Vol.66: e23220186, 2023
https://doi.org/10.1590/1678-4324-2023220186
ISSN 1678-4324 Online Edition

*Article - Agriculture, Agribusiness and Biotechnology*

# Chromosome Doubling in Endemic Iris sari Schott ex Baker with *In Vitro* Colchicine Treatments

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Editor-in-Chief: Bill Jorge Costa
Associate Editor: Bill Jorge Costa

Received: 22-Mar-2022; Accepted: 22-Dec-2022

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**HIGHLIGHTS**

- This the first report of the *in vitro* chromosome doubling of endemic *Iris sari* Schott ex Baker.
- In this study, 2 different colchicine doses and 3 different treatment times are testing.
- Significant structural differences are obtained in the stomatal measurements of tetraploid plantlets.
- As a result of the applications, 3 tetraploids are obtained from 45 plantlets analyzed.

**Abstract:** *Iris sari* Schott ex Baker, an endemic plant, has a natural distribution in Turkey. The plant, also known as “Ana kurtkulağı, Bahar çiçeği” among the people, has showy flowers. Chromosome doubling in the plant was successfully performed with colchicine applied to the micro bulbs of the *I. sari* obtained *in vitro*. The different colchicine doses and treatment times tested with *I. sari* were: 0.1 and 0.5% (2, 4, and 6 hours). The resulting polyploid plantlets were determined by flow cytometric analysis. The highest tetraploid plant yield was observed after 4 h of treatment with 0.1% colchicine. From a total of 45 surviving plantlets, 3 were detected. At the end of the applications, the control and other groups were compared in terms of the viability rates of plants. After 2 subcultures of *in vitro* conditions, the highest viability rate in plantlets was observed with the control group and 0.1% colchicine for 4 h application, 62.97%, and 67.12%, respectively. Also, *I. sari* putative tetraploid plantlets had fewer and larger stomata than the control, confirming the data. Although the best ratios in terms of stomata length and width (49.04 and 30.52 mm) were come off after 4 h of 0.5% colchicine treatment, tetraploid was not obtained as a result of this application.

**Keywords:** *I. sari* Schott ex Baker; endemic plant; *in vitro*; colchicine; chromosome doubling.

## INTRODUCTION

*I. sari* Schoot ex Baker, which is called “Ana kurtkulağı, Bahar çiçeği” in Turkish name, grows naturally in the region where Gaziantep, Çankırı, Amasya, Elazığ, Erzurum, Kayseri, Niğde, Bayburt regions are located in our country. The Iris genus, which is a member of the Iridaceae family, is a rhizome, bulbous, and rarely tuber. It is known that there are 80 genera and 2315 species belonging to the Iridaceae family in the

world. In addition, the accepted 389 Iris L. taxon is distributed and reported to be 1268 synonyms [1]. It has been reported Iris genus has a wide distribution area in the Northern Hemisphere [2]. The Iridaceae family is represented by 24 endemic species and 71 taxa in our country [3]. The distinctive and spectacular flower arrangements, colors, and leaf arrangements are indicative of the high availability of the flower industry. As used in medical and cosmetic fields, iris plants, which are frequently used in the ornamental plant sector, draw attention to those who are closely related to the breeders and the industry to develop new varieties. *I. sari* is quite showy flowers and blooming in April-June, the plant is a rhizome geophytes plant. The chromosome number of this diploid species is known as 2n=20.

Plant tissue culture appears as a powerful alternative technique used in the preservation and reproduction of plants, which are especially rare and difficult to reproduce with conventional methods. In addition to classical breeding studies, biotechnological methods used in combination with tissue culture techniques provide ease for breeders in obtaining polyploid plants. Polyploidy breeding is another breeding technique frequently, used especially in ornamental plants. In this method, the polymerization of the plant is prevented by interfering with the mitosis stage and thus the chromosomes are pulled to the poles in the anaphase stage [4]. Mitotic polyploidization studies in plants first started in the 1930s. Blakeslee and Avery, (1937) reported that has been obtained using colchicine of polyploidy plants, successfully [5, 6]. Colchicine inhibits mitosis by binding to tubulin, the protein subunit of microtubules, inhibiting the formation of microtubules and the polar migration of chromosomes. The result is a cell with twice the number of chromosomes [7, 8].

Polyploid plants; stem, leaf, flower, and surface areas are larger than diploid ones. These plants have larger cells and more chlorophyll content, attracting attention with darker and lively colors [9, 10]. Furthermore, polyploid plants grow vigorously the extreme environments, different climate conditions, subarctic regions, and high altitudes. Therefore, according to many researchers, polyploid species are more resistant to stress factors than diploids due to their morphological, physiological, and developmental differences [11]. According to Van Tuyl and Lim (2003), chromosome doubling is accepted as a source of evolution of answering plants, and breeders benefit from it for the domestication of certain genotypes [12]. In fact, in the last 50 years, there have been many chromosome doubling studies with fruit species such as banana [13], grapes [14], blueberry [15], and ornamental plants, such as *Lilium* spp.[16], *Cyclamen* spp. [17], *Alocasia* spp. [18], *Rhododendron* spp.[19], *Gerbera* spp.[20].

Polyploid plants are available obtained using different plant organs such as seed, flower buds, apical meristem, root, rhizome, and leaf [21, 22, 23, 24, 25, 26].

Polyploidy agents generally used to modify the plant shape are valuable tools to get bigger flowers. There are many different chemical agents, such as colchicine, oryzalin, and trifluralin, which are used for chromosome doubling. Some studies were used to detect a range of treatment concentrations and exposure times of the chemical agents [27]. Colchicine is one of the chemical agents commonly used for the promotion of polyploidy in plants [28]. Colchicine is a natural alkaloid obtained from the plant *Colchicum autumnale* [29]. Besides its low doses are used being as the treatment of some diseases in medicine, its high doses are used as a mutagen for chromosome doubling in plant science [30]. The mitotic effect of colchicine was first discovered by Allen (1936) on animal tissues [31]. Afterward, Eigsti 0.J. (1938) began colchicine application studies on plant tissues [32]. From 1930 to 1960, polyploidization studies were carried out mainly using seeds as explants, under ex vitro conditions [33].

Colchicine has been used successfully to induce the formation of polyploidy in several plant species orchids, roses, petunia, barley, cotton, calendula, citrus, cassava, etc. [34, 35, 26, 36, 37, 38, 39, 40]. Stadler and coauthors (1989) emphasized that different plant species may require very several effective concentrations of colchicine [41]. Evans (1955), Speckman and coauthors (1965) stated that stomatal length is an accurate indicator of polyploidy levels in many plants [42, 43].

It has been stated that flow cytometry (FCM) can be used as a fast and simple marker, especially in polyploidization studies with chemical agents such as colchicine [44]. For this reason, in recent years, due to its ease, speed and sensitivity, Flow cytometry has been the preferred method in ploidy analysis and has been used successfully [45, 46].

In the present study, colchicine effects were investigated to provide *in vitro* polyploidization in *I. sari*. Flow cytometry analysis was performed to determine the *in vitro* chromosome doubling and compared with the stoma characteristics of the leaves.

## MATERIAL AND METHODS

### Surface disinfection of plant materials

Immature capsules containing immature embryos (approximately 1 mm, length [47]) of *I. sari* were collected within Ahır Dağı - Kahramanmaraş province (Turkey) in May. The seed capsules containing immature embryos were washed in detergent and surface-sterilized for 1 minute in 70% (v/v) ethanol and then for 25 min. in 20% commercial bleach (Axion) adding 1-2 drops Tween-20 with continuous shaking. Finally, the seeds were rinsed with sterile distilled water and dried on drying sterile paper.

### Media sterilization and culture conditions

All media used in the present study were adjusted to pH 5.8 before autoclaving at 121ºC for 15 minutes. Cultures were placed in growth room conditions with 2500-3000 lux light intensity and maintained at a temperature of 25ºC. The explants were cultured in magenta containing 40 ml media. Each treatment tree was repeated and each magenta contained five explants.

*I. sari* capsule was dissected longitudinally with a sterile lancet and the immature embryos were taken out and then transferred to MS [48] medium containing 1.0 mg/L Thidiazuron (TDZ) + 0.5 mg/L naphthalene acetic acid (NAA) + 3.0% sucrose, 0.8% agar (Sigma) Petri dishes for multiplication. Micropropagated 0.5-1.0 cm long *in vitro* micro bulbs obtained from immature embryos were used as the initial explants. For the regeneration of micro bulbs were cultured in magenta.

### Treatment with colchicine and planting

When a sufficient number and length (1.0-2.0 cm) of micro bulbs became available, colchicine treatments were initiated. Colchicine is known to dissolve in dimethyl sulfoxide (DMSO) [8]. Therefore, the colchicine (Sigma) was dissolved in distilled water plus 2% (dimethyl sulfoxide) DMSO and prepared into a solution through filter sterilization (0.22 µm).

In this study, micro bulbs were excised from a culture medium and placed in Petri dishes including a 10 ml colchicine solution (Figure 1). The concentrations of colchicine dissolved in 1% DMSO used were 0.00, 0.10, and 0.50% and the duration of treatments was 2, 4, and 6 hours. For the control treatments, a group of micro bulbs was immersed in distilled water. After the treatment, the explants were washed three or five times with sterile water and dried on fresh filter paper. Then clean micro bulbs with sterile water cultured on MS medium supplemented with 1.0 mg/L TDZ + 0.5 mg/L NAA. The number of explants per treatment was 15. After two subcultures, the treated micro bulbs were determined either survival rate or multiplication rate and screened for any ploidy changes using flow cytometry, and stomata measurements.

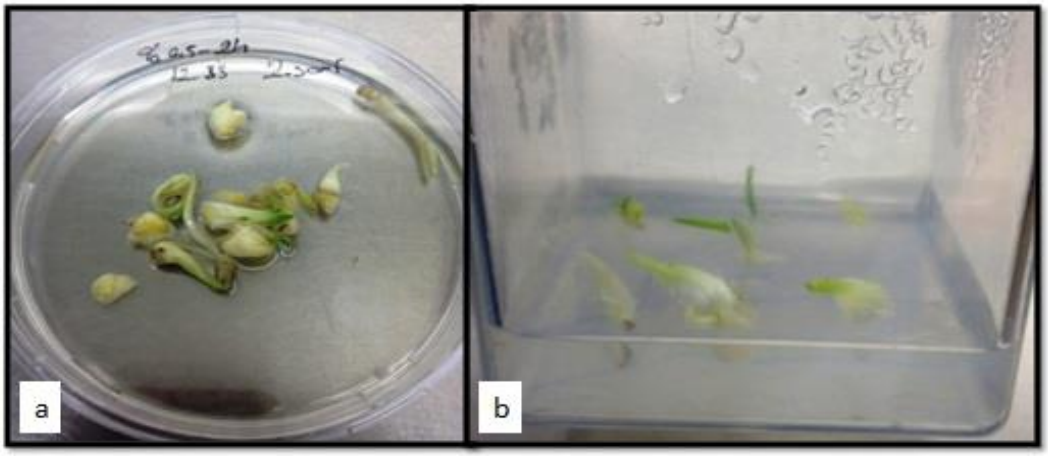


**Figure 1**. Colchicine treated *Iris sari in vitro* explant.

### Flow cytometry

For this purpose, candidate mutant plants obtained from 6 different applications were identified and sent to Namık Kemal University, Agricultural Faculty, Department of Field Crops, Cytology Labrotuvary. An analysis of plant leaf tissue (0.5 mg) was performed using the Partec - CyFlow space device.

Flow cytometry analysis and the amount of DNA passing through the reading device per unit time are made comparatively. Comparisons were made by correlating the amount of DNA passing through the optical

reader and the ploidy levels of the plants. All the viable explants were checked for changes in ploidy level. Young leaves treated with chemical mutagens (colchicine, oryzalin, trifluralin vs.) were preferred for flow cytometry analysis. The ploidy level was determined using the flow cytometer (Partec, CA), following the commercial indications, that is, approximately 0.5 $cm^2$ of fresh leaf tissue were chopped with a sharp razor blade submerged in 0.5 ml nucleus extraction buffer (HR A solution, Partec, CA) and then incubated in the same buffer during 1.5 min. After filtered, the solution was incubated for 1 min with HR B, Partec, CA [49]. The leaf sample from a known diploid plant of barley was used as the standard.

### Measurement of Stomata

Stomatal measurements were performed on the leaves of *Iris sari* plantlets exposed to chemical mutations. Fresh leaves were used for the stomata counts. Small areas determined on the leaf surface were dried with a thin layer of transparent nail polish. After, placed on a glass slide and removed swiftly treated area and then, observed through a microscope The pieces with the epidermal layer of the leaf were then placed on a glass slide and observed using 'Network-ağ Micrometer with the help of a 40x phase objective and 10x ocular micrometer. The number of stomata was determined by counting the two times for two different regions on an area of 1 $mm^2$. The diameter and length of the stoma were measured for all stomata in the unit area in 2 different regions of each leaf. All measurements related to stomata were evaluated by statistical analysis in comparison with the control group.

### Statistical analysis

In this study, different colchicine concentrations (0.00, 0.10, and 0.50%) and treatment duration (2, 4, and 6 h.) for chromosome doubling were tested. The experiment was carried out statistically completely randomized design consisting of each was three replicates consisting of five explants. In the stoma study, analysis was made with two measurements from two different areas. Data were statistically analyzed using the statistical program JMP 8.0. Means were separated according to the least significant difference (LSD) test at the 0.05 level of probability. The arcsin transformation values were calculated for the data in percentage (%). Polyploidization, survival rate and stomata measurements of plantlet leaves were evaluated by analyzing variance with the static program JMP 8.0 [50, 51].

## RESULTS AND DISCUSSION

### Colchicine inoculated and survival rate (%)

It is known that there are different techniques to induce *in vitro* polyploidy in plants. Dutt and coauthors (2010) reported liquid medium with colchicine using cell suspension culture of *Citrus reticulate* [75]. For *Rhododendron simsii*, Eeckhaut and coauthors (2001) mentioned the application of the rhododendron plant with colchicine solution dripped for 3 or 7 days on the cotyledons of the plantlets obtained *in vitro* [19]. Takamura and Miyajima (1996) stated that the tuber of *Cyclamen persicum* was immersed in colchicine solution without shaking for 1, 2, 4, and 7 days [76]. Vainola and Repo (2000) reported successfully applying the *in vitro* polyploidization protocol of micro shoots of Rhododendron hybrids with synchronized growth by submersion and shaking in colchicine solutions of different concentrations [77].

However, treatment times and obtained data may differ in chromosome doubling studies with different explant types. Atichart (2013), used protocorm-like bodies(PLBs) for polyploidization in *Dendrobium chrysotoxum* [53]. The researchers reported that *D. chrysotoxum* was treated with 0, 0.01, 0.02, 0.03, 0.04, and 0.05% colchicine (w/v) for 1, 2, 3, 4, and 5 days and the most effective dose was obtained with 47% tetraploid plant in 0.04% colchicine for 1 day.

In addition to the fact that genotype is known as an important factor in polyploidy studies in plants, mutagen concentration and application time are two effective criteria that guide results [78, 79]. Such that, the viability of plants after mutagen application is proportional to these two determinants. Some studies have indicated high concentrations and longer treatment time will decrease the survival and growth rates of explants [80, 81].

This study showed that obtaining polyploids is feasible by treating *in vitro I. sari* micro bulbs with colchicine. To achieve high efficiency in polyploidy plant formation through colchicine treatment, young tissues containing a lot of actively dividing cells are preferred [78]. In this study, *in vitro* micro bulbs (1.0-2.0 cm in length) obtained from the immature embryo culture were suitable explants for colchicine treatment.

In our study, for chromosome doubling, *Iris sari* explants (1.0-2.0 cm long micro bulbs) treated with colchicine were chemically purified and cultured in an MS medium containing 1.0mg/L TDZ + 0.5mg/L NAA.

The development of the cultivated plantlets per 6 weeks period (subculture) was observed. As can be seen in Table 1, while the development of the plantlets continued in the 1st subculture, it was observed that they gradually lost their vitality in the 2nd subculture. However, the plants that survived at the end of the 2nd subculture viability rates were determined. The highest viability rate was noted from the 0.1% colchicine treatment at 4 hours and in the control group plantlets were 67.12% and 62.97%, respectively (Table 1). Plantlets in these 2 applications (0.1% colchicine 4 h and control group) continued their normal *in vitro* growth, but the growth of plantlets in other applications was recorded as slow and growing leaves as weak and thin. Similar results were reported on *Bacopa monnieri* treatment using different colchicine doses (0.0, 0.1, 0.05, 0.01, and 0.001%) [52]. The researchers stated that they obtained polyploid plants by immersing the nodal segments in a solution of 0.001, and 0.01% colchicine for 24 or 48 h. Also, they stated these plants showed significant differences in size and color both in leaves and flowers compared to untreated controls.

Some researchers reported that increasing colchicine concentration and treatment time were significantly effective in reducing the plant viability of treated plants [54, 40]. On the other hand, Chen and coauthors (2011) in their study for chromosome doubling in *Anthurium andraeanum,* observed that while the duration of treatment increased at all mutagen concentrations, the viability rates decreased regularly [57].

In our study, Table 1 shows that the highest exposure times (6 h) of both colchicine concentrations (0.1 and 0.5%) showed the least survival rate. In this species, the survival rate was significantly reduced when 6 h in 0.1% colchicine. Some researchers reported similar findings indicating the detrimental effects of higher doses and treatment times of the antimitotic chemicals [55]. Çaglar and Abak, (1997) stated that haploid plants (*Cucumis sativus*) lost their viability as a result of the application of 4 h of 1.0% colchicine solutions [56]. Similarly, in our study, it was determined that the plantlets with the least survival rate of the plants in which 0.1% and 0.5% colchicine doses were treated for 6 hours. According to the viability of the plants after 2 subcultures, treatment with 0.1% colchicine for 4 hours was the best protocol to obtain tetraploid in *Iris sari.*

**Table 1.** Survival rates (%) of *I. sari* shoots at 2 subculture after colchicine treatment in *in vitro*

| | Colchicine dose and treatment time (%-hour) | 1. Subculture | 2. Subculture | Survival rate (%) |
|---|---|---|---|---|
| **1** | 0.1% - 2 h. | 22.66 AB | 8.00 BC | 35.23[36] B |
| **2** | 0.1% - 4 h. | 18.00 B | 11.66 B | 67.12[55] A |
| **3** | 0.1% - 6 h. | 17.00 B | 0.66 D | 3.17[6] D |
| **4** | 0.5% - 2 h. | 24.00 AB | 4.00 CD | 14.40[22] C |
| **5** | 0.5% - 4 h. | 18.66 B | 3.66 CD | 19.48[26] BC |
| **6** | 0.5% - 6 h. | 21.66 B | 2.66 CD | 11.89[17] CD |
| **7** | Control | 31.66 A | 20.00 A | 62.97[53] A |

Different upper case letters show highly significant differences in plant characteristics ($p < 0.05$).
**[]** Angle transformation valuue §

## Measurements of stomata

Chromosome doubling studies in plants have many advantages as well as disadvantages. An increase in the nuclear content of cells causes a raise in cell volume and which is reflected in its phenotypic characteristics [82]. Morphological features can be generally used for random identification of polyploidy, especially measurements of stomata and structures of leaves are also suitable for some plants. Stomata of polyploidy are generally larger and lower density than haploid or diploid plants. In this article, stomatal measurements made at the end of the treatments confirmed the tetraploid plants obtained. Flow cytometry analysis showed that plants determined to be polyploid were obtained by applying colchicine 0.1% for 4 h, and differences in stomata number, diameter, and length confirmed the polyploidy.

Some researchers have stated that stomatal measurements can be an indicator for determining ploidy levels and this indicator is also used in some plants [58, 55]. The stomatal length and diameter increase with the ploidy level. For this reason, plants with high ploidy levels have fewer stomata per unit area when compared to control plants. Large stomata are necessary for plants during photosynthesis [59]. Doheny-Adams and coauthors, (2012) supported a study, where plants with reduced transpiration, greater growth rates, and larger biomass [60].

In studies for polyploidization, chemical mutagen was applied and differences in stomata of possible polyploid plants were investigated by comparing them with the control group. When statistically analyzed, significant differences were observed between the stomata sizes of the colchicine-treated plants and the control group (Table 2).

By measurements of stomata, we confirmed that the tetraploid plants in the untreated diploid control plantlets were 6 number/mm$^2$ (Table 2), and all tetraploid plantlets had 3 number/mm$^2$ stomata number in the leaves. In *I. sari* number of the stomata density in chemical mutagen treated plants was the lowest stomata density significantly in plantlets treated with 0.1% colchicine for 4 and 6 h, 0.5% colchicine for 4 h with an average stomata number of 3 number/mm$^2$ compared with 6 number/mm$^2$ in control group plantlets (Table 2). As is known, the number of stomata of plants that have mutated is low, and the stoma size and diameters are higher than those of the control group. In this study, in the stoma measurements of *Iris sari* plants, the stoma diameter of the same 4 applications was the highest, 32.06 µm (0.1% colchicine 4h), 28.09 µm (0.1% colchicine 6h), 30.52 µm (0.5% colchicine 4h), and 27.79 µm (0.5% colchicine 6h) and the stoma length was 39.98, 43.68, 49.04 and 34.41 µm, respectively (Table 2).

Plants with polyploidy potential were observed in *Iris sari* with increased stomatal measurements and decreases stomatal density. There are also findings in the studies conducted by some researchers; on different plants (*Rye-grass; Glover and Legume; Zantedeschia sp., Gossypium arboreum; Catharanthus roseus*; *Zingiber officinale, Stevia rebaudiana)* that plant stoma measurements are a criterion for determining ploidy levels [61, 62, 63, 64, 65].

Xing and coauthors (2011) indicated in tetraploid *Catharanthus roseus* lines the stomata size and densities are greater than those of the control group, in tetraploid plants, stomata lengths were measured at 28.26 ± 2.51 µm, stomata diameter was measured at 20.35 ± 1.80 µm and total stomata area was measured at 1.76 ± 0.01%, while in control group plants 23.71 ± 1.83 µm, 17.11 ± 1.84 µm and 1.24 ± 0.02% [64]. These findings parallel the work of Moghbel and coauthors (2015), the length of stomata was observed at the 0.05 and 0.1% colchicine 24 h exposure time as 128.01, 181.86 nm, respectively, while in the control group was 84.7 nm [55].

Flow cytometry analysis showed that plants determined to be polyploid were obtained by applying colchicine 0.1% for 4 h, and differences in stomata number, diameter, and length confirmed the polyploidy.

**Table 2.** The effect of colchicine on the number and size of the leaf stomata of *I. sari*

| | Colchicine dose and treatment time (%-hour) | Number of stomata (number/mm$^2$) | Diameter of stomata (µm) | Lenght of stomata (µm) |
|---|---|---|---|---|
| **1** | 0.1% - 2 h. | 5.00 BC | 18.30 BC | 22.49 D |
| **2** | 0.1% - 4 h. | 3.00 D | 32.06 A | 39.98 BC |
| **3** | 0.1% - 6 h. | 3. 00 D | 28.09 A | 43.68 AB |
| **4** | 0.5% - 2 h. | 8.33 A | 14.52 C | 18.09 D |
| **5** | 0.5% - 4 h. | 3.00 D | 30.52 A | 49.04 A |
| **6** | 0.5% - 6 h. | 3.60 CD | 27.79 A | 34.41 C |
| **7** | Control | 6.00 B | 21.34 B | 31.56 C |

*Different upper case letters show highly significant differences in plant characteristics ($p < 0.05$).

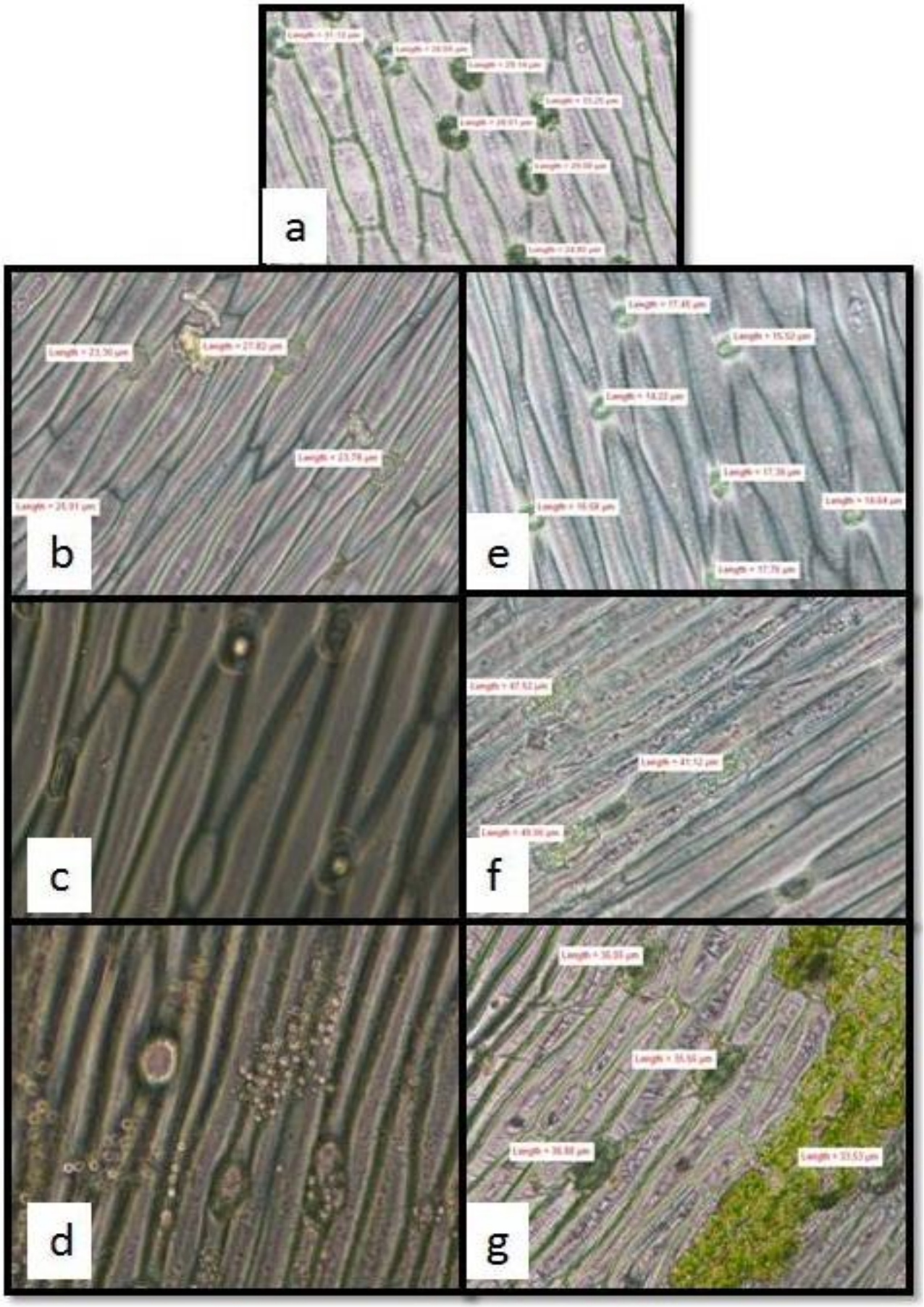


**Figure 2.** Stomata from the abaxial leaf epidermis: (**a**) control; (**b**) 0.1% colchicine for 2 h, (**c**) 0.1% colchicine for 4 h, (**d**) 0.1% colchicine for 6 h, (**e**) 0.5% colchicine for 2 h; (**f**) 0.5% colchicine for 4 h; (**g**) 0.5% colchicine for 6 h.

## Flow cytometry results of *Iris sari*

Flow cytometric analysis is known to be faster and more accurate to detect polyploidy compared to conventional methods [77,78]. In this study, the ploidy level of plantlets was determined by flow cytometry analysis. The results of flow cytometry indicated that diploid plants showed their (peak 1, 2) at channel 15-133 (mean=, Figure 3A), whereas the obtained putative tetraploid plants showed a peak (peak 3, 4) at channel 265-365 (mean=, Figure 3A) and mixoploid plants showed a peak (peak 3) at channel 499 (mean=, Figure 3B).

45 plantlets belonging to *Iris sari* were studied by flow cytometry analysis. 3 putative tetraploid plants were identified as a result of 4 h treatment of 0.1% colchicine applied to plants. As shown in Table 3 and Figure 4, the DNA content of the control group plants is determined as 21.86 - 23.01 Pg. the DNA content of the plants developed as a result of 4 h treatment of 0.1% dose of colchicine was determined in the range 44.26-47.18 Pg.

Furthermore, as a result of flow cytometry analysis, no increase in the amount of DNA of plants developed as a result of 0.5% colchicine treatment for 2 hours, but unlike normal shoot development when evaluated from a morphological point of view it has been observed to develop rather thick shoots, darker green leaves, fleshy and show the fewer number of proliferation (Figure 5). Zhou (2000) stated that the leaves of polyploidy plants belonging to *Zingiber officinale* were wider and thicker than those of plants with low ploidy levels [65]. In our study, when the plants belonging to both groups are compared, the leaves of the control group plants have a greater amount and longer, however, it has been observed that plants determined to be tetraploid have more fleshy structures but shorter leaves.

Nonetheless, the putative polyploid plants were determined based on morphology (different leaf structure, thick and curling), stomata measurements, and total DNA content analyzed by flow cytometry.

There have been studies with similar results in different plant species such as Anthurium, Pinellia, and Populus [57, 67, 68]. Also, measurements of stomata were significant difference from those of control plants. This result is similar to that of Zhou and coauthors (2020), in the polyploidy induction study of ginger (*Zingiber officinale*) [65].

**Table 3**. DNA quantities of *I. sari* plantlets in Flow cytometry Analyses

| Colchicine dose and treatment time (%-hour) | Fluorescence density of *Iris sari* | Fluorescence density of Barley | DNA content of *Iris sari* (Pg) | DNA content of Barley (Pg) |
|---|---|---|---|---|
| Control | 267.81 | 128. 09 | 22.26 | 10.65 |
| 0.1%-4 h. | 478.07 | 107.9 | 47.18 | 10.65 |
| 0.5%-2 h | 679.54 | 131.09 | 55.20 | 10.65 |

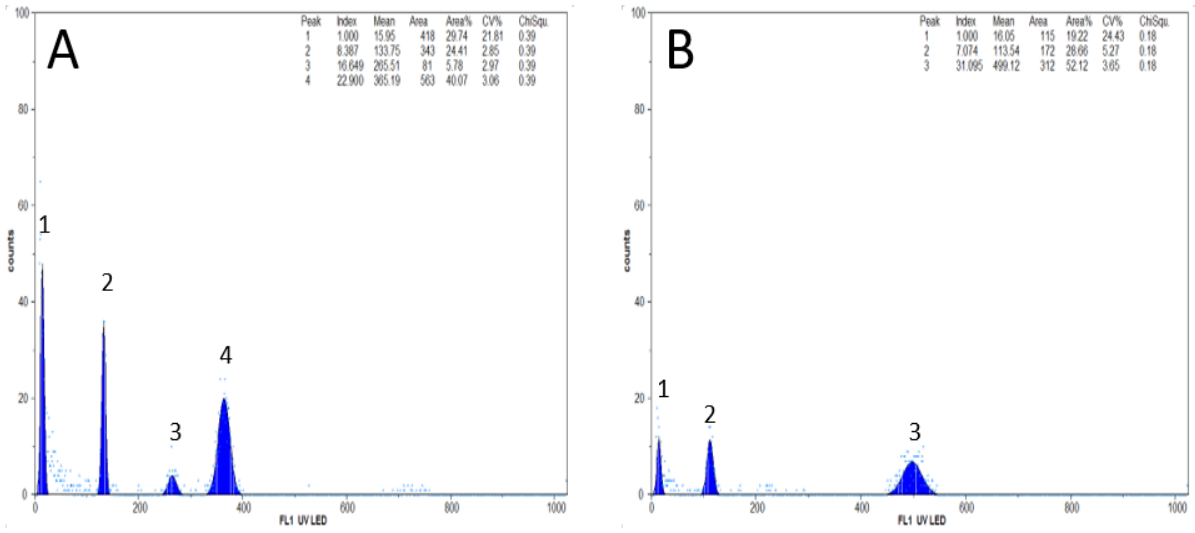


**Figure 3**. Flow cytometric histograms of (**A**) *I. sari* diploid (peak 3) and tetraploid plant (peak 4) (**B**): Mixoploid plant (pick 3)

Gangait and coauthors (2011) subjected 2 weeks *in vitro* shoots of *Gerbera jamesonii* to different colchicine doses (0.01, 0.05, 0.10, 0.50 or 1.0) and different treatment durations (2, 4, 8 h) [20]. As a result of the study, the highest rate of tetraploid plants was obtained with 64% in 8 h of application with 0.1% colchicine. The finding suggested that the optimal colchicine treatment for micro bulb explants was between 0.1% and 0.5% colchicine for the duration of 4 and 2 h. In our study, 3 tetraploid plants were obtained after 4 h of treatment at the same concentration (0.1% colchicine dose).

The findings obtained in this study were also compatible with similar studies by Beck and coauthors (2003) and Moghbel and coauthors (2015). These researchers reported that the effective mutagen dose was 0.1% in their study with colchicine in *Acacia mearnsii* and *Carthamus tinctorius* [69, 55].

This methodology has been used frequently by breeders for more than 50 years in many species such as banana, grapes, sugarcane, oil palm, and Zingiber [13, 14, 70, 71, 65]. Under *in vitro* conditions, chromosome doubling was applied in various ornamental plants, such as African violets, Cyclamen, Alocasia, Bacopa, and Gerbera [72, 17, 18, 73, 74].

It is known that there are different techniques to induce *in vitro* polyploidy in plants. Dutt and coauthors (2010) reported liquid medium with colchicine using cell suspension culture of *Citrus reticulate* [75]. For *Rhododendron simsii*, Eeckhaut and coauthors (2001) mentioned the application of the rhododendron plant with colchicine solution dripped for 3 or 7 days on the cotyledons of the plantlets obtained *in vitro* [19]. Takamura and Miyajima (1996) stated that the tuber of *Cyclamen persicum* was immersed in colchicine solution without shaking for 1, 2, 4, and 7 days [76]. Vainola and Repo (2000), reported successfully applying the *in vitro* polyploidization protocol of micro shoots of Rhododendron hybrids with synchronized growth by submersion and shaking in colchicine solutions of different concentrations [77].

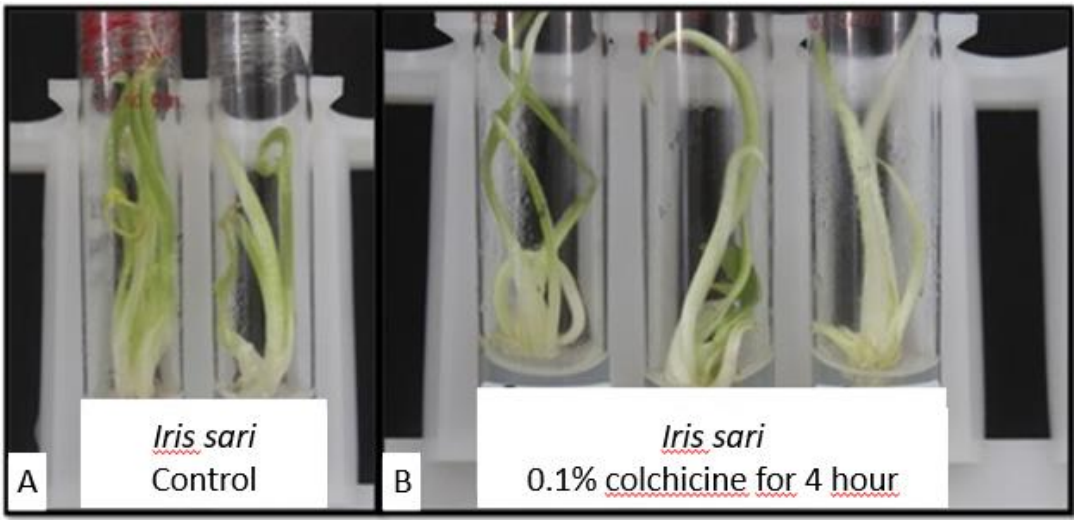


**Figure 4.** Morphological characteristic of conventional plantlets (**A**) control group of *Iris sari* (**B**) plantlets treated with 0.1% colchicine for 4 h.

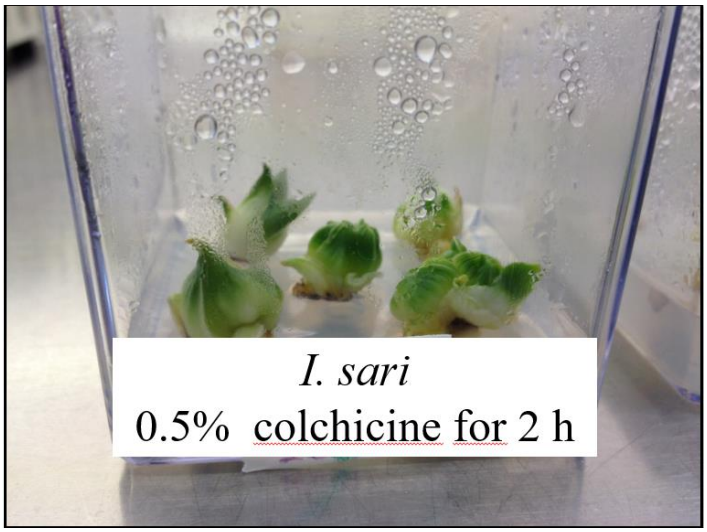


**Figure 5.** Differences in leaves as a result of colchicine application in *Iris sari.*

This study showed that obtaining polyploids is feasible by treating *in vitro I. sari* micro bulbs with colchicine. To achieve high efficiency in polyploidy plant formation through colchicine treatment, young tissues containing a lot of actively dividing cells are preferred [78]. In this study, *in vitro* micro bulbs (1.0-2.0 cm in length) obtained from the immature embryo culture were suitable explants for colchicine treatment.

In addition to the fact that genotype is known as an important factor in polyploidy studies in plants, mutagen concentration and application time are two effective criteria that guide results [78, 79]. Such that, the viability of plants after mutagen application is proportional to these two determinants. Some studies have indicated high concentrations and longer treatment time will decrease the survival and growth rates of explants [80, 81]. In this paper, the highest exposure times (6 hours) of both colchicine concentrations (0.1 and 0.5%) showed the least survival rate. In the study, the ideal application time of mutagen used for micro bulbs of the *I. sari* plant was 4 h.

Flow cytometric analysis is known to be faster and more accurate to detect polyploidy compared to conventional methods [77,78]. In this study, 3 putative tetraploids were obtained from 45 plantlets analyzed by flow cytometry.

Chromosome doubling studies in plants have many advantages as well as disadvantages. An increase in the nuclear content of cells causes a raise in cell volume and which is reflected in its phenotypic characteristics [82]. Morphological features can be generally used for random identification of polyploidy, especially measurements of stomata and structures of leaves are also suitable for some plants. Stomata of polyploidy are generally larger and lower density than haploid or diploid plants. In this article, stomatal measurements made at the end of the treatments confirmed the tetraploid plants obtained. Flow cytometry analysis showed that plants determined to be polyploid were obtained by applying colchicine 0.1% for 4 h, and differences in stomata number, diameter, and length confirmed the polyploidy. Moreover, polyploidy plants compared with the control group also had significant differences in stomata measurements.

In addition to stomatal measurement, which is an important criterion in determining ploidy plants, chloroplast counts will also support the findings. It would be appropriate to consider chloroplast measurements in future studies.

Zhou (2000) stated that the leaves of polyploidy plants belonging to *Zingiber officinale* were wider and thicker than those of plants with low ploidy levels [65]. In our study, when the plants belonging to both groups are compared, the leaves of the control group plants have a greater amount and longer, however, it has been observed that plants determined to be tetraploid have more fleshy structures but shorter leaves.

The putative tetraploid plantlets, which were obtained in limited numbers at the end of the applications, lost their vitality in the acclimatization stage.

## CONCLUSION

In this study, *in vitro* chromosome doubling of *I. sari* was conducted for the first time in the literature. In this method, the optimal mutagen concentration and duration of treatment to be applied to the specie were determined. The putative tetraploid plantlets, which were obtained in limited numbers at the end of the applications, lost their vitality in the acclimatization stage. In order to use the results more effectively, it is necessary to increase the number of explants applied in such studies.

Polyploidization is one of the most important methods of breeding plants and their rapid and effective. The data obtained from the article are a guide to breeding studies to be made with *Iris species.*

**Funding:** This research was funded by the Science Scientific Research Project, University of Kahramanmaras Sütcü Imam as a doctoral thesis project.

**Acknowledgments:** This study was carried out in the Department of Horticulture, Biotechnology Laboratory within Ph.D. thesis Project “Research on *In vitro* Regeneration and *In vitro* Polyploid Plant Formation in Some Endemic Iris Species of Turkey”. For his valuable ideas, help, and support, I would like to thank my Ph.D. Advisor, Prof. Dr. Gülat ÇAĞLAR, and Prof. Dr. Yeşim YALÇIN MENDİ.

**Conflicts of Interest**:The authors declare that they have no conflict of interest.

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