

INDUCTION OF AUTOTETRAPLOIDY IN DRAGONHEAD (*Dracocephalum moldavica* L.) BY COLCHICINE TREATMENT

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A B S T R A C T

The genome doubling agent colchicine was used effectively to obtain tetraploid plants in dragonhead. Treatment of apical meristem of seedlings was carried out in two stages. The first stage was when the cotyledon leaves emerged. The second stage was when the two true leaves emerged. Six levels of colchicine concentrations: 0, 0.05, 0.1, 0.2, 0.5, and 0.75% were applied in each of these stages. Seedling treatment in the stage of emergence of two true leaves with 0.1% colchicine solution proved to be the most effective in producing autotetraploids. Morphological, cytological and flow cytometry analyses showed the increase of chromosome numbers from $2n=2x=10$ to $2n=4x=20$. The increase of ploidy levels caused major changes in some morphological and physiological traits and active substances in dragonhead.

Key words: dragonhead, colchicine, tetraploidy, flow cytometry, chromosome counting

INTRODUCTION

Dragonhead (*Dracocephalum moldavica* L.) is an annual, herbaceous plant belonging to the *Lamiaceae* family (Omidbaigi, 2005) with chromosome number $2n=2x=10$ (Yan et al., 2000; Zhang, 1994). It originated in southern Siberia and off the slopes of

the Himalayas. The active substances of the vegetable organs of this plant have medicinal properties and are tranquillizing and appetizing. Its essential oil has antioxidant activity (Dastmalchi et al., 2005), antiseptic and antibacterial properties and is used for stomachache and bloat (Omidbaigi, 2005).

Within the last 30 years, the world has experienced an increased trend towards healthy diet and natural products. This has led to a growing demand for medicinal and aromatic plants (Gabler, 2002).

Together with requirements for safety, efficacy and stability of medicinal and aromatic plant products, the need for high quality raw materials is increasing. Breeding procedures for these plants is helping to spread and satisfy the demand for such materials (Bernath, 2002). The induction of artificial polyploidy may prove useful in increasing the quality and quantity of important medicinal compounds (Dhawan and Lavania, 1996).

Autopolyploidy can be induced by environmental factors and chemicals, and efficient techniques are required for high doubling rates. The most widely applied and best studied chemical inducing polyploidy is colchicine, an alkaloid extracted from seeds or corms of the autumn crocus (*Colchicum autumnale* L.). Colomid is its synthetic equivalent which is also often used. Colchicine disrupts mitosis by binding to tubulin, the protein subunit of microtubules, inhibiting the formation of microtubules and the polar migration of chromosomes. The result is a cell with double the chromosome number (Tambong et al., 1998). Colchicine was used for chromosome doubling of many crops including chickpea (*Cicer arietinum* L.) (Pundir et al., 1983), henbane (*Hyoscyamus niger* L.) (Lavania and Srivastava, 1991), hops (*Humulus lupulus* L.) (Roy et al., 2001), ginger (*Zingiber officinale* Roscoe) (Adaniya and Shirai, 2001),

tarragon (*Artemisia annua* L.) (Gonzalez and Weathers, 2003) and feverfew (*Tanacetum parthenium* L.) (Saharkhiz, 2007). There are several target tissues for colchicine treatment, such as meristems and seeds (Tamura et al., 1996).

In the present study an attempt was made to induce autotetraploidy in dragonhead using colchicine with the objective of creating more genetic variability. The derived tetraploid plants were traced by studying stomata characteristics, flow cytometric profiles and chromosome counting in root tips

MATERIAL AND METHODS

Plant materials

Seeds of *Dracocephalum moldavica* cv. SZK-1 provided from the Medicinal and Aromatic Plants Department of Corvinus University in Budapest, Hungary were used in this study. This is a diploid cultivar and its chromosome number is 10 ($2n=2x=10$). The flowers are violet and a thousand seed mass is equal to 1.29 g.

Experiments were carried out in 2007 in the greenhouse of the College of Agriculture, Tarbiat Modares University, Teheran, Iran.

Seedling treatment

Seeds were sown in a mixture containing soil, leaf mold and sand (1:1:2) in small plastic pots, under greenhouse conditions. The treatments were done at two stages of plant development: 1) at the emergence of cotyledon leaves and 2) at the emergence of two true leaves. One drop of aqueous solution of colchi-

cine was applied to the apex of seedlings for 3 successive days. Six levels of colchicine concentrations: 0, 0.05, 0.1, 0.2, 0.5 and 0.75% were applied at each of these stages. The solutions were supplemented with 2-4 drops of DMSO (dimethyl sulfoxide) and Tween 20 as a surfactant to facilitate better penetration of colchicine to plant cells. After the treatments the pots were placed in the greenhouse under natural day length. Average day and night temperatures in the green-house were 26 °C and 18 °C, respectively. The relative humidity varied between 50-55%.

At the 4th or 6th leaf stage, the plants were checked for the presence of different morphological characteristics. Some plants of the second treatment showed different appearances, compared to other seedlings of the same variant and to control plants (diploids). These seedlings often had 2 or 3 apical growth points. The leaves of the seedlings were thicker, dark green and deformed. These different plants were selected and sampled for stomata characteristics. Screening of mentioned characteristics (primary selection) revealed that some plants were presumably polyploids with higher ploidy levels; thus they were selected for flow cytometry analysis. The chromosome number of the starting material and tetraploid plant was determined in root tips of germinated seeds.

Stomata characteristics

In this investigation, the nail varnish technique was used to study stomata morphology. Three well

expanded leaves of each plant were removed from both control and treated plants. Clear fingernail polish was applied to the abaxial side of the leaf for a 1 × 1 cm square. After the polish was dried, it was removed with a pair of fine tip forceps. The polish strips were mounted on a microscope slide and then evaluated for the density and size of leaf stomata and stomatal guard cells under the light microscope (Olympus BX50) at 40X and 100X magnification.

Flow cytometric analysis

Cell nuclei were isolated from young leaves of the control and putative tetraploid plants. Approximately 1 cm² of leaf tissue from each sample and of parsley (*Petroselinum crispum* cv. Champion Moss Curled, (2C DNA content = 4.46 pg) (Yokoya et al., 2000) plants, used as an internal standard, were simultaneously chopped in a Petri dish with a sharp razor blade in 400 µL nuclei extraction buffer (CyStain UV Precise Partec GmbH-Munster, Germany). PVP-40 (polyvinylpyrrolidone-40) was added to the buffer to remove phenolic impurities. Nuclei suspensions were filtered through a 50 µm nylon mesh to remove large tissue debris. Staining solution (1600 µL), containing DAPI (4', 6 -diamidino-2-phenylindole), was added to the filtered suspensions. After 5 min nuclei were analyzed using a Partec-PA-I flow cytometer (Partec GmbH-Munster, Germany) equipped with a HBO-lamp and UV-laser. To estimate ploidy level, the peak position of the sample on a histogram was compared to that of a standard plant with

a known ploidy level. Each sample was analyzed 2-4 times.

Chromosome counting

Root tip meristems (5 mm in size) were obtained from germinated seeds and pre-treated in a saturated solution of α -bromonaphthalene for 3 h at 4 °C prior to fixing in Lewitsky's solution [(chromic acid : formaldehyde, 1:1 (v/v)] for 24 h at 4 °C. Hydrolysis was done in 1 N NaOH for 10 min at 60 °C and staining using Aceto-Iron-Hematoxylin for 16 h at 30-32 °C. Stained root tips were immersed in cytase enzyme to remove cell wall and organelles for 30-60 min at 30 °C. About 1 mm of root tips were excised, destained in a drop of 45% acetic acid, briefly flamed, and the cells spread by applying uniform pressure on the cover slip. The squashes were sealed with nail polish and examined under the light Olympus microscope at 100X under oil. The number of chromosomes was recorded for 2-4 roots taken from 10 diploid plants and 10 tetraploid plants.

Comparison of selected traits of diploid and tetraploid plants

After the ploidy levels of treated plants were identified using the above methods, some characteristics of 10 diploid parents were compared to 10 tetraploid plants. These characteristics included height of plant, leaf area (measured with a leaf area meter, Delta T. Image Devices), fresh and dry mass of plants, size of seeds and 1000 seed mass, essential

oil content and composition, were evaluated.

Isolation of essential oil

Shade dried aerial parts of both the control and tetraploid *Dracocephalum moldavica* L. (40 g samples in three replications) were subjected to hydro distillation in 600 ml of water for 3 h. An all glass Clevenger-type apparatus was used to extract oil according to the method recommended by the European Pharmacopoeia. The extracted oil was dried over anhydrous sodium sulphate and stored in a sealed vial at a low temperature before analysis.

Essential oil analyses

Essential oil analyses were performed by GC (gas chromatography) and GC/MS (gas chromatography/mass spectrometry). GC analysis was performed using a Shimadzu GC-9A gas chromatograph equipped with a DB-5 fused silica column. A GC/MS analysis was done using a Hewlett-Packard gas chromatograph HP 5973 Series II equipped with a DB-5 fused silica capillary column.

Statistical analysis

The experiment was arranged in a completely randomized design with 6 treatments and three replicates. Statistical analyses were carried out with the SPSS 10.0 for Windows software package (Statistica). Means were compared using Duncan's multiple range test and Student's t-test at $P > 0.01$.

RESULTS AND DISCUSSION

Results showed that dragonhead seedlings at the stage of the emergence of cotyledon leaves were very sensitive to colchicine treatment, which caused phytotoxicity and damping off. Most of the seedlings gradually died within 15 days. Similar results were obtained in treatment of chickpea seedlings at the stage of emergence of cotyledon leaves (Pundir et al., 1983; Saharkhiz, 2007). So, this stage was not suitable as an efficient and proper stage for treating dragonhead seedlings with colchicine.

As opposite, treatment of tip meristems at the stage of the emergence of two true leaves had remarkable results, as after treatment, many of the treated plants were alive and grew. Variations were also observed in characteristics of treated plants (Fig. 1).

Results of studying stomata morphology and using flow cytometry profiles indicated that the application of colchicine induced tetraploidy in seedlings.

Stomata characteristics were important indicators for the detection of new ploidy levels in dragonhead. Diploid plants had stomata and stomata guard cells with smaller diameter and smaller length of than tetraploid plants (Tab. 2, Fig. 2). Similar results were reported on hops (Roy et al., 2001), jujube (*Zizyphus jujuba* Mill.) (Gu et al., 2005) and feverfew (Saharkhiz, 2007).

Flow cytometry analysis of the selected plants clearly revealed the existence of three groups of ploidy levels:

1. The group of plants which presented a ploidy level very similar to the control (diploids).
2. A group of individuals with ploidy level higher by about two times compared to the control. This group was considered as plants with doubled chromosome number (tetraploids) (Fig. 3A).
3. The group of plants with two ploidy levels (diploid and tetraploid) in the same tissue, which means polyploidisation has not occurred in all cells of the treated tissues. These plants were classified as mixoploids (Fig. 3B). Only a few plants were found as mixoploids.

Flow cytometry was a helpful method for the determination of ploidy levels. It was convenient and rapid and therefore it is recommended for identifying ploidy levels in the plant breeding of polyploid *Dracocephalum* plants. An important advantage that flow cytometry has over other methods is its ability to identify mixoploids.

Polyploidisation efficiency (E) was calculated according to the following equation:

$$E (\%) = \text{doubled plants determined by flow cytometry (\%)} \times \text{survival rate (\%)}$$

The efficiency has a maximum of 100%, i.e. when all treated seedlings have a double chromosome number and all have survived. Polyploidisation efficiency is based on the number of double plants and also on the survival rate (Ajalin et al., 2002).

In this study, the best doubling efficiencies of the apex treatment were obtained with the colchicine at 0.1% concentration (Tab. 1).

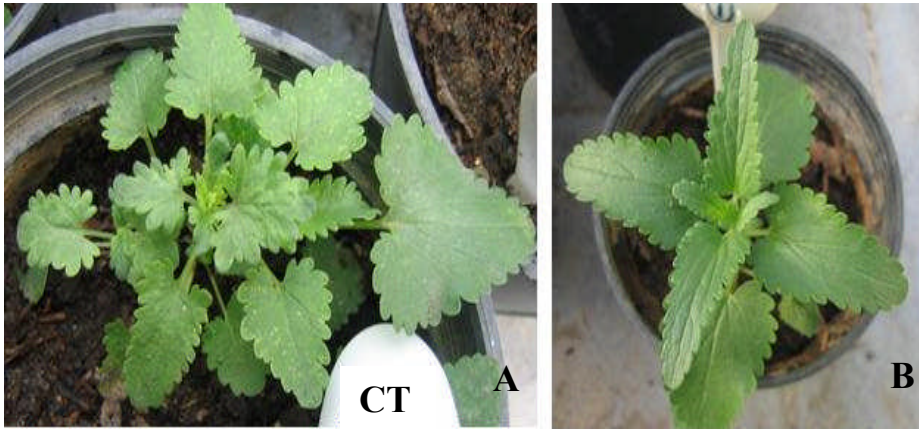


Figure 1. Seedling of dragonhead treated with colchicines (A) and untreated control (B)

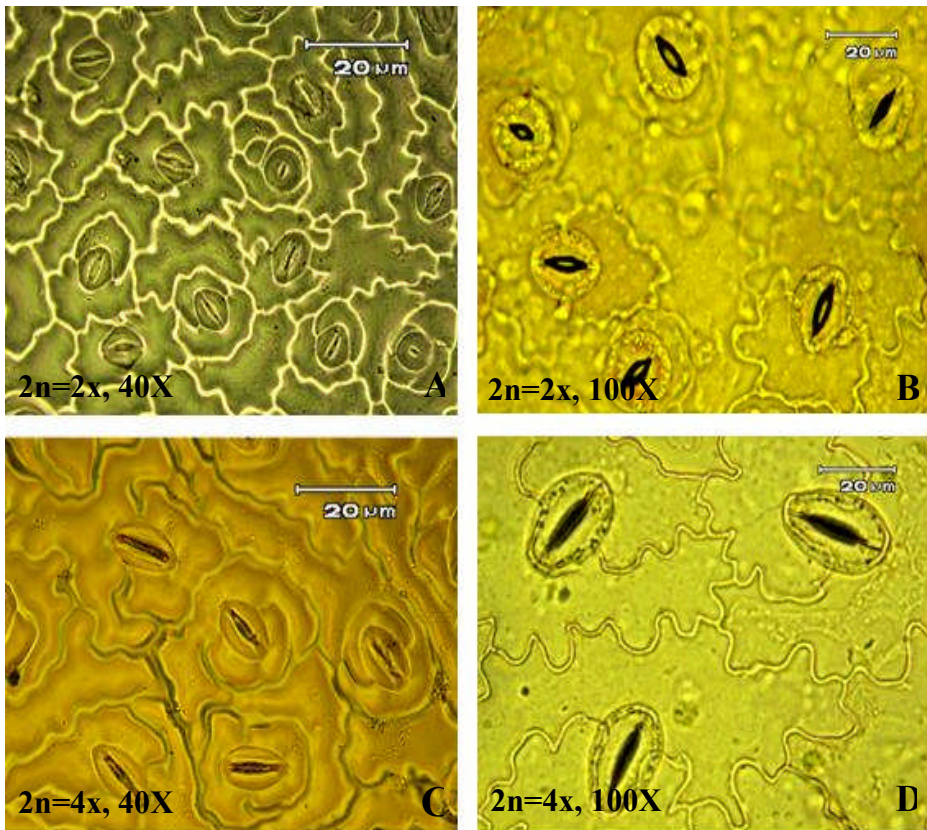


Figure 2. Comparison of density and size of leaf stomata and stomatal guard cells of diploid (A, B) and tetraploid (C, D) dragonhead plants

Induction of autotetraploidy (*Dracocephalum moldavica* L.) by colchicine

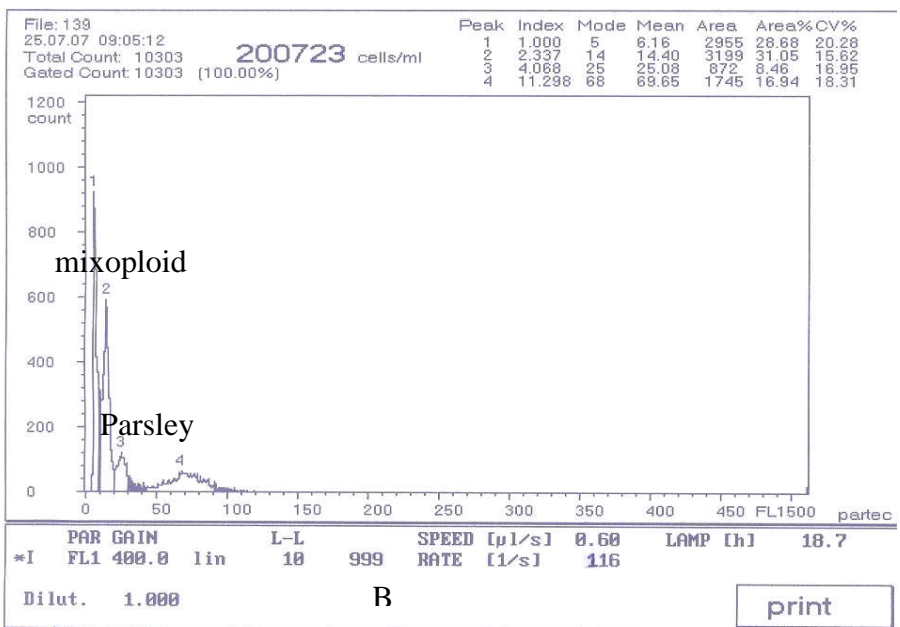
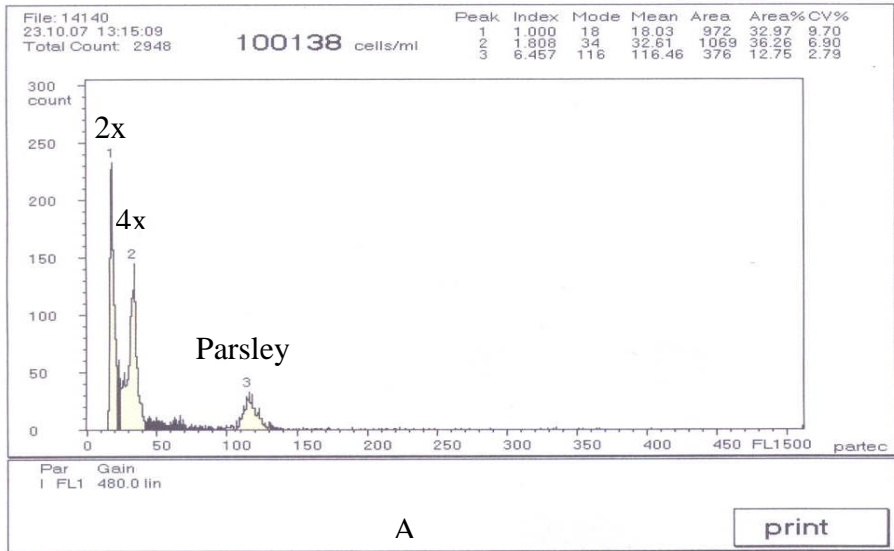


Figure 3. Histogram of ploidy level obtained by flow cytometry after simultaneous analysis of *Petroselinum crispum* cv. Champion Moss Curled (internal standard) and a mixed sample containing diploid and tetraploid *Dracocephalum moldavica* cv. SZK-1. (A) and mixoploid plant (peak 2, 3) (B)

Table 1. Effects of different concentrations of colchicine on polyploidisation efficiency in dragonhead after treatment of apical meristem at the stage of emergence of two true leaves

Colchicine concentration [%]	Survival rate [%]	Plants obtained by FCM [%]	Efficiency of polyploidisation [%]
0	82	0	0
0.05	44	4	1.76
0.1	45	16	7.2
0.2	47	6	2.82
0.5	39	6	2.34
0.75	37	8	2.96

Table 2. The characteristic of diploid and tetraploid dragonhead plants

Characteristics	Diploid (mean)	Tetraploid (mean)
Stomata length [μm]	13.09 \pm 0.36 b*	19.97 \pm 1.21 a
Stomata width [μm]	6.07 \pm 0.258 b	7.97 \pm 0.258 a
Stomatal guard cell length [μm]	21.74 \pm 0.45 b	31.3 \pm 1.23 a
Density of stomata [no. mm^{-2}]	395.633 \pm 2.236 a	195.684 \pm 5.673 b
Plant height [cm]	36.50 \pm 1.68 a	29.50 \pm 1.89 b
Leaf area [cm^2]	5.03 \pm 0.32 b	10.00 \pm 0.39 a
Fresh mass [g]	15.34 \pm 1.36 b	22.52 \pm 0.90 a
Dry mass [g]	5.15 \pm 0.76 b	8.50 \pm 0.38 a
1000 seed mass [g]	1.29 \pm 0.005 b	1.95 \pm 0.017 a
Essential oil content [%]	0.29 \pm 0.002 b	0.36 \pm 0.002 a

*Means followed by the different letters are significantly different at 1% level of probability using DMRT

Ploidy conversions from diploid ($2n=2x=10$) to tetraploid ($2n=4x=20$) were confirmed by chromosome counting in root tips (Fig. 4). Determination of chromosome number is difficult in dragonhead because of their small chromosome size.

Results of a comparison of the diploid parents' characteristics with tetraploid plants indicated that the increase of ploidy level in dragonhead caused a decrease in plant height (Fig. 5), an increase in leaf area, fresh and dry mass of plants, and size of seeds

(Fig. 6). Probably the increase of fresh and dry mass was due to an increase in the number of main plant stems to 2 or 3, increase of leaf area and change in quantity and quality of plants secondary metabolites. Many other researchers have reported that the increase of ploidy often causes anatomical and structural changes (Dhawan and Lavania, 1996; Adaniya and Shirai, 2001).

The weight of 1000 seeds was 1.29 g in controls and 1.95 g in derived tetraploids. Similarly, induction of autotetraploidy increased the size of

Induction of autotetraploidy (*Dracocephalum moldavica* L.) by colchicine

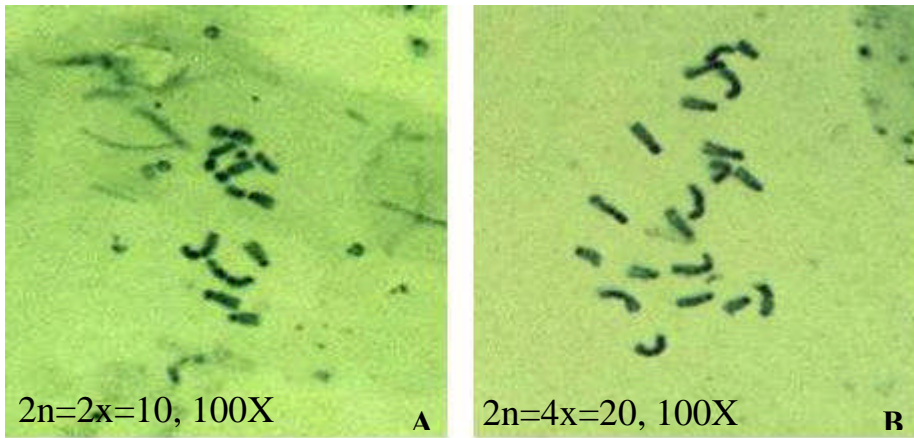


Figure 4. Comparison of chromosomes number in diploid (A) and tetraploid (B) dragonhead plants



Figure 5. Comparison of height and growth habit of diploid (left) and tetraploid plant (right) 60 days after treatment



Figure 6. Comparison of seed size of diploid (right) and tetraploid (left) plants

Table 3. Influence of ploidy level on relative content (%) of selected components of essential oil of *Dracocephalum moldavica*

Compounds	RI-DB5	Ploidy level	
		diploid (2x)	tetraploid (4x)
Z-citral	1223	14.10 ±0.057 b*	15.40 ±0.057 a
Geraniol	1249	16.30 ±0.057 b	19.50 ±0.057 a
Nerol	1241	5.80 ±0.057 a	5.50 ±0.057 b
E-citral	1255	9.90 ±0.057 a	9.10 ±0.057 b
Neryl acetate	1343	5.40 ±0.057 a	4.70 ±0.057 b
Geranyl acetate	1377	40.40 ±0.057 a	36.30 ±0.057 b

*Explanations: see Table 2

seeds and the 1000 seed mass in chickpea (Pundir et al., 1983) and caraway (*Carum carvi* L.) (Dijkestra and Speckmann, 1980). The effects of induction of autotetraploidy on some morphological and physiological characteristics in dragonhead are presented in Table 2.

The results showed that induction of autotetraploidy in dragonhead had a significant effect on content of essential oil. In this plant, the essential oil content of the vegetable organs increased by 27.5% in tetraploid plants. Dijkestra and Speckmann (1980)

reported that a ploidy level increase in caraway caused 35.6% increase of essential oil content. Similarly, induction of autotetraploidy in feverfew increased the content of essential oil up to 32% (Saharkhiz, 2007).

Induction of autotetraploidy had also effect on the essential oil compositions of dragonhead. The results of GC and GC/MS analyses showed that the essential oil of both diploid and tetraploid plants contained 30 constituents. An increase of ploidy level resulted in increase of the relative content of geraniol and Z-citral

increased but decrease of nerol, E-citral, neryl acetate and geranyl acetate contents (Tab. 3).

In conclusion, although the physiological effects of polyploidy are not generally predictable, and the responses are often species-specific, doubling the chromosome number of a plant increases the number of genes and thus changes enzymatic activity and isozyme diversity. This can affect the biosynthetic pathways of secondary metabolites. Induction of artificial autotetraploidy in many medicinal plants has often increased quantities of secondary metabolites and also altered them in a qualitative manner (Berteza et al., 2005; Dijkestra and Speckmann, 1980; Saharkhiz, 2007). The morphological features, high yielding potential and changed quality of essential oil of the tetraploid dragonhead suggest that induced polyploidy may have some application in plant improvement to enlarge diversity and yield potential.

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INDUKCJA AUTOTETRAPLOIDALNOŚCI U PSZCZELNIKA MOŁDAWSKIEGO (*Dracocephalum moldavica* L.) ZA POMOCĄ KOLCHICYNY

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S T R E S Z C Z E N I E

Kolchicyna, substancja powodująca podwojenie liczby chromosomów, została zastosowana do wytworzenia tetraploidalnych roślin pszczelnika mołdawskiego. Traktowanie merystemu apikalnego siewek przeprowadzono w dwóch fazach rozwojowych – pojawienia się liści zarodkowych oraz pojawienia się dwóch liści właściwych. W każdej z tych faz zastosowano sześć stężeń kolchicyny: 0; 0,05; 0,1; 0,2; 0,5 oraz 0,75%. Traktowanie w fazie dwóch liści właściwych przy stężeniu kolchicyny na poziomie 0,1% okazało się najbardziej efektywne dla otrzymania autotetraploidów. Analizy morfologiczne, cytologiczne oraz cytometria przepływową wykazały zwiększenie liczby chromosomów z $2n=2x=10$ do $2n=4x=20$. Wzrost poziomu ploidalności spowodował duże zmiany niektórych cech morfologicznych i fizjologicznych oraz w zawartości i składzie substancji aktywnych u pszczelnika mołdawskiego.

Słowa kluczowe: pszczelnik mołdawski, kolchicyna, cytometria przepływową, tetraploidalność, liczenie chromosomów