



# Nanoparticle-induced modulation of indole alkaloid biosynthesis and gene expression in *Catharanthus roseus* in vitro cultures

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## Abstract

*Catharanthus roseus* (L.) G. Don is a key medicinal plant producing pharmacologically important terpenoid indole alkaloids (TIAs). This study aimed to enhance the biosynthesis of three major TIAs; catharanthine (CAT), vincristine (VCR), and vinblastine (VBL) through a stepwise in vitro approach involving callus culture, hairy roots (HRs) induction via *Agrobacterium rhizogenes*, and elicitation with zinc oxide (ZnO) and chitosan (CH) nanoparticles (NPs) in solid and liquid media. Two HR lines (L1, derived from K599/p35SGFPUS<sup>+</sup>, and L2, from disarmed K599) and stem-derived calli were treated with 50 and 100 mg/L of ZnO- and CH-NPs. Nanoparticle treatments had limited effects on callus growth, except 100 mg/L CH-NPs, which reduced biomass in solid medium. In suspension culture, 50 mg/L ZnO-NPs enhanced fresh weight, whereas CH-NPs reduced it. Among HRs, the highest biomass occurred with 100 mg/L ZnO-NPs. HPLC analysis revealed higher VCR levels in cultured tissues than in field-grown leaves. In solid-grown HRs, CAT increased with 100 mg/L CH-NPs, and VCR rose significantly with 100 mg/L of either NP type. In liquid-grown HRs, CAT increased with 100 mg/L CH-NPs, VCR with 100 mg/L ZnO-NPs, and VBL with 50 mg/L CH-NPs. In callus cultures, ZnO-NPs promoted CAT and VCR accumulation in solid media, while VCR increased in suspension cultures treated with 100 mg/L ZnO-NPs or 50 mg/L CH-NPs. Gene expression analysis confirmed NP-induced modulation of *STR*, *DAT*, *PRX1*, and *SLS*, correlating with alkaloid accumulation. Overall, ZnO and CH nanoparticles effectively elicited TIA biosynthesis in *C. roseus* cultures, highlighting their potential for enhanced alkaloid production.

## Key message

Zinc oxide and chitosan nanoparticles differentially modulate terpenoid indole alkaloid production in *Catharanthus roseus* callus and hairy roots, depending on nanoparticle type, concentration, culture system, and target alkaloid.

**Keywords** Catharanthine · Callus culture · Hairy roots · Madagascar periwinkle · Nanoelicitation · Vinblastine · Vincristine · Terpenoid indole alkaloids

## Introduction

*Catharanthus roseus* (L.) G. Don is one of the most widely used medicinal plants due to its broad spectrum of therapeutic properties and its ornamental value, owing to year-round flowering. The plant is a principal natural source of terpenoid indole alkaloids (TIAs), particularly vinblastine (VBL) and vincristine (VCR), which are essential chemotherapeutic agents used in both traditional and modern medicine (Das et al. 2020). Although these TIAs are present in minute quantities among more than 130 alkaloids synthesized by the plant, they are especially valuable due to their strong anticancer properties (Abeed et al. 2021). Due

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to their highly complex chemical structures, the large-scale chemical synthesis of these compounds is not practically feasible (Almagro et al. 2015; Sun 2016; Tian 2015). Plant biotechnology approaches, including cell and tissue culture as well as genetic transformation, offer sustainable and controlled methods for secondary metabolite production (Bourgaud et al. 2001; Zhao et al. 2001a, b). Various techniques such as osmotic stress, light irradiation, medium optimization, large-scale bioreactor cultivation, hairy root induction, and cell immobilization have been successfully applied to increase TIAs accumulation (Iwase et al. 2005; Sharafi et al. 2013; Mirzaee et al. 2016). Notably, *A. rhizogenes*-mediated hairy root cultures of *C. roseus* have proven to be a rapid and reliable system for biomass production with enhanced alkaloid content (Hanafy et al. 2017; Elbouzidi et al. 2025).

Elicitation is a powerful strategy for boosting the synthesis of valuable bioactive compounds in medicinal plants. It can modulate the expression of genes involved in biosynthetic pathways, particularly those encoding key regulatory enzymes, thereby enhancing metabolite accumulation (Marchica et al. 2020; Bhaskar et al. 2022; Guru et al. 2022; Grzegorzczak-Karolak et al. 2024). The application of nanoparticles (NPs) is recognized as potent elicitors of secondary metabolite biosynthesis (Vyavahare et al. 2025). For example, zinc nanoparticles induced the upregulation of key TIAs pathway genes, such as Deacetylvindoline 4-O-Acetyltransferase (*DAT*) and class III peroxidase enzyme (*PRX1*) genes, which correlated with enhanced alkaloid biosynthesis (Perveen et al. 2022). Moreover, Zinc oxide nanoparticles have also been reported to enhance tropane alkaloid levels and antioxidant enzyme activities in *Hyoscyamus reticulatus* hairy roots (HRs) (Asl et al. 2019; Alizadeh et al. 2025). *C. roseus* cultures treated with copper and zinc nanoparticles synthesized using *Eriobotrya japonica* seed extract showed significantly increased VCR accumulation in callus and shoot cultures (Perveen et al. 2022).

While metallic nanoparticles have demonstrated strong elicitation potential, concerns related to their long-term environmental impact, phytotoxicity, and bioaccumulation have encouraged the search for safer, biodegradable, and biologically compatible elicitors. In this context, natural polymer-based elicitors, particularly chitosan and its nanoformulations, have gained considerable attention. These biogenic materials combine the elicitation efficiency of nanoparticles with the advantages of low toxicity, biocompatibility, and environmental safety, making them promising alternatives to metallic NPs for stimulating secondary metabolite biosynthesis. In *C. roseus* treatment with 100 mg/L chitosan significantly increased VBL and VCR levels compared to untreated controls (Plianpong et al. 2024). Moreover, the application of chitosan nanoparticles to *C. roseus* plants under drought conditions significantly

enhanced the expression of key alkaloid biosynthetic genes (*DAT*, *PRX1*, Strictosidine Synthase (*STR*), and geissoschizine synthase (*GS*)), which was associated with increased accumulation of alkaloid content compared to drought stress (Ali et al. 2021). Recent studies have also shown that the effects of nanoparticles in plant tissue culture are significantly influenced by the type of culture medium. For example, liquid media, especially in temporary immersion systems, promote better nanoparticle dispersion and uptake, leading to enhanced shoot proliferation and biomass accumulation. In contrast, semisolid media may restrict nanoparticle activity due to limited diffusion (Pérez-Caselles et al. 2023; Gunasena et al. 2024). Also, nanoparticles such as silver NPs have been found to reduce microbial contamination and stimulate callus growth, underscoring the importance of optimizing both medium composition and nanoparticle concentration for successful in vitro culture outcomes (Alfarraj et al., 2023). It is worth noted that the precise effects of NPs on secondary metabolism and the underlying mechanisms remain largely undefined (Hatami and Ghorbanpour 2014). These findings highlight the potential of nano-elicitation to modulate metabolic pathways and improve phytochemical yields in plant tissue culture systems. They also highlight the need for further research into optimal NP types, sizes, concentrations, uptake mechanisms, and elicitation responses.

The primary aim of this study is to evaluate the effects of ZnO-NPs and CH-NPs on biomass production and alkaloid accumulation—specifically catharanthine (CAT), VCR and VBL in hairy roots and tissue cultures of *C. roseus*. Furthermore, this study highlights the role of key TIA biosynthetic genes, including *PRX1*, *DAT*, Secologanin Synthase (*SLS*), and *STR*. This research also aims to provide insights into plant-nanoparticle interactions and shed light on the underlying mechanisms involved in TIAs accumulation in *C. roseus*. To the best of our knowledge, this is the first study to systematically compare the effects of metal-based and polymer-based nanoparticles across different in vitro culture systems (callus vs. hairy roots) and growth formats (solid vs. liquid, while simultaneously integrating biomass, metabolite profiling, and transcriptional regulation of the TIA biosynthetic pathway in *C. roseus*).

## Materials and methods

### Plant material and culture condition

*C. roseus* seeds were obtained from local market in Cairo, Egypt (coordinates: 30°2'40"N 31°14'9"E, and has an elevation of 25 m above sea level). The seeds were surface sterilized and germinated on a medium consisting of Murashige

and Skoog (MS) salts and vitamins (Murashige and Skoog 1962), supplemented with 30 g/L sucrose (w/v). The medium was adjusted to pH 5.8 before adding the gelling agent and then sterilized by autoclaving at 121 °C for 20 min. The seeds were germinated in a growth chamber at  $25 \pm 2$  °C under photoperiod (18 h light/6 h dark) using cool-white light (700–800 lx) for 3–4 weeks. The germinated seedlings were selected and used as a source of explants for both callus induction and *A. rhizogenes*-mediated transformation.

### Establishment of callus and suspension cultures

*C. roseus* callus was induced from stem explants (1–2 cm) derived from in vitro grown seedlings on MS basal medium supplemented with 3% sucrose, 1.0 mg/L 6-Benzylaminopurine (BAP) and 1.0 mg/L 1-Naphthaleneacetic acid (NAA) according to (Singh et al. 2011). The pH of the medium was adjusted to 5.8 and solidified with 0.2 (w/v) gelrite before autoclaving at 121 °C for 20 min. Five to six explants were cultured per Petri dish, and the cultures were incubated at  $25 \pm 2$  °C in a growth chamber with 16 h photoperiod using cool-white light (700–800 lx) for 3–4 weeks. The induced calli were subcultured every four weeks.

### Establishment of hairy roots (HRs) culture

#### Bacterial strain and hairy root induction

Two *A. rhizogenes* strains were used for transformation. The first strain was K599 carrying the p35SGFP<sub>GUS</sub>+ plasmid, which contains GFP-intron, *uidA* reporter genes driven by the CaMV 35 S promoter, as well as the *hptII* selectable marker within the T-DNA borders. This strain and plasmid were provided by Georgina Estrada-Navarrete (UNAM, Mexico). The second strain was a disarmed *A. rhizogenes* K599 obtained as chemically competent cells from Intact Genomics® (St. Louis, USA) and harboring the pCAMBIA1391z vector. Bacterial cultures were initiated from glycerol stocks on solid LB medium with appropriate

antibiotics and incubated at 28 °C in the dark. A single colony was grown overnight in liquid LB (180 rpm) to an OD<sub>600</sub> of 0.6, harvested by centrifugation, and resuspended in co-cultivation medium (half-strength MS liquid medium containing 60 g/L sucrose). The suspension was adjusted to an OD<sub>600</sub> of 0.8 for infection.

Two transformation methods were applied. In the first method, leaf and stem explants from three-week-old in vitro seedlings were immersed in the bacterial suspension for 30 min, co-cultivated on solid medium in the dark for 1–2 days, washed with cefotaxime (300 mg/L), and transferred to hormone-free MS medium containing cefotaxime. Hairy roots (HRs) emerged within four weeks and were subsequently cultured on MS medium with ticarcillin (300 mg/L) (Hanafy et al. 2016). The second method involved stem and leaf explants piercing or injection of 2–3-week-old seedlings with the bacterial suspension (OD<sub>600</sub> = 0.8). After 48 h of dark incubation, seedlings were transferred to the growth chamber with a 16 h photoperiod using cool-white light (700–800 lx). HRs appeared after approximately three weeks and were excised once they exceeded 5 mm in length, then cultured on MS medium containing ticarcillin (300 mg/L). Finally, two transgenic hairy root lines were selected for further experiments; L1, generated using strain K599 carrying p35SGFP<sub>GUS</sub>+ and L2, generated using the disarmed K599 strain from Intact Genomics®. Both lines were obtained via seedlings injection.

### Green Fluorescent Protein (GFP) expression and PCR analysis

GFP expression in transgenic HRs was visualized by fluorescence microscope (Olympus SZX7 connected with U-RFL-T) equipped with a filter set for excitation between 460 and 490 nm. Total genomic DNA was isolated from bacterial-free transgenic HRs and non transformed roots using Genomic Purification Kit according to the manufacturer's instructions (Thermos Scientific, USA). DNA samples were subjected to PCR to detect either the presence of *GFP*, *GUS*, and *HPTII* genes in the transgenic HR cultures (L1) or *rolA* gene in the HRs L2. The forward and reverse primer sequences were presented in Table 1. The total volume of each PCR reaction was 25 µl containing 12.5 µL 2X EmeraldAmp PCR master mix (Takara®, Japan), 10 µM forward and reverse primers and 150 ng genomic DNA. The PCR cycling conditions included an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30s at 94 °C, 40s at 55 °C (for *GUS*, *GFP* and *HPTII* genes) or 60 °C for *rolA* gene, and 1 min at 72 °C, with a final extension step at 72 °C for 5 min. Finally, the amplified products were separated on 0.8% (w/v) agarose gel containing ethidium bromide. The respective *A. rhizogenes* strain was used as a positive

**Table 1** The primers sequences used for the amplification of *GFP*, *GUS* and *HPTII* genes and the product sizes

| Gene         | Forward (5'-3')             | Reverse (5'-3')             | Product size (bp) |
|--------------|-----------------------------|-----------------------------|-------------------|
| <i>GFP</i>   | GTAAACGGCCACAAGT<br>TCAGCG- | TCGTCCATGCCGA<br>GAGTGATCC  | 641               |
| <i>GUS</i>   | GTAAACGGCCACAAGT<br>TCAGCG  | CATTCGGAATCTC<br>CACGTAC    | 750               |
| <i>HPTII</i> | CGATTGTGTACGCCCG<br>ACAGTC  | CGATGTAGGAGG<br>GCGTGGATATG | 812               |
| <i>rolA</i>  | GAAGAAGAAGGCCAA<br>ACGTG    | GGTCTGAATATTC<br>CGGTCCA    | 176               |

control and genomic DNA from wild-type *C. roseus* was used as a negative control in PCR analysis.

### Nanoparticles elicitation

Two types of nanoparticle elicitors were used in this study: zinc oxide nanoparticles (ZnO-NPs) with a particle size of less than 50 nm (purchased from Sigma-Aldrich), and chitosan nanoparticles (CH-NPs) with a particle size of 80 nm (purchased from Nanoshel-UK Ltd). Two concentrations of each elicitor (50 and 100 mg/L) were individually added to the MS culture medium. ZnO-NPs were dissolved in deionized water, followed by sonication for 30 min in a water bath sonicator (40 kHz frequency at 120 W ultrasonic power). A few drops of diluted NaOH were then added to adjust the pH of the solution to approximately 7.0, according to the methods described earlier (Bian et al. 2011; Zhou and Keller 2010). CH-NPs were prepared by dissolving them in 10 mL glacial acetic acid until a clear solution was achieved. This solution was then diluted to the desired final volume using deionized water, as described by Kim et al. (2023). The mixture was subsequently sonicated for 30 min. Each concentration of the nanoparticles elicitors was filter-sterilized (0.22 µm) and added to the culture medium before incubation in the growth chamber. Callus cultures were divided into equal pieces of 0.5 g and cultured on MS medium supplemented with the respective concentrations of NPs, 3% sucrose, 1.0 mg/L BAP, 1.0 mg/L NAA, and solidified with 0.2% (w/v) gelrite. The same medium without NPs served as the control. Fresh and dry weights of the callus tissues were measured at intervals of 0, 10, 20, and 30 days. For suspension cultures, 0.5 g of callus tissue was transferred to 50 mL of MS liquid medium containing the same supplements, except without the gelling agent. The cultures were incubated on an orbital shaker at 120 rpm. After 30 days in the growth chamber, fresh and dry weights were recorded. Subsequently, total alkaloids were extracted and quantified.

With regard to the hairy root cultures, 0.5 g of fresh HRs were weighed and cultured either in solid or liquid basal MS (50 mL) medium supplemented with the respective concentration of NPs and 300 mg/l ticarcillin. HRs cultures growing in liquid medium were incubated on orbital shaker (120 rpm). HRs fresh weight and dry weight were measured at intervals of 0, 10, 20, and 30 days. After 30 days of exposure to the different concentrations of nanoparticles, total alkaloids were extracted and analyzed. Each treatment included at least four petri dishes or flasks.

### Total alkaloid extraction

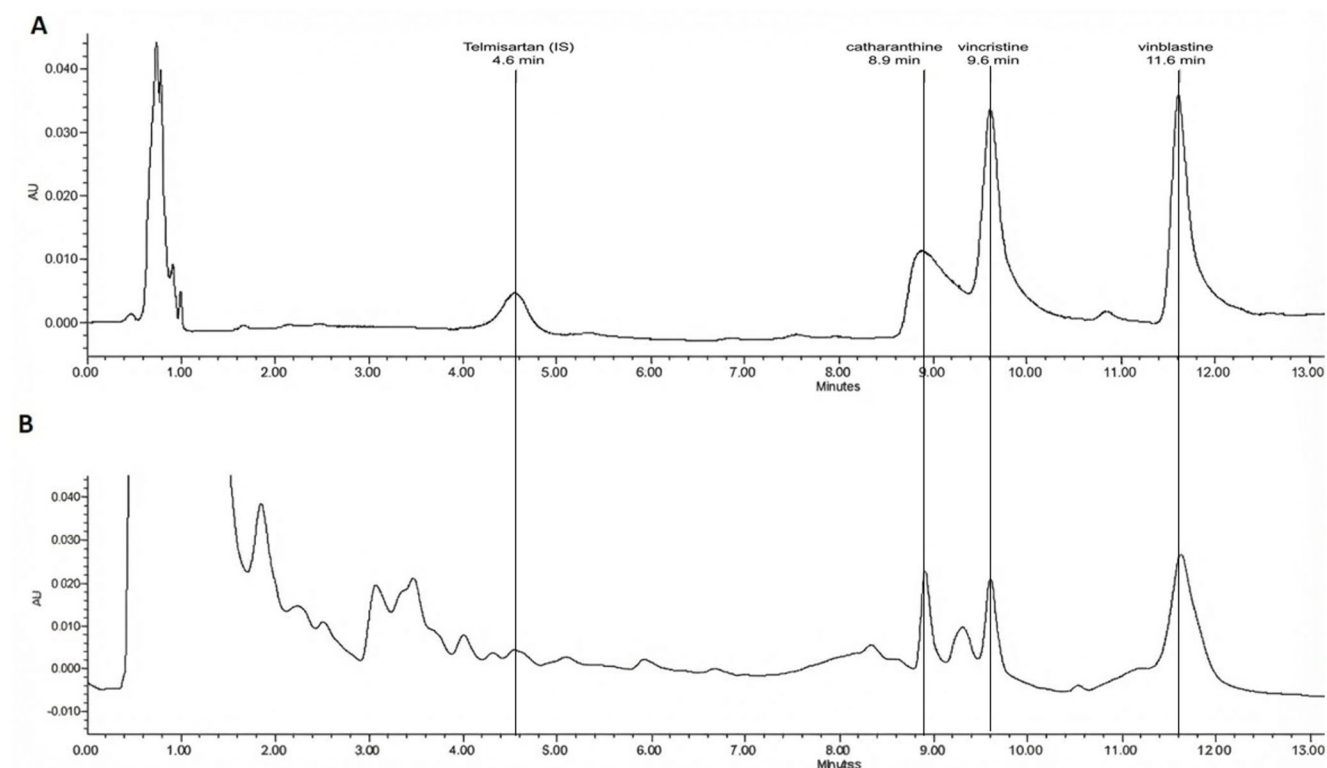
The extraction of alkaloids from leaves of in vivo wild type plants (control), HRs and callus cultures was carried out

following the method outlined by Zhou et al. (2012). Fresh *C. roseus* samples (1.0 g) were collected and ground into a fine powder using a mortar and pestle under liquid nitrogen. The powdered samples were extracted at room temperature in 1.0 mL of methanol (MeOH) for 60 min using a bath sonicator (40 kHz frequency at 120 W ultrasonic power). The resulting extract was centrifuged at 15,000xg for 5 min at 25 °C, and the supernatant was subsequently filtered (0.45 µm) and used for targeted alkaloid determination.

### HPLC method development and validation

The HPLC analysis for the three analytes (catharanthine (CAT), vincristine (VCR), and vinblastine (VBL) was carried out using a high-performance liquid chromatography system outfitted with a quaternary pump and a UV-Vis detector set to 254 nm for optimal detection of analytes (UHPLC Acquity Arc -PDA, Waters, USA) with Empower as data acquisition software. Telmisartan (Sigma-Aldrich) was used as an IS for quantification. To maintain consistency and stability in resolution, the column was kept at 35 °C. A reverse-phase C18 column (150 × 4.6 mm, 5 µm) was selected with a mobile phase consisting of 5 mM NaH<sub>2</sub>PO<sub>4</sub>-H<sub>3</sub>PO<sub>4</sub> buffer (pH 6) and acetonitrile (ACN) in gradient elution mode to ensure sharp peak resolution, reduced tailing, and shorter analysis times from 30% to 50% B in 10 min preceded by equilibration starting conditions for 3 min and maintained at the ending conditions for 6 min at 2.0 mL/min to strike a balance between efficient separation and reasonable run-time. This optimized method demonstrated superior performance compared to the original method by (Zhou et al. 2012), particularly in achieving shorter retention.

Calibration standards for CAT, VCR, VBL, and Telmisartan (used as the internal standard, IS) were prepared at concentrations ranging from 0.5 to 20 µg/mL in the mobile phase starting composition. Each standard was injected three times, and the peak areas were plotted against their corresponding concentrations to generate calibration curves. Linear regression equations and correlation coefficients ( $R^2$ ) were calculated for each analyte to confirm linearity. The limits of detection (LOD) and quantification (LOQ) were determined using the standard deviation of the response and the slope of the calibration curve. LOD was calculated as 3.3 times the standard deviation divided by the slope (3.3xSD/S), while LOQ was determined as 10 times the standard deviation divided by the slope (10xSD/S). The optimized HPLC-UV method was employed to quantify the concentrations of the three target alkaloids in the biological samples from 24 treatments, including leaves from in vivo-grown plants (control), non-elicited HR lines (two lines) and callus tissue, as well as elicited HRs and callus cultures grown in two culture systems (liquid and solid)



**Fig. 1** HPLC chromatograms demonstrating peak identification. (A) Standard mixture solution (10 µg/ml) showing Telmisartan (IS), catharanthine, vincristine, and vinblastine at retention times 4.6, 8.9, 9.6,

and 11.6 min, respectively. (B) Representative *C. roseus* hairy root extract showing alignment of sample peaks with standard retention times, confirming peak identity and method specificity in plant matrix

**Table 2** Analytical method validation parameters for Telmisartan (IS), catharanthine, vincristine, and vinblastine

|                     | Catharanthine          | vincristine            | vinblastine            |
|---------------------|------------------------|------------------------|------------------------|
| Regression equation | $y = 0.1941x + 0.0081$ | $y = 0.0862x + 0.0268$ | $y = 0.0918x + 0.0022$ |
| $R^2$               | 0.9972                 | 0.9962                 | 0.9968                 |
| Linearity Range     | 0.5–20 µg/mL           | 0.5–20 µg/mL           | 0.5–20 µg/mL           |
| LOD                 | 0.477 µg/mL            | 0.313 µg/mL            | 0.299 µg/mL            |
| LOQ                 | 1.444 µg/mL            | 0.947 µg/mL            | 0.907 µg/mL            |

(Supplementary Figure S1). All samples were extracted in triplicate, and the concentration of each alkaloid was determined individually. The mean values and standard deviations (SD) were calculated to ensure analytical precision.

### Optimized HPLC method

The HPLC method achieved baseline separation of Telmisartan (IS), CAT, VCR, and VBL, with retention times of 4.6 min, 8.9 min, 9.6 min, and 11.6 min, respectively (Fig. 1). The method demonstrated good linearity with correlation coefficients ( $R^2$ ) of 0.9972 for CAT, 0.9962 for VCR, and 0.9968 for VBL (Table 2). The LOD and LOQ for CAT were determined to be 0.477 µg/mL and 1.444 µg/mL, respectively. For VCR, the LOD and LOQ were 0.313 µg/mL and 0.947 µg/mL, while for VBL, these values were 0.299 µg/mL and 0.907 µg/mL, respectively (Table 2). This method provided well-resolved peaks with minimal baseline

noise, as shown in Fig. 1, which illustrates a chromatogram of a standard solution (10 µg/mL). Resolution ( $R_s$ ) for critical peaks (VCR and VBL) was 0.93, while achieving baseline resolution for other analytes. Representative chromatograms of *C. roseus* extracts demonstrated separation of the target alkaloids with retention times consistent with authentic standards (variation  $\leq 0.1$  min). The presence and stability of the internal standard (Telmisartan) in all sample injections confirmed method robustness and reliable peak identification across different sample matrices.

### Peak identification and method specificity

Peak identity for CAT, VCR, and VBL was confirmed through retention time matching with reference standards analyzed under identical chromatographic conditions. Standard solutions were injected individually and as mixtures to establish definitive retention times and peak characteristics.

Sample peaks were identified based on retention time matching ( $\pm 0.1$  min) with corresponding standards. The use of Telmisartan as an internal standard (IS) provided additional assurance of injection consistency and compensated for any minor variations in system performance across all sample analyses. The stability of the internal standard retention time (4.6 min) across all sample matrices confirmed the absence of chromatographic drift and supported reliable peak assignment in the complex plant extracts.

### RNA extraction and cDNA preparation

Total RNA was extracted from leaf tissues from in vivo wild-type (WT) plants, HRs, callus and cell cultures using TransZol Up kit (TransGen Biotech, Beijing, China Cat. No. ET111) according to the manufacturer instructions. To eliminate remaining genomic DNA, the extracted RNA samples were treated with RNase-Free DNase I (Fermentas). Nano-drop analysis was used for the determination of the quality and concentration of RNA samples. First strand cDNA was synthesized from 1.0  $\mu$ g total RNA of each sample using PrimeScript™ RT Reagent Kit (Takara®, Japan) according to the manufacturer structures in a final reaction volume of 20  $\mu$ L. The prepared cDNAs were stored at  $-80$  °C for quantitative real-time PCR (qRT-PCR).

### qRT-PCR analysis

qRT-PCR was performed to measure the expression of four genes involved in TIA biosynthesis: peroxidase 1, a gene encoding a class III peroxidase enzyme (*PRX1*), Deacetylindoline 4-O-Acetyltransferase (*DAT*), Secologanin Synthase (*SLS*) and Strictosidine Synthase (*STR*). The amplification process was carried out in a final volume of 25  $\mu$ L, which included 12.5  $\mu$ L of TB Green Premix EX Taq II (2x) (Takara, Japan), 2  $\mu$ L of cDNA (40 ng/ $\mu$ L), 1.0  $\mu$ L of each forward and reverse PCR primer (10  $\mu$ M) and 8.5  $\mu$ L of sterile nuclease free PCR-grade water. The PCR

conditions were set as follows: an initial denaturation at 95 °C for 30s, followed by 40 cycles of 95 °C for 15s, 50 °C for 20s, and 72 °C for 15s. The indigenous reference gene 40 S ribosomal protein S9 (*RSP9*) was used as an internal control, and the  $2^{-\Delta\Delta C_t}$  method was applied for relative quantification of gene expression (Livak and Schmittgen 2001). The primers used in qRT-PCR are listed in Table 3.

### Statistical analysis

Statistical analysis of the effects of NPs on biomass growth was performed according to Snedecor and Cochran (1989). Results of each treatment were presented as means of three biological replicates  $\pm$  standard error (SE). Data of TIAs contents and gene expression that violated assumptions of normality and homogeneity of variances, the non-parametric Kruskal-Wallis test (Kruskal and Wallis 1952) was employed. Following a significant Kruskal-Wallis result, Dunn's post-hoc test (Dunn 1964) with Bonferroni correction was applied to identify specific pairwise differences between treatment groups while controlling for family-wise error rate. For parametric data, one-way analysis of variance (ANOVA) was used, followed by Tukey's Honestly Significant Difference (HSD) post-hoc test (Tukey 1949). Statistical significance was defined at  $P \leq 0.05$  for all analyses. All statistical analyses were conducted using SciPy (Virtanen et al. 2020) and pandas (McKinney 2010). Data visualization was performed in Python (Van Rossum and Drake 2009) using matplotlib (Hunter 2007), with seaborn (Waskom, 2021) for enhanced graphical styling.

## Results

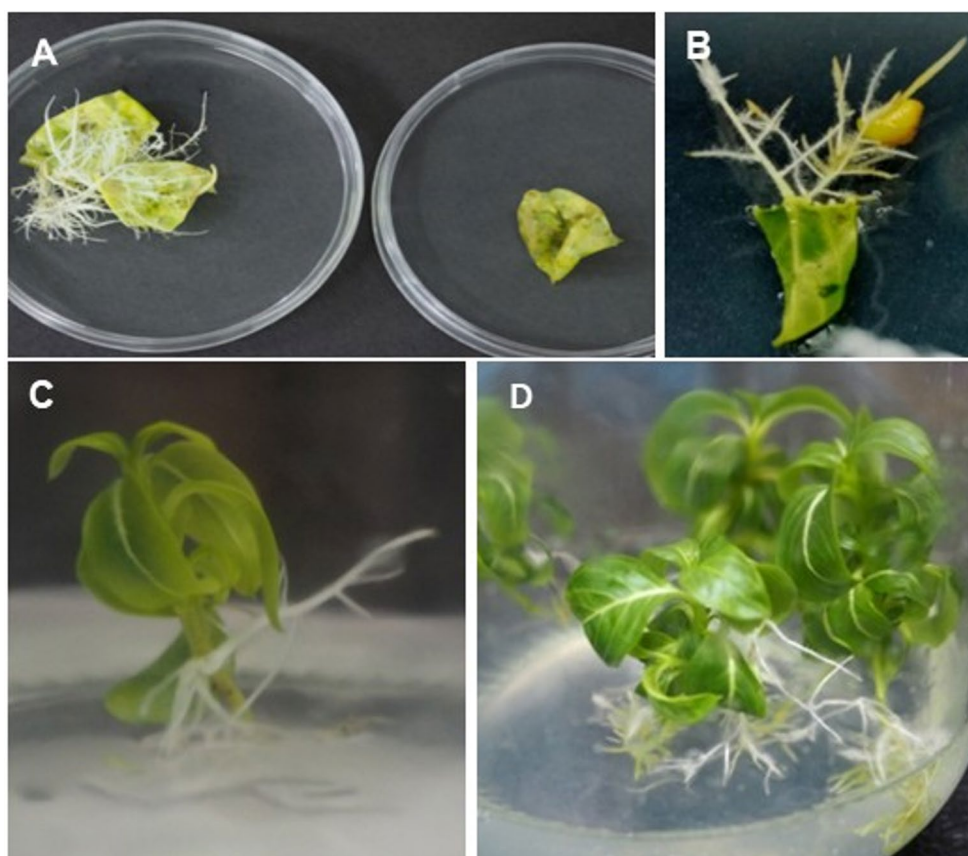
### Callus and hairy roots induction and growth

The results of the present study showed that callus formation was successfully induced from stem explants cultured on MS medium supplemented with 1.0 mg/L BAP and 1.0 mg/L NAA. In addition, both bacterial strains had a significant impact on inducing hairy roots from infected explants (leaves, stems, and seedlings) of *C. roseus*. Root initials developed within one week of co cultivation, emerging at the wounded edges of the leaf and stem explants. During the early stages of hairy roots (HRs) development, growth was slow, requiring approximately four weeks producing HRs of about 2–3 cm in length for both *Agrobacterium* strains. The developed HR cultures exhibited high degrees of branching and lacked geotropism, particularly those derived from leaf explants (Fig. 2). In contrast, stem explants showed poor responsiveness in terms of HRs induction and growth; thus, we discontinued further work

**Table 3** Primers used for the qRT-PCR analysis according to Tang et al. (2022)

| Gene | Forward (5'-3')       | Reverse (5'-3')         |
|------|-----------------------|-------------------------|
| RSP9 | GAGGGCCAAAACAACTTGA   | GAGGGCCAAAACAACTTGA     |
| PRX1 | CTGCTGCTTCTCTCATTTCC  | CAACATCGTTTGGGAAGACCT   |
| DAT  | AAACCTCTTCTCCAACCCCTC | CTTCCACGAAC TCAATTCCATC |
| SLS  | GTTCCTTC              | CCCATTGGGTC             |
|      | TCACCGGA              | AACATGTCA               |
|      | GTTG                  |                         |
| STR  | AAAATTCCCGATACTCCG    | ACCAATGGGC ACTTCCT      |

**Fig. 2** Hairy root induction after infection with *A. rhizogenes* of *C. roseus*. **(A)** infected leaf explants after four weeks (left) and un-infected control leaf explants (right), **(B)** growth pattern of the hairy roots. **(C and D)** Hairy roots emerging from the site of inoculation after about four weeks from injection with *A. rhizogenes*



with stem tissues. Leaf explants consistently demonstrated better performance in establishing HR cultures. Control explants, which were not inoculated with *A. rhizogenes*, failed to induce HRs and eventually died after several weeks (Fig. 2A, B). For the direct injection method, 2-3-week-old seedlings were injected with *A. rhizogenes* suspensions, as described in the materials and methods section. Several independent HR lines were successfully established (Fig. 2C, D) from the injected seedlings and were subsequently cultured in both solid and liquid media. It is noteworthy that direct injection of seedlings resulted in a greater number of HR lines compared to leaf explant injection (data not shown). Among the induced HR lines, two lines L1 belongs to the K599 p35SGFP<sub>GUS</sub>+ strain and L2 line belongs to the disarmed K599 strain were selected and advanced for further study.

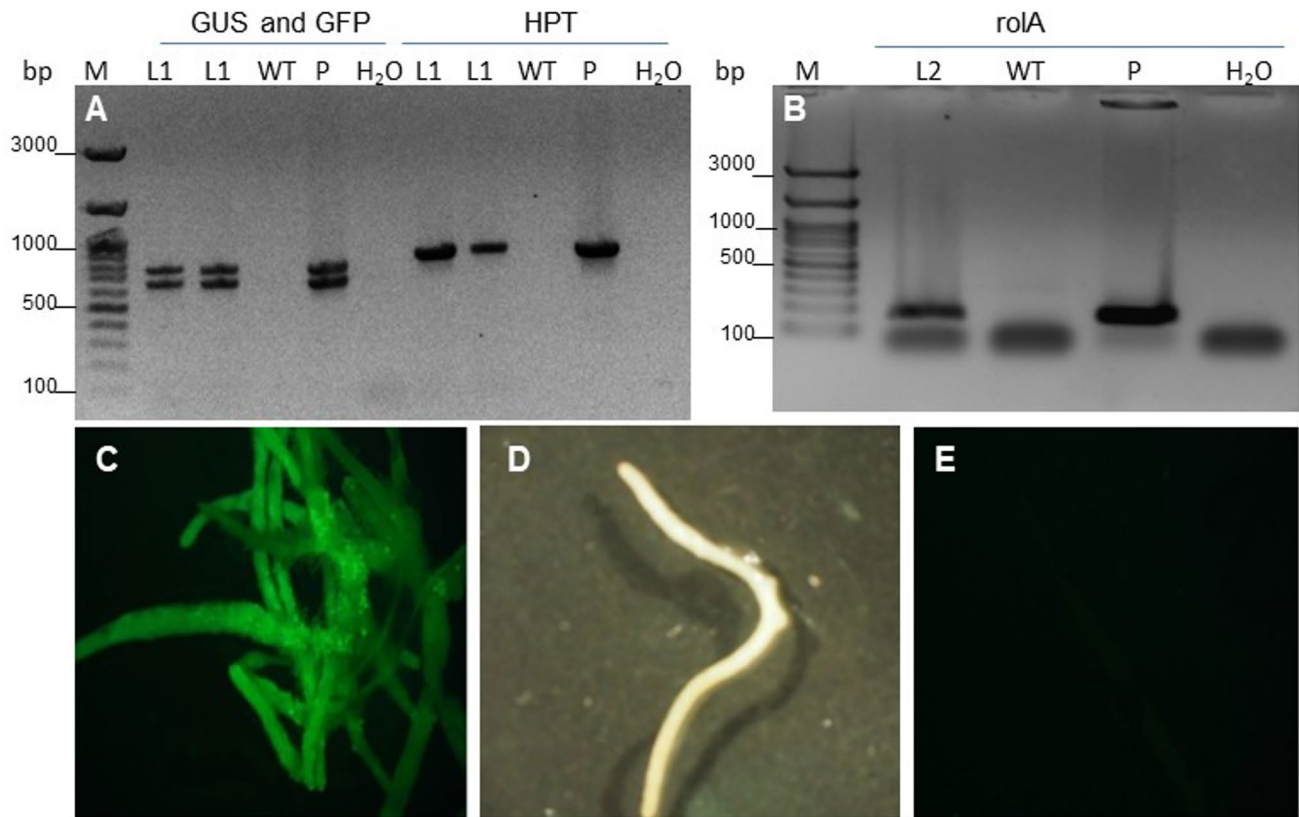
### Molecular characteristics of transformed hairy roots

The transgenic status of the developed bacterial free HRs was performed using PCR analyses of DNA isolated from HRs using forward and reverse primer amplifications to amplify *GUS*, *GFP*, *HPTII* and *rolA*, fragments. The PCR results showed the presence of *GUS*, *GFP* and *HPTII*, (Fig. 3A) bands of the expected sizes (750, 641 and 812 bp, respectively) in the transgenic hairy roots (L1) and in the positive

control (*A. rhizogenes* K599 p35SGFP<sub>GUS</sub>+plasmid). The absence of these fragments in the negative controls (non-transformed DNA samples), indicating the presence of integrated T-DNA in the hairy root cultures (Fig. 3A). Also, *rolA* fragment was amplified in HRs L2 with the expected product size of 176 bp (Fig. 3B). Moreover, GFP expression was monitored and confirmed the integration and expression of *GFP* in the HRs L1 (Fig. 3C). The WT roots were investigated under bright microscope (Fig. 3D) and under fluorescence microscope (Fig. 3E), in which no fluorescence was detected.

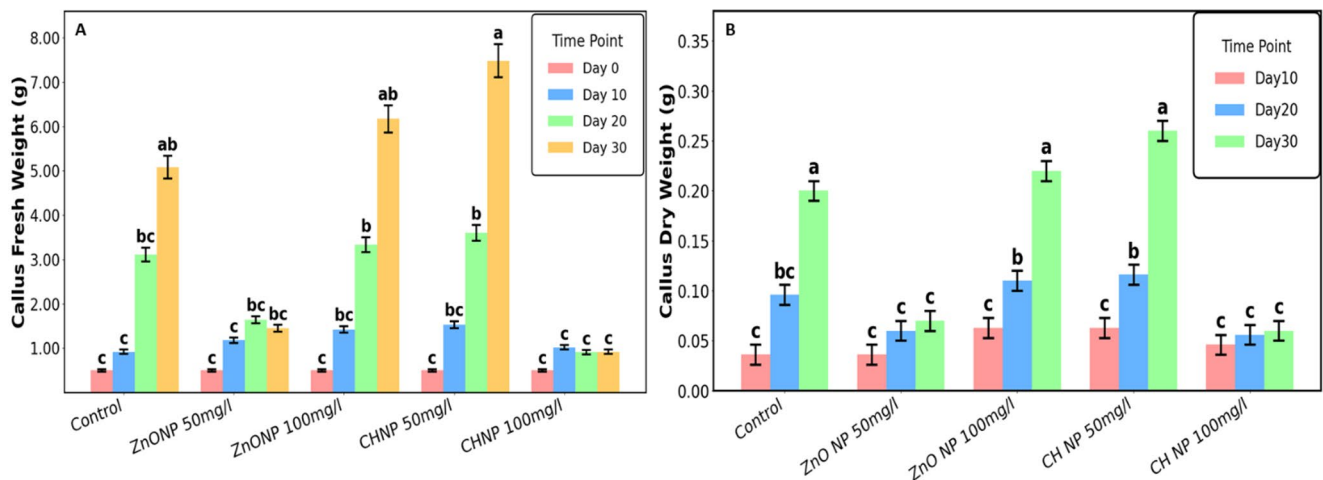
### Effect of NPs on callus growth

The effect of two different concentrations of ZnO-NPs and CH-NPs on callus growth of *C. roseus* was evaluated by measuring fresh and dry weights (FW, DW, respectively) of callus during 30 days of elicitation. The obtained results illustrated in Fig. 4 showed no significant differences among the elicitation treatments and the control except with the treatment with CH-NPs at 100 mg/L which caused a significant decrease in the FW and DW of the callus after 30 days of treatment, with fresh and dry weights dropping to 0.92 g and 0.06 g, respectively (4A and B). However, the treatments with ZnO-NPs at a concentration of 50 mg/L showed a significant decrease in the DW of the callus



**Fig. 3** PCR analysis for the presence of the transferred genes and detection of green fluorescent protein (GFP) in transgenic hairy roots of *C. roseus*. (A) PCR-amplified products of *GUS* (750 bp), *GFP* (641 bp) and *HPTII* (812 bp) genes in hairy roots (L1). (B) PCR-amplified prod-

ucts of *rolA* gene (176 bp) in hairy roots L2. (C) *GFP* visualization in transgenic hairy root L1. (D) Wild type roots under bright light. (E) Wild type roots under fluorescence microscope



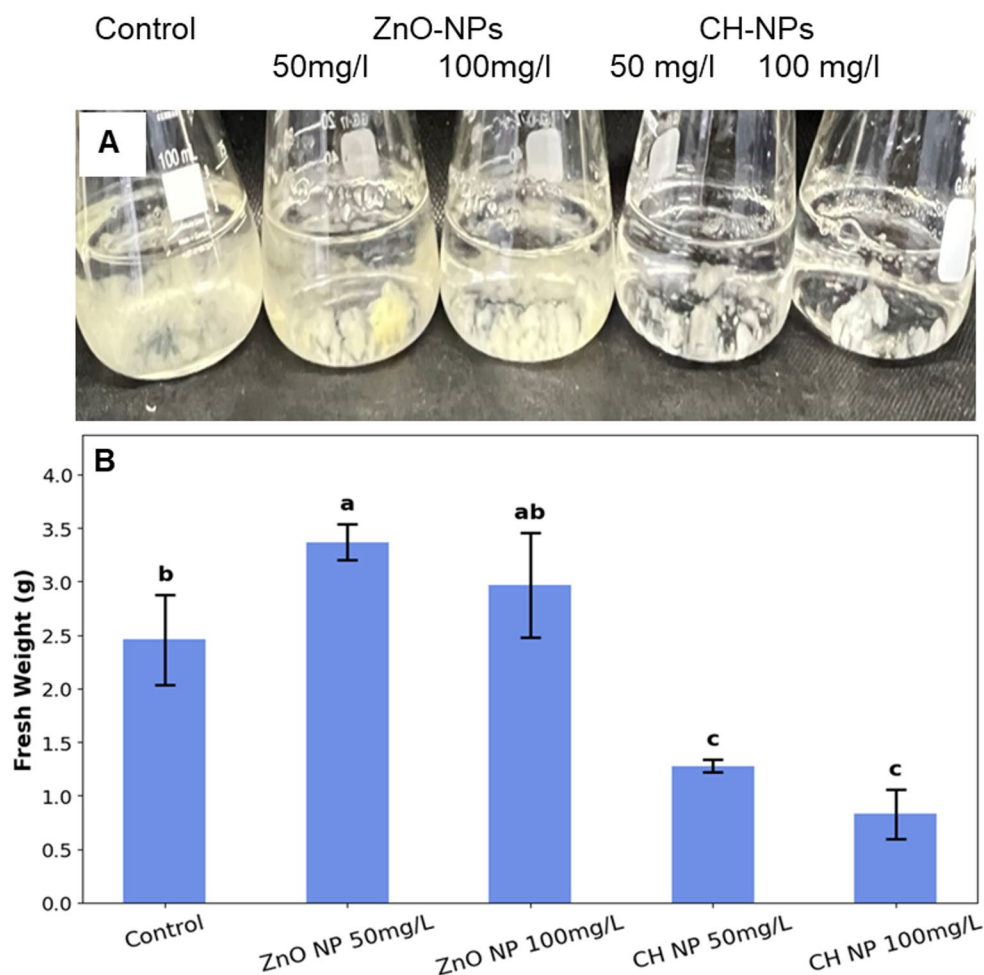
**Fig. 4** Effects of elicitation with two concentrations (50–100 mg/L) of ZnO-NPs and CH-NPs on (A) *fresh weight* and (B) *Dry weight* of the *C. roseus* callus over time on solid media. The data were scored after

0, 10, 20 and 30 days of treatment. Values represent mean  $\pm$  SE. Similar letters indicate the absence of a significant difference ( $p \leq 0.05$ ) after LSD range tests

tissues after 30 days of treatments (Fig. 4B). On the other side, Fig. 5A illustrated the effect of ZnO- and CH-NPs treatments on callus suspension cultures after 30 days of growth. Treatment with ZnO-NPs at 50 mg/L significantly

increased the FW (3.37 g) compared to the control (2.46 g) (Fig. 5B). Although ZnO-NPs at 100 mg/L also enhanced FW (2.97 g), the increase was not statistically significant relative to the control. In contrast, treatment with CHNPs

**Fig. 5** Effects of elicitation with two concentrations (50–100 mg/L) of ZnO-NPs and CH-NPs on (A) growth and (B) fresh weight of the *C. roseus* callus suspension culture after 30 day of elicitation. Values represent mean  $\pm$  SE. Similar letters indicate the absence of a significant difference ( $p \leq 0.05$ ) after LSD range tests



at both 50 and 100 mg/L resulted in a notable reduction in FW, with values recorded at 1.28 g and 0.83 g, respectively. The present study demonstrated that ZnO-NPs, particularly at a concentration of 50 mg/L, significantly enhanced FW production in *C. roseus* callus suspension cultures after 30 days, whereas the higher concentration (100 mg/L) did not result in a statistically significant increase in FW.

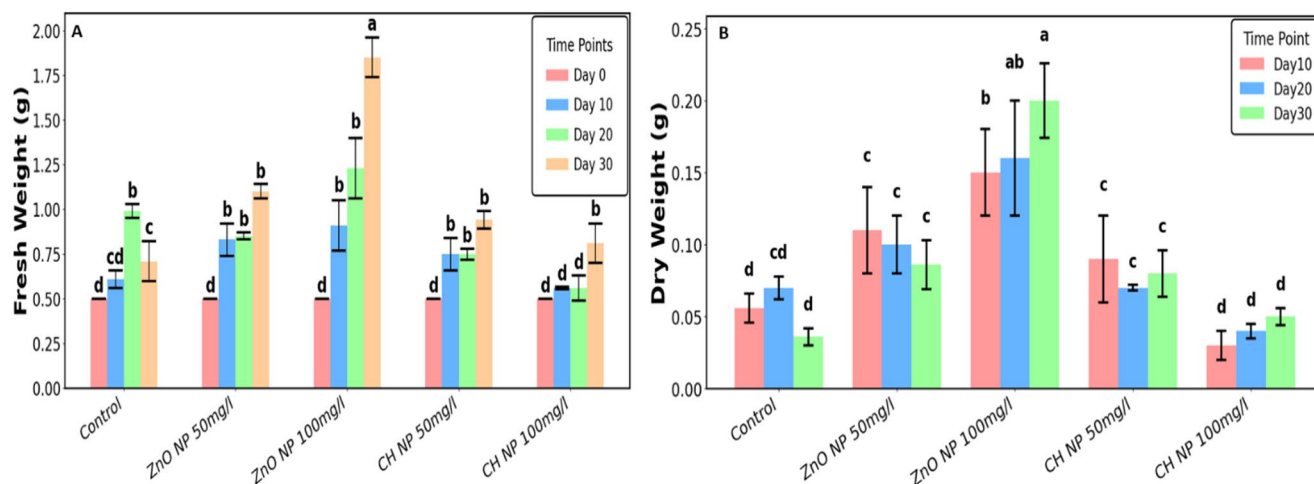
### The effects of ZnO-NPs and CH-NPs on biomass growth of HRs

In order to investigate the effect of ZnO-NPs and CH-NPs stimulator on hairy root biomass, the hairy roots FW and DW was measured over 30 days of culture on both solid and liquid cultures. The analysis of variance (ANOVA) showed that the effect of treatments was significant ( $p \leq 0.05$ ) depends on the type and concentration of the used NPs. The highest amount of FW and DW of the hairy roots on solid medium was detected in ZnO-NPs at concentration of 100 mg/L after 30 days of exposure (1.85 g and 0.2 g, respectively) (Fig. 6A, B) compared to the control group (0.71 g fresh and 0.036 g dry weight) after 30 days of growth. The

obtained data of FW and DW of the hairy roots grown in liquid media were aligned with the data of the HRs cultured on solid media (Fig. 7A). The highest amount of FW and DW was recorded in ZnO-NPs at concentration of 100 mg/L after 30 days of exposure (1.4 g and 0.19 g, respectively), compared to the control group (0.71 g fresh and 0.036 g dry weight) after 30 days of growth (Fig. 7B, C).

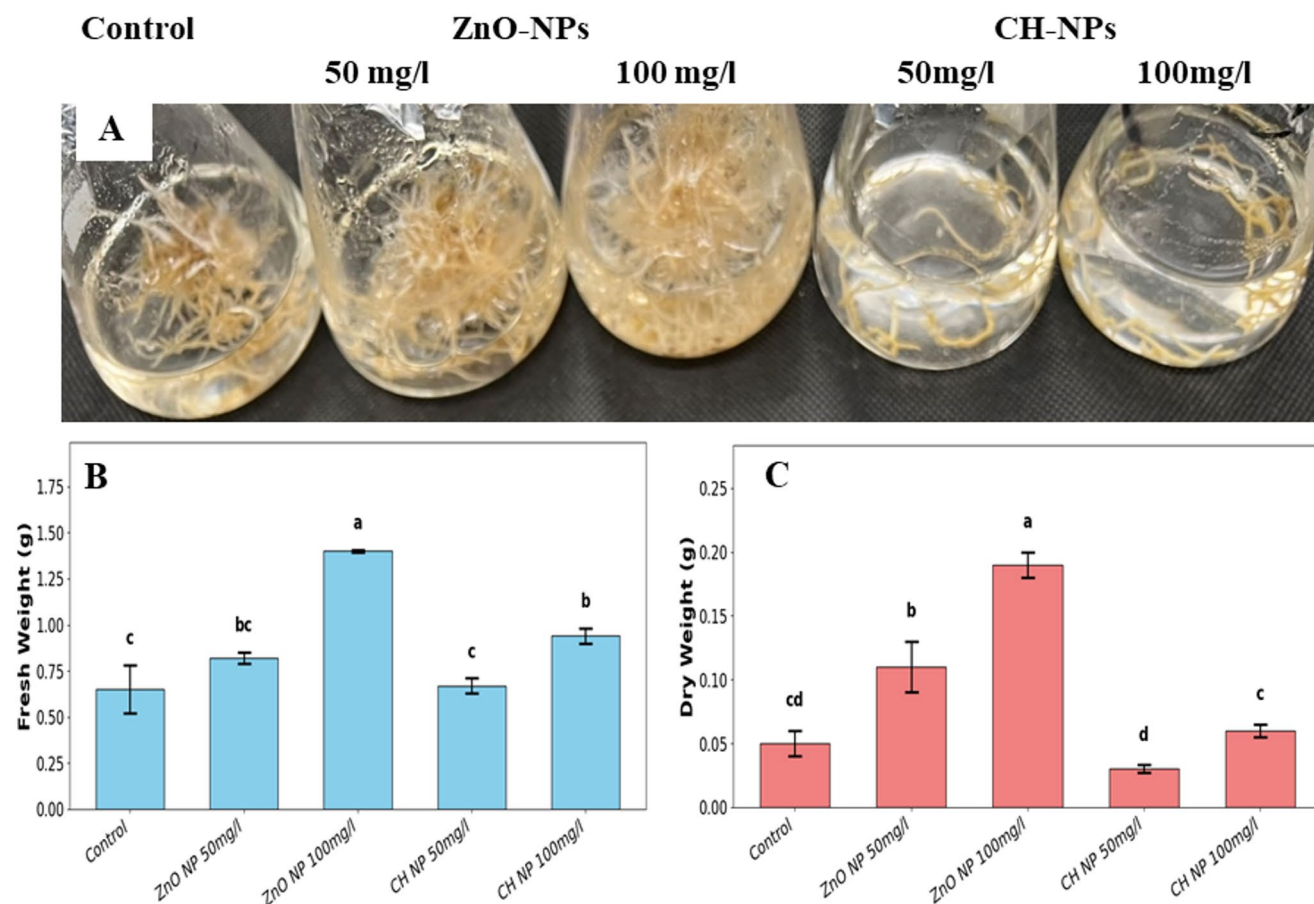
### TIA analysis

In the present study, HPLC analysis was employed to quantify the terpenoid indole alkaloids (TIAs); catharanthine (CAT), vincristine (VCR), and vinblastine (VBL) in the established HRs lines and callus tissues grown on solid culture media. No significant differences were observed in the levels of the target alkaloids in HRs line L2 compared with the control (Fig. 8A, C & E). Among these alkaloids, only VCR accumulated at significantly higher levels in HRs line L1 (18.12  $\mu\text{g/gFW}$ ) compared with its concentration in the leaves of two-week-old in vivo plants (9.5  $\mu\text{g/gFW}$ ) representing a 1.92-fold increase relative to the control (Fig. 8C). These results indicate that L1 outperformed L2 in the



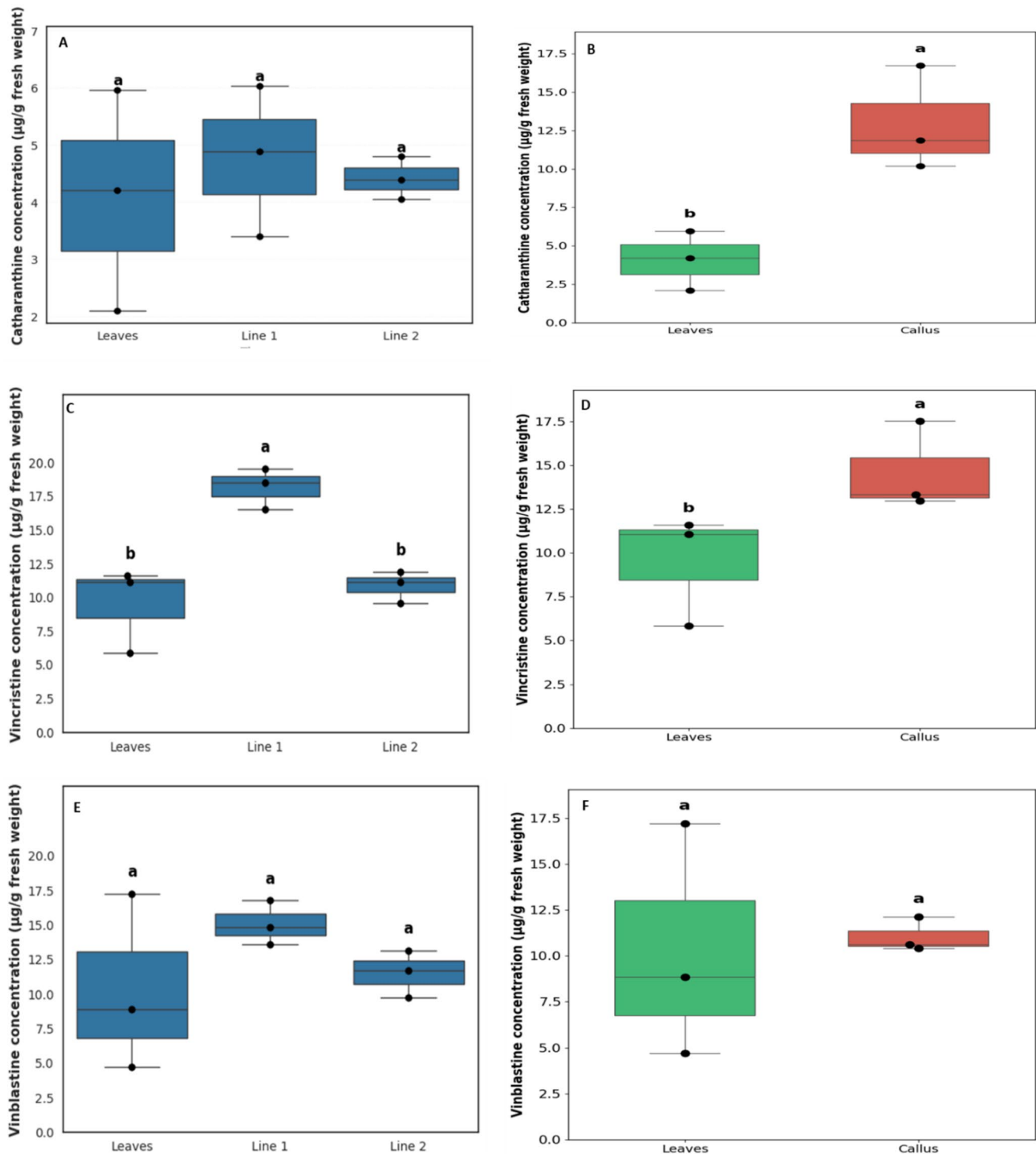
**Fig. 6** Effects of elicitation with two concentrations (50–100 mg/L) of ZnO-NPs and CH-NPs on (A) *fresh weight* and (B) *Dry weight* of the *C. roseus* hairy roots (L1) over time on solid media. The data

were scored after 0, 10, 20 and 30 day of treatment. Values represent mean  $\pm$  SE. Similar letters indicate the absence of a significant difference ( $p \leq 0.05$ ) after LSD range tests



**Fig. 7** Effects of elicitation with two concentrations (50–100 mg/L) of ZnO-NPs and CH-NPs on (A) *growth* (B) *fresh weight* and (C) *Dry weight* of the *C. roseus* hairy roots (L1) in liquid solid media. The data

were scored after 30 days of treatment. Values represent mean  $\pm$  SE. Similar letters indicate the absence of a significant difference ( $p \leq 0.05$ ) after LSD range tests



**Fig. 8** HPLC quantification of CAT, VCR, and VBL in transformed and non-transformed *C. roseus* tissues. Hairy root lines (L1 and L2) are shown in the left panel and callus tissue in the right panel, compared with wild-type (WT) leaves. (A, B) CAT, (C, D) VCR, and (E,

F) VBL contents in hairy roots and callus, respectively. Data are presented as box plots with individual biological replicates ( $n=3$ ). Different letters indicate significant differences ( $p<0.05$ )

accumulation of VCR, which represents the most pharmacologically important TIA. In contrast, the accumulation of CAT and VCR was significantly higher in the callus tissues (12.9  $\mu\text{g/g}$  and 14.6  $\mu\text{g/g}$ , respectively) than in the control leaves (4.08  $\mu\text{g/gFW}$  and 9.5  $\mu\text{g/gFW}$ , respectively), corresponding to a 3.16-fold increase for CAT and a 1.54-fold increase for VCR (Fig. 8B–D). Conversely, no significant difference was detected in the accumulation of VBL in the callus tissues compared with the control leaves (Fig. 8F).

### Effect of the elicitation with ZnO-NPs and CH-NPs on TIAs accumulation

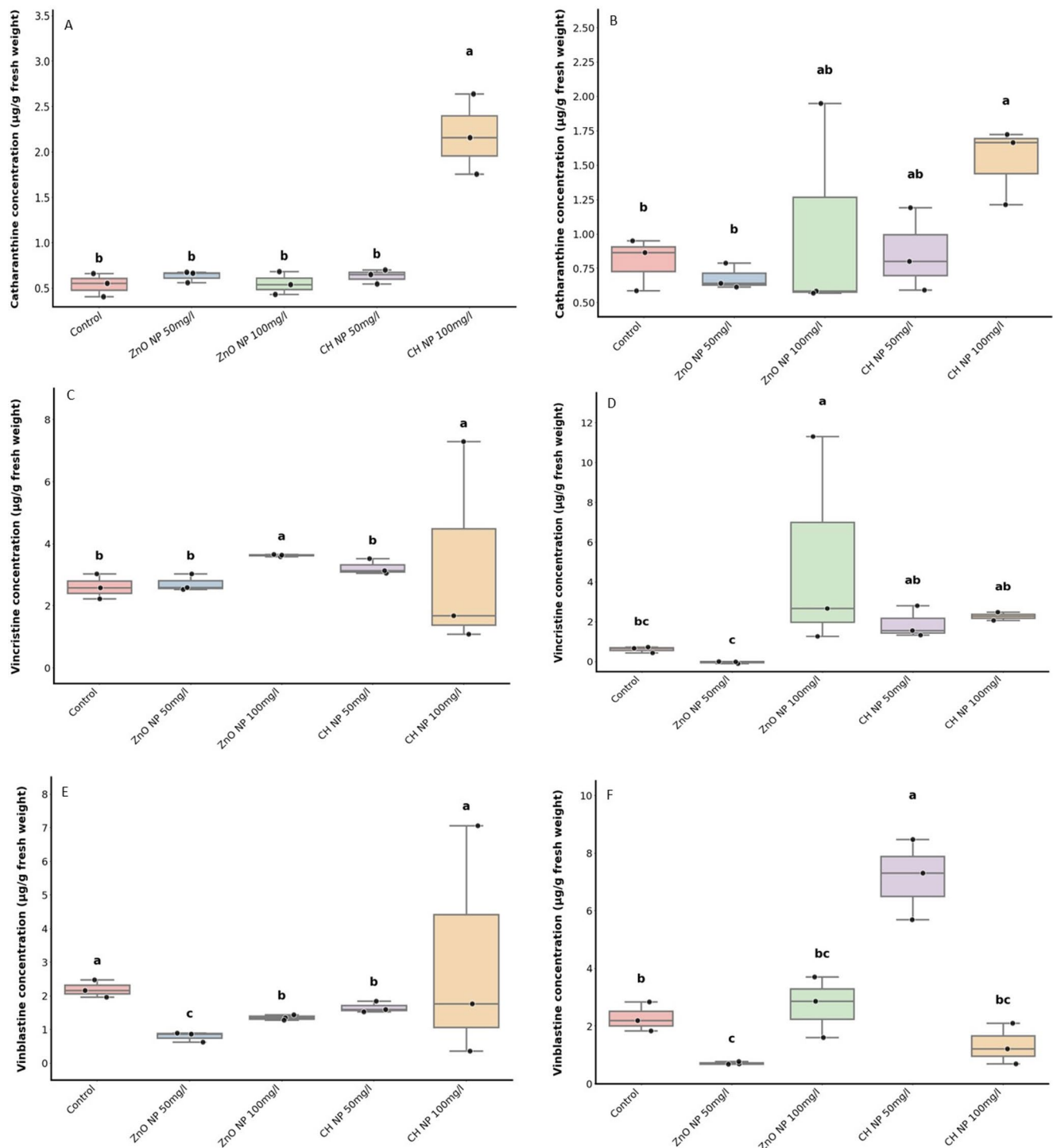
In this experiment, the contents of CAT, VCR, and VBL were quantified in extracts from both elicited and non-elicited hairy roots (HRs) of line L1 and callus cultures grown on solid or liquid media supplemented with either ZnO-NPs or CH-NPs using HPLC analysis. Figures 9 and 10 illustrate the differential capacities of *C. roseus* HRs and callus cultures to accumulate terpenoid indole alkaloids (TIAs) under various elicitation treatments. As shown in Fig. 9A, C, elicitation of HRs with CH-NPs at 100 mg/L on solid culture medium for 30 days resulted in significant increases in CAT and VCR contents (2.18  $\mu\text{g/gFW}$  and 3.35  $\mu\text{g/gFW}$ , respectively), corresponding to a 4.11-fold increase in CAT and a 1.29-fold increase in VCR compared with the control (0.53 and 2.60  $\mu\text{g/g FW}$ , respectively). Similarly, treatment with 100 mg/L ZnO-NPs increased the VCR to 3.26  $\mu\text{g/gFW}$ , representing a 1.25-fold increase relative to the control (Fig. 9C). Conversely, VBL content was significantly reduced in HRs elicited with ZnO-NPs at 50 and 100 mg/L, as well as with CH-NPs at 50 mg/L (Fig. 9E). In contrast, elicitation under liquid culture conditions led to significant increases in all three alkaloids (CAT, VCR, and VBL) after 30 days (Fig. 9B, D & F) depending of the type and concentration of NPs used. CAT content increased to 1.53  $\mu\text{g/gFW}$  following elicitation with CH-NPs (100 mg/L) (Fig. 9B), VCR reached 5.08  $\mu\text{g/gFW}$  after elicitation with ZnO-NPs (100 mg/L) (Fig. 9D), and VBL increased to 7.15  $\mu\text{g/gFW}$  after treatment with CH-NPs (50 mg/L) (Fig. 9F), compared with the control values of 0.8  $\mu\text{g/gFW}$ , 0.6  $\mu\text{g/gFW}$ , and 2.28  $\mu\text{g/gFW}$  for CAT, VCR, and VBL, respectively. With regard to the elicitation of callus cultures, Fig. 10A, C & E shows alkaloid accumulation in callus tissues grown on solid medium. The obtained data revealed that CAT content increased significantly following treatment with ZnO-NPs at 50 and 100 mg/L, reaching 1.99  $\mu\text{g/gFW}$  and 2.78  $\mu\text{g/gFW}$ , respectively, compared with the control (0.07  $\mu\text{g/gFW}$ ) (Fig. 10A). Similarly, VCR content increased significantly under both ZnO-NP concentrations (50 and 100 mg/L), reaching 3.95  $\mu\text{g/gFW}$  and 2.07  $\mu\text{g/gFW}$ , respectively, while it was not detected in the control culture

(Fig. 10C). Notably, although VBL content increased under ZnO-NPs (100 mg/L) to 4.16  $\mu\text{g/gFW}$  compared with the control (2.88  $\mu\text{g/gFW}$ ), this enhancement was not statistically significant (Fig. 10E).

Data presented in Fig. 10B, D & F illustrate the alkaloid contents in callus suspension cultures. Only minor and statistically insignificant changes in CAT concentration were observed under most elicitation treatments, except for CH-NPs at 100 mg/L, which caused a notable reduction (Fig. 10B). In contrast, VCR content was significantly elevated in response to 100 mg/L ZnO-NPs and 50 mg/L CH-NPs, with the highest VCR level (3.52  $\mu\text{g/gFW}$ ) recorded under CH-NPs at 50 mg/L compared with other treatments and the control (1.09  $\mu\text{g/gFW}$ ) (Fig. 10D). Similarly, ZnO-NPs at 100 mg/L increased VCR content to 2.59  $\mu\text{g/gFW}$  (Fig. 10D). However, neither ZnO-NPs nor CH-NPs caused significant changes in VBL accumulation in the elicited callus cultures (Fig. 10F).

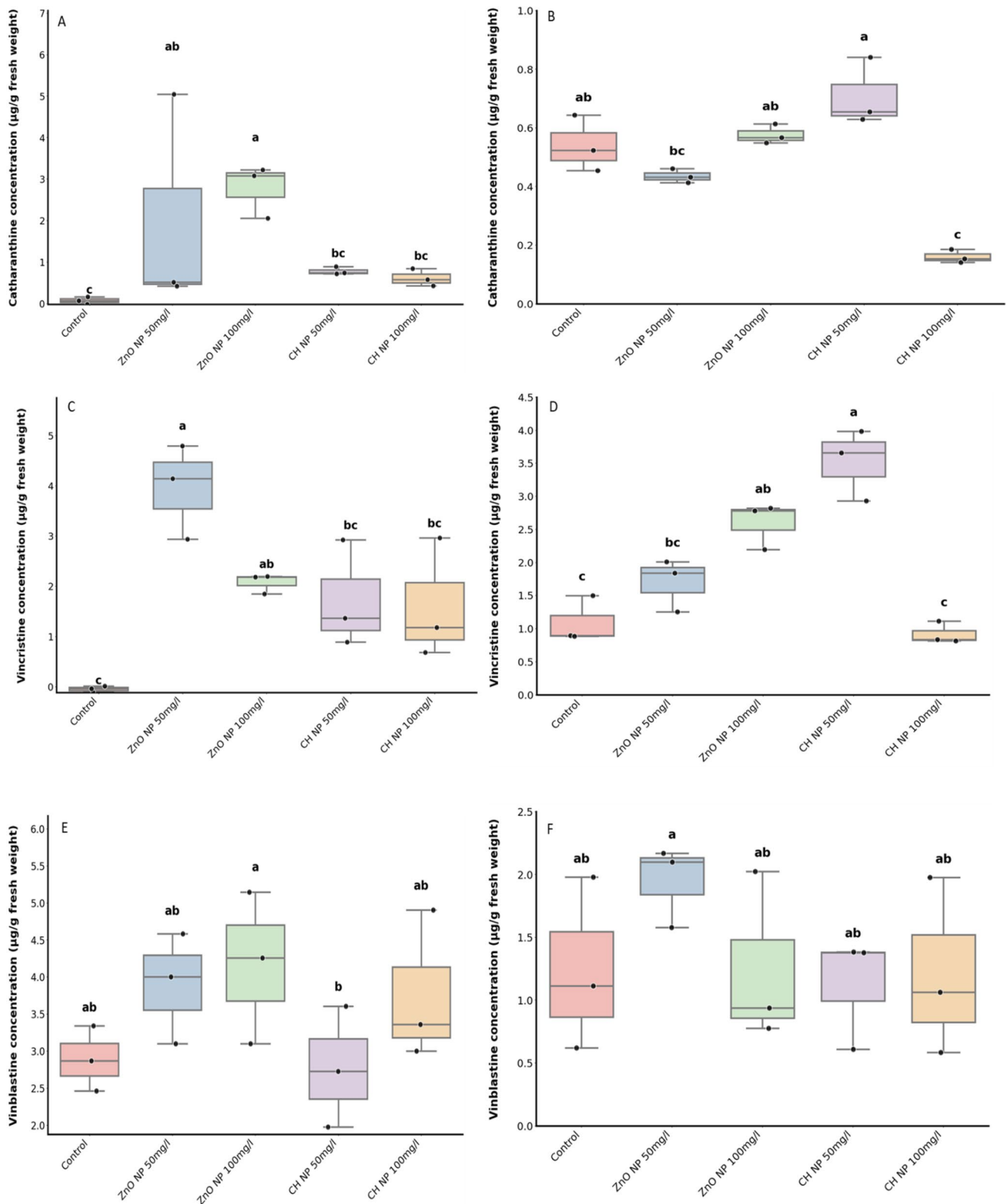
### Effect of nanoparticle elicitations on gene expression

Compared with the corresponding control, ZnO-NPs did not significantly alter the expression levels of the *PRXI*, *DAT*, and *STR* genes in HR line L1 grown on solid media (Fig. 11A, C & G). However, the expression level of *SLS* was significantly upregulated upon elicitation with ZnO-NPs at 50 mg/L and CH-NPs at 100 mg/L (Fig. 11E). In contrast, elicitation with CH-NPs at both concentrations resulted in a marked increase in *PRXI* and *DAT* expression (Fig. 11A & C). For the *STR* gene, treatment with 100 mg/L CH-NPs led to a significant upregulation compared to the control (Fig. 11G). When nanoparticles were applied in liquid cultures, the expression profiles of the studied genes differed from those observed in HRs grown on solid media. As shown in Fig. 11 (right panel), ZnO-NPs at 50 mg/L dramatically upregulated the expression of *PRXI*, *DAT*, *SLS*, and *STR* (Fig. 11B, D, F & H). Increasing the ZnO-NPs concentration to 100 mg/L enhanced *PRXI* expression (Fig. 11B), while the other genes remained unchanged (Fig. 11D, E & H). Elicitation with CH-NPs did not significantly affect *PRXI* and *SLS* expression (Fig. 11B & F). However, CH-NPs at 100 mg/L significantly increased *DAT* and *STR* expression (Fig. 11D & H), while the lower concentration (50 mg/L) also enhanced *STR* expression (Fig. 11H). Interestingly, elicitation of callus cultures on solid media with 100 mg/L ZnO-NPs resulted in a significant increase in *PRXI*, *SLS*, and *STR* expression compared with the control (Fig. 12A, E and G). The lower ZnO-NPs concentration (50 mg/L) significantly affected only *DAT* expression, which was notably higher than in the control (Fig. 12C). Treatment with 50 mg/L CH-NPs also led to



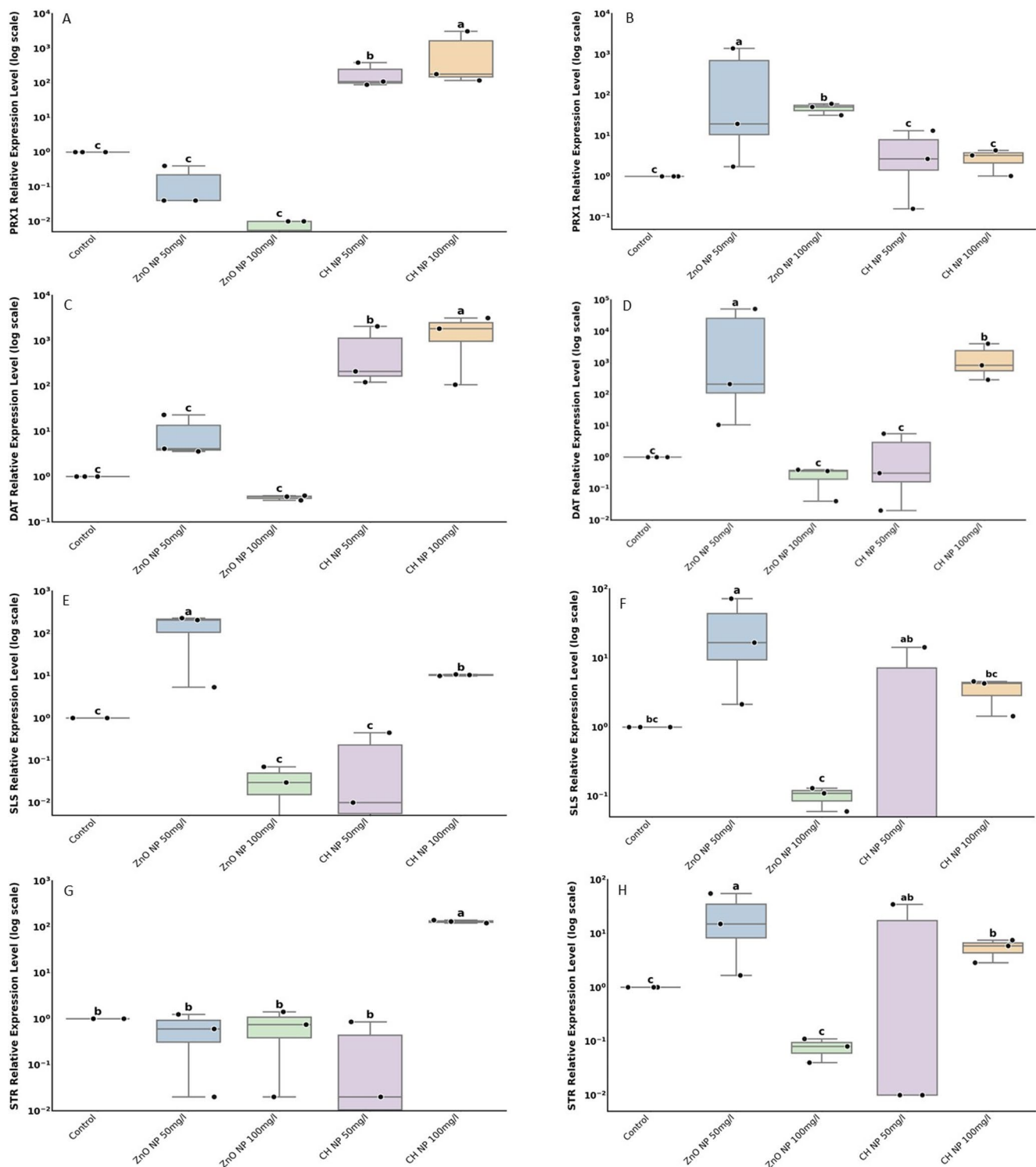
**Fig. 9** Effect of ZnO-NPs and chitosan NPs (CH-NPs) on terpenoid indole alkaloid accumulation in *C. roseus* hairy roots grown in solid (left panel) and liquid (right panel) culture systems. Box plots show HPLC quantification of CAT, VCR, and VBL in line L1 treated with ZnO-NPs or CH-NPs (50 and 100 mg/L), compared with untreated

controls. (A, B) CAT, (C, D) VCR, and (E, F) VBL contents in solid and liquid cultures, respectively. Data are presented as box plots with individual biological replicates ( $n=3$ ). Different letters indicate significant differences ( $p \leq 0.05$ )



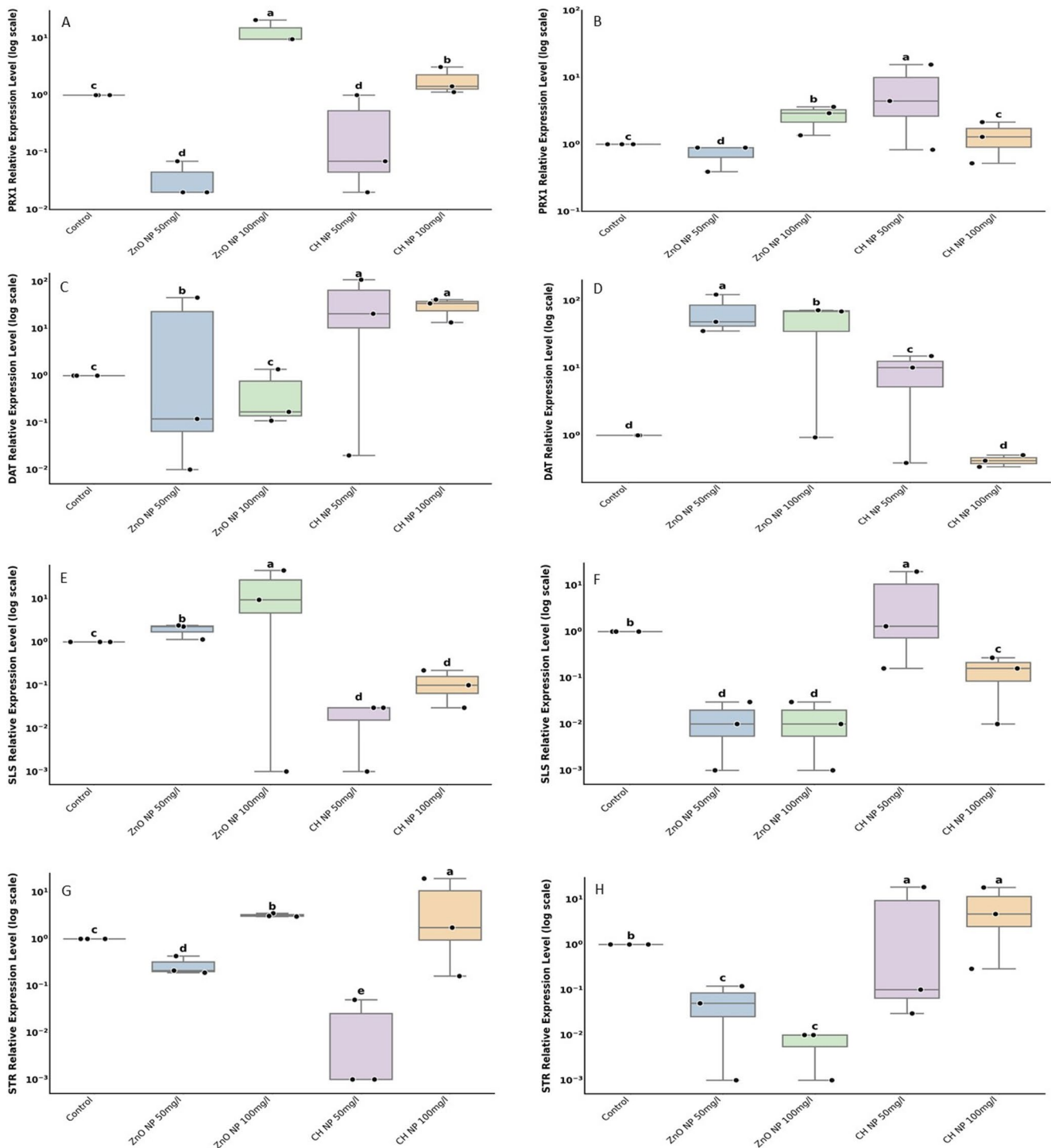
**Fig. 10** Effect of ZnO-NPs and chitosan NPs (CH-NPs) on terpenoid indole alkaloid accumulation in *C. roseus* callus cultures grown in solid (left panel) and liquid (right panel) systems. Box plots show HPLC quantification of CAT, VCR, and VBL in callus treated with ZnO-NPs or CH-NPs (50 and 100 mg/L), compared with untreated

controls. (**A, B**) CAT, (**C, D**) VCR, and (**E, F**) VBL contents in solid and liquid cultures, respectively. Data are presented as box plots with individual biological replicates ( $n=3$ ). Different letters indicate significant differences ( $p \leq 0.05$ )



**Fig. 11** Effects of ZnO-NPs and chitosan NPs (CH-NPs) on the relative expression of key TIA biosynthetic genes in *C. roseus* hairy roots (line L1) grown in solid (A, C, E, G) and liquid (B, D, F, H) culture systems. HRs were treated with ZnO-NPs or CH-NPs (50 and

100 mg/L). Relative expression of *PRX1* (A, B), *DAT* (C, D), *SLS* (E, F), and *STR* (G, H) is shown. Data are presented as box plots with individual biological replicates ( $n=3$ ). Different letters indicate significant differences ( $p \leq 0.05$ )



**Fig. 12** Effects of ZnO-NPs and chitosan NPs (CH-NPs) on the relative expression of key TIA biosynthetic genes in *C. roseus* callus cultures grown in solid (A, C, E, G) and liquid (B, D, F, H) systems. Cultures were treated with ZnO-NPs or CH-NPs (50 and 100 mg/L).

an increase in *DAT* expression (Fig. 12C). Increasing the CH-NPs concentration to 100 mg/L resulted in a significant upregulation of *PRX1*, *DAT*, and *STR* (Fig. 12A, C & G), whereas *SLS* expression was significantly lower than the

Relative expression of *PRX1* (A, B), *DAT* (C, D), *SLS* (E, F), and *STR* (G, H) is shown. Data are presented as box plots with individual biological replicates ( $n=3$ ). Different letters indicate significant differences ( $p \leq 0.05$ )

control (Fig. 12E). In callus suspension cultures, elicitation with 100 mg/L ZnO-NPs led to a dramatic increase in *PRX1* expression (Fig. 12B). Moreover, both ZnO-NPs concentrations significantly elevated *DAT* expression (Fig. 12D),

whereas *SLS* and *STR* expression were decreased (Fig. 12F & H). Interestingly, CH-NPs at 50 mg/L caused a significant increase in *PRX1*, *DAT*, *SLS*, and *STR* expression (Fig. 12B, D, F & H). However, increasing the CH-NPs concentration to 100 mg/L resulted in a significant upregulation of *STR* only (Fig. 12H), accompanied by a decrease in *SLS* expression (Fig. 12F) and no significant changes in *PRX1* or *DAT* (Fig. 12B & D).

## Discussion

In vitro culture systems, such as callus and hairy root cultures represent powerful platforms for the sustainable and large-scale production of high-value plant secondary metabolites for pharmaceutical and related industries (Su et al. 2021). Previous studies have demonstrated that the accumulation of these valuable compounds can be significantly enhanced through exposure the plants to signalling molecules and abiotic elicitors, including nanoparticles (NPs) (Fatima et al. 2020). Increasing evidence indicates that NPs can act as effective elicitors by modulating plant growth, development, and metabolic pathways, thereby stimulating secondary metabolite biosynthesis (Marslin et al. 2017; Mandal and Basu 2021; Perveen et al. 2022; Al-Theyab et al. 2023). However, despite their promising potential, the application of nanomaterials in plant biotechnology remains at an early stage of development, and their effects on specialized metabolite pathways are still not fully understood.

The current study aimed to investigate the effect of ZnO- and CH-NPs on TIAs accumulation in different in vitro culture systems of *C. roseus*, i.e. callus culture and hairy root. Stem-derived callus cultures were established on a media containing 1.0 mg/L BAP and 1.0 mg/L NAA, which is consistent with previous report indicating that balanced auxin-cytokinin combinations favor callogenesis in *C. roseus* (Singh et al. 2011). Hairy root induction was found to be influenced by both the *Agrobacterium rhizogenes* strain used and the transformation protocol applied. The injection-based method yielded higher transformation efficiency than the immersion method, likely due to localized bacterial delivery into wounded tissues. This observation agrees with earlier findings that transformation efficiency can vary depending on the inoculation strategy and plant species (Montazeri et al. 2019; Zhang et al. 2023; Jedličková et al. 2024). Our observations are consistent with previous reports on *C. roseus*, where hairy roots were induced using both immersion of explants (hypocotyls, leaves, and stems) (Jung et al. 1992; O'Keefe et al. 1997; Choi et al. 2004; Hanafy et al. 2016) and injection-based approaches (Rizvi et al. 2015). These findings highlight the importance of optimizing transformation methods and culture systems

to maximize hairy root induction and subsequent metabolite production in medicinal plants.

NPs have the potential to serve as effective elicitors in plants (Fatima et al. 2020), owing to their unique chemical and physical properties. These properties enable NPs to play important roles in various biological processes, including cellular uptake, bio-distribution, and release kinetics (Hu et al. 2020). Several factors influence the effects of nanoparticles on plants, including their type, concentration, size, dosage, and the plant species involved. These variables can determine whether nanoparticles will have beneficial or detrimental effects on plants (Ebadollahi et al. 2019; Rezaei et al. 2019; Sami et al. 2020; Rivero-Montejo et al. 2021; Perveen et al. 2022; Abed et al. 2023). To date, only a limited number of studies have explored the use of nanoparticles to induce stress in in vitro plant cultures for the enhanced production of secondary metabolites (Perveen et al. 2022). In this respect, silver nanoparticles have been reported to enhance alkaloid production in *C. roseus* suspension cultures, which was associated with the upregulation of strictosidine synthase (*STR*) and mitogen-activated protein kinase 3 (*CrMPK3*) expression (Fouad et al. 2021). Similarly, cobalt ions were found to be more effective than their biogenic nanoparticles in enhancing alkaloid accumulation, which was associated with increased *CrMPK3* gene expression and elevated activities of antioxidant enzymes such as superoxide dismutase (SOD) and ascorbate peroxidase (APX) in *C. roseus* cell suspension cultures (Fouad and Hafez 2018). Building on these findings, the current study evaluated the effects of ZnO- and CH-NPs at concentrations of 50 and 100 mg/L on callus and hairy root cultures of *C. roseus* over three growth periods (10, 20, and 30 days), grown on both solid and liquid media. The main objective was to assess their impact on biomass production and the accumulation of the TIAs, i.e. CAT, VCR and VBL.

NPs application exhibited differential effects on biomass depending on the culture system and nanoparticle type. Callus grown in liquid medium and treated with 50 mg/L ZnO-NPs for 30 days showed significant increase in fresh weight, whereas the other NP treatments either failed to enhance growth or caused a reduction in biomass compared with the control. In contrast, CH-NPs at a concentration of 100 mg/L negatively affected callus growth on solid media. On the other hand, hairy root cultures responded more positively, with the highest fresh and dry weights observed after the treatment with 100 mg/L ZnO-NPs on both solid and liquid media. The positive response of hairy roots in the present study may be attributed to their genetic stability, high metabolic activity, and enhanced ability to tolerate NP-induced stress. Several studies have reported that NPs significantly influence the growth of both callus and hairy roots. For example, the application of 10 mg/L ZnO-NPs has been

shown to enhance callus formation and shoot regeneration in rapeseed, whereas higher concentrations markedly inhibited callus development (Mousavi Kouhi and Lahouti 2018). Similarly, Javed et al. (2018) demonstrated that ZnO-NPs at 1.0 and 10.0 mg/L were the most effective in increasing the fresh weight of *Stevia rebaudiana* Bertonii callus compared to the control. In flax, 1.5 mg/L of Fe<sub>3</sub>O<sub>4</sub> NPs significantly promoted callus size (Kokina et al. 2017). In the case of hairy roots, Asl et al. (2019) reported that treatment with 100 mg/L ZnO-NPs significantly improved the fresh weight of *Hyoscyamus reticulatus* hairy roots by approximately 37% compared to untreated controls. However, biomass declined at higher concentrations (200 mg/L). These results highlight the potential role of nanoparticles as elicitors to support biomass accumulation in *C. roseus* callus and hairy root cultures (Kokina et al. 2017; Javed et al. 2018; Asl et al. 2019), and demonstrate that their effects are nanoparticle type-, dose- and culture system-dependent.

In the present study, hairy roots elicited with CH-NPs (100 mg/L) on solid medium showed the highest CAT and VCR contents, while VBL remained unchanged. In liquid cultures, CAT was enhanced by CH-NPs (100 mg/L), VCR by ZnO-NPs (100 mg/L), and VBL by CH-NPs (50 mg/L). In callus cultures, ZnO-NPs concentrations increased CAT and VCR on solid medium, whereas in liquid culture, CH-NPs (50 mg/L) and ZnO-NPs (100 mg/L) significantly enhanced VCR accumulation. These variations indicate that nanoparticle elicitation is strongly influenced by the physical culture environment, as previously reported for *C. roseus* (Hanafy et al. 2016; Mishra et al. 2018). Comparable results have been observed in other species, where NPs enhanced alkaloids, phenolics, and flavonoids, along with antioxidant enzyme activities (Mosavat et al. 2019; Iqbal et al. 2022; Khalifa et al. 2023). The TIAs of *C. roseus* derived from a very complicated pathway and the plant produces them in very limited amounts (Verma et al. 2017). In order to enhance TIAs production in this valuable plant species, their pathways, particularly the key enzymes involved and the metabolic regulatory mechanisms governing alkaloid synthesis in *C. roseus* has been detailed reported by Liu et al. (2016). Generally, it is revealed that TIA biosynthesis process is divided into three stages, upstream, midstream, and downstream. In the upstream stage, tryptamine and secologanin-derived from the shikimate and iridoid pathways-are coupled to form strictosidine, catalyzed by enzymes such as SLS and STR. In the midstream stage, strictosidine serves as a precursor for the synthesis of tabersonine and catharanthine. The downstream stage involves the conversion of tabersonine into vindoline through enzymes like T16H, D4H, and DAT, followed by the dimerization of vindoline and catharanthine, catalyzed by PRX1, to produce vinblastine and vincristine. Additionally, transcription factors such

as *ORCA3* play a vital role in regulating this pathway by activating key biosynthetic genes including *SLS*, *STR*, and *SGD* (Sharma et al. 2018; Liu et al. 2021).

In order to explore the effects of ZnO- and CH-NPs on the synthesis of TIAs in either callus or hairy roots cultures of *C. roseus* at the genetic level, qRT-PCR was used in the current study to detect the expression of the key genes in the synthesis pathway of CAT, VCR and VBL. The obtained results revealed that ZnO- and CH-NPs significantly modulate the expression of *STR*, *SLS*, *DAT*, and *PRX1*, which correlated with changes in alkaloid accumulation. The obtained data demonstrate that nanoparticle-mediated elicitation of TIAs biosynthesis in *C. roseus* is strongly influenced by both the type of in vitro culture system and the physical state of the growth medium. Distinct regulatory patterns were observed between hairy root and callus cultures, as well as between solid and liquid conditions, highlighting the complexity of nanoparticle-plant cell interactions. In hairy root cultures, a clear transcription-metabolite association was evident only under solid culture conditions. The enhanced accumulation of CAT and VCR in response to CH-NP treatment (100 mg/L) was accompanied by significant induction of *PRX1*, *DAT*, *STR*, and *SLS* transcripts relative to the control, indicating activation of the TIAs biosynthetic pathway at the transcriptional level. In contrast, this relationship was not maintained in liquid-grown hairy roots. Although ZnO-NPs at 50 mg/L caused the strongest upregulation of all four biosynthetic genes, this response did not result in maximal alkaloid accumulation, with each TIA reaching its highest level under a different treatment. This uncoupling suggests that, in liquid systems, transcriptional activation alone is insufficient to drive final metabolite accumulation and that additional regulatory constraints, such as post-transcriptional control, metabolic flux limitations, or intracellular transport processes, may be involved.

Callus cultures exhibited a similarly complex but system-specific response. In solid medium-grown callus culture, increased CAT levels under ZnO-NP (100 mg/L) corresponded with significant induction of *PRX1*, *SLS*, and *STR* relative to the control. Likewise, the higher VCR content observed under ZnO-NP (50 mg/L) was associated with significant upregulation of *DAT* and *SLS*. However, VBL levels remained unchanged across all treatments despite marked transcriptional responses, indicating the presence of a downstream regulatory bottleneck. In liquid medium-grown callus cultures, elevated VCR accumulation under CH-NP (50 mg/L) coincided with significant induction of *PRX1*, *DAT*, *SLS*, and *STR*, whereas CAT and VBL did not show significant changes.

Taken together, these findings indicate that nanoparticle elicitation of TIAs biosynthesis is governed by multiple regulatory layers that differ between culture systems

and growth conditions. While nanoparticles can effectively stimulate the expression of key biosynthetic genes, this activation does not always translate into proportional metabolite accumulation, particularly in liquid cultures. Such observations underscore the importance of considering post-transcriptional and metabolic constraints when optimizing in vitro systems for enhanced secondary metabolite production. These results align with previous studies reporting NP-mediated upregulation of TIA pathway genes (Ali et al. 2021; Perveen et al. 2022). In *H. reticulatus* hairy roots elicited by ZnO-NPs (100 mg/L) caused increased hyoscyamine and scopolamine with the activation of antioxidant enzymes and upregulation of alkaloid biosynthesis genes (Asl et al. 2019). Also, Perveen et al. (2022) reported that nano-zinc elicitation significantly upregulated the expression of *DAT* and *PRX1*. In the current study, gene expression analysis confirmed that the elicitation treatments modulated the expression of *STR*, *DAT*, *PRX1*, and *SLS*, aligning with the observed biochemical outcomes.

In conclusion, the present study demonstrates that ZnO- and chitosan nanoparticles act as potent elicitors of TIA biosynthesis in *C. roseus* callus and hairy root cultures. Their effects are highly dependent on nanoparticle type, dose, culture system, and medium state. The coordinated enhancement of key biosynthetic genes and alkaloid accumulation highlights the potential of nanoparticle-based strategies for improving the in vitro production of valuable anticancer alkaloids. This approach offers a promising and scalable alternative for the pharmaceutical exploitation of *C. roseus* secondary metabolites.

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**Author contributions** M.S.H. and S.E. conceived and designed the experiments. L.S. and R. H. conducted the optimization, validation of the HPLC method and data analysis. A. F. D. and M.S. H conducted experiments. M. S. H. drafted the manuscript. L.S., R.H., S.E., N.D., M.S.H. reviewed and revised the manuscript. M.S.H., S.E. and N.D. supervised the research work. All authors contributed to the article and approved the final submitted version.

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**Data availability** The data supporting the findings of this study are available within this paper and its supplemental information files.

## Declarations

**Ethical approval** Not applicable.

**Competing interests** There are no conflicts of interest to declare. All the co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report.

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