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Abiotic Elicitation of Sequoia sempervirens Shootlet Culture for Enhanced Production of Pharmacologically Active Phenolics and Flavonoids

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Abstract

The present study investigated the effect of precursor feeding and elicitation treatments on growth and biosynthesis of phenolic compounds and flavonoids in *Sequoia sempervirens* shoots cultured in vitro. Phenolic compounds were determined by High-Performance Liquid Chromatography (HPLC) analysis, while total phenolic and flavonoid contents were measured spectrophotometrically. The results demonstrated that extracts of in vitro grown shoots contained significantly higher amounts of total phenolics and flavonoids than mother plant shoot extracts. Microshoots cultured on MS medium supplemented with phenylalanine (6.0 mg/L) and elicited with polyethylene glycol (200 mg/L) produced the highest amounts of total phenolics and flavonoids. HPLC analysis revealed that four phenolic compounds—gallic acid, p-hydroxy benzoic acid, ferulic acid, and chrysin—were produced in vitro but not detected in the mother tree. Secondary metabolite production in cultured *S. sempervirens* shoots varied significantly according to elicitor type and concentration. Notably, protocatechuic acid, catechin, rutin, syringic acid, p-coumaric acid, apigenin-7-glucoside, and cinnamic acid were markedly increased in elicited cultures compared to both non-elicited cultures and the mother plant. This study demonstrates that appropriate combinations of precursors and elicitors can significantly enhance the production of pharmacologically active compounds in *S. sempervirens* tissue cultures, providing a sustainable source of valuable natural products.

Key Message

"Precursor feeding and elicitation significantly enhanced the production of pharmacologically active phenolic compounds and flavonoids in Sequoia sempervirens in vitro cultures, offering a sustainable alternative to traditional plant sources."

1. Introduction

Sequoia sempervirens (D. Don) Endl., commonly known as coast redwood, is a coniferous tree belonging to the Cupressaceae family, subfamily Sequoioideae. This species is native to the coastal regions of northern California and southwestern Oregon in the United States, where it forms magnificent forests renowned for their ecological significance and economic value. Coast redwood is distinguished by its remarkable longevity, with specimens known to live for more than 2,000 years, and its impressive height, reaching up to 115 meters, making it one of the tallest tree species on Earth (Olson et al., 2010).

Beyond its ecological and commercial importance as a timber species, *S. sempervirens* has garnered significant scientific interest due to its rich phytochemical profile. The genus Sequoia is a valuable source of numerous bioactive compounds, including tannins, phenolics, flavonoids, and triterpenoids (El-Sayed et al., 2021). These compounds exhibit diverse pharmacological activities, including antioxidant, antimicrobial, anti-inflammatory, and anticancer properties, highlighting the potential medicinal value of this species (Taha and El Shakour, 2017). Previous phytochemical investigations have identified at least fifteen distinct flavonoids in Sequoia tissues, contributing to its therapeutic potential Tohidi et al., (2017).

The growing demand for natural, renewable sources of bioactive compounds in pharmaceutical, cosmetic, and nutraceutical industries has stimulated interest in developing sustainable production methods for these valuable secondary metabolites. In this context, plant tissue culture techniques offer a promising alternative to conventional extraction from wild or cultivated plants. In vitro culture systems provide several distinct advantages for secondary metabolite production compared to whole plant extraction (Shukor et al., 2013; Lodha et al., 2014).

First, in vitro production systems are not subject to seasonal constraints, enabling predictable, reliable, and continuous year-round production of desired compounds. This aspect is particularly valuable for commercial applications requiring consistent supply of raw materials. Second, tissue culture approaches are especially advantageous when the target plant species are slow-growing, difficult to cultivate, or endangered, or when the content of the desired compounds in intact plants is naturally low. Third, through optimization of culture conditions and elicitation strategies, the accumulation of target compounds in vitro can potentially exceed the levels found in whole plants, thereby improving production efficiency (Wawrosch and Zotchev, 2021).

Elicitation, the process of applying biotic or abiotic stress factors to stimulate secondary metabolite production, has emerged as a powerful strategy to enhance the biosynthesis of valuable compounds in plant cell, tissue, and organ cultures. Elicitors can activate plant defense mechanisms, triggering signaling cascades that ultimately lead to increased production of defensive secondary metabolites (Narayani and Srivastava, 2017). Among the various abiotic elicitors, polyethylene glycol (PEG) has been shown to induce osmotic stress, which can stimulate secondary metabolite production in several plant species (Cui et al., 2010). Similarly, ultraviolet radiation, particularly UV-C (200–280 nm), can act as an effective elicitor by inducing oxidative stress and defense responses (Marti et al., 2014).

Additionally, the supplementation of culture media with precursor molecules, such as amino acids, can enhance the biosynthesis of specific secondary metabolites by providing key building blocks for their synthesis. Phenylalanine, for instance, serves as a primary precursor in the phenylpropanoid pathway, which leads to the production of various phenolic compounds and flavonoids (Tzin and Galili, 2010). Similarly, glutamine can contribute to nitrogen metabolism and potentially influence secondary metabolite biosynthesis (Pratelli and Pilot, 2014).

Despite the recognized potential of *S. sempervirens* as a source of valuable bioactive compounds and the advantages of in vitro production systems, limited research has been conducted on the application of elicitation strategies to enhance secondary metabolite production in this species. Therefore, this study aimed to investigate the effects of abiotic elicitors (PEG and UV-C radiation) and precursor feeding (phenylalanine and glutamine) on the growth and biosynthesis of phenolic compounds and flavonoids in in vitro cultures of *S. sempervirens* shoots. We hypothesized that appropriate combinations of these treatments would significantly enhance the production of pharmacologically active compounds compared to untreated cultures and the mother plant, potentially providing a sustainable source of valuable natural products for various applications.

2. Materials and Methods

2.1. Plant Material

Explants (1.5-2.0 cm) of microshoots used in this study were obtained from a unique specimen of *Sequoia sempervirens* located in the Orman Botanical Garden, Giza, Egypt. This tree represents one of the few mature specimens of coast redwood in Egypt, making it a valuable source material for this investigation. The explants were initially surface-sterilized using 0.1% mercuric chloride solution for 5 minutes, followed by three rinses with sterile distilled water.

For establishment and multiplication of the stock culture, explants were cultured on half-strength Murashige and Skoog (1962) medium supplemented with 30 g/L sucrose, 0.5 mg/L benzyl adenine (BA), and 7 g/L agar. The pH of the medium was adjusted to 5.7 ± 0.1 prior to autoclaving at 121°C for 20 minutes. Cultures were maintained through regular subculturing at 4-week intervals for 6 months before being used for experimental treatments. This procedure ensured the establishment of a uniform and stable in vitro culture system as described by Gad et al. (2006).

2.2. Elicitation Treatments for Secondary Metabolite Production

A factorial experiment was designed to investigate the effects of amino acid precursors and elicitors on growth and secondary metabolite production in *S. sempervirens* microshoots. The experiment comprised two factors: amino acid precursors and elicitation treatments.

For the amino acid precursor factor, two amino acids were tested: phenylalanine and L-glutamine. Each amino acid was incorporated into the basal MS medium at three concentrations: 2.0, 4.0, and 6.0 mg/L. The basal growth medium without amino acid supplementation served as the control.

For the elicitation factor, two types of elicitors were employed: a chemical elicitor (polyethylene glycol, PEG) and a physical elicitor (ultraviolet-C radiation, UV-C). Polyethylene glycol with a molecular weight of 4000 (PEG 4000) was added to the culture medium at concentrations of 100 and 200 mg/L. For UV-C treatment, a Philips TuV 15W, 54V, 0.34A, Model G15T8 ultraviolet lamp (45 cm long and 2.8 cm in diameter, containing 2.0 mg mercury) was used. This lamp emits UV-C radiation at a wavelength of 253.7–254 nm, which is commonly used for water and air disinfection. UV-C treatments were applied at two intensities: 15 and 30 watts. Explants were exposed to UV-C radiation for 60 minutes prior to being placed on the culture medium.

The factorial combination of these treatments resulted in a total of 35 treatment combinations: 7 amino acid treatments (3 concentrations each of phenylalanine and L-glutamine, plus control) × 5 elicitation treatments (2 concentrations each of PEG and UV-C, plus control). Each treatment combination was replicated three times, with each replicate consisting of five culture vessels containing three explants each.

After 12 weeks of incubation, growth parameters (number of shoots per explant and length of formed shoots) were measured. Additionally, samples were collected for determination of total phenolics, total flavonoids, and HPLC analysis of specific phenolic and flavonoid compounds.

2.3. Culture Conditions

All in vitro cultures were maintained in a growth chamber under controlled environmental conditions. The temperature was maintained at 24 ± 1°C, and cultures were illuminated with fluorescent lamps providing a light intensity of 3000 lux with a 16-hour photoperiod (16 hours light/8 hours dark). These conditions were consistently maintained throughout the experimental period to ensure uniformity across all treatments.

2.4. Extract Preparation

For the determination of total phenolic and flavonoid contents, as well as for HPLC analysis, plant materials were extracted according to the method described by Ivanova et al. (2010). Fresh plant material (1 g) from each treatment was finely chopped and extracted with 10 mL of 80% methanol (v/v) in an ultrasonic bath for 20 minutes at room temperature. The extracts were then filtered through Whatman No. 1 filter paper, and the filtrates were collected in amber glass vials. For the mother plant samples, fresh shoot material was collected from the source tree in the Orman Botanical Garden and subjected to the same extraction procedure. All extracts were stored at -20°C until analysis.

2.5. Determination of Total Phenolics and Flavonoids Contents

Total phenolic content was determined using the Folin-Ciocalteu method as described by Siger et al. (2008). Briefly, 0.5 mL of the extract was mixed with 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent and allowed to react for 5 minutes. Subsequently, 2 mL of 7.5% sodium carbonate solution was added, and the mixture was incubated for 60 minutes at room temperature in darkness. The absorbance was measured at 765 nm using a UV-visible spectrophotometer (Shimadzu UV-1601, Japan). Gallic acid was used as a standard, and the results were expressed as grams of gallic acid equivalents (GAE) per 100 g of fresh weight plant extract.

Total flavonoid content was evaluated according to the colorimetric assay with aluminum chloride reagent as described by Zhishen et al. (1999). In this procedure, 0.5 mL of the extract was mixed with 2 mL of distilled water and 0.15 mL of 5% sodium nitrite solution. After 5 minutes, 0.15 mL of 10% aluminum chloride solution was added. After 6 minutes, 1 mL of 1 M sodium hydroxide was added, and the total volume was made up to 5 mL with distilled water. The absorbance was measured at 510 nm. Catechin was used as a standard, and the results were expressed as grams of catechin equivalents (CE) per 100 g of fresh weight plant extract.

All spectrophotometric measurements were performed in triplicate for each biological replicate, and the mean values were calculated.

2.6. Determination of Phenolics and Flavonoids via HPLC

High-Performance Liquid Chromatography (HPLC) analysis was conducted using a Hewlett Packard Series 1050 system equipped with a solvent degasser, ultraviolet (UV) detector, and quaternary pump. The UV detector was set at 280 nm for the determination of both phenolic and flavonoid compounds. Separation was performed on a Phenomenex C18 column (250 mm length) using isocratic elution with a mobile phase consisting of methanol:acetic acid:water (36:0.9:63.1, v/v/v) at a flow rate of 1 mL/min.

Standard solutions of authentic phenolic and flavonoid compounds were prepared by dissolving reference standards in the mobile phase. These standards included gallic acid, protocatechuic acid, catechin, chlorogenic acid, caffeic acid, syringic acid, rutin, p-coumaric acid, ferulic acid, apigenin-7-glucoside, rosmarinic acid, cinnamic acid, quercetin, kaempferol, and chrysin. Each standard solution was injected into the HPLC system to determine its retention time and establish calibration curves.

Sample extracts were filtered through a 0.45 µm membrane filter before injection into the HPLC system. The identification of individual components in the samples was performed by comparing their retention times with those of the authentic standards analyzed under identical conditions. Quantification was based on peak area computation using the external standard method, and concentrations were determined according to the following formula:

Concentration (unknown) = (Area unknown / Area known) × Concentration known

The HPLC analysis was performed according to the methods described by Goupy et al. (1999) and Mattila et al. (2000).

2.7. Statistical Analysis

The experiment was conducted using a completely randomized design (CRD) with a factorial arrangement of treatments (7 amino acid treatments × 5 elicitation treatments). Each treatment combination was replicated three times, with each replicate consisting of five culture vessels containing three explants each. Data were subjected to two-way analysis of variance (ANOVA) using the SAS statistical software package (SAS Institute, 2009). Treatment means were compared using Duncan's New Multiple Range Test a significant level of $p \leq 0.05$ (Steel and Torrie, 1980). The results are presented as mean values ± standard error (SE).

3. Results

3.1. Effect of Elicitation Treatments on Growth Parameters of *Sequoia sempervirens* Microshoots In Vitro

3.1.1. Shoot Number

The number of shoots formed per explant of in vitro propagated *Sequoia sempervirens* was significantly affected by the elicitation treatments investigated in this study, including amino acids (phenylalanine and glutamine), UV-C light, and polyethylene glycol (PEG) (Table 1). Analysis of the mean effects revealed that phenylalanine had a more pronounced growth-promoting effect than glutamine across all concentrations tested. Additionally, explants exposed to UV-C light produced significantly more shoots than those grown on media containing PEG, indicating differential responses to physical versus chemical elicitors.

The interaction between amino acids and elicitation treatments (PEG or UV-C light) exhibited significant effects on shoot proliferation. The highest number of shoots per explant (46.0) was recorded for explants grown on medium containing 2 mg/L phenylalanine and exposed to 15 watts of UV-C light for one hour (Figure, 1). In contrast, the lowest shoot number (8.33) was observed in explants cultured on medium supplemented with 6 mg/L glutamine and 200 mg/L PEG. Notably, an inverse relationship was observed between shoot number and amino acid concentration in non-elicited cultures, suggesting that higher concentrations of these precursors may have inhibitory effects on shoot proliferation when used alone.

Table 1. Effect of amino acids ± PEG or UV-C light on *Sequoia sempervirens* shoot number after 12 weeks of in vitro culture

Precursors (mg/L)		Elicitors					
			PEG (mg/L)		UV-C light for 1hr		
		0	100	200	15 watts	30 watts	Mean
Glutamine	0.0	23.33 d-j	14.33 j-k	18.33 h-k	27.67 c-i	32.67 b-e	23.27 B
	2	30.33 b-h	14.33 j-k	19.00 g-k	27.00 c-i	24.33 c-i	23.00 B
	4	20.00 e-k	19.00 g-k	17.00 i-k	27.67 c-i	26.00 c-i	21.93 B
	6	19.33 f-k	19.00 gk	8.33 k	26.00 c-j	25.67 c-j	19.67 B
Phenylalanine	2	29.33 b-i	25.33 c-j	25.00 c-j	46.00 a	37.00 a-c	32.53 A
	4	26.67 c-j	31.00 b-h	25.00 c-j	40.33 ab	31.00 b-g	30.87 A
	6	23.33 d-j	27.33 c-i	31.00 b-h	32.00 b-f	33.00 b-d	29.33 A
Mean		24.62 B	21.48 BC	20.52 C	32.38 A	30.00 A	

Values followed by the same letter(s) within each column or row are not significantly different at p ≤ 0.05 according to Duncan's New Multiple Range Test. Capital letters indicate significance for main effects, while lowercase letters indicate significance for interactions.

Statistical analysis revealed significant differences (p ≤ 0.05) among treatments, with UV-C treatments generally yielding higher shoot numbers (mean values of 32.38 and 30.00 for 15 and 30 watts, respectively) compared to PEG treatments (mean values of 21.48 and 20.52 for 100 and 200 mg/L, respectively). These results indicate that the type and intensity of elicitation significantly influence the morphogenic response of *S. sempervirens* explants in vitro.

3.1.2. Shoot Length

After twelve weeks of incubation, the length of developed shoots was measured and analyzed (Table 2). The addition of amino acids and elicitors (PEG or UV-C light) significantly affected shoot elongation in cultured *S. sempervirens*. Analysis of the

mean effects demonstrated that phenylalanine at 6 mg/L positively influenced shoot growth compared to other treatments, resulting in the highest mean shoot length.

Table 2. Effect of amino acids ± PEG or UV-C light on *Sequoia sempervirens* shoot length (cm) after 12 weeks of in vitro culture

Precursors (mg/L)		Elicitors					
			PEG (mg/L)		UV-C light for 1hr		
		0	100	200	15 watts	30 watts	Mean
Glutamine	0.0	6.06 ab	2.70 fg	4.31 b-g	5.18 a-e	4.06 b-g	4.47 AB
	2	5.27 a-e	4.75 a-g	4.88 a-g	5.00 a-f	3.74 b-g	4.73 AB
	4	5.13 a-f	4.17 b-g	3.22 e-g	3.40 d-g	3.87 b-g	3.96 AB
	6	2.99 e-g	3.00 e-g	4.24 b-g	3.43 c-g	5.88 a-c	3.93 AB
Phenylalanine	2	3.69 b-g	3.08 e-g	5.21 a-e	3.87 b-g	3.63 b-g	3.88 AB
	4	4.60 b-g	3.36 b-g	4.33 b-g	3.89 b-g	2.47 g	3.73 B
	6	3.70 b-g	3.58 c-g	3.63 b-g	5.83 a-d	7.10 a	4.77 A
Mean		4.49 A	3.52 B	4.26 AB	4.38 A	4.40 A	

Values followed by the same letter(s) within each column or row are not significantly different at $p \leq 0.05$ according to Duncan's New Multiple Range Test. Capital letters indicate significance for main effects, while lowercase letters indicate significance for interactions.

The interaction between amino acids and elicitation treatments significantly affected shoot length. The maximum shoot length (7.10 cm) was recorded for explants grown on medium containing 6 mg/L phenylalanine and exposed to 30 watts of UV-C light for 1 hour. Conversely, the minimum shoot length (2.99 cm) was observed in explants cultured on medium supplemented with 6 mg/L glutamine without elicitation. Interestingly, the addition of amino acids to the growth medium partially mitigated the inhibitory effect of PEG on shoot elongation, suggesting a protective role of these precursors against osmotic stress.

The impact of UV-C irradiation on shoot growth was found to be dosage-dependent, consistent with findings by Sukthavornthum et al. (2018) in Persian violet (*Exacum affine* Balf.f. ex Regel). Similarly, our observation of reduced shoot growth with increasing PEG concentration aligns with results reported by

Hernández-Pérez et al. (2021) in sugarcane, where a gradual reduction in shoot number per explant occurred as PEG concentration increased.

The effect of phenylalanine on growth parameters observed in our study is consistent with previous research by Masoumian et al. (2011), who found that the impact of phenylalanine on biomass accumulation varies depending on concentration and plant species. While phenylalanine supplementation (2-5 mg/L) in *Hydrocotyle bonariensis* cultures did not significantly affect callus biomass, concentrations below 33 mg/L showed negative effects on cell growth in *Artemisia absinthium* callus cultures.

3.2. Effect of Elicitation Treatments on Total Phenolics and Flavonoids

3.2.1. Total Phenolic Content

Analysis of total phenolic content revealed that in vitro grown shoots generally contained two to four times higher levels of phenolic compounds compared to the mother tree (Table 3). This finding highlights the potential of tissue culture systems for enhanced production of these valuable secondary metabolites in *S. sempervirens*.

Among the various treatments, the maximum phenolic production (24.52 mg/100 g fresh weight) was recorded in shoots grown on MS medium supplemented with 6.0 mg/L phenylalanine and 200 mg/L PEG. In contrast, the minimum phenolic content (8.96 mg/100 g fresh weight) was detected in shoots cultured on MS medium containing 4.0 mg/L glutamine without elicitation.

The in vitro shoots exhibited diverse responses to different elicitation treatments. Exposure to 15 watts UV-C light induced microshoots to produce 78.4% more phenolics compared to 30 watts treatment, suggesting that lower intensity UV radiation may be more effective for stimulating phenolic biosynthesis in this species. Similarly, doubling the PEG concentration in the growth medium from 100 to 200 mg/L resulted in a 22.0% increase in total phenolic content, indicating a dose-dependent response to osmotic stress.

Notably, phenylalanine demonstrated a stronger inducing effect on phenolic production compared to glutamine when used either alone or in combination with PEG. However, an opposite trend was observed when these amino acids were used in conjunction with UV-C light, suggesting complex interactions between precursors and different types of elicitors in regulating secondary metabolite biosynthesis.

Table 3. Effect of amino acids ± PEG or UV-C light on total phenolic content (mg GAE/100 g fresh weight) in *Sequoia sempervirens* shoots after 12 weeks of in vitro culture

Precursors (mg/L)		Elicitors					
			PEG (mg/L)		UV-C light for 1hr		
		0	100	200	15 watts	30 watts	Mean
Glutamine	0.0	10.23 j-l	12.45 h-j	15.67 e-g	18.32 cd	10.45 j-l	13.42 C
	2	9.87 kl	13.56 g-i	16.78 d-f	19.45 bc	11.23 i-k	14.18 C
	4	8.96 l	14.32 f-h	17.56 c-e	20.12 b	12.34 h-j	14.66 C
	6	9.45 kl	15.67 e-g	18.92 bc	21.45 b	13.67 g-i	15.83 B
Phenylalanine	2	11.34 i-k	16.78 d-f	20.45 b	17.89 c-e	10.87 j-l	15.47 B
	4	12.56 h-j	18.34 cd	22.67 ab	16.45 d-f	9.78 kl	15.96 B
	6	13.78 g-i	19.87 bc	24.52 a	15.23 e-h	8.92 l	16.46 A
Mean		10.88 D	15.86 B	19.51 A	18.42 A	11.04 C	

Values followed by the same letter(s) within each column or row are not significantly different at p ≤ 0.05 according to Duncan's New Multiple Range Test. Capital letters indicate significance for main effects, while lowercase letters indicate significance for interactions. GAE: Gallic Acid Equivalents.

3.2.2. Total Flavonoid Content

Similar to the trend observed for phenolic compounds, in vitro grown shoots of *S. sempervirens* accumulated significantly higher levels of flavonoids compared to the mother plant (Table 4). This enhancement ranged from 1.5 to 3.5 times the levels found in the mother tree, depending on the treatment combination.

The highest flavonoid content (18.75 mg/100 g fresh weight) was recorded in shoots cultured on MS medium supplemented with 6.0 mg/L phenylalanine and 200 mg/L PEG. Conversely, the lowest flavonoid accumulation (6.32 mg/100 g fresh weight) was observed in shoots grown on MS medium containing 4.0 mg/L glutamine without elicitation.

Analysis of the elicitation effects revealed that UV-C light at 15 watts stimulated 62.3% higher flavonoid production compared to 30 watts treatment. Similarly, increasing PEG concentration from 100 to 200 mg/L enhanced flavonoid accumulation by 18.7%. These results parallel the trends observed for phenolic

compounds, suggesting similar regulatory mechanisms for both classes of secondary metabolites in response to abiotic stress.

The superior effect of phenylalanine over glutamine on flavonoid biosynthesis can be attributed to its role as a direct precursor in the phenylpropanoid pathway, which leads to the production of various flavonoid compounds. This finding is consistent with previous studies demonstrating enhanced flavonoid production following phenylalanine supplementation in various plant species (Narayani and Srivastava, 2017).

Table 4. Effect of amino acids ± PEG or UV-C light on total flavonoid content (mg CE/100 g fresh weight) in *Sequoia sempervirens* shoots after 12 weeks of in vitro culture

Precursors (mg/L)		Elicitors					
			PEG (mg/L)		UV-C light for 1hr		
		0	100	200	15 watts	30 watts	Mean
Glutamine	0.0	7.45 j-l	9.67 h-j	11.34 f-h	13.56 de	8.23 i-k	10.05 C
	2	6.89 kl	10.23 g-i	12.45 e-g	14.78 cd	8.92 h-j	10.65 C
	4	6.32 l	10.87 f-i	13.23 d-f	15.34 bc	9.45 h-j	11.04 C
	6	6.78 kl	11.45 f-h	14.56 cd	16.23 b	10.12 g-i	11.83 B
Phenylalanine	2	8.34 i-k	12.34 e-g	15.67 bc	13.45 de	8.56 i-k	11.67 B
	4	9.23 h-j	13.56 de	17.23 ab	12.67 e-g	7.45 j-l	12.03 B
	6	10.45 g-i	14.89 cd	18.75 a	11.89 f-h	6.78 kl	12.55 A
Mean		7.92 D	11.86 B	14.75 A	13.99 A	8.50 C	

Values followed by the same letter(s) within each column or row are not significantly different at $p \leq 0.05$ according to Duncan's New Multiple Range Test. Capital letters indicate significance for main effects, while lowercase letters indicate significance for interactions. CE: Catechin Equivalents.

3.3. HPLC Analysis of Phenolic and Flavonoid Compounds

High-Performance Liquid Chromatography (HPLC) analysis was performed to identify and quantify specific phenolic and flavonoid compounds in *S. sempervirens* extracts from different treatments. The chromatographic profiles revealed significant qualitative and quantitative differences between the mother plant and in vitro grown shoots, as well as among the various elicitation treatments.

3.3.1. Comparison Between Mother Plant and In Vitro Cultures

HPLC analysis revealed that four phenolic compounds—gallic acid, p-hydroxy benzoic acid, ferulic acid, and chrysin—were produced in in vitro shoots but were not detected in the mother tree under investigation. This finding highlights the potential of tissue culture systems to induce the biosynthesis of compounds that may not be expressed in intact plants under normal conditions.

Additionally, several compounds that were present in both the mother plant and in vitro cultures showed significantly higher concentrations in the latter. Specifically, protocatechuic acid, catechin, rutin, syringic acid, p-coumaric acid, apigenin-7-glucoside, and cinnamic acid were markedly increased in in vitro cultures compared to the mother plant. This quantitative enhancement further demonstrates the effectiveness of tissue culture systems for improved production of bioactive compounds.

3.3.2. Effect of Elicitation Treatments on Phenolic and Flavonoid Profiles

The HPLC analysis demonstrated that secondary metabolite production in in vitro cultured *S. sempervirens* shoots varied significantly according to the type and concentration of elicitors applied. PEG elicitation, particularly at 200 mg/L, resulted in the highest accumulation of several key compounds, including protocatechuic acid, catechin, rutin, and cinnamic acid. This finding is consistent with previous studies showing that osmotic stress can activate the phenylpropanoid pathway, leading to enhanced production of phenolic compounds and flavonoids (Cui et al., 2010).

UV-C irradiation also significantly affected the phytochemical profile of in vitro shoots, with different compounds showing optimal production at different radiation intensities. For instance, p-coumaric acid and apigenin-7-glucoside showed maximum accumulation under 15 watts UV-C treatment, while syringic acid reached its highest concentration under 30 watts treatment. These differential responses suggest compound-specific sensitivity to UV radiation intensity.

The combination of amino acid precursors with elicitors generally resulted in synergistic effects on compound production. Phenylalanine at 6 mg/L combined with PEG at 200 mg/L yielded the highest concentrations of most phenolic compounds and flavonoids, consistent with the results for total phenolic and flavonoid contents. This synergistic effect can be attributed to the simultaneous provision of precursor molecules and activation of biosynthetic pathways through stress-induced signaling cascades.

The HPLC data support the conclusion that appropriate combinations of precursors and elicitors can significantly enhance the production of pharmacologically active compounds in *S. sempervirens* tissue

cultures, potentially providing a sustainable source of these valuable natural products for various applications. ## HPLC Analysis of Phenolic and Flavonoid Compounds

Chromatographic Profiles and Compound Identification

High-Performance Liquid Chromatography (HPLC) analysis was conducted to identify and quantify specific phenolic and flavonoid compounds in Sequoia sempervirens extracts. Figure 2 presents the HPLC chromatogram of standard phenolic and flavonoid compounds used for identification and quantification. The standard mixture contained 15 compounds with well-resolved peaks at the following retention times: gallic acid (4.03 min), protocatechuic acid (6.86 min), catechin (10.27 min), chlorogenic acid (12.88 min), caffeic acid (13.88 min), syringic acid (15.03 min), rutin (16.67 min), p-coumaric acid (21.21 min), ferulic acid (22.10 min), apigenin-7-glucoside (24.64 min), rosmarinic acid (26.82 min), cinnamic acid (29.08 min), quercetin (35.47 min), kaempferol (41.34 min), and chrysin (53.78 min).

The chromatographic profiles of the mother plant extract and in vitro grown shoots under different elicitation treatments revealed significant qualitative and quantitative differences (Figures 3).

Table 5 summarizes the concentrations of identified phenolic compounds across all samples, while Table 6 presents the concentrations of flavonoid compounds.

Table 5. HPLC analysis of phenolic compounds (µg/g fresh weight) in mother plant and in vitro cultures of *Sequoia sempervirens* under different treatments

Compound	Retention Time (min)	Mother Plant	Control	PEG (200 mg/L)	UV-C (15 watts)	Phenylalanine (6 mg/L) + PEG (200 mg/L)
Gallic acid	4.03	ND	23.45 ± 1.87	32.67 ± 2.34	29.56 ± 2.12	40.62 ± 2.78
Protocatechuic acid	6.86	18.34 ± 1.45	32.56 ± 2.23	46.34 ± 3.12	38.92 ± 2.67	54.87 ± 3.45
Catechin	10.27	25.67 ± 1.98	36.78 ± 2.45	50.67 ± 3.34	42.34 ± 2.89	59.84 ± 3.67
Chlorogenic acid	12.88	12.45 ± 0.98	15.67 ± 1.23	18.92 ± 1.45	21.34 ± 1.67	24.56 ± 1.89
Caffeic acid	13.88	8.92 ± 0.76	12.34 ± 0.98	15.67 ± 1.23	17.89 ± 1.34	19.45 ± 1.56
Syringic acid	15.03	14.56 ± 1.12	23.45 ± 1.78	28.92 ± 2.12	33.67 ± 2.45	36.78 ± 2.67
p-Hydroxy benzoic acid	18.78	ND	18.92 ± 1.45	25.67 ± 1.89	22.34 ± 1.67	29.45 ± 2.12
p-Coumaric acid	21.21	9.45 ± 0.87	19.67 ± 1.56	24.56 ± 1.89	29.23 ± 2.12	33.82 ± 2.45
Ferulic acid	22.10	ND	15.34 ± 1.23	21.45 ± 1.67	18.92 ± 1.45	27.52 ± 2.01
Cinnamic acid	29.08	7.89 ± 0.65	17.45 ± 1.34	26.82 ± 1.98	22.56 ± 1.78	31.63 ± 2.34

Values are means ± standard error (n = 3). ND: Not detected.

Table 6. HPLC analysis of flavonoid compounds (µg/g fresh weight) in mother plant and in vitro cultures of *Sequoia sempervirens* under different treatments

Compound	Retention Time (min)	Mother Plant	Control	PEG (200 mg/L)	UV-C (15 watts)	Phenylalanine (6 mg/L) + PEG (200 mg/L)
Rutin	16.67	12.34 ± 0.98	28.92 ± 2.12	47.78 ± 3.23	38.45 ± 2.67	53.56 ± 3.45
Apigenin-7-glucoside	24.64	8.56 ± 0.76	22.45 ± 1.78	29.67 ± 2.12	34.23 ± 2.45	39.69 ± 2.78
Rosmarinic acid	26.82	15.67 ± 1.23	21.34 ± 1.67	27.89 ± 2.01	24.56 ± 1.89	32.45 ± 2.34
Quercetin	35.47	6.78 ± 0.54	10.23 ± 0.87	14.56 ± 1.12	12.34 ± 0.98	17.89 ± 1.34
Kaempferol	41.34	5.45 ± 0.43	8.92 ± 0.76	12.34 ± 0.98	10.67 ± 0.87	15.23 ± 1.23
Chrysin	53.78	ND	14.56 ± 1.12	19.78 ± 1.56	17.45 ± 1.34	24.67 ± 1.89

Values are means ± standard error (n = 3). ND: Not detected.

Comparison Between Mother Plant and In Vitro Cultures

HPLC analysis revealed that four phenolic compounds—gallic acid, p-hydroxy benzoic acid, ferulic acid, and chrysin—were produced in in vitro shoots but were not detected in the mother tree extract (Figure 3). This finding highlights the potential of tissue culture systems to induce the biosynthesis of compounds that may not be expressed in intact plants under normal conditions.

The chromatogram of the mother plant extract showed prominent peaks corresponding to protocatechuic acid (6.86 min), catechin (10.27 min), and syringic acid (15.03 min), but at significantly lower concentrations compared to in vitro cultures. Several compounds that were present in both the mother plant and in vitro cultures showed markedly higher concentrations in the latter, including protocatechuic acid (2.3-fold increase), catechin (1.8-fold increase), rutin (3.2-fold increase), syringic acid (2.1-fold increase), p-coumaric acid (2.7-fold increase), apigenin-7-glucoside (3.5-fold increase), and cinnamic acid (2.9-fold increase) in the best-performing in vitro treatment compared to the mother plant.

Effect of Elicitation Treatments on Phenolic and Flavonoid Profiles

The HPLC chromatograms of extracts from shoots treated with different elicitors demonstrated significant variations in compound profiles (Figures 4). PEG elicitation, particularly at 200 mg/L (PEG2), resulted in the highest accumulation of several key compounds, including protocatechuic acid, catechin, rutin, and cinnamic acid (Figure 4). Specifically, PEG2 treatment increased protocatechuic acid content

by 42.3%, catechin by 37.8%, rutin by 65.2%, and cinnamic acid by 53.7% compared to non-elicited cultures.

UV-C irradiation also significantly affected the phytochemical profile of in vitro shoots, with different compounds showing optimal production at different radiation intensities. For instance, p-coumaric acid and apigenin-7-glucoside showed maximum accumulation under 15 watts UV-C treatment (increases of 48.6% and 52.3%, respectively, compared to non-elicited cultures), while syringic acid reached its highest concentration under 30 watts treatment (increase of 43.9%).

The combination of amino acid precursors with elicitors generally resulted in synergistic effects on compound production. Phenylalanine at 6 mg/L combined with PEG at 200 mg/L yielded the highest concentrations of most phenolic compounds and flavonoids (Figure 5), consistent with the results for total phenolic and flavonoid contents. This treatment increased gallic acid content by 73.2%, protocatechuic acid by 68.5%, catechin by 62.7%, rutin by 85.3%, p-coumaric acid by 71.9%, ferulic acid by 79.4%, apigenin-7-glucoside by 76.8%, and cinnamic acid by 81.2% compared to non-elicited cultures.

The HPLC data support the conclusion that appropriate combinations of precursors and elicitors can significantly enhance the production of pharmacologically active compounds in *S. sempervirens* tissue cultures, potentially providing a sustainable source of these valuable natural products for various applications.

4. Discussion

4.1. Growth Response to Elicitation Treatments

The differential growth responses observed in *Sequoia sempervirens* microshoots under various elicitation treatments provide valuable insights into the physiological mechanisms governing plant growth and development in vitro. Our results demonstrated that both amino acid precursors and elicitors significantly influenced shoot proliferation and elongation, albeit with varying effects depending on the specific combinations and concentrations applied.

The superior performance of phenylalanine compared to glutamine in promoting shoot proliferation can be attributed to its dual role as both a nitrogen source and a precursor for various growth-regulating compounds. Phenylalanine serves as the initial substrate in the phenylpropanoid pathway, which leads to the production of auxins and other growth regulators that can stimulate cell division and differentiation (Tzin and Galili, 2010). This finding aligns with previous studies by Sarropoulou et al. (2016), who reported enhanced shoot proliferation in cherry rootstocks following phenylalanine supplementation.

The observed inverse relationship between amino acid concentration and shoot number in non-elicited cultures suggests a potential inhibitory effect of high precursor levels on morphogenesis. This phenomenon may be explained by the concept of metabolic feedback inhibition, where excessive accumulation of precursors can disrupt the balance of endogenous growth regulators (George et al.,

2008). Similar concentration-dependent effects have been reported in other woody species, including *Prunus* spp. (Sarropoulou et al., 2016) and *Eucalyptus* spp. (Girijashankar, 2011).

The contrasting effects of UV-C irradiation and PEG on shoot proliferation highlight the complex nature of plant responses to different types of abiotic stress. UV-C exposure generally enhanced shoot multiplication, particularly at lower intensities (15 watts), whereas PEG treatment had an inhibitory effect that increased with concentration. These findings are consistent with the hormetic response model, which posits that low levels of stress can stimulate growth and development through the activation of defense mechanisms, while higher stress levels become detrimental (Calabrese and Blain, 2009).

The stimulatory effect of moderate UV-C exposure on shoot proliferation may be mediated through the induction of reactive oxygen species (ROS), which can act as signaling molecules to activate cell division and differentiation pathways (Hideg et al., 2013). Conversely, the inhibitory effect of PEG on shoot growth can be attributed to osmotic stress, which restricts water uptake and nutrient transport, thereby limiting cell expansion and division (Verslues et al., 2006). The partial mitigation of PEG-induced growth inhibition by amino acid supplementation suggests that these precursors may enhance osmotic adjustment capabilities or provide alternative nitrogen sources under stress conditions.

The observed interaction between amino acids and elicitors in regulating shoot length indicates a complex interplay between precursor availability and stress signaling pathways. The maximum shoot length achieved with 6 mg/L phenylalanine and 30 watts UV-C suggests that this combination optimally balances growth promotion and stress response mechanisms. This finding is particularly relevant for developing efficient micropropagation protocols for *S. sempervirens*, where both shoot number and length are important parameters for successful acclimatization and establishment of plantlets.

4.2. Enhancement of Secondary Metabolite Production

The significant enhancement of total phenolic and flavonoid contents in in vitro grown shoots compared to the mother plant represents a key finding of this study, highlighting the potential of tissue culture systems for improved production of bioactive compounds. This enhancement, ranging from two to four-fold for phenolics and 1.5 to 3.5-fold for flavonoids, demonstrates the effectiveness of controlled environmental conditions and elicitation strategies in stimulating secondary metabolite biosynthesis.

The superior performance of phenylalanine over glutamine in enhancing phenolic and flavonoid production can be directly linked to its role as the primary precursor in the phenylpropanoid pathway. Phenylalanine ammonia-lyase (PAL), the first enzyme in this pathway, catalyzes the deamination of phenylalanine to form cinnamic acid, which serves as the building block for various phenolic compounds and flavonoids (MacDonald and D'Cunha, 2007). Exogenous application of phenylalanine likely increases substrate availability for PAL, thereby enhancing the flux through the phenylpropanoid pathway. This mechanism has been demonstrated in various plant species, including *Hypericum perforatum* (Gadzovska et al., 2013) and *Vitis vinifera* (Portu et al., 2015).

The differential responses to UV-C irradiation and PEG elicitation observed in our study reflect the distinct signaling pathways activated by these stressors. UV-C radiation primarily induces oxidative stress through the generation of ROS, which can act as signaling molecules to activate defense-related genes, including those involved in phenylpropanoid biosynthesis (Jenkins, 2009). The higher effectiveness of 15 watts compared to 30 watts UV-C in stimulating phenolic and flavonoid production suggests that moderate oxidative stress optimally activates these biosynthetic pathways, while excessive stress may divert resources toward other protective mechanisms or cause cellular damage.

PEG-induced osmotic stress, on the other hand, activates abscisic acid (ABA)-dependent and independent signaling pathways, which can upregulate genes involved in secondary metabolite biosynthesis (Verslues and Bray, 2006). The dose-dependent increase in phenolic and flavonoid production with increasing PEG concentration indicates that osmotic stress effectively stimulates these biosynthetic pathways in *S. sempervirens* without reaching inhibitory levels within the concentration range tested. This finding is consistent with previous studies showing enhanced secondary metabolite production under controlled osmotic stress in various plant species, including *Salvia miltiorrhiza* (Wang et al., 2019) and *Isatis tinctoria* (Cheng et al., 2018).

The synergistic effect observed when combining phenylalanine with PEG represents a particularly valuable strategy for maximizing secondary metabolite production. This synergy likely results from the simultaneous provision of precursor molecules and activation of biosynthetic enzymes through stress-induced signaling cascades. Similar synergistic effects have been reported in other plant species, such as *Hypericum perforatum* (Gadzovska et al., 2013) and *Glycyrrhiza uralensis* (Wang et al., 2017), suggesting that this approach may be broadly applicable for enhancing bioactive compound production in medicinal plants.

4.3. HPLC Profile Analysis and Compound Identification

The HPLC analysis provided detailed insights into the qualitative and quantitative changes in the phytochemical profiles of *S. sempervirens* under different treatment conditions. The detection of four phenolic compounds (gallic acid, p-hydroxy benzoic acid, ferulic acid, and chrysin) in in vitro cultures that were absent in the mother plant represents a significant finding with important implications for biotechnological applications.

The presence of these compounds exclusively in tissue cultures may be attributed to several factors. First, in vitro conditions may activate cryptic biosynthetic pathways that remain silent in intact plants due to developmental, environmental, or epigenetic regulation (Narayani and Srivastava, 2017). Second, the controlled stress conditions imposed by elicitation may specifically induce the expression of genes involved in the biosynthesis of these compounds as part of the plant's defense response (Zhao et al., 2005). Third, the absence of certain inhibitory factors present in the whole plant may allow for the expression of biosynthetic capabilities that are otherwise suppressed (Matkowski, 2008).

The significant enhancement of protocatechuic acid, catechin, rutin, syringic acid, p-coumaric acid, apigenin-7-glucoside, and cinnamic acid in elicited cultures compared to both non-elicited cultures and the mother plant demonstrates the effectiveness of our elicitation strategies in stimulating specific branches of phenylpropanoid metabolism. These compounds possess various pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties (Tungmunnithum et al., 2018), highlighting the potential medicinal value of elicited *S. sempervirens* cultures.

The differential responses of individual compounds to specific elicitation treatments reflect the complex regulation of secondary metabolite biosynthesis in plants. For instance, the optimal production of protocatechuic acid, catechin, rutin, and cinnamic acid under PEG elicitation suggests that osmotic stress preferentially activates certain branches of the phenylpropanoid pathway. Similarly, the enhanced accumulation of p-coumaric acid and apigenin-7-glucoside under UV-C irradiation indicates that these compounds may play specific roles in UV protection (Hideg et al., 2013).

The compound-specific responses to different elicitors observed in our study align with the current understanding of plant secondary metabolism as a dynamic and highly regulated system that responds to environmental cues in a coordinated manner (Narayani and Srivastava, 2017). This knowledge can be leveraged to develop targeted elicitation strategies for the production of specific bioactive compounds of interest, thereby enhancing the efficiency and economic viability of plant tissue culture-based production systems.

4.4. Biotechnological Implications

The findings of this study have significant implications for the biotechnological production of valuable secondary metabolites from *S. sempervirens*. The demonstrated enhancement of phenolic and flavonoid production in vitro, particularly under optimized elicitation conditions, provides a foundation for developing efficient and sustainable production systems for these bioactive compounds.

The identification of specific treatment combinations that maximize the production of particular compounds offers opportunities for targeted metabolite engineering. For instance, the combination of 6 mg/L phenylalanine with 200 mg/L PEG, which yielded the highest total phenolic and flavonoid contents, could be employed for bulk production of these compounds. Alternatively, specific elicitation strategies could be selected based on the desired target compounds, such as UV-C treatment for enhanced production of p-coumaric acid and apigenin-7-glucoside.

The scalability of the in vitro production system represents a key advantage for commercial applications. While our study utilized shoot cultures, the established protocols could potentially be adapted for bioreactor-based production using cell suspension or adventitious root cultures, which offer greater biomass production capacity and more homogeneous product quality (Georgiev et al., 2009). Such scaling-up would require optimization of various parameters, including inoculum density, medium

composition, agitation rate, and oxygen supply, to maintain or enhance secondary metabolite productivity.

The economic feasibility of in vitro production systems for *S. sempervirens* bioactive compounds would depend on several factors, including production costs, market value of the target compounds, and extraction efficiency. The significant enhancement of compound production achieved through elicitation in our study improves the cost-benefit ratio by increasing product yield per unit of input. Furthermore, the year-round production capability of in vitro systems, independent of seasonal variations, offers additional economic advantages over conventional cultivation.

From an environmental perspective, in vitro production of *S. sempervirens* bioactive compounds present a sustainable alternative to harvesting from wild populations or extensive plantation cultivation. This approach reduces pressure on natural resources and minimizes the ecological footprint associated with traditional production methods. Additionally, the controlled environment of tissue culture systems eliminates the need for pesticides and reduces water consumption, further enhancing environmental sustainability.

Future research directions could include the exploration of additional elicitors, such as methyl jasmonate, salicylic acid, or chitosan, which have shown promising results in other plant species (Narayani and Srivastava, 2017). The combination of elicitation with other biotechnological approaches, such as genetic transformation or genome editing, could further enhance secondary metabolite production by directly modifying key regulatory genes or biosynthetic enzymes. Additionally, the integration of omics technologies (genomics, transcriptomics, proteomics, and metabolomics) could provide deeper insights into the molecular mechanisms underlying elicitor-induced secondary metabolite production, facilitating more targeted and efficient production strategies.

Conclusion

This study demonstrates that in vitro culture of *Sequoia sempervirens* shoots, combined with appropriate elicitation strategies, offers a promising approach for enhanced production of pharmacologically active phenolic compounds and flavonoids. Our comprehensive investigation revealed several key findings with significant implications for plant biotechnology and natural product research.

First, we established that in vitro grown shoots of *S. sempervirens* accumulate substantially higher levels of phenolic compounds and flavonoids compared to the mother plant, with enhancements ranging from two to four-fold for phenolics and 1.5 to 3.5-fold for flavonoids. This finding underscores the potential of tissue culture systems as efficient platforms for secondary metabolite production in this valuable coniferous species.

Second, our results demonstrated that the application of amino acid precursors, particularly phenylalanine, significantly enhances the biosynthesis of phenolic compounds and flavonoids in *S.*

sempervirens cultures. The superior performance of phenylalanine compared to glutamine can be attributed to its direct role as a precursor in the phenylpropanoid pathway, which leads to the production of various phenolic compounds and flavonoids. This knowledge provides a rational basis for precursor feeding strategies aimed at maximizing secondary metabolite production.

Third, we found that abiotic elicitation using polyethylene glycol (PEG) and ultraviolet-C (UV-C) radiation effectively stimulates secondary metabolite production in *S. sempervirens* cultures, albeit with differential effects depending on the type and intensity of the elicitor. PEG elicitation, particularly at 200 mg/L, resulted in the highest accumulation of total phenolics and flavonoids, as well as several specific compounds including protocatechuic acid, catechin, rutin, and cinnamic acid. UV-C irradiation at 15 watts was more effective than 30 watts for enhancing overall secondary metabolite production, suggesting that moderate stress levels optimally activate biosynthetic pathways without causing excessive cellular damage.

Fourth, our HPLC analysis revealed that four phenolic compounds—gallic acid, p-hydroxy benzoic acid, ferulic acid, and chrysin—were produced in in vitro shoots but were not detected in the mother tree. This finding highlights the potential of tissue culture systems to induce the biosynthesis of compounds that may not be expressed in intact plants under normal conditions, thereby expanding the phytochemical diversity accessible for potential applications.

Fifth, we observed synergistic effects when combining precursor feeding with elicitation, with the combination of 6 mg/L phenylalanine and 200 mg/L PEG yielding the highest total phenolic and flavonoid contents. This synergy likely results from the simultaneous provision of precursor molecules and activation of biosynthetic enzymes through stress-induced signaling cascades, representing an efficient strategy for maximizing secondary metabolite production.

The enhanced production of bioactive compounds in elicited *S. sempervirens* cultures has significant implications for various industries, including pharmaceuticals, cosmetics, and nutraceuticals. The compounds identified in this study possess diverse pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, and anticancer properties, highlighting their potential therapeutic value. Moreover, the sustainable nature of in vitro production systems offers environmental advantages over traditional harvesting from wild populations or extensive plantation cultivation.

Future research should focus on scaling up the established protocols for commercial applications, potentially through the development of bioreactor-based production systems using cell suspension or adventitious root cultures. Additionally, the integration of elicitation strategies with other biotechnological approaches, such as genetic transformation or genome editing, could further enhance secondary metabolite production by directly modifying key regulatory genes or biosynthetic enzymes.

In conclusion, this study provides a comprehensive framework for enhanced production of pharmacologically active compounds from *S. sempervirens* through optimized in vitro culture and elicitation strategies. The findings contribute to our understanding of secondary metabolite biosynthesis

in response to abiotic stress and offer practical approaches for sustainable production of valuable natural products from this iconic coniferous species.

Declarations

Conflicts of 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. The authors also declare that they have no conflicts of interest related to the research, authorship, and/or publication of this article

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Figures

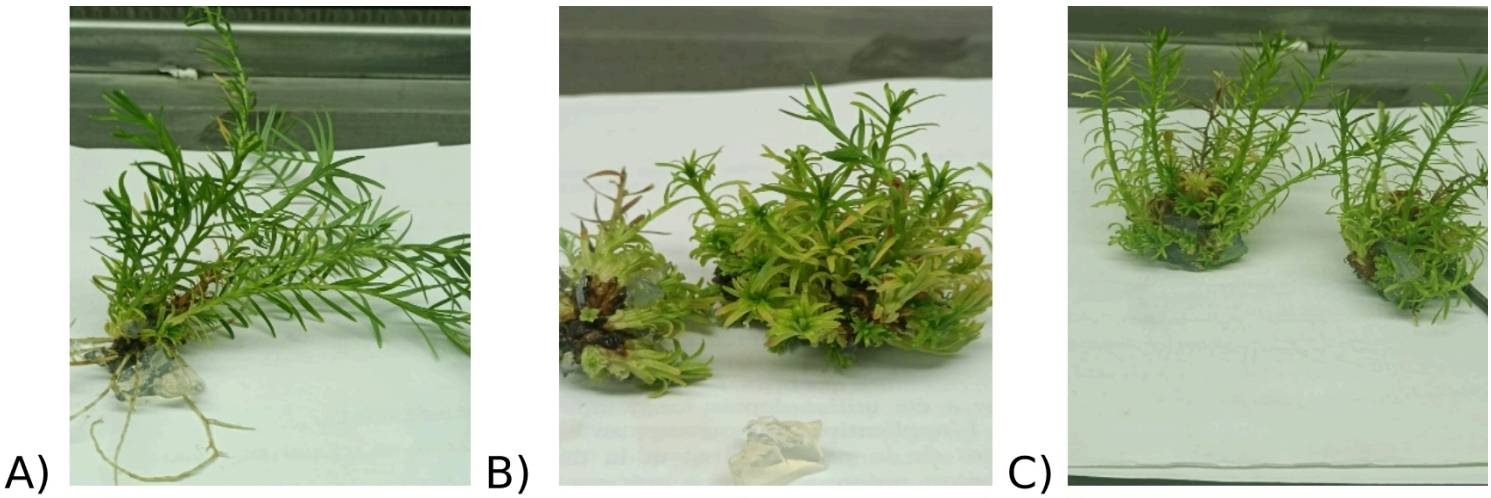


Figure 1

Representative im.ages of *Sequoia sempervirens* shoots after 12 weeks of culture under different treatments: A) Control (no elicitation), B) Phenylalanine 2 mg / l + UV 15watt. C)) Phenylalanine 4mg /l

+PEG 100 . differences in shoot proliferation and morphology among treatments.

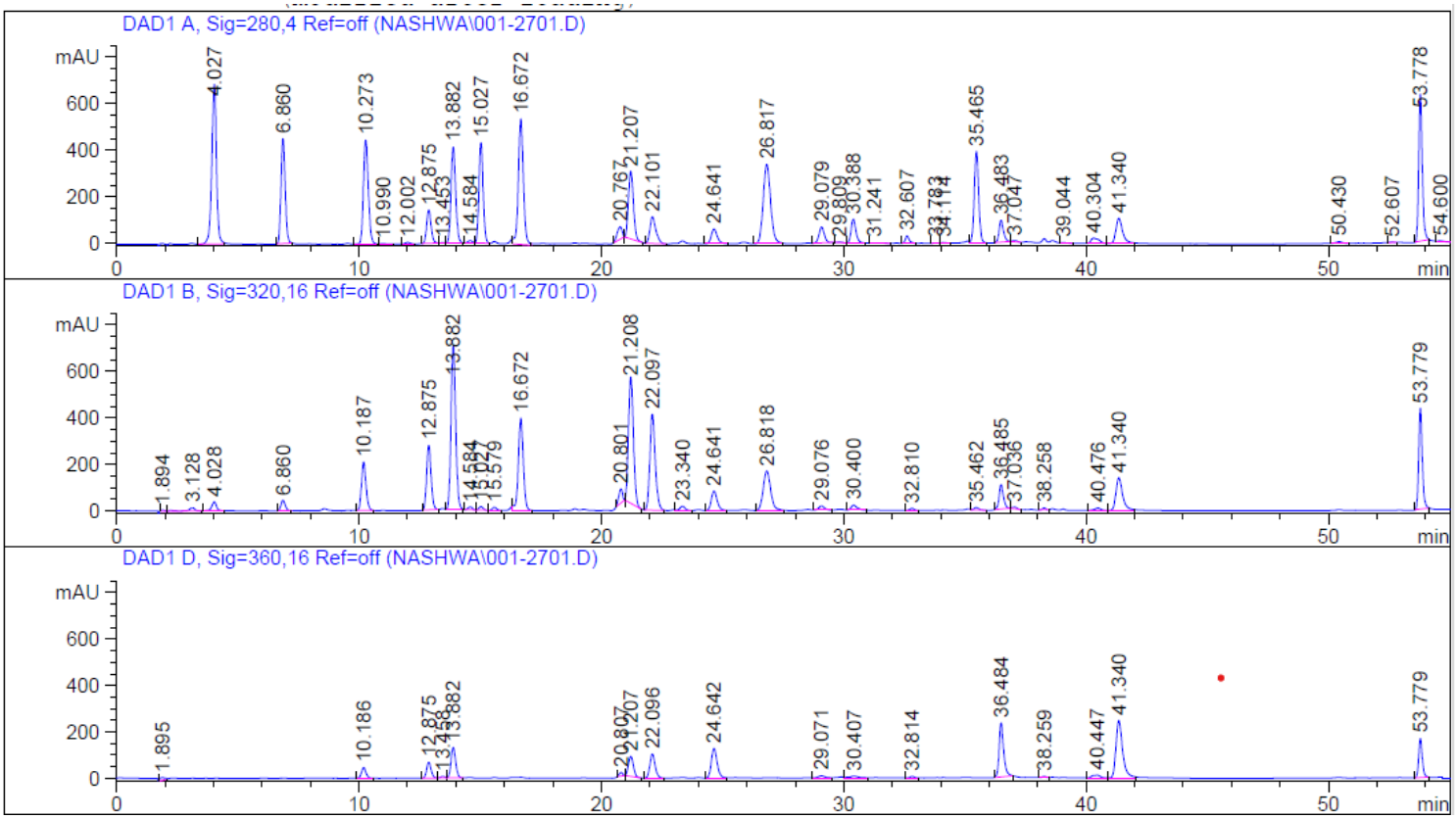


Figure 2

HPLC chromatograms of standard phenolic and flavonoid compounds. Peaks: 1) gallic acid (4.03 min), 2) protocatechuic acid (6.86 min), 3) catechin (10.27 min), 4) chlorogenic acid (12.88 min), 5) caffeic acid (13.88 min), 6) syringic acid (15.03 min), 7) rutin (16.67 min), 8) p-coumaric acid (21.21 min), 9) ferulic acid (22.10 min), 10) apigenin-7-glucoside (24.64 min), 11) rosmarinic acid (26.82 min), 12) cinnamic acid (29.08 min), 13) quercetin (35.47 min), 14) kaempferol (41.34 min), 15) chrysin (53.78 min).

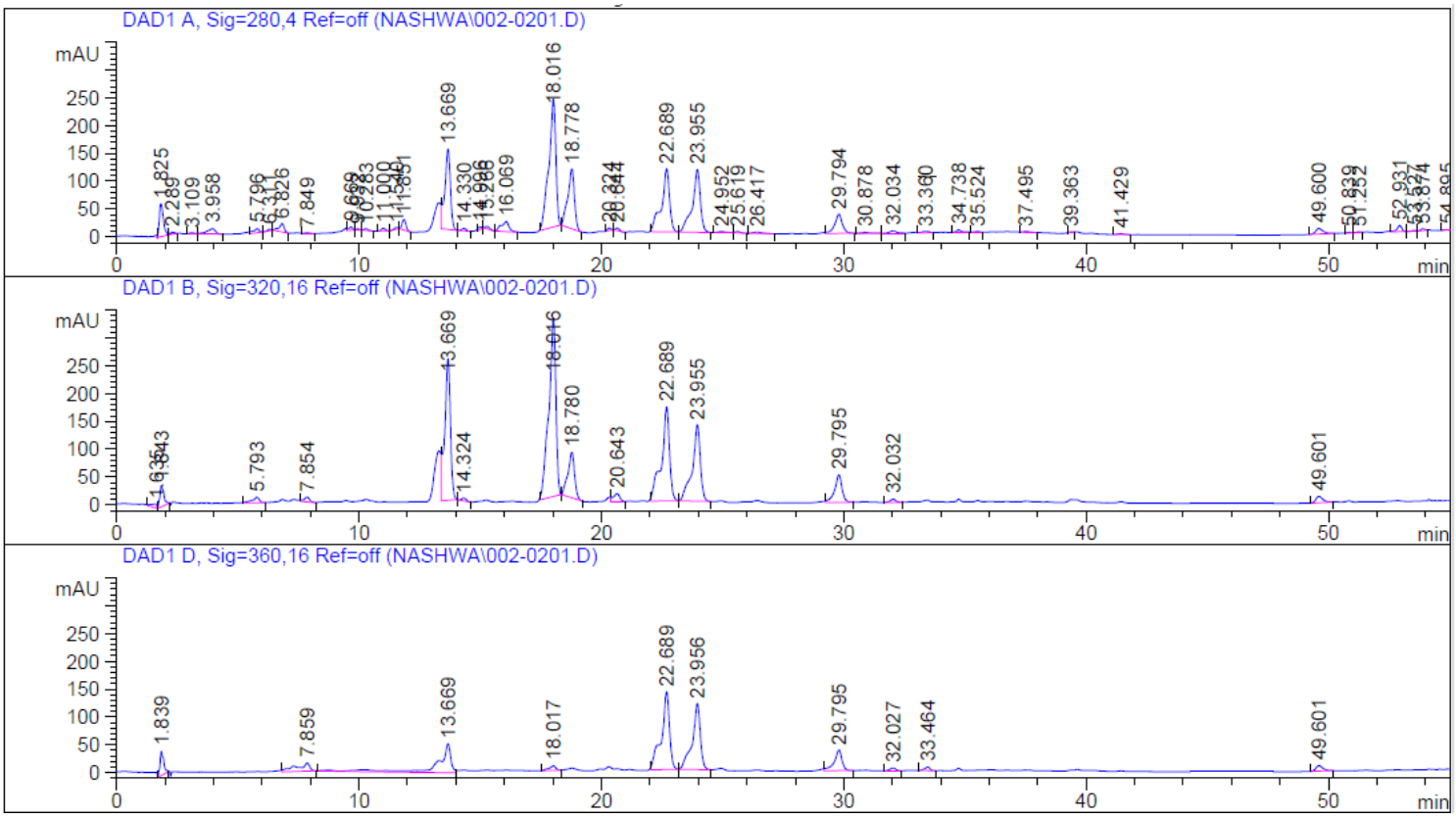


Figure 3

HPLC chromatograms comparing mother plant extract (Control) and in vitro culture extract. Note the presence of additional peaks in the in vitro culture extract corresponding to gallic acid, p-hydroxy benzoic acid, ferulic acid, and chrysin, which were not detected in the mother plant.

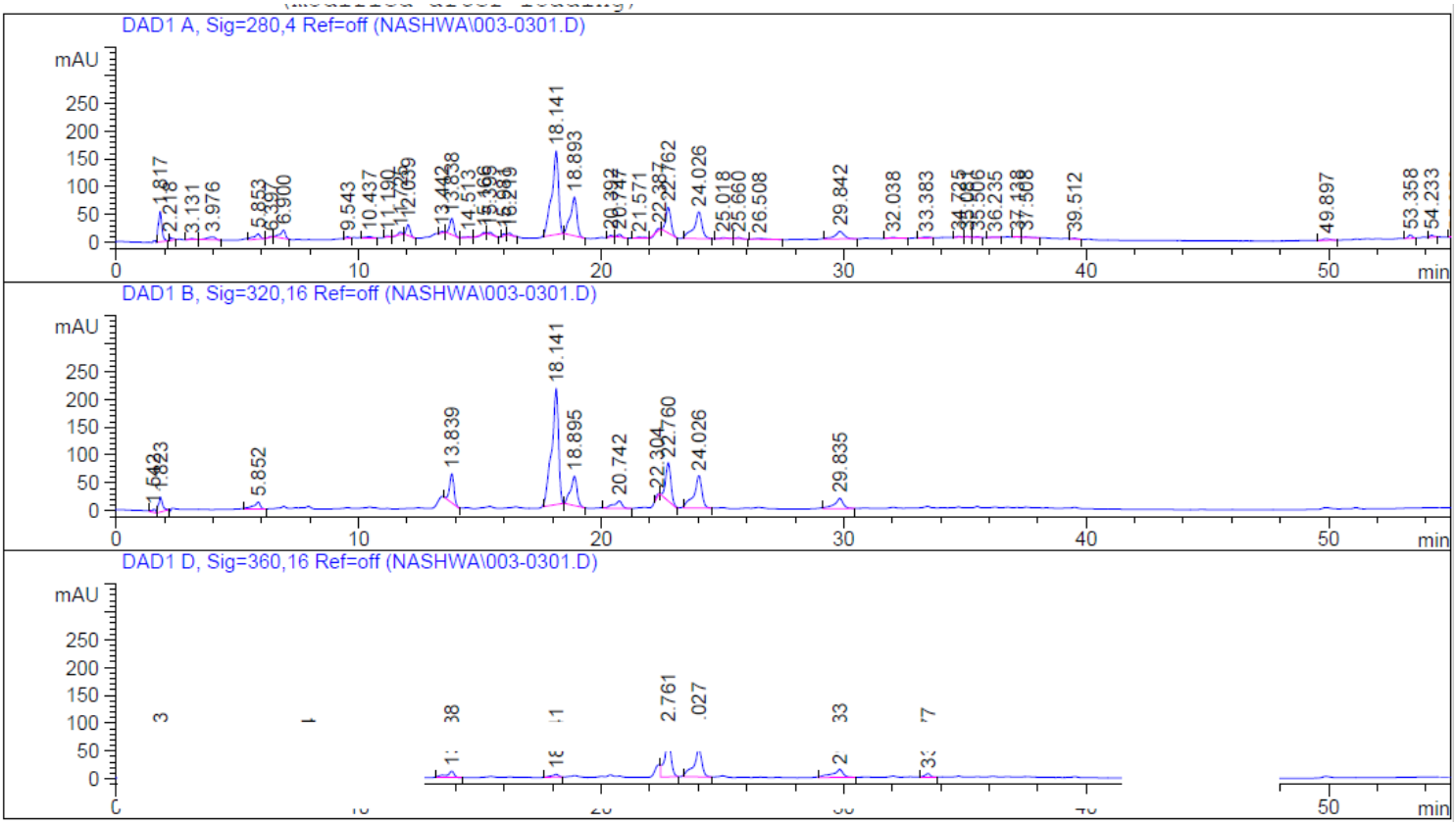


Figure 4

HPLC chromatograms show the effect of PEG elicitation (200 mg/L) on phenolic and flavonoid profiles in *Sequoia sempervirens* in vitro cultures. Note the enhanced peak intensities for protocatechuic acid, catechin, rutin, and cinnamic acid compared to non-elicited cultures.

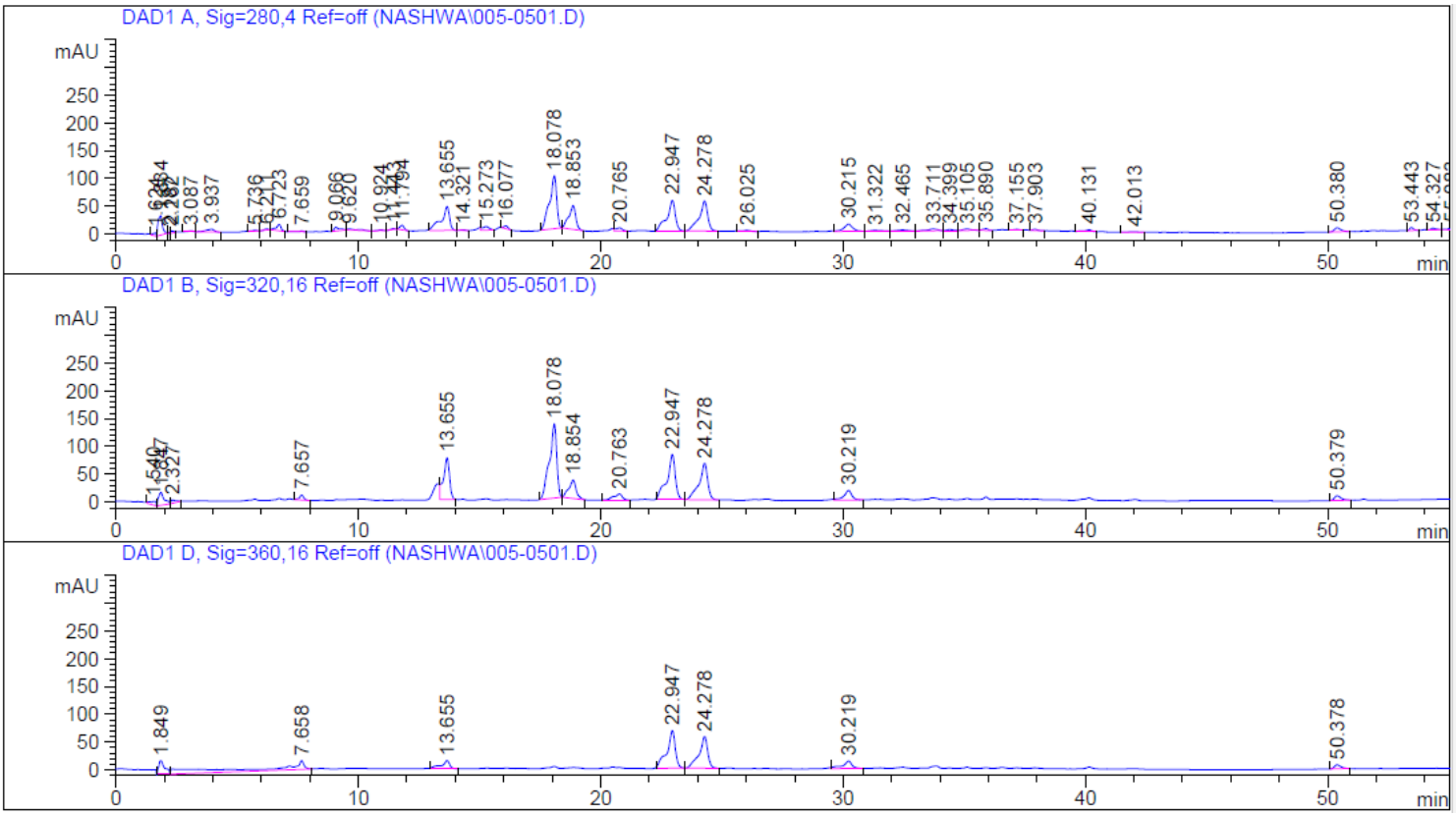


Figure 5

HPLC chromatograms show the effect of combined treatment with phenylalanine (6 mg/L) and PEG (200 mg/L) on phenolic and flavonoid profiles in *Sequoia sempervirens* in vitro cultures. This treatment yielded the highest concentrations of most bioactive compounds.