

Induction and identification of polyploidy in basil (*Ocimum basilicum* L.) medicinal plant by colchicine treatment

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Abstract

Basil (*Ocimum basilicum*) is one of the important medicinal plant species. In order to produce an autotetraploid population of basil (*Ocimum basilicum*) by colchicine, different concentrations (0.00, 0.05, 0.10, 0.20, 0.50 and 0.75%) and four treatment methods were examined (seed, the growing point of seedlings at the emergence of cotyledone leaves stage and emergence of true two type leaves stage, and root treatment) to determine the best treatment for the induction of tetraploid plants. Autotetraploid 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. The induced tetraploids in basil was accompanied by larger stomata and pollen grains, increase in chloroplast number in guard cells and decrease in stomata density, compared to diploid control plants. In order to distinguish the induced colchicine tetraploid plants from the diploids, morphological changes and techniques as stomata size, number of chloroplasts per guard cell, pollen grain diameter and flow cytometry were considered and proved that these methods are suitable, quick and easy methods for identification the ploidy level of *Ocimum basilicum* in various stages of the plant development of these species and among this methods flow cytometry as found to be the most efficient method for detecting induced changes in ploidy level.

Keywords: Colchicine; Cytological traits; Flow cytometry; *Ocimum basilicum*; Tetraploidy induction.

Introduction

Basil (*Ocimum basilicum* L.) is an annual plant belongs to the *Lamiacea* family which has been grown for its essential oil. The essential oil of basil is used to flavor foods, dental and oral products in fragrances, and in medicines (Omidbaigi, 2005; Simon et al., 1984). 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). Chromosome duplication using

colchicine has long been used in plant breeding programs. In the most plants, artificial polyploidy is often accompanied by increased cell size, leading to larger reproductive and vegetative organs (Adaniya and Shira, 2001). The induction of artificial polyploidy may prove useful in increasing the production of important medicinal compounds (Dhawan and Lavania, 1996). There are different methods to induction of polyploidy in plants 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 and tissue culture techniques (Roy et al., 2001), which revealed that the most efficient of treatment methods and colchicine concentrations to induction of polyploidy, are species-specific. Natural polyploidy is present within the Lamiaceae, for example within *Thymus* (Lopez-Pujol et al., 2004), *Glechoma* (Wide'n and Wide'n, 2000) and *Lavandula* (Upton and Andrews, 2004). *Ocimum basilicum* L. frequently has $2n = 2x = 48$ (Ryding, 1994; Xing-Hua et al., 1984). Chromosome number estimates in *O. basilicum* suggest that it is an ancient polyploid species. Basic chromosome number for both species has been suggested to be $x = 12$ with probable origin from primitive base number $x = 6$ through polyploidy (Mukherjee and Datta, 2005).

In breeding programs, it is important to determine the ploidy level in a quick and simple way in various stages of plant development. It has long been known that the classic method of counting chromosome number in mitotic cells of root-tips is an accurate procedure to determine the ploidy level, but it is time-consuming and requires much experience. Therefore, attempts have been made to find indirect methods for ploidy determination that these approaches can be used satisfactorily. In many of plant species, there are correlation between ploidy level and cytogenetic characteristics such as chloroplast number in guard cells, size of stomata cells, stomata density and pollen grain diameter. In pepper, stomata density and especially the number of chloroplast in guard cells seemed to be reliable for the estimation of ploidy level (Abak et al., 1998). Stomata size and changes in plant morphology were found useful indicators in the primary screening for new ploidy level in M_1 -generation of *Viola* \times *Wittrockiana* Gams (Ajalín et al., 2002). Among other cells that have been used to determine chromosome number by differences in size are the guard cells of the stomata which appear to be less influenced by environmental factors than many other cells of the plant. Furthermore in using by colchicine the size of the pollen offers a quick and fairly accurate way to locate polyploidy plants (Blakslee and Avery, 1937). Flow cytometry (FCM) would appear to offer advantages for plant breeding and might be used especially a very rapid and easy marker for ploidy manipulation such as polyploidisation by colchicine (Costich et al., 1993).

We conducted this experiment in order to obtain tetraploid plants of *Ocimum basilicum* using colchicine-treated diploid basils and induce further polyploidy in basil to produce higher oil-yield and more productive variety of basil. Furthermore, the present study aimed to identify morphological and cytological traits, such as stomata and guard cell size, number of chloroplasts per guard cell, pollen grain diameter; whose performance depend on plant ploidy and can be used for indirect identification of diploids and tetraploids in basil 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 basil (*Ocimum basilicum*) that were provided from the plants cultivated in experimental station of the Aromatic Plants Department of Corvinus University in Budapest, Hungary, were used to produce an autotetraploid population. Plants were grown under uniform condition at research station of Tehran university, suburb of Karaj, Iran (latitude 50° 57' E, longitude 35° 48' N) and experiments were conducted in 2008.

Doubling procedure

Our investigation consisted of following three experiments in order to induction of tetraploidy in basil.

Seed treatment

100 seed for each treatment, with three replications, were soaked in various concentration of colchicine (Sigma-Aldrich, St. Luis, Mo.) aqueous solutions (0, 0.05, 0.1, 0.2, 0.5 and 0.75% (w/v) pH=6) and 2% dimethyl sulfoxide (DMSO) and Tween '20' as a surfactant, at room temperature on a shaker at 150 r.p.m. for the periods of 6, 12, 24 and 36 h. Then the treated seeds were rinsed thoroughly with distilled water and planted carefully in the seedling pots filled with a mixture of one part mold leaf and one part sand and one part loam soil in greenhouse under normal condition (16 h light period, temperature 25-27 °C and humidity 65% and 60% sun light).

Tip meristem treatment of the seedlings

Seeds were planted in seedling pots filled with soil and farm yard manure. The apical meristem of 500 seedling at the emergence of cotyledone leave stage and 500 seedling at the emergence of true two type leaves stage were treated for 3 subsequently day by dropping method using an aqueous solution of various concentrations of colchicine (0, 0.1, 0.2, 0.5 and 0.75% (w/v) pH=6). Plants were covered with polyethylene plastic during treatment with colchicine. Subsequently the seedlings were thoroughly rinsed with water and planted in the field when they reach to 8 leaves stage.

Root treatment

when the plants reaches to 4-6 leaf stage, after washing the roots of plants with running water, 2-3 cm of root tips immersed in colchicine solutions (0, 0.05, 0.1, 0.2, 0.5 and 0.75% (w/v), pH=6), during periods of 6, 12, 24, 36 and 48 h.

One month after treatment, the treated seedlings were examined morphologically and also screened for any ploidy changes using examination of stomata and chloroplast (in guard cells) counts and finally flow cytometry.

Ploidy analysis

Selection of tetraploid plants was done on the basis of morphology, pollen size and followed by a selection on size of stomata and guard cells measurement, chloroplast number in guard cells and finally flow cytometry. The putative tetraploids were examined two months later to validate ploidy stability.

Pollen grain diameter

During flowering, another selection was applied among treated plants on the basis of the pollen characteristics. From each of control plants and putative polyploid plants, 5 plant and from each plant a few anthers were sampled, fixed in 3:1 absolute ethanol: acetic acid solution for 24 h, and stored in 70% ethanol at 4 °C. For examination of pollen size in diploid and tetraploid plants, the anther should be macerated to release pollen grains. After staining with aceto-orcein 2% (Funamoto et al., 2006). Microscope examination (at 1000 × magnification) of 100 pollen grains from each of control plants and putative polyploid plants, was carried.

Size and density of stomata and guard cells measurement

To estimate of suitability of the stomata size and density as criteria for determination of ploidy level in basil, stomatal measurement was done after morphological and pollen grain examination between diploid and autotetraploid regenerant plants. For this purpose, 10 plants of diploid control and 10 plants of putative tetraploids 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 (abaxial side) 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 (Hamill et al., 1992). After drying the polish, it was removed with a tip forceps then placed on a glass slide and observed through the light microscope (BX50; Olympus Optical Co. Ltd.) at 400 × magnification (for study stomata density) and 1000 × magnification (for study of stomata and guard cells measurement and chloroplast number).

Chloroplast number per guard cells

For study of the chloroplast number in the stomatal guard cells, samples of epidermal layer from abaxial side of diploid control plants and potential tetraploid plants's 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). The stomata characteristics and chloroplast number, were measured in the middle leaves from each of the tested branches both in control and putative tetraploids.

On the lower epidermis, 100 measurements of each of the parameters taken, in control and putative tetraploids, in five replications and each replication represented a different plant. Then ploidy level of all studied plants, putative polyploids, was confirmed by flow

cytometry. One-way ANOVA carried out to determine the differences in stomata size and density and chloroplast number between tetraploids and their respective controls.

Flow cytometry (FCM)

In order to confirmation of ploidy level in putative tetraploids, cell nuclei were isolated from upper young leaves and stained with 4', 6-diamino-2-phenylindole (DAPI) according to the manufacturer's instructions. The relative fluorescence intensity of DAPI-stained nuclei was measured by a Partec PA-I flow cytometer (Partec GmbH, Münster, Germany), provided with HBO-lamp, UV-laser; for DNA-content estimations.

Nuclei were released from 1 cm² of leaf tissue. For FCM analysis, rose (*Rosa hybrid a cv. Akito* 2n=4x=28) (khosravi et al., 2008) and untreated *Ocimum basilicum* nuclei were used as an internal standard, by chopping with a sharp razor blade simultaneously in the petri dish in 400 µl of extraction buffer (CyStain-UV precise Partec). For removing the phenolic impurities, PVP-40 was added to the buffer. Then the suspension was filtered into small tubes through a 30 µm Celltris disposable nylon filter and were kept on ice for 10 min. For the coloration of these samples, 1.6 ml of DAPI staining solution (4', 6-diamino-2-phenylindole) added and samples were put into the flow cytometer and the DNA histograms were created. The DNA content of each sample was evaluated in 10000 nuclei. Ten leaf samples of diploid control plants and putative tetraploids were analyzed alternately.

The experiment was repeated twice using different plants on different days.

Chromosome number determination

Before polyploidy was induced, the chromosome number of the primary materials was determined in root tips of germinated seeds to confirmed number of chromosome in diploid materials (2n=2x=48). Root tips (~2-3 mm), from actively growing roots were excised (about 19 p.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 30-36 h. The fixative was prepared by mixing equal volume of 1% chromic acid and 4% formaldehyde (10% formalin) just before using. After rinsing twice with distilled water, root tips were hydrolyzed with 1 N NaOH at 60 °C for 13 min and then rinsed in distilled water. Excess water was removed by blotting paper and the roots were stained for 16 h in Aceto-Iron-Hematoxylin 4% at 30-32 °C (Agayev, 2002). Then 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 (Olympus BX 50) at a magnification 1000X. At least twenty meristems were analyzed.

Statistical analysis

The statistical differences among means of the traits of control and autotetraploid plants were computed by the *t*-test and analysis of variance (ANOVA) by SPSS software.

Results

Seed treatment

The material however appeared to be extremely sensitive to the chemical because of almost all treated materials died in the germination and seedling stages except concentration 0.05 that about 10 plant survived that all of them proved to be diploid.

Root treatment

Root treatment didn't have any effects on elevation of ploidy level of the treated basil plants and after the treatment, the plants grew normally and no tetraploid plant was recognized by cytological study and flow cytometric analysis.

Tip meristem treatment of seedlings

Following the colchicine treatment, the plants were classified according to the chromosome number. Treatment of the seedlings at the emergence of true two type leaves didn't have any effects on ploidy level of the treated plants and all of them were diploid. Some of the plants that treated at the emergence of cotyledone leave stage were tetraploid. Distribution of plantlets that treated in this stage, with different ploidy is presented for each treatment in Table 1. Of the 5 colchicine levels applied, 0.1 and 0.5% of drug treatments appeared to be effective on the production of tetraploids and could be further used in breeding programs. This investigation demonstrated that, antimiotic agent of colchicine in basil can increase the ploidy level. In this study, the most efficient treatment method for chromosome doubling in *Ocimum basilicum* was seedling treatment in the emergence of cotyledone leave stage in concentration 0.5% (w/v) colchicine solution by dropping method.

Table 1. Tetraploid and mixoploid production (%), survival rate (%) after colchicine treatment (C_0 generation) of 500 basil seedlings at the emergence of cotyledon leave stage.

Colchicine concentration % (w/v)	Survival rate (%)	Tetraploid (%)	Mixoploid (%)
0	92	0	0
0.05	76	0	16
0.1	53	3	38
0.2	49	1	41
0.5	21	8	11
0.75	9	0	5

Confirmation and stability of population

On basis of this investigation, for the practical use of colchicine to induce polyploidy in *O. basilicum*, we recommended concentrations between 0.1 and 0.5% at the emergence of cotyledone leaves stage. Immediately after the treatment, the treated plants (C_0 plants) were examined for presence of different morphological characteristics. Putative mixoploids was selected visually. On the basis of changes on some of the characteristics of plant (Figure 1)

the first leaves appearing after treatment, revealed that 123 plants, or 59% of survived plants, (of 500 treated seedlings at the emergence of cotyledone leave stage) were presumably polyploid with higher ploidy-level that they were selected as potential mixoploid for cytological examinations such as size of stomata and guard cells and stomata density per unit of leaf surface, chloroplast number in guard cells, pollen size and finally flow cytometry (FCM) analysis. These selected plants were transplanted into the field along with the diploid control and given optimal culture care. Among putative tetraploid plants, 12 plant (9% of mixoploid plants) confirmed to be autotetraploid whereas remaining were diploid and mixoploid. The seeds obtained from isolated pollination C_0 tetraploid plants, was sown to order production of C_1 population and production of enough tetraploid population.

Distinguishing the induced colchicine tetraploid plants

In order to distinguish tetraploid plants from the diploids, characteristics as changes in treated plants morphology, stomata and guard cells size and density, number of chloroplasts per guard cells, pollen grain diameter and use of flow cytometry were considered.

*Morphological differences between diploid and tetraploid *O. basilicum**

More than 60% of the seedlings after treatment, became dark green and thick. The apical meristem remained stunted. The morphologically the C_1 generation tetraploid plants, were very similar to those of the C_0 generation. Morphological differences were observed between the diploid and tetraploid plants. The leaves of the tetraploid plants were dark green, more dissected and dentated at their margins and more thickness (Figure 1) and the tetraploids were more sturdier and had a little larger seeds than diploids (Figure 1), in the most of tetraploid plants, when compared with diploid plants.



Figure 1. Diploid (2x) (Left) and tetraploid (Right) (4x) leaf of *Ocimum basilicum*.

Pollen grain diameter

There was a clear differences between pollen size in diploid and tetraploid basils so that all or the majority of pollen grain in tetraploids were bigger than those from diploid plants (Table 2). Mean pollen diameter was 55% larger in tetraploids compared with diploids (Figure 2). The significant variation was not observed in pollen shape in two ploidy levels.

Most of the plants with bigger pollen grains proved to be tetraploid by stomatal measurement, chloroplast number and flow cytometry technique.

Table 2. Size of stomata, guard cells and; stomata density and chloroplast number in guard cells in diploid (2x) and confirmed tetraploid (4x) by FCM.

Characteristic	Diploid	Tetraploid
	mean	mean
Stomata length (μm)	$8.70 \pm 0.47^{\text{b1}}$	$16.45 \pm 0.97^{\text{a}}$
Stomata diameter (μm)	$2.97 \pm 0.17^{\text{b}}$	$11.99 \pm 0.93^{\text{a}}$
Stomata frequency / (mm^2)	$20.80 \pm 0.61^{\text{a}}$	$7.58 \pm 0.22^{\text{b}}$
Guard cell length (μm)	$13.88 \pm 0.78^{\text{b}}$	$24.62 \pm 0.75^{\text{a}}$
Chloroplast/ guard cell	$12.80 \pm 0.28^{\text{b}}$	$25.8 \pm 0.33^{\text{a}}$
Pollen diameter (μm)	$23.6 \pm 0.58^{\text{b}}$	$36.8 \pm 0.66^{\text{a}}$

¹Within each column values followed by different letters are significantly different ($P < 0.01$), by T- students.

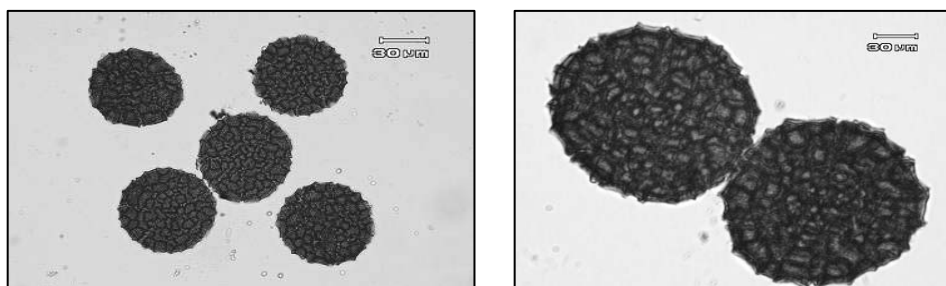


Figure 2. Difference in size of pollen between normal diploid (2x) and colchicine induced tetraploid (4x) basil. Left: haploid pollen; normal: diploid pollen; increased size.



Figure 3. Difference in stomata size and density between diploid and tetraploid basil. Left: diploid (2X); Right: tetraploid (4X).

Stomata counts and density

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 the the ploidy level, so stomata in autotetraploids were larger but fewer (in area unit of leaf) compared to the control parents.

The stomata length in tetraploids was about 89% larger than in diploids (Table 2). Also the stomata diameter in tetraploid plants was unexpectedly higher (more than tree time over diploids plants). The association between the density of stomata and the ploidy level was negative, where lower density values were obtained in tetraploid plants (7 (mm)^2) and stomata frequency in diploid plants was 63% more than tetraploids. Mean of guard cell length in tetraploids were about 77% more than diploids (Table 2).

Chloroplast number in guard cells

There was a clear difference between the chloroplast number in guard cells between diploid and tetraploid plants (about twice than diploid plants) (Figure 4). In guard cells of diploid plants, 12.80 Chloroplast were found but in tetraploids about 25.8 Chloroplast were found (Table 2).

One-way ANOVA, however showed significant differences ($P < 0.01$) in the stomata length and diameter, stomata density and chloroplast number in guard cells and pollen diameter between the control diploids and tetraploids (Table 2).

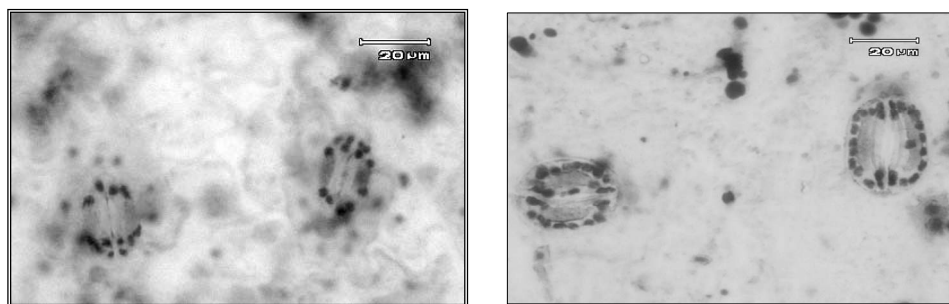


Figure 4. Difference in chloroplast number in guard cell between diploid and tetraploid basil. Left: diploid (2X); Right: tetraploid (4X).

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 G_1 nuclei of a plant with known ploidy with that of unknown sample (Figure 5). Tetraploids had double the amount of DNA confirming autotetraploidy and in this investigation, an important advantage of FCM over other methods is the ability to identify mixoploids and greatly increased the efficiency of screening ploidy levels at critical steps in the experiment.

Chromosome number determination

The diploid chromosome number was 48 (Figure 6). Due to the small size of chromosomes and low frequency of metaphase cells in root tips of *Ocimum basilicum*, counting of chromosome is difficult and time-consuming.

Discussion

Some of the plants that treated at the emergence of cotyledon leave stage, were tetraploid. This is supported by the findings of Srivastava and Srivastava (2002) in *Helianthus annuus* L. In using by colchicine the size of the pollen offers a quick and fairly accurate way to locate polyploidy in plants (Blakslee and Avery, 1937). Majority of pollen grain in tetraploids were bigger than those from diploid plants, comparable results have been reported for *Helianthus annuus* L. Var. morden (Srivastava and Srivastava, 2002), *Carum carvi* L. (Dijkstra and Speckmann, 1980). 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 plants supported by the findings of several researcher such as Gao et al., (2002) in *Scutellaria baicalensis* and Thao 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.

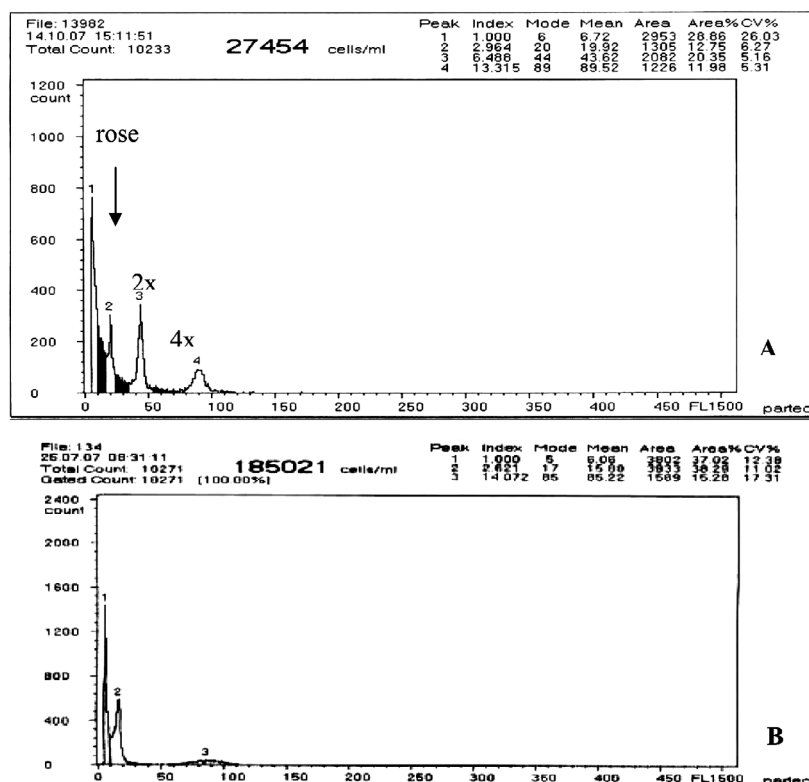


Figure 5. Flow cytometric histograms of (A): rose (Internal standard) (pick 2), diploid basil (pick 3) and tetraploid basil (pick 4) (B): Mixoploid plant (pick 2, 3).

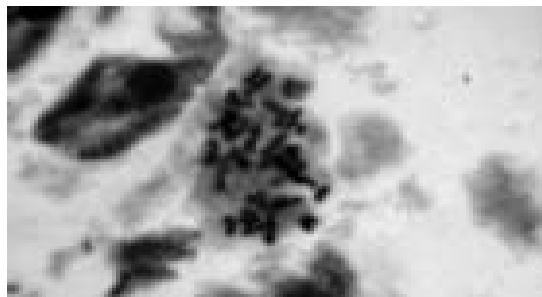


Figure 6. Estimation of the chromosome number in a diploid (2x) basil.

Clear differences between the chloroplast number in guard cells between diploid and tetraploid plants, is in agreement with results of took place on *Cucumis melo* L. (Yetisir and Sari, 2003). Although expensive, flow cytometry analysis of nuclear DNA content is being increasingly used for high-through ploid screening. This is supported by the findings of several researchers such as Beck et al., (2003), Madon et al., (2005). The reported diploid chromosome number in this report, ($2n=2x=48$), already reported by Ryding (1994) and Xing-Hua et al., (1984). Counting of chromosome is difficult and time-consuming furthermore this method is not suitable for detection of mixoploidy in tissues with low proportion of dividing cells such as leaves (Uhlík, 1981).

The results revealed that morphological changes in treated plants were not reliable and accurate indicators for identification of tetraploid plants but selection on pollen size, 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 *Ocimum basilicum* 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 and pollen diameter 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 (abnormality), pollen diameter and stomata counting and examination of chloroplast number in guard cells, is an effective method in primary screening of tetraploid *Ocimum basilicum* plants in polyploidisation breeding program and it is recommended flow cytometry to be used for accurate identification of ploidy level in mixoploid plants of basil.

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