Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Endophyte-Mediated Elicitation Enhances Picrosides Production in Suspension Cultures of *Picrorhiza kurroa* Royle ex Benth

Mahinder Partap
Institute of Himalayan Bioresource Technology
Ankita Thakur
Institute of Himalayan Bioresource Technology
Amit Kumar
Institute of Himalayan Bioresource Technology
Anish Tamang
Institute of Himalayan Bioresource Technology
Shiv Shanker Pandey
Institute of Himalayan Bioresource Technology
Ashish R. Warghat
ashish.warghat@csir.res.in

Institute of Himalayan Bioresource Technology

Research Article

Keywords: Picrorhiza kurroa, Endophytic fungi, Picrosides, Cell suspension culture, Secondary metabolite elicitation

Posted Date: November 2nd, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11232992/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

In the present investigation, three endophytic fungi isolated from rhizome tissues of wild *Picrorhiza kurroa Trichoderma* sp. (EE1), *Fusarium acuminatum* (EE2), and *Fusarium* sp. (EE3) were used to modulate picroside (P-I, P-II, P-III) biosynthesis in leaf- and root-derived cell suspension cultures. ITS-based molecular identification confirmed the taxonomic affiliation of the isolates, and mycelial extracts were applied as elicitors at 0.5–1.5 ml/L for up to 192 h. All three elicitors triggered concentration- and tissue-dependent shifts in metabolite accumulation, with pronounced stimulation of picroside accumulation and precursor biosynthesis under specific treatment regimes. EE1 elicitation produced moderate increases in picrosides, with maximal accumulation of picroside I and II at 1.5 ml/L in both leaf- and root-derived cultures. In contrast, picroside III peaked at 1.0 ml/L in leaf-derived cell cultures. EE2 exhibited the strongest elicitation potential, markedly enhancing metabolite production at 1.5 ml/L, including picroside I (4.71 mg/g DW), picroside II (3.93 mg/g DW), picroside III (0.48 mg/g DW), aucubin (0.4 mg/g DW), and catalpol (4.0 mg/g DW) in leaf-derived suspension cultures. In contrast, EE3 induced comparatively lower picroside accumulation but preferentially stimulated precursor metabolites, including catalpol, vanillic acid, and tissue-specific accumulation of caffeic and cinnamic acids. Vanillic acid was detected across all treatments, while p-hydroxybenzoic acid and 4-hydroxyacetophenone further indicated endophyte-driven remodelling of secondary metabolism. Collectively, these findings demonstrate that endophytic fungal elicitors exert distinct, dose-dependent, and tissue-specific effects on specialized metabolite biosynthesis in *P. kurroa*, identifying *F. acuminatum* (EE2) elicitation as a potential approach for enhancing picroside accumulation *in vitro*.

Introduction

In medicinal plants, the accumulation of secondary metabolites is often minimal or present only in trace amounts. This accumulation is influenced by various factors, including habitat, plant source, tissue type, developmental stage, physiological status, interactions with endophytes, stress-response mechanisms, and environmental conditions (Li et al. 2020). To address the challenge of low metabolite levels, researchers have relied on elicitor application to induce secondary metabolite production in tissue culture (Kumar et al. 2025a,b; 2026). Among these strategies, the use of endophytic fungal elicitors has gained prominence, as they have been shown to significantly boost the production of desired phytochemicals in medicinal plants (Gomes et al. 2018; Thakur et al. 2024).

Much of the prior literature has reported the effectiveness of fungal elicitors in increasing bioactive content production using *in vitro* culture techniques (Tamang et al. 2023). During plant-fungal associations, fungi secrete elicitor molecules that stimulate the host plant to produce phytochemicals, thereby enhancing their production. The process begins with an elicitor binding to receptors on the plant cell wall, triggering downstream signaling cascades. This activation involves the expression of structural and regulatory genes, transcription factors, and biosynthetic pathways (Bhaskar et al. 2022). A key initial response to elicitor binding is the alteration of ionic fluxes of Cl^-, K^+, H^+, and Ca^{2+}, leading to cytoplasmic acidification (Guru et al. 2022). This pH change affects various physiological responses within the cell,

including salicylic acid (SA), jasmonic acid (JA), ethylene, and calcium-dependent protein kinase pathways. These pathways, in turn, activate stress-associated genes, enhancing secondary metabolite production. Additionally, altered pH triggers NADPH oxidase activity and the production of reactive oxygen species (ROS), which further stimulate stress-associated gene expression (Nabi et al. 2021; Guru et al. 2022). This cascade activates transcription factors (TFs) that bind DNA, driving gene transcription and translation, ultimately boosting secondary metabolite production. In another pathway, mitogen-activated protein kinases (MAPKs) are activated, leading to the phosphorylation of TFs, which further enhance the production of JA, SA, and secondary metabolites, contributing to plant defense (Saijo et al. 2018; Lu et al. 2021).

Numerous examples show that fungal elicitation significantly increases phytochemical production in various medicinal plants. For instance, Li et al. (2011) observed increased diosgenin accumulation in *Dioscorea zingiberensis* suspension culture when *Fusarium oxysporum* was used as an elicitor. Likewise, the content of artemisinin in *Artemisia annua* was increased using an elicitor from *Colletotrichum spp.* (Wang et al. 2001). *Pythium aphanidermatum* was reported to stimulate the production of 4-hydroxybenzoic acid in *Daucus carota* cell culture (Schnitzler et al. 1992). Tonk et al. (2016) demonstrated that *Aspergillus flavus* positively influenced vinblastine and vincristine production in *Catharanthus roseus*. *Trichoderma harzianum* has been found to enhance asiaticoside content in shoot cultures of *Centella asiatica* L (Prasad et al. 2013). *Fusarium oxysporum* Schlecht (F9) has been observed to elevate alkaloid synthesis in cell suspension cultures of *C. roseus* (Tang et al. 2011). In hairy root cultures of *Salvia miltiorrhiza*, elicitation with *Trichoderma atroviride* D16 stimulated hairy root and tanshinone production (Ming et al. 2013). Fungal elicitors such as *F. oxysporum*, *Phoma exigua*, and *Botrytis cinerea* have been used to induce *Hypericum perforatum* cell suspensions, thereby scaling up the production of naphthodianthrones for medical applications (Gadzovska Simic et al. 2015).

Picrorhiza kurroa Royle ex Benth., a rhizomatous herb widely distributed in the Himalayan region at altitudes of 3,000–4,500 meters (Partap et al. 2020; 2022a). The pharmacological significance of *P. kurroa* is due to the presence of bioactive compounds, particularly picrosides (P-I, P-II, P-III) (Partap et al. 2022a,b). *P. kurroa* possesses many therapeutic properties, such as choleretic, anti-asthmatic, anti-cancerous, anti-malarial, and anti-liver disorder (Shitiz et al. 2013; Mehta et al. 2021) and is commercially available in various drug formulations. However, due to its high market demand and excessive wild harvesting, *P. kurroa* has been listed as an endangered species (Sharma et al. 2015). Overharvesting necessitated the development of novel conservation strategies to ensure the availability of high-quality raw materials and a reliable supply of valuable secondary metabolites. Numerous methods have been explored for the cultivation, conservation, and generation of desired bioactive compounds from *P. kurroa*. Among these, micropropagation of *P. kurroa* through shoot tissue is a robust approach for its large-scale production. More recently, a plant de-differentiated cell culture approach has also been used for the *in vitro* production of desired metabolites (Partap et al. 2020; 2022; 2023; 2025a,b). However, the *in vitro*-generated plants and de-differentiated cell culture systems often show reduced levels of therapeutically important secondary metabolites compared to their wild counterparts, a phenomenon not yet fully understood. Thus, the current investigation aims to explore the fungal endophytes associated with the

rhizomes of wild *P. kurroa* plants and their role in the plants' secondary metabolite biosynthesis. Thus, the current investigation is the first to demonstrate the role of endophytes in the production of picrosides and their precursor compounds in plant cells, offering novel insights into interactions between this medicinal plant and its beneficial endophytic microbial community.

Materials and methods

Collection of plant material

Wild specimens of *P. kurroa* Royle ex Benth. were collected from Rohtang Pass, Himachal Pradesh, at an elevation of 3992 meters. The collected plants were placed in sterile bags. Afterwards, the collected plants were transported to the CSIR-IHBT in Palampur for further processing. The plant material was authenticated by submitting a herbarium specimen to the Environment Technology Division, CSIR-IHBT, Palampur and was assigned accession number PLP16692 (Partap et al. 2022b). Following authentication, the plants were maintained under controlled conditions in the CSIR-IHBT growth chamber facility. Rhizomes from selected plants were excised under sterile conditions to isolate endophytic fungi, which were used in subsequent experiments.

Surface sterilization for isolation of endophytic fungi

Endophytic fungi were isolated from the rhizome tissues of the wild *P. kurroa* plants. The rhizome tissues underwent surface sterilization (Pandey et al. 2016; Nascimento et al. 2019). Initially, the rhizomes were rinsed with distilled water to eliminate surface contaminants. The tissues were then surface sterilized in 1% sodium hypochlorite solution (HiMedia, India) for 5 min, followed by 70% ethanol (Changshu Hongsheng Fine Chemicals, China) for 2 min. After sterilization, the rhizome tissues were washed five times with sterile distilled water and air-dried. To ensure sterility, the final rinse water was spread on agar plates and incubated at 28°C. Any microbial growth would indicate contamination.

Cultivation of endophytic fungi

Sterilized rhizome tissues were aseptically cut into 0.5 cm pieces. These tissue parts were cultured on potato dextrose agar (PDA) plates (HiMedia, India) augmented with (50 µg/mL) of streptomycin and (15 µg/mL) of chloramphenicol (HiMedia, India) to inhibit bacterial growth and promote fungal isolation (Pandey et al. 2016; Jain et al. 2021). Followed by incubation at 28°C for 7 days. During this incubation period, distinct fungal morphotypes were observed, isolated, and sub-cultured to obtain pure cultures. These purified fungal cultures were then preserved at -80°C in glycerol stocks for long-term storage.

Molecular characterizations of fungal isolates

Molecular characterizations of the isolated fungal endophytes were performed by extracting genomic DNA using the CTAB (cetyltrimethylammonium bromide) method, as reported by Sambrook et al. (1989). The primers used for ITS amplification were ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCG CTTATTGATATGC-3') (White et al. 1990). 100 nanograms of genomic DNA were used, and a PCR reaction was performed using GoTaq® master mix (Promega, USA) in a ProFlex™ thermal cycler. The PCR steps are as follows: Initial denaturation at 94ºC for 5 min, denaturation at 94ºC for 1 min, annealing at 55 ºC for 30 s, extension at 72ºC for 1 min, and a final extension at 72 ºC for 5 min. The amplified products were purified using ExoSAP-IT™ PCR Clean-Up Reagent (Applied Biosystems, USA) and subjected to Sanger sequencing. Sequencing PCR was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), and the sequencing reactions were analyzed on a 3730xl DNA Analyzer (Applied Biosystems, USA).

Fungal culture and elicitor preparation

Endophytic fungi were initially cultured on PDA medium for 7 days. Following this incubation period, the fungal cultures were carefully extracted by punching out the mycelial mats, which were then transferred into 250-mL flasks encompassing 100 mL of Potato Dextrose Broth (HiMedia, India). The flasks were incubated under static conditions at 28°C for 10 days for optimal growth. The preparation of the elicitor followed the protocol of Kushwaha et al. (2019) and Thakur et al. (2024), with minor alterations (Fig. 1). After the fungi reached maximum growth, the mycelia were detached from the culture filtrate using sterile filter paper (Filterking, India). The separated mycelia were rinsed two times with autoclaved distilled water to eliminate any remaining culture medium. The washed mycelia were then partially dried using sterile blotting paper. The partially dried mycelia were pulverized into a fine powder using liquid nitrogen and suspended in their respective filtered culture filtrates, then autoclaved at 15 psi and 121°C for 20 minutes, as described by Savitha et al. (2006). After autoclaving, the mixture was allowed to cool, followed by centrifugations at 5000 rpm for 10 min to separate the supernatant (Eppendorf, Germany). Finally, the supernatant was sterilized using a 0.22 µm syringe filter. Filtered supernatant was ready for treatment in the *P. kurroa* cell culture.

Maintenance of cell suspension cultures

As previously reported by Partap et al. (2020; 2022a,b), cell suspension cultures were established from differentiated callus cultures derived from leaf and rhizome tissue of wild *P. kurroa* plants. The callus cultures, which were one month old, served as the starting material for initiating rhizome suspension cultures (RHSC) and leaf suspension cultures (LFSC). These suspension cultures were maintained under optimized conditions and served as mother cultures for further experimentation (Partap et al. 2020; 2022a,b). The culture conditions were MS medium (Murashige and Skoog, 1962), fortified with 3% sucrose, 0.5 mg/L thidiazuron (TDZ) (HiMedia, India), and 0.3 mg/L indole-3-butyric acid (IBA) (HiMedia, India). The cultures were kept under the dark at 25°C. Key parameters for suspension culture growth, as established in Partap et al. (2022a), included an initial inoculum density of 3 g of cells per 75 mL of

medium, a pH of 5.8, and orbital shaking at 80 rpm. Cell suspension cultures were regularly subcultured by pouring at 4-week intervals to maintain culture vitality. Only mother cultures younger than 2nd months were used in subsequent experiments, as cell viability was observed to decline progressively from the 2nd to the 12th month of culture (Partap et al. 2022a). Therefore, all experiments were conducted using cell suspension cultures no older than 2 months.

Elicitor concentrations and exposure duration

Initial screening investigations were conducted using various concentrations (ml/L) of EE1 (0.25, 0.5, 1.0, 1.5, and 2.0), EE2 (0.25, 0.5, 1.0, 1.5 and 2.0), and EE3 (0.25, 0.5, 1.0, 1.5 and 2.0). Two separate experiments were designed to screen the elicitor concentrations and exposure duration. In the first experiment, all the filter-sterilized elicitor concentrations were added separately to the cooled, autoclaved liquid MS medium (pH adjusted to 5.8) fortified with sucrose (3%), TDZ (0.5 mg/L), and IBA (0.3 mg/L). After that, 3 g of cell biomass from the stock culture flask was inoculated into each flask containing 75 mL of liquid medium augmented with various elicitor concentrations. Subsequently, the cultures were kept on a shaker in the dark at 25°C for up to 28 days. In the second experiment, when cultures were at their maximum growth index, i.e., on the 21st day (Partap et al. 2022a,b), the LFSC and RHSC were treated with filter-sterilized different concentrations of elicitors as mentioned above. Afterwards, the elicitor-treated suspension cultures (LFSC and RHSC) were observed for up to 192 h of the culture period under optimized conditions. Based on morphological observations and the growth index of LFSC and RHSC, elicitor concentrations and exposure duration were further selected for treatments (Partap et al. 2022a,b).

Elicitor treatments

After screening elicitor concentrations and exposure durations, a further investigation was undertaken to determine the effects of different elicitors and concentrations on metabolite content. In brief, on the 21st day of the culture period (maximum GI), the suspension cultures of rhizome and leaf were treated with filter-sterilized elicitors at different concentrations. The EE1, EE2, and EE3 were used in the concentrations of 0.5 ml/L, 1.0 ml/L, and 1.5 ml/L respectively. The elicitor-treated suspension cultures were harvested at 12, 24, 48, 96, and 192 h for each concentration, in triplicate. According to our previous report by Partap et al. (2022a) on the cell suspension culture of *P. kurroa*, the cell suspension exhibits a sigmoidal growth curve with lag, log, and stationary phases (from 28 days onwards) under optimized parameters. Therefore, we selected 192 hours as the elicitor exposure time after 21 days of culture in the present investigation.

Secondary metabolite analysis

Harvested calli were dried in an oven at 37°C for a week to remove moisture and determine dry biomass. Furthermore, the dried calli were used to extract metabolites (Partap et al. 2022a,b). UPLC-PDA-ESI-

QTOF-MS was employed for both qualitative and quantitative analyses of 14 targeted compounds, following the method described by Partap et al. (2022b).

Statistical analysis

All data were statistically analysed using SPSS software. The analysis presents results as the mean ± standard error (SE) of three replicates per experiment. Differences among treatments were evaluated by one-way ANOVA followed by Duncan's and Dunnett's post hoc tests at $P \leq 0.05$.

Results

Isolation of endophytic fungus and molecular identification

From the collected rhizome tissues of wild *P. kurroa* plants, four distinct fungal colonies were successfully isolated. Among these, three isolates (EE1, EE2, and EE3) were further cultured and maintained as pure cultures. For molecular identification, genomic DNA was extracted, and ITS-based sequencing was performed. The fungal isolates were identified as follows: EE1 was classified as *Trichoderma* sp., EE2 as *Fusarium acuminatum*, and EE3 as *Fusarium* sp. The obtained nucleotide sequences were subsequently deposited in the GenBank database, and accession numbers were assigned to each isolate (Table 1).

Table 1
Molecular identification of the endophytes isolated from the rhizome tissue of *P. kurroa*

S/L No	Isolate name	Tissue source	Scientific name	Accession number	Class	Phylum
1	EE1 (PKRF1)	Rhizome	*Trichoderma harzianum*	PV528759	Sordariomycetes	Ascomycota
2	EE2 (PKRF2)	Rhizome	*Fusarium acuminatum*	PV528760	Sordariomycetes	Ascomycota
3	EE3 (PKRF3)	Rhizome	*Fusarium tricinctum*	PV528761	Sordariomycetes	Ascomycota

Screening of concentrations

In the first experiment, endophytic elicitors were introduced into the liquid culture medium at zero days with optimized cell inoculum density and incubated under standardized conditions. After the 14th day of elicitor treatment, the colour of the cell biomass and suspension of the LFSC and RHSC changed to brown, particularly at higher doses. In the second experiment, the elicitors were introduced into the liquid culture medium when the LFSC and RHSC achieved their maximum cell biomass, i.e., on the 21st day, and incubated under optimized conditions. The results revealed that no browning was observed in the cells or suspension of LFSC and RHSC treated with elicitors up to 192 h after the 21st day. However, slight browning and reduced growth were observed during longer exposure and at higher concentrations.

From the results, it was optimized that for further experiments, elicitors were introduced into the culture medium (LFSC and RHSC) on the 21st day. In addition, the exposure duration of elicitors was set to 192 h, i.e., up to 192 h after elicitor administration on the 21st day.

Effect of EE1 endophyte on metabolite content

The picrosides and their precursor metabolite content have been quantified in the LFSC and RHSC of *P. kurroa* cell culture at different endophyte concentrations over a culture period of up to 192 hr. P-I content in LFSC has been found in the range of 1.69 to 2.37 mg/g DW (EE1; 0.5 ml/L), 1.17 to 2.86 mg/g DW (EE1; 1 ml/L), and 1.89 to 2.89 mg/g DW (EE1; 1.5 ml/L). P-II content in LFSC has been found in the range of 1.43 to 1.59 mg/g DW (EE1; 0.5 ml/L), 1.86 to 1.93 mg/g DW (EE1; 1 ml/L), and 1.88 to 2.17 mg/g DW (EE1; 1.5 ml/L). P-III content in LFSC has been found in the range of 0.12 to 0.14 mg/g DW (EE1; 0.5 ml/L), 0.15 to 0.40 mg/g DW (EE1; 1 ml/L), and 0.15 to 0.35 mg/g DW (EE1; 1.5 ml/L) (Fig. 2a). P-I content in RHSC has been found in the range of 1.33 to 2.97 mg/g DW (EE1; 0.5 ml/L), 1.15 to 2.98 mg/g DW (EE1; 1 ml/L), and 2.14 to 2.84 mg/g DW (EE1; 1.5 ml/L). P-II content in RHSC has been found in the range of 1.40 to 1.72 mg/g DW (EE1; 0.5 ml/L), 1.33 to 1.63 mg/g DW (EE1; 1 ml/L), and 1.13 to 1.73 mg/g DW (EE1; 1.5 ml/L). P-III content in RHSC has been found in the range of 0.21 to 0.28 mg/g DW (EE1; 0.5 ml/L), 0.24 to 0.38 mg/g DW (EE1; 1 ml/L), and 0.23 to 0.39 mg/g DW (EE1; 1.5 ml/L) concentration (Fig. 2c). The results revealed that the maximum content of P-I and P-II in LFSC was recorded in (EE1; 1.5 ml/L) concentration, and P-III was found in (EE1; 1 ml/L) concentration. The study also reported that the highest content of P-I in RHSC was observed in the (EE1; 1 ml/L) concentration, while P-II and P-III were found in the (EE1; 1.5 ml/L) concentration. In LFSC, the maximum content of vanillic acid (0.1 mg/g DW) was detected in all three concentrations, while caffeic acid and cinnamic acid were not detected. The highest aucubin content (0.2 mg/g DW) was found at the EE1 concentration (1.5 ml/L). The highest catalpol content (2.7 mg/g DW) was found in (EE1; 0.5 ml/L) and (EE1; 1.5 ml/L) concentrations (Fig. 2b). In RHSC, the maximum content of vanillic acid (0.10 mg/g DW) was detected in all three concentrations, while caffeic acid (0.2 mg/g DW) and aucubin (0.24 mg/g DW) were detected in (EE1; 0.5 ml/L) concentration. The maximum content of cinnamic acid (0.05 mg/g DW) was found in (EE1; 1 ml/L) and (EE1; 1.5 ml/L) concentrations. The maximum content of catalpol (3.68 mg/g DW) was found in (EE1; 1.5 ml/L) concentration (Fig. 2d). The p-HA and 4-HAG have also been detected in LFSC and RHSC under different EE1 endophyte concentrations (Supplementary Fig. S1).

Effect of endophyte EE2 on metabolite content

The picrosides and their precursor metabolite content have been quantified in the LFSC and RHSC of *P. kurroa* cell culture at different endophyte concentrations over a culture period of up to 192 hr. P-I content in LFSC has been found in the range of 2.15 to 2.83 mg/g DW (EE2; 0.5 ml/L), 2.90 to 4.62 mg/g DW (EE2; 1 ml/L), and 2.51 to 4.71 mg/g DW (EE2; 1.5 ml/L). P-II content in LFSC has been found in the range of 1.40 to 2.19 mg/g DW (EE2; 0.5 ml/L), 2.16 to 2.70 mg/g DW (EE2; 1 ml/L), and 2.17 to 2.94 mg/g DW (EE2; 1.5 ml/L). P-III content in LFSC has been found in the range of 0.23 to 0.32 mg/g DW (EE2; 0.5 ml/L), 0.21 to 0.37 mg/g DW (EE2; 1 ml/L), and 0.27 to 0.38 mg/g DW (EE2; 1.5 ml/L) (Fig. 3a).

P-I content in RHSC has been found in the range of 2.15 to 2.88 mg/g DW (EE2; 0.5 ml/L), 2.26 to 2.95 mg/g DW (EE2; 1 ml/L), and 2.89 to 3.33 mg/g DW (EE2; 1.5 ml/L). P-II content in RHSC has been found in the range of 1.42 to 2.87 mg/g DW (EE2; 0.5 ml/L), 2.11 to 3.82 mg/g DW (EE2; 1 ml/L), and 1.86 to 3.93 mg/g DW (EE2; 1.5 ml/L). P-III content in RHSC has been found in the range of 0.25 to 0.26 mg/g DW (EE2; 0.5 ml/L), 0.28 to 0.47 mg/g DW (EE2; 1 ml/L), and 0.27 to 0.48 mg/g DW (EE2; 1.5 ml/L) concentration (Fig. 3c). The results revealed that in both LHSC and RHSC, the maximum content of P-I, P-II, and P-III was found in (EE2; 1.5 ml/L) concentration. In LFSC, the maximum content of vanillic acid (0.1 mg/g DW) was detected in all three concentrations, while caffeic acid and cinnamic acid were not detected. The highest aucubin content (0.4 mg/g DW) was found at the EE2 concentration (1.5 ml/L). The highest catalpol content (4.0 mg/g DW) was found in (EE2; 0.5 ml/L) concentration (Fig. 3b). In RHSC, the maximum content of vanillic acid (0.2 mg/g DW) was detected in (EE2; 1 ml/L) and (EE2; 1.5 ml/L) concentrations. While caffeic acid was not detected. The maximum content of cinnamic acid (0.1 mg/g DW) was detected in (EE2; 1 ml/L) and (EE2; 1.5 ml/L) concentrations. The highest aucubin content (0.4 mg/g DW) was found at the EE2 concentration (1.5 ml/L). The highest content of catalpol (3.7 mg/g DW) was found in (EE2; 0.5 ml/L) and (EE2; 1.5 ml/L) concentrations (Fig. 3d). The p-HA and 4-HAG have also been detected in LFSC and RHSC under different EE2 endophyte concentrations (Supplementary Fig. S2).

Effect of endophyte EE3 on metabolite content

The picrosides and their precursor metabolite content have been quantified in the LFSC and RHSC of *P. kurroa* cell culture at different endophyte concentrations over a culture period of up to 192 hr. P-I content in LFSC has been found in the range of 1.19 to 1.87 mg/g DW (EE2; 0.5 ml/L), 1.03 to 2.50 mg/g DW (EE3; 1 ml/L), and 1.53 to 2.29 mg/g DW (EE3; 1.5 ml/L). P-II content in LFSC has been found in the range of 1.40 to 2.19 mg/g DW (EE3; 0.5 ml/L), 1.62 to 2.91 mg/g DW (EE3; 1 ml/L), and 1.36 to 2.78 mg/g DW (EE3; 1.5 ml/L). P-III content in LFSC has been found in the range of 0.18 to 0.27 mg/g DW (EE3; 0.5 ml/L), 0.24 to 0.28 mg/g DW (EE3; 1 ml/L), and 0.25 to 0.28 mg/g DW (EE3; 1.5 ml/L) (Fig. 4a). P-I content in RHSC has been found in the range of 1.39 to 1.98 mg/g DW (EE3; 0.5 ml/L), 1.19 to 1.99 mg/g DW (EE3; 1 ml/L), and 1.17 to 1.98 mg/g DW (EE3; 1.5 ml/L). P-II content in RHSC has been found in the range of 1.09 to 2.02 mg/g DW (EE3; 0.5 ml/L), 1.29 to 2.11 mg/g DW (EE3; 1 ml/L), and 1.92 to 2.27 mg/g DW (EE3; 1.5 ml/L). P-III content in RHSC has been found in the range of 0.19 to 0.25 mg/g DW (EE3; 0.5 ml/L), 0.24 to 0.25 mg/g DW (EE3; 1 ml/L), and 0.24 to 0.28 mg/g DW (EE3; 1.5 ml/L) concentration (Fig. 4c). The results revealed that in LHSC, the maximum content of P-I and P-II was found in (EE3; 1 ml/L) concentration, while P-III was found in both (EE3; 1 ml/L) and (EE3; 1.5 ml/L) concentration. The results revealed that in RHSC, the maximum content of P-I was found in (EE3; 1 ml/L) concentration, while P-II and P-III were found in (EE3; 1.5 ml/L) concentration. In LFSC, the maximum content of vanillic acid (0.15 mg/g DW) was detected in both (EE3; 0.5 ml/L) and (EE3; 1.5 ml/L) concentrations, while caffeic acid (0.3 mg/g DW) was detected in (EE3; 1.5 ml/L) concentration. The maximum content of cinnamic acid (0.17 mg/g DW) and aucubin (0.36 mg/g DW) was found in (EE2; 0.5 ml/L) and (EE3; 1 ml/L) concentrations. The highest catalpol content (2.97 mg/g DW) was found in (EE3;

1.5 ml/L) concentration (Fig. 4b). In RHSC, the maximum content of vanillic acid (0.1 mg/g DW), aucubin (0.2 mg/g DW), and cinnamic acid (0.1 mg/g DW) was detected in all three concentrations, while caffeic acid was not quantified. The highest catalpol content (3.5 mg/g DW) was found in (EE3; 0.5 ml/L) and (EE3; 1.5 ml/L) concentrations (Fig. 4d). The p-HA and 4-HAG have also been detected in LFSC and RHSC under different EE3 endophyte concentrations (Supplementary Fig. S3).

Discussion

The current study demonstrates the significant role of endophytic fungi in modulating the biosynthesis of picrosides and precursor metabolites in leaf-derived (LFSC) and root-derived (RHSC) suspension cultures of *P. kurroa*. The three endophytes *Trichoderma* sp. (EE1), *Fusarium acuminatum* (EE2), and *Fusarium* sp. (EE3) elicited differential metabolic responses in both cell types, revealing their potential as biotic elicitors to enhance specialized metabolite production. The successful isolation and molecular identification of fungal endophytes from rhizome tissues of wild *P. kurroa* underline the plant's natural association with a diverse fungal microbiota. This is consistent with prior studies reporting endophytic fungi as crucial modulators of phytochemicals in medicinal plants (Kusari et al. 2012; Tamang et al. 2023). Specifically, members of the Trichoderma and Fusarium genera are well-documented as beneficial symbionts that induce both biotic stress responses and metabolic pathways in host plants (Vinale et al. 2008).

Previously, Tamag et al. (2023) reported that *in vitro*-propagated (Tc) *P. kurroa* plants exhibit reduced endophytic diversity compared to wild-type (Wt) plants, with dominant bacterial phyla including Proteobacteria and Firmicutes. This loss of microbial diversity was associated with a significant decline in picroside accumulation and in the accumulation of intermediate metabolites in Tc plants. A positive correlation between microbial abundance and host secondary metabolite production underscores the functional importance of endophytic communities. Moreover, predictive functional analysis revealed enrichment of key biosynthetic pathways (MVA/MEP and phenylpropanoid/shikimate) in the microbiome of Wt roots and rhizomes. Overall, the findings emphasize a strong host–endomicrobiome interaction influencing picroside biosynthesis. Among the tested isolates, *F. acuminatum* (EE2) exerted the most profound effect on picroside content. In both LFSC and RHSC, the maximum levels of P-I, P-II, and P-III were recorded at 1.5 ml/L EE2 concentration, indicating a strong elicitation potential. The results of the current investigations are comparable to earlier reports by Partap et al. (2025b), who demonstrated enhanced accumulation of picrosides in cell cultures by the biotic elicitation (yeast extract and chitosan). However, in the present study, direct inoculation with endophyte extract derived from *P. kurroa* rhizomes likely provided a more sustained and dynamic interaction with host cells, possibly explaining the greater picroside accumulation observed. *Trichoderma sp*. (EE1) also positively influenced picroside biosynthesis, albeit to a slightly lesser extent. The highest content of P-I (2.89 mg/g DW), P-II (2.17 mg/g DW), and P-III (0.40 mg/g DW) in LFSC under EE1 elicitation highlights the ability of Trichoderma to modulate secondary metabolism, which aligns with previous findings in *Withania somnifera* and *Catharanthus roseus*, where Trichoderma strains enhanced withanolide and vincristine and vinblastine yield content, respectively (Pandey et al. 2018; Farzaei and Sayyari 2024). Notably, catalpol content

peaked at 3.68 mg/g DW in RHSC under EE1 treatment, reflecting the influence of EE1 on iridoid glycoside biosynthesis, which shares biosynthetic intermediates with picrosides. *Fusarium* sp. (EE3), while less potent than EE2, still significantly enhanced picroside content compared to control levels, indicating that even different species within the same genus may have variable elicitation capabilities, possibly due to differential secretion of signaling molecules, endophytic colonization potential, or interaction with plant defence pathways (Kaul et al. 2013). Differences in metabolite accumulation between LFSC and RHSC highlight tissue-specific metabolic responses to endophytic interaction. For instance, EE2 induced greater accumulation of aucubin and catalpol in RHSC than in LFSC, indicating possible preferential uptake or metabolic routing in root-derived cultures. This observation aligns with Partap et al. (2020; 2025b), who noted that rhizome-derived cultures exhibited a more robust response to elicitation due to inherent tissue-specific gene expression patterns related to picroside biosynthesis. Moreover, the detection of precursor metabolites such as vanillic acid, cinnamic acid, and catalpol under different fungal treatments suggests a broader upregulation of the shikimate and iridoid biosynthetic pathways. Such systemic induction mirrors findings from previous omics-based studies on *P. kurroa*, which revealed elicitor-mediated upregulation of phenylpropanoid and monoterpenoid biosynthetic genes (Partap et al. 2022a,b).

Previous research on *P. kurroa* has revealed significant variation in picroside production in response to suspension culture, elicitor and precursor treatments, and light quality. Partap et al. (2022a) reported metabolite quantification in both LFSC and RHSC of *P. kurroa*. The results demonstrated that, during sequential scale-up in RHSC, the content ranges of P-I, P-II, and P-III were 1.41–1.51, 0.76–0.78, and 0.18–0.19 mg/g DW, respectively (Partap et al. 2022b). In contrast, LFSC exhibited higher P-I levels (2.15–2.36 mg/g DW), while P-II (0.26–0.28 mg/g DW) and P-III (0.08–0.09 mg/g DW) were comparatively lower. Additionally, the maximum accumulation of secondary metabolites, such as VA and AUB, was observed in RHSC and LFSC, respectively (Partap et al. 2022a). In our recent study, Partap et al. (2025b) investigated the effects of four elicitors, methyl jasmonate (Me-JA), salicylic acid (SA), chitosan (CHT), and yeast extract (YE), at concentrations ranging from 50–150 mg/L in the LFSC and RHSC of *P. kurroa*. The results indicated that, in LFSC, the highest accumulation of P-I and P-III occurred at 150 µM Me-JA, whereas P-II peaked at 50 µM Me-JA. In RHSC, treatment with 150 µM Me-JA resulted in the maximum levels of P-I, P-II, cinnamic acid, and aucubin. In our subsequent study, Partap et al. (2025a) also examined the coupled effects of light quality (red and blue) and precursor feeding of vanillic acid (VA), phenylalanine (PAL), and trans-cinnamic acid (TCNA) at 50–150 mg/L on both suspension cultures. Red light treatment yielded the highest accumulation of P-I (0.872 and 0.894 mg/g DW), P-II (0.892 and 0.837 mg/g DW), and P-III (0.286 and 0.288 mg/g DW) in LFSC and RHSC, respectively. Under precursor treatments in LFSC, the highest contents of picrosides were observed with 150 mg/L VA, yielding P-I (9.881 mg/g DW) and P-II (3.939 mg/g DW), along with increased levels of VA (0.236 mg/g DW). Several previous studies have reported that fungal endophytes significantly enhance secondary metabolite production in cell cultures of various medicinal plants (Tonk et al. 2016; Thakur et al. 2024). For instance, Salehi et al. (2019) demonstrated that elicitation with endophytic fungi isolated from *Corylus avellana* and *T. baccata* boosts paclitaxel production in suspension culture of *C. avellana*.

Similarly, Dehghan et al. (2025) investigated the elicitation potential of autoclaved cell extracts and culture filtrates derived from *F. graminearum* in suspension cultures of *T. baccata*. Supplementation with 10% autoclaved culture filtrate yielded the highest paclitaxel accumulation (9.438 µg/g DW). Conversely, autoclaved cell extracts most effectively stimulated 10-DABIII biosynthesis, with the 5% treatment yielding a 7.38-fold increase over the untreated control. The results suggested that fungal-derived elicitors differentially influence the biosynthesis of specific secondary metabolites. The extent and characteristics of these responses depend on the elicitor source, concentration, and the targeted metabolic pathway. Fungal elicitors trigger jasmonate-dependent signaling pathways and increase the activity of important biosynthetic enzymes, including tryptophan decarboxylase (TDC) and phenylalanine ammonia-lyase (PAL) (Miryousefzadeh et al. 2026). Additionally, Fungal elicitors stimulate a controlled oxidative and defence response, which may direct plant metabolism toward the production of more secondary metabolites. More recently, Miryousefzadeh et al. (2026) treated *C. roseus* cell suspension culture with the elicitation of *Piriformospora indica* extract to enhance the production of vincristine and vinblastine. Results showed that the elicitation enhanced the contents of vinblastine and vincristine.

This finding suggested that the endophytes also play a critical role in metabolite induction. Furthermore, the 1.5 ml/L concentration yielded the highest picroside level, suggesting an optimal threshold for elicitor–host interaction. Beyond this concentration, excessive fungal metabolite load could potentially trigger hypersensitive responses or metabolic feedback inhibition, as reported in another host-endophyte system (Lu et al. 2021). Moreover, time-course monitoring up to 192 hours enabled the identification of peak production windows, which are essential for developing scalable production protocols. Thus, optimizing the type, concentration and exposure duration of elicitors is crucial for effective elicitation while preserving cell viability in suspension cultures.

Conclusion

The present study demonstrates the significant potential of fungal endophytes, particularly *F. acuminatum*, as effective biotic elicitors to enhance the accumulation of picrosides and associated secondary metabolites in *P. kurroa* cell suspension cultures. Furthermore, these findings establish a foundation for the development of advanced elicitation approaches employing previously reported elicitation factors with endophyte extract in cell culture systems.

Declarations

Supplementary information

The data are available in the article and its online supplementary material.

Ethical Approval

This article followed the ethical standards of the institute.

Consent to Participate

Not applicable

Consent to Publish

Not applicable

Conflict of Interest

The authors declare no competing interests.

Funding

The authors acknowledge the Council of Scientific and Industrial Research (CSIR), Government of India, under the project “Development of industrially sustainable products and processes based on Himalayan Bioresources using advanced fermentation and phytofarming technologies” (MLP-002619), Understanding the molecular mechanism underlying cambial meristematic cells (CMCs) differentiation and their utilization for specialized metabolite production in *Picrorhiza kurrooa*” (GAP-0327) and the Phytopharma mission project (HCP-0010) for providing financial support.

Author Contribution

Author contributionsConceptualization: Mahinder Partap and Ashish R. Warghat; Methodology: Mahinder Partap, Ankita Thakur, Amit Kumar, Anish Tamang, Shiv Shanker Pandey and Ashish R. Warghat; Data analysis and investigation: Mahinder Partap, Ankita Thakur, Amit Kumar, Anish Tamang, Shiv Shanker Pandey and Ashish R. Warghat; Original manuscript preparation: Mahinder Partap, Ankita Thakur, Amit Kumar, Anish Tamang, Shiv Shanker Pandey and Ashish R. Warghat; Manuscript-review and editing: Shiv Shanker Pandey and Ashish R. Warghat

Acknowledgement

The authors are thankful to the Director, CSIR-IHBT, Palampur for providing the necessary facilities. The authors also acknowledge the Academy of Scientific and Innovative Research (AcSIR), Ghaziabad. Mahinder Partap also acknowledges CSIR, New Delhi, for providing Junior and Senior Research Fellowships (JRF and SRF). Mahinder Partap also acknowledges the Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, for PhD enrolment.

References

1. Bhaskar R, Xavier LSE, Udayakumaran G, Kumar DS, Venkatesh R, Nagella P (2022) Biotic elicitors: a boon for the in-vitro production of plant secondary metabolites. Plant Cell Tissue Organ Cult 149 1:7–24. https://doi.org/10.1007/s11240-021-02131-1
2. Dehghan AK, Zargar M, Bamneshin M, Mahmoudieh M, Safaie N, Yang JL, Naghavi MR (2025) The effects of *Fusarium graminearum* cell extracts and culture filtrates on the production of paclitaxel and 10-deacetylbaccatin III in suspension cell cultures of *Taxus baccata* L. BMC Plant Bio 25(1):229. https://doi.org/10.1186/s12870-025-06230-5
3. Farzaei L, Sayyari M (2024) Elicitation with sodium nitroprusside and *Trichoderma* improves vincristine and vinblastine yield in *Catharanthus roseus* cell suspension culture by modulating terpenoid indole alkaloid pathway genes. Plant Cell Tissue Organ Cult 157 2:37. https://doi.org/10.1007/s11240-024-02727-3
4. Gadzovska Simic S, Tusevski O, Maury S, Hano C, Delaunay A, Chabbert B, Lamblin F, Lainé E, Joseph C, Hagège D (2015) Fungal elicitor-mediated enhancement in phenylpropanoid and naphtodianthrone contents of *Hypericum perforatum* L. cell cultures. Plant Cell Tissue Organ Cult 122 1:213–226. https://doi.org/10.1007/s11240-015-0762-y
5. Gomes T, Pereira JA, Benhadi J, Lino-Neto T, Baptista P (2018) Endophytic and epiphytic phyllosphere fungal communities are shaped by different environmental factors in a Mediterranean ecosystem. Microb Ecol 76 3:668–679. https://doi.org/10.1007/s00248-018-1161-9
6. Guru A, Dwivedi P, Kaur P, Pandey DK (2022) Exploring the role of elicitors in enhancing medicinal values of plants under in vitro condition. S Afr J Bot 149:1029–1043. https://doi.org/10.1016/j.sajb.2021.10.014
7. Jain R, Bhardwaj P, Pandey SS, Kumar S (2021) *Arnebia euchroma*, a plant species of cold desert in the Himalayas, harbors beneficial cultivable endophytes in roots and leaves. Front Microbiol 16:12:696667. https://doi.org/10.3389/fmicb.2021.696667
8. Kaul S, Ahmed M, Zargar K, Sharma P, Dhar MK (2013) Prospecting endophytic fungal assemblage of *Digitalis lanata* Ehrh.(foxglove) as a novel source of digoxin: a cardiac glycoside. 3 Biotech 3 4:335–340. https://doi.org/10.1007/s13205-012-0106-0
9. Kumar A, Kanika, Rani S, Sharma A, Kumar A, Kumar D, Warghat AR (2026) Spectral regulation of growth, secondary metabolism, and iridoid pathway gene expression in in vitro cultures of *Valeriana jatamansi*. Vitro Cell Dev Biol - Plant 1–20. https://doi.org/10.1007/s11627-026-10647-4
10. Kumar A, Partap M, Warghat AR (2025a) Jasmonic acid: a versatile phytohormone regulating growth, physiology, and biochemical responses. J Plant Growth Regul 44 1:131–154. https://doi.org/10.1007/s00344-024-11376-x
11. Kumar A, Jaryal P, Rani S, Warghat AR (2025b) Biotechnological Interventions and Propagation Strategies of Some Endangered Plants of Himalaya. Biodiversity of the Himalaya 115–137. Apple Academic

12. Kusari S, Hertweck C, Spiteller M (2012) Chemical ecology of endophytic fungi: origins of secondary metabolites. Chem Biol 19 7:792–798. 10.1016/j.chembiol.2012.06.004
13. Kushwaha RK, Singh S, Pandey SS, Rao DV, Nagegowda DA, Kalra A, Vivek Babu CS (2019) Compatibility of inherent fungal endophytes of *Withania somnifera* with Trichoderma viride and its impact on plant growth and withanolide content. J Plant Growth Regul 38 4:1228–1242. https://doi.org/10.1007/s00344-019-09928-7
14. Li P, Mao Z, Lou J, Li Y, Mou Y, Lu S, Peng Y, Zhou L (2011) Enhancement of diosgenin production in *Dioscorea zingiberensis* cell cultures by oligosaccharides from its endophytic fungus *Fusarium oxysporum* Dzf17. Molecules 16 12:10631–10644. https://doi.org/10.3390/molecules161210631
15. Li Y, Kong D, Fu Y, Sussman MR, Wu H (2020) The effect of developmental and environmental factors on secondary metabolites in medicinal plants. Plant Physiol Biochem 1(80):9. https://doi.org/10.1016/j.plaphy.2020.01.006
16. Lu H, Wei T, Lou H, Shu X, Chen Q (2021) A critical review on communication mechanism within plant-endophytic fungi interactions to cope with biotic and abiotic stresses. J. Fungi. 1;7(9):719. https://doi.org/10.3390/jof7090719
17. Mehta S, Sharma AK, Singh RK (2021) Advances in ethnobotany, synthetic phytochemistry and pharmacology of endangered herb *Picrorhiza kurroa* (Kutki): A comprehensive review (2010–2020). Mini-Rev Med Chem 21 19:2976–2995. https://doi.org/10.2174/1389557521666210401090028
18. Ming Q, Su C, Zheng C, Jia M, Zhang Q, Zhang H, Rahman K, Han T, Qin L (2013) Elicitors from the endophytic fungus *Trichoderma atroviride* promote *Salvia miltiorrhiza* hairy root growth and tanshinone biosynthesis. J Exp Bot 64 18:5687–5694. https://doi.org/10.1093/jxb/ert342
19. Miryousefzadeh S, Hosseini B, Hemmaty S (2026) Fungal elicitation and precursor feeding: a novel approach to boost alkaloid yield in Catharanthus roseus cell suspension cultures. Sci Rep. https://doi.org/10.1038/s41598-026-65357-z
20. Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. https://doi.org/10.1111/j.1399-3054.1962.tb08052. .15:3
21. Nabi N, Singh S, Saffeullah P (2021) Responses of in vitro cell cultures to elicitation: regulatory role of jasmonic acid and methyl jasmonate: a review. Vitro Cell Dev Biol - Plant 57 3:341–355. https://doi.org/10.1007/s11627-020-10140-6
22. Nascimento FX, Glick BR, Rossi MJ (2019) Isolation and characterization of novel soil-and plant-associated bacteria with multiple phytohormone-degrading activities using a targeted methodology. Access Microbiol 1 7:000053. https://doi.org/10.1099/acmi.0.000053
23. Pandey SS, Singh S, Babu CSV, Shanker K, Srivastava NK, Shukla AK, Kalra A (2016) Fungal endophytes of *Catharanthus roseus* enhance vindoline content by modulating structural and regulatory genes related to terpenoid indole alkaloid biosynthesis. Sci Rep 6:126583. https://doi.org/10.1038/srep26583
24. Pandey SS, Singh S, Pandey H, Srivastava M, Ray T, Soni S, Pandey A, Shanker K, Babu CV, Banerjee S, Gupta MM (2018) Endophytes of *Withania somnifera* modulate in planta content and the site of

withanolide biosynthesis. Sci Rep 8:15450. https://doi.org/10.1038/s41598-018-23716-5

25. Partap M, Kumar P, Ashrita, Kumar P, Kumar D, Warghat AR (2020) Growth kinetics, metabolites production and expression profiling of picrosides biosynthetic pathway genes in friable callus culture of *Picrorhiza kurroa* Royle ex Benth. Appl Biochem Biotechnol 192 4:1298–1317. https://doi.org/10.1007/s12010-020-03391-x
26. Partap M, Rattan S, Kumar D, Warghat AR (2022a) Bioprocess development for enhancing cell biomass, differential picrosides accumulation, and gene expression profiling at shake flask levels in suspension cultures of *Picrorhiza kurroa*. Ind Crops Prod 187:115311. https://doi.org/10.1016/j.indcrop.2022.115311
27. Partap M, Chhimwal J, Kumar P, Kumar D, Padwad Y, Warghat AR (2022b) Growth dynamics and differential accumulation of picrosides and its precursor metabolites in callus cell lines of *Picrorhiza kurroa* with distinct anti-steatotic potential. Process Biochem 120:85–100. https://doi.org/10.1016/j.procbio.2022.06.002
28. Partap M, Warghat AR, Kumar S (2023) Cambial meristematic cell culture: a sustainable technology toward in vitro specialized metabolites production. Crit Rev Biotechnol 43 5:734–752. https://doi.org/10.1080/07388551.2022.2055995
29. Partap M, Kumar A, Kumar P, Kumar D, Warghat AR (2025a) Spectral light treatment and exogenous feeding of precursors enhanced the picrosides content in dedifferentiated cell culture of *Picrorhiza kurroa*. Biocatal Agric Biotechnol 64:103501. https://doi.org/10.1016/j.bcab.2025.103501
30. Partap M, Kumar A, Kumar P, Kumar D, Warghat AR (2025b) Elicitors mediated enhancement of Picrosides and their pathway precursors in dedifferentiated cell suspension culture of *Picrorhiza kurroa* Royle ex Benth. J Plant Growth Regul 44 1:247–265. https://doi.org/10.1007/s00344-024-11537-y
31. Prasad A, Mathur A, Kalra A, Gupta MM, Lal RK, Mathur AK (2013) Fungal elicitor-mediated enhancement in growth and asiaticoside content of *Centella asiatica* L. shoot cultures. Plant Growth Regul 69 3:265–273. https://doi.org/10.1007/s10725-012-9769-0
32. Saijo Y, Loo EPI, Yasuda S (2018) Pattern recognition receptors and signaling in plant–microbe interactions. Plant J 93 4:592–613. https://doi.org/10.1111/tpj.13808
33. Salehi M, Moieni A, Safaie N, Farhadi S (2019) Elicitors derived from endophytic fungi *Chaetomium globosum* and *Paraconiothyrium brasiliense* enhance paclitaxel production in *Corylus avellana* cell suspension culture. Plant Cell Tissue Organ Cult 136(1):161–171. https://doi.org/10.1007/s11240-018-1503-9
34. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press
35. Savitha BC, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA (2006) Different biotic and abiotic elicitors influence betalain production in hairy root cultures of *Beta vulgaris* in shake-flask and bioreactor. Process Biochem 41 1:50–60. https://doi.org/10.1016/j.procbio.2005.03.071

36. Schnitzler JP, Madlung J, Rose A, Ulrich Seitz H (1992) Biosynthesis of p-hydroxybenzoic acid in elicitor-treated carrot cell cultures. Planta 188 4:594–600. https://doi.org/10.1007/BF00197054
37. Sharma N, Chauhan RS, Sood H (2015) Seaweed extract as a novel elicitor and medium for mass propagation and picroside-I production in an endangered medicinal herb *Picrorhiza kurroa*. Plant Cell Tissue Organ Cult 122 1:57–65. https://doi.org/10.1007/s11240-015-0749-8
38. Shitiz K, Pandit S, Chauhan RS, Sood H (2013) Picrosides content in the rhizomes of *Picrorhiza kurroa* Royle ex Benth. traded for herbal drugs in the markets of North India. Int J Med Arom Plants 3 2:226–233
39. Tamang A, Swarnkar M, Kumar P, Kumar D, Pandey SS, Hallan V (2023) Endomicrobiome of in vitro and natural plants deciphering the endophytes-associated secondary metabolite biosynthesis in *Picrorhiza kurrooa*, a Himalayan medicinal herb. Microbiol Spectr 11 6:02279–02223. https://doi.org/10.1128/spectrum.02279-23
40. Tang Z, Rao L, Peng G, Zhou M, Shi G, Liang Y (2011) Effects of endophytic fungus and its elicitors on cell status and alkaloid synthesis in cell suspension cultures of *Catharanthus roseus*. J Med Plants Res 5 11:2192–2200
41. Thakur A, Thakur K, Kumar A, Warghat AR, Kumar D, Pandey SS (2024) Endophyte-based fungal elicitors for enhanced production of valepotriates and sesquiterpenoids in leaf cell suspension cultures of *Valeriana jatamansi* Jones. J Appl Microbiol 135 9:242. https://doi.org/10.1093/jambio/lxae242
42. Tonk D, Mujib A, Maqsood M, Ali M, Zafar N (2016) *Aspergillus flavus* fungus elicitation improves vincristine and vinblastine yield by augmenting callus biomass growth in *Catharanthus roseus*. Plant Cell Tissue Organ Cult 126 2:291–303. https://doi.org/10.1007/s11240-016-0998-1
43. Vinale F, Sivasithamparam K, Ghisalberti EL, Marra R, Woo SL, Lorito M (2008) Trichoderma–plant–pathogen interactions. Soil Biol Biochem 40 1:1–10. https://doi.org/10.1016/j.soilbio.2007.07.002
44. Wang JW, Zhang Z, Tan RX (2001) Stimulation of artemisinin production in *Artemisia annua* hairy roots by the elicitor from the endophytic *Colletotrichum* sp. Biotechnol Lett 23 11:857–860. https://doi.org/10.1023/A:1010535001943
45. White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: guide methods Appl 18:315–322

Figures

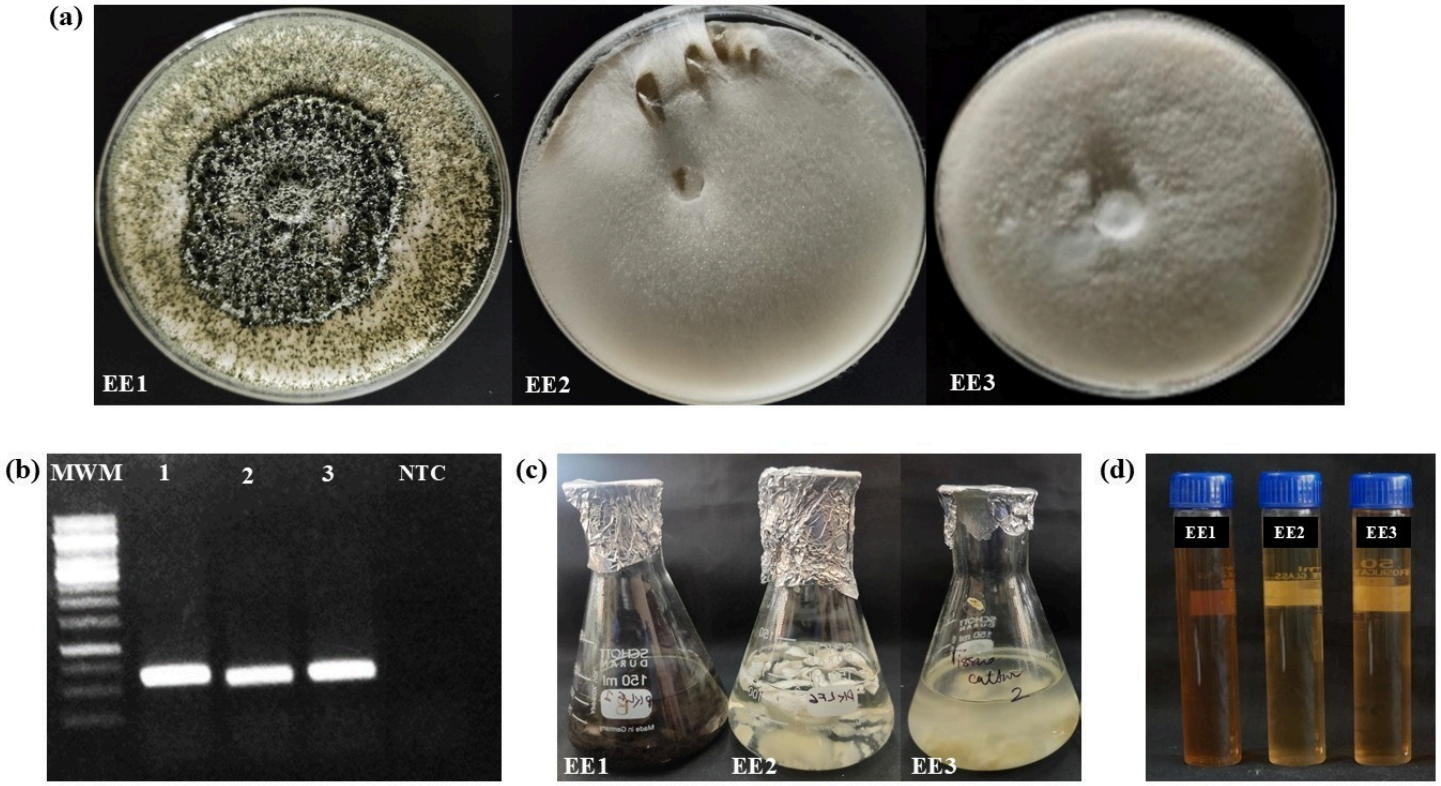


Figure 1

Isolation, identification, and cultivation of fungal endophytes isolated from the rhizome tissue of *P. kurroa* for elicitor treatment. (a) Isolated fungal endophytes on Potato Dextrose Agar (PDA) (b) Amplification for the ITS-based identification of the genomic DNA isolated from the fungal isolates: 1. EE1; *Trichoderma* sp., 2. EE2; *Fusarium acuminatum,* 3. EE3; *Fusarium*sp. NTC: no template control (c) Fungal cultures in Potato Dextrose Broth (PDB) (d) Filtered endophyte-based fungal elicitors.

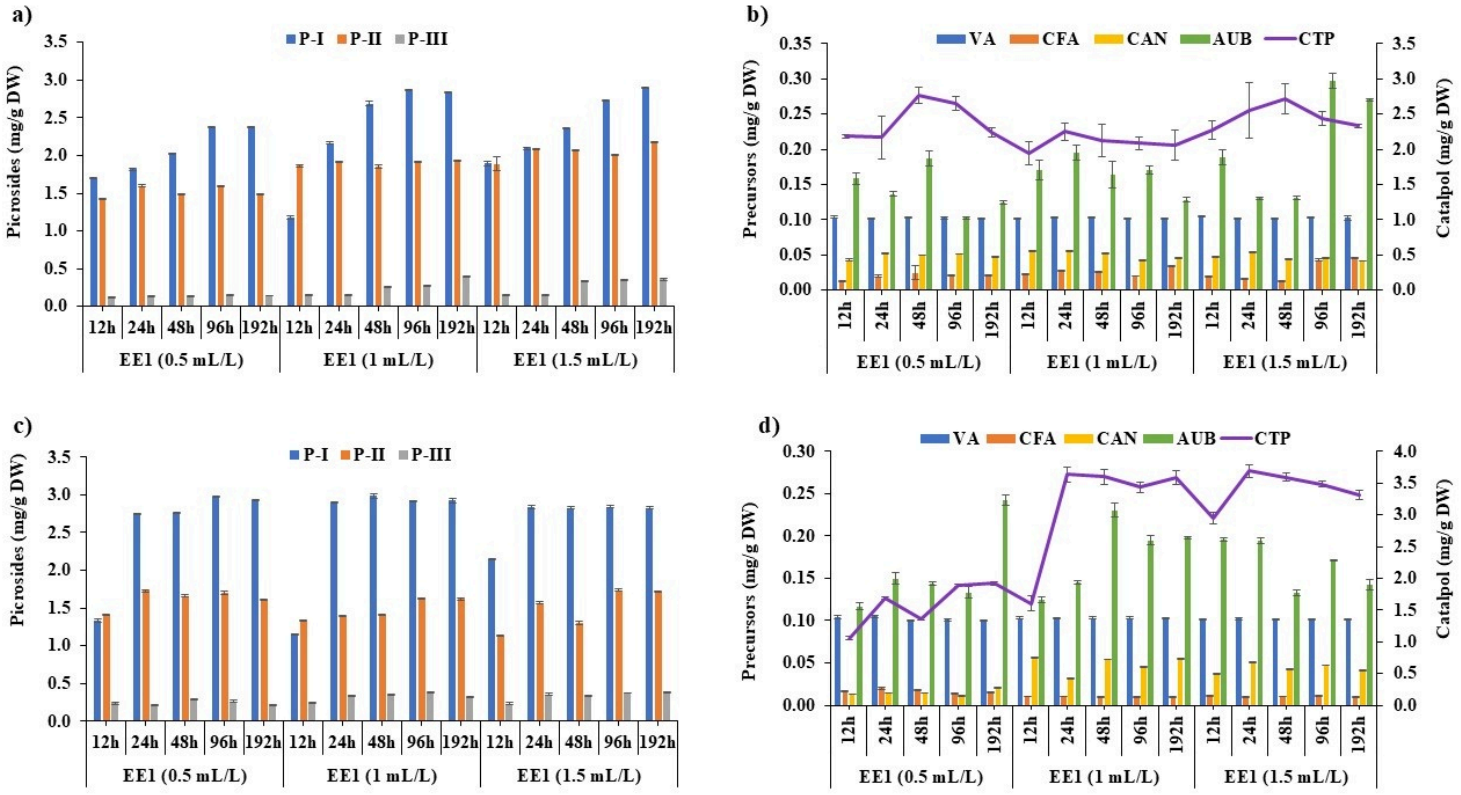


Figure 2

Effect of different concentrations (0.5-1.5 ml/L) of endophytic extract of *Trichoderma* sp. (EE1) on picrosides and precursor metabolites content enhancement in leaf cell suspension culture (LFSC) and rhizome cell suspension culture (RHSC) of *P. kurroa*. a) picrosides content in LFSC, b) precursor metabolites content in LFSC, c) picrosides content in RHSC, d) precursor metabolites content in RHSC. EE1, Endophytic extract 1; P-I, Picroside I; P-II, Picroside II; P-III, Picroside III; VA, Vanillic acid; CFA, Caffeic acid; CAN, Cinnamic acid; CTP, Catalpol; AUB, Aucubin. Data graph bars represent mean ± standard error (*n*=3).

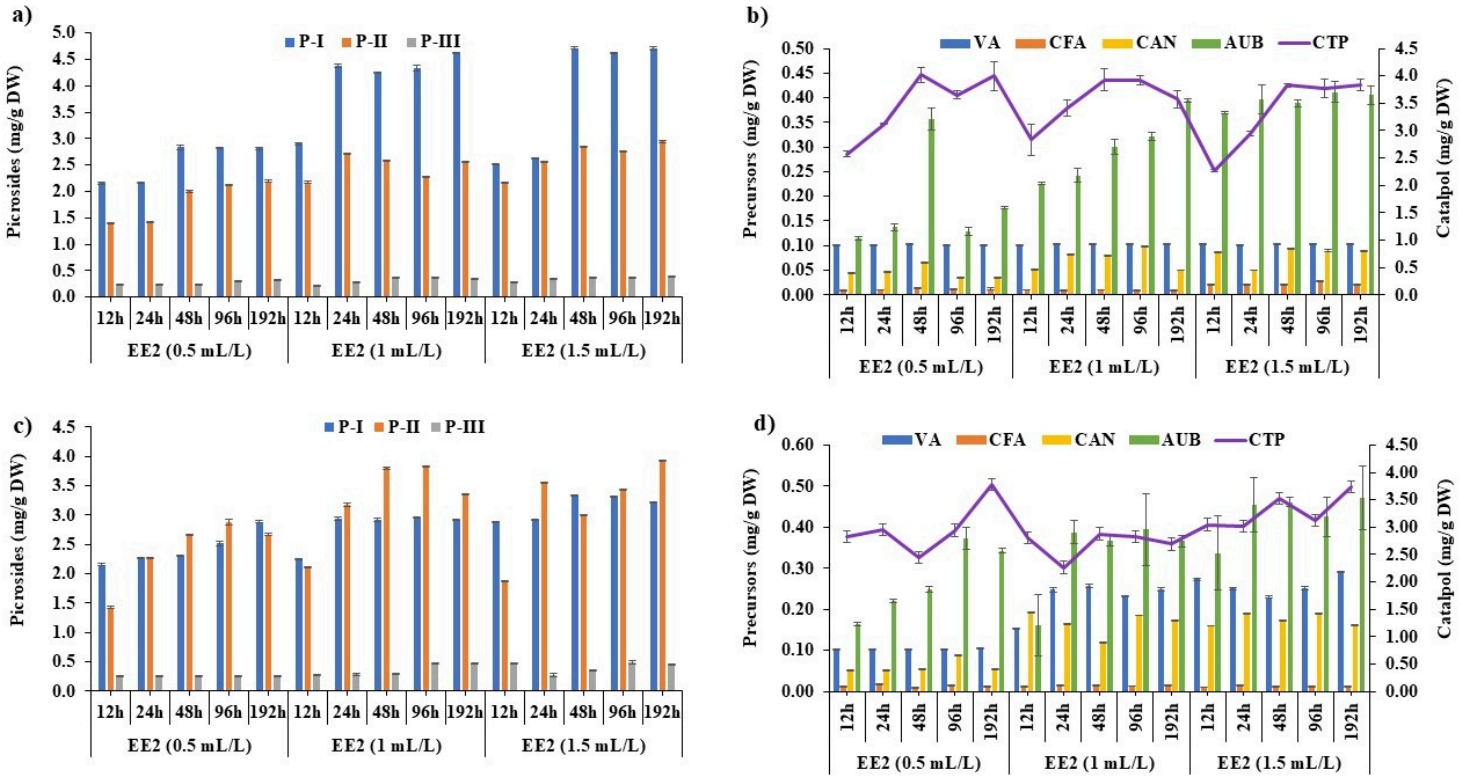


Figure 3

Effect of different concentrations (0.5-1.5 ml/L) of endophytic extract of *Fusarium acuminatum* (EE2) on picrosides and precursor metabolites content enhancement in leaf cell suspension culture (LFSC) and rhizome cell suspension culture (RHSC) of *P. kurroa*. a) picrosides content in LFSC, b) precursor metabolites content in LFSC, c) picrosides content in RHSC, d) precursor metabolites content in RHSC. EE2, Endophytic extract 2; P-I, Picroside I; P-II, Picroside II; P-III, Picroside III; VA, Vanillic acid; CFA, Caffeic acid; CAN, Cinnamic acid; CTP, Catalpol; AUB, Aucubin. Data graph bars represent mean ± standard error (*n*=3).

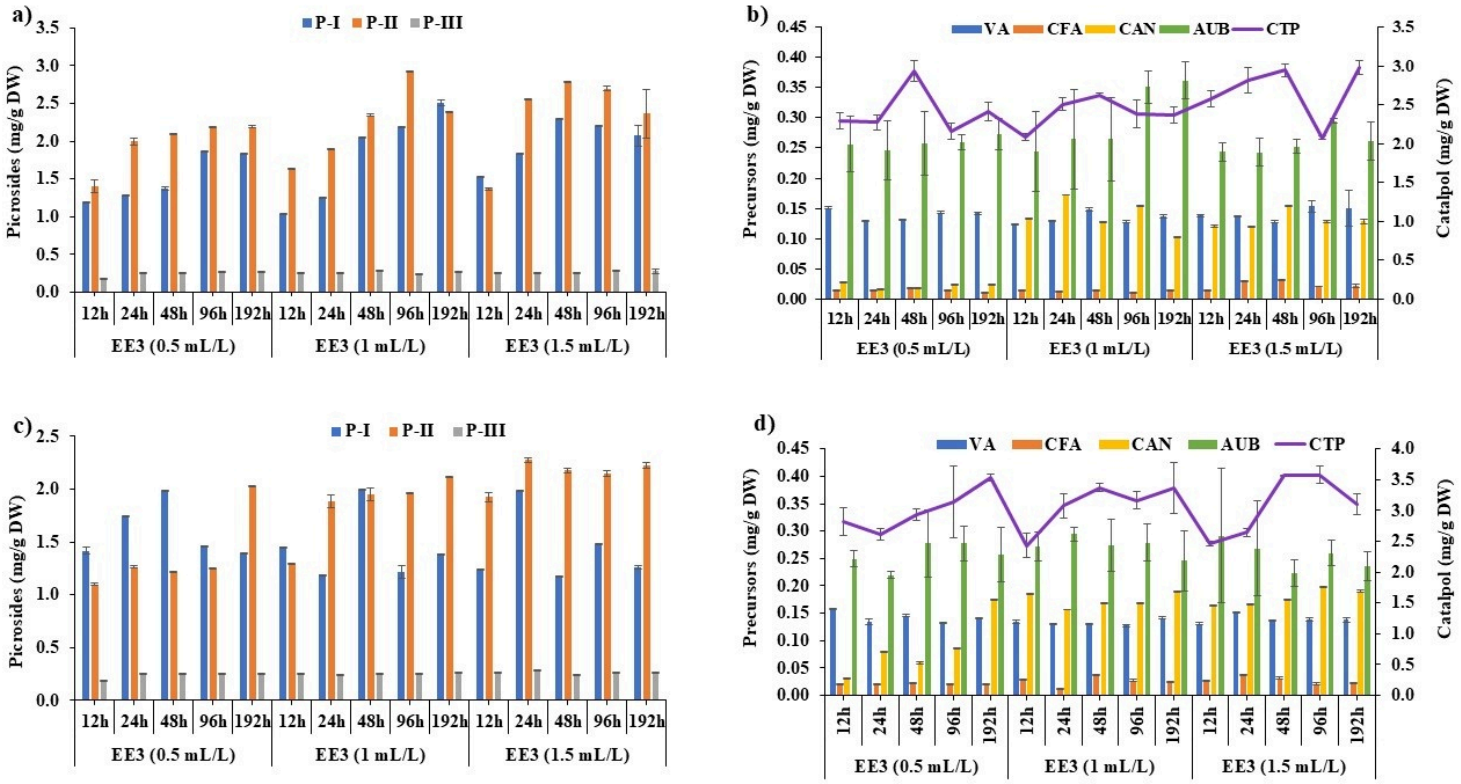


Figure 4

Effect of different concentrations (0.5-1.5 ml/L) of endophytic extract of *Fusarium* sp. (EE3) on picrosides and precursor metabolites content enhancement in leaf cell suspension culture (LFSC) and rhizome cell suspension culture (RHSC) of *P. kurroa*. a) picrosides content in LFSC, b) precursor metabolites content in LFSC, c) picrosides content in RHSC, d) precursor metabolites content in RHSC. EE3, Endophytic extract 3; P-I, Picroside I; P-II, Picroside II; P-III, Picroside III; VA, Vanillic acid; CFA, Caffeic acid; CAN, Cinnamic acid; CTP, Catalpol; AUB, Aucubin. Data graph bars represent mean ± standard error (*n*=3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.docx