Vol. 13, No. 3, pp. xxx-xxx, August 2025 DOI: https://doi.org/ 10.23960/jat.v13i3.11044

Jurnal Agrotek Tropika

P-ISSN: 2337-4993
E-ISSN: 2620-3138

Journal homepage: https://jurnal.fp.unila.ac.id/index.php/JA

PHENYLALANINE-INDUCED MODULATION OF CALLUS CHARACTERISTICS AND SECONDARY METABOLITE ACCUMULATION IN *Ocimum basilicum* L. UNDER IN VITRO CONDITIONS

MODULASI KARAKTERISTIK KALUS DAN AKUMULASI METABOLIT SEKUNDER YANG DIINDUKSI FENILALANIN PADA *Ocimum basilicum* L. DALAM KONDISI IN VITRO

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ARTICLE HISTORY:
Received: Tanggal Bulan Tahun
Peer Review: Tanggal Bulan Tahun
Accepted: Tanggal Bulan Tahun

KEYWORDS:
Herbal plant, Phenylpropanoids, secondary metabolism

ABSTRACT

Basil (Ocimum basilicum L.) is widely used in traditional medicine due to its rich content of phenolic and flavonoid compounds. However, the natural production of these metabolites is limited. Callus culture offers a controlled method to enhance their accumulation, with medium composition, particularly precursor supplementation, playing a critical role. Phenylalanine, an aromatic amino acid, is a key precursor in the biosynthesis of phenolics and flavonoids via the shikimate pathway. This study aimed to evaluate the effect of phenylalanine on callus growth and the accumulation of total phenolic and flavonoid compounds in basil. The experiment was conducted at the Plant Tissue Culture Laboratory, Faculty of Agriculture, Universitas Padjadjaran, using Murashige and Skoog (MS) medium with phenylalanine concentrations of 0, 1.3, 1.6, and 2 g.L^{-1}, each replicated six times. Results showed that phenylalanine treatments caused brown coloration, compact callus texture, and inhibited growth, as indicated by reduced fresh and dry weights. The 1.6 g.L^{-1} treatment produced the highest total phenolic content, while the 2 g.L^{-1} treatment yielded the highest flavonoid content. These findings suggest that phenylalanine can enhance secondary metabolite accumulation in basil callus, although it may suppress biomass growth.

KATA KUNCI:
Fenilpropanoid, metabolit sekunder, tanaman obat

ABSTRAK

Kemangi (*Ocimum basilicum* L.) banyak digunakan dalam pengobatan tradisional karena kaya akan kandungan senyawa fenolik dan flavonoid. Akan tetapi, produksi metabolit ini secara alami terbatas. Kultur kalus menawarkan metode terkontrol untuk meningkatkan akumulasinya, dengan komposisi medium, khususnya suplementasi prekursor, yang memainkan peran penting. Fenilalanina, asam amino aromatik, merupakan prekursor utama dalam biosintesis fenolik dan flavonoid melalui jalur shikimat. Penelitian ini bertujuan untuk mengevaluasi pengaruh fenilalanina terhadap pertumbuhan kalus dan akumulasi total senyawa fenolik dan flavonoid pada kemangi. Percobaan dilakukan di Laboratorium Kultur Jaringan Tanaman, Fakultas Pertanian, Universitas Padjadjaran, menggunakan medium Murashige dan Skoog (MS) dengan konsentrasi fenilalanina 0, 1,3, 1,6, dan 2 g.L^{-1}, masing-masing diulang enam kali. Hasil penelitian menunjukkan bahwa perlakuan fenilalanina menyebabkan warna cokelat, tekstur kalus kompak, dan menghambat pertumbuhan, seperti yang ditunjukkan oleh berkurangnya berat segar dan kering. Perlakuan 1,6 g.L^{-1} menghasilkan kandungan fenolik total tertinggi, sedangkan perlakuan 2 g.L^{-1} menghasilkan kandungan flavonoid tertinggi. Temuan ini menunjukkan bahwa fenilalanina dapat meningkatkan akumulasi metabolit sekunder dalam kalus basil, meskipun dapat menghambat pertumbuhan biomassa.

1. INTRODUCTION

Basil (*Ocimum basilicum* L.) is a herbaceous plant widely cultivated and utilized in Indonesia. Traditionally, basil leaves are harvested as vegetables, flavouring agents, and herbal medicine (Nurfitriyah *et al*., 2022). Beyond its traditional applications, basil has garnered attention in the pharmaceutical industry due to its rich content of bioactive compounds, particularly essential oils that contain various secondary metabolites (Ariani *et al*., 2020). These secondary metabolites include flavonoids, phenols, tannins, steroids, triterpenoids, saponins, and eugenol (Naya & Mardiyanti, 2021). The essential oil profile of basil is dominated by eugenol (78.02%), followed by α-cubebene (6.17%), nerol (0.83%), α-muurolene (0.74%), 3,7-dimethyloct-1,5-dien-3,7-diol (0.33%), and β-cubebene (0.30%) (Gebrehiwot *et al*., 2016). These compounds are known for their antioxidant, antimicrobial, anti-inflammatory, antifungal, insecticidal, larvicidal, and nematicidal properties (Skrypnik *et al*., 2019; Yaldiz *et al*., 2019). Among them, methyl eugenol—a derivative of eugenol—has notable uses as a mouthwash, toothache reliever, and tooth decay preventative (Maramis *et al*., 2023; Yuniarti, 2022), making basil a promising medicinal and industrial crop.

Despite its potential, the production of basil and its secondary metabolites is often constrained by environmental factors. Although basil grows well in tropical climates such as Indonesia, fluctuations in light, temperature, and soil nutrients can influence the quantity and quality of secondary metabolites produced (Utomo *et al*., 2020). Moreover, competition with weeds in open-field cultivation further limits optimal plant growth and metabolite accumulation (Giri *et al*., 2021). These limitations highlight the need for alternative production methods that are independent of environmental conditions. One such alternative is callus culture, a plant tissue culture technique that enables large-scale production of secondary metabolites under controlled conditions. By manipulating media components and growth environments, callus culture ensures consistent metabolite yields regardless of climate or location (Razavi, 2017). However, the success of this method depends on optimizing several factors, including the choice of basal media, the combination of plant growth regulators (PGRs), and the use of metabolic precursors to stimulate biosynthesis. Among the various strategies to enhance metabolite synthesis within callus cultures, the addition of precursor compounds such as phenylalanine has shown considerable promise.

Phenylalanine is an aromatic amino acid that plays a central role in plant metabolism and acts as a key entry point into the phenylpropanoid pathway, which leads to the production of flavonoids and phenolic compounds as two major classes of secondary metabolites in basil (Liunokas & Karwur, 2020; Pascual *et al*., 2016). In this pathway, phenylalanine is enzymatically converted into cinnamic acid, which undergoes further transformation into a range of bioactive compounds (Agar & Cankaya, 2020; Firmansyah, 2021). Previous studies have demonstrated that phenylalanine supplementation can significantly increase metabolite production across various plant species, making it a promising elicitor in in vitro culture systems. Therefore, this study aims to determine the optimal concentration of phenylalanine to enhance secondary metabolite accumulation in basil callus cultures, providing a scalable in vitro strategy for valuable compound production.

2. MATERIALS AND METHODS

2.1 Time and Place of Research

The study was conducted from November 2024 to January 2025 in the Tissue Culture Laboratory, Seed Technology Unit, Faculty of Agriculture, Universitas Padjadjaran, Jatinangor, Sumedang.

2.2 Experimental Design

This study was conducted using a Completely Randomized Design (CRD) consisting of four phenylalanine (Phe) treatment levels: A (0 $g.L^{-1}$ Phe), B (1.3 $g.L^{-1}$ Phe), C (1.6 $g.L^{-1}$ Phe), and D (2.0 $g.L^{-1}$ Phe). Each treatment was repeated six times, with each replicate comprising four callus samples, resulting in a total of 24 experimental units and 96 callus samples. The initial explants used for callus induction were young, healthy basil (*Ocimum basilicum* L.) leaf segments, approximately 0.5 × 0.5 cm in size.

2.3 Material and Tools

The materials used for callus induction were basil leaf explants aged 8 Weeks After Planting (WAP), and the materials used for treatment were basil callus aged 4 weeks after Acclimatization (WAA). Then, the materials used in making the media were the composition of chemical compounds of MS media, 6-Benzyl Amino Purine (BAP), Naphthalene Acetic Acid (NAA), Phenylalanine, 1 N NaOH, 1 N HCl, 95% Alcohol, and Gelzan. The tools used in the present experiment were Laminar Air Flow (LAF), petri dishes, culture bottles, scalpels, scissors, sterile paper, tweezers, culture racks, bunsen burners, plastic, rubber, measuring cups, beakers, hotplate magnetic stirrers, ovens, autoclaves, pH meters, tissues, Thermo hygrometers, millimetre blocks, colour charts, digital analytical scales, plastic covers, rubber, stationery, labels, and cellphone cameras.

2.4 Explants Preparation and Callus Induction

Callus induction was carried out using basil leaf explants of the Sariwangi variety aged 8 weeks after planting (WAP). The procedure began by cutting the leaf explant along with its stalk using sterilized scissors and tweezers. The explants were then trimmed into square pieces measuring 1 cm^2, using a millimetre block for accuracy. Prior to culturing, the explants were surface-sterilized to prevent contamination. The prepared explants were then planted onto Murashige and Skoog (MS) medium supplemented with a combination of growth regulators, including 2 $mg{\cdot}L^{-1}$ 6-benzylaminopurine (BAP) and 0.5 $mg{\cdot}L^{-1}$ α-naphthaleneacetic acid (NAA), which supported effective callus formation. In some cases, an alternative medium composition was also used, consisting of 1 $mg{\cdot}L^{-1}$ 2,4-D, 0.2 $mg{\cdot}L^{-1}$ BAP, and 1 $mg{\cdot}L^{-1}$ calcium phosphate (CaP), to optimize callus induction. The induction process was carried out over a four-week period to obtain sufficient healthy callus material for subsequent treatments.

2.5 Medium Preparation

The culture medium used in this study was based on Murashige and Skoog (MS) basal medium, which was supplemented with 30 $g.L^{-1}$ sucrose as a carbon source and solidified using 7 $g.L^{-1}$ agar. The medium was further enriched with 2 $mg.L^{-1}$ 6-benzylaminopurine (BAP) and 0.5 $mg.L^{-1}$ α-naphthaleneacetic acid (NAA) as plant growth regulators to promote callus induction and development. Four different treatments were applied by varying the phenylalanine (Phe) concentration added to the medium. The treatments included treatment A as the control (0 $g.L^{-1}$ Phe), treatment B (1.3 $g.L^{-1}$ Phe), treatment C (1.6 $g.L^{-1}$ Phe), and treatment D (2.0 $g.L^{-1}$ Phe). The phenylalanine was prepared as a stock solution, sterilized by filtration, and added to the medium after autoclaving to prevent thermal degradation. Prior to autoclaving, the pH of all media was adjusted to 5.8 using 1 N NaOH or 1 N HCl as needed. The media were then sterilized by autoclaving at 121°C and 15 psi for 15 minutes. After sterilization, the media were allowed to cool to approximately 45–50°C before the sterile phenylalanine stock solution was added aseptically. The

final media were poured into sterile culture vessels in a laminar airflow cabinet and left to solidify at room temperature under aseptic conditions before being used for inoculation.

2.6 Inoculation of Callus to Media Treatments and Incubation

Callus resulting from induction for 4 weeks after planting (WAP) was incubated in phenylalanine treatment media, with the size of each callus sample set at 1 cm^2, measured using a millimetre block. The calluses were placed in culture bottles and incubated in a controlled room for 5 weeks. During this period, incubation was carried out under stable environmental conditions, with the temperature maintained at 25 ± 2°C and relative humidity at 60–70%. These conditions were regulated to support optimal callus development and metabolite production. Additionally, the culture room was periodically sanitized to minimize the risk of microbial contamination throughout the treatment phase.

2.7 Parameter

The parameters observed in this study included the colour and size of the callus from 1 to 5 weeks after planting (WAP), the fresh and dry weight of the callus at 5 WAP, and the total phenolic and flavonoid content. Prior to these measurements, callus induction was carried out for approximately four weeks under controlled conditions (25 ± 2°C, 16-hour light/8-hour dark photoperiod), resulting in the formation of a friable, greenish-yellow callus with a soft texture. Only uniform and healthy callus tissue free from browning, contamination, or necrosis was selected for further phenylalanine treatments. These selected calluses were then transferred to fresh MS media containing the same concentrations of BAP and NAA, along with the assigned phenylalanine concentrations according to each treatment group. This preparation ensured that all samples had a consistent morphological and physiological baseline at the start of the treatment phase.

2.8 Analysis of Total Phenolic Compounds

Total phenolic compounds analysis was carried out using the Folin-Ciocalteu (FC) colourimetric method modified by Chutimanukul et al. (2022). Callus extract (200 µl) was mixed with 2000 µl of 1 N FC reagent and then incubated for 15 minutes at 25 °C. Furthermore, 600 µl of 7.5% sodium carbonate (Na2CO3) was added to neutralize the solution. The neutral solution was then measured for absorbance using UV-Vis spectrophotometry with a wavelength of 730 nm for 1 hour at room temperature to be calculated on the standard curve.

2.9 Analysis of Total Flavonoid Compounds

Total flavonoid analysis was performed using the modified colourimetric procedure of Chutimanukul et al. (2022). Initially, 350 µl of basil callus extract solution was mixed with 75 µl of 5% sodium nitrite (NaNO2) and centrifuged for 2 minutes at 25 °C and 12,000 rpm. The solution was then stored at room temperature for 5 minutes, followed by the addition of 75 µl of 10% aluminium chloride (AlCl3·6H2O). The mixture was homogenized and centrifuged. After another 5-minute storage at room temperature, 1 M NaOH was added, and the solution was centrifuged and stored at 25 °C for 15 minutes. Finally, the absorbance value was measured using a UV-Vis spectrophotometer at a wavelength of 515 nm, and the results were calculated using the standard curve.

2.10 Data Analysis

Data were analyzed qualitatively and quantitatively. Quantitative data were analyzed using SPSS Software with the following stages: Shapiro Wilk normality test, analysis of variance at a 95%

confidence level, and significantly different data were further tested using the Duncan Multiple Range Test (DMRT) at a 5% level of significance.

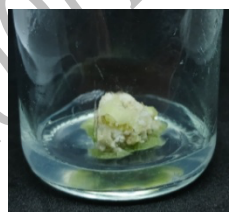
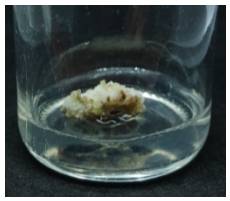
3. RESULT AND DISCUSSION

3.1 Callus Color

The colour produced by the callus indicates the compounds formed within it, such as green for chlorophyll compounds and brown for phenolic compounds (Zulaikha *et al*., 2022). This study described color descriptively based on visual observations using a color chart. In Table 1, callus culture with the addition of a phenylalanine precursor produced an average brownish-green colour, whereas callus grown on media without phenylalanine showed a bright green colour.

The colour of the callus reflects its physiological response to the composition of the incubation medium. In this study, the observed brownish-green colouration may be attributed to the accumulation of phenolic compounds within the callus tissue. According to Sitinjak *et al*., (2015) the browning of callus is caused by the oxidation of phenolic compounds. These compounds are secondary metabolites synthesized in plant cells and can be induced by phenylalanine, an aromatic amino acid (Arivianti & Parnanto, 2013). During phenolic compound biosynthesis, phenylalanine is converted via the cinnamic acid pathway with the assistance of specific enzymes, ultimately producing various phenolic compounds (Oosalo *et al*., 2022). This explanation is consistent with the findings of Duran (2024), who reported that the addition of phenylalanine to *Salvia officinalis*callus cultures (a species in the Lamiaceae family) resulted in a brownish-green colouration due to the accumulation of phenolic compounds such as rosmarinic acid.

Tabel 1. Callus Color in Response to Different Levels of Phenylalanine Addition

Treatment ($g.L^{-1}$)	Callus visualization	Mean Callus color
0 Phe		Green Group – 149D
1,3 Phe		Yellow green Group – 152D
1,6 Phe		Yellow green Group – 152D
2 Phe		Yellow green Group – 152D

3.2 Callus Texture

The control treatment formed a callus with a crumbly texture, while the callus grown on media supplemented with phenylalanine at all concentrations developed a compact texture (Figure 1). The difference in callus texture observed in this study is closely related to the callus's ability to bind water. Friable calluses tend to retain more water and have not undergone cell wall thickening or lignification, resulting in loosely arranged, easily separated cells. In contrast, compact callus has undergone lignification, a physiological process in which lignin, a complex phenolic polymer, is deposited into the plant cell walls. This deposition strengthens and hardens the tissues, reduces cell division activity, and increases structural rigidity (Ulva *et al*., 2019). Lignification is a sign of cellular maturation and is strongly associated with the biosynthesis of secondary metabolites, particularly phenolic compounds, which share biosynthetic pathways with lignin. This process plays a critical role in the plant's defense response against biotic and abiotic stress by forming physical barriers and producing antimicrobial compounds.

The expected callus texture for optimal secondary metabolite production is a compact callus. Indah & Ermavitalini (2013) explain that compact calluses can produce higher levels of secondary metabolites than friable ones due to increased metabolic activity related to stress responses and tissue differentiation. This finding is supported by Sugiyarto & Kuswandi (2014), who reported that compact callus texture in binahong (*Anredera cordifolia* L.) culture produced significantly more secondary metabolites than friable callus. The formation of compact callus is generally influenced by the composition of the culture media used during incubation, particularly the types and concentrations of plant growth regulators and added precursor compounds.

Phenylalanine is one of the factors that can cause compact callus formation in callus tissue culture. Gallardo *et al*., (2003) stated that phenylalanine is a biochemical precursor that can direct lignin formation in plant cells through the shikimate pathway secondary metabolite synthesis pathway. Lignin is one of the products of phenylpropanoid, which plays a role in making plant cells harder (Nobel, 2020). This statement was also reinforced by Higuchi (1966) that when lignification occurs in bamboo shoots, it was found that phenylalanine activity in the cells increases.

3.3 Callus Size

Figure 2 showed that the callus size at 3-5 WAP was significantly different based on the Duncan Test between the control and the addition of phenylalanine concentration. Callus size is an important indicator in callus culture because it indicates callus growth (Sukmadjaja & Mulyana, 2011). The increase in callus size is caused by actively dividing tissue around the callus. The increase in callus size is influenced by factors such as the composition of the planting medium used. This study showed that the control treatment produced the highest callus size compared to other treatments.

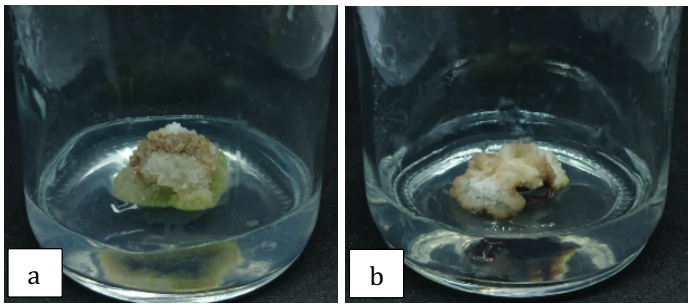


Figure 1. Basil Callus Texture : (a) Friable ; (b) Compact

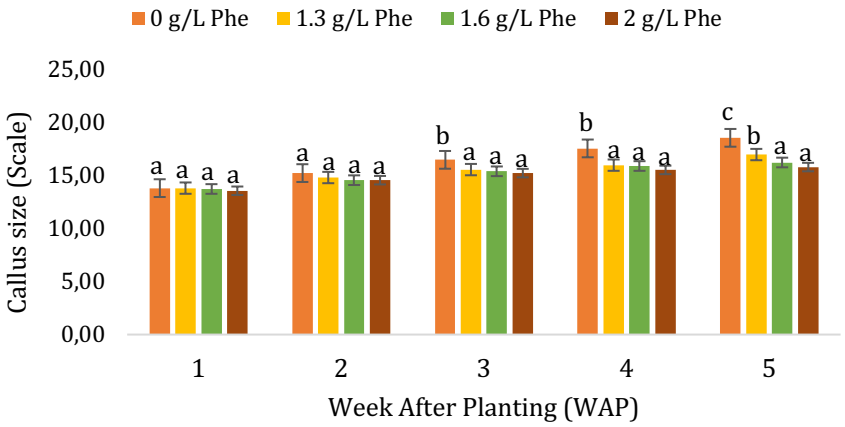


Figure 2. Basil Callus Size in Response to Different Phenylalanine Additions

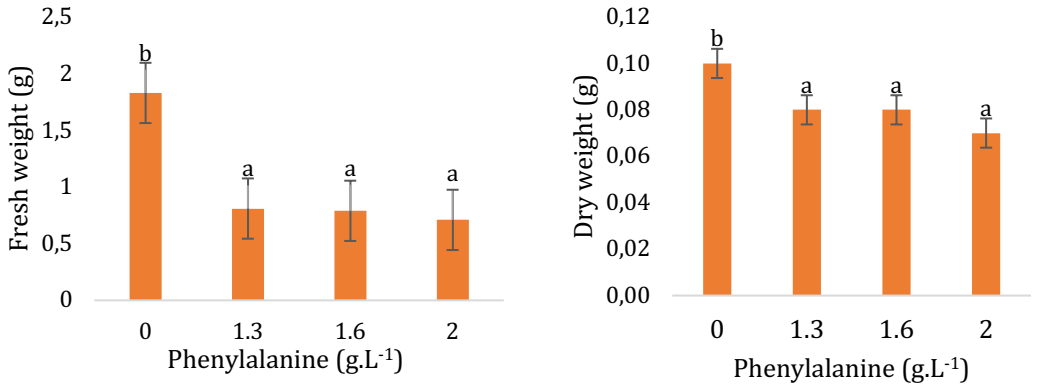
The addition of phenylalanine is also one of the factors that can affect the difference in callus size. Phenylalanine, when entering the plant, not only produces cinnamic acid but also undergoes oxidation and other enzymatic reactions so that it will produce one of the compounds that can inhibit growth, such as m-tyrosine compounds (Tyminski *et al*., 2021). M-tyrosine, if it enters the plant proteome, will interfere with cell metabolism, which causes a decrease in photosynthesis and respiration activity in plants that can affect the plant phenotype (Zer *et al.*, 2020). Yu *et al.* (2023) in their research also proved that the administration of exogenous phenylalanine at all concentrations (0.5, 2, 10, 50, and 100 µM) to tomato plants was able to inhibit the development of tomato plants compared to plants that were not treated with phenylalanine.

3.4 Fresh and Dry Weight of Callus

This study's fresh and dry weight parameters revealed a significant difference between callus treated with phenylalanine and those untreated. Callus without phenylalanine had the highest fresh and dry weights compared to all phenylalanine treatments, with values of 1.83 grams and 0.10 grams, respectively (Figure 3). The results align with those of Mahood *et al*. (2018), who found that increasing the phenylalanine concentration in Moringa oleifera L. callus culture reduced fresh weight. Barros & Dixon (2020) suggested that phenylalanine uses energy from sucrose to form secondary metabolites, diverting resources from primary metabolites. This result is supported by Skrzypczak *et al*. (2014), who reported that a combination of 1.6 g.L^{-1} phenylalanine and 6% sucrose reduced dry weight in Exacum affine in vitro cultures compared to explants incubated in 6% sucrose alone. Also, high phenylalanine concentrations can create osmotic pressure, stressing plant tissue and reducing cell metabolism (Kareem & Alwash, 2022).

3.5 Total Phenolic Compounds

The highest total phenolic compounds in this study were produced by callus incubated in media with the addition of a phenylalanine concentration of 1.6 g.L^{-1}, which was 18.90 mg.g^{-1} FW and was not significantly different from the phenylalanine treatment of 2 g.L^{-1}. The lowest total phenolic compounds were produced by calli incubated in the control treatment, which was 15.50 mg.g^{-1} FW (Figure 4). Hassan & Jassim (2018) stated that one way to increase the number of phenolic compounds in plants is to use phenylalanine, a precursor for secondary metabolite biosynthesis.



(a) (b)

Figure 3. Basil Callus Fresh (a) and Dry Weight (b) in Response to Different Concetration of Phenylalanine

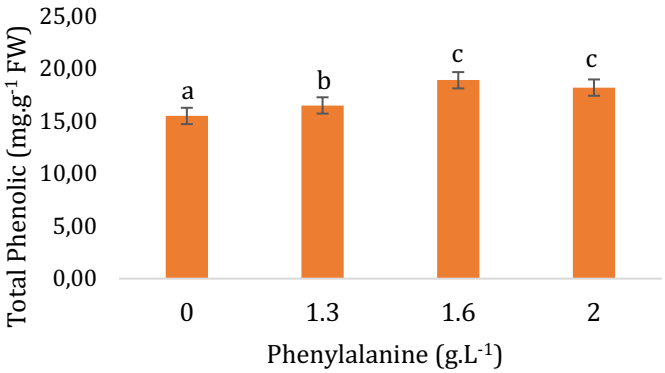


Figure 4. Basil Callus Total Phenolic Compounds in Response to Different Phenylalanine Additions

Research conducted by Burbulis *et al.* (2023) showed that adding exogenous phenylalanine to lemon and cinnamon basil varieties can increase total phenolic compounds. The general mechanism of phenylalanine in increasing total phenolic compounds is using the shikimic acid pathway, where phenylalanine can undergo an enzymatic reaction to form secondary metabolites. The key to the formation of secondary metabolite compounds in the shikimic acid secondary metabolite formation pathway is the enzyme Phenylalanine Ammonia Lyase (PAL) (Schuster & Retey, 1995). This enzyme plays an important role in phenylalanine metabolism. According to (Qiu *et al.*, 2024; Zheng *et al.*, 2024), the PAL enzyme is the mechanism of converting phenylalanine into cinnamic acid. Cinnamic acid will undergo another enzymatic reaction, eventually producing one of the phenylpropanoid derivative compounds, phenolic compounds.

Adding aromatic amino acid phenylalanine exogenously to plants can increase the activity of the PAL enzyme, increasing the production of secondary metabolites (Sagharyan & Sharifi, 2024). This condition aligns with the study's results, which show that the highest total phenolic compound content was observed in the treatment with 1.6 $g.L^{-1}$ phenylalanine, although it was not significantly different from the 3 $g.L^{-1}$ treatment. This suggests that total phenolic compound levels increased and that PAL enzyme activity in this study likely operated under optimal conditions for phenolic compound formation in basil callus.

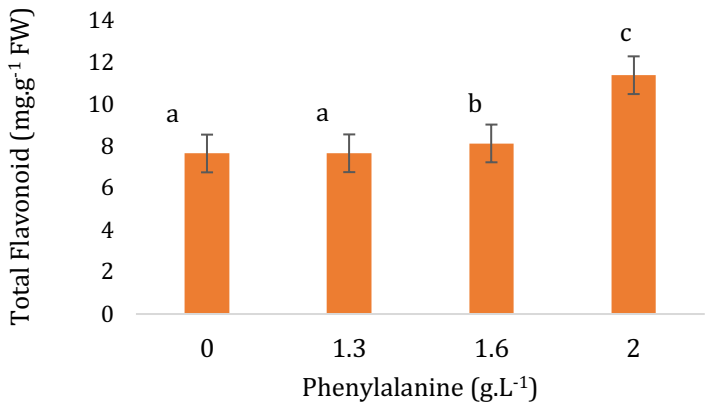


Figure 5. Basil Callus Total Flavonoid Compounds in Response to Different Phenylalanine Additions

3.6 Total Flavonoid Compounds

The research results show that the highest total flavonoid compounds were produced by callus treated with a 2 $g.L^{-1}$ phenylalanine concentration (11.41 $mg.g^{-1}$), significantly different from other treatments (Figure 5). Like total phenolic compounds, the increase in flavonoid compounds is also triggered by adding phenylalanine precursors, which can produce flavonoid compounds. Flavonoids are valuable compounds due to their antioxidant activity, making them important in the pharmaceutical field (Selonni, 2021). One method to increase total flavonoid compounds is called callus propagation and applying biochemical precursors such as phenylalanine. Widhalm *et al*. (2015) and Yang *et al*. (2022) explained that when phenylalanine is added exogenously, it is absorbed by plant cells, transported to plastids, and undergoes biosynthesis, resulting in secondary metabolite production.

The process of phenylalanine biosynthesis into flavonoid compounds, namely phenylalanine, will be assisted by the PAL enzyme in producing cinnamic acid as an enzymatic reaction. Phenylalanine releases cinnamic acid molecules to form flavonoid compounds (Perangin-angin et al., 2019). Phenylalanine is an amino acid that naturally appears when plants are stressed (Mardhiana *et al*., 2018). However, exogenous additions can also be carried out to increase total flavonoid compounds (Abdalla et al., 2022). This statement aligns with research conducted by Sajjalaguddam & Paladugu (2015), which found that adding exogenous phenylalanine to MS media is known to affect the increase in total flavonoid compounds in callus cultures.

4. CONCLUSION

The addition of phenylalanine to the basil (*Ocimum basilicum* L.) callus culture medium affected both callus morphology and biochemical composition. Phenylalanine functions as a precursor in the biosynthesis of secondary metabolites, particularly phenolic and flavonoid compounds. All treatments involving phenylalanine led to the formation of compact, brownish-green calluses, which were associated with a decrease in fresh and dry weight compared to the control. This suggests a trade-off between biomass accumulation and secondary metabolite production, where metabolic resources are redirected from growth toward biosynthesis.

Among the treatments, supplementation with 1.6 $g.L^{-1}$ phenylalanine resulted in the highest total phenolic content, although it was not significantly different from the 2.0 $g.L^{-1}$ treatment. In contrast, the highest accumulation of total flavonoid compounds was observed at 2.0 $g.L^{-1}$ phenylalanine, showing a significant difference compared to all other treatments. These findings indicate that phenylalanine supplementation, particularly in the range of 1.6–2.0 $g.L^{-1}$, can effectively enhance the biosynthesis of key secondary metabolites in basil callus culture under controlled conditions.

5. ACKNOWLEDGEMENT

This research was fully funded by Universitas Padjadjaran (Unpad), Indonesia, through an Associate Professor Acceleration Research grant fiscal year 2024 number 1603/UN6.3.1/PT.00/2024.

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