

Effects of 2-Hydroxypropyl- β -cyclodextrin and 28-Homobrassinolide on the Physiological Characteristics, gene expression and Terpenoid Indole Alkaloids Accumulation in *Catharanthus roseus* Cambial Meristematic Cell Cultures

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13 **Abstract:** Compared with callus culture, *Catharanthus roseus* (CAT) cambial meristematic cells (CMCs) cultured *in vitro* exhibit
14 high production of pharmacologically active terpenoid indole alkaloids (TIA). Elicitors can significantly promote the biosynthesis
15 of secondary metabolites in plant cells. However, the regulation of alkaloid biosynthesis in *C. roseus* CMCs by elicitors
16 2-Hydroxypropyl- β -cyclodextrin (H- β -CD) and 28-Homobrassinolide (HBL) is still unknown. Therefore, we established a new
17 cambial meristematic cell line, named *C. roseus* CMCs-2, and investigated the effects of H- β -CD and HBL individually or in
18 combination on the TIA content of CMCs in Erlenmeyer flasks (100, 250 and 500 mL) or in a 5-L airlift bioreactor. CMCs
19 cultured in the bioreactor and treated with 10.0 g/L H- β -CD and 150 μ g/L HBL gave the highest yields of vindoline (4.89 mg/L),
20 catharanthine (2.49 mg/L) vinblastine (3.96 mg/L) and ajmalicine (3.87 mg/L), concentrations that were 241, 277, 165 and 110 %
21 higher, respectively, than yields of CMCs cultured in 100-mL flasks without elicitors. These results indicated that CMCs cultured
22 in the bioreactor exhibited enhanced production of vindoline, catharanthine, vinblastine and ajmalicine than those cultured in
23 flasks. Quantitative RT-PCR showed that H- β -CD and HBL up-regulated transcription levels of genes related to the biosynthesis
24 of TIAs. These results imply that H- β -CD and HBL can boost TIA production in CMCs, thereby indicating that *C. roseus* CMCs
25 have considerable potential as an industrial platform for the production of bioactive TIAs.

26 **Key message**

27 H- β -CD and HBL enhance TIA production in *C. roseus* CMCs, highlighting their significant potential as an industrial platform for the
28 large-scale production of bioactive TIAs.

29 **Keywords** *Catharanthus roseus*; cambial meristematic cells; terpenoid indole alkaloids; 2-Hydroxypropyl- β -cyclodextrin;
30 28-Homobrassinolide

33 Introduction

34 The Madagascar periwinkle (*Catharanthus roseus*) is renowned for its production of over 130 terpenoid indole alkaloids (TIAs),
 35 including pharmaceutically significant compounds such as vincristine and vinblastine, which are extensively used in
 36 chemotherapy for treating various cancers, including leukemia and lymphomas (Guo, Liu et al. 2011, Kumar, Singh et al. 2022)
 37 and ajmalicine, which is a potent antihypertensive reagent (Kashyap, Kalaiselvan et al. 2020). The bioactive alkaloid content in *C.*
 38 *roseus* is notoriously low; for instance, only 1 gram of vinblastine can be extracted from approximately 500 kg of dry leaves. In
 39 contrast, undesired alkaloids are produced in abundance (Noble 1990). As a result, the extraction and purification of valuable
 40 TIAs from *C. roseus* leaves has traditionally been a labor-intensive and costly process. Despite their potential, the low natural
 41 abundance of these bioactive alkaloids in plants poses a significant challenge for large-scale production. This has driven research
 42 towards understanding the biosynthetic pathways of alkaloids in *C. roseus* and developing innovative approaches to enhance their
 43 yield.

44 The plant tissue culture applications introduced by Haberlandt (1902) have been used for over 100 years (Zhu, Liu et al.
 45 2021). Plant tissue culture system (PTCS), including callus, hairy root, and cambial meristematic cells (CMCs) have emerged as
 46 efficient platforms for the production of secondary metabolites, which have been widely explored to overcome the limitations of
 47 whole-plant extraction (Ozyigit, Dogan et al. 2023). Callus and hairy root cultures of *C. roseus* have great prospect to be used for
 48 vindoline, catharanthine and ajmalicine production (Salim and De Luca 2013). Callus tissue is composed of a group of
 49 dedifferentiated cells (DDCs) of various types, which may have undergone harmful genetic changes and epigenetic variations,
 50 hence callus tissue has poor growth characteristics and lower secondary metabolite content during long-term culture (Baebler,
 51 Hren et al. 2005, Roberts 2007). The distinguishing characteristics of CMCs proliferated from the cambium, in comparison to
 52 DDC culture, include numerous mitochondria, multiple vacuoles, genetic stability, self-renewal capacity, increased biomass, and
 53 stable metabolite accumulation (Partap, Warghat et al. 2023). Cultured CMCs from *Taxus. Cuspidata*, *Panax ginseng*, *Rehmannia*
 54 *glutinosa*, *Ocimum basilicum* and *Tripterygium wilfordii* Hook. f. may provide a cost-effective, environmentally friendly and
 55 sustainable source of paclitaxel, ginsenosides, catalpol, oleanolic acid and triptolide respectively (Lee, Jin et al. 2010, Song, Chen
 56 et al. 2019, A, Hafelder J et al. 2020, Zhou, Li et al. 2023). In our previous research, we observed long-term stable cell growth
 57 and production of vindoline, catharanthine, and ajmalicine in the *C. roseus* CMCs after 2 years of culture (Zhou, Yang et al. 2015,
 58 Zhou and Chen 2022). CMCs could offer a cost-effective and eco-friendly platform for the sustainable production of numerous
 59 vital plant natural products.

60 Elicitors can activate the defense mechanisms in PTCS, resulting in transcriptional alterations of biosynthetic genes and an
 61 increased production of secondary metabolites (Petrova, Miladinova-Georgieva et al. 2024). Among the recently identified
 62 inducers, β -cyclodextrin (β -CD) and brassinolide (BL) have demonstrated potential in stimulating the biosynthesis of secondary
 63 metabolites. β -CD functions not only as an elicitor but also as a solubilizing agent, which improves the bioavailability of
 64 hydrophobic precursors and metabolites. β -CD is a cyclic structure comprised of seven glucose units linked by α -1,4-glycosidic
 65 bonds. The molecular structure of β -CD is slightly conical, featuring a stereoscopic ring-shaped, hollow cylindrical form. This
 66 unique structure renders its exterior hydrophilic and its interior hydrophobic, enabling it to form inclusion complexes. The
 67 supplementation of 50 mM 2,6-dimethyl- β -cyclodextrins (DIMEB) has been shown to cause an early rise in intracellular
 68 carotenoid and quinone levels within *Artemisia annua* L. cell suspension cultures (Rizzello, De Paolis et al. 2014). Furthermore,
 69 the growth and yields of alkaloids, including vindoline, catharanthine, and ajmalicine, were enhanced in *C. roseus* cell cultures
 70 treated with β -CD compared to those of the control group (Zhou, Yang et al. 2015). Brassinosteroids (BRs) are a class of plant

hormones recognized for their role in regulating a variety of growth and developmental processes. The majority of research has concentrated on BR biosynthesis and function, with only a limited number of studies examining the effect of BRs on the biosynthesis of secondary metabolites. The application of BR at a concentration of 0.01 parts per billion (ppb) has been shown to significantly enhance the growth rate and shikonin content in cell cultures of *Onosma paniculatum* (Yang, Zhang et al. 1999). Additionally, BR has been found to increase glucosinolates biosynthesis in shoot cultures of Chinese cabbage (*Brassica rapa ssp. pekinensis*) (Sivanandhan, Moon et al. 2024). Trehalose and brassinolide have been observed to improve the accumulation of total phenolics and flavonoids, as well as the antioxidant activity in hairy root cultures of *Polygala tenuifolia* Willd (Ji, Yang et al. 2023). Notably, 2-hydroxypropyl- β -cyclodextrin (H- β -CD), a derivative with enhanced solubility, has been suggested as a superior alternative, yet the influence of H- β -CD and 28-Homobrassinolide (HBL) in promoting the biosynthesis of TIAs in PTCS remains undocumented. As a result, we are greatly interested in exploring the potential of H- β -CD and HBL as elicitors to stimulate the biosynthesis of TIAs in *C. roseus* CMCs.

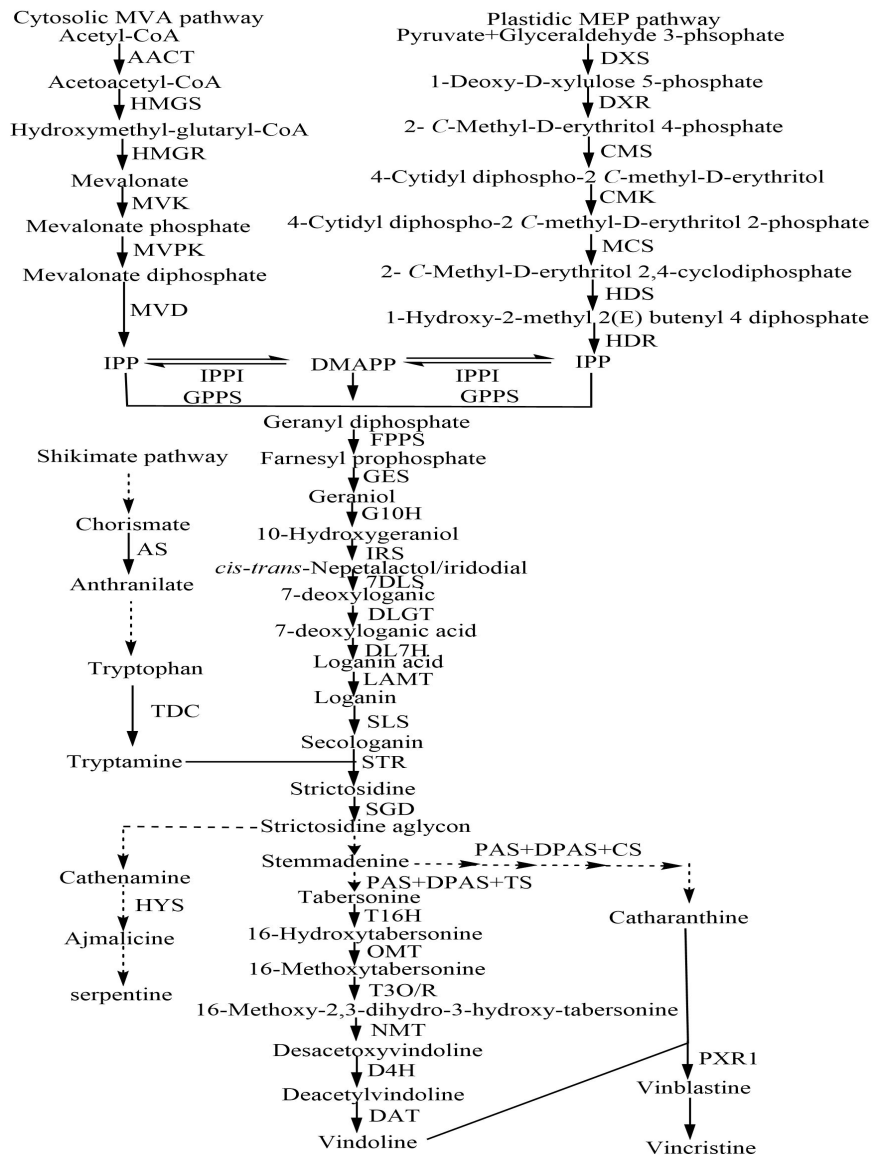


Fig. 1 The biosynthesis of TIA pathway in *C. roseus* (Zhu, Zeng et al. 2014, Caputi, Franke et al. 2018).

Abbreviations: AACT: acetoacetyl-CoA thiolase; HMGS: hydroxymethylglutaryl-CoA synthase; HMGR: hydroxymethylglutaryl-CoA reductase; MVK: mevalonate kinase; MVPK: mevalonate 5-phosphate kinase; MVD: mevalonate 5-diphosphate decarboxylase; DXS:

1-deoxy-D-xylulose-5-phosphate synthase; DXR: 1-deoxy-D-xylulose-5-phosphate reductoisomerase; CMS: 4-diphosphocytidyl-2-C-methyl-D-erythritol synthase; CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MCS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HDS: 1-hydroxy-2-methyl-2-butenyl 4-diphosphate synthase; HDR: 1-hydroxy-2-methyl-2-butenyl 4-diphosphate reductase; IPPI: isopentenylidiphosphate isomerase; G10H: geraniol 10-hydroxylase; IRS: iridoid synthase; 7DLS: 7-deoxyloganetic acid synthase; DLGT: 7-deoxyloganetic acid glucosyltransferase; DL7H: 7-deoxyloganic acid 7-hydroxylase; LAMT: loganic acid O-methyltransferase; SLS: secologanin synthase; STR: strictosidine synthase; SGD: strictosidine β -D glucosidase. T16H: tabersonine 16-hydroxylase; OMT: O-methyltransferase; T3O/R: tabersonine 3-oxygenase; NMT: N-methyltransferase; D4H: desacetoxyvindoline 4-hydroxylase; DAT: deacetylvindoline-4-O-acetyltransferase; PAS: precondylocarpine acetate synthase; DPAS: dihydroprecondylocarpine synthase; TS: tabersonine synthase; CS: catharanthine synthase; PRX1: peroxidase 1. AS: anthranilate synthase; TDC: tryptophan decarboxylase; HYS: heteroyohimbine synthase. Single arrows denote single steps and dotted arrows denote multiple or unidentified steps.

In *C. roseus*, elicitors have been shown to trigger the accumulation of terpenoid indole alkaloids (TIAs) in callus (Simkin, Miettinen et al. 2013), hairy root (Goklany, Loring et al. 2009), and CMC cultures (Zhou, Yang et al. 2015). This is achieved by enhancing the transcription of genes encoding enzymes integral to the TIA biosynthetic pathway. These enzymes include geraniol synthase (GES), geraniol 10-hydroxylase (G10H), iridoid synthase (IRS), loganic acid O-methyltransferase (LAMT), tryptophan decarboxylase (TDC), strictosidine synthase (STR), desacetoxyvindoline-4-hydroxylase (D4H), strictosidine β -D-glucosidase (SGD), and the transcription factor ORCA3 (van der Fits and Memelink 2000) (Fig. 1). The overexpression of ORCA3 in *C. roseus* CMCs has been found to increase the expression of *STR*, *SGD*, *TDC*, *D4H*, *LAMT*, desacetoxyvindoline-4-hydroxylase gene (*DPAS*), tabersonine synthase gene (*TS*), secologanin synthase gene (*SLS*), and *ORCA4*. Similarly, *ORCA4* overexpression leads to the upregulation of *STR*, *SGD*, *TDC*, *DPAS*, *TS*, catharanthine synthase gene (*CS*), *SLS*, and *ORCA3* (Yang, Ding et al. 2023). Furthermore, in in vitro shoots, zinc nanoparticles have been observed to significantly enhance vincristine production by a factor of 4.5, while also increasing the expression of *DAT* and *PRX1* genes by 14.3-fold and 3.7-fold, respectively. This indicates that the *DAT* and *PRX1* genes are crucial for the biosynthesis of vincristine (Perveen, Safdar et al. 2022).

In the current investigation, we examined the synthesis of vindoline, catharanthine, ajmalicine, vinblastine, and total alkaloids in *C. roseus* CMC cultures and subjected to treatment with H- β -CD and HBL, either separately or in tandem, within both Erlenmeyer flasks and 5-L bioreactors. Physiological traits were assessed, encompassing anti-oxidative enzymes such as catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), which are pivotal and early constituents of plant immune signaling pathways, as well as nitric oxide (NO) and calcium ions (Ca^{2+}) (Ma and Berkowitz 2016). The transcription levels of key biosynthetic genes and the transcription factor ORCA3 were also assessed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). By examining both gene transcription and TIA accumulation, a more comprehensive insight into the regulatory mechanisms of TIA biosynthesis is achieved. This study aims to bridge these research gaps by investigating the effects of H- β -CD and HBL on alkaloid biosynthesis in CMCs of *C. roseus*. The results indicate that combining *C. roseus* CMC cultures and elicitors with cost-effective bioreactor technology offers significant potential as a scalable strategy to enhance the commercial production of key alkaloids, including vindoline, catharanthine, ajmalicine, and vinblastine.

Materials and Methods

Chemicals

H- β -CD, bleomycin, fluorescein diacetate (FDA), methanol and acetonitrile (HPLC grade) were purchased from Aladdin Reagents Co.(Shanghai, China). Vindoline, catharanthine, ajmalicine, vinblastine and 28-Homobrassinolide were purchased from

Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China). Trizol, PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time), and SYBR® Premix Ex Taq™ (Tli RNaseH Plus) were obtained from Takara Bio, Inc. (Dalian, China). The assay kits for nitric oxide (NO)(A012-1), calcium ions (Ca²⁺) (C004-2-1), superoxide dismutase (SOD) (A001-3), peroxidase (POD) (A084-3-1), catalase (CAT) (A007-1-1), and hydrogen peroxide (H₂O₂) (A064-1-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). All other chemicals were of analytical grade.

Plant materials and culture conditions

The fresh twigs were harvested from two-year-old wild *C. roseus* plants growing in the medicinal botanical garden of Dongguan campus of Guangdong Medical University (Guangdong Province, China). The twigs were initially rinsed under running tap water for 30 minutes, followed by disinfection with 75% ethanol for 30 seconds, and six rinses with sterilized distilled water (dH₂O). The twigs were then immersed in 0.05% HgCl₂ for 7 minutes, rinsed six times with dH₂O, and given a final rinse with dH₂O containing 150 mg/L citric acid. The cambium, phloem, cortex, and epidermal tissues were carefully peeled from the xylem, with the epidermal tissue being placed onto pH-adjusted (5.80) Murashige and Skoog (MS) medium [26], supplemented with 2.0 mg/L α-naphthalene acetic acid (NAA), 20 g/L sucrose, and 3.6 g/L gelrite. Cell division was observed exclusively in the cambium within 8 to 12 days. Twenty days after culture initiation, a visible split appeared between the cambium cells, forming a flat plate of cells. The cambial cell layer was then transferred to 250-mL Erlenmeyer flasks containing 100 mL of MS medium supplemented with 2.0 mg/L NAA, 10 g/L sucrose, and 3.6 g/L gelrite. The CMCs were cultured at 25 °C in continuous darkness and subcultured every 12 days. Three months before the experiments, the CMCs were transferred to 100 mL of MS solid medium in a 250-mL Erlenmeyer flask and grown at 25 °C under a 12/12-hour light/dark photoperiod. Initial suspension cultures were established by inoculating CMCs into MS medium supplemented with 2.0 mg/L NAA and 20 g/L sucrose at a concentration of 50.0 g fresh weight/L. CMC growth was quantified by measuring the dry weight (DW) per liter. Growth rate = (final dry cell weight / initial dry cell weight) × 100%.

To identify the CMCs, 0.5 g of cells were collected and mixed with 5 milliliters of 0.9% normal saline, then pipetted evenly. Subsequently, four drops of the cell suspension were transferred onto a glass slide and examined under a microscope (Leica DM750). Suspension CMCs and DDCs of *C. roseus* were treated with radiomimetic drug bleomycin solution to attain final concentrations of 200 µg/mL. The treated suspension-cultured cells were subsequently incubated in darkness at a rotation speed of 120 r/min for durations of 0, 24, and 48 hours, respectively. FDA was added to achieve a final concentration of 200 ng/mL. The cells are then subjected to shaking at 120 r/min for a period of 20 minutes, followed by three washes with sterile PBS buffer. Subsequently, the cells are transferred onto a slide. The fluorescence of the cells is observed using a Zeiss Axioplan 2 multichannel fluorescence microscope (Oberkochen, Germany), employing an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Widholm 1972). Each sample was measured five times independently, with the highest and lowest values discarded, and the average mortality rate is computed. All experiments are conducted in triplicate.

Suspension cultures of CMCs were incubated on a HZT-2 gyrotory shaker (Donglian Electronic & Technol. Dev. Co., Beijing, China) at 25 °C and 120 rpm under continuous light. CMCs were subcultured every 12 days in 250-mL Erlenmeyer flasks by transferring 15 mL of 12-day-old suspension into 85-mL fresh MS medium. Suspension cultures were continuously maintained for 2 years. Roughly every 3 months, the cultures were harvested for determination of growth characteristics and TIA production. Cell viability was assayed using the fluorescein diacetate staining method (Durante et al. 2011).

Treatment with H-β-CD and HBL individually or in combination

Twelve-day-old *C. roseus* CMC suspensions were centrifuged at $1000 \times g$ for 10 minutes, and the supernatant was discarded. The CMCs (5.0-g fresh weight) were transferred into 250-mL Erlenmeyer flasks containing 100-mL MS liquid medium supplemented with varying concentrations of H- β -CD (1, 5, 10, 15, 20 g/L). The CMCs (5.0-g fresh weight) were transferred into 250-mL Erlenmeyer flasks containing 100-mL MS liquid medium supplemented with HBL (10, 50, 100, 150, 200 μ g/L). The cultures were maintained at 25 °C with continuous light and orbital shaking at 120 rpm and harvested after 12 days. Cell viability was assessed using the FDA staining method (Durante, Caretto et al. 2011). Subsequently, twelve-day-old CMC suspensions (50.0-g fresh weight /L) were cultivated under continuous light at 25 °C in Erlenmeyer flasks of 100, 250 and 500 mL, as well as in 5-L airlift bioreactors. These vessels contained 50-, 100-, 200-mL and 4-L liquid medium, respectively, with each medium supplemented with either 0 or 20 g/L H- β -CD. Flasks were maintained at 25 °C with constant light and orbital shaking at 120 rpm using an HZT-2 gyrotory shaker (Donglian Haer Instrument Manufacturing Co., Ltd. Beijing, China). The bioreactor was an MCF airlift bioreactor (Dibai Biotechnology Co., Ltd., Shanghai, China) equipped with inlet and outlet air sterilization filters (Midisart 2000, PTFE membrane, 0.2- μ m pore size, Sartorius).

All the experiments were conducted under continuous light at 25 °C with a constant pH of 5.8. The temperature in the bioreactor was regulated using a water bath, and aeration was maintained at 0.3 vvm (volume of air per volume of medium per min). Cultures were harvested at 3, 6, 9, 12 and 15 days. To evaluate the effects of H- β -CD and HBL, 12-day-old CMC suspensions (200.0-g fresh weight) were transferred to 5-L bioreactors containing 4L of MS liquid medium supplemented with 0 (control), 20 g/L H- β -CD and 150 μ g/L HBL, alone or in combination. Cultures were harvested on day 9, 12, 15. All CMC cultures were separated from the liquid media by vacuum filtration, rinsed with distilled water, and freeze-dried. Experiments were performed in triplicate to ensure reproducibility.

Measurement of NO, Ca²⁺, H₂O₂ and enzyme activity

C. roseus CMCs from a 12-day-old suspension culture were harvested and filtered after treatment with either H- β -CD (at varying concentrations: 0, 1, 5, 10, 15, or 20 g/L), 150 μ g/L HBL individually. The filtrate and cells were collected separately. For NO measurement, 1 mL of filtrate from each CMC suspension was filtered through 0.22- μ m nylon membrane filters. The extracellular NO concentration was determined using a NO assay kit, following the protocol described by Xu and Hu et al. (Hu, Neill et al. 2003, Xu and Dong 2005). The cells were ground in liquid nitrogen for 10 minutes, followed by the addition of 10 mL of PBS buffer. The mixture was centrifuged at $8000 \times g$ for 10 minutes at 4°C, and the supernatant was collected for subsequent analysis. The Ca²⁺ concentration in CMC cultures was measured using a Ca²⁺ assay kit (Merillon, Liu et al. 1991). The activities of SOD, POD, CAT and H₂O₂ of CMC cultures were analyzed using respective assay kits following the manufacturer's instructions. Absorbance values for NO, Ca²⁺, SOD, POD, CAT, and H₂O₂ were recorded at 550 nm, 610 nm, 405 nm, 450 nm, 420 nm and 405 nm, respectively. Each measurement was performed in triplicate using a Multiskan MK3 microplate reader (Thermo Fisher Scientific Inc., Massachusetts, USA) (Zhang, Han et al. 2010, Wang, Zhang et al. 2022).

Sample extraction and determination

Alkaloid extraction was carried out following the method described by Liu et al. (Liu, Zhu et al. 2014). The extracted alkaloids were dissolved in 1.0 mL of methanol and analyzed using HPLC and ion-pair extraction spectrophotometry, as outlined by Yang et al. (Yang L, Liu Y et al. 2008). Vindoline, catharanthine, ajmalicine and vinblastine were quantified at 25 °C using an Agilent 1260 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Phenomenex Gemini C18 column (250 mm $\times$ 4.6 mm, 5 μ m) (Phenomenex Inc., Torrance, CA, USA). The mobile phase consisted of methanol/acetonitrile/10 mM

ammonium acetate (15:40:45, v/v/v) at a flow rate of 1.0 mL/min. Detection was performed at a wavelength of 280 nm, with an injection volume of 10 µL. Prior to analysis, all samples were filtered through 0.22-µm nylon membrane filters. Alkaloids were identified and quantified by comparing their retention times and UV absorbance spectra with those of commercial standards. Each sample was analyzed in triplicate to ensure accuracy and reproducibility.

Monitoring gene expression using qRT-PCR

CMC cultures were frozen in liquid nitrogen, ground into a fine powder using a mortar and pestle, and RNA was extracted following the method described by Zhou et al. (Zhou, Yang et al. 2015).

The RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Total RNA (1 µg) was treated with DNase to eliminate genomic DNA using a PrimeScript™ reagent kit with gDNA Eraser (Takara Bio, Kyoto, Japan), and cDNA was subsequently synthesized according to the manufacturer's instructions. Transcript levels of the housekeeping gene 40S ribosomal protein S9 (RPS9), along with *GES*, *G10H*, *IRS*, *LAMT*, *TDC*, *STR*, *SGD*, *D4H*, *ORCA3*, *CS*, *TS* and *PXR1* were monitored in CMC cultures treated with 10 g/L β-CD and 150 µg/L HBL, individually and in combination. Primer sequences for *GES*, *G10H*, *IRS*, *LAMT*, *TDC*, *STR*, *SGD*, *D4H*, *ORCA3* (Zhou, Yang et al. 2015), *CS*, *TS* (Caputi, Franke et al. 2018) and *PXR1* (Tang, Liu et al. 2022) are listed in Table S1.

qRT-PCR reactions were prepared using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio, Kyoto, Japan) and run on a 48-well MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). Amplification conditions included an initial holding stage at 95 °C for 30 seconds, followed by 40 cycles of 95 °C for 5 seconds and 60 °C for 20 seconds. Melt curve analysis from 60 °C to 95 °C was performed to confirm the specificity of the amplicons. The stability of qRT-PCR results was analyzed using Bio-Rad CFX Manager Software (Bio-Rad, Hercules, CA, USA). All experiments were conducted in triplicate to ensure reproducibility and reliability.

Statistical analysis

All values are presented as mean ± standard deviation (SD). Statistical analyses were conducted using independent two-tailed Student's t-tests. All comparisons were made against untreated controls.

Results

Induction of *C. roseus* CMC

The sterilized periwinkle stems were cut into small sections of about 0.5-1 cm (Fig. 1a, b), then made a longitudinal cut, strip out the pith, expose the cambium layer, place the side with the cambium layer on the induction medium, and continue to culture in the dark. On the 8th to 12th day, cell division was observed exclusively in the cambium (Fig. 1c). Twenty days after culture initiation, a visible split appeared between the cambium cells, forming a flat plate of cells (Fig. 1d). The cambial cell layer was then transferred to 250-mL Erlenmeyer flasks containing 100 mL of MS medium supplemented with 2.0 mg/L NAA, 10 g/L sucrose, and 3.6 g/L gelrite. Following this, we established a new cell line, named *C. roseus* CMCs-2, based on our method reported by Zhou et al. in 2015 (Zhou, Yang et al. 2015). The CMCs were cultured at 25 °C in continuous darkness and subcultured every 12 days (Fig. 1e). Microscopic examination revealed that CMCs are considerably smaller and exhibit numerous, small vacuole-like structures, predominantly containing single cells or clusters of relatively few cells (Fig. 1f, g).

C. roseus DDCs and CMCs were treated with bleomycin at a concentration of 200 µg/mL. After 24 hours, the mortality rate was 34.7% for DDCs and 64.9% for CMCs. At 48 hours, the mortality rate increased to 58.9% for DDCs and 91.6% for CMCs (Fig. S1). The cell death percentage of CMCs was markedly higher than that of DDCs, highlighting the hypersensitivity of CMCs to the radiomimetic antibiotic zeocin.

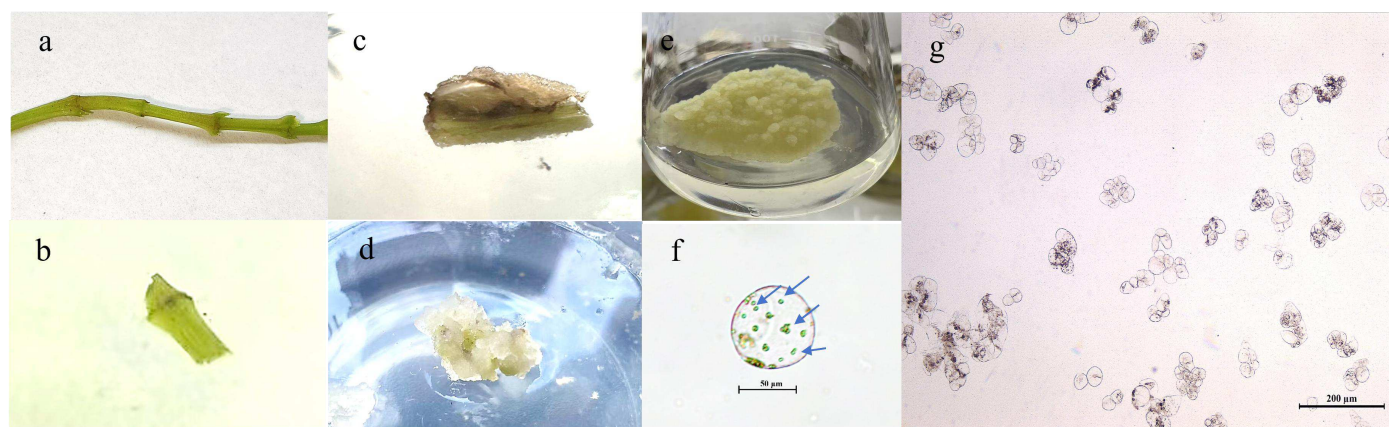


Fig. 2 Isolation and culture of *C. roseus* CMCs.

A twig of *C. roseus*. (b) Preparation of *C. roseus* explant by peeling off cambium, phloem, cortex and epidermal cells from the xylem. (c) Natural split of CMCs from DDCs induced from phloem, cortex and epidermal cells. (d) CMCs proliferated from the cambium. (e) 12-day-old *C. roseus* CMCs cultured in 250-mL Erlenmeyer flasks. (f) Micrographs of a CMC. CMCs are significantly smaller and possess characteristic numerous, small vacuole-like structures. The blue arrow indicates a vacuole-like structure. Scale bars, 50 µm. (g) Micrographs of a cambial meristematic cell. CMCs present as a single cell or small cell clusters.

Dose-dependent effect of H-β-CD on CMC growth, production of NO and Ca²⁺ and TIA

Increasing concentrations of H-β-CD did not inhibit CMC growth. On the contrary, CMCs treated with H-β-CD exhibited faster growth than the control cells (Fig. 3a). Additionally, H-β-CD induced the production of vindoline, catharanthine, and ajmalicine in a dose-dependent manner. Maximum production levels of vindoline (3.13 mg/L), catharanthine (2.06 mg/L), vinblastine (2.53 mg/L), and ajmalicine (2.64 mg/L) were achieved with 20 g/L H-β-CD, representing increases of 181%, 244%, 135%, and 164%, respectively, compared to control cultures (Fig. 3b–e).

The highest total alkaloid accumulation was observed on day 12 after treatment with 20 g/L H-β-CD, reaching 767.2 mg/L, a 64% increase compared to the control. Furthermore, 26% of the total alkaloids (175.6 mg/L) were secreted directly into the medium, representing a 408% increase relative to the control medium (Fig. 3f). To investigate the role of signaling molecules, we monitored the levels of NO and Ca²⁺, as common metabolic stimulators such as MeJA and β-CD are often associated with these molecules, which mediate the biosynthesis of plant secondary metabolites in response to elicitor-induced defense mechanisms. Interestingly, the maximum NO concentration (8.38 µmol/L) in the medium of H-β-CD-treated cells was nearly three times higher than in the control group (2.74 µmol/L) (Fig. 3g). Similarly, the maximum Ca²⁺ concentration (0.47 µmol/L) in the H-β-CD-treated cells was 1.8 times higher than that of the control (0.84 µmol/L) (Fig. 3h).

Dose-dependent effect of HBL on CMC physiological characteristics and TIA production

The highest growth rate was observed on day 12 after treatment with 150 µg/L HBL, reaching 15.39 g/L (DW), a 185% increase compared to the control. CMCs treated with HBL (10, 50, 100, 150 µg/L) exhibited faster growth than the control cells (Fig. 4a). Additionally, HBL induced the production of vindoline, catharanthine, and ajmalicine in a dose-dependent manner. Maximum

production levels of vindoline (3.07 mg/L), catharanthine (1.38 mg/L), vinblastine (2.75 mg/L), and ajmalicine (2.95 mg/L) were achieved with 150 $\mu\text{g/L}$ HBL, representing increases of 178%, 167%, 191%, and 150%, respectively, compared to control cultures (Fig. 4b–e). The highest total alkaloid accumulation was observed on day 12 after treatment with 150 $\mu\text{g/L}$ HBL, reaching 532.1 mg/L, a 37% increase compared to the control (Fig. 3f). Furthermore, HBL has not been found to promote alkaloid secretion into the medium.

The effects of varying concentrations of HBL on antioxidant enzyme activities were investigated. The results revealed that HBL treatment significantly enhanced the activities of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), and compared to those of the control (Fig. 5). Specifically, SOD activity in CMCs treated with 10, 50, 100, 150, and 200 $\mu\text{g/L}$ of HBL increased by 8.6%, 44.1%, 111.8%, 183.9%, and 102.1%, respectively. Similarly, POD activity rose by 27.3%, 63.3%, 102.2%, 140.6%, and 130%, while CAT activity increased by 10%, 47.9%, 102.2%, 140.6%, and 130% under the same treatment concentrations. In contrast, H_2O_2 content decreased progressively with increasing HBL concentrations, showing reductions of 11.6%, 24.2%, 41.6%, 58.7%, and 60% at 10, 50, 100, 150, and 200 $\mu\text{g/L}$, respectively.

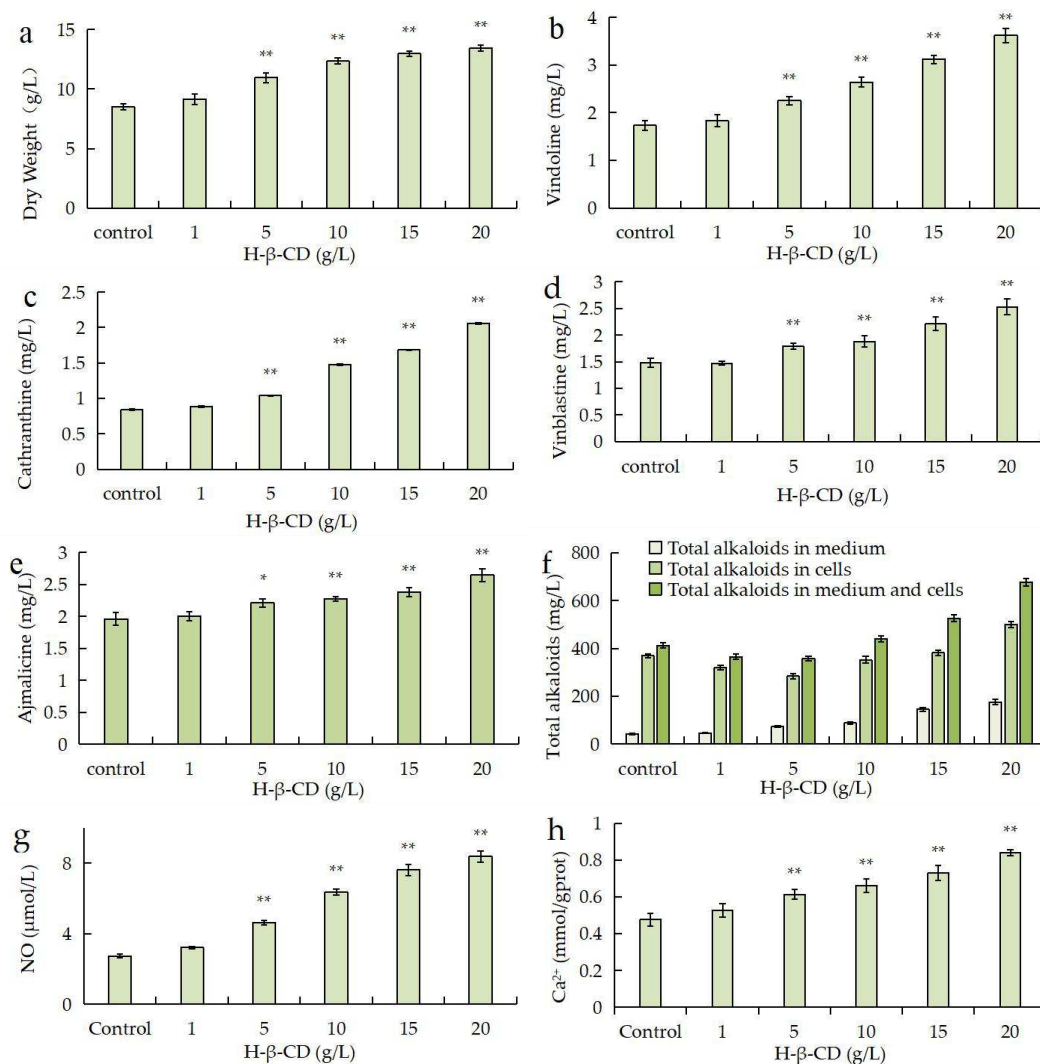


Fig. 3 Effects of different concentrations of H- β -CD (0, 1, 5, 10, 15 and 20 g/L) on growth (a), concentrations of vindoline (b), catharanthine (c), vinblastine (d) and ajmalicine (e), total alkaloids (f), NO release (g), Ca^{2+} (h) in *C. roseus* CMCs. Measurements were taken on day 12. Values are means $\pm$ SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

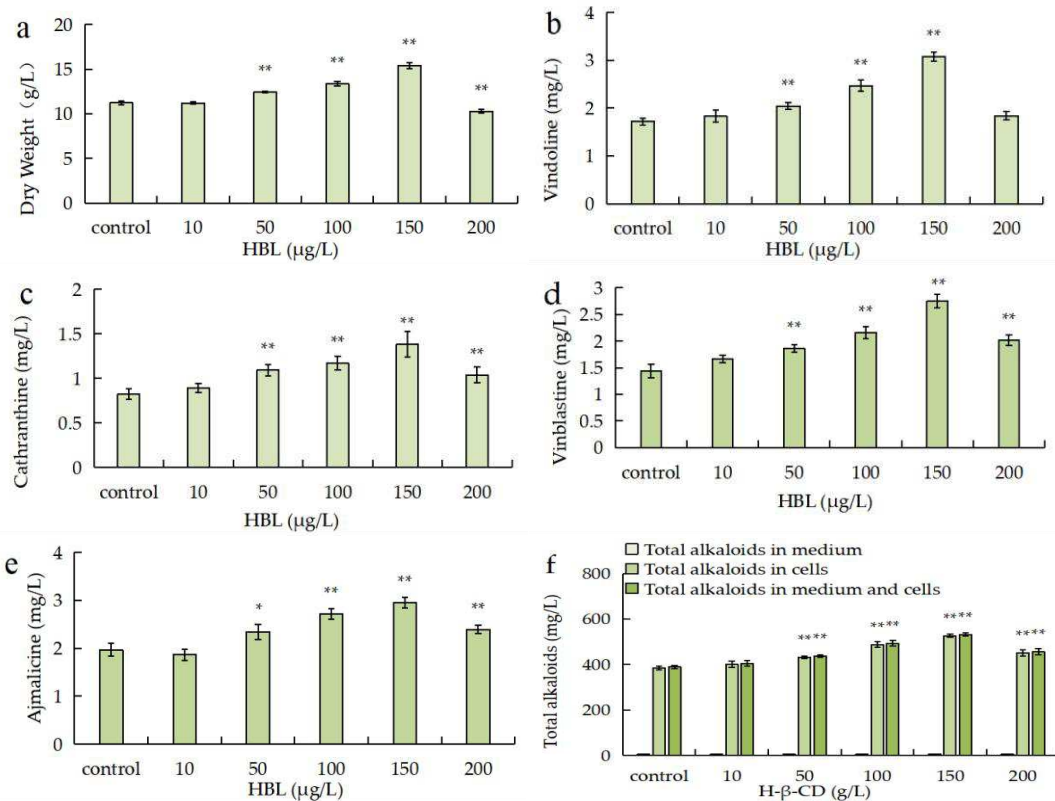


Fig. 4 Effects of different concentrations of HBL (0, 10, 50, 100, 150 and 200 µg/L) on growth (a), concentrations of vindoline (b), catharanthine (c), vinblastine(d), ajmalicine (e) and total alkaloids (f) in *C. roseus* CMCs. Measurements were taken on day 12. Values are means $\pm$ SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

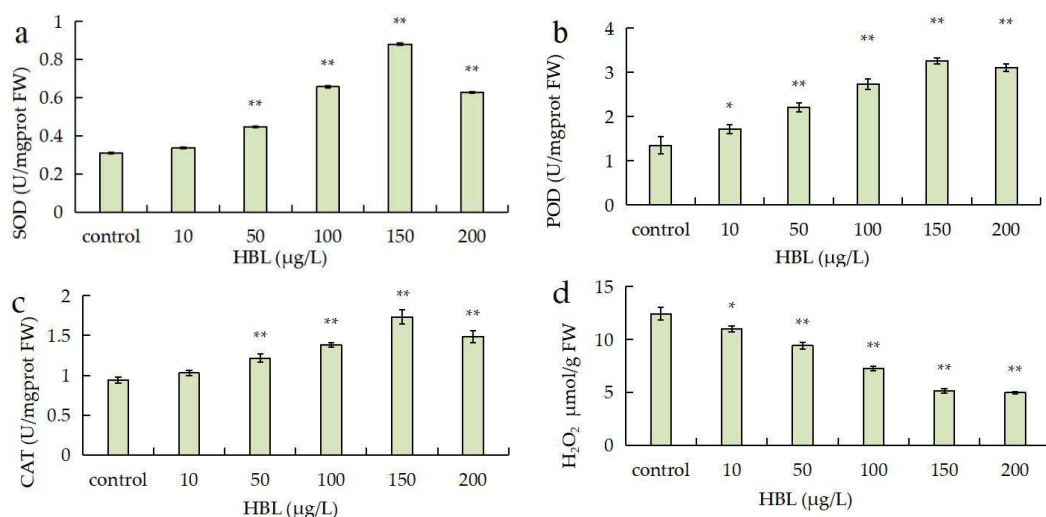


Fig. 5 Effects of different concentrations of HBL (0, 10, 50, 100, 150 and 200 µg/L) on the enzyme activity of SOD (a), POD (b), CAT (c) and the content of H_2O_2 (d). concentrations were determined on day 12. Data were analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

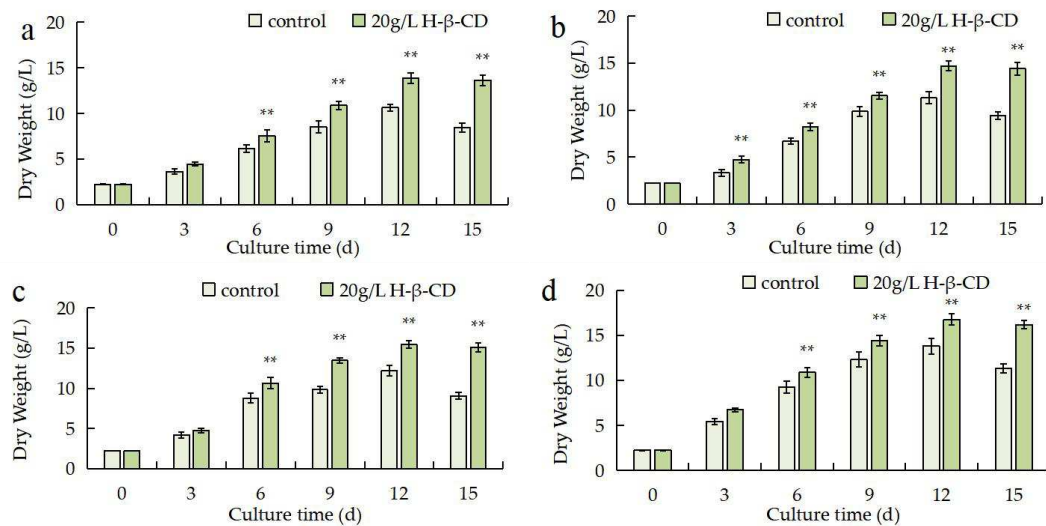


Fig. 6 Effect of 20 g/L H-β-CD on growth of *C. roseus* CMCs in 100-mL (a), 250-mL (b) and 500-mL (c) Erlenmeyer flasks and 5-L bioreactors (d). Measurements were taken 3, 6, 9, 12 and 15 d after treatment. Values are means ± SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

Comparison of *C. roseus* CMC growth and TIA production with 20g/L H-β-CD in Erlenmeyer flasks vs. Bioreactors

C. roseus CMC cultures were cultivated in both Erlenmeyer flasks and bioreactors under optimal growth conditions supplemented with 20 g/L H-β-CD to maximize yield and enhance biomass quality (Fig. S2). Growth analysis indicated that the addition of 20 g/L H-β-CD significantly improved the growth of treated CMCs compared to the control groups (Fig. 6). The maximal growth rates of CMCs treated with H-β-CD in 100-, 250-, and 500-mL Erlenmeyer flasks, as well as 5-L bioreactors, were 616%, 654%, 685%, and 744%, respectively. In contrast, the corresponding growth rates for the control groups were 473%, 503%, 540%, and 613%, respectively.

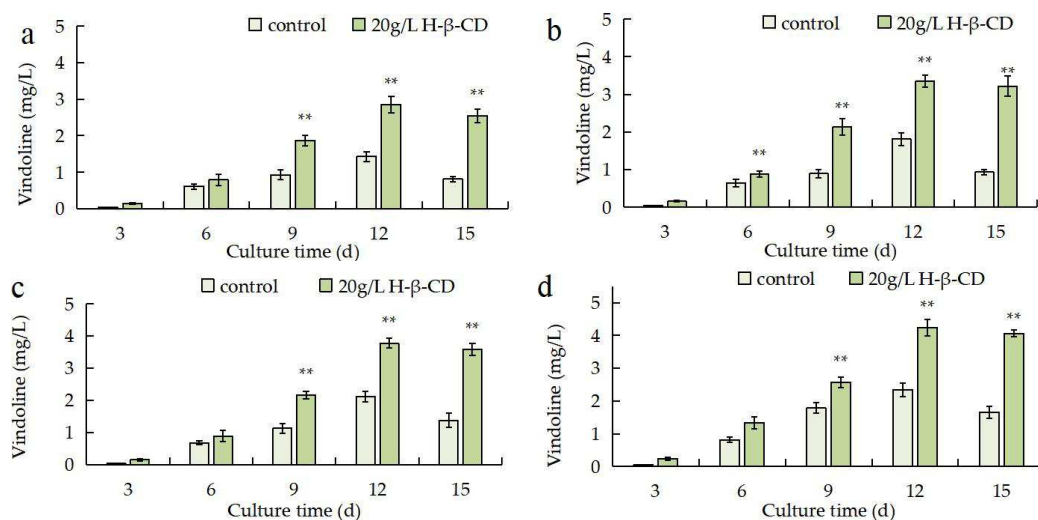


Fig. 7 Effect of 20 g/L H-β-CD on vindoline of *C. roseus* CMCs in 100-mL (a), 250-mL (b) and 500-mL (c) Erlenmeyer flasks and 5-L bioreactors (d). Measurements were taken 3, 6, 9, 12 and 15 d after treatment. Values are means ± SD of triplicate experiments. Data were

analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

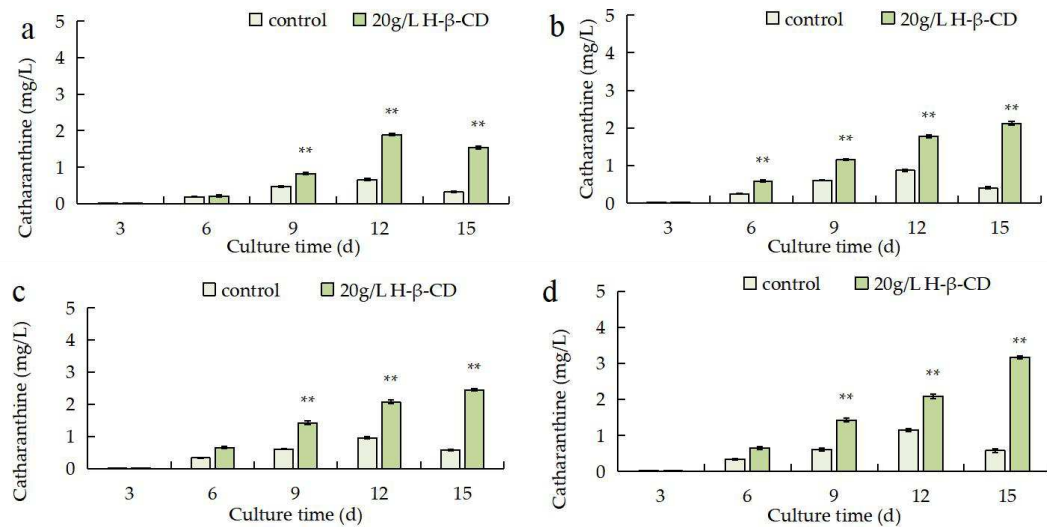


Fig. 8 Effect of 20 g/L H-β-CD on catharanthine of *C. roseus* CMCs in 100-mL (a), 250-mL (b) and 500-mL (c) Erlenmeyer flasks and 5-L bioreactors (d). Measurements were taken 3, 6, 9, 12 and 15 d after treatment. Values are means $\pm$ SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

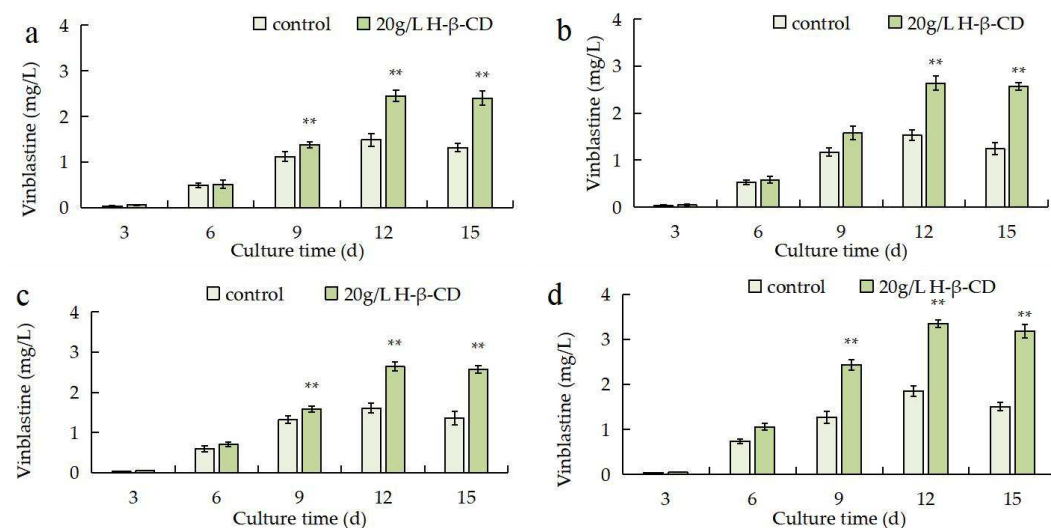


Fig. 9 Effect of 20 g/L H-β-CD on vinblastine of *C. roseus* CMCs in 100-mL (a), 250-mL (b) and 500-mL (c) Erlenmeyer flasks and 5-L bioreactors (d). Measurements were taken 3, 6, 9, 12 and 15 d after treatment. Values are means $\pm$ SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

The accumulation time course of each alkaloid (vindoline, catharanthine, vinblastine and ajmalicine) in CMCs treated with 20g/L H-β-CD are shown in Figs. 7-10, respectively. At all time points for each alkaloid, yields were higher in bioreactors than in Erlenmeyer flasks. In both vessels, the patterns of relative contents of each alkaloid were similar to those of the controls. In all

controls, vindoline production peaked on day 12, yielding 1.43, 1.81 and 2.12 mg/L in the 100-, 250- and 500-mL flasks, respectively, and 2.34 mg/L in the 5-L bioreactor. CMCs treated with 20g/L H- β -CD showed maximal vindoline production on day 12 in the flasks (2.86, 3.35 and 3.78 mg/L in the 100-, 250- and 500-mL flasks, respectively) and in the bioreactors (4.24 mg/L) (Fig. 7). Thus, H- β -CD increased vindoline content 78-100% across all scales of culture.

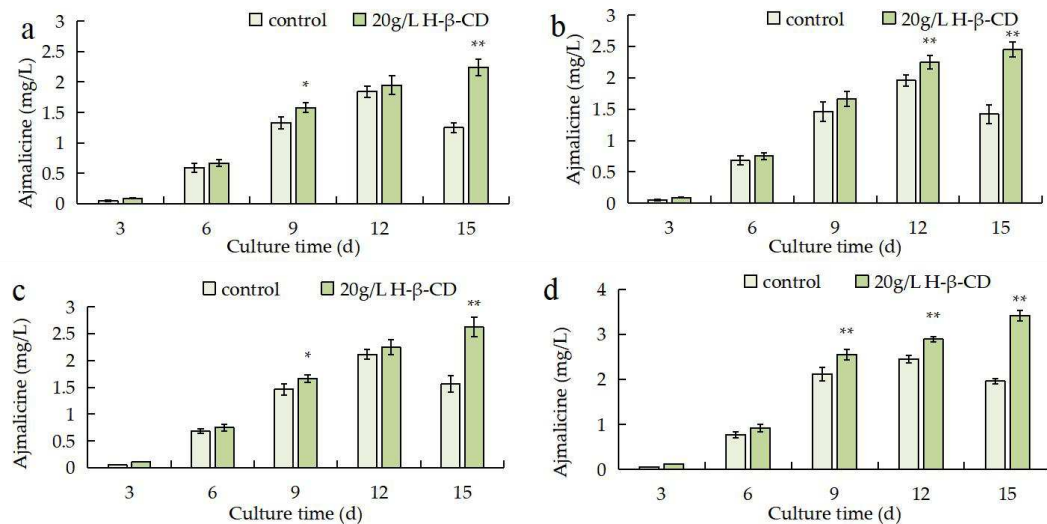


Fig. 10 Effect of 20 g/L H- β -CD on ajmalicine of *C. roseus* CMCs in 100-mL (a), 250-mL (b) and 500-mL (c) Erlenmeyer flasks and 5-L bioreactors (d). Measurements were taken 3, 6, 9, 12 and 15 d after treatment. Values are means $\pm$ SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as p < 0.05 (*) and p < 0.01 (**).

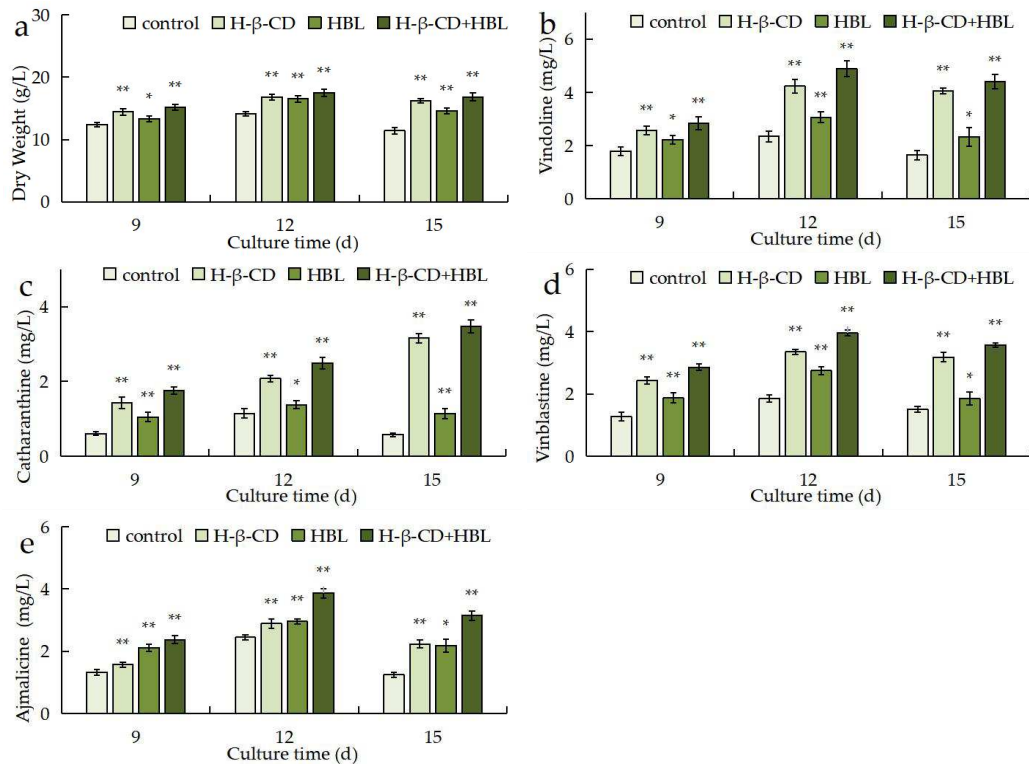


Fig. 11 Effects of 20 g/L H- β -CD and 150- μ M HBL individually or in combination on *C. roseus* CMC growth (a), vindoline production (b), catharanthine production (c), vinblastine (d) and ajmalicine production (e) in 5-L bioreactors. Measurements were taken 24 h after treatment

with MeJA on day 6, 9 and 12. Values are means $\pm$ SD of triplicate experiments. Data were analyzed by ANOVA followed by Student's t-test. Significant differences between treatments and the control are shown as $p < 0.05$ (*) and $p < 0.01$ (**).

Catharanthine production peaked on day 12 or 15. Treatment with H- β -CD increased catharanthine content 80–186 % over controls, yielding 1.89, 2.17 and 2.45 mg/L in the 100-, 250- and 500-mL flasks, respectively, and 3.16 mg/L in the 5-L bioreactor (Fig. 8). Treatment with H- β -CD increased vinblastine content 59–71 % over controls, yielding 2.45, 2.64 and 2.64 mg/L in the 100-, 250- and 500-mL flasks, respectively, and 3.35 mg/L in the 5-L bioreactor (Fig. 9). Maximum ajmalicine concentrations occurred on day 12 in control groups and on day 15 in H- β -CD-treated groups. Treatment with H- β -CD, however, increased ajmalicine yields by 21–39 % to 2.24, 2.45 and 2.62 mg/L in the 100-, 250- and 500-mL flasks and to 3.41 mg/L in the 5-L bioreactors (Fig. 10).

Overall, treatment of *C. roseus* CMCs with 20g/L β -CD induced increased cell growth and production of vindoline, catharanthine, vinblastine and ajmalicine, particularly in cells cultured in the bioreactors.

C. roseus CMC cultures were cultivated in 5-L bioreactors supplemented with 20 g/L H- β -CD, 150 μ g/L HBL, and a combination of H- β -CD/HBL. Maximum growth rates were observed on day 12 across all treatment groups. The maximal growth rates achieved by CMCs under H- β -CD, HBL, and combined H- β -CD/HBL treatments were 627%, 748%, 734%, and 778%, respectively. In the control and HBL treatment groups, the growth rate of CMCs significantly declined after 15 days. In contrast, the H- β -CD and H- β -CD/HBL treatment groups showed only a slight decrease in growth rate at the same time point. Growth analysis indicated that the addition of H- β -CD, HBL, a combination of H- β -CD/HBL significantly improved the growth of treated CMCs compared to the control groups.

Individual and combined effects of H- β -CD and HBL on regulation of CMC growth, TIA production and gene expression in 5-L bioreactor cultures.

The accumulation time courses of vindoline, catharanthine vinblastine and ajmalicine in each of the CMC culture treatments (H- β -CD, HBL or H- β -CD/HBL) and in the control are shown in Fig. 11b, c, d, e. All three elicitor treatments increased alkaloid yields relative to the controls. Maximal vindoline production was reached on day 12 in all groups, with 2.35 mg/L in the control group and 4.24, 3.07 and 4.89 mg/L in the H- β -CD, HBL and combined H- β -CD/HBL treatments, respectively. Thus, vindoline yields were 80, 31 and 108 % higher with added H- β -CD, HBL and H- β -CD/HBL (Fig. 11b). Maximal catharanthine yields were 2.08, 1.38 and 2.49 mg/L in the H- β -CD, HBL and combined H- β -CD/HBL treatments, respectively, representing increases of 79, 20 and 116 % relative to controls (Fig. 6c). Maximal vinblastine yields were 3.35, 2.75 and 3.96 mg/L in the H- β -CD, HBL and combined H- β -CD/HBL treatments, respectively, representing increases of 81, 48 and 114 % relative to controls (Fig. 11d). Similarly, maximal ajmalicine yields were 2.89, 2.96 and 3.87 mg/L in the H- β -CD, HBL and combined H- β -CD/HBL treatments, respectively, which were 18, 21 and 58 % greater than the respective yields in the control group (Fig. 11e).

Transcript levels of the genes involved in the biosynthesis of TIAs were previously examined in cell suspensions and hairy roots of *C. roseus* treated with MeJA (Goklany, Loring et al. 2009, Zhou, Yang et al. 2015). The combined effects of H- β -CD and HBL on the transcription of TIA biosynthesis genes in *C. roseus* CMC cultures, however, have not been reported. This research examined transcript levels of the genes involved in the methylerythritol 4-phosphate (MEP) pathway (*GES*, *G10H*, *IRS* and *LAMT*) and the early (*TDC*, *STR* and *SGD*) and late (*D4H*, *CS*, *TS* and *PXR1*) TIA-biosynthesis pathways, and of the transcription factor *ORCA3* in CMCs of *C. roseus* on day 12 and 15 treatment with 20g/l H- β -CD and 150- μ g/L HBL individually or in combination (Fig. S3). Maximal levels of *LAMT*, *TDC*, *SGD*, *D4H*, *ORCA3*, *TS* and *PXR1* transcription were reached on day 12 and were 5.38, 4.38, 5.87, 2.75, 6.85, 5.86 and 4.95 times higher than those of the control, respectively. Maximal levels of *GES*,

G10H, *IRS*, *STR* and *CS* transcription occurred on day 15 after elicitor addition and were 3.64, 2.88, 6.56, 3.26 and 3.12 times higher than those of the control, respectively. In summer, both elicitors were found to induce transcription of these genes, and the combination of H- β -CD and HBL together was more efficient than either alone.

Discussion

Over the past few decades, plant natural products (PNPs), including pharmaceuticals, spices, pigments, and fragrances, have generated immense economic and societal value (Zhu, Liu et al. 2021). While microbial cell factories or chemical and semi-synthetic approaches can produce simple PNPs in large quantities, they face challenges such as low or no yield and high costs when dealing with structurally complex PNPs (Liu, Ding et al. 2017, Ozyigit, Dogan et al. 2023). With the rapid advancements in plant physiology, genetics, and gene-editing technologies, researchers have shifted their focus from traditional microbial cell factories to complex plant cell cultures (PCCs), including callus culture, hairy root culture, and shoot tip culture (Zhu, Liu et al. 2021, Ozyigit, Dogan et al. 2023). PCCs provide a mature and efficient platform for the biosynthesis of structurally complex PNPs, some of which have already been commercially produced on a large scale (Krasteva, Georgiev et al. 2021). However, traditional PCC systems are limited by issues such as cell aggregation, low secondary metabolite content, and unstable yields, leading to low production efficiency (Ochoa-Villarreal, Howat et al. 2015, Ozyigit, Dogan et al. 2023). To address these challenges, and building on previous studies in our laboratory, we developed a new *C. roseus* CMC culture system that effectively resolves the issue of cell aggregation while ensuring high secondary metabolite content and stable yields (Zhou, Yang et al. 2015).

Since 1992, Elicitors have been employed in plant biotechnology to stimulate secondary metabolite pathways (Gundlach, Muller et al. 1992). Extensive research has confirmed their effectiveness across various plant cell and tissue cultures, boosting secondary metabolite production in cell cultures for biotechnological use (Almagro, Belchí-Navarro et al. 2015). Notably, the combined use of jasmonates and CDs exhibits synergistic effects, significantly increasing anthocyanin and stilbene levels (Lijavetzky, Almagro et al. 2008). The results of this study demonstrate that the elicitors H- β -CD and HBL, individually and in combination, significantly enhance the growth, NO and Ca²⁺, H₂O₂, enzyme activity, alkaloid production, and gene expression involved in TIA biosynthesis in *C. roseus* CMCs. By utilizing combined inducers, we effectively enhanced TIA content in bioreactors, paving the way for the large-scale production of bioactive alkaloids using *C. roseus* CMC cultures.

CMCs as a platform for the production of PNPs

In previous studies, researchers typically used periwinkle leaves as explants to induce the establishment of *C. roseus* callus and hairy roots for the production of alkaloids such as vindoline, catharanthine, and ajmalicine [2]. The stem tissue of *C. roseus* contains four distinct cell types: internal phloem-associated parenchyma, epidermal cells, idioblasts, and laticifers, which express several key enzymes involved in the TIA metabolic pathway. Furthermore, the biosynthesis of demethoxyvindoline, catharanthine, serpentine, and ajmalicine is most prominent in the stem regions that contain these cambial meristematic cells (CMCs).

A recently developed stem from a wild *C. roseus* plant (Fig. 2a) was used to establish CMCs. Microscopic analysis of a suspension culture of *C. roseus* CMCs revealed the presence of small, abundant vacuoles within the cultured cells (Fig. 2f). Most cells appeared as singletons or formed small aggregates consisting of 2–8 cells (Fig. 2g). The cell death percentage of CMCs was significantly higher than that of DDCs, indicating the hypersensitivity of CMCs to the radiomimetic antibiotic zeocin. This observation is consistent with findings from CMCs derived from *T. Cuspidata*, *Panax ginseng* and *R. glutinosa* (Fig. S1) (Lee, Jin et al. 2010, Zhou, Li et al. 2023). Notably, *C. roseus* CMCs demonstrated the ability to produce high levels of alkaloids, including vindoline, catharanthine, vinblastine, and ajmalicine (Zhou and Chen 2022). These findings support the conclusion that cultured CMCs exhibit stem cell-like properties, consistent with their identity as CMCs. Similar CMCs have been successfully established

from *T. cuspidata*, *Panax ginseng*, *R. glutinosa*, *O. basilicum*, and *T. wilfordii* (Lee, Jin et al. 2010, Ochoa-Villarreal, Howat et al. 2015, Zhou, Yang et al. 2015, A, Hafelder J et al. 2020, Partap, Warghat et al. 2023, Zhou, Li et al. 2023). These CMCs showed superior growth rates, smaller cell aggregations in bioreactors, and enhanced secondary metabolite production compared with DDCs, which may provide a cost-effective platform for the production of PNPs.

Enhancement of TIA production by H- β -CD

In plant cell culture, the biosynthesis of plant secondary metabolites often brings feedback inhibition and cytotoxicity to cells, leading to significant decreases of cell viability and enzymatic activities (Rao and Ravishankar 2002, Cardillo, Perassolo et al. 2021). β -CD and H- β -CD can form complexes with most secondary metabolites, thereby facilitating compounds removal. The addition of 50 mM Methyl- β -Cyclodextrin (MBCD) synergistically boosted paclitaxel biosynthesis to 402.4 μ g/L, representing a 5.8-fold increase (Farhadi, Moieni et al. 2020). CDs enhance the biosynthesis of polyphenols by *Bryophyllum* suspension cultured cells favoring their accumulation outside the cells (García-Pérez, Losada-Barreiro et al. 2019). CDs increase triterpene production in *Solanum lycopersicum* cell cultures by activating biosynthetic genes. The combined treatment of cells with MJ and MBCD synergistically induced the pterocarpan production (trifolirhizin, trifolirhizin malonate, and maackiain) in the cells (58 mg/g DW) and culture medium (222.7 mg/L) (Kim, Jeong et al. 2020). In addition, all of above reports indicate that CDs (such as β -CD, MBCD, γ -CD, etc.) have any negative effects on cell growth. Although there are a few reports on the addition to suspension cultures of callus cells, there is little information on the effect of CDs on secondary metabolites in CMCs. Like previous reports, our data (Zhou et al., unpublished) suggested that H- β -CD does not decrease CMC viability (Durante, Caretto et al. 2011). In the 100-, 250- and 500-mL flasks, the maximal growth rates of CMCs treated with H- β -CD in 100-, 250-, and 500-mL Erlenmeyer flasks increase 131 - 143% compared the control groups, while the maximal production of vindoline, catharanthine, vinblastine and ajmalicine increase 78 - 100%, 104–186 %, 59–67% and 21–25%, respectively. In *Corylus avellana* cell suspension culture, 78.6% of the total paclitaxel (316.5 μ g/L) was secreted into the culture medium, which was 146% higher than the control level (Farhadi, Moieni et al. 2020). In perfusion culture, CMCs from *T. cuspidata* produced a total of 264 mg of paclitaxel per kg of cells, 74 % of which was directly secreted into the medium (Lee, Jin et al. 2010). Similarly, *C. roseus* CMCs treated with 20g/L H- β -CD in our study yielded 767.2 mg/L total alkaloids (64% more than controls), 26% (175.6 mg/L) of which were directly secreted into the medium, which represented 408% more secretion than in controls (Fig. 3f). CDs induce the accumulation of NO and Ca^{2+} in the extracellular, and then NO will be released into the extracellular medium (Durante, Caretto et al. 2011, Belchi-Navarro, Almagro et al. 2013, Cardillo, Perassolo et al. 2021). In *C. roseus* CMCs, H- β -CD treatment increased extracellular NO levels by 3-fold and intracellular Ca^{2+} levels by 1.8-fold. In summary, cyclodextrin significantly promoted cell growth, enhanced NO release, increased Ca^{2+} levels, facilitated the secretion of intracellular alkaloids into the culture medium, and thereby boosting the production of TIAs.

Enhancement of TIA production by HBL

Brassinosteroids refer to a group of steroidal lactones with high plant physiological activity, which can stimulate cell elongation and division in plant. 24-epibrassinolide has been found to significantly increase the total amount of phenolic substances and total flavonoids in hairy root culture of *Echinacea purpurea* L. Moench and phenolic compounds in immobilized cell suspension cultures of *Vitis vinifera* L. cv. Cinsault, compared to the control (T., U. et al. 2020, Babalik 2021). BR added in M9 medium increased shikonin and its derivative content markedly by 31–87%, compared with its control (Yang, Zhang et al. 1999). HBL at a concentration of 10 μ g/L has no observable effect on the growth of CMC cells. However, treatments with 50, 100, and 150 μ g/L of HBL increased cell growth by 54 - 185%, whereas 200 μ g/L of HBL significantly inhibited growth. 150 μ g/L HBL treatment significantly increased the activities of SOD (184%), POD (140%), and CAT (185%) while reducing H_2O_2 content (59%)

compared to the control (Fig. 5). Additionally, 150 µg/L HBL treatment increased the production of vindoline, catharanthine, vinblastine and ajmalicine by 50 - 91%. In summary, an appropriate concentration of HBL can promote cell growth, enhance the activities of SOD, POD, and CAT enzymes, and reduce H₂O₂, thereby increasing alkaloid production.

Enhancement of TIA production by a combination of H-β-CD and HBL in bioreactors

Many types of bioreactors, such as stirred airlift and stirred tank bioreactors, have been successfully used for cultivating callus and transformed root cultures to obtain the chemical PNPs. Compared to 100 mL of flask cultivation, the maximum growth and the content of vindoline, catharanthine, vinblastine, and ajmalicine increased by 140, 63, 74, 24, and 33% in a 5-L bioreactor, respectively. It is worth highlighting that in this study, we employed a 5-L airlift bioreactor constructed with a heat-resistant glass vessel, complemented by an air pump and a sterile air filter to supply airflow to the system. The total cost of this setup was approximately 1,000 RMB. Remarkably, in our self-assembled, cost-effective bioreactor, the growth rate of CMCs was comparable to, if not better than, that achieved in our previous studies utilizing an expensive commercial 5-L stirred airlift bioreactor (Zhou, Yang et al. 2015). This demonstrates that *C. roseus* CMCs possess exceptional growth characteristics and strong adaptability, making them highly versatile for broader applications.

In 5-L bioreactor cultures, the maximum growth rate and production of vindoline, catharanthine, vinblastine, and ajmalicine in *C. roseus* CMCs treated with 20g/L H-β-CD increased by 271% (16.75 g/L), 196% (4.24 mg/L), 379% (3.16 mg/L), 125% (3.35 mg/L), and 85% (3.41 mg/L), respectively, relative to the yields of CMCs cultured in 100-mL flasks without elicitors; The maximum growth rate and production of vindoline, catharanthine, vinblastine, and ajmalicine in *C. roseus* CMCs treated with 150 µg/L HBL increased by 227% (16.52 g/L), 114% (3.07 mg/L), 109% (1.38 mg/L), 84% (2.75 mg/L), and 61% (2.96 mg/L), respectively. Furthermore, when CMCs were treated with 20 g/L H-β-CD and 150 µg/L HBL in a 5-L bioreactor, the maximum growth rate, as well as the contents of vindoline, catharanthine, vinblastine, and ajmalicine, reached 17.51 g/L, 17.51 mg/L, 4.89 mg/L, 2.49 mg/L, 3.96 mg/L, and 3.87 mg/L, respectively. These results represent remarkable increases of 335%, 54%, 241%, 277%, 165%, and 110% relative to yields of CMCs cultured in 100-mL flasks without elicitor, respectively, highlighting the significant enhancement achieved under these conditions. MeJA and β-CD have been shown to enhance the transcription of TIA biosynthetic genes, including *GES*, *G10H*, *TDC*, *STR*, *SGD*, and *D4H* in *C. roseus* (Goklany, Loring et al. 2009, Simkin, Miettinen et al. 2013, Zhou, Yang et al. 2015). ORCA3 is a jasmonate-responsive transcriptional regulator of TIA biosynthetic genes (van der Fits and Memelink 2000). The effects of H-β-CD and HBL, either individually or in combination, on the biosynthetic genes of alkaloids in CMCs remain unknown. The transcription levels of *GES*, *G10H*, *IRS*, *LAMT*, *TDC*, *STR*, *SGD*, *D4H*, *ORCA3*, *CS*, *TS* and *PXR1* peaked on day 12 or 15 after elicitor addition, reaching 275 - 685% increases compared to those of the control, which demonstrate that H-β-CD and HBL can significantly upregulate TIA genes.

These results confirmed that the application of H-β-CD and HBL, either individually or in combination, significantly enhances the growth of CMC cells, upregulates the expression of genes associated with the TIA biosynthetic pathway, promote extracellular transfer of the resulting compounds to reduce feedback inhibition and boosts the production of vindoline, catharanthine, vinblastine, and ajmalicine.

Conclusions

This study establishes the efficacy of H-β-CD and HBL as potent elicitors for enhancing TIA biosynthesis in *C. roseus* CMC cultures. The combined elicitor treatment not only improved alkaloid yields but also elucidated key molecular mechanisms driving these enhancements. These findings lay the groundwork for developing scalable and sustainable strategies for producing high-value alkaloids, contributing to advances in plant biotechnology and pharmaceutical production. *C. roseus* CMC cultures offer a cost-effective and environmentally friendly platform for sustainable production of a variety of TIAs.

Supplementary Materials The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Sequences for forward and reverse primers of the TIA biosynthetic pathway genes in *C. roseus* for qRT-PCR. Fig. S1: Levels of cell death in *C. roseus* cells after exposure to the bleomycin. Fig. S2: *C. roseus* CMCs cultured in the 500- (a), 250- (b) and 100-mL (c) Erlenmeyer flasks, respectively, and 5-L bioreactors on day 12 (d). Fig. S3: Effects of 20 g/L H- β -CD and 150- μ M HBL individually or in combination on expression of TIA genes in *C. roseus* CMCs cultured in 5-L bioreactors.

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Data availability All data generated or analysed during this study are included in this article.

Declarations

Conflicts of Interest The authors declare that they have no competing interests.

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