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Comparison of Four Different Treatment Methods with Colchicine to Induce Chromosome Doubling in Dragonhead (*Dracocephalum moldavica* L. 'SZK-1')

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ABSTRACT

In this investigation four different methods using aqueous solutions of colchicine were used to treat seed, roots and apical meristem in two stages – emergence of seed leaves (cotyledons) and the emergence of two true leaves – of dragonhead (*Dracocephalum moldavica* L.) to artificially induce autotetraploidy. Treatment of seedling apical meristems by applying the dropping method at the two-true-leaf stage of emergence was best as many of the treated plants developed and a diversity of characteristics was also observed in treated plants. Colchicine at 0.1% (w/v) most effectively produced autotetraploids, which were identified by flow cytometry.

Keywords: apical meristem, autotetraploidy, flow cytometry, root treatment, seed treatment

INTRODUCTION

Dragonhead (*Dracocephalum moldavica* L.) is an annual, herbaceous essential oil-bearing plant that belongs to the Lamiaceae family. This is a diploid plant with a chromosome number = 10 (2n=2x=10) (Yan *et al.* 2000; Yavari *et al*. 2009). This plant grows in the south of Siberia and the Himalayas. The essential oil of the vegetable organs is reported to have several therapeutic, including tranquilizing and appetizing (Borna-Nasrabadi *et al.* 2007), effects. Its active substances have antiseptic and antibacterial properties and are used for stomach-ache and bloat (Borna-Nasrabadi *et al.* 2007). The oil of dragonhead also has antioxidant activity (Dastmalchi *et al.* 2005).

Ploidy manipulation has been successfully used in plant breeding to induce diversity and to facilitate the production of superior cultivars in many plant species (Tambong *et al.* 1998). The induction of artificial polyploidy in medicinal and aromatic plants effects morphological and physiological characteristics, in many cases increasing the quantity and quality of important medicinal compounds. Polyploids are plants with multiple genomes. Chemicals such as the mitotic spindle inhibitor colchicine can be used to induce polyploidy, and has been successfully used to induce chromosome doubling in meristemic cells in many plants (Mensah *et al.* 2007; Saharkhiz 2007). There are different methods to induce polyploidy in plants such as the treatment of seed (Johnson *et al.* 2004; Quan *et al.* 2004), germinated seed (Urwin *et al*. 2007), flower buds (Wu *et al.* 2007), apical meristems (Lavania and Srivastava 1991; Hanzelka and Kobza 2001; Saharkhiz 2007; Yavari *et al.* 2009) and roots (Taira *et al*. 1991), using *in-vitro* tissue culture (Adaniya and Shirai 2001; Gu *et al*. 2005; Koutoulis *et al.* 2005), among others. The most effective treatment method and treatment duration, besides colchicine concentration, to induce polyploidy, are species-specific.

The main goal of this research was to compare the effectiveness of four different methods to induce autotetraploidy with colchicines, including treatment of seeds, roots and apical meristems (in two stages: emergence of seed leaves i.e. epicotyls, and of two true leaves).

MATERIALS AND METHODS

Plant materials and growth conditions

The seeds of dragonhead (*Dracocephalum moldavica* L. 'SZK-1') were provided by Dr. Reza Omidbaigi from the Department of Medicinal and Aromatic Plants of Corvinus University in Budapest, Hungary. This is a diploid cultivar and its chromosome number = 10 (2n=2x=10). Experiments were carried out in 2007 in the research greenhouse of the College of Agriculture, Tarbiat Modarres University, Tehran, Iran.

Seed treatment

The experiment was arranged in a completely randomized design (CRD) with 16 treatments and three replicates. Four colchicine concentrations (0, 0.05, 0.1 and 0.5%) were applied for 6, 12, 24 and 36 hrs. 0% colchicine solution used as control treatment. Colchicine was purchased from Sigma-Aldrich Co. Ripe seeds, 51 per treatment, were submerged in different concentrations of aqueous colchicine solutions. About 2-4 drops of dimethyl sulfoxide (DMSO) and Tween-20 as a surfactant were added to each solution to aid colchicine to penetrate the cells and glass containers holding these colchicine solutions and seeds were shaken at 25°C on a shaker at 105 rpm. After treatment, the seeds were thoroughly washed in running water and 5 seeds were sown in each plastic pot in depth of 2 to 3 cm. All pots were containing a mixture of 1:1:2 soil: decayed leaf mold: sand. The pots were placed in the greenhouse under natural conditions; the average day and night temperature was 26 and 18°C while the relative humidity varied between 50 and 55%. The seeds were irrigated as needed (Quan *et al.* 2004; Mensah *et al*. 2007; Saharkhiz 2007).

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Research Note

Root treatment

Seedlings at 12th or 15th leaf stage were removed from the soil and the roots were washed in running water. The roots of each plant were cut back to a 6-7 cm length and then immersed in beakers containing 0, 0.05, 0.1, 0.2, 0.5, 0.75, 1, 2.5, 5 and 7.5% colchicine solution, supplemented with 2-3 drops of DMSO and Tween-20. 0% colchicine solution used as control treatment. Treatments were conducted for 3, 6, 9, 12 and 24 hrs with three replicates each. The experiment was arranged in a CRD. After treatment, the roots were thoroughly washed in running water for 3 hrs. All plants were transplanted to pots containing a mixture of 1: 1: 2 soil: decayed leaf mold: sand and maintained under greenhouse conditions (Taira *et al.* 1991).

Seedling treatment

After seedlings emerged, apical meristems were treated with an aqueous solution of colchicine (containing 2-3 drops of DMSO and Tween-20) by applying a drop method at two stages: emergence of seed leaves i.e. epicotyls, and of two true leaves. One drop (about 5 µL) of colchicine aqueous solution was applied to the apex of each of seedlings every 3 days. The experiment was arranged in a CRD with 6 treatments and three replicates. 6 levels of colchicine concentrations including 0, 0.05, 0.1, 0.2, 0.5 and 0.75% were applied at each stage. 0% colchicine solution used as control treatment (Hanzelka and Kobza 2001; Saharkhiz 2007).

After treatment, in the 4th or 6th leaf stage, the plants were carefully checked to detect the presence of different morphological characteristics. Some plants (about 40%) of the treatment at stage of emergence of two true leaves were different in comparison with the other seedlings. Many of these seedlings had two or three apical growth points with thicker, greener and deformed leaves (**Fig. 1**). These different plants were selected for flow cytometric analysis in order to determine their ploidy levels (Urwin *et al*. 2007).

Flow cytometric analysis

Cell nuclei were isolated from young leaves of the diploid and probable tetraploid plants. Approximately 1 cm^2 of leaf tissue from each sample and 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 had been added to the buffer to remove phenolic impurities. Filtering nuclei suspensions through a 50 µm nylon mesh, fragments and large tissue debris were removed. Staining solution (1600 µL), containing 4′,6-diamidino-2-phenylindole (DAPI), was added to the filtered suspensions. After about 5 min, nuclei were analyzed using a Partec-PA-I flow cytometer (Partec Gmbh-Munster, Germany) equipped with a HBO-lamp and UV-laser flow apparatus and the precise analysis was repeated over each sample 2-4 times (Edmé *et al*. 2005; Saharkhiz 2007).

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

E-polyploidisation (%) = doubled plants obtained by flow cytometry (%) × survival rate (%) (Ajalin *et al*. 2002).

Statistical analysis

Statistical analysis was performed using SPSS (version 16.0) software with which gained means were compared using Duncan's new multiple range test (DNMRT) ($P < 0.01$).

RESULTS AND DISCUSSION

Seed treatment

Treatment of dragonhead seeds with colchicine is not the most appropriate method due since the plant in the germination stage is highly sensitive. A number of the treated seeds did not germinate (about 80%) while the remainder did; however, those that did died in the seedling cotyledon stage. Similar results were reported by Hanzelka and Kobza (2001) on *Callistephus chinensis* L., Quan *et al.* (2004) on *Arctium lappa* and Saharkhiz (2007) on *Tanacetum parthenium* L. These results are in contrast with those of Urwin *et al.* (2007) that reported the *Lavandula angustifolia* (lavender) seed germination in the presence of colchicine resulted in autotetraploid plants.

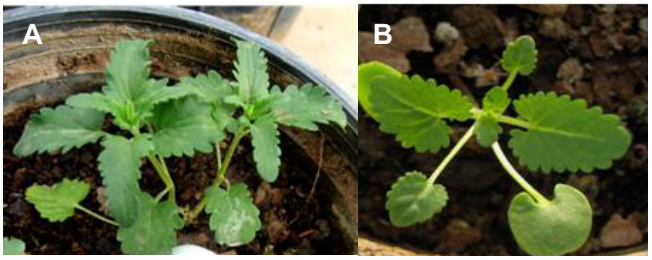


Fig. 1 Two apical growth points in tetraploid plants, in the stage of 6-8 true leaves (A); Untreated control (B).

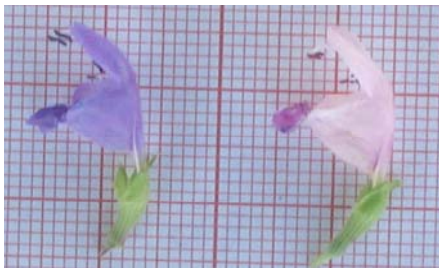


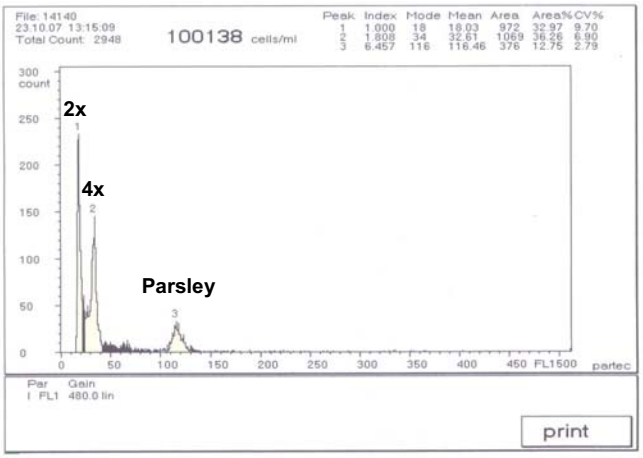
Fig. 2 Comparison of color of flowers in diploid plants (left) with some seedlings which treated with colchicine by root treatment method (right).

Fig. 3 Roots decline (A) and phytotoxicity (B) in seedlings which treated with colchicine in **the stage of emergence of seed leaves.**

Fig. 4 Tetraploid plant obtained of treatment of seedling with colchicine in the stage of emergence of two true leaves (A) or untreated control (B).

Root treatment

The root treatment method could not be used to effectively induce polyploidy in dragonhead. After treatment the plants began to grow normally and no tetraploid plants were recognized by flow cytometric analyses. The death of plants occurs with the increase in concentration or treatment period. Identical results were reported by Blakeslee and Avery (1937) on *Datura stramonium*, although a few plants



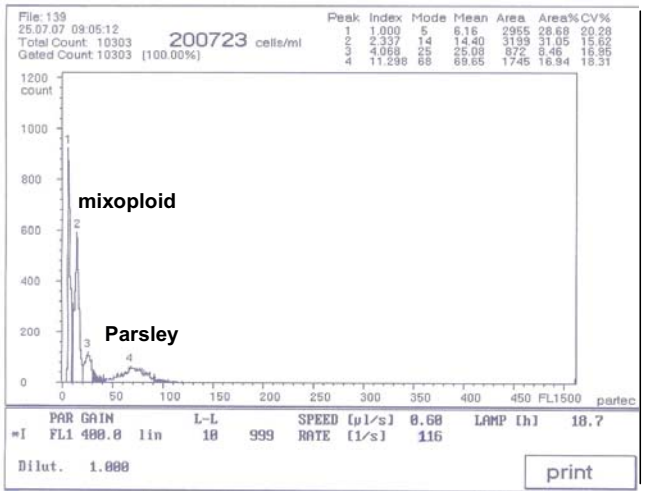


Fig. 5 Histogram of ploidy level obtained by flow cytometry. This was obtained after simultaneous analysis of *Petroselinum crispum* cv. 'Champion Moss Curled' (internal standard) and mixed sample containing diploid and tetraploid *Dracocephalum moldavica* L. 'SZK-1' (**A**) and mixoploid plant (peaks 2, 3) (**B**).

demonstrated variations in some characteristics compared with the control. For instance, unlike the control plants which had blue flowers, some of the treated plants had pink ones (**Fig. 2**) and also in some cases inflorescences emerged simultaneously. This obviously demonstrates that colchicine is a mutagenic agent (Mensah *et al*. 2007).

Seedling treatment

Dragonhead seedlings were exceedingly sensitive to colchicine at the stage of epicotyl emergence causing phytotoxicity as well as damping off. Therefore, most seedlings died gradually within 15 days (**Fig. 3**). A similar result was obtained when *Tanacetum parthenium* seedlings were treated at the same stage (Saharkhiz 2007). Therefore, this stage is not efficient or appropriate for treatment with colchicine.

The tip meristem method at the stage of emergence of two true leaves had desirable results: many of the colchicines-treated plants survived and developed. Flow cytometric profiles confirmed that colchicine induced tetraploidy in seedlings (**Fig. 4**), confirming that colchicine is both a polyploidising and mutagenic agent (Mensah *et al*. 2007).

Three groups of ploidy levels were identified in these analyses:

1. A group of individuals (55%) with a ploidy level very similar to control (diploids (2n=2x=10));

2. A group of individuals (40%) with a higher ploidy level approximately twice that of the control, i.e. double the chromosome number or tetraploids (2n=4x=20) (**Fig. 5**, top).

3. A group of individuals (5%) with two ploidy levels (diploid and tetraploid) in the same tissue. This implies that polyploidisation did not occur in all cells of the treated tissues. These plants were categorized as mixoploids (**Fig. 5**, bottom).

Table 1 Effect of different concentrations of colchicine on percentage of E-polyploidisation after treatment of apical meristem in the stage of emergence of two true leaves in dragonhead**.**

Treatment (%)[1]	Survival rate (%)	Tetraploid plants detected by FCM[2] (%)	E-polyploidisation (%)
0	82 a[3]	0 c	0 c
0.05	44 cd	4 bc	1.76 bc
0.1	45 bc	16 a	7.2 a
0.2	47 b	6 b	2.82 b
0.5	39 d	6 b	2.34 b
0.75	37 d	8 b	2.96 b

[1] 51 seedlings were used in each treatment
[2] Flow cytometry
[3] Means followed by the different letters are significantly different at 5% level of probability using DNMRT

In order to estimate ploidy level accurately, the peak position of the sample on a histogram was compared to that of standard plant, parsley, with known ploidy.

In this study, the best doubling rate involved the apex treatment with 0.1% colchicine resulting in a large percentage of polyploidisation efficiency (7.2%) (**Table 1**).

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