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Enhanced morphologic traits and medicinal constituents of octaploids in *Satureja mutica*, a high-yielding medicinal savory

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Abstract. *Satureja mutica* is a tetraploid and perennial semi-bushy plant cultivated for different medicinal purposes. To induce polyploidy, two-leafed seedlings were exposed to different concentrations (0.00, 0.05, 0.1, and 0.2 % w/v) and durations (6, 12, and 24 h) of colchicine. The seedlings were then transferred to a culture medium for recovery and propagation. After clones were prepared from each seedling, octaploid clones were identified using flow cytometry. Chromosome counting was also used to confirm flow cytometric results in tetraploid ($2n = 4x = 60$; 2C DNA= 1.90 ± 0.01 pg) and octaploid ($2n = 8x = 120$; 2C DNA= 3.82 ± 02 pg) plants. The highest polyploidy induction efficiency with 32% was related to 0.05 % colchicine and 12 h duration. The results showed that the phenotypic traits of anatomical (stomata size, leaf guard cell size), morphological (stem diameter, length, width, and leaf area), physiological (soluble sugars, phenols, and flavonoids), and phytochemical (essential oil yield, P-cymene, γ-Terpinene, α-Thujene, and α-Pinene) properties significantly increased in octaploid plants, while the density of leaf stomata decreased compared to tetraploid plants. Our results verified that octaploid induction in *Satureja mutica* is an effective breeding method, remarkably increasing the quantitative and qualitative characteristics, which could be used as a new genetic resource in future breeding programs.

Keywords: octaploid plants, polyploidy, phytochemical properties, genome size.

INTRODUCTION

Plant evolution includes polyploid development, which has facilitated adaptation and speciation. Researchers have since discovered that artificial polyploidy induction can be a useful method for the plant. Cell size and nuclear volume are directly related to ploidy levels in plants (Símová

and Herben, 2012; Robinson et al., 2018), but organ size varies due to regulatory mechanisms and a decrease in cell number (Tsukaya, 2008; Czesnick and Lenhard, 2015). In *Arabidopsis thaliana*, doubling the ploidy level increased cell size by 70% (4*x* versus 2*x* and 8*x* versus 4*x*), while organ size increased by only 20% (Robinson et al., 2018). Organ size depends on cell size and cell number. The number of cells is also affected by the regulation of cell proliferation and differentiation, so an increase in ploidy may not coordinate with organ size increment (Orr-Weaver, 2015). Also, ploidy does not affect nuclear size in all organisms and it is regulated by genetic factors (Vuković et al., 2016). For example, in *Saccharomyces pombe*, there is no difference between ploidy levels 2*x* to 32*x* in terms of nuclear volume and the cell area (Neumann and Nurse 2007).

Polyploidy induction is performed in plants with different purposes such as increasing the size of leaves, stems, inflorescences, flowers, fruits, and seeds (Wei et al., 2011; Huang et al., 2014; Shariat et al., 2021). In medicinal plants, ploidy induction is performed to increase the amount of *secondary* compounds, for example, the amount of parthenolide in tetraploid *Tanacetum parthenium* (Majdi et al., 2010); alkamines and caffeic acid derivatives in tetraploid *Echinacea purpurea* (Xu et al., 2014); wedelolactone in tetraploid *Eclipta alba* (Salma et al., 2018). Unexpectedly, the production of new secondary compounds or the reduction of active compounds due to polyploidy induction has also been reported, for example, loss of α-bergamotene and isocarveol geranial and a new component of β-bisabolene in tetraploid *Citrus limon* (Bhuvaneswari et al., 2020); Lack of α-terpineol in tetraploid *Trachyspermum ammi* L. (Noori et al., 2017); Loss of citral in triploid and lack of linalool in tetraploid *Lippia alba* (Julião et al., 2020), new compounds such as viridiflorol, α-terpineol and α-humulene in tetraploids *Tetradenia riparia* (Hannweg et al., 2016).

Species of savory are used in food and pharmaceutical industries due to their pleasant smell and medicinal properties such as antifungal, antibacterial, antiviral, antioxidant, and analgesic properties (Shariat et al., 2018a). Recognition of the therapeutic effects of *S. mutica* has led to increasing demand for fresh and dried leaves so that the amount of consumption is more than the amount of production. Due to the limited level of cultivation and agricultural inputs, cultivating high-yielding polyploid cultivars is a goal for a producer. Previous research on the species *mutica* has focused on its chemical composition and antimicrobial properties. To the best of our knowledge, breeding and polyploidy induction of *S. mutica* has yet to be reported. Among savory species, some species such as *S. mutica* and *S. spicigera* are naturally tetraploid and have larger bushes and more biomass than other *Satureja* species. The amount of essential oil yield in *S.mutica* populations varies from 0.17% to 4.22% (w / w) (Karimi et al., 2014). It should be noted that the population used in this study had an essential oil yield of 3.88% w/w.

Since *S. mutica* is tetraploid, polyploidy refers to upwards of tetraploidy. Questions addressed in this study include: (1) Is it possible to produce octaploid plants using colchicine? (2) Are the induced octaploids flowering? (3) Do octaploid plants exhibit superior morphological and physiological traits compared to tetraploids? (4) Do the content of essential oil (v/w) and yield (%) differ between tetraploid and octaploid plants?

The purpose of octaploid induction in this study was to increase the biomass, quantity, and quality of essential oil, along with an efficient protocol for inducing octaploidy in *S. mutica*.

MATERIALS AND METHODS

Plant material

The seeds were collected from mature plants of *S. mutica* in Keshanak, North Khorasan province, in the northeast of Iran. In order to surface-sterilize the seeds, the following solutions were used: (1) Thiram 0.2% (v / v) (10 min) as a fungicide, (2) Sodium hydrochloride 5% (2 min), (3) 70% alcohol (20 s). After each step, the seeds were rinsed with sterile water and then placed on a moistened filter paper in a Petri dish. Petri dishes were sealed with parafilm to retain moisture. The seeds were incubated at temperatures 25 ± 1 with controlled photoperiods (16 h: 8 h, light: dark interval) under standard cool white fluorescent tubes (35 μmol s^{-1} m^{-2}).

In vitro polyploid induction

After about eight days, the germinated seeds, which had reached the two-leaf stage, were transferred to Erlenmeyer containing different concentrations of colchicine. A factorial experiment with two factors was conducted in a completely randomized design (CRD) with three replications.

The first factor was different concentrations of colchicine (0.00, 0.05, 0.10, and 0.20% w/v), and the second factor was the duration (6, 12, and 24 h). The dimethyl sulfoxide (DMSO) solution was added to each treatment to increase the penetration of colchicine into plant tissue (Manzoor et al. 2018). After that, the Erlenmey-

ers containing the seedling samples were placed on a shaker with a rotation speed of 80 rpm. Each seedling was rinsed three times with sterile water after the treatment duration was over, then transferred to a small vial containing ½ Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The parameters of the growth chamber, including darkness, temperature, and light quality, were similar to those used for seed germination. A large number of seedlings were lost after one month, and the remaining seedlings were transferred to a suitable culture medium. The tissue culture medium used in the second month was a ½MS medium that four hormones were added [2-Isopentenyl-adenine (2iP) (0.3 mg l^{-1}), 1-Phenyl-3-(1,2,3-thidiazol-5-yl) urea (TDZ) (0.1 mg l^{-1}), Indole-3-butyric acid (IBA) (0.05 mg l^{-1}), 6-Benzylaminopurine (BAP) (0.5 mg l^{-1})]. After three regenerations (3 months), when the number of explants reached a sufficient number, the ploidy level was determined using flow cytometry, and octaploids were selected. Then, selected plants were subcultured to produce clones. As the next step, two rooting hormones were added to the MS medium [IBA (1 mg l^{-1}), Alpha-naphthalene acetic acid (NAA) (0.1 mg l^{-1})] (Shariat et al. 2016). After rooting proliferation (1 month), plantlets were transplanted in pots containing sterilized cocopeat and covered with polystyrene bags and then placed in a greenhouse at a temperature of 20 ± 1 °C with daylight intensity 8000–12000 lux.

Plastics were punctured after a week. This was done to reduce the humidity around the plant and make it more accustomed to the greenhouse environment. In the present study, to reduce mixoploidy results, Petri dishes containing sprouted seeds were kept in the refrigerator at 4°C for 24 h and then placed in the incubator at 25°C for 4 h under standard cool white fluorescent tubes (35 µmol s^{-1} m^{-2}). Then colchicine treatments were applied immediately. This method leads to synchronization of meristematic cell division and as a result, the percentage of mixoploid production is greatly reduced.

Flow cytometry analysis

It is necessary to measure the ploidy level of the seedlings before they can be propagated in large numbers and transferred to the greenhouse. Flow cytometry was used for this purpose. Maize (*Zea mays* CE-777, 2C DNA = 5.43 pg) was also selected as the standard reference plant (Doležel et al. 2007). 0.5 cm^2 of maize leaf with 1 cm^2 of savory leaves were placed in a Petri dish and 1 ml of WPB extraction buffer was added (2007 et al. Louirero). Crushing was performed with a sharp razor. The resulting mixture was passed through a 30 µm nylon mesh filter made by Partec. Then 50 µl of RNase solution (1 mg ml^{-1}, Sigma-Aldrich Corporation, MO, USA) and 50 µl of PI dye (1 mg ml^{-1}, PI, Fluka) were added to the solution (Shariat et al. 2018b) and then incubated for five min at room temperature and then injected into BD FACSCanto II flow cytometer (B.D. Biosciences, Bedford, MA, USA). The results were analyzed in FloMax 2.4e software and the value of CV value and mean G1 peak for each replication were calculated. Then, the amount of 2C DNA in each sample was calculated using the following formula:

$$Sample\ 2CDNA(pg) = \frac{Sample\ G1\ peakmean}{Standard\ G1\ peakmean} \times Standard\ 2CDNA(pg)$$

Chromosome counting

Flow cytometry confirmed the presence of octaploidy, so octaploid explants were transferred to MS medium containing rooting hormones as described before (Shariat et al. 2016). Root samples were collected before transferring the explants to the greenhouse. The roots were immersed in the following solutions respectively: (1) 2 h in α-bromonphthalein pretreatment solution (1 in 10,000 ml), (2) 16 h in Carnoy fixative solution (3 alcohols: 1 acetic acid), (3) Roots then kept in 70% alcohol at 4°C(4) 12 min at 60°C in 1 M HCl hydrolysis solution. It should be noted that root tips were rinsed with distilled water after each step (5) 6 h at 60°C in 4% hematoxylin as staining dye. A needle was used to crush the root tip on the slide, and a drop of 45% acetic acid was dropped on it. Slides were protected with lamellae and squashed (Shariat *et* al. 2013). Stomata photographs were captured using optical microscopy (Nikon Coolpix P90 digital camera interfaced to a BH2-RFCA Olympus microscope).

Anatomical and morphological analysis

Anatomical properties including abaxial and adaxial stomata length, width, area, and density were measured using the nail varnish technique (Smith et al. 1989). Stomata photographs were captured using a digital camera (Nikon Coolpix P90 digital camera interfaced with a BH2-RFCA Olympus microscope). Stem diameter, internode length, leaf length, width and area, floral leaf length, and width, corolla length, and width, calyx length, calyx lower, and upper teeth length, filament, and style length, peduncle length was measured with a caliper.

Measurement of physiological traits

The method of Anthron (Irigoyen, 1992) was used to measure soluble sugars. Proline and flavonoids were measured by using protocols described by Bates, 1973 and Kamtekar et al., 2014 respectively. Plant pigments were also measured using the acetone method (Lichtenthaler and Welburn 1983). Folin Ciocalteu phenol reagent was used to measure total phenol according to Singleton and Rossi's method (Singleton and Rossi, 1965).

Essential oil content and composition

The aerial parts of tetraploid and octaploid plants were harvested at the 50% flowering stage and air-dried in the shade (25°C). Oil was produced from the ground aerial parts of the plant (100 g) by hydro-distillation for 3 h using a Clevenger-type apparatus. The oil was dried over anhydrous calcium chloride and stored in a refrigerator at 4 °C before analysis. Oil yield was calculated as a weight percentage (w/w) according to the equation (Alizadeh et al., 2018):

$$Essential\ oil\ yield = \frac{Essential\ oil\ weight(g)}{Aerial\ biomass\ yield(g)} \times 100$$

Gas chromatography (GC) analysis was performed using a Shimadzu GC- 9A gas chromatograph (Japan). Gas chromatography coupled with a mass spectrometer (GC-MS) analyses were also carried out on a Varian 3400 GC- MS system (M.S. USA). Both systems were equipped with a DB-5 fused silica column (30 m × 0.25 mm i.d. film thickness 0.25 μm). In the present study, the protocol as described by Sefidkon and Jamzad was used (Sefidkon and Jamzad 2004). Compounds were identified using various indicators such as time and inhibition index, mass spectra, and comparison of these spectra with standard compounds (Adams 2007).

Data analysis:

The effect of different concentrations of colchicine and durations on seedling traits (seedling vigor, survival rate, and polyploidy induction efficiency) were analysed in a completely randomized design with three replications. Mean comparisons were performed by the least significant difference test (L.S.D.) at 1% and 5% levels of significance. Octaploid induction efficiency was calculated using the following formula (Tarkesh Esfahani et al. 2020):

$$Induction\ efficiency = \frac{Seedling\ survival(\%)}{octaploidy\ induction(\%)}$$

To calculate the octaploid induction efficiency, the genome size of the regenerated explants was determined by using flow cytometry, and the percentage of octaploid induction was calculated. Normalization of data was performed using Kolmogorov-Smirnov test. A significant comparison of anatomical, morphological, and physiological traits in tetraploid and octaploid plants was performed using the T-student test. Analyzes were performed using Excel 2016 software and IBM SPSS Statistics 24.

RESULTS

Polyploid Induction

Two-leafed seedlings were affected by colchicine toxicity, which was accompanied by symptoms such as necrosis of seedlings, blackening of roots, deformation of leaves and stems, or growth stops. At the same time, several seedlings were growing normally. The analysis of variance demonstrated that increasing the concentration of colchicine decreased seedling survival rate, while there was no significant relationship ($P > 0.05$) between treatment duration and survival rate. The highest survival rate among colchicine treatments was related to the concentration of 0.05% colchicine. The interaction between colchicine concentrations and durations on survival was not significant ($P > 0.05$) (Table 1). According to the ANOVA analysis, different concentrations of colchicine, durations, and the interaction between them had a significant impact ($P < 0.01$) on seedling vigor and polyploidy induction efficiency. In other words, colchicine concentrations at various durations did not affect the two traits in the same manner.

At the concentration of 0.05% colchicine solutions, survival rate and seedling vigor were 64 ± 2.5% and 59.5

Table 1. ANOVA results (mean square) for the influence of colchicine concentration and duration on seedling vigor, survival rate, and octaploid induction efficiency in *S. mutica.*

Source of Variation	Survival rate	Seedling vigour	Polyploid induction efficiency
Colchicine concentration (C)	6325.7**	14028.1**	1577.9**
Treatment duration (D)	3.5ns	40.8**	42.2**
C*D interaction	20.4ns	25.1**	1609**
Error	23.1	5.2	1.4

ns: Non significant, **: significant at 1% ($P < 0.01$).

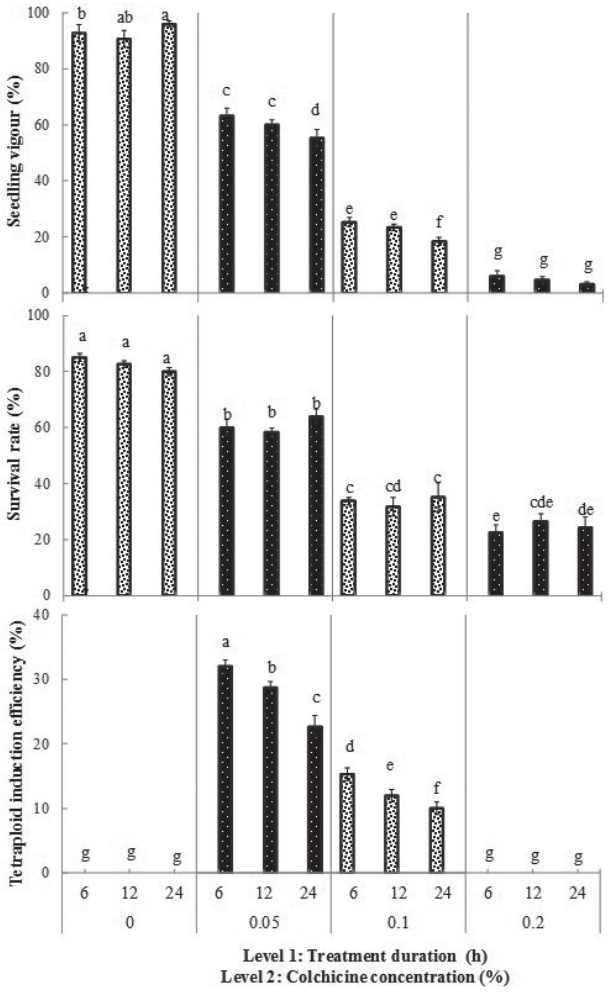

Figure 1. Effect of colchicine concentration and treatment duration on seedling vigor (a), survival rate (b), and polyploidy induction efficiency in *S. mutica* explants (mean ± standard error).

± 2.4%, respectively, while at the concentration of 0.2% was 22 ± 3.8%, and 4.5 ± 1.4% respectively (Figure 1). Therefore, increasing the concentration of colchicine leads to a sharp decrease in seedling vigor. Octaploony induction efficiency was the lowest in the presence of 0.2% colchicine treatment (15.3%) and was the highest in the presence of 0.05% colchicine treatment (32%).

Flow cytometric analysis

The genome size of plants treated with colchicine was determined by flow cytometry. The results classified the explants into three groups: tetraploid, octaploid, and mixoploid. The position of the standard maize plant relative to the tetraploid and octaploid plants is critical (Figure 2). The amount of 2C DNA in tetraploid *S. mutica* plants was 1.90 ± 0.01 and in induced octaploids was 3.82± 0.02 (Table 4). 15% Of individuals were mixoploid, Half of it was related to 0.05% colchicine treatment, and the rest was related to 0.1% and 0.2% colchicine treatments. In mixoploids, there are two types of cells with two ploidy levels. During plant growth, those cells with lower ploidy levels divide more rapidly, and after a while, the number of polyploid cells decreases or is eliminated. (Touchell et al., 2020). Therefore, due to the instability of mixoploids, they were excluded in this study and were not examined. It should be noted that only the percentage of octaploids obtained was used to calculate the efficiency of polyploidy induction.

Chromosome counting

To confirm the flow cytometry results, chromosome counting was performed using the microscopic method. Tissue culture seedlings (colchicine treated and control) were used for chromosome counting. The results of chromosomal counting showed that the number of chromosomes in control plants was $2n = 4x = 60$ and in octaploid plants was $2n = 8x = 120$ (Table 2, Figure 3).

Physiological traits

Octaploid induction had a significant effect ($P < 0.01$) on all measured physiological parameters including plant pigments (total chlorophyll and carotenoids), phenol content, flavonoids, and soluble sugars. Comparisons between traits measured in 4x and 8x plants were performed using the T-student test (Table 3). The amount of total phenol (according to the gallic acid standard) in the tetraploid plant increased from 20.34 ± 0.97 to 62.16 ± 1.90 μg g^{-1} DW in the octaploid (205% increase). The amount of flavonoids was also increased from 2.00 ± 0.04 to 6.58 ± 0.17 μg g^{-1} DW due to octaploid induction (229% increase). Total chlorophyll and carotenoids increased by 28% and 32% in octaploid plants, respectively. The amount of soluble sugars also increased from 939 ± 18 in tetraploids to 2,820 ± 62 μg g-1 DW in octaploids (200% increase)

Anatomical and morphological properties

Octaploid induction had a significant effect ($P < 0.05$) on the enlargement of stomata and morphological traits. Internode length was the only parameter that was not significant ($P > 0.05$) among the measured parameters. Vegetative leaf length increased from 19.70

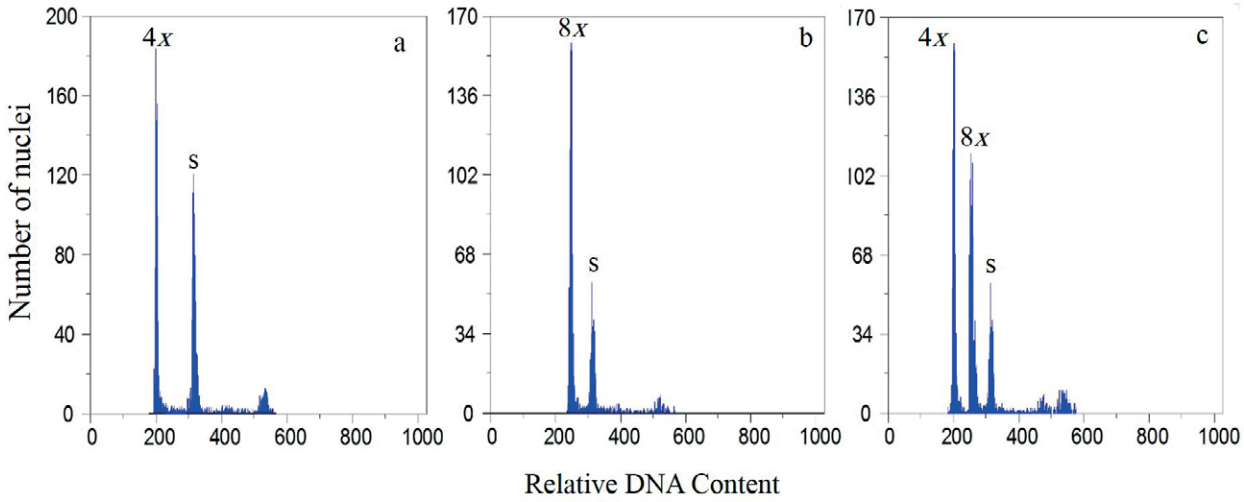

Figure 2. Flow cytometric histograms of nuclei isolated from in vitro-derived leaves of *S. mutica* tetraploid (a), octaploid (b), and mixoploid (c) plants. The right peaks (S) refer to the G1 of the standard maize reference plant (*Zea mays* CE-777, 2C DNA = 5.43 pg), the left peaks (4x, 8x) refer to the G1 of tetraploid and octaploid plants, respectively.

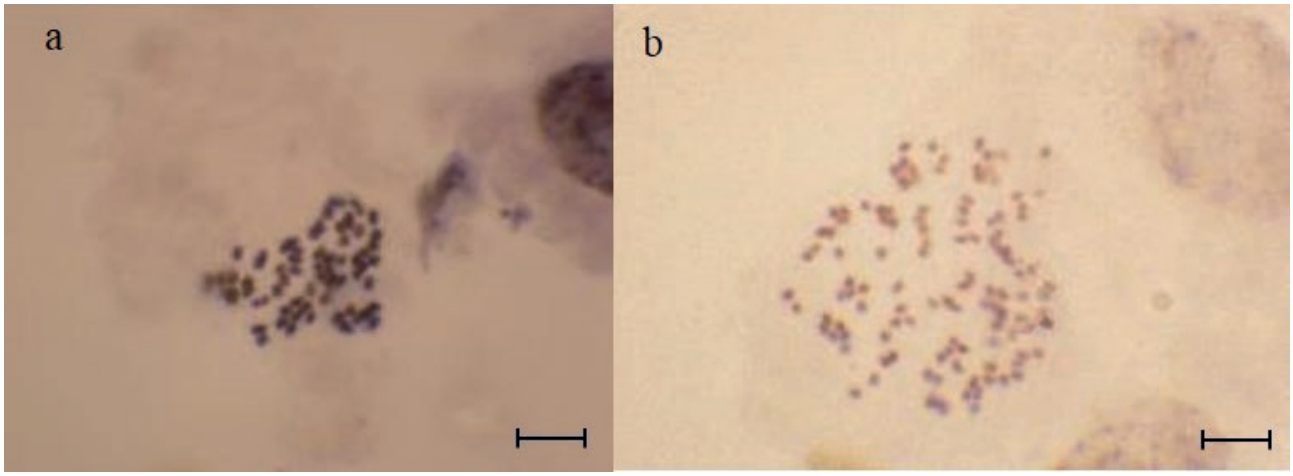

Figure 3. Chromosome numbers of *S. mutica*. Tetraploid control plant ($2n = 4x = 60$) (a) Octaploid plant ($2n = 8x = 120$) (b). Bars = 5 µm.

± 0.54 in tetraploids to 28.90 ± 0.60 mm in octaploids (46% increase), and leaf width increased from 3.96 ± 0.16 to 6.00 ± 0.16 mm. Leaf area was also 123% larger in octaploids ($P < 0.01$) (Table 3, Figure 4). Stem diameter increased from 0.97 ± 0.04 in tetraploids to 2.18 ± 0.05 in octaploids (124% increase) (Figure 5). An average of 50 stomata data was used to compare anatomical traits at two ploidy levels. The length of abaxial and adaxial stomata in octaploid plants increased by 62% and 58%, respectively.

The width of the abaxial and adaxial stomata also increased by 32 and 31%. The area of abaxial and adaxial stomata increased from 48.30 ± 2.74 and 69.24 ± 3.69 mm^2 in tetraploids to 97.60 ± 4.63 and 145.73 ± 8.18 mm^2 in octaploids, respectively. Therefore, the area of the abaxial and adaxial were 102 and 110% bigger (Figure 7). While the density of the abaxial and adaxial stomata decreased from 82.00 ± 1.26 and 68.25 ± 1.27 in tetraploids to 46.20 ± 1.36 and 37.40 ± 1.63 in octaploids. That means the number of stomata in the abaxial and adaxial stomata decreased by 44% and 46%, respectively ($P < 0.01$) (Table 3)

There was a significant increase ($P < 0.01$) in all flower characteristics (Table 3, Figure 6). Floral leaf length and width, calyx length, corolla length, and width, filament, and style length increased by 124%,

Table 2. Mean genome size (2C DNA) in the tested *S. mutica* (n = 5).

Ploidy level	2*n*	2C DNA value (pg) ± SE	1C DNAvalue (pg)	Holoploid genome size (1C DNA, Mbp)	Monoploid genome size (1Cx DNA,Mbp)
4*x*	60	1.901± 0.01	0.95	929.10	464.55
8*x*	120	3.82± 0.02	1.91	1866.02	466.51

Table 3. Effects of induced polyploidy on physiological and anatomical characteristics of *Satureja mutica*. Reported values are mean ± S.E., P-values based on t-test for independent samples (n = 10).

Traits (unit)	Abbr.	4x	8x	$t_{(df=8)}$	P-value
Phenol (μg g-1 DW)	Phenol	20.34 ± 0.97	62.16 ± 1.90	-19.61	< 0.001
Flavonoid (μg g-1 DW)	Flavonoid	2.00 ± 0.04	6.58 ± 0.17	-26.82	< 0.001
Soluble sugar (μg g-1 FW)	Ssugar	939 ± 18.0	2,820 ± 62.2	-29.04	< 0.001
Oil yield (%)	OilY	3.88 ± 0.07	5.68 ± 0.14	-11.83	< 0.001
Total chlorophyll (mg g-1 F.W.)	Chl	1.76 ± 0.03	2.27 ± 0.04	-9.70	< 0.001
Carotenoid (mg g-1 F.W.)	Car	14.02 ± 0.26	18.58 ± 0.59	-7.01	< 0.001
Stem leaf length (mm)	SLL	19.70 ± 0.54	28.90 ± 0.60	-11.41	< 0.001
Stem leaf width (mm)	SLW	3.96 ± 0.16	6.00 ± 0.16	-8.89	< 0.001
Leaf area ($mm^{2)}$	LA	38.80 ± 3.25	86.65 ± 2.45	-11.76	< 0.001
Internode length (mm)	InL	17.60 ± 1.44	21.00 ± 0.71	-2.13	0.07^{ns}
Stem diameter (mm)	SD	0.97 ± 0.04	2.18 ± 0.05	-19.14	< 0.001
Adaxial stomata length (μm)	AdSL	13.42 ± 0.35	21.30 ± 0.87	-8.38	< 0.001
Adaxial stomata width (μm)	AdSW	9.46 ± 0.42	12.44 ± 0.86	-3.10	< 0.05
Adaxial stomata area (μm^2)	AdSA	69.24 ± 3.69	145.73 ± 8.18	-8.52	< 0.001
Adaxial guard cell density	AdGCD	68.25 ± 1.27	37.40 ± 1.63	14.93	< 0.001
Abaxial stomata length (μm)	AbSL	11.20 ± 0.28	18.22 ± 0.73	-8.96	< 0.001
Abaxial stomata width (μm)	AbSW	7.94 ± 0.19	10.44 ± 0.28	-7.46	< 0.001
Abaxial stomata area (μm^2)	AbSA	48.30 ± 2.74	97.60 ± 4.63	-9.16	< 0.001
Abaxial guard cell density (no.)	AbGCD	82.00 ± 1.26	46.20 ± 1.36	19.30	< 0.001
Floral leaf length (mm)	FLL	17.80 ± 0.80	40.00 ± 1.05	-16.83	< 0.001
Floral leaf width (mm)	FLW	3.90 ± 0.09	7.02 ± 0.08	-26.00	< 0.001
Calyx length (mm)	CL	5.52 ± 0.17	6.36 ± 0.12	-4.10	< 0.01
Calyx lower teeth length (mm)	CLTL	2.66 ± 0.05	3.68 ± 0.07	-11.40	< 0.001
Calyx upper teeth length (mm)	CUTL	1.94 ± 0.04	2.79 ± 0.05	-13.12	< 0.001
Peduncle length 1 (mm)	PL1	1.82 ± 0.03	2.42 ± 0.03	-16.64	< 0.001
Peduncle length 2 (mm)	PL2	2.21 ± 0.03	3.05 ± 0.07	-10.98	< 0.001
Corolla length (mm)	CoL	44.60 ± 1.03	68.60 ± 1.03	-16.48	< 0.001
Corolla width (mm)	CoW	11.80 ± 0.46	16.80 ± 0.25	-9.45	< 0.001
Filament length (mm)	FL	8.34 ± 0.20	13.62 ± 0.24	-17.22	< 0.001
Style length (mm)	StL	16.00 ± 0.29	25.16 ± 0.40	-18.41	< 0.001

80%, 15%, 53%, 42%, 63%, and 57% in the induced octaploids, respectively (Table 3).

Phytochemical traits

The essential oil yield in tetraploid and induced octaploid plants was 3.88 ± 0.07 and 5.68 ± 0.14%. As a result, octaploid induction increased the essential oil yield by 49%. Identification of compounds in essential oils using GC and GC-MS devices led to the identification of 14 compounds, representing more than 99% of the oil (Table 4; Figure 8).

Table 4 shows the essential oil components of tetraploid and octaploid plants. The compounds are listed in order of their elution on the DB-5 column. In octaploid

Figure 4. Comparison of the morphology between tetraploid (a) and octaploid (b) *S. mutica*. Bars = 5 mm.

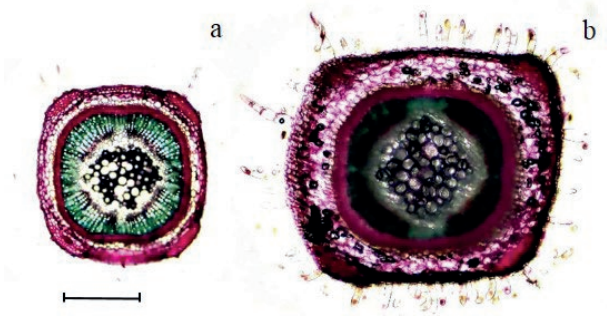

Figure 5. Comparison of the stem diameters between tetraploid (a) and octaploid (b) *S. mutica*, Tetraploid plant. Bars = 500μm.

plants several compounds such as α-thujene, α –pinene, sabinene, α –terpinene, ρ-cymene, limonene, 1,8-cineol, γ-terpinene, Methyl ether thymol increased but terpinolene, thymol, carvacrol, e-caryophyllene, germacrene D decreased (Table 4). The major components were p-cymene (5.9, and 17.7%), γ-terpinene (10.7, and 14.9%), thymol (47.7, and 29.2%) carvacrol (24.8, and 22.5%), in tetraploid and octaploid plants, respectively.

DISCUSSION

The purpose of polyploidy induction in medicinal plants with economic value, including *S. mutica*, is to improve the quantity and quality of essential oils and increase plant yield. In this study, successful octaploid induction was performed using a specific concentration and duration of colchicine. Colchicine is the most widely used chemical that is a mitotic spindle inhibitor and used for polyploidy induction (Tsai et al., 2021). Concentrations of 0.005–0.5% colchicine and durations of 6 h to 6 d are traditionally used for chromosomes duplication (Ahmadi and Ebrahimzadeh, 2020).

Researchers have shown that colchicine tolerance thresholds are not the same in different plant species and colchicine concentrations do not have the same effects on polyploidy induction in different plants. For example, the highest tetraploid induction efficiency (33%) in *Papaver bracteatum* Lindl, was obtained at a concentration of 0.05% colchicine with a duration of 24 hours (Tarkesh Esfahani et al., 2020). The highest polyploidy induction efficiency in *Rhododendron fortunei* Lindl (36.6 %), *Sophora tonkinensis* Gapnep (23.3%), *Thymus persicus* (26%) was obtained at a concentration of 0.1, 0.2, and 0.3% colchicine after 24, 30, and 12 h respectively (Tavan et al., 2015; Wei et al., 2018;

Figure 6. Comparison of the flower morphology between tetraploid (4*x*) and octaploid (8*x*) *S. mutica*. Bars = 5 mm.

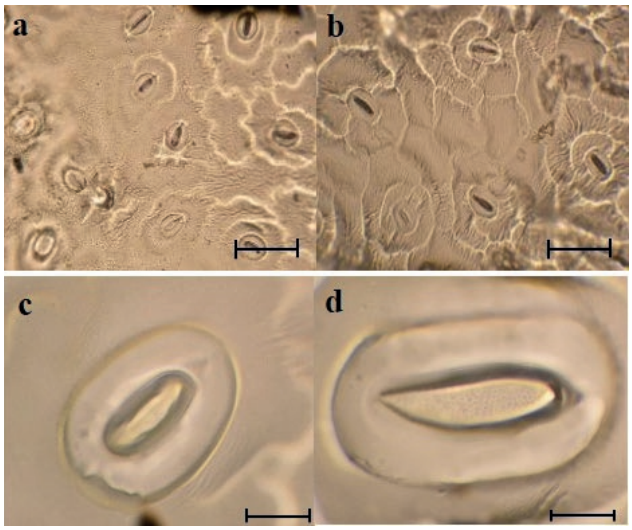

Figure 7. Abaxial stomata in leaves of *S. mutica*; stomatal density in tetraploid (a) and octaploid plant (b) (Bars = 50 μm); stomatal size in tetraploid (c) and octaploid plant (d) (Bars = 5 μm).

Table 4. Comparison between tetraploid and octaploid components of essential oils resulted by GC, and GC-MS.

Component	RI	Tetraploid	Octaploid
α-Thujene	929	0.4	1.8
α -Pinene	938	-	0.9
Sabinene	977	0.8	1.0
α -Terpinene	1032	1.6	2.8
ρ-Cymene	1044	5.9	17.7
Limonene	1046	0.2	0.5
1,8-Cineol	1050	-	0.2
γ-Terpinene	1075	10.7	14.9
Terpinolene	1090	1.1	0.6
Methyl ether thymol	1258	1.3	3.4
Thymol	1315	47.7	29.2
Carvacrol	1324	24.8	22.5
E-caryophyllene	1448	2.5	1.6
Germacrene D	1522	1.9	1.1

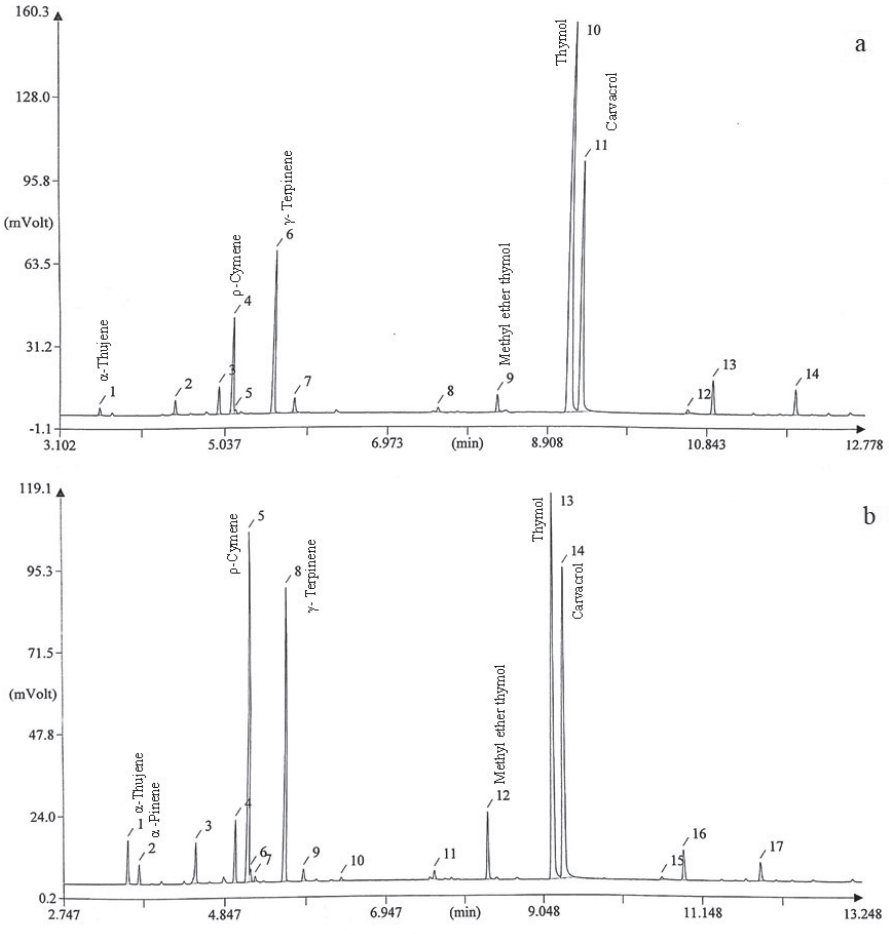

Figure 8. GC-MS chromatogram of tetraploid (a) and induced octaploid (b) of *Satureja mutica.*

Mo et al., 2020). In the present study, the highest polyploidy induction efficiency (32%) was obtained by 0.05% colchicine after 6 h. Prolonged exposure to colchicine decreased seedling vigor and polyploidy induction efficiency, but did not affect seedling viability.

Flow cytometry results showed that the genome size in *S. mutica* octaploid plants doubled. Several reports indicate a direct relationship between genome size and ploidy level. For example, in annual ryegrass (*Lolium multiflorum* Lamarck), tetraploid induction doubled genome size from 6.13 ± 0.36 in diploids to 12.30 ± 0.83 pg in tetraploid (Rios et al., 2015). Another study on polyploidy induction of two species of *Acacia* (*Acacia dealbata* Link. and *Acacia mangium* Willd.) showed that by increasing the ploidy level from diploid to triploid and tetraploid, the size of the genome increases by about 50%, respectively. Thus, the genome size of tetraploids is almost twice as large as diploids (Blakesley et al., 2002). Genome size in *D. rotundifolia* and *D. anglica* with three levels of diploid, tetraploid, and octaploid were 2.73, 5.34, and 11.12 pg, respectively, indicating a direct relationship between ploidy level and genome size (Rauf et al., 2021). However, there are reports that the size of the genome does not increase directly as the ploidy level increases. As an example, one study found that tetraploid species of *Rhododendron* had 2C DNA = 1.3-1.5 pg, but hexaploid species had 2CDNA = 4.27 pg (Kumar De et al. 2010).

In the present study, one of the consequences of octaploid induction was a significant reduction ($P < 0.01$) in the number of stomata per unit area. Stomatal density is not affected by external factors such as temperature and water content of tissues, so stomatal counting is a convenient and easy method that can be used to determine the ploidy level in a species (Silva et al., 2000). According to several studies, including Nianmaohuangqin (*Radix Scutellariae* Viscidulae) (Huang et al., 2014) and Stevia (*Stevia rebaudiana* Bertoni) (Zhang et al., 2018), stomatal size and density have been used as markers to differentiate polyploid seedlings and their control genotypes. However, stomatal size and density are not reliable factors in identifying chimer samples. As a result of polyploidy, plants are generally bigger and have thicker, darker leaves (Huang et al., 2014).

According to a study of rose chromosome duplication, polyploid roses possess a longer stem, more living pollen, and more leaflets with a greater width-to-length ratio (Kermani et al., 2003). In another study, polyploid lily plants also had larger leaves, roots, and stomata, but fewer stomata than diploid plants (Fang et al., 2009). An increase in leaf size and leaf area leads to an increase in biomass and yield. In medicinal plants, where leaves, stems, and flowers are all sources of active ingredients, biomass is an important characteristic. (Hannweg et al., 2016)

In the present study, Calyx length, Calyx lower and upper teeth length, Floral leaf length, and width, filament, and style length, corolla length, and width were significantly longer ($P < 0.01$) in octaploid plants. The results are compatible with those reported by other researchers for the African violet (Teixeira da Silva et al., 2017) and the chamomile (Majdi et al., 2010). Even though the overall flower size in tetraploid plants increased significantly in African violets, the inflorescence length and the number of petal buds per inflorescence decreased significantly which differs from our finding in this regard. Several reports confirm the increase in flower size and number per stem (Tulay and Unal, 2010), and some confirm the lengthening of the inflorescence stem (Takamura and Miyajima, 1996).

In addition to morphological characteristics, physiological traits including the number of plant pigments, soluble sugars, phenols, and flavonoids were also affected by octaploid induction in this study. The purpose of polyploidy induction in medicinal plants is to increase the yield of active ingredients. For example, tetraploid induction in *Dendrobium* hybrid increased the amount of shikunidine (Grosso et al., 2018), in *Bletilla striata*, superior phenolic and polysaccharide compounds (Li et al., 2018), in *Stevia rebaudiana* over accumulated stevioside (Hegde et al., 2015), in *Scutellaria baicalensis* higher baicalin (Gao et al., 2002), in *Dendrobium officinale* richer polysaccharides (Song et al., 2016) and in *Salvia officinalis* L. increased flavonoids, total phenol, and antioxidants such as polyphenol oxidase, catalase, and peroxidase (Hassanzadeh et al., 2020).

Secondary compounds increase in polyploid plants as cells multiply, leaves thicken, and roots develop (Hegde et al., 2015). Increasing gene expression can also result from the duplication of chromosomes, which leads to an increase in secondary compounds (Majdi et al., 2010). According to a study of *Arabidopsis thaliana*, increasing ploidy levels reduced cellulose and lignin in the cell wall, increasing saccharification function (Corneillie et al., 2019). Tetraploid induction in *Thymus vulgaris* L demonstrated that tetraploid and diploid plants contain similar levels of total terpenes but differ in the proportion of each terpene. Thus, the terpene ratios for the five compounds were higher, indicating that polyploidy induction had altered the quality of the essential oil (Navrátilová et al., 2021). There was an increase in fenchone content in *Tetradenia riparia* tetraploids. Furthermore, tetraploids contained several compounds (alpha-humulene, viridifloral, and alpha-terpinene) that were not present in diploids.

CONCLUSION

The present study is the first report of octaploid induction using colchicine in *S. mutica*. As *S. mutica* is tetraploid, it was expected that octoploid would not improve many traits, yet to our surprise, a plant with much higher potential than the original was produced, which was superior in terms of morphological, physiological, and phytochemical traits. Accordingly, it can be concluded that in the savory genus, doubling the chromosomes regardless of ploidy has a positive effect. It is evident that essential oils and extracts of octaploid plants have become more bioactive based on increases of 46% and 205% in essential oil and phenolic content, respectively. There is also a significant improvement in the performance of its major components, including α-thujene, α –pinene, sabinene, α –terpinene, ρ-cymene, limonene, 1,8-cineol, γ-terpinene, methyl ether thymol.

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