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Direct shoot regeneration and gene expression profiling for optimized picroside-I biosynthesis in *Picrorhiza Kurroa*

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***Picrorhiza kurroa*, an endangered medicinal herb, is known for its bioactive iridoid glycosides, the Picrosides, which possess a range of pharmacological properties, including hepatoprotective, anti-inflammatory, and antioxidant activities. As demand for this valuable plant increases, in vitro tissue culture conservation efforts have become critical. We attempted in vitro tissue engineering-assisted direct shoot regeneration from leaf explants of *P. kurroa* using various growth hormone combinations, for enhanced metabolite production. MS media, admixed with 0.5 mg/L thidiazuron and 1.5 mg/L kinetin, bypassed the callogenesis phase while offering 83% shoot regeneration efficiency. High-performance liquid chromatography analyses with shoots elucidated a high picroside-I content of up to 9.7 µg/mg. Gene expression analysis via qRT-PCR revealed a marked increase in the expression levels of critical genes associated with the Picroside-I biosynthesis pathway—namely HMGR, PMK, DXPS, G10H, DAHPS, and PAL—when compared to shoots generated through the callus-mediated process. Notably, a marked increase in the expression of geraniol synthase, a key gene in the iridoid pathway, was directly correlated with enhanced Picroside-I levels. This in vitro tissue engineering strategy enhances Picroside-I biosynthesis and sets the stage for future biotechnological and pharmaceutical applications in the conservation, genetic improvement, and commercial exploitation of *P. kurroa*.**

Plants produce a diverse array of secondary metabolites, many of which are highly valued in the pharmaceutical and nutraceutical industries. Plant cell cultures offer a standardized method of producing these compounds, independent of the biotic and abiotic factors that are difficult to control in conventional cultivation[1–4]. However, replicating the complex interactions of different biosynthetic pathways that occur in multiple cell types within real plant tissues is a challenge[5,6].

Picrorhiza kurroa Royle ex Benth. (abbreviated PK hereafter), commonly known as Kutki or Kutaki, is an indigenous medicinal herb belonging to the Scrophulariaceae (now classified under Plantaginaceae) family, primarily distributed in the North-Western Himalayan region[7,8]. This dicotyledonous plant is vegetatively propagated and holds significant economic value due to its bioactive iridoid glycosides[9]. Of these compounds, the picrosides contribute to its diverse pharmacological properties, including hepatoprotective, anti-inflammatory, antioxidant, immunomodulatory, and neuroprotective activities[7,9,10]. Escalating demand for PK, exceeding 415 MT/year, has resulted in its classification as an endangered species due to over-exploitation and anthropogenic pressures[11–13]. Conservation efforts are crucial, and in vitro tissue culture offers a promising solution for sustainable production and ex-situ conservation. Several studies have explored efficient tissue culture protocols for PK, investigating various explants, media formulations, and hormonal combinations[14–21]. While low-cost micropropagation and indirect shoot organogenesis have been established[22]optimizing regeneration protocols remains a key area of research. Other groups focused on developing propagation methods using ex vitro leaves and aseptic shoot cultures[19,20]while Bhat and colleagues explored direct organogenesis and Agrobacterium-mediated genetic transformation as strategies to enhance metabolite production[23,24]. However, these works

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haven't established a thorough connection between tissue culture conditions and picroside content in PK. Similarly, the use of elicitors, such as abscisic acid, hydrogen peroxide, salicylic acid, and methyl jasmonate, has been shown to potentially enhance picroside biosynthesis[24,25]. Additionally, genetic modifications and hairy root cultures have been established for increased production of picrotin and picrotoxinin[16,26]. However, in the dearth of technological advancements, integrating more modern approaches with plant-based cell culture systems has seen limited success, particularly in establishing consistent and reliable industrial mechanization for producing pharmaceutically relevant phytometabolites.

Picroside-I biosynthesis predominantly occurs in aerial tissues, such as shoots and leaves, which are highly amenable to in vitro manipulation via direct shoot regeneration. Conversely, Picroside-II biosynthesis presents a more complex challenge due to its predominant synthesis in stolons and roots, tissues requiring distinct culturing protocols and environmental conditions. The metabolic pathways and precursors involved in picroside-II production also differ significantly. To take this to a feasible resolution, molecular biology did facilitate a foundational understanding of the metabolic pathways involved in PK's secondary metabolite production[14,16,27]. Key genes responsible for picroside biosynthesis have been identified, providing insights into the underlying molecular mechanisms. Kumar and coworkers proposed a complete biosynthetic pathway in PK using a bio-retrosynthetic approach, assembling end products to precursors using four modules: MEP, MVA, shikimate/phenylpropanoid, and iridoid pathways for P-I and P-II production[28]. Comparative genomics approaches have led to the cloning of critical genes like HMGR, DXPS, DXPR, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, HDS, and acetyl-CoA reductase[29,30]. Other than these, transcriptome analyses using Illumina sequencing technology have further revealed the involvement of microRNAs, kinases, and transcription factors in regulating PK's secondary metabolite production. Specifically, a miRNA-4995 is known to regulate P-I biosynthesis, while in silico transcript abundance analysis identified key transcription factors[31]. Studies have established that picroside content varies from 3.7 to 10.9% in dry rhizomes, and primary metabolic pathways like photosynthesis, glycolysis, and the TCA cycle contribute precursors for secondary metabolite synthesis[32]. Furthermore, the temperature has been shown to regulate secondary metabolism pathways, influencing picroside-I production[33].

To align with these challenges and emerging insights, our study was designed to establish a regeneration protocol that not only enhances shoot proliferation in *P. kurroa*, but also directly correlates with elevated levels of the pharmaceutically significant metabolite—Picroside-I. By building upon our previous efforts in tissue culture optimization, metabolic profiling, and molecular investigation[22,25,34–37]the current work integrates regeneration efficiency with targeted secondary metabolite biosynthesis to address the urgent need for sustainable and scalable production systems.

Our present study focuses on optimizing Picroside-I production. This choice was driven by its broader pharmacological relevance and the feasibility of achieving high yields through shoot-based tissue culture techniques. At the outset, our work successfully led us to: (i) establish a rapid and effective in vitro regeneration protocol for PK, prioritizing enhanced shoot production while bypassing the callus phase through the strategic application of growth regulators like thidiazuron (TDZ); (ii) assess Picroside-I biosynthesis in direct versus indirect shoot regeneration systems using advanced chromatographic techniques; and (iii) link HPLC-quantified increases in Picroside-I content to the differential expression of critical biosynthetic pathway genes. Ultimately, this study lays a robust platform for future biotechnological integration, offering scalable, real-time monitoring systems for pharmaceutical development.

Results

Optimization of the direct regeneration regime

Out of the eight growth regulator combinations tested, Murashige and Skoog (MS) medium supplemented with either 0.25 mg/L thidiazuran (TDZ) + 1.5 mg/L kinetin (KIN) (Combination 5) and/or that with 0.5 mg/L TDZ + 1.5 mg/L KIN (Combination 3) resulted in direct shoot initiation from the abaxial surface of PK leaf explants without callus formation. The regeneration frequencies were 67% and 83%, respectively. Furthermore, MS medium with 0.5 mg/L TDZ + 1.5 mg/L KIN (Combination 3) showed the highest (7–8 numbers of) shoots regenerated per leaf explant (Figs. 1 and 2; and Table 1). The total time required for complete plantlet regeneration was only 45–50 days, a significant reduction compared to the indirect regeneration method traditionally used for in vitro PK plantlet development (Figs. 1 and 2). In contrast, the other growth regulator combinations (Table 1) resulted in significantly lower frequencies of shoot regeneration and/or exhibited increased callus formation. Notably, the synergistic effect of TDZ and KIN in combinations 3 and 5 led to markedly higher shoot induction without any callogenesis, coupled with a reduced overall regeneration period (Table 1) (Fig. 2).

Direct regeneration outperforms indirect protocols in *P. kurroa* micropropagation

Compared to the previously formulated callusing-driven indirect regeneration method for PK using MS medium supplemented with 0.5 mg/L TDZ[37]the hereby devised direct shoot regeneration approach (MS medium supplemented with 0.5 mg/L TDZ and 1.5 mg/L KIN) offers several advantages by optimizing the ratio of auxin to kinetin. In the indirect regeneration protocol, callus induction occurred within 15 days, followed by it proliferation in 30 days (Figs. 1 and 2). However, as reported previously it requires multiple subculturing steps to accommodate the varying media compositions necessary for different developmental stages, precisely productive callogenesis to effectuate further caulogenesis, and shoot multiplication[37]. Notably, shoot induction (caulogenesis) is observed after 50 days, while shoot multiplication is effective only upon transferring the shoots to appropriate growth media, and this takes 65 days. Overall, the indirect method ultimately produces fully developed plantlets in about 80–85 days, resulting a regeneration frequency of 88–90% (Table 1; Fig. 2). The direct organogenesis protocol thus offers a more rapid and efficient strategy for in vitro regeneration (Figs. 1

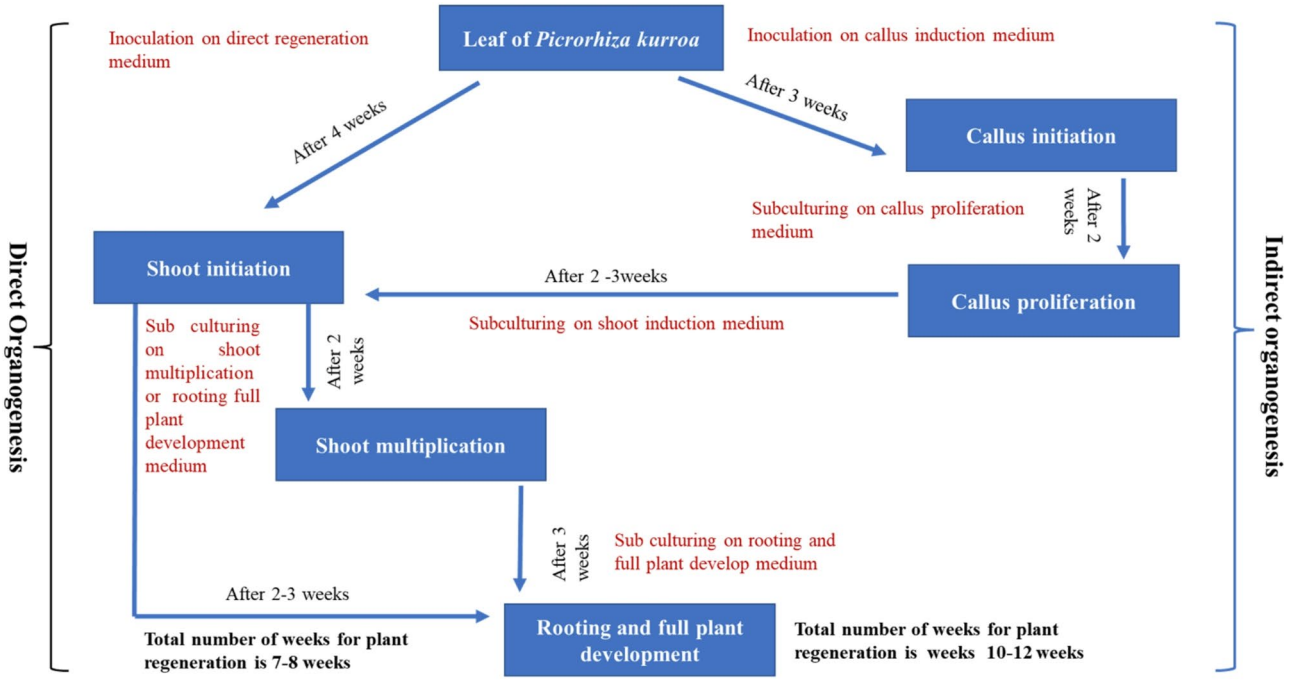

Fig. 1. Schematic representation comparing indirect and direct regeneration regimes in *Picrorhiza kurroa*. This workflow illustrates the procedural differences between previously established indirect regeneration[37] and the newly optimized direct shoot regeneration protocol developed in this study. The schematic emphasizes variations in subculturing steps, callus formation phases, regeneration timelines, and hormonal regimes, showcasing the efficiency and speed offered by bypassing the callogenic phase under direct organogenesis.

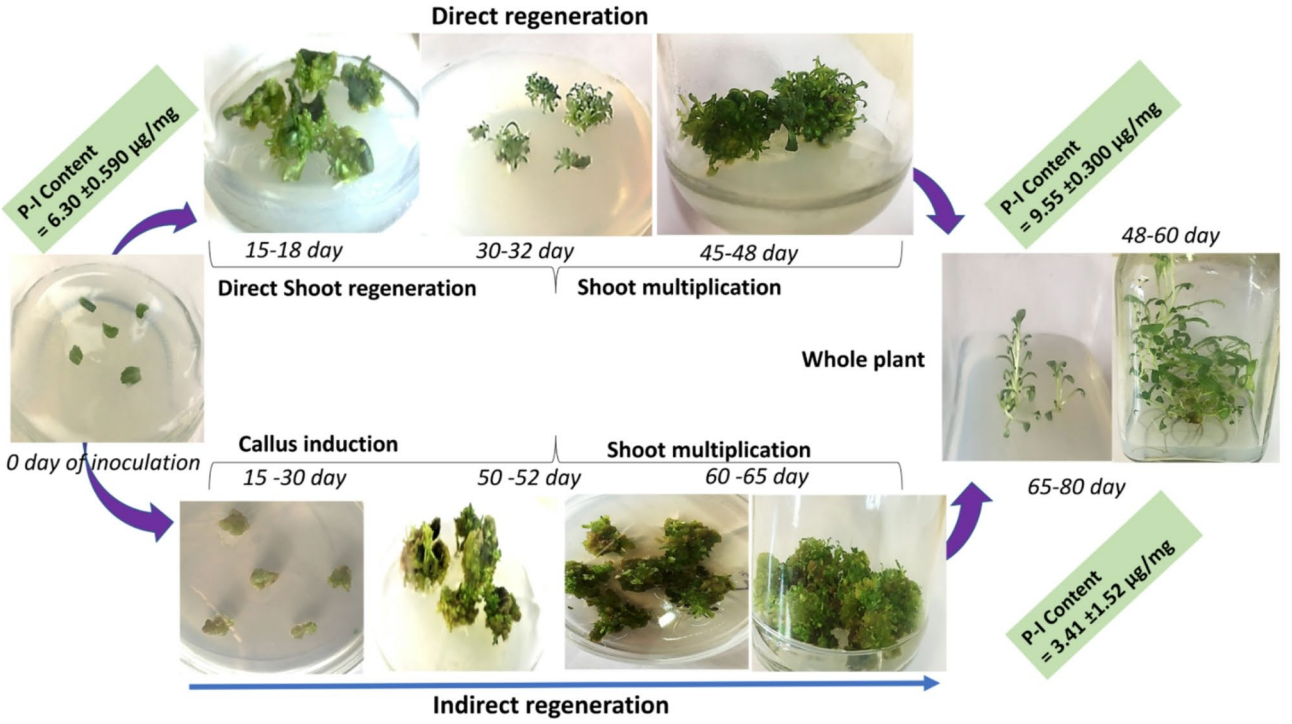

Fig. 2. Photographic comparison of shoot regeneration under indirect and direct organogenesis in Picrorhiza kurroa. Representative images depict morphological outcomes of leaf explants under the two regeneration protocols. Notable differences in shoot induction efficiency, callus development, and overall biomass formation are visually highlighted. This comparative pictographic evidence supports the higher regeneration rate and reduced culture duration achieved through direct organogenesis.

S. No.	MS media supplemented with growth regulator combinations			Shoot regeneration (%)	Callus induction	Shoots per explant
	TDZ (mg/L)	KIN (mg/L)	NAA (mg/L)			
1.	1.00	1.50	-	n.d.	++++	n.d.
2.	1.00	1.00	-	n.d.	++++	n.d.
3.	**0.50**	**1.50**	**-**	**83.10 ± 0.82**	**n.d.**	**7.20 ± 1.10[a]**
4.	0.50	1.00	-	54.80 ± 0.57	++	3.60 ± 1.14[b]
5.	**0.25**	**1.50**	**-**	**67.84 ± 0.59**	**n.d.**	**2.60 ± 0.89**
6.	0.25	1.00	-	63.00 ± 0.93	++	1.80 ± 0.84[cd]
7.	0.50	-	1.00	33.86 ± 0.54	+++	2.00 ± 0.70
8.	0.25	-	1.00	31.10 ± 0.51	+++	1.40 ± 0.54

Table 1. Effect of combinations of growth regulators on shoot regeneration in P. Kurroa. Measurements and observed effects recorded at 4 weeks from in vitro establishment. Here, callus induction is represented as maximum (++++), moderate (+++), and minimum callusing (++). Values represented with 'n.d.' denote effects none detected. Values are presented as means from 5 independent experiments with standard deviation (±). Means with the same letters do not significantly differ at the 95% level of confidence following duncan's multiple range test.

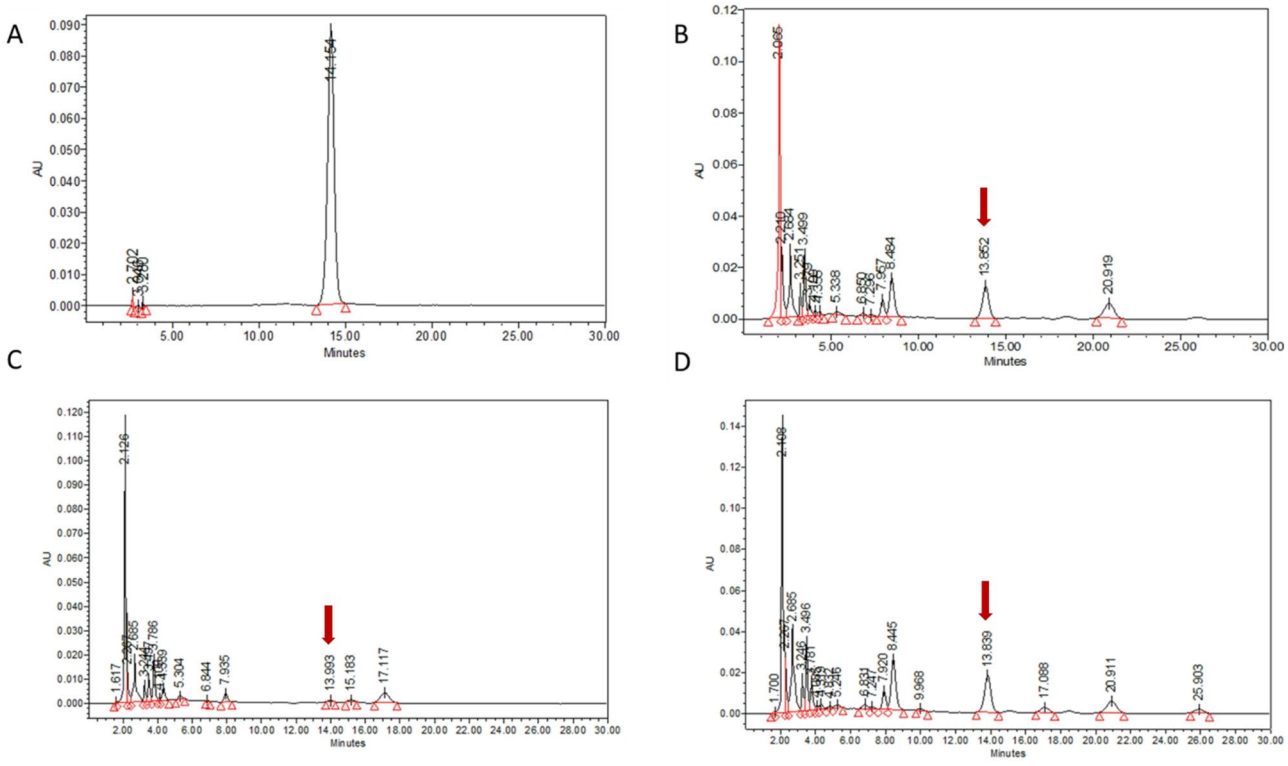

Fig. 3. HPLC chromatograms showing picroside-1 content. Here, A (P-1 standard), B (Control shoot), C (Indirect regenerated shoots), D (Direct regenerated shoots).

and 2). By requiring only 1–2 intermittent subculturing steps, compared to the 3–4 steps typical of indirect regeneration, this method accelerates the development of fully regenerated plantlets by approximately 2–3 weeks.

Picroside-I content in direct and indirect regenerated shoots

High-performance liquid chromatography (HPLC) estimations on sampled shoot biomass depicted a significant difference in Picroside-I (P-I) content amongst the direct and indirect regenerated shoots (Fig. 3). The shoots regenerated via direct organogenesis exhibited a higher P-I content compared to those regenerated through the callus-mediated method. Specifically, the mother plant showed a P-I content of 6.30 µg/mg, while callus-mediated, hence, indirectly regenerated shoots had a lower content of 3.41 µg/mg. In contrast, shoots regenerated through our newly devised direct regeneration regime recorded a relatively higher P-I content of 9.55 µg/mg (Figs. 1, 2 and 3).

Comparative gene expression analysis for P-I pathway genes

HPLC evidenced differential P-I content in in vitro PK shoot regenerants, following from the studied direct and indirect regenerants, necessitated analysis on any detectable difference in expression profiles for critical genes implicated in P-I biosynthesis. This could shed light on their contribution to P-I production under distinct culture conditions. Quantitative RT-PCR analyses over directly and indirectly raised regenerants revealed significantly upregulated RNAs corresponding to key genes in P-I biosynthesis, such as Phenylalanine Ammonia Lyase (PAL), 3-Hydroxy-3-Methylglutaryl-CoA Reductase (HMGR), and Geranyl Geranyl diphosphate Synthase (GS) in former (directly regenerated) shoots compared to those regenerated by latter regime (indirect) (Fig. 4a). These changes have been respectively annotated ahead over steps in the various P-I anabolic cascades (Fig. 4b).

Discussion

Our newly developed direct regeneration protocol for *Picrorhiza kurroa* is the result of extensive trials investigating the combinatorial effects of growth regulators—TDZ, KIN, and NAA—as well as various other permutations of phytohormones. These trials revealed distinct morphogenetic responses to varying phytohormone concentrations over time (Table 1). Among these, two specific combinations of TDZ and KIN demonstrated exceptional efficiency in direct shoot regeneration without an intervening callus phase, significantly reducing the regeneration timeframe. TDZ has been widely recognized in the literature as pivotal to shoot organogenesis and embryogenesis, with its effects amplified in combination with other growth regulators[38–45]. Our findings reaffirm its efficacy and further demonstrate its synergy with kinetin, which yielded an unprecedented shoot regeneration rate of 83% from leaf explants on MS medium supplemented with 0.5 mg/L TDZ and 1.5 mg/L KIN. This direct regeneration approach bypasses the callus phase, avoiding genetic and metabolic variability and mitigating the risk of somaclonal variations associated with callus-mediated systems. These findings highlight the potential of hormonal engineering to optimize tissue culture protocols for medicinal plant species.

The P-I content in directly regenerated shoots observed in our study was significantly higher than previously reported values[30,35–37]. This enhancement can be attributed to the elimination of the callus phase (which is known to disrupt metabolic pathways) and the reduction in subculturing steps, preserving the plant's secondary metabolite biosynthesis machinery. Additionally, the specific type and concentration of phytohormones used in direct regeneration play a critical role in promoting picroside biosynthesis, as corroborated by earlier studies[46]. Recent research has identified specific genes integral to the biosynthesis of picrosides, which are pivotal in regulating P-I levels in PK shoots. Studies by various groups[29,30,35] underscore the role of these genes in influencing the metabolic pathways leading to picroside production. In the present study, qRT-PCR analysis revealed differential expression of biosynthetic genes across the mevalonate (MVA) and iridoid pathways, closely

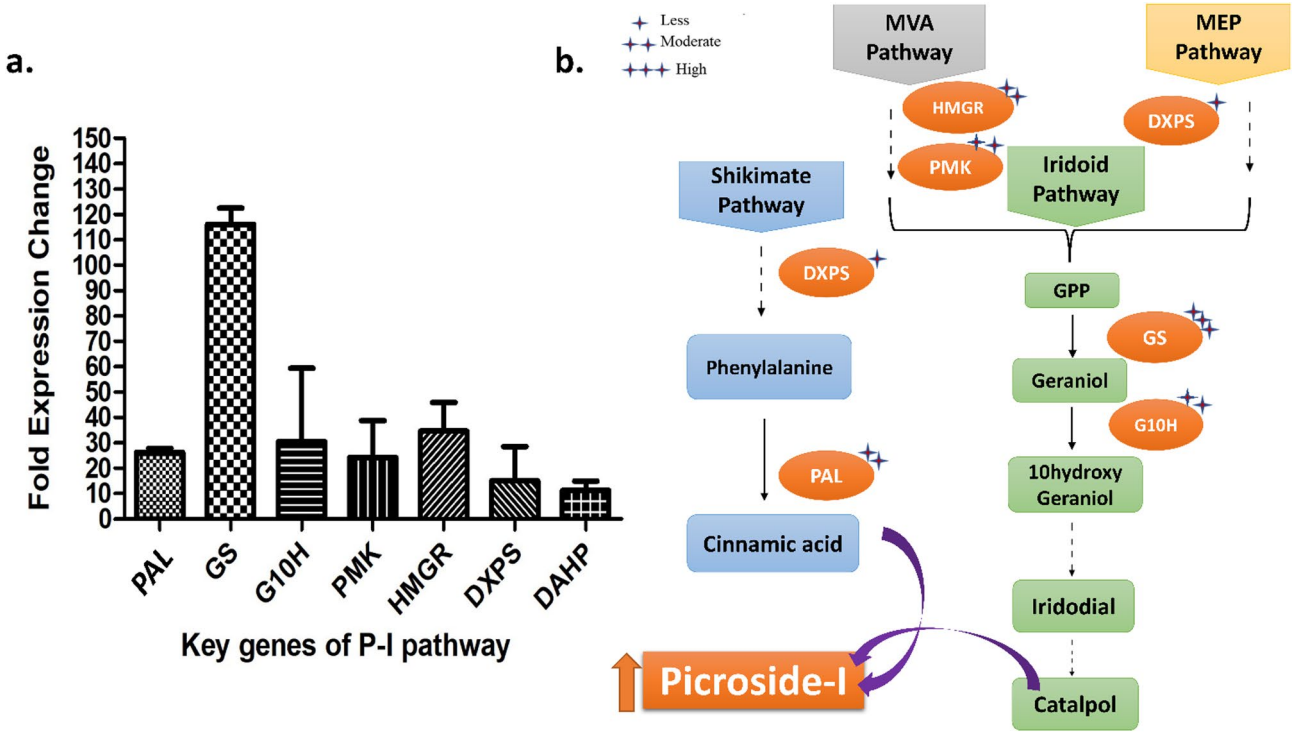

Fig. 4. Expression profiles of Picroside pathway genes. Here, **(a)** analysis of gene expression for critical genes implicated in the biosynthetic pathways of Picroside-I; and **(b)** the cascade representing key genes in Picroside-I biosynthesis under direct organogenesis. Note that in **b**, gene expressions were highlighted by blue stars with different ranges of up-regulation, while the orange-colored arrow indicates a highly significant increase in picroside-I content in direct regenerants[28].

correlating with the observed elevation in Picroside-I content in directly regenerated shoots. Specifically, *HMGR* (3-hydroxy-3-methylglutaryl-CoA reductase) and *PMK* (phosphomevalonate kinase), key enzymes in the MVA pathway, demonstrated markedly higher expression levels in shoot tissues derived via direct organogenesis. This suggests an active flux toward monoterpene biosynthesis, supporting findings by Pandit et al. (2013)[47]where tissue-specific upregulation of MVA genes was linked to picroside yield. Similarly, within the iridoid biosynthetic framework, genes such as *GS* (geraniol synthase) and *G10H* (geraniol-10-hydroxylase) showed significant transcriptional upregulation, reinforcing their roles in directing precursor molecules toward iridoid glycoside formation. Sharma et al. (2016)[35,37] previously reported 6–19-fold higher expression of these genes in shoot primordia, with direct correlation to Picroside-I biosynthesis. The observed expression patterns in our system not only validate the shoot-preferential activity of these pathways but also highlight the regulatory advantage of direct regeneration methods in preserving metabolite-linked transcriptomic signatures. This targeted gene-level activation supports the hypothesis that bypassing the dedifferentiation phase preserves metabolic integrity, thereby contributing to higher metabolite accumulation and paving the way for regeneration-linked biotechnological interventions.

Our results demonstrate a significant enhancement in P-I biosynthesis in directly regenerated shoots, corroborated by gene expression profiling. Notably, critical genes in the P-I biosynthetic pathway, including geraniol synthase (GS), exhibited upregulated expression in direct regeneration. These findings align with previous research by Bhat et al.[23,24] demonstrating the feasibility of manipulating plant biosynthetic pathways for improved secondary metabolite production. Additionally, key regulatory genes from the MVA (HMGR) and MEP (DXPS) pathways also showed elevated expression, further supporting their critical roles in the biosynthetic process. Our study correlates direct shoot regeneration in PK with elevated P-I production, surpassing the yields seen in indirect methods. The observed results are consistent with earlier reports on direct regeneration in medicinal plants. For example, Patial et al. showed that a 15-day TDZ exposure significantly enhances shoot regeneration in PK[20]. Sharma et al.[35,48] optimized direct regeneration protocols for *Gentiana kurroa* using a medium supplemented with 0.1 mg/L NAA and 0.75 mg/L TDZ. Similarly, Malik et al. (47) highlighted the importance of TDZ concentration and preculture duration in achieving efficient direct regeneration in *Arnebia euchroma*[49]. The findings of Pal and collaborators further demonstrated rapid, high-frequency regeneration in *Arnebia hispidissima*, while Hayta et al. standardized protocols for endangered species such as *Rhaponticoides mykalea*[46,50].

We conducted comparative studies on direct and indirect organogenesis, marking the first report of rapid, high-frequency direct regeneration of PK leaf explants focused on P-I production. In light of the previously established protocols[22,25,34–36]findings in this study revealed that directly regenerated shoots consistently exhibited higher P-I content compared to indirect regeneration. This superiority likely arises from the absence of dedifferentiation and re-differentiation phases, which typically alter genetic stability and disrupt metabolic integrity. Gene expression analysis further confirmed that directly regenerated shoots maintained elevated expression of GS, HMGR, and DXPS, indicating uninterrupted metabolic flux within the biosynthetic pathways. In contrast, callus-mediated systems exhibited negligible expression of these regulatory genes, reflecting the detrimental impact of the callus phase on metabolite production.

The study underscores the correlation between direct regeneration and upregulated expression of key biosynthetic genes, particularly GS, which plays a crucial role in the iridoid pathway. These findings are consistent with prior studies that identified GS as a key regulatory node in P-I biosynthesis[29]. Additionally, HMGR and DXPS genes from the MVA and MEP pathways exhibited significantly elevated expression, reinforcing their roles as critical metabolic regulators. Previous reports have suggested that the induction of shoot primordia activates essential biosynthetic enzymes[29]. In contrast, callus-based regeneration demonstrated negligible gene expression, necessitating subsequent reactivation. Our results indicate that direct regeneration sustains higher expression of key pathway genes, thereby preserving the metabolic integrity required for efficient secondary metabolite production.

Conclusions

In conclusion, our research has successfully advanced the protocol for direct shoot regeneration in *Picrorhiza kurroa* by leveraging the synergistic effects of TDZ and KIN, leading to rapid regeneration and significantly enhanced Picroside-I production. This optimized process bypasses the callus phase, avoiding metabolic inefficiencies and allowing for a more efficient metabolic flow toward secondary metabolite biosynthesis. The strong correlation between the regeneration mode and the upregulation of key biosynthetic genes, including HMGR, DXPS, and geraniol synthase, underscores the role of tailored growth hormone applications in maximizing phytochemical production. The significant increase in P-I content through direct regeneration highlights the interplay between regeneration techniques, growth conditions, and metabolite accumulation, paving the way for further exploration into the regulatory networks controlling P-I biosynthesis. Moving forward, efforts should focus on scaling up these findings for industrial applications by refining culture conditions, integrating genetic engineering, and adopting biotechnological innovations to optimize processes. The integration of plant cell and tissue culture systems with advanced biotechnological tools such as microfluidics, biosensors, and lab-on-a-chip (LOC) technologies would surely revolutionize the concept of plant tissue cultures as micromachines for the real-time monitoring and efficient production of pharmaceutically relevant metabolites[51–59]. This research aligns with multiple Sustainable Development Goals, such as SDG 3 (Good Health and Well-Being) by promoting the sustainable production of medicinal compounds like Picroside-I for pharmaceutical use; SDG 12 (Responsible Consumption and Production) by developing efficient, low-resource methods for producing high-value metabolites, supporting sustainable industrial practices; and SDG 15 (Life on Land) by contributing to the conservation and genetic improvement of endangered medicinal plant species. Thus, our study not only advances biotechnological approaches for the cultivation of PK but also contributes to the broader goal of

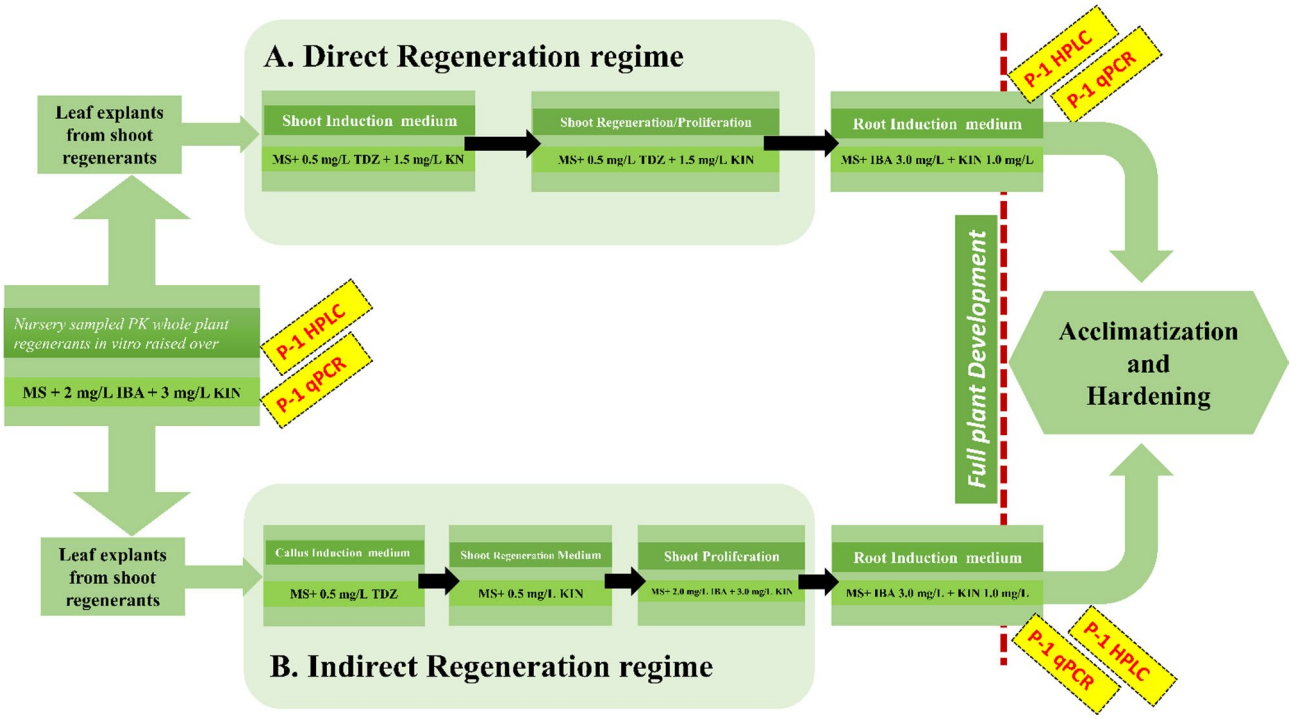

Fig. 5. Methodological scheme of work. A. a new direct regeneration method under optimization (this study); compared to that in B., a previously devised indirect regeneration regime used for PK[37].

ensuring sustainable production and conservation of valuable medicinal resources in alignment with global sustainability objectives.

Methods

Plant material and in vitro establishment

Picrorhiza kurroa (PK) plant material was obtained from the Himalayan Forest Research Institute (HFRI-ICFRE), Jagatsukh, Manali, Himachal Pradesh, India. Collection and identification of PK material was carried out by Prof. Hemant Sood, and a voucher specimen was deposited in the publicly available Herbarium Collections, at Jaypee University of Information Technology (Waknaghat, Solan, Himachal Pradesh, India; Deposit number: JUIT/HS/PK2018) and at ICAR - NBPGR (New Delhi, India; Accession No. IC0597952). Collection and use of plant materials adhered strictly to all applicable local, national, and international guidelines and regulations. Precisely, only leaf samples (and not the whole plants) were obtained exclusively from the sizeable nursery-cultivated populations maintained at the institute, with no materials sourced from wild or protected areas. Permissions were secured from the relevant municipal council and local authorities, ensuring compliance with educational and research-specific requisites. The study fully aligns with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora (CITES), ensuring no risk to the species' natural populations. The axenic shoot apices from nursery grown seedlings were directly excised rather than exhausting the whole plant(s) during PK sampling. These shoot tips were further used as explants for initiating regeneration using our previously worked PK micropropagation regime[47,60].

In brief, the explants were inoculated on MS medium supplemented with 3 mg/L indole-3-butyric acid (IBA), 1 mg/L kinetin (KIN), 30 g/L sucrose, and 9 g/L agar. The pH of the medium was adjusted to 5.8 before autoclaving. The cultures were maintained in a controlled environment growth chamber at 15 ± 2 °C a 16 h and under photoperiod with a photosynthetic photon flux density of 40 µmol/m2/s provided by fluorescent lamps (Philips, India), the relative humidity was maintained at 70% and subculturing routines performed at 4-week intervals to obtain sufficiently proliferated shoot biomass for further experiments. For comparative research, the leaf explants underwent in vitro regeneration under two techniques: direct regeneration (standardized in the present study) and indirect regeneration (previously standardized by our laboratory workers), and corresponding workflow and media compositions have been provided here (Fig. 5).

Direct and indirect regeneration trials

To determine the optimal conditions for callus formation followed by shoot regeneration, as well as direct shoot regeneration (without an intervening callus phase), leaf explants measuring 0.5–1 cm were excised from 3–4-week-old in vitro-regenerated PK plants and subjected to distinct experimental trials. Each trial involved culturing explants on MS basal medium supplemented with specific combinations of growth regulators, including TDZ paired either with kinetin KIN or NAA (as in Table 1). The influence of these growth regulators

on callus induction and shoot regeneration was rigorously evaluated. In each experimental setup, 5–6 leaf explants per culture vessel, positioned with their abaxial surfaces in contact with the medium, were incubated under controlled conditions (15 ± 2 °C temperature, a 16-hour photoperiod provided by cool white fluorescent light at 3,000 lx). Medium formulations lacking growth regulators served as controls. Key parameters assessed included the percentage of callus formation, rate of shoot regeneration, number of shoots per explant, time required for shoot regeneration, and the number of subculturing cycles. Each trial was performed in triplicate and repeated thrice to ensure the reliability and reproducibility of the results.

Rooting, acclimatization, and hardening of shoot regenerants

Shoot regenerants (3.5–4.5 cm) derived from both direct and indirect regeneration trials were transferred to a root-induction medium comprising MS basal salts supplemented with 3 mg/L IBA and 1 mg/L KIN (Fig. 5), to facilitate complete plantlet development[22,35,36]. Each trial consisted of 3 shoot regenerants, with 5 replicates per treatment, resulting in a total of 15 shoots per treatment group. For optimal root elongation, root-induced plantlets were subjected to weekly subcultures over at least three weeks on this same root-induction medium. To achieve acclimatization and hardening, the plantlets were carefully removed from culture vessels, thoroughly rinsed under running tap water to eliminate residual agar, and subsequently transplanted into pots containing a substrate mixture of sand, soil, and vermiculite in a 1:1:1 ratio. Each plantlet was placed in a separate pot, and 15 pots were used per treatment group. The potted plantlets were maintained under greenhouse conditions (temperature 25 ± 2 °C, relative humidity 80–90%) with exposure to natural light. During the initial acclimatization phase, the plantlets were enclosed in glass jars for one week to ensure a controlled microenvironment. The jars were then removed to permit hardening under ambient conditions over three weeks, after which the plantlets were transplanted into garden soil in the nursery. To mitigate the risk of fungal contamination, irrigation was supplemented with a 0.5% Bavistin solution during the acclimatization process.

Estimation of Picroside-I content

Shoot regenerants measuring 6.0–7.0 cm in length were selected for the quantification of picroside-I (P-1) content. Triplicate samples were individually harvested from each of the direct and indirect regeneration regimes during the rooting stage from fully developed plantlets (before initiating any acclimatization treatments) and subsequently stored at − 80 °C for HPLC analysis. Additionally, samples were collected from in vitro-raised regenerants of mother plants to assess variations in P-I content. Each shoot sample, weighing 200 mg, was finely pulverized using liquid nitrogen and subsequently resuspended in 80% methanol to ensure homogenization for further analysis. The resulting mixture was centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatant was filtered through 0.22 µm syringe filters. P-I quantification was performed using reverse-phase high-performance liquid chromatography (HPLC) (Waters 515) with a C18 column (5 µm, 4.6 × 250 mm Waters Symmetry). Detection was achieved using a photodiode array (PDA) detector (Waters 2996). Before injection, the samples were diluted and eluted using an isocratic mode with two solvent systems: 0.05% trifluoroacetic acid and a 1:1 mixture of methanol and acetonitrile. Compounds were identified by comparing retention times and UV spectra with authentic Picroside-I standards (ChromaDex, Inc.), following the previously described method[36].

Gene expression analysis in Picroside biosynthesis pathway

To analyze the expression profiles of critical genes associated with the picroside biosynthesis pathway in PK, previously identified target genes[29,30] were selected for evaluation in both direct and indirect in vitro regenerated PK shoots. These genes encode key enzymes across three major biosynthetic pathways: (i) Mevalonate (MVA) pathway: 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR) and phosphomevalonate kinase (PMK); (ii) Methylerythritol 4-phosphate (MEP) pathway: 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), 2-C-methylerythritol 4-phosphate cytidyltransferase (ISPD), and 4-(cytidine-5-diphospho)−2-C-methylerythritol kinase (ISPE); and (iii) Phenylpropanoid (PP) pathway: geraniol synthase (GS), geraniol-10-hydroxylase (G10H), 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase (DAHPS), and phenylalanine ammonia-lyase (PAL). Together, these pathways contribute to the biosynthesis of secondary metabolites, including picrosides, which are integral to the therapeutic efficacy of PK. Understanding the differential expression of these genes in direct and indirect regeneration conditions offers insights into the metabolic adjustments during tissue culture. For this, total RNA was extracted from the shoot tissues using TRIZOL® reagent (Invitrogen), following the manufacturer's protocol, and the quality of RNA was assessed using a Nanodrop 2000 spectrophotometer (Thermo Scientific, USA). High-quality RNA was converted to cDNA using the Verso cDNA synthesis kit (Thermo Scientific), ensuring optimal yields for downstream applications. All procedures adhered strictly to the kit manufacturer's instructions to ensure the reproducibility and accuracy of the results. Quantitative real-time PCR (qRT-PCR) was conducted using gene-specific primers (Table S1) and SYBR Green Mastermix (Bio-Rad®) on the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Each cDNA sample was analyzed in triplicate under identical conditions. The thermal cycling protocol included an initial denaturation step at 94 °C for 5 min, followed by 40 cycles consisting of denaturation at 94 °C for 20 s, annealing at 48–60 °C for 30 s (optimized for each primer pair), and extension at 72 °C for 20 s. Expression levels were normalized against the housekeeping gene 26 S ribosomal RNA, ensuring consistency across samples. Relative transcript levels were calculated using the 2^-ΔΔCT method, facilitating precise comparisons of gene expression under different experimental setups.

Statistical and computational methods

Each experiment was conducted in triplicate, with a minimum of three biological replicates included in each iteration. Results are expressed as mean ± standard deviation (SD), presented in tabular format, and depicted with error bars in graphical representations. Statistical analysis was performed using analysis of variance (ANOVA)

to identify significant differences between group means. For post-hoc comparisons, Duncan's multiple range test (DMRT) was employed at a significance threshold of 5% ($p < 0.05$). Graphical visualizations were created using Microsoft Excel, and composite figures were designed using Microsoft PowerPoint.

Data availability

The corresponding authors are committed to providing any raw data supporting the findings of this article upon formal request, without any undue reservation.

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Author contributions

Conceptualization, H.S. and S.S.; methodology, H.S., S.S.; software, H.S., G.A.; validation, G.A, S.S., G.M.; formal analysis, H.S., S.S., A.C.; investigation, S.S., H.S., G.M.; resources, S.S., H.S., G.M.; data curation, S.S., G.M., A.C.; writing—original draft preparation, H.S., S.S., G.M.; writing—review and editing, S.S., H.S., G.A., G.M., visualization, S.S., A.C., G.M.; supervision, H.S.; project administration, H.S.; supervision, software, writing—original draft preparation G.M., and G.A. All authors have read and agreed to the published version of the manuscript.

Declarations

Competing interests
The authors declare no competing interests.

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