

Modulation of aryl-naphthalene lignan accumulation in *Linum alpinum* in vitro root cultures by methyl jasmonate

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Received 23 January 2026 ♦ Accepted 11 March 2026 ♦ Published 27 March 2026

Citation: Zarev Y, Nedelcheva R, Ionkova I (2026) Modulation of aryl-naphthalene lignan accumulation in *Linum alpinum* in vitro root cultures by methyl jasmonate. Pharmacia 73: 1–6. <https://doi.org/10.3897/pharmacia.73.e186118>

Abstract

Linum alpinum L. is a promising source of aryl-naphthalene lignans, including justicidin B (JB). In vitro root cultures (RC) of *L. alpinum* were investigated for JB accumulation and its modulation by methyl jasmonate (MeJA). JB content was quantified by LC-HRESI-MS over a 30-day cultivation period. The modulatory effect of MeJA was evaluated after the 5th day of application at three different concentrations. Time-dependent increase in JB accumulation was observed, rising from 69.95 ng/mg DW on day 5 to 118.66 ng/mg DW on day 30. MeJA treatment significantly influenced JB accumulation in a non-linear manner, with the highest JB content observed at 100 µM MeJA (146.97 ng/mg DW), corresponding to a 23.8% increase compared with the untreated control, while higher concentrations resulted in no further enhancement in JB levels. These findings demonstrate that both cultivation duration and elicitor concentration critically affect JB biosynthesis in *L. alpinum* RC and highlight the species-specific nature of MeJA-mediated regulation.

Keywords

elicitation, in vitro root cultures, justicidin B, *Linum alpinum*, methyl jasmonate (MeJA), secondary metabolism

Introduction

Lignans represent a structurally diverse class of phenylpropanoid dimers with important pharmacological properties, including cytotoxic, antiviral, and antioxidant activities (Hemmati and Seradj 2016). Aryl-naphthalene lignans such as justicidin B (JB) are of particular interest due to their established antiproliferative potential and their occurrence in a limited number of *Linum* species (Schmidt et al. 2010). *L. alpinum* L. (Linaceae), a rare alpine species endemic to high-altitude European regions, has recently been recognized as a valuable source of these bioactive metabolites (Peruzzi 2011).

JB has attracted considerable attention because of its pronounced cytotoxic and antiviral activity and its promising effects against several human cancer cell lines. In addition to antiproliferative effects, aryl-naphthalene lignans exhibit anti-inflammatory and neuroprotective potential, suggesting multiple therapeutic applications. However, their low natural abundance and the ecological sensitivity of alpine habitats limit large-scale harvesting from wild plants, emphasizing the need for sustainable production through biotechnological methods (Hemmati and Seradj 2016).

Biotechnological approaches, especially in vitro culture systems, provide a sustainable alternative to field cultivation to produce plant secondary metabolites (Kumar and

Reddy 2011). Among these systems, root cultures (RC) are regarded as particularly efficient for continuous lignan biosynthesis because they combine high metabolic stability with sustained growth potential (Espinosa-Leal et al. 2018). Moreover, RC can be precisely manipulated under controlled conditions to study metabolic regulation and to enhance secondary-metabolite accumulation through elicitor treatment.

Metabolic activation, often achieved through elicitor application, involves the use of biotic or abiotic signal molecules that mimic stress stimuli and activate plant defence mechanisms, resulting in increased metabolite synthesis. Methyl jasmonate (MeJA) is one of the most potent abiotic signals known to regulate a wide range of secondary-metabolic pathways. It acts as a signalling compound in plant defence and development, modulating the expression of jasmonate-responsive genes associated with the phenylpropanoid pathway (Nyanasaigra et al. 2024).

Previous studies on other *Linum* species like *L. austriacum* have shown that MeJA can significantly enhance the production of aryl-naphthalene lignans, yet the response is often dose-dependent and nonlinear (Mascheretti et al. 2021). Excessive concentrations of MeJA may cause oxidative stress, inhibit growth, or suppress metabolite synthesis through feedback regulation (Wasternack and Hause 2013). These findings underline the need to define species-specific and concentration-specific elicitor conditions. Despite its rich lignan profile, *L. alpinum* remains unexplored, and no data are available on MeJA-induced metabolic changes in its *in vitro* RC.

JB is an aryl-naphthalene-type lignan lacking chiral centers and recognized as one of the most biologically active constituents within the genus *Linum*. It has demonstrated pronounced cytotoxic activity against multiple human cancer cell lines, including leukaemia and solid tumour models, primarily through induction of apoptosis and modulation of cell-cycle progression (Hemmati and Seradj 2016). In addition to its antiproliferative effects, JB exhibits antiviral, antifungal, anti-inflammatory, and anti-platelet activities, highlighting its broad pharmacological potential. Despite these promising properties, the natural abundance of JB in wild populations is relatively low and often influenced by environmental and developmental factors. Therefore, the establishment of controlled *in vitro* production systems represents a strategic approach to ensure sustainable and scalable supply of this pharmacologically relevant lignan.

Given these considerations, the present study aims to explore the dose-dependent modulation of aryl-naphthalene lignan accumulation by MeJA in *L. alpinum* RC. By applying MeJA at concentrations of 100, 150, and 200 μ M, the objective of this study is to identify the optimal elicitor concentration for maximizing JB production while maintaining culture viability. Additionally, the time-course analysis of lignan accumulation is assessed to reveal the progression of biosynthetic activation and potential stress adaptation over time. This work provides new insights into jasmonate-mediated control of phenylpropanoid

metabolism and supports the use of *L. alpinum* RC as an efficient biotechnological platform for the sustainable production of cytotoxic aryl-naphthalene lignans.

Materials and methods

Plant material

Shoot cultures (SC) of *L. alpinum* were established from surface-sterilized seeds following standard laboratory techniques (Ionkova et al. 2010). Seedlings were transferred to Murashige and Skoog (MS) basal medium (Murashige and Skoog 1962) for shoot proliferation. RC were subsequently obtained and maintained on a modified MS formulation supplemented with 5 mg/L indole-3-acetic acid (IAA), 2 mg/L kinetin, and 1 g/L casein hydrolysate (G56) (Nedelcheva et al. 2024).

General experimental procedures and analytical methods

All reagents and solvents were of analytical or higher grade (Fisher Scientific, Loughborough, UK). Quantitative analysis of JB was carried out by LC-HRESI-MS using a method based on previously optimized conditions. LC separation was performed with MS-grade solvents (Fischer) acetonitrile (ACN) and H₂O (Milli-Q). Gradient elution (0' 5% ACN, 3' 10% ACN; 16–17' 20% ACN; 19' 40% ACN, 20' 50% ACN, 22.50' 95% ACN, 24.50' 95% ACN) of filtered and degassed ACN/H₂O solution of FA 0.1% (v/v); column was maintained at 40 °C; a flow rate of 300 μ L/min was used during the analysis. Mass spectrometric detection was performed using a high-resolution ESI source operating in positive ion mode with optimized settings: spray voltage 3.5 kV, capillary temperature 320 °C, sheath gas 25 a.u., and auxiliary gas 5 a.u. Full-scan spectra were recorded over an *m/z* range of 100–1500 at 70 000 resolution, while MS/MS spectra were acquired at 17 500 resolution, with AGC target 1e5, maximum IT, a scan range of 100 to 1 500 *m/z*, an isolation window of 2.0 *m/z*, and (N) CE 20, 40, and 60. Nitrogen was used to atomize the samples. All the other parameters were set to obtain the most intense signal for [M+H]⁺. Data acquisition and processing were conducted with Xcalibur software (version 2.0, Thermo Fisher Scientific).

Extraction of plant material and subsequent quantification analysis of justicidin B

For the present analyses, powdered dried biomass obtained from *in vitro* RC was extracted exhaustively with 70% MeOH (3 $\times$ 30 mL), ultrasonicated at 40 °C. The combined extracts were diluted to a final volume of 5 mL when the extract mass was below 10 mg and to 10 mL when it exceeded 10 mg. Aliquots of these solutions were filtered, analysed by LC-HRESI-MS and JB was detected when

compared to reference standard by retention time and fragmentation patterns. The quantification of JB was performed by LC-HRESI-MS using purified JB previously isolated from *in vitro* hairy RC of *L. leonii* (Nedelcheva et al. 2024), dissolved in MeOH and diluted to five progressively increasing concentrations upon which was built calibration curve for quantitative assay.

Effect of elicitor

The effect of MeJA elicitation was evaluated based on JB accumulation. *In vitro* RC of *L. alpinum* were treated with MeJA at three concentrations (100, 150 and 200 µM) on day 25 of cultivation, and growth continued until day 30. Each MeJA treatment was performed in triplicate. Two control groups were included: an ethanol-treated control (used as the solvent for MeJA) and an untreated control. JB levels in MeJA-elicited cultures were compared with those of the control groups to assess the effectiveness of MeJA in enhancing JB production. MeJA was applied on day 25 because RC at this stage had reached stable growth and a measurable baseline of JB accumulation, allowing elicitation to be assessed without confounding effects of early culture establishment. The 5-day treatment window (days 25–30) was selected to capture the short-term metabolic response while maintaining culture integrity.

Results and discussion

Calibration model

The analytical method demonstrates linearity across the tested concentration range (Fig. 1). The correlation coefficient (r^2) for JB was 0.9963. Regression analysis was subsequently applied, and the resulting calibration equations and determination coefficients are summarized in Table 1.

Time-course analysis dynamics of JB accumulation

In vitro RC of *L. alpinum* were successfully cultivated for a period of 30 days and JB accumulation was monitored at five defined time points (days 5, 10, 20, 25, and 30) during the cultivation period. The quantification of JB in the extracts obtained from *in vitro* cultures of *L. alpinum* were performed using the established calibration equation $y = 0.506216x + 2E+06$ ($r^2 = 0.9963$). The concentration of JB in each sample was calculated based on this linear regression. *In vitro* RC of *L. alpinum* showed a steady and time-dependent increase in JB accumulation during the cultivation period (Fig. 2). JB content increased from 69.95 ng/mg DW on day 5 to 118.66 ng/mg DW on day 30, representing approximately 69.6% increase. The steady accumulation of JB suggests sustained activation of the lignan biosynthetic pathway during *in vitro* root maturation. Similar kinetics have been documented in *L. austriacum* (Mascheretti et al. 2021), where extended culture age favored higher lignan accumulation due to tissue differentiation and metabolic stability.

Effect of MeJA concentration on JB biosynthesis

Exposure to MeJA significantly influenced JB accumulation in *L. alpinum in vitro* RC, resulting in a non-linear response (Fig. 3). Treatment with 100 µM MeJA

Table 1. Determination coefficient and regression equations of JB.

Justicidin B	
Determination coefficient	$r^2 = 0.9963$
Linear range (ng/mL)	3.94-197.00
Regression equations	$Y = 0.506216x + 2E+06$
Number of standards	6
Rt (min)	3.76

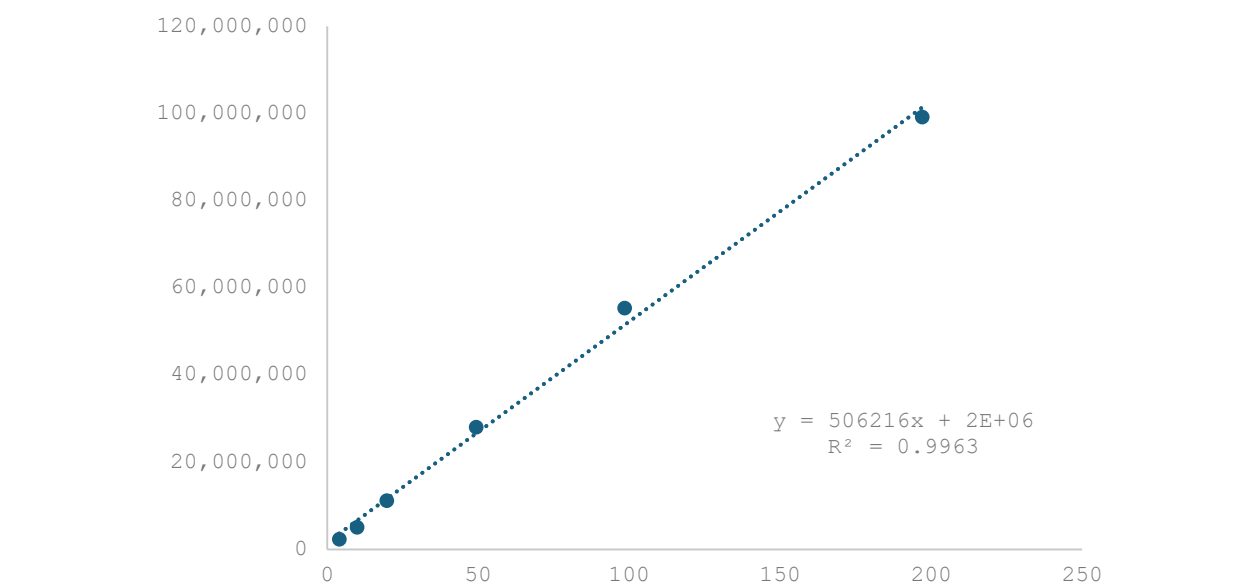


Figure 1. Calibration curve of JB.

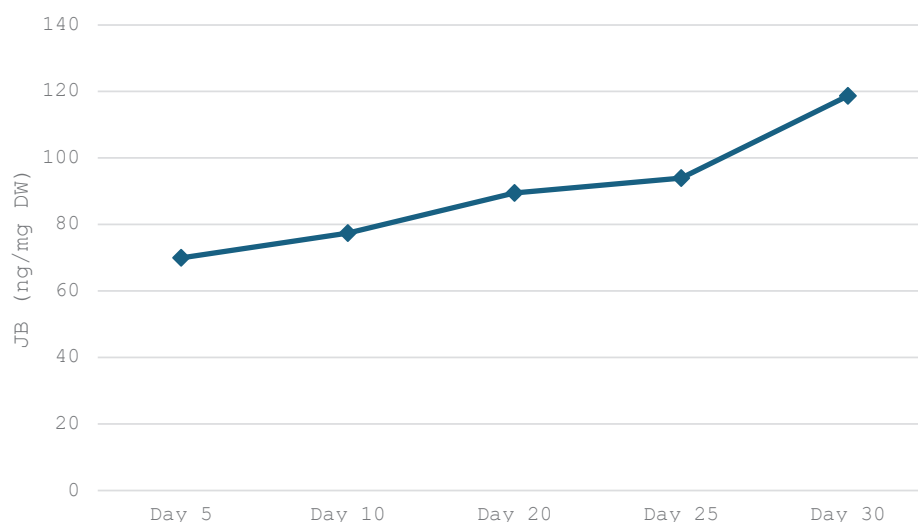


Figure 2. Time-course accumulation of JB in ng/mg DW in *L. alpinum* RC.

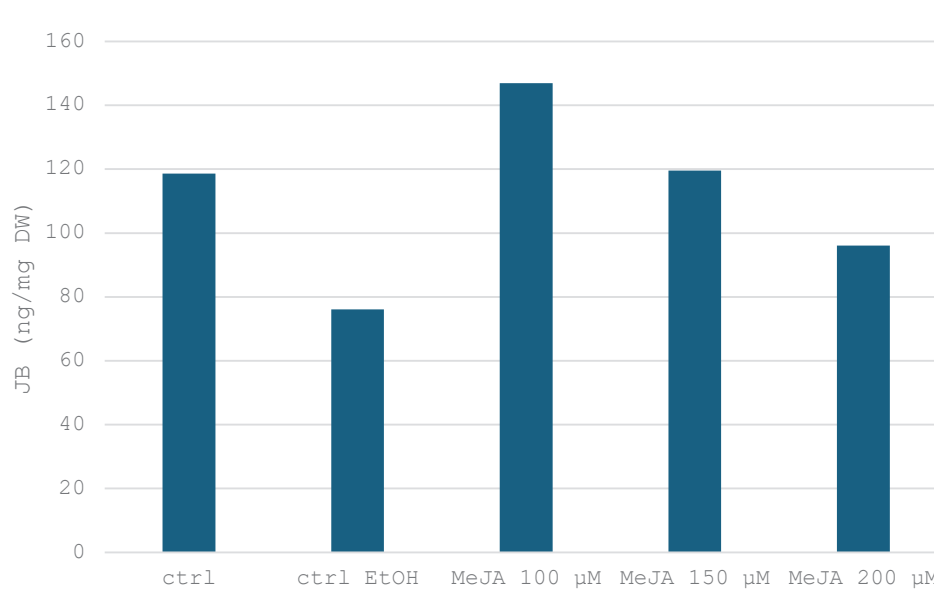


Figure 3. Effect of MeJA (EtOH, 100 µM, 150 µM, 200 µM) on JB content (ng/mg DW) after the 5th day of treatment.

produced the strongest effect, increasing JB content to 146.97 ng/mg DW, which corresponds to a 23.8% increase relative to the untreated control (118.66 ng/mg DW). Increasing the MeJA concentration to 150 µM resulted in JB levels (119.56 ng/mg DW) comparable to the control, indicating no further stimulation. At 200 µM MeJA, JB accumulation declined to 96.11 ng/mg DW, representing a 19.0% reduction compared with untreated cultures. The ethanol control (76.09 ng/mg DW) showed a pronounced 35.9% decrease, indicating that solvent exposure alone did not promote JB biosynthesis.

The initial stimulatory phase followed by inhibition is consistent with jasmonate-mediated regulation of secondary metabolism, in which moderate elicitor concentrations stimulate metabolite accumulation, whereas higher levels may attenuate the response. Comparable concentration-dependent effects of MeJA have been reported

in *L. thracicum* (Sasheva et al. 2015), *L. austriacum* (Mascheretti et al. 2021), and *L. lewisii* (Dougué Kentsop et al. 2022), highlighting interspecific differences in lignan responsiveness to jasmonate signaling.

The differential effect of MeJA concentration on JB accumulation may reflect the dual regulatory role of jasmonates in secondary metabolism. At moderate concentrations, MeJA acts primarily as a signalling molecule that activates defence-related metabolic pathways, whereas higher concentrations may result in metabolic constraints through feedback regulation mechanisms that limit pathway flow. Such concentration-dependent responses have been reported for other *Linum* species and are considered characteristic of jasmonate-mediated responses in plant *in vitro* systems (Wasternack and Hause 2013).

The decrease observed in the EtOH control suggests that the solvent may exert a mild stress effect or influence

carbon allocation in the cultures, which may reduce secondary-metabolite accumulation in some *in vitro* systems (Akula and Ravishankar 2011; Murthy et al. 2014). Such solvent-related responses have been reported in plant tissue cultures and are therefore not unexpected under *in vitro* conditions. This observation supports the inclusion of an EtOH control and confirms that the stimulatory effect observed at 100 μ M MeJA cannot be attributed to solvent exposure.

Overall, the results demonstrate that JB accumulation in *L. alpinum in vitro* RC is influenced by both cultivation time and elicitor concentration. The time-course experiment revealed a progressive increase in JB levels during root development, reaching the highest values after four weeks of cultivation. In parallel, MeJA treatment modulated JB accumulation in a non-linear manner, with 100 μ M emerging as the most effective concentration under the applied conditions, whereas higher concentrations resulted in reduced or only partially restored levels. This biphasic response indicates a finely regulated, species-specific jasmonate-mediated control of lignan biosynthesis, in which moderate elicitor levels are associated with favorable metabolic outcomes, while elevated concentrations may limit pathway efficiency. The combined time-course and elicitation data provide a useful framework for further optimization of culture conditions and elicitor regimes aimed at improving aryl-naphthalene lignan production in *L. alpinum in vitro* RC.

Despite the advantages of *in vitro* RC as controlled production systems, several limitations should be considered. Scale-up to bioreactor conditions may alter oxygen transfer, shear stress, and growth dynamics, which can affect metabolite profiles and reproducibility (Georgiev et al. 2009). In addition, *in vitro* cultures may differ from field-grown plants in hormonal balance, tissue differentiation status, and environmental signaling, all of which can influence phenylpropanoid flux and lignan biosynthesis (Murthy et al. 2014). Therefore, future work should include process optimization and validation of JB productivity under scaled cultivation conditions to assess robustness and industrial feasibility.

Conclusion

This study demonstrates that *L. alpinum in vitro* RC represents a stable and metabolically active system to produce aryl-naphthalene lignans. JB biosynthesis was shown to vary with both cultivation time and elicitor treatment, with a progressive increase observed during root development and the highest accumulation reached after four weeks of cultivation.

MeJA elicitation modulated JB accumulation in a non-linear manner, with a clear dependence on elicitor concentration. Under the applied experimental conditions, 100 μ M MeJA resulted in the highest JB

accumulation, whereas increasing the concentration to 150 μ M produced no further enhancement and higher levels (200 μ M) led to a reduction in JB content. This response indicates a species-specific and tightly regulated jasmonate-mediated control of lignan biosynthesis in *L. alpinum* RC.

Overall, these findings demonstrate that both culture duration and MeJA concentration are critical determinants of JB production in *L. alpinum*. The combined time-course and elicitation data provide a robust basis for optimizing *in vitro* culture conditions and support the use of *L. alpinum* RC as a sustainable biotechnological platform for aryl-naphthalene lignan production.

Acknowledgements

In this manuscript, the Bulgarian National Scientific Fund (BNSF), Contract No KP-06-H63/12/03.07.2023, is acknowledged.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statements

The authors declared that no clinical trials were used in the present study.

The authors declared that no experiments on humans or human tissues were performed for the present study.

The authors declared that no informed consent was obtained from the humans, donors or donors' representatives participating in the study.

The authors declared that no experiments on animals were performed for the present study.

The authors declared that no commercially available immortalised human and animal cell lines were used in the present study.

Use of AI

No use of AI was reported.

Funding

No funding was reported.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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