

IN VITRO APPLICATION OF PHENYLALANINE-INDUCED GROWTH, PHENOLIC AND FLAVONOID PRODUCTION IN *Ocimum basilicum* PLANTS

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Abstract

Ocimum basilicum is a medicinal plant known to have many health benefits. One way to propagate this plant is through in-vitro culture. In-vitro secondary metabolite production can be increased by adding elicitors such as phenylalanine. This study aims to determine the effect of phenylalanine on the growth and production of secondary metabolites of *Ocimum basilicum* in vitro. Experiments were conducted with various concentrations of phenylalanine (0, 200, 250, and 300 mg/150 mL) combined with 1.5 mg L⁻¹ Benzyl Amino Purine (BAP). The results showed that although phenylalanine combined with BAP inhibited the growth of *Ocimum basilicum* plants such as root growth in both the number of roots and root length, the number of leaves produced, and the height of the plantlets. However, phenylalanine has the potential to increase fresh weight at moderate concentrations and secondary metabolites such as total phenolics although it causes a decrease in total flavonoid content along with increasing phenylalanine concentration. Further investigation is needed to determine the specific cause of this response.

Key words: growth, in vitro, *Ocimum basilicum*, phenylalanine, secondary metabolite.

INTRODUCTION

Basil (*Ocimum basilicum* L.) is a widely used herbaceous plant with sweet and mildly spicy leaves, commonly consumed as a fresh vegetable. In addition to its culinary value, basil has significant health applications due to its rich content of polyphenols, phenolic acids, and flavonoids, which provide cardiovascular protection, antioxidant effects, and anticancer properties (Leri et al., 2020; Nasim et al., 2022; Romano et al., 2022). Furthermore, basil contains flavonoids, alkaloids, and polyphenols that exhibit antidiabetic activity by reducing glucose production, enhancing glycogen synthesis, suppressing gluconeogenesis, and stimulating pancreatic insulin secretion (Kheshti et al., 2022). Research by Widjaja et al. (2019) demonstrated that ethanolic basil leaf extracts significantly reduced blood glucose levels in

diabetic rats. Additionally, basil leaves are rich in essential oils containing secondary metabolites, such as alkaloids, tannins, phenols, flavonoids, and saponins.

Despite its benefits, basil propagation is challenging due to its low seed germination rate (34%) during physiological maturity (Wiendi & Putri, 2017). Tissue culture offers an effective alternative for basil propagation, involving the cultivation of plant tissues on synthetic nutrient media under controlled conditions of light, temperature, and humidity (Buzau et al., 2018). This method ensures rapid propagation, pest- and disease-free plants, and space efficiency in nurseries, thereby reducing transportation costs (Tegen & Mohammed, 2008). Tissue culture techniques, including cell, organ, and callus culture, also enhance secondary metabolite production through specific modifications (Ozyigit et al., 2023). For example, Gao et al. (1999) found

that *Fritillaria unibracteata* propagated via organ culture exhibited growth rates 30-50 times higher than natural conditions, alongside increased alkaloid and micronutrient content.

The success of tissue culture depends on factors such as genotype, media composition, and the application of plant growth regulators (PGRs) like auxins and cytokinins (Enkhbileg et al., 2019; Da Silva et al., 2017). These PGRs regulate growth and development while boosting biomass and secondary metabolite accumulation (Miri & Roughani, 2018; Nurokhman et al., 2019). For instance, Nazir et al. (2020) reported that callus cultures of Thai basil grown on media with 5 mg/L BAP and 1 mg/L NAA showed significantly increased biomass and secondary metabolite production, including rosmarinic and chicoric acids, compared to commercial Thai basil. Enhancing secondary metabolite content can also be achieved by applying environmental stressors or adding elicitors such as phenylalanine, which triggers the production of antimicrobial and antioxidant compounds and activates phenylalanine ammonia-lyase (PAL) enzymes (Gómez-Vásquez et al., 2004; Zhao et al., 2010). Phenylalanine plays a central role in the phenylpropanoid pathway, catalyzed by PAL enzymes, and serves as a precursor for various phenolic metabolites (Jakovljević et al., 2022). Its conversion through the shikimate pathway leads to the formation of hydroxycinnamic acids, which are further processed into hydroxybenzoic acids, flavonoids, tannins, and other phenolics (Szewczyk et al., 2021; 2023). According to Deng & Shanfa (2017), lower phenylalanine concentrations promote plant growth, while higher concentrations significantly enhance total phenolic and flavonoid content. This study was conducted to propagate basil plants in vitro, evaluate their growth and development under various phenylalanine concentrations, and identify the optimal media composition for enhancing secondary metabolite content to support their potential use as raw materials in pharmaceuticals.

MATERIALS AND METHODS

Experimental Design and Plant Preparation.

The experiment takes place at the Seed Technology Tissue Culture Laboratory in the

Faculty of Agriculture at Padjadjaran University, Jatinangor, Sumedang. The explants used in this experiment were sterilized shoots derived from seeds that had been germinated on MS0 medium for one week before being transferred to the treatment media. The seeds are sterilized by rinsing with sterile water, treated with bactericide and fungicide for 10 minutes, soaked in detergent for 5 minutes, 2% sodium hypochlorite (Clorox) for 15 minutes, and rinsed after each step. The explants are first planted for one week, then subcultured onto the treatment medium and incubated in the culture room with temperature range 22-25°C, for five weeks (Figure 1).

The experiment applied a quantitative experimental approach using a Completely Randomized Design (CRD) with five treatments, each combining Benzyl amino purine (BAP) and phenylalanine (Phe). The treatments included a control group and combinations of 1.5 mg·L⁻¹ BAP with different concentrations of phenylalanine: 0 mg·L⁻¹ (B), 30 mg·L⁻¹ (C), 37.5 mg·L⁻¹ (D), and 45 mg·L⁻¹ (E). Meanwhile, the control treatment (A) only used MS medium without the addition of BAP. Each treatment will be replicated five times with four units in each replication. The data obtained were analyzed using an F test, and if significant differences were found, further analysis was conducted using the Duncan's Multiple Range Test at a 5% significance level.

Data Collection and Analysis. Plant growth characteristics, such as number of leaves, number of shoots, root development, plant height, root length, and fresh and dry weight, were documented at the age of the plantlets 5 weeks after planting.

The quantity of leaves, shoots, and roots was enumerated separately for each plantlet, guaranteeing uniform and precise data gathering. The height of the plantlet was quantified as the distance from the stem base to the apex, whilst root length was assessed from the root base to the tip. The fresh weight was measured by measuring the complete plantlet on an analytical balance, whereas the dry weight was determined by drying the plantlets at 55-60°C for 72 hours.

The examination of secondary metabolites encompassed total flavonoid content (TFC) and total phenolic content (TPC).

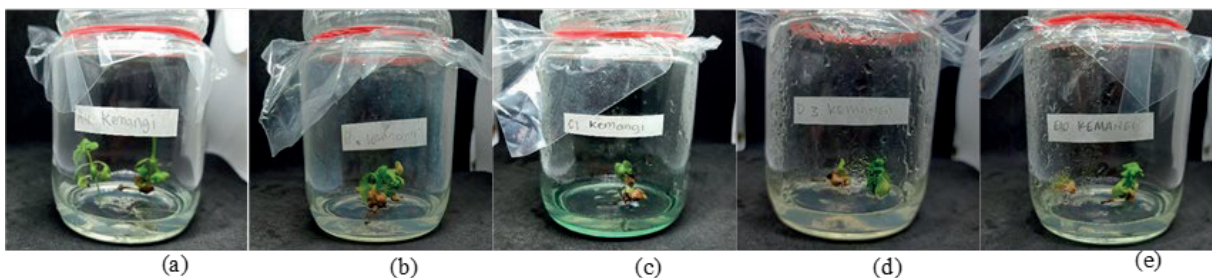


Figure 1. *Ocimum basilicum* plantlet 5 week ages: (a) Control; (b) 1.5 ppm BAP + 0 mg/150 mL phe ; (c) 1.5 ppm BAP + 200 mg/150 mL phe; (d) 1.5 ppm BAP + 250 mg/150 mL phe ; (e) 1.5 ppm BAP + 300 mg/150 mL phe

The TFC was quantified using a colourimetric technique adapted from Chutimanukul et al. (2022). A 350 μL extract sample was sequentially combined with sodium nitrite (NaNO_2), aluminium chloride (AlCl_3), and sodium hydroxide (NaOH), followed by centrifugation. Absorbance was recorded at 515 nm, and TFC was determined using a rutin standard curve, given as milligrammes of Quercetine equivalents per gramme of dry weight (mg QE/g DW). TPC was measured utilizing a modified Folin-Ciocalteu technique. An extract solution was combined with Folin-Ciocalteu reagent and sodium carbonate (Na_2CO_3), and absorbance was recorded at 730 nm following incubation. The TPC was determined utilizing a gallic acid standard curve and is given as milligrams of gallic acid equivalents per gramme of dry weight (mg GAE/g DW).

The data obtained were analyzed using SPSS software, using Duncan's multiple range test at a significance threshold of 5% to assess differences in treatment. The results issued are in the form of notations where each different notation letter indicates a significantly different response.

RESULTS AND DISCUSSIONS

Number of Shoots

Figure 2 illustrates the findings acquired at 5 WAP (Weeks After Planting). This study was structured with treatments including a control group and various combinations of 1.5 $\text{mg}\cdot\text{L}^{-1}$ BAP with differing amounts of phenylalanine. According to the findings, treatment B (1.5 $\text{mg}\cdot\text{L}^{-1}$ BAP + 0 $\text{mg}\cdot\text{L}^{-1}$ phenylalanine) exhibited the highest average shoot count (1.857), succeeded by treatment C (1.571) and treatment D (1.5). Simultaneously, the control treatment A exhibited the lowest average

number of shoots (1.071), followed by treatment E (1.143). The results suggest that phenylalanine at concentrations of 30 $\text{mg}\cdot\text{L}^{-1}$ (C) and 37.5 $\text{mg}\cdot\text{L}^{-1}$ (D) may promote shoot development when used in conjunction with BAP, but 45 $\text{mg}\cdot\text{L}^{-1}$ (E) seems to exert a less advantageous or perhaps inhibitory impact.

The findings align with the observations of Szewczyk et al. (2023), who indicated that phenylalanine elicitor treatments did not substantially influence the development of *Ruta graveolens* L. Furthermore, Szopa et al. (2020) discovered that phenylalanine concentrations ranging from 0.0165 to 0.165 $\text{g}\cdot\text{L}^{-1}$ did not impede development in *Aronia* spp., but higher doses (0.825 to 1.65 $\text{g}\cdot\text{L}^{-1}$) resulted in growth inhibition. This indicates that phenylalanine levels in this research may be inadequate to meaningfully affect the hormonal equilibrium required for promoting shoot development and, at elevated concentrations, may potentially impede it.

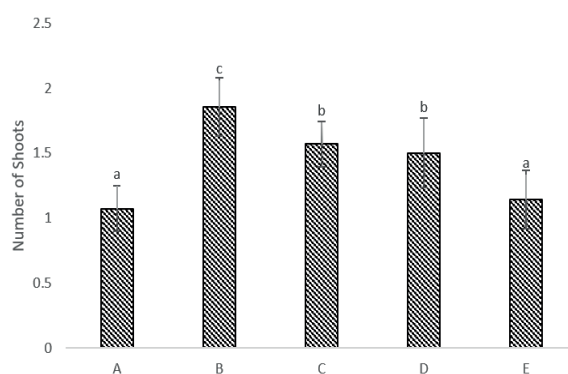


Figure 2. Effect of Various Concentrations of Phenylalanine on the Number of Shoot

Number of Roots

Data analysis (Figure 3) revealed substantial variations in root quantity among the treatments at 5 WAP. The data indicates that treatment A yielded the most significant average number of

roots (2.857), succeeded by treatment B (1.5). Treatments C, D, and E exhibited comparable and considerably reduced root counts (1.071). The results demonstrate that root development diminishes with increasing phenylalanine concentration and stabilizes at levels beyond 30 mg·L⁻¹.

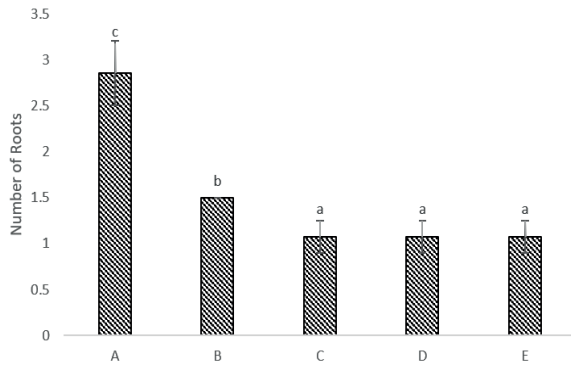


Figure 3. Effect of Various Concentrations of Phenylalanine on the Number of Root

The data indicate that phenylalanine may impede root development in *in-vitro*-cultivated basil plants. Bohdanovych (Bohdanovych, 2023) reported the same finding, noting that phenylalanine supplementation at different amounts impeded root development in *Artemisia tilesii*. Bohdanovych indicated that phenylalanine-tolerant plants demonstrated a higher root count than their more sensitive counterparts. Yu et al. (2023) elucidated that alterations in root quantity following phenylalanine treatment are probably associated with irregular auxin distribution, decreased amyloplast area, and elevated tyrosine levels. Phenylalanine functions as a precursor for the biosynthesis of tyrosine (Torrens-Spence et al., 2020) and auxin-like substances such as phenylpyruvate, while also supplying vital nitrogen for the creation of proteins, enzymes, and genetic material (Al-Mohammad, 2023). Thus, incorporating phenylalanine may impede root growth by disrupting the hormonal equilibrium and nutrient distribution essential for root elongation and development.

Number of Leaves

Based on data analysis (Figure 4), although the control treatment (A) had the highest number of leaves, no significant differences were found in all treatments at 5 MST. Among all the variations, treatment A showed the maximum number of leaves (7,000), far exceeding the

other treatments. The data indicates a steady decline in leaf count as phenylalanine concentrations increased, with the minimum leaf count recorded in treatment E (3,714). Treatments B, C, and D had intermediate values of 5,642, 5,143, and 5,214, respectively.

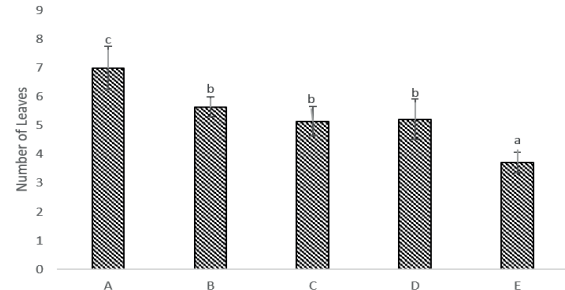


Figure 4. Effect of Various Concentrations of Phenylalanine on the Number of Leaves

The findings indicate that incorporating phenylalanine may impede leaf development in *in vitro*-cultivated basil plants. This discovery corresponds with research conducted by Jiao et al. (2018), which indicated suppressed leaf development in *Populus x canescens* after phenylalanine supplementation. The inhibitory action is presumably linked to the adverse influence of deaminated cinnamic acid derivatives, which are byproducts of phenylpropanoid metabolism. Evans et al. (2017) elucidated that these compounds, mediated by the enzyme phenylalanine ammonia-lyase (PAL), impede plant development by deaminating phenylalanine into cinnamic acid and ammonium. Jakovljević et al. (2022) similarly emphasized the significance of this enzyme route in the growth suppression of specific plant species.

Plantlet Height

Data analysis (Figure 5) revealed substantial variations in plant height among treatments at 5 WAP.

The results indicate a substantial reduction in plant height with elevated phenylalanine contents. The tallest plants were seen in the control treatment (A) with an average height of 2.193 cm, succeeded by treatment B (1.586 cm), treatment D (1.15 cm), treatment C (1.064 cm), and the most miniature plants in treatment E (0.921 cm). The heights of plants in treatments D and E exhibited no significant difference, indicating a cessation of growth at elevated phenylalanine concentrations.

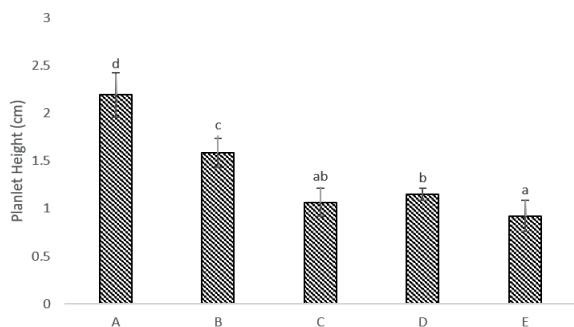


Figure 5. Effect of Various Concentrations of Phenylalanine on the Planlet Height

The data suggest that phenylalanine may impede plant height development, exhibiting a reaction analogous to that seen in root and leaf counts. This corresponds with the findings of Yang et al. (Yang et al., 2021), who indicated that elevated phenylalanine levels impeded the growth and development of *Arabidopsis thaliana* and ryegrass. Evans et al. (2017) suggest that this inhibition may result from phenylalanine modifying metabolic pathways, thus diminishing the availability of vital nutrients for development. Nutrient deficits may result in atypical growth, accounting for this study's reduced height. In conclusion, treatment A consistently exhibited superior performance across various growth parameters. In contrast, elevated phenylalanine concentrations adversely affected plant height, root quantity, and leaf count, corroborating the hypothesis that higher phenylalanine levels inhibit plant growth.

Root Length

Analysis of variance (Figure 6) revealed significant variations in root length among treatments at 5 WAP.

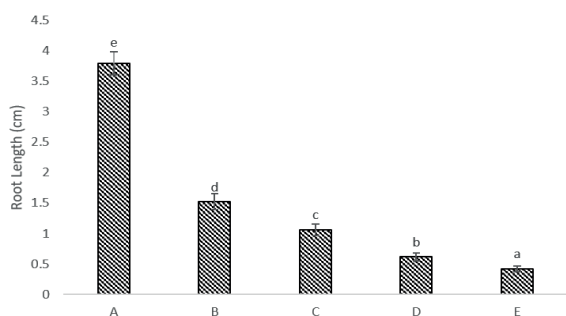


Figure 6. Effect of Various Concentrations of Phenylalanine on the Root Length

The study demonstrates a substantial reduction in root length with elevated phenylalanine

concentrations. The control treatment (A) had the longest roots, averaging 3.793 cm, whereas treatments B (1.521 cm), C (1.064 cm), D (0.614 cm), and E (0.421 cm) displayed progressively shorter roots. This pattern indicates a gradual reduction in root length as phenylalanine content rose, with the shortest roots observed in treatment E.

The results indicate that phenylalanine suppresses root development, especially at elevated quantities. This outcome aligns with research by Yu et al. (2023), which indicated that the administration of phenylalanine at different doses suppressed tomato root development. The inhibitory impact may be ascribed to the synthesis of tyrosine, a chemical that impedes root growth. Tyrosine production is enabled by phenylalanine, which acts as its precursor (Torrens-Spence et al., 2020). The buildup of tyrosine and its impact on metabolic pathways may restrict the resources and hormonal equilibrium necessary for root elongation, resulting in noted growth inhibition. In summary, treatment A consistently yielded the longest roots, whereas elevated quantities of phenylalanine markedly diminished root length. This corresponds with the pattern in other growth metrics, substantiating that elevated phenylalanine concentrations adversely impact root development in in-vitro basil production.

Fresh Weight

The analysis of variance and the corresponding bar chart (Figure 7) indicate significant variations in the fresh weight of basil plants at 5 WAP between treatments.

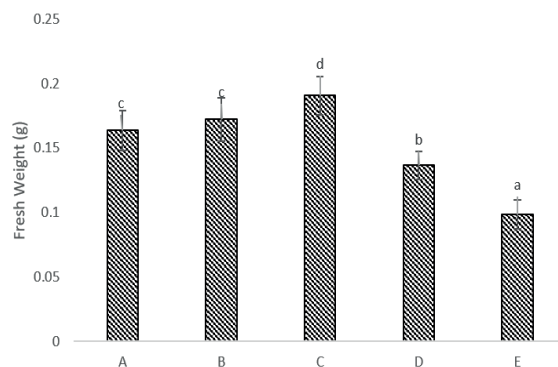


Figure 7. Effect of Various Concentrations of Phenylalanine on the Fresh and Dry Weight

Despite prior data indicating that phenylalanine may suppress plant development, fresh weight

increased with applying phenylalanine up to treatment C (30 mg·L⁻¹), which yielded the maximum fresh weight of 0.191 g. With further increase in concentration, fresh weight decreased significantly, as seen in treatments D (0.136 g) and E (0.099 g). Treatment A (0.164 g) and treatment B (0.172 g) exhibited intermediate results, notably lower than treatment C but higher than treatments D and E. The notable disparities across treatments indicate that moderate phenylalanine levels enhance fresh biomass development, but elevated amounts have an inhibitory impact. This tendency corresponds with the findings of Jiao et al. (2018), which emphasized the significance of phenylalanine in affecting carbon allocation and metabolic equilibrium. The inhibitory impact at elevated doses may be attributed to phenylalanine's involvement in tyrosine and phenylpropanoid metabolism, as shown by Torrens-Spence et al. (2020). In conclusion, treatment C demonstrated superior performance, but elevated phenylalanine concentrations (D and E) markedly decreased fresh weight, establishing a threshold effect in basil plants' sensitivity to phenylalanine.

Total Flavonoid Content

The analysis of variance and the associated bar chart (Figure 8) demonstrate substantial alterations in the TFC of *Ocimum basilicum* subjected to different phenylalanine and BAP treatments. The TFC was highest in treatment A (22.85 mg/g GAE FW) and exhibited a steady decrease across treatments, culminating in the lowest value in treatment E (3.89 mg/g GAE FW). The data indicate that a rise in phenylalanine and BAP concentrations corresponded with a substantial drop in TFC. This discovery indicates that elevated levels of phenylalanine and BAP adversely affect flavonoid production. Flavonoids are produced via the phenylpropanoid pathway, utilizing phenylalanine as a precursor (Nabavi et al., 2020). Excessive phenylalanine and BAP disturb the metabolic equilibrium by reallocating resources to alternative metabolic pathways or creating secondary metabolites such as phenolics. Dias et al. (2021) emphasized the essential functions of flavonoids in modulating cell proliferation, safeguarding against environmental stressors, and

neutralizing reactive oxygen species (ROS). Consequently, reducing TFC at elevated phenylalanine concentrations may impair the plant's capacity to withstand biotic and abiotic stressors. In summary, whereas phenylalanine serves as a precursor in the phenylpropanoid pathway, its elevated levels and BAP may inhibit flavonoid production. This underscores the necessity of adjusting phenylalanine and BAP concentrations to sustain or augment flavonoid content and related plant resistance.

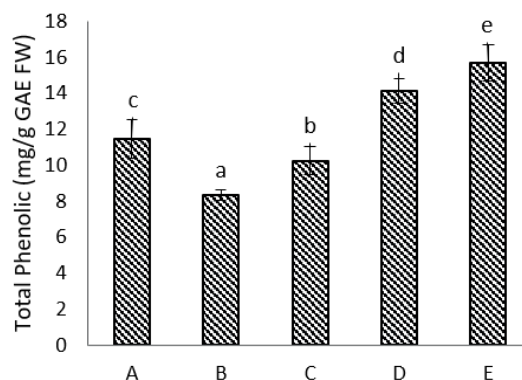


Figure 8. Effect of Various Concentrations of Phenylalanine on Total Flavonoid

Total Phenolic Content

The analysis of variance and bar chart (Figure 9) indicate substantial variations in the TPC of *Ocimum basilicum* across the treatments.

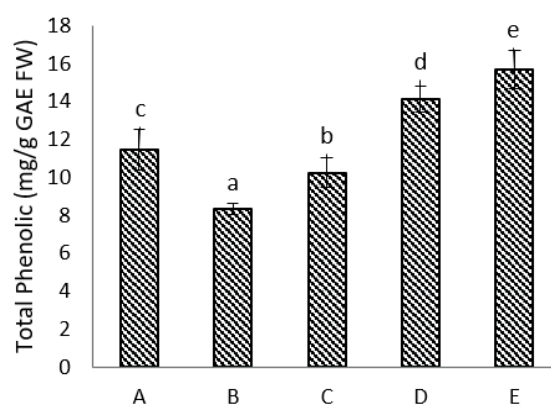


Figure 9. Effect of Various Concentrations of Phenylalanine on Total Phenolic

TPC in treatment A (11.48 mg/g GAE FW) diminished in treatment B (8.35 mg/g GAE FW) but progressively escalated with increased phenylalanine concentrations, attaining peak values in treatment D (14.15 mg/g GAE FW) and treatment E (15.68 mg/g GAE FW). The

findings indicate that modest levels of phenylalanine may inhibit TPC, as seen in treatments B and C. Nonetheless, elevated amounts, especially in treatments D and E, markedly increased TPC. This outcome corresponds with the research of Jakovljević et al. (2022), which illustrated that phenylalanine serves as a precursor in the phenylpropanoid pathway, wherein the enzyme phenylalanine ammonia-lyase (PAL) facilitates its transformation into trans-cinnamic acid, the primary step in the synthesis of phenolic compounds. Research conducted by Szewczyk et al. (2023) and Jańczak-Pieniążek et al. (2022) corroborates these findings, emphasizing that phenolic compounds augment antioxidant capacity through the scavenging of free radicals and the inhibition of ROS and reactive nitrogen species (RNS). Consequently, elevated phenylalanine levels may enhance the production of phenolic compounds, resulting in augmented antioxidant capacity. In conclusion, phenylalanine content markedly affects TPC in basil plants. Lower doses may inhibit phenolic production, but more significant quantities augment TPC, enhancing the plant's antioxidant capability and possible health benefits.

CONCLUSIONS

In conclusion, the application of phenylalanine during *in vitro* propagation had distinct effects on the growth development of basil. Phenylalanine concentration plays a crucial role in influencing different plant growth parameters. These results suggest that while phenylalanine can potentially promote certain growth aspects, it may also induce negative effects such as morphological damage and altered growth, highlighting the complex nature of its impact on plant development.

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