UDC 575.630
https://doi.org/10.2298/GENSR2201207A
Original scientific article

SOMACLONAL VARIATION IN POMEGRANATE (*Punica granatum* L.): ISSR AND CYTOLOGICAL EVIDENCES

Seyed Ardalan AZIZI[1], Farah FARAHANI[2*], Masoud SHEIDAI[3]

[1]Department of Genetic, Qom Branch, Islamic Azad University, Qom, Iran
[2]Department of Microbiology, Qom Branch, Islamic Azad University, Qom, Iran
[3]Department of Biology, Faculty of Biological Sciences, Shahid Beheshti University, Evin, Tehran, Iran

Azizi S. A., F. Farahani, M. Sheidai (2022). *Somaclonal variation in pomegranate (Punica granatum L.): ISSR and cytological evidences.* - Genetika, Vol 54, No.1, 207-217.

New advancements in tissue culture techniques have resulted in vitro propagation of several regenerations of horticultural crops. The somaclonal variation which occurs during plant tissue culture is a potential source for inducing new genetic variability in such crops. Several approaches have been utilized to ascertain the occurrence of somaclonal variation. The pomegranate plant can adapt to various environmental conditions and grow well in different kinds of soil and climate. Pomegranate Zagheh cultivar is mainly cultivated in the state of Esfehan, Kashan city in Iran. The present study was performed to investigate the potential occurrence of somaclonal variation in tissue culture regenerated plants of the cultivar Zagheh and provide morphological, cytological and molecular evidences for its occurrence. Nodal segments were chosen for the in vitro propagation of pomegranate. Explants were cultured on MS basal medium. We carried out both molecular (ISSR) and karyological studies in propagation collected. In the present study, we observed variation in both morphological (length of shoot, branch, internode, leaf and root and number of branch and root) and genetic features of the tissue culture regenerated plants. The regenerated plants of the third sub-culture differed from mother plants in the ISSR marker. We observed frequent occurrence of cells with different chromosome numbers, ranging from normal diploid 2n = 2x = 16 to tetraploid 2n = 32 chromosome number.

Keywords: Somaclonal variation, pomegranate, ISSR, cytological study

INTRODUCTION

New advancements in tissue culture techniques have resulted in to in vitro propagation of several regenerations of horticultural crops. In majority of cases, having a high degree of genetic uniformity amongst the regenerated plants is the main objective, particularly in cases of clonal propagation and preservation of elite genotypes, selected for their superior characteristics. However, in some instances, inducing new genetic variability is in demand, particularly in crops

Corresponding authors: Farah Farahani, Department of Microbiology, Qom Branch, Islamic Azad University, Qom, Iran. E-mail: farahfarahani2000@yahoo.com, Tel: +98912-2778171

with low and limited degree of genetic variability. Somaclonal variation which occurs during plant tissue culture is a potential source for inducing new genetic variability in such crops. Somaclonal variations occur as a result of gene mutation or changes in epigenetic marks, and may be utilized as a breeding method to achieve new genetic variation in crops with narrow and limited standing genetic diversity (SATO *et al.,* 2011a, b).

Different approaches have been used to investigate and confirm the presence of somaclonal variation in plants. These studies include morphological, physiological, biochemical, cytogenetics as well as using several molecular markers (SHEIDAI *et al.,* 2008).

Pomegranate is an ancient edible fruits, which is native and cultivated in Iran. It was later on cultivated in ancient Egypt and early in Greece, and more neighboring countries and in North Africa and Mediterranean Europe (MURASHIGE *et al.,* 1974).

At present, with respect to pomegranate export, Iran stands at the top of exporting countries with an annual export of 60,000 tones. India stands at second position with followed 35,176 tones. The usual method of propagation for this precious edible plant is by hardwood cutting after pruning, but the soft-wood cuttings are also practiced. Adding to these conventional aproaches, the micropropagation has been utilized in many places. In pomegranate (SINGH *et al.,* 2010).

The pomegranate plant can adapt to various environmental conditions and grow well in different kinds of soil and climate. It is a drought tolerant horticultural crop plant which has both food and medicinal properties. To enumerate some of the medicinal values of this plant, we can mention high fiber content and great amount of vitamin A and C (GLOZER and FERGUSON, 2011), as well as its antimicrobial phytochemicals (KAHRAMANOGLU and USANMAZ, 2016).

Due to great pharmacological and therapeutic properties, pomegranate plant is highly in demanded, but its propagation is difficult due to the following reasons: poor seed germination and dormancy, as well as its low germination rate due to hard seed coat, which are the setbacks for commercial pomegranate production. Instead, an in vitro regeneration of pomegranate can make commercialization a success (MATERECHERA and SEEISO, 2013).

Though somaclonal variations can be advantageous in horticultural crops (KARP, 1995) the resultant genetic variations should be detected and evaluated for crops improvement (YUSNITA *et al.,* 2005). Therefore, the present study was performed to investigate the potential occurrence of soma clonal variation in tissue culture regenerated plants of the cultivar Zagheh and provide morphological, cytological and molecular evidences for its occurrence.

Pomegranate Zagheh cultivar is mainly cultivated in the state of Esfehan, Kashan city. It has delicious fruits which are highly nutritive with high amount of protein, fat, fiber, carbohydrate etc. Moreover, we have very important processed products of this plant like, pomegranate paste, pomegranate juice and candy in Iran market. Its fruit and the bark are used as a traditional remedy against diarrhea, dysentery and intestinal parasites.

MATERIAL AND METHODS

Plant material

Nodal segments of pomegranate (*Punica granatum* L.) were collected from mature plants of three cultivars cultivated in Kashan Agriculture Research Center, Esfehan province, Iran, which are named locally as Zagheh cultivar (Figure1).

Figure 1. The tree and fruits of the pomegranate cultivar, Zagheh

Tissue culture and morphological study

Preparation of samples for tissue culture

We chose the nodal segments for *in vitro* propagation. The explants were washed and treated by a detergent for 5 min. Surface sterilization under aseptic conditions, bleaching (1% sodium hypochlorite), were performed as the earlier report (FARAHANI and MAJD, 2012). Then, the explants were cut into single node segments and cultured vertically on sterile nutrient medium.

Culture medium and conditions

Explants were cultured on MS basal medium (MURASHIGE and SKOOG, 1962), which is also supplemented, with 3% (w/v) sucrose and gelled with 5 g l^{-1} Agar (Duchefa, Haarlem, the Netherlands).

To optimize the hormonal requirements for bud sprouting and multiple shoot induction, we applied Benzyl adenine (BA), and naphtol acetic acid (NAA), (Sigma Cell Culture, min. 90%, St. Louis, USA).

The pH of the medium was 5.7 before autoclaving at a pressure of 1.2 Kg cm^{-2} and 121°C for 15 min. Obtained cultures were incubated in a culture room at 26 ± 2°C with a 16-h photoperiod under cool white fluorescent tubes. Finally, we added a combination of BA (2 mg l^{-1}), and NAA (0.5 mg l^{-1}), to the MS medium for the establishment and multiplication of shoots (PATIL *et al.*, 2011).

For indirect regeneration we used different combinations of cytokinin and auxin, as suggested by Zhang and Stoltz1 (ZHANG and STOLTZ, 1991).

Morphological characters

Morphological characteristics average length of shoot, branch, internode, leaf and root (cm) and number of branch and root were measured after 5 weeks of culture. The morphological traits of regenerated plantlets of pomegranate, Zagheh cultivar showed during five subcultures. There are several different ways to measure genetic diversity, among which morphological characteristics are the strongest determinants of the agronomic value and taxonomic

classification of plants, therefore morphological traits of regenerated plantlets used for somaclonal variation.

Molecular study

DNA Extraction and PCR amplification

For ISSR molecular studies, fresh leaves were collected and dried in silica gel powder. The genomic DNA was extracted using CTAB-activated charcoal protocol (SHEIDAI *et al.*, 2013). The extraction procedure was based on activated charcoal and poly vinyl pyrrolidone (PVP) for binding of polyphenolics during extraction and on mild extraction and precipitation conditions, promoting high-molecular weight DNA isolation without interfering contaminants. Quality of extracted DNA was examined by running on 0.8% agarose gel.

Ten ISSR primers; (AGC) 5GT, (CA) 7GT, (AGC) 5GG, UBC 810, (CA) 7AT, (GA) 9C, UBC 807, UBC 811, (GA) 9A and (GT) 7CA (MEHRABIAN *et al.*, 2011) commercialized by UBC (the University of British Columbia) were used. PCR reactions were performed in a 25 µL volume containing 10 mM Tris-HCl buffer at pH 8; 50 mM KCl; 1.5mM MgCl2; 0.2 mM of each dNTP (Bioron, Germany); 0.2 µM of each primer; 20 ng genomic DNA and 3 U of *Taq* DNA polymerase (Bioron, Germany). Amplification reactions were performed in Techne thermocycler (Germany) with the following program: 5 min initial denaturation step 94°C, 30 s at 94°C; 1 min at 57°C and 1 min at 72°C. The reaction was completed by final extension step of 7 min at 72°C. Amplification products were visualized by running on 2% agarose gel, following ethidium bromide staining. Fragment size was estimated by using a 100 bp molecular size ladder (Fermentas, Germany).

The ISSR bands obtained were treated as binary characters and coded accordingly (presence = 1, absence = 0). The number of private bands versus common bands was determined. Genetic differentiation of the studied populations was studied by AMOVA with 1000 permutations as performed in GenAlex 6.4 (PEAKALL and SMOUSE, 2006). Different ordination and clustering methods were applied on standardized data like PCoA (Principal Coordinate analysis) and NJ to group the plant specimens. Data analyses were performed by using PAST ver. 2.17 (HAMMER *et al.*, 2012).

Cytological study

We used the excised roots which were treated for one hour with 0.05% colchicine, for cytological investigation. These roots were washed throughly in distilled water and then transferred the fixative solution (3:1 absolute alcohol-glacial acetic acid), and stored at 4°C for 48 hours.

The root tips were squashed in 1% aceto-orcein., and the chromosome counts were made from these temporary slides using an oil immersion objective at a magnification of 100 x.

RESULTS

Morphological characteristics variation during subcultures

We studied morphological characteristics like, length of the shoot, branch, internode, size of leaf and root (cm) and the number of branches and roots in five subcultures in Zagheh cultivar.

The effect of long-term and several sub-culturing on morphologic characteristics is presented in Figure 2.

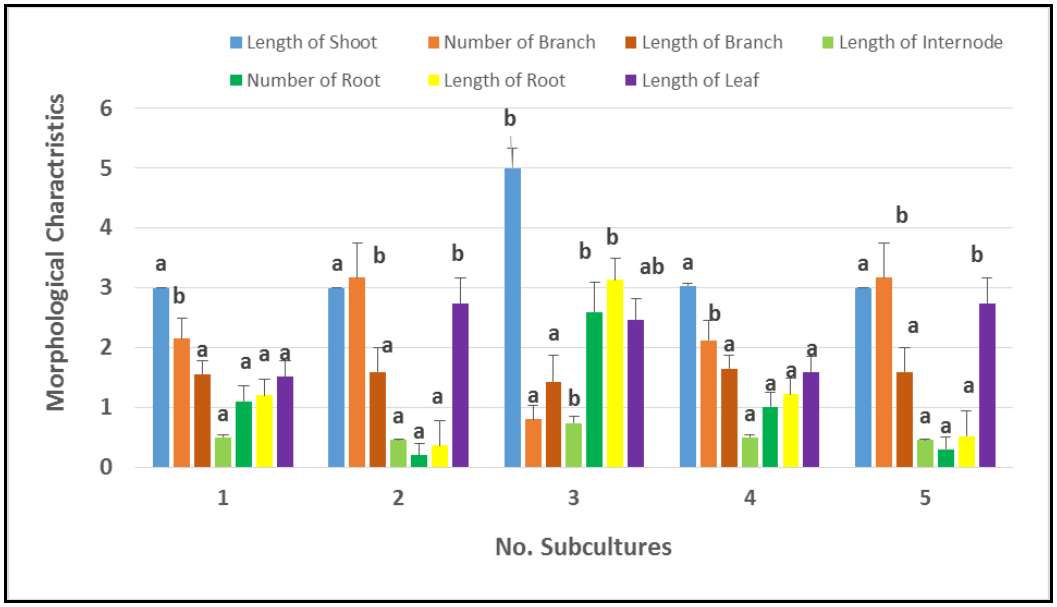

Figure 2. Morphological characters changes during five subcultures of pomegranate cultivar Zagheh

In the tissue culture medium which contained cytokinin, and benzyl adenin (BA), we obtained the regeneration of explants growth and shoots. In several subcultures, the studied plants were affected and the highest length of shoot (4.99 ± 0.35 cm), internode (0.74 ± 0.12 cm) and root (3.13 ± 0.31) were obtained in the third subculture.

Also, in response to shoot, the internode and root length were significantly decreased in some of the subcultures. We observed the best response to length of branch and internode changes (1.65 ± 0.22 cm) in the fourth subculture.

ISSR analyses

In total 44 ISSR bands were obtained in the studied mother plants and regenerated plants. Details of ISSR bands are provided in Table 1. The highest number of bands/ loci (33 bands) occurred in regenerated plants of the first sub-culture, while mother plants had the lowest number (23). This result indicates that some new ISSR loci have been formed during tissue culture probably by genetic restructuring and changes within regenerated plants. The mean value of gene diversity (He) also increased in tissue culture regenerated plants compared to that of mother plants. Some private bands also occurred in some of the sub-cultures. All these data indicate genetic changes and soma clonal variation in tissue-culture regenerated plants.

Table1. Detail of ISSR bands in pomegranate mother plants and tissue-culture regenerated plants

Group	Pop1	Pop2	Pop3	Pop4
No. Bands	23	33	31	26
No. Bands Freq. >= 5%	23	33	31	26
No. Private Bands	2	3	0	2
No. LComm Bands (<=50%)	5	9	11	7
Mean He	0.02	0.05	0.09	0.08

AMOVA revealed significant genetic difference among mother plants and regenerated plants of different sub-cultures (PhiPT = 0.84, P = 0.001). It also indicated that 74% of total genetic difference occurred due to among sub-cultures difference, while 24% of genetic variability was due to within sub-cultures.

Grouping of the mother plants as well as tissue culture regenerated plants by NJ tree and PCoA plot are provided in Figures 3 and 4.

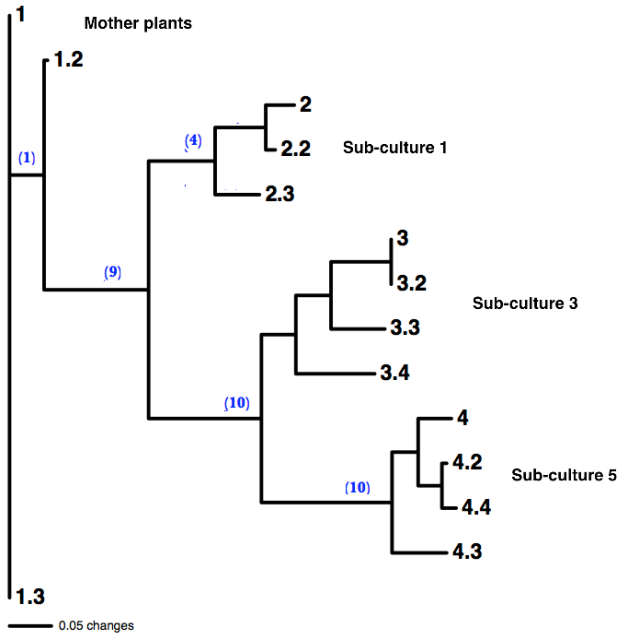

Figure 3. NJ tree of studied pomegranate plants based on ISSR data showing both among sub-cultures and within sub-cultures genetic variability

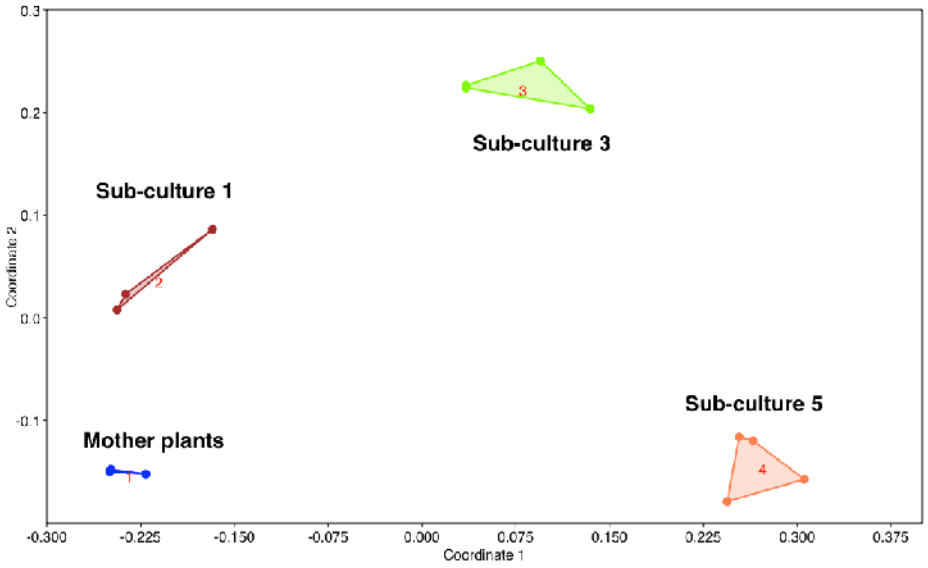

Figure 4. PCoA plot of pomegranate plants based on ISSR data showing genetic differences of different sub-cultures and the mother plants.

The NJ tree shows that tissue culture regenerated plants of the subsequent sub-cultures differ genetically from mother plants. The regenerated plants of the second sub-culture differed from mother plants by 9 ISSR bands/ loci. However, these plants showed some degree of genetic variability within themselves (differed in 4 ISSR bands).

The regenerated plants of the third sub-culture, differed from mother plants in 10 more ISSR bands, and showed a higher level of within sub-culture genetic variability (in 7 ISSR bands). Similarly, plants of the forth sub-culture, differed from mother plants in 10 more ISSR bands and showed a moderate level of within sub-culture genetic variability (in 4 ISSR bands). These results indicate that with increase in the number of sub-culture, a higher degree of genetic difference occurred from mother plants. The occurrence of within sub-culture genetic variability reveals that, individual regenerated plants of each sub-culture have gone through different mutations and DNA changes.

PCoA plot (Figure 4), supported AMOVA result and separated pomegranate plants in distinct groups due to their genetic differences.

Cytological result

During examination of many root tips clues of different regenerated plants, we observed frequent occurrence of cells with different chromosome numbers, ranging from normal diploid

2n = 2x = 16 to tetraploid 2n = 32 chromosome number. Many cells had aneuploidy chromosome number both hypo-ploidy and hyper-ploidy (Figure 5). These results indicate that, genetic changes occurred during tissue culture in both chromosome level and DNA sequences.

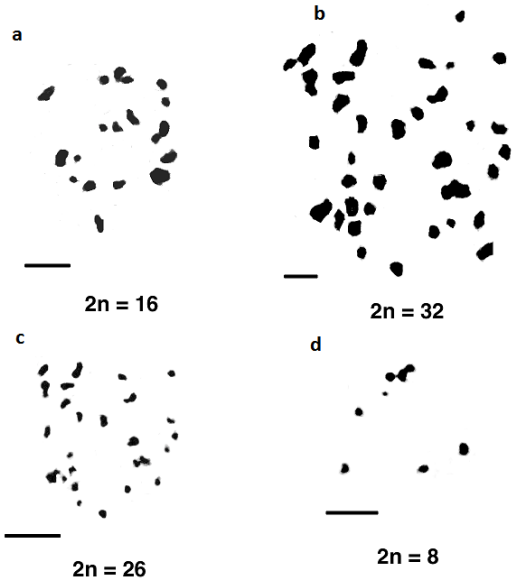

Figure 5. Somatic cells of pomegranate tissue culture regenerated plants showing different chromosome numbers. Scale = 5 micrometer

DISCUSSION

The present study revealed that somaclonal variation can be obtained at both morphological and molecular levels in pomegranate plant. In morphological characters studied, we observed only significant increase in the number of branch and root, particularly, in the third and fifth subcultures.

The time and concentration of auxins and cytokinins of the multiplication medium is an important factor affecting the extent of multiplication. Cytokinins are adenine derivatives which mainly affect the cell division, and modify the apical dominance and shoot differentiation in the tissue culture. Similarly, the cytokinin 6-benzylamino-purine (BAP) is the growth regulator which affect the shoot regeneration in plants (KUMAR *et al*., 2017).

Significant changes in morphological traits of pomegranate reported here, indicates that the pomegranate tree is an appropriate plant for a long-term vegetative propagation and that the stabilized favorable somaclonal variations may be obtained in it, as suggested by LEVA (2009). STIMELA *et al.* (2019), investigated the occurrence of somaclonal variation in pomegranate cultivar of Kenya, by using SSR (Simple Sequence Repeat) molecular markers. They reported

genetic similarity to range from 46-100% between tissues cultured regenerated plants and the mother plant.

It has been suggested that even though pomegranate breeding may benefit in future from the high throughput sequencing and mapping techniques, however, in the short term, breeding of this crop plant is yet based on classical breeding or on the marker-assisted selection (MAS). Therefore, finding the genetic markers or identifying important genes highly correlated with fruit quality, as well as the content of healthful compounds are considered as the immediate goals in pomegranate breeding (HOLLAND and YA'AKOV, 2019).

Though tissue culture is an efficient method of clonal propagation; a number of somaclonal variations do occur in this process. These variations occur due to newly generated mutations (SATO *et al.,* 2011 a,b). The factors leading to mutations may be related to numerous stress factors like, wounding, exposure to sterilants during sterilization, tissue being incomplete (protoplasts as an extreme example), imbalances of media components such as high concentration of plant growth regulators (auxin and cytokinins), etc. (SATO *et al.* 2011b).

In present study, we observed variation in both morphological and genetic features of the tissue culture regenerated plants. Much of the variability reported in regenerated plants may be due to, oxidative stress which occurs during in vitro culture. This in turn may result in elevation of pro-oxidants or reactive oxygen species (ROS) which changes the hyper and hypo-methylation of DNA; and the chromosome number. It may also lead to DNA base deletions and substitutions (CZENE and HARMS-RINGDAHL, 1995).

We observed changed in ISSR bands frequency and also new/ private ISSR band formation in some of the tissue culture regenerated plants. These molecular changes may be due to insertion/deletions or alteration in DNA sequences. It is also reported that tissue culture activates sillent transposable elements, resulting in somaclonal variations (TANURDZIC *et al.,* 2008).

In conclusion, the present study revealed that pomegranate plant may be subjected to somaclonal variation via tissue culture and that genetic as well as morphological changes occurred can be further evaluated for breeding purpose.

Received, September 20th, 2020
Accepted September 10th, 2021

REFERENCES

CZENE, M., M. HARMS-RINGDAHL (1995): Detection of single-strand breaks and formamidoprymidine-DNA glycosylase-sensitive sites in DNA of cultured human fibroblasts. Mutat Res, *336*: 235–242.

FARAHANI, F., A. MAJD (2012): Comparison of liquid culture methods and effect of temporary immersion bioreactor on growth and multiplication of banana (Musa, cv. Dwarf Cavendish). Afr. J. Biotechnol., *11*(33): 8302-8308.

GLOZER, K., L. FERGUSON (2011): Pomegranate production in Afghanistan. US Davis, Afghanistan old reference replace with newer one.

SHEIDAI, M., Z. NOORMOHAMMADI., A. SANEGHI., Z.H. SHAHREIYARI (2007): RAPD analysis of eleven Iranian pomegranate (Punica granatum L.) cultivars. Acta Biol. Szeged, *51*: 61-64

HAMMER, Ø., DAT. HARPER., P.D. RYAN (2012): PAST: Paleontological Statistics software package for education and data analysis. Palaeontologia Electronica, *4*: 9.

HOLLAND, D., I.B. YA'AKOV (2019): Pomegranate (Punica Granatum L.) Breeding. Advances in Plant Breeding Strategies: Fruits. pp. 601-647.

KAHRAMANOGLU, I., S. USANMAZ (2016): Pomegranate production and marketing (1st ed.). New York, London CRC Press.

KARP, A. (1995): Somaclonal variation as a tool for crop improvement. Euphytica, *85*:295-302.

KUMAR, R., M.L. JAKHAR, R. VERMA, H.R. JAT (2017): Pomegranate Micropropagation a Review. Int. J. Pure App. Biosci., *5 (5)*: 1138-1149.

MATERECHERA, S.A., T.M. SEEISO (2013): Seed Treatment to Improve Water Imbibition and Germination of Pomegranate (Punica granatum). Acta Hort., *979*: 713-722.

MURASHIGE, T., E. SKOOGE (1974): Plant Propagation through Tissue Culture. Ann. Rev. Plant Biol., *25*: 135 – 165.

PATIL, V.M., G.A. DHANDE, D.M, THIGALE, J.C. RAJPUT (2011): Micropropagation of pomegranate (*Punica granatum* L.) „Bhagava" cultivar from nodal explant. Afr. J. Biotech., *10*: 18130–18136.

PEAKALL, R., P.E. SMOUSE (2006): GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes, *6*: 288-295.

SATO, M., T. KAWABE, M, HOSOKAWA, F. TATSUZAWAM, M.,DOI (2011a): Tissue culture induced flower-color changes in Saintpaulia caused by excision of the transposon inserted in the flavonoid 39, 59 hydroxylase (F3959H) promoter. Plant Cell Rep., *30*: 929–939

SATO, M., M. HOSOKAWA, M. DOI (2011b): Somaclonal variation is induced de novo via the tissue culture process: a study quantifying mutated cells in Saintpaulia. PLoS ONE, *6*: e23541.

SINGH, S.K., A, SINGH, N.V. SINGH, D. RAMAJAYAM (2010): Pomegranate Tissue Culture and Biotechnology Fruit, Vegetable and Cereal Science and Biotechnology. Global Science Books.

SHEIDAI, M., A. SANEGHI, Z.H. SHAHREIYARI, Z. NOORMOHAMMADI, F. FARAHANEI, S.Z. TABATABAEI-ARDAKANEI (2008): RAPD and cytogenetic study of some pomegranate (*Punica granatum* L.) cultivars. Cytologia, *61*: 68-73.

STIMELA, T., R.W. KASILI, E.G. MAMATI (2019): Pomegranate Micropropagation, Callogenesis and Genetic Integrity Assessment Using Simple Sequence Repeat Markers. J. Agri. Sci *11 (1)*: 237-250.

TANURDZIC, M., M.W. VAUGHN, H. JIANG, T.J. LEE, R.K. SLOTKIN, B. SOSINSKI, W.F. THOMPSON, R.W, DOERGE, R.A. MARTIENSSEN (2008): Epigenomic consequences of immortalized plant cell suspension culture. PLoS Biol., *6(12):* 2880–2895.

YUSNITA, Y., W. WIDODO, S. SUDARSONO (2005): In vitro selection of peanut somatic embryos on medium containing culture filtrate of Sclerotium rolfsii and plantlet regeneration. HAYATI J Biosci., *12(2)*:50–56.

ZHANG, B., L.P. STOLTZ (1991): *In vitro* shoot formation and elongation of dwarf pomegranate. HortScience, *26*: 10–84.

SOMAKLONALNA VARIJACIJA KOD NARA (*Punica granatum* L.): ISSR I CITOLOŠKI DOKAZI

Seyed Ardalan AZIZI[1], Farah FARAHANI[2*], Masoud SHEIDAI[3]

[1]Departman za genetiku, Ogranak Qom, Islamski Azad Univerzitet, Qom, Iran
[2]Departman za mikrobiologiju, Ogranak Qom, Islamski Azad Univeryitet, Qom, Iran
[3]Departman za biologiju, Fakultet za biološke nauke, Šahid Behešti Univerzitet, Evin, Teheran, Iran

Izvod

Novi napredak u tehnikama kulture tkiva doveo je do *in vitro* propagacije nekoliko regeneracija hortikulturnih useva. Somaklonska varijacija koja se javlja tokom kulture biljnog tkiva je potencijalni izvor za izazivanje nove genetske varijabilnosti kod takvih useva. Nekoliko pristupa je korišćeno da bi se utvrdila pojava somaklonskih varijacija. Biljka nara se može prilagoditi različitim uslovima životne sredine i dobro raste u različitim vrstama podloge i klime. Sorta nara Zagheh se uglavnom gaji u državi Esfahan, gradu Kašan u Iranu. Ova studija je sprovedena da istraži potencijalnu pojavu somaklonalnih varijacija u regenerisanim biljkama kulture tkiva sorte Zagheh i pruži morfološke, citološke i molekularne dokaze za njenu pojavu. Za in vitro razmnožavanje nara izabrani su nodalni segmenti. Eksplanti su kultivisani na MS bazalnoj podlozi. Sproveli smo i molekularne (ISSR) i kariološke studije tokom razmnožavanja. U ovoj studiji uočili smo varijacije kako u morfološkim (dužina izdanaka, grana, internodija, lista i korena i broj grana i korena) tako i u genetskim karakteristikama regenerisanih biljaka u kulturi tkiva. Regenerisane biljke treće subkulture razlikovale su se od matičnih biljaka u ISSR markeru. Primetili smo čestu pojavu ćelija sa različitim brojem hromozoma, u rasponu od normalnog diploidnog 2n = 2k = 16 do tetraploidnog 2n = 32 broja hromozoma.

Primljeno 20.IX.2020.
Odobreno 10.IX. 2021.