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Research Article

Enhancement of Caffeine Concentration in Todolo Coffee Callus Cultures with L-Methionine and UV-Vis Spectrophotometry

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Abstract

Background and Objective: Todolo coffee (*Coffea arabica* L. var. typica) is the oldest commercially grown coffee in the Toraja region of South Sulawesi and is currently at risk of extinction. This study aims to induce callus from leaf explants of Todolo arabica coffee and improve the levels of caffeine compounds in Todolo coffee with the additional precursor L-methionine as elicitor.

Materials and Methods: This research was conducted at the Tissue Culture Laboratory, Faculty of Mathematics and Natural Sciences, Hasanuddin University. This experimental study used a Complete Randomized Design (CRD) approach. The five stages involved callus induction, elicitation with L-methionine precursor, caffeine extraction, qualitative testing using the Parry Method and quantitative analysis via UV-Vis spectrophotometry. Tools and materials were sterilized using autoclaving and media preparation followed standard procedures with varying L-methionine concentrations. Callus induction from sterilized leaf explants was done under aseptic conditions, followed by L-methionine treatment and caffeine content extraction. Caffeine was analyzed using UV-Vis spectrophotometry at 272 nm and statistical analysis was conducted using ANOVA and DMRT at a 5% significance level. **Results:** The friable callus structure has a shiny and wavy appearance and is more easily detached, which is highly correlated with the speed of callus growth. The callus on the treatment medium is slightly yellowish-white to somewhat brownish, indicating the production of secondary metabolites. The best concentration of L-methionine treatment was 25 mg/L with a percentage of caffeine content of 0.0078%. **Conclusion:** Results concluded that the addition of L-methionine has a significant effect on the percentage of caffeine production.

Key words: Caffeine biosynthesis, induced callus formation, micro-propagation, Tropical coffee varieties, ultraviolet spectrophotometer

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

INTRODUCTION

Indonesia is a country that is geographically ideal for the growth of various plants including coffee plants. Coffee is one of the plantation commodities that has a high selling value among other plantation crops¹. In addition, the coffee commodity also makes an important contribution as a source of foreign exchange. Indonesia is the third largest coffee producer in the world after Brazil and Vietnam, contributing around 6% of total world coffee production². Coffee is a plantation commodity that has a high selling value among other plantation crops. According to Martauli³, there are two types of coffee developed in Indonesia, namely Arabica coffee and Robusta coffee. Arabica coffee is a traditional type of coffee, considered to have the most delicious taste and Robusta coffee which has a higher caffeine content can be developed in an environment where Arabica coffee cannot grow, with a bitter and sour taste. However, the area of Robusta coffee plantations has decreased compared to Arabica coffee. This is because of the price Arabica coffee which is quite expensive and in great demand for consumption has resulted in the shift from planting Robusta coffee to Arabica³.

South Sulawesi is one of the provinces in Eastern Indonesia that has the potential for coffee development. This is indicated by a fairly large planting area and a very supportive agro-climatology. One of the areas that has the potential to produce coffee is the Luwu and Toraja regencies and this coffee has been widely recognized abroad under the names Kopi Toraja and Kopi Seko¹. Todolo coffee (*Coffea arabica* L. var. *typica*) is the oldest commercial coffee found in the Toraja Region, South Sulawesi and is threatened with extinction.

Coffee has many nutrients that are beneficial to the body. The active compounds of natural ingredients contained in coffee are polyphenols and alkaloids. The alkaloid compound in coffee is caffeine which is a xanthine-derived alkaloid that is naturally found in coffee and has benefits in the field of pharmacology. Caffeine is a heterocyclic alkaloid in the methylxanthine class, a nitrogen-containing organic compound with a two-ring or dual-cyclic structure⁴. This molecule naturally occurs in many types of plants as a secondary metabolite⁵. Caffeine is widely used as a central nervous system stimulant, accelerates metabolism, stimulates the heart muscle and relaxes bronchus smooth muscle so that we can think brilliantly, not sleepy and increase concentration. The pharmacological effects and side effects of caffeine vary depending on each person's sensitivity to caffeine. However, there have been no human or animal studies on the effects of caffeine on normal coffee consumption⁶. Caffeine is an organic

secondary metabolite compound synthesized by plants and is a source of medicinal compounds. To obtain secondary metabolites, plant samples are needed. The more compounds needed, the more plants are used for later extraction, so because of this it encourages humans to exploit plants which has an impact on the reduction of these plants⁷.

One way to get secondary metabolites without having to take plants in nature in large quantities is through the use of tissue culture techniques. Tissue culture is a technique for isolating parts of plants such as cells or tissues that are grown in aseptic conditions so that they can reproduce themselves and grow into complete plants^{8,9}. Based on the theory of cell totipotency, exclusive cells from plants have the inherited genetic potential to produce compounds *in vitro* and *in vivo* culture. In accordance with this theory, plants that contain certain chemical compounds in large quantities will also be able to produce the same compounds in large quantities when the plant is cultured *in vitro* conditions. So information about plant parts that have the desired compound content needs to be known before carrying out tissue culture activities to obtain secondary metabolite compounds. Parts that contain the desired compounds in high concentrations can then be used as a source of explants for the production of secondary metabolites⁹.

Utilization of this tissue culture technique provides an advantage in producing caffeine as a secondary metabolite no longer depends on the condition of the source plant and can provide material continuously without being disturbed by season, weather, temperature or environmental situation. The production of active compounds produced from tissue culture can be free from contaminants such as microbes and insects. Media is one of the major factors that support the success of tissue culture. Media is generally composed of macronutrients, micronutrients, growth regulators and amino acids.

Amino acids are constituents of proteins that function as support, transport other substances, coordinate organism activities, cell responses to stimuli, movement, protection against disease and accelerate chemical reactions selectively¹⁰. The amino acid used in this study is L-methionine. The L-methionine is an amino acid that contains sulfur (S) which functions in the formation of proteins. In addition, it can also be used to control cellular processes as a precursor of S-adenosylmethionine (SAM)¹¹. Giving methionine 50-100 mg/L in the media can produce endogenous cytokinins so that shoot growth will also be a lot¹².

In addition to amino acids, callus induction requires the supply of exogenous growth regulators, namely auxins and cytokinins, which can be used singly or in combination with the right concentration. The growth regulator 2,4-D is a

commonly used auxin for callus induction. Application of 2,4-D combined with cytokinin (BA or kinetin) will further enhance callus growth^{8,13}.

Based on various searches, research on the determination of caffeine levels in callus induction *in vitro* has been studied before¹⁴. While in the subculture of Arabica coffee callus *Coffea arabica* L. var. typica there has been no specific research. This study aims to produce caffeine from the callus of *Coffea arabica* var. typica and to determine the optimal concentration of L-methionine for enhancing caffeine productivity in the callus cultures of *Coffea arabica* L. var. typica.

MATERIALS AND METHODS

Study site and duration: This study was conducted from July to December, 2023. The study was carried out in the Plant Tissue Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences and Biochemistry Laboratory, Department of Chemistry, Universitas Hasanuddin, Indonesia.

Materials: The plant material used consisted of three young leaves of Arabica coffee (*Coffea arabica* L. var. typica) as explant which is in the second to third position from the top of the plant¹⁵ obtained from the forest area of Toraja. The medium used was Murashige and Skoog (MSP09, Caisson Labs), kinetin growth regulators (PhytoTech LABS) with concentrations 0.1, 0.5, 1.0 ppm and 2,4-Dichlorophenoxyacetic Acid (2,4-D) (Phytotech LABS) with concentration 1, 2, 3 ppm, agar (phytagel, Sigma), sucrose (Merck), alcohol 70 and 96% (Onemed), calcium hypochlorite 20% (Merck), solution 0.2% Dithane M-45 (Dow AgroSciences), HCl 1 N (Onemed), KOH 1 N (Medical and Laboratory Supplier), distilled water (Onemed), plastic wrap (Klinpak Cling Wrap), tissue (paseo), rubber (Ogawa), label paper (Fox), chloroform (Merck), reagent parry (Nitra kimia), dilute ammonia (Citra sari kimia), standardized caffeine solution (Sigma-Aldrich), L-methionine powder (Sigma-Aldrich), methylated spirits (Naga), plastic samples (C-tik) and matches (Tokai).

Methods: This research is included in experimental research with the Complete Randomized Design (CRD) method. This study consists of five stages: Callus induction, elicitation using L-methionine precursor, caffeine extraction, qualitative test using Parry Method¹⁶ and quantitative test using UV-Vis Spectrophotometry.

Sterilization of tools, materials and workplace: The tools used must be in a sterile state. Before being sterilized using an autoclave (Gea), glassware (Pyrex) and metal tools (Renz) are first washed with detergent and rinsed with running water until clean, then wrapped in plastic. Glassware, metal tools and distilled water were sterilized by autoclaving for 60 min and growth media for 15 min at 121°C. Planting tools after being sterilized in an autoclave, tools such as tweezers, scalpels and scissors were soaked with 96% alcohol and then heated over a bunsen flame to remain sterile during planting. Laminar air flow (LAF) (Labtech) before use was first sprayed with 70% alcohol and wiped using tissue. The UV light was turned on for 1-2 hrs before the LAF was used, then turned off. Furthermore, the blower was turned on for ± 5 min and after that, the lights were turned on.

Preparation of media

Preparation of methionine stock solution: Weighed 0.2 g of L-methionine powder then put it into a beaker glass and added 200 mL of sterile distilled water to make 1000 ppm L-methionine stock. The L-methionine stock was homogenized using a hot plate (Thermo Scientific) with a magnetic stirrer. Once homogeneous, L-methionine stock was poured into a 200 mL Erlenmeyer then covered with aluminum foil labeled and stored in the refrigerator.

Preparation of MS media+growth regulators (GR): Instant murashige-skoog (MS) medium was weighed as much as 4.43 g/L, sugar as much as 30 g/L and agar as much as 7 g/L each into a beaker glass. The 2,4-D as much as 2.0 mg and Kinetin as much as 0.5 mg were added to the beaker glass and then sterile distilled water was added to 1000 mL. In this study there were 9 treatments with a combination of using growth regulators 2,4-D and kinetin, namely A1 (1 ppm 2,4-D+0.1 ppm kinetin), A2 (1 ppm 2,4-D+0.5 ppm kinetin), A3 (1 ppm 2,4-D+1 ppm kinetin), A4 (2 ppm 2,4-D+0.1 ppm kinetin), A5 (2 ppm 2,4-D+0.5 ppm kinetin), A6 (2 ppm 2,4-D+1 ppm kinetin), A7 (3 ppm+0.1 ppm kinetin), A8 (3 ppm 2,4-D+0.5 ppm kinetin), A9 (3 ppm 2,4-D+1 ppm kinetin) and A0 as a control which is a medium with no addition of growth regulator 2,4-D or kinetin.

Preparation of MS media+growth regulators (GR)+

L-methionine: To facilitate the preparation of MS+GR+L-methionine solution, the L-methionine solution was divided into 6 different concentration levels (Table 1). Each medium was made as much as 250 mL per concentration. The

Table 1: Media with L-methionine treatment

Treatment	L-methionine					
	0 mg/L	5 mg/L	10 mg/L	15 mg/L	20 mg/L	25 mg/L
MS+GR	0 ppm	5 ppm	10 ppm	15 ppm	20 ppm	25 ppm

concentration of L-methionine for 5 ppm was 1.25 mL, for 10 ppm was 2.5 mL, for 15 ppm was 3.75 mL, for 20 ppm was 5 mL and for concentration of 25 ppm, 6.25 mL of L-methionine stock was taken. Homogenized by cooking until boiling and measuring the pH of the media solution to 5.8. The media mixture was poured into sterile culture bottles at the rate of ± 25 mL/bottle for each treatment and replicated. The culture bottles were closed with plastic seals and labeled. Medium sterilization was carried out using an autoclave with a temperature of 121°C for 15 min. After that, the culture bottles were kept in the culture room at 25°C before use.

Callus induction: The leaves were first cleaned with running water and sterilized in a 0.2% Dithane M-45 solution for 30 min, followed by thorough rinsing with sterile water. In a laminar airflow, the leaves were soaked in 70% alcohol for 3-5 min, then immersed in 20% calcium hypochlorite solution with 2-3 drops of tween for 7 min, being shaken every 5 min for 5 sec. Afterward, they were rinsed three times with sterile water. The leaves were cut into 1×1 cm pieces and the explants were placed in culture media. The cultures were incubated in darkness at 24°C with a relative humidity of approximately 60% for 2 months.

Elicitation using L-methionine precursor: Callus planting was done in laminar air flow (LAF) aseptically. Callus was subcultured into the treatment media with as many as 5 replicates so that there were 30 culture bottles. Then the bottle was closed again with a plastic seal and cling wrap and stored on a culture rack. Culture bottles containing callus were placed on a culture rack and incubated in a dark room at a temperature of $\pm 25^\circ\text{C}$ for ± 1 month. Maintenance was also carried out aseptically by separating bottles that had been contaminated by microorganisms from the incubation room. Periodic spraying of culture bottles was done using 70% alcohol. A callus that has been subcultured for approximately 1 month was observed for texture and color of the callus after being transferred into the treatment medium.

Caffeine extraction: Callus in the treatment (Table 1) was placed in a mortar until smooth, then the weight using an analytical balance. For each treatment 50 mL of hot water and CaCO_3 half of the weight of the callus was added into Erlenmeyer and then filtered. The dregs were rinsed with 50 mL of hot water twice and filtered until the filtrate volume

reached 150 mL. After adding 5 mL of 30% H_2SO_4 , the filtrate volume was reduced by heating to 50 mL. The filtrate was then added 2 mL of 10% NaOH and 75 mL of chloroform in a separating funnel, repeated 2 times. The chloroform fraction was evaporated at 70-80°C, then the result was added 50 mL of distilled water and homogenized.

Qualitative test with Parry Method¹⁶: Pipetted 1 mL of each sample filtrate into a test tube. Parry reagent was added as much as 3 drops and homogenized. Then dilute ammonia as much as 3 drops and homogenized. Observed the color change that occurs, if the solution is dark blue-green indicating a positive solution of caffeine.

Quantitative test using UV-Vis spectrophotometric method: Wavelength determination is done by detecting the absorbance of the standard solution in the wavelength range of 200-700 nm using a UV-Vis spectrophotometer instrument (Thermo Fisher Scientific). The maximum wavelength obtained based on wavelength determination is 272 nm. Caffeine levels in each sample were measured by UV-Vis spectrophotometry at a wavelength of 272 nm.

Preparation of caffeine standard solution: Preparation of 1000 ppm caffeine standard solution. Weighed as much as 250 mg of caffeine, put into a glass cup, dissolved with enough distilled water, put into a 250 mL volumetric flask then diluted with distilled water until the mark line and homogenized. The caffeine standard solution was pipetted as much as 2.5 mL, put into a 25 mL volumetric flask then diluted with distilled water to the mark line and homogenized.

Standard curve preparation: Sample measurements were preceded by measurements of caffeine standard solution to determine the maximum wavelength. Preparation of the standard solution was preceded by taking 0.05, 0.1, 0.2, 0.4 and 0.8 mL of 100 ppm caffeine standard solution and diluted to 5 mL so that the concentration of the standard solution obtained was 1, 2, 4, 8 and 16 mg/L.

Test of caffeine content of callus: The caffeine extract from each sample (Table 1) was put into a test tube as much as 5 mL, then determined the level with UV-Vis spectrophotometry at a wavelength of 272 nm.

Statistical analysis: Observational data were analyzed using Analysis of Variance (ANOVA) then if it gives a real effect, the Duncan's Multiple Range Test (DMRT) was conducted at the 5% level.

RESULTS AND DISCUSSION

Texture and callus color of typical Arabica coffee *Coffea arabica* L. var. typica: The appearance of a callus on the explant is characterized by enlargement at the incision site at the edge of the explant. It starts by appearing like a small clear dot and continues to grow over time. The day of callus appearance is counted as the first day of the appearance of a very small callus that grows at the edge of the former incision. According to Muthi'ah *et al.*¹⁷ callus formation begins with swelling of the explant surface and is followed by callus formation. A callus will form at the edge of the leaf where cells can divide and form callus¹⁸.

The fastest average time in the growth of callus is in the A2 treatment, namely the provision of 1 ppm 2,4-D and 0.5 ppm kinetin with an average time of 17.00 HST. As for the treatment that is not added growth regulators cannot produce callus up to 9 weeks of observation. This shows that the provision of 2,4-D and kinetin can trigger callus growth. This was in line with Ibrahim *et al.*¹⁹ which states that the development of callus from explants and until callus formation shows that in the induction medium contained growth regulators affect callus growth, explants begin to swell three weeks after culturing and visible efflorescence in the incision area. This is different from the control treatment where no callus was seen, only a slight swelling of the tissue.

Callus texture and color were observed at the end of the study visually using a loop directly. The resulting callus texture is relatively the same, namely the crumb structure. Direct observation of callus texture was carried out 77 days after subculture. The friable callus structure has a shiny and wavy appearance and is more easily detached which is highly correlated with the speed of callus growth so that the production of certain secondary metabolites that you want to obtain can be achieved more quickly. According to

Syahid *et al.*¹³ the application of a combination of auxin and cytokinins at the right concentration can produce callus with a crumb structure.

According to Bhandari and Brandizzi²⁰ injuries to plant cells or tissues will activate local and systemic self-defense mechanisms in these tissues or cells. This mechanism can be in the form of changes in the direction of metabolic pathways and the induction of the expression of certain genes. In damaged tissues, irregular cell structures will form, cells dedifferentiation, secrete stored compounds and lose a lot of water. This irregular cell structure will develop into callus²⁰.

Factors that influence the day of callus emergence are the composition of growth regulators added to the media¹³. The effectiveness of exogenous auxin and cytokinin growth regulators depends on the concentration of endogenous growth regulators in the plant tissue. In addition, the balance between the concentration of auxin and cytokinin in the media is also known to trigger callus formation through interactions in cell enlargement and division¹⁵.

Callus is formed because it is stimulated by 2,4-D (auxin group) which begins with the enlargement of the upper epidermal cells and then the cell divides into two. When the plant is wounded, callus will be formed due to cell damage and outolysis and the damaged cells will produce compounds that stimulate cell division in the next layer so that a clump of cells called callus is formed. In addition, the influx of water from organic ions and inorganic molecules continuously causes the number and size of cells to increase so that the weight of the callus also increases.

The L-methionine seemed to have no effect on the callus structure when compared to the control, but it did make a difference in the color of the callus. Giving L-methionine showed a difference in color change from slightly yellowish to brownish (Table 2). This was in line with Aguilar *et al.*¹⁵ that callus grown on medium with the addition of L-methionine showed a slightly browner color (Fig. 1b and 2b) when compared to callus without L-methionine in Arabica coffee culture (Fig. 1a and 2a).

Table 2: Callus color after 77 days after subculture typical Arabica coffee *Coffea arabica* L. var. typica

Treatment	L-methionine concentration (ppm)	Information
L0	0	White
L1	5	Yellowish white
L2	10	Yellowish white
L3	15	Yellowish white
L4	20	Yellowish white
L5	25	Yellowish white

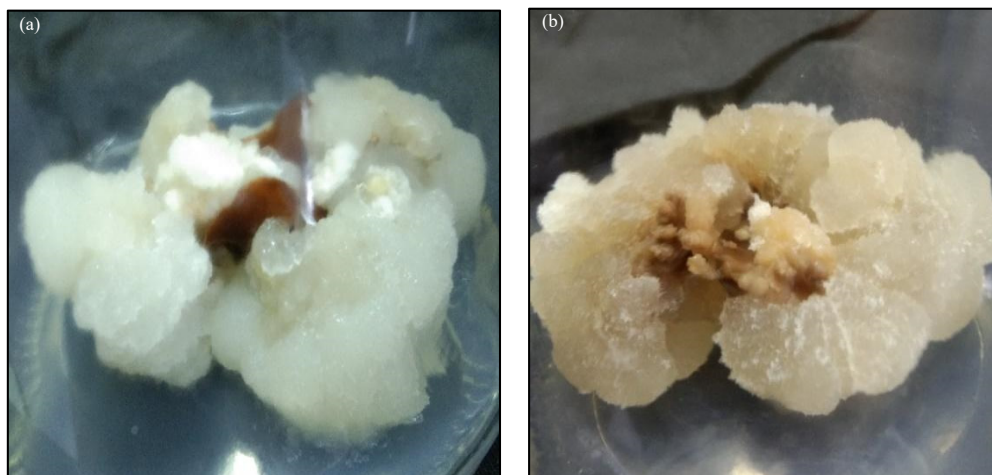


Fig. 1(a-b): Arabica coffee crumb callus texture 77 day after subculture, (a) Treatment without L-methionine control and (b) 10 ppm L-methionine treatment

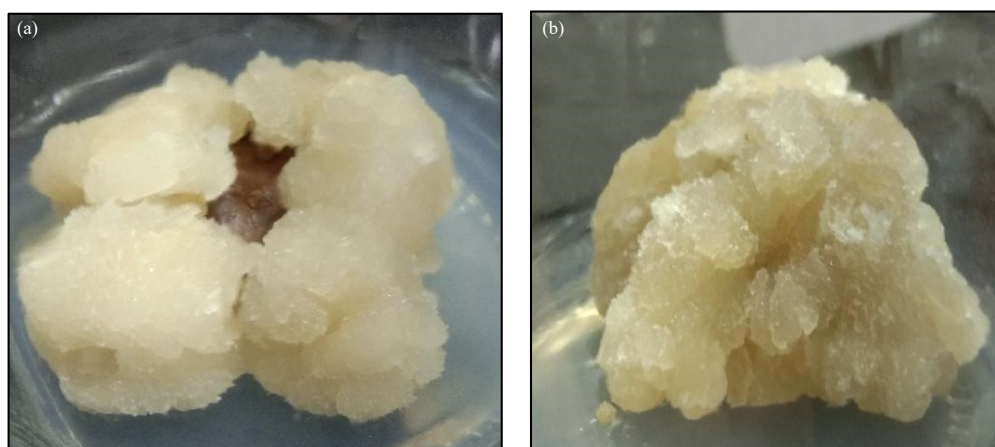


Fig. 2(a-b): Arabica coffee callus culture color 77 day after subculture, (a) Treatment without L-methionine and (b) 25 ppm L-methionine treatment

This color change can be caused by different levels of callus secondary metabolites. The color of the control callus can appear whiter than the color of the callus on the treatment medium with the addition of L-methionine. The callus on the treatment medium is slightly yellowish white to slightly brownish (Fig. 1b and 2b) which indicates that there is production of secondary metabolites. This is due to differences in the composition of the media for the treatment of L-methionine giving stress to the media. According to Hassanpour²¹, the stress exerted by the media on callus indicates that the callus will turn darker in color than fresh callus. Thus, the older the change in callus color in a medium indicates the higher and greater activity of secondary metabolite biosynthesis²¹.

Caffeine content of typical Arabica coffee *Coffea arabica* L. var. typical

Qualitative test of typical Arabica coffee *Coffea arabica* L. var. typica: The test for the presence of caffeine was carried out qualitatively and quantitatively. In order to qualitatively determine the presence of caffeine, a qualitative test was carried out with Parry's reagent. Each treatment was treated with solid-liquid extraction in order to obtain a filtrate/extract which was assumed to be a pure caffeine compound. Each of these extracts was tested with Parry's reagent. The presence of caffeine can be indicated by a change in the color of the solution. The results of the qualitative test can be seen in Table 3.

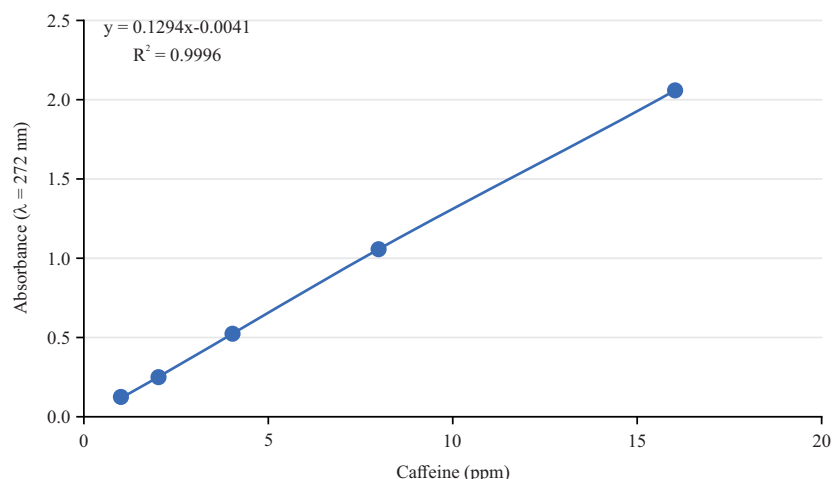


Fig. 3: Caffeine standard linear regression curve

Table 3: Qualitative test with Parry's reagent

Treatment	L-methionine concentration (ppm)	Color
L0	0	Dark blue/green
L1	5	Dark blue/green
L2	10	Dark blue/green
L3	15	Dark blue/green
L4	20	Dark blue/green
L5	25	Dark blue/green

A positive result for the presence of caffeine is indicated by the color of the solution turning dark blue/green. All treatments showed positive for caffeine which was tested with Parry's reagent. The Parry's reagent was prepared by reacting Cobalt Nitrate $\text{Co}(\text{NO}_3)_2$ with methanol CH_3OH . According to Koshiishi *et al.*²², cobalt (Co) ions in the reagent will form a dark blue/green complex. The cobalt ion has a positive double charge, making it possible to bind to the nitrogen group present in the caffeine compound. According to Aguilar *et al.*¹⁵ and Hassanpour²¹, caffeine compounds are found both in leaves and in callus. This could explain why each treatment including the control showed positive results, but the caffeine content in each treatment was unknown.

Quantitative test of caffeine content of typical Arabica coffee *Coffea arabica* L. var. typica:

To find out the real caffeine content, a quantitative test was carried out using the UV-Vis spectrophotometry method. Standard solution and sample measurements were carried out with the maximum wavelength obtained, namely 272 nm. Caffeine standard solutions were prepared at concentrations of 1, 2, 4, 8 and 16 mg/L which gave the equation $y = 0.1294x + 0.0041$ with a correlation coefficient of 0.9996. The measurement results of the standard caffeine solution can be seen in Fig. 3.

From the results of the quantitative analysis of callus extract using UV-Vis spectrophotometry, the six callus extract treatments namely L0, L1, L2, L3, L4 and L5 were read at a wavelength of 272 nm. The 50 mL sample solution was taken 5 mL each into a test tube. The final concentration value of each sample can be seen in Table 3.

Data analysis results using Analysis of Variance (ANOVA) with the treatment of L-methionine additionstration on caffeine production have a significant or significant effect. This shows that the concentration of L-methionine has a significant effect on caffeine production. This was also in line with Aguilar *et al.*¹⁵ that the addition of L-methionine had a significant effect on caffeine production in Arabica coffee callus culture. The data obtained were then analyzed with a follow-up test using the Duncan's Multiple Range Test (DMRT) at the 5% level so that the following Table 4 was obtained.

Based on the DMRT follow-up test at 5% level, it showed that the highest caffeine production was found in the L5 treatment (Table 5), which was significantly different from the other treatments, both the control and the addition of L-methionine. So it can be said that the best treatment for adding L-methionine is 25 mg/L (25 ppm) in culture media.

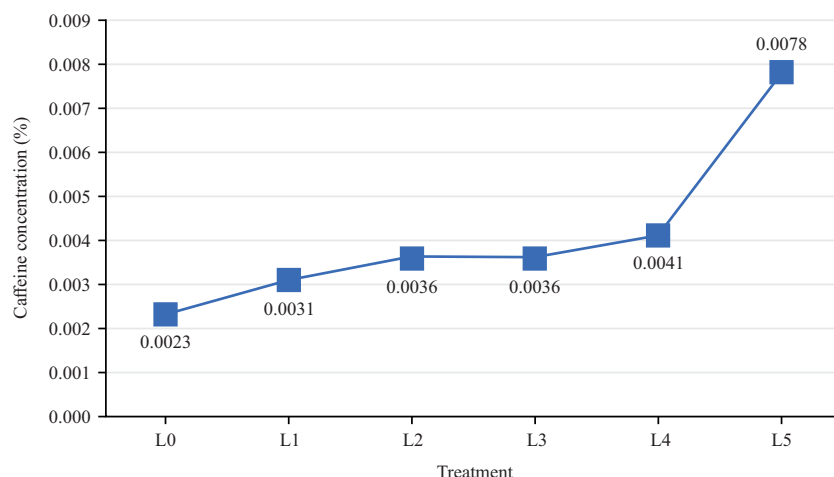


Fig. 4: Percentage of caffeine content per treatment of L-methionine

Table 4: Quantitative final analysis results of UV-Vis spectrophotometry method

Treatment	Sample mass (mg)	Extract volume (L)	Caffeine (mg/L)	Caffeine (%)
L0	5170.003	0.05	2.476	0.0023
L1	5253.640	0.05	3.229	0.0031
L2	5116.295	0.05	3.557	0.0036
L3	5067.332	0.05	3.664	0.0036
L4	5133.239	0.05	4.536	0.0041
L5	5233.664	0.05	8.181	0.0078

Table 5: 5% DMRT test of L-methionine addition on caffeine production

Treatment	Caffeine level (%) (Mean+SD)
L0	0.0023+0.00005 ^a
L1	0.0031+0.00015 ^b
L2	0.0036+0.00011 ^c
L3	0.0036+0.00011 ^c
L4	0.0041+0.00016 ^d
L5	0.0078+0.00013 ^e

Letters a, b, c, d and e indicate statistical grouping from the DMRT at a 5% significance level, where treatments sharing the same letter show no significant difference, while different letters represent significant differences in caffeine production

The percentage of caffeine production increased along with the increasing concentration of L-methionine in the treatment medium. In the caffeine biosynthesis process, a methylation step is required with the release of the CH₃ group by SAM facilitated by a specific enzyme N-Methyltransferase. After being methylated, SAM will produce S-adenosyl-L-homocysteine (SAH) products. The SAH is then hydrolyzed by SAH hydrolase to become adenosine and L-homocysteine. Adenosine will turn back into xanthosine into caffeine biosynthesis and then L-homocysteine will become methionine by methionine synthase. Methionine is then converted back to SAM by SAM synthase and then a continuous cycle is integrated into the caffeine biosynthetic pathway.

The increase in caffeine levels can increase with increasing concentrations of L-methionine because

L-methionine acts as a methylation precursor in the caffeine biosynthetic pathway. When the availability of L-methionine is abundant, the production of caffeine compounds will be stimulated which in this case will stimulate the S-adenosyl-L-methionine (SAM) cycle as a methylation group. Methyl groups which are abundant in cells will certainly methylate groups in biosynthetic complex pathways such as xanthosine, 7-methylxanthine and theobromine which then produce caffeine compounds.

The addition of 25 ppm L-methionine was the highest percentage of caffeine content (Fig. 4) in this study and was significantly different from other treatments. In this case, the cells can still synthesize caffeine which has not shown saturation with the given concentration. Cells can still be stimulated by addition of steering L-methionine with a higher concentration to produce caffeine compounds.

The treatment without L-methionine as a control showed the presence of caffeine content but had a lower percentage compared to all variations of the L-methionine treatment. This is because the coffee plant cells synthesize caffeine naturally, but not as much as when the cells are stimulated with L-methionine treatment. The L-methionine is one of the amino acids that acts as a methyl source group in the methylation process of caffeine synthesis. Naturally, methionine will be produced through a cyclic reaction of S-adenosyl-L-methionine (SAM) for the methylation process in three stages, namely 7-methyl xanthine, theobromine and the final stage becomes caffeine. Methionine itself is essential for the formation of caffeine as a methylation precursor. With the addition of exogenous L-methionine, the cells will be stimulated to produce more caffeine with its availability in the culture medium.

CONCLUSION

Caffeine production with the addition of L-methionine has been successfully carried out *in vitro* on typical Arabica coffee callus tissue culture *Coffea arabica* L. var. *typica* which is characterized by the results of qualitative and quantitative tests. The addition of L-methionine has a significant effect on the percentage of caffeine production. The best concentration of L-methionine treatment was 25 mg/L with a percentage of caffeine content of 0.0078%. Future studies should explore a broader range of L-methionine concentrations and investigate other factors influencing caffeine production in callus cultures. Additionally, scaling up the experiment and applying it to other coffee varieties may enhance the potential for commercial caffeine production. Understanding the mechanisms of L-methionine's role in caffeine biosynthesis could further optimize these processes.

SIGNIFICANCE STATEMENT

This study highlighted L-methionine as a novel elicitor to significantly enhance caffeine production in the callus cultures of *Coffea arabica* L. var. *typica*, focusing on the rare and threatened Todolo coffee from South Sulawesi. It successfully induced Todolo coffee callus and found that adding L-methionine, with 25 mg/L being the optimal concentration, significantly enhanced caffeine production. The callus color shifted from yellowish-white to brownish, correlating with increased metabolite production and the crumbly texture indicated rapid growth. This research underscores the importance of Todolo coffee for conservation

and caffeine production while offering an efficient *in vitro* method for secondary metabolite production, minimizing the need for extensive field cultivation.

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