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Comparative Growth and Bacoside Production in Diploid and Tetraploid *Bacopa monnieri* (L.) Wettst. Cultivated Indoors via Hydroponic and Soil Culture Systems

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Abstract: *Bacopa monnieri*, a cognitive-enhancing herb crucial in health supplements, faces quality variations and contamination by toxic substances in conventional field cultivation, which hinders industrial use. Here, indoor cultivation of diploid (2x) and tetraploid (4x) *B. monnieri* using hydroponic and soil systems was studied. Soil cultivation promoted longer shoot lengths but resulted in lower biomass and chlorophyll contents compared to hydroponic cultivation. Conversely, soil cultivation significantly elevated total phenolics, total triterpenoids, bacoside A3, and bacoside X contents in both lines, showing 1.7- to 3.3-fold increases over hydroponic cultivation. Furthermore, 4x plants grown in soil had higher bacoside II and total bacoside contents than hydroponically grown plants, with 2- and 1.5-fold increases, respectively. Yet, no significant differences were observed in growth and pigment between 2x and 4x lines under the same system. Similarly, no significant differences in bioactive compound productions were found between 2x and 4x hydroponically grown plants. However, in soil, 4x plants exhibited higher total phenolic content, bacoside II, and total bacoside contents compared to 2x plants. Interestingly, 2x plants grown in soil were the top performers for bacoside production per plant. These findings optimize cultivation practices to meet industry demands, warranting further research into large-scale production techniques.

Keywords: Brahmi; medicinal plant; secondary metabolites; indoor farming; LED light



Citation: Inthima, P.; Supaibulwatana, K. Comparative Growth and Bacoside Production in Diploid and Tetraploid *Bacopa monnieri* (L.) Wettst. Cultivated Indoors via Hydroponic and Soil Culture Systems. *Horticulturae* **2024**, *10*, 574. <https://doi.org/10.3390/horticulturae10060574>

Academic Editor: Saeid Hazrati

Received: 13 April 2024

Revised: 28 May 2024

Accepted: 29 May 2024

Published: 31 May 2024



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1. Introduction

Bacopa monnieri, commonly known as Brahmi, is an aquatic perennial or annual succulent herb characterized by its prostrate, glabrous stem and opposite, obovate green leaves [1]. Thriving in tropical regions, it is typically found in wetlands and along muddy shores [1]. Brahmi has garnered significant attention in the realm of herbal medicine for its cognitive enhancement properties deeply rooted in Indian Ayurvedic traditional medicine for more than 3000 years [1]. Extensive research has underscored its multifaceted pharmacological effects, ranging from neuroprotective [2], anti-anxiety [3], anti-Parkinson's [4], and antidepressant properties [5]. Central to its therapeutic efficacy are steroidal saponin glycosides, including bacoside A3, bacoside II, bacoside X, and bacosaponin C [6], which exert neuroprotective and cognitive-enhancing effects. Additionally, numerous phytochemicals have been found in *B. monnieri* such as flavonoids, phenolics, stigmastanol, apigenin, and nicotine [7,8].

In recent years, the demand for *B. monnieri* raw material has surged, particularly amidst the backdrop of the COVID-19 pandemic, as individuals increasingly seek natural health supplements to support mental well-being and cognitive function [8,9]. However,

traditional cultivation methods in waterlogged paddy fields face formidable challenges, such as limited suitable land availability and fluctuating quantity and quality of raw materials due to seasonal variations [10,11]. Additionally, the presence of harmful heavy metal and pesticide contamination in raw materials intended for health supplement production presents a serious concern [12]. Previous studies have indicated that *B. monnieri* exhibits a high tolerance to toxic heavy metals and can accumulate them [13–16]. Consequently, cultivation of *B. monnieri* in environments such as paddy fields or areas contaminated with heavy metals may yield undesirable raw materials.

In response to these challenges, indoor cultivation of *B. monnieri* has emerged as a promising alternative. By harnessing controlled environments and advanced cultivation techniques, indoor farming offers a sustainable solution to ensure consistent product quantity and quality while mitigating environmental risks associated with conventional agriculture [17,18]. Indoor cultivation, especially when employing vertically grown systems, offers significant benefits in land use efficiency and helps eliminate concerns related to pesticide and heavy metal contamination [19,20]. While indoor cultivation with artificial lighting has been utilized in various plant species, including vegetables and medicinal plants [17,21,22], there are no reported instances of its application in *B. monnieri*.

Among the various indoor cultivation methods, hydroponics stands out for its ability to precisely regulate nutrient delivery to plants, maximizing growth potential and yield. However, the complexity and initial investment required for hydroponic systems often render them inaccessible to small-scale growers and households [19]. Recognizing the need for a more accessible approach, our study attempts to explore the feasibility of soil-based indoor cultivation of *B. monnieri*. Nonetheless, while comparisons between hydroponic and open-field or greenhouse soil-grown plants have been documented in various plant species [23–25], no study has yet explored the potential of soil cultivation in indoor settings.

This study aims to investigate indoor cultivation, without temperature control, using both hydroponic and soil-based systems, with the goal of identifying an affordable method for small-scale farmers to produce high-quality *B. monnieri* raw materials. The findings shed light on how cultivation methods influence the growth and biochemical composition of diploid and autotetraploid *B. monnieri*, offering valuable insights for its cultivation and potential applications across different fields.

2. Materials and Methods

2.1. Cultivation of *B. monnieri*

Shoots measuring 5–7 cm in length from diploid (2x) and tetraploid (4x) line 82, as previously produced [26], were utilized as the plant materials in this study. For soil cultivation, commercial clay (for water lily cultivation) and coarse sand were sun-dried before being mixed at a 1:1 ratio by weight to obtain a soil mixture. The shoots were then grown in plastic pots (5 × 5 × 5 cm) filled with this soil mixture and supplemented with fertilizer (13-13-13 Osmocote®, 0.5 g per pot). Alternatively, for hydroponic cultivation, the shoots were grown in plastic pots filled with high-density hydroton (0.8 cm in diameter). The pots from both soil and hydroponic cultivation systems were placed in plastic containers (35 cm width × 50 cm length × 15 cm height) containing reverse osmosis water and Hoagland solution [27], respectively (Figure 1). These cultures were maintained indoors in a room without temperature control, with daytime ambient temperature ranging from 32 °C to 37 °C and nighttime temperature ranging from 28 °C to 32 °C. They were subjected to white LED light (80 µmol/m²/s) for 12 h a day. The reverse osmosis water was automatically refilled once a day to maintain the original volume of both cultivation systems.

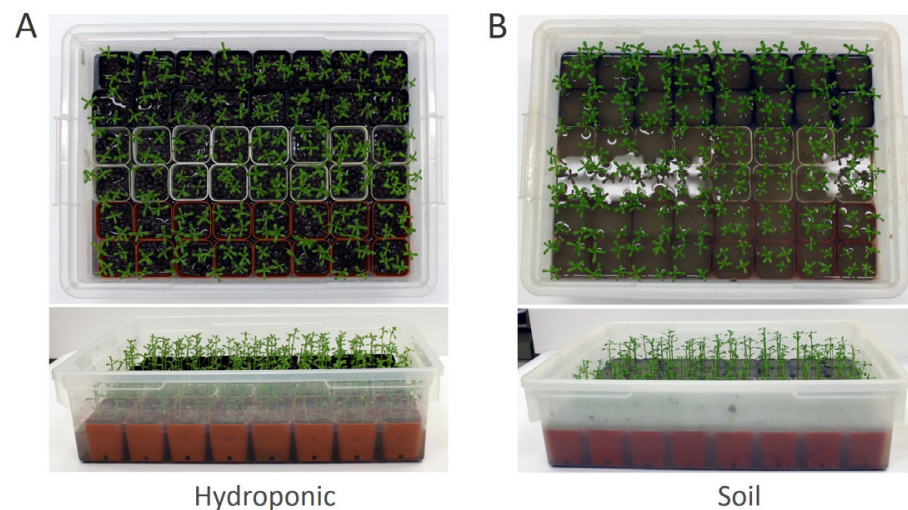


Figure 1. Cultivation of *B. monnieri* under the hydroponic (A) and soil (B) systems used in this study. The top panel shows the top view, and the bottom panel shows the side view.

2.2. Growth and Biomass Measurements of *B. monnieri*

The growth and biomass of *B. monnieri* were assessed 35 days after cultivation, at the vegetative stage. Shoot length was measured using a tape measure, and the shoots were then harvested by cutting at the base, just above the soil or hydroton. The fresh weight was promptly determined post-harvest, while the dry weight was obtained after drying in a hot air oven at 40 °C for 48 h. The leaf area of the 5th leaf was measured from digital scans using ImageJ software version 2.15.1 [28].

2.3. Analysis of Chlorophyll and Carotenoid Content in Leave of *B. monnieri*

Chlorophyll and carotenoid were extracted and quantified from the 5th to 6th leaf positions following the method outlined by Ramírez-Mosqueda and Iglesias-Andreu [29], with adaptation. Fresh leaves weighing 0.1 g were homogenized in a 2 mL tube containing stainless steel beads (0.5 mm in diameter) using a BeadBug™ homogenizer (Benchmark Scientific, Sayreville, NJ, USA). Subsequently, chlorophyll and carotenoid were extracted from these homogenized leaves using a 1 mL cold mixture of 80% acetone and absolute ethanol (1:1 v/v) by vortexing for 5 min, followed by centrifugation at 10,000 rpm for another 5 min. The resulting liquid extracts were collected and their absorbances at wavelengths of 441, 663, and 645 nm were measured using a microplate reader (BioTek Synergy H1, Agilent Technologies, Santa Clara, CA, USA). The contents (mg/gFW) of chlorophyll and carotenoid were calculated using the following formulas:

$$\text{Chlorophyll a} = [(12.25 \times A_{663}) - (2.55 \times A_{645})] \times [1/(100 \times W)]$$

$$\text{Chlorophyll b} = [(20.3 \times A_{645}) - (4.91 \times A_{663})] \times [1/(100 \times W)]$$

$$\text{Total chlorophyll} = [(7.34 \times A_{663}) + (17.76 \times A_{645})] \times [1/(100 \times W)]$$

$$\text{Carotenoid} = [((4.46 \times A_{441}) - (\text{Chlorophyll a})) + \text{Chlorophyll b}] \times [1/(100 \times W)]$$

Here, A_λ represents the absorbance value at λ nm and W denotes the leaf weight (g).

2.4. Extraction of *B. monnieri* Phytochemicals

Phytochemicals were extracted following the methods described by Nopparat et al. [8]. Initially, the dried plant sample was powdered. Subsequently, phytochemicals were extracted from a 0.05 g powder sample using 1 mL of methanol in a 2 mL tube. The extraction process involved vortexing for 1 min, followed by sonication for 15 min in an ultrasonic water bath, and centrifugation at 10,000 rpm for 5 min. The liquid portion was collected,

and the remaining powder sample underwent two additional extraction phases. All three liquid portions were combined and dried at 40 °C using a dry bath incubator. The resulting dry residues were then redissolved with 1 mL methanol by vigorously vortexing for 2 min to obtain the *B. monnieri* phytochemical extract (BPE), which was subsequently stored at −20 °C until further analysis.

2.5. Quantification of Total Phenolic Content in *B. monnieri*

The total phenolic content (TPC) was determined using the Folin–Ciocalteu assay, following the protocol outlined by Nopparat et al. [8]. A gallic acid solution (Bio Basic, Markham, ON, Canada) or BPE (15 µL) was mixed with the Folin–Ciocalteu reagent (15 µL) and deionized water (240 µL). The mixture was then gradually mixed and incubated for 5 min. Following this, 70 µL of 10% (*w/v*) Na₂CO₃ solution was added and continuously mixed in the dark at room temperature for 60 min. The absorbances at 750 nm were measured using a microplate reader (BioTek Synergy H1, Agilent Technologies, Santa Clara, CA, USA). The TPC was calculated using a standard calibration curve of gallic acid and expressed as milligrams of the gallic acid equivalent (GAE) per gram of dried plant sample (mg GAE/g).

2.6. Quantification of Total Flavonoid Content in *B. monnieri*

The total flavonoid content (TFC) was determined using the AlCl₃ colorimetric assay, following the methods described by Nopparat et al. [8]. A quercetin solution (Fisher Scientific, Hampton, NH, USA) or BPE (30 µL) was mixed with 10 µL of 5% (*w/v*) NaNO₂ and 120 µL of deionized water, followed by brief vortexing and was then incubated at room temperature for 5 min. Then, 10 µL of 10% (*w/v*) AlCl₃ was added to the mixture and incubated for an additional 6 min at room temperature. Afterward, 60 µL of 1 N NaOH and 70 µL of deionized water were added before quantifying the absorbances at 510 nm using a microplate reader (BioTek Synergy H1, Agilent Technologies, Santa Clara, CA, USA). The TFC was calculated using a standard calibration curve of quercetin and expressed as milligrams of the quercetin equivalent (QE) per gram of dried plant sample (mg QE/g).

2.7. Quantification of Total Triterpenoid Content in *B. monnieri*

The total triterpenoid content (TTC) was determined according to the protocol outlined by Nopparat et al. [8]. Ursolic acid (Sigma-Aldrich, St. Louis, MO, USA) or BPE (20 µL) was dried at 50 °C in a hot air oven. Next, it was combined with 10 µL of 5% (*w/v*) vanillin–acetic acid and 18 µL sulfuric acid solutions. The mixture was thoroughly mixed, incubated at 70 °C for 30 min, and then allowed to cool. Subsequently, 72 µL of glacial acetic acid was added before measuring the absorbances at 573 nm using a microplate reader (BioTek Synergy H1, Agilent Technologies, Santa Clara, CA, USA). The TTC was calculated based on a calibration curve of ursolic acid and expressed as milligrams of the ursolic acid equivalent (UAE) per gram of dried plant sample (mg UAE/g).

2.8. Quantification of Bacoside Content in *B. monnieri*

Bacoside content was quantified following the protocol outlined by Nopparat et al. [8]. The BPE underwent filtration through a 0.45 µm nylon syringe filter (Tianjin Fuji Science & Technology Co., Ltd., Tianjin, China) prior to HPLC analysis using the 1260 Infinity II LC System (Agilent Technologies, Santa Clara, CA, USA). The system was equipped with a LiChroCART® 4-4 Purospher® STAR RP-18 endcapped (5 µm) column (250 × 4.6 mm, Merck, Darmstadt, Germany) and a guard column. Chromatographic separation was achieved using a mobile phase consisting of 65% (*v/v*) acetonitrile and 35% (*v/v*) aqueous phosphoric acid (0.2% *v/v*, pH 3.0) at a flow rate of 1 mL/minute. Bacoside compounds were detected at 205 nm using a diode array detector, and their retention time was compared with authentic standards including bacoside A3, bacopaside II, bacopaside X (Sigma-Aldrich, USA), and bacopasaponin C (ChromaDex Standards, Los Angeles, CA, USA). The content of each bacoside compound in the BPE was determined using a linearity equation

derived from the standard curves of authentic standards. Consequently, the bacoside yields (mg/plant) were determined by multiplying the average content of each treatment by the dry weight of individual plants.

2.9. Quantification of DPPH Radical Scavenging Activity of *B. monnieri*

The DPPH radical scavenging activity was assessed following the protocol established by Nopparat et al. [8]. Initially, 50 µL of either methanol (control) or BPE was mixed with 100 µL of 0.1 mM DPPH solution. The resulting mixture was then incubated in darkness at 25 °C for 30 min. Following incubation, the absorbancy of the solution was measured at 517 nm using a microplate reader (BioTek Synergy H1, Agilent Technologies, Santa Clara, CA, USA). The inhibition percentage of DPPH radicals was calculated using the following formula:

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

where A_{control} is the absorbance value of the control (methanol) and A_{sample} is the absorbancy of the sample (BPE).

2.10. Analysis of Data

The experiment was conducted using a completely randomized design. Measurements of the shoot length, fresh weight, dry weight, and leaf area were taken from 20 plants. Chlorophyll and carotenoid contents as well as DPPH scavenging activity were analyzed from 5 plants. Bacoside contents and yields, TPC, TFC, and TTC were analyzed from 4 plants. Visualization of the data was conducted using the PlotsOfData web application [30]. To determine statistically significant differences among the treatments, a one-way analysis of variance (ANOVA) was employed, followed by Duncan's new multiple range test (DMRT) with a significance level set at $p \leq 0.05$.

3. Results

3.1. Growth and Biomass of *B. monnieri*

At 35 days after cultivation, the characteristics of the shoot and leaf (5th position) were examined in both hydroponic and soil cultivation systems for both 2x and 4x *B. monnieri*, as illustrated in Figure 2A and B, respectively. The shoot lengths were slightly longer in soil-grown plants, ranging from 16.5 to 24.0 cm, compared to those in the hydroponic system, which ranged from 15.0 to 21.0 cm, with this difference particularly noticeable in the 4x line (Figure 2C). However, no significant differences ($p \leq 0.05$) were observed in leaf area (Figure 2D) or fresh weight (Figure 2E) among both cultivation systems and plant lines. Interestingly, plants cultivated in the hydroponic system tended to exhibit a higher dry weight compared to those in the soil system (Figure 2F). Notably, a significant difference ($p \leq 0.05$) in dry weight between the two culture systems was evident in the 2x line, where hydroponically grown plants showed a 1.2-fold higher dry weight compared to soil-grown plants. However, no significant differences in dry weight were found among 4x plants grown under the same cultivation system.

When comparing different plant lines within the same cultivation system, both 2x and 4x plants showed no significant differences in shoot length, leaf area, fresh weight, and dry weight (Figure 2).

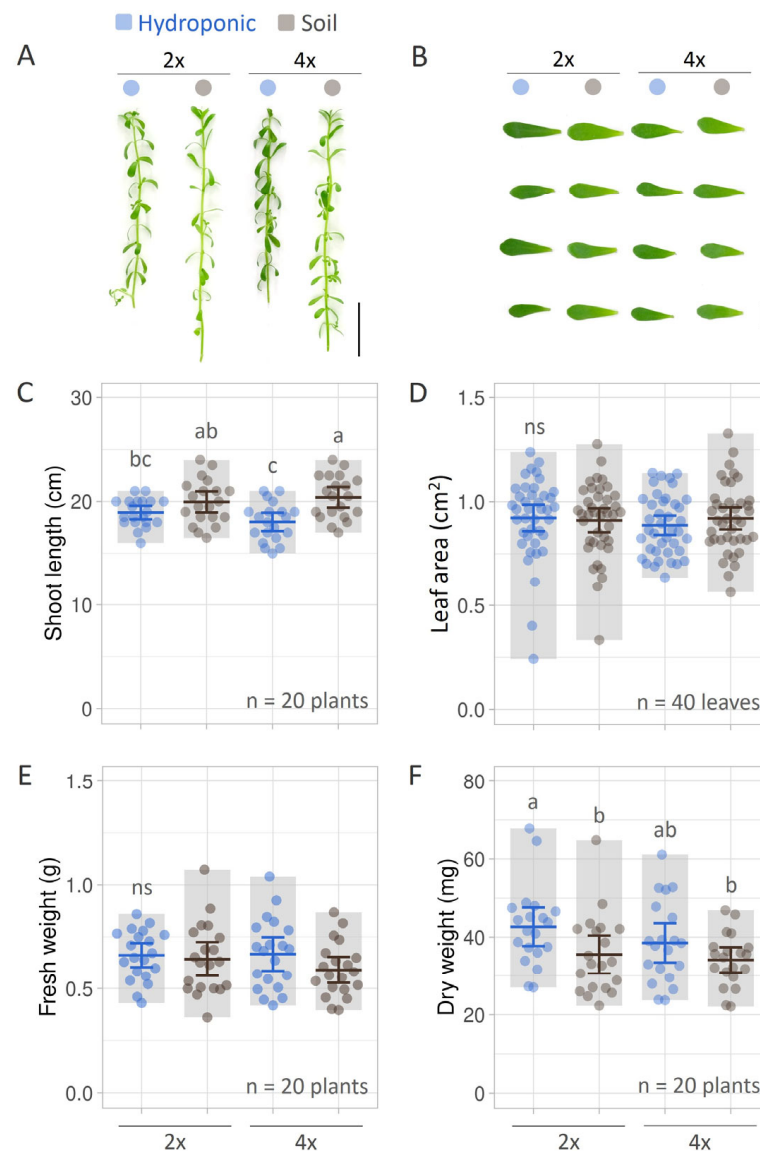


Figure 2. Shoot characteristics (A, bar = 5 cm), leaf characteristics (B, bar = 1 cm), shoot length (C), leaf area (D), fresh weight (E), and dry weight (F) of indoor-grown diploid (2x) and tetraploid (4x) *B. monnieri* using hydroponic and soil culture systems. Dots, horizontal lines, and vertical lines with caps and grey bars represent individual raw data, mean values, 95% confidence intervals, and data ranges, respectively. Different letters within the same figure indicate significant differences at $p \leq 0.05$ analyzed by DMRT. “ns” denotes non-significant differences.

3.2. Chlorophyll and Carotenoid Contents in Leaves of *B. monnieri*

Chlorophyll and carotenoid content were assessed 35 days after culture. It was observed that the contents of chlorophyll a in leaves of both 2x and 4x *B. monnieri* plants grown in hydroponic systems were slightly higher compared to those cultured in soil systems (Figure 3A). However, no significant differences were observed. In contrast, the chlorophyll b content in 2x plants grown in a hydroponic system (0.92 mg/gFW) was significantly higher than in those grown in a soil system by 2.1 times (Figure 3B). Additionally, the chlorophyll b content in 4x plants cultivated in a hydroponic system (0.67 mg/gFW) was also higher than in those grown in a soil system, although the difference was not statistically significant. Similarly, the total chlorophyll contents in *B. monnieri* plants grown in hydroponic systems were higher than those cultured in soil systems (Figure 3C). However, a statistically significant difference was only observed in the 2x line, with hydroponically grown 2x plants (2.27 mg/gFW) showing a 1.4-fold increase compared

to soil-grown 2x plants. In contrast, comparable levels of carotenoid (3.3 to 3.8 mg/gFW) were observed in all treatments, with no statistically significant differences (Figure 3D).

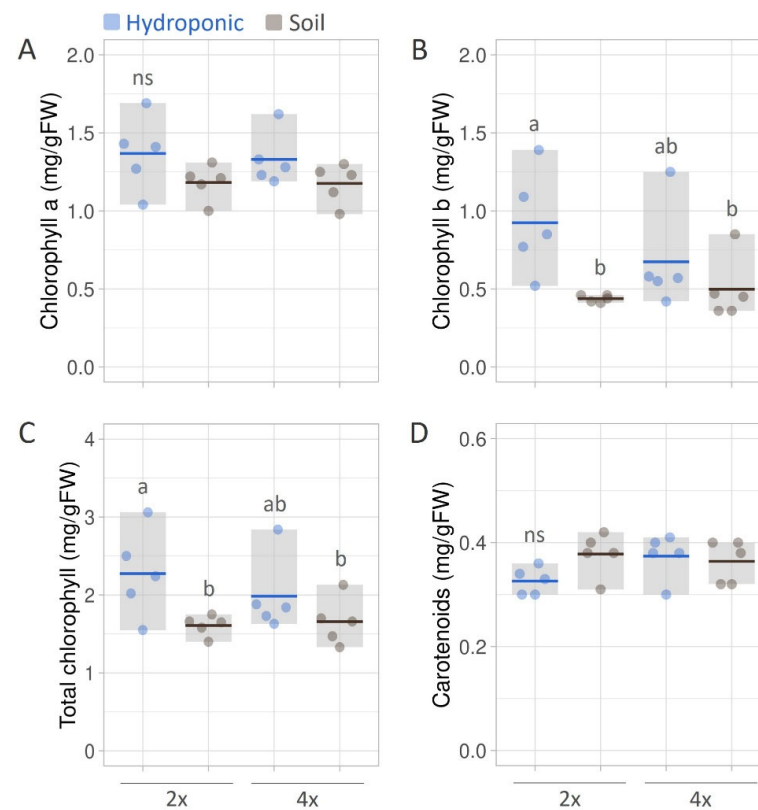


Figure 3. Chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), and carotenoid (D) contents of indoor-grown diploid (2x) and tetraploid (4x) *B. monnieri* using hydroponic and soil culture systems. Dots, horizontal lines, and grey bars represent individual raw data ($n = 5$ plants), mean values, and data ranges, respectively. Different letters within the same figure indicate significant differences at $p \leq 0.05$ analyzed by DMRT. “ns” denotes non-significant differences.

When comparing two plant lines, no statistically significant differences in chlorophyll a, chlorophyll b, total chlorophyll, and carotenoid contents were observed between 2x and 4x plants grown in the same cultivation system (Figure 3).

3.3. Total Phenolic, Total Flavonoid, and Total Triterpenoid Contents in *B. monnieri*

The total phenolic content (TPC), total flavonoid content (TFC), total triterpenoid (TTC), and DPPH radical scavenging activity in *B. monnieri* were analyzed 35 days after culture. TPCs were significantly higher in soil-grown plants compared to those in the hydroponic system for both plant lines (Figure 4A). In the soil system, TPCs in 2x (5.28 mg GAE/g) and 4x (8.80 mg GAE/g) plants were 1.7- and 3.7-fold higher, respectively, than those in the hydroponic system. On the contrary, no significant differences were observed in TFC among the treatments (Figure 4B). However, hydroponically grown plants exhibited slightly higher TFCs (2.60 and 2.50 mg QE/g for 2x and 4x plants, respectively) compared to soil-grown plants (2.21 and 2.29 mg QE/g for 2x and 4x plants, respectively). On the other hand, TTCs in plants grown in soil systems were markedly higher than those in the hydroponic system for both plant lines (Figure 4C). TTCs in 2x (10.49 mg UAE/g) and 4x (8.84 mg UAE/g) plants grown in soil were 3.3- and 2.2-fold higher, respectively, than those in the hydroponic system. Notably, relatively high DPPH radical scavenging activity was observed in *B. monnieri*, ranging from 70.0% to 73.7%. However, no significant differences ($p \leq 0.05$) were observed between both cultivation systems and plant lines (Figure 4D).

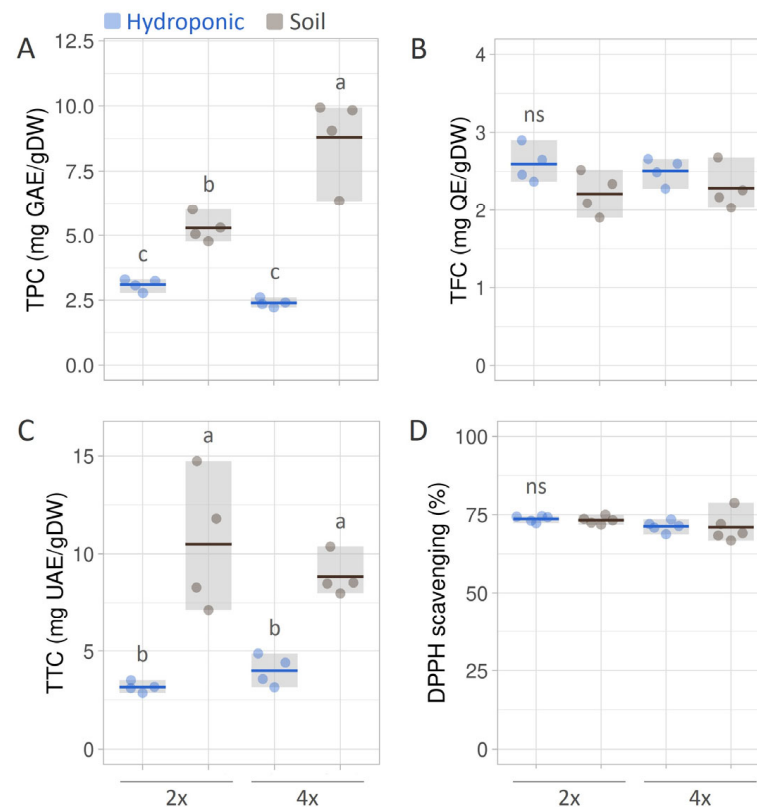


Figure 4. Total phenolic (TPC, (A)), total flavonoid (TFC, (B)), total triterpenoid (TTC, (C)) contents, and DPPH scavenging activity (D) of indoor-grown diploid (2x) and tetraploid (4x) *B. monnieri* using hydroponic and soil culture systems. Dots, horizontal lines, and grey bars represent individual raw data ($n = 4$ plants, except for DPPH $n = 5$ plants), mean values, and data ranges, respectively. Different letters within the same figure indicate significant differences at $p \leq 0.05$ analyzed by DMRT. "ns" denotes non-significant differences.

Regarding *B. monnieri* lines, there were no statistically significant differences in the TFC, TTC, and DPPH scavenging activity between 2x and 4x plants grown in the same culture systems (Figure 4B–D). However, differences between 2x and 4x plants were observed in TPC production under the soil system, where 4x plants produced 1.7-fold higher TPC than 2x plants (Figure 4A).

3.4. Bacoside Contents in *B. monnieri*

Figure 5 presents the HPLC chromatogram of the authentic bacoside mixture standards and the BPE. Each compound was successfully separated by the HPLC system, and the retention times of the compounds of interest in the BPE matched those of the authentic standards. The contents (% DW) of bacoside A3 were significantly higher in soil-grown plants compared to those in the hydroponic system for both plant lines (Figure 6A). The contents of bacoside A3 in 2x (1.33% DW) and 4x (1.43% DW) plants grown in soil were 1.9- and 1.7-fold higher, respectively, than those in the hydroponic system. On the other hand, divergent effects of cultivation systems between plant lines were evidenced in bacoside II content (Figure 6B). While there were no significant differences in bacoside II contents between the two cultivation systems in the 2x line (2.05% DW and 2.41% DW for hydroponic and soil systems, respectively), in the 4x line, plants grown in soil exhibited higher contents of bacoside II (3.43% DW) compared to those grown in the hydroponic system, by 2.1-fold. Likewise, the bacoside X content in 2x (0.49% DW) and 4x (0.61% DW) plants grown in the soil system were 2.5- and 3.2-fold higher, respectively, than in those grown in the hydroponic system (Figure 6C). In contrast, no significant differences were observed in bacosaponin C content among the treatments (Figure 6D). However, hydroponically

grown plants (2.26% DW and 2.36% DW for 2x and 4x plants, respectively) tended to produce slightly higher bacopasaponin C compared to soil-grown plants (1.94% DW and 2.01% DW for 2x and 4x plants, respectively). Notably, while no significant difference was observed in total bacoside content among cultivation systems in the 2x line, a distinction was reflected in 4x plants (Figure 6E). Specifically, 4x plants grown in soil exhibited higher contents of total bacoside compared to those grown in the hydroponic system, by 1.5 times.

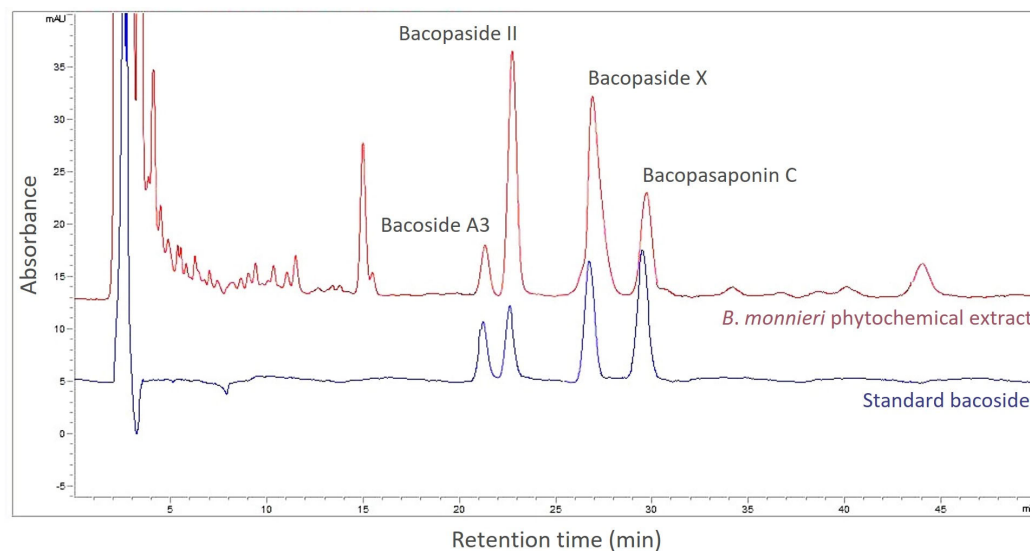


Figure 5. HPLC chromatogram of authentic standards bacoside mixture (blue line) and *B. monnieri* phytochemical extract (red line).

Upon analyzing the differences between the two plant lines, it was discovered that when grown in the hydroponic system, both 2x and 4x plants displayed no significant difference in the contents of all examined bacoside compounds (Figure 6). Likewise, under soil cultivation, the contents of bacoside A3, bacopaside X, and bacopasaponin C in 2x and 4x plants were found to be statistically similar. However, in the soil system, 4x plants exhibited 1.4- and 1.2-fold higher contents of bacopaside II and total bacoside, respectively, compared to 2x plants.

3.5. Bacoside Yields in *B. monnieri*

The yield of bacoside, calculated in terms of mg pre-planting and accounting for both dry biomass and bacoside content, is presented in Figure 7. The production yields of bacoside A3 (Figure 7A) in soil-grown 2x (0.67 mg/plant) and 4x (0.49 mg/plant) plants were 2.3 and 1.5 times higher than those in the hydroponic system (0.29 and 0.32 mg/plant for 2x and 4x plants, respectively). Similarly, the yield of bacopaside II (Figure 7B) in 2x (1.20 mg/plant) and 4x (1.17 mg/plant) plants grown in the soil system was 1.4 and 1.8 times higher, respectively, compared to those grown in the hydroponic system (0.87 and 0.64 mg/plant for 2x and 4x plants, respectively). Additionally, the yield of bacopaside X was significantly greater in soil-grown 2x (0.25 mg/plant) and 4x (0.21 mg/plant) plants compared to those in the hydroponic system (0.09 and 0.07 mg/plant for 2x and 4x plants, respectively), by 2.9 and 2.8 times, respectively (Figure 7C). Conversely, the yield of bacopasaponin C in the plants grown in the soil system did not differ significantly from those grown in the hydroponic system (Figure 7D). On the other hand, the yield of total bacoside was significantly higher in soil-grown 2x (3.09 mg/plant) and 4x (2.56 mg/plant) plants compared to those in the hydroponic system (2.22 and 1.95 mg/plant for 2x and 4x plants, respectively), exhibiting increases of 1.4 and 1.3 times, respectively (Figure 7E).

When comparing two plant lines, 2x and 4x plants cultivated in the hydroponic system exhibited no significant difference in yields of all determined bacoside compounds (Figure 7). Similarly, under the soil cultivation system, the yields of bacopaside II and bacopaside X in 2x and 4x plants showed no significant difference. However, under the soil cultivation system, 2x plants significantly yielded higher amounts of bacoside A3, bacopasaponin C, and total bacoside compared to 4x plants, by 1.4, 1.4, and 1.2 times, respectively.

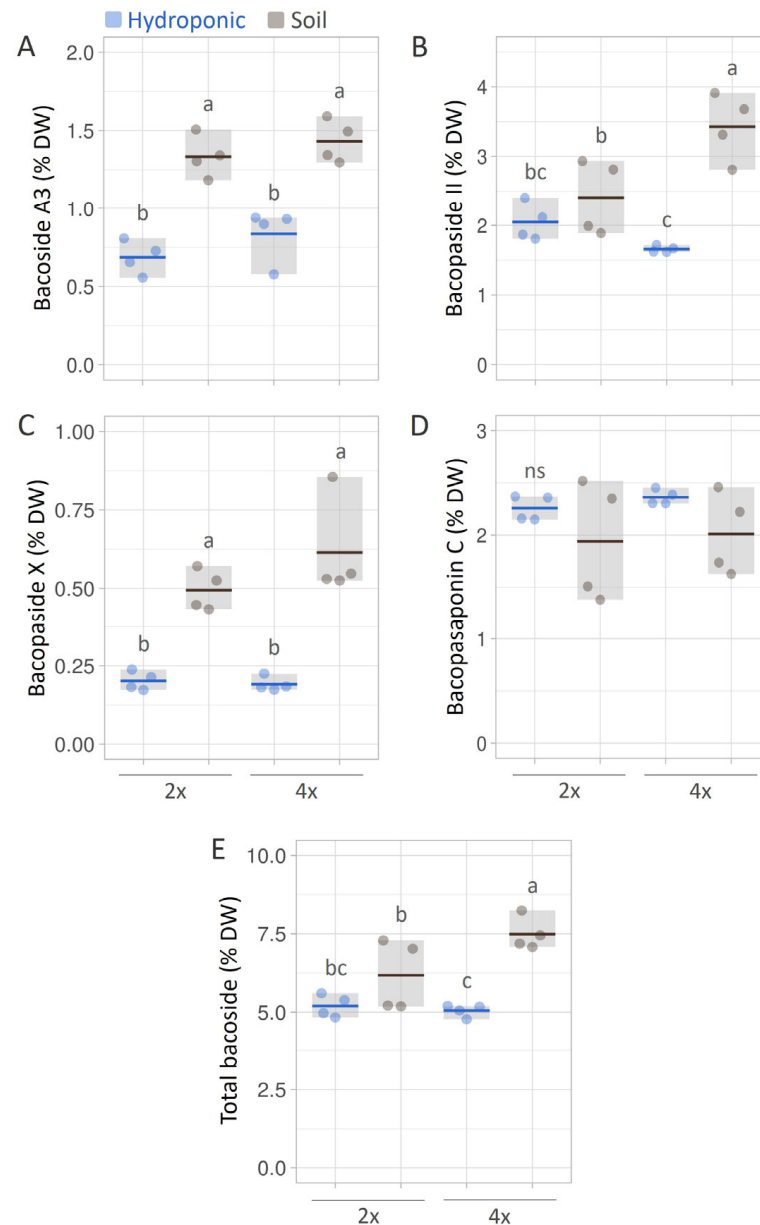


Figure 6. Bacoside A3 (A), bacopaside II (B), bacopaside X (C), bacopasaponin C (D), and total bacoside (E) contents (% DW) of indoor-grown diploid (2x) and tetraploid (4x) *B. monnieri* using hydroponic and soil culture systems. Dots, horizontal lines, and grey bars represent individual raw data ($n = 4$ plants), mean values, and data ranges, respectively. Different letters within the same figure indicate significant differences at $p \leq 0.05$ analyzed by DMRT. “ns” denotes non-significant differences.

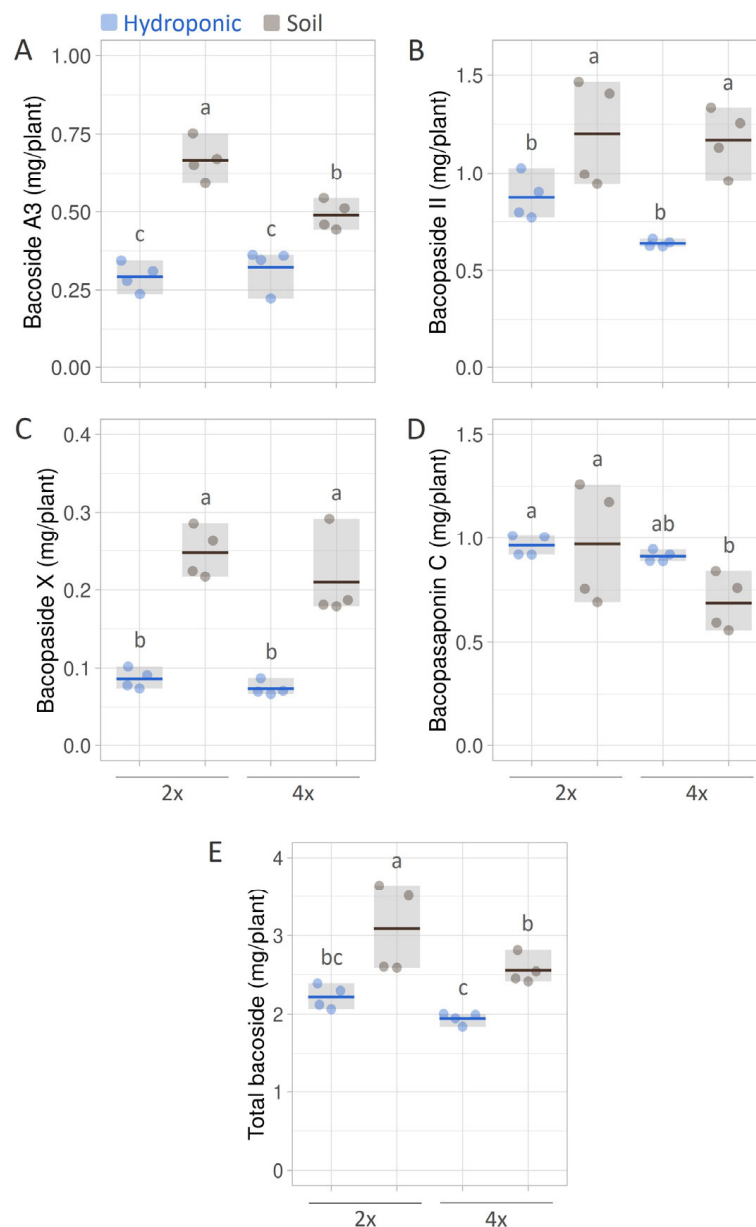


Figure 7. Bacoside A3 (A), bacopaside II (B), bacopaside X (C), bacopasaponin C (D), and total bacoside (E) yields (mg/plant) of indoor-grown diploid (2x) and tetraploid (4x) *B. monnieri* using hydroponic and soil culture systems. Dots, horizontal lines, and grey bars represent individual raw data ($n = 4$ plants), mean values, and data ranges, respectively. Different letters within the same figure indicate significant differences at $p \leq 0.05$ analyzed by DMRT.

4. Discussion

The variability in both the quantity and quality of herbal raw materials presents a significant challenge to commercial-scale production, primarily due to the complexities involved in ensuring product quality [31]. *B. monnieri*, like many other medicinal plants, exhibits considerable seasonal variation in its bioactive compounds when cultivated in open fields. Bansal et al. reported the variation of total bacoside A ranging from 2.64 to 6.32 mg/plant in *B. monnieri* accession BM1 during different seasons [11]. Similarly, variations in total bacoside saponins from 1.18 to 2.57% *w/w* dry weight in *B. monnieri* have been found [10]. Furthermore, contamination with harmful heavy metals and pesticides adds another serious concern for field-grown *B. monnieri* [12]. Previous studies have indicated that *B. monnieri* collected from heavy industrial areas contained chromium (Cr)

levels exceeding the limit set by the WHO for raw herbal materials, while cadmium (Cd) and lead (Pb) were also present, although below the specified limits [32]. Moreover, Mishra et al. reported that contamination of Cr, Cd, and Pb was found in all 12 samples of three Ayurvedic formulations containing *B. monnieri*, albeit below the WHO limit. Additionally, some pesticides were detected in some of those samples [33]. Furthermore, various experiments have demonstrated that *B. monnieri* has the capacity to accumulate harmful heavy metals and displays a notable tolerance to them [13–15]. For these reasons, conventional open-field cultivation of *B. monnieri* may result in the production of undesirable raw materials. Our study offers an alternative cultivation system that limits the impact of environmental factors, eliminates the use of pesticides and herbicides, and provides relatively high-quality *B. monnieri* with total bacoside content ranging from 5.05 to 7.49% DW (Figure 6E). However, the stability of both the quantity and quality of *B. monnieri* across different batches needs to be further examined.

The observed differences in shoot length and dry biomass between hydroponic and soil cultivation systems suggest that the choice of cultivation method can significantly impact the growth of *B. monnieri*, although with variations depending on plant lines (Figure 2). Notably, a significant difference in shoot length between soil and hydroponic cultivation was observed only in the 4x line (Figure 2C), while differences in dry biomass were evident only in the 2x line (Figure 2F), indicating an interplay of genotype. Despite previous reports suggesting differences in fresh biomass and leaf size between soil-grown and hydroponically grown plants [34–36], our results revealed no significant differences in either fresh biomass or leaf area between the two cultivation methods (Figure 2D). These disparate findings may be attributed to variations in climate conditions, as previous studies compared open-field soil-grown plants with greenhouse hydroponically grown ones, whereas our study maintained similar climate conditions for both cultivation methods. Additionally, differences in root growth and mineral absorption between hydroponic and soil cultivation methods may have contributed to these variations [35]. However, it is worth noting that *B. monnieri* is an aquatic plant well-adapted to low oxygen levels, as evidenced by its successful growth in both the static hydroponic and flooded soil systems employed in this study. Interestingly, differences in mineral composition among hydroponic and soil cultivations in this study slightly impacted the overall growth of both 2x and 4x *B. monnieri*, suggesting that a low mineral culture system, such as soil cultivation employed in this study, may be sufficient for this plant species.

While prior reports indicated that hydroponically grown plants produced higher chlorophyll a and chlorophyll b content in leaves compared to soil-grown plants [35,37], in this study, only the chlorophyll b contents in both 2x and 4x *B. monnieri* were significantly higher in the hydroponic system compared to soil cultivation (Figure 3). This discrepancy could be attributed to the higher availability of nitrogen in the Hoagland nutrient used in the hydroponic cultivation systems in this study, as it has been reported that high nitrogen levels induce high chlorophyll accumulation [38,39].

Several studies have reported the role of rhizomicrobes in inducing secondary metabolites in plants, such as induced essential oil production in oregano [40] and lemon balm [41], increased accumulation of flavonoids and triterpenoids in *Cyclocarya paliurus* plants grown under a low-level organic fertilizer [42], and increased production of bacoside A in *B. monnieri* [43]. Additionally, a previous study has shown that *Curcuma longa* cultivated in natural soil exhibits higher curcumin content compared to sterile soil. Moreover, the application of a bacteria consortium to natural soil further enhances curcumin levels [44]. Therefore, the significant increase in biochemical production in *B. monnieri* grown in soil, as opposed to hydroponic cultivation, suggests that the soil used in this study may contain some microorganisms, and their activity may play a pivotal role in inducing secondary metabolites in this study. However, further studies are needed to elucidate the presence of microbial activity underlying these differences.

When comparing the biochemical contents of soil-grown 4x *B. monnieri* with previously reported commercial products available in Thai online markets [8], several interesting

observations emerge. The TFC and bacopaside X levels in soil-grown 4x *B. monnieri* were relatively similar to those reported for commercial products. However, soil-grown 4x *B. monnieri* exhibited lower TTC levels compared to the commercial products. Notably, soil-grown 4x *B. monnieri* contained higher levels of bacoside A3, bacopaside II, and total bacosides, and slightly higher levels of bacopasaponin C compared to the reported commercial products. This suggests that cultivating 4x *B. monnieri* in soil under indoor conditions produces high-quality plant material, potentially surpassing the quality of dried plant and dried plant powder commercial products available online in Thai markets. This suggests its promising potential for use in the development of health supplement products. However, it is worth mentioning that the DPPH radical scavenging activity of soil-grown 4x *B. monnieri* (73.7%, Figure 4D) was slightly lower than that reported for commercial products, which ranged from 82.8% to 87.2%. This indicates that although soil-grown 4x *B. monnieri* shows promise as a high-quality source of bioactive compounds, there may be factors influencing its antioxidant properties that require further investigation.

Under soil cultivation, although the content (% DW) of total bacoside was higher in 4x plants compared to 2x plants (Figure 6), the yield (mg/plant) of total bacoside per plant was lower in 4x plants (Figure 7). This discrepancy in total bacoside yield can be attributed to the higher dry weight of 2x plants observed in Figure 2F. It is noteworthy that while tetraploid (4x) plants generally exhibit larger cell and organ sizes, leading to a higher biomass compared to diploid (2x) plants [45,46], some studies have reported no difference in size and biomass, and even lower growth in tetraploid plants [47,48]. Liu et al. [49] suggest that slower growth in tetraploid plants may be attributed to the increased energy required to maintain larger cell sizes. This indicates a complex relationship between ploidy level, growth, and biochemical composition, highlighting the need for further investigation into the underlying mechanisms governing these phenomena.

5. Conclusions

The cultivation method significantly influenced the growth, pigment composition, and phytochemical content of *B. monnieri*. While soil cultivation generally promotes longer shoot lengths and higher production of bioactive compounds, hydroponic cultivation offers better dry weight and pigment contents. Genetic factors also play a role, as evidenced by the higher production of certain bioactive compounds in 4x plants under soil cultivation. Notably, 2x plants grown in the soil system emerge as the best performers for bacoside production per plant. In this study, soil cultivation proves to be an effective method for producing harmful substance-free *B. monnieri* with high concentrations of bioactive compounds under indoor conditions. Its simple operation makes it an affordable choice for small-scale growers. However, before cultivation, thorough assessments of soil quality, including the determination of harmful heavy metals, are imperative to ensure the production of uncontaminated *B. monnieri*. Furthermore, exploring scale-up systems is crucial to assess the feasibility of larger-scale production. Additionally, a comprehensive examination of the stability of both the quantity and quality of *B. monnieri* in each batch is essential for maintaining consistency and reliability. By addressing these considerations, we can optimize cultivation practices and enhance the commercial availability of *B. monnieri* production for medicinal and commercial purposes.

Author Contributions: Conceptualization, P.I.; Investigation, P.I.; Methodology, P.I.; Data curation, P.I.; Formal Analysis, P.I.; Validation, P.I. and K.S.; Visualization, P.I.; Writing—original draft, P.I.; Writing—review and editing, P.I. and K.S.; Resources, P.I.; Project administration, P.I.; Funding acquisition, P.I.; Supervision, K.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by Grant No. RGNS 63–132 from the Office of the Permanent Secretary, Ministry of Higher Education, Science, Research, and Innovation, Thailand.

Data Availability Statement: The data presented in this study are available on request from the corresponding author due to technical/time limitations.

Acknowledgments: The authors acknowledge the Department of Biology and Science Lab Centre, Faculty of Science, Naresuan University, and the Center of Excellence in Research for Agricultural Biotechnology, Naresuan University, for their valuable facility support.

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

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