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DNA Polymorphism Changes in *Bacopa monnieri* (L.) Wettst Polyploids Affect Phytochemical and Antioxidant Profiles

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Abstract

This study aimed to evaluate the morphological, genetic, and phytochemical variations between diploid and colchicine-induced tetraploid *Bacopa monnieri* plants to explore the effects of polyploidy on plant traits and metabolite synthesis. Tetraploid lines were regenerated and acclimatized for four weeks in ½ HS medium. Morphological traits were measured, and cluster analysis was performed to assess phenotypic divergence. Genetic diversity was examined using RAPD markers, with data analyzed by UPGMA clustering. Phytochemical profiles, including bacosides, phenolics, flavonoids, triterpenoids, and antioxidant activity, were quantified by HPLC and spectrophotometric assays. Correlation analysis further revealed that bacoside accumulation is strongly linked to leaf and node morphological traits rather than overall plant size, suggesting genetic or metabolic regulation beyond simple vegetative growth. In contrast, phenolics, flavonoids, and triterpenoids showed positive correlations with growth traits and antioxidant activity, with triterpenoids exhibiting the strongest free radical scavenging effect. The average polymorphism rate correlated positively with bacoside content but not with other phytochemicals, indicating that genetic diversity plays a major role in bacoside biosynthesis. Results showed that most tetraploids had reduced length and biomass compared to diploids, except line 4-9, which exhibited increased growth and fresh weight. Morphological and genetic cluster analyses consistently identified three distinct groups, with lines 4-9 forming a unique cluster. Tetraploids, especially lines 4-1, 4-5, 4-7, and 4-9, accumulated significantly higher levels of bacosides and secondary metabolites, with line 4-9 yielding the highest bacoside compound (6.10 ± 1.11 mg/plant). Interestingly, some tetraploid lines showed reduced antioxidant activity despite increased phytochemical levels, possibly due to shifts in compound proportions or metabolic regulation. This study demonstrates that colchicine-induced tetraploidy enhances secondary metabolite production and genetic variability in *B. monnieri*, highlighting polyploid induction as a novel and promising approach to improving medicinal plant quality and diversity.

Keywords: Bacopa monnieri; Polyploid; RAPD; Bacoside; Antioxidant; Correlation.

1. Introduction

Bacopa monnieri (L.) Wettst., commonly known as Brahmi, is renowned for its cognitive and memory-enhancing properties, attributable to a diverse array of phytochemicals. The principal compounds include bacoside A-comprising bacoside A3, bacopaside II, bacopasaponin C, and the jujubogenin isomer of bacopasaponin C [1]- as well as bacoside B [2], bacopaside I [3, 4], bacopasaponins K and L, bacopasides IV and VII, and bacopasaponins E and F [4]. Brahmi's

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pharmacological actions extend beyond cognitive benefits [5], encompassing anticancer activity [6], amelioration of amyloid-β-induced Alzheimer's disease [7], neuroprotection, and cognitive enhancement [8], and vasorelaxant properties [9]. Notably, emerging clinical and preclinical investigations have further illuminated Brahmi's neuroprotective mechanisms. A comprehensive systematic review (2024) underscores its ability to attenuate inflammation, oxidative stress, mitochondrial dysfunction, and apoptosis-thereby supporting cognitive enhancement and neuroprotection [10]. In another study, bacopaside I displayed strong inhibitory affinity toward BACE1, suggesting potential utility in Alzheimer's therapy as a safer alternative to synthetic inhibitors [11]. By overexpressing squalene synthase and silencing G10H in *B. monnieri*, this study enhanced bacoside content and demonstrated improved neuroprotective effects in a Parkinson's disease rat model [12].

Beyond genetic and metabolic enhancements, environmental and cultivation factors also significantly influence bacoside accumulation and antioxidant activity. For instance, water stress and methyl jasmonate application have been shown to elevate specific bacoside levels [13, 14]. Polyploid induction has likewise increased bioactive compound content in various species: *Artemisia annua* [15], autotetraploid *B. monnieri* [16], *Papaver bracteatum* [17], and *Linum album* [18]. Morphological shifts accompanying polyploidy-such as increased leaf size or altered stomatal anatomy-have been observed in *Melissa officinalis*, *A. annua*, and *Arabidopsis thaliana* [15, 19, 20].

Among molecular marker techniques, RAPD (Random Amplified Polymorphic DNA) has been widely utilized due to its simplicity, cost-effectiveness, and the advantage of not requiring prior genome sequence information [21]. Recent studies have confirmed the efficacy of RAPD markers in detecting genetic polymorphisms in various plant species, including *B. monnieri* [22-24]. In the present study, we primarily employed RAPD markers to investigate the genetic polymorphism associated with polyploidy induction in *B. monnieri.* This approach provides valuable insights into the genetic diversity underlying phenotypic and phytochemical variation, laying the groundwork for future research incorporating complementary marker systems to further elucidate these relationships.

However, the relationships among polyploidy-induced genetic polymorphism, morphological traits, and phytochemical accumulation in *B. monnieri* remain poorly understood. Although increasing evidence suggests that both genetic manipulation (e.g., polyploidy, metabolic engineering) and cultivation practices can enhance the phytochemical profile of *Bacopa*, the correlation between polyploidy-induced genetic variation, morphological changes, and resultant phytochemical composition has not been thoroughly characterized. This study aims to address this knowledge gap by investigating the effects of polyploid induction on DNA polymorphism, phytochemical content, and antioxidant activity in *B. monnieri*, thereby elucidating the links among genotype, phenotype, and pharmacological function.

The following sections describe the procedures for regenerating colchicine-induced tetraploid lines of *B. monnieri* (e.g., 4-1, 4-5, 4-7, and 4-9), their acclimatization, and the methods used for morphological measurement, RAPD-based genetic analysis, and quantification of phytochemical compounds such as bacosides, phenolics, flavonoids, and triterpenoids. This is followed by the presentation of results on morphological divergence, genetic clustering, and phytochemical variation, highlighting the unique performance of line 4-9 in terms of growth and bacoside accumulation. The discussion then interprets the effects of polyploidy on plant traits and metabolite synthesis, considering its potential for enhancing the quality and diversity of medicinal plants. The article concludes by summarizing the principal outcomes and outlining directions for future research in polyploid induction for phytochemical improvement.

2. Material and Methods

2.1. Study Design

This study examines morphological, genetic, and phytochemical differences between diploid and colchicine-induced tetraploid *B. monnieri* to evaluate the impact of polyploidy on traits and metabolite production. Accordingly, the study was planned as outlined in the following chart (Figure 1).

2.2. Cultivation of Polyploid B. *monnieri* and Phenotypic Observation

One diploid (coded as 2) and tetraploid lines (coded as 4-1 to 4-9) of *B. monnieri*, induced via colchicine treatment and confirmed for ploidy levels by flow cytometry, were obtained from a previous study [16]. Ploidy levels of the tetraploid lines were verified by flow cytometry, showing a DNA content peak at channel 200 (4x)-approximately twice that of the diploid control peak at channel 100 (2x), consistent with genome duplication. All *in vitro* cultures of *B. monnieri* were acclimatized and transferred to sponges as supporting materials, supplemented with half-strength Hestrin and Schramm (1/2HS) basal medium. The plants were then cultured in a greenhouse at 25 ± 2°C for 30 days prior to harvesting. Phenotypic traits, including plant height, fresh weight, node size, and leaf shape, were recorded and subjected to statistical analysis.

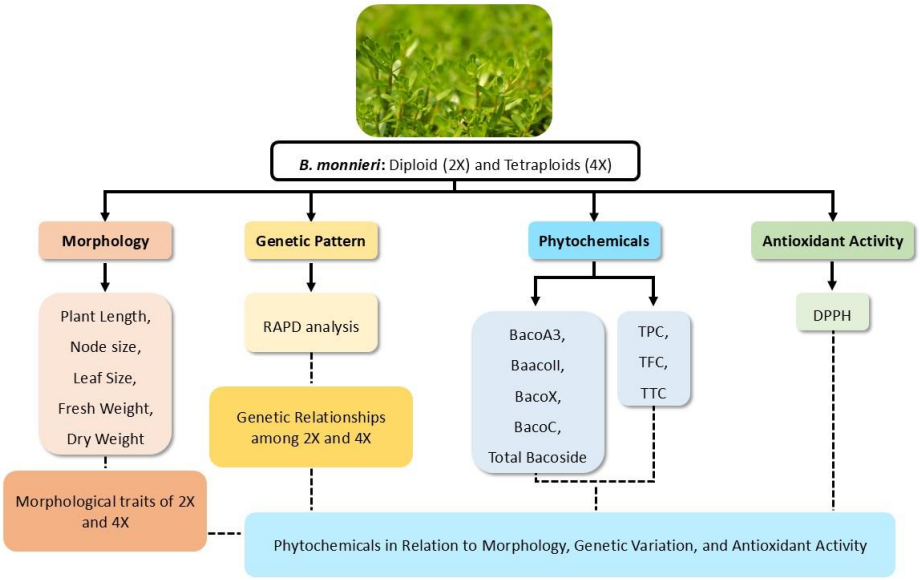

Figure 1. Overview of the research workflow

2.3. DNA Extraction

Genomic DNA was extracted from the diploid and tetraploid *B. monnieri* plants (five individuals per group) using the CTAB method [25]. The extraction buffer (1X CTAB) was prepared in a microcentrifuge tube, and 3 µl of β-mercaptoethanol was added before incubating the mixture at 60 °C. Fresh leaves were placed into a bead tube, immersed in liquid nitrogen, and ground into a fine powder with a bead beater. One milliliter of extraction buffer and 1 µl of RNase A were added to the powdered tissue, and the mixture was gently inverted to ensure thorough mixing. The samples were incubated at 60 °C for 60 minutes with gentle mixing every 10 minutes, then subjected to centrifugation at 14,000 × g for 10 minutes. The resulting supernatant was carefully moved to a fresh microcentrifuge tube, followed by the addition of 600 µl chloroform:isoamyl alcohol (24:1, v/v). The microcentrifuge tubes were gently inverted and then centrifuged at 14,000 × g for 10 minutes. Subsequently, 400 µl of the upper aqueous phase was transferred to a new tube, mixed with 800 µl of isopropanol (2X volume), and incubated at -20 °C for 30 minutes to DNA precipitation. After incubation, the samples were centrifuged at 14,000 × g for 10 minutes, and discard the supernatant. The DNA pellet was washed with 1 ml of 75% ethanol, followed by centrifugation at 14,000 × g for 5 minutes and the ethanol wash was repeated twice. DNA pellets were allowed to air-dry at room temperature before being dissolved in 50 µl of TE buffer. DNA concentration was determined using a NanoDrop spectrophotometer, and integrity was assessed by agarose gel electrophoresis.

2.4. RAPD Analysis

DNA fingerprints of diploid and tetraploid samples were analysed using 20 RAPD primers (Table 1) via the polymerase chain reaction. DNA concentrations were adjusted to 50 ng/µl using diethyl pyrocarbonate (DEPC)-treated water. Each PCR reaction (total reaction volume: 25 µl) comprising 12.5 µl of 1× DreamTaq Green PCR Master Mix, 1 µl of a 10 µM RAPD primer (final concentration 0.2 µM), 10.5 µl of nuclease-free water, and 1 µl of DNA template. PCR reactions were performed in 0.2 ml tubes using a MiniAmp Plus thermal cycler with the following program: initial 5 minutes denaturation at 95°C; 40 seconds 40 cycles of denaturation at 95°C, 1 minute annealing at 36°C, and 90 seconds extension at 72°C; followed by a final 7 minutes extension at 72°C. Amplified products were separated on a 1.5% agarose gel for 25 min by electrophoresis and visualized under UV light. A 3 kb DNA ladder served as the molecular size marker.

Table 1. Sequence of RAPD primers used in this study

Primer Name	Sequence 5′ > 3′	Primer Name	Sequence 5′ > 3′
SBS –A1	CAGGCCCTTC	SBS –I19	AATGCGGGAG
SBS –A8	GTGACGTAGG	SBS –M5	GGGAACGTGT
SBS –A9	GGGTAACGCC	SBS –M15	GACCTACCAC
SBS –A12	TCGGCGATAG	SBS –N3	GGTACTCCCC
SBS –A15	TTCCGAACCC	SBS –N5	ACTGAACGCC
SBS –A19	CAAACGTCGG	SBS –N10	ACAACTGGGG
SBS –A20	GTTGCGATCC	SBS –Q4	AGTGCGCTGA
SBS –I2	GGAGGAGAGG	SBS –Q5	CCGCGTCTTG
SBS –I10	ACAACGCGAG	SBS –Q7	CCCCGATGGT
SBS –I18	TGCCCAGCCT	SBS –Q11	TCTCCGCAAC

2.5. Polymorphism and UPGMA Cluster Analysis

The results of gel electrophoresis were analysed based on band classification. Clear, distinct, and reproducible bands generated by each primer were scored as 1, while their absence was scored as 0. A genetic similarity matrix was constructed to compare diploid and tetraploid plants, or among tetraploids [26]. Similarity coefficients were calculated and analysed by the unweighted pair-group method with arithmetic mean (UPGMA), and the resulting cluster analysis was performed using SYSTAT software.

2.6. Plant Extraction for Phytochemical Analysis and Antioxidant Assay

The diploid and tetraploid samples were dried at 40-50 °C and ground into powder. A 0.05 g portion of each sample was extracted with 1 ml of methanol, vortexed for 1 minute, sonicated for 15 minutes, and then centrifuged at 10,000 rpm for 5 minutes. The resulting supernatant was transferred to a new tube. The extraction process was repeated twice, and the combined extracts were dried at 40 °C. All dried extracts were stored at -20 °C until subsequent phytochemical analysis and antioxidant assays.

2.7. Bacoside Analysis by HPLC

The extracts were diluted with methanol, filtered through 0.45 µm nylon membranes, and analysed for phytochemical compounds using HPLC. Standard solutions of bacoside A3, bacopaside II, bacopaside X, and bacopasaponin C were prepared by dissolving the compounds in absolute methanol at concentrations ranging from 0 to 500 µg/mL, followed by filtration through 0.45 µm nylon membranes. The HPLC analysis was performed using an Agilent 1260 Infinity HPLC system equipped with a LiChroCART® Purospher® STAR RP-18 endcapped column (5 µm, 250 × 4.6 mm) and a LiChroCART® 4-4 Purospher® STAR RP-18 endcapped guard column (5 µm, Merck, Germany). The column temperature was maintained at 35 °C. The mobile phase consisted of 0.2% phosphoric acid in deionized water and acetonitrile (65:35, v/v), with a flow rate of 1.0 mL/min. Each injection was run for 50 minutes, and detection was carried out at a wavelength of 205 nm.

2.8. Quantification of Total Phenolic Content (TPC), Total Flavonoids Content (TFC), Total Triterpenoids Content (TTC), and 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The quantification of total phenolic content (TPC), total flavonoid content (TFC), total triterpenoid content (TTC), and DPPH radical scavenging activity was conducted following the methods described by Nopparat et al. (2024) and others [27-31].

Total Phenolic Content (TPC): TPC was determined using the Folin-Ciocalteu method. Briefly, 15 µL of *B. monnieri* extracts were added to a 96-well plate, followed by 240 µL of deionized water and 15 µL of 0.2 N Folin-Ciocalteu reagent. After gentle mixing, 70 µL of 10% (w/v) Na_2CO_3 was added. The sample was kept in darkness at ambient temperature for 60 minutes, after which absorbance was recorded at 765 nm using a microplate reader. Phenolic content was quantified using a gallic acid standard curve (0-400 µg/mL) and expressed as milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g DW).

Total Flavonoid Content (TFC): TFC was measured using an aluminum chloride colorimetric assay. In brief, 30 µL of *B. monnieri* extracts were added to a 96-well plate and combined with 10 µL of 5% (w/v) $NaNO_2$ and 120 µL of deionized water. After a 5-minute incubation at room temperature,10 µL of 10% $AlCl_3$ was added, followed by a 6-minute incubation. A volume of 60 µL of 1 N NaOH and 70 µL of deionized water were then added to the reaction mixture. Absorbance measurements were taken at 510 nm. Results were reported as milligrams of quercetin equivalents per gram of dry weight (mg QE/g DW).

Total Triterpenoid Content (TTC): For TTC determination, 20 µL of *B. monnieri* extracts were added to a 96-well plate and dried completely at 50-60 °C. Next, 10 µL of 5% vanillin in acetic acid and 18 µL of sulfuric acid were added, mixed, and incubated at 70 °C for 30 minutes. After cooling on ice, 72 µL of glacial acetic acid was added. Absorbance was measured at 573 nm. TTC was calculated using a standard curve of ursolic acid (0-400 µg/mL) and reported as milligrams of ursolic acid equivalents per gram of dry weight (mg UAE/g DW).

The DPPH radical scavenging activity was assessed by combining 50 µL of *B. monnieri* extracts with 100 µL of 0.1 mM DPPH reagent in 96-well plates. The reaction mixtures were kept in the dark at ambient temperature for 40 minutes, followed by absorbance measurement at 517 nm. The inhibition percentage was then calculated and reported.

2.9. Hierarchical Clustering Analysis (HCA)

The detected phytochemicals were analyzed for correlations with various traits of diploid and tetraploid *B. monnieri*, including morphological characteristics (length, weight, node size, leaf size, and leaf area), antioxidant activity, and the average polymorphism rate. The phytochemicals included bacoside contents (BacoA3, BacoII, BacoX, BacoC, and total bacosides), TPC, TFC, and TTC. Correlation analysis was conducted using HCA in Origin software version 2022. The dataset was grouped based on Pearson correlation with average linkage clustering to construct the clustering hierarchy. This method facilitated the analysis, visualization, and identification of patterns within the entire dataset. The resulting heat map illustrated the relationships between the detected compounds and other traits, with correlation strength represented by a gradient color scale ranging from blue (negative) to red (positive).

3. Results

3.1. Growth Characteristic of B. *monnieri* Diploid and Tetraploids

Four weeks after transferring the diploid and tetraploid plants to new supporting material supplemented with ½ HS medium, plant morphology, including plant length, weight, and the characteristics of leaves and nodes, were recorded and subjected to statistical analysis (Figures 2 and 3).

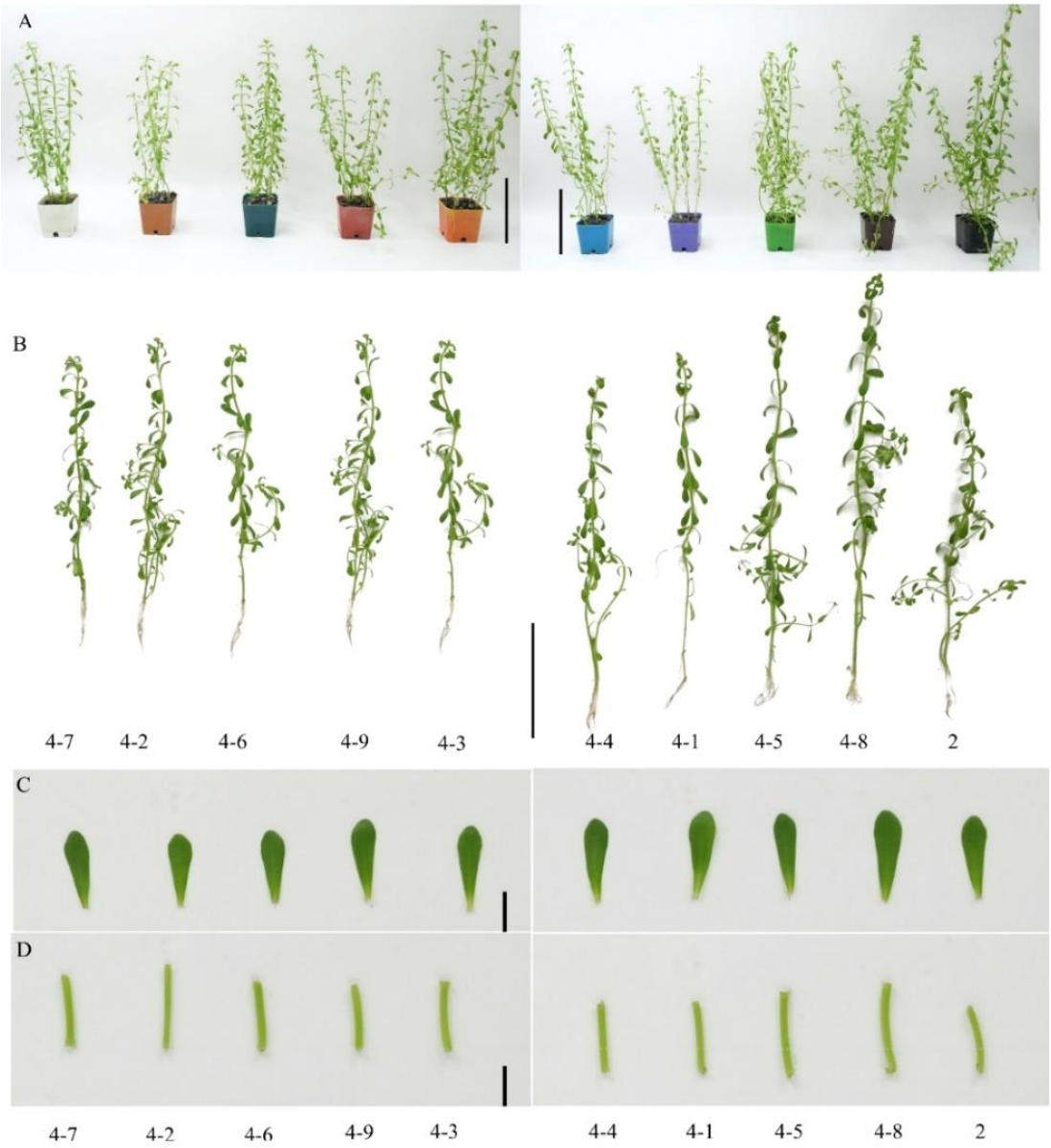

Figure 2. Effect of colchicine on shoot proliferation and phenotypic diversity in regenerated *B. monnieri*. Plants in pots and overall plant characteristics are shown in panels A and B. Scale bars represent 10 cm. The fifth leaves (C) and fifth nodes (D) are shown, with scale bars representing 1 cm.

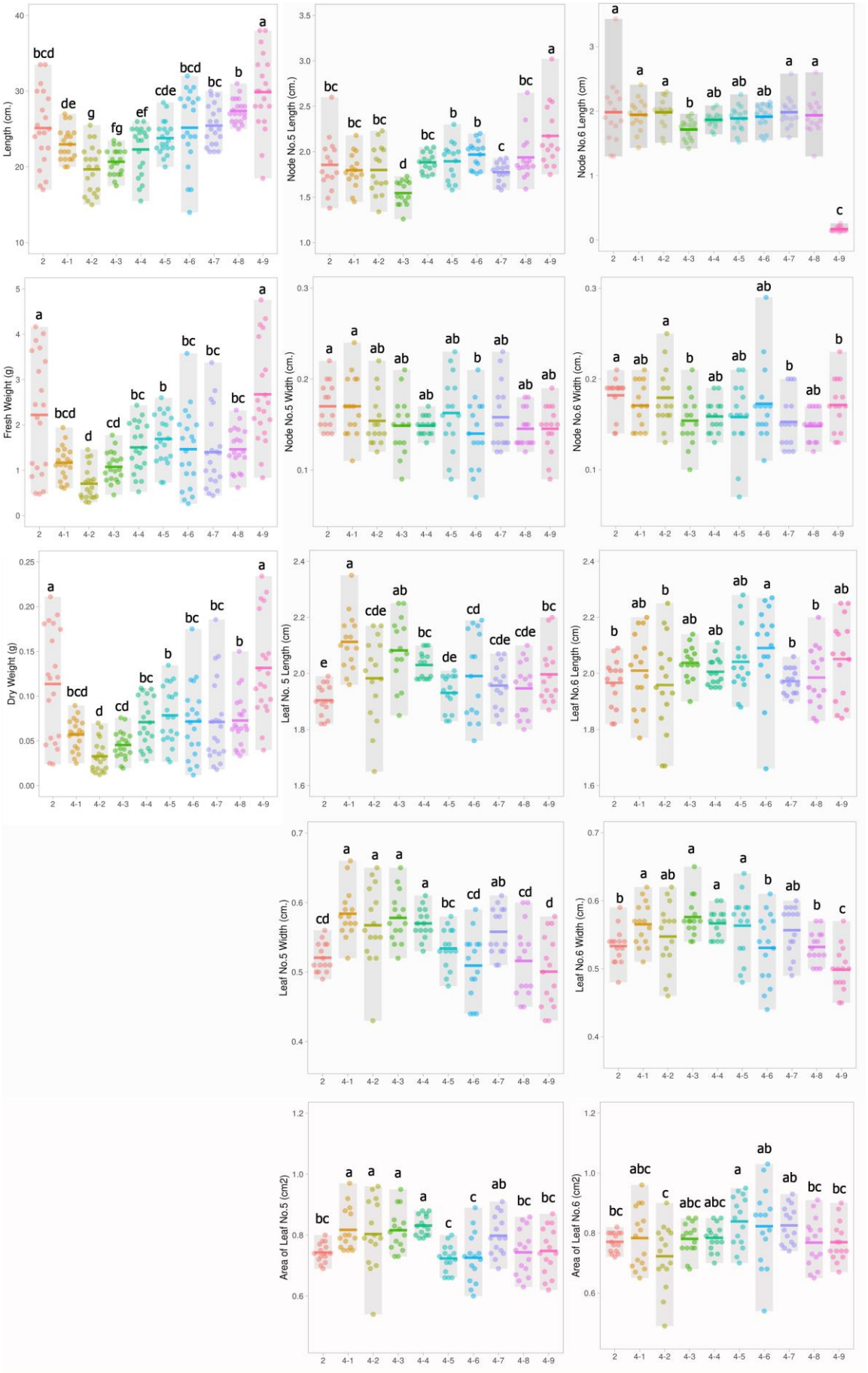

Figure 3 Plant length (n=21), fresh and dry weight (n=21), internode (5th-6th) length/width (n=15), and leaf (5th-6th) length/width/area (n=15) of *B. monnieri* cultured in ½ HS medium for 30 days. Data analysed by one-way ANOVA with Duncan's multiple range test (DMRT), $p \leq 0.05$.

Variations in length between diploid and tetraploid plants were observed. Some tetraploids (4-2, 4-3, and 4-4) were shorter than the diploid (2), while one tetraploid (4-9) was longer (Figure 3). The results for fresh and dry weight showed that all tetraploids exhibited lower values than the diploid, except for plant 4-9. The lengths of both the fifth and sixth nodes followed a similar pattern, with plant 4-3 being shorter than the other diploid and tetraploid plants. However, plant 4-9 showed a distinct pattern, with a longer fifth node and a shorter sixth node compared to the others (Figure 3). The width of the fifth node in tetraploid 4-6, and the sixth node in 4-3, 4-7, and 4-9, was lower than that of the diploid at the same positions. The width and length of the fifth and sixth leaves in the tetraploids were either larger than or not significantly different from those of the diploid, except for the width of the sixth leaf in plant 4-9, which was smaller (Figure 3). The leaf area measurements in all tetraploids followed the same trend as leaf size, with all tetraploids having larger or not significantly smaller leaf areas than the diploid.

To group morphologically similar polyploid plants, a dendrogram was constructed using UPGMA cluster analysis based on various morphological characteristics, including plant length; fresh and dry weights; the length and width of the fifth and sixth internodes; and the length, width, and area of the fifth and sixth leaves (Figure 4). These traits were used to compare diploid and tetraploid *B. monnieri* plants. The Dice similarity coefficient, calculated from the morphological data, ranged from 0.11 to 1.85 and was used for dendrogram construction. The resulting dendrogram revealed the relationships between the induced tetraploid regenerants and the diploid donor plant, grouping the samples into three clusters at 70% similarity. Cluster I included samples 2, 4-1, 4-4, 4-5, 4-6, 4-7, and 4-8, with the diploid plant (sample 2) also placed in this cluster (Figure 4). Among them, samples 4-6 and 4-7 were the most closely related to each other and showed the greatest similarity to the diploid plant. Cluster II comprised samples 4-2 and 4-3, while sample 4-9 formed a separate group in Cluster III, indicating a distinct morphological profile (Figure 4).

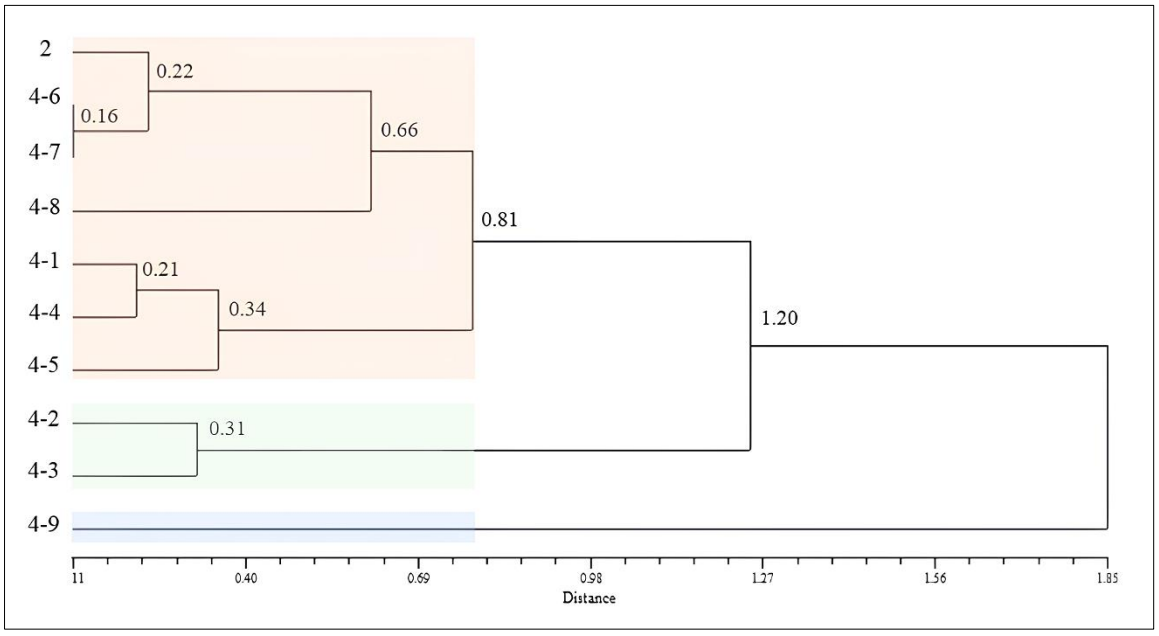

Figure 4 UPGMA dendrogram based on morphological traits of diploid and tetraploid *B. monnieri* plants

3.2. Clonal Fidelity Test

The genomic DNA of diploid and tetraploid *B. monnieri* were extracted for PCR amplification by using 20 random RAPD primers (10-mer) for initial screening. Among them only 6 primers presented clear and showed the polymorphic loci between diploid (2) and tetraploid (4-1 to -9) plants (Table 2 and Figure 5). From 6 RAPD primers, produced 25 reproducible and scorable bands averaging of 4.2 bands per primer. Each primer generated a distinct pattern set of amplification products ranging in size from 500-3000 bp (Table 2). The SBS A9 and SBS N10 yielded the highest number of scorable bands, totalling 9, whereas the fewest bands, only 4, were produced by SBS Q5 (Table 2 and Figure 5). The RAPD profiles for the 6 primers (SBS A9, SBS I2, SBS M5, SBS N5, SBS N10, and SBS Q5) generated a total of 25 polymorphic bands. A minimum of 3 polymorphic band was scored for SBS M5, SBS N5 and SBS Q5 and the maximum of 6 polymorphic band was observed for SBS A9 resulting in 51.39% degree of polymorphism (Table 2).

Table 2 The size of the amplicons produced and the level of polymorphism detected in the propagated diploid and tetraploid *B .Monnieri*

Primer code	No .of scorable bands	No .of monomorphic bands	Degree of monomorphism (%)	No .of polymorphic bands	Degree of polymorphism (%)	Amplification fragment size)bp(
SBS A1	5	5	100	0	0	700-2500
SBS A9	9	3	33	6	67	600-3000
SBS I2	8	3	38	5	63	500-2500
SBS M5	6	3	50	3	50	600-2500
SBS N5	6	3	50	3	50	1000-2500
SBS N10	9	4	44	5	56	500-3000
SBS Q5	4	1	25	3	75	900-3000
Total 7	47	22	48.61	25	51.39	

The RAPD analysis revealed clear genetic variation among the regenerant plants, highlighting the effectiveness of this molecular marker technique in detecting polymorphisms. Notably, 14 primers (SBS A1, SBS A8, SBS A12, SBS A15, SBS A19, SBS A20, SBS I10, SBS I18, SBS I19, SBS M15, SBS N3, SBS Q4, SBS Q7, and SBS Q11) produced a 100% monomorphic banding pattern, demonstrating total genetic uniformity between the diploid and tetraploid plants for these loci. This finding suggests that while some genomic regions remain conserved following polyploidization, other regions exhibit polymorphism, reflecting genetic divergence among regenerants. The observed polymorphic bands likely represent genetic rearrangements, mutations, or epigenetic changes induced by the colchicine treatment or tissue culture process, which are common phenomena during polyploid induction and plant regeneration. Conversely, the overall RAPD profiling successfully distinguished all regenerant plants, confirming the technique's sensitivity and efficacy in detecting genetic polymorphisms induced during the regeneration and polyploidization processes. These polymorphisms likely reflect chromosomal rearrangements, mutations, or epigenetic modifications that occurred during tissue culture or colchicine treatment, which are known sources of somaclonal variation.

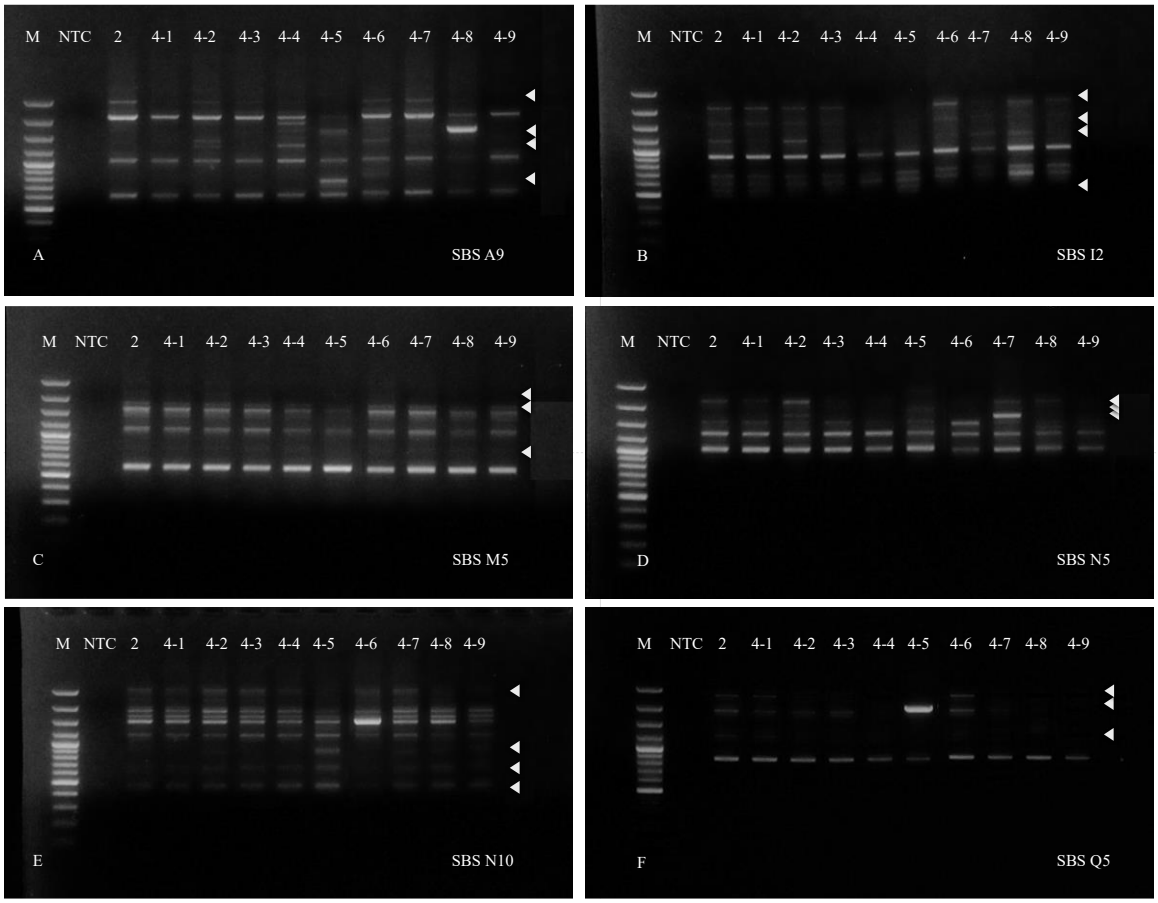

Figure 5. Clonal fidelity of diploid and tetraploid *B. monnieri* assessed by RAPD markers. Banding patterns generated using primers SBS A9 (A), I2 (B), M5 (C), N5 (D), N10 (E), and Q5 (F). M: 1 kb ladder; NTC: negative control; 2: diploid plant; 4-1 to 9: tetraploid plants.

The genetic similarity matrix, calculated using Nei and Li's coefficient, exhibited a broad range of similarity values among the plants, from 0.62 to 0.97 (Table 3), indicating varying degrees of genetic divergence. The tetraploid plant 4-5 showed the lowest similarity to all other plants, suggesting it underwent the most extensive genetic changes or mutations following polyploid induction. This divergence could affect phenotypic traits and adaptability, highlighting 4-5 as a genetically distinct individual within the tetraploid group. In contrast, plants 4-2 and 4-3 displayed the highest genetic similarity to each other (0.91) and also showed close genetic affinity to the diploid control, with similarity indices of 0.93 and 0.97, respectively (Table 3). These results imply that these two tetraploid regenerants retained genomic integrity more closely resembling the diploid progenitor, which might correlate with stable morphological and biochemical characteristics. On the other hand, plant 4-4 exhibited the lowest genetic similarity to the diploid (0.66), suggesting greater genomic divergence possibly due to extensive genomic rearrangements or mutations (Table 3).

Table 3 Gene similarity matrix for pairs of combinations of clones of *B .monnieri*

Clone	2	4-1	4-2	4-3	4-4	4-5	4-6	4-7	4-8	4-9
2	1.00									
4-1	0.87	1.00								
4-2	0.93	0.82	1.00							
4-3	0.97	0.87	0.91	1.00						
4-4	0.66	0.70	0.74	0.74	1.00					
4-5	0.70	0.79	0.70	0.70	0.70	1.00				
4-6	0.79	0.74	0.79	0.82	0.74	0.62	1.00			
4-7	0.81	0.85	0.89	0.89	0.77	0.77	0.77	1.00		
4-8	0.76	0.81	0.81	0.81	0.85	0.77	0.81	0.87	1.00	
4-9	0.82	0.81	0.72	0.81	0.77	0.72	0.77	0.79	0.83	1.00

Using the genetic similarity coefficients derived from RAPD data, a UPGMA dendrogram was constructed to visualize the genetic relationships among the regenerants (Figure 6). The dendrogram resolved three major clusters at 78% similarity threshold, reflecting distinct genetic groupings within the regenerant population. The first cluster included plants 2, 4-1, 4-2, 4-3, and 4-7, with 4-6 forming a separate subcluster within this group (Figure 6). This cluster likely represents a group of regenerants with relatively conserved genetic backgrounds and close relationships, possibly sharing similar phenotypic traits. The second major cluster grouped regenerants 4-4, 4-8, and 4-9, indicating a moderate level of genetic divergence from the first cluster, which may be associated with distinct phenotypic or biochemical characteristics (Figure 6). Lastly, plant 4-5 was positioned alone in the third cluster, reinforcing its status as the most genetically divergent regenerant (Figure 6). This isolation in the dendrogram suggests that 4-5 could serve as an important candidate for further studies on the effects of polyploidy and somaclonal variation on genetic and phenotypic diversity.

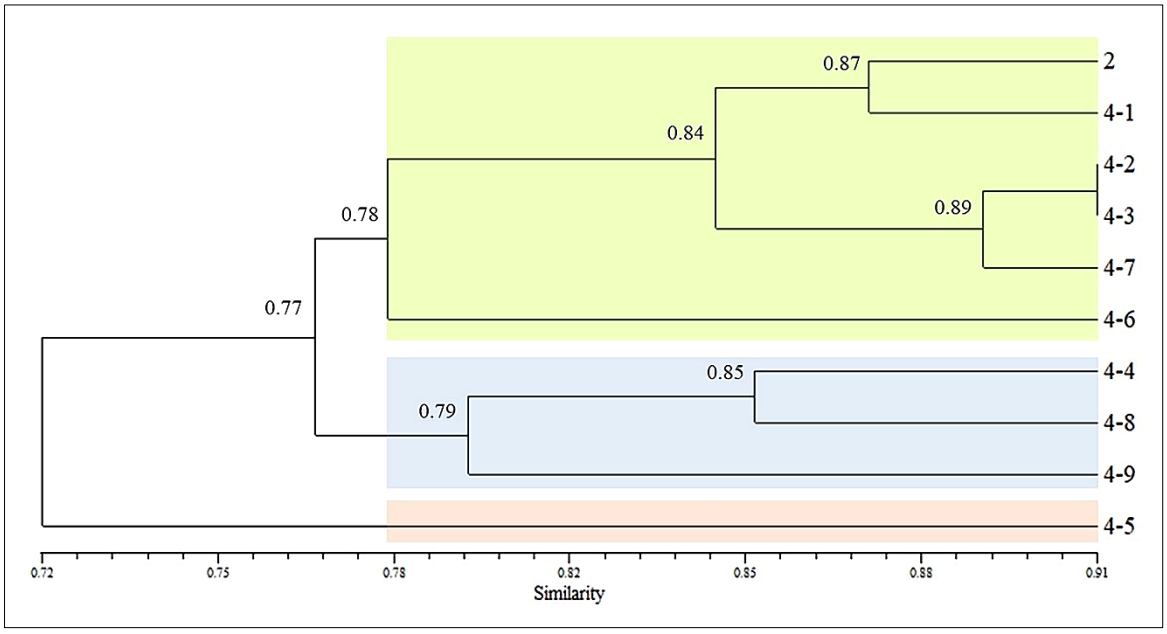

Figure 6. Dendrogram from UPGMA cluster analysis of RAPD data showing genetic relationships among diploid and tetraploid *B. monnieri* (L.) plants.

Overall, these results provide comprehensive insight into the genetic diversity generated by tetraploid induction and tissue culture regeneration in this species. The genetic variation detected among the regenerants may have important implications for breeding programs, as it offers the potential to select plants with desirable traits linked to improved agronomic performance or secondary metabolite production. Further analysis correlating these genetic differences with phenotypic and biochemical traits will be critical to fully understand the practical implications of the observed variation.

3.3. HPLC Determination of Bacoside A3, Bacopaside II, Bacopaside X, Bacopasaponin C, and Total Bacoside

The statistical analysis of bacoside A3, bacopaside II, bacopaside X, bacopasaponin C, and total bacoside contents and yields, determined by the HPLC, are illustrated in Figure 7. Overall, the data indicate a clear trend of increased accumulation of these key bacosides contents in tetraploid *B. monnieri* lines compared to their diploid counterparts. This enhancement suggests that polyploidization may stimulate secondary metabolite biosynthesis pathways, potentially due to gene dosage effects or altered regulatory mechanisms affecting the expression of biosynthetic enzymes.

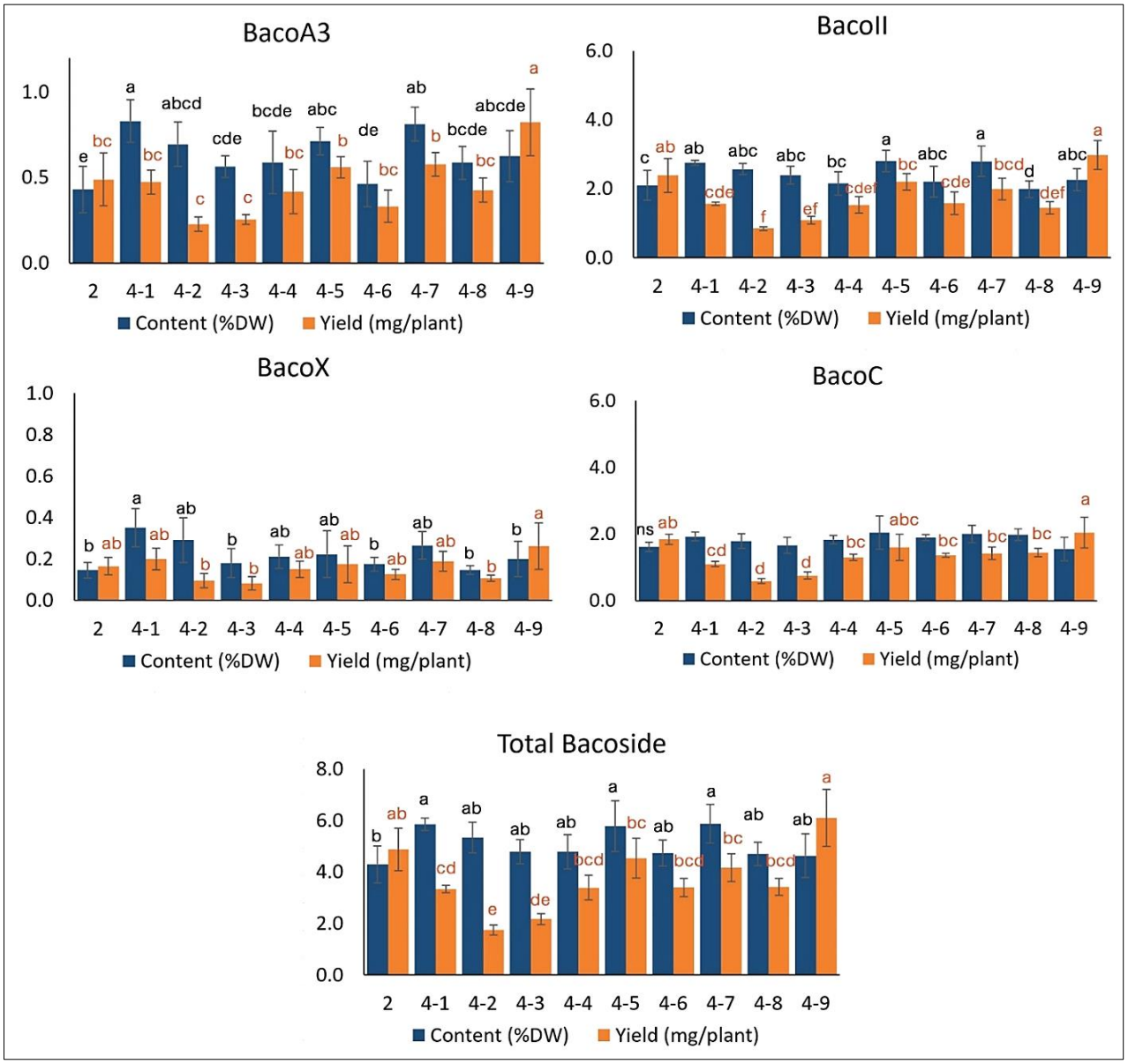

Figure 7. Bacoside contents and yield in diploid and tetraploid *B. monnieri* determined by HPLC. Statistical analysis was performed using one-way ANOVA with Duncan's multiple range test at $p \leq 0.05$. BacoA3 = Bacoside A3; BacoII = Bacopaside II; BacoX = Bacopaside X; BacoC = Bacopasaponin C; Total bacosides.

Among the tetraploid lines, line 4-1 exhibited the highest bacoside A3 content at 0.83 ± 0.12 %DW, Figure 7, which represents a significant improvement over typical diploid levels reported in previous studies. Similarly, bacopaside II content peaked in lines 4-5 (2.80 ± 0.31 %DW) and 4-7 (2.82 ± 0.44 %DW), indicating that different tetraploid lines

may differentially regulate specific bacoside compounds (Figure 7). This line-specific variation highlights the importance of selective breeding or screening to identify elite genotypes for pharmaceutical applications. Bacopaside X, though present in smaller quantities, was notably highest in line 4-1 (0.35 ± 0.09 %DW), supporting the observation that some tetraploid lines simultaneously enhance multiple bacoside components (Figure 7). Total bacoside content, a critical measure of overall therapeutic potential, was significantly elevated in lines 4-1 (5.85 ± 0.24 %DW), 4-5 (5.78 ± 0.99 %DW), and 4-7 (5.78 ± 0.99 %DW), reaching values of approximately 5.8 %DW (Figure 7). These findings are consistent with the hypothesis that tetraploidy can lead to increased metabolite production, likely through combined effects of gene duplication and cellular metabolic shifts.

When evaluating the yields of these compounds per plant, a more complex pattern emerged. While certain tetraploid lines demonstrated higher yields of bacoside A3, bacopaside II, bacopaside X, bacopasaponin C, and total bacosides compared to diploids, other lines showed either no significant difference or lower yields. This variability underscores the influence of additional factors such as plant size, biomass, and physiological traits that impact compound accumulation on a per-plant basis. Notably, tetraploid line 4-9 stood out as the most promising candidate, exhibiting the highest yields per plant across all measured bacosides (Figure 7): bacoside A3 (0.83 ± 0.20 mg/plant), bacopaside II (2.98 ± 0.42 mg/plant), bacopaside X (0.26 ± 0.11 mg/plant), bacopasaponin C (2.04 ± 0.46 mg/plant), and total bacosides (6.10 ± 1.11 mg/plant). This superior yield suggests that line 4-9 combines both enhanced biosynthetic capacity and favorable growth traits, making it an excellent candidate for further development in pharmaceutical cultivation. These results collectively demonstrate that colchicine-induced tetraploidy in *B. monnieri* can significantly influence both the concentration and total yield of pharmaceutically important bacosides. The observed increases in bacoside content and yield have important implications for improving the efficacy and economic viability of *B. monnieri* as a medicinal plant. However, the variation among tetraploid lines also highlights the necessity for careful selection and characterization of elite genotypes to fully harness the benefits of polyploid breeding.

3.4. TPC, TFC, TTC and Antioxidant Activities

The induction of tetraploidy in *B. monnieri* resulted in notable biochemical changes, particularly in the accumulation of secondary metabolites such as phenolics, flavonoids, and triterpenoids. Our results demonstrate a general trend of TPC in tetraploid lines compared to the diploid progenitor (Figure 8). Specifically, seven out of nine tetraploid lines (excluding 4-1 and 4-2) showed a statistically significant enhancement in TPC, ranging from 1.53- to 2.09-fold higher than the diploid control. This increase suggests that genome duplication may upregulate the biosynthetic pathways responsible for phenolic compound production, potentially through increased gene dosage or altered regulatory mechanisms. Phenolic compounds are well-known for their roles in plant defense and antioxidant activity; thus, their elevation may contribute to improved stress tolerance or bioactivity in these tetraploid lines.

Similarly, TFC was significantly elevated in most tetraploids (except lines 4-2, 4-6, and 4-7), with increases ranging from 1.28- to 1.55-fold relative to the diploid (Figure 8). Flavonoids are crucial antioxidants and have diverse pharmacological properties, including anti-inflammatory and neuroprotective effects, which are characteristic of *B. monnieri*. The observed rise in flavonoid levels in tetraploids may reflect enhanced metabolic flux through the phenylpropanoid pathway, often influenced by polyploidy. However, the lack of increase in certain lines indicates potential genotype-specific responses or differential regulation of flavonoid biosynthesis after chromosome doubling.

Regarding TTC, a significant elevation was detected in tetraploid lines 4-7, 4-8, and 4-9 compared to both the diploid and other tetraploids (Figure 8). Triterpenoids, known for their pharmacological relevance including anti-cancer and hepatoprotective activities, are synthesized via distinct pathways from phenolics and flavonoids. The marked increase in TTC in these specific tetraploid lines may suggest that tetraploidy can selectively enhance certain metabolite pathways, possibly due to structural genomic changes or altered enzyme activities.

Interestingly, despite the increased levels of these phytochemicals, antioxidant activity assessed by the DPPH radical scavenging assay did not uniformly improve in tetraploid lines. Six tetraploids showed no significant difference from the diploid in antioxidant capacity, while lines 4-1, 4-2, and 4-6 exhibited significantly reduced activity, ranging from 0.77 to 0.99 times that of the diploid (Figure 8). This discrepancy implies that antioxidant activity is influenced not only by total metabolite content but also by the specific composition, synergistic interactions, and bioavailability of these compounds. For example, the quality and structure of phenolics and flavonoids, rather than quantity alone, may govern antioxidant efficacy. Furthermore, tetraploid induction might induce physiological or metabolic trade-offs affecting antioxidant systems beyond mere metabolite accumulation.

Overall, these findings highlight that tetraploidization in *B. monnieri* can enhance the biosynthesis of certain bioactive compounds, but the relationship between metabolite levels and functional antioxidant capacity is complex and likely involves multiple regulatory layers.

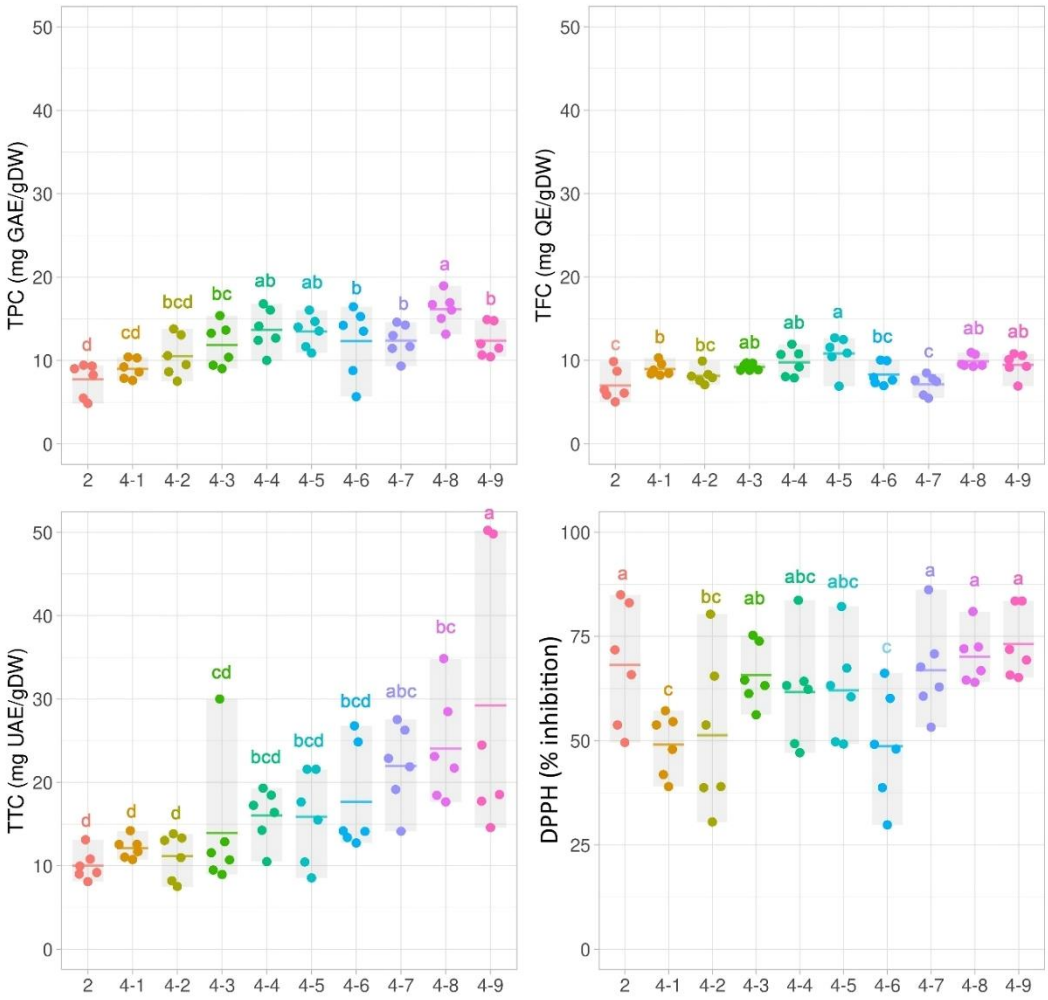

Figure 8. Total phenolic content (TPC), total flavonoid content (TFC), total triterpenoid content (TTC), and DPPH antioxidant activity in diploid (2) and tetraploid (4-1 to 4-9) *B. monnieri*

3.5. Correlation Analysis Between Phytochemicals and Other Characteristics

Heat map visualization of the correlations between phytochemicals and other plant traits was performed using hierarchical cluster analysis (HCA) and displayed as a continuous colour gradient ranging from blue (negative correlation) to red (positive correlation) (Figure 9). This approach enabled the identification of trait-compound associations and provided an intuitive representation of their strength and direction. Analysis of the relationships between phytochemical contents and morphological parameters-including plant length, fresh weight, dry weight, node size, leaf size, and leaf area-revealed distinct correlation patterns. Bacoside compounds (BacoA3, BacoII, BacoX, BacoC, and total bacosides) exhibited no significant correlation with overall plant length or biomass production, as reflected by the predominance of blue zones in these regions of the heat map. This suggests that bacoside accumulation in *B. monnieri* is not necessarily linked to plant size or vegetative growth, indicating that their biosynthesis may be more strongly influenced by genetic or metabolic regulation rather than by morphological vigour. Interestingly, positive correlations were detected between bacoside contents and traits related to node size, leaf size, and leaf area, as indicated by the red zones (Figure 9). These associations imply that morphological structures with larger photosynthetic surfaces or greater metabolite storage capacity may facilitate the biosynthesis or accumulation of bacosides. Such findings are consistent with previous studies showing that secondary metabolite content can be influenced by specific organ size and developmental stage rather than total plant biomass.

In contrast, TPC, TFC, and TTC were positively correlated primarily with plant length and biomass-related traits (Figure 9). This suggests that phenolics, flavonoids, and triterpenoids may be linked to growth-associated metabolic processes, with triterpenoids potentially contributing to membrane stability, regulation of signalling pathways, and chemical defence against pathogens and herbivores, particularly during active vegetative growth. Regarding genetic traits, the average polymorphism rate was positively associated with bacoside contents but showed no meaningful correlation with TPC, TFC, or TTC (Figure 9). This finding suggests that genetic diversity within the studied *B. monnieri* populations may contribute to variations in bacoside biosynthesis, supporting the hypothesis that bacoside production has a stronger genetic basis compared to other phytochemicals measured.

Finally, correlation analyses between TPC, TFC, TTC, and DPPH radical scavenging activity revealed predominantly positive associations, indicating that higher levels of these phytochemicals are generally linked to stronger antioxidant capacity (Figure 9). Among these, TTC exhibited the highest correlation with DPPH activity, suggesting that triterpenoids may play a more substantial role than phenolics or flavonoids in mediating free radical scavenging in *B. monnieri*. This finding aligns with previous reports highlighting the antioxidant potential of triterpenoid in medicinal plants. Interestingly, when comparing diploid and tetraploid lines, some tetraploids (e.g., 4-1, 4-2, and 4-6) displayed lower antioxidant activity than their diploid counterparts, despite potential increases in certain phytochemical levels typically associated with polyploidization. This discrepancy may be attributed to differential metabolic regulation in polyploid genomes, shifts in the relative proportions of active compounds, or possible changes in compound bioavailability. It is also plausible that antioxidant activity is influenced by synergistic interactions among multiple phytochemical classes, and that an increase in one group (e.g., TTC) may not fully compensate for decreases in others (e.g., TPC or TFC). Collectively, these correlation trends provide new insight into how morphological, genetic, and biochemical traits interact in shaping the phytochemical profile and functional properties of *B. monnieri*.

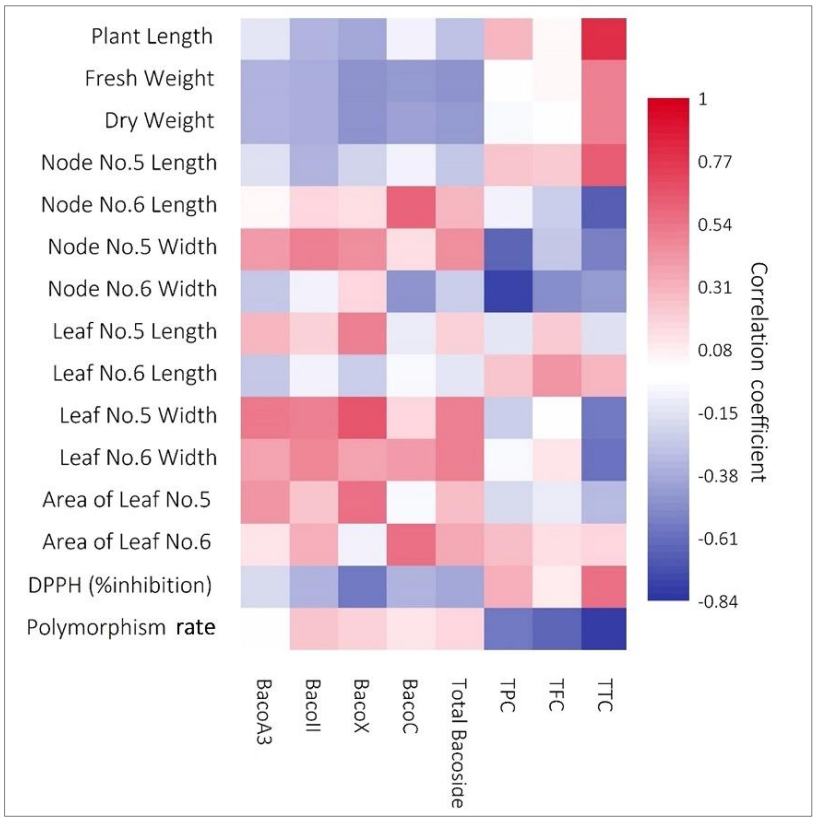

Figure 9. Heat map visualization of the correlations between phytochemicals in *B. monnieri* (2x and 4x) and other characteristics, including morphology, antioxidant activity, and polymorphism. The colours represent correlations, ranging from dark blue (negative) to red (positive).

4. Discussion

The results of this study provide important insights into the effects of colchicine treatment on *B. monnieri*, encompassing not only morphological traits, phytochemical composition, and bioactivity, but also alterations in DNA patterns. Morphological observations revealed that certain tetraploid lines (e.g., 4-1, 4-2, 4-3, 4-4) exhibited reduced plant length and biomass compared to the diploid control, while one line (4-9) outperformed the diploid in these metrics, highlighting intra-group variability among the tetraploids. Such variability is consistent with findings in other species, where colchicine-induced tetraploids display decreased biomass.

For example, in various cultivars and breeding lines of *Lactuca sativa* L., specific tetraploid lines showed lower leaf fresh/dry weights and total plant fresh/dry weights compared to diploids [32]. Similarly, tetraploids of Java Ginseng (*Talinum paniculatum* (Jacq.) Gaertn.) exhibited darker and thicker leaves, larger fruits, larger stomata with lower stomatal density, along with a significantly reduced aboveground biomass relative to diploids [33]. Despite the overall reduction in biomass observed in most tetraploid lines, leaf characteristics; specifically, area, width, and length, were generally comparable to or greater than those of diploids, except in line 4-9. These findings are consistent with observations in birch (*Betula pendula*), where increases in leaf area among tetraploids were attributed to cell expansion rather than increased cell proliferation [34].

Cluster analysis of morphological traits revealed three distinct groups: most tetraploids clustered with the diploid control, while lines 4-2, 4-3, and 4-9 formed separate clusters. This dendrogram thus illustrates both morphological similarity and notable divergence among the tetraploid lines. Overall, our findings support the notion that tetraploidization probably enhances leaf size and alters internode characteristics. However, the effects on biomass and morphology remain heterogeneous among regenerated tetraploids, with some lines even underperforming relative to diploids. These inconsistencies likely reflect genotype-specific responses to colchicine-induced polyploidy.

From the perspective of genomic pattern alterations, RAPD analysis between diploid and colchicine-induced tetraploid *B. monnieri* revealed moderate genetic variation. RAPD analysis remains a valuable tool for assessing genetic relationships and managing plant germplasm, despite concerns regarding the homology of co-migrating bands [35]. It is particularly effective for evaluating somaclonal variation and genetic stability in micropropagated plants [36]. Out of 20 primers screened, six generated 25 reproducible and polymorphic bands, reflecting a polymorphism rate of 51.39%. No unique bands were found. These results support previous findings on the effectiveness of RAPD in monitoring genetic stability [37]. Twenty RAPD primers were initially tested, but only six are reported because the other 14 produced no amplicons or showed minimal variation, as shown in the picture below. This may be due to the low genetic divergence among the tested plants, which belong to the same species and were subjected to induced DNA duplication, as described by Choi & Lee (2021) [38].

Previous studies have similarly investigated the genomic impacts of colchicine-induced polyploidy. In *Lilium davidii* var. *unicolor* (Lanzhou lily), colchicine treatment followed by morphological, cytological, and genomic DNA analyses revealed substantial variation [39]. Inter-simple sequence repeat (ISSR) assays indicated 75% polymorphism between diploid and tetraploid plants, demonstrating that chromosome doubling via colchicine can lead to significant changes at the DNA sequence level. In another study, colchicine treatment of *Lilium leichtlinii* var. *Maximowiczii* resulted in 11 mutant lines (Nos. 1-11), comprising both diploids and aneuploids [40]. ISSR marker analysis of mutant No. 2 (a diploid) revealed a genetic variation rate of 18.99% compared to the control.

Although tetraploidy was not achieved in this case, the findings confirmed that colchicine can still induce detectable genetic variation. Among the six effective primers used in the current study, SBS A9 yielded the highest number of polymorphic bands, whereas SBS Q5 produced the fewest, suggesting variable sensitivity of primers to colchicine-induced genomic changes. The presence of unique RAPD banding patterns in some polyploid plants further implies that colchicine treatment may lead to chromosomal rearrangements [41] or other mutations during the polyploidization process. In contrast, 14 primers showed a completely monomorphic banding pattern, indicating that many genomic regions remained conserved between diploid and tetraploid plants. This mixed pattern of polymorphism and monomorphism further supports the hypothesis that colchicine-induced polyploidy does not uniformly affect the entire genome, but rather induces changes in a locus-specific manner. These results validate the usefulness of RAPD markers as a cost-effective and reliable tool for assessing genomic changes and clonal fidelity following polyploidization.

Colchicine, a well-known agent for inducing polyploidy, is also associated with genome-wide mutations, leading to morphological and phytochemical variations [42-44]. Despite a high genetic similarity (0.66-0.91) between diploid and colchicine-induced tetraploid lines, the tetraploids displayed distinct morphological characteristics and elevated levels of bacosides, phenolics, flavonoids, and triterpenoids. Polyploidization significantly enhanced the accumulation of key bioactive compounds in *B. monnieri*, particularly bacoside A3, bacopaside II, bacopaside X, bacopasaponin C, and total bacoside content. While bacoside yields varied across tetraploid lines, most showed either reduced or statistically insignificant differences compared to their diploid progenitors. Notably, line 4-9 consistently exhibited higher or comparable yields. Given the central role of phenolics, flavonoids, and triterpenoids in antioxidant activity, their increased accumulation in tetraploids suggests enhanced antioxidant potential [45-48]. Nevertheless, previous studies have shown that in some species, diploids may outperform polyploids in phenolic and flavonoid production, correlating with stronger antioxidant and anticancer activities [49].

The significant increase in key phytochemical content was observed only in certain tetraploid (4x) lines, not across all lines. Considerable variation in plant traits among the tetraploid lines was found, with some 4x lines exhibiting lower phytochemical levels than diploid (2x) plants. This suggests that the relationship between ploidy and phytochemical accumulation is not strictly linear. Such variation could arise from genetic and epigenetic changes induced during polyploidization and subsequent plant development. In particular, the process of inducing polyploidy can cause chromosomal abnormalities, including structural rearrangements, aneuploidy, or other genomic instabilities [50, 51]. These chromosomal changes may be considered a form of somaclonal variation, which can alter gene expression patterns and metabolic pathways beyond the simple doubling of chromosome number. Therefore, the observed enhancement of phytochemicals in some tetraploid lines likely arises from a combination of factors: the increased chromosome number, which may enhance biosynthetic gene expression, and additional genetic and epigenetic variations caused by chromosomal instability and cellular responses to polyploid induction. To clearly differentiate the effects of ploidy from other sources of variation, further investigations such as gene expression profiling and detailed molecular genetic analyses are recommended.

The correlation study between phytochemicals and other plant traits was visualized using a heat map generated by HCA, which effectively illustrates both the strength and direction of correlations. This study's heat-map analysis revealed that bacoside compounds-including BacoA3, BacoII, BacoX, BacoC, and total bacosides-do not correlate significantly with overall plant length or biomass, but show strong positive associations with morphological traits such as node size, leaf size, and leaf area, suggesting that bacoside accumulation is more closely linked to organ-specific architecture than to vegetative growth vigor. This aligns with earlier findings [52], who reported that bacoside biosynthesis in *B. monnieri* is more dependent on developmental stage and organ-specific metabolism than on overall plant size. In contrast, TPC, TFC, and TTC contents were positively correlated with plant length and biomass, indicating their involvement in growth-associated metabolism such as structural integrity or defense during active growth, consistent with observations [53] who demonstrated that phenolic and flavonoid accumulation increased with enhanced vegetative growth in medicinal plants. Additionally, the positive correlation between average polymorphism rate and bacoside contents suggests that genetic diversity plays a stronger role in bacoside biosynthesis compared to other phytochemicals, supporting hypotheses proposed on the genetic regulation of secondary metabolite pathways. These findings illuminate the differential drivers underlying secondary metabolite accumulation in *B. monnieri* and suggest that morphological development and genetic diversity may selectively influence biosynthesis of specific compounds.

The DPPH assay revealed lower antioxidant activity in some tetraploid lines, which may be attributed to alterations in phytochemical composition following polyploidization. Antioxidant activity in plants results from multiple classes of bioactive compounds, including total phenolic content (TPC), total flavonoid content (TFC), total triterpenoid content (TTC), and other metabolites. Correlation analyses between TPC, TFC, TTC, and DPPH radical scavenging activity typically show positive relationships, with TTC exhibiting the strongest correlation, followed by TPC and TFC. When comparing diploid and tetraploid lines, some tetraploid lines (e.g., 4-1, 4-2, and 4-6) exhibited lower antioxidant activity than diploid counterparts. Although TTC-showing the strongest positive correlation with antioxidant activity-did not differ significantly between these lines, 4-6 and 4-1 displayed significantly higher TPC and TFC levels compared to diploids. This suggests that additional phytochemical classes beyond phenolics, flavonoids, and triterpenoids may contribute to total antioxidant capacity.

In *Bacopa* species, bacosides, which are triterpenoid saponins, have been reported to possess antioxidant properties [54]. Interestingly, line 4-1 contained significantly higher bacoside content relative to diploid lines, yet its DPPH activity remained lower. This discrepancy suggests that diploid lines may contain other antioxidant constituents-possibly non-bacoside triterpenoids or synergistic minor metabolites-that enhance radical scavenging capacity, which may be reduced or compositionally altered in certain tetraploid lines. Although polyploidization often increases metabolite content, this does not always translate into enhanced bioactivity, some reports have even shown this expectation to be a myth [49]. In conclusion, this study demonstrates that colchicine-induced tetraploidy in *B. monnieri* enhances the biosynthesis of key phytochemicals and induces genetic variation. This represents a novel and promising strategy for improving the pharmaceutical value of this important medicinal plant.

5. Conclusion

This study demonstrates that colchicine-induced tetraploidy in *B. monnieri* generates significant morphological, genetic, and phytochemical variation among regenerant lines. Morphological assessment revealed that while most tetraploid lines exhibited reduced biomass relative to the diploid parent, one line (4-9) displayed superior growth performance, underscoring the potential for selecting elite variants. Cluster analysis based on morphological traits grouped the tetraploids into three distinct clusters, with some lines (e.g., 4-6 and 4-7) closely resembling the diploid, highlighting intra-tetraploid diversity. Genetic analysis via RAPD markers confirmed this diversity and partial similarity, with line 4-5 showing the greatest divergence, reflecting the polymorphic nature induced by chromosome doubling.

Phytochemical profiling revealed that several tetraploid lines (notably 4-1, 4-5, 4-7, and 4-9) accumulated higher levels of key bacosides, such as bacoside A3, bacopaside II, and bacopaside X, compared to diploids. Among them, line 4-9 stood out with the highest total bacoside yield per plant (6.10 ± 1.11 mg), making it an elite candidate for commercial development. Correlation analysis further revealed that bacoside accumulation is linked to leaf and node morphological traits rather than overall plant size, suggesting genetic or metabolic regulation beyond simple vegetative growth. In contrast, phenolics, flavonoids, and triterpenoids correlated positively with growth traits and antioxidant activity, with triterpenoids exhibiting the strongest free radical scavenging effect. The average polymorphism rate was positively associated with bacoside contents but not with other phytochemicals, indicating genetic diversity plays a major role in bacoside biosynthesis. Interestingly, some tetraploid lines showed reduced antioxidant activity despite increased phytochemical levels, possibly due to shifts in compound proportions or metabolic regulation. Collectively, these findings establish induced polyploidy as an effective approach to enhance the pharmaceutical value of *B. monnieri*, by boosting bioactive compounds and generating genetic diversity, thus paving the way for advanced breeding and commercialization of this medicinal plant.

6. Declarations

6.1. Author Contributions

Conceptualization, P.I. and K.P.; methodology, P.I. and K.P.; software, P.I. and K.P.; validation, P.I. and K.P.; formal analysis, N.P., K.P., and A.R.; investigation, P.I. and K.P.; resources, P.I. and K.P.; data curation, P.I. and K.P.; writing—original draft preparation, N.P., K.P., and A.R.; writing—review and editing, P.I., A.R., and K.P.; visualization, N.P., A.R., and K.P.; supervision, P.I. and K.P.; project administration, K.P.; funding acquisition, K.P. All authors have read and agreed to the published version of the manuscript.

6.2. Data Availability Statement

The data presented in this study are available in the article.

6.3. Funding

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6.4. Acknowledgments

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6.5. Institutional Review Board Statement

Not applicable.

6.6. Informed Consent Statement

Not applicable.

6.7. Declaration of Competing Interest

The authors declare that there are no conflicts of interest concerning the publication of this manuscript. Furthermore, all ethical considerations, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancies have been completely observed by the authors.

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