 µg/mL of propidium iodide and 50 µg/mL of RNase solution were added to the filtered suspension. The mixture was then kept for 20 min in the dark on ice. In the end, leaf nuclei suspensions were evaluated in Flow Cytometer (BD LSRFortessa™ Cell analyzer, BD Biosciences, USA).

2.9. Induction and proliferation of adventitious roots

To induce adventitious roots, *in vitro*-derived shoots (refer to Section 2.4) were utilized. The shoots were cut to a size of 0.5–1 cm from the base (Fig. 6A). Subsequently, they were inoculated in Petri plates with MS media supplemented each with 0.5, 1.0, and 1.5 mg/L IBA or NAA (Fig. 6B), whilst the control group used media without auxins. Following that, the culture was incubated for 5 weeks at 22±2°C in the dark. In order to accumulate adventitious root biomass, 0.1 g of induced adventitious roots from the previous 5-week experiment were placed in 25 mL of liquid MS medium with different strengths (¼, ½, ¾, 1) and 1.5 mg/L NAA. For five weeks, the cultures were incubated at 22±2°C in the dark and agitated at 70 rpm on an orbital shaker. Three replications of the experiment were conducted, using ten samples for each replicate. At the end of 5 weeks, the fresh weight (FW), dry weight (DW), total phenolic content (TPC), and total flavonoid content (TFC) of the roots were measured.

2.10. Elicitation of adventitious root culture

After the proliferation experiment, the 0.5 g of proliferated roots were placed in 25 mL of ¼ MS liquid media supplemented with 1.5 mg/L NAA and varying concentrations of MeJ (200, 400, and 600 µM) and SA (100, 200, and 300 µM). The cultures were then incubated for five weeks at 22±2°C in the dark on an orbital shaker set to revolve at 70 rpm. Three replicates of the experiment were conducted, using ten samples each time. Biomass accumulation (FW and DW), TPC and TFC, and were measured at the end of the fifth week.

2.11. Analysis of phytochemicals

2.11.1. Plant material and extract preparation

Rhizomes from conventionally propagated plants and adventitious root cultures that were 5-week-old were collected. Tap water was used to rinse them in order to get rid of impurities and dirt particles adhered to the rhizome and roots. After that, the rhizomes and roots were dried for 48 h at 40°C in a hot air oven. They were then powdered using a mortar and pestle and stored in air-tight containers until further use. The extract was prepared by adding 100 mL of 50 % methanol to 0.1 g of powdered samples. The solution was then shaken on an orbital shaker at 125 rpm at room temperature for 24 h. The mixture was filtered using Whatman's No. 1® filter paper before being concentrated at low pressure on a rotary evaporator. The crude extracts were then kept at 4°C until further study. The total phenolic and flavonoid content was determined using a method established by Phuyal et al. (2020).

2.11.2. Estimation of total phenolic content

The gallic acid standard was prepared by dissolving 1 mg of gallic acid in 1 mL of methanol. Gallic acid was diluted to various concentrations (25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL) from the stock solution. The tests were performed in triplicate, and the calibration curve was plotted using the absorbance of different concentrations of gallic acid. The TPC was estimated using the Folin-Ciocalteu colorimetric technique. Once 100 µL of the crude extract was taken, it was mixed with 500 µL of 10 % Folin-Ciocalteu (v/v) reagent and 400 µL of 7.5 % sodium carbonate (w/v). The solution was mixed thoroughly, and 100 µL of it was pipetted into 96 wells of microplate and incubated at room temperature and in darkness for 30 min. Because of the Folin-Ciocalteau's reaction with the extract's phenols, the solution took on a blue colour. After incubation, the absorbance was measured at 760 nm. The results were expressed as mg gallic acid equivalents (GAE)/g DW of the plant material. The following formula was used to determine the TPC: C= c (V/m), where C is the total phenolic content, c is the concentration of the gallic acid obtained from the calibration curve, m is the mass of the extract in grams and V is the volume of the extract in mL.

2.11.3. Estimation of total flavonoid content

To make a stock solution, 1 mg of quercetin was dissolved in 1 mL of methanol for the purpose of obtaining the quercetin standard. Quercetin was diluted to various concentrations (25 µg/mL, 50 µg/mL, 75 µg/mL, and 100 µg/mL) from the stock solution. The calibration curve was plotted using the absorbance of various quercetin concentrations, and the tests were run in triplicate. The TFC was ascertained by using the colorimetric technique with aluminum chloride. After taking 100 µL of the crude extract, 30 µL of 5 % (w/v) sodium nitrite was added to it. For 5 min, the mixture was left to stand. Subsequently, 30 µL of 10 % (w/v) aluminum chloride and 200 µL of 1 M NaOH were added to the mixture. After that, 100 µL of this solution was pipetted into each of the 96 microplate wells. The absorbance was measured at 510 nm. The results were expressed as mg quercetin equivalents (QE)/g DW of the plant material. The following formula was used to determine the TFC: C= c (V/m), where C is the TFC, c is the concentration of the quercetin

obtained from the calibration curve, m is the mass of the extract in grams, and V is the volume of the extract in mL.

2.12. Statistical analysis

The experiments used a completely randomized design, replicated thrice (or ten-times for acclimatization) with ten explants per replication. ANOVA was used to analyze the results, which were presented as mean ± standard deviation. Significant differences between mean values were concluded via Duncan's multiple-range test (Duncan, 1955) at $P \leq 0.05$ and R software.

3. Results and discussion

3.1. Sterilization and the effect of explant size in the establishment of in vitro cultures

The high contamination rates were one of the most significant barriers to micropropagation of Zingiberaceae members. The sterilization process adopted in this study reduced the contamination rate of explants during establishment, resulting in a 94 % survival. Another essential factor was the size of the explants, which appeared to have an impact on their capacity to withstand sterilization. The results indicate that, of the three sizes of explants that were tested—1, 2, and 3 cm—the explants with size 3 cm had the highest percentage of survival (94.00 %), whereas the explants with size 1 cm had the lowest percentage of survival (63.34 %) (Table 1). This could be because the explant is too young to resist the effects of a strong sterilant. The survival percentage of 1 cm and 2 cm long explants, however, did not differ substantially. Also, explants measuring 1 and 2 cm showed signs of bacterial and fungal infection. It was also discovered that the explant of size 3 cm required fewer days (10.33) for the induction of new shoots. Similar findings were observed in studies by Park et al. (2021) on *Kaempferia parviflora* micropropagation and Zuraida et al. (2016) on *Z. officinale* micropropagation. This was because larger explants had nutrient reserves that, when combined with nutrients in the media, improved the growth of new shoots. Shoot induction was further aided by adding 2.0 mg/L of BAP to the medium. Regardless of the explant size, new shoots developed in the establishing media supplemented with 2.0 mg/L BAP. Explants measuring 3 cm produced an average of 2.66 buds (Fig. 1D). Nevertheless, across all tested explant sizes, the number of shoots per explant was not statistically significant. Finally, at the end of the 6-week period, the total number of shoots produced throughout the establishment of cultures was noted. Compared to other explant sizes, the 3 cm explant produced a larger total number of shoots (75.01) due to its higher survival.

Studies on Zingiberaceae members like *H. coronarium* (Mohanty et al., 2013) and *Z. officinale* (Zuraida et al., 2016) have examined the impact of explant size on micropropagation. While this was the case in the studies by Zuraida et al. (2016), where larger explants (3 cm) had a greater survival percentage, the current study found that explants of 3 cm had higher survival (94 %) than smaller (1-2 cm) ones. The outcome aligned with studies conducted by Mohanty et al. (2013), which demonstrated that 1.5 cm buds responded more strongly (96 %) to shoot induction than 0.5 cm buds, which did not produce any new shoots. Nevertheless, in the same study, the induction frequency of shoots was reduced to 80 % by a further size increase to 2 cm.

Table 1
Effect of explant size on establishment of *C. zedoaria in vitro* cultures after 6 weeks of growth stage

Explant size (cm)	Survival (%)	Days for shoot induction	Shoots per explant	Total number of shoots
1	63.34 ±5.00 b	17.33±1.67 b	1.66±0.57 a	31.54±0.00 c
2	80.67 ±5.00 b	13.67±1.57 a	2.00±0.17 a	48.40±0.00 b
3	94.00 ±5.00 a	10.33±1.57 a	2.66±0.57 a	75.01±0.80 a

Data indicates mean±standard deviation values of 3 replicates (each with 10 explants) per treatment. Data represented in each of the *column* with different letters of *alphabets* are significantly varied according to DMRT at $P \leq 0.05$

3.2. Efficacy of cytokinins in the production of multiple shoots

Even after 6 weeks of culture, buds grown on MS medium without PGR failed to initiate shoots. Conversely, cytokinin-fortified medium, regardless of cytokinin type or concentration, promoted multiple shoot production 7–14 days after induction. The following section discusses the findings for each cytokinin separately.

3.2.1. Effect of BAP on shoot induction and multiplication

In MS medium enriched with BAP, fresh shoot development was seen 2 weeks after incubation. A 100 % shoot induction (Fig. 1F) was noted for all tested BAP concentrations. As shown in Table 2 and Fig. 1G, the medium supplemented with 1.0 mg/L produced the largest (2.50) number of shoots, while the medium supplied with 1.5 mg/L produced the maximum (1.93) number of leaves and the longest shoots (4.01 cm). The number of shoots (1.30) and shoot length (2.16 cm) were lowest in medium supplemented with 0.5 mg/L BAP as well as 2.5 mg/L with 1.40 shoots having 2.12 cm shoot length.

3.2.2. Effect of mT on shoot induction and multiplication

Similar to BAP, 100 % shoot induction was also obtained using MS

Table 2
Multiple shoot production in *C. zedoaria* influenced by different concentration of *meta*-Topolin (*m*T) and 6-benzylaminopurin (BAP) after 6 weeks of inoculation

Cytokinins	Concentration (mg/L)	% shoot bud induction	No. of shoots	Shoot length (cm)	No. of leaves
Control	0	0 b	0.00 ±0.00 f	0.00 ±0.00 f	0.00 ±0.00 c
*m*T	0.5	100 a	2.60 ±0.58 cd	2.77 ±0.37 de	2.17 ±0.12 a
	1.0	100 a	3.20 ±0.46 bc	4.17 ±0.91 ab	2.15 ±0.23 a
	1.5	100 a	5.40 ±0.50 a	4.57 ±0.30 a	2.19 ±0.10 a
	2.0	100 a	4.00 ±0.92 b	3.59 ±0.10 bc	2.01 ±0.25 ab
	2.5	100 a	3.50 ±0.50 bc	3.20 ±0.11 cd	2.03 ±0.15 ab
BAP	0.5	100 a	1.30 ±0.31 e	2.16 ±0.19 e	1.58 ±0.23 b
	1.0	100 a	2.50 ±0.45 cd	3.09 ±0.73 cd	1.52 ±0.48 b
	1.5	100 a	2.10 ±0.23 de	4.01 ±0.20 ab	1.93 ±0.09 ab
	2.0	100 a	2.00 ±0.92 de	2.97 ±0.25 cd	1.50 ±0.51 b
	2.5	100 a	1.40 ±0.42 e	2.12 ±0.61e	1.53 ±0.44 b

Data indicates mean±standard deviation values of 3 replicates (each with 10 explants) per treatment. Data represented in each of the *columns* with different *alphabets* are significantly varied according to DMRT at $P \leq 0.05$

medium supplemented with *m*T. After a week of inoculation, it was discovered that all of the *m*T concentrations examined helped to induce new shoots. The medium augmented with 1.5 mg/L *m*T (Fig. 1H) produced the highest (5.40) number of shoots with a maximum (4.57 cm) shoot length and leaves (2.19), whereas the MS medium treated with 0.5 mg/L *m*T produced the lowest (2.60) number of shoots and minimum (2.77 cm) shoot length.

3.2.3. Comparison of effect of BAP and mT on multiple shoot production

C. zedoaria showed 100 % shoot induction in response to both cytokinins, BAP and *m*T. When the optimal concentrations of BAP (1.0 mg/L) and *m*T (1.5 mg/L) were examined for their effects on the duration for shoot induction and multiple shoot production, it was found that *m*T induced shoots more quickly than BAP. Shoot induction started after the first week of culture in media enhanced with *m*T, but it started after the second week in media supplemented with BAP. Moreover, it was discovered that the quantity of shoots generated by *m*T (5.40) was double that of shoots generated in media supplemented with BAP (2.50). A reduction in shoot regeneration was seen when the concentration of BAP and *m*T was increased above their optimal levels. This was consistent with a study by Zafar et al. (2019), who found that cells destined to create new shoots were less likely to regenerate themselves when exposed to increased cytokinin concentrations.

Given that they have a significant impact on the growth and development of plants in culture media, cytokinins are essential to the micropropagation of plants. Previous studies on *C. zedoaria* revealed that BAP was significantly more successful in producing multiple shoots. The results of this study showed that *m*T was significantly more effective at promoting shoot multiplication than BAP. These findings are consistent with reports on *Cannabis sativa* (Lata et al., 2016), *Daphne mezereum* (Nowakowska and Pacholczak, 2020), *Oxystelma esculentum* (Jayaprakash et al., 2021), *Vanilla planifolia* (Manokari et al., 2021), *Gerbera jamesonii* (Mahanta et al., 2023), *Stevia rebaudiana* (Subrahmanyeswari et al., 2023), and *Solanum tuberosum* (Char et al., 2023). The presence of a hydroxyl group in *m*T is thought to provide it an advantage over other cytokinins because it aids in the synthesis of stable O-glucosides, which can be converted into cytokinins when needed. Moreover, these O-glucosides are capable of quicker translocation, which increases their availability to plant cells (Gantait and Mitra, 2021).

It was also investigated if the *in vitro* shoots could generate increasing number of multiple shoots throughout 6 subculture cycles. Hence, the *in vitro* shoots were grown in MS medium + 1.5 mg/L *m*T and MS medium + 1.0 mg/L BAP. Every subsequent subculture cycle saw an increase in the average number of shoots produced per explant in the MS medium + 1.5 mg/L *m*T. The decrease in apical dominance, which promoted the growth of new shoots, was the cause of this notable rise in the quantity of shoots produced (Kudikala et al., 2020). The fourth subculture cycle produced the greatest number of shoots (20.40); but, following that subculture cycle, the number of shoots produced began to decline. An identical pattern was also noted in BAP-supplemented media, where the quantity of shoots began to decline after the fourth subculture (Fig. 2). According to Konar et al. (2019), the accumulation of inhibitory compounds and a lowered metabolism resulted in a loss in the explant's regeneration capability, resulting in the reduction in number of shoots. After 36 weeks, or six subcultures, 620 shoots were generated in *m*T (1.5 mg/L) medium, while 275 shoots were generated in MS medium + 1.0 mg/L BAP.

3.3. Effect of media type and IBA on in vitro formation of roots

It was observed that in Zingiberaceae members, roots and shoots grew concurrently in the cytokinin-supplemented media. The cytokinin medium did not, however, develop enough roots to support the survival of the plant during acclimatization. Consequently, in order to facilitate the development of a robust root system, the plants were moved to a

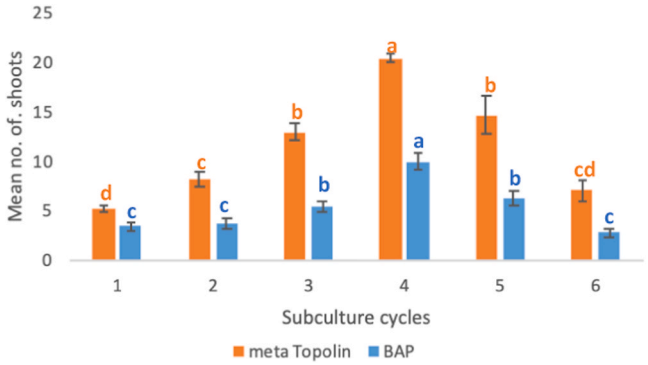

Fig. 2. Shoot regeneration potential of *in vitro* shoots of *C. zedoaria* at various subculture cycles. Each of the *columns* with error bar represents mean±standard deviation value of 3 replicates (each with 10 explants) per treatment. The bars with different *alphabets* are significantly varied according to DMRT at $P \leq 0.05$

medium supplemented with auxins. This improved the formation of *in vitro* roots and made it simpler for the *in vitro* plants to adapt to *ex vitro* environment, potentially improving their survival. When placed in semi-solid and liquid MS media supplemented with different concentrations of IBA (1.0, 1.5, and 2.0 mg/L), the *in vitro* shoots were successfully rooted (Table 3). The presence of endogenous auxins was believed to be the cause of the root induction seen in MS medium lacking auxin (control). Nevertheless, compared to the roots generated in MS medium enriched with auxins, the quantity of roots developed in control medium (2.70) were considerably less. Regardless of the kind of medium (liquid or semi-solid), 1.5 mg/L of IBA produced the greatest number of roots among the various IBA concentrations tested. Table 3 and Fig. 1J indicate that the maximum (10.30) number of roots with the maximum root length of 5.26 cm were produced most effectively in MS semi-solid media containing 1.5 mg/L IBA. While the maximum (7.30) roots with the maximum root length of 3.20 cm were produced in MS liquid medium containing the same concentration of IBA. Nevertheless, the maximum root length was found in semi-solid media with 1.5 mg/L IBA. In the medium supplemented with 2.0 mg/L IBA, a significant reduction in root number (5.30) was noted. This resulted from increased quantities of exogenous auxins inhibiting endogenous auxin. Baradwaj et al. (2017) noted a similar pattern in *Alpinia galanga*, where the development of *in vitro* roots was negatively impacted by 2.0 mg/L IBA.

Table 3
Effect of varying concentrations of indole-3-butyric acid (IBA) on *in vitro* root formation in *C. zedoaria* after 4 weeks of inoculation

Media type	IBA (mg/L)	Root number	Root length (cm)	Root diameter (mm)	Root volume (unit)
Semi-solid	0	2.70±0.60 d	2.30 ±0.30 c	0.88±0.08 ef	0.19±0.02 a
	1.0	6.60±1.20 b	3.82±1.50 b	1.18±0.12 bc	0.16±0.03 a
	1.5	10.30 ±2.10 a	5.26±0.60 a	1.31±0.08 b	0.20±0.03 a
	2.0	5.30±0.60 bc	3.27±0.65 bc	1.50±0.06 a	0.24±0.07 a
Liquid	0	2.30±0.50 d	2.00 ±0.90 c	0.69±0.04 g	0.06±0.02 a
	1.0	5.70± 0.60 bc	2.13 ±0.20 c	0.79±0.10 fg	0.09±0.01 a
	1.5	7.30±2.10 b	3.20±0.98 bc	1.02±0.02 de	0.15±0.03 a
	2.0	3.70 ±0.60 cd	2.80±0.10 bc	1.15±0.09 cd	0.39±0.09 a

Data indicates mean±standard deviation values of 3 replicates (each with 10 explants) per treatment. Data represented in each of the *columns* with different *alphabets* are significantly varied according to DMRT at $P \leq 0.05$

Research on *C. longa* by Bandara et al. (2021) similarly discussed the detrimental effects of increased auxin concentration on root production. In contrast, the largest root diameter (1.50 mm) was observed in semi-solid MS medium that was enhanced with 2.0 mg/L IBA. The *in vitro* roots volume was not significantly influenced by the kind of media or IBA.

3.4. Effect of different potting media on acclimatization of *C. zedoaria* plants

Acclimatization is the progressive transition of *in vitro* propagated plantlets to *ex vitro* settings. Acclimatization of *in vitro* grown plantlets improves their ability to endure severe *ex vitro* circumstances, which is necessary for successful plant growth in the field (Shamsudheen et al., 2018).

The study revealed that the plants that were acclimated to rice husk (3:1) had the highest survival (86 %) (Fig. 1K). The potential of rice husk to retain water and their ability to offer adequate aeration were the reasons behind the increased survival percentage of plantlets. According to reports on *C. zedoaria* (Loc et al., 2005) and *K. marginata* (100 %) (Saensouk et al., 2016), the use of soil and rice husk mixture as media is suitable for increased survival of *in vitro* plants during acclimatization. Plants that were acclimatized in soil had a 60 % survival. With their leaves becoming yellow and the majority of the plants dying within 2 weeks of acclimatization, plants acclimatized in soil: sand (2:1) were unable to survive and had the lowest survival (Fig. 3). The reason for the plant death was that the sand substratum impeded the proper movement of nutrients and oxygen to the roots. Similar findings were reported for *K. galanga* (Senarath et al., 2017) and *Lavandula angustifolia* (Kirimer et al., 2017).

3.5. Genetic fidelity

3.5.1. Using molecular markers

C. zedoaria plants that had been regenerated *in vitro* were tested for genetic variation. There were distinct and repeatable bands from all 5 ISSR primers, which could be scored and had sizes between 250 and 3000 bp. However, out of the five SCoT primers, only three (SCoT-1, SCoT-2, and SCoT-3) yielded well-resolved bands. The SCoT primers generated distinct scorable bands, with sizes varying from 400 to 3000 bp. Three DAMD (M13, INS, URP9F) and CDDP (KNOX-02, ERF1, ERF2) primers yielded distinct and scoreable bands with sizes ranging from 250 bp to 2800 bp (Table 4). *In vitro*-regenerated *C. zedoaria* plants produced a total of 756 bands, all of which were monomorphic, suggesting that there was no genetic variation (Fig. 4). This shown that even after six subculture cycles, the plants developed via this technique are genetically homogeneous.

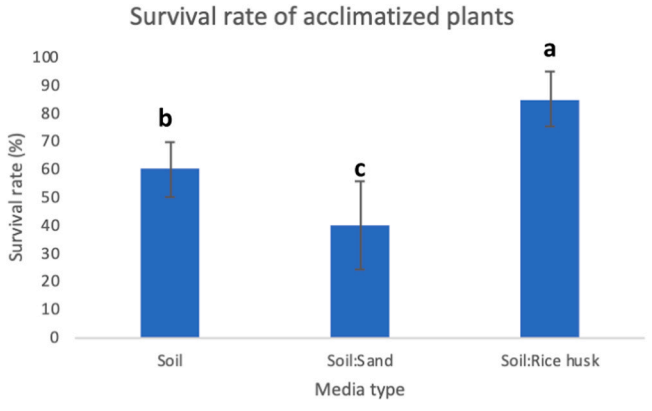

Fig. 3. Survival rate of *in vitro*-propagated *C. zedoaria* plants acclimatized in different potting media after 4 weeks. Each of the *columns* with error bar represents mean±standard deviation value of 10 replicates (each with 10 plantlet) per treatment. The bars with different *alphabets* are significantly varied according to DMRT at $P \leq 0.05$

Similar investigations were carried out on *Rheum rhabarbrum* (Clapa et al., 2020) and *Simmondsia chinensis* (Kumar et al., 2011), where genetic fidelity after many subcultures was assessed using random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers. For *R. rhabarbrum* and *S. chinensis*, polymorphism was not found until the ninth and twelfth subculture cycles, respectively. On the other hand, genetic variation was observed to set in after the eighth subculture cycle and continued to increase with each successive cycle in the instance of Banana (Khan et al., 2011). The regeneration capacity of *Hibiscus sabdariffa* (Konar et al., 2019) was investigated up until the eighth subculture cycle. Following the fifth subculture cycle, changes were noted in the ISSR and RAPD banding profiles, while the bands produced by DAMD markers remained monomorphic until the eighth subculture.

3.5.2. Flow cytometry

FCM is typically used to evaluate the DNA content, ploidy stability, and genome size in order to further establish the genetic stability of the *in vitro*-propagated plants. Therefore, in order to verify the genetic stability of *C. zedoaria in vitro* regenerants, FCM was used in this investigation. It was discovered that the histogram peaks of DNA content of plants grown conventionally and those grown under *in vitro* condition were the same (Fig. 5). It could be said that the *in vitro*-regenerated plants were genetically analogous to the plants that were conventionally propagated because no change in ploidy levels was found. This result was in contrast to Jena et al. (2020) earlier study on the micropropagation of *C. zedoaria*, which showed a minor difference in the 2 C DNA content between the field-grown plant and the *in vitro*-propagated plant. In plants like *Jatropha curcas* (Rathore et al., 2014), Sugarcane (Nogueira et al., 2015), *R. serpentina* (Zafar et al., 2019), and *Juniperus phoenica* (Loureiro et al., 2007), the use of FCM for genetic fidelity assessment of plants after multiple subculture cycles has been reported. The *in vitro*-propagated plants in these situations had the same histogram peaks as the field-grown plants following several subculture cycles. After 20 subculture cycles, an examination using FCM showed that the genetic makeup of the *in vitro* propagated *Jatropha* plants remained unchanged. Similarly, in *R. serpentina* (Zafar et al., 2019), up until the fifth subculture, the 2 C DNA content of the *in vitro*-propagated plant was the same as the mother plant.

3.6. Effect of auxins on induction of adventitious roots

Study was conducted on the impact of different NAA and IBA concentrations on induction of adventitious roots from *C. zedoaria in vitro* shoot. The amount of time taken to induce adventitious roots, quantity of roots,root response, and FW were found to be strongly impacted by the auxins NAA and IBA, at various concentrations. The optimum auxin concentration for initiating early root production (10.00 days) with 100 % response and a maximum number of roots (7.60) was found to be 1.5 mg/L NAA, out of all the concentrations of IBA and NAA examined (Fig. 6C). Additionally, the medium supplemented with 1.5 mg/L of NAA showed increased accumulation of FW (0.23 g) (Table 5). In all examined IBA concentrations, root response, number of roots, and FW were significantly lower than those of NAA; the lowest root response (50 %) and lowest FW (0.07 g) were seen in 0.5 mg/L IBA. The amount of FW generated, root response, and root number did not change substantially in medium supplemented with 0.5 or 1.0 mg/L NAA. However, in comparison with the control medium (MS media lacking auxins), NAA and IBA enhanced root induction. The control medium showed the lowest root response (42 %) and the longest induction period (22.00 days). This control media also had the lowest measured FW (0.13 g) and number of roots (3.33).

The induction and differentiation processes in rooting were exogenously stimulated by the addition of certain auxins. In several plants,

Table 4
ISSR, SCoT, DAMD, CDDP primers used for evaluation of genetic fidelity of the micropropagated plants of *C. zedoaria*, their sequence, melting temperature (T_m), number of bands, number of polymorphic bands and size of amplified products.

Markers	Primers	Sequence (5′-3′)	T_m (°C)	Total no. of bands	No. of polymorphic bands	Size (bp)
ISSR	UBC866	CTCCTCCTCCTCTCTCTC	57	32	0	1000–400
	UBC825	ACACACACACACACACT	51	40	0	900–400
	ISSCR5	CACACACACACAAC	52	72	0	1300–300
	UBC847	CACACACACACACACARC	52	72	0	3000–300
	C6	GAACGGACTC	52	80	0	2500–250
SCoT	SCoT−1	CAACAATGGCTACCACCA	52	32	0	3000–800
	SCoT−2	CAACAATGGCTACCACCC	52	48	0	3000–400
	SCoT−3	ACGACATGGCGACCCACA	52	40	0	2800–850
DAMD	M13	GAGGGTGGCGGCTCT	55	72	0	2000–200
	INS	ACAGGGGTGGGG	48	48	0	1000–250
	URP9F	ATGTGTGCGATCAGTTGCTG	52	38	0	2800–650
CDDP	KNOX−02	CACTGGTGGGAGCTSCAC	55	46	0	2500–700
	ERF1	CACTACCGCGGSCTSCG	58	56	0	2800–350
	ERF2	GCSGAGATCCGSGACCC	55	80	0	2600–380

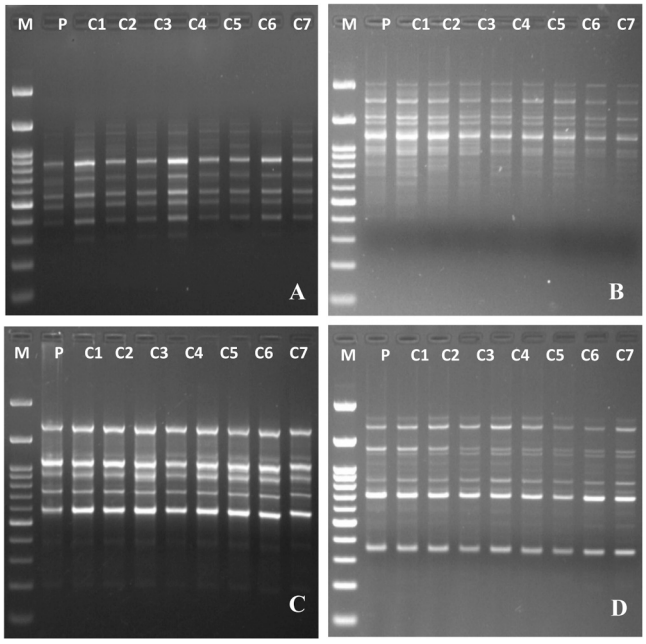

Fig. 4. Representative banding profiles of mother and micropropagated plants of *C. zedoaria* using (A) ISSR primer (ISSCR 5); (B) SCoT primer (SCoT 2); (C) DAMD primer (M13); (D) CDDP primer (ERF1); Lane P - mother plant, Lane C1–C7 micropropagated plants. M is 100 bp ladder

NAA proved successful in establishing adventitious roots. According to Khan et al. (2017), NAA has a greater capacity for induction than IBA. This was consistent with the study's findings, which showed that NAA was superior to IBA in inducing adventitious roots in *C. zedoaria*. This is because, in comparison to other auxins, NAA was more stable and persistent and hence more effective at inducing the cellular responses necessary for root growth and development (Hao et al., 2021). According to Saeed et al. (2017) and Khan et al. (2017), for instance, *Ajuga bracteosa* and *Fagonia indica*, respectively, had root induction rates of 47 % and 58 %. NAA increased both the number of roots obtained from each explant as well as the root response in *Boerhaavia diffusa* (Jenifer et al., 2012) and *Aloe vera* (Lee et al., 2011). It has also been observed that plants grown in culture medium can rapidly absorb NAA (Saeed et al., 2017). Peeters et al. (1991), who identified that tobacco absorbed NAA six times more quickly than other auxins, supported this. Additionally, plants such as *Tripterygium wilfordii* (Zhang et al., 2020) and *Andrographis paniculata* (Praveen et al., 2009) showed improved biomass accumulation when treated with NAA. While a 4-fold rise in root biomass of *Prunella vulgaris* was reported by Fazal et al. (2014) after treatment with 1.0 mg/L of NAA and a 2.3-fold increase in *Gynura procumbens* (Saiman et al., 2012) was observed in media containing 5.0 mg/L of NAA.

3.7. Effect of MS strength on the proliferation of adventitious root culture

Both the establishment and proliferation of adventitious roots and the accumulation of phytochemicals were facilitated to a great extent by the salt content of the media. Thus, induced roots weighing 0.1 g from the prior experiment were inoculated into media containing 4 different MS salt strengths (¼, ½, ¾, and 1) in order to ascertain the impact of salt strength on root growth and proliferation including accumulation of total phenol and flavonoid content. After five weeks of inoculation, the root fresh and dry biomass in MS media with ¼ strength had the highest mean values, 1.98 g and 0.99 g, respectively (Fig. 6G); this was followed by ½ MS strength, which produced 1.58 g FW and 0.78 g DW, respectively (Table 6). With an average of 0.50 g FW and 0.24 g DW, respectively, there was a noticeably decreased amount of root biomass observed in full strength MS media (Fig. 6F). In addition to root development, salt strength affected the adventitious root culture's accumulation of phytochemical content. The TPC ranged from 3.41 to 4.36 mg GAE/g DW. The medium with ¼ salt strength had the highest TPC (4.36 mg GAE/g DW), and there was no significant difference between the TPC measured in the other salt strengths. The TFC ranged from 5.16 to 7.43 mg QE/g DW, with ¼ salt strength yielding maximum (7.43 mg QE/g DW) flavonoid content. TFC too was found to not significantly differ in ½, ¾ and 1x salt strength.

Root biomass accumulation was found to decrease considerably as salt strength increased. The outcome supported the hypothesis put forth by Danesh et al. (2006), according to which MS medium with higher salt strength meant higher nitrogen content, which raised the media's pH and inhibited root growth and caused browning. Conversely, reduced salinity enhanced root development by making more ions and mineral nutrients from the medium available. Comparable to the outcomes of this study, ¼MS strength was also optimal for biomass accumulation in *Morinda citrifolia* (Baque et al., 2010), *Plumbago rosea* (Silja and Satheeshkumar, 2015), and *B. rotunda* (Yusuf et al., 2018). For enhanced root biomass, ½MS strength was ideal in plants such as *C. mangga* (Soundar Raju et al., 2015) and *Hypericum perforatum* (Cui et al., 2010). While it was previously recognized that increased salt strength could impede root growth by creating stress, maximum root growth was observed in media with full MS strength for *B. diffusa* (Jenifer et al., 2012) and *A. vera* (Lee et al., 2011). The media with the lowest salt strength (¼MS) in this investigation showed the maximum root biomass and phytochemical accumulation. The maximum root biomass and phytochemical accumulation were found in the media with the same salt strength (¼MS) in *B. rotunda* (Yusuf et al., 2018) and *M. citrifolia* (Baque et al., 2010). However, this was not same in the case of *Plumbago rosea*,

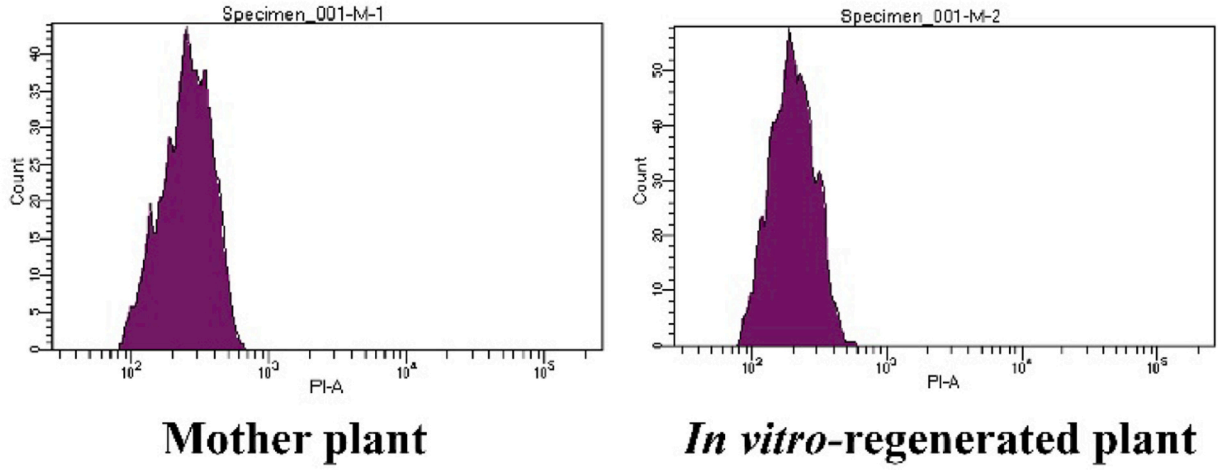

Fig. 5. Flow cytometry analysis of *C. zedoaria* plantlets showing their ploidy levels**.** Histograms of relative nuclear DNA content isolated from leaves of mother plant and *in vitro*-regenerated plant

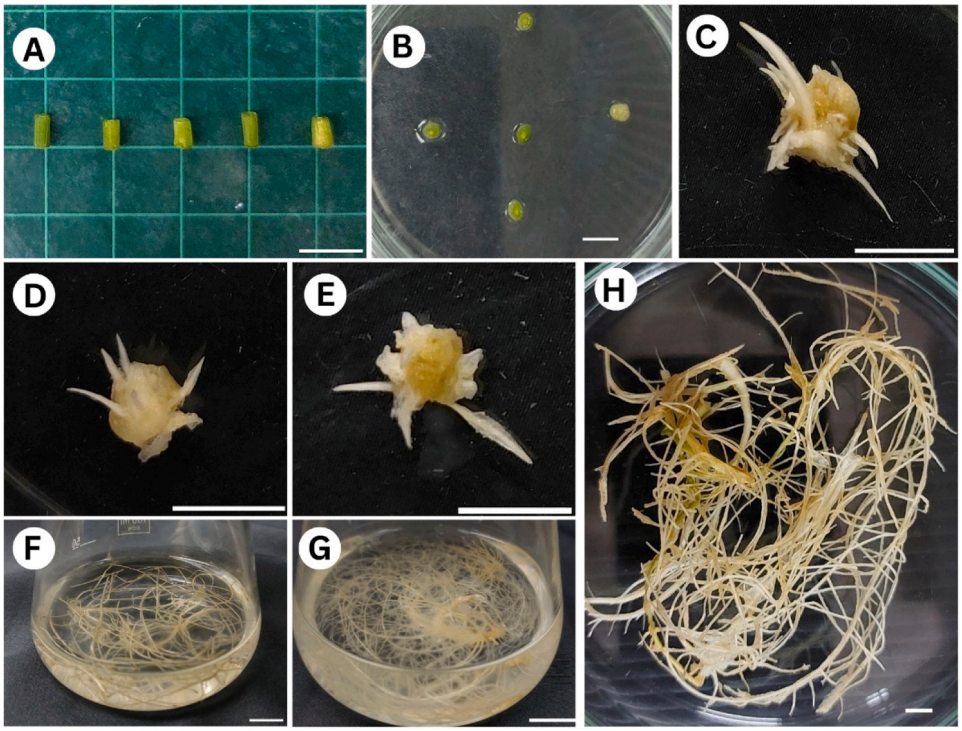

Fig. 6. Adventitious root culture of *C. zedoaria*. (A) *In vitro* shoots cut to a size of 0.5 cm for induction of adventitious roots; (B) explants inoculated in root induction media; (C—E) induction of adventitious roots at 2 weeks in MS medium + 1.5 mg/L NAA, 1.5 mg/L IBA and control; (F) proliferated roots in ¼MS medium + 1.5 mg/L NAA after 5 weeks; (G) proliferated roots in MS medium + 1.5 mg/L NAA; (H) proliferated roots obtained from ¼MS medium + 1.5 mg/L NAA after harvested after 5 weeks. (Scale bar = 1 cm)

where ¼MS salt strength was ideal for increasing biomass content, but the maximum phytochemical accumulation was recorded in ¾MS strength medium (Silja and Satheeshkumar, 2015). Similar results were observed in the case of *Tripterygium wilfordii*, where ½MS medium improved root growth, but the celastrol level was higher in full strength MS medium (Zhang et al., 2020). On the contrary, a reduced (½MS) medium strength resulted in improved Withenolide production from *Withania somnifera* (Sivanandhan et al., 2012). In the present study, ¼MS medium provided the maximum root biomass, TPC, and TFC, so it was employed in the future experiment for eliciting root culture to further boost the phytochemical content.

3.8. Elicitation of adventitious root culture for enhanced production of phytochemicals

Elicitors were exogenously added to adventitious root cultures to improve the biosynthesis of phytochemicals in the roots. We investigated the impact of two elicitors on phytochemical accumulation in adventitious roots of *C. zedoaria*: MeJ (200, 400, 600 μM) and SA (100, 200, 300 μM). Table 7 shows a considerable increase in the root biomass (FW and DW) following the application of elicitors. In comparison to the control treatment, the biomass produced by each of the elicitor treatments examined was higher. The root FW (1.95 g) was substantially higher in medium with MeJ at 400 μM than in the control medium

Table 5
Induction of adventitious roots of *C. zedoaria* in MS medium supplemented with different concentrations of α-naphthalene acetic acid (NAA) and indole-3-butyric acid (IBA)

Auxins (mg/L)		Root response (%)	Days for induction	No. of roots	Fresh weight (g)
	0	42.00±2.00 e	22.00±1.00 e	3.33 ±0.57 f	0.13±0.02 bcd
NAA	0.5	77.00±5.00 b	17.00±1.70 cd	5.00±1.00 bc	0.16±0.06 b
	1.0	83.00±5.00 b	12.00±2.00 ab	5.30±0.57 b	0.17±0.02 b
	1.5	100.00±0.00 a	10.00±0.00 a	7.60±0.57 a	0.23±0.01 a
	2.0	81.00±2.00 b	13.00±1.40 abc	4.28±0.28 cde	0.14±0.01 bc
IBA	0.5	50.00±1.00 d	19.00±2.40 de	3.99±0.06 def	0.07±0.01 e
	1.0	53.00±1.00 d	19.00±1.63 de	4.37±0.08 cde	0.08±0.02 de
	1.5	57.00±0.80 cd	18.00±0.00 de	4.88±0.10 bcd	0.11±0.03 cde
	2.0	62.00±2.00 c	16.00±3.70 cde	3.71±0.11 ef	0.12±0.01 bcde

Data indicates mean±standard deviation values of 3 replicates (each with 10 explants) per treatment. Data represented in each of the *columns* with different *alphabets* are significantly varied according to DMRT at $P \leq 0.05$

Table 6
Effect of MS media with varying strength supplemented with 1.5 mg/L α-naphthalene acetic acid (NAA) on proliferation of adventitious root culture of *C. zedoaria* after 5 weeks of inoculation

Treatments	Fresh weight (g)	Dry weight (g)	TPC (mg GAE/g DW)	TFC (mg QE/g DW)
¼ MS	1.98±0.29 a	0.99±0.15 a	4.36±0.25 a	7.43±0.63 a
½ MS	1.58±0.21 b	0.78±0.11 b	3.58±0.32 b	5.48±0.05 b
¾ MS	1.09±0.09 c	0.53±0.03 c	3.65±0.23 b	5.24±0.30 b
Full MS	0.50±0.04 d	0.24±0.03 d	3.41±0.12 b	5.16±0.52 b

Data indicates mean±standard deviation values of 3 replicates (each with 10 explant) per treatment. Data represented in each of the *columns* with different *alphabets* are significantly varied according to DMRT at $P \leq 0.05$

Table 7
Effect of elicitors (in ¼MS medium with 1.5 mg/L α-naphthalene acetic acid) i.e. methyl jasmonate (MeJ) and salicylic acid (SA), on biomass accumulation, total phenolic content (TPC), total flavonoid content (TFC) in the root culture of *C. zedoaria*

Treatment		Fresh weight (g)	Dry weight (g)	TPC (mg GAE/g)	TFC (mg QE/g)
Control		0.99±0.15 b	0.49±0.02 b	3.45±0.20 d	5.48±0.15 c
MeJ	200 μM	1.92±0.17 a	0.70±0.04 a	3.60±0.10 d	7.72±0.40 b
	400 μM	1.95±0.02 a	0.87±0.06 ab	5.55±0.19 a	9.85±1.40 a
	600 μM	1.63±0.02c	0.83±0.08 b	4.61±0.13 b	7.3±1.23 b
SA	100 μM	1.89±0.13 a	0.69±0.10 ab	3.52±0.11 bc	6.94±0.18 bc
	200 μM	1.87±0.13 a	0.82±0.09 ab	3.71±0.1 bc	6.99±0.56 bc
	300 μM	1.44±0.15 d	0.84±0.12 c	4.14±0.1 bc	6.36±0.23 bc

Data indicates mean±standard deviation values of 3 replicates (each with 10 explants) per treatment. Data represented in each of the *columns* with different *alphabets* are significantly varied according to DMRT at $P \leq 0.05$

(0.99 g). FW (1.92 g) was similarly increased by the addition of 200 μM MeJ. The biomass generated in the 200 μM and 400 μM MeJ-supplemented media did not, however, differ significantly. In cultures treated with 600 μM MeJ, a significantly lesser root FW (1.63 g) was observed. Nevertheless, it remained significantly higher than the root FW found in the control media. In the root cultures of *C. zedoaria*, different concentrations of SA also had a favourable impact on the accumulation of root biomass. Root cultures supplemented with 100 μM and 200 μM SA produced the maximum fresh root biomass (1.89 g and1.87 g, respectively), whereas 300 μM SA produced a lowe root biomass (1.44 g) (Table 7). MeJ and SA both improved root growth; cultures treated with 200 and 400 μM of MeJ and 100 and 200 μM of SA showed increased FW. By raising the elicitor concentration, the root growth in both cases of elicitors declined. Following MeJ elicitation, the TPC in the adventitious root culture varied from 3.60 to 5.55 mg GAE/g DW. 400 μM MeJ produced the highest TPC (5.55 mg GAE/g DW). The medium with 200 μM MeJ showed the lowest TPC of 3.60 mg GAE/g DW. There was no significant difference seen between the TPC derived from 200 μM MeJ (3.60 mg GAE/g DW) and control medium (3.45 mg GAE/g DW). The phenolic content was shown to increase when the concentration of MeJ was raised from 200 to 400 μM, but to decrease when the concentration was raised to 600 μM. After being elicited with SA, the TPC in the adventitious root culture varied between 3.52 and 4.14 mg GAE/g DW. They did not, however, prove to be substantially different across the range of SA concentrations examined. In adventitious root cultures, TFC concentrations in MeJ augmented media, varied from 7.30 to 9.85 mg QE/g DW. The medium that had 400 μM MeJ added to it had the highest TFC (9.85 mg QE/g DW). There was no significant difference in TFC at 200 μM (7.72 mg QE/g DW) and 600 μM MeJ (7.30 mg QE/g DW). The flavonoid content was shown to increase when the concentration of MeJ was raised from 200 to 400 μM, but to decrease when the concentration was raised to 600 μM. There was no significant difference between the TFC derived from the control media and that derived from the media supplemented with various concentrations of SA. The media augmented with 400 μM MeJ exhibited the highest total phenolic and flavonoid content. Thus, in the adventitious root culture of *C. zedoaria*, 400 μM of MeJ can be utilized to enhance the accumulation of root biomass and phytochemical content. Additionally, compared to the total flavonoid and phenol content found in the rhizome, the increase in total flavonoid and phenol accumulation in root culture following elicitation was two-fold.

Eleutherococcus koreanum (Lee et al., 2015), *P. rosea* (Silja and Satheeshkumar, 2015), and *F. indica* (Khan et al., 2017) have all been shown to exhibit MeJ-induced increases in root biomass. On treating *Artemisia absinthium* with 2.0 mg/L MeJ, Ali et al. (2015) observed a three-fold increase in biomass. The triggering response caused by the presence of MeJ on endogenous indole-3-acetic acid synthesis can be connected to biomass buildup following elicitation (Saeed et al., 2017). Nevertheless, a number of investigations found that root growth was reduced by a higher MeJ concentration. Alwakil et al. (2022), for instance, observed that in *Z. zerumbet*, the production of root biomass dropped by 66 % at higher doses of MeJ (600–1200 μM). Furthermore, studies in *Ajuga bracteosa* (Saeed et al., 2017) and *F. indica* (Khan et al., 2017) also found that greater concentrations of MeJ inhibited biomass accumulation. According to Zhang et al. (2020), SA, was found to have no effect on root growth at lower concentrations but to restrict biomass accumulation in *T. wilfordii* at higher concentrations. *Ajuga bracteosa* (Saeed et al., 2017) and *S. rebaudiana* (Kazmi et al., 2019) have both shown improvements in their total phenolic and total flavonoid contents upon elicitation of their root cultures with MeJ. In general, higher total phenolic and flavonoid content is associated with higher levels of certain bioactive chemicals. In *C. longa*, there was found to be a positive association between the curcumin content and total phenolic content (Muflihah et al., 2021). Likewise, in *Phaleria macrocarpa*, a positive linear association was found between mangiferin and TPC and TFC (Lim et al., 2019). According to Wu et al. (2008), MeJ enhanced the ginsenoside content in ginseng by eight times while having no effect on the total phenolic and flavonoid content. According to Zhang et al. (2020), MeJ also caused a 2.3-fold increase in the celastrol level of *T. wilfordii*. According to Rodríguez-Sánchez et al. (2020), SA increased the amount of 5-hydroxymethylfurfural, phenol, and (*Z*)-9-octadecenamide content

in *Piper cumanense*. Root culture stimulated with SA was found to enhance withenolide in *W. somnifera* (Sivanandhan et al., 2012) and bacoside in *Bacopa monnieri* (Sharma et al., 2015) by 1.32 folds. Moreover, zerumbone and α-humulene production in *Z. zerumbet* was found to be enhanced by the synergistic action of MeJ and SA (Alwakil et al., 2022).

4. Conclusion

A protocol for the rapid propagation of *C. zedoaria* was established. The use of the newly discovered cytokinin *m*T, which was found to be more promising than BAP at encouraging multiple shoot development in *C. zedoaria* is described in this article for the first time. The maximum (5.4) shoot count was observed in MS medium + 1.5 mg/l *m*T and at the end of 36 weeks yielded an average of 620 shoots from a single explant, whereas BAP only produced an average of 275 shoots. Even after six subculture cycles, *in vitro* plants remained genetically similar, according to genetic fidelity studies conducted using FCM and molecular markers. This indicates the viability of this propagation procedure in large-scale production of genetically homogenous *C. zedoaria*. Moreover, this work is the first account of adventitious root culture establishment in *C. zedoaria*. Direct adventitious root formation from shoot explants requires auxin. MS medium + 1.5 mg/L NAA produced the best rooting response and highest number of roots. The best conditions for accumulation of biomass and total phenolic and flavonoid content were found in liquid MS medium with ¼ strength supplemented with 1.5 mg/L NAA. In adventitious root cultures of *C. zedoaria*, elicitation was found to be a successful method for increasing the phytochemical content, wherein, higher levels of total phenolic and flavonoid contents were achieved using MeJ and SA. Thus, this research paved way for mass production of roots by adventitious root culture, where, the phytochemical composition of roots was enhanced using elicitors.

Funding

This research was funded by the Ministry of Higher Education Malaysia under the Fundamental Research Grant Scheme (FRGS) FRGS/1/2020/STG03/UPM/01/1.

CRediT authorship contribution statement

Juju Nakasha Jaafar: Supervision, Methodology. **Saikat Gantait:** Writing – original draft, Supervision, Methodology, Formal analysis, Data curation, Conceptualization. **Uma Rani Sinniah:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization. **Mohd Firdaus Ismail:** Supervision, Methodology. **Meenakshi Subramanian:** Writing – original draft, Methodology, Formal analysis, Data curation.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgement

The authors are grateful to the Department of Crop Science, Faculty of Agriculture of Universiti Putra Malaysia for the facilities and to Ms. Tsama Subrahmanyeswari and Mr. Sandipan Bandyopadhyay for their help in genetic fidelity analysis.

Data availability

Data will be made available on request.

References

Ali, M., Abbasi, B.H., Ali, G.S., 2015. Elicitation of antioxidant secondary metabolites with jasmonates and gibberellic acid in cell suspension cultures of *Artemisia absinthium* L. Plant Cell Tissue Organ Cult. 120, 1099–1106. https://doi.org/10.1007/s11240-014-0666-2.

Alwakil, N.H., Mohamad Annuar, M.S., Jalil, M., 2022. Synergistic effects of plant growth regulators and elicitors on α-humulene and zerumbone production in *Zingiber zerumbet* Smith adventitious root cultures. Molecules 27, 4744. https://doi.org/10.3390/molecules27154744.

Bandara, M.M.N.T., Dahanayake, N., Subasinghe, S., Perera, P.C.D., 2021. A review on *in vitro* propagation of turmeric (*Curcuma longa* ln.). J. Univ. Ruhuna 9, 39–46. https://doi.org/10.4038/jur.v9i1.7975.

Banisalam, B., Sani, W., Philip, K., Imdadul, H., Khorasani, A., 2011. Comparison between *in vitro* and *in vivo* antibacterial activity of *Curcuma zedoaria* from Malaysia. Afr. J. Biotechnol. 10, 11676–11681.

Baque, M.A., Hahn, E.J., Paek, K.Y., 2010. Growth, secondary metabolite production and antioxidant enzyme response of *Morinda citrifolia* adventitious root as affected by auxin and cytokinin. Plant Biotechnol. Rep. 4, 109–116. https://doi.org/10.1007/s11816-009-0121-8.

Baradwaj, R.G., Rao, M.V., Senthil, K.T., 2017. Curbing actinomycetes and thidiazuron enhanced micropropagation in the rare *Alpinia galanga*–a medicinal zingiber. Asian J. Pharm. Clin. Res. 10, 153–158.

Behera, S., Kar, S.K., Rout, K.K., Barik, D.P., Panda, P.C., Naik, S.K., 2019. Assessment of genetic and biochemical fidelity of field-established *Hedychium coronarium* J. Koenig regenerated from axenic cotyledonary node on meta-topolin supplemented medium. Ind. Crop. Prod. 134, 206–215. https://doi.org/10.1016/j.indcrop.2019.03.051.

Behera, S., Monalisa, K., Meher, R.K., Mohapatra, S., Madkami, S.K., Das, P.K., Naik, P. K., Naik, S.K., 2022. Phytochemical fidelity and therapeutic activity of micropropagated *Curcuma amada* Roxb.: a valuable medicinal herb. Ind. Crop. Prod. 176, 114401. https://doi.org/10.1016/j.indcrop.2021.114401.

Bharalee, R., Das, A., Kalita, M.C., 2005. *In vitro* clonal propagation of *Curcuma caesia* Roxb and *Curcuma zedoaria* Rosc from rhizome bud explants. J. Plant Biochem. Biotechnol. 14, 61–63. https://doi.org/10.1007/BF03263228.

Char, M., Subrahmanyeswari, T., Bhattacharyya, S., Gantait, S., 2023. *meta*-Topolin-induced *in vitro* propagation, field evaluation, flow cytometry and molecular marker-based genetic stability assessment of potato cv. Badami alu. Plant Cell Tissue Organ Cult. 155, 809–826. https://doi.org/10.1007/s11240-023-02601-8.

Chong, Y.H., Khalafalla, M.M., Bhatt, A., Chan, L.K., 2012. The effects of culture systems and explant incision on *in vitro* propagation of *Curcuma zedoaria* Roscoe. Pertanika J. Trop. Agric. Sci. 35, 863–874.

Clapa, D., Borsai, O., Hârța, M., Bonta, V., Szabo, K., Coman, V., Bobiș, O., 2020. Micropropagation, genetic fidelity and phenolic compound production of *Rheum rhabarbarum* L. Plants 9, 656. https://doi.org/10.3390/plants9050656.

Cui, X.H., Murthy, H.N., Wu, C.H., Paek, K.Y., 2010. Sucrose-induced osmotic stress affects biomass, metabolite, and antioxidant levels in root suspension cultures of *Hypericum perforatum* L. Plant Cell Tissue Organ Cult. 103, 7–14. https://doi.org/10.1007/s11240-010-9747-z.

Danesh, Y.R., Goltapeh, E.M., Alizadeh, A., Sanavy, M.M., 2006. Optimizing carrot hairy root production for monoxenic culture of arbuscular mycorrhizal fungi in Iran. J. Biol. Sci. 6, 87–91. https://doi.org/10.3923/jbs.2006.87.91.

Dey, A., Nandy, S., Nongdam, P., Tikendra, L., Mukherjee, A., Mukherjee, S., Pandey, D. K., 2020. Methyl jasmonate and salicylic acid elicit indole alkaloid production and modulate antioxidant defence and biocidal properties in *Rauvolfia serpentina* Benth. ex Kurz. *in vitro* cultures. S. Afr. J. Bot. 135, 1–17. https://doi.org/10.1016/j.sajb.2020.07.020.

Duncan, D.B., 1955. Multiple range and multiple F tests. Biometrics 11, 1–42. https://doi.org/10.2307/3001478.

Elayaraja, D., Subramanyam, K., Vasudevan, V., Sathish, S., Kasthurirengan, S., Ganapathi, A., Manickavasagam, M., 2019. Meta-Topolin (*m*T) enhances the *in vitro* regeneration frequency of *Sesamum indicum* (L.). Biocatal. Agric. Biotechnol. 21, 101320. https://doi.org/10.1016/j.bcab.2019.101320.

Fazal, H., Abbasi, B.H., Ahmad, N., 2014. Optimization of adventitious root culture for production of biomass and secondary metabolites in *Prunella vulgaris* L. Appl. Biochem. Biotechnol. 174, 2086–2095. https://doi.org/10.1007/s12010-014-1190-x.

Gantait, S., Mitra, M., 2021. Role of meta-topolin on *in vitro* shoot regeneration: an insight. In: Ahmad, N., Strnad, M. (Eds.), *Meta*-topolin: a growth regulator for plant biotechnology and agriculture. Springer, Singapore, pp. 143–168. https://doi.org/10.1007/978-981-15-9046-7_12.

Hao, N., Piao, Z., Zang, J., Li, H., Zhou, R., 2021. Establishment of adventitious root cultures and assessment of secoiridoid production in the Chinese medicinal plant *Gentiana scabra*. In Vitro Cell. Dev. Biol. Plant 57, 864–873. https://doi.org/10.1007/s11627-021-10186-0.

Jadhao, A.S., Bhuktar, A.S., 2019. Phytochemical screening of rhizome extract of *Curcuma zedoaria* (Christm) Roscoe by HRLC-MS technique. Int. J. Life Sci. A 13, 53–57.

Jayaprakash, K., Manokari, M., Badhepuri, M.K., Raj, M.C., Dey, A., Shekhawat, M.S., 2021. Influence of *meta*-topolin on *in vitro* propagation and foliar micro-morpho-anatomical developments of *Oxystelma esculentum* (Lf) Sm. Plant Cell Tissue Organ Cult. 147, 325–337. https://doi.org/10.1007/s11240-021-02126-y.

Jena, S., Ray, A., Sahoo, A., Sahoo, S., Kar, B., Panda, P.C., Nayak, S., 2018. High-frequency clonal propagation of *Curcuma angustifolia* ensuring genetic fidelity of micropropagated plants. Plant Cell Tissue Organ Cult. 135, 473–486. https://doi.org/10.1007/s11240-018-1480-z.

Jena, S., Ray, A., Sahoo, A., Sahoo, S., Dash, B., Kar, B., Nayak, S., 2020. Rapid plant regeneration in industrially important *Curcuma zedoaria* revealing genetic and biochemical fidelity of the regenerants. 3 Biotech 10, 17. https://doi.org/10.1007/s13205-019-2009-9.

Jenifer, U., Francina Cecilia, K., Ravindhran, R., 2012. *In vitro* adventitious root and hairy root cultures in *Boerhaavia diffusa* L. Int. J. Curr. Res. 4, 65–67.

Kayum, M.A., Qaiyyum, I.A., Jabeen, A., Nawab, M., 2021. A review on pharmacological and therapeutic profile of zaranbad (*Curcuma zedoaria* Rosc.). World J. Pharma. Sci. 9, 60–66. ⟨https://wjpsonline.com/index.php/wjps/article/view/80⟩.

Kazmi, A., Khan, M.A., Mohammad, S., Ali, A., Kamil, A., Arif, M., Ali, H., 2019. Elicitation directed growth and production of steviol glycosides in the adventitious roots of *Stevia rebaudiana* Bertoni. Ind. Crop. Prod. 139, 111530. https://doi.org/10.1016/j.indcrop.2019.111530.

Khan, T., Abbasi, B.H., Khan, M.A., Azeem, M., 2017. Production of biomass and useful compounds through elicitation in adventitious root cultures of *Fagonia indica*. Ind. Crop. Prod. 108, 451–457. https://doi.org/10.1016/j.indcrop.2017.07.019.

Khan, S., Saeed, B., Kauser, N., 2011. Establishment of genetic fidelity of in vitro raised banana plantlets. Pak. J. Bot. 43, 233–242.

Khanam, M.N., Anis, M., Ahmad, S., 2018. Establishment of adventitious root cultures of *Allamanda cathartica* L. for the production of iridoid glycosides and its identification using HPTLC MS. Ind. Crop. Prod. 125, 198–206. https://doi.org/10.1016/j.indcrop.2018.08.044.

Kirimer, N., Mokhtarzadeh, S., Demirci, B., Goger, F., Khawar, K.M., Demirci, F., 2017. Phytochemical profiling of volatile components of *Lavandula angustifolia* Miller propagated under in vitro conditions. Ind. Crop. Prod. 96, 120–125. https://doi.org/10.1016/j.indcrop.2016.11.061.

Konar, S., Adhikari, S., Karmakar, J., Ray, A., Bandyopadhyay, T.K., 2019. Evaluation of subculture ages on organogenic response from root callus and SPAR based genetic fidelity assessment in the regenerants of *Hibiscus sabdariffa* L. Ind. Crop. Prod. 135, 321–329. https://doi.org/10.1016/j.indcrop.2019.04.018.

Krishna Vrundha, C.P., Aswathi, N.V., Thomas, T.D., 2021. The role of meta-topolin in plant morphogenesis in vitro. In: Ahmad, N., Strnad, M. (Eds.), Meta-topolin: a growth regulator for plant biotechnology and agriculture. Springer, Singapore, pp. 93–118. https://doi.org/10.1007/978-981-15-9046-7_10.

Kudikala, H., Jogam, P., Sirikonda, A., Mood, K., Allini, V.R., 2020. In vitro micropropagation and genetic fidelity studies using SCoT and ISSR primers in *Annona reticulata* L.: an important medicinal plant. Vegetos 33, 446–457. https://doi.org/10.1007/s42535-020-00128-3.

Kumar, S., Mangal, M., Dhawan, A.K., Singh, N., 2011. Assessment of genetic fidelity of micropropagated plants of *Simmondsia chinensis* (Link) Schneider using RAPD and ISSR markers. Acta Physiol. Plant. 33, 2541–2545. https://doi.org/10.1007/s11738-011-0767-z.

Lan, T.T.P., Huy, N.D., Luong, N.N., Quang, H., Tan, T.H., Thu, L.T.A., Huy, N.X., Loc, N. H., 2019. Effect of salicylic acid and yeast extract on curcuminoids biosynthesis gene expression and curcumin accumulation in cells of *Curcuma zedoaria*. J. Plant Biotechnol. 46, 172–179. https://doi.org/10.5010/JPB.2019.46.3.172.

Lata, H., Chandra, S., Techen, N., Khan, I.A., ElSohly, M.A., 2016. *In vitro* mass propagation of *Cannabis sativa* L.: A protocol refinement using novel aromatic cytokinin *meta*-topolin and the assessment of eco-physiological, biochemical and genetic fidelity of micropropagated plants. J. Appl. Res. Med. Aromat. Plant. 3, 18–26. https://doi.org/10.1016/j.jarmap.2015.12.001.

Lee, E.J., Park, S.Y., Paek, K.Y., 2015. Enhancement strategies of bioactive compound production in adventitious root cultures of *Eleutherococcus koreanum* Nakai subjected to methyl jasmonate and salicylic acid elicitation through airlift bioreactors. Plant Cell Tissue Organ Cult. 120, 1–10. https://doi.org/10.1007/s11240-014-0567-4.

Lee, Y.S., Yang, T.J., Park, S.U., Baek, J.H., Wu, S., Lim, K.B., 2011. Induction and proliferation of adventitious roots from *Aloe vera* leaf tissues for in vitro production of Aloe-emodin. Plant Omics 4, 190–194. ⟨https://search.informit.org/doi/10.3316/informit.307923778436899⟩.

Lim, Y.P., Pang, S.F., Yusoff, M.M., Mudalip, S.K.A., Gimbun, J., 2019. Correlation between the extraction yield of mangiferin to the antioxidant activity, total phenolic and total flavonoid content of *Phaleria macrocarpa* fruits. J. Appl. Res. Med. Aromat. Plant. 14, 100224. https://doi.org/10.1016/j.jarmap.2019.100224.

Lobo, R., Prabhu, K.S., Shirwaikar, A., Shirwaikar, A., 2009. *Curcuma zedoaria* Rosc. (white turmeric): a review of its chemical, pharmacological and ethnomedicinal properties. J. Pharma. Pharmacol. 61, 13–21. https://doi.org/10.1211/jpp.61.01.0003.

Loc, N.H., Duc, D.T., Kwon, T.H., Yang, M.S., 2005. Micropropagation of zedoary (*Curcuma zedoaria* Roscoe) – a valuable medicinal plant. Plant Cell Tissue Organ Cult. 81, 119–122. https://doi.org/10.1007/s11240-004-3308-2.

Loureiro, J., Capelo, A., Brito, G., Rodriguez, E., Silva, S., Pinto, G., Santos, C., 2007. Micropropagation of *Juniperus phoenicea* from adult plant explants and analysis of ploidy stability using flow cytometry. Biol. Plant. 51, 7–14. https://doi.org/10.1007/s10535-007-0003-2.

Mahanta, M., Gantait, S., Mukherjee, E., Bhattacharyya, S., 2023. *meta*-Topolin-induced mass propagation, acclimatization and cyto-genetic fidelity assessment of gerbera (*Gerbera jamesonii* Bolus ex Hooker f.). S. Afr. J. Bot. 153, 236–245. https://doi.org/10.1016/j.sajb.2022.11.032.

Manokari, M., Priyadharshini, S., Jogam, P., Dey, A., Shekhawat, M.S., 2021. Meta-topolin and liquid medium mediated enhanced micropropagation via *ex vitro* rooting in *Vanilla planifolia* Jacks. ex Andrews. Plant Cell Tissue Organ Cult. 146, 69–82. https://doi.org/10.1007/s11240-021-02044-z.

Mendoza, D., Cuaspud, O., Arias, J.P., Ruiz, O., Arias, M., 2018. Effect of salicylic acid and methyl jasmonate in the production of phenolic compounds in plant cell suspension cultures of *Thevetia peruviana*. Biotechnol. Rep. 19, e00273. https://doi.org/10.1016/j.btre.2018.e00273.

Mishra, J., Bhardwaj, A., Misra, K., 2018. *Curcuma* sp.: the nature's souvenir for high-altitude illness. In: Misra, K., Sharma, P., Bhardwaj, A. (Eds.) Management of high altitude pathophysiology, Academic Press, pp. 153-169. https://doi.org/10.1016/B978-0-12-813999-8.00008-2.

Mohanty, P., Behera, S., Swain, S.S., Barik, D.P., Naik, S.K., 2013. Micropropagation of *Hedychium coronarium* J. Koenig through rhizome bud. Physiol. Mol. Biol. Plant. 19, 605–610. https://doi.org/10.1007/s12298-013-0199-x.

Muflihah, Y.M., Gollavelli, G., Ling, Y.C., 2021. Correlation study of antioxidant activity with phenolic and flavonoid compounds in 12 Indonesian indigenous herbs. Antioxidants 10, 1530. https://doi.org/10.3390/antiox10101530.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Nogueira, G.F., Pio, L.A.S., Pasqual, M., Amaral, A., Scherwinski-Pereira, J.E., 2015. An approach on the *in vitro* maintenance of sugarcane with views for conservation and monitoring of plant nuclear DNA contents via flow cytometry. Vitro Cell. Dev. Biol. -Plant 51, 220–230. https://doi.org/10.1007/s11627-014-9660-y.

Nowakowska, K., Pacholczak, A., 2020. Comparison of the effect of meta-topolin and benzyladenine during *Daphne mezereum* L. micropropagation. Agronomy 10, 1994. https://doi.org/10.3390/agronomy10121994.

Park, H.Y., Kim, K.S., Ak, G., Zengin, G., Cziáky, Z., Jekő, J., Adaikalam, K., Song, K., Kim, D.H., Sivanesan, I., 2021. Establishment of a rapid micropropagation system for *Kaempferia parviflora* wall. Ex Baker: phytochemical analysis of leaf extracts and evaluation of biological activities. Plants 10, 698. https://doi.org/10.3390/plants10040698.

Peeters, A.J., Gerards, W., Barendse, G.W.M., Wullems, G.J., 1991. *In vitro* flower bud formation in tobacco: interaction of hormones. Plant Physiol 97, 402–408. https://doi.org/10.1104/pp.97.1.402.

Phuyal, N., Jha, P.K., Raturi, P.P., Rajbhandary, S., 2020. Total phenolic, flavonoid contents, and antioxidant activities of fruit, seed, and bark extracts of *Zanthoxylum armatum* DC. Sci. World J. 2020, 8780704. https://doi.org/10.1155/2020/8780704.

Praveen, N., Manohar, S.H., Naik, P.M., Nayeem, A., Jeong, J.H., Murthy, H.N., 2009. Production of andrographolide from adventitious root cultures of *Andrographis paniculata*. Curr. Sci. 96, 694–697. ⟨http://www.jstor.org/stable/24104564⟩.

Rahmat, E., Kang, Y., 2019. Adventitious root culture for secondary metabolite production in medicinal plants: a review. J. Plant Biotechnol. 46, 143–157. https://doi.org/10.5010/JPB.2019.46.3.143.

Ranghoo-Sanmukhiya, V.M., 2021. Somaclonal variation and methods used for its detection. In: Siddique, I. (Ed.), Propagation and Genetic Manipulation of Plants. Springer, Singapore, pp. 1–18. ⟨https://doi.org/10.1007/978-981-15-7736-9_1⟩.

Rathore, M.S., Yadav, P., Mastan, S.G., Prakash, C.R., Singh, A., Agarwal, P.K., 2014. Evaluation of genetic homogeneity in tissue culture regenerates of *Jatropha curcas* L. using flow cytometer and DNA-based molecular markers. Appl. Biochem. Biotechnol. 172, 298–310. https://doi.org/10.1007/s12010-013-0517-3.

Rodríguez-Sánchez, L.K., Pérez-Bernal, J.E., Santamaría-Torres, M.A., Marquínez-Casas, X., Cuca-Suárez, L.E., Prieto-Rodríguez, J.A., Patiño-Ladino, O.J., 2020. Effect of methyl jasmonate and salicylic acid on the production of metabolites in cell suspensions cultures of *Piper cumanense* (Piperaceae). Biotechnol. Rep. 28, e00559. https://doi.org/10.1016/j.btre.2020.e00559.

Saeed, S., Ali, H., Khan, T., Kayani, W., Khan, M.A., 2017. Impacts of methyl jasmonate and phenyl acetic acid on biomass accumulation and antioxidant potential in adventitious roots of *Ajuga bracteosa* Wall ex Benth., a high valued endangered medicinal plant. Physiol. Mol. Biol. Plant. 23, 229–237. https://doi.org/10.1007/s12298-016-0406-7.

Saensouk, P., Muangsan, N., Saensouk, S., Sirinajun, P., 2016. In vitro propagation of *Kaempferia marginata* Carey ex Roscoe, a native plant species to Thailand. J. Anim. Plant Sci. 26, 1405–1410.

Saiman, M.Z., Mustafa, N.R., Schulte, A.E., Verpoorte, R., Choi, Y.H., 2012. Induction, characterization, and NMR-based metabolic profiling of adventitious root cultures from leaf explants of *Gynura procumbens*. Plant Cell Tissue Organ Cult. 109, 465–475. https://doi.org/10.1007/s11240-011-0111-8.

Sarma, I., Deka, A.C., Sarma, S., Sarma, T.C., 2011. High frequency clonal propagation and microrhizome induction of *Curcuma longa* L. (cv lakadong)-a rich source of curcumin of North East India. BioScan 6, 11–18.

Senarath, R.M.U.S., Karunarathna, B.M.A.C., Senarath, W.T.P.S.K., Jimmy, G.C., 2017. *In vitro* propagation of *Kaempferia galanga* (Zingiberaceae) and comparison of larvicidal activity and phytochemical identities of rhizomes of tissue cultured and naturally grown plants. J. Appl. Biotechnol. Bioeng. 2, 00040. https://doi.org/10.15406/jabb.2017.02.00040.

Shamsudheen, K.M., Mehaboob, V.M., Thiagu, G., Shajahan, A., 2018. High frequency shoot multiplication of *Alpinia galanga* (L.) willd. using rhizome buds. Res. J. Life Sci. Bioinformat. Pharma. Chem. Sci. 4, 579–585. https://doi.org/10.26479/2018.0404.51.

Sharma, M., Ahuja, A., Gupta, R., Mallubhotla, S., 2015. Enhanced bacoside production in shoot cultures of *Bacopa monnieri* under the influence of abiotic elicitors. Nat. Prod. Res. 29, 745–749. https://doi.org/10.1080/14786419.2014.986657.

Silja, P.K., Satheeshkumar, K., 2015. Establishment of adventitious root cultures from leaf explants of *Plumbago rosea* and enhanced plumbagin production through elicitation. Ind. Crop. Prod. 76, 479–486. https://doi.org/10.1016/j.indcrop.2015.07.021.

Sivanandhan, G., Arun, M., Mayavan, S., Rajesh, M., Mariashibu, T.S., Manickavasagam, M., Selvaraj, N., Ganapathi, A., 2012. Chitosan enhances withanolides production in adventitious root cultures of *Withania somnifera* (L.) Dunal. Ind. Crop. Prod. 37, 124–129. https://doi.org/10.1016/j.indcrop.2011.11.022.

Soundar Raju, C., Varutharaju, K., Thilip, C., Aslam, A., Shajahan, A., 2015. Rhizogenesis in cell suspension culture from Mango Ginger: a source of isosorbide and n-hexadecanoic acid. Adv. Bot. 2015, 942761. https://doi.org/10.1155/2015/942761.

Subrahmanyeswari, T., Gantait, S., Kamble, S.N., Singh, S., Bhattacharyya, S., 2023. *meta*-Topolin-induced regeneration and ameliorated rebaudioside-A production in genetically uniform candy-leaf plantlets (*Stevia rebaudiana* Bert.). S. Afr. J. Bot. 159, 405–418. https://doi.org/10.1016/j.sajb.2023.05.045.

Syahid, S.F., Heryanto, R., 2017. Morpho-agronomic characteristics of twelve accessions of white turmeric (*Curcuma zedoaria*) germplasm. Biodiversitas 18, 269–274. https://doi.org/10.13057/biodiv/d180134.

Waman, A.A., Bohra, P., Devi, R.K., Pixy, J., 2021. *In vitro* multiplication protocol for *Curcuma mangga*: studies on carbon, cytokinin source and explant size. J. Hortic. Sci. 16, 69–76. https://doi.org/10.24154/JHS.2021.v16i01.008.

Wu, C.H., Murthy, H.N., Hahn, E.J., Paek, K.Y., 2008. Establishment of adventitious root co-culture of Ginseng and Echinacea for the production of secondary metabolites. Acta Physiol. Plant. 30, 891–896. https://doi.org/10.1007/s11738-008-0181-3.

Yusuf, N.A., Rahim, N.S.M., Azhar, S.Z.A., Ghani, K.A., Sommano, S., Khalid, N., 2018. Adventitious root cultures of *Boesenbergia rotunda* as a source of pinostrobin. Int. J. Adv. Sci. Eng. Info Technol. 8, 377–383.

Zafar, N., Mujib, A., Ali, M., Tonk, D., Gulzar, B., Malik, M., Sayeed, R., Mamgain, J., 2019. Genome size analysis of field grown and tissue culture regenerated *Rauvolfia serpentina* (L) by flow cytometry: histology and scanning electron microscopic study for *in vitro* morphogenesis. Ind. Crop Prod. 128, 545–555. https://doi.org/10.1016/j.indcrop.2018.11.049.

Zahid, N.A., Jaafar, H.Z.E., Hakiman, M., 2021. Micropropagation of ginger (*Zingiber officinale* Roscoe) 'Bentong'and evaluation of its secondary metabolites and antioxidant activities compared with the conventionally propagated plant. Plants 10, 630. https://doi.org/10.3390/plants10040630.

Zeeshan, U., Barkat, M.Q., Mahmood, H.K., 2018. Phytochemical and antioxidant screening of *Cassia angustifolia*, *Curcuma zedoaria*, *Embelia ribes*, *Piper nigrum*, *Rosa damascena*, *Terminalia belerica*, *Terminalia chebula*, *Zingiber officinale* and their effect on stomach and liver. Matrix Sci. Pharm. 2, 15–20. https://doi.org/10.26480/msp.02.2018.15.20.

Zhang, B., Chen, L., Huo, Y., Zhang, J., Zhu, C., Zhang, X., Ma, Z., 2020. Establishment of adventitious root cultures from leaf explants of *Tripterygium wilfordii* (thunder god vine) for the production of celastrol. Ind. Crop. Prod. 155, 112834. https://doi.org/10.1016/j.indcrop.2020.112834.

Zuraida, A.R., Mohd Shukri, M.A., Erny Sabrina, M.N., Ayu Nazreena, O., Che Radziah, C.Z., Pavallekoodi, G., Sreeramanan, S., 2016. Micropropagation of ginger (*Zingiber officinale* var. Rubrum) using buds from microshoots. Pak. J. Bot. 48, 1153–1158.