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Effects of shoot tip colchicine applications on some grape cultivars

Zeki Kara[1] **Kevser Yazar**[1,*]

[1]Selçuk University, Faculty of Agriculture, Department of Horticulture, Konya, Turkey

[*]Corresponding Author: kyazar@selcuk.edu.tr

Abstract

Polyploidization can provide changes in vital features such as growth, development, environmental stress tolerance in plants. Colchicine is one of the most commonly used chemicals as a polyploidization agent. In this study, 2-year-old 'Ekşi Kara', 'Gök Üzüm' and 'Trakya İlkeren' (2x, *Vitis vinifera* L.) saplings grown on their own roots were used. When the enforced shoots reached about 15 cm length, colchicine applied (0, 2.5 g L^{-1}, 5 g L^{-1}, 7.5 g L^{-1}) 24 and 48 hours to the lateral shoot tips. The effects of treatments were evaluated by shoot tip viability, stoma size and density, chloroplast counts, and flow cytometry (FC) analysis, and 'Kyoho' (4x) were used as the control. The maximum stomatal variations were determined in Ekşi Kara cultivar at 2.5 g L^{-1} 24-h application. Based on morphological differences, FC analysis was performed only in 'Ekşi Kara' but there was no genomic duplication. Since the morphological differences were not sufficient in the diagnosis of polyploid in grape cultivars, FC analysis should be performed to achieve confirmed results.

Keywords: Grapevine, Cultivar development, Breeding, Chemical mutagen, Autotetraploidy

Introduction

Grape (*Vitis vinifera* L.) is one of the most important fruit species grown globally as producing table grape, wine, raisin and fruit juice. Approximately 36% of the world production, and 56.1% in Turkey's grape production is used as table grape (OIV, 2019). New grape cultivars are needed to ensure high adaptation to changing environmental conditions for sustainable viticulture and to meet market demands.

Polyploidization, is an important tool employed to create new genetic resources in many plant species, to shorten the time needed for breeding and to obtain properties that cannot be achieved through hybridization (Yue et al., 2017). Mutation, in plants can be stimulated with many chemicals and physical mutagens, colchicine is the most commonly used chemical mutagen for this purpose. This antimitotic agent promotes polyploidy in the cells by blocking the mitosis in the metaphase stage (Planchais et al., 2000). In previous studies reported that there is increasing fruit quality and developing the stress tolerance in polyploid grapes (Notsuka et al., 2000; Park et al., 2004; Yamada and Sato, 2016). This method has been used in grape breeding since 1937 and interest has been increasing in recent years (Olmo, 1937). Polyploid grape cultivars are used commercially and cv. Kyoho (4x) constitutes 44% of the total vineyard area in China, which ranks the first in the world grape production (Olmo, 1937).

'Ekşi Kara' and 'Gök Üzüm' are ancient and autochthonous grape cultivars grown extensively in Central Taurus Region (Kara et al., 2017a). In order to meet the pollen needs of 'Ekşi Kara' (Kara et al., 2017b), and to increase its marketability without loss of adaptation ability to the area where traditional cultivar is grown, its fruit quality characteristics must be improved (Kara et al., 2017b). Previous studies conducted on chromosome doubling differ in terms of application doses, durations, tissue types, and application methods for polyploidy

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ORCID: Zeki Kara: https://orcid.org/0000-0003-1096-8288 and Kevser Yazar: https://orcid.org/0000-0002-0390-0341
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stimulation (Değirmenci-Karataş et al., 2010; Dhooghe et al., 2011; Kuliev, 2011; Ma et al., 2014; Kara et al., 2018). Studies are needed to determine the proper colchicine application methods with different cultivars, and to obtain cultivars with increased ploidy levels.

In this study, the effects of different doses and durations of colchicine applications on shoot tips were tested in order to provide genomic duplication in 'Ekşi Kara', 'Gök Üzüm' and 'Trakya İlkeren' grape cultivars *in vivo* conditions.

Materials and Methods

Plant Material

In this study, Ekşi Kara and Gök Üzüm autochthonous cultivars which were obtained from Selçuk University Faculty of Agriculture Department of Horticulture, and cv. Trakya İlkeren obtained from Tekirdağ Viticulture Research Institute, were used. 'Ekşi Kara' and 'Gök Üzüm' are well-adapted and the most intensely grown cultivars in Konya-Karaman provinces and Central Taurus Region. Since 'Ekşi Kara' has functional female flowers, 'Gök Üzüm' is used as a pollinator. Both cultivars are used as table and as raisin locally (Kara et al., 2017a). 'Trakya İlkeren' is preferred in early ecology in terms of its early yield, it can also be successful in short-vegetation areas (Köse and Ateş, 2017; Gülcü et al., 2020). Plants obtained from cuttings were used in study. Thermotherapy was applied for 30 minutes to the cuttings that would be used in the study at 50°C before rooting (Waite and Morton, 2007). After thermotherapy, cuttings were rooted in the mixture (peat: perlite 2:1 v:v) in the greenhouse. Rooting plants were planted in pots that containing a 2: 1 peat-perlite mixture. The chloroplast counts in stoma guard cells of plants were compared to 'Kyoho' (4x) (Yamada and Sato, 2016).

Chemical Mutagen Colchicine Applications

Colchicine (Sigma-Aldrich) applications were made to stimulate the polyploidy, which is effective even in low doses (0.5 mM< dose) in plants (Allum et al., 2007). In the present study, 1% dimethyl sulfoxide (DMSO) dose was used as the solvent for colchicine (Yang et al., 2006). 2-year-old potted plants grown on their own roots in greenhouse conditions, pinching was applied in early active growth period, when the shoots reached at 15 cm length. Lateral shoots tips were exposed to colchicine, 3 different doses (2.5 g L^{-1}, 5 g L^{-1}, 7.5 g L^{-1}) and 2 different time which 24 [3 times in 24 hours, (morning, noon and evening)]-48 hours [3 times in every 24 hours, (morning, noon and evening)]. After 24 hours from the first application, shoot tips were washed with sterile water. Control plants underwent washing only with a sterile water.

Determination of plant growth and ploidy level after colchicine applications

Survival rates of shoot tips (%)

Two weeks after the applications, the number of surviving shoot tips was determined by proportioning the number of alive shoot tips to all shoots of treated plants (%) (Kara et al., 2018).

Stoma Length (μm), Stoma Width (μm) and Stoma Density (stoma mm^{-2}) Observations

Two mounts after the treatments, the leaf epidermal traces of the plants that underwent the application were examined at the abaxial side of the fourth leaf from the end on the developing shoots after the application was carried out. The lower epidermis was removed by pasting with transparent nail polish from three different areas, and placed on the slide to determine the width and length of the stoma with a ×400 microscope (Moghbel et al., 2015).

Chloroplast Count (pcs $stoma^{-1}$)

Two mounts after the treatments, the changes in chloroplast counts were examined in stoma guard cells in all treated plants survived shoots. In the leaves that were taken for the stoma sample, the colour of the leaf sections was decolorized with Carnoy's Solution (3-part ethyl alcohol: 1-part glacial acetic acid v/v). The leaf sections that were taken out of the solution were kept in sterile water for 2-5 minutes, and then stained with 1% I-KI for 30 seconds. A total of 30 stoma chloroplast counts were performed in each sample. Chloroplast numbers were detected with ×400 microscope (Yuan et al., 2009), and were compared to diploid parents and tetraploid 'Kyoho'.

Flow Cytometry (FC) Analysis

Fresh leaf samples (3-4 weeks) were taken to a petri dish of 0.5 cm^2 for each application, 500 µL isolation buffer (Partec-Nuclei Buffer Extraction) was added, and the leaf texture was divided into small pieces with razor blades. The samples in the petri dish were shaken for 10-15 seconds, filtered with Partec-CellTrics 30 µm- green filter into the tube (Partec-Sample Tubes, 3.5 ml, 55x12 mm). A total of 1600 µL staining solution [Partec-DAPI (4.6 diamidino-2-phenylinole) Staining Buffer] was added to the tubes and was kept for 5 minutes in a medium isolated from light. Then the samples were analysed with the FC device. Samples were compared based on peak channels formed by diploid parents and tetraploid (4x) control in the FC device (Pazuki et al., 2018).

Statistical Analysis

The experiment was conducted in completely randomized design, with 3 repetitions, and with 10 shoot tips per repeat. The effects of the applications dose and duration interaction were compared in the JMP 13.0 Statistical Program with the Tukey test at $p<0.05$ significance level (Yue et al., 2017).

Results and Discussion

Survival Rates of Shoot Tips (%)

The shoot tip viability rates were varied according to the interaction of the cultivar, dose and duration. The colchicine doses and application times tested in this study affected the survival rates of the shoot tips in varying degrees according to the cultivars. The minimum shoot viability rates in 'Ekşi Kara' (83.67%) as a result of the toxic effect was recorded in 2.5 g L^{-1} 24-h, while in the control all of them were alive. The lowest shoot tip viability rates in 'Trakya İlkeren' and 'Gök Üzüm' were detected in 2.5 g L^{-1} 48-h (86.22%) and 2.5 g L^{-1} 24-h (84.89%) applications, respectively. It was observed that in 'Gök Üzüm' shoot tip viability rates were higher than other cultivars (Table 1).

Since the microtubules are in different tubule compounds in explant sources like shoot tips, the sensitivity levels of explant sources to chemical mutagens might vary. Sekiguchi et al. (1971) indicated that shoots did not grow as a result of the

Table 1. Effects of applications on survival rates of shoot tips (%)*

Time		Ekşi Kara	Gök Üzüm	Trakya İlkeren
24 h.	Control	100.00±0.00	100.00±0.00	100.00±0.00
	2.5 g L^{-1}	83.67±1.53	84.89±2.70	87.62±2.27
	5 g L^{-1}	87.72±1.55	95.03±2.27	88.48±1.34
	7.5 g L^{-1}	84.94±2.84	93.86±1.97	86.27±2.23
48 h.	2.5 g L^{-1}	84.45±0.57	92.88±2.83	86.22±3.36
	5 g L^{-1}	83.69±3.49	90.40±1.20	89.15±1.82
	7.5 g L^{-1}	86.00±3.61	92.85±1.90	89.84±2.64

*Colchicine applications and time interactions are non-significant at $p<0.05$

damage to the shoot tip area due to applications with mutagenic effects in some species. Also, it was reported that the shoot tips dyed at varying rates in antimitotic applications made to different types of tissues in rootstocks and grape cultivars due to the toxic effect of the chemical used (He et al., 2016; Kara et al., 2018). The findings from the present study are similar to these results.

Stoma Length (*µm*), Stoma Width (*µm*) and Stoma Density (stoma mm^{-2}) Results

The effects of the applications varied according to the cultivars, and the increases in stoma lengths were determined. Applications of 2.5 g L^{-1} and 7.5 g L^{-1} doses in 'Ekşi Kara' for 24 and 48 h caused elongation in stoma length compared to the controls (19.73 µm), while 5 g L^{-1} application caused decreases (19.10 µm). Similarly, in 'Trakya İlkeren' the 2.5 g L^{-1} and 7.5 g L^{-1} for 24-h and 48-h applications increased the stoma length. The longest stoma was recorded in the 2.5 g L^{-1} 24-h (26.23 µm) application in 'Gök Üzüm'. In the 'Trakya İlkeren' stoma length was increased in the 7.5 g L^{-1} 24-h (24.17 µm) application.

The stoma widths significantly ($p<0.05$) affected by colchicine applications, varied according to the cultivars. The 7.5 g L^{-1} colchicine for 48-h application in 'Ekşi Kara' and 'Trakya İlkeren' increased stoma width as 17.16 µm, 16.56 µm, respectively. In 'Gök Üzüm', the highest width was achieved in the 2.5 g L^{-1} 24-h (17.61 µm) (Figure 2).

The effects of colchicine dose and time of applications combinations in all grape genotypes in terms of stoma count per unit area were statistically important ($p<0.05$). Stoma densities were decreased depending on colchicine applications in all cultivars. The lowest stoma density values were determined in 'Ekşi Kara' the 5 g L^{-1} 24-h (297.10 stoma mm^{-2}), in 'Trakya İlkeren' the 7.5 g L^{-1} 48-h (408.11 stoma mm^{-2}) and in 'Gök Üzüm' with 5 g L^{-1} 48-h (433.91 stoma mm^{-2}) applications (Figure 3).

Stoma data, ensures approximate identification of genome size for the autopolyploid stimulated genotypes (Yang et al., 2006). The increase in cell size causes depending on the response of the species and cultivars, increasing occur in the shoot diameter, pollen, leaf and stoma sizes in tetraploid plants (Motosugi et al., 2002; Sattler et al., 2016). As a result of the increase in stoma size, decreases are detected in stoma count per unit area ($mm^{2)}$ (Ma et al., 2014; Xie et al., 2015). According to the findings in present study, stoma data can be used for pre-evaluation of the ploidy detection, the stoma data obtained outside the full genome folding might vary, and be affected by environmental conditions.

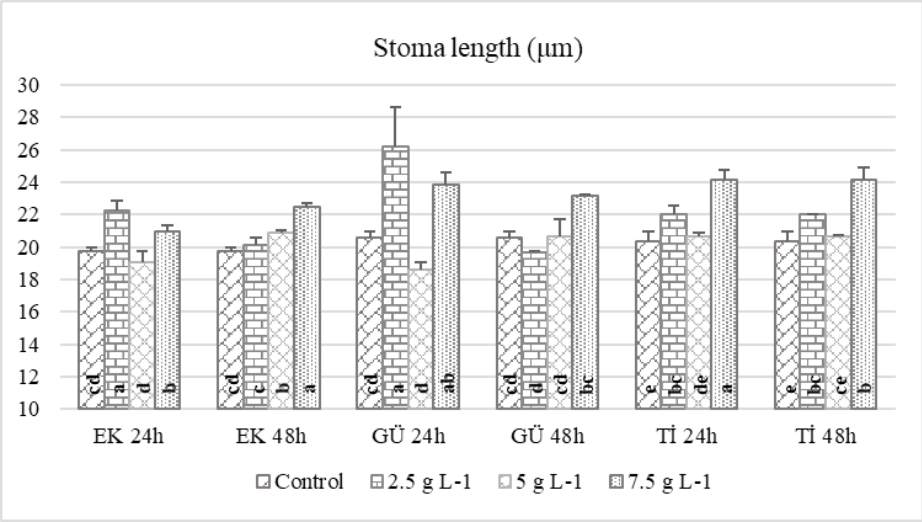

Figure 1. Effects of applications on stomata length (µm) (EK: Ekşi Kara, GÜ: Gök Üzüm, Tİ: Trakya İlkeren)

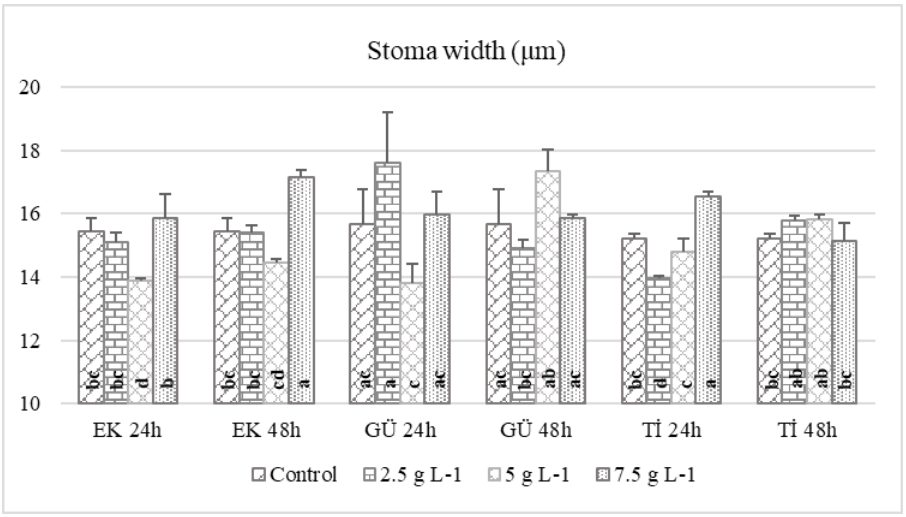

Figure 2. Effects of applications on stoma width (µm) (EK: Ekşi Kara, GÜ: Gök Üzüm, Tİ: Trakya İlkeren)

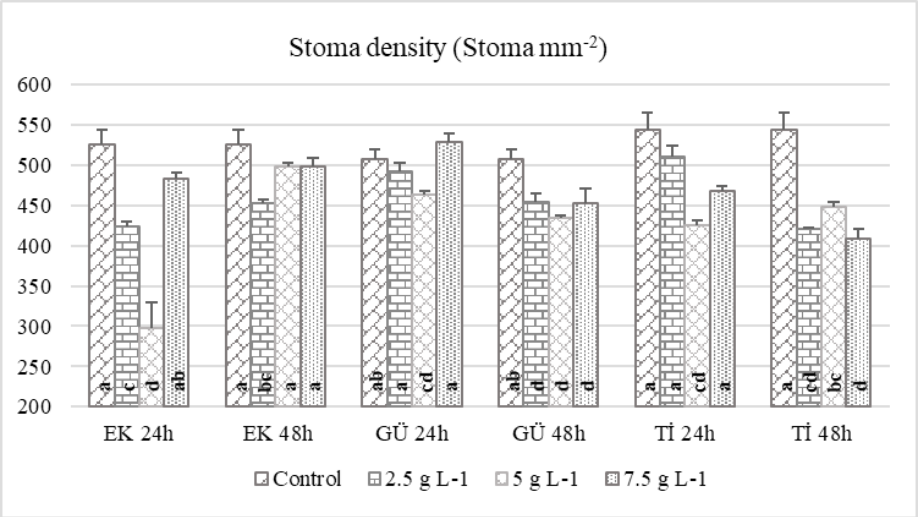

Figure 3. Effects of applications on stomata density (stoma mm^{-2}) (EK: Ekşi Kara, GÜ: Gök Üzüm, Tİ: Trakya İlkeren)

Chloroplast Count (pcs stoma^{-1}) Results

Chloroplast counts of stoma guard cells differed in 'Ekşi Kara' which were colchicine applied, and in control 'Kyoho'. The range of chloroplast count was between 18-28 in mutagen applied grape cultivars, and 38-40 in tetraploid 'Kyoho' (Table 2). The chloroplast numbers of the colchicine applied samples in Trakya İlkeren and 'Gök Üzüm' were similar to those of the controls (18-20); however, that was increased in 'Ekşi Kara' a dose-dependent, and the maximum value was 24.92 in the 2.5 g L^{-1} 24-h application.

The previous studies were reported that there is an association between the chloroplast counts and ploidy levels in stoma guard cells (Chen et al., 2009). Xie et al. (2015) indicated that chloroplast counts were made easier and earlier in stoma guard cells compared to the chromosome counts and FC analysis. In the present study, chloroplast counts increased compared to the original diploids; however, its frequency remained low comparted to the tetraploid control 'Kyoho'.

Flow Cytometry (FC) Analysis Results

Based on the numerical differences occurring in chloroplast counts, FC analysis was performed on plants whose ploidy levels were estimated to be different. As a result of FC analysis, it was determined that the ploidy levels of the samples examined did not change and they maintained their diploid forms (Figure 4).

In previous studies, FC analyses were used to determine the change in the ploidy levels of plants. In our study, FC analysis results were similar to the literature (Yang et al., 2006; Dhooghe et al., 2011; Acanda et al., 2013; Acanda et al., 2015).

Table 2. Effects of applications on chloroplast number (stoma mm^{-2})*

		Ekşi Kara		Gök Üzüm		Trakya İlkeren	
	Number of stoma	Average	Range	Average	Range	Average	Range
Kyoho	30	38.50±0.50 a	38-40	38.33±0.58 a	38-40	38.75±1.09 a	38-40
Control	30	19.73±0.28 d	18-20	20.05±0.23 cd	18-22	20.53±0.42 b	18-22
2.5 g L^{-1} 24h	30	24.92±1.01 b	18-28	19.71±0.25 d	18-22	20.45±0.08 b	18-22
2.5 g L^{-1} 48h	30	20.42±0.05 de	18-22	20.88±0.39 bc	18-22	20.25±0.12 b	18-22
5 g L^{-1} 24h	30	21.34±0.15 d	18-22	21.76±0.48 b	18-22	20.91±0.21 b	18-22
5 g L^{-1} 48h	30	20.26±0.09 de	18-22	20.06±0.26 cd	18-22	20.29±0.15 b	18-22
7.5 g L^{-1} 24h	30	23.11±0.12 c	18-26	20.03±0.20 cd	18-22	20.20±0.00 b	18-22
7.5 g L^{-1} 48h	30	19.58±0.04 d	18-20	20.83±0.15 bc	18-22	19.84±0.01 b	18-22

*Mean separation within columns by Tukey multiple test at, 0.05 level

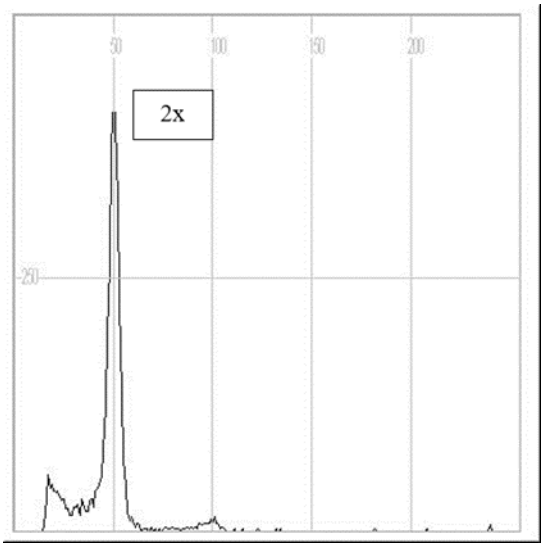

Figure 4. FC analysis result of 'Ekşi Kara' 2.5 g L^{-1} 24-h application (diploid, 2n = 2x)

Conclusion

The polyploidy breeding method is used in the breeding of economically important plants since its provides potentially beneficial results. Although differences were detected in the morphological and stoma sizes of the grape cultivar that underwent colchicine applications, it was determined with the chloroplast counts and FC analyses in stoma guard cells that these changes did not cause differences at the genome level. It was observed in the study that the reactions to *in vivo* applications varied on cultivar basis, and that the colchicine applications had limited effects on the development of grape cultivars that had domestic and regional importance. It is considered that future studies should focus on different cultivars and tissue types, dose and duration combinations.

Compliance with Ethical Standards

Conflict of interest

The authors declared that for this research article, they have no actual, potential or perceived conflict of interest.

Author contribution

The contribution of the authors to the present study is equal. All the authors read and approved the final manuscript. All the authors verify that the Text, Figures, and Tables are original and that they have not been published before.

Ethical approval

Not applicable.

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Data availability

Not applicable.

Consent for publication

Not applicable.

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