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Research Article

Biotic and Abiotic Elicitation Enhances Silymarin Biosynthesis in *Silybum marianum* Callus Cultures: Integrating HPLC Profiling and Gene Expression Analysis

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Abstract

Background and Objective: *Silybum marianum* (milk thistle) is a valuable medicinal plant that produces silymarin, a complex of flavonolignans with well-documented hepatoprotective, antioxidant and anti-inflammatory properties. However, silymarin production from field-grown plants is limited by low yield, environmental variability and inconsistent metabolite accumulation. This study aimed to evaluate the effects of biotic and abiotic elicitors on biomass growth, silymarin biosynthesis and the expression of key biosynthetic genes in *S. marianum* callus cultures. **Materials and Methods:** Callus cultures were established from sterile explants and treated with biotic elicitors (yeast extract and chitosan) and abiotic elicitors (methyl jasmonate and silver nanoparticles) at different concentrations under optimized *in vitro* conditions. Biomass accumulation, total flavonolignan content and the expression of the phenylpropanoid pathway genes PAL, CHS and TAT were analyzed. High-Performance Liquid Chromatography (HPLC) was employed to quantify the major silymarin constituents, including silybin A, silybin B, silychristin, silydianin and isosilybin. **Results:** Elicitor treatments significantly enhanced silymarin biosynthesis compared with the untreated control. The combined treatment of methyl jasmonate (100 μ M) and chitosan (50 mg/L) produced the highest response, resulting in a 10.4-fold increase in total silymarin content (14.08 mg/g DW) and an 18.42-fold upregulation of CHS expression. The HPLC analysis confirmed increased accumulation of the major silymarin flavonolignans, demonstrating a synergistic effect of combined biotic and abiotic elicitation on secondary metabolite production. **Conclusion:** Combined biotic and abiotic elicitation effectively enhanced silymarin biosynthesis in *S. marianum* callus cultures, with increased expression of selected phenylpropanoid-associated genes and higher flavonolignan accumulation. The findings support combined elicitation as a promising controlled *in vitro* approach, but validation at larger culture scales and across broader molecular targets is required before industrial application.

Key words: *Silybum marianum*, silymarin, callus culture, biotic elicitors, abiotic elicitors, methyl jasmonate, chitosan, flavonolignans, HPLC, gene expression

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Silybum marianum (L.) Gaertn., commonly recognized as milk thistle, is an annual or biennial herb belonging to the Asteraceae family. For centuries, extracts from its fruits (achenes) have been used in preparations for liver-related disorders^{1,2}. The active therapeutic fraction is silymarin, a complex mixture of flavonolignans that includes silybin A, silybin B, isosilybin A, isosilybin B, silychristin and silydianin, together with the precursor flavonoid taxifolin^{3,4}. Silymarin has been extensively investigated for hepatoprotective, antioxidant and related pharmacological activities, although clinical evidence and standardization remain important considerations^{5,6}. Field-derived *S. marianum* material can also exhibit substantial variation in silymarin composition among populations and locations, which may complicate the production of chemically consistent raw material⁷. Furthermore, agricultural space competing with food crops restricts extensive expansion. To bypass these limitations, plant cell and tissue culture systems offer an alternative. *In vitro* micropropagation and callus cultures facilitate the synthesis of valuable secondary metabolites in a controlled environment, independent of seasonal or geographical barriers^{8,9}. Despite these advantages, unelicited *in vitro* cultures frequently demonstrate low, unstable, or economically unfeasible baseline yields of secondary metabolites, as these defense-associated compounds are often enhanced under specific cellular stress conditions^{10,11}.

To overcome this biosynthetic bottleneck, the technique of elicitation is widely applied. Elicitation involves exposing plant cells to low concentrations of chemical or physical agents to mimic ecological stress, thereby triggering defense mechanisms and stimulating specialized secondary metabolism^{12,13}. Elicitors are generally divided into biotic and abiotic categories. Biotic elicitors include chitosan and yeast extract, whereas abiotic elicitors include signaling compounds such as methyl jasmonate and physical or nanomaterial-associated stressors such as silver nanoparticles. These treatments can stimulate defense-associated signaling and secondary-metabolite accumulation in plant cell cultures¹⁴⁻¹⁷.

Beyond evaluating final chemical outputs, understanding the genetic pathways associated with elicitor-mediated accumulation is important. Flavonolignan biosynthesis in *S. marianum* involves interconnected branches of the phenylpropanoid and flavonoid pathways. The PAL catalyzes the entry of phenylalanine into phenylpropanoid metabolism, while CHS contributes to formation of flavonoid precursors. Gene-expression and metabolite-profiling studies in *S. marianum* have identified PAL, CHS,

and other pathway genes as components associated with flavonolignan biosynthesis¹⁸⁻²¹. TAT catalyzes an early step in tyrosine metabolism and represents a relevant enzyme of aromatic-amino-acid metabolism²². Elicitation with jasmonates is also widely used to stimulate secondary-metabolite biosynthesis and associated transcriptional responses in plant cultures^{23,24}. Variations in the transcription of pathway-associated genes may therefore be associated with changes in precursor availability and flavonolignan accumulation, although such associations do not by themselves establish direct causal regulation.

Despite extensive research on elicitation strategies in medicinal plants, the response of *S. marianum* cultures to different elicitors can vary with culture system, elicitor type, concentration and exposure period^{25,26}. Previous work in *S. marianum* has demonstrated that yeast extract and methyl jasmonate can stimulate silymarin accumulation and alter CHS-related responses, supporting the value of elicitation for improving flavonolignan production²⁵. The combined use of different elicitors remains of particular interest because interactions between elicitation signals may alter both metabolite accumulation and gene-expression responses. Recent studies of plant cell cultures have shown that combined elicitation can modify phenolic and flavonoid production²⁷, while studies in *S. marianum* have documented relationships between flavonolignan composition and biosynthetic characteristics²⁸. Accordingly, the present study correlates quantitative HPLC profiles of silymarin isomers with expression patterns of PAL, CHS and TAT in *S. marianum* callus cultures.

Accordingly, this comprehensive study is designed to critically evaluate and compare the individual and combined effects of selected biotic (YE, CH) and abiotic (MeJA, AgNPs) elicitors on both biomass stability and individual silymarin isomer accumulation in *S. marianum* callus cultures, directly correlating metabolic yield changes with the real-time expression profiling of phenylpropanoid biosynthetic genes.

MATERIALS AND METHODS

Study area and duration: The study was conducted under controlled *in vitro* tissue-culture conditions from March 2024 to December 2025. The 22-month study period included callus establishment and maintenance, elicitor screening, optimization of elicitor concentrations, HPLC profiling and gene-expression analyses.

Plant material and explant sterilization: Healthy, uniform seeds of *Silybum marianum* were collected from wild plants at Borg Alarab Alexandria, Egypt and thoroughly washed

under running tap water for 30 min to eliminate surface debris, followed by transferring to a sterile environment inside a horizontal laminar flow hood. The seeds were briefly immersed in 70% (v/v) ethanol for 60 seconds for initial wetting and surface disinfection, then immediately transferred into a 20% (v/v) commercial sodium hypochlorite solution (containing approximately 5% active chlorine) supplemented with 3 drops of surfactant (Tween-20) for 15 min under continuous, gentle agitation. Following chemical treatment, the seeds were rinsed thoroughly five consecutive times with sterile, double-distilled water to eliminate all trace chlorine residues. Finally, the sterilized seeds were transferred onto hormone-free Murashige and Skoog (MS) basal medium²⁹ solidified with 0.8% (w/v) agar for sterile germination.

Callus induction and maintenance: Hypocotyl and cotyledon tissue fragments (~0.5-1.0 cm) were carefully excised from 14-day-old, sterile, uniformly germinated seedlings to serve as explants. These explants were inoculated onto fresh MS basal medium supplemented with 30 g/L sucrose, 8 g/L analytical-grade agar and optimized combinations of the plant growth regulators 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin) to promote cell dedifferentiation. The initial pH of the media configurations was adjusted precisely to 5.7-5.8 using 1N NaOH or 1N HCl prior to sterilization by autoclaving at 121°C and 15 psi pressure for 20 min. Explant cultures were incubated at 25±2°C in a specialized climate growth chamber, maintained either under continuous dark conditions or an automated 16 hrs light/8 hrs dark photoperiod provided by cool-white fluorescent lamps. To establish stable, fast-growing cell lines, homogeneous callus tissues were subcultured onto freshly prepared media every 4 weeks. Callus establishment and maintenance were performed using Murashige and Skoog basal medium as the culture foundation, with the present hormonal combinations optimized for *S. marianum* callus induction²⁹.

Elicitor formulation and treatment execution: Primary concentrated stock solutions of all selected biotic and abiotic elicitors were prepared. Chitosan (CH) stocks were prepared by dissolving high-purity chitosan powder (Sigma-Aldrich, USA) in a 1% (v/v) aqueous acetic acid solution, followed by adjusting the final solution pH to 5.6. Yeast extract (YE) was prepared by dissolving analytical-grade yeast extract powder (Sigma-Aldrich, USA) directly in distilled water. Both biotic elicitor solutions were sterilized using 0.22 µm membrane filters before addition to the media. Methyl jasmonate (MeJA) was dissolved in absolute ethanol to create a stock solution, while silver nanoparticles (AgNPs) (20-30 nm particle size, Sigma-Aldrich, USA) were suspended in sterile water and

subjected to 20 min of intense sonication to avoid particle aggregation. Optimized volumes of these stocks were added to autoclaved, cooled MS media to achieve specific targeted treatment concentrations: YE (250, 500, 1000 mg/L), CH (50, 100, 200 mg/L), MeJA (50, 100, 200 µM) and AgNPs (10, 25, 50 mg/L). Actively growing, uniform callus tissues (approx. 1.0 g starting fresh weight) were transferred into treatment vessels and grown across designated exposure timelines (12, 24, 48 and 72 hrs) to track physiological and molecular shifts. Based on preliminary screening, optimal concentrations were selected for detailed analysis.

Quantitative HPLC analysis of flavonolignans: Harvested elicited and control callus tissues were carefully removed from cultures, blotted dry on filter paper to record total fresh weight and then dried to a constant weight at 40°C in a forced-air oven to establish dry-weight profiles. Dried biomass samples were finely ground using a mortar and pestle. A precise quantity of the resulting dry powder (100 mg) was first defatted by washing with n-hexane to eliminate lipid components. The remaining solid residue was subjected to ultrasonic-assisted extraction using high-purity methanol for 45 min at 35°C. The extracted fluid was filtered through a 0.45 µm syringe filter, concentrated under reduced pressure using a rotary evaporator and re-solubilized in a fixed volume of HPLC-grade methanol. The extraction and chromatographic procedures were based on established approaches for silymarin/flavonolignan profiling, with modifications to suit the present callus material and analytical system^{25,28}.

The separation and quantification of silymarin components (including silybin A, silybin B, isosilybin A, isosilybin B, silychristin, and silydianin) were executed using an Agilent Technologies 1260 Infinity HPLC system equipped with a Quaternary Pump and a Diode Array Detector (DAD). Chromatographic separation was achieved on a reversed-phase C18 column (250×4.6 mm, 5 µm particle size; Phenomenex, USA) maintained at a constant column oven temperature of 30°C. The mobile phase consisted of a binary gradient elution system utilizing solvent A (0.1% phosphoric acid in water, v/v) and solvent B (100% methanol, HPLC grade). The gradient profile was initiated at 20% B, increasing linearly to 50% B over 20 min, then progressively reaching 80% B at 35 min, followed by a 5 min re-equilibration step. The flow rate was maintained at 1.0 mL/min and the sample injection volume was 20 µL. Flavonolignans were monitored and quantified at an absorption wavelength of 288 nm. Target peaks were identified and verified by direct comparison of retention times and UV-Vis spectra with certified reference standards (Sigma-Aldrich, USA). Calibration curves were constructed using authentic standards ($R^2 \geq 0.999$) for quantitative determination.

Total RNA isolation and gene expression analysis (qRT-PCR):

Total cellular RNA was isolated from fresh control and elicited callus tissues harvested at targeted intervals (12, 24, 48 and 72 hrs) using the GeneJET Plant RNA Purification Kit (Thermo Scientific, USA) according to the manufacturer's protocol. The purity and concentration of the isolated RNA were verified using a micro-volume spectrophotometer (A260/A280 ratio 1.8-2.0) and structural integrity was checked by 1% agarose gel electrophoresis. For cDNA synthesis, 1 µg of purified RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. RT-qPCR assays were performed and reported with attention to established minimum reporting principles for quantitative PCR 30.

Quantitative real-time PCR (qRT-PCR) assays were performed to monitor the transcriptional activity of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and tyrosine aminotransferase (TAT) genes using sequence-specific forward and reverse primers designed from conserved regions of *S. marianum* sequences available in GenBank. Amplification reactions were carried out in a StepOnePlus real-time thermal cycler (Applied Biosystems, USA) using PowerUp SYBR Green Master Mix (Applied Biosystems, USA). The following primer sequences were used:

- PAL: F: 5'-TGA GGT GCT GAC AAG CAG AAG-3', R: 5'-TTG AGC AGG ATC TTC ACG TCC-3'
- CHS: F: 5'-GTC CTC CTC GTC GTC TCT CC-3', R: 5'-ACC AAG TGC GTC CCA GAG AT-3'
- TAT: F: 5'-GAA GTC CTA CGG CGT GGA GT-3', R: 5'-GGC GAG AAG AGC ATC ACG TT-3'
- Actin: F: 5'-GCC GAC GAA ACG AAG AGG GAA-3', R: 5'-GCC GCA TCC AGG GAA ACA TAG-3'

The thermal cycling protocol consisted of initial denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec. Melting-curve analysis was performed to confirm primer specificity.

Relative gene-expression fold changes were calculated using the 2- $\Delta\Delta C_t$ comparative method, with Actin as the internal reference gene, following the approach described by Livak and Schmittgen³².

Experimental design and statistical analysis: The study was conducted using a Randomized Complete Block Design (RCBD) with three blocks. The experiments were performed as sequential experimental phases rather than as a single continuous treatment period. The experimental unit was

defined as an individual culture vessel containing approximately 1.0 g of actively growing callus. The study comprised two sequential experimental phases. In Phase I (elicitor screening), 13 treatment groups were evaluated: One untreated control and three concentrations of each elicitor, namely yeast extract (YE; 250, 500 and 1000 mg/L), chitosan (CH; 50, 100 and 200 mg/L), methyl jasmonate (MeJA; 50, 100, and 200 µM), and silver nanoparticles (AgNPs; 10, 25 and 50 mg/L). All 13 treatments were represented once in each of the three blocks, resulting in 39 experimental units (13 treatments×3 blocks). Based on the screening results, the optimized concentrations of YE (500 mg/L), CH (50 mg/L), MeJA (100 µM), and AgNPs (25 mg/L) were selected for Phase II, together with the untreated control and a combined treatment of MeJA (100 µM)+CH (50 mg/L), giving six treatment groups. Each of these six treatments was represented once in each of the three blocks, resulting in 18 experimental units (6 treatments×3 blocks). Thus, 57 experimental units were used across the two sequential experimental phases. The two phases were treated as separate experiments for purposes of experimental-unit counting and statistical analysis. Each biological replicate consisted of one independent experimental unit, whereas duplicate technical measurements were performed for analytical and molecular assays where applicable; technical replicates were not treated as independent biological replicates.

Quantitative data were subjected to Analysis of Variance (ANOVA) appropriate for the RCBD using CoStat statistical software (Version 6.4, CoHort Software, USA). Treatment means were statistically compared using Duncan's Multiple Range Test (DMRT) at a significance level of $p \leq 0.05$. Standard errors ($\pm$ SE) were calculated for the three biological replicates. For temporal CHS-expression data, measurements obtained at 12, 24, 48 and 72 hrs were analyzed according to the experimental structure and Pearson's correlation coefficient was used to assess the association between gene expression and silymarin accumulation.

RESULTS

Impact of selected elicitors on callus biomass accumulation:

The accumulation of dry and fresh weight of *S. marianum* callus was carefully monitored across varied elicitor treatments to determine whether higher elicitor doses caused cellular toxicity or reduced tissue growth rates. The data presented in Table 1 demonstrate distinct responses among the different elicitor treatments.

Table 1: Effects of elicitor treatments on biomass accumulation in *S. marianum* callus cultures

Elicitor type	Concentration	Exposure duration (Days)	Fresh weight (g)	Dry weight (g)	Growth index (GI)
Control (Untreated)	0.0	28	8.42±0.31 ^e	0.87±0.04 ^{cd}	7.42±0.31 ^e
Yeast extract (YE)	250 mg/L	28	9.86±0.42 ^c	1.04±0.06 ^b	8.86±0.42 ^c
Yeast extract (YE)	500 mg/L	28	10.93±0.38 ^a	1.18±0.05 ^a	9.93±0.38 ^a
Yeast extract (YE)	1000 mg/L	28	7.15±0.29 ^a	0.72±0.03 ^e	6.15±0.29 ^a
Chitosan (CH)	50 mg/L	28	9.12±0.35 ^d	0.95±0.04 ^c	8.12±0.35 ^d
Chitosan (CH)	100 mg/L	28	6.84±0.27 ^h	0.68±0.03 ^{ef}	5.84±0.27 ^h
Chitosan (CH)	200 mg/L	28	4.53±0.19 ^j	0.43±0.02 ^h	3.53±0.19 ^j
Methyl jasmonate (MeJA)	50 µM	28	7.86±0.33 ^f	0.79±0.03 ^{de}	6.86±0.33 ^f
Methyl jasmonate (MeJA)	100 µM	28	6.42±0.28 ^h	0.61±0.03 ^{fg}	5.42±0.28 ^h
Methyl jasmonate (MeJA)	200 µM	28	4.18±0.18 ^k	0.39±0.02 ^h	3.18±0.18 ^k
Silver nanoparticles (AgNPs)	10 mg/L	28	8.95±0.37 ^d	0.92±0.04 ^{cd}	7.95±0.37 ^d
Silver nanoparticles (AgNPs)	25 mg/L	28	7.63±0.31 ^f	0.75±0.03 ^e	6.63±0.31 ^f
Silver nanoparticles (AgNPs)	50 mg/L	28	5.21±0.22 ⁱ	0.50±0.02 ^{gh}	4.21±0.22 ⁱ

*Growth index (GI) is calculated using the formula: $GI = \frac{\text{Final fresh weight} - \text{Initial fresh weight}}{\text{Initial fresh weight}}$ initial fresh weight was standardized at 1.0 g for all treatments. Values represent Mean ± Standard Error (n = 3). Different letters within each column indicate statistically significant differences at $p \leq 0.05$ according to Duncan's multiple range test

Table 2: Quantitative HPLC analysis of silymarin isomers in *S. marianum* callus cultures under different elicitor treatments

Treatment group	Silybin A (mg/g DW)	Silybin B (mg/g DW)	Silychristin (mg/g DW)	Silydianin (mg/g DW)	Isosilybin (mg/g DW)	Total silymarin (mg/g DW)
Control (Untreated)	0.38±0.02 ^a	0.25±0.01 ^a	0.41±0.02 ^a	0.19±0.01 ^f	0.12±0.01 ^f	1.35±0.07 ^a
YE optimized group (500 mg/L)	1.86±0.09 ^d	1.12±0.06 ^d	1.54±0.08 ^d	0.87±0.04 ^c	0.58±0.03 ^c	5.97±0.30 ^d
CH optimized group (50 mg/L)	2.74±0.14 ^c	1.58±0.08 ^c	2.21±0.11 ^c	1.23±0.06 ^b	0.79±0.04 ^b	8.55±0.43 ^c
MeJA optimized group (100 µM)	3.42±0.17 ^b	2.06±0.10 ^b	2.68±0.13 ^b	1.52±0.08 ^b	0.96±0.05 ^b	10.64±0.53 ^b
AgNPs optimized group (25 mg/L)	2.21±0.11 ^d	1.35±0.07 ^d	1.89±0.09 ^d	1.04±0.05 ^c	0.67±0.03 ^c	7.16±0.36 ^d
Combined Elicitation Strategy (MeJA 100 µM+CH 50 mg/L)	4.58±0.23 ^a	2.87±0.14 ^a	3.41±0.17 ^a	1.98±0.10 ^a	1.24±0.06 ^a	14.08±0.70 ^a

*Values represent Mean ± Standard Error (n = 3). DW = Dry Weight. Different letters within each column indicate statistically significant differences at $p \leq 0.05$ according to Duncan's Multiple Range Test

Biomass accumulation analysis: The results reveal a clear concentration-dependent response pattern across all elicitor treatments. Moderate concentrations of yeast extract (500 mg/L) produced the maximum biomass enhancement, yielding a fresh weight of 10.93 g (GI = 9.93), representing a 29.8% increase over the untreated control (8.42 g). This stimulatory effect suggests that YE at optimal concentrations provides additional nutritional components and signaling molecules that promote cellular proliferation without inducing significant stress responses.

Chitosan treatments demonstrated a sharp threshold effect, where the 50 mg/L concentration marginally enhanced biomass (9.12 g, GI = 8.12), while concentrations of 100 and 200 mg/L caused progressive biomass reductions of 18.8 and 46.2%, respectively, compared to the control. This inhibitory pattern indicates that chitosan triggers dose-dependent defense responses that redirect metabolic resources toward secondary metabolite production at the expense of primary growth.

Methyl jasmonate exhibited the most pronounced growth-suppressive effects among all elicitors tested. At 200 µM, the fresh weight dropped to 4.18 g (GI = 3.18),

representing a 50.4% reduction compared to the untreated control. This substantial biomass reduction is consistent with the well-established role of jasmonates as potent stress signaling molecules that prioritize defense-related secondary metabolism over cellular division and expansion.

Silver nanoparticles displayed a moderate inhibitory profile, with the highest concentration (50 mg/L) reducing biomass by 38.1% compared to the control. The intermediate concentration (25 mg/L) caused a more modest 9.4% reduction, suggesting that AgNPs exert their effects through controlled oxidative stress mechanisms that do not completely arrest growth at lower concentrations.

Quantitative HPLC profiling of individual silymarin isomers:

Table 2 presents the chemical profiles and individual concentrations of key flavonolignans alongside the total accumulated silymarin yields within the callus tissue extracts following the optimized treatment configurations. Based on preliminary screening, optimal concentrations were selected for each individual elicitor: YE (500 mg/L), CH (50 mg/L), MeJA (100 µM) and AgNPs (25 mg/L).

Table 3: Relative expression profiles of biosynthetic genes in *S. marianum* callus cultures under elicitor treatments (48 hrs peak values)

Treatment type	Applied concentration	Relative mRNA expression fold change (PAL)	Relative mRNA expression fold change (CHS)	Relative mRNA expression fold change (TAT)
Control (Untreated)	0.0	1.00±0.05 ^f	1.00±0.05 ^f	1.00±0.05 ^f
Yeast extract	500 mg/L	3.87±0.19 ^d	5.67±0.28 ^d	3.12±0.16 ^d
Chitosan	50 mg/L	5.98±0.30 ^c	8.93±0.45 ^c	5.21±0.26 ^c
Methyl jasmonate	100 µM	7.89±0.39 ^b	11.54±0.58 ^b	6.87±0.34 ^b
Silver nanoparticles	25 mg/L	5.23±0.26 ^c	7.82±0.39 ^c	4.56±0.23 ^c
Combined strategy	MeJA 100 µM+CH 50 mg/L	12.64±0.63 ^a	18.42±0.92 ^a	10.87±0.54 ^a

*Values represent Mean ± Standard Error (n = 3). The 2 $\Delta\Delta C_t$ method was used for relative quantification, with Actin serving as the internal reference gene. Different letters within each column indicate statistically significant differences at $p \leq 0.05$ according to Duncan's multiple range test

Table 4: Temporal expression patterns of CHS gene under different elicitor treatments

Treatment type	Applied concentration	CHS at 12 hrs	CHS at 24 hrs	CHS at 48 hrs	CHS at 72 hrs
Control (Untreated)	0.0	1.00±0.05 ⁱ	1.08±0.06 ⁱ	1.12±0.06 ⁱ	1.06±0.05 ⁱ
Yeast extract	500 mg/L	2.84±0.15 ^f	4.12±0.21 ^e	5.67±0.28 ^d	3.89±0.19 ^e
Chitosan	50 mg/L	3.56±0.18 ^e	6.84±0.34 ^c	8.93±0.45 ^c	5.42±0.27 ^d
Methyl jasmonate	100 µM	4.87±0.24 ^d	8.26±0.41 ^b	11.54±0.58 ^b	7.31±0.37 ^c
Silver nanoparticles	25 mg/L	2.98±0.15 ^f	5.43±0.27 ^d	7.82±0.39 ^c	4.76±0.24 ^d
Combined strategy	MeJA 100 µM+CH 50 mg/L	6.34±0.32 ^c	12.87±0.64 ^b	18.42±0.92 ^a	11.95±0.60 ^b

*Values represent Mean ± Standard Error (n = 3). The 2 $\Delta\Delta C_t$ method was used for relative quantification, with Actin serving as the internal reference gene. Different letters within each column indicate statistically significant differences at $p \leq 0.05$ according to Duncan's Multiple Range Test

Silymarin accumulation analysis: The HPLC quantification reveals that all elicitor treatments significantly enhanced the accumulation of individual silymarin isomers compared to the untreated control, albeit with marked differences in efficacy and isomer specificity.

Silybin A and Silybin B: These diastereomeric flavonolignans represent the most abundant and pharmacologically active components of silymarin. The combined elicitation strategy (MeJA+CH) achieved the highest concentrations of silybin A (4.58 mg/g DW) and silybin B (2.87 mg/g DW), representing 12.1-fold and 11.5-fold increases over the control, respectively. Methyl jasmonate alone (100 µM) demonstrated impressive silybin accumulation (3.42 mg/g DW for silybin A), surpassing chitosan (2.74 mg/g DW), AgNPs (2.21 mg/g DW) and yeast extract (1.86 mg/g DW). This hierarchy suggests that jasmonate signaling pathways are particularly effective at activating the specific enzymatic machinery required for silybin biosynthesis.

Silychristin and silydianin: These isomeric flavonolignans showed similar accumulation patterns across treatments. The combined strategy produced the highest yields (3.41 mg/g DW for silychristin and 1.98 mg/g DW for silydianin), followed by MeJA (2.68 and 1.52 mg/g DW), CH (2.21 and 1.23 mg/g DW), AgNPs (1.89 and 1.04 mg/g DW), and YE (1.54 and 0.87 mg/g DW). The proportional increases in silychristin and silydianin relative to the control (8.3-fold and 10.4-fold for the combined treatment) indicate that elicitors broadly upregulate the flavonolignan pathway rather than selectively enhancing specific branches.

Isosilybin: As the least abundant isomer in the control (0.12 mg/g DW), isosilybin exhibited the highest relative increase (10.3-fold) under the combined elicitation strategy (1.24 mg/g DW). This substantial enhancement suggests that specific transcriptional reprogramming may favor the accumulation of this isomer under certain stress conditions.

Total silymarin: The total silymarin content followed the order: Combined (14.08 mg/g DW)>MeJA (10.64 mg/g DW)>CH (8.55 mg/g DW)>AgNPs (7.16 mg/g DW)>YE (5.97 mg/g DW)>Control (1.35 mg/g DW). The combined elicitation strategy achieved a remarkable 10.4-fold increase in total silymarin content, demonstrating the synergistic potential of integrating biotic and abiotic elicitors.

Expression profiles of phenylpropanoid biosynthetic genes:

To clarify the molecular basis associated with enhanced silymarin accumulation, the expression levels of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and tyrosine aminotransferase (TAT) were monitored via RT-qPCR at different time points (12, 24, 48 and 72 hours) following elicitor treatment. The 48 hrs expression values are presented in Table 3.

The temporal changes in CHS expression at 12, 24, 48 and 72 hrs are presented in Table 4.

Gene expression analysis: The qRT-PCR analysis reveals dynamic and treatment-specific temporal patterns of biosynthetic gene expression, providing molecular insight into the mechanisms underlying the observed metabolic enhancements.

Kinetics of gene activation: All elicitor treatments induced a time-dependent increase in transcript levels for all three genes (PAL, CHS and TAT), with peak expression consistently observed at the 48 hrs sampling interval. This temporal pattern indicates a coordinated transcriptional response requiring de novo protein synthesis and signal transduction cascades. The decline in expression at 72 hrs suggests either the activation of negative feedback mechanisms or the depletion of signaling intermediates. Exposure to abiotic elicitors induced a rapid upregulation of PAL transcription within 24 hrs of treatment introduction, showing a fivefold increase relative to the untreated control. Conversely, CHS expression reached peak levels at a later time point (48 hrs), indicating a sequential activation of pathway enzymes.

Elicitor efficacy hierarchy: The maximum gene expression levels followed a consistent hierarchy across all three genes: Combined>MeJA>CH>AgNPs>YE>Control. For CHS, the values were: Combined (18.42-fold)>MeJA (11.54-fold)>CH (8.93-fold)>AgNPs (7.82-fold)>YE (5.67-fold)>Control (1.12-fold). This hierarchy perfectly correlates with the total silymarin accumulation data (Table 2), establishing a direct link between transcriptional activation of biosynthetic genes and metabolic output. The correlation coefficient between CHS expression and total silymarin content was $r = 0.94$ ($p < 0.001$), confirming CHS as a reliable molecular marker for pathway activation.

Synergistic effects of combined strategy: The combined elicitation strategy (MeJA+CH) achieved expression levels significantly higher than individual treatments for all three genes. For CHS, the combined treatment produced 1.6-fold higher expression than MeJA alone and 2.1-fold higher than CH alone at the 48 hrs peak. This synergistic effect suggests that biotic and abiotic elicitors activate distinct but complementary signaling pathways that converge on the transcriptional machinery regulating phenylpropanoid biosynthesis.

Sequential activation pattern: The differential temporal expression patterns of PAL and CHS provide insight into the biosynthetic sequence. The PAL expression peaked earlier (24-48 hrs), followed by CHS (48 hrs), reflecting the sequential nature of the phenylpropanoid pathway where upstream enzymes are activated before downstream commitment steps. TAT expression showed a similar temporal pattern to PAL, consistent with its role in providing coniferyl alcohol precursors for flavonolignan formation.

DISCUSSION

The present study demonstrated that biotic and abiotic elicitors produced distinct concentration-dependent effects on *S. marianum* callus biomass and silymarin accumulation. Yeast extract at 500 mg/L produced the highest biomass among the tested elicitors, with fresh weight increasing by 29.8% relative to the untreated control. In contrast, higher concentrations of chitosan, methyl jasmonate and silver nanoparticles reduced biomass, indicating increasing physiological stress at elevated elicitor levels. At the optimized concentrations used for biochemical and molecular analyses, MeJA (100 μ M) produced 10.64 mg/g DW total silymarin, while the combined treatment of MeJA (100 μ M)+CH (50 mg/L) produced the highest total silymarin content (14.08 mg/g DW), representing a 10.4-fold increase over the control. The combined treatment also produced the highest expression levels of PAL, CHS and TAT, with CHS reaching an 18.42-fold increase at 48 hrs. Temporal analysis showed that CHS expression peaked at 48 hrs under the combined treatment. Together, these findings indicate that the optimized combined elicitation strategy enhanced silymarin accumulation and was associated with strong transcriptional activation of selected biosynthetic genes. The inverse relationship between biomass accumulation and silymarin production across several elicitor treatments is consistent with the metabolic trade-off commonly observed when plant cells allocate resources toward defense-related secondary metabolism. Methyl jasmonate at 200 μ M reduced fresh biomass by 50.4% relative to the control, whereas the optimized MeJA treatment increased silymarin accumulation. Similarly, increasing chitosan and AgNP concentrations reduced biomass. These observations suggest that stronger elicitation can redirect cellular resources away from growth and toward specialized metabolite accumulation. This interpretation is consistent with established concepts of elicitor-induced secondary metabolism²⁶⁻³².

The enhanced response observed with the combined MeJA+chitosan treatment may reflect complementary elicitation effects. Chitosan is widely recognized as a biotic elicitor capable of modifying defense-associated responses, whereas jasmonates are important regulators of stress-related signaling and secondary metabolism^{12,16}. Previous studies have proposed receptor-mediated signaling, calcium fluxes, MAPK cascades, jasmonate signaling and downstream transcriptional regulation as components of elicitor responses. However, the present study did not directly measure these upstream signaling components. Therefore, such pathways

are presented here only as possible explanations based on previous literature, whereas the experimental evidence directly supports the association between combined elicitation, increased PAL, CHS and TAT expression and increased silymarin accumulation.

The HPLC profiling showed that the combined treatment increased all measured silymarin constituents, with the highest concentrations of silybin A, silybin B, silychristin, silydianin and isosilybin. Total silymarin increased from 1.35 mg/g DW in the control to 14.08 mg/g DW under combined elicitation. Isosilybin showed a 10.3-fold increase relative to the control. These results indicate that the combined treatment enhanced accumulation across the measured flavonolignan profile. Although changes in precursor availability and downstream oxidative coupling could contribute to this pattern, the present study did not directly quantify the enzymes or genes responsible for these downstream steps. Therefore, mechanistic interpretations concerning pathway branching or precursor limitation should be considered tentative and require direct experimental validation.

Study limitations. Several limitations should be considered when interpreting the present findings. First, the experiments were conducted entirely under controlled *in vitro* callus-culture conditions; therefore, the results should not be assumed to translate directly to whole plants, field production, or industrial manufacturing. Second, only three biosynthetic genes (PAL, CHS and TAT) were analyzed, whereas silymarin biosynthesis involves additional structural genes, downstream enzymes and regulatory components that were not examined. Third, the proposed upstream signaling mechanisms were inferred from previous literature rather than directly measured in the present experiment. Fourth, the optimized elicitation strategy was not validated at larger production scales or in bioreactor systems. In addition, the present study establishes associations between transcriptional responses and metabolite accumulation but does not demonstrate direct causal control of silymarin biosynthesis by the measured genes. Future work should therefore include broader transcriptomic and/or proteomic profiling, direct validation of upstream and downstream pathway components, evaluation of additional reference genes and qPCR performance parameters and scale-up validation in controlled bioreactor systems.

CONCLUSION

This study demonstrates that combined biotic and abiotic elicitation can enhance silymarin accumulation in *Silybum marianum* callus cultures under controlled *in vitro* conditions.

The combined treatment of 100 μ M methyl jasmonate and 50 mg/L chitosan produced the highest total silymarin content (14.08 mg/g DW), corresponding to a 10.4-fold increase over the untreated control and was associated with an 18.42-fold increase in CHS expression at 48 hrs. The results support combined elicitation as a promising approach for increasing flavonolignan accumulation *in vitro*. However, the findings should be interpreted within the limitations of the controlled callus-culture system and the limited number of genes examined. Validation in larger-scale culture systems, together with broader molecular analysis and direct testing of additional pathway and regulatory components, is required before conclusions regarding scale-up or industrial application can be made.

SIGNIFICANCE STATEMENT

This study demonstrates that combined methyl jasmonate and chitosan elicitation can substantially enhance silymarin production in *Silybum marianum* callus cultures. The associated upregulation of selected biosynthetic genes and increased flavonolignan accumulation highlight the potential of combined elicitation as an *in vitro* strategy for sustainable silymarin production, while larger-scale validation remains necessary.

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