

<http://www.pjbs.org>

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSI*net*

Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Tetraploid Induction of *Hyoscyamus muticus* L. using Colchicine Treatment

¹F. Shahriari-Ahmadi, ¹E. Dehghan, ¹M. Farsi and ²M. Azizi

¹Department of Biotechnology and Plant Breeding, College of Agriculture,
Ferdowsi University of Mashhad, P.O. Box 91775-1163, Mashhad, Iran

²Department of Horticulture, College of Agriculture, Ferdowsi University of Mashhad,
P.O. Box 91775-1163, Mashhad, Iran

Abstract: In this study the tip of meristem in diploid plants ($2n = 2x = 28$) were treated with a 0.2% colchicine solution and flowcytometry was used to determine the DNA ploidy level of different tissues in individual plants. Morphological characteristics such as size and thickness of leaves, flowers, seeds, epidermis and guard cells, as well as the number of chloroplast in guard cells were considered as other parameters for identifying putative tetraploid plants. We show that, the DNA ploidy of *Hyoscyamus muticus*, treated with Colchicine can be evaluated using flow cytometry. In addition we also report reliable morphological markers to identify putative tetraploids in this species. Although, tetraploidy was induced using this technique, mixoploid plants were also observed among the Egyptian henbane plants. The results of this study also indicate that colchicine treatment can efficiently be used to improve *Hyoscyamus muticus* for commercial and pharmaceutical applications of its vegetative organs.

Key words: Egyptian henbane, flowcytometry, mixoploidy, tetraploid induction

INTRODUCTION

Egyptian henbane (*Hyoscyamus muticus*) is one of the most important medicinal plant of the Solanaceae family. It contains valuable tropane alkaloids, such as hyoscyamin and traces of hucin and atropine (Lavania, 1986).

Tropane alkaloids, especially hyoscyamin and scopolamine, are widely used in medicine for their mydriatic, antispasmodic, anticholinergic, analgesic and sedative properties (Zehra *et al.*, 1998). These alkaloids are synthesized in roots and then transported to aerial parts of the plant (Pal Bais *et al.*, 2001). The synthetic production of these alkaloids is more expensive than their extraction from plant materials and they are, therefore, currently industrially extracted from various Solanaceous plants belonging to the genera *Atropa*, *Duboisia*, *Datura* and *Hyoscyamus*.

Polyploidy manipulation has been successfully used in plant breeding to facilitate the production of superior cultivars in many crop species. Polyploids often show novel phenotypes or exceed the trait range of their diploid ancestors in characteristics such as increased drought tolerance, apomixis, pest resistance, enhanced biomass and increased concentration and/or qualitative changes

in active plant principles-thus enhancing their chances of being selected in agriculture (Lavania, 2005).

The most widely applied and best studied chemical that induces polyploids is colchicine, an alkaloid extracted from seeds or corms of the autumn crocus (*Colchicum autumnae*) or Colemid, its synthetic equivalent (Hassawi and Liang, 1991; Pehlman, 1987).

Colchicine disrupts mitosis by binding to tubulin, inhibiting the formation of microtubules and the polar migration of chromosomes, resulting in cells with doubled chromosome number (Panda *et al.*, 1995).

Colchicine has been used for chromosome doubling in many crops including banana (Hamill *et al.*, 1992), *Zizyphus jujube* (Gu *et al.*, 2005), mulberry (Chakraborti *et al.*, 1998) and few cases of medicinal plants (Gao *et al.*, 1996). Tropane alkaloids, terpenoids and isoflavonoids are among the most important medicinal compounds found in plant roots. Thus, induction of artificial polyploidy may prove useful in increasing the production of these important medicinal compounds (Dhawan and Lavania, 1996). It has been reported that the tetraploid plant, *Hyoscyamus muticus*, had nearly 1.5 times higher economic production potential compared with that of its diploid counterpart (Lavania, 1986). In addition, the leaf, stem and root which can be useful parts in most

medicinal plants are usually bigger in polyploidy plants than in diploid plant. Thus, the polyploid plants may increase biomass or product yields (Gao *et al.*, 1996).

Since, the plant meristem consists of many cells, in addition to tetraploids, it is possible to obtain mixoploids (chimeras consisting of diploid and tetraploid tissue) after colchicine treatment (Koutoulis *et al.*, 2005). On the other hand, high chromosome numbers in tetraploid *H. muticus* ($2n = 4x = 56$) (Lavania, 1986) make microscopic cytological analysis of this species unfeasible. Therefore, flow cytometric analysis of the DNA content of leaves in Egyptian henbane was applied in the present experiment to determine ploidy level. Flow cytometry is a rapid and exact method for estimating nuclear DNA content (Galbraith *et al.*, 1983). It can be efficiently used for ploidy determination in plants growing in fields or in the greenhouse (Dolezel *et al.*, 1989; Joachimiak *et al.*, 2001).

The aim of this study is to present a reliable protocol for obtaining tetraploid plants in the commercial exploitation of *Hyoscyamus muticus* by colchicine treatment. A further objective of the present study is to produce raw material for future study on different ploidy levels in transgenic hairy root culture of this species. Furthermore, this study reports on the DNA ploidy level of plantlets treated with colchicine using flow cytometry and finally on finding reliable morphological markers to identify putative tetraploids in these species.

MATERIALS AND METHODS

Plant material and polyploidy induction: This study was conducted for two years (2007, 2008) in the agricultural research glasshouse at Ferdowsi University of Mashhad-Iran. Diploid seeds of Egyptian henbane (*Hyoscyamus muticus*), which were imported from Finland were used as a source for polyploidy induction. The seeds were first surface sterilized with 1% sodium hypochlorite for 10 min, washed two times with sterile water and placed in filter paper. Afterwards pre-germinated seeds with newly emerged radicles were transferred to small pots containing vermiculite-perlite-peat mixture. The cultured plantlets were incubated in a growth chamber at a photoperiod of 16/8 h light/dark and irrigated with 50% Hogland solution.

The meristem tip of plantlets in their 6-8 leaf stage were first covered with cotton (Fig. 1) and then 0.2% aqueous solutions of colchicines (Sigma) was used as droplet on cotton for 24 h with interval times of 4 h. Afterwards, the plantlets were completely washed with sterile distilled water and after one month Colchicine treatment, plantlets with new organs emerged from the treated section were transferred to big pots and grown in glasshouse for acclimatization under a photoperiod of 16/8 h light/dark.



Fig. 1: Treatment of tip meristem by the cotton plug method

Measurement of few morphological characteristics: The characteristics of treated plantlets (C_0) and their progeny (C_1) were compared with non-treated ones (control). These characteristics included visual comparison of plant body size, leaf, flower and seed size. For the stomata measurements of the above plantlets, the upper epidermis of leaves was removed and stuck on microscopic slides. The number of chloroplasts in 8 guard cells of stomata was counted by removing the upper epidermis of leaves. For this purpose, three plantlets were chosen from both the diploid control and tetraploid groups.

Flow cytometry analysis of ploidy levels: The ploidy level of treated plants near their flowering stage, was measured by flow cytometry (Partec PA, Germany). Fresh leaves (nearly one month old) were first harvested and nuclei suspensions were prepared by chopping the leaf tissue (50 mm^2) of colchicines treated and diploid (control) plants, with a fresh razor blade in $400 \mu\text{L}$ of nuclei extraction buffer (Kit A, suggested by Partec PA, Germany) for 30-60 sec. After filtration through a $30 \mu\text{m}$ Cell-Trice disposable filter Partec, $1600 \mu\text{L}$ 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.

In this research to investigate the stability of the induced tetraploids, flow cytometry measurement was conducted again from leaves in C_1 and C_2 generations.

Biomass comparison: Four months after planting, the aerial organs of diploid and tetraploid plants were collected for measuring their wet and dry weight.

Statistical analysis: Where appropriate, statistical analysis was performed using student t-test for morphological and biomass comparison between tetraploid and diploid types (control).

RESULTS

Survival and growth of colchicine treated plantlets: The effects of colchicine on the growth of plantlets were assessed one month after treatment. The plant survival was not affected by the concentration and time of exposure of colchicines. Although, all of the plantlets were affected by the colchicines, small number of plantlets showed some browning and burning at the place of treatment.

An inverse relationship has been reported between colchicine concentration and explant survival with *ex vitro* (Lavania, 1986; Lavania and Srivastava, 1991) and *in vitro* (Hamill *et al.*, 1992; Chakraborti *et al.*, 1998) studies using other plant types.

The first visible effect of colchicines was the delayed growth of treated plantlets. The initiation of growth occurred about 10 days after treatment, whereas untreated plantlets continue to grow without any delay. After about 40 days of growth, all colchicines treated plantlets had significantly shorter shoots than untreated plantlets.

Morphological differences between diploid and tetraploid Egyptian henbane: The morphological features of tetraploid and diploid plants were evaluated to determine whether they could be used as markers for identifying putative tetraploids in these species. In most cases, the first leaves of tetraploids had a distorted appearance, but the subsequent leaves appeared normal at the place of treatment. The body size of diploids and tetraploids were significantly different, tetraploids being stronger than diploids one (Fig. 2a-g). The epidermic cells of tetraploids as shown in Fig. 3a-d were bigger than diploids, so, that visual comparison was possible.



Fig. 2: Representative body size, flowers, seed sizes and leaves from diploid and colchiploid plants of Egyptian henbane (*Hyoscyamus muticus*) (a, b) body size, (c, d) relative size of flowers, (e, f) relative size of seeds and (g, h) leaves of diploid and tetraploid, respectively

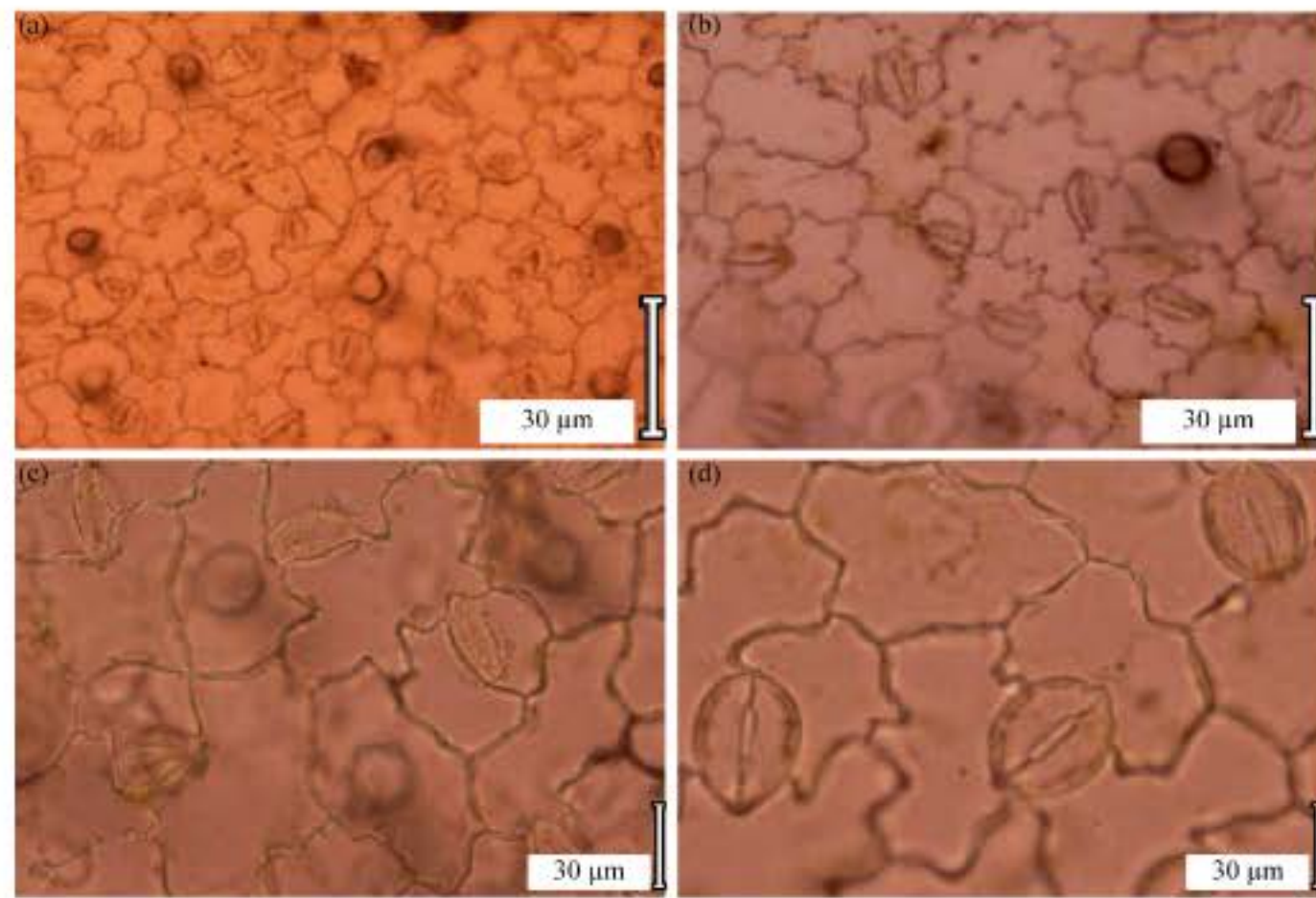


Fig. 3: The stomata frequency and sizes of diploid (a, c) and tetraploid (b, d) Egyptian henbane (*Hyoscyamus muticus*)

Flow cytometric profiles of colchicine-treated plants: All colchicine-treated plants, subjected to flow cytometry were classified as diploid, mixoploid and tetraploid, according to the peaks obtained by the flow cytometry (Fig. 4a-c). According to the classification of Koutoulis *et al.* (2005), mixoploids were graded base on the relative amounts of diploid and tetraploid nuclei. In grade 1, the number of diploid nuclei is more than tetraploids, whereas in grade 2, the number is equal. In grade 3, the situation is completely opposite and so, they are most similar to tetraploid plants.

In this study, the peak that represents the G_1 phase of the tetraploid cell cycle was located on channel 180, whereas for control this peak was on channel 90. The G_2 peak of tetraploids is approximately on channel 360, while diploids showed a G_2 peak on channel 180. Mixoploid plants had a peak on channel 360 with $8n$ nuclei that corresponds to the G_2 phase of tetraploid cell cycle (Fig. 4a-c). Flow cytometry was also individually performed for stems and roots in few diploid plants. The stem histograms showed no significant difference with those of leaves, while the root histogram showed a higher difference. In root samples, the proportion of G_1 and G_2 nuclei were nearly balanced and had higher debris, while in leaf and stem, the proportion of G_2 nuclei was higher than those of G_1 nuclei and had a small amount of debris. Also, no $8n$ nuclei peak in the flow cytometry histogram of control root samples was observed, whereas leaf and stem samples of this plants, in some cases showed a small

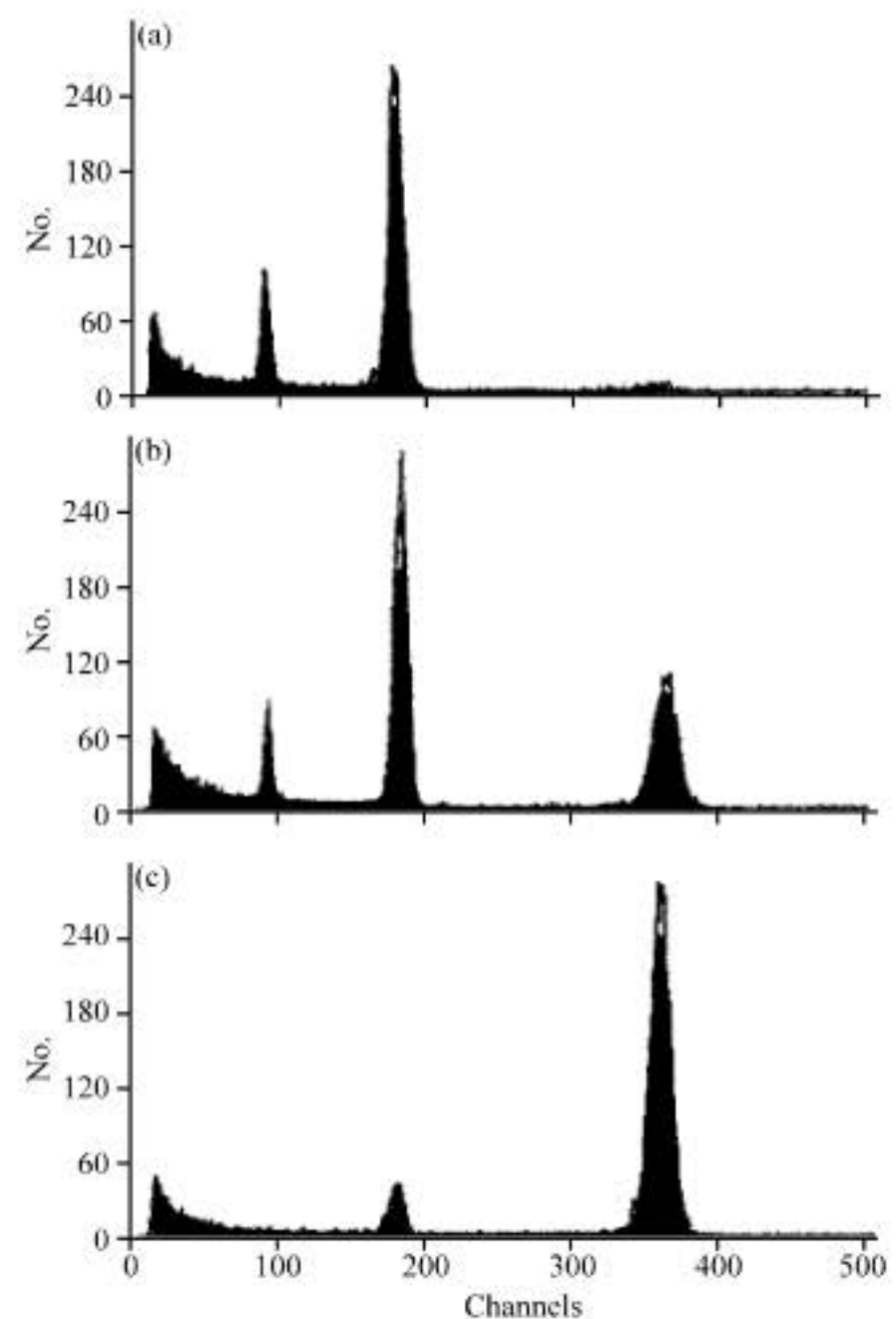


Fig. 4: Flow cytometric histograms representing Egyptian henbane (*Hyoscyamus muticus*) seedling with (a) a diploid profile ($2n$), (b) mixoploid ($2n + 4n$) profile and (c) a tetraploid ($4n$) profile

percentage of 8n nuclei peaks. This result shows that endoreduplication possibly does not occurred in the root of this species.

In this experiment, the most of treated plantlets were affected by the time of exposure and the concentration of applied colchicine and therefore, there were only two diploid plants after treatment. Out of 50 plantlets, five pure tetraploid plants were seen and the rest of them were mixoploids which were mostly in grade 3.

Regarding to the mixoploidy production in colchicines treated meristems and remaining of diploid cells that proliferate at higher rates than tetraploid ones (Mergen and Lester, 1971) it is possible that selected plants, partially or totally reversed to the diploid condition after successive cell division cycles. For this reason, stability analysis by flow cytometry was conducted in C_1 and C_2 generations. It was also found that all of the peak values were nearly twice that of the diploid control, indicating the stability of tetraploids.

Comparison of chloroplast numbers: Increased numbers of chloroplast in the stomata guard cells of tetraploid plants compared to diploid ones is another consequence of polyploidy induction (Gu *et al.*, 2005), which was quite evident in the present study. The average number of chloroplast in stomata guard cells of diploid plants were 30, whereas tetraploid plants produced by colchicine treatment exhibited about 55 chloroplasts in the mentioned cells (Fig. 5).

Table 1: Yield comparison (wet weight, dry weight and percentage of dry matter (measured as Mean \pm SE) between diploid and tetraploid *Hyoscyamus muticus*

Characteristics	Diploid	Tetraploid	Increase tetraploid (%)
Wet weight (g plant ⁻¹)	91.35 \pm 6.20b	125.08 \pm 11.70a	36.91
Dry weight (g plant ⁻¹)	17.25 \pm 1.01b	20.18 \pm 0.70a	16.95
Percentage of dry mater	18.09 \pm 1.21a	16.14 \pm 1.03b	-14.63

Within each row Mean values followed by the same letter(s) are not significantly different at the p = 0.05 level by students t-test

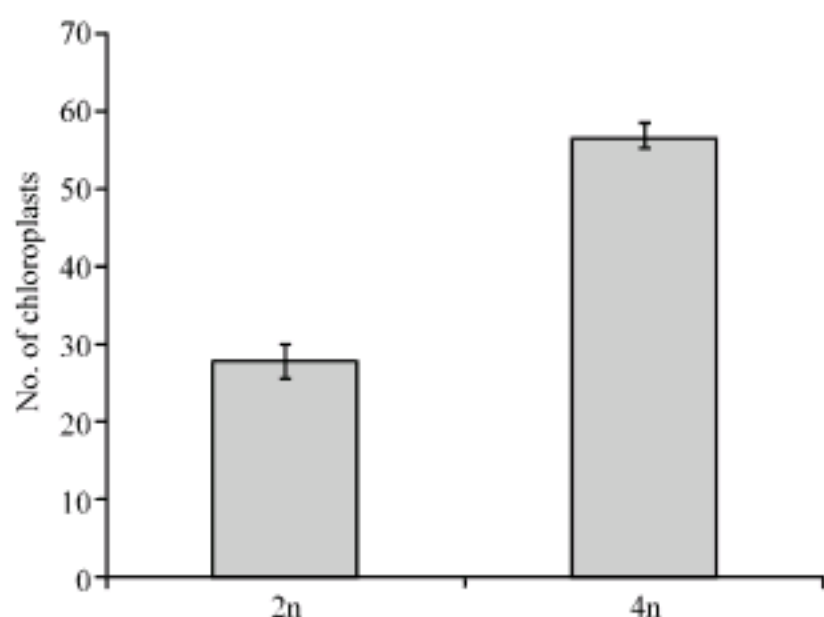


Fig. 5: Histogram comparison of average number of chloroplast between diploid and tetraploid cells

Comparison of yield components: Wet and dry weight was increased by tetraploid induction (Table 1), as expected. Although, an increase in wet weight was noticeable in the present study, a 17% increase of dry weight in tetraploids compared to diploids is quite in agreement by Lavania (1986) Lavania and Srivastava (1991).

DISCUSSION

Solid, stable autotetraploids as well as mixoploids were produced by treating Egyptian henbane plantlets with colchicine. Increased size of stomata guard cells is one of the general effects of tetraploidy induction in the Egyptian henbane, as shown in Fig. 3. This is actually a genetic effect which can be seen in all organs, such as seeds, flowers, leaves and shoot. Visual observations also indicated strong correlation between increased polyploidy level and bigger vegetative organs, for those plantlets grown in greenhouse. Similar results also had been observed in other studies by Lavania (1986) and Lavania and Srivastava (1991). Stomata characteristics (stomata size, stomata frequency, chloroplast number) previously have been used as useful parameters for distinguishing ploidy level (Cohen and Yao, 1996; Compton *et al.*, 1996; Gu *et al.*, 2005). As for this day, no reports of a relationship between stomata chloroplast number and ploidy level in Egyptian henbane have been made. This study showed that stomata characteristics markedly differed between diploid and tetraploid plants. Thus, as a simple and efficient method, the stomata size and chloroplast number can be used as markers to identify the ploidy level of this plant before flow cytometric analysis.

Flow cytometric profile confirmed the production of tetraploids and mixoploids. Using colchicines for ploidy level change. Usually leading to mixoploidy (Roy *et al.*, 2001; Koutoulis *et al.*, 2005; Wan *et al.*, 1989; Thiem and Sliwiska, 2001). Since, the plant meristem consists of many cells, as well as obtaining tetraploids, it is possible to obtain mixoploids (chimeras consisting of diploid and tetraploid tissue) after colchicine treatment (Koutoulis *et al.*, 2005). However, this agent works effectively only on dividing cells, thus polyploidization generally does not equally occur in all explant cells, leading to the occurrence of mixoploids and chimeras (Wan *et al.*, 1989). In flow cytometry profile of root samples, the proportion of G_1 and G_2 nuclei were nearly balanced, while in leaf and stem, the proportion of G_2 nuclei was higher than those of G_1 . This higher proportion of 4n nuclei, as mentioned by Thiem and Sliwiska (2001) could be due to higher cell cycle activity or endoreduplication, which often occurs in older plant

tissue. Biradar *et al.* (1993) indicated that nuclear DNA amounts vary in somatic tissues due to endoreplication resulting to polyploid nuclei. Presence of an inhibitor agent could be another possible explanation for high proportion of nuclei in G₂ phase of cell cycle in these species. Tambong *et al.* (1998) reported that colchicine-treated plantlets had nuclei arrested at all the three phases suggesting that the cells were actively dividing with significant amounts of the cell population at G₂ phase. Vera *et al.* (1994) reported that quiescent pericycle cells of transgenic tobacco were developmentally arrested at G₂ and rapidly progressed into M phase upon mitogenic stimulation with colchicine. Since, key facts with regards to the physiology of this plant are unknown, however, basic physiological studies are still required to understand this stringent process in *Hyoscyamus muticus*. To our knowledge, this is the first estimation of genome size for *hyoscyamus muticus*. The information provided in this study may be helpful in genome research as well as in study of the relationship between DNA content, plant physiology and ecology.

A common model for explaining changes, originating from tetraploid induction, relies on the assumption that the lower ratio of nuclear membrane to chromatin (the volume of tetraploid cells is typically about double and their surface area is about 1.5 times), results in more chromatin coming into contact with the nuclear membrane, thereby enhancing gene activity, improving water relationships, hormonal status and photosynthetic rate on a per cell basis (Lavania, 2005), coupled with enlarged cell size, all of these factors could have a positive impact on secondary metabolism and the production of active phyto-pharmaceuticals (Dhawan and Lavania, 1996). Thus, the measurement of alkaloids and its possible raising should be considered in polyploidy induction.

Male sterility was a visible effect of tetraploid induction of *H. muticus*. Although, the seed is not the ultimate economic product in this species, it is still required for propagation. Therefore, if seeds are required for multiplication, it is possible to search those genotypes with high fertility as reported by Lavania (1986). The flower structure of the Egyptian henbane is as heterostylous and so open pollination leads to increased heterozygosity, resulting bivalent formation in meiosis and high fertility.

The overall conclusion is that the method used is reliable for routine *in vivo* production of polyploids in Egyptian henbane. And this study showed that flow cytometry can be used as an efficient method for screening of *H. muticus* plant with different ploidy level and the results of flow cytometry and morphological

characteristics paralleled to each other. The positive and significant correlation between ploidy level and biomass production, is economically important for medicinal plants, such as the Egyptian henbane.

ACKNOWLEDGMENTS

This study was carried out with the financial assistance of the Institute of Biotechnology and the Deputy Research of Ferdowsi University in Mashhad-Iran.

REFERENCES

- Biradar, D.P., A.L. Rayburn and D.G. Bullock, 1993. Endopolyploidy in diploid and tetraploid maize (*Zea mays* L.). *Ann. Bot.*, 71: 417-421.
- Chakraborti, S.P., K. Vijayan, B.N. Roy and S.M.H. Qadri, 1998. In vitro induction of tetraploidy in mulberry (*Morus alba* L.). *Plant Cell Rep.*, 17: 799-803.
- Cohen, D. and J.L. Yao, 1996. In vitro chromosome doubling of nine *Zantedeschia* cultivars. *Plant Cell Tiss. Organ Cult.*, 47: 43-49.
- Compton, M.E., D.J. Gray and G.W. Elmstrom, 1996. Identification of tetraploid regenerants from cotyledons of diploid watermelon cultured *in vitro*. *Euphytica*, 87: 165-172.
- Dhawan, O.P. and U.C. Lavania, 1996. Enhancing the productivity of secondary metabolites via induced polyploidy. *Euphytica*, 87: 81-89.
- Dolezel, J., P. Binarova and S. Lucretti, 1989. Analysis of nuclear DNA content in plant cells by flow cytometry. *Biol. Plant*, 31: 113-120.
- Galbraith, D.W., K.R. Harkins, J.R. Maddox, N.M. Ayres, D.P. Sharma and E. Firoozabady, 1983. Rapid flow cytometric analysis of the cell cycle in intact plant tissues. *Science*, 220: 1049-1051.
- Gao, S.L., D.N. Zhu, Z.H. Cai and D.R. Xu, 1996. Autotetraploid plants from colchicine-treated bud culture of *Salvia miltiorrhiza* Bge. *Plant Cell Tiss. Organ Cult.*, 47: 73-77.
- GU, X.F., A.F. Yang, H. Meng and J.R. Zhang, 2005. In vitro induction of tetraploid plants from diploid *Zizyphus jujube* Mill. cv. Zhanhua. *Plant Cell Rep.*, 24: 671-676.
- Hamill, S.D., M.K. Smith and W.A. Dodd, 1992. In vitro induction of banana autotetraploids by colchicine treatment of micropropagated diploids. *Aust. J. Bot.*, 40: 887-896.
- Hassawi, S.D. and G.H. Liang, 1991. Antimitotic agents: Effects of double haploid production in wheat. *Crop Sci.*, 31: 723-726.

- Joachimiak, A., A.E. Kula, A. Sliwiska and S.K.A. Sobieszczan, 2001. C-banding and nuclear DNA amount in six *Bromus* species. Acta Biol. Crac. Ser. Bot., 43: 105-115.
- Koutoulis, A., A.T. Roy, A. Price, L. Sheriff and G. Leggett, 2005. DNA ploidy level of colchicine-treated hops (*Humulus lupulus* L.). Sci. Hortic., 105: 263-268.
- Lavania, U.C., 1986. Genetic improvement of Egyptian henbane, *Hyoscyamus muticus* L. through induced tetraploidy. Theor. Applied Genet., 73: 292-298.
- Lavania, U.C. and S. Srivastava, 1991. Enhanced productivity of tropane alkaloids and fertility in artificial autotetraploids of *Hyoscyamus niger* L. Euphytica, 52: 73-77.
- Lavania, U.C., 2005. Genomic and ploidy manipulation for enhanced production of phyto-pharmaceuticals. Plant Genet. Resour., 3: 170-177.
- Mergen, F. and D.T. Lester, 1971. Colchicine induced polyploidy in *Abies*. For. Sci., 7: 314-319.
- Pal Bais, H., V.M. Loyola-Vargas, H.E. Flores and J.M. Vivanco, 2001. Root-specific metabolism: The biology and biochemistry of underground organs. *In vitro* Cell Dev. Biol. Plant, 37: 730-741.
- Panda, D., B.L. Goode, S.C. Feinstein and L. Wilson, 1995. Kinetic stabilization of microtubule dynamics at steady state by tau and microtubule binding domain of tau. Biochemistry, 34: 11117-11127.
- Poehlman, J.M., 1987. Breeding Field Crops. 3rd Edn., Van Nostrand Reinhold, New York.
- Roy, A.T., G. Leggett and A. Koutoulis, 2001. *In vitro* tetraploid induction and generation of tetraploids from mixoploids in hop (*Humulus lupulus* L.). Plant Cell Rep., 20: 489-495.
- Tambong, J.T., V.T. Sapra and S. Garton, 1998. *In vitro* induction of tetraploids in colchicine-treated cocoyam plantlets. Euphytica, 104: 191-197.
- Thiem, B. and E. Sliwinska, 2001. Flow cytometric analysis of nuclear DNA content in cloudberry (*Rubus chamaemorus* L.) *in vitro* cultures. Plant Sci., 164: 129-134.
- Vera, P., C. Lamb and P.W. Doerner, 1994. Cell-cycle regulation of hydroxyproline-rich glycoprotein HRGPnt3 gene expression during the initiation of lateral root meristems. Plant J., 6: 717-727.
- Wan, Y., J.F. Petolino and J.M. Widholm, 1989. Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus. Theor. Applied Genet., 77: 889-892.
- Zehra, M., S. Banerjee, A.A. Naqvi and S. Kumar, 1998. Variation in growth and alkaloid production ability of the hairy roots of *Hyoscyamus albus*, *H. muticus* and their somatic hybrid. Plant Sci., 136: 93-99.