Original Research Article

The Impact of 6-Benzylaminopurine (BAP) on Plant growth, Micro-Rhizome Induction and Phytochemical Content of Black Ginger (*Kaempferia parviflora*) Using Two In-Vitro Culture System

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| This study investigates the impact of 6-Benzylaminopurine (BAP) on shoot multiplication, micro-rhizome induction, and 5,7,4-trimethoxyflavone content in *Kaempferia parviflora*, commonly known as kunyit hitam or black ginger. Explants were cultivated using two culture systems, conventional semi-solid medium and the temporary immersion system (RITA®) with varying BAP concentrations (0 to 7.5 mg/L). Results revealed that BAP concentration significantly influenced shoot and root development. Optimal shoot proliferation was achieved at 2.5 mg/L BAP, while root growth decreased at higher concentrations. The semi-solid culture system outperformed RITA® in overall plantlet development and micro-rhizome fresh weight. Phytochemical analysis indicated that antioxidant activity, total phenolic content, and total flavonoid content were enhanced under higher BAP concentrations and were further increased through drying. Additionally, the semi-solid system yielded higher levels of 5,7,4-trimethoxyflavone compared to RITA®. These findings demonstrate that BAP optimization is critical for efficient propagation and secondary metabolite enhancement in *K. parviflora*, offering insights for improved cultivation and phytochemical enrichment strategies.  |

*Keywords: Black ginger, Direct regeneration, RITA, Multiplication, Temporary Immersion System (TIS).*

1. INTRODUCTION

In Malaysia, *Kaempferia parviflora* is commonly referred to as black ginger or kunyit hitam. It is a member of the ginger family, which comprises approximately 60 species within the same genus (Boonma *et al*., 2020). The plant propagates through the division of its rhizome. However, the availability of rhizomes is limited due to their demand in the production of commercial products. Traditional propagation methods for *K. parviflora* face challenges such as hibernation periods lasting up to three months and rhizome rot disease, leading to significant losses of planting materials (Mongkolchaipak *et al*., 2006).

To address these limitations, in vitro propagation techniques have been employed for large-scale multiplication of *K. parviflora*. Although various strategies for in vitro regeneration using rhizome bud explants have been explored, a standard semi-solid culture method remains prevalent (Prathanturarug *et al*., 2007; Zuraida *et al*.,2015). This method, characterized by multiple culture containers and limited media contact, requires substantial labor for periodic cleaning and subculturing. Alternatively, the temporary immersion system (TIS), such as RITA®, provides continuous exposure of explants to liquid media, enhancing nutrient absorption while mitigating hyperhydricity and suffocation (Bayraktar, 2019).

This study aims to optimize the concentration of 6-Benzylaminopurine (BAP) for effective shoot multiplication across various culture systems. Furthermore, it examines the induction of micro-rhizomes from shoot explants under different BAP concentrations and quantifies the presence of 5,7,4-trimethoxyflavone in these micro-rhizomes. The role of plant growth regulators, particularly BAP, has gained increasing interest due to their impact on agricultural productivity and phytochemical biosynthesis. BAP has been shown to help promote cell division and shoot growth in a variety of plant species (Kafindra *et al*., 2015; Khairudin *et al*., 2020; Labrooy *et al*., 2020). Understanding how BAP modulates these biological pathways holds promise not only for boosting yields but also for enriching the plant’s bioactive profile.

2. material and methods

**2.1 Planting Materials**

The rhizomes of *Kaempferia parviflora* were sourced from a germplasm collection (accession number 14-[KH-MJ]) at MARDI Jerangau, Terengganu (Jerangau accession). All experiments were conducted in the cryopreservation laboratories at the MyGeneBank Complex, MARDI Serdang, Selangor (coordinates: 2°58’42.3”N, 101°41’17.4”E). To remove dirt and residue, the rhizomes were thoroughly cleaned using Decon 90 with a soft brush and then rinsed under running tap water for one hour. Following this, they were immersed in a 25% antiseptic solution (Dettol) for 30 seconds to one minute and then soaked in a 0.05% (w/v) Kenlate fungicide solution for two hours. To prevent contamination, the rhizomes were cultivated on sterilized perlite. Rhizomatous buds were excised from the cleaned rhizomes and used as explants for the experiments. This procedure was designed to minimize the presence of soil-borne bacteria and fungi.

**2.2 Initial Culture**

The rhizome buds, measuring 1.5–2.0 cm, were initially cleaned with Decon 90 and then rinsed under running tap water for 45 minutes. They were subsequently treated with Dettol for 1 minute, followed by immersion in 0.05% (w/v) Kenlate fungicide for 30 minutes, with continuous agitation on a shaker platform. The explants were then transferred to a laminar airflow chamber. Disinfection was carried out using different concentrations of Clorox® (60%, 70%) and 20% Clorox® combined with a few drops of Tween 20, followed by rinses with double-distilled water. The cleaned explants were then cultured on Murashige and Skoog (MS) (1962) medium containing 3% (w/v) sucrose and solidified with 0.3% (w/v) GelriteTM to establish initial cultures of *K. parviflora*.

2.3 Effects of Tissue Culture Systems and BAP Levels on Explant Growth

Shoot tips from the initial cultures were further propagated using two culture systems: a conventional semi-solid culture jar with a 240 ml capacity and a temporary immersion system RITA® container (900 ml capacity) from Cirad, France. Both systems utilized MS medium supplemented with 3% (w/v) sucrose and different concentrations of 6-Benzylaminopurine (BAP): 0, 2.5, 5.0, and 7.5 mg/L. Each experimental condition included five replications, with two samples per replication. Cultures were maintained at 25 ± 2°C under an 18/6-hour light/dark photoperiod. After six weeks of cultivation, data on shoot number, shoot length, root number, and root length were recorded.

2.4 Effects of Tissue Culture Systems and BAP Levels on Micro-rhizome Induction

To stimulate the growth of micro-rhizomes, aseptic shoots (2 ± 0.5 cm long) produced from the shoot multiplication study were utilised as explants. The shoots were acquired by using different levels of BAP ranging from 0 mg/L to 7.5 mg/L. Following the transfer of the explants to a culture jar and RITA system,1 mg/L BAP and 1 mg/L Naphthaleneacetic acid (NAA) were added, together with 6% (w/v) sucrose without gelling agent. A controlled environment was established for the cultures, maintaining a temperature of 25 ± 2°C and a light-dark cycle of 18 hours of light and 6 hours of darkness for a duration of 12 weeks. Shoot explants in culture jars filled with liquid media were kept on an orbital shaker. The cultures were moved to fresh growth medium for every 4 weeks. Data was collected at the end of a 12-week cultural period.

**2.4.1 Assessment of phytochemical and bioactivity (antioxidant) of micro-rhizome**

To eliminate surface contaminants, fresh plant materials were collected and rinsed under running tap water. Samples were cut into thin slices, processed to a fine powder with a grinder, and stored in airtight containers. The dried sample was dried using a hot air oven.  The materials were crushed into a fine powder with a moisture content of 8-10% dry basis and stored in an airtight container until extraction. The materials were extracted with 70% methanol by immersing them in the appropriate solvents (1:10) and continuously shaking on an orbital shaker (Protech, Malaysia) set to 150 rpm for roughly 24 hours. Extraction was repeated three times under the same conditions. The filtrates were mixed to produce crude extracts. Crude extracts were kept at 4°C till analyse.

A final volume of 7 µL was mixed with 280 µL of methanolic solution containing 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, yielding a final concentration of 0.06 mM. The reaction mixture was vigorously agitated and allowed to stand for 30 minutes in the dark. Ascorbic acid was employed as the positive control. The negative control contained all reagents, and methanol was used as a blank. The DPPH radical scavenging activity was evaluated by measuring absorbance at 517 nm with a microplate reader (Aeon Biotek, VT, USA). The % inhibition of DPPH radicals was estimated as follows:

Inhibition (%) = (Absorbance of control − Absorbance of test sample) / (Absorbance control) x 100%

The results were reported as an IC50 value, which represents the inhibitory concentration required to scavenge DPPH radicals by 50%. All treatments were performed in triplicate with minimal exposure to light. The ferric reducing antioxidant power (FRAP) assay measured the reduction of ferrictripiridyltriazine (Fe3+-TPTZ) to a blue ferrous form (Fe2+-TPTZ) (Mirfat *et al.*, 2020). The functioning FRAP reagent was made by combining 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution (Sigma, USA), and 20 mM FeCl3.6H2O (Sigma, USA) at a ratio of https://doi.org/10.26656/fr.2017.8(S4).6 Copyright 2024 The Authors. Published by Rynnye Lyan Resources. Mirfat et al. / Food Research 8 (Suppl. 4) (2024) 57–64 59 Prior to usage, mix 10:1:1 and warm in a water bath at 37℃. Incubate 7 µL sample and 20 µL distilled water with 200 µL of FRAP reagent at 37℃ for 4 minutes. The reaction mixture's absorbance was measured at 593 nm and compared to a blank. A calibration curve was constructed using ferrous sulphate heptahydrate (FeSO4.7H2O) as a reference standard (100-1000 mM). The results were expressed as the concentration of antioxidants with ferric-reducing activity in Fe μmol/mg sample.

The total phenolic content of the test samples was measured using the Folin-Ciocalteu colorimetric method (Mirfat et al., 2020). In brief, a 50 µL test sample was combined with 100 µL of Folin Ciocalteu's phenol reagent. After 3 minutes, 100 µL of 10% Na2CO3 was added to the reaction mixture and left in the dark for 60 minutes. The analysis was carried out in triplicate with little light exposure. The resulting blue complex was detected at 725 nm against a blank. Gallic acid was employed as a reference standard, and the total phenol concentration was reported as gallic acid equivalents (GAE) in milligrams (mg) per gram (g) of sample using the calibration curve.

The total flavonoids were determined using the aluminium chloride technique (Mirfat *et al*., 2020). An aliquot of 30 µL extract was diluted with 120 µL distilled water. Initially, 9 µL of 5% NaNO2 solution was introduced and left to react for 5 minutes. Then, 9 µL of 10% AlCl3 solution was added and allowed to stand for 5 minutes. Finally, add 60 µL NaOH and 72 µL dH2O, and mix thoroughly with a vortex. All samples were tested in triplicate, and the absorbance was measured immediately at 510 nm against a blank. The total flavonoid content was estimated using the calibration curve and rutin as a standard reference. The results were expressed as rutin equivalents (RE) in mg per g samples.

Experiments were performed in triplicate and results were provided as means ± standard deviations. The data were analysed statistically using SPSS software, version 20 (SPSS Inc; Chicago, IL, USA), using analysis of variance (ANOVA) and Tukey's post hoc test. A P-value of <0.05 was judged statistically significant.

**2.4.2** **Quantification of 5,7,4-trimethoxyflavone**

The micro-rhizomes of *K. parviflora* produced from this study were washed with running tap water and cut into thin slices. They were then homogenized at 10,000 rpm using a SASTEC homogenizer prior to extraction. The samples were extracted with 70% methanol (1:10) under sonication (JAC Ultrasonic, Korea) with a fixed power of 300 W for 1 hour. Following that, the samples were subjected to centrifugation using a Heraus Multifuge X3R from Jerman at 10,000 revolutions per minute (rpm) for 10 minutes in order to separate the liquid portion from the solid particles. Extraction was repeated three times under identical conditions. The filtrates were combined to obtain the crude extracts. The crude extracts were stored at 4°C till the following determinations.

Prior to the quantification of 5,7,4-trimethoxyflavone, the methanolic liquid extracts were filtered using a nylon membrane filter with a pore size of 0.22 micrometers. A high-performance liquid chromatography (HPLC) analysis was conducted on the chemical 5,7,4-trimethoxyflavone using an Agilent chromatography 1290 Infinity Series equipment. The HPLC system was comprised of a column evaporator, a DAD detector, an auto-sampler, a vacuum degasser, and a quaternary pump. The chemical was maintained at a temperature of 40 °C and subjected to chromatographic separation using an XBRIDGE column (150 mm x 4.6 mm x 3 μm) from Waters. The mobile phase A was composed of a linear binary gradient between water and acetonitrile (0.1% formic acid). At various time intervals during the run, the mobile phase composition was altered as follows: 0 minutes, 11% B; 3 minutes, 30% B; 15 minutes, 30% B; 23 minutes, 50% B; 27 minutes, 90% B; 2 hours, 90% B; and 3 hours, 11% B. The flow rate was adjusted to 1 milliliter per minute and the injection volume was recorded as 1 microliter. The UV–vis absorption chromatogram was monitored at a wavelength of 265 nm using a DAD detector. The regression equation of the peak area of the extracts to the peak area of known concentration of the standard from the calibration curve was employed to determine the quantity of 4,5,7-trimethoxyflavone present.

Experiments were performed in triplicate and results were provided as means ± standard deviations. The data were analysed statistically using SPSS software, version 20 (SPSS Inc; Chicago, IL, USA), using analysis of variance (ANOVA) and Tukey's post hoc test. A P-value of <0.05 was judged statistically significant.

**2.5 Statistical Analysis**

Before conducting ANOVA, all the data except phytochemical and bioactive compound were performed a square root transformation. An analysis of variance (ANOVA) was conducted using the Statistical Analysis System (SAS) software version 9.4. The experiment followed a factorial design incorporated within a completely randomized design (CRD). Mean differences in BAP concentrations were examined for significance using Duncan's Multiple Range Test (DMRT), with statistical significance set at P < 0.05. In contrast, mean differences in culture systems were evaluated for significance using Least Significant Difference (LSD), with P < 0.05.

3. results and discussion

3.1 Effects of Tissue Culture Systems and BAP Levels on Explant Growth

In a comparative study, the rhizome buds of Kaempferia parviflora were cultivated in two different culture systems on Murashige and Skoog (MS) medium supplemented with varying concentrations of 6-Benzylaminopurine (BAP), ranging from 0 to 7.5 mg/L (Figure 1). six weeks of culture, significant interactions were observed between the culture systems and BAP concentrations regarding the shoot number, root number and root length suggesting a combined influence of tissue culture systems and BAP concentrations on these traits (Table 1). Analysis of variance (ANOVA) revealed that the culture systems significantly influenced shoot length, root number, and root length (P<0.01), while showing a moderate effect on shoot number and leaf number (Table 1). BAP concentration had a highly significant effect on shoot number, root number, and root length, but not on shoot length or leaf number (Table 1).

Semi-solid system performed better than RITA in most parameters, with higher shoot length, root number, and root length (Table 2). RITA culture system had slightly higher leaf numbers, but its root and micro-rhizome induction were lower, indicating that the semi-solid system is more effective for overall plantlet development (Table 2).

While for BAP concentration, 2.5 mg/L BAP resulted in the highest shoot number and root number, but root length was significantly lower than in 0 mg/L BAP. Higher BAP concentrations (5.0 mg/L and 7.5 mg/L) resulted in a decline in root development, indicating potential toxicity or inhibition at elevated levels (Table 2).

Fig. 1 *K. parviflora* multiplication of shoots under different culture system. (a) RITA® (b) Conventional semi-solid.

Table 1 Mean square ANOVA effect of tissue culture systems (Cs) and levels of BAP (Conc) on the explant growth of *K. parviflora*.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Shoot number  | Shoot length (cm) | Leave number  | Root number | Root length (cm) |
| Cs | 0.13 | 1.98\* | 0.08 | 15.37\*\* | 14.59\*\* |
| Conc | 3.86\*\* | 0.62 | 0.11 | 4.31\*\* | 5.50\*\* |
| Cs x Conc | 2.68\*\* | 0.51 | 0.48 | 6.55\*\* | 0.95\* |
| Grand Mean | 3.35 | 6.85 | 2.84 | 5.26 | 2.97 |
| CV | 39.85 | 26.37 | 26.55 | 43.19 | 38.18 |

*\*Square-root-transformed mean followed by \* indicate significant difference at P<0.05*

*Square-root-transformed mean followed by \*\* indicate significant difference at P<0.01*

Table 2 Evaluation of tissue culture systems and BAP concentrations on the development of K. parviflora explants

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Factors | Shoot number  | Shoot length (cm) | Leave number  | Root number | Root length (cm) |
| Culture System | Semi-solid | 2.43 a | 7.58 b | 2.80 a | 6.65 b  | 4.21 b  |
| RITA | 2.36 a | 6.21 a | 2.92 a | 3.97 a | 1.77 a |
| BAP Concentrations | 0 mg/L | 1.00 a | 7.36 a | 2.79 a | 6.16 bc | 5.93 b |
| 2.5 mg/L | 3.00 b | 6.07 a | 2.75 a | 7.45 c | 2.66 a |
| 5 mg/L | 2.80 b | 7.52 a | 3.10 a | 3.55 a | 1.81 a |
| 7.5 mg/L | 2.70 b | 6.68 a | 2.80 a | 4.20 ab | 1.76 a |

*\* Mean followed by the same letter in a column were not significantly different at P ≤0.05 by LSD (culture system) and DMRT (BAP concentration).*

Table 3 Analysis of the correlation between the number of shoots and shoot height, number of leaves, roots, and root length.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | Shoot number  | Shoot length | Leave number  | Root number | Root length  |
| Shoot number | 1 | 0.28\* | 0.55\*\* | 0.24\* | -0.21ns |
| Shoot length |  | 1 | 0.64\*\* | 0.44\*\* | 0.40\* |
| Leave number |  |  | 1 | 0.42\*\* | 0.30\*\* |
| Root number |  |  |  | 1 | 0.68\*\* |
| Root length |  |  |  |  | 1 |

*\* Square-root-transformed mean followed by \* indicate significant difference at P<0.05*

*Square-root-transformed mean followed by \*\* indicate significant difference at P<0.01*

Shoot number correlated significantly (P<0.01) with the number of leaves and roots, indicating that an increase in shoots also leads to a higher number of leaves and roots (Table 3). Root number and root length were significantly correlated (P<0.01), reinforcing that strong root development is linked to overall plant vigour (Table 3). However, shoot number had no significant correlation with root length, implying that while shoot growth is promoted, root elongation is not necessarily enhanced (Table 3).

Park *et al*. (2021) reported similar findings for the number of shoots (2.6-4.2) and the number of roots (3.6-5.3) of *K. parviflora* within the range of BAP concentrations of 2 to 4 mg/L using the conventional culture system. In contrast, the report found that using a higher level of BAP (8 mg/L BAP) resulted in a more significant number of shoots (6.3). While in this study, the highest level of BAP (7.5 mg/L) resulted in reduced shoot number produced in both culture systems. Thus, no research has been found on the RITA system for *K. parviflora*. The correlation analysis revealed a substantial negative association between root length and shoot number (Table 3). This indicates that increasing the shoot number can limit root growth. This is comparable to how high cytokine concentrations restrict root growth while increasing the shoot number (Laplaze *et al*.,2007)

**3.2 Effects of Tissue Culture Systems and BAP Levels on** **Micro-rhizome Induction**

The culture system had a significant effect on micro-rhizome fresh weight (P<0.05), with the semi-solid system yielding higher fresh weight (Table 4). BAP concentrations significantly influenced micro-rhizome weight (P<0.01) (Table 4). The ANOVA analysis revealed that culture system and BAP concentration had no influence on the shoot numbers (Table 4). There was no significant interaction between culture system and BAP concentration on both parameters. 5.0 mg/L BAP yielded the highest number of new shoots and micro-rhizome fresh weight, followed by 2.5 mg/L and 7.5 mg/L BAP, while 0 mg/L BAP had the lowest induction (Table 5). Semi-solid system significantly outperformed RITA in micro-rhizome induction and fresh weight (Table 5). RITA culture led to lower fresh weight production, suggesting that while it is effective for shoot propagation, it may not be ideal for micro-rhizome formation. A strong positive correlation (r = 0.90, P<0.01) was observed between new shoot number and micro-rhizome fresh weight. This implies that higher shoot proliferation directly enhances micro-rhizome formation, confirming that BAP concentration plays a crucial role in both parameters. The application of BAP plays a pivotal role in micro-rhizome induction in K. parviflora, demonstrating its ability to enhance vegetative propagation. BAP, a synthetic cytokinin, promotes cell division and differentiation, which are essential for micro-rhizome development. Optimal BAP concentrations stimulate vigorous growth and robust root structures, with synergistic effects observed when combined with other growth regulators (Abdullah *et al*., 2019). Conversely, the absence of BAP results in slower growth and reduced micro-rhizome yield. These findings underscore the importance of BAP in optimizing shoot multiplication and micro-rhizome induction, ultimately advancing sustainable agricultural practices for K. parviflora.

Table 4 Mean square ANOVA effect of tissue culture systems (Cs) and levels of BAP (Conc) on the micro-rhizome induction of *K. parviflora*

|  |  |  |
| --- | --- | --- |
|  | New shoots number | Micro-rhizome fresh weight (g) |
| Cs | 1.27 | 0.98\* |
| Conc | 1.85 | 3.0\*\* |
| Cs x Conc | 0.42 | 2.82  |
| Grand Mean | 2.81 | 10.85 |
| CV | 32.70 | 41.03 |

*\*Square-root-transformed mean followed by \* indicate significant difference at P<0.05*

*Square-root-transformed mean followed by \*\* indicate significant difference at P<0.01*

Table 5 The impact of different levels of BAP on the explant employed in micro-rhizome induction of *K. parviflora* using semi-solid culture system.

|  |  |  |
| --- | --- | --- |
| Factors | New shoots number (per explant) | Micro-rhizome fresh weight (g/explant) |
| Culture System | Semi-solid | 12.21 a | 3.43 b |
| RITA | 5.00 a  | 0.38 a |
| BAP Concentrations | 0 mg/L BAP | 2.00 a | 0.33 a |
| 2.5 mg/L BAP | 15.17 b | 3.57 b |
| 5.0 mg/L BAP | 17.75 b | 4.91 b |
| 7.5 mg/L BAP | 12.67 b | 3.88 b |

*\* Mean followed by the same letter in a column were not significantly different at P ≤0.05 by LSD (culture system) and DMRT (BAP concentration).*

A

B

Figure 2 Micro-rhizome induction from different culture system. A) Semi-solid culture B) RITA

* + 1. **Antioxidant and Phytochemical Content of Micro-rhizome**

**Semi-solid culture produced higher free radical scavenging activity (DPPH values) compared to RITA (Table 6). The activities varied from 83.962 mg/mL to 477.099 mg/mL, with micro-rhizome induced in 2.5 mg/L BAP in RITA system being the strongest (IC50 83.962 mg/mL) (P<0.05). Meanwhile, the lowest scavenging activity was observed in micro-rhizome induced in 7.5 mg/L BAP in semi-solid culture system (IC50 477.099 mg/mL) (P<0.05). These antioxidant activities are inversely proportional to the IC50 values. BAP concentration influenced antioxidant content**, with the highest **total phenolic and flavonoid content** observed at **higher BAP levels (7.5 mg/L)**. **RITA showed the highest antioxidant potential compared to the semi-solid system**. The highest **FRAP value** was recorded in the **RITA culture at 7.5 mg/L BAP**, suggesting it may be more efficient at enhancing iron-reducing capacity.

Table 6 Comparison of antioxidants and phytochemical content in two culture systems using fresh *K. parviflora* micro-rhizomes developed from shoot explant grown from varying BAP concentrations.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Concentration of BAP in shoot multiplication stage | Culture system | Free radical scavenging activity DPPH, IC50 (mg/mL) | Ferric reducing antioxidant power (FRAP) (µmol Fe/g) | Total phenolic content (mg GAE/g) | Total flavonoid content (mg RE/g) |
| 0 mg/L BAP | Semi solid | 368.551 e | 0.047 c | 7.327 a | 1.078 c |
|  | RITA | 120.356 bc | 0.042 bc | 10.509 b | 0.867 b |
| 2.5 mg/L BAP | Semi solid | 303.648 e | 0.011 a | 11.119 b | 0.898 b |
|  | RITA | 83.962 a | 0.053 c | 11.661 bc | 0.949 cb |
| 5.0 mg/L BAP | Semi solid | 189.284 d | 0.022 ab | 12.769 cd | 0.691 a |
|  | RITA | 107.916 b | 0.031 abc | 13.498 c | 0.793 ab |
| 7.5 mg/L BAP | Semi solid | 477.099 f | 0.013 a | 13.455 c | 0.653 a |
|  | RITA | 128.225 c | 0.082 d | 15.191 d | 1.055 c |

*\* Means followed by the same letter in a column are not significantly different at P≤0.05 by Tukey*

**Dried samples had significantly higher phenolic and flavonoid content than fresh samples**, supporting the hypothesis that drying enhances secondary metabolite concentration (Table 7). **Free radical scavenging activity (DPPH) was lower in dried samples**, indicating some loss of antioxidant activity during drying. The activities varied from 67.125 **mg/mL to 477.099 mg/mL (Table 7). FRAP values ranged from 0.011 µmol Fe/g to 0.059 µmol Fe/g (Table 7). However, dried samples at 2.5 mg/L and 7.5 mg/L BAP exhibited the greatest values, indicating that drying enhances iron-reducing capacity.** **Higher BAP concentrations generally resulted in higher phenolic content, which was consistent with trends reported in fresh samples; however, higher BAP concentrations resulted in lower flavonoid content (Table 7).**

Table 7 Comparison of antioxidant activity and phytochemical content of fresh and dried samples using a semi-solid culture system

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Concentration of BAP in shoot multiplication stage | Sample condition | Free radical scavenging activity DPPH, IC50 (mg/mL) | Ferric reducing antioxidant power (FRAP) (µmol Fe/g) | Total phenolic content (mg GAE/g) | Total flavonoid content (mg RE/g) |
| 0 mg/L BAP | Fresh | 368.55 d | 0.047 cd | 7.327 a | 1.078 c |
|  | Dried | NA | NA | NA | NA |
| 2.5 mg/L BAP | Fresh | 303.648 d | 0.011 a | 11.119 ab | 0.898 b |
|  | Dried | 67.125 a | 0.059 d | 16.089 d | 2.766 d |
| 5.0 mg/L BAP | Fresh | 189.284 c | 0.022 b | 12.769 b | 0.691 a |
|  | Dried | 90.729 b  | 0.036 c | 13.861 c | 3.427 e |
| 7.5 mg/L BAP | Fresh | 477.099 e | 0.013 a | 13.455 c | 0.653 a |
|  | Dried | 74.076 a  | 0.056 d | 15.362 cd | 2.531 d |

*\* Means followed by the same letter in a column are not significantly different at P≤0.05 by Tukey.*

**3.2.2 Quantification of 5,7,4-trimethoxyflavone**

The rhizomes of *K. parviflora* exhibit various biological activities, which are associated to the presence of various types of flavonoids including methoxyflavones. Previous research has shown that *K. parviflora* and its methoxyflavones have a variety of pharmacological benefits, including antioxidant, antiallergic, anti-inflammatory, antitumor, anticancer, antimicrobial, anti-osteoarthritis, neuroprotective, vascular relaxation and cardioprotective, cellular metabolism-regulating, sexual enhancement, and transdermal permeable properties. Among the primary methoxyflavones that contribute to these activities are 5,7,4′-trimethoxyflavone (TMF), 5,7-dimethoxyflavone (DMF) and 3,5,7,3′,4′-pentamethoxyflavone (PMF) (Chen *et al*., 2018; Huo *et al*., 2023;). TMF has potential vasodilator effects, as indicated by its ability to elicit concentration-dependent vasorelaxation in the rat aorta, mediated by endothelial-derived nitric oxide, which may contribute to improved exercise performance and overall physical fitness (Tan *et al*., 2024).

In the present study, the identification of 5,7,4-trimethoxyflavone (TMF) was achieved by comparing the retention times of the peaks in the sample with those of known standard. The results indicated that the extracts obtained from the semi-solid culture system exhibited a greater concentration of TMF compared to those obtained from the RITA culture method. The TMF concentration in the semi-solid culture system ranged from 1.551 to 2.763 µg/mg. These findings suggest that the MS media with BAP concentration of 7.5 mg/L used in the semi-solid culture system successfully enhanced the TMF content in *K. parviflora*. Meanwhile, the TMF in the RITA culture system varied between 0.110 to 0.275 µg/mg.

4. Conclusion

**Semi-solid culture system is more effective than RITA for plantlet growth and micro-rhizome induction** in Kaempferia parviflora**. BAP concentration plays a critical role in shoot proliferation, root formation, and antioxidant potential,** with **5.0 mg/L being optimal for overall growth and micro-rhizome development. RITA system is useful for shoot multiplication but less effective for root and rhizome development. Antioxidant and phytochemical content are significantly influenced by BAP concentration, with drying improving total phenolic and flavonoid content.** Future studies should explore **alternative elicitors or environmental conditions** that further enhance secondary metabolite production in K. parviflora**.**

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