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Induction of polyploidy in grapevine (*Vitis vinifera* L.) seedlings by in vivo colchicine applications

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Abstract: Polyploidization is an important technique used in grape breeding to create new genetic resources and can be induced using some antimitotic agents, including colchicine. Plants with increased ploidy can gain better quality than their original diploids in some characteristics. In this study, which was carried out to obtain polyploid genotypes under in vivo conditions, the effects of six (1 g L^{-1}, 2 g L^{-1}, 3 g L^{-1}, 4 g L^{-1}, 5 g L^{-1}, 6 g L^{-1}) doses of colchicine were applied to the shoot tip meristem regions of the seedlings obtained from the seeds of grapevine cv. Ekşi Kara and cv. Trakya İlkeren, for three consecutive days and twice a day (at 08.30 and 18.00 h) were investigated. The effects of mutagen were evaluated by following the treated seedlings' morphological changes and comparing them with the 'Kyoho' (4x) by flow cytometry (FC) analysis. In surviving plants after treatment, stomatal characteristics differed to varying degrees from their original diploids, and stomatal density decreased inversely with the increase in stomatal size in polyploid genotypes. Among cv. Ekşi Kara seedlings, 1 tetraploid (5 g L^{-1}), and cv. Trakya İlkeren seedlings, 1 mixoploid (2 g L^{-1}), and 1 tetraploid (6 g L^{-1}) genotypes were selected by stomatal characteristics, chloroplast numbers, and confirmed by FC analysis. It was determined that colchicine is an effective mutagen in the breeding of polyploid grapevine, and stomatal observations, chloroplast numbers, and FC analysis are useful methods for obtaining confirmed results in the selection of polyploid genotypes.

Key words: Grape breeding, artificial polyploidy, autotetraploidy, mixoploidy, whole genome doubling

1. Introduction

Grapevine (*Vitis vinifera* L.) is one of the world's oldest plants with economic and cultural importance. In today's conditions where consumer and market demand change, there is a need to develop new genotypes by improving the characteristics of high popularity grape varieties. For this purpose, improvements in ecological responses with phenotypic and genotypic characteristics can be achieved by creating variation in the genome by hybridization and polyploidy breeding methods (Eng and Ho, 2019; Fox et al., 2020; Rezende et al., 2020). In studies of changing and improving highly heterozygous plants such as grapevine by crossbreeding, changes other than expected characters may occur (Di Genova et al., 2014). Polyploidy methods are being tested to develop new varieties with high adaptability, to shorten the time required in traditional breeding studies, and to obtain agronomic characters that cannot be acquired by combination breeding (Yue et al., 2017; Kara and Yazar, 2018; Fox et al., 2020; Hoang et al., 2020).

Polyploidization is the doubling of the complete set of chromosomes of a particular species to obtain a new genotype (Corneillie et al., 2019; Scholes, 2020). Genome doubling can occur in plants by two mechanisms, allopolyploidy, and autopolyploidy, depending on their genomic origins (Soltis et al., 2016; Van de Peer et al., 2017). The ploidy change of allopolyploid plants according to the donor plant and the results of crossing must be evaluated together (Corneillie et al., 2019). However, autotetraploid plants differ from their original parents in terms of genome size (Cohen et al., 2013; Sattler et al., 2016), and for this reason, they are often preferred to examine phenotypic and genetic changes in genome doubling studies (Sattler et al., 2016; Cimen, 2020; Niazian and Nalousi, 2020). Somatic chromosome doubling can be artificially induced using many physical and chemical mutagens (Germanà, 2012; Eng and Ho, 2019). Colchicine ($C_{22}H_{25}O_6$) is the most widely used chemical antimitotic for this purpose. This agent blocks the formation of microtubules by binding to the β tubulin in the metaphase stage of mitosis, thus enabling the formation of polyploid cells (Planchais et al., 2000; Lu et al., 2012). In genome doubling studies, young tissues with actively dividing cells are generally used to increase the effectiveness of antimitotic chemicals (Huy et al., 2019). Although colchicine can be applied to seedlings at different developmental stages, successful results can

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be obtained at the stage when the cotyledons expand and the apical meristem is exposed (Castro et al., 2018; Kara and Yazar, 2018). In this period, since the number of cells in the active division and the surface area that interacts with the antimitotic agent in the apical meristem increase, success in mutation induction can be achieved (Castro et al., 2018).

Although polyploidy has been used in grape breeding studies since 1937 (Olmo, 1937), it has attracted closer attention in recent years to reach triploid and autopolyploid genotypes (Yamada and Sato, 2016). Plants with increased ploidy levels may have better quality than some of the original diploids in terms of berry size, leaf size, and phylloxera resistance (Notsuka et al., 2000; Park et al., 2004; Yamada and Sato, 2016). Previous studies show that the efficiency of colchicine for induction of polyploidy in grapevine varies according to the tissue types and the methods used, and the polyploidy frequency is generally low (Xie et al., 2015; Ekbic and Tangolar, 2016; Kara and Yazar, 2020). For these reasons, new studies should be conducted to determine the colchicine application method that is effective in different grape varieties and tissues to obtain cultivars with high ploidy levels.

In this study, it was aimed to improve the quality characteristics, increase the ploidy level of the cultivars, and develop new genotypes with increased adaptability. In this context, the effects of colchicine applications on artificial polyploid induction in seedlings at the cotyledon stage obtained from the seeds of Ekşi Kara and Trakya İlkeren cultivars were investigated in vivo.

2. Materials and methods

2.1. Plant material

In the study, seeds of autochthonous 'Ekşi Kara' (2n = 2x) (Kara et al., 2017) and crossbred 'Trakya İlkeren' (2n = 2x) were used. The chloroplast numbers of the mutagen-treated seedlings were compared with their diploid parents and tetraploid cv. Kyoho (2n = 4x) (Yamada and Sato, 2016).

2.2. Mutagen application to seedlings at the cotyledon stage

In order to stimulate germination in the seeds of two grape varieties, they were stratified at +4 °C for 90 days (Sabır and Kara, 2011). Then, the seeds were washed in tap water and sowed in viols (32 wells) containing a 2:1 peat: perlite mixture under greenhouse conditions. Colchicine (Sigma-Aldrich CAS Number 64-86-8) was dissolved in a dose of 1% Dimethyl sulfoxide (DMSO CAS Number 67-68-5). Six doses (1 g L^{-1}, 2 g L^{-1}, 3 g L^{-1}, 4 g L^{-1}, 5 g L^{-1}, 6 g L^{-1}) of colchicine were applied as one drop to the shoot tip meristem regions of the seedlings for three consecutive days and twice a day (at 08:30 and 18:00). Only distilled water was dripped onto control plants' shoot tip meristems (Kara and Yazar, 2018).

2.3. The effects of colchicine applications on plant growth and ploidy level

2.3.1. Shoot tip viability rate (%)

It was determined by dividing the number of shoot tips of surviving plants after colchicine applications by the number of shoot tips of all treated plants (Kara et al., 2018).

2.3.2. Shoot length (cm)

Shoot lengths were determined in surviving seedlings by measuring with a tape measure at the end of vegetation (Kara et al., 2018).

2.3.3. Stoma observations [stoma density (pcs mm^{-2}), stoma width (µm), stoma length (µm)]

Leaf epidermal traces of the plants were obtained by applying clear nail polish to three different areas on the abaxial side of the 4th leaf from the tip when 6 leaves were formed on the shoot. The lower epidermis was peeled off and placed on a slide, and the stomatal width and length in the samples were counted by magnifying ×400 times under the microscope (Moghbel et al., 2015).

2.3.4. Chloroplast count (number $stoma^{-1}$)

The changes in the number of chloroplasts in the stomatal guard cells of all surviving plants 2 months after the treatments were examined. Chloroplast observations were also made in leaf samples taken for stoma observations. Leaf sections were bleached by soaking in Carnoy solution (3 parts ethyl alcohol: 1 part glacial acetic acid). The leaf sections removed from the solution were kept in sterile water for 2–5 min and then stained with 1% I-KI for 30 s. Chloroplasts were counted in 30 stomata in each sample. Chloroplasts were counted under a ×400 magnification microscope (Yuan et al., 2009), compared with their diploid parents and cv. Kyoho.

2.3.5. Flow cytometry (FC) analysis

Sections of approximately 0.5 cm^2 prepared from fresh (3–4 weeks old) leaf samples for each application were placed in a petri dish and 500 µL of isolation buffer (Partec-Nuclei Extraction Buffer) was added, and the leaf tissue was cut into small pieces with a razor blade. The samples in the petri dish were shaken for 10–15 s and transferred to tubes (Partec-Sample Tubes, 3.5 mL, 55×12 mm) filtered with a Partec-CellTrics 30 µm-green filter. 1600 µL of staining solution [Partec-DAPI (4,6 diamidino-2-phenylinole) Staining Buffer] was added to the tubes and kept in a light-isolated environment for 5 min. Samples were compared based on the peak channels formed by diploid parents and Kyoho in an FC device (Pazuki et al., 2018).

2.4. Statistical analysis

Colchicine applications were arranged according to a completely randomized design plan with 3 replications and

32 seedlings in each replication. The data obtained from the surviving plants were compared with the Duncan multiple comparison test in the SPSS 22.0 statistical program (SPSS Inc, Chicago, IL, USA) at a $p < 0.05$ significance level (Yue et al., 2017).

3. Results and discussion

3.1. Shoot tip viability rate (%)

The effects of in vivo colchicine applications on shoot tip viability were significant ($p < 0.05$). Shoot tip viability varied according to the cultivars and decreased with increasing colchicine doses, except applications 'Ekşi Kara' 1 g L^{-1} and 'Trakya İlkeren' 5 g L^{-1}. The lowest shoot tip viability rates in Ekşi Kara seedlings compared to the control (100%) were determined at the doses of 4 g L^{-1} (31.77%), 6 g L^{-1} (47.26%), and 3 g L^{-1} (51.84%), respectively. The shoot tips of Trakya İlkeren seedlings were less dry than Ekşi Kara seedlings. In cv. Trakya İlkeren seedlings, the highest shoot tip viability was recorded in the control (100%), while that was 60.17% in 3 g L^{-1} colchicine application (Figure 1a).

Colchicine concentration, application time, and treated tissue types are important factors affecting the survival rates and polyploidy success of the mutagen-induced plants (Sattler et al., 2016). The penetration of colchicine into plant cells is lower than in animal cells (Sivakumar et al., 2017; Ebrahimzadeh et al., 2018). For this reason, it is recommended to apply colchicine at increasing doses and times to achieve polyploidization targets in plant cells (Eng and Ho, 2019; Niazian and Nalousi, 2020). However, colchicine is a highly toxic chemical and causes plant cell death at high concentrations (Manzoor et al., 2018). On the other hand, solvents (DMSO) used to increase the penetration of colchicine into plant cells can also reduce the viability rate (Xie et al., 2015; Eng and Ho, 2019). Previous studies (Ekbic and Tangolar, 2016; Kara and Yazar, 2020) determined that the doses of colchicine applied to different tissues in grapes affect the viability rates, and the tissues' viability decreases with increasing mutagen doses. Kara and Yazar (2018) emphasized that in cotyledon stage treated plants with 1.0 g L^{-1} colchicine, the shoot tip dried, the seedlings could not survive, blind seedlings were formed, and ploidy success might increase at lower concentrations. In our study, in both cultivars, the polyploid seedlings were obtained (Figure 1a), and shoot tip viability rates decreased due to the increase in colchicine concentrations, like the literature.

3.2. Shoot length (cm)

The shoot length of the surviving plants was determined at the cessation of shoot development (90th day). Morphological differences were determined in the survived seedlings, especially in the first-third leaves. The selected seedlings, which were determined to be polyploid in the first vegetation period after the applications, developed more stable and weaker than the control. While the shoot length of control Ekşi Kara seedlings reached 40 cm at the end of the vegetation period, the shortest shoot length was measured as 14 cm in seedlings treated with 5 g L^{-1} colchicine. Similarly, in control seedlings of cv Trakya İlkeren, the minimum shoot length was determined at 2 g L^{-1} application (30.79 cm) (Figure 1b).

In a previous study, Hassan et al. (2020) reported that the size of pointed gourd seedlings (4x) developed after colchicine application was shorter than the control at the first growth stage, but the plant size increased after 4 months. Similarly, Liu et al. (2007) reported that leaf and stem growth inhibition was temporary by applying colchicine to the apical growth point of seedlings at the cotyledon stage. On the contrary, Tsukaya (2008) stated that the size of octoploid plants with high ploidy syndrome is smaller than tetraploids. Finally, tetraploid genotypes obtained by genome doubling in grapes have shorter internode lengths and correspondingly shorter shoot lengths compared to original diploids (Ekbic and Tangolar, 2016).

3.3. Stoma length (µm), width (µm), and density (pcs mm^{-2}) observations

It was confirmed by microscopic measurements that colchicine applications significantly affected stomatal properties ($p < 0.05$). Differences in stoma length (Figure 1c) and width (Figure 1d) were limited between treatments in Ekşi Kara seedlings. There was a significant difference in stomatal length in Trakya İlkeren seedlings. While the average stomatal length in the control was 20.82 µm, the highest stomatal length was in the 2 g L^{-1} (29.41 µm) application (Figure 1c).

The average stomatal width of Trakya İlkeren control seedlings was 16.17 µm, and those induced seedlings were listed as 2 g L^{-1} (19.81 µm), 6 g L^{-1} (18.39 µm), and 5 g L^{-1} (17.95 µm). In these samples, stomatal width (µm) values increased similarly to stomatal length (Figure 1d).

Similarly, stoma densities in colchicine-induced seedlings decreased due to the increase in stomatal length and width. The lowest stomatal density was in Ekşi Kara seedlings with 5 g L^{-1} (305.15 mm^{-2}) and in Trakya İlkeren seedlings with 6 g L^{-1} (275.51 mm^{-2}) applications (Figure 1e).

In previous polyploidy studies, stomatal sizes are often compared to quickly assess the variation in genome size. In this way, the selection of genotypes thought to be polyploid from large plant populations is facilitated and time and area are saved (Huy et al., 2019). However, it is recommended to be used with other validation techniques to obtain more precise results (Kara and Yazar, 2020). Although it varies according to the response of the species and variety, stomatal sizes increase in parallel with the increase in cell size in genotypes in which autotetraploidy is induced

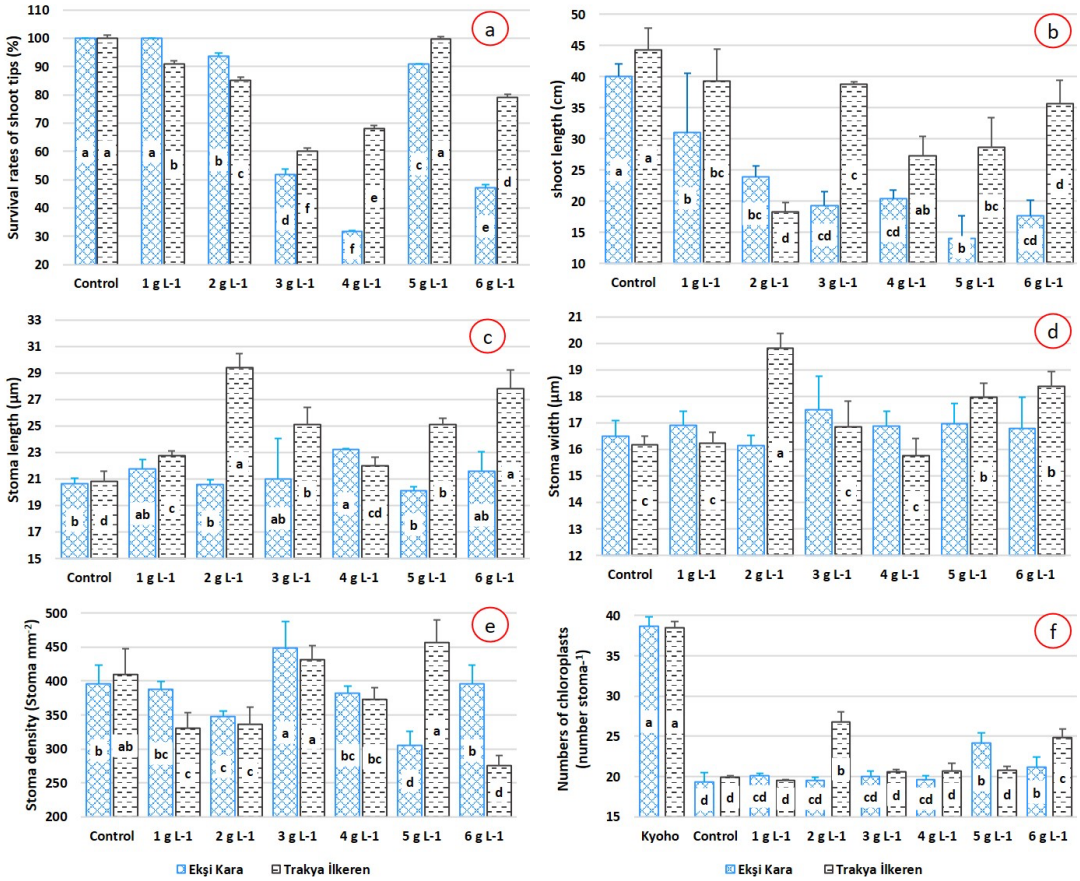

Figure 1. Effect on the shoot viability (a), growth (b), and stomatal characteristics (c-f) *,** According to Duncan's multiple comparison test, there is a 5% difference between the means expressed with different letters. Figure, 1f (cv. Kyoho was used as the tetraploid control in both cultivars).

(Manzoor et al., 2018; Eng and Ho, 2019). Similarly, changes in stomatal density in plants are considered one of the most important indicators of polyploidy (Gomes et al., 2014; He et al., 2016; Hoang et al., 2020). In previous studies on grapevine genotypes, it was reported that the number of stomata per unit area (mm^2) decreased as stoma sizes increased (Xie et al., 2015; Kara and Yazar, 2020). The findings obtained in our study are like previous studies, and a significant decrease was observed in stoma density due to the increase in stoma size, especially in Trakya İlkeren seedlings.

3.4. Change in the number of chloroplasts (number stoma^{-1})

The difference between the chloroplast numbers in stomatal cells of the colchicine-treated genotypes was significant ($p < 0.05$). The range of the chloroplast numbers of the diploid donors, 'Kyoho' and autotetraploid genotypes were 18–20, 38–40, and 18–38, respectively. While the average number of chloroplasts was 24.19 stomata in Ekşi Kara seedlings treated with 5 g L^{-1} colchicine, it was 38.00 stomata in the selected tetraploid seedling. The chloroplast numbers in the mixoploid genotype selected from Trakya İlkeren seedlings which 2 g L^{-1} colchicine-treated was 38, and the same chloroplast number was in selected tetraploid seedling from the 6 g L^{-1} (Figure 1f).

In previous similar studies, changes in the number of chloroplasts in stoma guard cells are frequently used for the preliminary detection of ploidy as a complement to stomal observations (Eng and Ho, 2019). Moreover, in ploidy studies in some species and cultivars, correlations were determined between chloroplast numbers and ploidy levels in stomatal guard cells (Talebi et al., 2017; Kara and Yazar, 2020). Additionally, Xie et al. (2015) determined correlations between stoma data (stoma size and numbers, chloroplast numbers in stoma guard cells) and ploidy level after polyploidy stimulation with colchicine and oryzalin in grapevine. In our study, genotypes thought to be polyploid based on changes in chloroplast numbers were selected for FC analysis.

3.5. Flow cytometry (FC) analysis

FC analyses were performed on diploid parents, 'Kyoho', and selected genotypes based on chloroplast and stomal

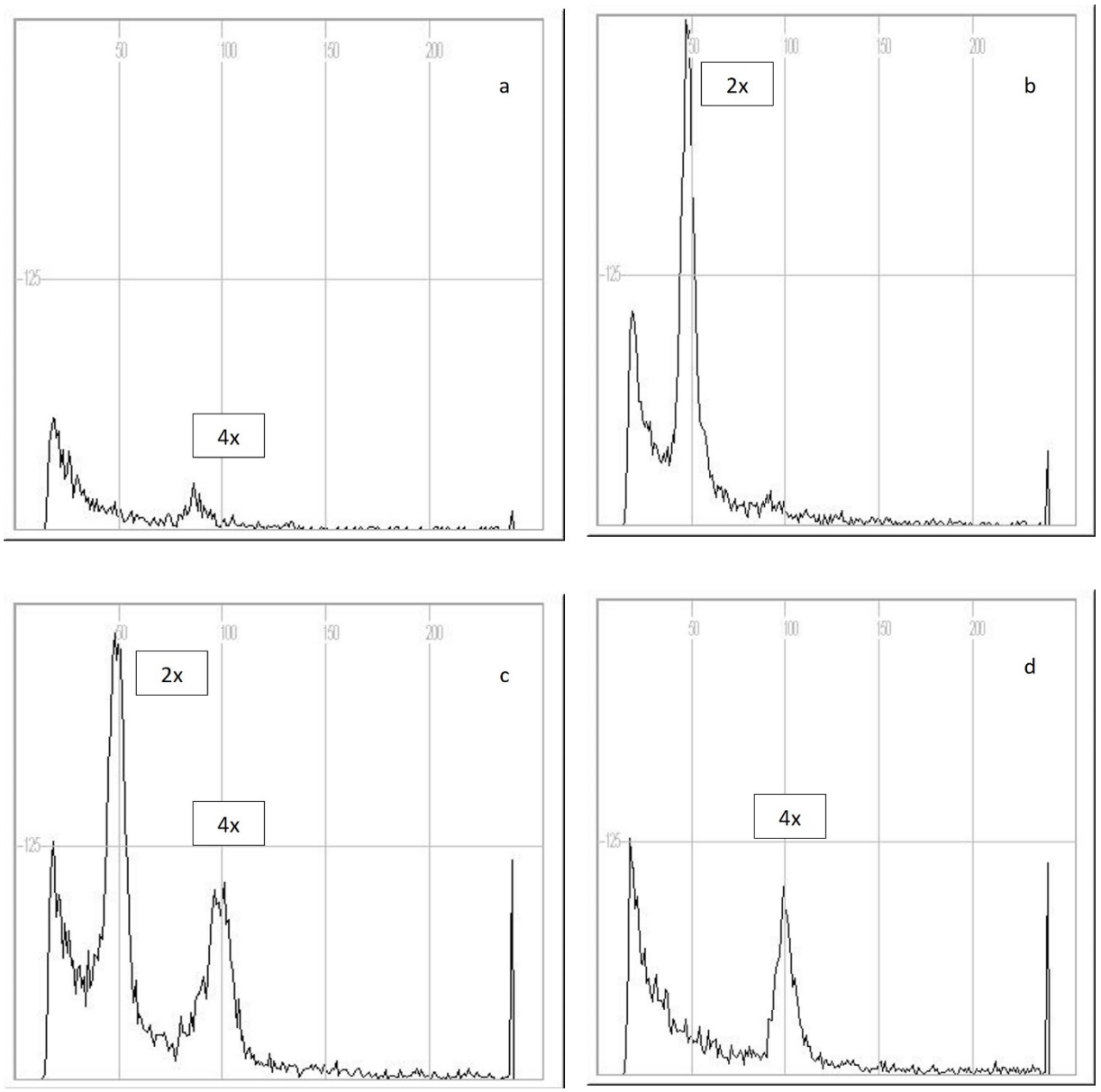

Figure 2. FC histograms of tetraploid Ekşi Kara seedling (a), diploid cv. Trakya İlkeren (b), mixoploid (c), and tetraploid Trakya İlkeren seedlings (d).

observations. Peaks of diploid grapevines appeared in channel 50, and peaks of the 'Kyoho' appeared in channel 100. The selected Ekşi Kara seedling from 5 g L^{-1} application was confirmed to be tetraploid (2n = 4x) (Figure 2a,b).

It was confirmed that the genotype selected from the Trakya İlkeren seedlings treated with 2 g L^{-1} was mixoploid (2n = 2x + 4x) (Figure 2c) and the genotype selected among those treated with 6 g L^{-1} was tetraploid (2n = 4x) (Figure 2d).

In previous studies, many plants and antimitotic agents were used in artificial autotetraploid stimulation, and timesaving, applicability, practicality, and feasibility were important in the method preference of researchers for ploidy success (Manzoor et al., 2018; Eng and Ho, 2019; Huy et al., 2019). FC analysis provides unique possibilities to measure the immediate results of genome duplications, especially when the chromosome sizes of the studied species are small (Castro et al., 2018; Julião et al., 2020). In previous studies on the grapevine, researchers examined the change in ploidy levels with FC analyses (Acanda et al., 2015; Luo et al., 2018). FC analyses and chloroplast number changes showed parallelism (Xie et al., 2015; Panpan et al., 2018). Moreover, mutation-induced damage in plants can be localized in tunica (L1, L2) or corpus (L3)

cells, dispersed, or evenly distributed throughout the meristem, although this differed between species, and the periclinal chimera was generally determined by the L1/L2 ratio in grapevine (Walker et al., 2006). On the other hand, Eng and Ho (2019) noted that mixoploid stimulation is an undesirable phenomenon in terms of solid tetraploid formation. They also reported that mixoploid genotypes show genetic instability and may revert to the original ploidy level. Again, Dhooghe et al. (2011) noted that in cases where colchicine concentration and application time are insufficient, the success of polyploidy decreases, and mixoploid genotypes emerge, however, autotetraploidy induction can be achieved depending on the increase in lethal colchicine dose. In this study, obtaining mixoploid genotypes at low colchicine doses and tetraploid genotypes at increasing colchicine doses confirmed the literature.

4. Conclusions

In this study, in vivo colchicine was applied to seedlings obtained from two grape cultivars, mutagen was effective in the induction of polyploidy, and this activity varied according to the applications and genotypes. It is thought that the effectiveness of colchicine applications varies according to the grapevine genotypes, the ambient conditions where the application is made, and the application time. Changes in stomatal characteristics and chloroplast numbers of Ekşi Kara and Trakya İlkeren grape seedlings were the leading indicators of changes at the genome level. FC analyses confirmed that chloroplast counts and stomatal observations can be used to predict polyploidy. In addition, the tetraploid control and diploid parents were a useful choice to check the effectiveness of FC assays in detecting ploidy levels from colchicine-treated seedlings. It was confirmed that colchicine can be a successful antimitotic agent in increasing the ploidy level of native and hybridized grape varieties. It is thought that successful results can be achieved by testing different doses and application times of colchicine in future studies to increase the ploidy levels of grapevine genotypes.

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