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Artificial Autotetraploidy Induction Possibility of Two Iranian Endemic Mint (*Mentha mozaffarianii*) Ecotypes

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Abstract

The present study was conducted to polyploidy possibility induction of two Iranian endemic mint (*Mentha mozaffarianii*) ecotypes. For this purpose, three experiments were done. The first experiment was factorial, based on completely randomized design with three factors and three replications that rhizomes were used for treatment. The first factor including different colchicine concentrations (0, 0.025, 0.05, 0.1 and 0.2% that 0 as control). The second factor including two *Mentha* ecotypes (Ecotype A: Kamarej region and Ecotype B: Pirmohlat region) and the third factors consist of two soaking time (6 h and 12 h). In second experiment, apical meristem was treated. The factorial experiment based on randomized completely design with two factors and five replications. The factors including different colchicine concentrations (0, 0.035, 0.07, 0.15, 0.3 and 0.7%) and two ecotypes. In the third experiment, colchicine as combined with irrigation was applied. For this purpose, a factorial experiment in randomized complete design with two factors (colchicine concentrations: 0, 0.025, 0.012, 0.006% and two mint ecotypes) and three replications was conducted. At the end of all experiments, survival rate and tetraploidy percentage (by morphological change, stomata traits, flow cytometry and chromosome counts) were measured. The results showed that different treatment had significant effects on survival percent on all experiments and increasing of colchicine concentration caused decreasing plants survival. On the other hand, tetraploidy changes only in the first experiments were observed. Only in 0.025% colchicine treatment with 6 h soaking time on ecotype A, 12.66% tetraploidy was identified. Totally, it is seems that *Mentha mozaffarianii* hardly response to colchicine for tetraploidy induction.

Keywords: colchicine, flow cytometry, polyploidy, stomata cells

Introduction

Lamiaceae family consists of more than 4000 species in 200 genera. Many species within this family are medicinal plants that apply in human disease therapy as well as food in raw and cooked forms.

The genus *Mentha* consists of more than 25 species and are well known for monoterpenes like menthol, menthone, carvone and pulegone (Arumugam *et al*., 2006). *Mentha mozaffarianii* Jamzad is an endemic mint species from Iran. Six species and several subspecies of this genus are found in Iran, among which just Mentha mozaffarianii Jamzad is endemic (Mozaffarian, 2004; Mozaffarian, 2013). Distribution of this species is exclusive to Hormozgan province (South of Iran) and Fars province (Southwest of Iran). Natural habitat of this species in Hormozgan province were placed in highland locations, Altitude domain 500-1000 m and south gradient (20-50%) including Siyahu, Qotbabad, Sikhouran and Dam Tang regions (Soltanipour, 2004). In Iran, this plant has been used for treating digestive disorders, headache, emphysema and bellyache (Soltanipour, 2004; Mozaffarian, 2013). Chromosome counts for this genus suggest a basic number of x= 12 with a range of numbers from diploid (2n=24 in *Mentha longifolia* or *Mentha spicata*) to octaploid (2n=96 in *Mentha aquatica*) (Gobert *et al*., 2002).

Ploidy manipulation is considered as a valuable tool in genetic improvement of many plants (Madon *et al*., 2005). Polyploidy often generates variants that may possess useful characteristic and by doubling the gene products, polyploids also provide a wider germplasm base for breeding studies (Thao *et al*., 2003).

Artificial polyploidy induction may establish valuable 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.) (Tambong *et al*., 1998). In the most plants, artificial polyploidy is often accompanied by increased cell size, leading to larger reproductive and vegetative organs (Adaniya and Shira, 2001).

For polyploidy induction in plants, there are different methods such as seed treatment (Hanzelka and Kobza, 2001; Quan *et al.,* 2004), flower bud (Wu *et al.,* 2007), apical meristem (Lavania and Srivastava, 1991; Saharkhiz, 2007) and root (Taira *et al.,* 1991). *In vitro* techniques for polyploidy induction discovered that the most efficient of treatment methods and colchicine concentrations are species-specific (Roy *et al.,* 2001). Also natural polyploidy is present within the Lamiaceae family, for example within thyme (Lopez-Pujol *et al.,* 2004), Glechoma (Wide'n and Wide'n, 2000) and Lavandula (Upson and Andrews, 2004).

Literature showed, 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), hop (*Humulus lupulus* L.) (Roy *et al*., 2001), ginger (*Zingiber officinale* Roscoe) (Adaniya and Shirai, 2001), tarragon (*Artemisia annua* L.) (Gonzalez and Weathers, 2003), feverfew (*Tanacetum parthenium* L.) (Saharkhiz, 2007), Dragonhead (*Dracocephalum moldavica* L.) (Omidbaigi *et al*., 2010b) and balm (*Ocimum basilicum* L.) (Omidbaigi *et al*., 2010a; Malekzadeh *et al*., 2012).

Since the *Mentha mozaffarianii* Jamzad (Iranian endemic mint) is one of the valuable herbs and spices plant that is endemic in Iran, the present study focuses on artificial autotetraploidy induction possibility of two *Mentha mozaffarianii* Jamzad ecotypes by colchicine.

Material and methods

Plant Materials

Mentha mozaffarianii rhizomes from two natural habitats were collected from Kazeroon, Fars province (South West of Iran. Ecotype A: Kazeroon, Kamarej, 51′ 28° E, 29′ 36° N; and ecotype B: Kazeroon, Pirmohlat, 51′ 34° E, 29′ 30° N) in Jan 2012.

The plant species were identified and authenticated by M. R. Joharchi, a plant taxonomist, at Ferdowsi University of Mashhad Herbarium (FUMH), Mashhad, Iran. A voucher specimen (FUMH, no. 44666 and 44667) has been deposited in the herbarium. In May 2012, ten pots of each ecotype consist of three rhizomes (5-7 cm length) on Mashhad climate conditions (Khorasan Razavi province) were planted. All pots were placed on natural conditions.

Doubling procedure

Our research consisted of following three experiments in order to tetraploidy induction in two *Mentha mozaffarianii* ecotypes.

Rhizome treatment

For this purpose, a factorial experiment was conducted out based on completely randomized design with three replications. The first factor including different colchicines concentrations (0.025, 0.05, 0.1, 0.2% and 0 as control). The second factor included two *Mentha* ecotypes (Ecotype A: Kamarej region and Ecotype B: Pirmohlat region) while the third factors consisted of two soaking time (6 and 12 h). In each replication, four rhizomes were soaked in various concentrations of colchicine and 2% dimethyl sulfoxide (DMSO) and Tween '20' as a surfactant, at room temperature (25±2 °C on a shaker at 200 r.p.m.) for the period mentioned. After soaking, they were placed in tap water for 2 h due to eliminate colchicine remains. The rhizomes treated were planted in the pots filled with a mixture of cocopeat-perlite (1:1) and were placed in green-house under normal condition. Plants nutrition was done with complete fertilizer (300 ml) once a week.

Tip meristem treatment

In the second experiment, apical meristems were treated. The factorial experiment based on completely randomized design with five replications. The factors including different colchicine concentrations (0, 0.035, 0.07, 0.15, 0.3 and 0.7%) and the two previous mentioned ecotypes. In each replication approximately eight tip meristem of plants were treated for three subsequently day by dropping method using an aqueous solution of various concentrations of colchicine. The plants were covered with polyethylene plastic during treatment.

Colchicine usage accompanied with irrigation

For this purpose, colchicine as combined with irrigation was applied. A factorial experiment was conducted based on a complete randomized design with two factors (colchicine concentrations: 0, 0.025, 0.012, 0.006% and two mint ecotypes: A and B) and three replications. In each replication ten observation (including three seedlings planted in small plastic pots) were selected. The pots filled with cocopeat-perlite (1:1) and for three weeks irrigation with colchicine solution (30 ml) once a week were done. Also twice a week, the pots were irrigated with complete fertilizer. In all experiments survival percentage was measured after 6-8 weeks from the start of experiment to the end of experiment.

Identification of tetraploid plants

Recognition of tetraploid plants was done on the basis of morphology, stomata cells measurement, flow cytometry and finally chromosome counting.

Stomata traits

In this research, the nail varnish method was used to study stomata morphology. Three well expanded leaves of each plant were removed from both control and treated plants. A small area of abaxial side of leaves was covered with a thin layer of clear nail polish and left to dry (Hamill *et al*., 1992). 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 (Zeiss axiophot-West Germany) at 40X and 100X magnification.

Flow cytometric analysis (FCM)

The ploidy level of treated plants was measured by flow cytometry (Partec PA, Germany). Fresh leaves (nearly one month old) were first harvested and nuclei suspen-sions were prepared by chopping the leaf tissue (50 mm^2) of colchicine treated and diploid (control) plants, with a fresh razor blade in 400 µL of nuclei extraction buffer (Kit A, suggested by Partec PA, Germany) for 30-60 sec. After filtration through a 30 µm Cell-Trice disposable filter Partec, 1600 µL of 4-6 diamidino 2-2 phenylindole (DAPI, provided by Partec PA, Germany as Kit B) was added. A minimum of 5000 nuclei were measured per sample and histograms of DNA content were generated using the Mode Fit software.

Chromosome counts

Before tetraploidy was induced, the chromosome number of the primary materials was determined in root tips of rooted cuttings in hydroponic system to confirmed number of chromosome in diploid materials (2n=2x=24). Root tips (~2-3 mm), from actively growing roots were excised (about 9 a.m.) and pretreated in a saturated α-bromonaphthalene solution for 4 h at 4 °C to accumulate cells in metaphase. Root tips were then washed in distilled water for 10-15 min and fixed over night in Lewitski fixative at 4 °C for 32 h. The fixative was prepared by mixing equal volume of 1% chromic acid and 4% formaldehyde (10% formalin) just before using. Following, the roots were placed in tap water for 3 h and then were hydrolyzed with 1 N NaOH at 60 °C for 15 min and then rinsed in distilled water. Additional water was removed by blotting paper and the roots were stained for 16h in Aceto-Iron-Hematoxylin 4% at 30-32 °C (Agayev, 2002). In the following about 1 mm the root of tips were cut and transferred a drop of 45% acetic acid on a slide for 3-5 min and squashed in beneath a cover slip. The preparations were observed with an optical microscope (Zeiss axiophot-West Germany) at a magnification 1000X.

Statistical analysis

Statistical analysis was performed by MINITAB and MSTAT-C software and means were compared by using Duncan multiple tests at a significance level of 5%.

Results and Discussion

Rhizome treatment

Data analysis variance of this experiment showed that some factors had significant effects (P<0.01) on survival plants and tetraploidy induction (Tab. 1). In addition, response of ecotypes to treatments was differing. According with results presented in Fig. 1, different colchicine concentrations had significant effect on survival rate so that, the maximum and minimum survival percentage (91.67 and 52.64%) related to control and 0.025% colchicine treatments respectively (however among 0.025, 0.05 and 0.1% colchicine treatments did there were no statistically significant differences). Also it is determined that ecotype B was more sensitive than ecotype A to colchicine (Fig. 2).

Tab. 1. Analysis of variance for survival rate and tetraploidy percentage of two *Mentha mozaffarianii* ecotypes affected by colchicine concentrations and soaking time

Source of variation	DF	Survival rate
Colchicine concentrations (factor A)	4	16.72**
Soaking time (factor B)	1	2.15^{ns}
Ecotype (factor C)	1	19.32**
A × B	4	1.05^{ns}
A × C	4	1.92^{ns}
B × C	1	16.11**
A × B × C	4	1.47^{ns}

DF: degrees of freedom; **significant at p< 0.01; *significant at p< 0.05^{ns}, not significant

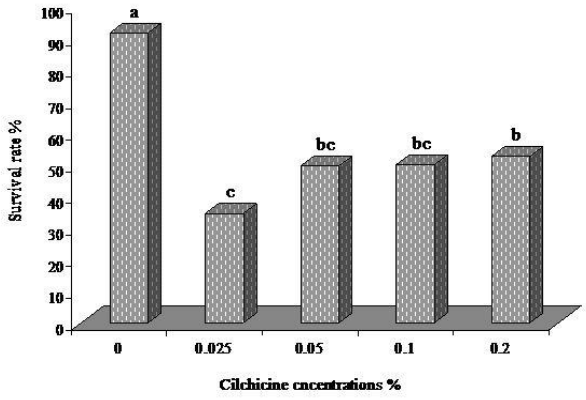

Fig. 1. The effect of different colchicine concentrations on survival rate

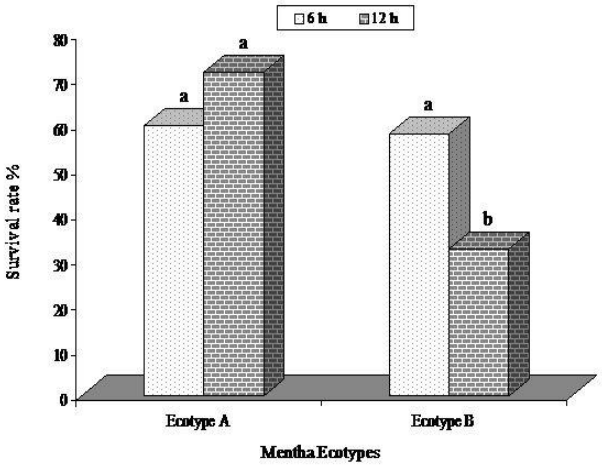

Fig. 2. Interaction between two *Mentha* ecotypes and soaking time on survival rate

Increasing in soaking time decreased survival rate, especially in ecotype B. The minimum survival percent (32.67%) observed in 12 h soaking in ecotype B (Fig 2).

As shown in Tab. 2, only in 0.025% colchicine treatment with 6 h soaking time on ecotype A, 12.66% tetraploid plants was identified. Soaking time had significant effect ($P<0.05$) on tetraploidy induction (Tab. 1). It is seems increasing in soaking time via reduction survival rate cause decline of tetraploidy induction. Also tetraploidy induc-tion affected by ecotype factor so that the ecotype B had not positive response to different colchicine treatments and soaking time as related to tetraploidy induction (Tab. 2).

Tab. 2. The effect of colchicine concentration and soaking time on tetraploidy induction (%) of two Iranian mint ecotypes (*Mentha*

Colchicine concentra-tions	6 h soaking		12 h soaking		Mean
	Ecotype A	Ecotype B	Ecotype A	Ecotype B	
0	0^{b}	0^{b}	0^{b}	0^{b}	0
0.025	12.66^{a}	0^{b}	0^{b}	0^{b}	3.17^{a}
0.05	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}
0.1	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}
0.2	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}

Means with similar letter in 5% level of Duncan test are not significant

Tip meristem treatment

In terms of tip meristem treatment, colchicine concen trations had significant effect on damaged shoot apex (due to colchicine treatment) and survival rate ($P<0.01$) on two ecotypes. In two ecotypes, the minimum damaged shoot apex (0%) were observed in control treatment (without colchicine usage) while with addition colchicine concentrations damaged shoot apex were increased and in 0.7% treatment in all ecotypes was reached to 100% (Tab. 3).

Totally, ecotype B as compared with ecotype A is more sensitive than (55.74 and 22.98% damaged shoot apex, respectively). In this experiment, survival percentage in ecotype B, also affected by colchicine concentrations. In ecotype A, although shoot apex damaged but other buds beginning to growth and the plants survived. Generally, the high mortality rates observed in ectype B (Decreasing plant survival from 100% to 7% from 0% to 0.7% colchicine concentrations), indicates that this ecotype has a low level of resistance to the general toxicity of colchicine. Tip meristem treatment did not have any effects on elevation of ploidy level of the treated mint plants and after the treatment; no tetraploid plant was recognized by stomata traits study and flow cytometry analysis.

Colchicine usage accompanied with irrigation

Treatment of the seedlings with colchicine combined with irrigation did not have any effects on ploidy level of the treated plants and all of them were diploid. These treatments were impressed survival percentage in all ecotypes and increasing colchicine concentrations incur plant survival reduction (Fig. 3).

Tab. 3. The survival rate and damaged shoot apex of two Iranian mint ecotypes (*Mentha mozaffarianii* Jamzad.) under affecting of apical meristem, colchicine treatments

Colchicine concentra-tions	Damaged shoot			Survival		
	Apex (%)		Mean	Percentage (%)		Mean
	Ecotype A	Ecotype B		Ecotype A	Ecotype B	
0	0^{e}	0^{e}	0^{d}	100^{a}	100^{a}	100^{a}
0.035	2.86^{e}	29.67^{cd}	16.26^{cd}	100^{a}	80^{b}	90^{b}
0.07	$5.4d^{e}$	51.43^{bc}	28.41^{c}	100^{a}	60^{c}	80^{c}
0.15	$5.8d^{e}$	60^{b}	32.9^{c}	100^{a}	60^{c}	80^{c}
0.3	23.8^{de}	93.34^{a}	58.57^{b}	100^{a}	5^{d}	52.5^{d}
0.7	100^{a}	100^{a}	100^{a}	100^{a}	7^{d}	53.5^{d}
Mean	22.98^{b}	55.74^{a}	-	100^{a}	52^{b}	-

Means with similar letter (small letters are for interactions and capital letter are for means) in 5% level of Duncan test are not significant

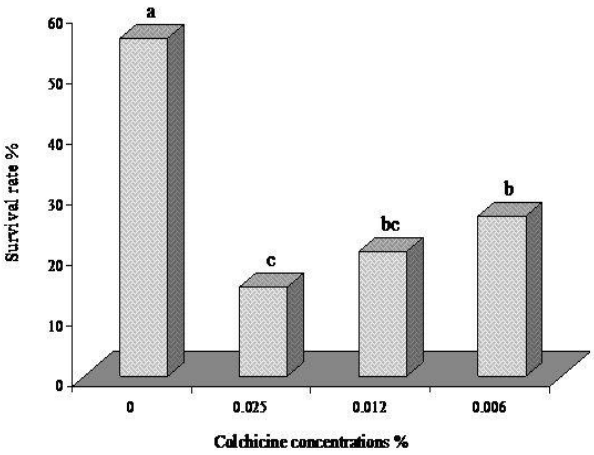

Fig. 3. The survival rate under affecting of colchicine-irrigation treatments

Stomata characteristics

The results suggested that tetraploid plants could be identified with a fair amount of certainty when the screening was based on the size of stomata and density of stomata.

The stomata length and diameter increased with increasing the ploidy level, so stomata in autotetraploids were larger but fewer (in area unit of leaf) compared to the control parents (Fig. 4).

Flow cytometry (FCM)

Ploidy level assessed by flow cytometry (FCM) of nuclei showed that the putative the measured fluorescence is correlated with the DNA content of the stained nuclei.

Ploidy level can be deduced by comparing peak position of G1 nuclei of a plant with known ploidy with that of unknown sample (Fig. 5).

Tetraploids had double the amount of DNA confirming The G2 peak of tetraploid plant is approximately on channel 100, while diploid plant showed a G2 peak on channel 50.

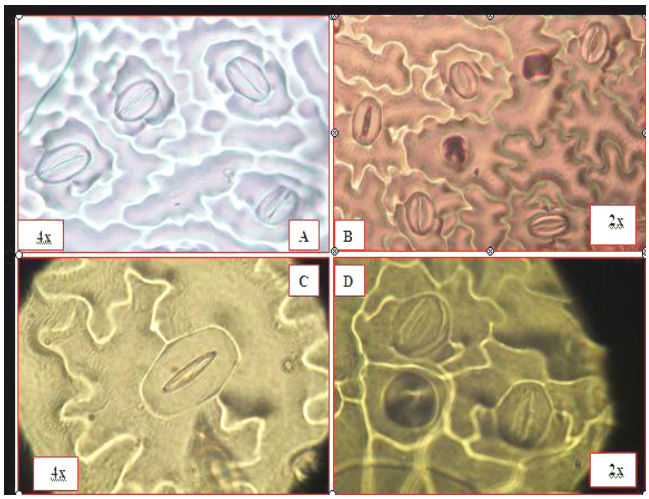

Fig. 4. Comparison of stomata density and size of tetraploid (A, C) and diploid (B, D) plants (A, B 40X and C, D 100X magnification)

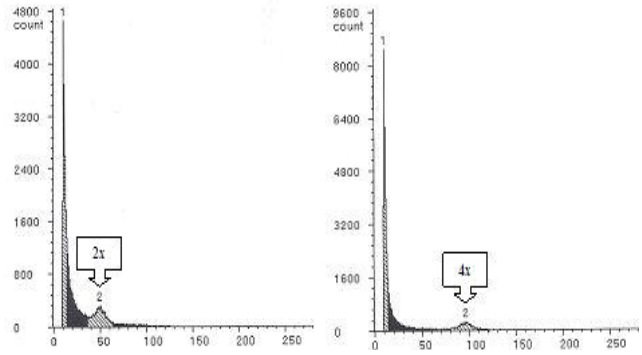

Fig. 5. Flow cytometric histograms of diploid (left) and tetraploid (right) Iranian mint

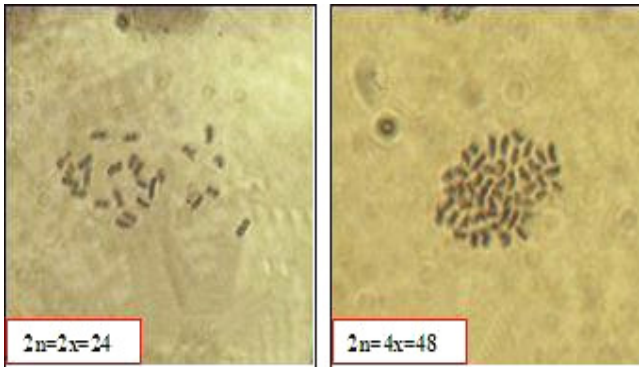

Fig. 6. Chromosomes number in diploid (2n=2x=24) and tetraploid (2n=4x=48) Iranian mint

Chromosome counts

Ploidy conversions from diploid (2n=2x=24) to tetraploid (2n=4x=48) were confirmed by chromosome counts in root tips (Fig. 6). Determination of chromosome number is difficult in *Mentha* genus because of their small chromosome size and time-consuming.

Some morphology changes including leaf thickness, leaf enlargement as diameter, leaf darkness, leaf brittle, decreasing plant height were observed in some plants that these plants were suspected to tetraploidy.

Morphological variations between diploid and tetraploid plants were also observed in Fig. 7.

Colchicine, acts as an antimitotic agent and, as such, has been widely used to induce polyploidy in plant breeding (Kunitake *et al.*, 1998; Nigel *et al.*, 2007; Kermani *et al.*, 2003). In the process of polyploid induction, the combination of colchicine concentration and treatment duration is critical (Ye *et al.*, 2010). Typically, high concentrations of colchicine coupled with short durations of treatment or, conversely, low concentrations of colchicine coupled with long durations of treatment are preferred. In Lespedeza formosa, 0.2% colchicine applied with a 24 h treatment duration and 0.1% colchicine concentration with a 36 h treatment duration had similar success in polyploid induction (Li *et al.*, 2007). In *Colophospermum mopane,* the frequency of induced tetraploids was increased to 100% when seeds were treated with 0.05% colchicine for 96 h, a level that was also achieved following treatment with 0.1% colchicine for 48 h, or 1.0% colchicine for 24 h (Rubuluza *et al.*, 2007). The results from *Lagerstroemia indica* treated, indicated that a concentration of 0.2% colchicine applied for 96 h or 0.5% colchicine applied for 48 h had similar levels of effectiveness for polyploid induction (Ye *et al.*, 2010).

Ramachandran (1982) produced tetraploid ginger via *in vivo* conditions; however, *in vivo* induction of tetraploid ginger is not easy; he obtained only four tetraploids (4.4%) from 90 pieces of diploid rhizomes treated with 0.25% colchicine. Previously, poliploid *Mentha longifolia* plants were obtained via in vitro conditions and it was found that treatment dosage ranges between 100-150 mg/l colchicine were appropriate; both the shoot tip and single node explants which are prepared from sterile plants developed in culture conditions could be used for this purpose and it is possible to say that an average period of 7 days can be

Fig. 7. Morphological changes between diploid (right) and tetraploid (left) Iranian mint

selected as the period of treatment (Tepe *et al.*, 2002).

Determination of the best treatments (colchicine concentration, tissue treated, growth stage and etc) was differing between different plants, species and even genotype or ecotype. Omidbaigi *et al.* (2010a) was reported that autotetraploid basil (*Ocimum basilicum*) plants were produced only by treatment of growing point of seedlings, at the emergence of cotyledone leaves stage, and treatment with 0.5% proved to be the most effective in producing autotetraploids. In another research, the best results to induce polyploidy in *Ocimum basilicum* plants obtained in 0.05% colchicine concentration for six hours when the treatment was treated using cotton plug (Malekzadeh *et al.,* 2012). While in Moldavian balm (*Dracocephalum moldavica*) the best doubling efficiencies of the apex treatment were obtained with the colchicine at 0.1% concentration in two true leaves stage (Omidbaigi *et al.,* 2010b).

An inverse relationship has been reported between colchicine concentration and explant survival with ex vit-ro (Lavania and Srivastava, 1991; Saharkhiz *et al.,* 2007; Omidbaigi *et al.,* 2010a; Omidbaigi *et al.,* 2010 b) and in vitro (Tepe *et al.,* 2002; Bennici *et al.,* 2006; Shahriari-Ahmadi *et al.,* 2009) studies using other plant types. Increased size of stomata guard cells is one of the general effects of tetraploidy induction in the most plants. This is actually a genetic effect which can be seen in all organs, such as seeds, flowers, leaves and shoot. Diploid plants had stomata and stomata guard cells with smaller diameter and smaller length than of tetraploid plants. Stomata characteristics (stomata size, stomata frequency, chloroplast number) previously have been used as useful parameters for distinguishing ploidy level in some plant including *Humulus lopulus* (Roy *et al.,* 2001), jujube (*Zizyphus jujuba* Mill.) (Gu et al., 2005), feverfew (Saharkhiz, 2007; Majdi *et al.,* 2010), basil (Omidbaigi *et al.,* 2010a; Malekzadeh *et al.,* 2012) Moldavian balm (Omidbaigi *et al.,* 2010b).

Conclusion

The results showed that different treatment had significant effects on survival rate on all experiments and increasing of colchicine concentration caused decreasing plants survival. On the other hand, tetraploidy changes only in the first experiments were observed. Only in 0.025% colchicine treatment with 6 h soaking time on ecotype A, 12.66% tetraploidy was identified. In the first experiment rhizome treatment were used because of *Mentha* genus propagated with this asexual organs and any change in this organ can be sustained in the next generation. In this research, tip meristem treatment due to damaged shoot apex and lateral buds growth, this method was not successful. Also, in the third experiment, innovative procedure was used that colchicine were used as combined with irriga tion solution with this hypothesis that rhizome dormant buds during the time affected by colchicine and polyploidy induction happen. Totally, it is seems that *Mentha mozaffariani* hardly response to colchicine for tetraploidy induction. For next investigations, in vitro polyploidy induction for this species or other species of this genus by colchicine or other mutagenic agent is recommended.

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