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Influence of Culture Conditions on Growth and Daidzein and Genistein Production in Hairy Root Cultures of *Pueraria candollei* var. *mirifica*

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Abstract: *Pueraria candollei* var. *mirifica* produces and accumulates various phytoestrogen compounds in its tuberous roots, including daidzein and genistein. Plant cell culture methods have been established to alleviate the problems associated with producing valuable phytochemicals from natural or field-cultivated plants, and hairy root culture is one of the most promising methods for the in vitro production of plant secondary metabolites. Thus, this study aimed to produce daidzein and genistein from hairy root cultures of *P. candollei* var. *mirifica*. The influences of cultivation parameters, including the culture medium, light conditions, sugar content in the culture medium, incubation temperature, and agitation speed, on biomass and daidzein and genistein production in hairy root cultures of this medicinal plant were investigated. The results revealed that the optimal cultivation conditions for biomass and bioactive compound production were Murashige & Skoog (MS) medium, a sucrose concentration of 30 g/L, a 16/8 h light/dark photoperiod, an incubation temperature of 26 °C, and an agitation speed of 90 rpm. The highest biomass and daidzein and genistein contents achieved in this study were 17.76 g/L, 6.85 mg/g DW, and 0.96 mg/g DW, respectively. Interestingly, the daidzein and genistein contents obtained from hairy roots were approximately 45.7- and 12.0-fold greater than those obtained from normal roots, respectively, suggesting that hairy root culture is a suitable method for the sustainable production of phytoestrogen, daidzein, and genistein from this medicinal plant.

Keywords: in vitro production; phytoestrogen; secondary metabolites; tuberous roots

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1. Introduction

Pueraria candollei Graham ex. Benth. var. *mirifica*, recently reclassified as *P. mirifica* (Airy Shaw & Suvat.) Niyomdham (hereafter referred to as *P. mirifica*), also known as white Kwao Krua, is an important indigenous herb of Thailand. Traditionally used as a dietary supplement or in combination with other medicinal plants, it is valued for its rejuvenating and anti-aging properties [1]. This woody climber plant belongs to the Fabaceae family and is native to Southeast Asia. It primarily grows in the deciduous forests of northern, western, and northeastern Thailand. The plant is characterized by its bluish-purple, legume-shaped flowers and white tubers [2,3].

P. mirifica exhibits various biological activities in vitro, including antioxidant, antimicrobial, anticancer, anti-aging, anti-osteoporosis, and antiproliferative properties [4,5]. Recently, the plant has gained popularity as a functional ingredient in cosmeceutical products, particularly those targeting anti-aging effects. It is also used in dietetic products or supplements to alleviate menopausal symptoms and treat conditions associated with

estrogen deficiency, such as vasomotor symptoms, depression, musculoskeletal pain, hair loss, wrinkles, hyperpigmentation, and sagging breasts. Furthermore, *P. mirifica* has been employed for breast augmentation and aesthetic enhancement purposes [5–7]. Various product formulations are available in the market, including lotions, gels, creams, sprays, tablets, and capsules [5].

The tuberous roots of *P. mirifica* produce and accumulate three significant groups of phytochemical compounds [1,8] with structures and functions similar to those of mammalian estrogenic hormones [9]. These bioactive constituents include chromenes (miroestrol, deoxymiroestrol, and isomiroestrol), isoflavonoids (kwakhurin, puerarin, daidzin, daidzein, genistin, genistein, mirificin, and puemiricarpene), and coumestans (coumestrol, mirificoumestan, mirificoumestan glycol, and mirificoumestan hydrate). These compounds show promising potential in treating various diseases and symptoms, including breast and prostate cancers, cardiovascular disease, osteoporosis, and menopausal diseases [5,10–12].

The mass production of bioactive compounds from *P. mirifica* currently relies on natural or field-cultivated plants; however, the quality and quantity of the bioactive compounds produced by natural plants often vary due to several environmental factors, such as geographical area, weather, and climate changes. Moreover, plant pathogens can lead to yield loss and unsafe products for consumers. These challenges underscore the need for more efficient and controlled production methods. In vitro plant cell culture techniques have been successfully established to alleviate problems associated with the production of materials from natural plants. These biotechnological techniques offer a significant advantage and allow for the continuous and reliable production of stable bioactive compounds independent of seasonal and environmental factors. Various plant cell culture techniques, such as callus cultures [13,14], cell suspension cultures [15,16], somatic embryo cultures [17], and hairy root cultures [18], have been utilized for biomass and plant secondary metabolite production.

Hairy root culture systems are considered promising for the mass production of valuable bioactive compounds from various medicinal plants owing to their rapid growth and physiological, biochemical, and genetic stability [18]. In addition to the ability to grow in a culture medium without exogenous plant hormones, hairy roots also exhibit greater efficiency in producing secondary metabolites than normal roots [18–20]. The successful production of several plant secondary metabolites using hairy root cultures has been previously reported, such as isoflavonoid production in *P. candollei* [21], puerarin and daidzin production in *P. lobata* [22], scopolamine production in *Atropa belladonna* [23], picroliv production in *Picrorhiza kurroa* [24], caffeoylquinic acid derivative and flavonoid production in *Rhaponticum carthamoides* [25], and phenolic and flavonoid production in *Verbascum erianthum* and *V. stachydiforme* [26].

Various environmental factors influence biomass and secondary metabolite production of in vitro hairy root cultures. These include the types and growth stages of the explants, carbon sources and concentrations, culture medium, and cultivation conditions [14,15,17,21,22,24–28]. As different plant species respond differently to each environmental factor, it is necessary to optimize these factors for the in vitro production of biomass and secondary metabolites. *P. mirifica* has garnered significant interest in recent years. However, research on optimizing its hairy root cultures remains limited. To date, only one study has examined the impact of medium types and sucrose concentrations on growth and isoflavonoid accumulation in *P. candollei* hairy root cultures [15]. The effects of light conditions, cultivation temperature, and agitation speed on biomass and secondary metabolite production in *P. mirifica* hairy root cultures remain unexplored. This study aims to determine the effect of cultivation parameters, including culture medium, light conditions, sugar content, incubation temperature, and agitation speed, on growth and daidzein and genistein production in hairy root cultures of *P. mirifica*. Additionally, this research compares the daidzein and genistein contents produced by hairy root cultures with those derived from normal roots of field-cultivated plants as well as those produced using other platforms, such as microbial fermentation, biotransformation by microbial

enzymes and engineered microorganisms. These findings could potentially enhance the efficiency of secondary metabolite production in this valuable medicinal plant.

2. Materials and Methods

2.1. Chemicals

Murashige & Skoog (MS) basal medium, Gamborg (B5) medium, Woody Plant medium (WPM), Schenk & Hildebrandt (SH) medium (Supplementary Materials: Table S1), and a gelling agent (Phyto agar) were obtained from PhytoTech Labs (Lenexa, Kansas, MO, USA). Daidzein and genistein were purchased from Sigma Aldrich Corporation (Burlington, MA, USA). Sucrose was purchased from KemAus^{TM} (Cherrybrook, NSW, Australia). Other chemicals (analytical grade) were acquired from a local supplier in Khon Kaen Province, Thailand.

2.2. Bacterial Strain and Culture Conditions

Agrobacterium rhizogenes ATCC15834 used to induce hairy roots in this study was obtained from the Department of Biotechnology, Faculty of Technology, Khon Kaen University, Thailand, and was cultured on yeast extract broth (YEB) agar (5.0 g/L beef extract, 1.0 g/L yeast extract, 5.0 g/L peptone, 0.5 g/L $MgCl_2$, 5.0 g/L sucrose, 15 g/L agar) and maintained at 4 °C. For inoculum preparation, a single colony of a bacterial strain grown on YEB agar at 26 °C for 72 h was inoculated into liquid YEB medium (5.0 g/L beef extract, 1.0 g/L yeast extract, 5.0 g/L peptone, 0.5 g/L $MgCl_2$, 5.0 g/L sucrose) (pH 7.2) and incubated on a controlled incubation shaker (JSR, Gongju, Republic of Korea) at 160 rpm and 26 °C for 24 h. The active bacterial cells were collected via centrifugation (Sorvall LYNX 4000, Thermo Fisher Scientific Inc., Langenselbold, Germany) at 10,000 rpm and 4 °C for 5 min, washed twice with sterile distilled water, resuspended in fresh YEB medium, and used as a starter culture for hairy root induction in subsequent experiments.

2.3. Plant Material and Preparation of Sterilized Plantlets

P. mirifica used in the current study was kindly provided by Dr. Prasarn Chaladkid from the Institute of Agricultural Technology, Suranaree University of Technology, Thailand. The plant specimens (No. 11348) were deposited at the herbarium of the Department of Biology, Faculty of Science, Khon Kaen University, Thailand. The sterilized plantlets were prepared using a procedure described by Thanonkeo and Panichajakul [13], with slight modifications. Briefly, seeds from 2-year-old plants were thoroughly washed with running tap water. The resulting seeds were subjected to surface sterilization by shaking them in 10% sodium hypochlorite solution containing 10 µL of Tween 20 for 20 min. The seeds were then washed three times with sterile distilled water and germinated on semisolid hormone-free MS basal media supplemented with 30 g/L sucrose (pH 5.6). After incubating in a standard culture room at 26 ± 2 °C with a 16/8 h light/dark photoperiod at a light intensity of 3000 lux for 7 days, the regenerated plantlets were used as plant materials for hairy root induction.

2.4. Induction of Hairy Roots Using Cocultivation Techniques

The hairy roots of *P. mirifica* were induced using the method of Habibi et al. [29], with slight modifications. Briefly, shoots were excised from 7-day-old sterile plantlets and cut into 3 cm segments. The resulting explants were immersed in an *A. rhizogenes* suspension supplemented with 200 µM acetosyringone and cocultivated in a controlled incubator shaker at 100 rpm and 26 °C. After 20 min of incubation, the explants were placed on sterile filter paper and transferred to semi solid hormone-free MS basal medium supplemented with 30 g/L sucrose. After 4 days of cocultivation at 26 °C, the explants were washed three times with sterile distilled water and soaked in 1000 mg/L cefotaxime solution for 20 min to remove excess *A. rhizogenes* cells. Subsequently, the resulting explants were briefly blotted on dry sterile filter paper, transferred onto a semisolid, hormone-free MS basal medium containing 30 g/L sucrose and 250 mg/L cefotaxime, and incubated in the

cultivation room at 26 ± 2 °C with a 16 h light and 8 h dark photoperiod at a light intensity of 3000 lux. The generated hairy or transformed roots were excised from the infected explants and transferred to fresh MS basal agar medium supplemented with 30 g/L sucrose and 250 mg/L cefotaxime without phytohormones. The hairy roots were subcultured onto the abovementioned medium every two weeks until all the *A. rhizogenes* cells were removed. The resulting sterile hairy roots were then cultured in 100 mL of hormone- and cefotaxime-free MS liquid medium and incubated on a rotary incubator shaker at 110 rpm in the cultivation room under the same conditions described above. The morphology and growth performance of the obtained hairy roots in the liquid MS medium were monitored.

2.5. Confirmation of Transformed Roots Using Polymerase Chain Reaction (PCR)

Genomic DNA was isolated from hairy roots and normal roots of *P. mirifica* using a DNeasy® Plant Mini Kit (Macherey-Nagel, MN, Düren, Germany). Roots were ground to a fine powder under liquid nitrogen using a mortar and pestle, and approximately 20 mg of tissue powder was transferred to a 1.5 mL microcentrifuge tube. The sample was mixed with 400 µL of Buffer AP1 and 4 µL of RNase A (100 mg/mL), then vortexed and incubated at 65 °C for 10 min. After adding 30 µL of Buffer P3, the mixture was incubated on ice for 5 min and centrifuged at 14,000 rpm and 4 °C for 5 min. The supernatant was processed through a QIAshredder Mini spin column (Macherey-Nagel, MN, Düren, Germany) and centrifuged at 14,000 rpm and 4 °C for 2 min. The flow-through was mixed with 1.5 volumes of Buffer AW1 and transferred to a DNeasy Mini spin column (Macherey-Nagel, MN, Düren, Germany). After centrifugation at 8000 rpm and 4 °C for 1 min, the column was washed twice with 500 µL of Buffer AW2. Genomic DNA was eluted with 100 µL of Buffer AE and stored at −20 °C.

Plasmid DNA from *A. rhizogenes* was extracted using a Plasmid DNA Mini Kit (Qiagen, Macherey-Nagel, MN, Düren, Germany). Bacterial cells were grown in liquid YEB medium at 160 rpm and 26 °C overnight, then harvested via centrifugation at 8000 rpm and 4 °C for 15 min. The pellet was resuspended in 250 µL of Buffer A1, lysed with 250 µL of Buffer A2, and neutralized with 300 µL of Buffer A3. After centrifugation at 12,000 rpm and 4 °C for 10 min, the supernatant was processed through a NucleoSpin® plasmid column. After centrifugation at 12,000 rpm at 4 °C for 1 min, the column was washed with 600 µL of Buffer A4, dried, and the plasmid DNA was eluted with 50 µL of Buffer AE. The extracted plasmid DNA was stored at −20 °C.

The quality and quantity of the isolated genomic and plasmid DNA were determined using spectrophotometry and agarose gel electrophoresis following standard procedures [30]. PCR was used to detect the rooting locus B (*rolB*) and C (*rolC*) genes in the hairy and normal roots of *P. mirifica* and the plasmid DNA of *A. rhizogenes*. The following primers were used: *rolB*-F (5′-GCTCTTGCAGTGCTAGATTT-3′) and *rolB*-R (5′-GAAGGTGCAAGCTACCTCTC-3′) for the *rolB* gene and *rolC*-F (5′-CTCCTGACATCAAACTCGTC-3′) and *rolC*-R (5′-TGCTTCGAGTTATGGGTACA-3′) for the *rolC* gene [31]. The PCRs were prepared using PCR Master Mix (Fermentas, Hanover, MD, USA) according to the manufacturer's instructions. PCR reactions were prepared using PCR Master Mix (Fermentas, Hanover, MD, USA). Each 25 µL reaction mixture contained 10 ng DNA template, 1.6 nmol/µL each of forward and reverse primers, 12.5 µL 2× *Taq* DNA polymerase buffer, 4 mM $MgCl_2$, 0.4 mM dNTPs, and 0.625 units *Taq* DNA polymerase. PCR amplification was performed using a PCR thermocycler (Icycler, Bio-Rad, Hercules, CA, USA) with 35 cycles of denaturation at 94 °C for 1 min, annealing at 53 °C for 2 min, and extension at 72 °C for 5 min. The amplified PCR products were examined using 1.0% agarose gel electrophoresis.

2.6. Effect of Culture Conditions on Growth and Daidzein and Genistein Production in Hairy Root Culture of P. mirifica

Two-week-old hairy roots were cut into pieces 4 cm in length and transferred to 250 mL Erlenmeyer flasks containing 50 mL of hormone-free liquid cultivation medium. Each flask was inoculated with 3 hairy root explants, with an initial fresh weight of approximately 0.2 g.

The effects of various cultivation conditions, including culture medium, light conditions, initial sugar concentration, incubation temperature, and agitation speed, on growth and daidzein and genistein production by *P. mirifica* hairy root cultures were tested in this study. For the culture medium test, hairy roots were transferred to liquid MS, WPM, SH, or B5 media and cultivated on a rotary incubator shaker at 26 ± 2 °C and 110 rpm with a 16 h light and 8 h dark photoperiod at a light intensity of 3000 lux. For the light test, hairy roots were inoculated into liquid MS medium and cultivated on a rotary incubator shaker at 26 ± 2 °C and 110 rpm without exposure to light (dark conditions) and with a 16 h light and 8 h dark photoperiod at a light intensity of 3000 lux. For the initial sugar concentration of the culture medium test, hairy roots were transferred to a liquid MS medium supplemented with 15, 30, or 45 g/L sucrose and then cultivated under the same conditions as those used for the culture medium. For the incubation temperature test, hairy roots were placed into liquid MS medium and then cultivated on a rotary incubator shaker at various incubation temperatures, including 22, 26, and 30 °C. The agitation speed was 110 rpm, and the photoperiod was 16/8 h light/dark at a light intensity of 3000 lux. For the agitation speed test, hairy roots were transferred to liquid MS medium and cultivated on a rotary incubator shaker at 26 ± 2 °C with various agitation speeds (90, 110, and 130 rpm). The cultures were exposed to a 16/8 h light/dark photoperiod at a light intensity of 3000 lux. All experiments were performed twice and in triplicate. During hairy root cultivation, samples were randomly collected, and the growth of hairy roots and daidzein and genistein production were analyzed.

2.7. Analytical Procedure

The growth of hairy roots was determined by measuring dry cell weight (DW). Biomass was dried at 60 °C until a constant weight was reached [13]. The extraction and quantification of daidzein and genistein were performed using the methods of Thanonkeo and Panichajakul [13]. Briefly, dried cells were ground to a fine powder using a mortar and pestle. One gram of resulting powder was mixed with 1 g of celite, and the resulting mixture was packed in a 16-mL glass column (Bio-Rad, Bangkok, Thailand). The column was eluted with 50 mL of 80% (*v*/*v*) methanol, and one milliliter of flow-through was filtered using a 0.45 µm nylon membrane. The filtered sample (20 µL) was then subjected to analysis. Daidzein and genistein contents were determined via high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). The HPLC system was equipped with a reversed-phase column (150 mm × 3.9 mm, 4 µm diameter) (Nova-Pak C18, Waters, Munich, Germany) and an ultraviolet (UV) detector set at a wavelength of 254 nm [13]. The mobile phase consisted of 33% (*v*/*v*) acetonitrile and 67% (*v*/*v*) aqueous 1% (*v*/*v*) acetic acid, with a flow rate of 0.8 mL/min. All samples were analyzed in triplicate, and the results are expressed as mg/g DW.

2.8. Statistical Analysis

A completely randomized design (CRD) was used in this study, and all experiments were carried out twice and in triplicate. The data were statistically analyzed using SPSS (IBM SPSS Statistics 28, IBM Corporation, Armonk, NY, USA). The experimental data are expressed as the means ± standard deviations (SDs).

3. Results and Discussion

3.1. Induction of Hairy Roots from P. mirifica by A. rhizogenes ATCC15834

Hairy roots can be induced from plants using the Gram-negative soil bacterium *A. rhizogenes*, which harbors a root-inducing plasmid (Ri plasmid), and the induction efficiency varies depending on the plant species, explant type, plant age, and bacterial strain [18–20,22,26,29]. Several strains of *A. rhizogenes*, such as A4, LBA9402, MTCC532, KCCM11879, ATCC43057, and ATCC15834, are available for hairy root induction. However, *A. rhizogenes* ATCC15834 is the most effective and widely used strain due to its high

efficiency in inducing hairy roots from various plants [18,26]. Therefore, this study used *A. rhizogenes* ATCC15834, which is an agropine strain, to produce hairy roots from *P. mirifica*.

After cocultivating the shoot explants from 7-day-old sterile plantlets with an *A. rhizogenes* ATCC15834 cell suspension for four days, hairy roots were directly generated at the wounded sites of the explants after cultivation on semisolid, hormone-free MS medium for two weeks (Figure 1). The induction efficiency of hairy roots was 39.18%, which is within the range reported for hairy root induction of *V. erianthum* and *V. stachydiforme* by Amini et al. [26]. However, this finding was in contrast with that of a study by Habibi et al. [29], who reported the highest hairy root induction efficiency of 91.3% in *Origanum vulgare* after the inoculation of plant shoots with *A. rhizogenes* ATCC15834 and K599 and cocultivation on MS medium supplemented with low salt concentrations. These results implied that different plant species respond differently to *A. rhizogenes* and that low salt concentrations in the culture medium are preferable for hairy root induction.

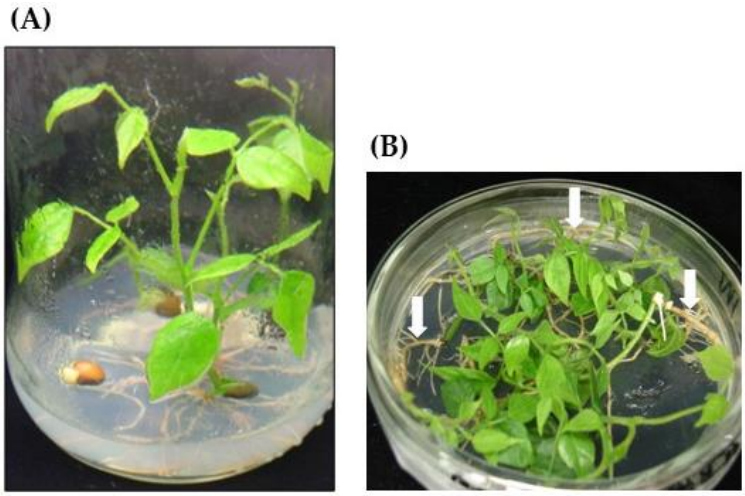


Figure 1. Seven-day-old sterile plantlets of *Pueraria candollei* var. *mirifica* grown on semisolid, hormone-free Murashige & Skoog (MS) basal medium (**A**) and hairy roots of *P. candollei* var. *mirifica* after induction with *Agrobacterium rhizogenes* ATCC15834 (**B**). Arrowheads indicate the hairy roots generated from the infected explants.

3.2. Confirmation of Hairy Roots Using PCR

The genetic transformation process of plant cells by *A. rhizogenes* is well established and involves several genes and proteins [32]. The *rol* genes *rolB* and *rolC* are among the essential elements for hairy root induction. To confirm that the hairy roots obtained from cocultivated explants were due to *A. rhizogenes*-mediated transformation, the *rolB* and *rolC* genes were detected via PCR amplification using genomic DNA isolated from randomly selected hairy roots as a template. The genomic DNA isolated from normal or nontransformed roots and plasmid DNA isolated from *A. rhizogenes* were also used as negative and positive controls, respectively. As illustrated in Figure 2, PCR products of approximately 450 and 600 bp were detected on the gel after PCR amplification using primers specific for the *rolB* and *rolC* genes, respectively. The size of these PCR amplicons coincided with the expected size of the *rolB* and *rolC* genes based on the amplification size of the primers. No PCR products of the negative control (normal roots) were visualized on the gel, while PCR products of approximately 450 and 600 bp were detected in the positive control (plasmid DNA of *A. rhizogenes*). These results indicated that the *rolB* and *rolC* genes responsible for hairy root induction were integrated into the chromosomal DNA of *P. mirifica*. Notably, the intensities of the PCR products on the gel varied among the different hairy root samples, indicating differences in the copy numbers of the *rolB* and *rolC* genes in the genomes of the transformed plants since the ability of *A. rhizogenes* to infect

plant cells is random and depends on various factors, such as culture medium, incubation temperature, cocultivation time, genotype, and bacterial strain [29,33].

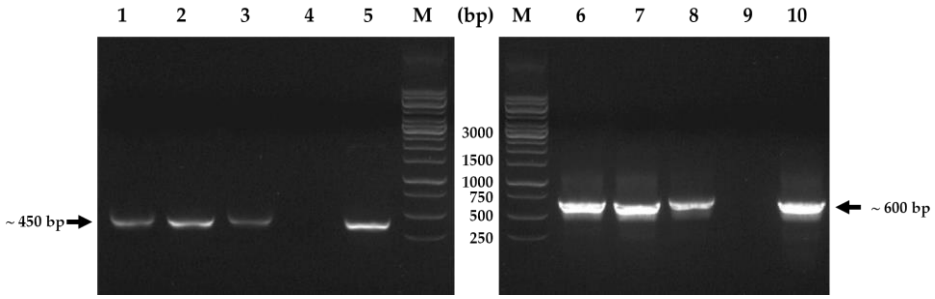


Figure 2. PCR amplification of *rolB* (lanes 1 to 5) and *rolC* genes (lanes 6–10) in hairy and normal roots of *Pueraria candollei* var. *mirifica*. Lanes 1–3 and 6–8 indicate PCR products of hairy root samples, 4 and 9 indicate PCR products of normal roots (negative control), 5 and 10 indicate PCR products of plasmid DNA isolated from *Agrobacterium rhizogenes* ATCC15834 (positive control), and M indicates a DNA marker.

3.3. Effect of Culture Conditions on Growth and Daidzein and Genistein Production in Hairy Root Cultures of P. mirifica

The growth performance and morphology of the hairy roots obtained in this study varied. However, one hairy root cell line (Pc-HR-10) was selected based on its stable growth performance and high branching ability in a hormone-free, liquid MS medium after subculturing for 20 cycles. The effect of culture conditions, including the culture medium, light conditions, initial sugar concentration in the culture medium, incubation temperature, and agitation speed, on biomass, daidzein, and genistein production in this cell line was determined. The results are summarized in Figures 3–7.

3.3.1. Effect of Culture Medium on Growth and Daidzein and Genistein Production

The biomass and secondary metabolite production of hairy roots depend on the cultivation media [18,26]. In this study, the effects of various culture media, including MS, WPM, SH, and B5, on growth and daidzein and genistein production were assessed, and the results are summarized in Figure 3. The highest biomass production was recorded in the MS medium, accounting for 16.66 g/L, followed by WPM (15.46 g/L). The lowest biomass accumulation was detected in the B5 medium (7.54 g/L) (Figure 3A). Hairy roots grown in liquid MS and WPM media exhibited greater growth and luxuriant branching morphology than those grown in other media (Figure 3D). This finding suggested that the MS medium was preferable for the growth and development of *P. mirifica* hairy roots, possibly because this medium contains higher concentrations of trace elements, such as nitrate, phosphate, and potassium, than other media. A similar result was also reported by Udomsuk et al. [21], who demonstrated that the MS medium promoted the biomass production of *P. candollei* hairy root cultures. In contrast to our study, Amini et al. [26] showed that the B5 medium, which contains less salt than the MS medium, increased the biomass production of *V. erianthum* and *V. stachydiforme* hairy roots. It could be concluded from this information that the biomass production of hairy roots depends on the plant species and type of culture medium.

In terms of secondary metabolite production, the MS medium yielded greater daidzein and genistein contents than did the other media. The maximum concentrations of daidzein and genistein produced by hairy root cultivation in the MS medium were 4.78 and 0.81 mg/g DW, respectively. The SH and B5 media yielded relatively low concentrations of these bioactive compounds. These results indicated that the MS medium promoted secondary metabolite production in the hairy root cultures of *P. mirifica*. One possible explanation is that high salt concentrations in the MS medium may cause stressful conditions for plant cells and, as a result, stimulate the expression of genes and enzyme activity involved

in stress response mechanisms, such as phenylalanine ammonia-lyase (PAL), leading to the synthesis of plant secondary metabolites, including daidzein and genistein. Another possibility is that trace elements in the culture medium can act as cofactors of enzymes in the biosynthesis pathway, specifically the phenylpropanoid pathway, and, as a result, enhance the production of these bioactive compounds [26].

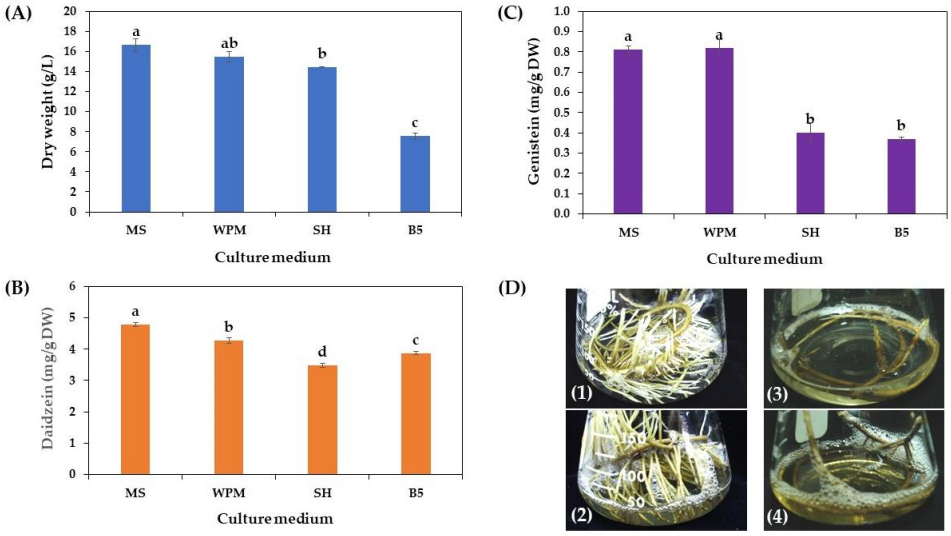


Figure 3. Effects of culture media on the growth (**A**), daidzein (**B**) and genistein (**C**) production, and morphology (**D**) of hairy roots of *Pueraria candollei* var. *mirifica* after cultivation for 30 days. Hairy roots of *P. candollei* var. *mirifica* cultured in Murashige & Skoog (MS) (**D-1**), Woody Plant Medium (WPM) (**D-2**), Schenk & Hildebrandt (SH) (**D-3**), and Gamborg (B5) (**D-4**) media. The bars followed by the same letters in each graph are not significantly different at $p \leq 0.05$ based on DMRT analysis.

3.3.2. Effects of Light Conditions on Growth and Daidzein and Genistein Production

Light is another factor affecting the formation and biomass production of hairy roots. A previous study by Kim et al. [22] reported that dark conditions significantly enhanced hairy root formation in *P. lobata* after inoculation with *A. rhizogenes* KCTC2744. However, no information is available on the effect of light conditions on biomass and secondary metabolite production in hairy root cultures of *P. mirifica*. Therefore, the effects of cultivation under dark (24 h/day) and light (16 h/8 h light/dark photoperiod) conditions on biomass, daidzein, and genistein accumulation in hairy root cultures of this medicinal plant were determined. As illustrated in Figure 4A, the biomass production of hairy roots under light conditions was significantly greater than that under dark conditions. The maximum biomass accumulations were 17.76 and 15.12 g/L for the light and dark cultivations, respectively. Hairy roots cultured under light conditions exhibited more branching roots than did those cultured under dark conditions (Figure 4D). This result was similar to that of Liu et al. [34], who reported that a 16/8 h light/dark photoperiod significantly increased the biomass production of *Artemisia annua* hairy root culture. Notably, increased growth performance of cells cultured under dark conditions, such as in the case of *Echinacea purpurea* [35], was also recorded. Thus, different plant species or types of culture exhibit different abilities to manipulate plants under light/dark conditions.

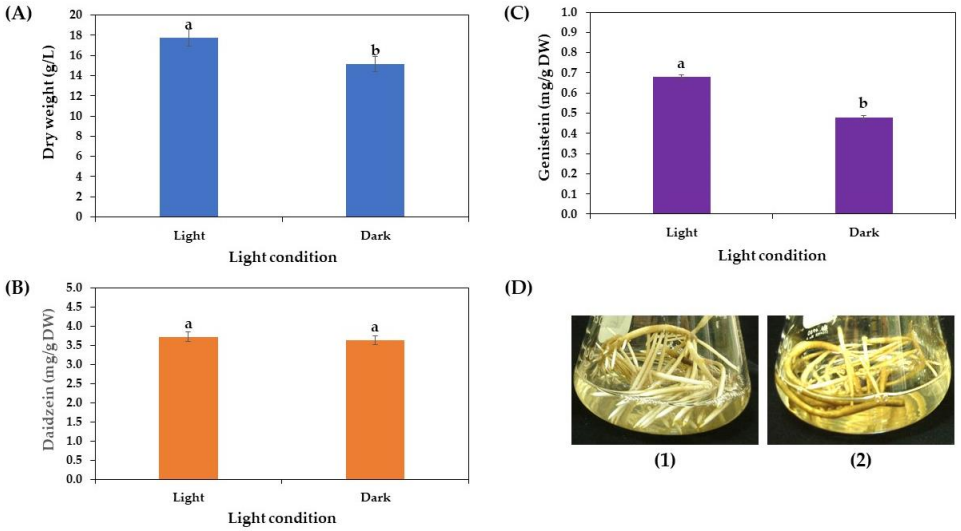


Figure 4. Effects of light and dark conditions on the growth (**A**), daidzein (**B**) and genistein (**C**) production, and morphology (**D**) of hairy roots of *Pueraria candollei* var. *mirifica* after cultivation in Murashige & Skoog (MS) medium for 30 days. Hairy roots of *P. candollei* var. *mirifica* cultured under light (**D-1**) and dark (**D-2**) conditions. The bars followed by the same letters in each graph are not significantly different at $p \leq 0.05$ based on DMRT analysis.

For bioactive compound production, light conditions did not improve daidzein production since the contents of this compound obtained from hairy roots cultivated under light and dark conditions were almost the same. The concentration of daidzein in hairy roots cultivated under a 16/8 h light/dark photoperiod was 3.72 mg/g DW, which was slightly greater than that in hairy roots cultivated under dark conditions (3.63 mg/g DW) (Figure 4B). In contrast, cultivation under light conditions significantly promoted the production and accumulation of genistein compared to cultivation under dark conditions. The highest genistein content under light conditions was 0.68 mg/g DW, while that under dark conditions was 0.48 mg/g DW (Figure 4C). These findings suggested that light could promote hairy root growth and bioactive compound production in hairy root cultures of *P. mirifica*. Previous studies have shown that light can promote enzyme activity, such as that of cytochrome P450 and chalcone synthase, which are involved in the biosynthesis of plant secondary metabolites, as well as other enzymes involved in plant stress response mechanisms and, as a result, can induce secondary metabolite production [36,37].

3.3.3. Effect of the Sucrose Concentration on Growth and Daidzein and Genistein Production

Sucrose is the most widely used sugar for in vitro plant cell culture because it is inexpensive, readily available, and more stable during high-temperature sterilization without causing caramelization of the culture medium [38]. This disaccharide sugar serves as a carbon and energy source for cell growth, differentiation, and development. Sucrose can act as a signal molecule that controls various plant developmental processes and metabolic activities involved in primary and secondary metabolite production [39,40]. Previous studies demonstrated that sucrose, especially at high concentrations, promoted secondary metabolite production in hairy root cultures of several plant species, for instance, pyranocoumarins in *Angelica gigas* [41], ginsenosides in *Panax quinquefolium* [42], baicalein in *Scutellaria baicalensis* [43], and caffeoylquiic acid derivatives and flavonoids in *Rhaponticum carthamoides* [25]. Thus, the effects of sucrose concentrations (15, 30, and 45 g/L) on biomass, daidzein, and genistein production in hairy root cultures of *P. mirifica* were assessed in this study. The results revealed that a low sucrose concentration (15 g/L) yielded the lowest biomass (8.58 g/L) of hairy roots, most likely due to insufficient carbon and energy sources for plant growth and development. Increasing sucrose concentrations from 15 g/L to 30 and 45 g/L significantly increased biomass production. The highest biomass (19.5 g/L) was

recorded in the hairy roots cultivated in MS medium supplemented with 45 g/L sucrose (Figure 5A). This result indicated that sucrose was an essential element for the growth and development of hairy roots of *P. mirifica*, similar to other reports in the literature [21,25,44]. The optimum concentration of sucrose that yields the greatest biomass accumulation varies depending on the plant species and type of culture medium. For instance, Petrova et al. [45] reported that the MS medium supplemented with 30 g/L sucrose produced the most biomass in hairy root cultures of *Arnica montana*. Beigmohamadi et al. [46] noted that the growth of *Plumbago europea* hairy roots was maximized in MS medium supplemented with 30 g/L sucrose. A recent study by Skala et al. [25] demonstrated that WPM containing 30 g/L sucrose yielded the highest biomass of *R. carthamoides*-transformed roots.

Notably, increasing the sucrose concentration to 60 g/L resulted in a remarkable decrease in biomass production. Necrosis and browning of hairy roots were observed after 21 days of cultivation, which differed from the results obtained for hairy roots cultured in MS medium supplemented with 15 to 45 g/L sucrose (Figure 5D), and almost all of the hairy roots died after 24 days of cultivation. This might be due to the osmotic stress caused by high sugar concentrations, which can generate reactive oxygen species (ROS). A high level of ROS can damage several macromolecules, such as proteins, nucleic acids, and lipids, causing physiological and morphological changes, leading to cell death [47,48]. Another possibility is that the high viscosity of the culture medium due to a high sugar concentration limits nutrient uptake by plant cells, resulting in a reduction in cell growth and development, which in turn may also cause cell death [49]. The results of this study were in accordance with those of Makowczuńska et al. [50] and Skala et al. [25], who demonstrated that high sucrose concentrations reduced the biomass of hairy root cultures of *Codonopsis pilosula* and *R. carthamoides*, respectively.

The sucrose concentration also impacted the production of daidzein and genistein in the hairy root culture of *P. mirifica*. Like biomass production, the lowest content of daidzein (2.06 mg/g DW) was detected in MS medium supplemented with 15 g/L sucrose. The production of daidzein significantly increased and reached its highest level (6.85 mg/g DW) when hairy roots were cultured in the medium supplemented with 30 g/L sucrose; this value was approximately 3.3 times greater than that obtained for hairy roots cultured in the medium supplemented with 15 g/L sucrose. However, increasing the sucrose concentration to 45 g/L caused a significant reduction in daidzein production. The daidzein content in the medium supplemented with 45 g/L sucrose was 3.68 mg/g DW (Figure 5B), approximately 1.8-fold lower than the maximum value. Similar results were also observed for the production of genistein. The lowest genistein content was detected in hairy roots cultivated in the MS medium supplemented with 15 g/L sucrose, while the highest value was detected in hairy roots cultivated in the medium supplemented with 30 g/L sugar. Increasing the sugar concentration to 45 g/L led to a slight decrease in genistein production. Therefore, a sucrose concentration of 30 g/L was the optimal concentration for daidzein and genistein production in hairy root cultures of *P. mirifica*. This result aligned with a study by Li et al. [51], who reported that 30 g/L sucrose enhanced chlorogenic acid production in *Lonicera macrantha* cell cultures.

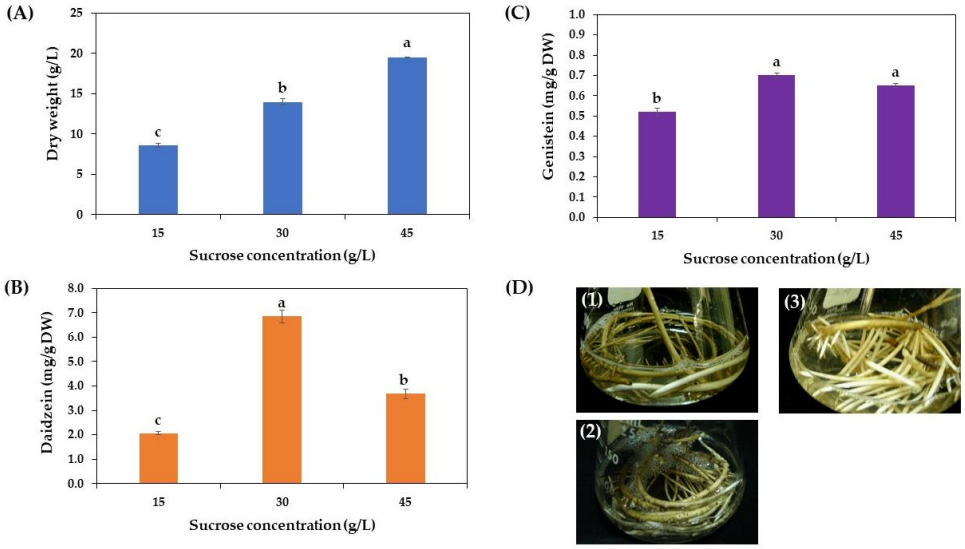


Figure 5. Effects of sucrose concentration on the growth (**A**), daidzein (**B**) and genistein (**C**) production, and morphology (**D**) of hairy roots of *Pueraria candollei* var. *mirifica* after cultivation in Murashige & Skoog (MS) medium for 30 days. Hairy roots of *P. candollei* var. *mirifica* cultured in MS medium supplemented with 15 g/L (**D-1**), 30 g/L (**D-2**), or 45 g/L sucrose (**D-3**). The bars followed by the same letters in each graph are not significantly different at $p \leq 0.05$ based on DMRT analysis.

Notably, the contents of daidzein and genistein decreased under high sugar concentrations. This might be attributed to osmotic stress, which may disturb the expression of key genes involved in the biosynthetic pathway of daidzein and genistein production. The negative effect of high sugar concentrations on secondary metabolite production has also been reported in other plants, such as *Morinda citrifolia* [52], *Gynura procumbens* [53], and *R. carthamoides* [25].

The present study demonstrated that a high sucrose concentration (45 g/L) was the best for biomass accumulation, while a low sucrose concentration (30 g/L) was favorable for daidzein and genistein production. A similar phenomenon was also recorded in hairy root cultures of *R. carthamoides*, in which high biomass accumulation was detected in medium supplemented with 30 g/L sucrose, while high concentrations of quercetin and quercetin hexosides were detected in medium supplemented with 10 g/L sugar [25]. This information suggests that biomass and secondary metabolite production require different sugar concentrations depending on the plant species and culture conditions. To increase the production of daidzein and genistein in the hairy root cultures of *P. mirifica*, a two-step cultivation technique may be needed, i.e., a biomass production step using a cultivation medium with a relatively high sugar concentration and a bioactive compound production step using a medium with a low sugar concentration.

3.3.4. Effect of Incubation Temperature on Growth and Daidzein and Genistein Production

Temperature is considered one of the important physical factors affecting plant growth and secondary metabolite production. Temperature can act as an abiotic elicitor that plays a vital role in in vitro cell culture for biomass and phytoconstituent production [28,54]. The expression of genes or the activity of enzymes involved in plant physiological and biochemical processes is also closely linked with temperature, and as previously reported, the optimum temperature for plant growth and development, as well as plant secondary metabolite production, is plant species-specific. Thus, the temperature for in vitro plant growth and development and secondary metabolite production needs to be optimized. Based on the literature review, no information is available regarding the optimum temperature for growth and bioactive compound production in hairy root cultures of *P. mirifica*. Hence, this study investigated the effect of incubation temperatures of 22, 26, and 30 °C

on biomass and daidzein and genistein production in hairy root cultures of this medicinal plant.

No significant difference in hairy root growth was observed at the various incubation temperatures tested in this study (Figure 6A). The dry cell mass was in the range of 13.06–13.86 g/L, in which the maximum biomass yield (13.86 g/L) was recorded at 26 °C. All of the hairy roots cultivated at different temperatures exhibited similar morphologies, i.e., high branches with relatively large hairy roots (Figure 6D). These results suggested that hairy roots of *P. mirifica* grow well at incubation temperatures in the range of 22–30 °C. A similar result was noted in the somatic embryo culture of *Eleutherococcus senticosus*, in which the optimum temperature for growth was 24 °C [17]. Furthermore, a temperature in the range of 25–30 °C yielded relatively high biomass contents of the callus culture of *Solanum nigrum* [14].

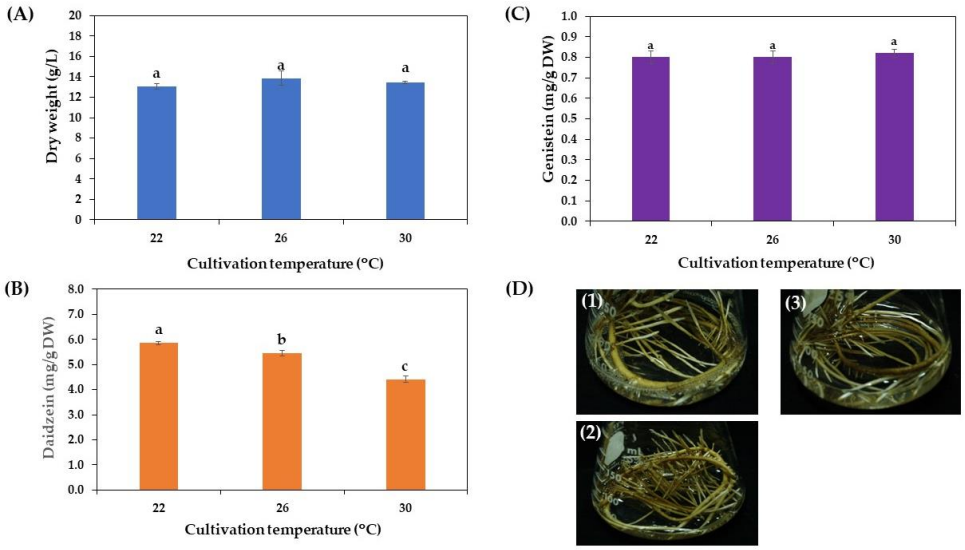


Figure 6. Effects of incubation temperature on the growth (**A**), daidzein (**B**) and genistein (**C**) production, and morphology (**D**) of hairy roots of *Pueraria candollei* var. *mirifica* after cultivation in Murashige & Skoog (MS) medium for 30 days. Hairy roots of *P. candollei* var. *mirifica* were cultured in MS medium and incubated at 22 °C (**D-1**), 26 °C (**D-2**), or 30 °C (**D-3**). The bars followed by the same letters in each graph are not significantly different at $p \leq 0.05$ based on DMRT analysis.

Figure 6B summarizes the production of daidzein from a hairy root culture of *P. mirifica* at various incubation temperatures. A low incubation temperature yielded a significantly greater daidzein content than a high temperature. The maximum concentration of daidzein was recorded at 5.86 mg/g DW at 22 °C. Increasing the incubation temperature to 30 °C significantly reduced daidzein production, resulting in the lowest value of 4.41 mg/g DW. The reduction in daidzein production under high-temperature conditions may be attributed to the downregulation of genes involved in the biosynthetic pathway of this bioactive compound. As previously reported, relatively high temperatures can downregulate the expression of genes involved in the biosynthesis of some bioactive compounds, such as ascorbic acid, in tea plants [55]. Notably, the results obtained in this study were in accordance with those of a study by Chan et al. [56], who reported the maximum anthocyanin production in the cell culture of *Melastoma malabathricum* at a low temperature of 20 °C compared to that under high-temperature conditions (26 and 29 °C).

Unlike genistein production, the incubation temperature in the range of 22 to 30 °C did not affect the production performance of this bioactive compound. The genistein content produced by the hairy root culture of *P. mirifica* was in the range of 0.80–0.82 mg/g DW (Figure 6C). This finding differed from that reported for genistein production in the callus culture of *P. candollei*, in which high-temperature conditions (35 °C) promoted the production of this phytoconstituent [13]. This information suggested that the optimum

temperature for genistein production varied depending on the cell culture system. Based on the results obtained in this study, a temperature of 26 °C should be used to cultivate *P. mirifica* hairy root culture since it provides relatively high biomass and daidzein and genistein production.

3.3.5. Effect of Agitation Speed on Growth and Daidzein and Genistein Production

Agitation speed is another physical factor that affects the growth and secondary metabolite production of in vitro plant cell culture. This is mainly attributed to the mixing behavior of the culture system. A good agitation speed can facilitate homogenous nutrient uptake by plant cells and provide sufficient oxygen and carbon dioxide for cell growth, development, and metabolic processes related to primary and secondary metabolite production [15,57]. Previous studies have shown that plant cells are sensitive to hydrodynamic stress caused by agitation, and the severity of the stress depends on several factors, specifically the plant species, culture type (cell suspension or hairy root culture), medium volume, and culture vessel configuration [15,23]. Therefore, optimizing the agitation speed for in vitro cell cultures is necessary. Several studies have reported on the effect of agitation speed on biomass and secondary metabolite production in the in vitro cell culture of many plants, but no information is available regarding the hairy root culture of *P. mirifica*. Thus, the effect of agitation speed on growth and daidzein and genistein production in the hairy root culture of this plant was evaluated in this study, and the results are illustrated in Figure 7.

Although the biomass production was not significantly different at various agitation speeds tested in this study, hairy roots cultivated at 110 rpm yielded the highest biomass content (15.30 g/L). A slight decrease in biomass was recorded for the hairy roots cultured at 90 rpm (14.00 g/L) and 130 rpm (14.20 g/L) (Figure 7A). A similar morphology of hairy roots cultivated at various agitation speeds was observed (Figure 7D). Thus, it can be concluded that 110 rpm is the best agitation speed for the biomass production of *P. mirifica* hairy root culture. These results were in close agreement with those of studies by Singh and Chaturvedi [57] and Mishra et al. [58], who reported the optimum agitation speed for biomass production of *Spilanthes acmella* and *Decalepis hamiltonii* cell suspension cultures at 120 rpm.

Considering the production of bioactive compounds, a low agitation speed was suitable for daidzein production, while a high agitation speed was preferable for genistein production. The highest daidzein content (5.29 mg/g DW) was recorded at an agitation speed of 90 rpm, and the lowest content (3.82 mg/g DW) was detected at 130 rpm (Figure 7B), approximately 1.4-fold lower than that at 90 rpm. In contrast, the genistein content peaked (0.96 mg/g DW) at an agitation speed of 130 rpm, and the lowest value (0.86 mg/g DW) was found at 90 rpm (Figure 7C), which was not significantly different among the agitation speeds tested. Relatively low agitation speeds have been shown to be suitable for biomass and secondary metabolite production in several plants, such as hairy root cultures of *A. belladonna* [23] and cell suspension cultures of *S. acmella* [57] and *D. hamiltonii* [58]. Thus, based on the overall content of daidzein and genistein produced in this study, an agitation speed of 90 rpm may be suitable for their production. In addition to providing relatively high biomass production, in terms of commercial production, this agitation speed is considered a cost-effective approach due to the low energy required compared to high agitation speeds.

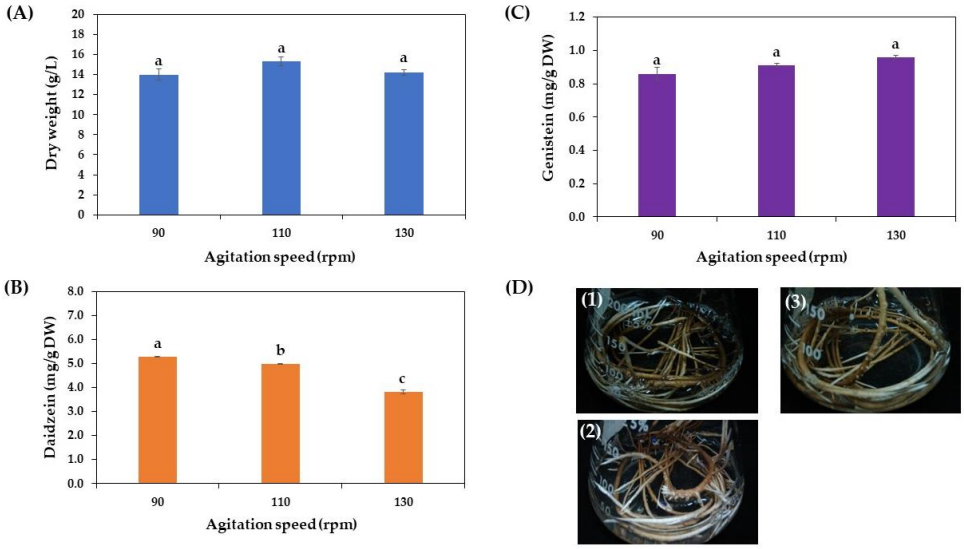


Figure 7. Effects of agitation speed on the growth (**A**), daidzein (**B**) and genistein (**C**) production, and morphology (**D**) of hairy roots of *Pueraria candollei* var. *mirifica* after cultivation in Murashige & Skoog (MS) medium for 30 days. Hairy roots of *P. candollei* var. *mirifica* were cultured in MS medium with agitation speeds of 90 rpm (**D-1**), 110 rpm (**D-2**), and 130 rpm (**D-3**). The bars followed by the same letters in each graph are not significantly different at $p \leq 0.05$ based on DMRT analysis.

Thanonkeo and Panichajakul [13] reported that the concentrations of daidzein and genistein in the tuberous roots of 16-month-old field-cultivated plants were 0.15 and 0.08 mg/g DW, respectively. Thus, based on the current results, the maximum daidzein and genistein contents produced by the hairy root culture of *P. mirifica* were approximately 45.7- and 12.0-fold greater than those in tuberous roots. The concentrations of daidzein and genistein produced from the hairy root culture of *P. mirifica* were comparable with other production platforms, such as microbial fermentation and biotransformation using microbial enzymes or genetically engineered microorganisms. For instance, Jiyeon et al. [59] demonstrated that 1.18 mg/g daidzein and 2.00 mg/g genistein could be obtained after fermentation of soymilk using a mixture of *Lactobacillus paraplantarum* and *Enterococcus faecium*. Another study by El-Shazly et al. [60] reported a maximum concentration of daidzein (0.51 mg/g) and genistein (0.18 mg/g) after fermentation of soymilk using *L. rhamnosus* and *L. casei*, respectively. Shin et al. [61] reported the production of daidzein and genistein via the biotransformation process of Korean wild soybean extracted using a thermostable β-galactosidase enzyme isolated from *Thermoproteus uzoniensis*, with maximum concentrations of 3.05 mg/g of daidzein and 3.10 mg/g of genistein. Liu et al. [62] demonstrated that genetically engineered *Saccharomyces cerevisiae* could produce 2.85 mg/g daidzein and 1.12 mg/g genistein from glucose. These findings suggest that hairy root culture is a promising platform for the production of these phytochemicals.

However, despite the significantly higher contents of daidzein and genistein produced by hairy root culture compared to tuberous roots from field-cultivated plants, the production capacity remains low (less than 1% of the dry biomass). This might not be beneficial for commercial production. In contrast, chemical synthesis processes can produce up to 0.59 g of daidzein and 0.62 g of genistein from one gram of raw material, which is approximately 86 and 645 times greater than those obtained from in vitro plant cell culture [63]. To improve the production efficiency of in vitro hairy root culture, several techniques have been successfully utilized to enhance secondary metabolite production in plant cell cultures. These include elicitation using biotic and abiotic elicitors, addition of precursors in the biosynthesis pathway of plant secondary metabolite production, application of appropriate bioreactors for large-scale production, and genetic engineering to up- and downregulate corresponding genes in the biosynthesis pathway [15,16,18,20,23,64]. Further investigations based on these findings should be performed in hairy root cultures

of *P. mirifica*. Additionally, the literature review indicates that *P. mirifica* produces and accumulates various bioactive compounds, such as kwakhurin, puerarin, daidzin, genistin, miroestrol, and deoxymiroestrol, which possess several health benefits [65]. Therefore, future studies should also focus on the accumulation of these phytoconstituents in hairy root cultures of *P. mirifica*.

4. Conclusions

This study demonstrated that various cultivation conditions affect the growth and production of daidzein and genistein in hairy root cultures of *P. mirifica*. These conditions include culture medium, light conditions, sucrose concentration, incubation temperature, and agitation speed. The MS medium with 30 g/L sucrose proved optimal for growth and bioactive compound production. Furthermore, cultivating hairy root cultures under specific light/dark (16/8 h) photoperiods in a controlled incubator shaker at 26 °C and an agitation speed of 90 rpm proved to be suitable for promoting plant growth and daidzein and genistein production, giving a significantly greater yield of bioactive compounds than those obtained from normal roots.

The experimental data established in this study hold great promise for further large-scale investigations of sustainable daidzein and genistein production using *P. mirifica* hairy root cultures. This can significantly expand the application of these phytochemicals as functional ingredients in cosmeceutical and nutraceutical products that may address various health concerns, including aging effects, menopausal symptoms, and conditions associated with estrogen deficiency.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10080788/s1, Table S1: Chemical compositions of plant cell culture media used in this study.

Author Contributions: Conceptualization, S.T., P.T. and P.K.; methodology, S.T., P.T. and P.K.; software, P.T.; validation, S.T. and P.K.; formal analysis, S.T., P.T. and P.K.; investigation, S.T., T.P. and P.K.; resources, S.T. and P.K.; data curation, S.T., P.T. and P.K.; writing—original draft preparation, S.T., P.T. and P.K.; writing—review and editing, S.T., P.T. and P.K.; visualization, S.T., T.P. and P.T.; supervision, S.T., P.T. and P.K. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The experimental data are contained within the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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