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Research Article

Using Elicitors to Induce Curcuminoid Biosynthesis in *Curcuma longa* Via *In Vitro* Micropropagation

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ABSTRACT

Turmeric (*Curcuma longa*) is an important herbal plant used in the cosmetic and medical industries. It contains two important products: curcuminoids and volatile oils. Three types of curcuminoids exist: curcumin (the major compound), monodesmethoxycurcumin, and bisdes-methoxycurcumin. Curcuminoids can remedy various diseases such as peptic ulcers, gastric ulcers, dyspepsia, and metabolic and inflammatory conditions. However, monoculture cropping and the practice of rhizome propagation can lead to pathogen contamination, reducing the quality and quantity of curcuminoids. This study aimed to propagate in vitro *C. longa* plants and improve curcuminoid yield. Rhizomes were propagated using a micropropagation technique, and multiple shoot growth was induced on Murashige and Skoog media supplemented with 5 mgL^{-1} kinetin and 1 mgL^{-1} BA (8.00±0.58 shoots). Shoots grown on MS media with the addition of 5 µM phenylalanine, 5 µM malonic acid, and a growth retardant (ancymidol) exhibited the highest curcuminoid accumulation (2.31±0.061 µgg-*1* dry weight). It can be concluded that the addition of phenylalanine and malonic acid can stimulate curcuminoid biosynthesis. Therefore, supplementing certain precursors, such as leguminous plants and green grasses, as planting material before cultivation can help increase curcuminoid levels.

Key words: Bulb stem, growth retardation, multiple shoot propagation, precursor, turmeric

INTRODUCTION

Turmeric (*Curcuma longa* L.) is an underground rhizome plant belonging to the Zingiberaceae family. It is used as a food additive and in plant-based cosmetics and pharmaceutical products. The rhizome of *C. longa* is a valuable source of natural products, including curcuminoids, yellow polyphenolic pigment, and volatile oil. Curcumin is the main compound of curcuminoids, at approximately 75–80%; however, other derivative curcuminoids also exist: demethoxycurcumin (15–20%); bis-demethoxycurcumin (3–5%); cyclic curcumin (Alvarado *et al*., 2023). In the pharmaceutical industry, the demand for curcumin has been increasing due to its medicinal properties. For example, curcumin can reduce cholesterol levels and has anti-tumour, anti-inflammatory, anti-irritant, and antioxidant properties. Notably, medicinal products account for 50% of the global curcumin market. Moreover, curcumin is used in pharmaceutical-grade skin care products and cosmetics due to the increasing demand for raw plant materials (Pandey *et al*., 2020; Sharifi-Rad *et al*., 2020). In skin care products, curcumin is used as an active anti-aging compound and to reduce reactive oxygen species (Sharifi-Rad *et al*., 2020). Therefore, the demand for curcuminoids is growing.

Curcumin is a secondary metabolite produced via the curcuminoid biosynthesis pathway. This pathway requires phenylalanine as a precursor to form p-coumaroyl-CoA and feruloyl-CoA. Then, malonyl-CoA and two specific enzymes, dilketide-CoA synthetase and curcumin synthase, are required in the final step (Figure 1). Previous studies have shown that adding elicitors as precursors could improve secondary metabolite production. For example, adding jasmonic acid improves the production of cyanidin 3-glucoside, a cherry pigment, during callus culture (Jirakiattikul *et al*., 2021), and adding shikimic acid enhances the production of polyphenols, catechins, caffeine, and other secondary components (Alvarado *et al*., 2023).

Curcuma longa is widely planted in various regions, especially in tropical and subtropical countries such as Brazil, India, Sri Lanka, China, Indonesia, and Taiwan. It is normally cultivated using conventional methods with previously cultivated rhizomes to produce the next generation (Tian *et al*., 2025). However, this method results in slow growth rates and a high likelihood of plant pathogen infection with *Phytium* sp. and *Coleotrichum* sp., known as rhizome deterioration (Khan *et al*., 2023). Moreover, monoculture cropping can increase pathogen levels in the soil (Belete & Yadete, 2023). Previous studies applied plant tissue culture techniques to increase curcuminoid content and produce healthy *in vitro* plants, which were subsequently used as parental plantlets. Additionally, Pikulthong *et al*. (2016) transformed the diketide CoA synthase (*DCS*) and curcumin synthase (*CURS*) genes, involved in curcuminoid biosynthesis in turmeric, into somatic embryos of *C. longa* and *Curcuma manga* induced via plant tissue culture. Although this study involved the plant tissue culture of *C. longa*, inducing curcuminoid production involves a different method. Therefore, the present study aimed to establish *in vitro C. longa* explants with multiple shoots and then stimulate curcuminoid biosynthesis by adding the precursors phenylalanine and malonic acid via plant micropropagation.

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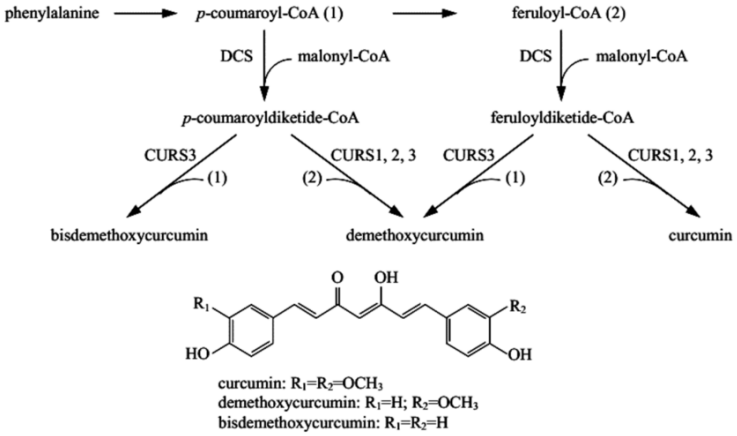

Fig. 1. Curcuminoid biosynthesis pathway in *C. longa* (Lan *et al*., 2018).

MATERIALS AND METHODS

Disinfection of the plant material

Curcuma longa L. rhizomes were cultivated on coconut coir in a greenhouse and watered two times per week until sprouting to approximately 3–4 cm. Shoots were cleaned with running tap water for 30 min and then soaked in 2% benomyl, a fungicide solution, for 30 min before washing with running tap water. The explants were then surface sterilised with 0.01% $HgCl_2$ and 2–3 drops of Tween 20 for 10 min and washed with sterile distilled water for 10 min five times.

Multiple shoot production

The sterilised explants were inoculated onto Murashige and Skoog (MS) medium (Kaewthip *et al*., 2021) supplemented with 1 mg/L benzyl adenine (BA) and kinetin (0, 1, 3, 5, 7, or 10 mgL^{-1}) (Table 1). This medium was adapted from the experiment of Soundar *et al*. (2015). The culture medium also contained 40 gL^{-1} sucrose and was solidified with 8 gL^{-1} agar. The explants were incubated at 25 ± 2°C under illumination (200 µ mol m-2s-1) with a 16-hr photoperiod. Shoots were monitored for 2 months, and any contaminated shoots were eliminated. The regenerated plantlets were recorded after 4 and 8 weeks.

Table 1 Growth regulator concentrations for multiple shoot induction

Treatment	Kinetin (mL^{-1})	BA (mgL^{-1})
1	0	0
2	1	1
3	3	1
4	5	1
5	7	1
6	10	1

Curcuminoid induction

Eight-week-old plants with multiple shoots were divided into five groups, with approximately 20 plantlets per group. Each group was treated with different curcuminoid precursors (see Table 2), and MS medium served as the basal medium for supplement addition. All groups also received 0.5 mgL^{-1} ancymidol (ANC), a growth retardant. The plants were incubated at 25 ± 2°C under illumination (200 µ moL m-2s-1) with a 16-hr photoperiod for 6 months.

Curcuminoid analysis

Standard curcumin was prepared in different combinations. Briefly, 0.01 mg of curcuminoid (Merck, USA) was dissolved in 25 mL 95% ethanol to be diluted to 0.8, 2.4, 4.8, and 6 mgL^{-1}, and the dilution was measured at a wavelength of 427 nm using a UV-visible spectrophotometer. The bulb stems of explants from each treatment were cut up and dried at 55°C for 38 hr before blending. Dried bulb stem samples (0.3 mg) were dissolved in 10 mL 95% ethanol and incubated in the dark at room temperature with shaking for 24 hr before filtering. The solution-to-ethanol solvent ratio was 1:4 (v/v). All analyses were performed in triplicate, and curcuminoid content was calculated from a standard curve (Figure 2).

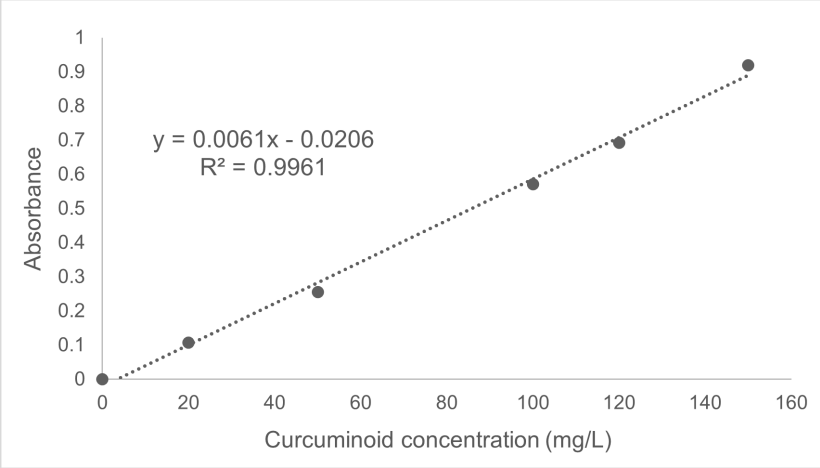

Fig. 2. Calibration curve of the curcuminoid standard in 95% ethanol determined by a spectrophotometer.

Statistical analysis

All data were analysed using one-way analysis of variance (ANOVA), and the difference between means was determined using Duncan's multiple range test (DMRT) at $p \leq 0.05$ using IBM SPSS Statistics 26. The experiments were repeated at least three times, and all results are given as mean ± standard error (SE) for multiple shoot induction, curcuminoid content, and bulb stem fresh weight.

RESULTS

Tissue culture is an effective technique for rapid plantlet production, producing healthy plants, and improving yield. In this technique, parts of tissue are grown on synthetic media under specific conditions. This study used sprouted shoots of *C. longa* L. for propagation and induced curcuminoids by adding elicitors.

Multiple shoot induction

The young shoots used for multiple shoot induction were approximately 3–4 cm in length. Shoot growth was then stimulated under various concentrations of kinetin, with BA concentration at 1 mgL^{-1}. These treatments were compared with a control group that did not receive plant growth regulators. The results indicate that treatment 4, which consisted of 5 mgL^{-1} kinetin and 1 mgL^{-1} BA, resulted in the highest number of shoots ($p \leq 0.05$) even though increasing kinetin concentrations did not affect shoot induction (Table 2).

Table 2. Effects of different growth regulator concentrations on multiple shoot induction

Treatment	Kinetin (mgL^{-1})	BA (mgL^{-1})	Number of shoots Mean ± SE
1	0	0	1.80 ± 0.37^{c}
2	1	1	2.60 ± 0.45^{c}
3	3	1	4.00 ± 0.82^{b}
4	5	1	8.00 ± 0.58^{a}
5	7	1	6.8 ± 0.37^{a}
6	10	1	5.8 ± 0.68^{b}

Data are presented as mean ± SE. Mean values followed by the same letter are not significantly different (Duncan's test at $p \leq 0.05$). Treatments were performed in triplicate.

Curcuminoid induction and quantity analysis

Two-month-old shoots were individually separated and transferred to a curcuminoid induction medium containing different concentrations of the elicitors phenylalanine and malonic acid (Table 2).

The basal medium for this experiment was MS medium supplemented with 1 mgL^{-1} kinetin and 1 mgL^{-1} BA, which was used for the main study on curcuminoid production in tuberous stems or microrhizomes. In the preliminary test, the plantlets were induced to form microrhizomes and produced curcuminoids with excessive sucrose (90 gL^{-1}) and elicitor. The results indicate that the plantlets could form microrhizomes (Figure 3a) but could not accumulate curcuminoids within them.

Fig. 3. (a) Microrhizome after induction with 90 gL^{-1} of sucrose. Curcuminoid accumulation under growth retardant and different concentrations of elicitors. (b) Control with no elicitors. (c) 5 µM malonic acid and 5 µM phenylalanine. Scale bars: 10 mm.

Consequently, 0.5 mgL^{-1} ANC, a plant growth retardant, was added to the curcuminoid induction medium instead of excessive glucose for curcuminoid accumulation in the bulb stem. Strong yellow or orange colour indicated curcuminoid accumulation. Therefore, the colour of the control stem indicated no curcuminoid accumulation, whereas the colour of treatment 4 stems showed clear curcuminoid accumulation (Figures 3-b & c). Moreover, the colour of the bulb stem-treatment 4 was more intense than that of the other treatments.

The fresh bulb stems of each treatment were sliced and dried at 55°C for 3 hr before measuring curcuminoid accumulation. Table 3 presents curcuminoid production results following elicitor treatments. Treatment 4, which contained 5 µM malonic acid and 5 µM phenylalanine, resulted in the highest curcuminoid yield of 11.57 μgg^{-1} dry weight, approximately 30% higher than the control. Moreover, adding high concentrations of substrates did not affect yield production. These results are similar to those indicated by curcuminoid colour in the stem (Figures 3-b & c). However, bulb fresh weight was also measured, with the results showing no difference between the experimental groups and the control group.

Table 3. Elicitor concentrations in each treatment

Treatment	Malonic acid (µM)	Phenylalanine (µM)	Curcuminoid content (μgg^{-1}) Mean ± SE	bulb stem Fresh weight (g/plantlet) Mean ± SE
1	0	0	3.87 ± 0.07^{e}	1.30 ± 0.11
2	1	1	5.08 ± 0.07^{d}	1.33± 0.08
3	2.5	2.5	6.17 ± 0.06^{c}	1.40 ± 0.12
4	5	5	11.57 ± 0.06^{a}	1.50 ± 0.00
5	10	10	8.70 ± 0.00^{b}	1.43 ± 0.05

Data are presented as mean ± SE. Mean values followed by the same letter are not significantly different (Duncan's test at $p \leq 0.05$). Treatments were performed in triplicate.

DISCUSSION

Curcuma longa is a valuable medicinal plant grown in various countries in Asia and Southeast Asia. It is used in home cooking and as a home remedy due to its high concentration of curcuminoids. For decades, *C. longa* has also been an important component in the cosmetic and pharmaceutical industries (Pandey *et al*., 2020). However, *C. longa* production cannot meet the increasing demand for curcuminoids due to rhizome deterioration. In conventional *C. longa* cultivation, rhizomes are collected to produce the next generation of plants. However, monoculture cropping can decrease rhizome quality. These problems result in pathogen accumulation in the rhizome and soil, causing rhizome deterioration and the subsequent reduction of curcuminoid quality and quantity.

In the present study, rhizomes were pre-treated with 2% fungicide solution (benomyl) to reduce the fungal infection rate, which is often high in underground stems (Labrooy *et al*., 2020). Benomyl eliminates a wide range of fungi and has no phytotoxic effect (Jain *et al*., 2018). Moreover, 0.01% $HgCl_2$ was used to sterilise explants instead of sodium hypochlorite (NaOCl), which can damage explants and lead to cell death (Kaewthip *et al*., 2021). Previous studies have shown that $HgCl_2$ is more suitable than NaOCl for application to shoots and shoot buds to eliminate bacteria, fungi, and endophytic microorganisms (Musfir *et al*., 2019; Boruah, 2020).

For multiple shoot induction, explants were placed on MS media containing different concentrations of kinetin and BA. The maximum BA concentration was 1 mgL^{-1} since higher concentrations are known to reduce the number of shoots in *C. longa*, *C. amada*, *Zingiber officinale*, and *Phoenix dactylifera* (Soundar *et al*., 2015). Different rhizomatic plant species have different requirements for growth regulation and proliferation. Ginger (*Zingiber officinale*) sprout induction requires less Naphthaleneacetic acid (NAA) than BA: approximately 0.5 mgL^{-1} and 1 mgL^{-1}, respectively (Musfir *et al*., 2019). Although *C. aromatica* Salisb. proliferated on MS with 5 mgL^{-1} BA alone, the medium contained a high amount of sucrose (60 gL^{-1}) to produce microrhizomes (An *et al*., 2020). This is similar to the results of Labrooy *et al*. (2020), who found that 8 mgL^{-1} µM BA resulted in the highest number of shoots (22.4 shoots), while high sucrose (6% (w/v)) resulted in a high number of microrhizomes (5.2±0.78) with thick reddish tuberous roots. Baghel and Yogendra (2015) induced shoot production in *C. longa* with thidiazuron (TDZ), resulting in seven shoots per sprout.

In vitro propagation is useful for secondary metabolite induction. Previous studies improved curcumin production by adding yeast extract, salicylic acid, and chitosan, and even by changing gene transcription (Muthukrishnan *et al*., 2016). As previously described, the curcuminoid biosynthesis pathway converts phenylalanine to coumaroyl-CoA and feruloyl-CoA. Thereafter, malonyl-CoA is required for the final conversion to curcumin and its derivatives.

In this study, phenylalanine and malonic acid were applied at different concentrations to increase curcuminoid quantity. Thus, phenylalanine is the appropriate precursor in this pathway, while the added malonic acid must first be converted into malonyl-CoA by the enzyme malonyl-CoA synthetase. Moreover, ANC was added instead of excessive sucrose to encourage curcuminoid storage in the bulb stem. Excessive sucrose has been used for microrhizome induction in *C. aromatica*, *Kaempferia parviflora*, and *Z. officinale* Rosc. (Labrooy *et al*., 2020; Yu *et al*., 2024) since sucrose is a significant source of carbon and energy for organogenesis and microrhizome induction (Muthukrishnan *et al*., 2016; Labrooy *et al*., 2020). However, excessive sucrose can inhibit tubulous formation, leading to bud decay (Archana *et al*., 2013). In this study, a high sucrose level (90 gL^{-1}) was initially used to produce microrhizomes, and the results indicate that excessive sucrose addition resulted in many microrhizomes without curcuminoid accumulation (Figure 3a).

Subsequently, ANC was used instead of sucrose to stimulate curcuminoid accumulation in the bulb stem. ANC, paclobutrazol (PAC), and uniconazole (UCZ) are plant growth retardants (PGRs) widely used in agriculture since they inhibit gibberellin synthesis. Moreover, cycocel, PAC, and succinic acid-2,2-dimethyl hydrazine (SADH) have been used within *in vitro* cultures to produce bulb yield of Asiatic *Lilium* and *Polianthes tuberosa* (Malik *et al*., 2021; Kumar *et al*., 2022). Based on *in vitro* culture propagation results, although PGRs at minimal concentrations inhibited leaf development and could lead to dwarfism, they induced bulb formation (Malik *et al*., 2021; Kumar *et al*., 2022).

Furthermore, previous studies have shown that PGRs increased the accumulation of secondary metabolites since PGRs acted as stress-inducing agents that activated the plant's natural defensive systems by stimulating secondary compounds in response to those conditions. Additionally, existing studies have shown that ANC affects *Hypericum perforatum* L. by promoting the accumulation of hypericin, a secondary metabolite of the plant. This is because ANC interferes with the gibberellin (GA) pathway, which causes increases in other terpenoid metabolites due to the excessive precursors (Rao *et al*., 2010). A study on *Stevia rebaudiana* noted that applying PGRs increased both steviol glycosides (SGs) and other economically important secondary compounds, such as polyphenols (Radić & Marko, 2016; Ashrafi *et al*., 2022).

Although there is still no evidence regarding the effect of PGRs on curcumin accumulation via the curcumin biosynthesis pathway, in this study, it was possible that ANC served a crucial role not only in inhibiting the GA pathway and promoting bulb stem formation but also in activating secondary metabolites. In contrast, excessive sucrose promoted microrhizomes but did not promote secondary metabolite accumulation in *Z. officinale Rosc*. or *C. aromatica* (Archana *et al*., 2013).

Table 2 presents the curcuminoid content after induction. The experimental results indicate that when using 10 µM malonic acid and 10 µM phenylalanine, the curcumin content is lower than 5 µM because curcumin is synthesised via the curcuminoid biosynthesis pathway. Previous studies have shown that excessive elicitors caused cytotoxicity and suppressed secondary metabolite production since an elicitor that promotes one pathway might be diverted to another, resulting in such inhibition (Thakur & Sohal, 2013; Khaldari *et al*., 2025).

CONCLUSION

It is concluded that rhizome propagation of *C. longa* faces limited success due to the susceptibility of rhizomes to pathogens, especially *Phytium sp*., which causes rot disease, and *Coleotrichum sp*., which causes challenges during storage. Micropropagation, or tissue culturing, is a widely used technique for producing plantlets and uncontaminated plants. This technique also allows the addition of precursors that can increase metabolite production. Notably, the present study added elicitors, ANC, and hormones (1 mgL^{-1} kinetin & 1 mgL^{-1} BA) to the culture medium so that PGRs could promote bulb stem formation by inhibiting the GA pathway, thereby making it possible for PGRs to contribute to secondary metabolite accumulation. Moreover, appropriate elicitor concentrations (5 µM phenylalanine and 5 µM malonic acid) are an important factor to consider because high concentrations can inhibit secondary metabolite production. This is a preliminary study on the *in vitro* propagation of *C. longa*, which induced curcuminoid production via the addition of precursors from the curcuminoid biosynthesis pathway. In the *C. longa*-pot experiment, the use of legumes and green grasses as ground planting materials will be a future study to improve curcuminoid content.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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