

Fungal Elicitation and Precursor Feeding: A Novel Approach to Boost Alkaloid Yield in *Catharanthus roseus* cell suspension cultures

Shabnam Miryousefzadeh

Urmia University

Bahman Hosseini

b.hosseini@urmia.ac.ir

Urmia University

Syavash Hemmati

Urmia University

Research Article

Keywords: Catharanthus roseus, HPLC, Piriformospora indica, Precursor feeding, Terpenoid indole alkaloids

Posted Date: May 9th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-6402841/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: No competing interests reported.

Abstract

Background: Medicinal plants are a crucial source of bioactive compounds with pharmaceutical applications. *Catharanthus roseus* is widely recognized for its production of terpenoid indole alkaloids, including vincristine and vinblastine, which are used in cancer treatments. However, their natural production is low, necessitating alternative strategies to enhance their yield.

Results: This study investigates the effect of *Piriformospora indica* extract elicitation and tryptophan precursor feeding on the production of vincristine and vinblastine in *C. roseus* suspension cultures. Cell suspensions were treated with different concentrations of *P. indica* extract (0%, 2%, 4%, and 6% v/v) for 48 and 72 hours. Growth efficiency, total phenolic (TPC) and total flavonoid (TFC) content, and alkaloid levels were analyzed. The results revealed that 4% *P. indica* extract for 48 hours significantly increased fresh and dry weight. The highest TPC (8.82 mg GAL/g FW) and TFC (6.24 mg GAL/g FW) were observed at 6% *P. indica* extract during 48 hours exposure time. The maximum vinblastine (0.31 µg/g) and vincristine (634.7 µg/g) accumulation was achieved at 4% and 2% *P. indica* extract, respectively, for 48 hours.

Conclusion: These findings suggest that *P. indica* extract elicitation, coupled with precursor feeding, can enhance the biosynthesis of pharmacologically valuable alkaloids in *C. roseus* suspension cultures, providing an effective biotechnological approach for sustainable alkaloid production.

Background

In recent decades, there has been growing interest in medicinal plants and herbal medicines, leading to a significant increase in their usage. Given the current and future demand for herbal medicines, cultivating medicinal plants as an alternative to synthetic chemical compounds has created valuable opportunities in the agricultural sector [1]. The World Health Organization (WHO) has predicted that by 2050, the global market for medicinal plants will reach 5 trillion dollars [2]. As a result, the consumption of medicinal plants has expanded widely in both developing and developed countries. The presence of bioactive plant-derived compounds in 25–50% of prescription drugs worldwide highlights the substantial economic value of medicinal plants in human society [3]. *Catharanthus roseus* is particularly notable for its broad range of alkaloids, including vincristine and vinblastine, which have demonstrated significant efficacy in treating various cancers such as leukemia and lymphoma [4]. This plant is cultivated extensively in regions across Africa, India, Thailand, Australia, the Americas, Germany, Hungary, Italy, England, Russia, and Palestine [5]. The concentration of terpenoid indole alkaloids varies across different parts of the plant, with levels ranging from 0.78–1.22% in the root, 0.26–0.31% in the stem, 0.6–6.7% in the leaves, and 0.007–0.005% in the flowers [6]. Due to the high demand for these alkaloids, their complex chemical synthesis, low natural production, and costly extraction process, significant efforts have been made to enhance their production through various biotechnological approaches, including cell culture, callus and hairy root culture, metabolic engineering, stimulation, and precursor feeding [7]. One of the most advanced and effective strategies to enhance the yield of valuable compounds such as

indole alkaloids is the stimulation of synthetic pathways in cell suspension and hairy root cultures. The production and accumulation of secondary metabolites in plant cells are part of their defense response against pathogens, as well as physical and chemical stressors. These responses are triggered by various internal or external molecules known as elicitors, which are widely used to induce plant metabolite production [8]. Fungal stimulants are commonly applied to enhance the synthesis of therapeutic compounds such as terpenoids, coumarin derivatives, alkaloids, and flavonoids in plant cell cultures [9]. *Piriformospora indica*, a mycorrhizal fungus, has gained attention in recent years due to its unique ability to promote plant growth and enhance resistance to environmental stressors [10]. The application of *P. indica* cell extracts and filtered cultures has been widely used to increase the production of secondary metabolites in cell suspension and hairy root cultures [11]. For example, research on the effects of *P. indica* stimulation on growth, phenolic compound production, antioxidant activity, and gene expression in the flavonoid biosynthesis pathway of *Ficus carica* hairy root cultures showed that the highest phenolic compound accumulation occurred after 72 hours of treatment with 2% *P. indica*. Additionally, the maximum antioxidant activity was observed after 48 hours of treatment with 6% *P. indica* extract. Gene expression analysis revealed that fungal elicitation led to increased expression of key biosynthetic genes, including phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT), and the transcription factor MYB312 [12]. A study on the impact of endophytic fungi *Stemphylium amaranthi* and *Gliomastix masseei* on the production of ajmalicine and vinblastine in *C. roseus* cell cultures (cv. Ice Pink) found that *S. amaranthi* contained high levels of total phenols and flavonoids. Extracts of these fungi were shown to enhance the production of ajmalicine and vinblastine [13]. Another study demonstrated that *Aspergillus flavus* could increase the production of terpenoids and indole alkaloids in *C. roseus*, presenting an alternative biotechnological approach for obtaining bioactive alkaloids [14]. Furthermore, stimulating *C. roseus* cultures with *A. flavus* was found to enhance callus biomass growth and increase vinblastine and vincristine levels [15]. Precursors are biological compounds that serve as intermediates in the biosynthetic pathways of secondary metabolites. Incorporating these compounds into culture media can significantly enhance the production of desired metabolites [16]. Among these, amino acids are commonly used as precursors to stimulate secondary metabolite biosynthesis [17]. Tryptophan, an essential amino acid, plays a crucial role in alkaloid biosynthesis in *C. roseus*. Studies have shown that tryptophan acts as a precursor in the production of indole alkaloids, serving as a fundamental component in the formation of key biosynthetic intermediates such as strictosidine, a pivotal compound in the synthesis of terpenoid indole alkaloids [18]. In growth media supplemented with tryptophan, researchers observed a significant increase in alkaloid production in *C. roseus* callus cultures. This amino acid not only enhances alkaloid synthesis but also influences the expression of key enzymes involved in the biosynthetic pathway [19]. Given the high pharmacological value of *C. roseus* and the crucial role of vincristine and vinblastine in anticancer therapies, understanding the factors that regulate their production and accumulation is essential. Therefore, this study investigates the effects of *P. indica* extract elicitation and tryptophan precursor feeding on vincristine and vinblastine production in *C. roseus* suspension cultures.

Methods

In Vitro Germination of Seeds and Suspension Culture Establishment

The seeds of *Catharanthus roseus*, variety Red Really (*Vinca Pacifica XP Really Red* – PanAmerican Seed Company, USA), were used to obtain sterile seedlings. Surface disinfection was carried out by immersing the seeds in 70% ethanol for 1 minute, followed by treatment with a 2.5% sodium hypochlorite solution for 10 minutes. After each stage, the seeds were rinsed three times with sterile distilled water. They were then transferred to Murashige and Skoog (MS) medium [20]. and incubated at 25°C under a 16-hour light/8-hour dark photoperiod to promote germination and seedling growth. For callus induction, leaves were excised from 20-day-old seedlings, wounded, and placed on MS culture medium supplemented with 1 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D). The cultured explants were maintained in complete darkness for one month. To establish a suspension culture, 1 gr of vigorous, friable, and white callus was selected and transferred to flasks containing 50 mL of liquid MS medium supplemented with 1 mg/L 2,4-D. The flasks were then placed on an orbital shaker at 100 rpm under dark conditions at 25°C. A stable cell suspension culture was established after three subcultures, with each subculture performed at three-week intervals (Fig. 1).

Determination of Suspension Cell Growth Rate

The percentage of settled cell volume (SCV) was used to determine the growth rate of the cell suspension culture. For this purpose, 1 gr of cell mass was inoculated into 100 mL Erlenmeyer flasks containing 50 mL of MS liquid culture medium supplemented with 1 mg/L 2,4-D. The flasks were incubated in a shaker at 25°C and 100 rpm. Every three days, 10 mL of the flask contents were transferred to a graduated Falcon tube and allowed to settle for 30 minutes. The volume of settled cells was then recorded as a percentage. This process was repeated over a period of 40 days [21].

Biotic Elicitor (*P. indica* Extract) Treatment

A cell extract of *Piriformospora indica* was used as a biotic elicitor. The elicitor was added at different concentrations (0%, 2%, 4%, and 6% v/v) to the cell suspension culture medium (MS + 1 mg/L 2,4-D + 300 mg/L tryptophan) at the late growth phase (28 days). The cultures were exposed to the elicitor for 48 and 72 hours. Untreated cell suspension cultures served as control samples. After the 48-hour and 72-hour exposure periods, samples were collected, and the indole terpenoid alkaloid content was measured.

Estimation of Total Phenolic Content (TPC) and Total Flavonoid Content (TFC)

The total phenolic content (TPC) was measured using the Folin-Ciocalteu method [22], with slight modifications. Briefly, 600 µL of extract was mixed with 1200 µL of 10% Folin-Ciocalteu reagent in glass tubes. Then, 960 µL of 7% sodium carbonate solution and 180 µL of distilled water were added. The tubes were incubated in the dark at room temperature for 30–40 minutes. Absorbance was measured at 760 nm using a spectrophotometer (UNICO, Model: UV2100 PC). The total phenolic content was expressed as mg of gallic acid equivalents per gram of fresh weight (mg GAE/g FW). To determine the total flavonoid content (TFC), 500 µL of methanolic extract was mixed with 150 µL of 5% sodium nitrite

solution and incubated in the dark for 5–15 minutes. Next, 300 µL of 10% aluminum chloride solution and 1000 µL of 1 M potassium acetate were added. The absorbance was measured at 380 nm, and the total flavonoid content was expressed as mg of quercetin equivalents per gram of fresh weight (mg QE/g FW) [23].

Extraction of Terpenoid Indole Alkaloids and HPLC Analysis

For alkaloid extraction, 1 gr of powdered plant sample was placed in a test tube, and 7 mL of a solvent mixture of chloroform and methanol (15:6 ratio) was added. The test tubes were then sealed and subjected to ultrasonic treatment (Eurosonic 4D) for 15 minutes. After sonication, the samples were centrifuged at 3000 rpm for 5 minutes at 4°C, and the supernatant was collected in a separate test tube. The remaining residue was re-extracted with the same solvent mixture, and the process was repeated. After a second centrifugation, the supernatants were combined. The extracted solution was then evaporated to dryness using a rotary evaporator (Heidolph) at 50°C. The dried residue was dissolved in 2 mL of 3% HCl and mixed with 5 mL of chloroform. The solution was centrifuged at 3000 rpm for 5 minutes at 4°C, forming two distinct phases. The aqueous phase (upper layer) was carefully separated and transferred to a clean test tube, while the chloroform phase (lower layer) was discarded. The pH of the aqueous phase was adjusted to 8.5 using ammonia, followed by the addition of 2 mL of chloroform. After another round of centrifugation (3000 rpm, 4°C, 5 minutes), the chloroform phase was collected, and the aqueous phase was discarded. To remove residual moisture, 0.5 g of anhydrous sodium sulfate was added to the chloroform phase. After filtration to remove the sodium sulfate, the final chloroform extract was evaporated to dryness using a rotary evaporator at 50°C. The dried residue was fully dissolved in 0.5 mL of HPLC-grade methanol and stored in Eppendorf tubes at -20°C until HPLC analysis. Separation, identification, and quantification of terpenoid indole alkaloids (vindoline, catharanthine, vinblastine, and vincristine) were performed using high-performance liquid chromatography (HPLC, Agilent 1100 series, USA). The system was equipped with a 20 µL injection loop, a four-solvent gradient pump, a degassing system, a column oven (set at 25°C), and a diode array detector (set at 205 nm). Separation was conducted on a ZORBAX Eclipse XDB octadecyl silane column (25 cm length, 4.6 mm internal diameter, 5 µm particle size, Dr. Mainsch, Germany). The mobile phase consisted of 30% 5 mM phosphate buffer (pH 6) (solvent A) and 70% acetonitrile (solvent B), with a flow rate of 1 mL/min. Vindoline, catharanthine, vincristine, and vinblastine standards obtained from Sigma-Aldrich were used to generate the calibration curve.

Statistical Analysis

The experiment followed a completely randomized factorial design with three replications. Data obtained from the experiments were analyzed statistically using SAS software version 9.2. Mean comparisons were performed using Duncan's multiple range test at a significance level of $P \leq 0.01$.

Results

Suspension Cell Growth Rate

The results indicated that following cultivation, the fresh weight of the cells began to increase, exhibiting a logarithmic growth pattern by the third week. However, after this phase, cell growth started to decline, and by the fifth week, the growth trend became imperceptible. At the end of the logarithmic phase and near the onset of the stationary phase (days 26–27), when the biomass of the cell suspension had reached an optimal level, this time point was selected for the application of *P. indica* extract treatment (Fig. 2).

Growth Efficiency of Cell Suspension Treated with *P. indica*

Different concentrations of *P. indica* extract and exposure durations had a significant effect ($p \leq 0.05$) on the fresh and dry weight of *C. roseus* cells. The highest fresh weight (8.26 g) and dry weight (0.31 g) were recorded in cells treated with 4% v/v *P. indica* extract for 48 hours. In contrast, the lowest fresh weight (5.85 g) and dry weight (0.21 g) were observed in the control group (Fig. 3).

Effect of *P. indica* Elicitation on Total Phenolic and Flavonoid Content

The results showed that different levels of concentration and exposure time had a significant effect on the content of total phenol and total flavonoid in the cell suspension cultures of *C. roseus* at $P \leq 0.01$. The highest content of total phenol and total flavonoid was obtained after 48 hours of treatment with a concentration of 6% v/v of *P. Indica* extract. 72 hours exposure time caused decreasing TPC and TFC of cells, especially in high concentrations of elicitor (fig. 4).

Effect of Tryptophan Precursor and *P. indica* Extract on Alkaloid Production in *C. roseus* Cell Suspension Culture

The interaction between concentration and exposure time had a significant impact on indole terpenoid alkaloid production ($p < 0.01$). The highest catharanthine production (1.73 $\mu\text{g/g}$) was recorded at 6% v/v *P. indica* after 48 hours, while the lowest amount (0.03 $\mu\text{g/g}$) was observed after 72 hours (Fig. 5A). The maximum vindoline content (18.53 $\mu\text{g/g}$) was obtained in cultures treated with 4% v/v *P. indica* for 48 hours, whereas the lowest (0.76 $\mu\text{g/g}$) was recorded after 72 hours (Fig. 5B). The highest vinblastine production (0.31 $\mu\text{g/g}$) was observed in cultures elicited with 4% and 6% v/v *P. indica* after 48 hours, while the lowest (0.05 $\mu\text{g/g}$) was recorded after 72 hours (Fig. 5C). The maximum vincristine content (634.7 $\mu\text{g/g}$) was documented in cultures treated with 2% v/v *P. indica* for 48 hours, whereas the control group had the lowest vincristine level (89.28 $\mu\text{g/g}$) (Fig. 5D). This represents an approximately sevenfold increase in vincristine content compared to the control treatment.

Discussion

Phenolic acids constitute an important group of plant metabolites with diverse and valuable therapeutic properties. They are derived from the phenylpropanoid pathway and are widely distributed in the plant kingdom. Phenolic acids are known for their antioxidant, antimicrobial, and anti-inflammatory properties, making them valuable for human health and industrial applications [24]. The production of phenolic

acids in whole plants can be affected by different factors such as, genetic and genotype of species, environmental condition, biotic and abiotic stress and the presence of a nutrient precursor etc. The primary function of phenolic and flavonoid compounds as free radical scavengers has been well documented [25]. Research indicates a direct correlation between the content of phenolic and flavonoid compounds and antioxidant activity. Phenolic compounds act as electron donors and metal ion chelators, contributing to free radical scavenging or inhibition [26]. Flavonoids are synthesized in the cytoplasm and on the cytoplasmic surface of the endoplasmic reticulum, playing a protective role against biotic and abiotic stress due to their antioxidant activity [27, 28]. Plant tissue culture is considered a viable alternative for the economical production of these pharmaceutical compounds under *in vitro* condition [29]. raising the phenolic acids production and accumulation in plants using biotechnology methods is a key area of research in metabolic engineering and synthetic biology. These methods aim to enhance the biosynthesis of phenolic acids for applications in nutraceuticals, pharmaceuticals, and sustainable agriculture [30]. Stimulation and feeding with precursors are one of the most effective methods of increasing the production of such as alkaloids, flavonoids, terpenoids, and phenolic acids in plant tissue culture. By supplying precursors as starting molecules for metabolic pathways, researchers can enhance the flux through specific biosynthetic pathways, leading to increased production of desired secondary metabolites. studies have been demonstrated that the use of precursors can enhance the accumulation of natural compounds in plant cell cultures [31, 32, 33].

Biological stimuli are generally recognized by specific receptors on the cell membrane, initiating signal transduction pathways that ultimately lead to the production of phytoalexins [34]. In this context, the application of external biological stimuli likely enhances phenols and flavonoid biosynthesis by triggering plant defense responses [35]. The simultaneous use of elicitors that target different metabolic pathways and perform various functions may exert a synergistic effect, thereby activating otherwise limited enzymatic functions and improving metabolite production [36, 37]. Fungal elicitors have been widely employed to enhance the accumulation of natural compounds in plant cell cultures [38]. This strategy has been shown to increase the production of bioactive compounds such as phenols, flavonoids, anthocyanins [39, 40, 41], alkaloids [42], and glycosides [43]. The enhancement of total phenolic content (TPC) and total flavonoid content (TFC) observed in this study is consistent with these findings. Furthermore, the results of the present study revealed that vincristine content in *C. roseus* cell cultures treated with *P. indica* extract was significantly higher than in control cultures. This finding suggests that fungal extract has a direct stimulatory effect on the biosynthesis and accumulation of vincristine, a valuable medicinal compound. Elicitors act as stress factors by inducing osmotic pressure, which activates key enzymes in the biosynthetic pathways of natural metabolites [44]. Stimulating factors and precursor molecules enhance the expression of relevant pathway genes through cellular signaling, ultimately leading to increased production of secondary metabolites in plant cells [45]. By stimulating alkaloid biosynthetic pathway genes, reinforcing plant cell wall synthesis, and activating defense mechanisms, fungal extracts enhance the accumulation of important biomolecules such as phytoalexins, flavanols and alkaloids [46]. Additionally, studies have shown that fungal elicitors promote vinblastine production by demethylating vincristine [47]. Research further suggests that the activity of

tryptophan decarboxylase (TDC) significantly increases in fungal-treated cultures compared to controls [48]. Tang *et al.* [49] reported that biological stimuli enhance cell density and upregulate TDC and PAL genes, leading to a significant increase in alkaloid production in cell suspensions. Similarly, Kumar *et al.* [50] observed a 1.4-fold increase in cell growth and a 13-fold enhancement in lignan content in *Linum album* hairy root cultures treated with *P. indica*. The findings of the present study align with previous research on *L. album* [51], *Centella asiatica* [52] and *Ficus carica* [53], further supporting the efficacy of fungal elicitation in promoting bioactive compound synthesis in plant cell cultures.

Conclusion

This study demonstrated that *Piriformospora indica* extract elicitation and tryptophan precursor feeding significantly enhance the biosynthesis of vincristine and vinblastine in *Catharanthus roseus* suspension cultures. The results revealed that 4% *P. indica* extract for 48 hours maximized fresh and dry biomass accumulation, while 6% extract induced the highest phenolic and flavonoid content. Furthermore, the elicitation treatment notably increased antioxidant activity, reinforcing the role of biotic stress in secondary metabolite production. Notably, vinblastine and vincristine accumulation peaked at 4% and 2% *P. indica* extract, respectively, highlighting the efficiency of fungal elicitors in promoting alkaloid biosynthesis. These findings provide a promising biotechnological approach for sustainable production of pharmacologically valuable alkaloids, offering an alternative to conventional extraction from whole plants. Future studies should focus on optimizing elicitation conditions, exploring molecular mechanisms underlying alkaloid enhancement, and scaling up production in bioreactors for commercial applications.

Declarations

Acknowledgments

The authors of this article would like to thank all the staff of Horticultural Science Department of Urmia University, Faculty of Agriculture

Author Contributions

Sh. MY. Investigation, Methodology, Writing an original draft. B. H. Investigation, Methodology, Formal analysis, Review & Editing. S. H: Conceptualization, Statistical analysis, Review & Editing. All authors read and approved the final manuscript.

Funding

No funding was received for this work.

Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This article did not include any research involving human subjects, animals, or endangered species.

Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publication

Not applicable

References

1. Fitzgerald M, Heinrich M, Booker A. Medicinal plant analysis: A historical and regional discussion of emergent complex techniques. *Front Pharmacol*. 2020;10:1480. doi:10.3389/fphar.2019.01480.
2. Parvin S, Reza A, Das S, Miah MMU, Karim S. Potential role and international trade of medicinal and aromatic plants in the world. *Eur J Agric Food Sci*. 2023;5(5):89-99.
3. Lowe H, Steele B, Bryant J, Fouad E, Toyang N, Ngwa W. Antiviral activity of Jamaican medicinal plants and isolated bioactive compounds. *Molecules*. 2021;26(3):607. doi:10.3390/molecules26030607.
4. Zhu J, Wang M, Wen W, Yu R. Biosynthesis and regulation of terpenoid indole alkaloids in *Catharanthus roseus*. *Pharmacogn Rev*. 2015;9(17):24-28. doi:10.4103/0973-7847.156323.
5. Mozaffarian V. Plant classification. Tehran: Amir Kabir Publications; 2004. [In Persian]
6. Beigi R. Production and processing of medicinal plants. Tehran: [Publisher Name]; 2006. [In Persian]
7. Fazili MA, Bashir I, Ahmad M, Yaqoob U, Geelani SN. *In vitro* strategies for the enhancement of secondary metabolite production in plants: a review. *Bull Natl Res Cent*. 2022;46(1):35. doi:10.1186/s42269-022-00717-z.
8. Ramadani MRN, Jadid N. A comprehensive review of *in vitro* precursor feeding strategies for the overproduction of high-value plant secondary metabolites. *Arab J Chem*. 2024:106018.
9. Niazian M, Sabbatini P. Traditional *in vitro* strategies for sustainable production of bioactive compounds and manipulation of metabolomic profile in medicinal, aromatic and ornamental plants. *Planta*. 2021;254(6):111. doi:10.1007/s00425-021-03771-5.
10. Shahabivand S, Parvaneh A, Aliloo AA. Root endophytic fungus *Piriformospora indica* affected growth, cadmium partitioning and chlorophyll fluorescence of sunflower under cadmium toxicity. *Ecotoxicol Environ Saf*. 2017;145:496–502.

11. Biswas D, Chakraborty A, Mukherjee S, Ghosh B. Hairy root culture: a potent method for improved secondary metabolite production of Solanaceous plants. *Front Plant Sci.* 2023;14:1197555. doi:10.3389/fpls.2023.1197555.
12. Amani S, Mohebodini M, Khademvatan S, Jafari M, Kumar V. *Piriformospora indica*-based elicitation for overproduction of phenolic compounds by hairy root cultures of *Ficus carica*. *J Biotechnol.* 2021;327:43-53.
13. Hemmati N, Azizi M, Spina R, Dupire F, Arouei H, Saeedi M, Laurain-Mattar D. Accumulation of ajmalicine and vinblastine in cell cultures is enhanced by endophytic fungi of *Catharanthus roseus* cv. Icy Pink. *Ind Crops Prod.* 2020;158(2):1-10.
14. Liang C, Chen C, Zhou P, Xu L, Zhu J, Liang J, Yu R. Effect of *Aspergillus flavus* fungal elicitor on the production of terpenoid indole alkaloids in *Catharanthus roseus* cambial meristematic cells. *Molecules.* 2018;23(12):3276.
15. Tonk D, Mujib A, Maqsood M, Ali M, Zafar N. *Aspergillus flavus* fungus elicitation improves vincristine and vinblastine yield by augmenting callus biomass growth in *Catharanthus roseus*. *Plant Cell Tissue Organ Cult.* 2016;126:291-303.
16. Rao RS, Ravishankar GA. Plant tissue cultures; chemical factories of secondary metabolites. *Biotechnol Adv.* 2002;20:101-153.
17. Shinde AN, Malpathak N, Fulzele DP. Enhanced production of phytoestrogenic isoflavones from hairy root cultures of *Psoralea corylifolia* L. using elicitation and precursor feeding. *Biotechnol Bioprocess Eng.* 2009;14:288-294.
18. Kumar K, Kumar SR, Dwivedi V, Rai A, Shukla AK, Shanker K, Nagegowda DA. Precursor feeding studies and molecular characterization of geraniol synthase establish the limiting role of geraniol in monoterpene indole alkaloid biosynthesis in *Catharanthus roseus* leaves. *Plant Sci.* 2015;239:56-66.
19. Zhu W, Yang B, Komatsu S, Lu X, Li X, Tian J. Binary stress induces an increase in indole alkaloid biosynthesis in *Catharanthus roseus*. *Front Plant Sci.* 2015;6:582. doi:10.3389/fpls.2015.00582.
20. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant.* 1962;15(3):473–497.
21. Farjaminezhad R, Zare N, Zakaria RA, Farjaminezhad M. Establishment and optimization of cell growth in suspension culture of *Papaver bracteatum*: a biotechnology approach for thebaine production. *Turk J Biol.* 2013;37(6):689-697.
22. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* 1999;299:152-178.
23. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. *J Food Drug Anal.* 2002;10:178-182.
24. Roumani M, Duval RE, Ropars A, Risler A, Robin C, Larbat R. Phenolamides: plant specialized metabolites with a wide range of promising pharmacological and health-promoting interests. *Biomed Pharmacother.* 2020;131:110762.

25. Lata R, Chowdhury S, Gond SK, White JF Jr. Induction of abiotic stress tolerance in plants by endophytic microbes. *Lett Appl Microbiol.* 2018;66(4):268-276.
26. Teofilović B, Grujić-Letić N, Gligorić E, Rašković A, Igić R, Vastag G, Gadžurić S. Experimental and computational evaluation of extraction procedure and scavenging capacity of sweet basil extracts (*Ocimum basilicum* L.). *Plant Foods Hum Nutr.* 2021;76(2):240-247.
27. Pourcel L, Routaboul JM, Cheynier V, Lepiniec L, Debeaujon I. Flavonoid oxidation in plants: from biochemical properties to physiological functions. *Trends Plant Sci.* 2007;12(1):29-36.
28. Gautam H, Sharma A, Trivedi PK. The role of flavonols in insect resistance and stress response. *Curr Opin Plant Biol.* 2023;73:102353.
29. Prashant SP, Bhawana M. An update on biotechnological intervention mediated by plant tissue culture to boost secondary metabolite production in medicinal and aromatic plants. *Physiol Plant.* 2024;176(4):e14400. doi:10.1111/ppl.14400.
30. Ma XH, Ma Y, Tang JF, et al. The biosynthetic pathways of tanshinones and phenolic acids in *Salvia miltiorrhiza*. *Molecules.* 2015; 20(9): 16235-16254. doi:10.3390/molecules200916235.
31. Skrzypczak-Pietraszek E, Słota J, Pietraszek J. The influence of L-phenylalanine, methyl jasmonate, and sucrose concentration on the accumulation of phenolic acids in *Exacum affine* Balf. f. ex Regel shoot culture. *Acta Biochim Pol.* 2014;61(1).
32. Chandra HM, Shanmugaraj B, Sharma A, Ramalingam S. Elicitation and precursor induced approaches for the enhancement of α -tocopherol production using suspension cultures of *Solanum lycopersicum*. *Curr Plant Biol.* 2024;39:100377.
33. Ramadani MRN, Jadid N. A comprehensive review of *in vitro* precursor feeding strategies for the overproduction of high-value plant secondary metabolites. *Arab J Chem.* 2024:106018.
34. Baenas N, García-Viguera C, Moreno DA. Elicitation: a tool for enriching the bioactive composition of foods. *Molecules.* 2014;19(9):13541-13563.
35. Jiao J, Gai QY, Yao LP, et al. Ultraviolet radiation for flavonoid augmentation in *Isatis tinctoria* L. hairy root cultures mediated by oxidative stress and biosynthetic gene expression. *Ind Crops Prod.* 2018;118:347-354.
36. Zong G, Fu J, Zhang P, et al. Use of elicitors to enhance or activate the antibiotic production in *Streptomyces*. *Crit Rev Biotechnol.* 2022;42(8):1260-1283. doi:10.1080/07388551.2021.1987856.
37. Zhai X, Jia M, Chen L, et al. The regulatory mechanism of fungal elicitor-induced secondary metabolite biosynthesis in medicinal plants. *Crit Rev Microbiol.* 2017;43(2):238-261.
38. Sák M, Dokupilová I, Kaňuková Š, et al. Biotic and abiotic elicitors of stilbenes production in *Vitis vinifera* L. cell culture. *Plants (Basel).* 2021;10(3):490. doi:10.3390/plants10030490.
39. Gadzovska Simic S, Tusevski O, Maury S, et al. Fungal elicitor-mediated enhancement in phenylpropanoid and naphthodianthrone contents of *Hypericum perforatum* L. cell cultures. *Plant Cell Tissue Organ Cult.* 2015;122:213-226.

40. Mendhulkar VD, Vakil MM. Elicitation of flavonoids by salicylic acid and *Penicillium expansum* in *Andrographis paniculata* (Burm. f.) Nees. cell culture. *Res Biotechnol.* 2013;4(2).
41. Selwal N, Supriadi K, Rahayu F, et al. Elicitation strategies for enhanced secondary metabolite synthesis in plant cell cultures and its role in plant defense mechanism. *Plant Gene.* 2024;100485.
42. Taha HS, El-Bahr MK, Seif-El-Nasr MM. *In vitro* studies on Egyptian *Catharanthus roseus* (L.) G. Don. IV: manipulation of some amino acids as precursors for enhanced indole alkaloids production in suspension cultures. *Aust J Basic Appl Sci.* 2009;3(4):3137-3144.
43. Nadeem M, Abbasi BH, Garros L, et al. Yeast-extract improved biosynthesis of lignans and neolignans in cell suspension cultures of *Linum usitatissimum* L. *Plant Cell Tissue Organ Cult.* 2018;135:347-355.
44. Kim BR, Kim SB, Moon DG, et al. Strategies in using elicitors for the production of bioactive secondary metabolites in plant cell cultures. *Trends Agric Life Sci.* 2023;61:13-22.
45. Neumann G. The role of ethylene in plant adaptations for phosphate acquisition in soils—a review. *Front Plant Sci.* 2016;6:1224.
46. Ramirez-Estrada K, Vidal-Limon H, Hidalgo D, et al. Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules.* 2016;21(2):182.
47. Linh TM, Mai NC, Hoe PT, et al. Development of a cell suspension culture system for promoting alkaloid and vinca alkaloid biosynthesis using endophytic fungi isolated from local *Catharanthus roseus*. *Plants (Basel).* 2021;10(4):672. doi:10.3390/plants10040672.
48. Tonk D, Mujib A, Maqsood M, et al. *Aspergillus flavus* fungus elicitation improves vincristine and vinblastine yield by augmenting callus biomass growth in *Catharanthus roseus*. *Plant Cell Tissue Organ Cult.* 2016;126:291-303.
49. Tang Z, Rao L, Peng G, et al. Effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis in cell suspension cultures of *Catharanthus roseus*. *J Med Plants Res.* 2011;5(11):2192-2200.
50. Kumar V, Rajauria G, Sahai V, Bisaria V. Culture filtrate of root endophytic fungus *Piriformospora indica* promotes the growth and lignan production of *Linum album* hairy root cultures. *Process Biochem.* 2012;47:901-907.
51. Baldi A, Farkya S, Jain A, et al. Enhanced production of podophyllotoxins by co-culture of transformed *Linum album* cells with plant growth-promoting fungi. *Pure Appl Chem.* 2010;82:227-241.
52. Jisha S, Gouri PR, Anith KN, Sabu KK. *Piriformospora indica* cell wall extract as the best elicitor for asiaticoside production in *Centella asiatica* (L.) Urban, evidenced by morphological, physiological and molecular analyses. *Plant Physiol Biochem.* 2018;125:106-115.
53. Amani S, Mohebodini M, Khademvatan S, et al. *Piriformospora indica* based elicitation for overproduction of phenolic compounds by hairy root cultures of *Ficus carica*. *J Biotechnol.* 2021;327:43-53.

Figures

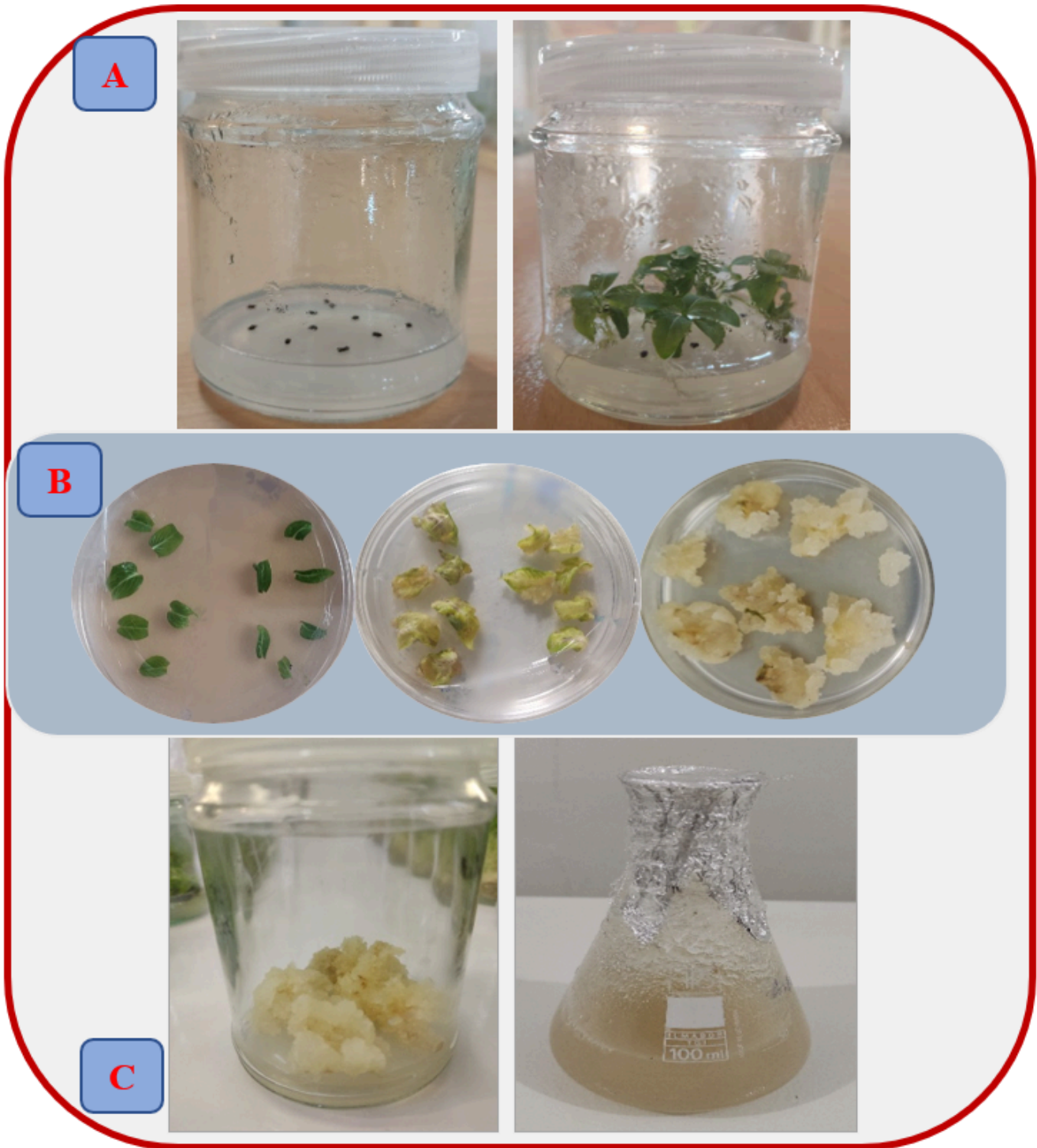


Figure 1

Developmental Stages of *C. roseus* from Seed Cultivation to Cell Suspension culture. (A) Seed culture and germination in MS media (B) Stages of Callus Induction from Leaf in MS Media Containing 1 mg/l 2,4-D and (C) frible Callus formation and Cell Suspension culture.

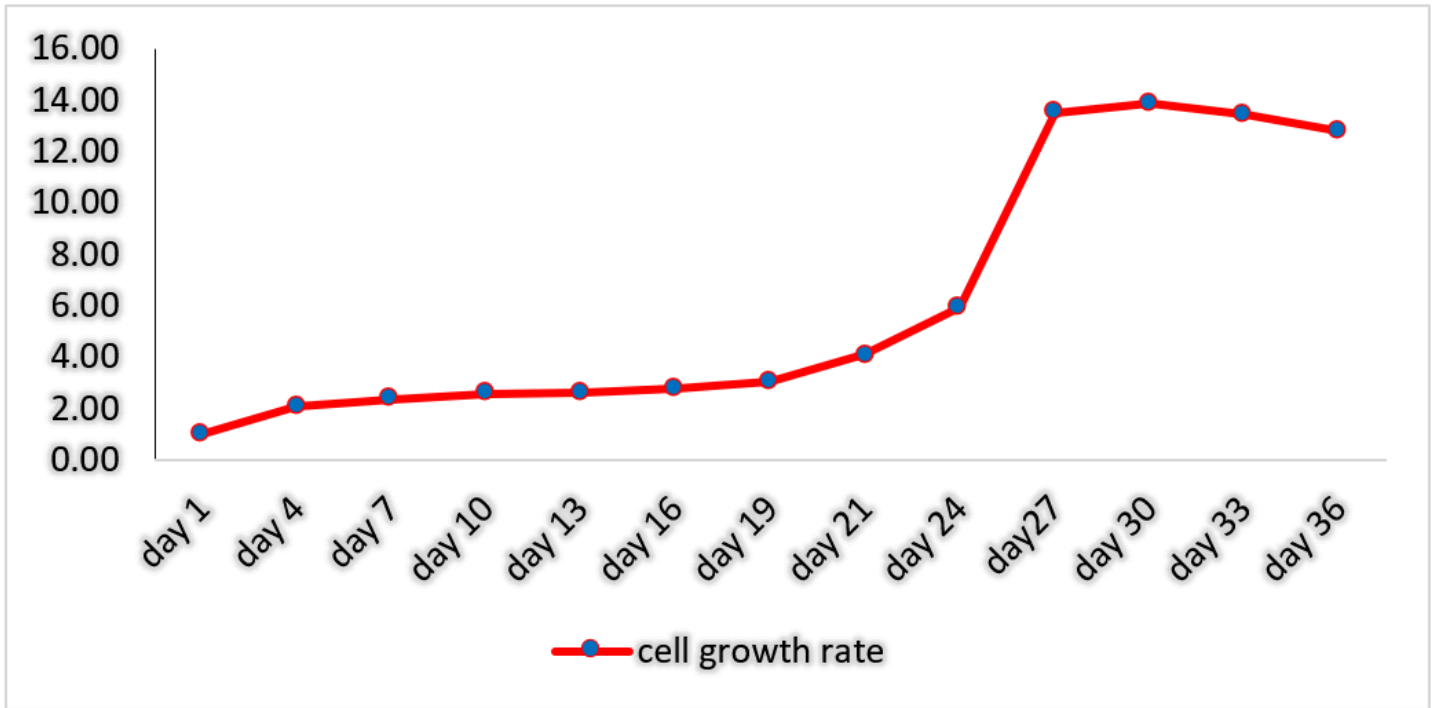


Figure 2

Cell growth rate in cell suspension culture of *C. roseus* based on SCV.

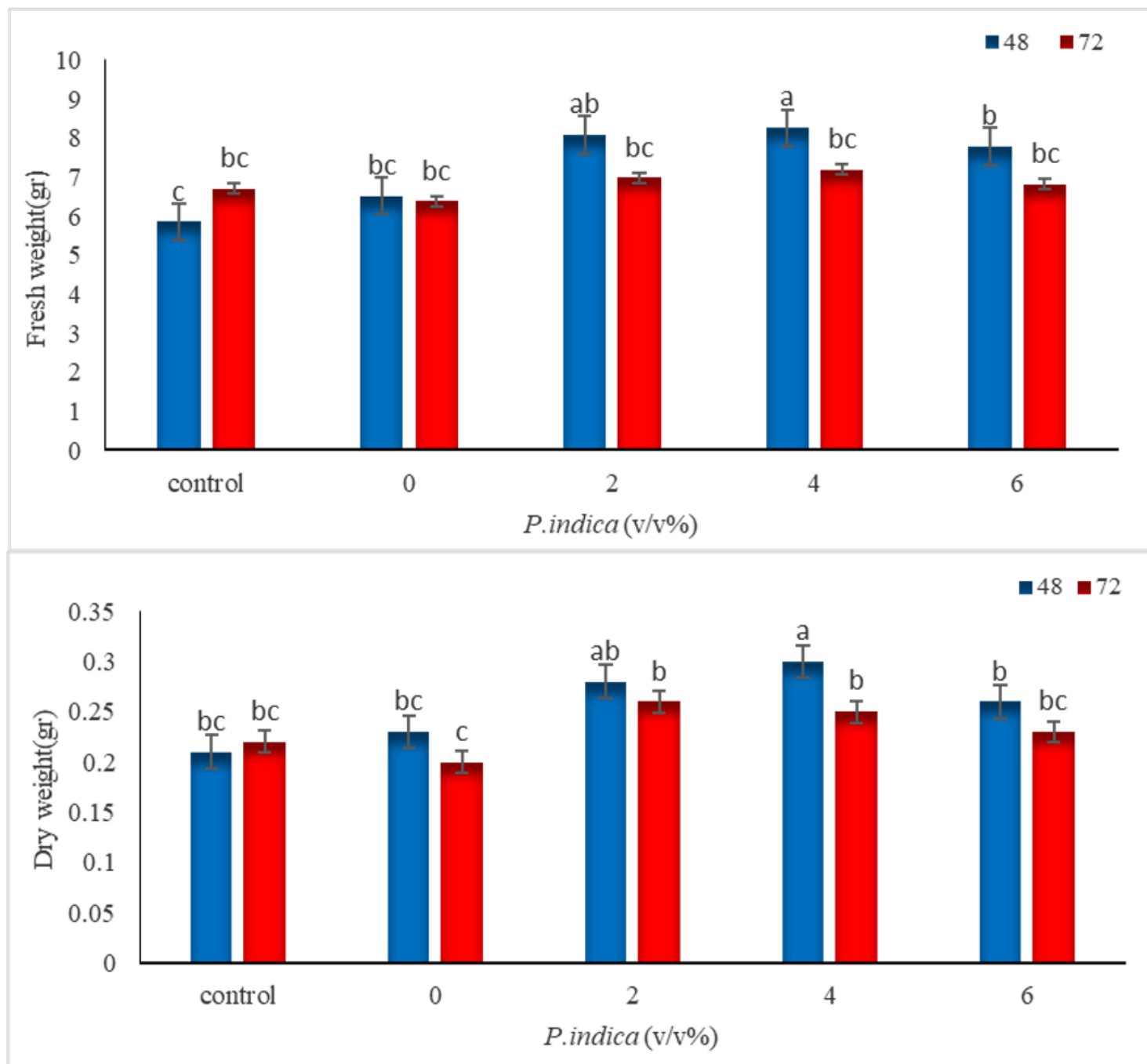


Figure 3

Effect of different concentrations of *P. Indica* at different exposure times on the FW and DW of *C. roseus* cell suspension culture. Mean values marked with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.01$).

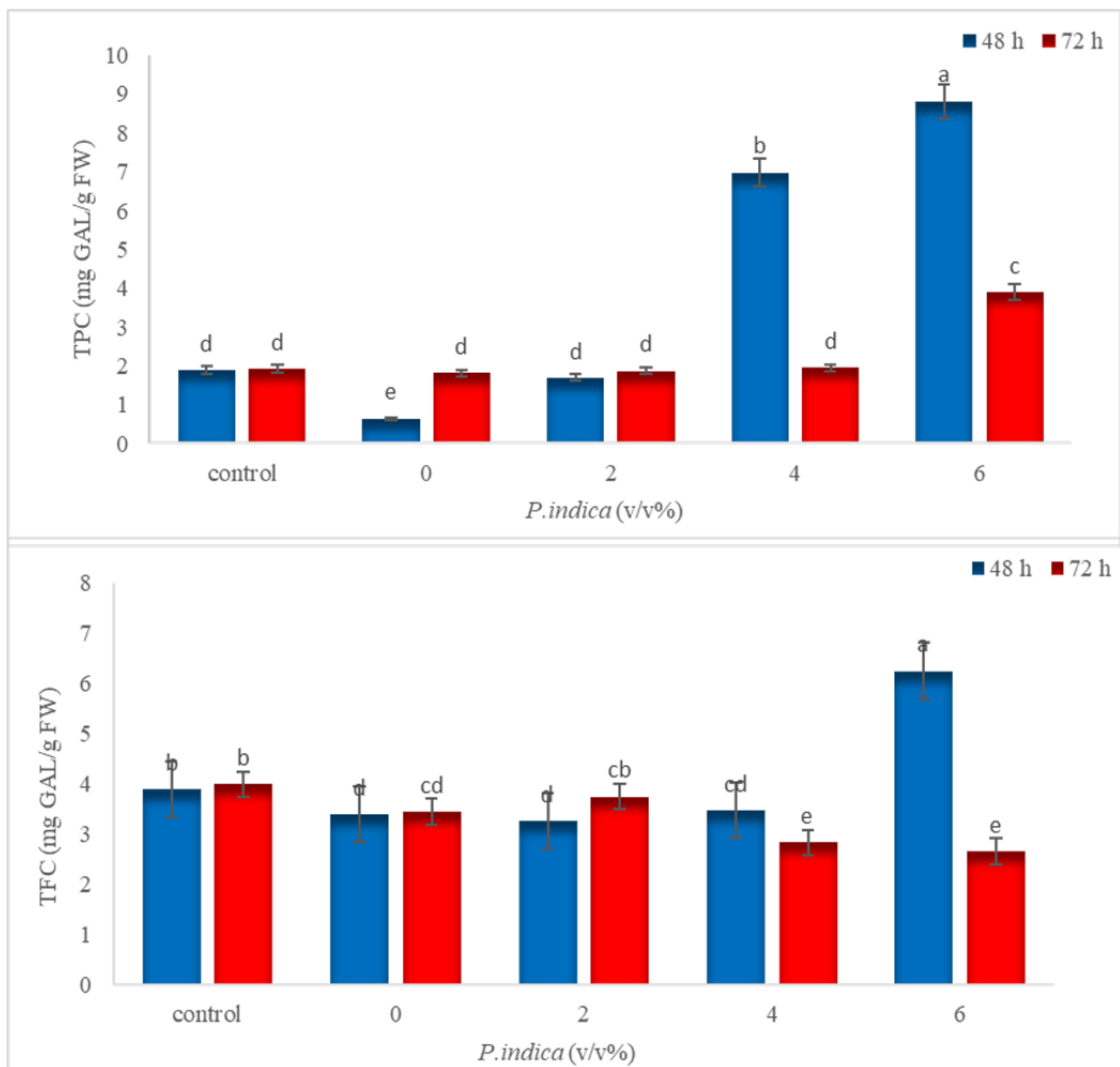


Figure 4

Effect of different concentrations of *P. Indica* at different exposure times on TPC and TFC content in *C. roseus* cell suspension culture. Mean values marked with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.01$).

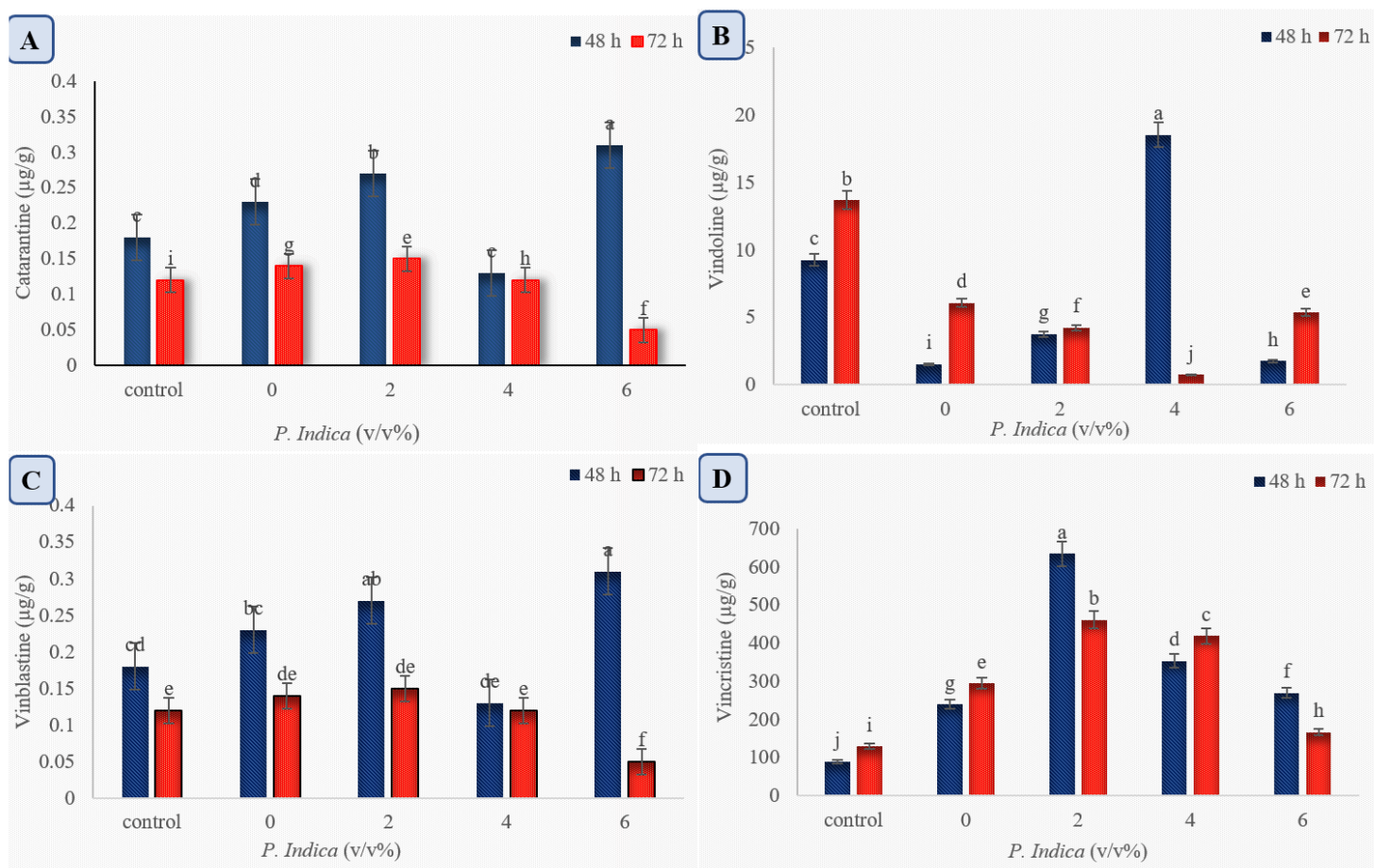


Figure 5

Effect of different concentrations of *P. Indica* at different exposure times on the Catharanthine (A), Vindoline (B), Vinblastine (C) and Vincristine (D) content (μg/g) in the *C. roseus* cell suspension culture. Mean values marked with different letters are significantly different according to Duncan's multiple range test ($P \leq 0.01$).