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Effect of Phenylalanine and Methyl Jasmonate on Secondary Metabolite Production by Shoot Cultures of Holy Basil, Purple-Type (*Ocimum sanctum* L.)

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ABSTRACT

Holy basil, purple-type (*Ocimum sanctum* L.) is a medicinal plant that accumulates a range of secondary metabolites with important antioxidant capacities. Secondary metabolite production may be enhanced by precursor feeding and elicitation under aseptic conditions and is used to supply medicinal and pharmaceutical needs. This study investigated the roles of phenylalanine (Phe), a precursor, and methyl jasmonate (MeJA), an elicitor, in the enhancement of eugenol, total phenolic and flavonoid accumulation, and antioxidant activity in shoot cultures of holy basil, purple-type. Single-node segments were cultured on Murashige and Skoog (MS) medium supplemented with Phe at 25 and 50 µM, and MeJA at 50 and 100 µM, both alone and in combination. MS medium supplemented with only 6-benzyladenine (BA) (2.22 µM) was used as a control. Shoots were harvested at three weeks and analyzed for secondary metabolite accumulation and antioxidant activity. MeJA alone was shown to be more effective than Phe, and no synergistic effects (synergistic coefficients = 0.47-0.96) were observed. The highest accumulation of eugenol (208.84±9.07 µg/g dry extract), total phenolics (190.38±5.76 mg GAE/g dry extract), and total flavonoids (260.43±7.28 mg CE/g dry extract) were recorded following treatment with 100 µM MeJA. These were 14.57, 2.94, and 4.74 times greater than the control, respectively. This treatment also produced the greatest enhancement in DPPH radical scavenging, with an EC_{50} value of 8.26±1.09 µg/mL. The study demonstrated MeJA to be an effective elicitor of secondary metabolites and antioxidants in shoot cultures of holy basil, purple-type.

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Keywords: Elicitor; Eugenol; Flavonoids; Phenolic; Precursor

1. Introduction

Holy basil (*Ocimum sanctum* L. or *O. tenuiflorum* L.) is a medicinal plant of the family Lamiaceae. This species is native to tropical regions. It is an erect plant, aromatic, highly branched, and 30-60 cm in height, with green (white or green type) or purple (red or purple-type) leaves [1]. Kumar [2] reported that the purple-type had higher antioxidant activity than the green type, making it an excellent source of secondary metabolites. Reported medicinal properties include efficacy as an anti-asthmatic, anti-malarial, anti-diarrhoeal, anticarcinogen, anti-stress, anti-allergic, neuro-protectant, and antioxidant [1, 3, 4]. Eugenol is a major aromatic compound found in holy basil leaves. Other secondary metabolites extracted from its leaves include phenolic compounds [5] and flavonoids [2]. Eugenol, a common ingredient in medicinal, pharmaceutical, and cosmetic products, is mainly harvested from *Eugenia caryophyllata* [6]. *E. caryophyllata* has not traditionally been widely cultivated in Thailand and several years of growth are needed before yields can be harvested [7]. Holy basil is commonly and widely grown in Thailand, offering an alternative source of eugenol. However, conventional agricultural production is hampered by environmental change and insect or pathogen damage, leading to poor quality and reduced yield.

Plant secondary metabolites can also be produced from tissue cultures. This technique allows conditions to be optimized, shortening production time, and guaranteeing quality and yield. Production can run continuously throughout the year, regardless of environmental variations [8, 9]. *In vitro* propagation of holy basil and other species of *Ocimum* has been reported [10-13]. From our preliminary study, *in vitro* regenerated shoots had lower eugenol content than field-grown leaves, but higher total phenolics and flavonoids. Several approaches to improvement of secondary metabolite accumulation have been proposed. In precursor feeding, a precursor or intermediate compound in the biosynthetic pathway is supplemented, with the aim of increasing the final product yield [8]. Rastogi et al. [14] reported that eugenol, phenolic compounds, and flavonoids originate from phenylalanine (Phe) in the phenylpropanoid pathway. A number of studies have demonstrated the use of Phe as a precursor for enhanced secondary metabolite production. It has been applied to phenolic compounds in callus cultures of *Daucus carota* [15], flavonoids in callus cultures of *Hydrocotyle bonariensis* [16], and eugenol in shoot cultures of *O. basilicum* and *O. tenuiflorum* [17]. Elicitation is another effective method for enhancing secondary metabolite accumulation. Abiotic or biotic elicitors are introduced into the plant cells, triggering a defense response [8, 18, 19]. Methyl jasmonate (MeJA) is a biotic elicitor that has been used to enhance production of flavonoids, terpenoids, alkaloids, and phenylpropanoids [19, 20]. MeJA has been shown to increase yields of secondary metabolites including eugenol, phenolic compounds, and flavonoids from medicinal plants such as *Dianthus caryophyllus* [21], *Ophiorrhiza mungos* var. *angustifolia* [22], *Thevetia peruviana* [23], *Mentha spicata* [24], and *Boesenbergia rotunda* [25]. A combination of Phe and MeJA has also been applied to several plant species, enhancing production of secondary metabolites in *Vitis vinifera* [26, 27] and *Exacum affine* [28]. However, there are no reports of the application of Phe and MeJA to enhancement of secondary metabolite accumulation in holy basil, purple-type under aseptic conditions. The current study is the first to elucidate the effect of applying Phe and MeJA, both individually and in combination, on secondary metabolite accumulation and antioxidant activity of *in vitro* regenerated

shoots of purple-type holy basil.

2. Materials and Methods

2.1 Plant materials and shoot multiplication

Young axillary shoots were collected from Holy basil, purple-type which has purple leaves, stems, petioles, and inflorescence. The mother plants were grown in the greenhouse of the Department of Agricultural Technology, Thammasat University. Single-node segments approximately 1 cm in length were used as explants after surface sterilization using sodium hypochlorite with a few drops of Tween 20. The explants were cultured on semi-solid Murashige and Skoog (MS) medium supplemented with 4.44 µM 6-benzyladenine (BA) and 3% (w/v) sucrose for shoot induction. The regenerated shoots required a low BA concentration for further growth and proliferation. They were subcultured on MS medium supplemented with 2.22 µM BA and 3% (w/v) sucrose at 4-week intervals for shoot multiplication. The pH of the medium was adjusted to 5.6–5.8 with 1 N NaOH, followed by the addition of 0.8% agar and autoclaving at 121°C (15 psi) for 15 min. The cultures were incubated in a temperature-controlled room at 25±2°C with a 16-h photoperiod under cool white fluorescent light.

2.2 Phe and MeJA treatments

The *in vitro* shoots were cut into node segments approximately 0.5 cm in length. The segments were cultured on semi-solid MS medium supplemented with 2.22 µM BA in combination with Phe at individual concentrations of 25 and 50 µM and individual MeJA concentrations of 50 and 100 µM. They were also tested with the following combinations: 25 µM Phe with 50 µM MeJA, 25 µM Phe with 100 µM MeJA, 50 µM Phe with 50 µM MeJA, and 50 µM Phe with 100 µM MeJA. Each culture bottle contained 12.5 mL of medium and four node segments were cultured in each container. As the 100 µM MeJA-treated shoots regenerated poorly at 2 weeks and turned brown at 4 weeks, harvesting was carried out at 3 weeks. MS medium supplemented with 2.22 µM BA was used as a control. The fresh weight of the regenerated shoots was recorded after treatment and the shoots were dried for secondary metabolite analysis.

2.3 Preparation of extracts

Extraction for eugenol analysis used a method modified from Sharma et al. [17]. The treated regenerated shoots were freeze-dried, and 6 g of powdered sample was extracted using 100 mL of HPLC grade methanol in a Soxhlet extractor. The process was run for 6 h, the product was filtered and evaporated until dry, and then stored at -20°C until use. Plant extraction for analysis of total phenolics and flavonoids and for DPPH radical scavenging was performed following Jirakiattikul et al. [25]. The dried extracts were kept at -20°C for further use.

2.4 Secondary metabolite analysis

2.4.1 Eugenol content

Eugenol content was determined following Inam et al. [29] with minor modification. Extracts of 10 mg were dissolved in 1 mL of 99.9% HPLC grade methanol and sonicated for 1 min. The solution was passed through a filter membrane with pore size 0.22 µm. Ultra-high-performance liquid chromatography (UHPLC, Shimadzu brand, Nexera LC-30 A model) was performed using a Zorbax Eclipse XDB-C_{18} column (150 mm x 4.6 mm, 5 µm) with a guard column. The isocratic system was used and a mobile phase of methanol: acetonitrile: HPLC grade water at a volume ratio of 10: 50: 40 was delivered at a flow rate of 0.7 mL/ min. A column oven was heated to 30°C. The 10 µL sample solution was directly injected into the system. The UV detector was used to detect the peak of eugenol at 280 nm and a retention time of 5.132 min (Fig. 1)

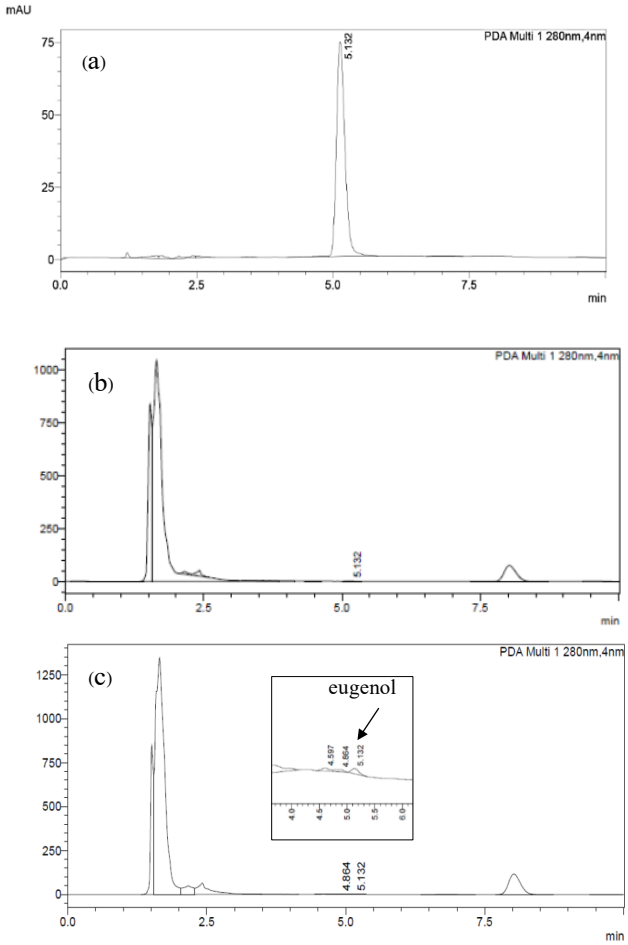

Fig. 1. HPLC chromatograms of eugenol: (a) a standard; (b) *in vitro* regenerated shoots of holy basil, purple-type culture on MS medium supplemented with 2.22 µM BA or control (c) *in vitro* regenerated shoots treated with 100 µM MeJA.

2.4.2 Total phenolic and flavonoid content, and antioxidant activity

The protocols for analysis of total phenolics and flavonoids, and for assessment of antioxidant activity, were modified from Jirakiattikul et al. [25]. The Folin–Ciocalteu method was used to determine total phenolic content. The absorbance value of samples was read from a microplate reader (PowerWave XS-BT-MQX200R) at 765 nm. The total phenolic content was reported in mg of gallic acid equivalent per gram of dry extract (mg GAE/g dry extract). Flavonoid content was measured using the aluminum chloride colorimetric method. Absorbance was measured at 510 nm. The total flavonoid content was reported in mg of catechin equivalent per gram of dry extract (mg CE/g dry extract). Antioxidant activity was assessed using DPPH radical scavenging activity assays. Absorbance was measured at 520 nm and the effective concentration required to obtain a 50% antioxidant effect (EC_{50}) was calculated using a regression equation.

2.5 Statistical analysis

The experiment used a completely randomized design with 9 treatments, done in triplicate. Data were subjected to analysis of variance (ANOVA). The means were compared by Tukey' HSD to determine significant differences ($p < 0.05$).

3. Results and Discussion

3.1 Shoot biomass

The control regenerated shoots had the highest fresh weight of 47.95±2.76 mg/shoot (Fig. 2). The lowest fresh weight was observed in specimens receiving 25 µM Phe and 100 µM MeJA (22.96±2.65 mg/shoot), but was not significantly different from those receiving 100 µM MeJA alone (23.43±1.58 mg/shoot), or a combination of 50 µM Phe and 100 µM MeJA (23.59±3.78 mg/shoot). The dry weights of all treated shoots (2.71±0.27 to 3.52±0.33 mg/shoot) were significantly lower than control (4.52± 0.41 mg/shoot). This suggested that all treatments had a negative effect on shoot biomass. Decreases in biomass production after treatments combining Phe and MeJA have also been reported for *V. vinifera* [27] and *E. affine* [28]. Numerous studies have reported that Phe has no effect on cell growth unless administered at high concentrations [16, 30-32]. The adverse effect of Phe on shoot growth maybe due to Phe inhibiting assimilation of intracellular ammonium into amino acids, as was shown in cells grown on a culture medium containing nitrate [33]. The application of MeJA may result in plant stress, an increase in reactive oxygen species, and damage to the cell membrane [34]. Chen and Chen [35] suggested that the decrease in primary metabolic activity reflects a distribution of substrate [36] or energy [37] by the plant cell into the production of secondary metabolites. MeJA has also been reported to have a negative effect on biomass production in several plant species including *B. rotunda* [25], *Hypericum perforatum* [38], and *Talinum paniculatum* [39].

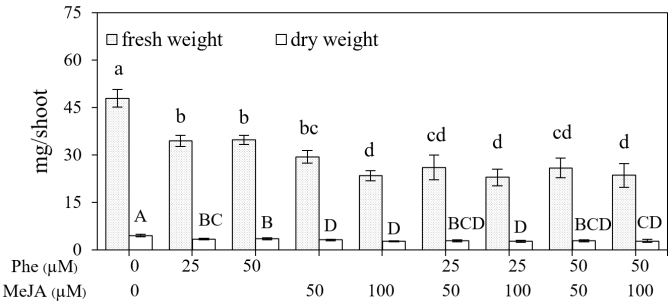

Fig. 2. Fresh and dry weight of shoots at 3 weeks cultured on medium supplemented with Phe and MeJA, individually and in combination at different concentrations, plus control, where different lowercase letters above columns of fresh weight, different uppercase letters above columns of dry weight show significant differences at $p < 0.05$ and error bars indicate ± SD.

3.2 Secondary metabolite contents and DPPH antioxidant activity

Eugenol content varied significantly among treatments (Fig. 3a). When Phe was applied at concentrations of 25 and 50 µM, the eugenol yields of 70.90±12.40 and 46.82±9.44 µg/g dry extract were 4.95 and 3.26 times that of the control, respectively. The maximum eugenol content was observed in shoots treated with a combination of 25 µM Phe and 100 µM MeJA (223.62±4.97 µg/g dry extract or 15.61 times that of the control). This was not significantly different from treatment with 100 µM MeJA alone (208.84±9.07 µg/g dry extract or 14.57 times that of the control) or a combination of 50 µM Phe and 100 µM MeJA (200.44±4.76 µg/g dry extract or 13.99 times that of the control).

Significant differences in total phenolic content were also noted among treatments. Treatments using 100 µM MeJA alone or combinations 25 µM Phe with 50 µM MeJA and 25 µM Phe with 100 µM

MeJA showed a significant increase in total phenolics. These were recorded as 190.38±5.76, 191.40±9.53, and 192.70±5.00 mg GAE/g dry extract, respectively, or 2.94, 2.96, and 2.98 times the control (Fig. 3b). When applied alone, Phe produced no significant change in total phenolic content, at either concentration, compared with control. In addition, combination treatments that used 50 µM Phe reduced total phenolic accumulation.

The highest total flavonoid content of 260.43±7.28 mg CE/g dry extract, or 4.74 times that of the control, was recorded after elicitation with 100 µM MeJA (Fig. 3c). When used alone, Phe had no effect on total flavonoid content. Combinations using MeJA with high concentrations of Phe also yielded low total flavonoids (146.66±2.50–171.21±1.30 mg CE/g dry extract).

No significant difference in antioxidant activity was found between control (EC_{50} of 20.64±0.37 µg/mL) and extracts following treatment with 25 or 50 µM Phe (21.20±0.62 and 20.22±1.10 µg/mL). DPPH radical scavenging was enhanced in shoot cultures treated with 100 µM MeJA (8.26±1.09 µg/mL) or a combination of 25 µM Phe with 50 µM MeJA (7.45±0.71 µg/mL) (Fig. 3d).

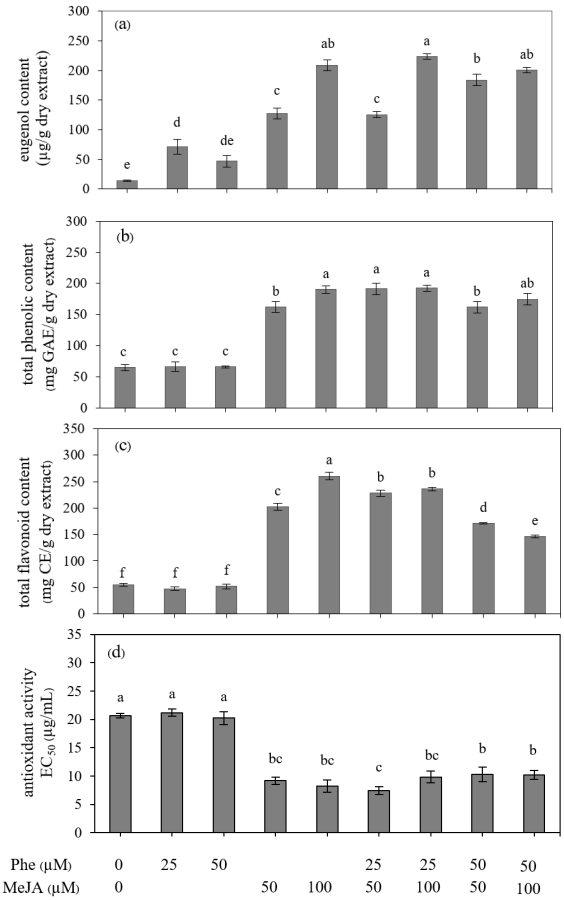

Fig. 3. Contents of (a) eugenol; (b) total phenolic compounds; (c) total flavonoids; (d) antioxidant activity (EC_{50}) at three weeks of shoots cultured on medium supplemented with Phe and MeJA, individually and in combination at different concentrations, plus control, where EC_{50} is 50% antioxidant effect, CE is catechin equivalents, GAE is gallic acid equivalents, different lowercase letters above columns indicate a significant ($p < 0.05$) different and error bars indicate ± SD.

Strong positive correlations were found among eugenol, total phenolic and flavonoid contents (0.826-0.950). This indicated that an increase in eugenol contents was associated with increases in total phenolic and flavonoid contents. However, the contents of these three compounds had a significant negative correlation with EC_{50} values of DPPH radical scavenging activity (-0.843 - -0.969) (Table 1). This suggested that an increase in eugenol, total phenolics and flavonoids increased antioxidant capacity, reflected in the lower EC_{50} values.

Table 1. Pearson's correlation coefficient of eugenol, total phenolic and flavonoid contents, and EC_{50} values of antioxidant activity of *in vitro* shoots of Holy basil, purple-type after treated with Phe and MeJA for three weeks.

parameter	total phenolic content	total flavonoid content	antioxidant activity
eugenol content	0.901**	0.826**	-0.843**
total phenolic content	-	0.950**	-0.969**
total flavonoid content	-	-	-0.939**

** significantly different at $P < 0.01$

These results suggested that the application of MeJA to purple-type holy basil significantly improved secondary metabolite accumulation and antioxidant activity, particularly when applied individually at 100 µM. Although Phe is the precursor of the phenylpropanoid pathway, its use alone exhibited less enhancement of eugenol accumulation than MeJA alone or combinations of Phe and MeJA. Phe had no effect on total phenolic and flavonoid content, nor on antioxidant activity. It is possible that the Phe concentrations used in this study were suboptimal for the species and especially for the purple-type variant. Rahimi et al. [40] noted that the choice of precursor concentration is a key factor in successful feeding. A wide range of Phe concentrations have been used in previous studies. Sharma et al. [17] reported that *in vitro O. sanctum* shoots treated with 5 mg/L Phe (30.26 µM) had higher eugenol content than field-grown shoots. Masoumian et al. [16] reported that a lower Phe concentration was optimum for *H. bonariensis.* The flavonoid content of their *H. bonariensis* callus tissue was enhanced by treatment with 3 mg/L Phe (18.16 µM). Higher concentrations of Phe have also been investigated. Skrzypczak-Pietraszek et al. [28] applied 1.6 g/L Phe (9.68 mM) in combination with MeJA and sucrose to *E. affine* shoots, enhancing phenolic acid content. Meena et al. [41] reported that elicitation of cell cultures of *Citrullus colocynthis* with 0.5 g/L Phe (3.03 mM) gave the highest quercetin content of 7.25 mg/g DW. These studies suggest that the optimum Phe concentration varies from species to species.

To identify possible synergistic effects between Phe and MeJA, we applied the synergistic coefficient. This was calculated from the secondary metabolite content after combined Phe and MeJA treatment, divided by the sum of the contents when used individually [26]. If the coefficient exceeds 1, synergistic effects are likely to present. In the current study, the coefficients were less than 1 (0.47-0.96) suggesting that Phe and MeJA did not have synergistic effects on secondary metabolite accumulation. This finding is inconsistent with that from Qu et al. [26], who reported that the anthocyanin content of cell suspension cultures of *V. vinifera* was maximized following treatment with a combination of 5 mg/L Phe with 50 mg/L MeJA. Skrzypczak-Pietraszek et al. [28] also reported that 1.6 g/L Phe with 100 µM MeJA and 6% sucrose increased the phenolic acid content in shoot cultures of *E. affine.* Andi et al. [27] reported that

accumulation of total phenolics, flavonoids, and stilbenes by *V. vinifera* cell suspension cultures under dark conditions was maximized when using a combination of 1 mM Phe with 25 µM MeJA. The current study also found that Phe and MeJA combinations at higher concentrations reduced secondary metabolite accumulation, particularly total phenolics and flavonoids (Fig. 3b and 3c). This suggests that increasing the precursor and elicitor dosage may reduce enhancement. Our results confirm those of Koca and Karaman [42], who found the lowest total phenolic and total flavonoid contents in sweet basil leaves following treatment with Phe and MeJA at high concentrations of 0.5 mM and 0.5 mM.

Our results confirmed that MeJA is an effective elicitor, enhancing both secondary metabolite accumulation and antioxidant activity by *in vitro* regenerated shoots of purple- type holy basil. MeJA is a signal transducer that regulates defensive gene expression and induces specific enzymes to synthesize secondary metabolites [20, 43]. It has been reported to increase the activity of phenylalanine ammonia lyase (PAL) [38, 44]. PAL is the first enzyme in phenylpropanoid biosynthesis and is involved in the phytochemical synthesis of phenolics, flavonoids, terpenoids, alkaloids, and phenylpropanoids [20]. MeJA has been extensively used to enhance accumulation of secondary metabolites by plants under aseptic culture conditions. Studies include eugenol in callus cultures of *D. caryophyllus* [21], total phenolic, total flavonoids, and DPPH antioxidant activity in callus cultures of *Phyllanthus pulcher* [45], flavonoids in cell suspension cultures of *H. perforatum* [38], phenolic compounds in cell suspension cultures of *T. peruviana* [23], and phenolic acid in cell suspension cultures of *M. spicata* [24].

4. Conclusion

In the current study, MeJA at a concentration of 100 µM was the most effective elicitor for shoot cultures of purple-type holy basil, enhancing accumulation of eugenol, total phenolic compounds, and total flavonoids by *in vitro* shoot cultures. Antioxidant capacity was also increased. The protocol developed for secondary metabolite production in this study may be applied to production of many medicinal, pharmaceutical, and cosmetic formulations.

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