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Biomass and Phenolic Acid Accumulation in *Salvia austriaca* Hairy Roots Grown in Temporary Immersion and Mist-Trickling Bioreactors

Łukasz Kuźma [1,*], Anna Karolina Kiss [2], Ireneusz Pieszyński [3], Dymitr Mojsiejew [4], Justyna Pękala [1] and Jadwiga Nowak [5]

[1] Department of Biology and Pharmaceutical Botany, Medical University of Lodz, 1 Muszyńskiego St., 90-151 Lodz, Poland
[2] Department of Pharmaceutical Biology, Faculty of Pharmacy, Medical University of Warsaw, 1 Banacha St., 02-097 Warsaw, Poland
[3] Department of Medical Rehabilitation, Faculty of Health Sciences, Medical University of Lodz, 251 Pomorska St., 92-213 Lodz, Poland
[4] National Seminar for Older Candidates Ordination in Lodz, 14 Saint Stanislaw Kostka St., 90-457 Lodz, Poland
[5] Department of Pharmacology and Therapeutics, School of Biomedical Sciences, College of Health Sciences, Makerere University, Kampala P.O. Box 7062, Uganda
* Correspondence: lukasz.kuzma@umed.lodz.pl

Abstract: Transformed roots of *Salvia austriaca* were cultivated for 45 days in various systems, including Erlenmeyer flasks, a temporary immersion system (TIS) bioreactor, and a mist-trickling bioreactor, under controlled light conditions. The mist-trickling bioreactor yielded the highest biomass, with fresh and dry weights of 155.4 g/L and 10.2 g/L, respectively. Quantitative UHPLC analysis of hydromethanolic extracts revealed the biosynthesis of significant phenolic acids: caffeic acid, rosmarinic acid, and salvianolic acid A. Among these, rosmarinic acid was the most abundant, with its concentration varying based on the cultivation system. The highest total phenolic acid content, 165 mg/L, was obtained in the mist-trickling bioreactor, demonstrating its superiority in both biomass production and phenolic acid biosynthesis. This study highlights the potential of mist-trickling bioreactors for optimizing growth and metabolite production in *S. austriaca* transformed root cultures.

Keywords: *Salvia austriaca* hairy roots; phenolic acids; temporary immersion and mist-trickling bioreactors

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1. Introduction

The genus *Salvia* (Lamiaceae) comprises approximately 900 species, many of which are utilized in folk and official medicine, as well as for culinary purposes [1]. Numerous *Salvia* species are recognized for their therapeutic properties and are employed in the treatment of common colds, bronchitis (both acute and chronic), various gastrointestinal disorders, and cardiovascular conditions [1–3]. Among these, *Salvia austriaca* stands out due to its medicinal significance. Essential oils [4], diterpenoids [5,6], triterpenoids [7], and phenolic acids [8] have been identified as active secondary metabolites in this species.

Phenolic acids, a prominent group of secondary metabolites in *Salvia* species, contribute significantly to their pharmacological activity. These compounds exhibit a range of beneficial properties, including antioxidant, anti-inflammatory, anticancer, antimicrobial, antiallergic, antiviral, antithrombotic, and hepatoprotective effects [9]. The search for new, efficient plant sources of these valuable metabolites remains an area of active research.

In *Salvia austriaca*, phenolic acids such as rosmarinic acid and salvianolic acid K, along with other organic compounds like malic acid, trihydroxyoctadecadienoic acid, and tri-coumaroylspermidine, have been identified in soil conditions [9].

In plant biotechnology, conventional in vitro culture systems often utilize glass or polypropylene vessels, such as test tubes or Erlenmeyer flasks. The use of these vessels and solid or semi-solid media can be labor-intensive and costly. Scaling up cultivation through the use of bioreactors has been proposed as a means to enhance efficiency and reduce costs [10]. Various types of bioreactors, including mechanically or pneumatically agitated, gas-phase, and modified systems, are used depending on the in vitro plant culture and its specific needs [10]. This study investigates the scaling-up of *Salvia austriaca* hairy root culture using shake Erlenmeyer flasks and two types of bioreactors: the Plantform temporary immersion system and a mist-trickling bioreactor. Additionally, it explores the qualitative and quantitative aspects of phenolic acid biosynthesis in these transformed root cultures, an area not previously studied.

2. Materials and Methods

2.1. Plant Material

Salvia austriaca transformed root cultures were initiated by infecting shoot culture with *Rhizobium rhizogenes* strain A4, as detailed previously [6]. The transformation was confirmed through molecular techniques [6]. The roots were cultured in 300 mL Erlenmeyer flasks containing 80 mL of hormone-free Schenk and Hildebrandt (SH) liquid medium, supplemented with 3% sucrose [11]. The cultures were maintained in a growth chamber on a rotary shaker (70 rpm) at 24 ± 2 °C, with a 16 h light/8 h dark photoperiod provided by white fluorescent lamps (35 μmol/m^2 s). Subcultures were performed every 35 days, with root fragments transferred to fresh sterile SH liquid medium. The average fresh and dry weights of the inoculum were approximately 0.33 g and 0.02 g per flask, respectively.

2.2. Bioreactor Studies

To scale up the culture, *Salvia austriaca* transformed roots were cultivated in a 5.0 L mist-trickling system and a temporary immersion bioreactor (TIB, Plantform, Stockholm, Sweden) (see Figure 1), maintaining the same light and temperature conditions as those used for flask-grown cultures. The mist-trickling bioreactor, made from polypropylene, featured three tubes: one for sterile air supply, one for liquid culture medium dispersion through a stainless-steel nebulizer nozzle, and one for excess gas removal. The peristaltic pump delivered the liquid medium to the nozzle at a rate of 10 mL every 10 s, with a 3 min interval between nutrient supply cycles. This operation was controlled by a temporary electric relay.

Both bioreactors were aerated in the same way with sterile-filtered air for 10 min every 180 min, and in the TIB (Plantform) bioreactor, the air also served as a factor in raising the liquid column of the culture medium, thus regularly feeding the root culture. Each bioreactor, containing 0.5 L of sterile SH medium, was inoculated with 35-day-old roots from shake flask cultures (passage 158, representing 158 consecutive 35-day cultivation cycles). The average fresh weight of the inoculum was approximately 5.3 g, and the dry weight was around 0.29 g. After 45 days of culture, the roots were harvested for biomass measurement and phenolic acids analysis.

The growth index (Gi) for all cultivation systems, including flasks, Plantform, and mist-trickling bioreactors, was calculated using the formula: Gi = (final fresh biomass − initial fresh biomass)/initial fresh biomass.

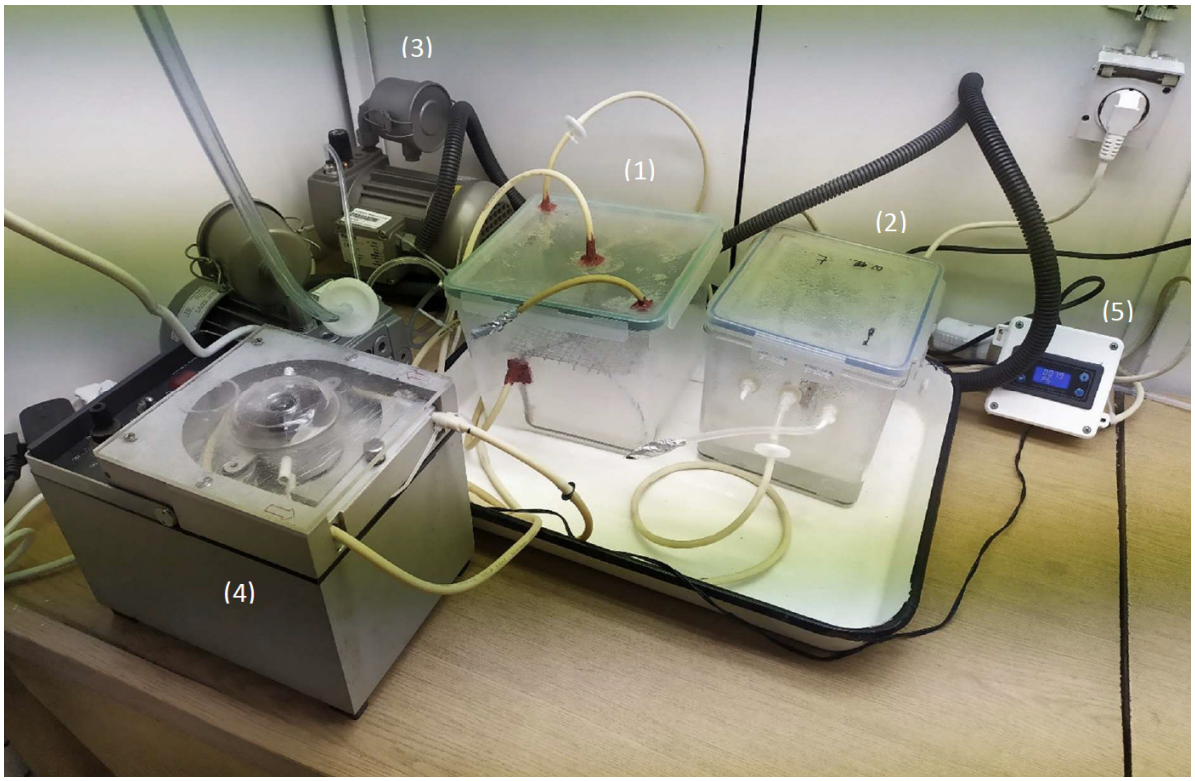


Figure 1. *S. austriaca* hairy root cultivation bioreactors: (1)—mist-trickling bioreactor; (2)—temporary immersion bioreactor (Plantform); (3)—air compressor; (4)—peristaltic pump; (5)—temporary electric relay.

2.3. Phytochemical Analysis

Phenolic acids were identified using LC-MS. The crude hydromethanolic extract from *S. austriaca* hairy roots was obtained through a 45 min extraction in an ultrasonic bath at room temperature. For qualitative analysis, the dry residue was dissolved in methanol and analyzed using UHPLC-DAD-ESI-MS/MS. Chromatographic separation was performed on a UHPLC-3000 RS system (Dionex, Germering, Germany) equipped with a DAD detector and an AmaZon SL ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). The separation was achieved on a Zorbax SB-C18 column (150 × 2.1 mm, 1.9 µm) (Agilent, Santa Clara, CA, USA) with a mobile phase consisting of water (A) and acetonitrile (B) and the following gradient: 0–60 min, 5–40% B. The ESI interface parameters were as follows: nebulizer pressure at 40 psi, drying gas flow rate at 9 L/min, nitrogen gas temperature at 300 °C, and capillary voltage at 4.5 kV. Mass scanning was conducted over a range of 100 to 2200 m/z, with UV spectra recorded from 200 to 400 nm and detection at 325 nm. Compounds were identified by comparing the mass spectrometric data with literature references. Table 1 presents data on the identification of phenolic acids in the tested material, while Figure 2 displays the corresponding UHPLC chromatogram from this analysis.

Table 1. The data of identified phenolic acids (Retention time, λ_{max}, and MS spectroscopic data) in *S. austriaca* transformed roots.

Peak No.	Compound	Retention Time (min)	λ_{max} (nm)	Negative Ion Mode	
				$[M-H]^-$	Major Fragments MS^2
1	Caffeic acid	16.0	323	179	135
2	Rosmarinic acid	30.1	327	359	197, 179, 161
3	Salvianolic acid A	30.7	312	493	359

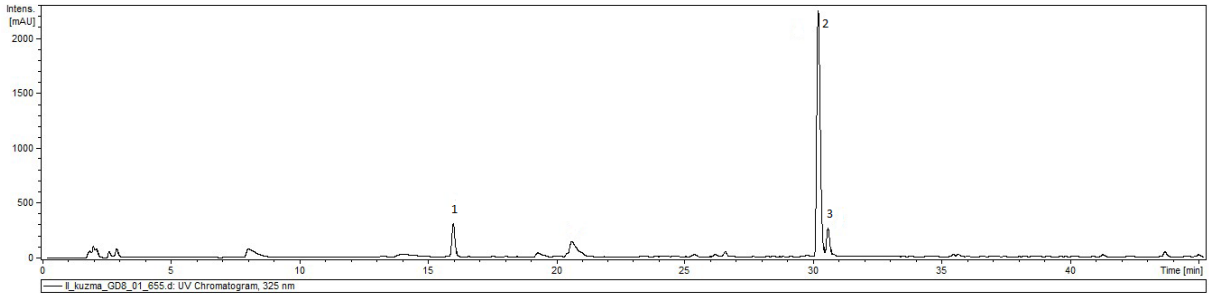


Figure 2. UHPLC chromatogram of hydromethanolic extract of *S. austriaca* transformed roots maintained in SH liquid medium.

2.4. Quantitative Analysis

Quantitative analysis was conducted using UHPLC with analytical-grade solvents. Acetonitrile and water were sourced from J. C. Baker (Mallinckrodt Baker B.V., Deventer, The Netherlands). The analysis utilized a hydromethanolic extract from 100 mg of lyophilized and micronized *S. austriaca* hairy roots. To remove non-polar contaminants, an initial extraction with *n*-hexane (3 × 20 mL) was performed, followed by extraction with methanol–water (8:2, *v*/*v*) using an ultrasonic bath for 45 min at room temperature. After the first extraction with *n*-hexane, the plant material was washed three times with *n*-hexane, filtered, and dried in a vacuum. The pre-cleaned material was extracted with 10 mL of a methanol–water mixture (8:2, *v*/*v*) via ultrasonication for 45 min at room temperature. The extracts were then centrifuged (12,000 rpm for 5 min) and filtered (0.2 µm pore size) into the HPLC vials before analysis.

The UHPLC analysis was carried out using a 1290 Infinity UHPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD) and gradient elution on a Zorbax Eclipse Plus C18 column (100 × 3.0 mm i.d., 1.8 µm). The mobile phases consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The gradient elution profile was as follows: 0–15 min, 10–20% A; 15–17 min, 20–25% A; 17–19 min, 25–29% A; 19–21 min, 29% A and 21–21.1 min, 29–95% A. The flow rate was 0.5 mL/min, and the injection volume was 10 µL. Detection was performed at 328 ± 2 nm.

Phenolic acid concentrations were determined from peak areas and calibration curves, previously prepared using solutions of standard substances of phenolic acids (Sigma, St. Louis, MO, USA) dissolved in methanol (HPLC purity) in the concentration range of 5–100 µg/mL. Calibration curves were drawn using 6 concentration levels, and each of them was repeated 3 times. Calibration UHPLC analyses were performed under the same conditions as described above for UHPLC analyses of extracts. Results were expressed as mg per liter of culture medium, calculated as the average dry weight (g/L) multiplied by the average compound content (mg/g dry weight).

2.5. Statistical Analysis

The average root biomass for the three cultivation models is presented in Table 2. Descriptive statistics for the quantitative analysis of phenolic acids in the roots are detailed in Table 3. Since the phenolic acid content in the samples did not follow a normal distribution (as confirmed by the Shapiro–Wilk test), the Kruskal–Wallis test was used, with 95.0% Bonferroni intervals (this refers to Bonferroni-adjusted confidence intervals), to assess the statistical significance of differences in phenolic acid content across the different cultivation models. The significance level was set at $\alpha = 0.05$. The analysis was performed using STATGRAPHICS Centurion version 18.1.12 (Statgraphics Technologies, Inc., The Plains, VA, USA).

Table 2. The biomass of 45-day-old *S. austriaca* hairy roots grown in the flasks, Plantform, and mist-trickling bioreactors.

Liquid Medium	Growth Environment	FW (g/L)	DW (g/L)	Gi
SH	Flask	149.29	9.72	35.19
	Plantform bioreactor	45.89	2.85	3.25
	Mist-trickling bioreactor	155.43	10.16	13.66

FW—fresh weight, DW—dry weight, Gi—growth index.

Table 3. Descriptive statistics for the yield of phenolic acids in 45-day-old *Salvia austriaca* hairy roots cultivated across three distinct cultivation systems.

Selected Descriptive Statistics of the Analyzed Variables									
	Caffeic Acid (n = 25)			Rosmarinic Acid (n = 25)			Salvianolic Acid A (n = 25)		
	F	Ptf	M-t	F	Ptf	M-t	F	Ptf	M-t
Average	1.73	0.47	2.37	4.38	25.52	158.74	1.91	1.31	4.18
Median	1.70	0.49	2.36	4.60	24.57	156.83	1.87	1.26	4.06
Standard error	0.03	0.01	0.07	0.26	0.56	2.98	0.07	0.03	0.11
Minimum	1.56	0.41	1.94	0.20	22.15	132.62	0.57	1.06	3.41
Maximum	2.04	0.51	3.00	5.86	30.98	175.50	2.61	1.68	5.03
Lower quartile	1.65	0.45	2.04	4.38	23.48	148.20	1.78	1.24	3.76
Upper quartile	1.79	0.50	2.56	4.77	26.47	172.67	2.08	1.42	4.69
Interquartile range	0.14	0.05	0.52	0.39	2.99	24.47	0.30	0.18	0.93

Phenolic acid yields were measured in mg/L and denoted as follows: F for Flask, Ptf for Plantform bioreactor, and M-t for Mist-trickling bioreactor.

3. Results and Discussion

3.1. Identification of Polyphenolic Compounds in Hydromethanolic Extract of Salvia austriaca Hairy Roots

The hydromethanolic extract of *Salvia austriaca* transformed roots was analyzed for polyphenolic compounds using UHPLC-PDA-ESI-MS (Figure 2 and Table 1).

Three phenolic acids were identified by comparing their UV-Vis absorption spectra and MS/MS patterns with literature data [12]. The mass spectra of peak 1 revealed a pseudomolecular ion $[M\text{-}H]^-$ at m/z 179 and a major fragment ion at m/z 135, consistent with caffeic acid [12]. Peak 2 exhibited a pseudomolecular ion $[M\text{-}H]^-$ at m/z 359 and three major fragment ions at m/z 197, 179, and 161, corresponding to rosmarinic acid [12]. Peak 3, with a pseudomolecular ion $[M\text{-}H]^-$ at m/z 493 and a major fragment ion at m/z 359, identified salvianolic acid A [12]. The chemical structure of the identified phenolic acids is shown in Figure 3.

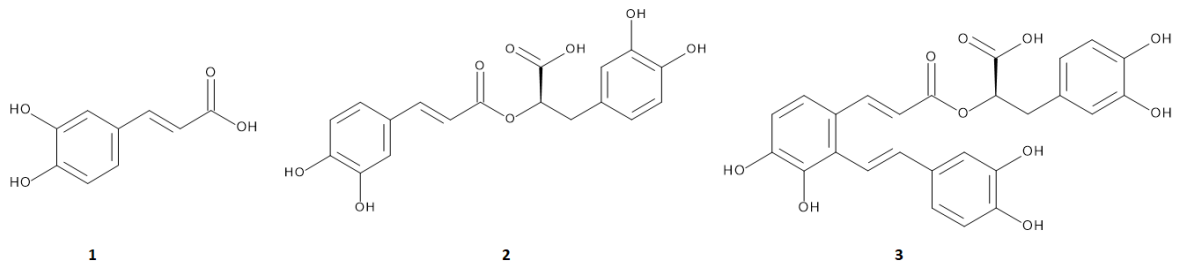


Figure 3. The chemical structure of caffeic acid (1), rosmarinic acid (2), and salvianolic acid A (3).

Caffeic acid has previously been detected only in the aerial parts of the plant grown in field conditions [13]. Notably, the transformed roots were cultured under light conditions. Açikgöz et al. (2018) observed that light conditions enhanced caffeic acid derivative biosynthesis in *Echinacea purpurea* callus cultures [14]. Similarly, light exposure increased

solasodine biosynthesis in *Solanum khasianum* hairy root cultures [15]. It is likely that light positively influenced caffeic acid biosynthesis in *S. austriaca* hairy roots, although rosmarinic and salvianolic acid A were also detected. While the presence of rosmarinic and salvianolic acids has been confirmed in *S. austriaca* [8], salvianolic acid A has not been previously reported in this species. Similar phenomena of novel derivative appearance have been noted in other in vitro cultures, such as new isomers, *iso*-sphaeralcic acid, and 8-methyl-iso-sphaeralcic acid, detected in *Sphaeralcea angustifolia* transformed roots [16].

3.2. Effect of Cultivation Environment on Biomass and Phenolic Acid Production in S. austriaca Hairy Roots

The morphology of *S. austriaca* hairy root cultures after a 45-day growth period is illustrated in Figure 4.

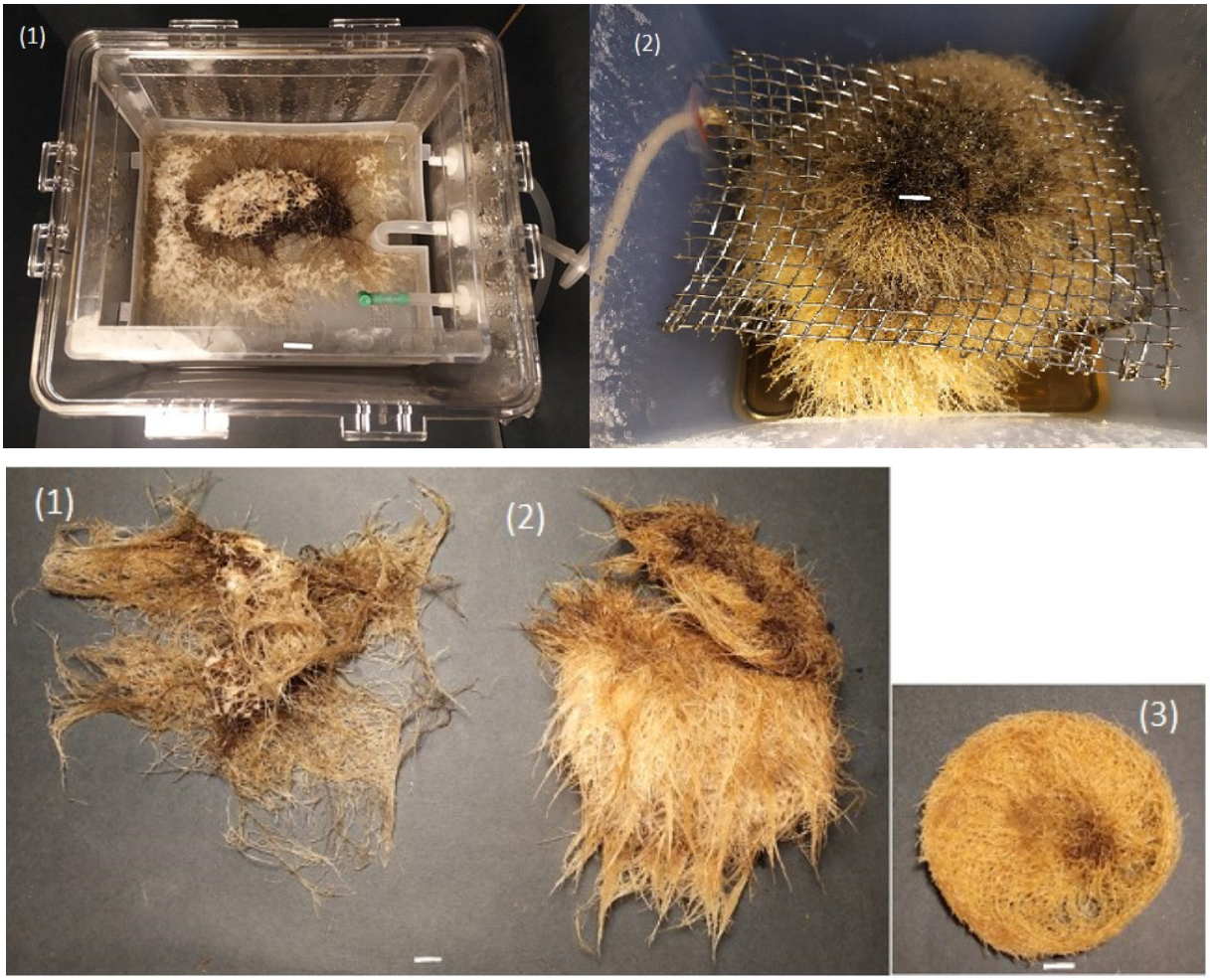


Figure 4. Forty-five-day-old *S. austriaca* transformed roots grown in Plantform (**1**), mist-trickling (**2**) bioreactors, and the Erlenmeyer flask (**3**) in SH liquid medium (bar 1 cm).

The duration of the culture was based on prior studies [17]. Roots cultured in SH liquid medium in shake flasks displayed typical growth patterns, oriented perpendicular to gravity. This pattern was also observed in the Plantform bioreactor (Figure 4). In contrast, roots grown in the mist-trickling bioreactor primarily grew in the direction of gravity (Figure 5).

Similar growth patterns were observed in other nutrient-sprinkle bioreactors for transformed roots of *Salvia sclarea* [18], *Platycodon grandiflorum* [19], and *Leonurus sibiricus* [20].



Figure 5. Forty-five-day-old *S. austriaca* transformed roots grown in mist-trickling bioreactor (bar 1 cm).

A bioreactor of similar design has previously been employed for the cultivation of *Salvia austriaca* transformed roots [17]. However, the biomass parameters observed in that study were significantly lower than those recorded in the current investigation. This discrepancy may be attributed to excessive shear forces in the earlier system, which likely caused mechanical damage to the fragile root structures due to the sprinkler mechanism employed [10]. In response to this challenge, the present study introduced a newly designed bioreactor, developed by the primary author. To mitigate the adverse effects of shear forces on root growth, this bioreactor was equipped with a nozzle featuring an outlet diameter of 0.4 mm. This modification effectively reduced the particle size of the sprayed liquid medium, thereby significantly diminishing the unfavorable mechanical forces exerted on the roots. This improvement is likely responsible for the enhanced biomass accumulation and increased biosynthesis of secondary metabolites observed during cultivation in the newly proposed bioreactor design. Table 2 shows the *S. austriaca* hairy root biomass data after a 45-day growth period.

The mist-trickling bioreactor was more effective for biomass production, achieving 10.2 g dry biomass per liter of culture (155.4 g/L FW) after 45 days, compared to 9.7 g (149.29 g/L FW) from the Erlenmeyer flasks. The Plantform bioreactor resulted in the lowest biomass yield, with 2.9 g and 45.9 g per liter of culture of the dry and fresh weight, respectively. Roots grown in the Plantform bioreactor exhibited larger darkening zones, indicating less favorable growth conditions (Figure 4).

The highest growth index (Gi) value, based on fresh biomass, was observed for roots cultivated in flasks. In comparison, a lower Gi was recorded for roots grown in the mist-trickling bioreactor. The lowest Gi value (Gi = 3.25) was noted for roots cultivated in the Plantform bioreactor. This system also exhibited a relatively large dark zone, potentially indicative of root necrosis (Figure 4).

The UHPLC chromatograms of the hydromethanolic extract confirmed the biosynthesis of the identified phenolic acids in all culture systems (Figure 6).

Table 3 shows the *S. austriaca* hairy root yield of phenolic acids after a 45-day growth period. The mist-trickling bioreactor exhibited the highest total phenolic acid biosynthesis, yielding 165 mg per liter of culture after 45 days. In contrast, roots in the Plantform bioreactor produced six times fewer phenolic acids (27 mg/L), while those in flasks yielded only 8 mg/L. Although the growth index (Gi) was significantly lower for roots in the mist-trickling bioreactor compared to flask cultures, the nebulized delivery of the liquid culture medium and the distinct growth conditions within the mist-trickling system greatly enhanced phenolic acid biosynthesis.

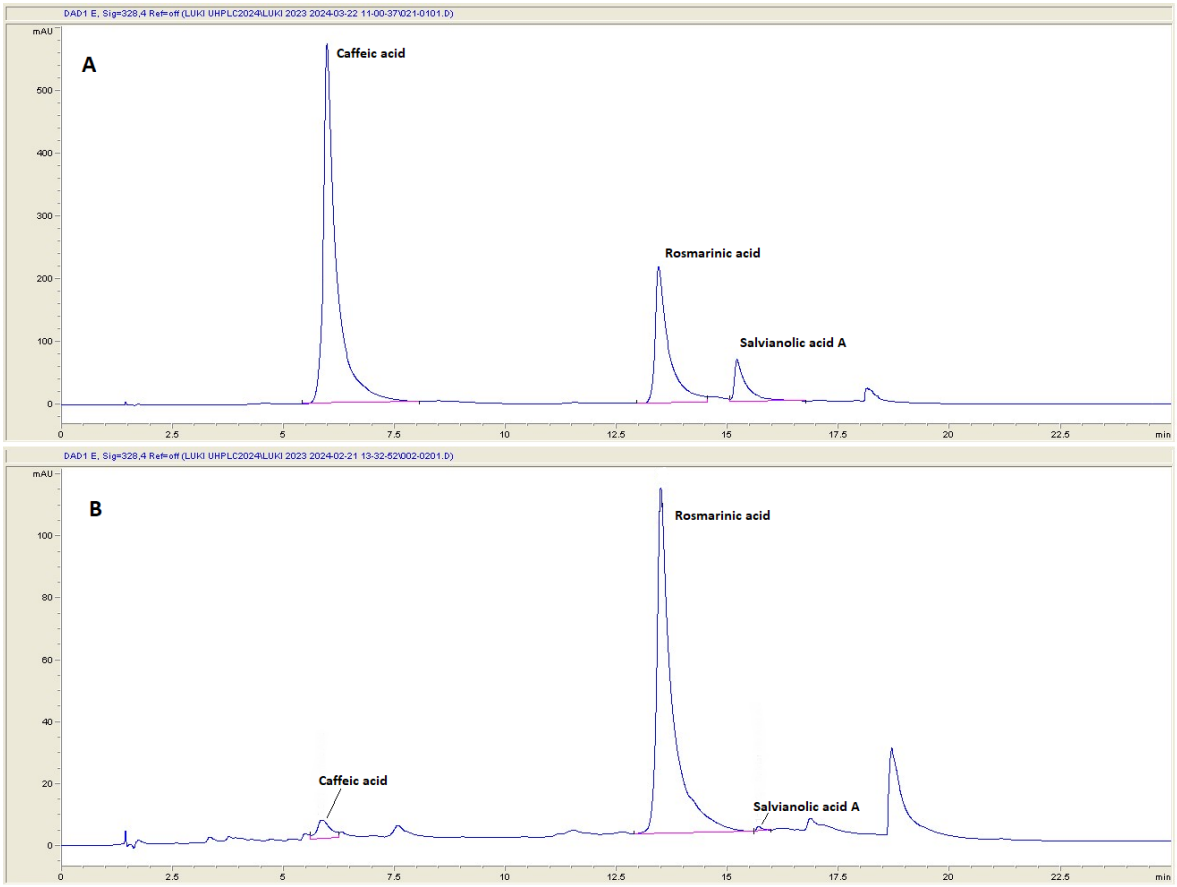


Figure 6. The UHPLC chromatograms of the standard compound mixture (**A**) and the hydromethanolic extract from *S. austriaca* hairy roots (**B**).

The Kruskal–Wallis test revealed significant differences between the medians of phenolic acid content across different growth models (F—flask, Ptf—Plantform bioreactor, M-t—mist-trickling bioreactor)—with a p-value < 0.0001 for each phenolic acid measured in all cultivation environments. The Bonferroni procedure further indicated that there were statistically significant differences in the concentrations of all analyzed phenolic acids between culture models, as illustrated in Figure 7.

Rosmarinic acid was the predominant metabolite, accounting for approximately 54% of the total phenolic acids in flask-grown roots and 93% and 96% in the Plantform and mist-trickling bioreactors, respectively. Caffeic acid was the least abundant, with 22%, 1.73%, and 1.43% synthesized in flask, Plantform, and mist-trickling systems, respectively.

In conclusion, the mist-trickling bioreactor proves to be the most effective cultivation system for *S. austriaca* hairy roots, delivering both high biomass yield and efficient phenolic acid biosynthesis. The system presented in this study is structurally simple, making it cost-effective and easy to operate. By dispersing the culture medium as a fine mist, the system reduces shear forces, which are less harmful to root growth compared to other methods [10,21]. This makes it a promising option not only for laboratory research but also for a wide range of applications.

While temporary immersion systems (TIS) like the Plantform bioreactor have been successful for some species [22], they may not be optimal for *S. austriaca* and other plant species [23], as indicated by unfavorable morphological changes and lower metabolite yields. This suggests that, for each in vitro plant culture, the selection of a bioreactor for scaling up must be carefully evaluated and preceded by a thorough experimental investigation. Future research should further investigate the impact of light quality and other environmental factors on the growth and secondary metabolism of *S. austriaca* hairy roots.

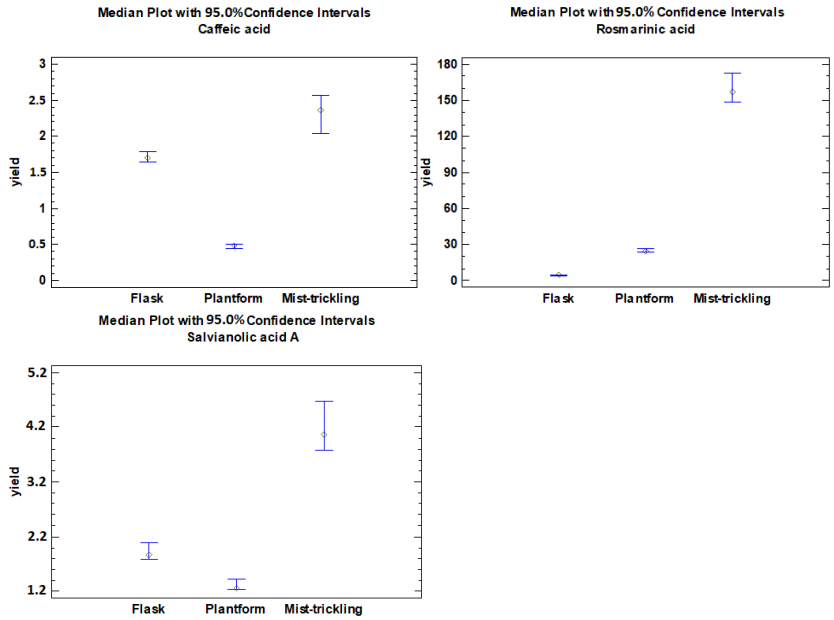


Figure 7. Median values of the determined phytocompounds measured in all three growth models, with yield expressed in mg/L.

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