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Polyploidy Induction by Sodium Azide and Ethyl Methane Sulfonate in Grape Genotypes

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Abstract

Continuous improvement of vine rootstocks and grape varieties is necessary for the sustainability of viticulture. In this context, grapevine breeding and especially the development of polyploid grapevine genotypes offer opportunities. This study investigated the effectiveness of sodium azide (SA, five dozes and two treatment duration) and ethyl methane sulfonate (EMS, four dozes and two treatment duration) in inducing polyploidy. In Ekşi Kara and Gök Üzüm grape cultivars and 41B and Fercal grapevine rootstocks, different parameters were studied, such as seedling growth, stoma, chlorophyl and chloroplast observations, and cytogenetic analysis. In the experiment, each mutagen dose was applied to twenty seeds, 300 for each genotype and 1200 seeds in total. As a result of both mutagen treatments, a total of 268 seedlings survived: 39 in cv. Ekşi Kara, 48 in cv. Gök Üzüm, 78 in 41B and 103 in Fercal. A total of 50 seedlings, 8 in Ekşi Kara, 17 in Gök Üzüm, 9 in 41B and 16 in Fercal, were selected through preliminary screening by observing shoots, leaves, chlorophyll, stomata and chloroplasts, and the polyploidy level was examined by flow cytometry (FC) analysis. In the FC analysis, only one seedling selected from the Gök Üzüm SA 0.05% 2-h treatment was

confirmed to be triploid. In addition, in the chromosome count performed on the root tip-sample of the seedling selected by FC analysis, 57 chromosomes were counted that this was exactly triploid with 3n = 57 chromosomes, and ploidy verification was repeated. The triploid Gök Üzüm seedling obtained in this study is the first productive result in which polyploidy was induced with SA in grapevine genotypes. In subsequent studies to induce polyploidy in grapevine, SA 0.05% 2-h treatment may be recommended.

Keywords
Grapevine; improve; polyploidy; mutation; triploid

1. Introduction

A significant portion of today's viticulture needs are met through grape breeding efforts [1-6], and polyploidization is also used for this purpose [7, 8]. Polyploid varieties contribute to improved yield and quality, increased tolerance to biotic and abiotic stresses and seedless fruit production [9-11]. Most essential traits are assumed to remain intact while the plant phenotype changes with polyploidization. Since the effects are independent in grafted species, the chance of finding successful combinations increases [12]. Three thousand two hundred seventy-five mutant varieties have been obtained in sixty countries in the last seventy years by inducing genetic diversity in plants with natural or synthetic mutagens. Since mutagens affect different points in the plant cell cycle [13], various compounds (colchicine, oryzalin, trifluralin, nitrous oxide, SA, EMS) have been used to induce polyploidy [14-19]. As chemical mutagens, SA [19-24] and EMS [25-29]) have been determined to have mutagenic effects in different species, but grapevines have not been studied. The world vineyard area of tetraploid grape varieties continues to increase [30]. Since the first Kyoho variety was obtained in 1939 [31], more than thirty new tetraploid varieties have been developed with different breeding methods [31-33]. Moreover, seedless berries can be obtained without requiring GA treatments with triploid grape varieties [31]. This study investigated the polyploidy-inducing SA and EMS activities treated to grapevine genotypes at different doses and durations by stoma observations, chloroplast counts, FC analyses, and chromosome counts in root tips.

2. Material and Method

In this study, the polyploidy induction effects of SA (five doses and two treatment duration) and EMS (four doses and two treatment duration) treatments were tested on seeds of four grapevine genotypes, two of which were local grape cultivars and two were grapevine rootstocks. Shoot and leaf dimensions, stomatal characteristics, leaf chlorophyll contents, chloroplast numbers, FC analyses, and chromosome counts evaluated the effects of mutagen treatments.

2.1 Plant Materials

Cv. Ekşi Kara and cv. Gök Üzüm (both are *Vitis vinifera* L. and 2n = 38) seeds, which have been grown since ancient times in the mountainous areas of the Central Taurus region of Konya-Karaman provinces, were obtained from the 'clone vineyard' created by the selection studies carried out at

the Horticulture Department of Selcuk University, Faculty of Agriculture, and the vine rootstocks 41B [*Vitis vinifera* (cv. Chasselas) × *Vitis berlandieri*], and Fercal (B.C.n°1B × 31 Richter) [33], (both are 2n = 38) seeds were obtained from Manisa Viticulture Research Institute. Each mutagen dose was treated to 20 cold-stratified seeds stimulated for germination in the experiment. Three hundred seeds were used for each vine genotype and 1200 in total.

2.2 Chemical Mutagens

Sodium azide (SA, NaN_3, Cas No. 26628-22-8) was purchased from the German company Merck, and ethyl methane sulfonate (EMS), $CH_3SO_3C_2H_5$, Cas No. 62-50-0) was obtained and used from China (Shanghai Huirui Chemical Technology Co., Ltd.).

2.3 Method

2.3.1 Preparation of Seeds

Ripe bunches were harvested to obtain the seeds, and the seeds were separated by squeezing, washed with tap water and pure water, dried in the shade, placed in cotton bags, and stored in room conditions until stratification time. Since mutagens are known to cause chromosome doubling by blocking spindle fibers in actively dividing cells [34], stratified seeds (120 days at +4°C) and stimulated for germination were used to promote active cell division during the mutagen treatment process.

2.3.2 Mutagen Treatments and Seed Germination

Seeds of 4 grapevine genotypes were washed after stratification, with SA doses (0%, 0.01%, 0.05%, 0.1%, 0.2%) for 2-h and 4-h, and EMS doses (0%, 0.01%, 0.02%, 0.5%) for 6-h and 10-h, it was added to the seeds placed in falcon tubes, and the treatments were made by waiting on the shaker (IKA KS 4000 i control). Seeds that underwent control measurements of mutagen treatments were kept in distilled water for 2-h in SA treatments and 6-h in EMS treatments. Following mutagen treatments, the seeds were placed in Petri dishes on moistened paper towels to germinate at 22°C, 16-h of daylight, and 8-h of darkness. When the root tips reached approximately 2 mm in length, they were considered germinated and transferred to the growth greenhouse supplemented with nutrients.

2.3.3 Evaluation of the Morphological Effects of Mutagen Treatments

Shoot lengths, leaf numbers, and shoot diameters were determined after six months of growth in plastic containers of 8*8*10 cm in peat medium supplemented with nutrients (Klasmann turf) under greenhouse conditions. Since tetraploid plants have broader and thicker leaves than diploids [35], leaf thickness was examined under a ×100 magnification microscope (BX50; Olympus Optical Co. Ltd.) in leaf samples taken from the middle 1/3 of the developing shoots after ninety days of growth. Leaf epidermal scars were removed by applying transparent nail polish to 3 different areas on the lower surface of the 4th leaf from the tip in ninety-day-old plants. The lower epidermis was stripped and placed on a slide, and the stoma density (mm^{-2}), width (µm), and length (µm) of the samples were determined under a ×1000 magnification microscope (BX50; Olympus Optical Co. Ltd.)

[36]. Chloroplast counts were performed in the same samples [37]. Since it has been reported that the chlorophyll contents of tetraploid plants are higher than diploids, SPAD values were examined with Minolta Spad Meter 520 in 4-6th leaf samples at the shoot tip of approximately ninety-day-old seedlings [35, 38].

Chloroplast counts were made in the stomatal guard cells of the same leaves. For this purpose, fresh leaves were bleached in Carnoy solution (3-parts ethyl alcohol: 1-part glacial acetic acid), leaf particles removed from the solution were kept in sterile water for 2-5 minutes and then stained in 1% I-KI solution for 30 seconds. Chloroplasts (number stoma cells^{-1}) were counted under a microscope (BX50; Olympus Optical Co. Ltd.) with ×400 magnification [37].

2.3.4 Flow Cytometry

FC allows the analysis of different types of tissues and cell layers [13]. FC analysis was performed as in previous studies [8]. Fresh leaf tissues and healthy eaves were taken from young (3-4 weeks old). It was cut into approximately 0.5 cm^2 pieces, placed in a petri dish, and 500 µL of isolation buffer (Partec-Nuclei Extraction Buffer). Leaf tissue was shredded with a razor blade for about 1 minute until broken into small pieces [8]. Thus, leaf tissue cells were separated from each other, cell nuclei were released, the nuclear membrane was broken down with the buffer used, and openings were created in the nuclear membranes [39]. Prepared samples were shaken in petri dishes for 10-15 seconds. The samples were 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 they were kept in Styrofoam boxes for 5 minutes in a dark environment [13]. The samples were then analysed in the FC instrument.

2.3.5 Chromosome Count

Since FC analyses can classify ploidy levels according to their DNA content [13], chromosome counting was performed to confirm chromosome folding data in the selected seedling with the results of chromosome counting [13, 40] and FC analysis [17, 41]. For this purpose, cuttings were prepared and rooted from the shoot tips of the seedlings predicted by FC analysis to have different ploidy levels, and the root tips of the freshly developed seedlings were taken between 10:30-11:00 in the morning [42], then transferred to 0.002 M 8-hydroxyquinoline solution at +4°C. Then, it was washed with distilled water. Then, the root tissues were stained by keeping them in 2% aceto-orcein at +4°C for 2 days, and 0.5-1 mm root tips were transferred to the slide, and a crushed preparation was prepared with 45% acetic acid [43] chromosomes were counted.

2.3.6 Characteristics of Selected Triploid Gök Üzüm Seedling (3n)

In this study, the triploid Gök Üzüm seedling was selected by pre-selection based on plant development results and stomata and chloroplast analyses. FC analysis and chromosome counting were performed to verify chromosome doubling in this genotype. Analysis results were compared with the parent cv. Gök Üzüm (2n) and reference tetraploid cv. Kyoho (4n).

2.3.7 Statistical Analysis

Mutagen treatments were arranged according to a completely randomized design plan with counting replications and twenty 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 [44].

3. Results

3.1 Effects of Mutagen Treatments on Seedlings

As a result of both mutagen treatments, 268 seedlings survived, including 39 cv. Ekşi Kara, 48 cv. Gök Üzüm, 78 41B and 103 Fercal rootstocks, and the effects of the mutagenic treatments were evaluated on these seedlings. Effects of treatments on shoot length, shoot diameter, leaf width, leaf length, number of leaves, leaf thickness, stomata density, stoma length, stoma width, chlorophyll content, and chloroplast numbers were evaluated by ANOVA. The changes in the numerical values obtained with all mutagen treatments, determined by ANOVA, are shown as the least significant difference (LSD) in Table 1. Mutagenic treatments caused substantial ($p < 0.05$) variation in all parameters examined, except for the features specified as ns in Table 1. However, the significant differences revealed in the variance analysis were insufficient for the definitive determination of the polyploid genotypes.

Table 1 Mutagenic variation results determined by ANOVA as LSD.

	EMS				SA			
	Ekşi Kara	Gök Üzüm	Fercal	41B	Ekşi Kara	Gök Üzüm	Fercal	41B
Shoot length	4.99	10.24	11.61	8.11	12.59	13.25	10.07	9.26
Shoot diameter	ns	0.31	0.53	0.43	0.42	0.41	0.32	0.30
Number of leaves	0.81	2.02	1.17	1.17	1.99	3.04	1.45	1.10
Leaf width	1.59	4.61	3.80	1.78	6.35	3.56	4.81	2.17
Leaf length	3.82	2.21	2.05	1.97	5.20	2.52	2.86	2.14
Leaf thickness	1.53	9.98	8.68	ns	14.79	13.32	9.51	15.21
Stoma density	23.42	2.02	10.20	15.79	19.39	17.40	15.65	16.92
Stoma length	2.52	2.42	1.28	1.68	1.81	2.01	1.74	1.73
Stoma width	1.40	0.86	1.36	1.74	1.70	1.38	1.65	1.03
Chlorophyll content	ns	2.22	3.19	3.95	6.18	3.37	1.17	2.44
Chloroplast numbers	ns	2.56	2.73	ns	145	2.44	1.92	3.00

ns: non-significant.

3.2 FC Analysis

With the preliminary selection considering the mutagenic variation results, 50 seedlings, including 8 cv. Ekşi Kara, 17 cvs. Gök Üzüm, 9 41B and 16 Fercal rootstock were selected for FC analysis due to suspicion of polyploidy. As a result of FC analysis, it was determined that only one Gök Üzüm seedling treated with SA 0.05% 2-h was triploid (Figure 1). As a result of FC analysis, it

was confirmed that the significant differences observed in the growth, stomata, and chloroplast observations of mutagen-treated grapevine seedlings and the failure to produce polyploid mutants were attributed to the effectiveness of the genome repair process in grapevine genotypes [45]. Grape cultivars and grapevine rootstocks, like other woody perennial species, have proven to be recalcitrant to develop polyploid offspring under chemical mutagenic stimulation [46].

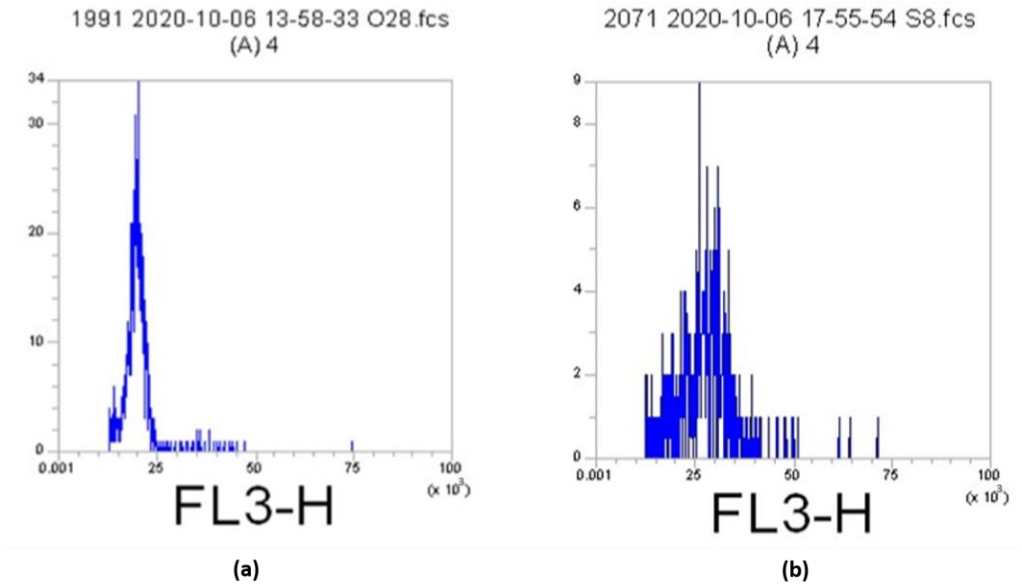

Figure 1 DNA histograms of FC analysis result. a) diploid parent cv. Gök Üzüm, b) Triploid Gök Üzüm offspring.

The most reliable method to determine the ploidy level is chromosome counting. In addition to being laborious and time-consuming, it requires actively dividing cells [47]. Chromosome number can be specified in cells undergoing mitotic or meiotic cell division. Counting mitotic chromosomes is easier and faster. Root tips are the most suitable source of mitotic cells. Although young buds, leaves, or calluses can be used when roots are unavailable [48-51], root tips are more common [42]. In our study, chromosome counts were performed on fresh root tip samples of rooted cuttings, and 57 chromosomes were counted, which showed that this was precisely triploid with 3n = 57 chromosomes.

3.3 The Selected Triploid Gök Üzüm Seedling

The leaf chlorophyll content of the selected Gök Üzüm seedling was compared with the parent cv. Gök Üzüm and reference tetraploid cv. Kyoho (Table 2). As a result, the leaf chlorophyll content of the selected triploid Gök Üzüm seedling was lower than that of its parent and cv. Kyoho and the number of chloroplasts was higher than that of the parent Gök Üzüm but lower than that of cv. Kyoho.

Table 2 Comparison of some characteristics of selected Triploid Gök Üzüm Seedling (3n = 57) with parent cv. Gök Üzüm (2n = 38) and cv. Kyoho (4n = 76).

	Selected triploid Gök Üzüm seedling (3n)	Gök Üzüm (2n)	Kyoho (4n)
Stoma length (µm)	10.66 ± 0.58	7.66 ± 1.15	10 ± 1.00
Stoma width (µm)	3.33 ± 0.58	3.33 ± 0.58	3.33 ± 0.58
Stomata density (stoma mm^{-2})	3.66 ± 1.53	5.33 ± 1.53	6.66 ± 0.58
Chlorophyll (as SPAD value)	25.93 ± 1.94	1.94 ± 3.50	29.36 ± 0.90
Chloroplast numbers (chloroplast stoma $cell^{-1}$)	29.33 ± 3.06	20.66 ± 1.15	40 ± 0.90

4. Discussion

Developing high-quality, large seedless genotypes is one of the most important goals of table grape breeding programs. Almost all commercial diploid seedless grape varieties have stenospermocarpic berries. Recently, researchers have been trying to develop triploid seedless varieties [52]. The traditional method of breeding triploid plants by crossing diploid and tetraploid varieties is long and laborious [53]. Regeneration of triploid plants from endosperm culture provides an easy and direct approach to triploid breeding [54]. Triploid grape genotypes were developed by crossing diploid seedless varieties as female parents and tetraploid seedless grape varieties as male parents and using embryo rescue technique [55-57]. In this study, 57 chromosomes were counted in the root tip cells of seedlings. Only one study in the literature reported that a triploid/nearly triploid genotype was obtained by applying N_2O to Gök Üzüm offspring [58]. In this study, the triploid Gök Üzüm generation obtained by SA 0.05% 2-h treatment to Gök Üzüm seeds was confirmed by FC analysis and root tip chromosome count, and the first result was the determination of the triploid induction efficiency of SA in grapevine genotypes.

5. Conclusions

This is the first fruitful result in which polyploidy was induced by using SA as a mutagen in grapevine genotypes. In developing grape rootstock and grape varieties for today's needs, mutation breeding and especially polyploidy breeding can offer additional improvement opportunities without losing the gains of conventionally used genotypes. For subsequent studies of mutation and polyploidy induction in grapevine, the treatment of SA 0.05% 2-h, confirmed by FC analysis and chromosome count, can be recommended.

Abbreviations

°C	Degree Celsius
2n	diploid genome constitution
3n	triploid genome constitution
41B	[*Vitis vinifera* (cv. Chasselas) × *Vitis berlandieri*]
4n	tetraploid genome constitution
ANOVA	Analysis of variances

cm	centimetre
cm^2	square centimetres
cv	cultivar
DNA	Deoxyribonucleic acid
EMS	Ethyl methane sulfonate, $CH_3SO_3C_2H_5$
FC	flow cytometry
GA	Gibberellic acid
h	hour
I-KI	Iodized Potassium iodide
LSD	limited significant difference
M	molar
ml	millilitre
mm	millimetres
N_2O	Nitrous oxide
ns	non-significant
SA	Sodium azide, NaN_3
µL	microliter

Author Contributions

Conceptualization: Z.K., Data collection: A.B., Z.K., Data analysis: Z.K., A.B., Methodology design: Z.K., A.B., Written and edited by: Z.K., A.B.

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Competing Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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