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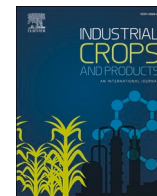
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Overexpression of the DXR reductoisomerase (DXR) gene increases the production of valuable biologically-active compounds in the transgenic hairy roots of *Plectranthus venter* (van Jaarsv. & Hankey)

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ABSTRACT

Transgenic hairy root cultures offer a sustainable alternative for producing high-value bioactive compounds. This study aimed to generate *Plectranthus venter* hairy roots overexpressing the 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) gene of the methylerythritol phosphate pathway (MEP) to enhance diterpenoid biosynthesis. Stable integration of the DXR transgene was confirmed by molecular analyses. DXR-overexpressing hairy root clones showed increased accumulation of valuable diterpenoids compared with unmodified controls transformed with a pGFPGUSplus vector. The best-growing clone was scaled up in a 10 L sprinkle bioreactor, resulting in markedly higher levels of selected metabolites. The averaged peak area of coleon U-quinone isomer 1 was **17.3-fold** higher in the bioreactor culture and **10.5-fold** higher in the Erlenmeyer flask culture relative to the unmodified sample, while coleon U isomer 2 levels were **3.66-fold** and **5.19-fold** higher in the bioreactor and Erlenmeyer cultures, respectively. High-resolution mass spectrometry also identified dihydroxyroyleanone, royleanone derivatives, horminone, and 7 α -hydroxyroyleanone, compounds known to have anticancer, antimicrobial, and anti-inflammatory activities. These findings demonstrate that combining DXR overexpression with bioreactor cultivation significantly enhances diterpenoid production in *P. venter* hairy roots and may serve as an efficient platform for sustainable production of pharmacologically relevant metabolites.

1. Introduction

The endogenous pathways of plants can be modified to generate new compounds, or enhance or delay the production of existing ones, an approach known as metabolic engineering (Lu et al., 2016; Zhou and Pichersky, 2020). Unlike simple genetic engineering, metabolic engineering enables the manipulation of entire bioprocesses by treating the metabolic and cellular system as a whole (Lu et al., 2016; Markus Lange and Ahkami, 2013; Nagegowda and Gupta, 2020). This approach allows tremendous changes to plants to be made on the metabolic level, and can be an effective means of enhancing the production of secondary metabolites that have medicinal value (Lu et al., 2016; Pickens et al., 2011).

For example, previous research has found such manipulation to increase the yields of phenolic acids and pentacyclic triterpenes (Kowalczyk et al., 2021b, 2020a; Sitarek et al., 2018b, 2018a).

One of the most varied classes of secondary products, with over 40,000 known examples, is that of the terpenoids (Lu et al., 2016). These compounds originate from two precursor molecules: isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) (Böttger et al., 2018). In higher plants, these are synthesised via the mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway. The MVA pathway is found mainly in the cytosol, while the MEP pathway is located in plastids. Regarding the latter, the enzymes of the MEP pathway are encoded in the nuclear genome; they are

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synthesised and transported into plastids (Markus Lange and Ahkami, 2013; Pulido et al., 2013; Silva et al., 2020; Simkin et al., 2011) where they occupy a sub-plastid localization and facilitate terpenoid production (Joyard et al., 2009; Nagegowda and Gupta, 2020; Vranová et al., 2013). The key rate-limiting enzymes of the MEP and MVA pathways are 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and hydroxymethylglutaryl-CoA reductase (HMGR), respectively. These enzymes are responsible for catalyzing the second step of terpene biosynthesis in chloroplasts (Markus Lange and Ahkami, 2013; Tholl, 2015).

An important enzyme acting at the beginning of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway is 1-Deoxy-d-xylulose 5-phosphate reductoisomerase (DXR) (Markus Lange and Ahkami, 2013; Nagegowda and Gupta, 2020). The DXR gene catalyzes the rearrangement and reduction of 1-Deoxy-d-xylulose-5-phosphate (DXP) to 2-C methyl-D-Erythritol 4-phosphate (MEP) in an NADP-dependent manner; this is a key component of the MEP pathway that supports the major C5 units in the formation of mono- and diterpenes (Banerjee et al., 2013; Nagegowda and Gupta, 2020; Wang et al., 2019). As DXRs play such an important role in the MEP pathway, there has been growing interest in studying their function with the aim of increasing terpenoid yield (Tong et al., 2015; You et al., 2020; Zhang et al., 2021).

A number of terpenoids have been extracted from medicinal plants such as *Taxus chinensis*, *Artemisia annua* or *Salvia miltiorrhiza*. While many of these compounds have excellent therapeutic properties and have been used to treat various conditions, such as malaria (artemisinin) and cancer (paclitaxel), their low yield from natural sources prevents widespread implementation (Li et al., 2025; Lu et al., 2016; Mandić et al., 2025; Nagegowda and Gupta, 2020). Several plant biotechnology strategies have been developed to increase the yields of valuable secondary metabolites. Among them, the most promising strategy involves the use of highly productive transgenic hairy roots. These cultures combine fast growth without exogenous regulators, high genetic and biochemical stability, and the capacity to synthesize target metabolites at elevated levels, which makes them particularly suitable for scale-up in bioreactor systems (Aghaali et al., 2024; Georgiev et al., 2012; Giri and Narasu, 2000; Mirmazloum et al., 2024). The efficiency of hairy root cultures has been further enhanced by introducing transgenes to boost specific biosynthetic pathways. For instance, Tong et al. (2015) cloned and characterized the *DXS* and *DXR* genes from *Tripterygium wilfordii* and demonstrated that *DXR* overexpression resulted in a significant increase in triptophenolide accumulation. Triptophenolide is a bioactive diterpenoid with notable anti-inflammatory and antitumor properties. These findings provide molecular evidence that enhancing the expression of MEP pathway enzymes can effectively boost the production of valuable secondary metabolites and support the use of *DXR* as a metabolic engineering target for improving diterpenoid yields (Tong et al., 2015).

An interesting plant with a wide spectrum of activity is *Plectranthus venterii*, of the *Lamiaceae* family. Its native range extends from southern Zimbabwe to the Limpopo (Leolo Hills). It is a semi-succulent sub-shrub and is found mainly in the sub-tropical biome. Many species of *Plectranthus* have been used in Ayurvedic medicine to address various conditions, such as problems with the digestive system, skin, respiratory system, and genitourinary system. Numerous studies have shown it to possess a range of compounds with *inter alia* analgesic, anti-cancer, antimicrobial, and anti-inflammatory properties. This plant is an endemic species that accumulates high levels of abietane-type diterpenoids, which are biosynthesized via the plastidial methylerythritol phosphate (MEP) pathway involving 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). The ability of hairy root cultures to enhance diterpenoid biosynthesis in related *Plectranthus* species has already been demonstrated (Lukhoba et al., 2006; Maree et al., 2014; Rabe and Van Staden, 1998; Rice et al., 2011).

This present study examines the effect of cultivation scale and biotechnology strategy on productivity in transgenic HRDXR clones of *Plectranthus venterii*. The primary objective of the study was to acquire

transgenic hairy roots of *P. venterii* (van Jaarsv. & Hankey) with confirmed overexpression of the *DXR* gene. It also examines the influence of culture scale (Erlenmeyer flask or 10-L bioreactor) on *DXR* gene expression and biomass production in the transformed culture. Finally, it compares the profiles of selected metabolites. Our results provide only an initial evaluation of the feasibility of obtaining efficient hairy root culture: further analyses are needed to confirm that the selected enzymes play a role in enhancing terpenoid biosynthesis.

2. Materials and methods

2.1. Plant in vitro cultures and explant preparation

The *in vitro* plant cultures used for genetic transformation were obtained as previously described for *Leonotis nepetifolia* (Kowalczyk et al., 2021a). Briefly, *P. venterii* seeds were surface sterilised in 70 % ethanol and then in 4 % NaClO with the addition of a drop of Tween-20. The seeds were rinsed thoroughly multiple times with sterile distilled water. The sterilized and dried seeds were placed on sterile 1/2 MS medium without sucrose for germination. The 2–3 cm seedlings were then transferred to agar-solidified (0.8 %) MS medium (Murashige and Skoog, 1962) containing 3 % sucrose in sterile glass jars. The seedlings were allowed to grow in the glass jars at 22 °C with a photoperiod of 8/16 h (day/night) for one month. These were used as starting material for transformation (Fig. 2A).

2.2. Construction of a recombinant binary vector for plant transformation

The cultures were transformed using a recombinant vector based on the pGFPGUSPlus plasmid (Fig. 1B); this was obtained as a gift from Claudia Vickers (Addgene plasmid # 64401; <http://n2t.net/addgene:64401>; RRID:Addgene_64401). Complete CDS of 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR, GenBank accession number KF941189.1), was synthesised and provided by Biomatik Co. (Canada). The pUC57-DXR plasmid was hydrolysed using the *Xba*I and *Sac*I restriction enzymes, which were then used to clone the DXR coding sequence into the binary vector. In the pGFPGUSPlus vector, the sequence encoding the EGFP protein was removed and replaced by the DXR coding sequence, resulting in the production of a vector carrying the DXR cds under the control of the CaMV35S promoter and harboring the NOS terminator (Fig. 1A).

2.3. Preparation of *Rhizobium rhizogenes* competent cells and bacterial transformation

Rhizobium rhizogenes (A4 strain) was pre-cultured in 5 mL of YMB (Yeast Mannitol Broth, 0.1 % Yeast Extract, 0.02 % MgSO₄, 0.05 % KH₂PO₄, 0.01 % NaCl and 1 % mannitol) liquid culture medium, incubated at 28 °C for 48 h with continuous agitation at 200 rpm. Following this, 100 µL of the 10x diluted culture was plated on a Petri dish with YMB medium solidified with 1.5 % agar and cultured at 28 °C for 48 h. Bacteria derived from single colonies were cultured in liquid YMB medium until reaching the appropriate optical density ($A_{600}=0.8$). The bacterial cultures were harvested by centrifugation (1000 rpm/10 min.) and then washed and finally resuspended in 20 mM CaCl₂. The bacteria were incubated on ice for one hour and used for genetic transformation. The bacterial cells were transformed with recombinant and non-recombinant vectors as described previously (Kowalczyk et al., 2020a).

2.4. Plant genetic transformation for hairy root induction

Hairy root induction was carried out as follows: the transgenic *R. rhizogenes* (A4 strain), harboring both the recombinant and original pGFPGUSPlus vectors, was cultured in liquid YMB with kanamycin (50 mg/L); bacteria without the plasmid were cultured without

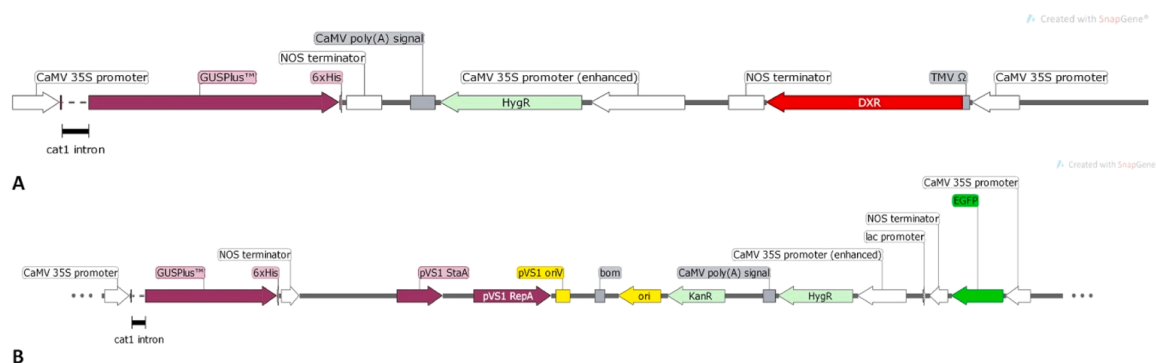


Fig. 1. Schematic representation of expression cassettes used in plant transformation. A- cassette containing the DXR gene, B- cassette present in the original pGFPGUSPlus vector. SnapGene® software (from Dotmatics; available at snapgene.com).

antibiotic. All cultures were incubated at 28 °C with vigorous shaking (200 rpm) until reaching OD₆₀₀ = 0.5.

The bacterial culture was centrifuged (10 min, 4000 rpm), and the pellet was suspended in an appropriate volume of MS medium to obtain OD₆₀₀ values of 0.4, 0.6, and 0.8 with an appropriate concentration of acetosyringone (0, 50, 100, and 200 µM). For each experiment, 30 plant explants were used in triplicate. The stems and leaves of the *in vitro* grown seedlings were infected by the modified dipping method, i.e. they were wounded manually with a 24 gauge medical needle, immersed in a bacterial suspension, and then incubated for 15 min (Kowalczyk et al., 2021a).

In addition, a vacuum infiltration method was also tested. The plant explants were placed in a 50 mL tube with 25 mL of the respective bacterial suspensions. The open tubes were placed inside a vacuum desiccator, and a vacuum was applied three times for six minutes each, with two-minute breaks between each application. Following this, all explants were dried on sterile paper, divided into four groups, and co-cultivated on MS basal medium at 25 °C for one, three, five, or seven days. Sterile MS medium without *Rhizobium* cells was used as a control. After co-cultivation, the explants were washed with sterile distilled water containing 500 mg/L cefotaxime to eliminate the bacteria. (Bio-taxime, Polpharma, Poland), dried on sterile paper and transferred to MS medium supplemented with 3 % sucrose and 250 mg/L cefotaxime. The explants were kept at 16 h light and 8 h dark cycles, with passages every seven days, until hairy roots appeared.

The transgenic hairy roots were selected as follows. Briefly, 2–3 cm roots were placed onto MS solid medium (1.5 % agar) supplemented with 3 % sucrose, 250 mg/L cefotaxime, and 25 mg/L hygromycin B. All root cultures were incubated in the dark at 25 °C, and transferred into new medium every 14 days. After the fourth passage, the roots were transferred to liquid MS medium and cultured at 25 °C at 80 rpm on an orbital shaker in the dark; the antibiotic concentrations were steadily reduced (250 mg/L, 125 mg/L, 62.5 mg/L, 31.25 mg/L and 0 mg/L for cefotaxime and 25 mg/L, 12.5 mg/L and 0 mg/L for hygromycin B).

2.5. DNA isolation and PCR analysis

Hairy root DNA was extracted using a Genomic Mini AX DNA purification kit (A&A Biotechnology, Gdansk, Poland) following the manufacturer's instructions. The isolated DNA samples were analysed by PCR using specific primers (Table 1 Supplementary Data). The PCR reaction mixture was composed of 100 ng of template DNA, 1 µL of each 10 µM primer, 12.5 µL of PCR Mix Plus master mix (A&A Biotechnology, Gdańsk, Poland) and 11 µL of PCR Ultra-Pure Water (Bliert). The reaction conditions were as follows: 5 min at 94 °C then 30 cycles: 45 s at 94 °C, 45 s at (see Table 1 Supplementary Data), and 45 s at 72 °C, with a final extension of 72 °C for 5 min. DNA amplification was performed in a Techne TC-3000X PCR Thermal Cycler. The reaction products were visualized on 1.5 % agarose gel stained with EtBr.

2.6. GUS assay

Histochemical testing of hairy roots for β-glucuronidase (GUS) activity was performed on previously PCR-selected and hygromycin B-resistant hairy root lines. For the histochemical determination of β-glucuronidase, 14-day-old hairy root cultures were used. For this purpose, root fragments were incubated (37 °C for 24 h) in a solution containing the substrate for GUS: 100 mM sodium phosphate buffer (pH 7.0) with 2 mM X-Gluc (Duchefa Biochemie, Haarlem, the Netherlands), 0.05 mM potassium ferrocyanide, 0.05 mM potassium ferrocyanide and 0.1 % (w/v) Triton X-100. After this time, the solution was discarded, and the plant tissue was rinsed with 70 % (v/v) ethanol.

2.7. RNA extraction, cDNA synthesis and RT-PCR

The DXR expression profiles of the transgenic *Plectranthus venterii* roots were determined using RT-PCR. Total RNA was extracted from each of five clones using a Plant RNA Mini Kit (A&A Biotechnology, Poland) according to the manufacturer's guidelines; RNA sample quality and quantity were assessed using a spectrophotometer. Following this, 1 ng of RNA was used to synthesise the first strand of cDNA using the TransScriba kit (A&A Biotechnology, Poland), all according to the manufacturer's instructions. Quantitative real-time PCR was then performed using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, USA) as follows: 10 µL of mixture contained 1 µL of cDNA, 0.313 µM of each primer (F: 5'-CAGAAGGATGGTGGAGCAAA-3' and R: 5'-ATGAC-CAGGCTCTACAACCG-3') and 5 µL of Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, USA); 10 min of initial denaturation at 95 °C, followed by 40 cycles of 30 s at 95 °C and 60 s at the primer annealing temperature (T_m). Each reaction was performed in triplicate. Elongation factor 1α (EF-1α) served as the reference gene. Relative DXR expression was determined using the 2^{-ΔCt} method, where ΔCt represents the difference between the threshold cycle (C_t) values of the target gene and those of EF-1α (F: 5'-TGAGATGCACCACGAAGCTC-3', R: 5'-CCAACATTGTCACCAGGAAGTG-3') (Schmittgen and Livak, 2008).

2.8. Hairy root cultures in Erlenmeyer flasks and sprinkle bioreactor

The most vigorously-growing hairy root clone was selected for further cultivation. The selected two-week-old clone, confirmed as GUS-positive, had been grown in Erlenmeyer flasks with intensive shaking (150 rpm, 25 °C in the dark). It was used to initiate two sets of cultures. The first set was performed in Erlenmeyer flasks, each with 50 mL of liquid Schenk and Hildebrandt medium with 3 % (w/v) sucrose (intensive shaking, 150 rpm, 25 °C in the dark). The second set was carried out in a sterilized 10-liter sprinkle bioreactor with 2 L of medium according to Kowalczyk et al. (2021b); the liquid medium was dispensed through a nozzle for 30 s with an interval of 180 s, which allowed approximately 60 mL of medium to be delivered to the growing roots

Table 1
The efficiency of hairy root induction depending on transformation conditions.

	Leaves					Stems				
	Acetosyringone concentration (μM)									
	0	50	100	200		0	50	100	200	
Modified DM	9.33 ± 2.51 ^a	10.0 ± 1.0 ^a	24.0 ± 1.53 ^b	15.67 ± 2.03 ^c		9.0 ± 1.0 ^a	12.33 ± 3.29 ^{ab}	19.67 ± 0.88 ^b	17.67 ± 0.88 ^b	
Vacuum infiltration	5.67 ± 1.53 ^a	8.0 ± 1.73 ^a	21.33 ± 0.88 ^b	14.33 ± 1.67 ^c		9.33 ± 1 ^a	9 ± 0.58 ^a	17.33 ± 1.33 ^b	18.67 ± 0.33 ^b	

per one sprinkle cycle. Again, the second culture was conducted at 25 °C in the dark.

2.9. Determination of fresh and dry weight of normal and hairy roots

Root biomass was harvested at the end of the cultivation period and gently blotted on sterile filter paper to remove excess of culture medium. The fresh weight (FW) was immediately recorded using an analytical balance. Subsequently, the roots were dried at 50 °C for 48 h in a drying oven until a constant weight was achieved. The dry weight (DW) was then measured. Biomass growth was calculated by weighing the roots after the cultivation cycle was completed and after drying. All results came from three independent cultivations.

2.10. HPLC-MS-ESI-QTOF-MS fingerprinting of the *Plectranthus venteri* root extracts with overexpression of *DXR* gene

The root compounds were extracted with three solvents: ethyl acetate, ethanol (96 % v/v) and 50 % ethanol extracts. Their contents were compared using an Agilent Technologies 1200 Series HPLC chromatograph (Santa Clara, California, USA), including a degasser, a binary pump, a low flow-rate pump for a calibration mixture, an autosampler, a UV detector, and a column thermostated chamber. Additionally, an ESI-QTOF-MS/MS detector (G6530B) was attached to the system. The obtained extracts were separated on an RP-18 Phenomenex chromatographic column (150 mm×2.1 mm, pore size of 5.0 μm) at 20 °C, flow rate 0.3 mL/min; the extraction used the following gradient acetonitrile with 0.1 % formic acid (solvent B) in 0.1 % aqueous solution of formic acid: 0 min – 1 % of B, 10 min – 20 % of B, 15 min – 40 % of B, 17–22 min – 95 % of B, 22.1–30 min – 1 % of B. The following gradient was used for the positive ion mode determinations: 0–2 min – 1 % of B, 3 min – 30 % of B, 13 min – 45 % of B, 18 min – 65 % of B, 28 min – 80 % of B, 30–35 min – 95 % of B, 36–45 min – 1 % of B. The mass detector settings were as follows: positive and negative ion modes, with 1000 V of nozzle voltage setting, 3000 V of capillary voltage, 110 V of fragmentor voltage, 65 V of skimmer voltage, 275 and 325 °C of gas and sheath gas temperatures, respectively, 12 L/min gas flows and 35 psig nebulizer pressure. The collision energies were selected as 10 and 20 V.

The MS/MS spectra were recorded twice for a given *m/z* feature and the fragmented signal was later excluded for the following 0.3 min. The injection volume was set at 10 μL. The analysis was performed on a freshly-calibrated apparatus; the sample was co-injected with a constant on-line flow of the calibration mixture (0.04 mL/min) to ensure *m/z* stability and accuracy. The data were processed by Mass Hunter Workstation (version B.10.00, Agilent Technologies, Santa Clara, CA, USA).

The positive ion mass chromatograms were found to be richer in metabolites. Both negative and positive ion injections were later analyzed with Mass Hunter Profinder 10.0 and Mass Profiler Professional (v.15.1 Agilent Technologies, Santa Clara, CA, USA) software. The molecular features of the extracts were tentatively identified according to the inbuilt Metlin database. The MS/MS fragmentation patterns were compared with other databases (Metlin, HMDB) and existing scientific literature.

2.11. Statistical analysis

Statistical analysis was performed with the SigmaPlot. 11.0 software package (SigmaPlot. 11.0; Systat Software, Inc., San Jose, CA). The significance of differences was analyzed using Student's t-test. Differences in values were considered to be significant at $p < 0.05$. The results were depicted with Graph Pad Prism v8.0.1 software (GraphPad Software, Inc., San Diego, California, USA). Statistically significant data are indicated by asterisks (***) $p < 0.001$, * $p < 0.05$.

3. Results

3.1. Establishment of hairy root cultures from *Plectranthus venteri*

The bacterial transformation procedure resulted in the successful creation of transgenic hairy root cultures (Fig. 2B and C). The best transformation efficiency was obtained using the modified dipping method (DM) in combination with 100 μ M acetosyringone and a cultivation time of five days (Table 1).

Efficiency of hairy root induction (mean $\pm$ SE) under different transformation conditions using the Modified DM and Vacuum infiltration methods. Different letters (a, b, c) indicate statistically significant differences ($p < 0.05$) within the same explant type and transformation method, based on Tukey's post hoc test.

3.2. PCR-based characterization of hairy root lines

The PCR results confirmed the presence of *R. rhizogenes*-specific genes in the genomic DNA of the obtained hairy root lines (Fig. 2D). As expected, bands were detected in all hairy root lines (Fig. 3) but none in the normal roots (NR, control). These results confirm that the roots obtained are hairy roots and contain the desired T-DNA fragment with the CDS for DXR in their genome.

3.3. Determination of gene overexpression DXR gene in hairy root lines

The DXR gene profile varied between clones 1–4 (HRDXR 1–4). The highest level of DXR gene expression was observed for clone 3 (HRDXR 3), and the lowest for clone 4 (HRDXR 4) (Fig. 4). Based on DXR gene expression and the rate of root growth (growth intensity), HRDXR3 clone was selected for further work; the clone was then grown in parallel in a bioreactor and an Erlenmeyer flask.

3.4. Histochemical GUS assay

The hairy root clones containing the DXR gene (HRDXR 1–4) and the clone transformed with unmodified vector (HRDXR-) were tested for the activity of β -glucuronidase encoded by the GUSPlus gene present in the

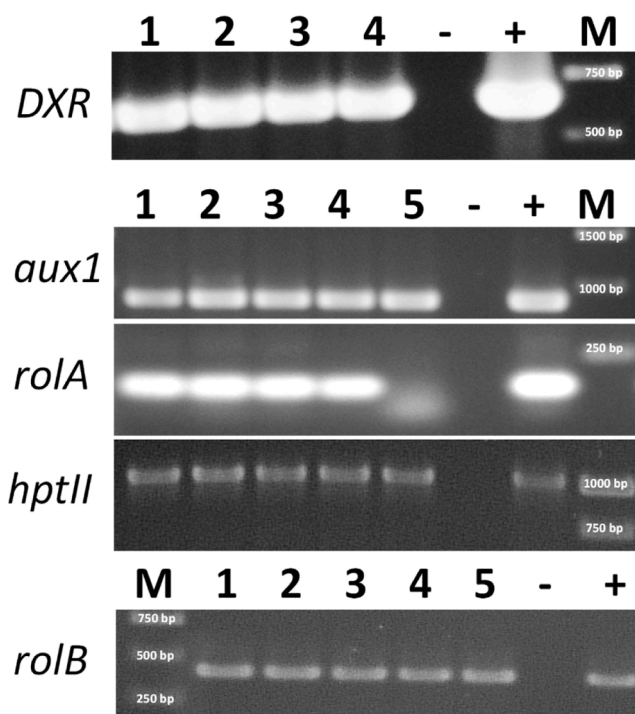


Fig. 3. Electrophoresis gel confirming the presence of the tested genes: *dxr*, *aux1*, *rolA*, *hptII* and *rolB*. For DXR: Columns 1–4 indicate respective independent clones of hairy root with overexpression of DXR (HRDXR1–4), Column (-) shows the PCR results obtained using DNA isolated from normal roots; Column (+) indicates genes associated with transgenic *R. rhizogenes*; For *aux1*, *rolA*, *hptII*, *rolB*: Columns 1–5 indicate respective independent clones of hairy root with overexpression of DXR (HRDXR1–4); Column 5 indicates hairy roots obtained by transformation with original pGFPUSPlus vector without the intended genes; Column (-) shows the PCR results obtained using DNA isolated from normal roots; Column (+) indicates genes associated with transgenic *R. rhizogenes* M: DNA molecular weight marker.

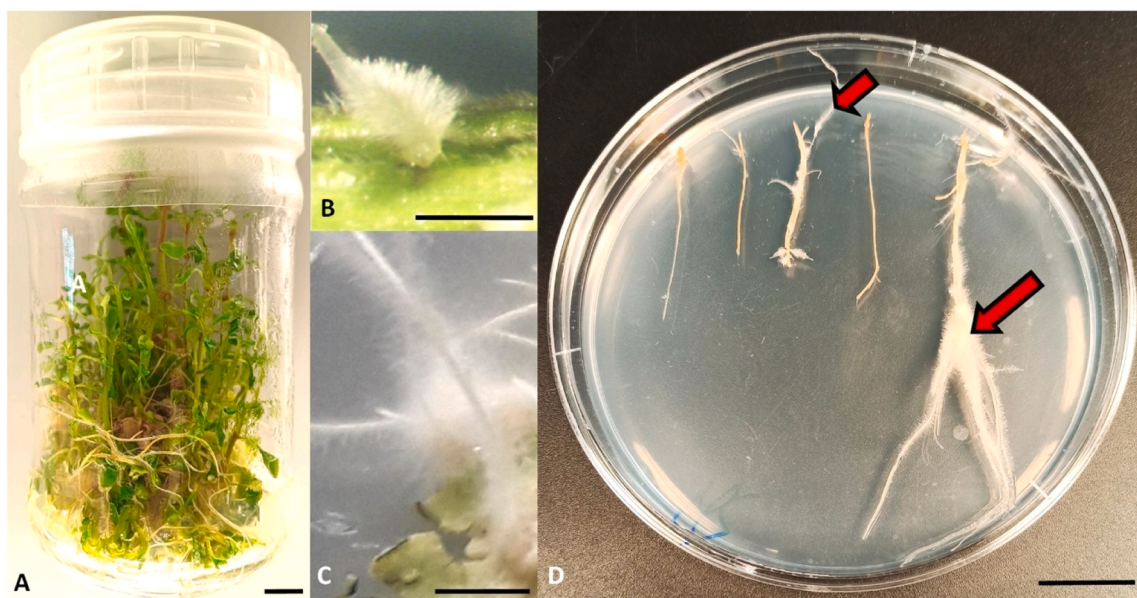


Fig. 2. Subsequent stages of hairy root (HRDXR clones) induction on plant material. A- *Plectranthus venteri* in vitro culture, B- Forming hairy roots on stem at the bacterial penetration site 14 days after inoculation, C- Forming hairy roots on leaf at the bacterial penetration site 14 days after inoculation D- Hairy roots on selection medium (supplemented with Hygromycin B). Red arrows indicate transgenic hairy roots, bar= 0.5 cm for B,C, bar= 1 cm for A,D.

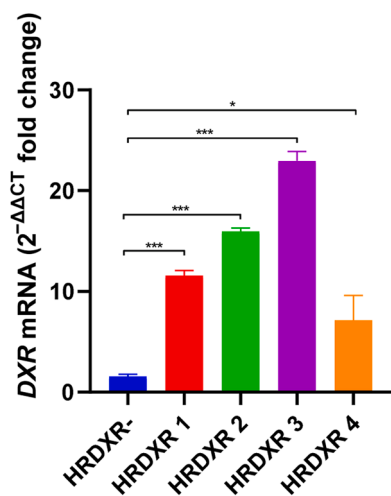


Fig. 4. Relative mRNA expression of DXR in five independent transgenic clones. Control (HRDXR-, hairy roots clone obtained by empty plasmid vector *R. rhizogenes* transformation). Results are expressed as the mean fold change, with error bars representing the standard deviation. All experiments were carried out in triplicate. Elongation factor 1 α (EF-1 α) served as the reference gene. Statistics were performed using paired *t*-test. **p* < 0.05, ***p* < 0.001, ****p* < 0.001.

pGFPGUSPlus vector; a positive result was confirmed by the formation of a blue product (5,5'-dibromo-4,4'-dichloro-indigo). As expected, all tested HRDXR lines were found to exhibit β -glucuronidase activity, but not the untransformed control lines (Fig. 5).

3.5. Hairy root growth in Erlenmeyer flasks and 10-L bioreactor

The hairy root clones were cultured in Erlenmeyer flasks (Fig. 6B) for 21 days. The greatest increase in fresh and dry weight was observed for clone HRDXR3 in Erlenmeyer flasks, increasing from 1 g of starting material to 21.53 ± 0.98 g fresh weight and 2.39 ± 0.26 g dry weight. Biomass yields are given in Table 2.

Based on this performance, clone HRDXR3 was selected for culture in the 10-L bioreactor (Fig. 6A,C). After 21 days, the bioreactor culture yielded 170.3 ± 1.33 g fresh matter and 16.53 ± 1.35 g dry matter. For normal roots, the following values were obtained 39.23 ± 1.74 g fresh

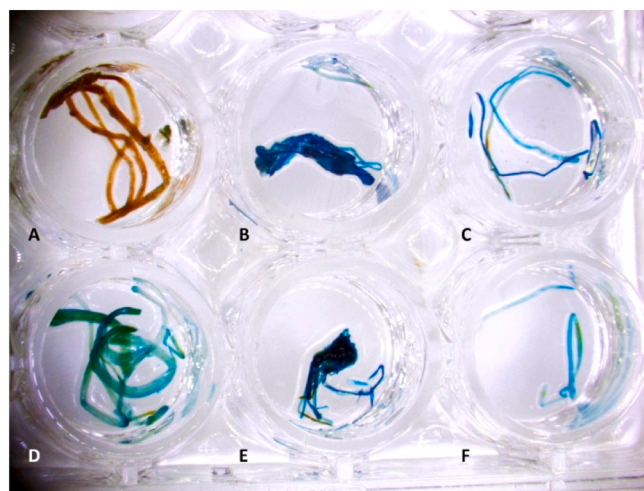


Fig. 5. GUS detection in transgenic hairy roots. A- normal roots (NR), B,C,D,E- transgenic hairy roots transformed with pGFPGUSPlus-DXR vector (HRDXR 1-4), F- transgenic hairy roots transformed with unmodified pGFPGUSPlus vector (HRDXR-).

matter and 3.57 ± 0.20 .

Biomass accumulation of normal roots and HRDXR (1–4) transgenic root lines cultured in Erlenmeyer flasks. Data represent mean values $\pm$ standard deviation (SD) for fresh weight (FW) and dry weight (DW), calculated from *n* = 3 independent experiments. Values within each column followed by different letters are significantly different from each other at *p* < 0.05 according to unpaired Student's *t*-test.

3.6. Compositional studies by HPLC-ESI-QTOF-MS/MS fingerprinting

The HPLC-MS analysis revealed some differences between the tested extracts from three different root lines in terms of cultivation conditions and origin (Figure S1 and S2, Supplementary Data). The results confirm that the transformation procedure was effective. The HPLC-ESI-QTOF-MS/MS analysis performed in both positive and negative ion mode suggested the presence of both phenolic compounds and terpenes; these were clearly visible in the negative ion mode. Based on the results and previous data on different *Plecranthus* species, the primary phenolics were found to be rosmarinic acid, as the major component, chlorogenic acid and coumaroylquinic acid. In addition, the main triterpenes were found to be coleon-U isomers, coleon-U-quinone isomers, epoxycoleon-U-quinone, nepetoidin B, and royleanone type isomers (Table 3).

This recorded terpene profile was similar to that noted in a previous study on *Plecranthus forsteri* (Nicolas et al., 2023), although higher levels of terpenoids were observed in the genetically-modified lines, particularly in the bioreactor culture (Table 4).

Unfortunately, it was not possible to purchase specific terpenoids as standards to complete the quantitative analysis of terpenoids' content in the investigated samples. Therefore, to select the clone with the highest amount of pharmacologically potent metabolites, the recorded peak areas of the compounds were compared between different injections (see Figure S3, Supplementary Data). This information helped us to differentiate between modified and unmodified root cultures. Comparing the peak areas of single components between the injections of extracts prepared at the same concentration lets us discuss whether a given plant matrix is a better or worse source of this compound. The obtained information is insufficient to perform an objective, quantitative analysis of these metabolites in the extracts, but it only allows comparisons of the three types of plant material used in the study in terms of higher or lower concentrations of selected metabolites.

As a result of this approach, we noticed that higher levels of terpenoids were obtained in the ethanolic extracts. These included epoxide of coleon-U-quinone, dihydroxyroyleanone, and the royleanone type triterpenes (horminone or 7α -hydroxyroyleanone). These extracts were therefore taken for further semi-quantitative analysis.

For the sake of comparing the samples, the averaged peak areas (*n* = 3) of the investigated components were determined from the injections of each ethanolic extract. Then, the highest peak area of a given product was taken as 100 % (Table 4), and the values for the remaining two were shown as percentages of this value, i.e. as the relative abundance.

The extracts from the HRDXR3 clones (Erlenmeyer flask and bioreactor) demonstrated generally higher concentrations of secondary metabolites of interest than the HRDXR-unmodified line (Table 4); the unmodified line contained only 5.78 ± 0.31 % of the coleon U isomer content of the bioreactor line. However, nepetoidin B was found to be more plentiful in the non-modified line: 4.94 times higher than in the bioreactor line and 3.63 times higher than the Erlenmeyer flask extracts.

The sprinkle bioreactor HRDXR3 clone demonstrated a higher triterpene content than the flask cultures, with higher levels of four of the tested compounds: two coleon U-quinone isomers, an epoxycoleon U quinone derivative and dihydroxyroyleanone (Table 4). Dihydroxyroyleanone was not identified in the unmodified lines.

Interestingly, markedly higher levels of coleon U-quinone derivatives were observed in the Erlenmeyer flasks and the bioreactor than in the unmodified lines. The level of both isomers were around 10 times

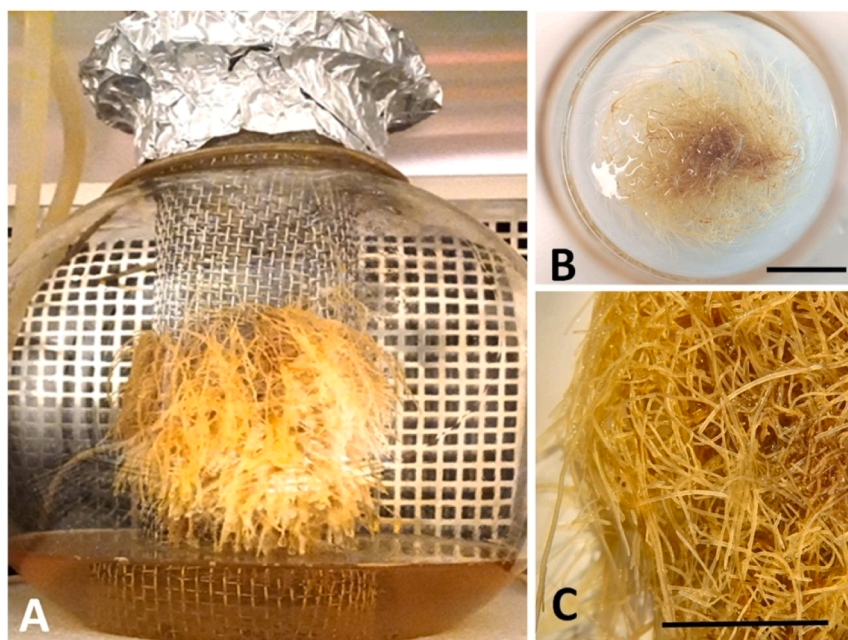


Fig. 6. Example photo of HRDXR1 hairy root culture of *P. venteri* in A- 10 L bioreactor, B- Erlenmeyer flask; C- General morphology of hairy roots growing in a bioreactor after 21 days of culture. Bar= 1 cm.

Table 2

Increase in biomass of hairy roots after 21 days of cultivation in 250 mL Erlenmeyer flasks containing 50 mL of medium.

Clone/root variant	Fresh mass (g) (FW)	Dry mass (g) (DW)
	Erlenmeyer flask	
Normal roots	4.81 ± 0.27 ^a	0.44 ± 0.039 ^a
HRDXR 1	17.63 ± 2.26 ^b	1.84 ± 0.09 ^b
HRDXR 2	17.30 ± 1.71 ^b	2.03 ± 0.08 ^b
HRDXR 3	21.53 ± 0.98 ^b	2.39 ± 0.26 ^b
HRDXR 4	16.97 ± 3.0 ^b	1.97 ± 0.16 ^b

higher in the Erlenmeyer flask culture and more than 14 times higher in the bioreactor extracts (17.3 and 14.49 times for isomer 1 and isomer 2, respectively). The HRDXR3 clone cultured in the Erlenmeyer flask was a better source of coleon-U-isomers than the sample from the bioreactor, especially for one of the isomers. Notably, higher levels of phenolic acids, such as chlorogenic and rosmarinic acid, in both constructs were noted, indicating the potential applications of this modified material.

4. Discussion

wide range of procedures have been developed for improving secondary metabolite yields, with many of them targeting cellular metabolic pathways (Ashrafizadeh and Seifollahi, 2021; Venayak et al., 2015). While such procedures can be used to transfer components of specialized metabolic pathways between plants (Courdavault et al., 2021; Farré et al., 2014), they can also facilitate the generation of new bioactive compounds, or improve the activity or efficiency of existing ones (Kalia and Saini, 2017; Pickens et al., 2011; Zhu et al., 2021). It is also important to highlight that numerous secondary metabolites act as pesticides, albeit with significantly less activity than traditional synthetic pesticides (Ribera and Zuñiga, 2012; Souto et al., 2021; Yang et al., 2018).

Numerous studies have explored methods to boost productivity by modifying plant metabolic pathways. These approaches include enhancing or suppressing genes encoding specific enzymes, integrating genetic engineering with elicitation, utilizing transcription factors (TFs),

and introducing new genes encoding particular secondary metabolites. (Aharoni and Galili, 2011; Kowalczyk et al., 2020b, 2020a; Sitarek et al., 2018b; Yuan and Grotewold, 2015).

Biotechnology has often been used to enhance the potential of plants as natural biofactories for producing valuable bioactive compounds, with transformed root cultures being used to produce large amounts of valuable metabolites (Alamgir, 2018; Balestrini et al., 2021; Kowalczyk et al., 2022). Such cultures grow rapidly or remain genetically stable, enabling the efficient production of large amounts of material in a short time while preserving the environment (Dhiman et al., 2018; Srivastava and Srivastava, 2007). Furthermore, their cultivation in bioreactors offers greater production efficiency (Kowalczyk et al., 2021b; Sitarek et al., 2018a). For this reason, the aim of the present study was to compare the productivity of different transgenic HRDXR clones of *Plectranthus venteri* cultured in different scales using various biotechnological techniques.

The DXR gene encodes a key enzyme in terpenoid biosynthesis, and its overexpression has been shown to affect terpenoid synthesis in different plants. In *Chrysanthemum indicum* var. aromaticum, CidXR expression, activated by methyl jasmonate treatment, was found to increase terpenoid content (Gao et al., 2018). Overexpression of NtDXR1 in *Nicotiana tabacum* led to increased production of photosynthetic pigments, hormones and membrane-associated diterpenoids (Zhang et al., 2015). In *Litsea cubeba*, overexpression of LcDXR significantly increased monoterpene content (Zhao et al., 2023).

Although these results demonstrate that it is possible to influence terpenoids biosynthesis, the present work is the first to explore the potential of genetic modification of *Plectranthus venteri* hairy root culture in this regard. To date, studies have only reported the transformation of *Coleus forskohlii* (syn. *Plectranthus barbatus*) and *Coleus blumei* (syn. *Plectranthus scutellarioides*) root cultures using *Rhizobium rhizogenes* (Khah et al., 2019; Rajiv et al., 2014). The use of *R. rhizogenes* is the most widely-used strategy for obtaining transgenic plant material, particularly hairy roots.

There are many factors influencing the efficiency of transgenesis (Azizi-Dargahlou and pouresmaeil, 2024), with an important role played by the phenolic compounds secreted by wounded plant tissues (Levengood et al., 2024). Hence, many *in vitro* strategies employ the

Table 3
The metabolites identified by HPLC-ESI-QTOF-MS/MS analysis in negative ionisation mode (Diff – error of measurement, DBE – double bond and ring number, tr – traced with no MS/MS spectra registered).

No	Ion	Retention time [min]	Compound	Neutral formula	Theoretical mass	Precursor ion m/z	Diff [ppm]	DBE	Product ions [m/z]
1	[M-H] ⁻	5.13	Epoxycoleon U-quinone	C ₁₈ H ₁₈ O ₉	377.0878	377.0860	4.78	10	-
2	[M-H] ⁻	18.0	Chlorogenic acid	C ₁₆ H ₁₈ O ₉	353.0878	353.0877	0.3	8	191.0551 179.0336 173.0437 161.0234
3	[M-H] ⁻	18.5	12-Hydroxyjasmonic acid glucoside	C ₁₈ H ₂₈ O ₉	387.1661 [*]	387.1649	2.98	5	-
4	[M-H] ⁻	21.9	Rosmarinic acid	C ₁₈ H ₁₆ O ₈	359.0772	359.0778	-1.55	11	197.0455 179.0348 161.0242 135.0450 298.0481 283.0247 161.0244
5	[M-H] ⁻	23.4	Nepetoidin B	C ₁₇ H ₁₄ O ₆	313.0718	313.0721	-1.08	11	280.9828
6	[M-H] ⁻	25.3	Coleon U isomer 1	C ₂₀ H ₂₆ O ₅	345.1707	345.1702	1.58	8	-
7	[M-H] ⁻	29.8	Coumaroylquinic acid	C ₁₆ H ₁₈ O ₈	359.0772	359.0782	-2.66	11	-
8	[M-H] ⁻	25.96	Horminone or 7α-hydroxyroyleanone isomer	C ₂₀ H ₂₈ O ₄	331.1915	331.1915	-0.05	7	-
9	[M-H] ⁻	25.9	Coleon U isomer 2	C ₂₀ H ₂₆ O ₅	345.1707	345.1701	1.87	8	280.9839
10	[M-H] ⁻	26.5	Dihydroxyroyleanone	C ₂₀ H ₂₈ O ₅	347.1864	347.1865	-0.29	7	329.1753
11	[M-H] ⁻	26.67	Horminone or 7α-hydroxyroyleanone isomer	C ₂₀ H ₂₈ O ₄	331.1915	331.1925	-3.06	7	-
12	[M-H] ⁻	26.9	Coleon U-quinone isomer 1	C ₁₈ H ₁₆ O ₈	359.0772	359.0767	1.5	11	315.0831 341.0795 197.0449 179.0348 315.0884 341.0801 197.0449 179.0348
13	[M-H] ⁻	27.55	Coleon U-quinone isomer 2	C ₁₈ H ₁₆ O ₈	359.0772	359.0759	3.72	11	-
14	[M-H] ⁻	29.03	Formyloxy-hydroxyroyleanone	C ₂₀ H ₂₈ O ₄	331.1915	331.1909	1.75	7	-

Table 4
The relative amounts of the identified components in each experimental variant; out of the three tested variants, the highest peak area (green) was taken as 100 %, while the other two are measured relative to this; yellow indicates second highest value, red indicates lowest value (ND – not detected).

Compound number	Compounds	HRDXR-	HRDXR3 (bioreactor)	HRDXR3 (Erlenmeyer flask)
4	Rosmarinic acid	29.56 ± 0.91	70.96 ± 0.67	100.00 ± 3.04
2	Chlorogenic acid	10.88 ± 1.02	100.00 ± 2.85	72.31 ± 2.11
3	12-Hydroxyjasmonic acid glucoside	21.23 ± 1.37	87.17 ± 3.08	100.00 ± 4.44
5	Nepetoidin B	100.00 ± 3.24	20.24 ± 1.63	27.52 ± 1.67
6	Coleon U isomer 1	20.06 ± 0.87	0.78 ± 0.078	100.00 ± 3.65
9	Coleon U isomer 2	19.26 ± 1.21	70.46 ± 4.23	100.00 ± 6.61
12	Coleon U-quinone isomer 1	5.78 ± 0.31	100.00 ± 2.87	60.80 ± 2.91
13	Coleon U-quinone isomer 2	6.91 ± 0.30	100.00 ± 5.50	63.41 ± 1.12
1	Epoxycoleon U quinone	63.98 ± 3.27	100.00 ± 6.00	47.80 ± 2.76
10	Dihydroxyroyleanone	0	100.00 ± 8.75	88.82 ± 5.15

phenolic acetosyringone to increase transformation efficiency (Cheng et al., 2021; Marimuthu et al., 2014), as also used in the present work. Our results confirm that acetosyringone has a positive effect on hairy root formation, further confirming also the transformed nature of hairy roots by PCR (Foti and Pavli, 2020; Kowalczyk et al., 2021a).

Previous data indicates that transgenic *Leonurus sibiricus* root cultures with overexpression of the *AtPAP1* gene showed increased phenolic acid production in the phenylpropanoid pathway (Sitarek et al., 2018b), and transgenic *Senna obtusifolia* root cultures over-expressing the *PgSS1* gene increased production of pentacyclic

triterpenes such as betulinic acid (Kowalczyk et al., 2020a). However, the present study is the first to manipulate the terpenoid metabolic pathway in a species of *Plectranthus*. A key finding is that the transformed cultures demonstrated increased expression of *DXR*, a gene involved in the MEP pathway. Similarly, previous research on *Salvia miltiorrhiza* found hairy root cultures transformed with the *DXR* gene to demonstrate greater *DXR* transcription and increased accumulation of tanshinones after exposure to hyperosmotic stress and yeast elicitor (Wu et al., 2009), as well as increased *DXR* expression and tanshinone production after elicitation (Shi et al., 2014).

Transformed *Salvia sclarea* roots have been found to display elevated *DXR* protein production and increased dry weight (DW), as well as a higher abietane diterpene content (Vaccaro et al., 2019, 2014). These findings are consistent with ours, and confirm the formation of stable transgenic root cultures with *DXR* gene overexpression.

In the present study, the most productive clone in terms of growth rate and *DXR* expression (HRDXR 3), was selected for cultivation in a bioreactor to optimize secondary metabolite production. Bioreactor cultures represent an innovative technology that merges high productivity with minimal environmental impact; as such, they create new possibilities for sustainable bioactive compound production. Indeed, bioreactor cultivation have been found to increase the expression of genes involved in metabolic pathways, thus providing an excellent production platform for pharmacologically-valuable compounds (Kowalczyk et al., 2020a; Sitarek et al., 2018a).

In the present work, the combination of transgenesis and bioreactor-based cultivation enhanced the production of selected valuable terpenoids and phenolics. The investigated extracts were characterised by coleon-U isomers and quinone derivatives, as well as an epoxyl form of coleon-U-quinone. These compounds have been noted previously in *Plectranthus* spp., with their structures confirmed by NMR (Jovanović Stojanov et al., 2023; Ntungwe et al., 2019). Previous research has also found coleon-U to fragment into 341 and 313 *m/z* derivatives (Rodríguez-Ferreiro et al., 2022); both were observed among the coleon-U isomers in the present study, viz. *m/z* of 315.0831 and 341.0795.

As reported previously for *P. neochilus*, the *P. venter* extracts contained small quantities of royleanone diterpenes: namely, dihydroxyroyleanone or isomers of horminone/7 α -hydroxyroyleanone (Matias et al., 2019; Nicolas et al., 2023). Our tests also suggest the presence of hydroxyjasmonic acid glucoside, which was also noted in *Plectranthus neochilus* (Rodríguez-Ferreiro et al., 2022).

However, the dominant phenolic compound present in the investigated extracts was rosmarinic acid (RA), with a similar MS/MS spectrum fragmentation pattern as that noted previously (Serrano et al., 2021). The RA fragmentation spectra included characteristic signals at *m/z* of 197.0455 (2-hydroxy derivative of hydro-caffeic acid), 179.0348 (caffeic acid), 161.0242, and 135.0450 Da (Hahm et al., 2021). Chlorogenic acid was also identified; it also demonstrated a typical fragmentation pattern including an *m/z* of 191.0551 (quinic acid) and 179.0336 (caffeic acid moieties) (Fetene et al., 2024), which were observed in the internal library of compounds created after the injection of the authentic standard.

It is worth noting that coleon-U and its derivatives demonstrate strong pharmacological potential. They have been found to have anticancer properties against non-small-cell lung carcinoma, particularly the multidrug resistant type, owing to its ability to inhibit P-glycoprotein (Jovanović Stojanov et al., 2023). Also, coleon-U and coleon-U-quinones have been found to demonstrate antibacterial activity against *Pseudomonas syringae*, *Staphylococcus aureus*, and *Enterococcus faecalis* (Nicolas et al., 2023). As such, these metabolites merit further investigation. The root cultures prepared in the present study are able to deliver sufficient quantities of these rare triterpenes for further research. Additionally, the extracts were found to contain nepetoidin B, an anti-inflammatory compound which has been demonstrated to alleviate reperfusion injury and liver ischemia in a mouse model (Yu et al.,

2024).

Regarding the phenolics, both rosmarinic acid, predominating in the HRDXR 3 Erlenmeyer flask sample, and chlorogenic acid, present in the bioreactor cultures. These have been found to possess various valuable activities, such as antiradical and anti-inflammatory properties, and memory-enhancing activity (Sun and Shahrajabian, 2023).

Our findings confirm the creation of stable transgenic root cultures of *P. venter* with overexpression of *DXR*. This offers promise for further studies aimed at using such approaches to increase the production of valuable secondary metabolites. The HRDXR 3 transgenic root culture from the bioreactor was found to offer more promise than the flask culture, producing higher levels of terpene compounds and a greater variety of metabolites with pharmacological properties. Hence, the present strategy appears to be a promising solution for satisfying the growing need for highly-productive plant culture. The ever-expanding range of precise tools for modifying the genome, and technical solutions for intensive cell, tissue and plant growth, offers hope for the development of efficient production strategies that can fully make use of the natural treasure trove of plant secondary compounds.

5. Conclusions

Independent transgenic hairy root lines of *Plectranthus venter* overexpressing the *DXR* gene, a key enzyme of the MEP pathway, were successfully generated and confirmed by molecular analyses. Among the obtained lines, clone HRDXR3 showed both the highest biomass accumulation and the strongest *DXR* overexpression. When scaled up in a 10 L sprinkle bioreactor, HRDXR3 produced substantially higher levels of royleanone-type diterpenoids compared with unmodified controls. In particular, the peak area of coleon U-quinone isomer 1 increased **17.3-fold** in the bioreactor culture and **10.5-fold** in Erlenmeyer flasks, while coleon U isomer 2 levels increased **3.66-fold** and **5.19-fold**, respectively. These diterpenoids, including horminone and 7 α -hydroxyroyleanone, possess reported anticancer and antimicrobial activities. The results demonstrate that combining *DXR* overexpression with bioreactor cultivation is an efficient strategy to enhance the production of pharmacologically relevant diterpenoids in *P. venter* hairy roots. This approach provides a promising platform for sustainable and scalable production of valuable secondary metabolites for potential medical applications.

CCRediT authorship contribution statement

Patricia Rijo: Writing – review & editing, Investigation. **Laurent Picot:** Writing – review & editing, Investigation. **Tomasz Kowalczyk:** Writing – original draft, Visualization, Supervision, Methodology, Investigation, Conceptualization. **Virginia Kukula-Koch:** Writing – review & editing, Supervision, Methodology, Investigation. **Ewelina Synowiec:** Writing – review & editing, Methodology, Investigation. **Tomasz Śliwiński:** Writing – review & editing. **Przemysław Sitarek:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of Competing Interest

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.indcrop.2025.122188.

Data availability

Data will be made available on request.

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