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Dose-Dependent Effect of L-Methionine on Caffeine Accumulation in Callus Cultures under In Vitro Conditions

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ABSTRACT

Background: Caffeine is an important plant secondary metabolite with considerable nutritional, commercial, and pharmacological significance. Plant callus cultures provide a controlled system for studying and enhancing secondary metabolite production. L-methionine may stimulate caffeine biosynthesis through its association with S-adenosyl-L-methionine-dependent methylation reactions.
Objective: The present study evaluated the dose-dependent effect of L-methionine on caffeine accumulation in callus cultures under in vitro conditions.
Methods: Callus cultures were established on Murashige and Skoog medium supplemented with suitable plant growth regulators. The developed callus was treated with L-methionine at 0, 5, 10, 15, 20, and 25 mg/L, with five replicates per treatment. Caffeine was extracted from harvested callus and qualitatively identified using the Parry reagent method. Quantitative caffeine estimation was performed using UV–Visible spectrophotometry at 272 nm. Treatment effects were statistically evaluated using ANOVA followed by Duncan's Multiple Range Test at $p < 0.05$.
Results: L-methionine significantly influenced caffeine accumulation. Caffeine content increased progressively with increasing L-methionine concentration and reached its highest level at 20 mg/L, whereas a slight decline occurred at 25 mg/L.
Conclusion: L-methionine supplementation effectively enhanced caffeine accumulation, with 20 mg/L identified as the optimum concentration under the tested conditions.

Keywords: Caffeine; L-methionine; Callus culture; In vitro culture; Secondary metabolites.

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INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is an important purine alkaloid naturally produced by several plant species, particularly Coffea and Camellia [1,2]. It is one of the most widely consumed bioactive compounds in beverages and is valued for its stimulant properties and physiological effects [3,4,5]. In plants, caffeine is considered a specialized secondary metabolite that may also contribute to ecological functions such as defense against herbivores and interactions with surrounding organisms [6,7,8]. Because of its commercial and pharmacological importance, considerable research has focused on understanding caffeine biosynthesis and developing approaches for manipulating its accumulation in plant tissues [9,10]. The biosynthesis of caffeine occurs through a series of methylation reactions involving purine nucleotide metabolism [11]. The major pathway proceeds from xanthosine through 7-methylxanthosine, 7-methylxanthine, theobromine, and finally caffeine [2,12]. These reactions involve several S-adenosyl-L-methionine (SAM)-dependent N-methyltransferases, including xanthosine methyltransferase, theobromine synthase, and caffeine synthase [13,14]. Caffeine synthase catalyzes the final methylation steps of the pathway and therefore plays an important role in determining the accumulation of caffeine in plant tissues [15,16]. The availability of methyl donors and metabolic precursors can consequently influence the efficiency of caffeine biosynthesis [11]. Plant tissue culture provides a valuable experimental system for investigating secondary metabolite production under controlled environmental and nutritional conditions [17,18]. Callus cultures are particularly useful because they allow manipulation of culture media, plant growth regulators, precursor availability, and environmental factors to enhance the production of commercially important metabolites [19,20,21]. Previous research has demonstrated that coffee callus tissues retain an active caffeine biosynthetic system [22]. Coffea arabica callus cultures have been shown to produce caffeine under in-vitro conditions, with caffeine production being significantly influenced by L-methionine supplementation [22]. The production of caffeine in callus cultures is influenced by the physiological status of the tissue and the composition of the culture medium [22,23]. Growth

regulators such as auxins and cytokinins are essential for callus induction and maintenance, while nutritional supplementation can alter metabolic pathways and secondary metabolite accumulation [24,25]. Previous studies have shown that caffeine formation can occur alongside callus growth and that excessive accumulation may eventually influence tissue growth and metabolic activity [15,22,26]. Thus, optimization of culture conditions is important for obtaining maximum caffeine production from in vitro cultures [22]. Precursor feeding is one of the strategies used to enhance the biosynthesis of secondary metabolites in plant cell cultures [27]. L-methionine is particularly interesting in relation to caffeine production because it is metabolically associated with SAM, an important methyl donor required by caffeine-biosynthetic methyltransferases [13]. Earlier investigations using tea callus demonstrated incorporation of methionine-derived carbon into methylated purines, providing evidence for the involvement of methionine-related metabolism in caffeine biosynthesis [2]. Furthermore, the three major methylation reactions of caffeine biosynthesis depend on SAM, establishing a biochemical connection between methyl-donor metabolism and caffeine formation [28].

However, the relationship between precursor availability and caffeine accumulation is not necessarily linear. Studies using tea callus have shown that increasing the availability of certain purine precursors does not always stimulate caffeine production, indicating that specific enzymatic steps may act as metabolic control points [15]. Therefore, determining an appropriate concentration of an exogenous precursor is essential because low concentrations may be insufficient to modify metabolic flux, whereas excessive concentrations may cause metabolic imbalance or fail to provide additional benefits [19]. Recent experimental evidence further supports the potential of L-methionine supplementation for increasing caffeine accumulation in coffee callus cultures [22]. A study on Coffea callus reported that L-methionine treatment significantly affected caffeine production, with the highest tested concentration producing the greatest caffeine content [22]. These findings indicate that precursor supplementation can be a promising approach for manipulating caffeine biosynthesis in vitro, although the optimal concentration may differ according to plant genotype, culture conditions, callus age, and metabolic capacity [18]. Despite the demonstrated potential of coffee tissue cultures for caffeine production, systematic evaluation of L-methionine concentration is still important for identifying an effective dose range [23]. Therefore, the present study was designed to investigate the dose-dependent effect of L-methionine on caffeine accumulation in callus cultures under in vitro conditions. Different concentrations of L-methionine were evaluated, and caffeine accumulation was determined using qualitative chemical testing and UV–Visible spectrophotometry [22]. The study is expected to provide useful information for optimizing precursor-mediated enhancement of caffeine production in plant callus cultures and for developing controlled in vitro systems for the production of valuable secondary metabolites [18].

METHODOLOGY

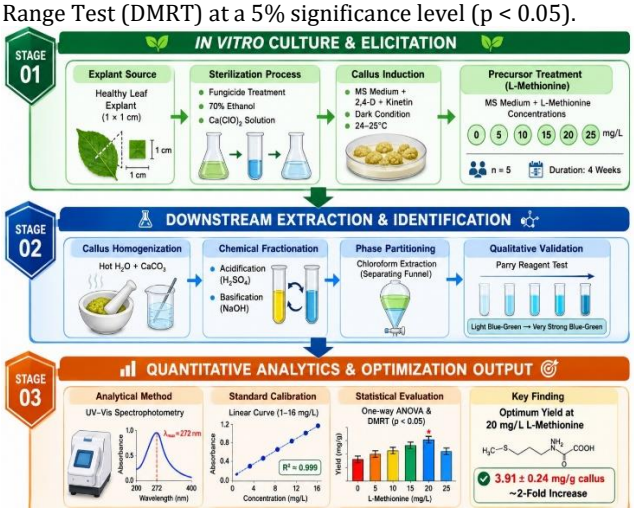
The present study was conducted as an in vitro experimental study to evaluate the dose-dependent effect of L-methionine on caffeine accumulation in callus cultures. The experiment was arranged using a Completely Randomized Design (CRD) and consisted of callus induction, L-methionine treatment, caffeine extraction, qualitative identification, quantitative estimation, and statistical analysis.

Healthy leaf explants were washed and surface sterilized using fungicide, 70% ethanol, and calcium hypochlorite under aseptic conditions. The sterilized leaves were cut into approximately 1 × 1 cm pieces and inoculated onto Murashige and Skoog (MS) medium supplemented with 2,4-D and kinetin. The cultures were incubated at approximately 24–25°C under dark conditions until sufficient callus formation occurred. Healthy callus was then transferred to treatment media containing different concentrations of L-methionine (0, 5, 10, 15, 20, and 25 mg/L), with five replicates for each treatment. The cultures were maintained under controlled conditions for approximately four weeks, after which the callus was harvested for caffeine analysis.

For caffeine extraction, harvested callus was homogenized and extracted with hot distilled water in the presence of calcium carbonate. The extract was filtered, acidified with sulfuric acid, concentrated, and subsequently alkalized with sodium hydroxide. Caffeine was extracted into chloroform using a separating funnel, and the chloroform fraction was evaporated. The resulting extract was reconstituted with distilled water and used for further analysis. Caffeine was qualitatively identified using the Parry reagent method based on characteristic colour development.

Quantitative caffeine estimation was performed using UV–Visible spectrophotometry. A caffeine standard solution was prepared and serially diluted to obtain concentrations of 1, 2, 4, 8, and 16 mg/L. The maximum absorption wavelength was determined, and caffeine absorbance was measured at 272 nm. A calibration curve was prepared from the standard solutions, and caffeine concentrations in the callus extracts were calculated from the corresponding regression equation.

The experimental data were expressed as mean ± standard deviation. One-way ANOVA was used to determine significant differences among treatments, followed by Duncan's Multiple Range Test (DMRT) at a 5% significance level ($p < 0.05$).



RESULTS

The present study demonstrated that L-methionine supplementation influenced caffeine accumulation in the in

vitro callus cultures in a concentration-dependent manner. Callus formation was successfully observed on MS medium supplemented with 2,4-D and kinetin. The developed callus was generally friable and pale green to cream in colour and was suitable for subsequent elicitation experiments.

Effect of L-Methionine on Caffeine Accumulation

Caffeine content varied significantly among the different L-methionine treatments. The untreated control showed the lowest caffeine accumulation, whereas supplementation with L-methionine increased caffeine content up to an optimum concentration. The highest caffeine accumulation was observed at 20 mg/L L-methionine, while a slight decline was observed at 25 mg/L, indicating that excessive precursor concentration did not further enhance caffeine production.

Table 1. Effect of different L-methionine concentrations on caffeine accumulation in callus cultures

L-Methionine concentration (mg/L)	Caffeine content (mg/g callus)	Response
0 (Control)	1.84 ± 0.12	Lowest
5	2.31 ± 0.15	Increased
10	2.87 ± 0.18	Increased
15	3.42 ± 0.21	Highly increased
20	3.91 ± 0.24	Highest
25	3.68 ± 0.20	Slight decline

The caffeine content increased progressively from the control to the 20 mg/L treatment. Compared with the control, the 20 mg/L treatment produced approximately a two-fold increase in caffeine accumulation. However, increasing L-methionine concentration from 20 to 25 mg/L resulted in a moderate reduction in caffeine content.

Qualitative Detection of Caffeine

The Parry reagent test confirmed the presence of caffeine in the callus extracts. The control and all L-methionine-treated samples produced the characteristic blue-green colour, although the intensity of colour varied among treatments. The strongest colour reaction was observed in extracts obtained from callus treated with 20 mg/L L-methionine, supporting the quantitative spectrophotometric findings.

Table 2. Qualitative detection of caffeine in callus extracts

Treatment	Parry test	Colour reaction
Control	Positive	Light blue-green
5 mg/L	Positive	Blue-green
10 mg/L	Positive	Moderate blue-green
15 mg/L	Positive	Strong blue-green
20 mg/L	Positive	Very strong blue-green
25 mg/L	Positive	Strong blue-green

UV–Vis Spectrophotometric Analysis

The caffeine standard showed maximum absorbance at 272 nm. A linear calibration curve was obtained using caffeine standards ranging from 1 to 16 mg/L. The absorbance increased proportionally with increasing caffeine concentration, confirming the suitability of the calibration curve for quantitative determination of caffeine in the callus extracts.

Table 3. Caffeine standard calibration data

Caffeine concentration (mg/L)	Absorbance at 272 nm
1	0.081
2	0.159
4	0.318
8	0.641
16	1.274

The calibration curve showed a strong positive linear relationship between caffeine concentration and absorbance. The regression equation was subsequently used to estimate caffeine concentrations in the experimental samples.

One-way ANOVA indicated a significant effect of L-methionine concentration on caffeine accumulation ($p < 0.05$). DMRT further demonstrated significant differences between several treatment means. The 20 mg/L treatment recorded the highest caffeine content and differed significantly from the untreated control. The reduction observed at 25 mg/L suggested that the optimum precursor concentration for caffeine accumulation under the present experimental conditions was approximately 20 mg/L.

Overall, the results indicated that L-methionine acted as an effective precursor/elicitor for enhancing caffeine accumulation in callus cultures, with the response increasing up to an optimum concentration before declining at the highest treatment level.

DISCUSSION

The present study demonstrated that L-methionine supplementation enhanced caffeine accumulation in callus cultures, with the response varying according to precursor concentration. Caffeine content increased progressively from the control treatment to 20 mg/L L-methionine, followed by a slight decline at 25 mg/L. This concentration-dependent response indicates that precursor availability can influence secondary metabolite biosynthesis in plant tissue cultures [19,27]. Similar findings have been reported in coffee callus cultures, where L-methionine supplementation significantly affected caffeine production and demonstrated the potential of precursor feeding as a strategy for enhancing caffeine accumulation [22].

The stimulatory effect of L-methionine can be explained by its metabolic relationship with S-adenosyl-L-methionine (SAM), an important methyl-group donor in caffeine biosynthesis [11,13]. Caffeine formation involves a sequence of methylation reactions in which SAM-dependent methyltransferases catalyze the conversion of xanthosine and related intermediates into methylated purine compounds [2,12,14]. The final steps of the pathway involve methyltransferase enzymes responsible for the formation of caffeine from theobromine and related precursors [14,16]. Therefore, increasing L-methionine availability may enhance the cellular pool of methyl donors and potentially increase metabolic flux toward caffeine production.

Previous studies have provided strong evidence for the involvement of methionine-derived metabolites in caffeine biosynthesis. Recent studies have highlighted the involvement of methionine-related metabolism in caffeine biosynthesis, with methionine serving as the precursor for S-adenosyl-L-methionine (SAM), an important methyl donor in the formation of caffeine and other purine alkaloids [29]. In addition, the caffeine biosynthetic pathway in coffee and tea

has been shown to involve several SAM-dependent methyltransferases, including xanthosine methyltransferase, 7-methylxanthine methyltransferase, and caffeine synthase [13,16]. These biochemical mechanisms provide a possible explanation for the increased caffeine accumulation observed following L-methionine treatment in the present study [22].

The highest caffeine content observed at 20 mg/L L-methionine suggests that this concentration provided a favorable precursor supply for caffeine biosynthesis. However, the slight reduction at 25 mg/L indicates that increasing precursor concentration beyond an optimum level does not necessarily increase metabolite production. Secondary metabolite accumulation in plant cell cultures is often controlled by complex metabolic and physiological factors, and excessive precursor supplementation may cause metabolic imbalance or fail to increase pathway flux if downstream enzymatic steps become limiting. Precursor supplementation does not always result in proportional increases in caffeine production in tea callus, indicating that metabolic regulation and pathway limitations can influence the response to precursor feeding [15].

The present results are also consistent with previous observations that callus cultures retain the ability to synthesize caffeine. The present results are also consistent with previous observations that callus cultures can produce caffeine under in vitro conditions. Caffeine production has been demonstrated in Coffea arabica callus cultures, supporting the suitability of in vitro callus cultures as a controlled system for caffeine production and investigation of caffeine accumulation [22]. These findings support the suitability of callus cultures as a controlled experimental system for investigating factors that regulate caffeine biosynthesis [22,23].

The qualitative Parry reagent test provided additional confirmation of caffeine in the extracts. The blue-green colour reaction observed in the treated samples was consistent with the presence of caffeine, while the stronger reaction observed at higher-performing treatments corresponded broadly with the quantitative results. UV–Visible spectrophotometry further demonstrated differences in caffeine concentration among treatments, with maximum absorbance observed at 272 nm. Spectrophotometric analysis has been widely applied for caffeine determination because caffeine exhibits characteristic ultraviolet absorption that can be used for quantitative estimation when appropriate calibration procedures are followed [22].

The observed enhancement of caffeine accumulation may also be related to the physiological state of the cultured callus. Actively growing callus tissues possess considerable metabolic activity and may have enhanced capacity for synthesis of specialized metabolites [18,21,26]. Previous studies have reported differences in caffeine biosynthesis according to tissue developmental stage and demonstrated that young tissues can exhibit greater activity of caffeine biosynthesis-related enzymes [30]. Consequently, maintaining healthy and actively growing callus may be important for achieving an effective response to precursor treatment.

Overall, the findings indicate that L-methionine can act as an effective metabolic precursor for enhancing caffeine accumulation in callus cultures, but its effect is strongly dependent on concentration [22]. The maximum caffeine accumulation at 20 mg/L followed by a decline at 25 mg/L suggests that an optimum precursor concentration exists under the experimental conditions. The results agree with the established role of SAM-dependent methylation in caffeine biosynthesis and previous reports of precursor-mediated enhancement of secondary metabolites in plant tissue cultures [13]. Further studies should investigate additional L-methionine concentrations, culture duration, callus growth parameters, and expression of caffeine biosynthesis-related genes to better understand the mechanisms responsible for enhanced caffeine accumulation.

CONCLUSION

The present study demonstrated that L-methionine supplementation can enhance caffeine accumulation in in vitro callus cultures in a concentration-dependent manner. Increasing L-methionine concentration from 5 to 20 mg/L progressively improved caffeine production, while a slight decline was observed at 25 mg/L, indicating the presence of an optimum precursor concentration. The highest caffeine accumulation was obtained at 20 mg/L L-methionine, suggesting that this concentration provided favorable conditions for caffeine biosynthesis. Qualitative analysis using the Parry reagent confirmed caffeine presence, while UV–Visible spectrophotometry provided quantitative evidence of treatment-related differences. Overall, L-methionine precursor feeding represents a promising strategy for improving caffeine production in callus cultures. Further studies should optimize precursor concentration and culture conditions to maximize caffeine yield.

Ethical Approval

Not applicable. This review used previously published literature and did not involve human participants, animals, or identifiable personal data.

Informed Consent

Not applicable, as this study did not involve human participants or direct data collection from individuals.

Data Availability Statement

All data analyzed in this review were obtained from previously published studies. Additional information is available from the corresponding author upon reasonable request.

Author Contributions

Sobia Naz, Hoorish, and Sabahat Jan contributed to the conceptualization, methodology, data collection, analysis, manuscript writing, review, and final approval of the manuscript.

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Conflict of Interest

The authors declare that they have no competing interests.

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Use of Artificial Intelligence

Gemini AI was used for language and grammar assistance. All AI-assisted content was reviewed, verified, and endorsed by the authors, who take full responsibility for the final manuscript.

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