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ASSESSMENT OF SOMACLONAL VARIATION IN MICROPROPAGATED GRAPEVINE CULTIVARS USING MOLECULAR MARKERS

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Abstract

Micropropagation of five grapevine cultivars (Fetească albă, Cabernet Sauvignon, Merlot, Riesling Italian and Traminer roz) was performed using axillary shoots as a source of explant from field-grown plants. Shoot initiation and proliferation were performed on Murashige and Skoog (MS) medium with 0.5 mg/L NAA and BAP (0.5, 1.0 and 2.5 BAP mg/L). Subsequently, proliferated shoots from V3 were used for grapevine callus induction. The callus was then subcultured on fresh MS medium supplemented with 1.0 mg/L BAP +5.0 (V4) and 10 mg/l NAA (V5). Embryogenic calli grown on V5 were transferred and subcultured on MS medium supplemented with 0.5 mg/L NAA and three different concentrations of TDZ (0.5; 1.0; 2.0 mg/L) for shoot regeneration. RAPD assay was performed after the 12th subculture using in vitro-raised plants from V6 culture medium (0.5 mg/L NAA+0.5 mg/L TDZ) and the mother plants grown in the field and used as control. The results of this study reveal that the highest number of somaclones (5) was regenerated from the Merlot variety and the lowest number (1) from the Feteasca albă and Traminer roz varieties. For practice, the induction of somaclonal variation and the selection of valuable somaclones are important goals for future grapevine breeding programs.

Key words*: Vitis vinifera*, *in vitro*, callus, somaclones, RAPD.

INTRODUCTION

Grapevine (*Vitis vinifera* subsp. *vinifera*) is one of the oldest and the most important fruit crops in the world (Butiuc-Keul and Coste, 2023). There are currently over 15000 named grapevine cultivars held in *Vitis* collections worldwide (Panara et al., 2018). However, the identification and characterization of grapevine cultivars have been one of the points of interest in viticulture (Jackson et al., 2014). This objective has usually been achieved by evaluating the agrobiological and ampelographic characteristics of grapevine varieties (Stavrakaki and Biniari, 2017; Maistrenko et al., 2020). Thus, distinguishing clones of the same variety or closely related varieties by the previously mentioned characteristics was sometimes very difficult and required the existence of qualified personnel in the field of viticulture (Biniari and Stavrakaki, 2019). To complement the morphological characterization of grapevine varieties, biotechnological approaches, such as DNA-based molecular markers techniques have been developed to analyse the diversity of grape germplasm resources (Milišić et al., 2021). In this regard, several molecular markers have been used for characterization of grapevine cultivars, clone's identification and the detection of synonymies, including random amplified polymorphic DNA (RAPDs) (Karataş & Ağaoğlu, 2010), amplified fragment length polymorphisms (AFLPs) (Stajner et al., 2009), simple sequence repeats (SSRs) and single nucleotide polymorphism (SNPs) (Emanuelli et al., 2013; Villano et al., 2014).

In addition, biotechnological researches were used to improve the quality of grape cultivars (Butiuc-Keul and Coste, 2023). In this regard, the development of *in vitro* plant regeneration methods has played a crucial role in the conservation and propagation of valuable genotypes, as well as in increasing genetic variability (Butiuc-Keul et al., 2008; Fehér, 2019; Nuzzo and Perrone, 2022). To date, *in vitro* grapevine regeneration has been achieved

by organogenesis and somatic embryogenesis (Fehér, 2019; Campos et al., 2021).

Organogenesis is defined as the development of organs, such as roots, shoots, and flowers, either directly from an explant (direct organogenesis), or from the callus culture (indirect organogenesis). It is noteworthy that indirect organogenesis induces somaclonal variation involving both genetic and epigenetic changes in *in vitro* regenerated plants that could be useful for the selection of improved grapevine genotypes (Schellenbaum et al., 2008). Moreover, it has been shown that PCR-based molecular markers such as RAPD have been effective for detection of occurred grapevine somaclonal variations (Pop, 2008). Thus, the aim of the present research was to study the influences of different experimental factors such as growth regulators, type of *in vitro* culture, length of culture, and genotype on somaclones obtained from five grapevine cultivars. A comparative RAPD analysis was performed between callus-regenerated somaclones from five grapevine cultivars and their field-grown mother plants.

MATERIALS AND METHODS

Plant material and *in vitro* culture

Micropropagation of five grapevine cultivars: Cabernet sauvignon, Fetească albă, Merlot, Riesling Italian and Traminer roz (Gewürztraminer) was performed using as explant sources nodal segments from field-grown plants.

Shoot initiation and proliferation were performed on Murashige and Skoog (MS) medium supplemented with 0.5 mg/L NAA + 0.5 mg/L BAP (V1); 0.5 mg/L NAA + 1.0 mg/L BAP (V2) and 0.5 mg/L NAA + 2.5 mg/L BAP (V3). The proliferated shoots on V3 were used for the induction of the grapevine callus. Calli were then subcultured on fresh MS medium supplemented with 1.0 mg/L BAP +5.0 mg/l NAA (V4) and 1.0 mg/L BAP +10 mg/l NAA (V5). The morphogenic calli grown on V5 were transferred and sub-cultured on MS medium supplemented with 0.5 mg/L NAA and three different concentrations of TDZ (0.5 mg/L -V6; 1.0 mg/L - V7; 2.0 mg/L- V8) for shoot regeneration. The *in vitro* plants regenerated via callus after the 12th subculture (8 weeks/subculture) were acclimatized under greenhouse conditions in plastic pots (Ø = 9 cm) filled with perlite. The rooted plants were then hardened under field conditions.

Genetic analysis using RAPD markers

RAPD analysis was performed after the 12th subculture using the *in vitro*-raised plants from V6 culture medium (0.5 mg/L NAA+0.5 mg/L TDZ). Field-grown plants of each cultivar analysed were used as controls.

Before DNA isolation, harvested leaves from each cultivar were dried, ground into a fine powder (TissueLyser II, Qiagen, Germany), and stored at 4°C until genetic analyses were performed.

Total genomic DNA was isolated from 0.15 g of dry powder using a protocol based on the CTAB (cetyltrime-thylammonium bromide) method published by Lodhi et al. (1994) and improved by Pop et al. (2003) and Bodea et al. (2016). DNA concentration (ng/ µL) and purity (260/280 nm) were determined with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Before performing PCR (polymerase chain reaction) amplifications, all DNA samples were diluted to 50 ng/µL, using sterile double-distilled water. For the RAPD analysis, the PCR amplification reactions were performed using the protocol described by Williams et al. (1990) modified by Pop et al. (2003). To amplify the DNA samples, a number of 9 RAPD primers (Microsynth AG) were used. PCR amplifications were performed in 25 µl reaction volume containing 50 ng of template, 200 µM dNTP, 0.2 µM primer, 2.5 mM $MgCl_2$, 2.5 mM 10 x buffer, 1 U Taq DNA Polymerase (Promega, USA) and 2% PVP in a 96 Well Gradient Palm-Cycler CG1-96 (Corbett Research). PCR conditions were: 1 cycle of 95ºC for 3 minutes for initial denaturation, followed by 45 cycles of 1 minute at 93ºC for denaturation, primer annealing at 34ºC for 1 minute, and primer extension at 72ºC for 1 minute. After a final extension cycle (10 min. at 72ºC) the samples were stored at 4ºC. The PCR amplifications were repeated twice for each RAPD primer to ensure the reproducibility of the results. Separation of the PCR amplified products was carried out by electrophoresis on 1.4% agarose gels (Promega,

Madison, WA, USA) stained with ethidium bromide solution in 1X TBE (Tris Borate-EDTA buffer), at 110 V and 136 mA for 2.5-3 h. The electrophoretic profiles were visualized under UV (ultraviolet) in UVP Biospectrum AC Imaging System (Upland, CA, USA). The list of RAPD primers used in this study is shown in Table 1.

Table 1. The list of RAPD primers used

Primer name	The 3'-5'nucleotide sequence of the primer
OPA 01	CAG GCC CTT C
OPA 03	AGT CAG CCA C
OPA 04	AAT CGG GCT G
OPAB 11	GTG CGC AAT G
OPAB 18	CTG GCG TGT C
AB 11	GTG CGC AAT G
OPAL 20	AGG AGT CGG A
OPX 03	TGG CGC AGT C
OPE 14	TGC GGC TGA G

Statistical analysis

The data were analysed using ANOVA PoliFact software (UASVM Cluj-Napoca, 2015) with Duncan test for multiple comparisons among the experimental variants (three repetitions x 5 vessels x 5 inoculum/ variant) (p-value < 0.05). Gel images were analyzed using TL120 software (Nonlinear Dynamics). Amplified bands were scored present (1) or absent (0) and data entered into a binary matrix. The genetic distances between analysed genotypes were calculated using Euclidean coefficient of similarity. Cluster analysis was conducted with an UPGMA algorithm using PAST software (PAle-ontological STatistics Version 4.11, Natural History Museum, Norway). Its consistency was assessed using bootstrap method in 10000 repetitions.

RESULTS AND DISCUSSIONS

Multiplication by axillary shoots

The results of this study revealed that MS media supplemented with 0.5 mg/L 1-naphthaleneacetic acid (NAA) and different combinations of benzyl-adenine (BA) (0.5 mg/L; 1.0 mg/L and 2.5 mg/L) have influenced the average number of proliferated shoots/explant in the analysed grapevine genotypes. Thus, the highest number of proliferated shoots/explant was recorded for Cabernet Sauvignon (7.7) multiplied on the V3 proliferation medium variant (Fig.1). Our results were in agreement with those reported by Laslo et al. (2010) who stated that Cabernet sauvignon cv. showed a high *in vitro* regeneration capacity only on media supplemented with a high concentration of BA (5 mg/L). The lowest number of proliferated shoots/explant (1.18) was recorded in Fetească albă proliferated on the V1 culture medium variant. Our results are consistent with those reported by Butiuc-Keul et al. (2007) in a previous study concluding that Fetească albă cv. was the most recalcitrant cultivar to the *in vitro* multiplication conditions.

It is noteworthy that MS media supplemented with the highest concentration of BA (2.5 mg/L) generated in this study the highest number of proliferated shoots/explants for all five analysed grapevine genotypes as shown in Figure 1.

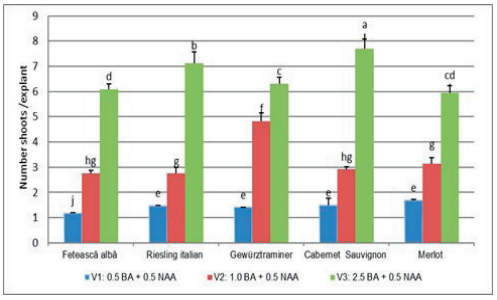

Figure 1. The influence of proliferation media variants (V1-V3) on the average number of proliferated shoots/explant in five grapevine genotypes. Bars with the same letters are not significantly different according to Duncan's multiple range test, p = 0.05. Error bars represent standard error of the mean (SEM)

However, the highest values of average length (cm) of proliferated shoots/explant were recorded on V1 culture media supplemented with the lowest concentration of BA (0.5 mg/L) (Figure 2).

Regarding the average length (cm) of proliferated shoots/explant (Figure 2), it can be seen that the longest shoots were obtained at genotype Gewürztraminer (8.22 cm), followed by Cabernet sauvignon cv. (7.14 cm) and Riesling italian cv. (6.89 cm) on the V1-variant of the proliferation culture media, while the shortest proliferated shoots were recorded in

Feteasc alba (4.52 cm) on the V3-variant of the proliferation culture media (Figure 2).

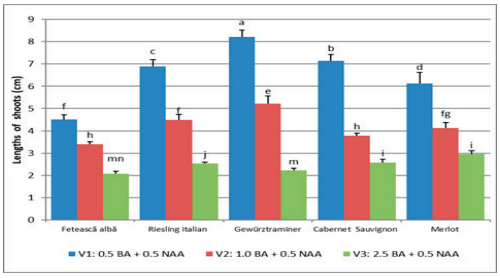

Figure 2. The influence of proliferation media variants (V1-V3) on the average length (cm) of proliferated shoots/explant in five grapevine genotypes. Bars with the same letters are not significantly different according to Duncan's multiple range test, p = 0.05. Error bars represent standard error of the mean (SEM)

An explanation of these results is due to the particularities of *in vitro* vegetative development of the analysed genotypes. According to Reisch (1986) and Garcia et al., 2023) *in vitro* proliferation of grapevine is genotype-dependent.

Callus induction

In terms of callus morphology, the results of this study revealed that explants from nodal fragments cultured on MS - variant V4 generated non-friable, white or pale-brown calli in all analysed varieties that were unusable for further experiments (Figures 3 and 4).

Figure 3. Non-morphogenic callus from Cabernet Sauvignon cv. grown on MS-V4

Figure 4. Non-morphogenic callus from Feteasc albă cv. grown on MS-V4

Noticeably, the callus mass initiated from nodal segments and grown on V5 culture medium (10 mg/l NAA concentration) showed morphogenic characteristics (compact consistency, high optical density and different colors such as milky-white, yellow, yellow-green to red-violet pigmentation) as can be seen in Figures 5a and 5b. The amount of morphogenic callus from V5 was used for previous indirect organogenesis experiments.

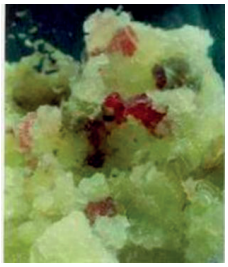

Figure 5a. Morphogenic callus from Cabernet sauvignon cv. grown on MS-V5

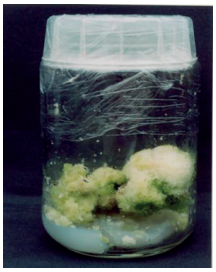

Figure 5b. Morphogenic callus from Feteasc albă cv. grown on MS-V5

The results of this study showed that callus induction was successfully achieved by using the most commonly used auxins such as NAA and BA (cytokinin) for *in vitro* tissue cultures (Pop, 2008).

The influence of culture media variants (V4-V5) on the average percent (%) of induced calli in five grapevine genotypes are presented in Figure 5.

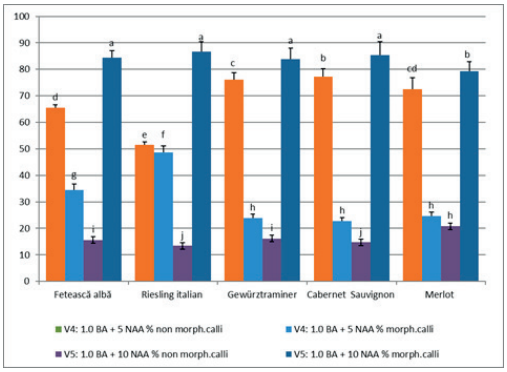

Figure 5. The influence of culture media variants (V4-V5) on the average percent (%) of induced calli in five grapevine genotypes. Bars with the same letters are not significantly different according to Duncan's multiple range test, p = 0.05. Error bars represent standard error of the mean (SEM)

In this study, morphogenic calli were induced in all five grapevine cultivars analysed on the MS-V5 culture medium variant, but with statistically non-significant differences between

grapevine cultivars in terms of the percentage of morphogenic calli, except Merlot cv., as previously shown in Figure 5. These results suggest that callus induction was influenced by genotype and the combinations between the phytohormones used. In agreement with the results of this study, Yun-Zhu et al. (1985) also reported callus development in *in vitro* cultured grapevine cultivars by using different concentrations of BA and 2,4-D.

In the experiments of this study, we studied whether the plants regenerated via callus and subcultured for a long period of time were suitable for morphological and genetic changes. As shown in Figure 6, the highest average number of plantlets regenerated from morphogenic calli was recorded on MS media supplemented with 0.5 mg/L NAA + 0.5 mg/L TDZ (V6) at Riesling Italian cv. (11.6 plantlets) and Cabernet sauvignon (10.50 plantlets). The lowest average numbers were recorded at Riesling Italian cv. (3.80 plantlets) and Merlot cv. (3.97 plantlets) cultured on MS media and supplemented with 0.5 mg/L NAA + 2.0 mg/L TDZ (V8).

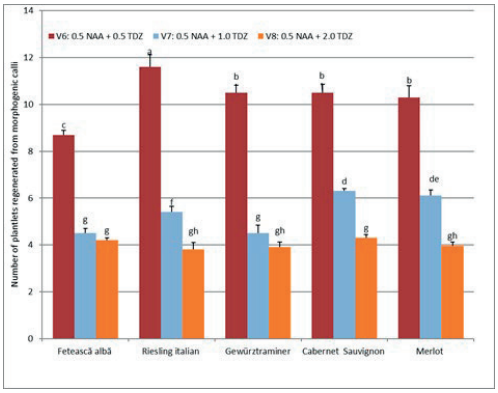

Figure 6. The influence of culture media variants (V6-V8) on the average number of plantlets regenerated from morphogenic calli in five grapevine genotypes. Bars with the same letters are not significantly different according to Duncan's multiple range test, p = 0.05. Error bars represent standard error of the mean (SEM)

These results confirm the statement that the response of grapevine to *in vitro* culture is usually related to genotype, culture medium, and plant endogenous phytohormones (Nookaraju et al., 2008; Melyan et al., 2015). Our results are consistent with other studies (Olah et al., 2003; Morariu et al., 2011) which concluded that the addition of TDZ to the culture media in concentrations ranging from 0.5-3 mg/L had a stimulatory effect on the regeneration of plants from calli, especially on woody species and recalcitrant plants.

Genetic analysis based on RAPD molecular markers

The results of the present study show that RAPD markers were suitable to assess the somaclonal variations on five grapevine cultivars. Out of the nine primers screened for their ability to amplify the DNA samples from *Vitis vinifera* subsp. *vinifera* genotypes, all revealed reproducible and consistent results. The levels of polymorphism detected with RAPD primers are presented in Table 2.

Table 2. The level of polymorphism detected with RAPD primers in somaclones from five grapevine genotypes (NPB-number of polymorphic bands; NTB-number of total bands; PPB-percent of polymorphic bands)

Primer name	Size of bands (bp)	NPB	NTB	PPB (%)
OPA 01	400-800	2	4	50
OPA 03	500-1600	4	6	66.6
OPA 04	600-1800	5	7	71.4
OPAB 11	500-2000	7	8	87.5
OPAB 18	500-1800	2	4	50.0
AB 11	600-1200	9	9	100.0
OPAL 20	500-2000	7	8	87.5
OPX 03	300-2000	7	9	77.7
OPE 14	500-1000	4	5	80.0
Total		47	60	
Average		5.2	6.6	74.5

The nine RAPD primers amplified 60 reproducible fragments ranging from 500 to 2000 bp, out of which 47 bands were polymorphic bands (5.2 bands/ primer). The number of polymorphic bands for each primer ranged from 2 to 9. The highest number of polymorphic bands (9) was generated by AB 11. The lowest number of amplified polymorphic bands (2) was obtained with the primers OPA 01 and OPAB 18. The electrophoretic profile generated with primer OPA 03 is show in Figure 7.

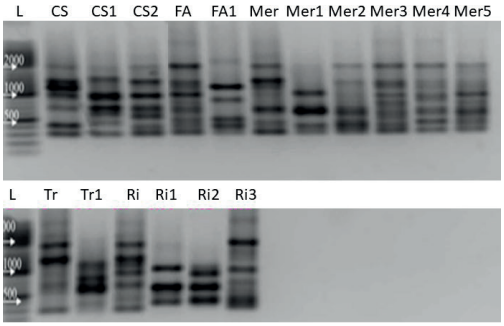

Figure 7. The genetic RAPD profiles of somaclones obtained with primer OPA 03 and generated from five grapevine genotypes. L-100 bp Ladder (Promega); CS -mother plant Cabernet Sauvignon; CS1-CS2 - somaclones from Cabernet Sauvignon; FA - mother plant Fetească albă; FA1 - somaclone Feteasca albă; Mer - mother plant Merlot; Mer1-Mer5 - somaclones from Merlot; Tr - mother plant from Traminer roz; Tr1 - somaclone from Traminer roz; Ri - mother plant from Riesling Italian; Ri1-Ri3 - somaclones from Riesling Italian

The percentage of polymorphism (no. of polymorphic bands/no. of total bands x 100) ranged from 50.0% (OPA 01 and OPAB 18) to 100.0% (AB 11) with a mean value of 74.5%.
The UPGMA dendrogram, built based on Euclidean distances, grouped the grapevine genotypes into two main clusters as shown in Figure 8. The first main cluster included one distingly sub-clusters including two somaclones (CS1 and CS2) from Cabernet Sauvignon cv. and their mother plant (CS). The second main cluster grouped five somaclones from Merlot (Mer1-Mer5) and their mother plant (Mer), one somaclone from Gewürztraminer (Tr1) and mother plant (Tr) and one somaclone from Fetească alba (FA 1) and mother plant (FA). Noteworthy is the grouping of the Italian Riesling somaclones together with their mother plant in a distinct sub-cluster compared to the other analyzed genotypes of the second main group.
This clustering pattern suggests that somaclonal variability was detected at the DNA molecular level by using RAPD molecular markers.

CONCLUSIONS

The results of this study confirm the hypothesis that after successive subcultures somaclonal variations were detected. On the other hand, micropropagation of the analyzed grapevine cultivars produced healthy and vigorous plants, which allowed them to acclimatize to *ex vitro* conditions. The callus that was induced in this study represents a valuable plant material to produce clones and make genetic improvements in *Vitis vinifera* subsp. *vinifera*.

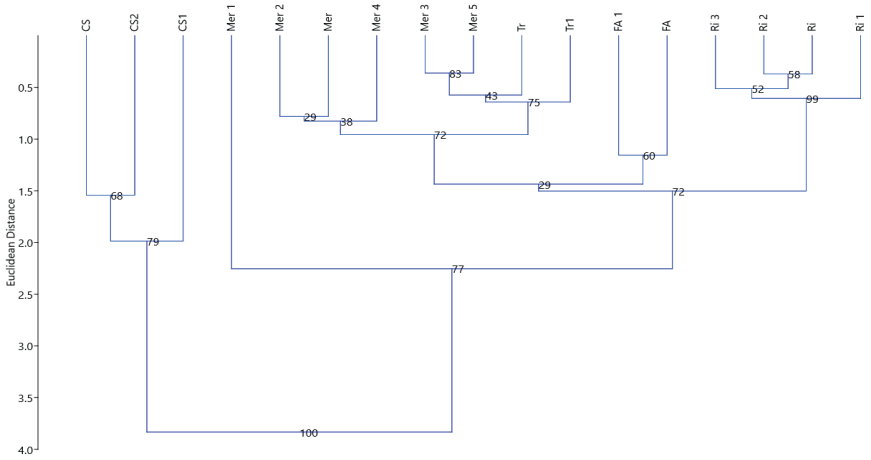

Figure 8. UPGMA dendrogram generated by RAPD markers, showing the relationships between grapevine somaclones and their mother plants based on Euclidean's distance index. Numbers on the branches show bootstrap values, computed from 10000 replications

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