



The Effects of Nitrogen Protoxide and Orizalin on Promotion of Polyploidy in Grapes

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ABSTRACT

The effort to reach higher yield and quality in grape production without losing the current quality has led researchers to induce polyploidy with chemical mutagens that cause less damage to the environment and researchers to obtain polyploid grape genotypes. The fact that the number of polyploid grape varieties and their production continue to increase creates synergy for research in this direction. In this study, Nitrous Protoxide, Oryzalin and their combined applications were tested to induce polyploidy in seeds and seedlings whose germination was exponentially induced of cvs. Ekşi Kara and Gök Üzüm (both *Vitis vinifera* L.). After 48-h, N₂O at 5 bar pressure was applied to seeds that germinated until root tips were visible, as well as 48-h and/or 96-h 0 (control), 25 µM and 100 µM oryzalin were applied to seedlings whose cotyledon leaves reached full size. Ploidy verification was made by flow cytometry (FC) analysis in selected seedlings by examining the effects of the treatments on the surviving seedling rate, stoma density and size, and the number of chloroplasts. While the morphological and cytological effects of all mutagen applications were found to be significant, only one seedling selected from 25 µM 48-h oryzalin application in Ekşi Kara cultivar was confirmed by FC analysis. Plant survival rate was lower and morphological differences were more pronounced in the combination of N₂O and oryzalin. In future studies, more polyploid induction can be tried with different dose and application time combinations of N₂O and oryzalin.

1. Introduction

In the grape marketing sector, it is important to develop high quality grape varieties with good market value. In grape breeding, it is aimed to develop varieties that can adapt well to different conditions, have high stress tolerance, good yield, and quality characteristics. Stimulated polyploidization technique is a widely used in plant breeding, as it provides various morphological differences in plant growth and development and generally strong environmental adaptation (Esmaeili et al 2020; Wang et al 2020). Although colchicine is effective in autopolyploid induction in vine, it has been sought for alternative mutagens due to its intensive labour requirement, dangerous and high price of higher concentrations (Kihara & Tsunewaki, 1960; Kitamura et al 2009), chimera regeneration, formation of aneuploids, abnormalities in plant development, and low seed productivity (Hassawi & Liang, 1991).

Compounds such as dinitroaniline and phosphonodiamidite have been used in polyploidization

studies in recent years (Ramulu et al 1991; Tosca et al 1995; Hansen & Andersen, 1996; Väinölä, 2000; Simmonds et al 2001; Kermani et al 2003; Eeckhaut et al 2004; Pintos et al 2007; Touchell et al 2020). When colchicine, APM and different dinitroanilines (oryzalin, ethalfluralin, trifluralin, pendimethalin, benefin) were compared, a higher polyploidization potential was determined for all dinitroanilines compared to colchicine (Mitrofanova et al 2003).

Antimicrotubular herbicides were thought to be alternatives since they inhibit microtubule in a similar way to colchicine (Yemets & Blume, 2008). It has been reported in different species that nitrous oxide (N₂O) gas is effective in the doubling of the chromosome number in the cell and the formation of polyploid genotypes by preventing the formation of spindle fibers and the retraction of chromosomes during the cell division process (Kihara & Tsunewaki, 1960; Kitamura et al 2009).

In this study, the effects of combined and separate applications of Nitrous Oxide (N₂O) and Oryzalin on ploidy promotion in cvs. Ekşi Kara and Gök Üzüm (both *Vitis vinifera* L.) were investigated at the morphological and cytological phase.

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2. Materials and Methods

As the plant material, seeds of cvs. Ekşi Kara and Gök Üzüm which are the most important cultivars of vineyards in the Central Taurus Mountains, were germinated after 90 days of stratification at +4 °C and their root tips became visible. Tetraploid (4x) 'Kyoho' was used as control.

The application doses and durations of Nitrous oxide (N₂O) and Oryzalin as chemical mutagens are presented in Table 1.

In the experiment of induction of polyploidy with N₂O, germinated seeds applied 0, 48-h and 96-h N₂O under 5 bar pressures were sown in production pans in the greenhouse. In the trial of polyploidy induction with oryzalin, two application times (0 (control), 48-h and 96-h), two doses of oryzalin [0 (control), 25 µM and 100 µM] were applied to the seedlings that reached the cotyledon stage. Pure water was applied to control seedlings.

The experiment was set up in a randomized block design with 3 replications, and the number of plants in each replication was 12. In total, morphological, and cytological observations were made on 720 seedlings.

The rate of conversion to plant was calculated by dividing the number of shoot tips applied and viable to the total number of plants. Shoot length was measured

Table 1

Effects on morphological traits on mutagen treated seedlings

N ₂ O	Oryzalin	Duration	Seedling's mortality	Shoot length	cv. Ekşi Kara			
					Number of stomata	Stoma length	Stoma width	Number of chloroplasts
0 Bar	25 µM	Control	97.00±2.65 a	5.57±0.09 a	254.93±5.49 a	27.86±0.43 d	17.82±0.20 d	19.92±0.07 e
		48-h	83.17±1.65 b	3.02±0.09 de	213.61±1.85 cd	33.17±1.06 c	21.05±0.76 c	23.13±0.81 ab
		96-h	74.03±2.31 c	3.25±0.10 d	232.27±9.51 b	35.46±0.93 ac	22.58±0.67 ab	23.27±0.77 ab
	100 µM	48-h	66.63±5.25 d	2.83±0.10 f	232.64±11.20 b	37.70±0.71 ab	22.86±0.26 ab	24.05±0.47 a
		96-h	58.83±4.52 e	2.77±0.09 f	207.30±5.25 d	35.40±0.90 ac	22.00±0.65 b	22.45±0.22 bc
		Control	76.00±1.56 c	4.76±0.25 b	227.24±12.76 bc	37.24±1.51 ab	22.87±0.25 ab	23.52±0.20 ab
5 Bar	25 µM	48-h	70.03±4.05 cd	4.83±0.27 b	209.08±7.87 d	38.28±2.82 a	23.06±0.65 a	21.19±0.96 d
		96-h	66.07±4.31 d	3.98±0.24 c	212.61±10.60 cd	35.28±1.76 bc	22.59±0.47 ab	21.90±0.24 cd
		Control	57.47±0.98 e	3.71±0.26 c	204.39±3.57 d	36.19±2.92 ab	22.38±0.35 ab	21.59±0.72 cd
	100 µM	48-h						
		96-h						
		Control						
LSD _{≤0.05}		6.16	0.29	14.84	2.60	0.91	1.00	
					cv. Gök Üzüm			
0 Bar	25 µM	Control	100.00±0.00 a	5.71±0.28 a	251.01±5.40 a	31.25±0.42 d	20.37±0.52 c	20.48±0.17 d
		48-h	88.89±1.92 b	3.58±0.24 e	237.97±7.36 b	32.09±0.47 cd	18.62±0.30 e	20.60±0.53 d
		96-h	76.90±1.03 d	2.97±0.21 f	227.27±5.65 bc	31.83±0.35 d	19.26±0.33 de	21.93±0.46 bc
	100 µM	48-h	70.10±5.11 e	5.00±0.22bc	204.03±5.74 e	35.73±0.95 a	20.82±0.22 bc	22.62±0.56 b
		96-h	55.93±0.64 g	4.67±0.33 bd	223.67±7.07 cd	35.60±0.22 a	21.59±0.73 b	24.50±0.44 a
		Control	82.96±5.13 c	5.17±0.26 b	214.86±8.05 de	35.76±0.22 a	23.78±0.28 a	22.33±0.61 bc
5 Bar	25 µM	48-h	69.26±1.28 ef	4.83±0.35 bd	227.00±6.14 bc	33.11±0.95 bc	22.82±0.71 a	20.63±0.13 d
		96-h	64.45±3.85 f	4.38±0.26 d	208.10±1.66 e	35.89±0.63 a	19.98±0.73 cd	24.89±0.51 a
		Control	46.67±4.41 h	4.62±0.22 cd	205.08±6.16 e	34.16±0.72 b	20.61±0.70 bc	21.41±0.90 cd
	100 µM	48-h						
		96-h						
		Control						
LSD _{≤0.05}		5.31	0.49	11.04	1.08	0.98	0.89	

3.1. Vitality Rate (%)

The viability rate of the plants treated with oryzalin and/or N₂O decreased (p<0.05). In the Oryzalin study, increasing doses and application times decreased the surviving plant rate. The viability rate of seedlings treated with N₂O was higher than those treated with Oryzalin. In cv. Ekşi Kara, the highest rate of viability was obtained in the control (85 ± 1.73%), while the highest seedling death was in the application of 5 bar-

with a tape measure at the end of vegetation. The width and length of the stoma were examined with a 40x magnification objective binocular microscope by peeling the lower epidermis of the leaf and placing it on the slide. In addition, chloroplasts were counted in all seedlings and compared with tetraploid (4x) 'Kyoho' grape variety. Flow cytometry (FC) analysis was performed on the selected material by determining the morphological difference. Data were subjected to analysis of variance (ANOVA), means were compared with Duncan multiple comparison test.

3. Results and Discussion

According to the results obtained from the multivariate analysis, the viability rate (%), shoot length (cm), stomatal density (number stoma mm⁻²) and stomatal size (µm) of the examined traits in seedlings treated with N₂O and oryzalin were significantly higher than chemical mutagen applications (p<0.01). affected (Table 1). The shoot length difference between the two grape cultivars treated with mutagen was significant (p<0.01). Viability rates were affected by the increase in application time (p<0.01). Stoma densities (number stoma mm⁻²) were significantly affected (p<0.01) according to cultivar, mutagen doses and application times (Table 1 and Table 2).

48-h N₂O + 100 µM 96-h oryzalin (57.47 ± 0.98% survival).

The highest survival rate in cv. Gök Üzüm was determined in the control (90 ± 2%) and the lowest (46.67% ± 4.40) 5 bar 48-h N₂O + 100 µM 96-h oryzalin application (Table 1 and Table 2).

In a previous study using oryzalin in artificial induction of polyploidy in grapevine, the survival rates of

autotetraploid plants ranged from 8.6 to 30%, depending on the variety (Bosco et al 2017). In another study of chromosomal folding in grapevine, while the linear effect of oryzalin on plant survival was determined, it was determined that increasing doses of oryzalin were more effective on plant regeneration than the application time (Sinski et al 2014). Similarly, 120 μM 24-s (33% of surviving explants were tetraploid) was reported to be the most effective of the in vitro applied

The ANOVA of seedlings differences that treated with N_2O and oryzalin

SV	df	Seedling's mortality	Shoot length	Number of stomata	Stoma length	Stoma width	Number of chloroplasts
Cultivar	1	44.374 ^{ns}	4.647 ^{**}	0.077 ^{ns}	0.053 ^{ns}	3.480 [*]	1.188 ^{ns}
Dosage	3	1252.006 ^{**}	2.411 ^{**}	774.881 ^{**}	29.165 ^{**}	23.026 [*]	3.442 ^{**}
Duration	1	1109.673 ^{**}	0.058 ^{ns}	56.587 ^{ns}	1.364 ^{ns}	0.551 ^{ns}	6.538 ^{**}
Cul. x Dos.	1	24.160 ^{ns}	5.368 ^{**}	374.270 [*]	4.043 ^{ns}	4.076 [*]	7.583 ^{**}
Cul. x Dur.	1	31.924 ^{ns}	0.468 [*]	91.650 ^{ns}	0.189 ^{ns}	0.204 ^{ns}	8.178 ^{**}
Dos. x Dur.	3	7.357 ^{ns}	0.113 ^{ns}	78.300 ^{ns}	3.007 ^{ns}	1.245 ^{ns}	8.631 ^{**}
Cul.xDos.xDur.	1	4.646 ^{ns}	0.125 ^{ns}	2072.711 ^{**}	7.205 ^{ns}	2.388 ^{ns}	1.955 ^{ns}
Failure	40	16.062	0.313	162.343	6.414	4.207	4.792

ns: Not significant, *: Significance level 5%, **: Significance level 1%, Cul.: Cultivar, Dos.: Dosage, Dur.: Duration, SV: Source of variation, df: Degree of freedom

3.2. Shoot Length

The effects of mutagen treatments on shoot length were significant ($p < 0.05$). Although less in N_2O applied plants; Increasing mutagen dose and application times caused a decrease in shoot length. While the shoot length was 5.57 ± 0.09 cm in cv. Ekşi Kara control seedling, the lowest shoot length was 2.77 ± 0.09 cm in 100 μM 96-h oryzalin treated seedlings. The shoot length was 5.71 ± 0.28 cm in the control seedlings of cv. Gök Üzüm, and the lowest shoot length was 2.96 ± 0.50 cm in those treated with 25 μM 96-h oryzalin (Table 1 and Table 2).

In previous studies of chromosome folding in grapevine in vitro, significant delays in growth and development of explants were reported (Xie et al 2015). Moreover, autotetraploid plants obtained from artificial polyploidy stimulation with in vitro oryzalin in grapevine had shorter internodes, thicker stems and more compact root systems (Sinski et al 2014).

3.3. Stoma Data

The effects of mutagen applications on stomatal length, width and density were significant ($p < 0.05$). All applications increased the stoma length and width. In cv. Ekşi Kara, stomatal length was 27.86 ± 0.43 μm in control seedlings; It was measured as 38.28 ± 2.82 μm in the application of 5 bar N_2O + 25 μM 96-h oryzalin. The stomatal length was 31.24 ± 2.20 μm in the control seedlings of the cv. Gök Üzüm, while it was 36.55 ± 2.64 μm in those treated with 5 bar N_2O + 100 μM 48-h oryzalin. While the stomatal width of cv. Ekşi Kara control seedlings was 17.82 ± 0.20 μm ; The largest stomata were 23.06 ± 1.14 μm with 5 bar N_2O + 25 μM 96-h oryzalin applied plants. While the stomatal width was measured as 20.36 ± 1.47 μm in the control seedlings of the cv. Gök Üzüm; The largest stomata were in seedlings treated with 23.78 ± 0.81 μm and 5 bar N_2O + 25 μM 48-h oryzalin (Table 1 and Table 2).

oryzalin doses in *Watsonia lepidota* (Ascough et al 2008). In addition, it has been suggested that the dose and application times that reduce the plant survival rate may provide an advantage by reducing the number of diploid and mixoploid plants. It was also reported that tetraploid plants obtained with high mutagen doses could show abnormalities such as a decrease in growth rate (Väinölä & Repo, 2000).

All mutagen applications reduced stoma density. In cv. Ekşi Kara, the densest stoma was 254.93 ± 14.81 mm^{-2} in the control, the least stoma was 204.39 ± 3.57 mm^{-2} in 5 bar N_2O + 100 μM 96-h oryzalin application. In cv. Gök Üzüm, the highest stomatal density was 251.008 ± 13.54 mm^{-2} in control seedlings, and the minimum 204.02 ± 5.73 mm^{-2} in 100 μM 48-h oryzalin application.

In a similar previous study, the stomatal length of tetraploid plants was one-third longer than diploids (Xie et al 2015). In another grapevine polyploidy study, researchers reported that the stomatal length and width values of the tetraploid plants they obtained showed a positive correlation with the chromosome numbers and a negative correlation with the stomatal density (Sinski et al 2014).

3.4. Chloroplast Count Data

Both mutagen treatments increased chloroplast numbers significantly ($p < 0.05$) in seedlings of both grape cultivars. In cv. Ekşi Kara, 19.92 ± 0.07 chloroplasts per cell were determined in control seedlings, while the maximum number of chloroplasts was 24.05 ± 0.47 in 100 μM +48-h oryzalin applied seedlings. The number of chloroplasts per cell in cv. Gök Üzüm ranged from 20.48 ± 0.16 units in control seedlings to 24.88 ± 7.32 units in the application of 5 bar N_2O + 100 μM 48-h oryzalin (Table 1 and Table 2).

In a previous study, it was reported that the number of chloroplasts in stomatal guard cells was determined as 14.4 ± 1.81 in diploids and 23.5 ± 1.78 in tetraploids, as an effective and economic indicator for distinguishing tetraploids (Xie et al 2015). Other previous studies have suggested that stomatal size measurement and chloroplast count in stomatal guard cells are the most economical and efficient methods for detecting polyploidy (de Carvalho et al 2005; Yang et al 2006). In addition, it has been reported that chloroplast densities in stomatal guard cells are directly proportional to the

number of chromosomes (Sinski et al 2014; Lan et al 2020). In the polyploid induction made in *Alocasia* plant, the best result was obtained with a 24-hour application of 0.01% oryzalin, with a rate of 15.4% tetraploid plant (Thao et al 2003).

3.5. Flow Cytometry (FC) Analysis

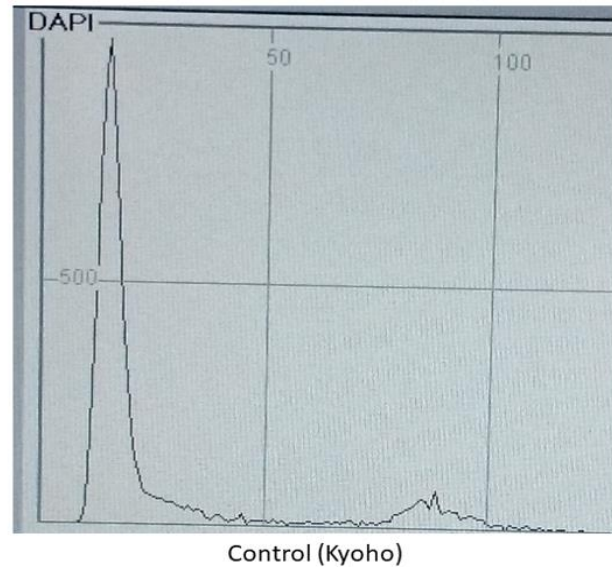
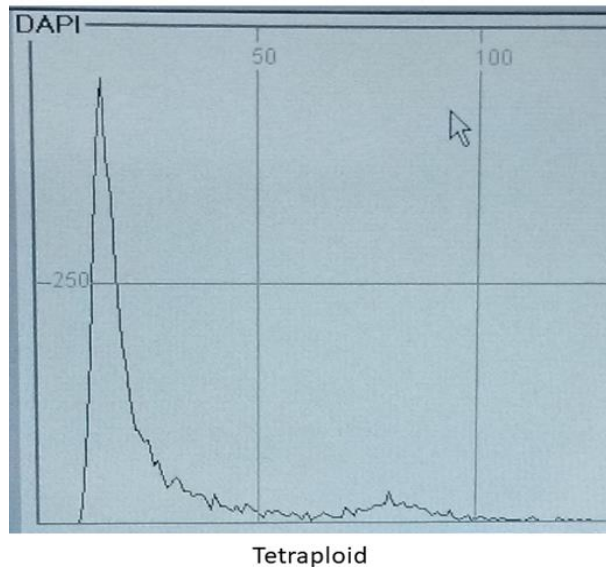


Figure 1
FC histograms of selected tetraploid cv. Ekşi Kara seedling (left) and tetraploid cv. Kyoho (right)

4. Conclusion

Our study is the first report in which N_2O and oryzalin were used together to obtain polyploidy in grapevine *in vivo*. By the N_2O and Oryzalin and combined mutagen applications, significant changes were recorded in morphological analyses in all the seedlings that survived and developed. In combination with N_2O + oryzalin, plant survival rate was lower and morphological differences were more pronounced. It was confirmed that changes in stomatal density and size, and changes in the number of chloroplasts could be used in the preselection of polyploidy prior to flow cytometry. In future studies, more polyploid induction can be tried with different dose and administration time combinations of N_2O and oryzalin. All surviving plants in our study are being preserved and monitored.

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Morphological differences were determined by all observations, and seedlings suspected of being polyploid were selected and flow cytometry analysis was performed on them. Selected 1 Ekşi Kara seedling treated with 25 μM 48-h oryzalin was confirmed to be tetraploid (Figure 1).

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