UDC (UDK) 634.64(496.5)

Ariola BACU,
***Valbona MATA, Efigjeni KONGJIKA*[1]**

POMEGRANADES OF ALBANIA, THE MOLECULAR EVALUATION OF THEIR GENETIC DIVERSITY AND POSSIBLE *IN VITRO* PROPAGATION OF BEST VARIETIES

SUMMARY

The pomegranate (*Punica granatum*, Punicaceae) is cultivated and naturalized over the whole Mediterranean region since ancient times, and is also present in different areas of Albania. While there is enough evidence on the morphological discrimination among varieties of pomegranade around the world, on contrary the number of studies on molecular analysis of the genetic differences among them are more restricted. The same situation is present for studies regarding the pomegranade of Albania. It is considered as a local fruit of low use compared to other species, however, during the last years it has been much more present in the markets and in the consumers tables. For this reason, research is being conducted on the establishment of the genetic relationships among varieties of pomegranade of Albania and on the micropropagation, which could help in overcoming difficulties of vegetative propagation, production of true to-type plants and rapid and mass production of planting materials. The genetic relations among 10 local varieties presented here were based on RAPDs. From the random decamer primers used, 187 fragments were produced, of each 60 polymorphic. Estimates of genetic relationships, were conducted using Jaccard's similarity coefficient. It is also represented the work conducted on the micropropagation of three local varieties. The data on the response of the explants of the three cultivars were compared and the differences in shoots number, leaves number and shoot length parameter were observed and analysed. This work will be continued for a better evaluation of the genetic relatedness among more cultivars and for the improvement of the methodology of micropropagation.

Keywords: pomegranate, RAPDs, micropropagation, vegetative propagation.

INTRODUCTION

Pomegranate (*Punica granatum* L.) is generally known as a distinct family (*Punicaceae*), which comprises only one genus (*Punica*) and two species *P. granatum* and *P. protopunica* (Samir, 2010). *Punica granatum* was cultivated and naturalized over the whole Mediterranean region since ancient times. It is an economically important species of the tropical and subtropical regions of the world

[1] Ariola BACU, (corresponding author: ariolabacu@yahoo.com), Valbona MATA Department of Biotechnology, Faculty of Natural Sciences, University of Tirana, Efigjeni KONGJIKA, Academy of Sciences of Albania, Albania

due to its delicious edible fruits and pharmaceutical and ornamental usage (Jayesh and Kumar, 2004). The pomegranate is one of the oldest known edible fruits, and an excellent tree for growing in arid zones for its resistance to drought conditions. It is largely used as a dessert. The seeds along with the fleshy pulp are dried and used as condiment. The fruit juice is a good source of sugars, vitamin C, vitamin B, pantothenic acid, potassium, antioxidant polyphenols, and a fair source of iron. Some parts of the pomegranate tree (leaves, immature fruits, fruit rind, flower buds) have been used traditionally for their medicinal properties and also for tanning of leather.

The several uses of the minor fruit plants (alimentary, ornamental, forestry and medicinal), where the pomegranate belong, as well as the socio-economic impact, justifies the need to preserve these traditional genetic resources, that include conservation of agro-biodiversity, production of organic food, conservation of traditional landscapes and the related bio-diversity. In this contex, the pomegranate represents a very interesting fruit plant for both economic and scientific reasons. While there is enough evidence on the morphological discrimination among varieties of pomegranade around the world, on contrary, the number of studies on molecular analysis of the genetic differences among them are more restricted. DNA markers have proved to be valuable tools for the identification of plant varieties because they are more precise, while not susceptible to environmental effects. Randomly amplified polymorphic DNA (RAPD) is one of the most widely-used techniques in genetic diversity studies (Garcia et al., 2002; Joung and Roh, 2004), and for genetic mapping (Joobeur et al., 2000; Dettori et al., 2001; Carlier et al., 2004). Talebi Baddaf et al, (2003) report the evaluation of genetic diversity in 28 genotypes of Iranian pomegranate, from the Yazd pomegranate collection, using 13 RAPD primers. They noticed a low level of polymorphism between the cultivars studied and concluded that extensive morphological variation in pomegranates in Iran could be related to the vegetative (clonal) propagation of this plant and its behaviour at anthesis. Z. Zamani et al, (2006), revealed genetic relationships among 24 genotypes of pomegranades of Iran (different from those considered by Talebi Baddaf et al. 2003) and reported a very low correlation between the similarity matrices obtained based on fruit characteristics and RAPD markers for the pomegranate genotypes studied. Work is also conducted for the propagation of pomegranate vegetatively or through micropropagation. It is propagated vegetatively by the rooting of hard wood cuttings, although the establishment of new plants requires one year. Micropropagation, on the other side, is reported to help in overcoming difficulties of vegetative propagation, producing true to-type plants, and rapid and mass production of planting materials (Samir et al., 2009). Media have been reported for some of these plants included pomegranate (Dražeta, 1997; Naik et al., 1999).

MATERIAL AND METHODS

Plant material. Leaf samples from 10 pomegranate genotypes (ten paralels each) were collected from different locations in Central and Northeastern Albania. (Tab1).

DNA extraction. Genomic DNA was extracted from pomegranate leaves according to the instructions of the SIGMA Plant Genomic DNA Extraction Kit.

The purity and quantity of the genomic DNA was determined spectrophotometrically.

RAPD markers. Twenty Operon 10-mer primers (Operon Technologies, Alameda, CA, USA) were used in this study. Polymerase chain reactions (10 µl) each contained 5 ng template DNA, 1PCR buffer (SIGMA), 3,5mM $MgCl_2$, 200 µM each dNTP, 0.2 µM each decamer primer, and 1 Unit of *Taq* DNA polymerase (SIGMA). Amplification reactions were performed in a thermocycler (Mastercycler Eppendorf) programmed as follows: 94°C for 1 min, followed by 35cycles of 94°C for 10 sec, 42°C for 1 min, 72°C for 2 min and a final extension at 72°C for 5 min. Amplified products were separated by electrophoresis in 1.5% (w/v) agarose gels in Tris-borate-EDTA (TAE) buffer, pH 8.0, visualized by ethidium bromide staining and photographed under UV light with a Gel Doc system (UVP; 3UV Transilluminator). For data analysis, each gel was analysed by scoring for the presence (1) or absence (0) of each polymorphic band in each sample lane. NTSYS software (version 2.02) was used to estimate genetic similarities using Jaccard's similarity coefficient (**Rohlf, 1998**). The matrix of similarities thus produced was analysed by the unweighted pair-group method with arithmetic average (UPGMA), using the SAHN clustering module.

Table 1. List of the analyzed cultivars

No.	Analyzed cultivars	No.	Analyzed cultivars
1.	Devedishe e ëmbël	6.	Sheqerja
2.	Devedishe e thartë	7.	Shegë vendi
3.	Shega e Pllanës	8.	Shega pikaloshe
4.	Hibrid	9.	Shega Zejmen
5.	Shega gjigante	10.	Shega Laknas

In vitro Propagation

Plant Material. Buds were collected from one year old mother plants of three different pomegranate plant cultivars (Qenam Majhoshe e hershme, Majhoshe e Vonshme and Zejmen) from the collection of Botanical Garden, Tirana University.

Sterilization of explants. Apical and lateral buds were used as primary explants. The buds were washed carefully with water and than were shaken for 5 min. in 70% ethanol, followed by 20 min. treatment with $HgCl_2$ 0.01% and two drops of Tween 20. Finally the explants were rinsed three times with with autoclaved distilled water.

Explant size is not as important for micropropagation as purposes as for obtaining disease-free plants. The buds were dissected up to 3 mm by removing the outer scales and showed no sign of contamination after over one year of continued culture.

After inoculation in micropropagation media, the cultures were incubated at 25 °C (±2 °C) under a 16 hour photoperiod for germination.

Culture media. MS media was tested supplemented with MS vitamins. Sucrose was added at 30.0 g/l and myoinositol at 0.1 g/l. The pH of the prepared media was adjusted between 5.6 to 5.8 and agar-agar was added as 6.0 g/L for media solidification.

For establishment stage was added BAP 0.3 mg/l, IBA 0.1 mg/l and GA3 0.3 mg/l. While for proliferation stage was added BAP 0.5 mg/l and NAA 0.1 mg/l.

Statistical analyses. Data collections in experiment were subjected to Analyses of Variance and Student's Test. Those were evaluated by computer using the statistical evaluation program JMP 7.0.

RESULTS AND DISCUSSION

I. Genetic diversity

According to the results of the RAPDs analysis, none of the collected varieties represent homonymous genotypes. The results show that 60 out of 187 bands produced were polymorphic, demonstrating this way a considerable level of genetic differences among the ten varieties. The dendrogram of similarity (Fig.1) built on the RAPDs results demonstrates the clustering of the varieties into two big groups, which share very low similarity (~15%), of which the biggest cluster contains eight varieties grouped into two sub-clusters. According to different reports, RAPDs technique is useful to identify pomegranate genotypes, and more precise information on the genetic background of pomegranate cultivars can be obtained by this technique compared to morphological fruit characteristics. However, the interpretation of these results based on morphological traits of fruits or geographical origin of the samples was not was not possible, because in Albania it is usual the local cross-breeding of different cultivars in order to produce fruits of higher quality, and the distribution of the newly created cultivars is realized by the farmers themselves.

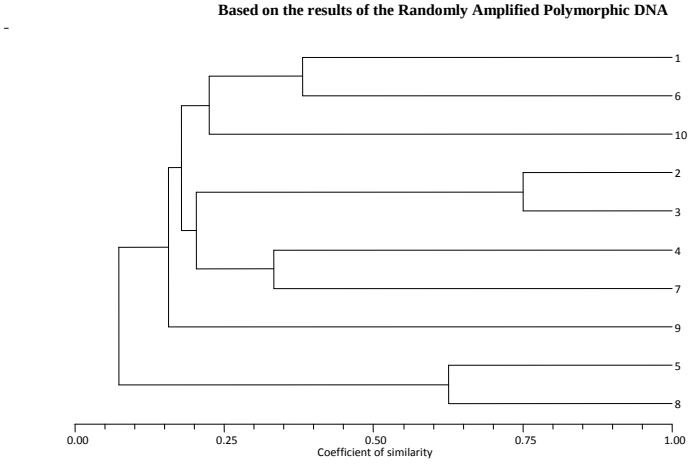
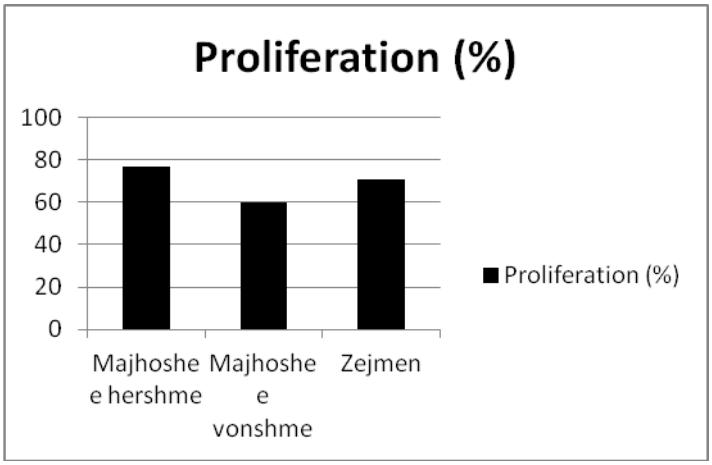


Figure 1. Dendrogram of similarity (Jaccard coefficient of similarity) among ten pomegranate varieties based on RAPDs.

Considering the lack of certainty on the origine of the cultivars and the reports on post-transcriptional effects, and epi-genetic inheritance, which can cause a lack of fit between morphological and molecular markers, we consider the use of RAPDs as a reliable tool for the observation of genetic differences among pomegranate cultivars of Albania.

In vitro Propagation

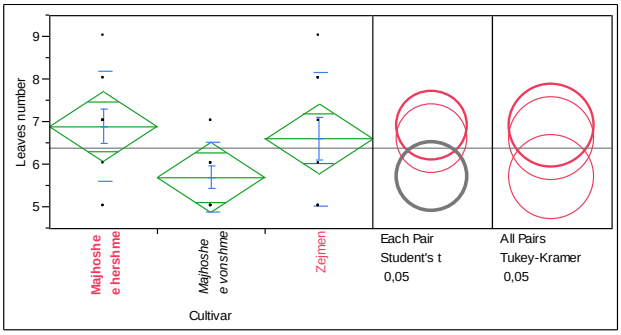
The proliferation percentage of the populations within the species was different. This parameter varies too much for the three populations of pomegranate (Graph. 1). Majhoshe e hershme cultivar gives better results on the proliferation response.



Graphic 1. Proliferation percentage for the three pomegranate plant cultivars

Differences in proliferation of three populations are related with specific reaction of initial explants of different populations inside one species to the conditions of nutrient medium.

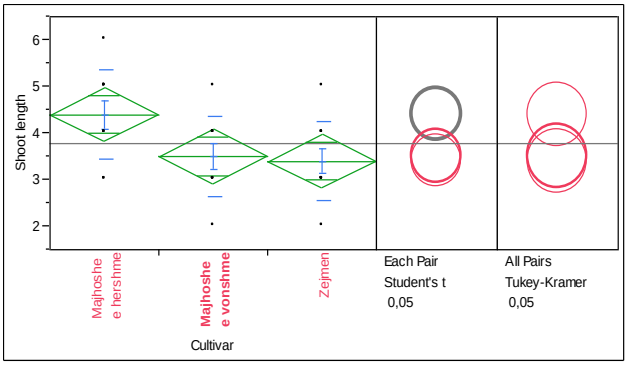
Figure 1. *In vitro* propagated pomegranate plants



Levels not connected by same letter are significantly different.

Level			Mean
Majhoshe e hershme	A		6,9000
Zejmen	A	B	6,6000
Majhoshe e vonshme		B	5,7000

Graphic 2a. Oneway Analysis and Student's test of parameter of leaves number of three pomegranate plant cultivars



Levels not connected by same letter are significantly different.

Level			Mean
Majhoshe e hershme	A		4,4000
Majhoshe e vonshme		B	3,5000
Zejmen		B	3,4000

Graphic 2b. Oneway Analysis and Student's test of parameter of shoot length of three pomegranate plant cultivars.

As pomegranate shoots grow during elongation stage to several cm with some leaves they are ready to pass to the first subculture. The leaves number and shoot length was also evaluated for the three pomegranate plant cultivars in order

to specify if there is or not any statistical difference between them regarding to these parameters. During subcultures, (Graph. 2a, b), the explants of different populations were characterized from specific reaction to micropropagation medium with a higher quantity of cytokinin BAP in comparison with the proliferation medium.

CONCLUSIONS

The aim of this study was to reveal genetic relationships among different varieties of pomegranate *(Punica granatum* L.*)*, of Albania, as well as to develop protocols for the *in vitro* propagation of some of them. The response of the plants to the conditions of micropropagation was used as an extra parameter for the detection of differences among cultivars. As a result, the RAPDs showed a level of similarity which varies from 15-77%, and clearly separate the cultivars under study from each-other. The dendrogram of similarity (Fig. 1) built on the RAPDs results demonstrates the clustering of the varieties into two big groups, which share very low similarity, of which the biggest cluster contains eight varieties; grouped into two sub-clusters. A similar situation of non-similarity results from the comparison of different parameters of plant micropropagation at different cultivars. The chosen protocol results effective in obtaining high percentage of micropropagated plants from three cultivars. There is difference in shoots number, leaves number and shoot length parameter, and the explants of the three cultivars demonstrate different percentage of proliferation and better results for the leaves number and shoot length. As a result, we conclude that based on the present study, the ten cultivars under investigation represent a considerable level of polymorphism, and that upon the careful work to be conducted regarding the labeling of specimens collected in private collections, or anywhere else, we should continue the research for the complete determination of the genetic variability of pomegranates via molecular tools in the whole territory of Albania, in order to have a complete view of the interspecies biodiversity. Also, due to the fact that the cultivation of pomegranate is limited to marginal areas, in order to overcome the risk of losing the genetic resources, there is need to develop efficient micropropagation methods. This is considered as an economic method of propagating woody plants in large numbers, and can be used in order to preserve juvenility (Osterc, 2004; Chaturvedi, 2004).

REFERENCES

Carlier, J.D., Reis,A., Duval, M.F., Conppens, G. and Leitao, J.M. (2004). Genetic maps of RAPD, AFLP, and ISSR markers in Ananas bracteatus and Ananas comosus using the pseudo-test-cross strategy. Plant Breeding, 123, 186–192.

Dettori, M.T., Quarta, R. and Verde, I. (2001). A peach linkage map integrating RFLPs, SSRs, RAPDs and morphological markers. Genome, 44, 783–790.

Garcia, M.G., Ontivero,M., Diaz Ricci, J.C. and Castagnaro, A. (2002). Morphological traits resolution RAPD markers for the identification of the

main strawberry varieties cultivated in Argentina. Plant Breeding, 121, 76–80.

Jayesh K.C., Kumar, R. (2004). Crossability in pomegranate (*Punica granatum* L.). Indian J. Horticulture. 61: 3.

Joobeur,T.,Periam,N.,Devecente,M.C.,King,G.J. and Arus, P. (2000). Development of a second generation linkage map for almond using RAPD and SSR markers. Genome, 43, 649–655.

Joung, Y. and Roh, M. S. (2004). Paternity determination of *Ornithogalum* seedling using DNA markers. Journal of Horticultural Science & Biotechnology, 79, 316–321.

Samir, Z. (2010). *In vitro* Salt and Drought Tolerance of Manfalouty and Nab El-Gamal Pomegranate Cultivars. Australian. Basic Appl. Sci. 4(6): 1076-1082.

Samir, Z., El-Agamy, Rafat, A.A., Mostafa, Mokhtar M. Shaaban, Marwa, T., El-Mahdy (2009). *In vitro* Propagation of Manfalouty and Nab Elgamal Pomegranate Cultivars Research. J. Agric. Biol. Sci. 5(6): 1169-1175.

Zamani, Z. Genetic relationships among pomegranate genotypes studied by fruit characteristics and RAPD markers. Journal of Horticultural Science & Biotechnology (2007) 82 (1) 11–18.

Talebi Baddaf, M., Sharifineia, B. and Bahar, M. (2003).Analysis of genetic divesity in pomegranate cultivars of Iran, using Random Amplified Polymorphic DNA (RAPD) markers. Proceedings of the Third National Congress of Biotechnology, Mashad, Iran. 2, 343–345.

Dražeta, L. 1997. Pomegranate (*Punica granatum* L.) propagation by *in vitro* method of tissue culture. Rev. of Res. Work at the Faculty of Agriculture, Belgrade. 42, (1), p. 49-59.

Naik, S. K., Pattnaik, S., & Chand, P.K., 1999. *In vitro* propagation of pomegranate (Punica granatum L. cv. Ganesh) through axillary shoot proliferation from nodal segments of mature tree. Science Horticulture, 79, (3/4), p. 175-183.

Rohlf, F.J. 1998. NTSYSpc. Numerical Taxonomy and Multivariate Analysis System 2.02i. Exter Software, New York.

Chaturvedi, H. C., 2004. Plant Tissue Culture. Indian Journal of Biotechnology, 3 (2), p. 159-315.

Osterc, G., 2004. Importance of micropropagation in the mass propagation of woody (fruit) plants: the main method of tree nursery production for the future? Slovenskega sadjarskega kongresa z mednarodno udeležbo, Krško, Slovenia, 24-26 marec, 2: 593-599.

Ariola BACU,
Valbona MATA, Efigjeni KONGJIKA

NAR U ALBANIJI, MOLEKULARNA PROCJENA GENETIČKOG DIVERZITETA I MOGUĆNOST IN VITRO RAZMNOŽAVANJA NAJBOLJIH VARIJETETA

SAŽETAK

Nar (*Punica granatum*, Punicaceae) se od davnina gaji i naturalizovan je u cijelom regionu Mediterana, a prisutan je u različitim oblastima Albanije. Iako postoji veliki broj dokaza o morfološkm razlikama između sorti nara širom svijeta, s druge strane broj istraživanja o molekularnoj analizi genetičkih razlika među njima nije veliki. To važi i za istraživanja o naru u Albaniji. On se smatra lokalnom vrstom voća slabe upotrebe, međutim, u poređenju sa drugim vrstama, poslednjih godina je mnogo više prisutan na tržištu i u potrošačkim tabelama. Iz tog razloga, sprovedeno je istraživanje o uspostavljanju genetičkih veza između sorti nara u Albaniji i o mikro-propagaciji, koje bi moglo pomoći u prevazilaženju teškoća kod vegetativnog razmnožavanja, proizvodnje originalnih sorti i brze i masovne proizvodnje sadnog materijala. Genetičke veze između deset, ovdje predstavljenih, lokalnih sorti zasnovani su na slučajnoj amplifikaciji uzoraka polimorfne DNA (RAPD). Od korišćenih slučajnih prajmera, proizvedeno je 187 fragmenata, od kojih je 60 polimorfnih. Proračun genetičkih veza je izvršen upotrebom Žakarovog koeficijenta. Takođe, predstavljen je rad na mikropropagaciji tri domaće sorte. Upoređeni su podaci o reagovanju eksplantata tri sorte, a posmatrana je i analizirana razlika u broju izdanaka, broju listova, kao i dužina izdanka. Ovaj rad će biti nastavljen sa ciljem da se ostvari bolja procjena genetičke srodnosti između više sorti, radi unapređenja metodologije mikropropagacije.

Ključne riječi: nar, RAPD, mikro-propagacija, vegetetivno razmnožavanje