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Phytochemicals and Antioxidant Activities of Conventionally Propagated Nodal Segment and In Vitro-Induced Callus of *Bougainvillea glabra* Choisy Using Different Solvents

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Abstract: *Bougainvillea*, popularly known as 'Bunga kertas' in Malaysia, is thoroughly explored for nutritional and medicinal purposes. *Bougainvillea* has been shown to possess alkaloids and flavonoids which are widely used in folk medicine to treat different illnesses such as inflammatory, diarrheal, ulcer, and diabetic. Despite its major conventional therapeutic importance, only limited attempts have been made to investigate this species' chemical and pharmacological properties in relation to its medicinal uses. Therefore, this study was conducted to determine the effect of in vitro-induced callus under different light conditions and plant growth regulators on phytochemical and antioxidant activities using different extraction solvents. Based on the results, the maximum days (17.67) to callus initiation were recorded when nodal was cultured on woody plant medium (WPM) supplemented with 7.5 µM 2,4-D + 0.5 µM BAP under light condition. On the contrary, the minimum days (7) to callus initiation were obtained when nodal was treated with 2.5 and 5 µM 2,4-D + 1 and 1.5 µM BAP under dark conditions. However, higher fresh and dry weight of callus was obtained when nodal was cultured on woody plant medium fortified with 7.5 µM 2,4-D + 1.5 µM BAP under dark and light conditions. In the analysis of the phenolics content and antioxidant activities, aqueous extract of conventionally propagated nodal part exhibited the highest phenolic content and antioxidant activities. However, the highest iron (II) chelating activity was produced from the aqueous extract of the calli induced under a dark condition. Hence, it can be concluded that the callus culture of *Bougainvillea* produced plant secondary metabolites and antioxidant activities comparable to the mother plants.

Keywords: ornamental plant; cytokinin; auxin; incubation condition; callus induction; antioxidant; phenolic

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1. Introduction

Medicinal plants or herbs are enriched in various phytochemicals, which have been shown to have various biological effects and should be researched further [1,2]. The World Health Organization (WHO) recognized the importance of medicinal plants in millions of people's primary care. It is estimated that more than 80% of the world's population relies on these resources as their primary source of health-related problems [3]. One of the medicinal plants that has currently gain much attention because of its therapeutic constituents is *Bougainvillea glabra* [4]. The genus of *Bougainvillea* belongs to the family Nyctaginaceae, which is one of the utmost valuable ornamental and medicinal plants native to South America (Brazil, Peru, and northern Argentina). The name derives from the French navigator Louis Antoine de Bougainville, who was the first to discover this plant in Brazil in 1786 [5,6]. *B. glabra* is a perennial and evergreen shrub that is widely growing in

warm climates like Indonesia, Ethiopia, Philippines, Thailand, Malaysia, Vietnam, Taiwan, India, Australia, Mexico, South Africa, United States, Central America, Caribbean, and the Mediterranean [7].

Species of the *Bougainvillea* genus are frequently explored for their nutritional and therapeutic properties. The plants of the *Bougainvillea* species are considered to be utilized in traditional medicine for a variety of ailments, as phytopharmacological studies have reported, including anti-inflammatory and antipyretic [8], antidiarrheal, pain ailments and antiulcer [9], antimicrobial [10], antidiabetic [11], immunomodulatory [12], hypoglycaemic [13], antihyperlipidemic [14], anti-cough, sore throat, blood vessel troubles, leucorrhoea, hepatitis [15], anti-skin problems (tyrosinase) [16], analgesic [17], antiviral [18], antifungal [19], neuroprotective [4], and anthelmintic [20]. Various parts of the *Bougainvillea* plants are used to treat various ailments. In Panama, the flowers are used for treating hypotension. In India, leaves, flowers, and stem barks treat many illnesses, including stomach acidity, diarrhea, cough, blood vessel problems, sore throat, and hepatitis. In Thailand, the flowers treat stomachache, nausea, and diarrhea [1].

According to the report by Abarca-Vargas and Petricevich [15] on *Bougainvillea glabra*, 35 volatile compounds, 4 phenolic compounds, and 21 flavonoids compounds were extracted from leaves, bracts, and branched. Thus, the demand for raw materials is increased in tropical areas with temperate and cool weather. *B. glabra* is vegetatively propagated by stem cuttings. However, in the traditional propagation method, the production of secondary metabolites is known to be unsuitable due to external factors in the environment such as climate, plant pests and diseases, and fertilizer application. Therefore, the plant tissue culture technique is the most suitable technique for the consistent production of secondary metabolites under a controlled condition. A massive amount of raw materials is needed to extract the high amount of secondary metabolites which is not possible to be produced through conventional methods due to land availability. This problem can be overcome by producing the secondary metabolites in the laboratory by plant tissue culture technique [21]. Plant tissue culture is the most effective way to propagate rare, endangered, and valuable medicinal and commercial plant species on a wide scale in a short time while also protecting them [22]. Furthermore, callus formation investigations are helpful in understanding the metabolic pathways of secondary metabolites. On the other hand, the selection of plant growth regulators (PGRs) and environmental (cultural) conditions have an impact on callus growth in culture. Specific PGRs at appropriate concentrations can play an important role during callogenesis. Auxin and cytokinin type and concentration are essential determinants of in vitro callogenesis and regeneration. The ratio of auxin to cytokinin is the most crucial factor in this case [23]. Because the extraction of secondary metabolites requires a considerable amount of biomass, tissue culture using multiple shoots and callus induction is one of the methods used.

Extraction is the first and vital step in analyzing secondary metabolite constituents from plant materials [24]. Due to the presence of diverse molecules with varying chemical features, the polarity of the solvent used for extraction and the method of extraction play critical roles in both the efficiency and efficacy of plant secondary metabolites [24–28]. Moreover, extraction from plant products is complicated and difficult due to the vast range of structures and polarity of chemical compounds. Solvent, time, solid-to-solvent ratio, number of extractions, temperature, and partial size of the sample material are all important extraction parameters [29].

As far as we know, there are not enough studies on the influence of white light and dark on callus induction and phytochemical/antioxidant activity in *B. glabra.* Hence, we studied the effect of light quality on morphological and biochemical components of in vitro grown node-derived callus cultures of *B. glabra*. This research will help understand the effect of light on the production of commercially essential secondary metabolites and their optimization in the in vitro cultures of *B. glabra*.

2. Materials and Methods

2.1. In Vitro Callus Induction

2.1.1. Plant Materials and Sterilization

The nodal segments of conventionally propagated plants of *Bougainvillea glabra* Choisy were collected from the campus, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia. The samples of *B. glabra* plants used in the study were deposited at Biodiversity Unit, Institute of BioScience, Universiti Putra Malaysia with a plant confirmation voucher number MFI 0197/21.

For the sterilization process, after removing the leaves from cut branches, nodal segments were cut into pieces (0.5–1 cm) and the explants were washed under running tap water containing a few drops of detergent for 30 min to remove dust particles. After that, the explants were washed with sterile distilled water once and put in a glass jar containing 500 mg/250 mL (*w/v*) streptomycin + 500 mg/250 mL (*w/v*) bavistin for pre-treatment of the explants for one hour [30]. Then, the explants were disinfected by immersion in 70% ethanol for 25 s and washed two times with autoclaved distilled water. Then, the explants' surface was sterilized with a bleaching agent with a concentration of 30% of Clorox® (5.25% (*w/v*) of sodium hypochlorite and a few drops of tween-20 as an emulsifier per 100 mL solution for 15 min by using a shaker with 300 rpm [31]. After that, the explants were washed three times with autoclaved distilled water and prepared for culturing.

Chemicals and Reagents

Folin-Ciocalteu reagent, potassium persulphate, iron (II) chloride, sodium hydroxide, ammonium chloride, gallic acid, sodium nitrite, sodium carbonate, dimethyl sulfoxide (DMSO), hexane, methanol, acetone, and ethyl alcohol were purchased from (R&M Chemical, Selangor, Malaysia). 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) ABTS was purchased from (Alfa Aesar, Mumbai, India). 2,2-diphenyl-1-picrylhydrazyl DPPH (Sigma-Aldrich, Baden-Württemberg, Germany). Ferrozine reagent (Acros Organics, Burgenland, Austria), rutin hydrate (Sigma, China), Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) (Sigma-Aldrich, Shanghai, China). N6-benzylaminopurine (R&M Chemical, Cardiff, UK), 2,4-dichlorophenoxyaceticacid (BDH, Poole, UK), streptomycin sulfate (Sigma, St. Louis, MO, USA), gelrite (Duchefa Biochemie, Haarlem, The Netherlands), Clorox® (Clorox Sdn. Bhd., Kuala Lumpur, Malaysia), Polysorbate 20 or Tween 20 (YKL Multi Sdn. Bhd., Bukit Mertajam, Malaysia) were also used in the study. All of the chemicals and reagents were of analytical grade.

2.1.2. Preparation of Basal Medium, Aseptic Condition and Glassware

A basal medium was prepared using a formulation described by Lloyd and McCown (WPM) (1981). The basal medium was fortified with 30 g/L sucrose, 3.0 g/L gelrite as a gelling agent, and pH was adjusted to 5.7 ± 0.5 using 1 M sodium hydroxide (NaOH) or 1 M hydrochloric acid (HCl). Then, the basal medium was autoclaved at a temperature of 121 °C for 20 min at a pressure of 1.05 kg/cm^2. The laminar airflow chamber was exposed to ultraviolet (UV) light for 30 min to sterilize the surface of the working area.

The glassware used for the culture comprised of 35 mL culture tubes for surface sterilization with 10 mL of solid WPM medium and 150 to 250 mL conical flasks, 300 mL modified jars with transparent polypropylene caps with 30 mL of solid WPM medium for the callus induction experiment. Before using the glassware, they were thoroughly washed under running tap water with liquid detergent and then rinsed with distilled water. Next, the clean glassware was sterilized by autoclaving at 121 °C and 104 kPa pressure for 20 min.

2.1.3. Callus Induction as Affected by Cytokinin and Auxin

The survived nodal explants were selected after four weeks for callus induction under different culture conditions. The prepared basal medium was supplemented with the combination of auxin; 2,4-dichlorophenoxyacetic acid (2,4-D) at a concentration of (2.5, 5,

and 7.5 µM) and cytokinin; 6-benzylaminopurine (BAP) at a concentration of (0.5, 1, and 1.5 µM). The WPM basal medium without PGRs served as control to find out and compare the influence of PGRs on callus induction of *B. glabra* under different cultural conditions. Each treatment of this experiment was replicated three times, with 9 explants per replication and every three explants cultured in a 250 mL conical flask (Figures 1 and 2A) for callus initiation, but after the callus initiated, only two calluses derived from nodal segment cultured in a 250 mL conical flask (Figures 1 and 2B–E) in order to have better access to the media and space. The data on days to callus initiation were taken every day but the data such as callus frequency, callus morphology, fresh and dry weight of the callus were taken after every four weeks of incubation four times for, in total, 16 weeks. The following Equation (1) was used for determining the callus induction frequency as given below [32]:

$$\text{Callus induction frequency } (\%) = \frac{\textit{Number of explants induced callus}}{\textit{Number of explants cultured}} \times 100 \quad (1)$$

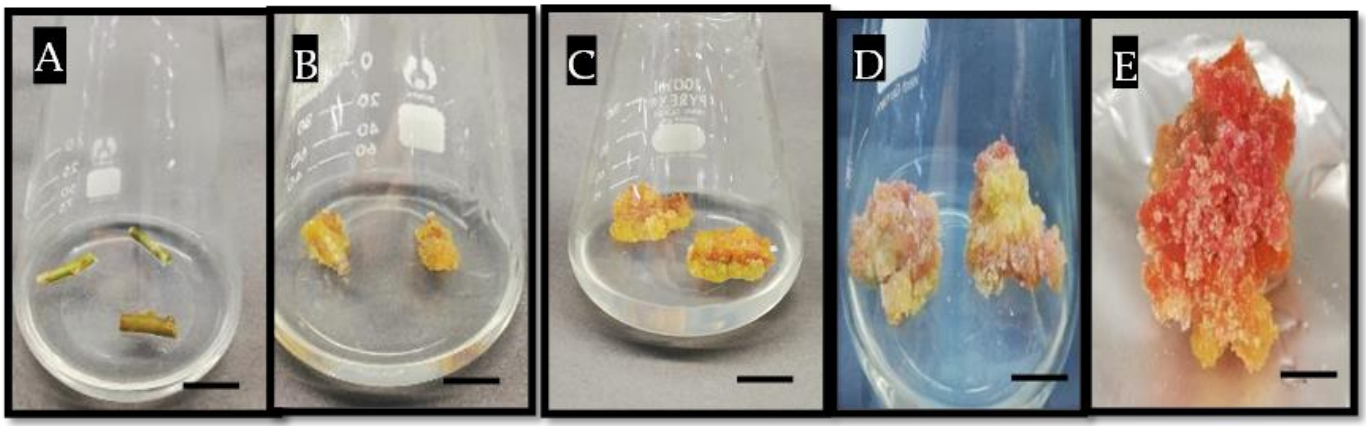

Figure 1. In vitro callus induction of *Bougainvillea glabra* Choisy under light incubation condition. (**A**) Sterilized nodal segment on WPM basal medium; (**B**) callus induction from a node section on WPM medium supplemented with 5 µM 2,4-D + 1 µM BAP after 4 weeks; (**C**) callus after 6 weeks in the same medium and PGRs condition; (**D**) callus after 8 weeks; (**E**) callus induction from a node section on WPM medium fortified with 7.5 µM 2,4-D + 1.5 µM BAP after 10 weeks with a red spot.

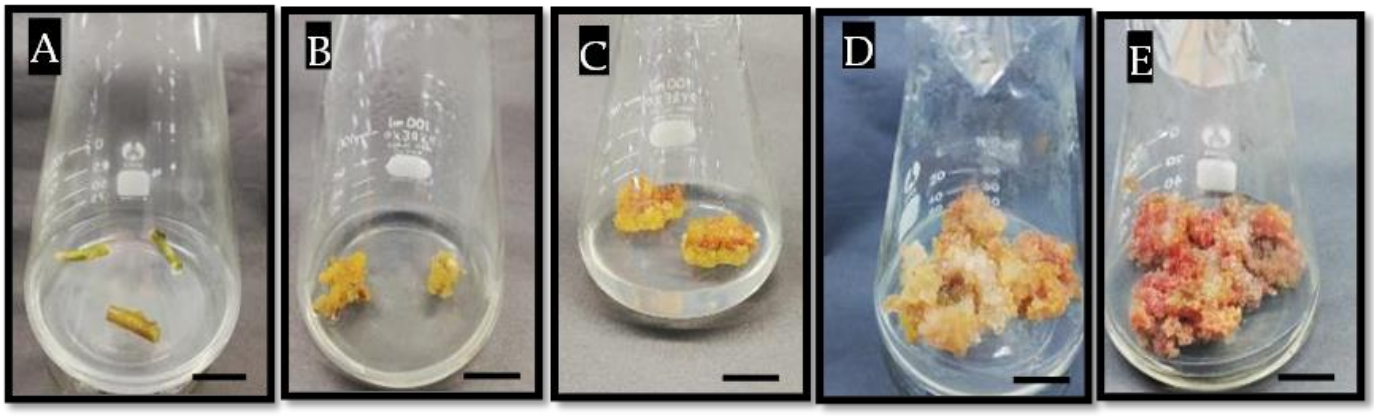

Figure 2. In vitro callus induction of *Bougainvillea glabra* Choisy under dark incubation condition. (**A**) Sterilized nodal segment on WPM basal medium; (**B**) callus induction from a node section on WPM medium supplemented with 2.5 µM 2,4-D + 1.5 µM BAP after 3 weeks; (**C**) callus after 4 weeks in the same medium and PGRs condition; (**D**) callus after 8 weeks; (**E**) callus induction from a node section on WPM medium fortified with 7.5 µM 2,4-D + 1.5 µM BAP after 10 weeks with a red spot.

2.1.4. Culture Maintenance

All the cultures were incubated in a culture room maintained at a temperature of 25 ± 2 °C under 16 h light and 8 h dark using white fluorescence light irradiation of 45 µmol/m^2/s or complete dark. Subculture of experimental materials (developing culture) was done every four weeks after each culture. Similar basal medium and PGRs composition from previous culture was used.

2.1.5. Fresh and Dry Weight of Callus

Growth parameters were made in terms of an increase in the fresh and dry weights of cultured tissue. The cultured tissue was carefully taken from the culture vessel and cleaned of agar particles that had adhered at the point of contact. After that, the tissue was placed on pre-weighed aluminum foil and the weight was calculated using a single pan digital balance of fresh weight of callus which presented as gram (g). The tissues were oven-dried at 55 °C to a consistent weight on the same foils for estimation of their dry weight as milligrams (mg) after recording their fresh weight.

2.2. Quantification of Phenolic Content and Antioxidant Activities of B. glabra Nodal Segments and In Vitro-Induced Calli

2.2.1. Planting Materials and Preparation of Extract

The nodal segments of conventionally propagated plants of *B. glabra* were collected from the Faculty of Agriculture, Universiti Putra Malaysia. In addition, the treatment of 7.5 µM 2,4-D + 1.5 µM BAP under dark and light incubation conditions which exhibited the highest biomass accumulation in vitro were obtained after 16 weeks in the Tissue Culture Laboratory, Faculty of Agriculture, Universiti Putra Malaysia were selected as a suitable treatment and quantified for phenolic content and antioxidant activities. The collected samples were oven-dried at a temperature of 55 °C for 48 h or until the weight remained constant. The dried nodes and in vitro-induced calluses were used to determine phenolic content and antioxidant properties. Furthermore, the extraction was conducted following the method employed by Hakiman and Maziah [33] with minor modifications. Dried nodes and in vitro-induced callus samples of *B. glabra* were ground using a commercial blender (Brand: Panasonic). An amount of 1 g dry weight of each sample was weighed and placed in a 150 mL conical flask. Each solvent, such as distilled water, ethanol, acetone, and hexane, received a total volume of 50 mL, and the flasks were covered with aluminum foil. The samples were placed in conical flasks on an orbital shaker at room temperature for 1 h in the dark. The samples were filtered with Whatman No. 1 filter paper and the extract was used for further analysis.

2.2.2. Total Phenolic Acids Content

Total phenolic acid content was evaluated following a method proposed by Singleton and Rossi [34]. To begin, test tubes were filled with 0.5 mL of extracts and 4.5 mL of distilled water. Then, 0.5 mL of Folin–Ciocalteu phenol reagent was added and thoroughly mixed using a vortex machine. After 5 min, 5 mL sodium carbonate (7%) was added. By adding 2 mL of distilled water, the final volume was adjusted to 12.5 mL. At room temperature, the reaction mixtures were incubated for 90 min. At 750 nm, the absorbance was measured. By establishing a standard curve of absorbance against various amounts of gallic acid, the total phenolic acids content was measured. The total phenolic acids content of the extract was measured in milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

2.2.3. Total Flavonoids Content

Total flavonoids content was generated using the method established by Marinova et al. [35]. First, 0.5 mL of extracts was added to 2 mL of distilled water in test tubes. After that, 150 µL of 5% sodium nitrite was added to the mixture, which was then incubated for 5 min after 150 µL of 10% aluminum chloride was added. After that,

1 mL of 1 M sodium hydroxide and 1.2 mL distilled water were added at the sixth minute. A spectrophotometer was used to measure absorbance at 510 nm after the mixture was properly mixed. To assess the level of the total flavonoid content of the extract, a standard curve of absorbance against various concentrations of rutin was established. The extract's total flavonoids concentration was calculated as mg rutin equivalents per gram dry weight of the sample (mg RE/g DW).

2.2.4. DPPH Free Radical Scavenging Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay was determined to quantify the ability of the DPPH molecule to neutralize free radicals by donating a hydrogen atom. Thus, the color shift from purple to violet is visible. DPPH free radical scavenging assay was performed according to the procedure explained by Wong et al. [36]. The DPPH was first generated in methanol at a concentration of 0.1 mM, and the initial absorbance of methanolic DPPH was measured with a spectrophotometer at 515 nm. The extracts were then combined in 1.5 mL of 0.1 mM methanolic DPPH solution. After shaking the mixture and incubating it at room temperature for 30 min, the absorbance was measured at 515 nm. The control was Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), and the DPPH value was measured in mg Trolox equivalent per gram dry weight of the sample (mg TE/g DW)

2.2.5. ABTS Scavenging Activity

The ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) scavenging activity was determined following Re et al. [37]. Before the experiment, 7 mM ABTS stock solution was mixed in a ratio of 1:1 with 2.45 mM potassium persulfate and incubated at room temperature for 16 h in the dark. The $ABTS^+$ solution was diluted with ethanol after incubation until the absorbance was detected at 0.700 ± 0.05 at 734 nm. The extract was then combined with 0.9 mL of diluted $ABTS^+$ solution. After 15 min, the absorbance of the reaction mixture was measured at 734 nm. As a control, Trolox was utilized. The ABTS scavenging activity was measured in milligrams of Trolox equivalent per gram of dry weight (mg TE/g DW).

2.2.6. Iron (II) Chelating Activity

Iron (II) chelating activity was conducted by the procedure described by Dinis et al. [38] with minor modifications. An amount of 400 µL of the extract was mixed with 50 µL of 2 mM ferrous chloride. The reaction was started by adding 200 µL of 5 mM ferrozine to the reaction liquid and incubating it at room temperature for 10 min. The mixture's absorbance was measured at 562 nm in comparison to a blank sample. The decrease in absorbance was due to increased iron chelating activity. The following Equation (2) was used to calculate the iron (II) chelating activity:

$$\text{Chelating (\%)} = (1 - \text{A562 Sample}/\text{A562 Control}) \times 100 \quad (2)$$

2.3. Experimental Design and Statistical Analysis

The data were analyzed using the Statistical Analysis System (SAS) ver. 9.4. All of the experiments were performed in triplicates in a two or three-factorial Completely Randomized Design (CRD) and the results are expressed as mean $\pm$ SE. Duncan's Multiple Range Test (DMRT) was used to compare means at a $p = 0.05$ level. Pearson's correlation analysis was used to determine the correlation between variables with indicator $r < 0.25$ indicating a weak correlation, $r < 0.75$ indicating an intermediate correlation, $r < 1$ indicating a strong correlation, $r = 1$ indicating perfect correlation.

3. Results

3.1. In Vitro Callus Induction of Bougainvillea glabra via Nodal Segment

3.1.1. The Main Effect of 2,4-D, BAP and Light Regimes on Callus Induction of *B. glabra*

Results from the present study showed that from the different concentrations of 2,4-D, the minimum days for callus initiation was obtained in the treatment of 5 µM 2,4-D but it was not significantly different from the treatment of 2.5 µM 2,4-D. Meanwhile, the maximum days for callus initiation were recorded from the treatment of 7.5 µM 2,4-D. However, for the treatments of BAP, the minimum days for callus initiation was exhibited from the 1.5 µM BAP but it was not significantly different from 0.5 and 1 µM BAP, respectively. The type of explants to induce callus is also an important factor that needs to be considered in callus induction experiments due to various factors affecting the development of cell culture systems, such as genotype, explant type, plant growth regulators (PGR), culture medium, and culture condition. Based on the results in (Table 1), with WPM medium supplemented with various concentrations of 2,4-D, an increment of the callus frequency was observed as 2,4-D concentration increased until 5 µM. The highest callus frequency was recorded using 5 µM 2,4-D, which is significantly different from 7.5 µM 2,4-D, but it was not significantly different from 2.5 µM 2,4-D. On the other hand, there was no callus formation on the control media without plant growth regulators, indicating that growth regulators had a significant effect on callus induction on the nodal explant. Following that, there was a decrease in percent callus induction growth, followed by an increase of the concentrations of 2, 4-D, and BAP.

Table 1. Main effects of 2,4-D and BAP on callus induction in the nodal segment of *Bougainvillea glabra* under different light conditions.

Treatment	Days to Callus Initiation	Callus Frequency (%)	FW of Callus (g)	DW of Callus (mg)
Control	—	—	—	—
2,4-D (µM)				
2.5	12.09 ± 0.72 b	88.54 ± 1.89 a	2.98 ± 0.17 c	122.12 ± 3.76 c
5	11.72 ± 0.76 b	90.08 ± 1.57 a	3.85 ± 0.15 ab	164.58 ± 8.90 a
7.5	14.26 ± 0.82 a	76.04 ± 0.77 b	4.06 ± 0.26 a	150.09 ± 6.77 b
BAP (µM)				
0.5	13.02 ± 0.85 a	86.39 ± 2.56 a	3.15 ± 0.19 b	132.89 ± 8.13 b
1	12.79 ± 0.79 a	85.67 ± 1.69 a	3.51 ± 0.12 b	140.39 ± 19.10 b
1.5	12.73 ± 0.86 a	81.77 ± 2.56 b	4.23 ± 0.24 a	167.50 ± 12.48 a
Culture Condition				
Light	14.73 ± 0.73 a	75.69 ± 3.81 b	3.52 ± 0.23 a	141.56 ± 17.44 a
Dark	8.45 ± 0.73 b	81.03 ± 4.05 a	3.43 ± 0.20 b	132.77 ± 8.63 b
F-value				
2,4-D	744.57 ***	3020.89 ***	121.66 ***	143.7 ***
BAP	6.72 ***	17.97 ***	16.42 ***	15.05 ***
Culture Condition	1836.00 ***	117.57 ***	5.91 **	4.35 *
2,4-D*BAP	22.19 ***	83.51 ***	31.82 ***	42.68 ***
2,4-D*Condition	53.02 ***	4.19 **	0.40 ns	0.81 ns
BAP*Condition	27.05 ***	1.38 **	2.48 ns	1.56 ns
2,4-D*BAP*Condition	7.5 ***	6.35 ***	0.96 ns	0.53 ns
CV (%)	5.58	2.77	12.56	12.15

Values are means ± SE. the same letter within the same column for each factor indicates no significant difference ($p < 0.05$). The means separation is done by using Duncan's multiple range test (DMRT). F value represented * = <0.05, ** = $p < 0.01$, *** = $p < 0.001$ and ns = no significant differences. 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyl amino purine (BAP), FW (Fresh Weight), and DW (Dry Weight).

In terms of biomass accumulation, maximum callus biomass was recorded when the nodal explants were cultured on WPM medium supplemented with 5 µM 2,4-D. However, for the treatments of BAP, 1.5 µM BAP exhibited significantly higher callus biomass, respectively. Based on the results, it was seen that there was an increase in the fresh and dry weight of callus as the concentration of BAP was increased up to 1.5 µM, but for the

treatments of 2,4-D, the callus biomass increased as the 2,4-D increased until 5 µM and significantly decreased with 7.5 µM. Primary metabolism, particularly carbon and nitrogen metabolism, is closely linked to biomass accumulation. Carbon metabolism meets the energy requirements resulting from carbohydrate synthesis, and hence, contributes to cell growth and structural components [39]. The products of nitrogen metabolism, on the other hand, are primarily amino acids and proteins, which are then used to regulate cellular processes [40]. Over all, both cellular development and division result in increased fresh and dry weight.

The callus induction of *B. glabra* was also evaluated under two growth conditions, dark and light. The results showed that the dark condition was more favorable for earlier callus initiation and higher callus frequency than the light condition. However, the calluses induced under light incubation conditions produced significantly higher callus fresh and dry weight than the calluses induced under complete dark incubation conditions. Based on the observation in this experiment, the nodal segments incubated under complete dark conditions grew faster than in a light-containing photoperiod. In the dark condition, the minimum days for callus initiation was (8.45), which is significantly different from the light condition with (14.73) days. In addition, the higher callus frequency was also obtained from the cultured incubated under the dark condition, which is significantly different from the cultured incubated under the light condition, respectively. However, callus grown under a set photoperiod presented the higher callus fresh and dry weight than callus incubated under the complete dark condition.

Based on the observation in this experiment, the interaction effects of various concentrations of 2,4-D and BAP were significant ($p < 0.05$) for all parameters, but the interaction effect between 2,4-D *Condition and BAP *Condition were only significant for the parameters such as days to callus initiation and callus frequency; also, the statistical analysis indicated that the triple interaction of 2,4-D, BAP, and culture condition was significantly different for days to callus initiation and callus induction rate, but was not significantly different for the biomass production (Table 1). A similar trend also was observed in the WPM medium supplemented with different concentrations of BAP. The maximum callus frequency was obtained from 0.5 µM and 1 µM BAP, respectively. However, by increasing the BAP concentration, the callus frequency decreased.

3.1.2. Synergistic Effect of Cytokinin, Auxins, and Light Regime on Callus Induction

The results in Table 2 exhibit that the minimum days of callus initiation were recorded from the treatments of 2.5 µM 2,4-D + 1.5 µM BAP and 5 µM 2,4-D + 1 µM BAP under the dark incubation condition, respectively. However, the maximum days for callus initiation were recorded from the treatment of 7.5 µM 2,4-D + 0.5 µM BAP under light incubation conditions. As shown, the period of callus induction and growth of callus varied. They depend on the type and concentration of growth regulators and lighting conditions. Meanwhile, the highest callus induction rate was produced from the combination of 2.5 µM 2,4-D + 1.5 µM BAP under a dark incubation condition and 5 µM 2,4-D + 0.5 µM BAP under both light and dark incubation conditions, respectively. However, the lowest callus induction rate was obtained from the treatments of 7.5 µM 2,4-D + 1.5 µM BAP under both light and dark conditions.

In this experiment, for the WPM medium fortified with various concentrations of 2,4-D and BAP, a different trend of callus fresh and dry weight was observed. The callus fresh and dry weight was drastically increased by three-fold in the treatment of 7.5 µM 2,4-D + 1.5 µM BAP under dark incubation condition but it was not significantly different from the same treatment under the light condition. As the concentration decreased to 2.5 µM 2,4-D and 0.5 µM BAP, the callus fresh weight was decreased under dark and light incubation conditions, respectively. Overall, no significant biomass differences were observed for light regimes.

Table 2. Interaction effects of 2,4-D and BAP on callus induction in the nodal segment of *Bougainvillea glabra* under different light conditions.

Condition	2,4-D (µM)	BAP (µM)	Days to Callus Initiation	Callus Frequency %	FW of Callus (g)	DW of Callus (mg)	Callus Morphology
Light	Control	0	—	—	—	—	—
	2.5	0.5	15.17 ± 0.44 fg	78.33 ± 1.67 de	2.53 ± 0.15 hi	120.00 ± 11.54 fg	Y, B, & C
		1	15.33 ± 0.44 ef	76.67 ± 1.67 def	3.21 ± 0.24 fgh	141.67 ± 20.48 de	Y, B, & C
		1.5	14.12 ± 0.56 g	93.33 ± 1.67 b	3.03 ± 0.03 gh	93.33 ± 3.33 i	Y, B, & C
	5	0.5	16.01 ± 0.18 def	100.00 ± 0 a	3.67 ± 0.17 defgh	165.00 ± 7.63 bc	Y, B, & C
		1	13.00 ± 0.58 h	86.67 ± 1.67 c	3.7 ± 0.53 defg	113.33 ± 8.82 ghi	Y, B, & C
		1.5	16.00 ± 0.58 def	80.67 ± 1.2d	3.77 ± 0.34 defg	156.00 ± 7.81 bcd	Y, B, & C
	7.5	0.5	17.67 ± 0.88 ab	73.33 ± 1.67 f	3.00 ± 0.06 gh	116.67 ± 8.81 fgh	R, Y, & C
		1	16.73 ± 0.50 bcd	73.33 ± 1.67 f	3.55 ± 0.24 efgh	146.67 ± 9.28 de	R, Y, & C
		1.5	17.27 ± 0.43 bcd	73.33 ± 1.67 f	5.23 ± 0.16 a	221.67 ± 13.01 a	R, Y, & C
Dark	Control	0	—	—	—	—	—
	2.5	0.5	10.17 ± 0.30 j	86.67 ± 1.67 c	2.11 ± 0.39 i	100.00 ± 5.7 hi	Y, B, & F
		1	10.58 ± 0.33 ij	80.00 ± 1.53 d	3.68 ± 0.16 defgh	159.00 ± 2.3 bc	Y, B, & F
		1.5	7.00 ± 0 k	100.00 ± 0 a	3.30 ± 0.3 fgh	118.00 ± 18.9 fgh	Y, B, & F
	5	0.5	8.00 ± 0.12 k	100.00 ± 0 a	4.36 ± 0.46 bcd	182.00 ± 9.6 bc	W, B, & F
		1	7.00 ± 0 k	93.33 ± 1.2 b	2.93 ± 0.03 h	126.67 ± 3.38 ef	R, Y, & F
		1.5	10.28 ± 0.17 j	93.33 ± 0.88 b	4.71 ± 0.54 abc	185.33 ± 12.81 b	R, Y, & F
	7.5	0.5	11.11 ± 0.11 ij	80.00 ± 1.53 d	3.25 ± 0.05 fgh	113.67 ± 3.18 fgh	Y, B, & F
		1	11.08 ± 0.22 ij	80.00 ± 1.53 d	3.98 ± 0.02 cdef	154.67 ± 3.18 cd	R, Y, & F
		1.5	11.72 ± 0.03 i	73.33 ± 0.67 f	5.33 ± 0.16 a	230.67 ± 5.20 a	W, B, & F

Values are means ± SE. the same letter within the same column for each factor indicates no significant difference ($p < 0.05$). The means separation is done by using Duncan's multiple range test (DMRT). 2,4-dichlorophenoxyacetic acid (2,4-D), and 6-benzylaminopurine (BAP). W white, Y yellow, R red, B brown, F friable, and C compact.

Based on the results, the morphogenesis responses based on the intensity of callus formation, texture, types, and concentration of PGRs, cultural condition, and in vitro morphogenesis were different according to the treatment used and are presented in (Table 2) and (Figures 1 and 2A–E). The cultural condition was capable of inducing a callus that could be classified into two types. The first type, which was induced under light incubation conditions, was compact and yellow to brown and red (Figure 1A–E). The second type, which was induced under dark incubation conditions, was friable and yellow to white and brown (Figure 2A–E).

3.2. Quantification of Phenolics Contents and Antioxidant Activities of In Vitro-Induced Calli and Conventionally Propagated Plant of Bougainvillea glabra

3.2.1. Total Phenolic Acid and Total Flavonoid Content

The results showed that the content of total phenolic acid, total flavonoid, and antioxidant properties except for iron (II) chelating activity of conventionally propagated nodal segment of *B. glabra* were significantly different ($p \leq 0.05$) from the in vitro-induced calli under light and dark incubation conditions (Tables 3–5). The aqueous extract of the node recorded the highest total phenolic acid followed by aqueous extract of the calli induced under photoperiod. In addition, there were no significant differences between the aqueous extract of calli induced under the dark condition and the ethanol extract of node and calli induced under photoperiod and dark conditions. The results also showed no significant differences between acetone extract of the node and calli induced under photoperiod and dark conditions. Meanwhile, the lowest amount of TPC was obtained from hexane extract of the node, but there were no significant differences between hexane extract of node and calli induced under photoperiod and dark conditions.

Table 3. Total phenolic acids and flavonoids contents of node and in vitro-induced calli of *Bougainvillea glabra*.

Source of Sample	Type of Solvent									
	Phenolic Acids (mg GAE/g DW)					Flavonoids (mg RE/g DW)				
	Aqueous	**Ethanol**	**Acetone**	**Hexane**	**Mean**	**Aqueous**	**Ethanol**	**Acetone**	**Hexane**	**Mean**
Node	21.88 ± 0.57 a	3.33 ± 0.38 cd	1.84 ± 0.22 ef	0.25 ± 0.05 g	6.82 ± 1.87 A	42.05 ± 0.18 a	21.46 ± 0.31 b	8.92 ± 0.04 d	1.00 ± 0.04 i	18.36 ± 4.67 A
Callus induced in light	6.43 ± 0.26 b	2.73 ± 0.26 d	1.52 ± 0.03 ef	0.20 ± 0.02 g	2.72 ± 0.7 B	10.30 ± 0.11 c	8.46 ± 0.22 e	7.34 ± 0.14 f	0.92 ± 0.11 i	6.75 ± 1.07 B
Callus induced in dark	3.90 ± 0.17 c	2.63 ± 0.08 d	1.00 ± 0.27 f	0.25 ± 0.07 g	1.95 ± 0.43 C	6.67 ± 0.11 g	6.67 ± 0.15 g	4.46 ± 0.07 h	0.88 ± 0.17 i	4.67 ± 0.72 C
Mean	10.74 ± 1.83 A	2.90 ± 0.17 B	1.45 ± 0.16 C	0.24 ± 0.03 D		19.67 ± 5.62 A	12.20 ± 2.33 B	6.91 ± 0.65 C	0.93 ± 0.06 D	

Values are means ± SE, the same letter within the same column for each factor indicates no significant difference ($p < 0.05$). The means separation is done using Duncan's multiple range test (DMRT).

In total flavonoid content analysis, the highest value was recorded from aqueous extract of the node, followed by ethanol extract of the node. On the other hand, the lowest TFC was obtained from hexane extract of the node and calli induced under photoperiod, and dark conditions ranged between 0.88 and 1 mg RE/g DW, respectively.

3.2.2. Antioxidant Activities

The antioxidant activities of *B. glabra* nodal extracts, propagated via conventional method and in vitro-induced calli via tissue culture techniques, were measured using three in vitro antioxidant assays including 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging activity, 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and iron (II) chelating activity. All antioxidant assays were measured using a UV-Vis spectrophotometer at a specific absorbance. The results showed that DPPH free radical scavenging activity and ABTS scavenging activity of the conventionally propagated nodal segment were significantly different at $p \leq 0.05$ than in vitro-induced calli under dark and light incubation condition. However, the highest iron (II) chelating activity was obtained from in vitro-induced calli under a dark incubation condition. The DPPH free radical scavenging activity recorded from sources of samples and extraction solvents ranged between 0.14 to 7.64 mg TE/g DW. The highest DPPH free radical scavenging activity was obtained from aqueous extract of the node, followed by ethanol extract of the node, and aqueous extract of the calli induced under photoperiod. In addition, there were no significant differences between hexane extract with all of the samples. The lowest DPPH free radical scavenging activity was recorded from hexane extract of calli induced under photoperiod and dark conditions.

Table 4. 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical scavenging activity and 2,2-azino- bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity of node and in vitro-induced calli of *Bougainvillea glabra*.

Source of Sample	Type of Solvent									
	DPPH (mg TE/g DW)					ABTS (mg TE/g DW)				
	Aqueous	Ethanol	Acetone	Hexane	Mean	Aqueous	Ethanol	Acetone	Hexane	Mean
Node	7.64 ± 0.01 a	2.98 ± 0.11 b	1.01 ± 0.01 e	0.24 ± 0.08 gh	2.97 ± 0.68 A	1.51 ± 0 a	0.72 ± 0.01 d	0.58 ± 0.01 ef	0.46 ± 0.02 gh	0.82 ± 0.12 A
Callus induced in light	2.12 ± 0.03 c	1.37 ± 0.06 d	0.89 ± 0.05 f	0.19 ± 0.08 hi	1.14 ± 0.56 B	1.12 ± 0 b	0.68 ± 0.01 de	0.54 ± 0.03 fg	0.40 ± 0.01 j	0.69 ± 0.08 B
Callus induced in dark	0.80 ± 0.18 f	0.59 ± 0.07 g	0.40 ± 0.06 gh	0.14 ± 0.02 ij	0.48 ± 0.44 C	0.98 ± 0 c	0.40 ± 0.01 hi	0.30 ± 0.01 ij	0.16 ± 0.01 jk	0.46 ± 0.09 C
Mean	3.52 ± 0.64 A	1.64 ± 0.46 B	0.76 ± 0.31 C	0.19 ± 0.33 D		1.20 ± 0.08 A	0.60 ± 0.05 B	0.48 ± 0.04 C	0.34 ± 0.05 D	

Values are means ± SE, the same letter within the same column for each factor indicates no significant difference ($p < 0.05$). The means separation is done using Duncan's multiple range test (DMRT).

Table 5. Iron (II) chelating activity of node and in vitro-derived calli of *Bougainvillea glabra*.

Source of Sample	Type of Solvent				
	Iron (II) Chelating Activity (%)				
	Aqueous	Ethanol	Acetone	Hexane	Mean
Node	29.64 ± 0.77 b	20.08 ± 1.31 de	6.64 ± 2.18 h	17.02 ± 1.24 ef	18.35 ± 2.55 B
Callus induced in light	26.87 ± 2.45 bc	20.78 ± 0.73 de	5.39 ± 1.4 h	15.25 ± 1.12 fg	17.07 ± 2.47 B
Callus induced in dark	43.30 ± 0.13 a	22.78 ± 0.53 cd	11.96 ± 0.56 g	22.92 ± 1.7 cd	25.24 ± 3.44 A
Mean	33.27 ± 2.64 A	21.21 ± 0.61 B	8.00 ± 1.27 C	18.40 ± 1.35 D	

Values are means ± SE, the same letter within the same column for each factor indicates no significant difference ($p < 0.05$). The means separation is done using Duncan's multiple range test (DMRT).

In the analysis of ABTS scavenging activity, there was a significant interaction between the sources of samples and extraction solvents on ABTS scavenging activity. The highest ABTS scavenging activity was significantly obtained by aqueous extract of the node followed by aqueous extract of the calli induced under the photoperiod, and aqueous extract of the calli induced under the dark condition Meanwhile, there were no significant differences between ethanol extract of the node and calli induced under photoperiod conditions. In addition, there were no significant differences between ethanol, acetone, and hexane extract of the calli induced under dark conditions. On the other hand, the lowest ABTS scavenging activity was recorded from hexane extract of calli induced under dark conditions. Thus, all of these extracts had some ABTS radical scavenging abilities in their phytochemical components, and the contents may have some ABTS radical scavenging ability equivalence.

In the present study on iron (II) chelating activity, a significant interaction was found between extraction solvents and sources of samples. In contrast to other antioxidant activities conducted in which aqueous and other node extracts produced higher antioxidant activities, the iron (II) chelating activity was produced from the aqueous extract of the calli induced under dark conditions. The highest iron (II) chelating activity was produced from an aqueous extract of the calli induced under the dark condition. Meanwhile, there were no significant differences between the aqueous extract of the node and calli induced under photoperiod and hexane extract of the calli induced under dark conditions. In addition, there were no significant differences between ethanol extract of the node, calli induced under photoperiod, and dark conditions. Furthermore, the lowest iron (II) chelating activity was produced by the acetone extract of calli induced under the photoperiod

3.2.3. Correlation Analysis between Variables

Pearson's correlation analysis was used to examine the relationship between phenolic contents (phenolic acids and flavonoids) and antioxidant activities, such as DPPH free radical scavenging activity, ABTS scavenging activity, and iron (II) chelating activity (Table 6).

Table 6. Pearson's correlation analysis between variables.

Variable	TPC	TFC	ABTS	DPPH	Fe^{2+}
TPC	1				
TFC	0.92 **	1			
ABTS	0.90 **	0.80 **	1		
DPPH	0.79 **	0.87 **	0.84 **	1	
Fe^{2+}	0.46 **	0.30 ns	0.54 **	0.24 ns	1

Notes: **: Significant correlation at $p < 0.05$; ns: Non-significant correlation; TPC: Total phenolic acids content; TFC: Total flavonoids content; DPPH: DPPH free radical scavenging activity; ABTS: ABTS scavenging activity; $Fe2^{+}$: Iron (II) chelating activity, respectively.

The correlation showed that all variables except iron (II) chelating activity were positively correlated ranged from intermediate positive to a high positive correlation. The variables of total phenolic acids, flavonoids, DPPH free radical scavenging activity, and ABTS scavenging activity produced a highly significant strong correlation ($r > 0.75$). Meanwhile, iron (II) chelating activity showed a low positive correlation against TPC and ABTS ($r < 0.75$) and a non-significant correlation against TFC and DPPH free radical scavenging activity. The Pearson's correlation analysis showed that the phenolic compounds present in *B. glabra* nodes and calli induced under different light condition extract are strong as scavenging and chelating agents.

4. Discussion

4.1. In Vitro Callus Induction of B. glabra

Bougainvillea glabra is an important medicinal plant with high medicinal values. Plants propagated via traditional methods are vulnerable to a variety of diseases and pests, as well as weather and land availability, all of which have an adverse effect on the medicinal qualities of the harvested plants [41]. The overall objective of this study was to induce calli under different PGRs and cultural conditions and evaluate the secondary metabolites content of the in vitro-induced calli and conventionally propagated plant. In the in vitro callus induction, plant growth regulators are one of the most important factors affecting the growth of explants. Synthetic auxins, such as 2,4-D, are important PGRs that are applied in a variety of embryogenic cell and tissue culture methods, as well as callus production and cell suspension culture [42]. Meanwhile, some research has confirmed the positive effect of 2,4-D on callus formation during the physiological and molecular process in many circumstances, and those studies have shown that 2,4-D regulates the endogenous IAA metabolism, promotes specific proteins, and controls DNA methylation [43]. On the other hand, cytokinin plays an important part in inducing callus by promoting cell division and differentiation [44].

In the present study, the minimum days of callus initiation (7) was recorded from the treatments of 5 µM 2,4-D + 1 µM BAP under a dark incubation condition; however, the maximum days for callus initiation (17.67) was recorded from the treatment of 7.5 µM 2,4-D + 0.5 µM BAP under light incubation conditions. The study results also showed that the highest callus induction rate (100%) was produced from the combination of 2.5 µM 2,4-D + 1.5 µM BAP under a dark incubation condition; however, the lowest callus induction rate (73.33%) was obtained from the treatments of 7.5 µM 2,4-D + 1.5 µM BAP under both light and dark conditions. Similarly, the synergistic effect of plant growth regulators and cultural condition has been studied by Renu et al. [45] in the nodal segment of *Catharanthus roseus*, revealing the minimum days for callus initiation and higher callus frequency obtained when WPM basal medium supplemented with BAP 0.5 mg + 2,4-D 2 mg under dark condition.

Meanwhile, a study conducted by Behbahani et al. [46] showed that the earlier callus initiation and higher callus frequency obtained when the nodal segment was cultured on a WPM basal medium fortified with 1 mg 2,4-D under dark incubation condition. Moreover, previous studies by Azad et al. [47] of *Phellodendron amurense*, Hoque et al. [48] of *Trapa* sp., Thammina et al. [49] of *Euonymus alatus*, Hesami and Daneshvar. [42] of *Ficus religiosa*, Pandey et al. [50] of *Boerhaavia diffusa* reported that the explants cultured on basal medium supplemented with 2.2–8.8 µM 2,4-D + 1.1–4.4 µM BAP significantly increased the callus induction rate and minimized the callus initiation under a dark condition. Meanwhile, they have reported that a higher concentration of 2,4-D with a higher concentration of BAP significantly reduced the callus frequency and increased the days for callus initiation. It was noted that a combination of 2,4-D with lower BAP concentrations was found to increase the callus induction frequency. This improved effect could be related to BAP, which helps 2,4-D influence rapid cell division and the synthesis of zeatin-like hormones in the plant for effective callus induction [51]. Furthermore, the frequency of callus induction decreased with the increase in the concentration of 2,4-D. The increase in the concentration of 2,4-D causing a significantly delayed response. Bhojwani and Dantu [52] reported that 2,4-D may have herbicidal properties at high concentrations, which could reduce callus formation.

Other than PGRs, light is one of the most important elicitors, as it affects several physiological processes such as photosynthesis; hence, light can influence the growth, development, and morphogenesis of a variety of plant species in vitro [53,54]. Light, on the other hand, is known to affect cell division rates and ethylene evolution, which can affect callogenesis and rhizogenesis. Therefore, the duration and timing of light exposure are critical in explant morphogenesis [55,56]. In our experiments, the explants exposed to light in the early stages of culture developed more severe browning than those exposed to darkness, implying that darkness is preferable for callus induction in the early stages. According to Habibah et al. [56], in a dark environment, it is thought that the release of phenolic substances can be inhibited, and the rate of callus induction can be increased. However, dark incubation conditions may be associated with negative effects in some parameters such as callus biomass. Overall, callus initiation was faster in the dark than in the light, but the callus deteriorated when sub-cultured in the dark continuously, independent of media.

Based on the callus biomass accumulation, a different trend of callus fresh and dry weight was observed. The callus fresh weight was drastically increased by three-fold in the treatment of 7.5 µM 2,4-D + 1.5 µM BAP with 5.33 g. As the concentration decreased to 2.5 µM 2,4-D and 0.5 µM BAP, the callus fresh weight was decreased to 2.11 g and 2.53 g under dark and light incubation conditions, respectively. The yield of biomass and secondary metabolites can be used to determine the success of callus culture. These can be achieved with the proper balance of plant growth regulators, nutritional media, and growing conditions. The primary metabolism, particularly carbon and nitrogen metabolism, is closely linked to biomass accumulation. Carbon metabolism meets the energy requirements resulting from carbohydrate synthesis, and hence, contributes to cell growth and structural components [39]. The products of nitrogen metabolism, on the other hand, are primarily amino acids and proteins, which are then used to regulate cellular processes [40].

4.2. Quantification of Secondary Metabolites and Antioxidant Activity of In Vitro-Induced Calli and Conventionally Propagated Nodal Segment of B. glabra

The results of phytochemicals and antioxidant activities analysis revealed that total phenolic acid, total flavonoid content, DPPH free radical scavenging activity, and ABTS scavenging activity of aqueous extract of the conventionally propagated nodal segment were significantly different from other extraction solvents of in vitro-induced calli of *B. glabra*. The interrelating effect of the extraction solvent and source of the sample has been studied by Mahendra et al. [57] in conventionally propagated plant parts and calli induced under the light regime of *Decalepis arayalpathra*. They revealed maximum TPC and TFC from aqueous extract of the conventionally propagated nodal segment and calli

induced under photoperiod, respectively. Similarly, the current study results confirm the findings of Esmaeili et al. [58] that high polar solvents such as water of conventionally propagated plant parts produced higher TPC, and TFC compared with in vitro induced calli. In addition, node cultures of *Eucalyptus camaldulensis* produced under a 16-h light photoperiod revealed an increase in the level of phenolic compounds [59]. On the other hand, a study conducted by Zahid et al. [60] indicated the hexane extract of micropropagation and conventional propagated 'Bentong' ginger *Zingiber officinale* Roscoe produced the lowest amount of TPC and TFC compared to other extraction solvents, which are in agreement with our findings.

In the antioxidant activities, the aqueous extract of conventionally propagated nodal segment produced higher DPPH free radical scavenging activity and ABTS scavenging activity compared to in vitro-induced calli. This is in agreement with the findings of Esmaeili et al. [58] that the highest antioxidant properties was obtained from the aqueous extract of field-grown plant parts compared to the in vitro induced calli. Moreover, previous studies by Islam et al. [61] on *B. glabra*, Murali and Prabakaran [62] on *Ociumum basilicum* L., and Mahendra et al. [57] on *Salacia macrosperma* showed that the aqueous and ethanol extracts of the conventionally propagated plant parts exhibited the higher DPPH free radical scavenging and ABTS scavenging activity compared to the in vitro induced calli of less polar solvents such as acetone and hexane. Furthermore, studies conducted by López-Laredo et al. [63] on *Tecoma stans*, Shah et al. [64] on *Silybum marianum*, Mohammad et al. [65] on *Olea europaea* L., and Rameshkumar et al. [51] on *Nilgirianthus ciliate* indicated that the calli induced under the photoperiod produced a higher antioxidant activity than the calli induced under dark incubation conditions which are in line with our findings. On the other hand, a study conducted by Zahid et al. [60] indicated that the hexane extract of micropropagated and conventional propagated 'Bentong' ginger *Zingiber officinale* Roscoe produced the lowest inhibition of DPPH than other extraction solvents, which are also in agreement with our findings.

In contrast to other antioxidant activities conducted in which aqueous and other node extracts produced higher antioxidant activities, the iron (II) chelating activity was produced from the aqueous extract of the calli induced under dark conditions. It is clear that the chelating powers of water extracts of the calli induced under dark conditions were higher than the other three extracts and sources of samples. Similarly, a study conducted by Hakkim et al. [66] reported that the in vitro induced callus had a higher iron (II) chelation than that of the field-grown stem, leaves, and inflorescence of *Ocimum sanctum* L. Furthermore, according to Costa et al. [67], the synergistic effect of solvents and the source of the sample exhibited that the aqueous extract of the in vitro-induced callus showed higher iron (II) chelation than conventionally propagated plant parts and other extraction solvents of *Thymus lotocephalus*. In addition, study conducted by Song et al. [68] confirmed that shoot of the field-grown plant of *Mertensia maritima* L. produced higher TPC, TFC, DPPH free radical scavenging activity, and ABTS scavenging activity. However, the in vitro-induced calli chelate had more iron (II) than field-grown shoots, supporting our findings. So, the in vitro-induced callus can be a good source of iron (II) chelation, and this indicates that polyphenols are not primary chelating particles [69].

5. Conclusions

The results of this study demonstrated that the growth of *B. glabra* calli is greatly influenced by the combination of plant growth regulators and cultural conditions. No callus was induced on a PGR-free medium. The media composed of WPM supplemented with 2.5 µM 2,4-D + 1.5 µM BAP and 5 µM 2,4-D + 1 µM BAP significantly reduced the days for callus initiation (7 days) under a dark incubation condition, while the maximum days (17.67) was recorded when WPM fortified with 7.5 µM 2,4-D + 0.5 µM BAP under light incubation condition. Meanwhile, 5 µM 2,4-D + 0.5 µM BAP significantly increased the callus frequency (100%) under both light and dark incubation condition. By increasing the PGRs concentration and combination, the callus frequency significantly decreased.

Moreover, the light regime did not have any influence on biomass accumulation, while the PGRs significantly influenced and the maximum biomass was recorded when the WPM basal medium was supplemented with 7.5 µM 2,4-D + 1.5 µM BAP under both dark and light conditions with FW: 5.33 g per callus and DW: 230.67 mg per callus, respectively. Furthermore, the morphology of the callus was influenced by PGRs and cultural condition; the calli that induced under a light condition had good texture and were compact in nature with yellow to brown and red colors, while the calli induced under a dark condition were friable with yellow to brown and white colors.

The study on phenolics content found that the aqueous extract of the conventionally propagated node exhibited the highest phenolic acids (21.88 mg GAE/g DW) and flavonoids content (42.05 mg RE/g DW). Furthermore, in antioxidant activities conducted, the highest DPPH free radical scavenging activity (7.64 mg TE/g DW), and ABTS scavenging activity (1.51 mg TE/g DW), were also recorded from aqueous extract of the conventionally propagated node. On the other hand, the highest iron (II) chelating activity was exhibited from the aqueous extract of the calli induced under the dark condition with 43.3% inhibition, respectively. In conclusion, higher phenolics content and antioxidant properties recorded from the conventionally propagated nodal part showed that the plant propagated through conventional propagation technique has the potential to produce high secondary metabolites. Meanwhile, both conventionally propagated parts and callus samples suggest that in addition to the plant material, the callus may also be used as a supplement raw material to obtain secondary metabolites for the pharmaceutical industry. Thus, the in vitro-derived *B. glabra* callus could plausibly act as a novel source of metabolites for food and pharmaceutical industries in their preparation of food preservatives and medicines, respectively.

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