USE RAPD TECHNIQUE FOR REVEALING MOLECULAR POLYMORPHISM OF SOME GRAPEVINE SOMACLONES

DETERMINAREA POLIMORFISMULUI LA NIVEL MOLECULAR PRIN TEHNICA RAPD A UNOR SOMACLONE DE VIȚĂ DE VIE

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Abstract. *In vitro grapevine regenerated shoots obtained from callus culture were analyzed in order to reveal the molecular polymorphism. Somaclonal variation is so often associated by direct callus or cell suspensions regenerations. The samples were analyzed using RAPD Markers (Random Amplified Polymorphic DNA). Total DNA was extracted using the protocol published by Lodhi et al. (1994) and modified by our research team (Pop R. et al., 2003). For DNA isolation we used regenerated shoot provided by in vitro subcultivated grapevine cultivars such as: Cabernet Sauvignon, Fetească albă, Merlot, Traminer rose and Riesling italian. PCR amplification reactions were carried out using the same 22 arbitrary primers which generated polymorphic bands in case of parental cultivars collected by experimental field. Genetic distances between analyzed samples were calculated based on Jaccard coefficient and the dendrogram was constructed with RAPDistance 1.04 soft. The built dendrogram shows that there are differences at molecular level among somaclones and among grapevine parental cultivars and somaclones.*

Rezumat. *In acest studiu a fost testat heteromorfismul molecular la regeneranții de viță de vie obținuți in vitro prin cultura de calus. Variabilitatea somaclonală este asociată adesea cu regenerarea directă din calus sau din suspensii de celule. Pentru testarea heteromorfismului la nivel molecular probele au fost analizate cu ajutorul markerilor RAPD (Random Amplified Polymorphic DNA). Extracția ADN-ului a fost realizată prin metoda Lodhi şi colab.(1994) modificată de noi (Pop R şi colab., 2003), din regeneranții obținuți prin subcultivări repetate in vitro la soiurile Cabernet Sauvignon, Fetească albă, Merlot, Traminer roz şi Riesling italian. Pentru amplificarea PCR a probelor s-au folosit un număr de 22 de primeri decameri, primeri care au dat benzi polimorfice şi în cazul extracției de ADN din soiurile de viță de vie recoltate din câmp. Distanțele genetice dintre probele analizate au fost calculate pe baza coeficientului Jaccard, iar dendrograma a fost realizată cu ajutorul programului RAPDistance 1.04. Dendrograma obținută relevă faptul că somaclonele obținute se diferențiază la nivel molecular de cultivarele parentale din care au provenit.*

Key words: grapevine, somaclones, molecular polymorphism, RAPD

Somaclonal variation is an important source of variability for *in vitro* cultivated plants. At the present moment, this type of variation is considered an aleatory process which can be successfully use in some of plant breeding strategies. In the last two decades, plant tissue culture proved to be a potential source of genetic variation, and

therefore, a possible mean for selecting new valuable genotypes among the regenerated somaclones exhibiting stable modification of a trait (Popescu et al., 2002).

In the case of grapevine, somaclonal regenerants of different types have been reported with regard to plant morphology. On the basis of ampelographical criteria it has been concluded that *in vitro* regenerated somaclones are very similar, but this has not been proved at the genetic level (Popescu et al., 2002). If morphogenetic changes are not apparent, other plant screening procedures must be applied: comparative biochemicals analysis (e.g. isozyme analysis after protein electrophoresis separation) or cytological studies reffering to changes in number and structure of the chromosomes.

During the last years, especially after the improvement of PCR (Polimerase Chain Reaction) method, molecular techniques became increasingly used in genus *Vitis* for identification of species, cultivars and somaclonal variations (Saiki and all., 1988, cited by Pamfil, 1999). Various molecular techniques (RFLP-Restriction Fragment Length Polymorphism, AFLP- Amplified Fragment Length Polymorphism, RAPD- Random Amplified Polymorphic DNA, microsatellites and ISSR- Inter Simple Sequence Repeats etc.) are used to characterize genetic grapevine diversity. Among these molecular techniques, RAPD is simple, quick, easy to perform and require small amount of DNA for analysis.The major advantage of RAPD is that no prior sequence information is required. These benefits justify the frequent application of the technique in genetic variability studies (Loureiro et al., 1998, cyted by Pamfil, 1999; Pop Rodica et al.,2005, Popescu et al., 2002).

MATERIALS AND METHODS

Grapevine varieties included in this study were collected from Blaj, Recas, Iasi, Valea Calugareasca-Odobesti and were represented of the following cultivars: Feteasca alba, Feteasca regala, Riesling Italian, Traminer, Cabernet Sauvgnion, Muscat Ottonel, Merlot, Cetatuia, Napoca and Timpuriu de Cluj. Somaclonal variation study was performed using plantlets regenerated via callus. In our studies the callus was induced using the plantlets micropropagated *in vitro*.

The morphogenetic callus, obtained from nodal fragments, was subcultured in Murashige-Skoog medium supplemented with different concentrations of TDZ - thidiazuron (N-phenyl-N'-1,2,3,-thidiazol-5-yl urea) in combination with 0,5 mg/l NAA (naftil acetic acid). All the variants of culture media were supplemented with 3% sucrose and 0,75% agar. The pH was adjusted to 5,8 with 0,1 N NaOH solution, before autoclaving at 121^0C, for 20 minutes. We used three variants of culture media: MS– TDZ – 0,5 mg/l TDZ + 0,5 mg/l ANA, MS –TDZ – 1,0 mg/l TDZ+ 0,5 mg/l ANA and MS– TDZ – 2,0 mg/l TDZ+ 0,5 mg/l ANA. The experiment was made in three replications and interpreted as a bifactorial test with: factor A (cultivar) with ten graduations and factor B (culture media) with three graduations. The obtained data were computed according to analysis of variance in bifactorial experiments (Ardelean et al., 2002).

The micropropagated plants were subsequently maintained *in vitro* by periodical transfer to the same variants of culture media in order to obtain enough material for DNA isolation. In this study, we used for genomic DNA isolation leaves harvested from plantlets obtained after 5, 10 and 15 subcultures on fresh culture media. We analyzed somaclones provided from different grapevine cultivars.

Genomic DNA was extracted from *in vitro* plantlets regenerated via callus using a modified version (Pop Rodica et al., 2003) of the protocol publised by Lodhi et al. in 1994. Extraction buffer has the following composition: 100mM Tris-HCl, 20 mM EDTA, pH =8.0, 1.4 M Na Cl, 2% (w/v) CTAB and 2% PVP-40 and 0.2% β-mercaptoethanol. This buffer was also supplemented with 5 mM ascorbic acid and 4 mM DIECA. DNA concentration and the absorbance ratio at A_{260}:A_{280} was quantified in a BioPhotometer Eppendorf. Reaction mixture for PCR in 25 µl volume consisted of 50 ng DNA, 200 µM of each dNTP (Promega), 0,2 µM primer (UBC, Pharmacia Biotech, see table no. 1), 2,5 mM $MgCl_2$, 2,5 mM 10 x Buffer, 1 U Taq DNA Polymerase (Promega), 2% PVP (Sigma),

bidistillated sterile water. Amplification was performed in a Eppendorf Mastercycler Gradient programmed for the following thermal profile: an initial denaturation step – 3 min at 95°C, followed by 45 cycles of 1 min. at 93°C, 1 min. at 34°C, 1 min. at 72°C. A final extension step at 72°C was performed for 10 minutes. Amplification products were separated in 1.4 agarose (Sigma) gel at 55 V for 2.5 h in 0.5 x TBE Buffer and visualized under UV light after staining with ethidium bromide using Alpha Innotech system. For the comparation among results, in agarose gels, after DNA Ladder we loaded in second lane DNA sample provided from *in vivo* plant control (leaves of parental cultivar collected by experimental field) following by DNA samples isolated from each somaclones of the same cultivar. RAPD bands were scored as present (1) or absent (0). The gel image was recorded with AlphaImager 2.200 system. Genetic distances were established using Jaccard's coefficient. The dendrogram was constructed with RAPDistance 1.04 software using Neighbor-Joining method. Table 1 shows the nucleotide sequences of RAPD primers used in DNA amplification of regenerants obtained *in vitro*.

Table 1

Primers used in DNA amplification of regenerants obtained

No	*Primer*	*Sequence (5' – 3')*	*No*	*Primer*	*Sequence (5' – 3')*
1.	UBC 228	GCT GGG CCG A	12.	OPAB 18	CTG GCG TGT C
2.	UBC 245	CGC GTG CCA G	13.	OPE 14	TGC GGC TGA G
3.	UBC 563	CGC CGC TCC T	14.	AB 11	GTG CGC AAT G
4.	UBC 584	GCG GGC AGG A	15.	OPA 04	AAT CGG GCT G
5.	UBC 599	CAA GAA CCG C	16.	OPA 03	AGT CAG CCA C
6.	PB 1	GGT GCG GGA A	17.	OPAL 20	AGG AGT CGG A
7.	PB 3	GTA GAC CCG T	18.	OPX O3	TGG CGC AGT C
8.	PB 4	AAG AGC CCG T	19.	OPA 01	CAG GCC CTT C
9.	PB 5	AAC GCG CAA C	20.	70.08	CTG TAC CCC C
10.	PB 6	CCC GTC AGC A	21.	70.03	ACG GTG CCT G
11.	OPAB 11	GTG CGC AAT G	22.	MIC – 07	TGT CTG GGT G

RESULTS AND DISCUSSIONS

The values of the calculated F test for all of analysed experimental factors were much higher than the theoretical values (25,4>2,04;2,72, 21484,8>3,15;4,98, 22,2>1,81;2,32). That means that the differences between repetitions and variants were statistical assured in experiment shows in Fig 1.

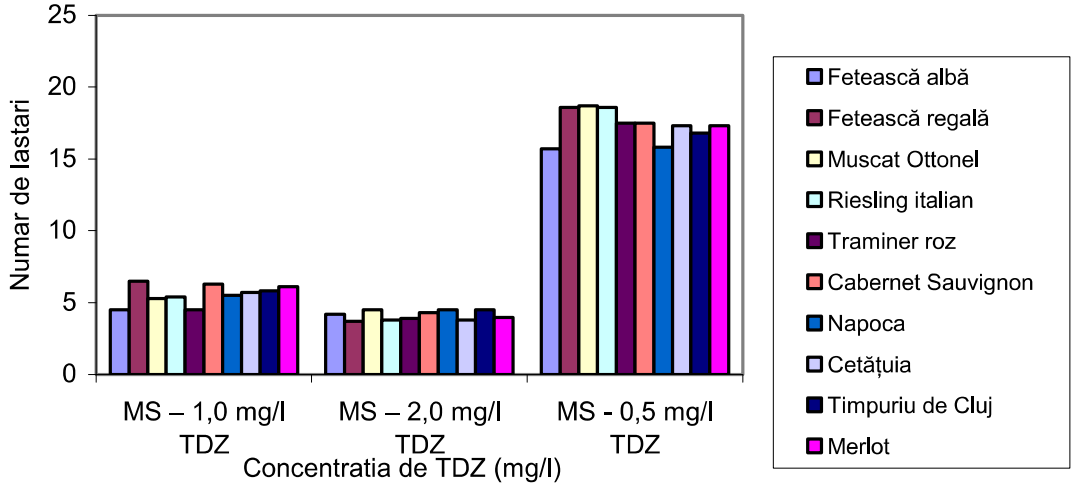


Fig. 1. Number of shoots in the to way experiment (10x3) with cultivars of Vitis vinifera and culture media used in plantlets regeneration from callus

As it can be seen in Fig.1, data obtained by Duncan test shows that the highest mean values of shoots number (18,7) was obtained using MS culture media supplemented with thidiazuron in concentration 0,5 mg/l .

Fig. 2 shows the influence of culture media and cultivars upon the mean length of regenerated shoots after 8 week *in vitro* culture. In this case, F test shows that only culture media generated the differences statistical assured between experimental variants (value of F calculated 954,6>3,15;4,98).

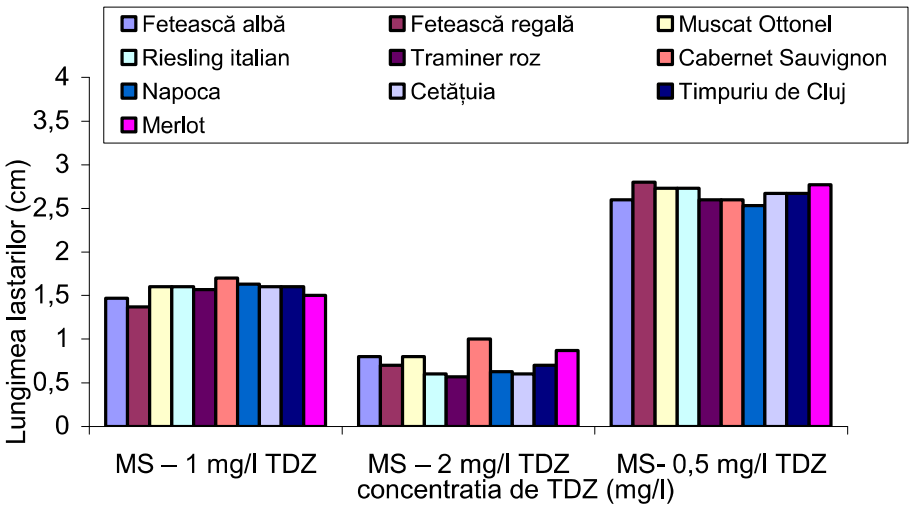


Fig. 2. Length (cm) of shoots in the to way experiment (10x3) with cultivars of Vitis vinifera and culture media used in plantlets regeneration from callus

As it can be seen in this graphic the lowest mean of shoots length was obtained using MS culture media supplemented with thidiazuron in concentration 1,0 mg/l and 2,0 mg/l.

The interaction between these two analysed factors shows that the highest mean of length shoots (2,53-2,80) was obtained when regenerants were provided by Traminer roz cultivar and were subcultivated on MS culture media supplemented with 0,5 mg/l thidiazuron.

Our results suggest that, at phenotypic level, there are not significant differences among plantlets regenerated from morphogenetic calli.

On the other hand, we observed that the somaclonal variation were identified at regenerants obtained after 15 *in vitro* subcultures and for only five analyzed cultivars. Merlot was the cultivar with the high number (5) of somaclones.

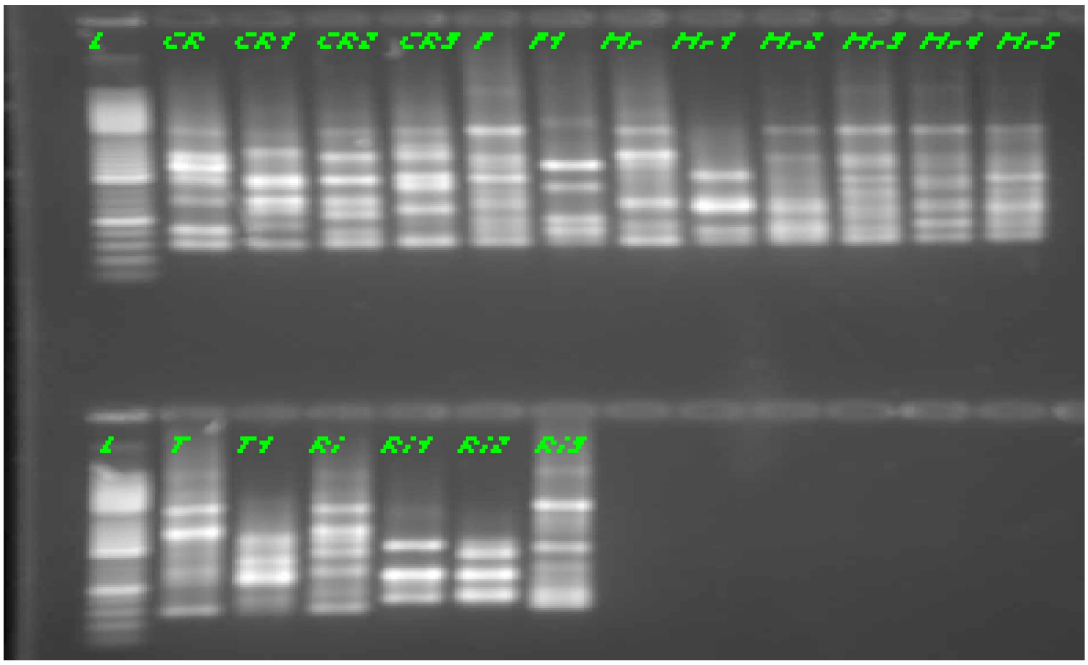
The genetic variability among *in vitro* selected somaclones was analyzed using RAPD molecular marker technique.

DNA fragments generated after PCR amplifications with RAPD primers had the length comprised between 200-2000 bp, respectively 300-1400 bp for the majority.

The primer OPX 03 produced maximum 12 polymorphic bands; primer OPE 14 generated 11 polymorphic bands; OPA 04 and PB 6 primers gave 10 polymorphic bands, PB 1 9 polymorphic bands.

The others primers used generated 6-9 polymorphic bands. An interesting RAPD profile was obtained with primer OPX-03 (Fig.3).

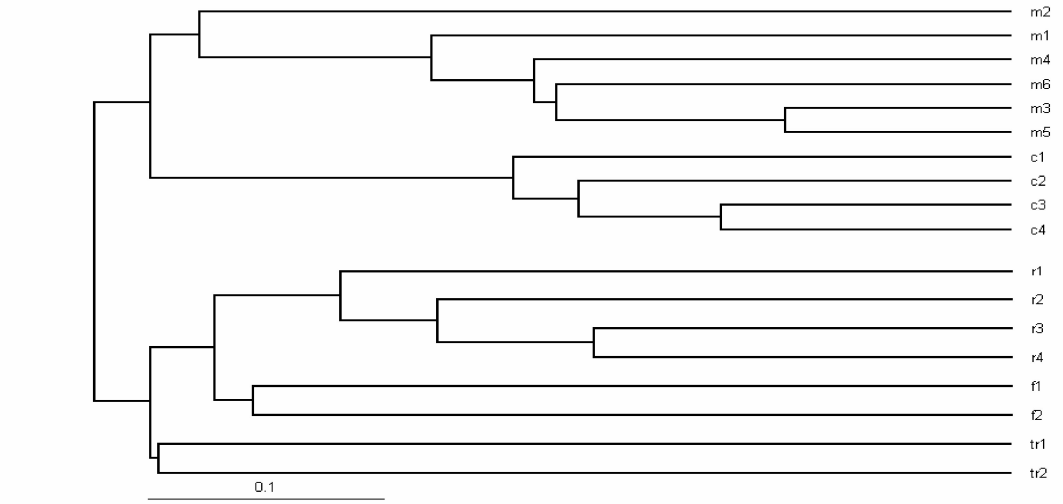
Fig. 3. RAPD profile obtained with primer OPX 03 of parental cultivars(controls) and regeretated somaclones



L- DNA Ladder, CR-parental cultivar Cabernet Sauvignon, CR1, CR2, CR3-somaclones; F-parental cultivar Feteasca albă,F1-somaclone; Mr-parental cultivar Merlot, M1,M2,M3, M4, M5-somaclones; T- parental cultivar Traminer, T1-somaclone; Ri parental cultivar Riesling italian, R1,R2,R3-somaclones.

The agarose gels analysis reveal genetic differences among all of the regenerated grapevine somaclones.

The built dendrogram shows that there are differences at molecular level among somaclones and also grapevine parental cultivars.



Dendrogram shows the genetic reletionship between the parental cultivars (controls) and regenerants obtained in different *in vitro* experimental variants.

Fig.4 Dendrogram of somaclones obtained using Neighbor Joining method

Based on this dendrogram it can be observed that parental cultivar and somaclones provided from this are very closely grouped.

Cabernet Sauvignon and Merlot cultivars were grouped in dendrogram in the same secondary branching closely grouped with regenerated somaclones. This result is in concordance with our *in vivo* preliminary data referring to parental cultivars dendrogram's. This situation is also valuable in case of Fetească albă and Riesling italian cultivars and your's somaclones.

CONCLUSIONS

- Murashige-Skoog culture media supplemented with thidiazuron in concentration 0,5 mg/l can be successfully used of grapevine somaclones induction and previous morphologycal development. In this case, we obtained a lot of neoplantlets, with good caulogenesis development but without radicular system.

- Moderate or high level of TDZ concentration in culture media had the negativ influence upon number and length of grapevine regenerated shoots.

- The grapevine cultivar had not significant influence upon number and length of grapevine regenerated shoots.

- Dendrogram shows the genetic reletionship between the parental cultivar (used as control) and regenerants selected on different experimental variants. Based on this dendrogram we can observe that parental cultivar and somaclones provided from this are very closely grouped.

- The RAPD analysis reveal genetic differences among all of the regenerated grapevine somaclones and it can be considered a valuable tool for revealing molecular polymorphism of some grapevine somaclones.

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