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INDUCTION AND IDENTIFICATION OF AUTOTETRAPLOID FOR THE AUGMENTATION OF TANSHINONE IIA IN *SALVIA MILTIORRHIZA* BUNGE BY *IN VITRO* TREATMENT WITH COLCHICINE

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Summary

Induction and Identification of Autotetraploid for the Augmentation of Tanshinone IIA in *Salvia miltiorrhiza* Bunge by *in vitro* Treatment with Colchicine

Polyploidization technique has been successfully used for increasing the levels of quantitative and qualitative patterns of secondary metabolite production and exhibit enhanced vigour and superior performance in different medicinal plants. A protocol for the *in vitro* induction of *Salvia miltiorrhiza* Bunge tetraploids has been optimized to enhance the Tanshione IIA content, a major component of *Salvia miltiorrhiza* Bunge, inhibits platelet activation. *In vitro* leaves were used for treatment with different concentration of colchicine (0, 0.01, 0.02, 0.05, 0.1 w/v) along with treatment durations (7, 14, 21 and 28 days). The treated explants were then incubated on Murashige and Skoog (MS) medium having 0.5 mg/L N6-benzylaminopurine for shoot regeneration. The mutant types were isolated based on morphological characteristics and flow cytometry assays plantlets at *in vitro* and *in vivo* conditions. The tetraploids of *S. miltiorrhiza* were proficiently induced by the treatment of 0.05% colchicine for 14 days. The resulting tetraploid plants showed significantly enhanced agronomic traits, including the size of stomata and leaflet as well as root diameter, and fresh weight of root. In addition, an obvious reduction of length to width ratio was found in the 4x plants, including stomata frequency, leaflets, and roots. High-performance thin-layer chromatography showed a significant enhancement in the bioactive compound tanshinone IIA content of tetraploid plants (0.22% of dried sample) in comparison to diploid plants (0.14% of dried sample), signifying the prospective of this technique for the trade value improvement.

Keywords: *Colchicine treatment, Salvia miltiorrhiza Bunge, Tetraploid, Tanshinone IIA.*

1. Introduction

The root of *Salvia miltiorrhiza* Bunge has been used for thousands of years as a top-grade traditional Chinese medicine since it had been documented in Shen Nong Materia Medica. The main bioactive compounds in the dried root of *Salvia miltiorrhiza* Bunge are tanshinones and related quinones. They are mainly used for diseases of cardiovascular system, respiratory system, liver and kidney [1]. The major clinical indication is coronary heart disease such as angina [2]. They also have been used for the treatment of hyperlipemia, atherosclerosis and cerebrovascular disease [3],[4].

Salvia miltiorrhiza Bunge has originated from China. In the 60s - 70s of the 20th century, the National Institute of Medicinal Materials studied the importation and production of *S. miltiorrhiza* at Bac Ha Pharmaceutical Farm - Lao Cai. After many years of research, it has been determined that *S. miltiorrhiza* is suitable for production in some regions of Vietnam [5],[6].

Polyploids can occur naturally but they can also be the result of artificial induction using antimitotic agents. Due to the effects of polyploidization on plant growth and development, chromosome doubling has been applied in plant breeding to increase the levels of target compounds and improve morphological characteristics. It is a dual beneficial breeding strategy [7]. Polyploidy plants cannot always be segregated phenotypically from their diploid parents and could have a different phenotype; therefore, they are not restricted by the traits of ancestral diploids and may have different levels of resistance to drought and insects, biomass production, and quality and concentration of bioactive plant compounds [8]. Since the past century, artificial polyploidy induction has evolved to be a potent technique in plant improvement programs. Polyploidization generally improves the dynamism of determinate plant parts. Therefore, determinate tissues in plants that store the secondary metabolites can be greatly valuable for improving biomass content the phytochemicals [7],[9]. The process is easy to carry out *in vitro* and proficiently induces polyploidy on account of the controlled environment. A literature survey further revealed that accumulation of some secondary metabolites can be potentially increased by genome multiplication when the compounds occur in the determinated parts, such as andrographolide, artemisinin, baicalin, diosgenin, ginsenosides

Rg1, tanshinone content *Artemisia annua* [10], *Scutellaria baicalensis* [11], *Dioscorea zingiberensis* [12], *Panax ginseng* [13], *Salvia miltiorrhiza* [14] plants, correspondingly. In Vietnam, some plants, such as citrus, watermelon and China pink, have been polyploids [15]. Murashige and Nakano (1966) [16] were the first to report the uses of mitotic polyploidization in *in vitro* cultures. Among the reagents used for polyploid induction, colchicine, a toxic alkaloid is the most frequently used [17]. Tetraploid induction by tissue culture has a huge advantage due to its ease and high efficacy [18].

In this report, we have established a protocol for the induction of tetraploidy in *S. miltiorrhiza* for the first time in Vietnam. We also intended to examine the effect of polyploidization on morphological traits and secondary metabolite accumulation in *S. miltiorrhiza* and compared it with the corresponding diploid plantlets.

2. Materials and methods

Plant material and in vitro propagation

Ten- months old diploid *Salvia miltiorrhiza* Bunge plants grown in the medicinal field of National Institute of Medicinal Materials were employed to raise the *in vitro* culture following the procedure standardized by Ta Nhu Thuc Anh et al. 2014 [19]. The shoots were cultured in the multiplication medium consisting of Murashige and Skoog medium (MS) fortified with 1.25 mg L^{-1} BAP, 0.1 mg L^{-1} IBA, 30 g L^{-1} sucrose, 8 g L^{-1} agar, and pH 5.8, and incubated at 25±2 °C temperature under a 16-h photoperiod. Leaf segments (approximately 1 cm^2) were used as explants for testing the effects of colchicine on polyploidy induction. The explants were taken from plantlets which had been cultured for approximately 2 to 3 months.

Induction of polyploidy via direct shoot formation

The explants were cultured in MS medium containing 0.5 mg L^{-1} BAP, 30 g L^{-1} sucrose, 8 g L^{-1} agar and various concentrations filter-sterilized colchicine (0, 0.01, 0.02, 0.05, 0.1%; w/v) along with 2% (v/v) dimethyl sulfoxide at 25 °C for 7, 14, 21 and 28 days for the polyploidy shoot induction experiments. Twenty explants were used per treatment and each treatment was done in three replications (a total of 60 explants per treatment). Explants were maintained by subculturing after every 3 weeks and the regenerated shoots were subsequently inoculated in 0.05 mg L^{-1} IBA enriched ½MS medium to form roots. The data on the rate of morphological differentiation shoot formation, shoot tetraploid induction were recorded after 60 days of culture. For *ex vitro* transfer, the plantlets were removed from the culture vessels, washed with sterilized water and hardened in soil and sand mixture (1:1; w/w) at the greenhouse.

Flow cytometry (FCM) analysis

FCM was executed for accurate confirmation using leaf tissue (1.0 cm^2) taken from *in vitro* colchicine-treated regenerants and control. In the presence of 500 µL of DAPI (Sysmex Partec, Goerlitz, Germany), the materials were chopped by a razor blade thoroughly and filtered by a 30 µm nylon mesh to remove cell debris. The samples were then analyzed with a CyFlow® Ploidy (Sysmex Partec GmbH, Goerlitz, Germany) and DNA histograms were made.

Analysis of stomata

To compare the difference of stomata characteristics between tetraploid and diploid plants, fully expanded leaves were used. The leaf tissue was taken from the third node of plantlets (plantation approximately 3-month-old). For counting of stomata frequency, the epidermis was peeled and then observed with a light microscope. For evaluating the length and width of the randomly selected stomata.

Evaluation of bioactive compounds

The procedure for determination of bioactive compounds was performed as in Vietnamese pharmacopeia V [20]. Root were taken from plants after 10 months of plantation. The materials were harvested separately for evaluation. Bioactive compound tanshinone IIA (Chemfaces, CAS: 568-72-9, purity: 98%, Lot: CFS202003) was assayed to compare the difference between diploid and tetraploid plants.

Statistical analysis

Experimental data were processed according to the Microsoft Excel 2016.

3. Results and discussion

The leaf explants have adventitious shoots rate of colchicine-treated leaf explants and morphological differentiation shoots

In vitro polyploidization offers efficient methods to increase the production of plant material, improve the production of valuable compounds and morphological differentiation [21] in comparison to conventional breeding programs which is highly influenced by environment. An *in vitro* chromosome doubling technique depends on various factors which include the proper dosage of antimitotic agent along with its exposure time. The influence of

diverse concentrations and durations of colchicine treatment was assessed after 60 days on the percentage of leaf explants that have adventitious shoots (Tables 1). The results demonstrated that the leaf explants have adventitious shoots percentage of treated leaf explants was inversely proportional to the colchicine concentration and duration of exposure, i.e., it decreased significantly with the increase in colchicine level along with treatment duration. Colchicine at 0.1% along with treatment durations of 21 and 28 days totally inhibited shoot formation from the leaf explants. However, adventitious shoots could be obtained from the leaf explants in several treatments, including 0.01, 0.02, 0.05% colchicine along with treatment durations 7, 14, 21 and 28 days and 0.1% colchicine along with treatment durations 7 and 14 days and the highest percentages of leaf explants have adventitious shoots were 74.00% at 0.01% colchicine treated for 7 days (Table 1). Simultaneously with the decrease in viability and shoot formation, morphological differentiation shoots appeared in all of these experimental treatments. The concentration of 0.05% colchicine treated in 14 days caused the highest morphological differentiation shoots rate (75.33%). The recorded inverse correlation between the leaf explants have adventitious shoots percentage of plants and colchicine dosage also supports the report on several other species, such *Hyoscyamus reticulatus* [18], *Trachyspermum ammi* [22] and *Linum album* [23]. Poorer leaf explants have adventitious shoots was possibly due to the condensed rate of cell division due to colchicine-mediated spindle inhibition, resulting in the physiological disturbance.

Polyploid induction and its verification

The *in vitro* polyploidy of plants could be induced using antimitotic agents, the most used being colchicine [17]. The proper combination of colchicine concentration and duration of treatment is the chief factor that significantly induced tetraploids in the current study (Table. 1). The ploidy level of the regenerated *S. miltiorrhiza* morphological differentiation shoot was confirmed by flow cytometric analysis (FCM) after 60 days of culture. FCM predominantly gave two kinds of peaks at different positions determining that chromosome duplication was achieved by colchicine treatment (Fig. 1). Peak position in the tetraploid plants was twice that of the diploid plants which corresponds to 4x (Fig. 1A) and 2x (Fig. 1B), respectively. To verify the ploidy induction, FCM is one of the quick and reliable methods used widely in different medicinal plants [24,26]. The flow cytometric analysis proved that the tetraploids could be obtained in 14 treatments and the highest percentages of tetraploids were 19.08% at 0.05% colchicine for 14 days (Table 1). According to Javadian et al. (2017) [23], to conclude the best treatment for the tetraploidization, the most suitable parameter is to compute tetraploid induction efficiency as it considers both survival and tetraploid production frequencies. To examine the stability of the tetraploids, FCM was repeated at three months intervals with the samples collected from both *in vitro* and *ex vitro* tetraploid plants. The peak of the FCM was found constant at 4x position, indicating the stability of the ploidy level. Our results clearly demarcated that higher colchicine concentration affects the survival rate; Thus, lower concentration together with extended treatment duration is optimum for polyploidy induction which is also supported by the reports in *Thymus persicus* [24, *Trachyspermum ammi* [22] and *Salvia miltiorrhiza* [14] etc.

Table 1. Effect of different concentrations and durations of colchicine treatment on *in vitro* leaf explants for tetraploid induction in *S. miltiorrhiza* after 60 days of culture

Colchicine (%)	Duration (day)	Leaf explants have adventitious shoot (%)	Morphological differentiation shoot (%)	Tetraploid shoot (%)
0	7	86.00	0.00	0.00
0	14	84.33	0.00	0.00
0	21	83.33	0.00	0.00
0	28	83.33	0.00	0.00
0.01	7	74.00	8.67	2.08
0.01	14	62.67	13.33	2.99
0.01	21	58.00	22.67	5.89
0.01	28	53.33	30.67	7.16

0.02	7	47.33	40.00	10.93
0.02	14	44.00	48.00	11.52
0.02	21	38.67	55.33	15.86
0.02	28	36.67	58.67	15.65
0.05	7	31.33	67.33	16.38
0.05	**14**	**25.33**	**75.33**	**19.08**
0.05	21	17.33	18.00	4.92
0.05	28	12.67	10.67	2.42
0.1	7	4.00	4.67	1.12
0.1	14	0.67	1.33	0.35
0.1	21	0.00	0.00	0.00
0.1	28	0.00	0.00	0.00

Morphological characterization

The extensive morphological disparity was evidenced among the diploid and tetraploid plantlets (Table 2; Fig. 2). The initial noticeable outcome of colchicine-treated plants was the slower growth which may be due to the physiological change that retarded the cell division rate. It is also assumed that the meristematic zones of the newly emerged plant part might be harmed by the colchicine residue. The reduced growth rate in induced polyploids was also confirmed by the other reports [24],[26]. For classifying plants of higher ploidy levels, irregular leaf shape occasionally produced by higher ploidy plants also serves as a suitable identification technique. In 3-month-old *in vitro* grown plantlets, the growth behaviors, including Length and width of the leaves and root diameter of tetraploid plants were all significantly higher than diploid plants. By contrast, the root length of tetraploid plants was significantly lower than diploid plants. The roots of tetraploid plants were darker, shorter and thicker than diploid plants (Fig. 2 C, D). After 3 months of plantation, the plants grew well with a 100% survival rate. The leaflet of tetraploids not only had greater length, but also had greater width and area than did diploids. The ratio of leaflet length to width in tetraploids was approximately 1.19 (length/width = 3.64/3.08) which was lower than diploids with a ratio of 1.68 (length/width = 4.64 /2.79) (Table 2). In addition, the shape of the leaflet was dramatically changed by polyploidy level. The leaflet shape of diploid plants was mostly ovate, but the tetraploid plants were orbiculate in the first leaflet and elliptical in the rest of the leaflets (Fig. 2 E, F). The petiole of the tetraploid plants was shorter and thicker than the diploid plants (Fig. 2 E, F). The leaflet length of tetraploid plants was significantly shorter than diploid plants, but the leaflet width and area of tetraploid plants were significantly larger than diploid plants (Table 2). After 10 months of plantation, the diameter and length of roots were significantly higher in tetraploids (18.78 ± 2.83, 2.01 ± 0.28 cm, respectively) than in diploids (12.22 ± 1.79, 1.37 ± 0.20 cm, respectively) (Table 3). The enhanced diameter and length of roots, which was the most useful part for medicinal purpose of *S. miltiorrhiza* could be commercialized in pharmaceutical industries. The vigorous morphological features in comparison to the diploids for leaf size recorded here in tetraploid *S. miltiorrhiza* corroborates the former reports in *Pogostemon cablin* [27], *Trachyspermum ammi* [22] and *Salvia miltiorrhiza* [14]. Also, the obtained tetraploids plants were found fertile in nature. Contrary to our findings, some researchers reported leaves were found smaller in length and insignificant leaf width in tetraploids in comparison to diploids [28]. However, Shao et al. (2003) [28] accounted that enlarged length-to-width leaf ratios, are essential markers for selection of putative tetraploids. Ploidy level is by and large interconnected with cell size, and organ size is the direct link to degree of polyploidy as well as cell size [30]. An increase in the organ size is obvious, because the cells had to incorporate a large number of chromosomes complement for which it consequently grows bigger and more expression of proteins may likely to occur.

Stomatal size analysis

Stomatal size variation significant differences were observed for stomatal size and density between tetraploid and diploid plantlets (plantation approximately 3-month-old) has been tracked (Table 4, Fig. 3). An assessment on stomatal features showed that the size of stomata and stomatal frequencies were inversely proportional in relation to the ploidy difference. The mean length and width of stomata in tetraploids (56.94 ± 1.55 µm; 46.45 ± 3.12µm, Fig. 3 B) was significantly larger than the diploids (51.24 ± 2.07 µm; 34.73 ± 2.77 µm, Fig 3 A), whereas the average stomatal frequency in tetraploid plants was lower than diploids (Table 4). However, no morphological differences in stomatal shape were noticeable between the tetraploid and diploid plantlets. These findings demarcated that the doubling of genome can chiefly alter the stomata characteristics and appear to be an essential marker to discriminate tetraploids. The lower frequency of stomata in tetraploids was probably due to the larger epidermal and guard cells [31] and the results of our study validates several reports [14,22,31]. The size and the ratio of length to width of stomata were both considerably increased in the tetraploid plants of *S. miltiorrhiza*. Therefore, the stomatal morphology was proposed as a reliable selection indicator for polyploidy in *S. miltiorrhiza.*

Table 4. Effect of ploidy level on stomatal status of *S. miltiorrhiza* after plantation approximately 3-month-old.

Character	Tetraploid	Diploid
Stomata length (µm)	56.94 ± 1,55	51.24 ± 2.07
Stomata width (µm)	46.45 ± 3.12	34.73 ± 2.77
Stomata length/ width	1.23 ± 0.08	1.48 ± 0.15
Stomatal frequency (no./ 450µm²)	41.67 ± 3.44	77.33 ± 3.33

Data in each column represents mean ± standard deviation.

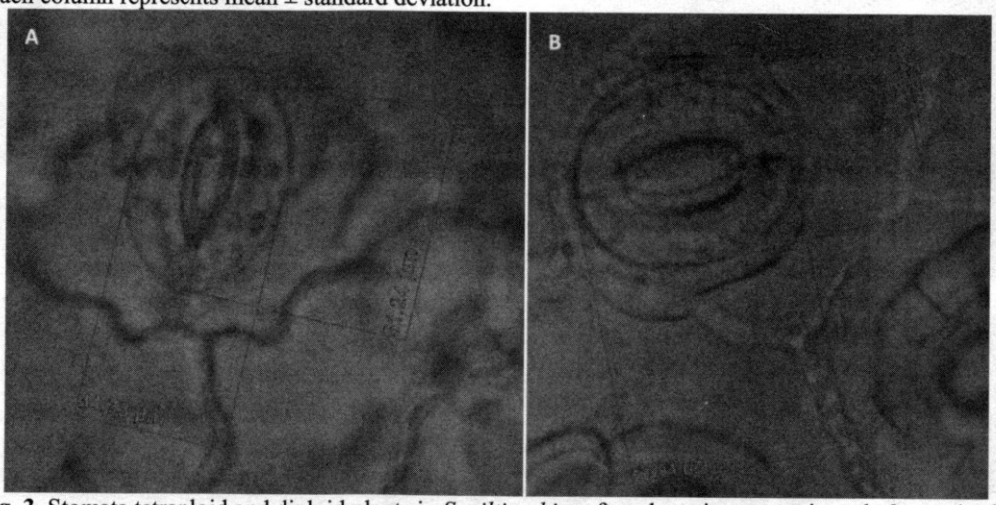

Fig. 3. Stomata tetraploid and diploid plants in *S.miltiorrhiza* after plantation approximately 3 month-old. (A) diploid plant. (B) tetraploid plant.

Evaluation of major chemical compounds through HPLC

The content of effective compounds is very important in the medicinal plant. With the aim of preliminary evaluating the phytochemical profile of *S. miltiorrhiza*, HPLC was performed (Fig. 4). The chromatogram of standard tanshinone IIA was found to overlap with the extracts obtained from diploid and tetraploid plants after 10 months of plantation, exhibiting one characteristic peak of tanshinone IIA at 270 nm (Fig. 4d). However, the peak area calculation revealed that there was a deviation in the tanshinone IIA content among the diploid and tetraploid plants (Fig. 5a-c). The tanshinone IIA was recorded to be accumulated at a preliminary much higher quantity in the tetraploid (0.22% dry weight) than in the diploid (0.14% dry weight). An affirmative connection between the higher ploidy level and enhanced secondary metabolite content has been established in numerous artificially induced tetraploid plants, such as,

Table 2. Effect of ploidy level on morphological characteristics of *S. miltiorrhiza* after 3 months of plantation

Treatment	Leaf			Leaflet		
	Length (cm)	Width (cm)	Length /Width	Length (cm)	Width (cm)	Length /Width
Control	7.4 ± 0.26	4.33 ± 0.15	1.71 ± 0.03	4.64 ± 0.46	2.79 ± 0.28	1.68 ± 0.1
Tetraploid	7.67 ± 0.15	6.47 ± 0.47	1.19 ± 0.06	3.64 ± 0.30	3.08 ± 0.32	1.19 ± 0.06

Data in each column represents mean ± standard deviation.

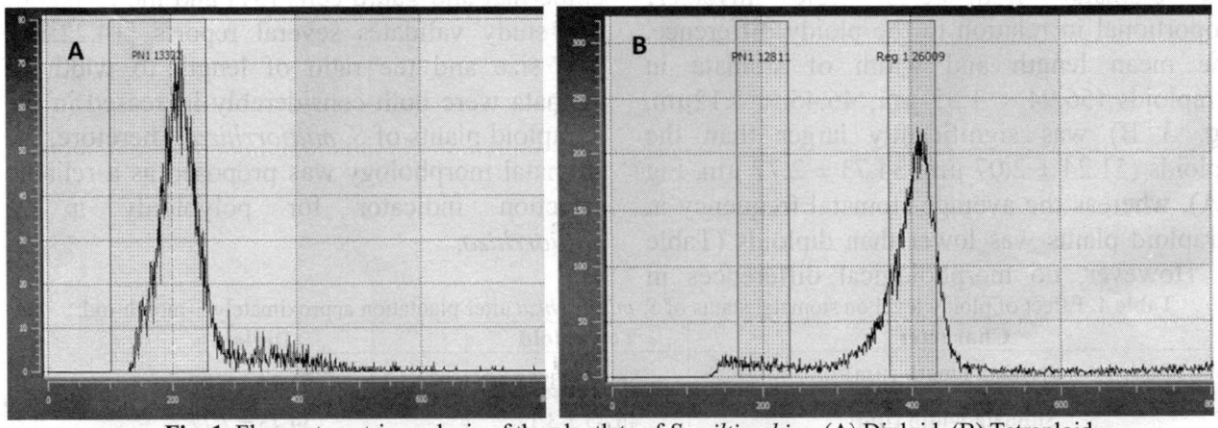

Fig. 1. Flow cytometric analysis of the plantlets of *S. miltiorrhiza*. (A) Diploid. (B) Tetraploid

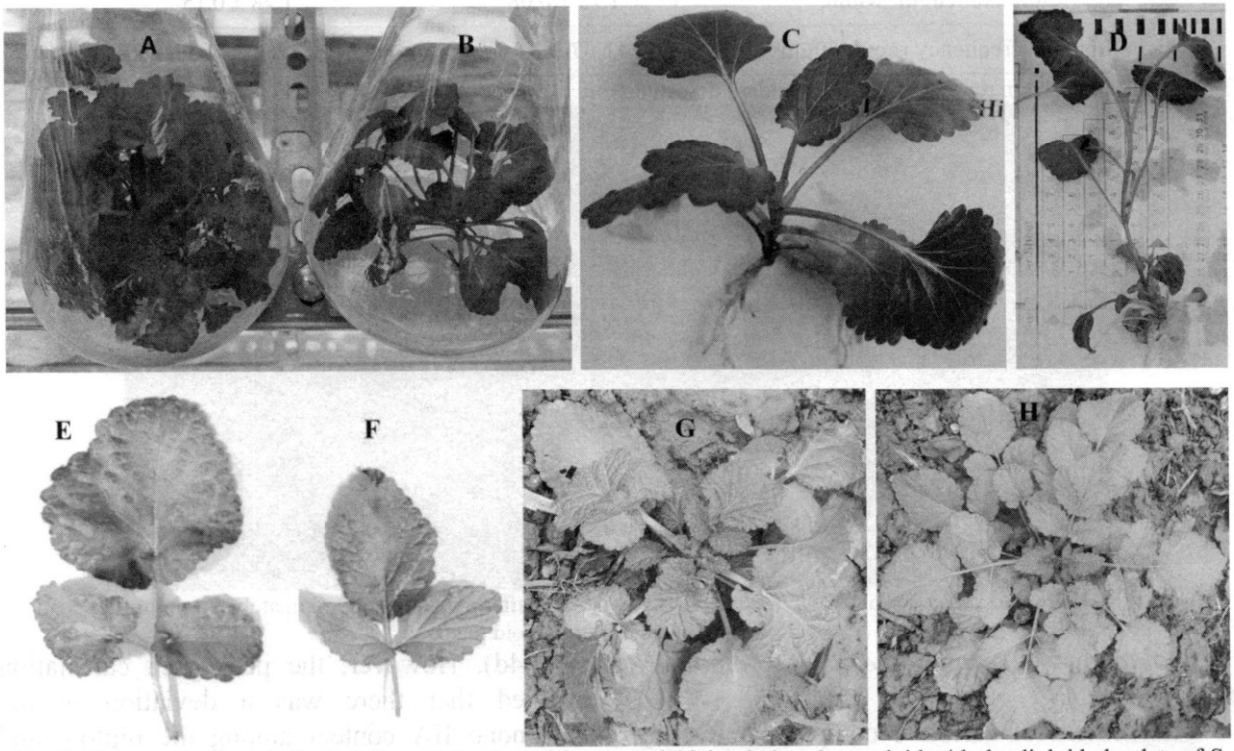

Fig. 2. Comparative morphological characterization of *in vitro* colchicine-induced tetraploid with the diploid plantlets of *S. miltiorrhiza*.

Variation in size of plantlets between *in vitro* tetraploid (A) and diploid (B); 3-month-old *in vitro* grown plantlets of tetraploid (C) and diploid (D); Leaf size of tetraploid (E) and diploid (F); Variation in size of plants between tetraploid (G) and diploid (H) after 3 months of plantation.

Table 3. Effect of polyploidy in biomass of plants in *S. miltiorrhiza* (After 10 months of plantations)

Treatment	Root Length (cm)	Root Diameter (cm)	Root Fresh Weight (g)
Diploid	12.22 ± 1.79	1.37 ± 0.20	195.00 ± 14.73
Tetraploid	18.78 ± 2.83	2.01 ± 0.28	296.33 ± 10.97

Data in each column represents mean ± standard deviation.

27.5% more essential oil content in *Dracocephalum moldavica* [33], as high as 40% more accumulation *Scutellaria baicalensis* [11], 8.66% more scopolamine content in *H. reticulatus* [18], etc. The augmentation of the tanshinone IIA content in the tetraploids is probably attributable to increased metabolic activity and over-expression of genes following chromosome doubling [34]. Hence, in *Salvia* spp., tanshinones are the major bioactive terpenoids which play important roles in the growth and development of plants [35]. Consequently, in this study, a noteworthy improvement in tanshinone IIA production, as well as several agronomic traits of 4x plants including length, diameter and fresh weight of root, which was the most useful part for medicinal purpose, in *S. miltiorrhiza*, has been achieved through the artificial polyploidy technology which could be commercialized in pharmaceutical industries for different herbal formulations.

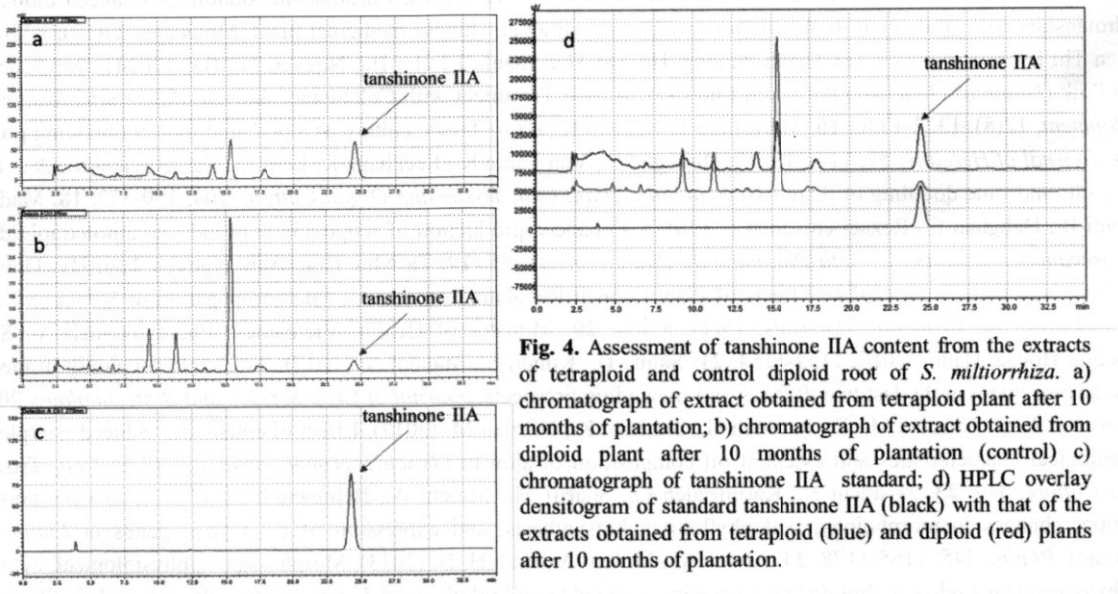

Fig. 4. Assessment of tanshinone IIA content from the extracts of tetraploid and control diploid root of *S. miltiorrhiza*. a) chromatograph of extract obtained from tetraploid plant after 10 months of plantation; b) chromatograph of extract obtained from diploid plant after 10 months of plantation (control) c) chromatograph of tanshinone IIA standard; d) HPLC overlay densitogram of standard tanshinone IIA (black) with that of the extracts obtained from tetraploid (blue) and diploid (red) plants after 10 months of plantation.

4. Conclusion

An efficient technique for *in vitro* colchicine-mediated tetraploidization of *S. miltiorrhiza* had been established for the first time in Vietnam. The tetraploids of *S. miltiorrhiza* were proficiently induced by the treatment of 0.05% colchicine for 14 days. The tetraploids demonstrated noteworthy variations in their morphological traits in comparison to diploid plants; for instance, larger leaves, greater size of stomata but reduced stomatal density in the leaves. Due to the doubled chromosome number, the tetraploid is probably accumulable 1.6-fold more tanshinone IIA than the diploids. The obtained results signify that the established tetraploids can be effectively used for the potential supply in pharmaceutical application. These results provide an efficient platform to aid breeding programs and provide materials for further genetic studies in *S. miltiorrhiza*.

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