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In vitro colchicine-induced polyploids from different explant segments of *Bacopa monnieri*

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ABSTRACT: *Bacopa monnieri* (*Bmo*) is a highly valued medicinal plant, widely used for several pharmacological products in Thailand. The diploid progenitor of *Bmo* grown in the wild as a natural source has low biomass and bacoside contents. Fifteen treatment combinations between three *Bmo* explant types (apical shoot, node and leaf segments) and five various colchicine concentrations (0.00, 0.05, 0.075, 0.1, and 0.5% w/v) were determined for a number of polyploid inductions, including bacoside contents and gene expression. Results showed that low concentration (0.05%) of colchicine was more effective in inducing multiple shoots (17.88 regenerants per explant) and tetraploid plantlets (15%) from leaf segments than other treatment combinations. Tetraploid clone (no.4x-3) showed the highest *BmoOSC* gene expression, bacoside-A3 (4.276 ± 0.019 mg/g dry weight) and bacoside-C contents (5.040 ± 0.078 mg/g dry weight) compared to diploid progenitors and mixoploid plants. These findings indicated that colchicine-induced tetraploids could have beneficial uses for genetic improvements to increase the medicinal value of local *Bmo* herbal medicine powder production in Thailand.

KEYWORDS: *Bcopa monnieri*, colchicine, polyploidization, bacoside content, gene expression

INTRODUCTION

Bacopa monnieri(*Bmo*) belongs to the Scrophulariaceae family, and is a highly valued medicinal plant in India, China and Thailand [1]. Recent publications have reported that this species contains high content of bacoside saponins which appears to play an important role in ameliorating Alzheimer's disease [2] by promoting free radical scavenging activity and protecting cells in the prefrontal cortex, hippocampus, and striatum [3], while enhancing memory retention through brain rejuvenation and activating neuron transmissions [4]. Being bacosides, dried *Bmo* powder is extensively used in commercial products and functional foods that are widely available in Thai markets [5].

The *Bmo* demand in the pharmaceutical industry is increasing today, but most of its plant materials are harvested from natural sources [6]. Overexploitation has led to rapid depletion of *Bmo* in wild populations. The *Bmo* grown in the wild also has low biomass and bioactive compound contents [7]. Therefore, new varieties of *Bmo* with improved agronomic traits are needed to fulfil the increasing market demand. However, genetic improvement through conventional breeding is time-consuming, and very difficult since the plant has a very small flower with tiny anthers, and has numerous small chromosomes (2n=2x=64) [8].

One strategy, which has successfully overcome this limitation, is chromosome doubling using polyploidy-inducing agent, such as colchicine, which is proven effective in generating high ploidy levels [9]. This antimitotic agent interrupts mitotic cell division during both stages of the late metaphase by binding to the protein tubulin, and the early anaphase by inhibiting spindle-fiber formation, resulting in inseparable chromosomes in cells with a doubled chromosome number [10]. Most polyploid plants contain more than two complete sets of chromosomes, with heritable and more superior agronomic characters than their diploid counterparts [6, 11–13]. Previous reports noted that the *Bmo* polyploid showed increased values of various agronomic traits such as flower size [14], shoot growth [15], and biomass [6]. Genetic improvement through polyploidization increased the drought resistance of *Ocimum basilicum* [16], produced higher yield levels in *Trifolium pretense* [17], produced wider and thicker leaves in *Limonium bellidifolium* [11], increased size of medicinal valuable rhizomes of *Paris* plants [13], and increased root size for easy cultivation in *Echinacea purpurea* [18]. However, the correct choice of explant type is an important first step in plant polyploidization. Different explant sources (such as apical shoot, node and leaf segments) affected on polyploidization depending on the permeability potential of the antimitotic agent through the nuclear membrane [19]. Moreover, these explants presented different properties of totipotent cells that had significant influence on further regeneration and proliferation after colchicine treatment [20]. Of these, the shoot tip of *Thymus persicus* [21], nodal segment of *Bacopa monnieri* [22], and leaf of *Pogostemon cablin* [23] have been employed for successful polyploidization.

Thus, the main objective of this study was to determine *in vitro* colchicine-induced polyploid plantlet regeneration from different explant types (apical shoot, node and leaf segments) of *Bacopa monnieri*. Moreover, these plantlets were used to determine a profile of bacoside contents and expression of *BmoAACT* and *BmoOSC* genes. Results will be beneficial for further genetic improvement to increase the medicinal value of a local *Bmo* for herbal medicine powder production in Thailand.

MATERIALS AND METHODS

Plant materials and *in vitro* multiplication

Diploid *Bacopa monnieri* (*Bmo*) mother plants were grown in plastic pots (10 inches diameter), containing clay soil and a fertilizer tablet (N:P:K=15:15:15) per pot and irrigated using tap water. All plants were cultivated under greenhouse conditions for six weeks at the Biology Department, Science Faculty, Naresuan University, Thailand. For *in vitro* cultures, shoots with 3–4 nodes were collected from healthy six-week-old plants, washed in running tap water for 3–5 min and then immersed in distilled water supplemented with 1 ml/l Tween-20 for 20 min. The explants were surface sterilized with 0.1% mercuric chloride ($HgCl_2$) solution with shaking for 5 min. Finally, the explants were thoroughly washed with sterilized distilled water repeated four times following Kharde et al [6] with minor modifications. The shoot explants were excised to approximately 1 cm length and transferred into a 35 ml screw-capped glass bottle containing ½MS [24] supplemented with 0.2 mg/l BAP, 3.0% sucrose, with pH adjusted to 5.8 before gelling with 0.7% agar. Cultures were incubated at 25 ± 2 °C under 10 h photoperiod with light intensity of 20 μmol/m^{2}s PAR provided by warm white LED lamps following the method of Sharma et al [25] with minor modifications. At eight weeks, the explants had regenerated and proliferated many plantlets.

Colchicine-treated explant segments

A stock solution (10% w/v) was prepared by dissolving colchicine (1 g) (Sigma-Aldrich, USA) in absolute ethanol (2 ml) with heating to 60 °C, yielding a clear to slightly hazy yellow to yellow-green solution. The final volume (10 ml) was adjusted with sterilized distilled water, the mixture was sterilized using a 0.2 μM syringe filter (Sigma-Aldrich, USA), and the eluted solution was collected in a new tube and stored at −20 °C until required for experimental use. This stock solution was further diluted by adding the appropriate amount of sterilized distilled water.

For the colchicine treatment, shoot, node or leaf segments were excised from eight-week-old regenerated plantlets and subjected to ½MS liquid medium supplemented with individually different colchicine concentrations (0.00, 0.05, 0.075, 0.1, and 0.5% w/v) and with colchicine-untreated explants as the control. All cultures were incubated by shaking at 100 rpm for 48 h at 25 ± 2 °C, and 10 h photoperiod with light intensity of 20 μmol/m^{2}s PAR provided by warm white LED lamps. All colchicine-treated explants were rinsed with sterilized distilled water five times and then transferred to solidified ½MS medium according to Sharma et al [25]. The cultures were kept un-

der the aforementioned condition for eight weeks. Numbers of regenerants per explant from individual treatments, performed as eight biological replicates, were recorded for further statistical analysis.

Analysis of ploidy levels through flow cytometry assay

Ploidy levels of the putative polyploid plants were assessed by flow cytometry assay. Nuclear DNA contents were measured using the method, slightly modified, from Pfosser et al [26]. Briefly, a chopping buffer (500 ml) was freshly prepared by mixing 200 mM Tris, 4 mM $MgCl_2 \cdot 6\,H_2O$, and 0.5% (v/v) Triton X-100, adjusted to pH 7.5 with either cool HCl (10 N) or NaOH (10 N), and then stored at 4 °C until required for further use. A young leaf sample (approximately 0.5 cm × 0.5 cm) was gently chopped with a sharp razor blade in cool chopping buffer solution (1 ml) in a Petri dish on ice. The suspension (500 µl) was pipetted and the nuclei were filtered through a 40 µM nylon net filter (Merck Millipore Ltd., Germany). The nuclear suspension (100 µl) was stained using Muse™ Cell Cycle Kit (Merck KGaA, Darmstadt, Germany), followed by incubating on ice in the dark for at least 30 min. The nuclear DNA contents were stained with propidium iodide for ploidy level by a Guava® easyCyte Flow Cytometer with InCyte™ software version 2.7 (Merck KGaA, Darmstadt, Germany).

Quantification of bacoside contents using HPLC assay

Diploid, mixoloid and tetraploid plantlets of *Bmo* were grown in ½Hoagland solution (HS) [27] under greenhouse condition for four weeks. Bacoside contents were quantified using the modified method of Bansal et al [28]. In brief, aerial parts of *Bmo* seedlings were individually dried at 45–48 °C for two days in a hot-air oven (1375 FX, ShelLab, USA), and then ground to fine powder using a mortar and pestle. The fine powder (100 mg) was transferred into a 15 ml amber centrifuge tube containing 3 ml methanol, mixed thoroughly, and incubated at room temperature for 1 h. The mixture was sonicated in an ultrasonic water bath (S50R Elmasonic, Elma, Germany) for 15 min and then incubated in the dark at 4 °C for 5 min. The final volume was adjusted to 10 ml with methanol and filtrated through a 0.45 µm nylon syringe filter (Tianjin Fuji Science & Technology Co., Ltd., China). The solution was stored at −80 °C until required for injection into the chromatographic system.

A stock solution of the calibrating reference standard was prepared by weighing bacoside A (mixture of Bacoside A3, bacoside II, Bacoside X and bacopasaponin C, Sigma-Aldrich, USA) 5 mg, dissolved in methanol 1 ml. The stock solution was further diluted with methanol to give standard solutions of 40, 60, 120, and 240 mg/l bacoside concentrations. Each standard solution (60 µl) was injected into the HPLC system (Shimadzu, Japan) equipped with a Purospher®STAR-RP-18 endcapped (5 µM) LiChroCART®250-4.6 HPLC cartridge (150 × 4.4 mm) (Merck, Germany), an LC-10AD VP pump, and a Rheodyne injector (20 µl loop) (Shimadzu, Japan). Chromatographic conditions were used to quantify the bacoside contents as follows: (i) the mobile phase consisted of phosphoric acid (0.2%) dissolved in a mixture of water and acetonitrile (65:35 v/v) adjusted pH to 3.0 with 5 M NaOH; (ii) the flow rate was 1 ml/min for saturation time 30 min; and (iii) the bacoside contents were detected at 205 nm using a SPD-10A VP UV-Vis detector (Shimadzu, Japan) and its contents were calculated by comparing relative retention times with standard samples.

Quantification of gene expression using qRT-PCR assay

The same plant materials used in the experiment of bacoside content quantification were extracted for total RNA using Total RNA Extraction Kit Maxi (RBC Real Genomics, Taiwan), according to the manufacturer's instructions. The quantity was estimated by absorbance at 260 nm and 280 nm with a Synergy H1 Hybrid Multi-Model Microplate Reader (BioTek Instruments, USA). For first-strand cDNA synthesis, total RNA (1 µg) was reverse transcribed in 20 µl reaction mixture using a Tero cDNA Synthesis Kit (Bioline, UK) containing random hexamer (1 µl), 10 mM dNTP mix (1 µl), 5X RT buffer (4 µl), RiboSafe RNase Inhibitor (1 µl), and 200 U/µl Tero Reverse Transcriptase (1 µl), and adjusted to a final volume of 20 µl with nuclease-free water (Life Sciences, USA). The reaction mixture was incubated at 25 °C for 10 min, 45 °C for 30 min, and 85 °C for 5 min. The first-strand cDNA was immediately subjected to RT-PCR amplification and stored at −20 °C until required for future use.

The qRT-PCR analyses for the *BmoAACT* (acetyl-CoA C-acetyltransferase), *BmoOSC* (oxidosqualene cyclase), and *Bmo18S-rRNA* (18S-ribosomal RNA) genes were performed using total reaction mixture of SensiFAST™ SYBR No-ROX (10 µl) (Bioline, UK), containing 10 µM of each forward and reverse

primer (0.4 µl), first-strand cDNA (1 µl), and final volume adjusted to 20 µl with nucleic acid-free water. The *Bmo18S-rRNA* gene was used as the internal control for normalization of all the reactions. Sequences of all gene-specific primers are shown in Table S1. The qRT-PCR reaction was carried out under the following conditions: 1 cycle of 95 °C for 2 min, followed by 45 cycles of 95 °C for 5 s, 55.6 (*BmoAACT*) or 60 °C (*BmoOSC* and *Bmo18s-rRNA*) for 10 s, and 72 °C for 20 s. Finally, a melting curve was realized by progressively heating the reaction mixture from 75 °C to 95 °C using 1.0 °C increments every 10 s to check the purity of the qRT-PCR product. All reactions were run in triplicate and repeated twice.

For statistical analysis, the baseline correction was automatically calculated to determine the cycle threshold (Ct) value in each reaction. Data were normalized with *Bmo18S-rRNA* as the endogenous control (set to 1). Relative expression of the target gene was analyzed using the comparative Ct method ($2^{-\Delta\Delta Ct}$). Briefly, relative expression of the target gene was analyzed using the comparative Ct method (ΔCt1, ΔCt2, and $2^{-\Delta\Delta Ct}$). Whereas the ΔCt1 represented different expression between target and reference gene in tetraploid samples, ΔCt2 represented different expression between target and reference gene in diploid samples, and ΔΔCt showed different expression levels between ΔCt1−ΔCt2. Normalized target gene expression level was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$) using program gene expression levels.

Statistical analysis

Data (regenerant numbers, bacoside contents, and gene expression) were statistically assessed using one-way analysis of variance (ANOVA). Mean comparisons between multiple treatments were assessed by Tukey's HSD (honest significant difference) test at p-value $\leqslant 0.01$ statistical significance using the Statistical Product and Service Solution version 17.0 software (SPSS Inc., Chicago, USA). All values were expressed as mean ± standard error (SE) of two–four biological replicates.

RESULTS AND DISCUSSION

Regenerant induction from different colchicine-treated explant types

The original diploid plants proliferated into multiple plantlets. Average numbers of shoot multiplications were highest (363 and 317 from a grand total of 1482 regenerated shoots) in low colchicine concentrations (0.05 and 0.075%, respectively) and this applied to colchicine-treated shoot, node and leaf segments compared to other treatments (Table 1).

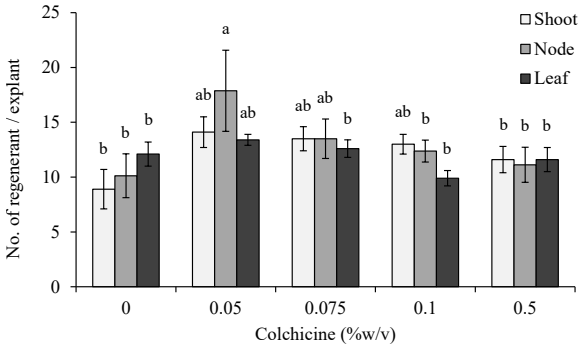

Fig. 1 Average number of regenerants per explant for different treatment combinations. Data are expressed as means ± standard error of eight biological replicates of eight-week-old plantlets after culturing. Different letters within the 15 treatment combinations indicate significant differences as assessed by Tukey's HSD test at $p \leqslant 0.01$.

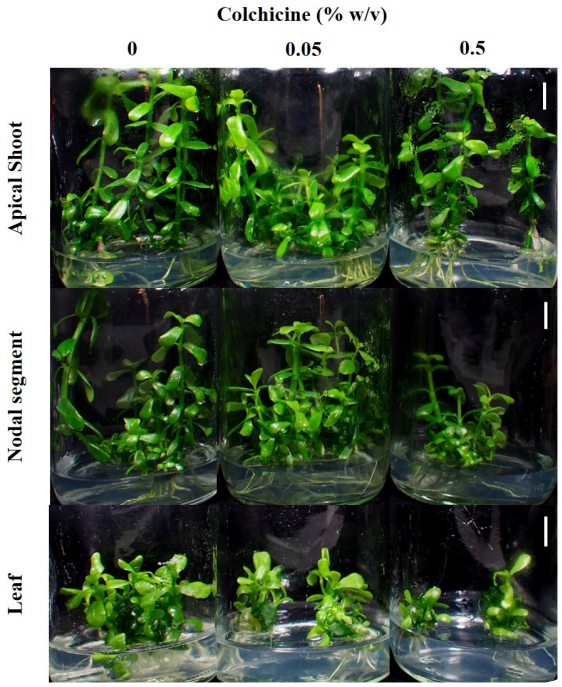

Fig. 2 Six representatives (from 15 treatment combinations) of regenerants from apical shoot (top panel), node (middle panel), and leave (bottom panel) segments of *Bacopa monnieri*. After colchicine-treated, the explant segments were cultured on ½MS agar medium for eight weeks, and their plantlets were photographed. Scale bar indicates 1 cm.

Table 1 Effect of different colchicine concentrations and explant segments on polyploidy induction in *Bacopa monnieri* assessed by flow cytometry assay.

Colchicine (% w/v)	Type of plant material[w]	Total no. of regenerants[x]	Flow cytometry analysis				
			No. putative regenerants[y]	Mixoploid		Tetraploid	
				No.	%[z]	No.	%
0	Shoot	71	24	0	0	0	0
	Node	81	26	0	0	0	0
	Leaf	97	24	0	0	0	0
Treatment total		249	74	0		0	
0.05	Shoot	113	24	0	0	4	3.5 (16.7)
	Node	143	35	0	0	15	10.5 (42.9)
	Leaf	107	24	0	0	16	15.0 (66.7)
Treatment total		363	83**	0		35	
0.075	Shoot	108	39	3	2.8 (7.7)	5	4.6 (12.8)
	Node	108	33	0	0	11	10.2 (33.3)
	Leaf	101	26	0	0	13	12.9 (50.0)
Treatment total		287	90**	5		23	
0.5	Shoot	93	32	0	0	3	3.2 (9.4)
	Node	89	32	2	6.3	13	14.6 (40.6)
	Leaf	89	27	0	0	4	4.5 (14.8)
Treatment total		271	91**	2		20	
Grand total		1482	362	10		107	

[w] Individual treatment combinations were performed with eight biological replicates.
[x] Total numbers of shoot inductions were generated from *in vitro* culture at different concentrations (0, 0.05, 0.075, 0.1, and 0.5%) of colchicine-treated shoot, node and leaf segments.
[y] Numbers of tested regenerants were selected from colchicine treatment based on morphological criterial presented in Fig. 2. ** indicates a highly significant difference between colchicine treatment and untreated-control treatment by student's *t*-test at $p \leqslant 0.01$.
[z] Percentage was calculated per number of mixoploids (or tetraploids) divided by total regenerants (out bracket) and putative regenerants in bracket).

Results revealed that the 0.05% colchicine-treated node segment was the optimal condition, and induced the maximum number of shoot regenerations per original explant (143/8=17.88 regenerants per explant) with significant difference at $p \leqslant 0.01$ compared to control treatments (Fig. 1). The next highest average number of shoot multiplication (113/8=14.13 regenerants per explant) was found in the 0.05% colchicine-treated shoot segment (Fig. 1).

By contrast, high concentration (0.5%) of colchicine produced less shoot multiplication from leaf segments compared to the untreated control, however, with only slight significant difference (Table 1, Fig. 1 and Fig. 2). Increasing concentrations of colchicine tended to inhibit growth and development, as well as multiple shoot induction from explants such as those of *Bacopa monnieri* [6, 16] and *Fagopyrum tataricum* [29], with decreased numbers of lateral buds per explant of *Pogostemon cablin* [30]. Moreover, colchicine-treated explants of *Pogostemon cablin* under high concentration showed significantly decreased numbers of lateral buds per explant compared to untreated plants [31].

In summary, the treatment of the node segment with low colchicine concentration (0.05%) was more effective for successful multiple-shoot induction than other treatment combinations (Fig. 1). This indicated that both different colchicine concentrations and types of explants had important effects on successful multiple shoot induction under *in vitro* conditions. Our results concurred with previous publications that multiple shoot regeneration of *Bacopa monnieri* was significantly influenced by different concentrations of colchicine

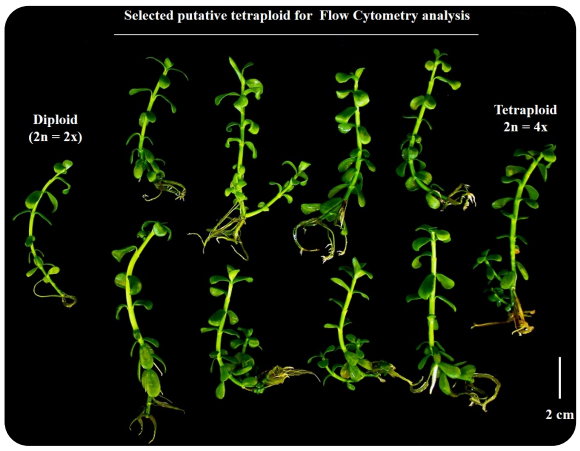

Fig. 3 Morphological characteristics of putative polyploid regenerants selected for flow cytometry analysis (in the middle group), compared to the progenitor diploid plant control (on the left) and identified tetraploid plant by flow cytometric assay (on the right). Scale bar indicates 2 cm.

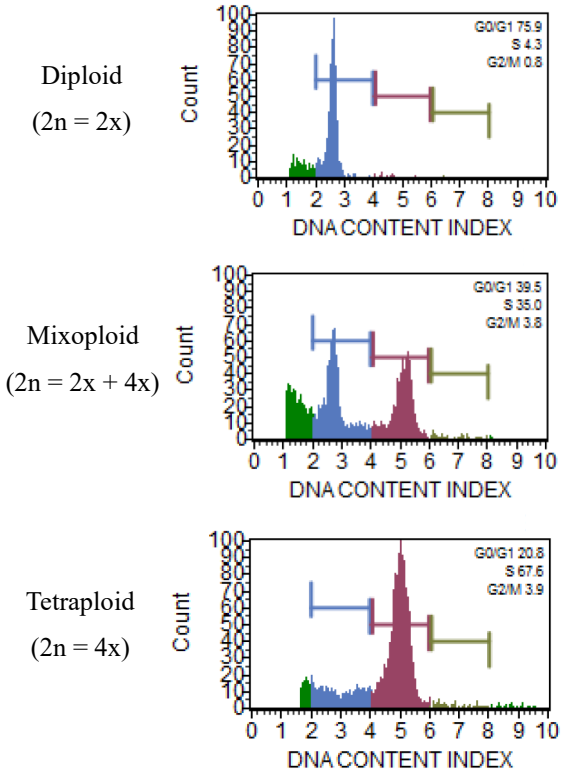

Fig. 4 Flow cytometry histograms showing nuclear DNA content index isolated from diploid (top panel), mixoploid (middle panel) and autotetraploid (bottom panel) plantlets of *Bacopa monnieri* cultured on ½MS [24] for eight weeks.

treatment [6, 31] in various segmental tissues [15]. Moreover, colchicine-treated leaf segments of hybrid sweetgum (*L. styraciflua* × *L. formosana*) produced visibly higher shoot induction than petiole segments [32, 33].

Polyploidy identification using flow cytometric assay

At the end of treatment, putative-polyploid plantlets were preliminary screened during *in vitro* culture using the criteria of vegetative characteristics with regard to bigger and thicker leaves and stems, compared to diploid plantlets (Fig. 3). The 362 putative clones (from colchicine-treated explants) were subsequently confirmed as either mixoploids or tetraploids by flow cytometry assay, comparing with 74 standard diploid clones (from untreated explants) (Table 1). To set the DNA-content standard peak, nuclear DNA contents were isolated from leaves of diploid plants followed by staining with propidium iodide (PI) and subjected to flow cytometry. The standard peak of diploid control samples (2n=2x=64) was set at channel 2.5 (Fig. 4 top panel) and the DNA-content index peak corresponding to tetraploid samples (2n=4x=128) was measured at a channel around 5.0 (Fig. 4 bottom panel) with two-fold PI intensity compared to diploid explant. The DNA-content index peak of mixoploid explant (containing diploid and tetraploid chimera) was measured between channel 2.5 to 5.0 (Fig. 4 middle panel). An individual clone was subjected to three biological replicates. Results showed that 10 and 107 clones (out of 362 putative clones) were identified as mixoploid and tetraploid clones, respectively (Table 1). Among the 15 colchicine treatment combinations, the efficiency (%) of tetraploid induction (range 3.2–15.0%) was higher than mixoploid induction (ranged 0.0–5.1%) with significant difference at $p \leqslant 0.01$ (Table 1, Fig. 1).

In this study, optimal effectiveness of colchicine application for polyploidization induction in *Bmo* was 0.05% for all segments (shoot, node and leaf). This provided the highest percentage of tetraploid plantlets without mixoploids. Of all treatment combinations, the most effective condition for tetraploid production was the treatment of leaf segments with 0.05% colchicine, producing 15% (16/107 tetraploid plants) without mixoploids (Table 1). Tetraploid plants showed higher genetic stability (regarding DNA content and chromosome number) over next generations than mixoploid plants of

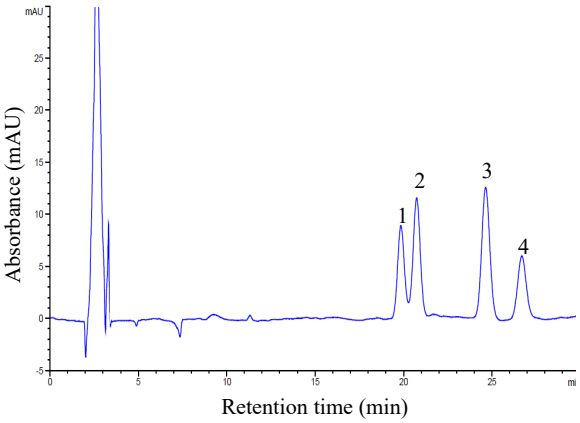

Fig. 5 HPLC chromatograms of standard bacoside mixtures at 205 nm obtained showing bacoside A3 (peak 1), bacopaside II (peak 2), bacopaside X (peak 3) and bacopasaponin C (peak 4) from the standard. Extracted saponins were separated by the mobile phase sodium sulfate (0.05 M) buffer pH 2.3 and acetonitrile (50:50, v/v) in the ratio of 70:30 (v/v) at a flow rate of 1 ml/min. Bacosides were detected at a wavelength of 205 nm and quantified against the standards. Peak assignments were matched with compounds.

Lolium multiflorum [33], probably because mixoploidized plants consisted of chimeras with both diploid and tetraploid sectors [34].

Accumulation of polyploid bacoside contents

The results showed that bacoside mixture concentrations of 120 mg/l were calibrated as the best separation efficiency in peak shape (symmetrical and no tailing peak) and retention times relative to individual bacoside. As shown in Fig. 5, the HPLC-chromatographic performance of standard bacoside mixtures detected at 205 nm showed four major peaks calibrated as the best separation efficiency in both peak shape (symmetrical and no tailing peak) and retention time relative to standard bacoside. These peaks (no. 1–4) corresponded to bacoside A3 (peak 1), bacopaside II (peak 2), bacopaside X (peak 3), and bacopasaponin C (peak 4) at different retention times of 20, 21, 24.5, and 27 min, respectively.

Three clones with the best growth (assessed by naked eye) were selected among mixoploid and autotetraploid plants. The one-month-old seedlings were used to examine bacoside mix contents (bacoside-A3, bacoside-II, bacoside-X, and bacosidesaponin-C) using HPLC assay with two–three biological replicates. Overall, both mixoploids and tetraploids showed a significant increase in the content of bacoside-A3, bacoside-II, and bacosidesaponin-C compared to its diploid progenitor. By contrast, only bacoside-X content was not significantly different among mixoploids, tetraploids and diploids (Table 2). Out of the studied clones, tetraploid clone number 4x-3 had maximum amounts of bacoside-A3 and bacoside-C contents at 4.276 ± 0.019 and 5.040 ± 0.078 mg/g dry weight, respectively. Meanwhile, tetraploid clone number 4x-1 had maximum amount of bacoside-II content at 5.009 ± 0.092 mg/g dry weight, but was not significantly different from mixoploid clone number mixo-1, containing bacoside-II content of 4.752 ± 0.096 mg/g dry weight (Table 2). This finding suggested that the polyploidy enabled an increase in bacoside content in *Bmo*. One possible explanation is that doubling chromosome number in plants leads to increased gene copies associated with secondary metabolite biosynthesis. As supported by previous publications, the productivity of triterpenoids in *Jatropha curcas* [12], patchouli alcohol in *Pogostemon cablin* [30], bacoside in *Bacopa monnieri* [6, 31], and flavonoid in *Fagopyrum tataricum* [29] was more in tetraploids than in their diploid plants.

BmoAACT and *BmoOSC* polyploid gene expression patterns

To examine polyploid gene expression patterns, three mixoploid and three tetraploid clones with the best growth were selected and grown in ½HS for six weeks under greenhouse conditions. These seedlings were used for evaluating expression patterns of *BmoAACT* and *BmoOSC* genes, associated with initial and late steps of triterpenoid saponin biosynthesis, respectively. The results showed that the *BmoAACT* gene was not significantly different in expression among all mixoploids, tetraploids and diploid *Bmo* at $p \leqslant 0.01$ (Table 3). However, expression level of the *BmoOSC* gene strongly increased in all tetraploid clones, but only slightly increased in mixoploid clones compared to their diploid progenitor (Table 3). Of the three tetraploid clones, the clone number 4x-3 gave the highest up-regulated expression level of the *BmoOSC* gene.

In this study, results indicated that the *BmoOSC* gene expression in polyploids (both mixoploids and autotetraploids) was higher than its diploid ancestor. Increasing proportional gene dosages (with more than two copies) caused by polyploidization might have significantly affected important changes in the differentially transcriptional profile compared

Table 2 Accumulation of bacoside contents in polyploidy determined by HPLC assay.

Ploidy level[y]	Content (mg/g dry weight)[z]			
	Bacopaside-A3	Bacopaside-II	Bacopaside-X	Bacopaside-C
2x	1.967 ± 0.098^{d}	2.033 ± 0.042^{d}	1.594 ± 0.016^{ns}	2.623 ± 0.046^{f}
mixo-1	2.956 ± 0.054^{c}	4.752 ± 0.096^{ab}	1.585 ± 0.027	3.311 ± 0.033^{e}
mixo-2	3.339 ± 0.035^{cb}	3.174 ± 0.106^{c}	1.631 ± 0.058	3.408 ± 0.125^{e}
mixo-3	3.562 ± 0.044^{cb}	4.362 ± 0.192^{b}	1.539 ± 0.050	3.885 ± 0.100^{d}
4x-1	3.352 ± 0.066^{b}	5.009 ± 0.092^{a}	1.549 ± 0.086	4.465 ± 0.170^{c}
4x-2	3.689 ± 0.094^{b}	3.546 ± 0.123^{c}	1.571 ± 0.026	5.573 ± 0.068^{b}
4x-3	4.276 ± 0.019^{a}	3.257 ± 0.049^{c}	1.709 ± 0.100	5.040 ± 0.078^{a}

Data represent mean ± SE of two–three biological replicates. Different letters within the same column indicate significant differences analyzed by Tukey's HSD test at $p \leqslant 0.01$.

[y] Individual clones of *Bacopa monnieri* were grown in ½HS for 30 days under greenhouse condition.

[z] Bacoside content (mg/plant) was calculated on a dry weight basis for individual plants.

Table 3 Expression patterns of *BmoOSC* and *BmoAACT* genes in different plant polyploids.

Clone	Relative gene expression (mean fold)	
	BmoAACT	*BmoOSC*
2x	1.000 ± 0.000^{ns}	1.000 ± 0.000^{c}
mixo-1	1.054 ± 0.129	1.295 ± 0.083^{c}
mixo-2	1.131 ± 0.194	1.496 ± 0.103^{b}
mixo-3	1.090 ± 0.013	1.637 ± 0.187^{b}
4x-1	1.025 ± 0.065	1.783 ± 0.229^{ab}
4x-2	1.157 ± 0.125	1.393 ± 0.176^{ab}
4x-3	1.158 ± 0.169	2.155 ± 0.116^{ab}

Values of qRT-PCR are expressed as means ± standard error of triplicate experiments. Mean values with different letters (in the same gene) are significantly different at $p \leqslant 0.01$.

to diploids [35]. Moreover, allele-dosage effects in polyploidy, in case of positively correlated with up-regulated gene expression, might be associated with epigenetic changes that fundamentally activated or suppressed gene transcriptions [32, 36]. Previous experimental results reported that 45.28% (calculated by 96 of 212 tested genes) showed up-regulated expression in tetraploids of *Citrus junos* [37]. Similarly, two candidate genes with potential regulatory roles of cell division and differentiation, i.e. expansin B3 (*EXPB3*) and *TCP* gene, resulted in the up-regulated expression in tetraploids of *Dendrocalamus latiflorus* compared to their diploid [38]. Higher expressions of two genes, phenylalanine ammonia-lyase (*PAL*) and pinoresinol-lariciresinol reductase (*PLR*), showed a more significant increase in its tetraploid *Linum album* than in its diploid counterpart [39]. Four genes (*Os04g21590*, *Os01g68560*, *Os11g38620*, and *Os11g38630*) that encoded stage-specific proteins during meiosis gave up-regulated expression in autotetraploids of *Oryza sativa* L. subsp. japonica compared to its diploid [40, 41]. Among 62 validated genes, only 6.8% produced up-regulated expression in autopolyploids of *Chrysanthemum lavandulifolium* [42]. We also found that up-regulation of *BmoOSC* expression in tetraploid clones (4x-3) was greater than in diploid plants; and this led to increased bacoside production. The *BmoOSC* gene may play an important role in construction of triterpenoid skeletons in the bacoside synthesis pathway.

CONCLUSION

Low concentration (0.05%) of colchicine-treated node and leaf segments was the most effective condition to induce multiple shoots and tetraploid plantlets. Our findings revealed that the positive relation of the *BmoOSC* gene expression and bacoside content in the tetraploid clone (4x-3) was higher than in the diploid plant. The *BmoOSC* gene may play an important role in construction of basic triterpenoid skeletons, leading to increased bacoside production.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2021.003.

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Appendix A. Supplementary data

Table S1 List of primer sequences used in this study.

Primer name	Primer sequence (5′-3′)
BmoAACT-F	GACTACGGCATGGGAGTTTG
BmoAACT-R	ATTCCACGCTCAAAACTTTGG
BmoOSC-F	GCATGTGGAATGCACTGCTTCTGT
BmoOSC-R	TGCCTTCGCCACGGAGATTTCTAT
Bmo18S-rRNA-F	GCACGCGCGCTACACCGAAG
Bmo18S-rRNA-R	GTCTGTACAAAGGGCAGGGACG

Individual primers for determining gene expression of *BmoAACT*, *BmoOSC*, and *Bmo18S-rRNA* were designed from GenBank loci FJ947159, HM769762 and JN148054, respectively, [43, 44].