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ORIGINAL ARTICLE

Molecular and biochemical insights into tryptophan-induced indole alkaloid biosynthesis in *Isatis tinctoria* L. root cultures

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Abstract

This study was carried out to investigate the effects of tryptophan (Trp) application on indigotin (IND) and indirubin (INR) production, as well as on the expression levels of *Isatis tinctoria Tryptophan Synthase Alpha (It-TSA)* and *Cytochrome P450* (*CYP79B2)* genes involved in their biosynthesis, in *Isatis tinctoria* L. root cultures. Three-week-old root cultures were treated with Trp at concentrations of 100, 200, and 300 mg/L for 48 h, after which the IND and INR contents in the harvested roots were quantified. Concurrently, changes in the expression levels of *It-TSA* and *CYP79B2* genes, which are responsible for indole alkaloid biosynthesis, were determined. The findings revealed that all analyzed parameters significantly varied depending on the Trp concentration. Notably, the 200 mg/L Trp treatment resulted in both the highest accumulation of IND and INR and the maximum expression levels of the target genes. A striking positive relationship was found between the increase in gene expression and metabolite accumulation. These results clearly demonstrated that Trp acts as an effective precursor for increasing secondary metabolite production in *I. tinctoria* root cultures. By providing crucial data for optimizing the production of plant-derived valuable metabolites through in vitro techniques, this study makes valuable contributions to the field of plant biotechnology.

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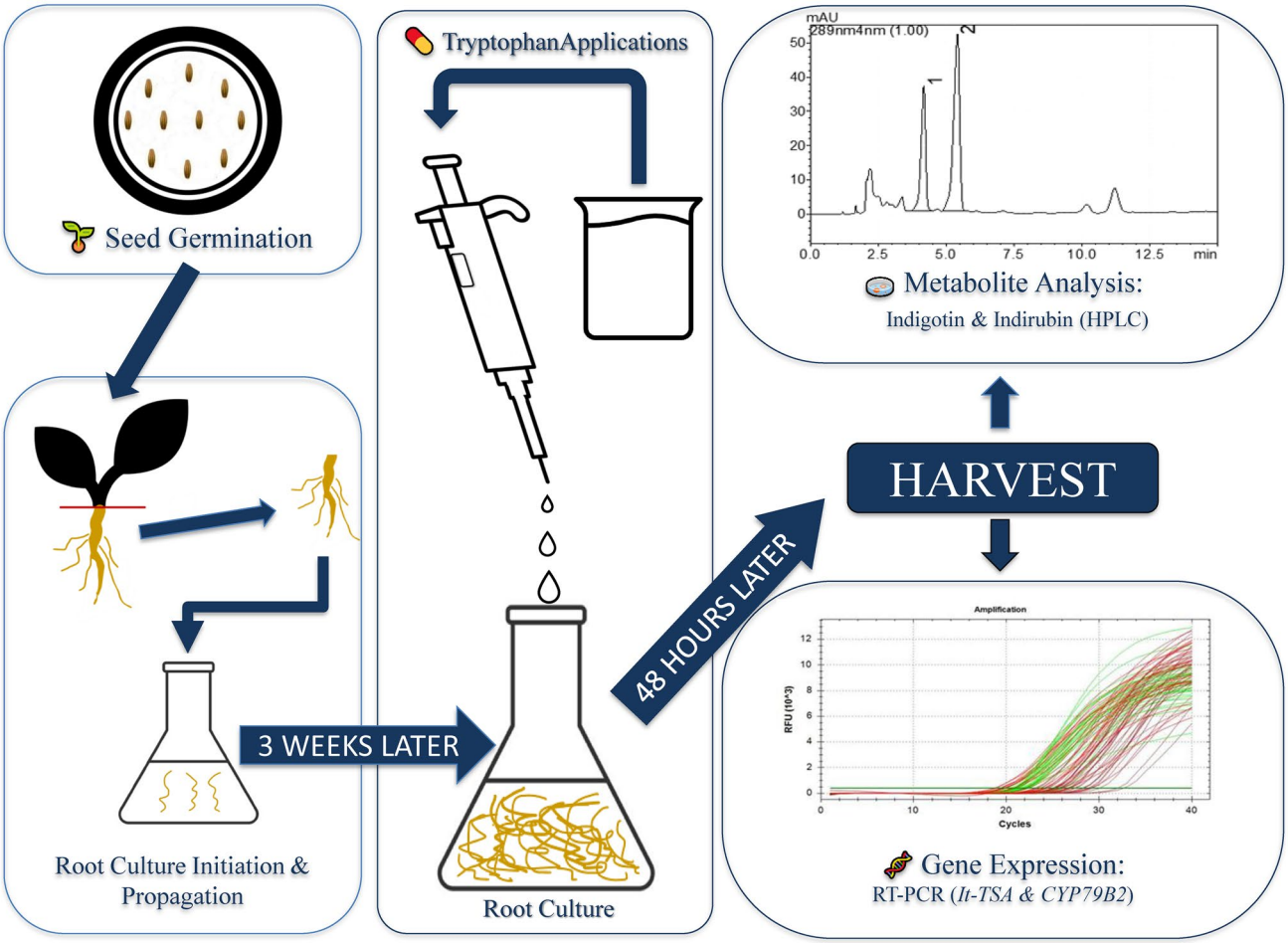
Graphical abstract



Key message
Tryptophan increased the production of indole alkaloids, and the expression levels of gene related the indole alkaloid biosynthesis when applied the appropriate concentrations.

Keywords *Isatis tinctoria* · Root culture · Tryptophan · Indigotin · Indirubin · Gene expression

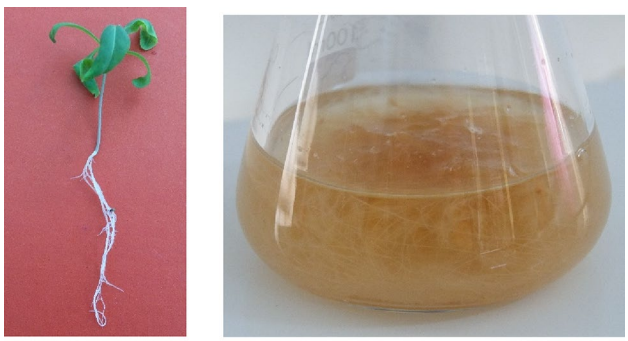
Introduction

Isatis tinctoria L., belonging to the Brassicaceae family, is known as a natural source of indole alkaloids that are of great importance in the dye and pharmaceutical industries (Speranza et al. 2020). In traditional Chinese medicine, *I. tinctoria* has historically been used to treat a variety of ailments, including gastrointestinal disorders, skin diseases and asthma. Its extracts are also used in the cosmetics industry to produce soaps, essential oils and creams (Spataro et al. 2007). In modern applications, it is evident that *I. tinctoria* stands out as a source of raw materials for medical drugs and dyes, particularly due to its components such as the blue pigment indigotin (IND) and the red pigment indirubin (INR). The rarity of blue pigments in nature enhances the commercial importance of the IND component (Kızıl and Arslan 2001). *I. tinctoria* extracts were reported to exhibited not only antioxidant properties but also anticancer effects by suppressing the growth of various cancer cell lines (Taviano et al. 2018; Speranza et al. 2020). Notably, INR, an active compound used to treat leukemia symptoms, was shown to inhibit the proliferation of human lung and breast cancer cells (Marko et al. 2001; Gaboriaud-Kolar et al. 2015).

The uncontrolled collection of *I. tinctoria* from nature to meet demands in various industrial sectors leads to an inability to obtain metabolites of standard quantity and quality due to ecological factors (Atanasov et al. 2015), as well as putting its populations at risk of extinction (Wawrosch and

Zotchev 2021). In vitro techniques offer a sustainable alternative for obtaining high-value plant metabolites by eliminating these negativities (Fazili et al. 2022). However, in some cases, the inability to obtain metabolites at the desired level with plant cell and tissue cultures is the most important challenge encountered in in vitro secondary metabolite production. Therefore, various biotechnological strategies can be implemented to enhance secondary metabolite production efficiency. These include culture medium optimization, modification of cultivation conditions, application of growth regulators, elicitor treatments, cell immobilization techniques, as well as genetic and metabolic engineering approaches (Zhao et al. 2005). One of the methods used to increase secondary metabolite production under in vitro conditions is the addition of precursors or intermediates involved in the biosynthesis of the desired compound to the nutrient medium (Mohamad Zuldin et al. 2013; Ramadani and Jadid 2024). Precursor feeding refers to the incorporation of the primary building block that initiates the biosynthesis pathway of secondary metabolites into the synthesis step (Hussain et al. 2012). Thus, the addition of appropriate precursor compounds to the nutrient medium at the initial stage of culture or during specific developmental periods can effectively enhance the yield of metabolites in cultured plant tissues, cells, and organs (Isah et al. 2018).

Tryptophan (Trp) is an aromatic, plant-derived amino acid containing an indole ring that is essential for protein biosynthesis. Beyond its role as a fundamental amino acid required for normal growth, Trp serves as a precursor for various bioactive compounds (Friedman 2018). Notably, it functions as a key metabolic precursor for secondary metabolites in plants, particularly indole glucosinolates and indole phytoalexins in Brassicaceae family members (Ishihara et al. 2011). Its metabolism is highly complex and multifunctional, contributing to the formation of bioactive molecules that exert effects in different organs by following various biochemical pathways (Modoux et al. 2021).



(A) (B)

Fig. 1 In vitro seedlings of *I. tinctoria* (**A**) and roots propagated in liquid media (**B**)

In *I. tinctoria*, the *Tryptophan Synthase Alpha (It-TSA)* gene encodes the enzyme catalyzes the conversion of indole-3-glycerol phosphate (IGP) to indole and glyceraldehyde-3-phosphate, representing the fundamental stage in plant indole alkaloid synthesis (Salvini et al. 2008). The *Cytochrome P450* (*CYP79B2)* gene, belonging to the *CYP79* gene family, encodes a cytochrome P450 enzyme that catalyzes the conversion of Trp to indole-3-acetaldoxime - a crucial precursor for indole glucosinolates. Both Trp and indole-3-acetaldoxime serve as metabolic intermediates for various bioactive compounds, including indole acids, glucosinolates, as well as medicinally important alkaloids such as IND and INR (Mikkelsen et al. 2000).

This research was carried out to investigate the effects of Trp applied at different concentrations to in vitro root cultures of *I. tinctoria* on the accumulation of IND and INR, and to determine the activities of the *It-TSA* and *CYP79B2* genes involved in their biosynthesis pathway. Thus, it will be possible to establish an effective protocol to produce IND and INR from endangered *I. tinctoria* without damaging their natural habitats and to elucidate the molecular mechanism between metabolite accumulation and the related genes.

Materials and methods

Seed sterilization and germination

In the sterilization step, the seeds of *I. tinctoria* were first immersed in 70% ethanol for 1 min and then rinsed with sterile distilled water. The seeds were then shaken in a 15% solution of commercial bleach containing 5% sodium hypochlorite supplemented with 1–2 drops of Tween 20 for 10 min for surface sterilization. Afterward, they were rinsed 5 times with sterile distilled water and prepared for planting. The surface-sterilized seeds were transferred to a solid Murashige and Skoog (MS) (Murashige and Skoog 1962) nutrient medium containing 30 g/L sucrose and 6 g/L agar and cultured in a growth chamber at 24 ± 1 °C with 16 h of light (at an intensity of 50 µmol/m²s) and 8 h of darkness (Gai et al. 2015). For germination, 2.400 seeds were used, evenly distributed across three replicates, with 800 seeds per replicate. The average germination rate of the seeds was determined as 64.5 ± 4.32%.

Initiation and propagation of root cultures

Following a 2-week germination period (Fig. 1A), approximately 0.3–0.5 g root segments from in vitro seedlings were carefully excised and transferred to 100 ml flasks containing 30 mL of liquid ½ MS nutrient medium with 20 g/L sucrose and cultured for 3 weeks under dark conditions in a climate

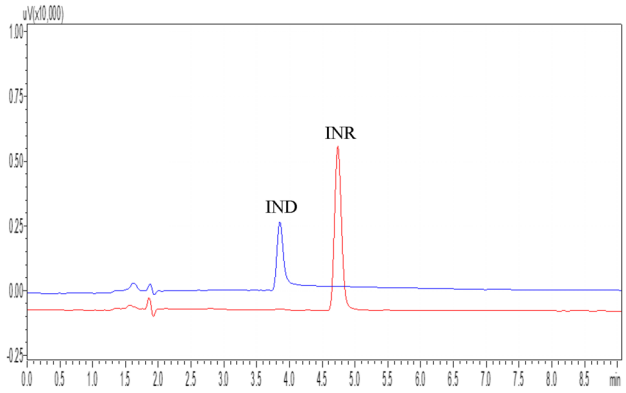


Fig. 2 HPLC chromatogram of IND (1) and INR (2)

chamber set to a temperature of 25 °C ± 1 with a shaking speed of 90 rpm. The roots were similarly subcultured twice in nutrient media to obtain enough for Trp applications (Fig. 1B).

Trp applications

For Trp applications, 0.3 g of roots were initially transferred into 100 ml flasks containing 30 ml of liquid ½ MS medium supplemented with 30 g/L sucrose. The roots were cultured for 3 weeks under dark conditions where the temperature was set to 25 ºC with shaking at 90 rpm. At the end of this period, a stock solution of Trp prepared with distilled water, sterilized by passing through a 0.22 µm sterile filter added to the nutrient media at concentrations of 100, 200, and 300 mg/L. Controls were given sterile distilled water in volumes matching experimental groups. The experiment was conducted using a randomized design with three replications, each consisting of five flasks. After the treatments, the roots were harvested 48 h later to determine the effects of Trp applications on both metabolite accumulation and gene activities. Following the harvest, the roots were washed with sterile distilled water, and excess water was absorbed using paper towels before being used for analysis.

Determination of IND and INR by HPLC

The extraction of root samples was made using the method of Cessur et al. (2023). For this purpose, approximately 0.5 g of root sample, completely dried and powdered, was subjected to maceration by adding 25 ml of 80% methanol and incubating at 50 °C for 15 h. Then, the samples were treated in an ultrasonic water bath at 50 °C for 25 min. After filtration, the samples were completely dried in a rotary evaporator at 45 °C and dissolved in HPLC grade methanol. Samples passed through a 0.45 µm filter were used for HPLC analysis.

Analyses of IND and INR were performed by HPLC (Shimadzu, Japan) with some modifications in the method used by Zhang et al. (2012). In this method, a 80% methanol-distilled water mixture was used as the mobile phase, with a flow rate of 1.2 mL/min and a column temperature set to 60 °C. Analyses were conducted using an Agilent-C18 (250 × 4.6 mm, 5 µm) column, and readings were taken at a wavelength of 289 nm using an HPLC/DA detector. The detection chromatogram was presented in Fig. 2. Using a calibration curve constructed with standards obtained from Sigma (Sigma-Adrich, St. Louis, MI, USA), IND ($y = 1701x + 62.92$, $r^2 = 0.998$) and INR ($y = 3661.7x + 2167.50$, $r^2 = 0.996$) amounts in samples were calculated as µg/g DW.

Gene expression analyses by RT-PCR

Total RNA isolation from roots harvested 48 h after Trp treatments was performed using PureZol (BioRAD, USA) solution according to the manufacturer's recommendations. To prevent genomic DNA contamination in the isolated RNA, DNase I treatment (RNAse-Free DNase Set, QIAGEN) was applied following the manufacturer's instructions. The RNA quality and quantity were evaluated by performing 1% agarose gel electrophoresis and measuring with a NanoDrop-2000 spectrophotometer (Thermo Scientific, Germany). cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis Kit (BioRAD, USA) by reverse transcription (RT) according to the manufacturer's protocol. The expression levels of *It-TSA* (Salvini et al. 2008) and *CYP79B2* (Thomas and Kropp 2009), genes related with the biosynthesis of IND and INR, were detected by RT-PCR using gene-specific primers (Table 1). The amplification of cDNAs was carried out in a 10 µL reaction mixture consisting of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA), 100 ng of cDNA template, and 10 µM of each gene-specific primer. RT-PCR was performed in a CFX Connect Real-Time System (Bio-Rad,

Table 1 Primer nucleotide sequences used in RT-PCR

Genes	Accession number (Gen Bank)	Forward and reverse primer sequences
It-TSA (*Trp synthase alpha*)	AJ841705.1	F: 5"- GTCCAGTTTCGCTGTTCACG-3' R: 5" CCACAAGTCCCTGTACACCA"3
CYP79B2(Cytochrome p450)	EU684741.1	F: 5'-CCGCCGATGAAATCAAACCC-3' R: 5'-TTTGTTCACCATCTCCGCCA-3'
β-actin	AY870652.1	F: 5'-' ACTGGAATGGTGAAGGCTGG-3' R: 5'-' TCTGACCCATCCAAACCGTG-3'

USA). The RT-PCR was run at 95 °C (30 s); 40 cycles at 95 °C (10 s), 55.7 °C (10 s), and melting curve. In the study, β-actin served as the reference gene to normalize changes in gene expression. The normalized gene expression levels were calculated relative to the control group using the comparative CT method ($2^{-\Delta\Delta Ct}$) as described by Livak and Schmittgen (2001) in the CFX Manager TM software (Bio-Rad, USA). RNA isolation was conducted in triplicate for each treatment, and RT-PCR reactions were performed in duplicate.

Statistical analysis

All statistical analyses were performed with the use of JMP software version 17 (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was conducted at $p \leq 0.05$ based on Tukey test to determine significant differences between the treatments. Box plots graphs were made using ggplot2 (v3.3.3) in R Studio (R Studio Team, 2023; R Core Team, 2023, Vienna, Austria).

Results

Effects of Trp applications on the accumulation of IND and INR

The application of Trp at different concentrations to the in vitro roots of *I. tinctoria* significantly changed the accumulation of IND and INR ($p \leq 0.05$) (Fig. 3). At a concentration of 100 mg/L, Trp did not cause a significant difference in IND accumulation compared to the control. However, as the concentration increased to 200 and 300 mg/L, IND accumulation was found to rise compared to the control. The highest IND content was received from roots treated with 200 mg/L Trp, reaching 851.81 µg/g, while the accumulation of IND decreased when the concentration was raised to 300 mg/L (Fig. 3A). In *I. tinctoria* roots treated with Trp, INR accumulation followed a similar trend to IND. The 100 mg/L application did not have a significant effect on INR accumulation compared to the control. However, with 200 mg/L Trp treatment, INR accumulation increased five-fold compared to the control, reaching 11.67 µg/g. The application of 300 mg/L Trp significantly increased INR accumulation more than control but resulted in lower INR accumulation than the 200 mg/L application (Fig. 3B). These results indicate that Trp applications have a dose-dependent effect in *I. tinctoria* root cultures, with 200 mg/L being the optimal concentration for both IND and INR.

Effects of Trp applications on the gene expression levels

The activities of the *It-TSA* and *CYP79B2* genes, which are involved in the biosynthetic pathway of indole alkaloids, were also determined by RT-PCR in *I. tinctoria* roots treated with different concentrations of Trp. It was found that Trp applications significantly increased the expression levels of these genes (Fig. 4). The highest *It-TSA* gene activity was obtained from the 200 mg/L Trp treatment, followed by 300 mg/L and 100 mg/L Trp applications, respectively (Fig. 4A). The activity of another gene involved in indole alkaloid biosynthesis, *CYP79B2*, also showed a significant increase compared to the control with Trp treatments. The highest levels of *CYP79B2* gene activity were reached with 100 and 200 mg/L Trp applications, while a decrease in gene activity was observed when the Trp concentration was increased to 300 mg/L (Fig. 4B). These results indicate that

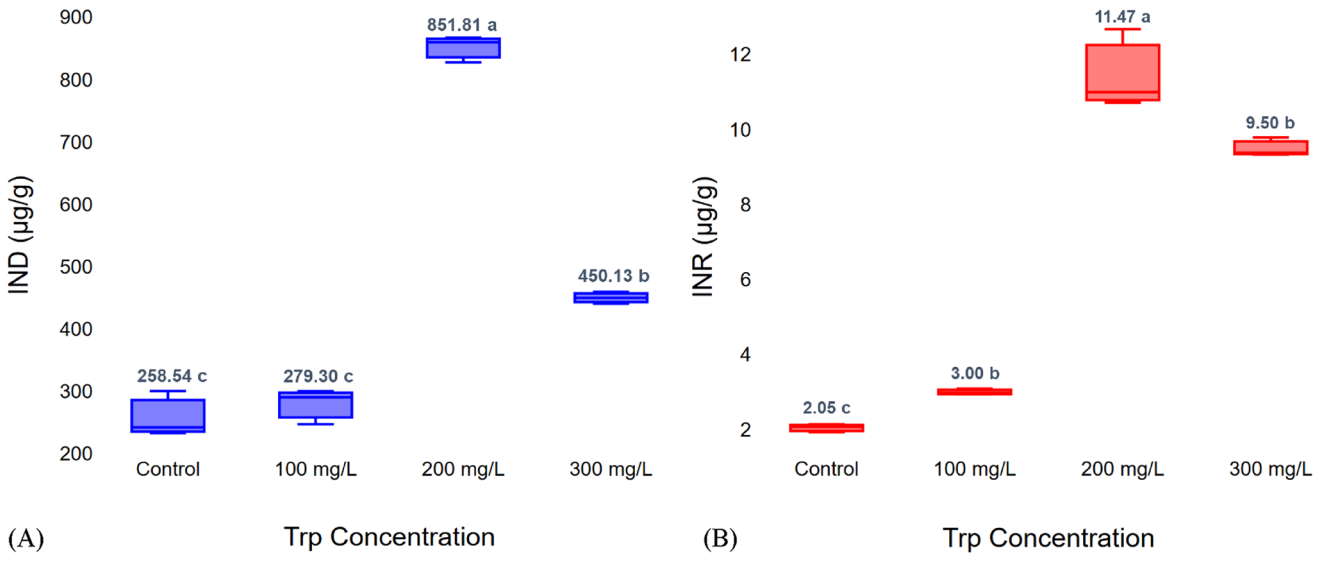


Fig. 3 Effects of Trp applications on the accumulation of IND (**A**) and INR (**B**) in *I. tinctoria* roots

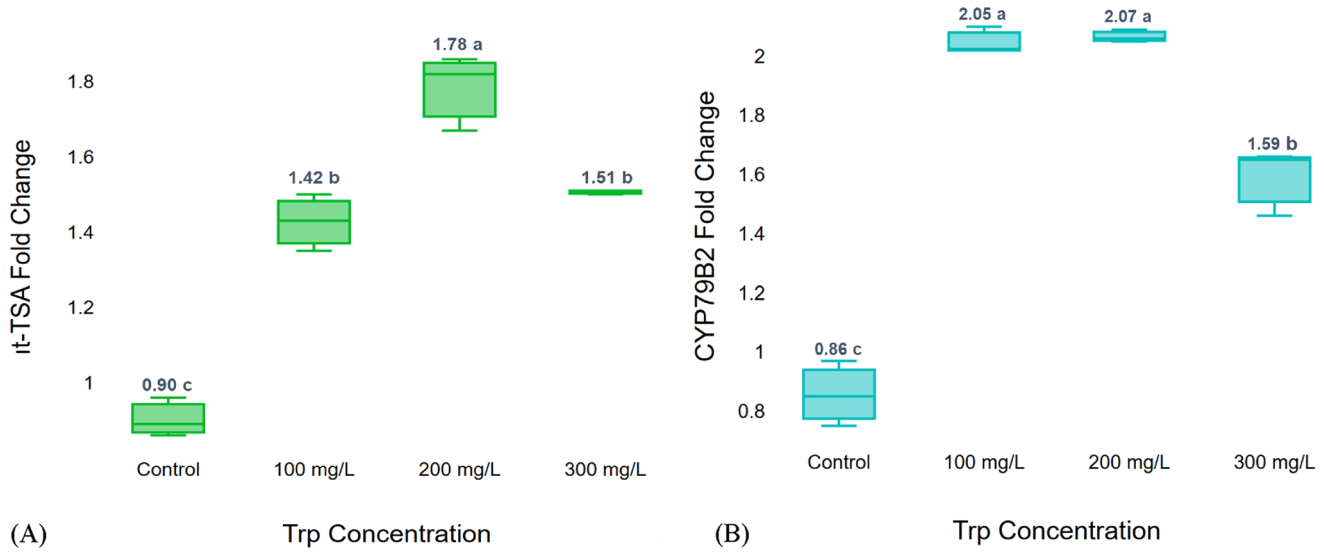


Fig. 4 Effects of Trp applications on the expressing of*It-TSA* (**A**) and *CYP79B2* (**B**) in *I. tinctoria* roots

Trp applications, which are precursors in the biosynthesis of indole alkaloids, significantly increased the expression of the *It-TSA* and *CYP79B2* genes in *I. tinctoria* roots, but this increase changing depending on the concentrations.

Discussion

This study explored, for the first time, the effects of exogenous tryptophan application on the production of two pharmacologically significant indole-derived compounds, IND and INR, in *I. tinctoria* root cultures. The primary objective was to develop a controlled and reproducible secondary metabolite production system for generating root-derived secondary metabolites in *I. tinctoria*. In addition to assessing metabolite accumulation, the research investigated the expression levels of *It-TSA* and *CYP79B2* genes, key players in the indole alkaloid biosynthesis pathway, to uncover the metabolic regulation mechanisms triggered by Trp application. By examining how Trp feeding influenced both metabolic flux and gene expression, this research provided valuable insights into the regulatory mechanisms of plant secondary metabolism and presented a novel strategy for metabolic engineering in plants.

Trp, an essential amino acid involved in protein synthesis, is also a precursor compound responsible for the synthesis of various groups of secondary metabolites in plants (Ishihara et al. 2011). Similarly, previous studies demonstrated that when applied to in vitro cultures at appropriate concentrations Trp increased the alkaloid production in as an effective precursor. In one of the studies, Krishnan et al. (2023) reported that the application of 1 mM and 2 mM Trp significantly increased the accumulation of camptothecin, a quinoline alkaloid, in *Nothapodytes nimmoniana* cell suspension cultures by 6.26-fold and 9.43-fold, respectively, compared to the control. Similarly, Sing et al. (2017) found that Trp supplementation enhanced the accumulation of pyrroloquinazoline alkaloids in *Adhatoda vasica* cell cultures. This research is the first study to investigate the effects of Trp applications on secondary metabolite production in *I. tinctoria* root cultures. In this study, IND and INR accumulation was found to vary depending on Trp concentration, with the highest alkaloid yield in *I. tinctoria* root cultures observed at 200 mg/L Trp treatment. Similarly, Jassim and Ameen (2014) reported that among 200, 300, and 400 mg/L Trp applications in *Catharanthus roseus* callus cultures, the highest vincristine accumulation occurred at 200 mg/L. Supporting this dose-dependent effect of Trp on alkaloid accumulation, Majumder (2012) found that among 100, 250, and 500 mg/L Trp treatments in *Podophyllum hexandrum* cell suspension cultures, the 250 mg/L treatment resulted in the highest podophyllotoxin accumulation, increasing levels by 2.7-fold compared to the control. Additionally, in *Catharanthus roseus* callus cultures, 175 mg/L Trp significantly enhanced catharanthine alkaloid accumulation compared to 200 and 225 mg/L treatments (Rachmi et al. 2006). Precursor applications at high concentrations can induce feedback inhibition in secondary metabolite biosynthetic pathways, ultimately reducing metabolite levels (Ouyang et al. 2005; Liu et al. 2007). In line with this, the present study found that increasing the Trp concentration to 300 mg/L led to a significant decline in IND and INR accumulation. These findings highlight that dosage is a crucial factor in Trp applications. Indeed, when applied at appropriate concentrations, Trp has been shown to significantly enhance the accumulation of key alkaloids, including reserpine in *Rauvolfia tetraphylla*

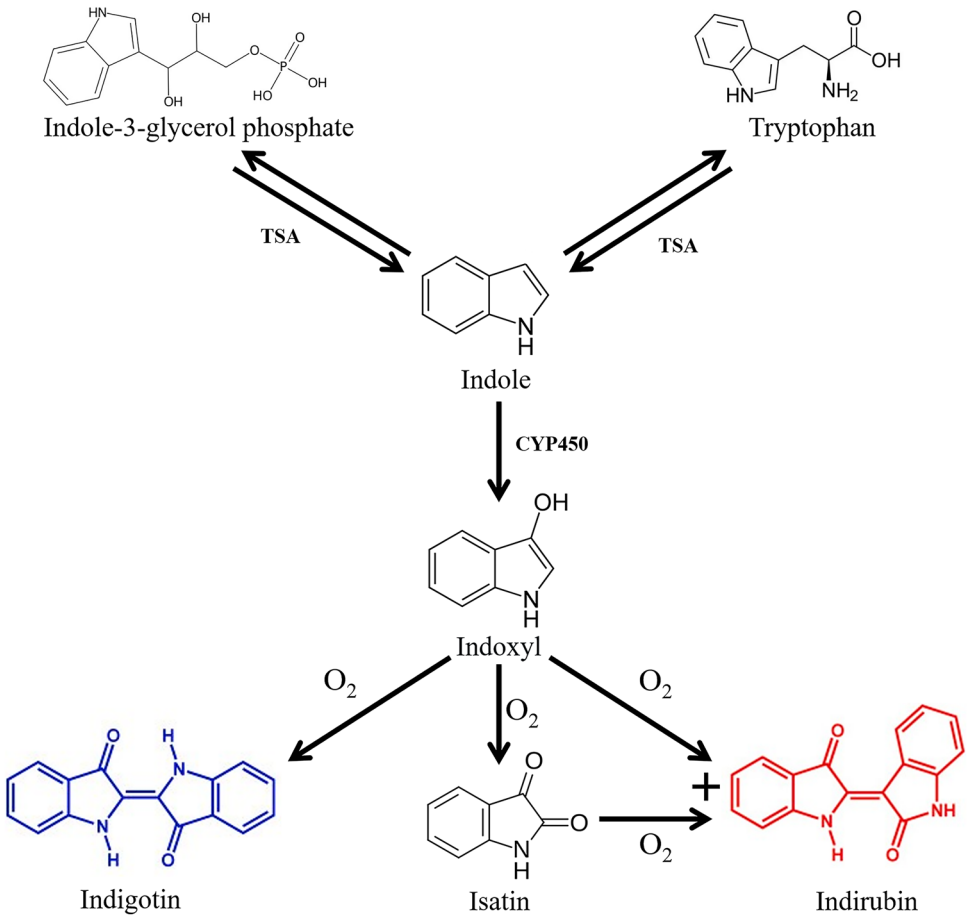


Fig. 5 Diagram of IND and INR biosynthesis pathway (Wang et al. 2019)

L. (Anitha and Ranjitha 2006; Rohela et al. 2021), mitragynine in *Mitragyna speciosa* (Mohamad Zuldin et al. 2013), vincamine in *Vinca minor* (Verma et al. 2014), and camptothecin in *Camptotheca acuminata* (Noé et al. 1984; De Luca et al. 1989; López-Meyer and Nessler 1997).

This study also investigated the effects of Trp treatments on the expression levels of *It-TSA* and *CYP79B2* genes, which play key roles in the IND and INR biosynthesis pathways in *I. tinctoria* roots.

The *TSA* gene encodes the enzyme responsible for converting indole-3-glycerol phosphate (IGP) into indole, and it also regulates the interconversion processes among IGP, tryptophan, and indole. Genes belonging to the cytochrome P450 monooxygenase family, of which *CYP79B2* is a member, encode enzymes that catalyze the conversion of indole to indoxyl. (Wang et al. 2019) (Fig. 5). IND and INR are alkaloids derived from indole, and the biosynthesis of indole directly influences the accumulation of these compounds. The synthesis of indole alkaloids is a process that begins with Trp (Xiao et al. 2023) and occurs through a three-step biosynthetic pathway. In the first step, Trp is oxidized by the enzyme Trpase to form indole. In the second step, indole undergoes further oxidation by heterologous oxygenases, producing intermediates such as 3-hydroxyindoxyl, isatin, or 2-oxindole. Finally, in the third step, two molecules of indoxyl combine with oxygen to form IND, while INR is synthesized through the condensation of indoxyl with 2-oxindole or isatin (Zhang et al. 2014).

In this study, Trp treatments significantly increased the expression levels of *It-TSA* and *CYP79B2* genes depending on its concentrations. The highest gene activity was observed with 200 mg/L Trp application, while an increase in concentration to 300 mg/L led to a decrease in gene activity. In one of the few studies examining the expression levels of genes related to secondary metabolism, it was found that the application of 300 and 500 mg/L Trp to *Catharanthus roseus* shoot cultures resulted in a significant increase in the expression of the *CrSGD* and *CrPRX1* genes, which are responsible for triterpene indole alkaloid biosynthesis. However, when the application concentration was increased to 1000 mg/L, gene expression levels decreased (Sharma et al. 2019). These results are in strong agreement with the findings of this study, which also showed that the effect of Trp applications on gene activity varies dose-dependently, with high Trp concentrations causing a reduction in gene activity.

Precursor compounds, when used in combination with other signaling molecules, can also affect the expression of

genes related with secondary metabolite synthesis. Jiao et al. (2022) found that applying the indole alkaloid precursors Trp and secologanin along with MeJA to protocorm-like bodies of *Dendrobium officinale* significantly upregulated the expression of *DoDXS*, *DoSTR*, *DoSLS*, and *DoTDC* involved in alkaloid biosynthesis, compared to the control.

Plant secondary metabolism is regulated by multiple genes and their encoded enzymes, which catalyze biochemical reactions (Shuilin et al. 2002). Variations in the expression of key biosynthetic enzyme genes directly affect metabolite production, playing a crucial role in pathway regulation (Vom Endt et al. 2002). Sivanandhan et al. (2014) also stated that precursor compound applications are one of the approaches used to overexpress genes involved in secondary biosynthesis pathways. Similarly in this study, a positive relationship was found between accumulations of IND and INR and the activities of *It-TSA* and *CYP79B2* genes, with increased gene expression leading to higher accumulation of IND and INR. Based on the findings of this study and existing literature, it has been demonstrated that Trp plays a significant role in regulating secondary metabolite biosynthesis and gene expression, offering substantial potential for biotechnological applications. Based on these findings and existing literature, it was demonstrated that Trp regulates secondary metabolism and gene expression, making it a promising tool for biotechnological applications.

Conclusion

This study investigated the effects of Trp applications on IND and INR accumulation, as well as on the expression levels of *It-TSA* and *CYP79B2* genes involved in their biosynthesis, in *I. tinctoria* root cultures. The 200 mg/L Trp treatment resulted in the highest metabolite accumulation and gene expression levels. A positive correlation between gene expression and metabolite levels suggested that Trp is an effective precursor for indole alkaloid production in *I. tinctoria.* Comprehensive literature reviews revealed no prior studies on Trp-derived alkaloid production or related molecular mechanisms in *Isatis tinctoria* roots. As the first study of its kind, this research significantly contributed to the development of strategies for secondary metabolite production in plant biotechnology and highlighted the need for further investigations in this field.

Author contributions AC: Conceptualization, Methodology, HPLC analysis, Gene expression analysis, Writing– original draft. UT: Methodology, HPLC analysis, Gene expression analyses, Writing. IA: Methodology, HPLC analysis, Gene expression analyses. NGB: Conceptualization, Design, Formal analysis, Data curation, Writing–review & editing, Supervision. All authors contributed to the writing and approved the final version of the manuscript.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

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