Journal of Medicinal Plants Research Vol. 5(19), pp. 4892-4898, 23 September, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Doubling the chromosome number of *Salvia hains* using colchicine: Evaluation of morphological traits of recovered plants

Mohammad Sadat Hosseini Grouh[1]*, Heidar Meftahizade[2], Naser Lotfi[3], Vahid Rahimi[4] and Behdad Baniasadi[1]

[1]Department of Plant Sciences, Faculty of Agriculture, University of Jiroft, Jiroft, Iran.
[2]ACECR Medicinal Plants Research Center, Ilam, Iran.
[3]Department of Medicinal Plants, College of Miandoab, University of Uremia, Uremia, Iran.
[4]Department of Horticulture, Abouraihan Campus, University of Tehran, Tehran, Iran.

Accepted 20 July, 2011

This study demonstrates a protocol of polyploidy induction in *Salvia hains*, in order obtained tetraploid five concentrations (0.1, 0.3, 0.4, 0.5 and 0.7%) of colchicine were used to determine the best treatment for the induction of tetraploid plants. Treatment seeds with colchicine for 24 h product tetraploid plants. Ploidy level of the resulting plantlets was determined by morphological study, chromosome counting and flow cytometry. Tetraploid plants were obtained from *S. hains* after applied concentrations 0.3, 0.4 and 0.5% of colchicine. Plants obtained from 0.1% colchicine were all diploids. The induced tetraploid in S. hains was accompanied by larger stomata increase in chloroplast number in guard cells and decrease in stomata density, compared to diploid (control) plants. The techniques used to induce tetraploid *S. hains* plants by colchicine were successful and could be used in breeding programs.

Key words: Autotetrapolid, flowcytometry, polyploid, *Salvia hains,* chromosome counting.

INTRODUCTION

Polyploidy is widely acknowledged as a major mechanism of adaptation and speciation in plants (Osborn et al., 2003). Polyploidy is such a common component of plant evolution that it must be part of any general theory of the evolutionary ecology and genetics of plant (Thompson et al., 2004). The stages in polyploid evolution include frequent fertility bottlenecks and infrequent events such as gametic nonreduction and interspecific hybridization, yet little is known about how these and other factors influence overall rates of polyploid formation. In addition to naturally occurring polyploids, many insights have emerged from recent explorations using laboratory generated or synthetic polyploids. Study of these experimental polyploids has revealed extensive and rapid genomic changes in some groups, including sequence rearrangements, homoeologous recombination, sequence elimination, and changes in deoxyribonucleic acid (DNA) methylation (Osborn et al., 2003; Liu and Wendel, 2003). Some genomic changes have arisen immediately with the onset of polyploidy, whereas others have occurred within a few generations. Chromosome doubling using colchicine has long been used in plant breeding programs and progress in genetic study (Hancock, 1997). The first applications of mitotic polyploidization in agriculture were described in the 1930s (Blakeslee and Avery, 1937). This initial polyploidization was induced on plants established in soil. Colchicine was the most commonly used antimitotic agent. Roots were soaked in or axillary buds were wetted with a colchicine solution (Pei, 1985). Blakeslee (1939) reported the successful production of polyploids in 48 different species using colchicine; for agricultural crops, *in vivo* chromosome doubling was successfully applied for sugar and fodder beet, ryegrass and red clover (Adaniya and shira, 2001).

*Corresponding author. E-mail: hosseinim@alumni.ut.ac.ir or msadat.1983@gmail.com. Tel: +98-348-3260062. Fax: +98-348-3260065.

Abbreviations: DNA, Deoxyribonucleic acid; **DAPI,** 4, 6-diamino-2-phenylindole.

Despite the effectiveness of the method, low rates of polyploidized plants were frequent and high numbers of mixoploids were retrieved (Pei, 1985). Polyploid plants may be found in agriculture and horticulture as they often possess superior agronomic traits over their diploid counterparts. For example, ploypoids may have larger leaves and flowers, thickerstems and roots, darker green leaves, an increased width-to-length ratio of the leaves, increased cell size, leading to larger reproductive and vegetative organs a more compact growth habit and a higher tolerance to environmental stress (Kehr, 1996; Kermani et al., 2003; Shao et al., 2003; Adaniya and Shira, 2001). The induction of artificial polyploidy may prove useful in increasing the production of important medicinal compounds (Dhawan and Lavania, 1996). Together with requirement for safety, efficacy and stability of medicinal and aromatic plant products, the need for high quality raw materials increasing. Breeding procedures that is, induction autotetraploidy for these plants is helping to spread and satisfy the demand for such materials (Bernath, 2002). Resulting polyploidy plants often have larger leaves, flowers, fruit and seed (Hartwell et al., 2004). In polyploid plant are usually bigger than those of the diploid plant. Thus, the polyploid plants may increase the biomass or product yields (Adaniya and shira, 2001). There are numbers of factors that may provide polyploids with adaptive and evolutionary advantages. Perhaps most importantly, polyploids can be significantly more heterozygous than their diploid counterparts. Polyploids can have 4 different genes (alleles) present at any given locus (location on a chromosome). The degree of heterozygosity may be a key factor in the growth, performance, and adaptability of a polyploid. The genus *Salvia* commonly called sage is one the largest members of the family Lamiaceae that contains nearly 900 species spread throughout the world (Lu and Foo, 2002).

In the Flora of Iran, the genus is represented by about 58 species of which 17 species are endemic (Mozaffarian, 1994). They are well known among people and widely used as flavoring agents or fragrances and for various medicinal purposes (Nickavar et al., 2007: Nickavar et al., 2005). *Salvia* species are rich sources of polyphenolic flavonoids and phenolic acids (Lu and Foo, 2002; Nickavar et al., 2007). The genus contains a wide variety of flower colors, morphological characters and ecotypes. Some *Salvia* species have a long history of utilization as medicinal plants, not only as ornamental plant. Chromosome numbers of *Salvia* species are unusual in their extreme variability. Published counts range from a low of 2n = 12 in *Salvia hispanica* (Harley and Heywood, 1992) to a high of 2n = 88 in the octoploid *Salvia guaranitica* (Alberto et al., 2003). In addition to wide variation in ploidy level in *Salvia*, the basic number of chromosomes is also wide-ranging with x reported as 6, 7, 8, 9, 10, 11, and 15 for species within the genus (Goldblatt and Johnson, 1979; Harley and Heywood, 1992). In the present research attempt was made to induce autotetraploidy in *Salvia hains* (2n = 2x =1 6) using colchicine with the objective of creating more genetic variability higher oil-yield, more productive variety and used in breeding programs. Furthermore, the present study aimed to identify morphological and cytological traits, such as stomata and guard cell size, number of chloroplasts per guard cell; whose performance depend on plant ploidy and can be used for indirect identification of diploids and tetraploids in sage medicinal plant. It also compares these traits with flow cytometry to determine whether any markers can be used to identify putative tetraploids in this species.

MATERIALS AND METHODS

Plant material

Seeds of *S. hains* provided from the ACECR (Medicinal Plants research Center, Ilam, Iran) used in this work, this is the diploid genus and chromosome number is 8 (2n = 2x = 16), experiment were carried out in 2010 in the greenhouse of the Medicinal Plants research Center, Ilam, Iran.

Tetraploid induction

The seeds of *S. hains* were sterilized in 1% sodium hypochlorite (NAOH) for 23 min, and then rinsed twice with sterile distilled water for 4 min and seeds then were sown in Petri-dishes (0.2 g per- dish ~70 seeds) on filter paper. Six levels of colchicine concentrations (0, 0.1, 0.3, 0.4, 0.5 and 0.7%) were applied at each Petri-dish. For each application rate ~250 seeds were treated. The cultivations were supplement with 2 to 3 drops of DMSO and Tween 20 as a surfactant to facilitate better penetration of colchicin to seeds. After 24 h, the seeds were washed with sterile water and transferred to fresh filter paper, soaked distilled water. Petri-dishes were sealed with parafilem and incubation at 24°C and 16 h photoperiod for germination and plant regeneration.

Morphological observations

Selection of tetraploid and diploid plants was done on the basis of morphology followed by a selection on size of stomata and gradual cells measurement, chloroplast density in guard cells, the putative tetraploid were examined two months later to validate ploidy stability.

Size and density of stomata an guard cells measurement

For this purpose, 15 plants of diploid control and 15 of tetraploid plants randomly were selected. Measurement and scoring were performed for five well expanded leaves of each plant. Three samples of epidermal cells were obtained from lower surface by nail varnish technique. A small area of abaxial side of leaves was covered with a thin layer of clear nail polish and left to dry (Hamil et al., 1992). After drying the polish, it was removed with a tip forced then placed on a glass slide and observed through the light microscope at 1000 × magnification.

Chloroplast number for guard cells

For study of the chloroplast number in the stomatal guard cells,

Table 1. Effect of different concentration of colchicine on germinated seed of *Salvia hians* and induced of tetraploid plants. ~250 seed were exposed to each concentration of colchicine.

Concentration of colchicine % (w/v)	Germination rate (%)	Day of germination	Number of tetraploid obtained[a]	Surviving of tetraploid obtained[b]	Confirmed polyploidy[c]
0 (no treated)	72.0	3	0	0	0
0.1	66.8	4	3	1	0
0.3	52.8	4	5	2	1
0.4	44.0	5	9	4	2
0.5	36.8	5	13	6	4
0.7	21.6	6	2	0	0

[a]Number of tetraploid obtained were determinate according to chloroplast number in gradual cell, stomatal size and stomata frequency, [b] number of surviving tetraploid after 45 day, [c] number of plants confirmed as polyploid (tetraploid).

samples of epidermal layer from abaxial side of diploid control plants and tetraploid plants leaves were obtained and this epidermal layer was stained with lugol's iodine solution 1% and observed by light microscope at 1000 × magnification (Guimaraes and Stotz, 2004). Other major morphological traits observations and growth habit characteristics measured in the 45 day after regenerations plants in greenhouse. Dimensions measured were the plant height, intermodal distance of stem; number and length of branches (only those over 4 cm included); length and width of the leaf; fresh and dry weight of leaves (taken from ten leaves from each individual); fresh mass and dry mass; according to Guofeng et al. (2007).

Flow cytometeric analysis

Flow cytometry was used for determination of the ploidy level of samples prepared from young leaves of the diploid (control) and tetraploid plants. Nuclei were released from 0.5 cm^2 of leaf tissue by chopping with a razor blade for 25 s in 500 µl of modified Galbrith's nuclei isolation buffer (Galbrith et al., 1983; Sadat et al., 2011) containing 200 mM Tris, 4 mM MgCl2.6H2O, pH = 7.5, 0.5% Triton X-100 (CyStain UV Precise Partec Gmbh-Munster, Germany). Then, 500 µl of staining solution 4, 6-diamino-2-phenylindole (DAPI) was added for DNA staining. After 3 min incubation, nuclei were passed through a 30 µm nylon filter to eliminate cell debris. The samples were analyzed using a Partec PA-I flow cytometer (Partec, Munster, Germany).

Chromosome counting

Chromosome number was counted in root tip cells obtained from plantlets were recovered, using the standard Feulgen technique (Lillie, 1951). The root samples were pre-treated with α-bromonaphtalene for 4 h at 4°C. The samples were fixed for 24 h in 3:1 ethanol: acetic acid, and then stored in 70% ethanol at 4°C until viewing. Fixed root tips were placed in aceto-iron-hematoxylin dye for staining chromosomes (Lu and Raju, 1970). The fixed root tips were hydrolyzed in 1N HCl for 8 min at 60°C and squashed in a drop of 45% (v/v) acetic acid.

RESULTS AND DISCUSSION

The approach we used to induce tetraploidy in *S. hains* was to germinate seed in the presence of colchicine at various concentration on filter paper in Petri dishes for 6 day (Table 1). In this study, the best doubling efficiencies of the seed treatment were obtained with the colchicine at the 0.5% concentration (Table 1) colchicine inhibits the formation of spindle fibers and temporarily arrests mitosis at the anaphase stage. At this point, the chromosomes have replicated, but cell division has not yet taken place resulting in polyploid cells (Ranney, 2006). Colchicine in high concentration had very effect on germination of seed and survival plants recovered (Table 1). Seedling tetraploids were visibly affected by high concentration of colchicine, being shorter and having broader stems than diploid (control), (Table 2).

These results are in agreement with the observations in Lavandula angustifolia (Nigel et al., 2007). Plants with increased ploidy levels are sometimes apparent by their distinct morphology. Increasing ploidy often results in increased cell size that in turn results in thicker, broader leaves and larger flowers and fruit. Shoots are often thicker and can have shortened internodes and wider crotch angles. Results of studying stomata morphology and flow cytometry profiles indicated that the application of colchicine induced teraploidy in seedling. Result showed that stomata characteristics were important indicators for the determination of ploidy level in *S. hains*. Other effective, but more time-consuming, measures that indicate polyploidy include larger pollen size, greater number of chloroplasts per guard cell (Solov'eva, 1990), and larger guard cells and stomates. Diploid plants rather than tetraploids had stomata and stomata guard cells with smaller diameter, reduced frequency of stomata per unit leaf area, frequency of chloroplast number was lower, and smaller length (Table 3 and Figures 1 and 2). Our results are similar and consistent with those published by Roy et al. (2001) for hops (*Humulus lupulus* L.), by Gu et al. (2005) for jujube (*Zizyphus jujube* L.) and by Omidbaigi et al. (2010) for dragonhead (*Dracocephalm moldavica* L.). The utility of stomata size in distinguishing plants with different ploidy levels has been used in other plant types that increase in stomata size in tetraploid plants as compared to diploid such as Gao et al. (2002) in *Scutellaria baicalensis* and Thao

Table 2. Comparison of plant morphological traits of diploids (control) and tetraploid induced from *Salvia hians*

Characteristic	Diploid			Tetraploid		
	Dip1[a]	Dip2	Dip3	Tet1[b]	Tet2	Tet3
Plant height (cm)	24.75[a]	25.50[a]	26.75[a]	16.00[b]	16.25[b]	15.75[b]
Length of branches (cm)	8.75[a]	8.50[a]	8.50[a]	5.25[b]	5.00[b]	4.75[b]
Number of branches	12[a]	10[a]	11[a]	7	6[b]	5[b]
Length of leaf (cm)	3.00[a]	2.50[a]	3.00[a]	6.57[b]	6.7[b]	6.25[b]
Width of leaf (cm)	2.50[a]	2.75[a]	2.25[a]	4.50[b]	4.50[b]	4.25[b]
Leaf index (length/width)	1.2[a]	0.9[a]	1.33[a]	1.46[b]	1.50[b]	1.47[b]
Internodal distance of stem (cm)	2.50[a]	2.25[a]	2.25[a]	6.00[b]	6.25[b]	5.50[b]
Fresh mass (g)	15.50[a]	16.75[a]	16.00[a]	23.50[b]	23.25[b]	23.75[b]
Dry mass (g)	6.75[c]	7.50[cb]	6.25[c]	10.00[a]	9.75[ab]	9.75[ab]

[a]Dip 1, Dip2 and Dip3 represent diploid plants obtained from 0, 0.1 and 0.3% colchicin treated, respectively, [b]Tet1, Tet2 and Tet3 represent tetraploid plants derived from apply 0.3, 0.4 and 0.5% colchicin treated, respectively, Significant different of characters between diploid and tetraploid determinate by Tukey test at the $p = 0.05$ level by ANOVA analysis (SAS, ver 9.1) of three diploid and three tetraploid samples.

Table 3. Size stomata, stomata density and chloroplast number in gradual cell in diploid (control, 2x) and confirmed tetraploid (4x).

Characteristic	Diploid (mean)	Tetraploid (mean)
Stomata length (µm)	11.60[b]	16.40[a1]
Stomata width (µm)	5.50[b]	8.40[a]
Stomata index	2.07	1.95
Stomata frequency/(mm)^2	19.20[a]	8.20[b]
Chloroplast number in gradual cell	8.60[b]	15.00[a]

[1]Within each column values followed by different letters are significantly different ($P = 0.05$), by tukey.

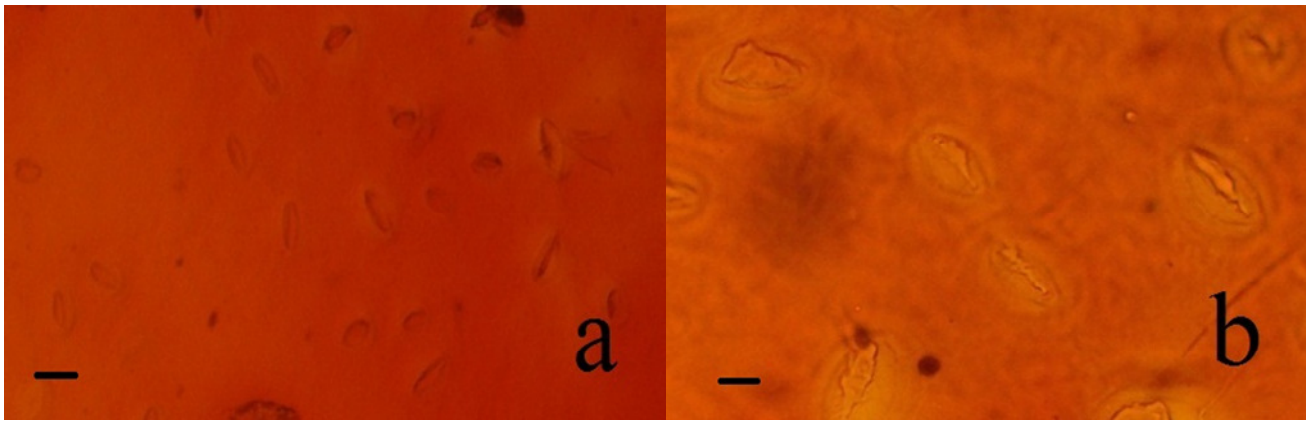

Figure 1. Diffrence in stomate size and density between of *S. hians,* (a) diploid (2x) and (b) tetraploid (4x), Bar = 10 µm.

et al. (2003) in ornamental Alocasia and so comparable results that stomata diameter, guard cell length increase with higher ploidy level, has been reported by Yetisir and Sari (2003) in *Cucumis melo* L. Measurement of morphological evaluation were taken when all plants were 45 day after induced, total plant height was lower in three tetraploid plants compared to the three diploids, and this reduced height caused due to shorter internodal distance (Table 2) the branch numbers was also reduced in the tetraploid plants and the length of branches was shorter than diploid plants (Table 2).

These results are in agreement with the observations in

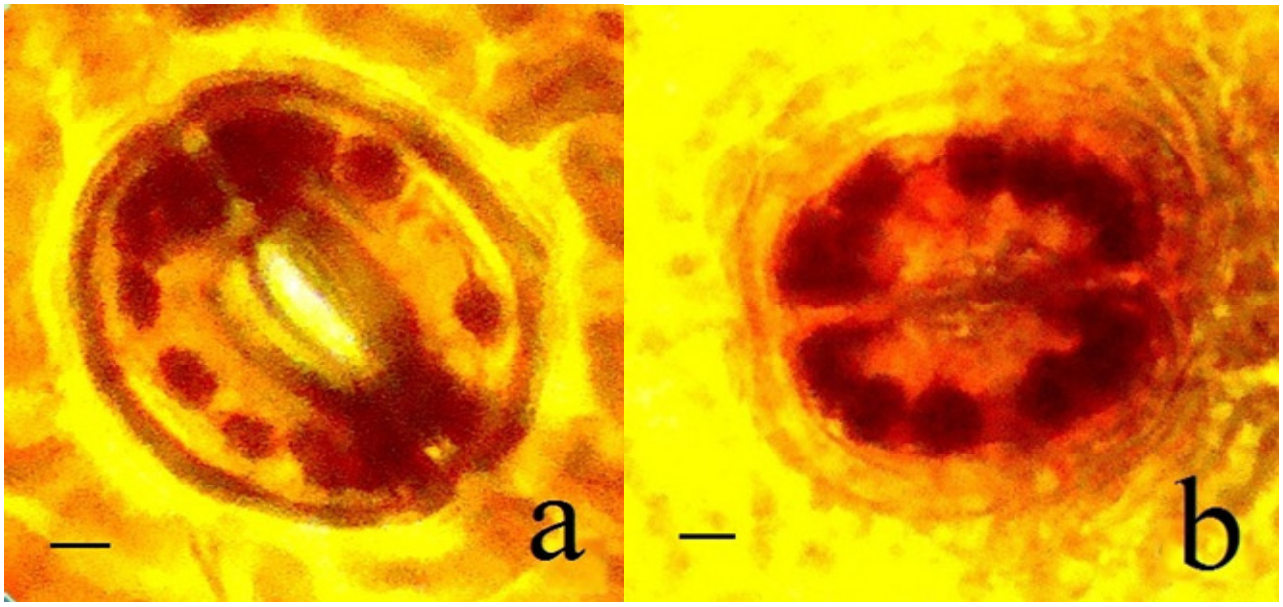

Figure 2. Diffrence in chloroplast number in gradual cell between of *S. hians*, (a) diploid (2x) and (b) tetraploid (4x), Bar = 10 µm.

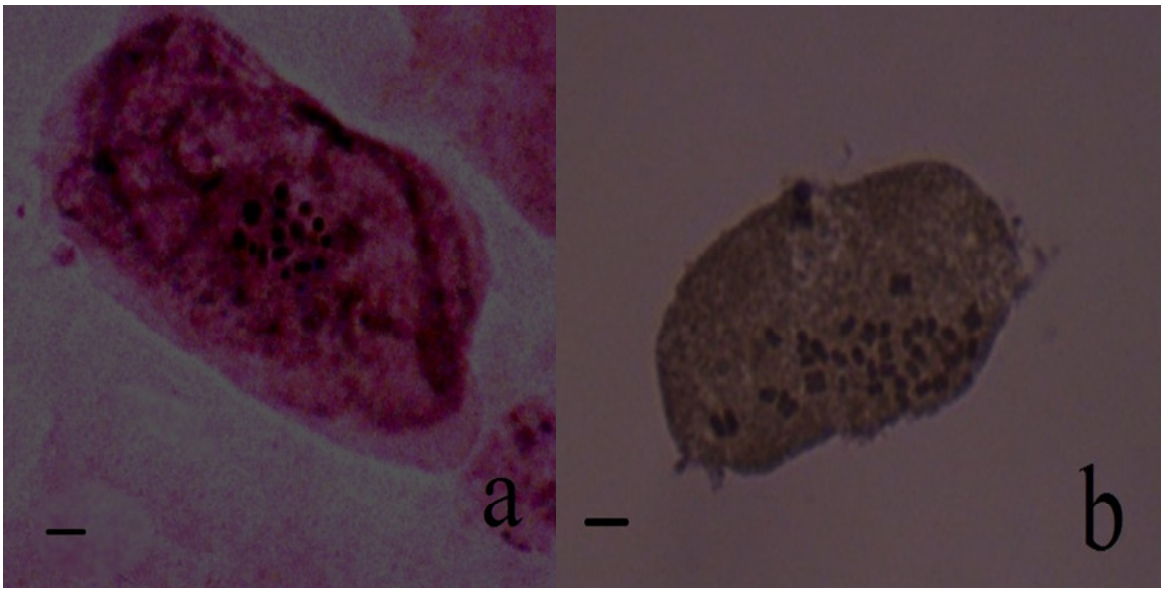

Figure 3**.** (a) Diploid cell (2n = 2x = 16) observed in a root apex of *Salvia hians* and (b) Tetraploid cell (2n = 4x = 32) observed in a root apex of *S. hians,* Bar = 10 µm.

Dracocephalm moldavica L. (Omidbaigi et al., 2010) and *Platanus acerifolia* (Guofeng et al., 2007), in which poliploids than diploids had were length of branches was shorter, and lower number branches. In addition morphological studying, for determination of ploidy level we are used of chromosome counting and flow cytometry (as described in Materials and Methods), tetraploid status was determined by chromosome counting and flow cytometry (Figures 3 and 4). All the plants recovered from apply 0 and 0, 1% concentrations colchicine were diploid, tetraploid plants were obtained apply 0.3, 0.4 and 0.5% colchicine concentrations were used.

No tetraploid plants were recovered from the 0.7 colchicine concentration. In total, 7 tetraploid plants were obtained in this research (Table 1), in addition, the best concentration of colchicine for tetraploid production in *S. hains* was 0.4 and 0.5%, In comparison, 0.1% gave the best result for dragonhead (Omidbaigi et al., 2010)

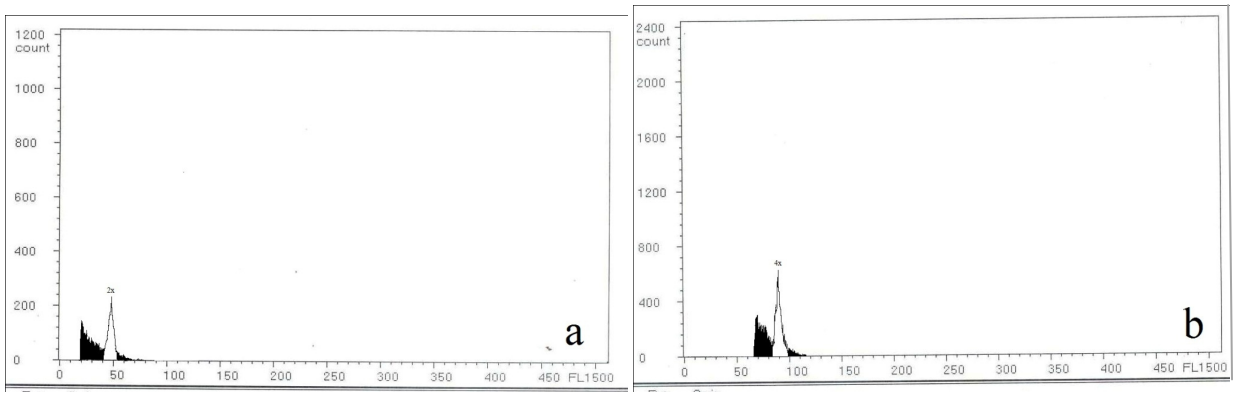

Figure 4. Flow cytometry of diploid and tetraploid plants of *S. hians*. DAPI (4, 6-Diamidino-2-phenylindole) fluorescence of nuclei (*x* axis) versus number of nuclei counted (*y* axis) in diploid (a) and tetraploid (b) plants.

and 0.4% were the best for *P. acerifolia* (Guofeng et al., 2007). In near Future experiment our could test 0.45, 0.55 and 0.6% concentration to determine the capacity of these treatments to produce more tetraploid plants in *S. hains.* The results revealed that morphological changes specially Stomata characteristics in treated plants were reliable and accurate indicators for identification of tetraploid plants and selection on stomata and guard cells size, stomata density and chloroplast number in guard cells and flow cytometry, proved to be an effective way to identify the tetraploid plant and these methods are suitable, quick and easy methods for identifying the ploidy level of *S. hians* in various stages of the plant development. Although there was significant differences (*P<0.01*) in the stomata length and diameter, stomata density, chloroplast number in guard cells between the control diploids and tetraploids, but for confirm ploidy level of mixoploid and tetraploid plants, flow cytometric analysis was be required. Therefore estimation of morphological changes and stomata counting and examination of chloroplast number in guard cells, is an effective method in primary screening of tetraploid Salvia hians plants in polyploidisation breeding program and it is recommended flow cytometry to be used for accurate identification of ploidy level in mixoploid plants of sage.

ACKNOWLEDGMENT

We thank the University of Jiroft for their financial supports and Medicinal Plants research Center, Ilam, Iran (ACECR) for providing the plant materials.

REFERENCES

Adaniya S, Shira D (2001). *In vitro* induction of tetraploid ginger (*Zingiber officinali* Roscoe) and its pollen fertility and germinability. Sci. Hort., 88: 277-287.

Alberto CM, Sanso AM, Xifreda CC (2003). Chromosomal studies in species of *Salvia* (Lamiaceae) from Argentina. Bot. J. Linn. Soc., 141: 483-490.

Dhawan OP, Lavania UC. (1996). Enhancing the productivity of secondary metabolites via induced polyploidy: a review. Euphytica., 87: 81-89.

Galbrith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983). Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Sci., 220: 1049-1051.

Gao SL, Chen BJ, Zhu DN (2002). *In vitro* production and identification of autotetraploids of Scutellaria baicalensis. Plant. Cell. Tiss. Org. Cult., 70: 289-293.

Goldblatt P, Johnson DE (1979). Index to plant chromosome numbers. Missouri Bot. Garden, St. Louis, MO.

Gu XF, Yang AF, Meng H, Zhang JR (2005). In vitro induction of tetraploid plants from diploid *Zizyphus jujuba* Mill. cv. Zhanhua. Plant. Cell. Rep., 24: 671-676.

Guofeng L, Zhineng L, Manzhu B (2007). Colchicine-induced chromosome doubling in Platanus acerifolia and its eVect on plant morphology. Euphytica., 157: 145-154.

Hancock JF (1997). The colchicine story. Hortsci., 32: 1011-1012.

Harley RM, Heywood CA (1992). Chromosome numbers in tropical American Labiatae,. In: Harley, R.M. and T. Reynolds (eds.). Advances in labiate science. Royal Botanic Gardens, Kew, UK., pp. 211-246.

Hartwell LH, Hood L, Goldberg ML, Reynolds AE, Silver LM, Veres RC (2004). Genetics from Genes to Genomes, 2nd edn. McGraw Hill, Boston.

Kehr AE (1996). Woody plant polyploidy. Am. Nurseryman, 183: 38–47.

Kermani MJ, Sarasan V, Roberts AV, Yokoya K, Wentworth J, Sieber VK (2003). Oryzalin-induced chromosome doubling in Rosa and its eVect on plant morphology and pollen viability. Theor. Appl. Genet., 107: 1195-1200.

Lillie RD (1951). Simplification of the manufacture of Schiff reagent for use in histochemical procedures. Stain. Technol., 26:163-165.

Liu B, Wendel JF (2003): Epigenetic phenomena and the evolution of plant allopolyploids. Mol. Phylogenet. Evol., 29: 365-379.

Lu BC, Raju NB (1970). Meiosis in Coprinus. II. Chromosome pairing and the lampbrush diplotene stage of meiotic prophase. Chromosoma., 29: 305-316.

Lu Y, Foo LY (2002). Polyphenolics of *Salvia*. Phytochem., 59: 117-140.

Mozaffarian V (1994). A Dictionary of Iranian Plant Names. Farhang Moaser Publishers, Tehran. pp. 477-480.

Nickavar B, Kamalinejad M, Izadpanah H (2007). *In vitro* free radical scavenging activity of five *Salvia* species. Pak. J. Pharm. Sci., 20: 291-294.

Nickavar B, Mojab F, Asgarpanah J (2005). Volatile composition of the essential oil of *Salvia hypoleuca v* Benthh. Int. J. Aromather., 15: 51-53.

Nigel AR, Urwin J, Therese M (2007). Generation and characterization

of colchicine autotetraploid *Lavandula angustifolia*. Euphytica., 156: 257-266.
Osborn TC, Pires JC, Birchler JA, Auger DL, Chen ZJ, Lee H-S, Comai L, Madlung A, Doerge RW, Colot V (2003). Understanding mechanisms of novel gene expression in polyploids. Trends. Genet., 19: 141-147.
Pei XS (1985). Polyploidy induction and breeding. Shanghai Science and Technology Press, pp. 95-98.
Roy A, Legget G, Koutoulis A (2001). In vitro tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). Plant Cell Rep., 20: 489-495.
Sadat HGM, Vahdati K, Lotfi M, Hassani D, Birvand N (2011). Production of Haploids in Persian Walnut through Parthenogenesis Induced by Gamma-irradiated Pollen. J. Am. Soc. Hort. Sci., 136(3): 198-204.
Shao J, Chen C, Deng X (2003). In vitro induction of tetraploid in pomegranate (*Punica granatum*). Plant. Cell. Tiss. Org. Cult., 75: 241-246.
Thao NTP, Ureshino K, Miyajima I, Ozaki Y, Okubo H (2003). Induction of tetraploids in ornamental Alocasia through colchicine and oryzalin treatments. Plant cell. Tiss. Org. Cult., 72: 19-25.
Yetisir H, Sari N (2003). A new method for haploid muskmelon (*Cucumis melo* L.) dihaploidization. Sci. Hor., 98: 277-283.