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Methyl Jasmonate Elicitation Enhanced Biomass, Phenolic, and Flavonoid Production of Adventitious Root Culture of *Gynura procumbens* in Balloon Type Bubble Bioreactor

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ABSTRACT

Secondary metabolites in the medicinal plant *Gynura procumbens* have various beneficial pharmacological activities. However, the limited availability of these beneficial compounds has constrained the possibilities for therapeutic applications. Exogenously applied methyl jasmonate (MeJa) as an elicitor has been reported to trigger the accumulation of secondary metabolites in plant tissues cultured *in vitro*. This study aimed to assess how varying periods of MeJa elicitation affect the growth and accumulation of phenolic and flavonoid compounds in adventitious root cultures of *G. procumbens* within a balloon-type bubble bioreactor (BTBB). Adventitious roots were induced from leaf and stem explants obtained from established *in vitro* shoots. Upon transfer to the BTBB, the cultures were supplemented with 100 µM MeJa and sampled at 1, 2, 3, and 4 weeks. The results revealed that including MeJa in the culture medium significantly boosted growth and the accumulation of phenolic and flavonoid compounds in *G. procumbens*. The highest biomass and concentrations of phenolic and flavonoid compounds were observed after 3 weeks of elicitation. These findings highlight the potential of MeJa elicitation for enhancing the production of valuable bioactive compounds in *G. procumbens* adventitious root cultures, showcasing its utility for industrial applications.

Keywords: balloon-type bubble bioreactor; flavonoid; *Gynura procumbens*; methyl jasmonate; phenol

INTRODUCTION

Gynura procumbens is an indigenous Indonesian plant that has been traditionally used as a vegetable, anesthesia drug, and herbal medicine to treat various symptoms, such as fever, high cholesterol level, dysentery, tumors, and kidney problems. Previous works have reported the potential use of *G. procumbens* for cancer (Nurulita et al., 2012) and diabetic treatments (Algariri et al., 2013). It has also been widely used as an immunomodulator agent (Takanashi et al., 2019), vasodilatation agent (Hoe et al., 2011; Ng et al., 2013), antiviral agent (Jarikasem et al., 2013), anti-inflammatory agent (Iskander et al., 2002) and antioxidant agent (Krishnan et al., 2015). These beneficial pharmacological properties are mainly because of the flavonoid compounds present in the leaf and root organs of *G. procumbens* (Tan et al., 2016). These compounds include kaempferol, quercetin (Kim et al., 2011), and flavanon (Manogaran et al., 2019). Despite the potential of these compounds, their limited availability in nature has constrained the possibilities of these compounds for therapeutic applications.

*G. procumbens*is conventionally propagated by cuttings. This method is slow and time-consuming since the growth of plants can be affected by season and environment. To meet the growing demand for bioactive compounds sourced from plants, developing an efficient in vitro system using tissue culture techniques is crucial. Numerous plant secondary metabolites have been successfully generated through *in vitro* systems. (Cui et al., 2010a), such as caffeic acid, anthraquinones, phenolics, and flavonoids. Numerous secondary metabolites are biosynthesized by specialized cells. For example, adventitious root cultures have demonstrated the ability to yield a greater quantity of secondary metabolites than those obtained from cell cultures. Adventitious roots are frequently employed at an industrial scale to produce these safe and non-

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carcinogenic secondary metabolites (Baque et al., 2014; 2013; Murthy et al., 2016). For instance, the FDA has approved using bioactive compounds derived from adventitious roots of *Panax ginseng* and *Echinacea purpurea* in the pharmaceutical and cosmetic industries. (Baque et al., 2014). Adventitious roots from other plant species, such as *Hypricum perforatum* (Cui et al., 2010a), *Astragalus membranace*ous (Wu et al., 2011), and *G. procumbens* (Saiman et al., 2012), have also been reported.

In vitro plant culture systems employing bioreactors are commonly employed to augment the production of secondary metabolites. A bioreactor is an engineered system capable of sustaining plant growth within varying chemical conditions, utilizing liquid culture media, and controlling physical factors such as airflow, aeration, temperature, pH, and dissolved oxygen levels. (Karuppusamy, 2009). Different types of bioreactors have been employed in plant research. Of these, balloon-type bubble bioreactor (BTBB) is frequently used for adventitious root cultures (Baque et al., 2014; Choi et al., 2000; Lee et al., 2014; Lee & Paek, 2012; Paek et al., 2001). In our previous works, we have enhanced the biomass of *G. procumbens* adventitious root cultures in BTBB by 13-fold by adding 50% sucrose into the media (Manuhara et al., 2017; Muthoharoh et al., 2019). Besides sucrose, various biotic elicitors (microbial extract, polysaccharide, and oligosaccharide) and abiotic elicitors (heavy metal, pesticide, UV light, cold shock, hyperosmotic stress, and ultrasound) have been used to enhance the production of secondary metabolites. For example, heavy metal ($CuSO_4$) and low concentration of *Saccharomyces cerevisiae* extract have been added to *G. procumbens* cultures to improve biomass and flavonoid production (Faizah et al., 2018). The addition of jasmonic acid (JA) or its methyl ester variant methyl jasmonate (MeJa) into *in vitro* plant cultures has been shown to effectively increase the secondary metabolite accumulation (Foyer & Harbinson, 2019; Ho et al., 2020; Rahimi et al., 2015). This promotive effect of JA or MeJa treatment has been shown in adventitious root cultures of *Panax ginseng* (Ali et al., 2007; Kim et al., 2004), *Pseudostellaria heterophylla* (Wang et al., 2015), *Eleutherococcus koreanum* (Lee et al., 2015) and *Oplopanax elatus* (Jiang et al., 2017).

In this study, we assessed how varying periods of MeJa elicitation affect the growth, as well as the production of phenolic and flavonoid compounds in adventitious roots of *G. procumbens* within a BTBB. Additionally, we examined changes in malondialdehyde (MDA) and proline levels in MeJa-treated adventitious roots, which serve as indicators of cellular oxidative stress. Furthermore, we analyzed alterations in pH, sugar levels, and electrical conductivity of the culture media, providing valuable insights into the system's overall biochemical and environmental dynamics.

MATERIALS AND METHOS

Materials

Leaf and stem tissues of *Gynura procumbens* (Lour.) Merr obtained from Purwodadi Botanical Garden, Pasuruan, East Java, Indonesia (Botanical Garden identification number/IPH.06/HM/XI/2015) were used to establish adventitious root cultures. Murashige and Skoog's solid medium were used to induce adventitious roots with 7 g/L agar.

Methods

Induction of adventitious roots

The leaves and stems of *G. procumbens* were cleaned with detergent for 5 minutes and then thoroughly rinsed with tap water. Subsequently, the cleaned leaves were immersed in a 10% (v/v) Clorox solution for 10 minutes (commercial bleach with 5.25% w/v sodium hypochlorite), followed by three washes with sterile distilled water. The plant explants were then sectioned into 1 cm^2 pieces and placed on Murashige and Skoog (MS) solid medium supplemented with 5 mg/L indole-3-butyric acid (IBA), 30 g/L sucrose, and 7 g/L agar for leaf-derived explants. In contrast, stem-derived explants were cultured on MS solid medium supplemented with 2 mg/L indole acetic acid (IAA), 4 mg/L 6-benzylaminopurine (BAP), 30 g/L sucrose, and 7 g/L agar. The pH of the medium was adjusted to 5.8 and autoclaved at 121°C for 15 minutes. The cultures were then incubated in darkness at 25±2°C. After 21 days of culture, the adventitious roots were collected for subsequent BTBB culture.

Adventitious roots culture in BTBB

Approximately 2 grams of adventitious roots were placed in a BTBB with a maximum capacity of 1 liter, containing 500 mL of liquid MS medium supplemented with 5 mg/L IBA and 30 g/L sucrose. The cultures were maintained in darkness at a temperature of 25±3°C for 7 weeks. Weekly measurements of fresh and dry weights of the adventitious roots were taken for analysis of growth rates, and growth curves were constructed using data from two replications. At the 4-week mark of the culture period, 100 µM MeJa, sterilized through a 0.2-µm filter, was introduced into the BTBB culture and incubated for durations of 1, 2, 3, and 4 weeks.

Biomass measurement of adventitious roots

The fresh weight of the harvested adventitious roots (1, 2, 3, and 4 weeks of culture) was obtained by gently blotting dry the adventitious roots on a paper towel for 15 min and weighing using an analytical balance. The adventitious roots were dried at room temperature (31-34^{o}C) for 2-3 days to get the dry weight then ground into powder using a pestle and mortar. The growth index of adventitious root cultures was calculated using equation (1):

$$\text{Growth index} = \frac{\text{Final dry weight} - \text{Initial dry weight}}{\text{Initial dry weight}} \quad (1)$$

The growth rate of the cultured adventitious roots was calculated using equation (2):

$$\text{Growth rate} = \frac{\ln(\text{final dry weight}) - \text{an}(\text{ initial dry weight})}{\text{length of culture periods (days)}} \quad (2)$$

Quantification of total phenolic and flavonoid contents

The maceration method was used to extract adventitious roots to get the total phenolic compound and flavonoid. One gram (1g) of adventitious root powder was mixed with 20 mL methanol and then incubated for 48 hours in a shaker incubator at 7-10 rpm. Using filter paper, the liquid extract was separated from solid debris and concentrated by evaporation to 2 mL at room temperature. For phenolic compound measurement, 200 µL of the methanol extract was mixed with 1 mL of 1:10 Folin-Ciocalteau reagents and incubated for 5 minutes at room temperature. Next, the solution was added 0.8 mL of 7.5% (w/v) natrium carbonate (Na_2CO_3) then incubated for 30 minutes at room temperature The absorbance of the solution was measured at 765 nm using a UV spectrophotometer (BOECO S-22, Germany). Determining the total phenolic compounds was done by regression analysis from the absorbance rate of standard phenolic acid. Results were expressed as mg GEA (gallic acid equivalent) per gram of dry weight (Kaewseejan et al., 2015).

The concentration of flavonoids was measured using 250 µL of the methanol extract mixed with 125 mL distilled water and 75 µL of 5% (w/v) sodium nitrate, and then the mixture was incubated at room temperature for 6 minutes. Next, the solution was added to 150 µL of 10% (w/v) aluminum chloride and then incubated for 5 minutes. One hundred and fifty microlitres of sodium hydroxide were added and topped with distilled water to 2.5 mL total volume. The absorbance of the solution was measured using a UV spectrophotometer at 510 nm. The flavonoid concentration was determined by regression analysis from the absorbance rate of standard quercetin and kaempferol (Kaewseejan et al., 2015).

Malondialdehyde and proline measurement

MDA and proline levels in MeJa-treated adventitious root cultures were determined following the procedure outlined by Cui et al., 2010. Initially, 0.2 g of fresh adventitious roots were homogenized by adding 4 mL of 0.1% (w/v) trichloroacetic acid (TCA) using a mortar and pestle. The resulting mixture was then centrifuged at 3,000 rpm for 10 minutes. The supernatant obtained was combined with 0.25 mL of 200% TCA, 0.25 mL of butylated hydroxytoluene (BHT), and 0.5 mL of 0.65% (w/v) thiobarbituric acid (TBA). This mixture was incubated at 95°C in a water bath for 30 minutes, followed by rapid cooling on ice. Subsequently, the solution was centrifuged again at 3,000 rpm for 10 minutes. The absorbance of the resulting supernatant was measured using a UV spectrophotometer (Multiscan Go - Thermosci) at wavelengths of 523 nm and 600 nm. The concentration of MDA was calculated using equation (3).

$$\text{Malondialdehyde (MDA)} = \frac{\text{Absorbance value at } \lambda_{523} - \text{Absorbance value at } \lambda_{600}}{155\ \text{mM}^{-1}\text{cm}^{-1}(\text{Coefficient value MDA} - \text{TBA})} \quad (3)$$

Lipid peroxidation was calculated according to Puangpronpitag et al., (2010).The absorbance of MDA for each sample (A_{sample}) and phosphate buffer ($A_{control}$) was measured at 532 nm and calculated using equation (4):

$$\text{Inhibition (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \cdot 100\% \quad (4)$$

The concentration of proline was performed according to Bates et al., 1973. First, 0.5 g of fresh adventitious roots were homogenized using a mortar and pestle, adding 10 ml of 3% (v/v) sulfosalicylic acid. The mixture was centrifuged at 6,000 rpm for 10 min. The supernatant was mixed with 2 mL ninhydrin acid and 2 ml glacial acetate before being incubated at 100^{o}C in a water bath for 1 hour, followed by immediate cooling on ice. Next, the solution was added 4 mL of toluene and mixed thoroughly by vortexing for 15-20 seconds. The chromophore was measured using a UV spectrophotometer at 520 nm with pure toluene as a blank solution. Proline concentration was calculated based on the L-proline standard solution.

Measurement of physical and chemical conditions of the media

The changes of pH, total sugar level (Atago, Master 10T), and electrical conductivity (Ezdo, Condo5021) of culture media were determined at

different stages of the experiment: pre- and post-sterilization and at the end of the culture period.

RESULTS

Quantitative analysis of *G. procumbens* adventitious roots growth rate

Adventitious roots derived from both leaves and stems of *G. procumbens* were cultured and maintained in a BTBB. The fresh and dry adventitious roots weights were documented at 7-day intervals to ascertain the appropriate starting time for MeJa elicitation (see Figure 1). During the initial two weeks of culture, the growth of adventitious root cultures was sluggish, indicating a lag phase where the initial explants were acclimating to the culture conditions. Subsequently, a notable surge in fresh and dry weights was observed in the third and fourth weeks of culture, signifying a logarithmic or exponential growth phase.

However, by the fifth week of culture, the adventitious roots' growth rate decelerated, indicating a transition towards a stationary phase of growth. The peak biomass was recorded in the sixth week of culture, yielding 35.8 g of fresh weight and 2.005 g of dry weight. After the sixth week, fresh and dry weights of adventitious roots declined. This growth pattern aligns with previous reports on the hairy root culture of *Glycyrrhiza* reported by Wongwicha et al., (2011). Given that MeJa elicitation is recommended during the exponential growth phase, we introduced the MeJa elicitor in the fourth week of culture for subsequent experiments.

The effect of different MeJa elicitation periods on the growth of *G. procumbens* adventitious roots

After adding MeJa to the culture medium, the fresh and dry weights of *G. procumbens* adventitious roots were increased from 0 days (M0) to the third week of elicitation (M3) (Figure 2A). These results showed that MeJa positively affects the biomass of *G. procumbens* adventitious roots. The growth index of MeJa-treated *G. procumbens* adventitious roots, however, was negatively correlated with its growth rate (Figure 2B).

The effect of the MeJa elicitation period on phenol and flavonoid production

To assess the impact of MeJA on the production of phenolic and flavonoid compounds, a concentration of 100 μM MeJa was administered to 4-week-old adventitious root cultures. Our findings revealed that MeJa elicitation significantly heightened the accumulation of phenolic and flavonoid compounds, including kaempferol and quercetin, within G. procumbens adventitious roots in the BTBB (see Figure 3). The peak concentrations of phenolic compound (484.49 mg/L/10 g DW), kaempferol (3137.29 mg/L/10 g DW), and quercetin (882.67 mg/L/10 g DW) were noted during the third week of MeJa elicitation (see Figure 3).

The effect of MeJa elicitation periods on the concentration of MDA and proline

The concentration of MDA and proline of MeJa-treated adventitious root cultures was measured to examine the level of cellular stress. We found that the highest level of MDA (80432.61 nmol/0.5 g FW) and proline (6665.32 nmol/0.5 g FW) was recorded after 1 week of elicitation (Figure 4). The concentration of MDA and proline in MeJa-treated adventitious root cultures after 1, 2, 3, and 4 weeks of elicitation was generally higher than 0 days.

In this study, during elicitation from the start culture, until week four (M0 – M4) MDA and proline profiles have the same patterns, except in week three (M3). This is due to the lack of repetitions so less data is obtained. Accumulation of MDA in the adventitious root of *G. procumbens* was followed by proline accumulation. This result indicated that the accumulation of proline was a response of MeJa treatments to peroxidation lipid activity. A higher concentration of MeJa triggered this accumulation because proline accumulation is found when plants get various kinds of abiotic stress; most previous studies have reported that the concentration of proline usually increases when plants have abiotic stresses (Yue et al., 2019).

This study has established a culture protocol for *G. procumbens* adventitious roots in BTBB (Figure 5). The culture was started to induce adventitious roots from leaves explant in MS solid medium supplemented with 5 mg/L indole-3-butyric acids (IBA). In contrast, the stem explants were cultured on MS solid medium supplemented with 2 mg/L indole acetic acid (IAA), 4 mg/L 6-benzylaminopurine (BAP), then adventitious roots were culture in Erlenmeyer with 100 mL liquid MS medium supplemented with 5 mg/L IBA. Cultured was transferred to BTBB and various concentrations of MeJa added the medium.

DISCUSSION

Jasmonic acid is an important plant signaling molecule for plant growth and development as well as defense responses. In this study, we added 100 μM MeJa based on the previously reported concentration (Jiao et al., 2018; Mangas et al., 2006; Yi et al., 2019) into the

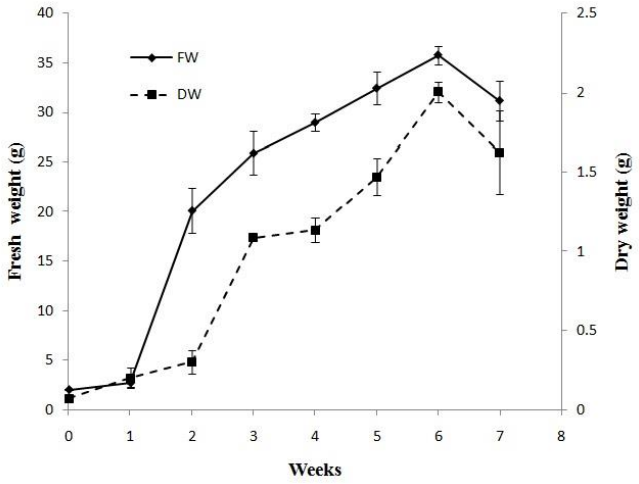
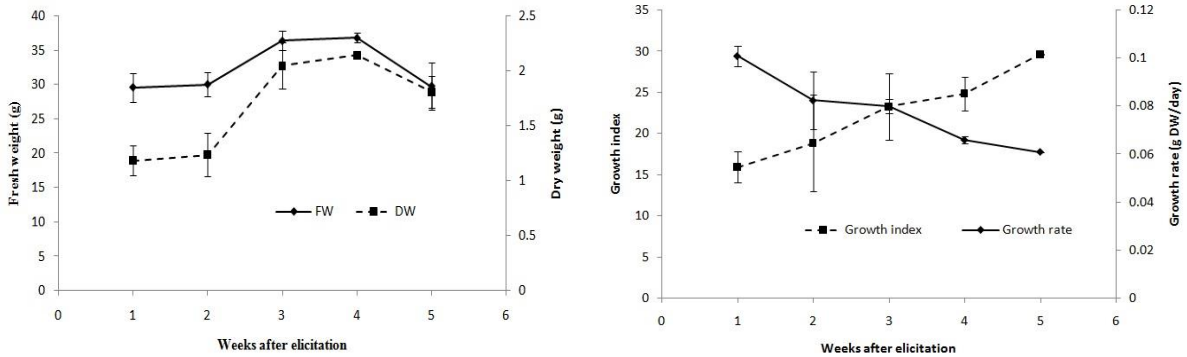


Figure 1. The growth trajectory of *G. procumbens* adventitious root culture within BTBBs



(A) **(B)**

Figure 2. *G. procumbens* adventitious root growth elicited with 100 µM MeJa at different periods (A: Fresh and dry weights of MeJa-treated adventitious roots at different elicitation periods; B: Growth index and growth rate of MeJa-treated adventitious roots at different elicitation periods; M0: 0 days of MeJa elicitation (4 week-old culture); M1-M4: one to four weeks of MeJa elicitation)

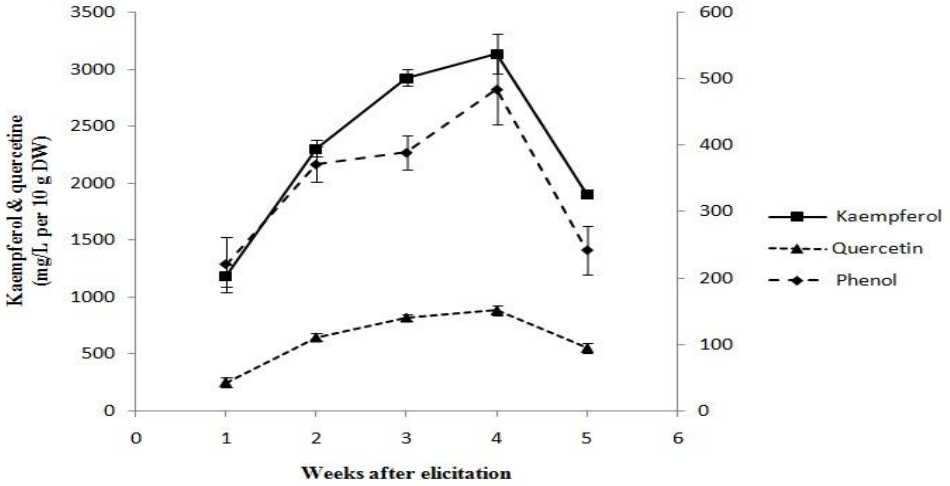


Figure 3. The concentration of phenolic and flavonoids (kaempferol and quercetin) in MeJa-treated (Each data point represents the mean ± standard error value (n=2)

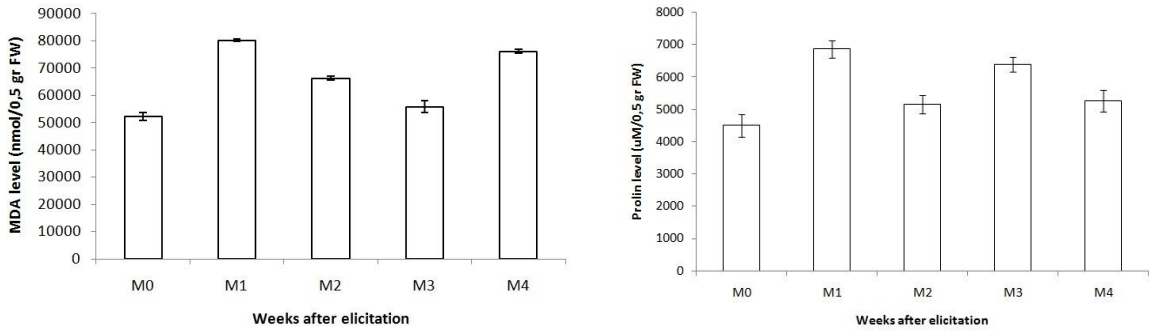


Figure 4. The concentration of MDA and proline in *G. procumbens* adventitious root cultures treated with MeJa for different elicitation periods

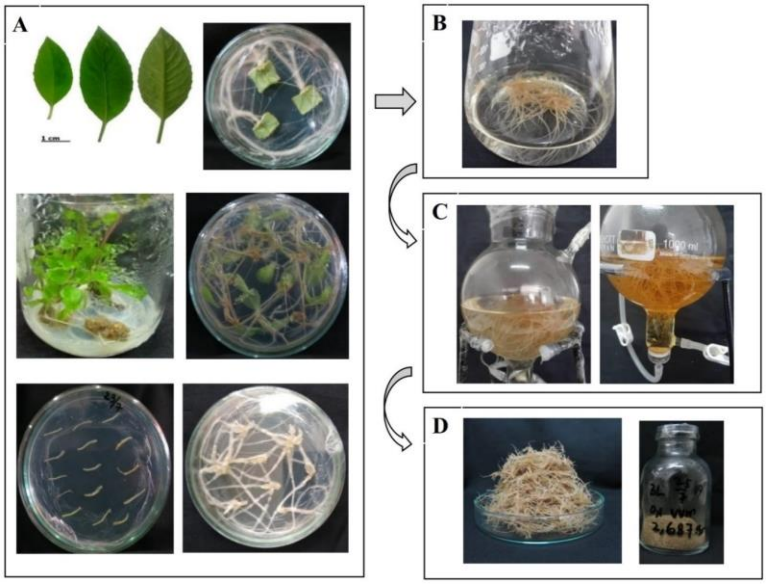


Figure 5. The overview of culture protocol for *G. procumbens* adventitious roots in BTBB (A: Leaf and stem explants of *G. procumbens* were used to induce adventitious roots; B: Adventitious root cultures were cultured in liquid MS medium supplemented with 5 mg/L IBA; C: Adventitious root cultures were transferred to BTBB and incubated in liquid MS medium supplemented with 100 µM MeJa (right) and without MeJa(left); D. Fresh (left) and dry (right) weights of adventitious roots collected from BTBB)

culture media containing 4-week-old adventitious roots. We found that the fresh and dry weights of MeJa-treated adventitious root cultures were increased for the first three weeks of culture. Supplementation of MeJa in culture medium has been reported to improve the plant growth in several plant species, such as cell suspension culture of *Melastoma malabathricum* (See et al., 2011), callus culture of Carnation (Matter et al., 2017) and adventitious root culture of *Centella asiatica (L.) Urb* (Nguyen et al., 2019). Nevertheless, Meja could negatively affect plant growth, as reported in the cell suspension culture of *Thevetia peruviana* (Pers.) *K. Schum* (Mendoza et al., 2018a), Taxus (Patil et al., 2014), and strawberries (Miyanaga et al., 2000).

MeJA has been extensively employed in numerous plant species as an elicitor to augment flavonoid production in cell culture systems. For instance, MeJa has been introduced into culture media to elevate flavonoid production in Hypericum *perforatum* cell cultures (Cui et al., 2010), ginsenoside in American ginseng root hair cultures (Kochan et al., 2018), and triterpenoid in *Centella asiatica* (L.) Urb root hair cultures (Nguyen et al., 2019). In this investigation, we found that adding MeJa to the culture media for one to three weeks notably enhanced the

biomass of phenolic compounds and flavonoid accumulations in adventitious root cultures of *G. procumbens*. However, a more prolonged exposure time (4 weeks of elicitation) resulted in reduced biomass accumulation and concentration of phenolic compounds and flavonoids. Comparable results were documented in *Thevetia peruviana* cell suspension cultures, where the highest phenol and flavonoid concentrations were achieved after 72 and 96 hours of MeJa elicitation (Mendoza et al., 2018). Extended exposure times (7 days) with a high concentration of MeJa were observed to enhance the production of saponin and ginsenoside in American ginseng root hair cultures (Kochan et al., 2018), while a lower MeJa concentration (100 µM) increased the accumulation of ginsenoside in *Panax ginseng* adventitious roots (Kim et al., 2004). Prolonged exposure to MeJa has bfeen reported to inhibit the production of secondary metabolites in *Centella asiatica* (L.) Urb. root hairy cultures (Nguyen et al., 2019) and *Thevetia peruviana* cell suspension cultures (Mendoza et al., 2018). This adverse effect may be attributed to the direct toxic impact of MeJA or a loss of culture viability.

In our research, we observed an increase in both MDA and proline levels following MeJa elicitation. Adventitious root cultures can face stress due to the high levels of agitation and aeration present in an in vitro system like the BTBB. This *in vitro* environment induces oxidative stress in plant cells due to the heightened presence of reactive oxygen species (ROS). ROS compounds are detrimental to plant cells and can lead to the degradation of lipid membranes, proteins, and DNA (Davey et al., 2005; Esfandiari et al., 2007). MDA, a byproduct of lipid peroxidation caused by ROS, serves as a marker for oxidative stress. An elevation in MDA concentration was noted in adventitious roots of *Hypericum perforatum* when cultured in MS media containing a high nitrate to ammonium ratio and high sucrose concentration (5-9%) (Baque et al., 2014)

Proline, a proteinogenic amino acid crucial for plant primary metabolism, acts as an osmolyte protectant. Additionally, proline stabilizes proteins and mitigates the detrimental effects of ROS under stressful conditions (Kishor, 2015). It has been documented that proline accumulates in liquid culture media when plant cells or tissues are exposed to mineral stress (Baque et al., 2014), osmotic stress (Lee & Paek, 2012), or addition of hormones into the media. Furthermore, a high concentration of inorganic salts in culture media resulted in increased proline concentration in *Spathiphyllum cannifolium* (Dewir et al., 2005).

CONCLUSION

Overall, we have established a simple and robust *in vitro* system for *G. procumbens* adventitious roots using BTBB. The growth of adventitious roots of *G. procumbens* and its biomass were influenced by MeJa elicitation. The highest biomass was attained at 3 weeks of MeJa elicitation compared to six weeks of culture without MeJa treatment in BTBB. The concentration of phenol, kaempferol, and quercetin MeJa-treated adventitious root cultures were 2.2-, 2.6-, and 3.5-fold higher than non-treated control after 3 weeks of culture. MDA and proline concentrations in MeJa-treated adventitious root cultures were higher than in non-treated control, indicating the importance of MeJa in alleviating oxidative stress. The pH, total sugar level, and electrical conductivity of the culture media were decreased at the end of the culture period. Our research outcomes carry significant implications for forthcoming biotechnological applications, particularly for large-scale production of valuable bioactive compounds intended for industrial use.

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