Volume 17(4), 99- 106, 2013
JOURNAL of Horticulture, Forestry and Biotechnology
www.journal-hfb.usab-tm.ro

Assessment of somaclonal variation of regenerated *Ducrosia anethifolia* plants using AFLP markers

Lia Shooshtari[1], Mansour Omidi[2], Eslam Majidi[3], Mohammadreza Naghavi[2], Mansour Ghorbanpour[4]*, Alireza Etminan[1]

[1]Department of Plant breeding, Kermanshah branch, Islamic Azad University, Kermanshah, Iran; [2]Department of Plant Breeding, Faculty of Agriculture and Natural Resources, University of Tehran, Iran; [3]Department of Plant breeding, Science and Recearch branch, Islamic Azad University, Tehran, Iran; [4]Department of Medicinal Plants, Faculty of Agriculture and Natural Resources, Arak University, Arak, Iran.

*Corresponding author: E-mail: m_ghorbanpour@yahoo.com

Abstract *Ducrosia anethifolia* is a medicinal plant of Apiaceae family. The plant contains active constituents with antimicrobial activity which makes it as a valuable crude drug for use in pharmaceutical and food industries. Besides others, *in vitro* production of secondary products is an appropriate way of production of theses group of metabolites. In these cases, many factors such as number of subcultures, explant, types of culture media and their constituent's especially exogenous hormones may lead to genetically and epigenetically changes along with DNA methylation which can be exploited as a desirable phenomenon. The present study was done to investigate somaclonal variation in regenerated *D. anethifolia*) plants and to study importance of DNA methylation in occurrence of somaclonal variation. To do this, eight abnormal plants regenerated from different explants calli along as well as a normal plant were selected and their genomic DNA were extracted using modified Delaporta method. AFLP analysis of selected plants was performed using two different digestion systems. Digested DNA fragments were ligated to adapters and amplification was done using suitable primers. Using two different digestion systems, considerable polymorphism was observed. Considering obtained polymorphism and differences of used enzymes to methylation, it can be concluded that observed variations in regenerated plants are resulted from genome methylation. Additionally, the applied method has the potential to be used for assessment of somaclonal variations for *in vitro* regenerated plants.

Key words

Ducrosia anethifolia, Methylation, Somaclonal variation, AFLP

Abbreviation

AFLP: Amplified Fragment Length Polymorphism; ISSR: Inter Simple Sequence Repeat; PCR: Polymerase Chain Reaction; 2,4-D: 2,4-Dichlorophenoxyacetic acid; KIN: Kinetin; NAA: 1-Naphthaleneacetic acid; BA: N6-benzyladenine; UPGMA: Unweighted Pair-Group Method Arithmetic average; PCA: Principal Coordinated Analysis

Ducrosia anethifolia, with the Persian name of 'Moshgak or Moshk bou', is a medicinal plant of Apiaceae family (Mozaffarian, 1996). Oxygenated aliphatic compounds present in the essential oil of this plant have antimicrobial activity against Gram-positive, yeasts and fungi (Jonson et al., 1984). Aerial parts of this plant contain an essential oil which effectively prevents the growth of some parasite fungi such as *Candida albicans* (Mostafavi and Afzali, 1385). As an active constituent of *Ducrosia anethifolia*, myrcene is applied in medicine to increase absorption of pharmaceuticals in body especially is used in the case of indomethacin and diazepam (Jonson et al., 1984). Considering the presence of many secondary metabolites in *Ducrosia anethifolia* and the need of pharmaceutical and cosmetic industries, propagation of this plant in large scale using in vitro culture techniques is important.

Genetic diversity is a necessary component of breeding programs designed for improving plant characteristics. In recent years, cell suspension cultures have been considered as a potential source of useful genetic diversity. Genetic variations that occur in the processes of plant tissue cultures are considered as somaclonal variation and result from a vast array of mutations including point mutations, chromosomal rearrangements, inversion, insertion, deletion and polyploidy. The extent of genetic variations is usually

related to the culture time and many factors such as number of subcultures, explants, types of culture media and their constituents' especially exogenous hormones. Somaclonal variation, a common phenomenon in plant cell cultures, includes all types of variations among plants or cells and derives from all kinds of tissue cultures (Skirvin et al., 1993). Somaclonal variation not only affect genes and therefore plant morphology but also can alter gene expression (Gaj, 2004). Many events are related to genetic variations and in fact with changes in the gene expression patterns and may lead to genetically instability. One of the most important of these events in plants is methylation and demethylation of coding DNA (Lamb, 1997). In addition to methylation, environmental changes and different stresses can also lead to genetically and epigenetically changes (Loknez and Zhan, 2007). Somaclonal related methylation usually occur in cytosine containing nucleotides (Negzhayo et al., 2007). Considering somaclonal variation occurs mainly as the result of mutations, appropriate techniques should be served for detection of occurred mutations in *in vitro* culture procedures. Although there are many techniques for detection of occurred variations, molecular markers are considered as one of the most appropriate tools due to their high accuracy and low performing time for evaluation of many samples (Martinez et al., 2004). The occurrence of genetic variation associated with indirect regeneration can be detected by different DNA techniques. RAPD analysis, initially employed, is currently being replaced by more reproducible techniques such as AFLP or SSR.

In this respect, ISSR markers, which are considered as PCR-based markers, are suitable for evaluation of somaclonal variations. So far, different band pattern resulting from somaclonal variations has been revealed as a result of serving PCR-based markers in calli obtained in different culture media or different plant explants. It has been revealed that extend of genetic variations is related to the number of performed subcultures and also hormonal composition of culture medium (Poorjabar et al., 1389). It has been shown that PCR-based molecular markers have been effective for detection of occurred somaclonal variations. One of the most effective techniques in detecting genome methylation is a modified AFLP technique which is known as the methylation-sensitive amplification polymorphism (Li et al., 2006). Using methylation–sensitive enzymes in comparison to their non-sensitive counterparts, a high polymorphism has been observed in in vitro culture regenerated oil palm plantlets. In a performed study, AFLP markers revealed 2/94 polymorphisms in *in vitro* regenerated asparagus plants (Pentaroli et al., 2005).

In general, it has been reported that abnormal phenotypes in regenerated plants are not related to the major changes in the genome and this phenomenon is due to methylation of some parts of it (Li et al., 2006). Genome methylation may create heritable phenotypic variations with the exploitation potential (Lokenz and Gan, 2007). There are still no reports about somaclonal variation of *D. anethifolia.* The present study was done to investigate the somaclonal variation in regenerated *D. anethifolia* plants and to study the importance of DNA methylation in occurrence of somaclonal variation.

Materials and Methods

Callus induction and regeneration

In this study, leaves and lateral buds of a wild growing *Ducrosia anethifolia* plant were collected from the area located at the institute of medicinal plants (ACECR), Karaj, Iran. They were then surface-disinfected by 70% ethanol for 30 s and then by 1% (w/v) sodium hypochlorite with 3 drops of Tween-20 for 10 min. Finally they were washed three times with sterile, distilled water, and then were served for calli production under *in vitro* culture. For callus production, explants were transferred to the MS media containing different concentrations of plant hormones NAA, BA, 2,4-D and KIN (Table 1). To study the effect of subculture numbers on somaclonal variation, calli obtained from both explants were subcultured each 35 days. After 3 times subcultures, half of obtained calli from 12 different treatments (two types of explants and six different hormonal compositions) were transferred to MS medium supplemented by 2 mg/l BA for regeneration. Remained calli were subcultured for 3 additional times and subsequently transferred to the regeneration medium as described above. Finally, 24 regenerated plants from two different subculture times (3 and 6 times), two different explants (leaves and lateral buds) and six hormonal compositions with abnormal phenotype were selected (Table 2) and subjected to DNA extraction.

Table 1

Regeneration media containing different concentrations of 2,4-D, NAA BA and Kin

Regeneration media	Growth regulators (mg/l)			
	2,4-D	NAA	BA	Kin
A	-	1	0.5	-
B	-	2	2	-
C	2	-	-	1
D	-	2	1	-
E	2	-	-	2
F	1	-	-	0.5

2,4-D: 2,4-Dichlorophenoxyacetic acid; KIN: Kinetin; NAA: 1-Naphthaleneacetic acid; BA: N^6-benzyladenine

Table 2

Type of explants, the number of subculture, media culture and seedling status used in callusing stage of *D. anethifolia*

Samples	Explants	No. subculture	Media	Seedling status
1	bud	3	E	abnormal
2	bud	6	C	abnormal
3	bud	6	C	abnormal
4	-	-	-	normal
5	leaf	6	E	abnormal
6	leaf	3	E	abnormal
7	leaf	6	B	abnormal
8	leaf	6	F	abnormal
9	leaf	6	D	abnormal

DNA extraction

For extraction of DNA from one normal plant along with eight abnormal plants the method illustrated by Dellaporta et al. (1983) was used with minor modification. Afterwards, quality and quantity of extracted DNA were evaluated using electrophoresis in 0.8% agarose gel.

AFLP markers

AFLP analysis was carried out according to Vos et al. (1995). Genomic DNA (about 250 ng) was subjected to double digestion using two restriction enzymes with sticky ends. Digestion was performed using two sets of restriction enzymes including *Mse*I and *EcoR*I and also *Bgl*II and *Mse*I (Fermentas). For double digestion, buffer recommended by Fermentas Company was used. After digestion, appropriate adapters were ligated to digested DNA fragments using T4 DNA ligase (Fermentas). For ligation, the reaction mixture was kept for 3 h in 37 ºC and then in room temperature (25 ºC) overnight. The adapter ligated DNA fragments were diluted (3:1 ratio) and used as template for preselective amplification. Selective amplification was performed using 5 M and E (for DNA fragments resulted from *Mse*I and *EcoR*I) or M and B primer combinations (for DNA fragments digested with *Bgl*II and *Mse*I) (Table 3). A thermal cycler (I Cycler, Bio-Rad) was served for PCR and all materials needed were prepared from Fermentase. Finally, PCR products were run on a denaturing polyacrylamide gel and visualized by the silver staining method. In this study, an electrophoresis (Bio-Rad) apparatus with dimensions 30×38 was used.

Data analysis

Jaccard's coefficient of similarity (Sneath and Sokal 1973) was calculated for the samples data. A dendrogram was generated by cluster analysis using the unweighted pair group method of the arithmetic averages (UPGMA). Principal coordinated analysis (PCA) was also carried out to show the distribution of the samples in a scatter-pot (NTSYS-pc, version 2.1) (Rohlf 2000).

Table 3

The sequences (5' – 3') of adapters and primers used for AFLP analysis

Adaptors	Sequence (5' – 3')
E adaptor1	CTCGTAGACTGCGTACC
E adaptor2	AATTGGTACGCAGTCTAC
M adaptor1	GACGATGAGTCCTGAG
M adaptor2	TACTCAGGACTCAT
B adaptor1	CATCTGACGCATGGCTAG
B adaptor2	CTCGTAGACTGCGTACC
Primers	**Sequence (5' – 3')**
M_{000}	GATGAGTCCTGAGTAA
M20	GATGAGTCCTGAGTAACAT
M17	GATGAGTCCTGAGTAACAA
M-GC	GATGAGTCCTGAGTAAGC
M35	GATGAGTCCTGAGTAAGAG
M22	GATGAGTCCTGAGTAACCC
E_{000}	GACTGCGTACCAATTC
E-CG	GACTGCGTACCAATTCCG
B_{000}	GACTGCGTACCGATCT
B-CG	GACTGCGTACCGATCTCG

Results and Discussions

High quality of leaf and lateral bud explants were regenerated depending on the concentration and combination of hormones used (Figure 1). There was no bacterial or fungal contamination observed and the cultures obtained were 100% contamination free. The 10 AFLP primers are $M_{000,}$ M20, M17, M-GC, M35, M22, E_{000}, E-CG, B_{000} and B-CG. Also, their sequence information was listed in table 3. The level of polymorphism can be observed in table 4. Out of 29 obtained polymorphic bands, the maximum polymorphic bands were observed in primer combination E-CG/M-35 with 8 polymorphic bands. In average, each primer combination produced 5.8 bands. In addition, PIC values for used primers ranged from 0.38 to 0.46 with the mean value of 0.41 (Table 4). In general, the higher polymorphic information content reveals the superior power of served primer combination in separation of studied genotypes (Labersted et al., 2000).

Table 4

List of AFLP primers, the number of polymorphic bands and olymorphism information content (PIC) value

Primers pair combinations	Number of polymorphics bands	PIC
E-CG/M-35	8	0.39
E-CG/M-22	6	0.46
E-CG/M-GC	4	0.38
E-CG/M-17	7	0.41
E-CG/M-20	4	0.43

Because tested DNA samples were double digested by *Mse*I and *Eco*RI enzymes and then ligated to their appropriate adaptors, it can be concluded that these enzyme combination digested DNA of various plants differentially. Since all tested samples were collected from a given plant, differences in their DNA digestion patterns can be related to the events that may be occurred in their genomes during tissue culture procedure. The extent of genetic changes in the cell nucleus is usually related to the culture time and number of subcultures and is affected by culture media composition especially plant hormones.

According to the results of cluster analysis based on the Jaccard's similarity coefficient, all tested plants divided into two main group. While the first group contained only one plant (normal plant), other plants (abnormal plants) were placed in the second group (Figure 2). Principal coordinate analysis (PCA) also confirmed the result of the cluster analysis (Figure 3).

In this study, the four-base restriction enzyme *Mse*I was served in both systems. Therefore, differences in

digestion patterns of tested DNA samples can be related to the differences between six-base restriction enzymes *Eco*RI and *Bgl*II. In fact, *Eco*RI is a methylation-sensitive enzyme and if methylation occurred in its recognition sequence, methylated site cannot be recognized. In contrast, *Bgl*II is not a methylation-sensitive enzyme and if methylation occurred in its recognition site, methylated site can be digested. It has been revealed that genome methylation is inheritable and a cause of phenotypic variations (lokenz and Gan, 2007).

It is reported that tissue culture procedure leading to great cellular reprogramming level may be a consequence of increased somaclonal variations (Morcillo et al. 2006). Earlier, Labra et al. (2004) reported various somaclonal variations in transgenic *Arabidopsis thaliana* plants resulted from callus formation. According to Kaeppler et al. (2000), somaclonal genetic variation results from micropropagated plant cultures. In plant tissue culture system the rate of somaclonal variation enhances with the increase of subcultures in micropropagation protocols (Gaafar et al. 2006). Considering this fact and characters of served enzymes in this study, phenotypic variations in abnormal plants that are resulted from somaclonal variation can be related to genome methylation. These results are in agreement with the results of previous studies on grape (Skilnbon et al., 2008), oil palm (li et al., 2006), banana (Praza et al., 2001) and potato (Kumar et al., 2007).

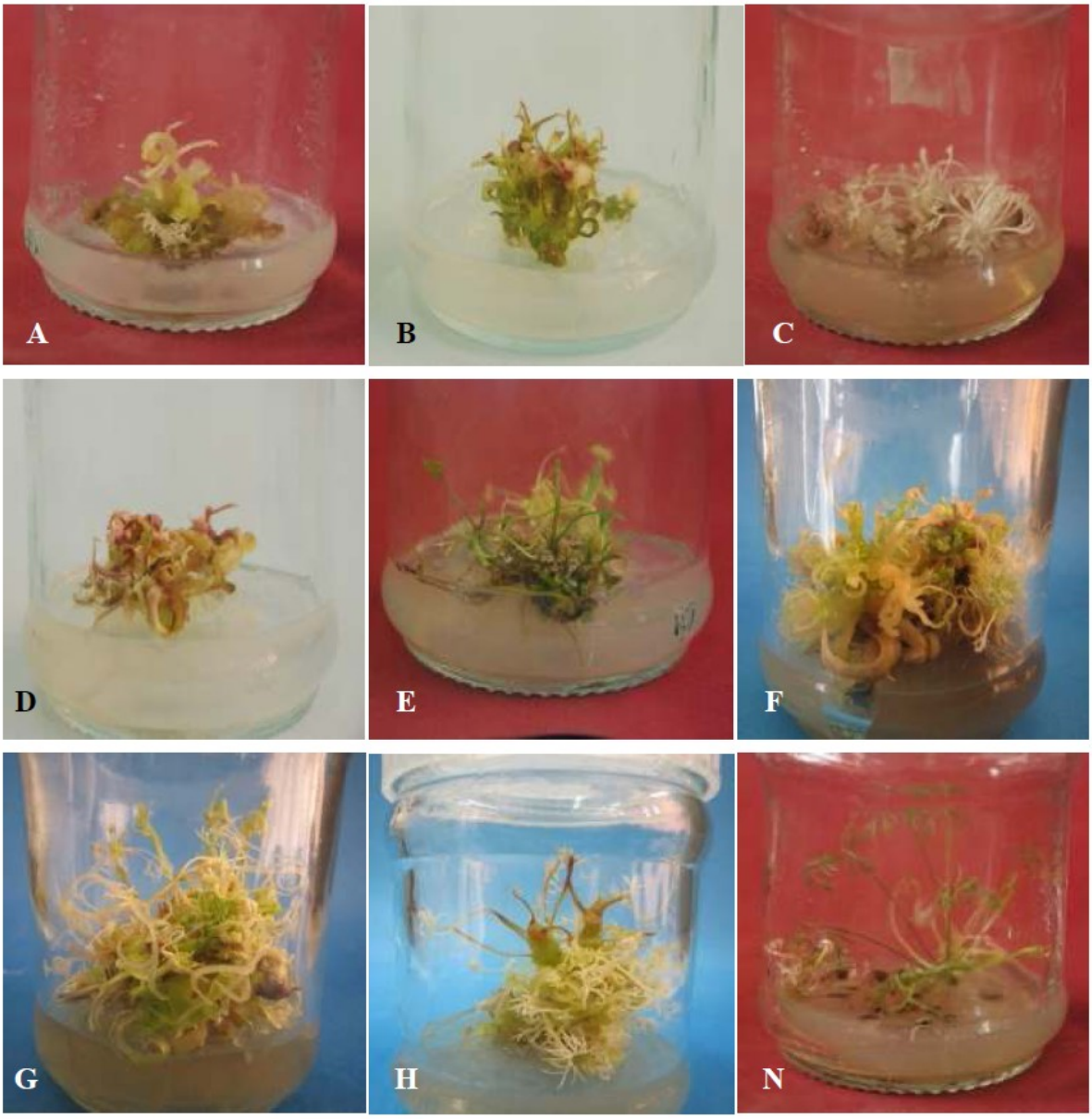

Fig. 1. *D. anethifolia* regenerated from bud explant (A,B,C), and leaf explant (D-N). A-H (abnormal seedlings), N (normal seedling) from bud and leaf tissue culture by manipulation of the make-up of the MS media with different hormonal concentration based on table 2.

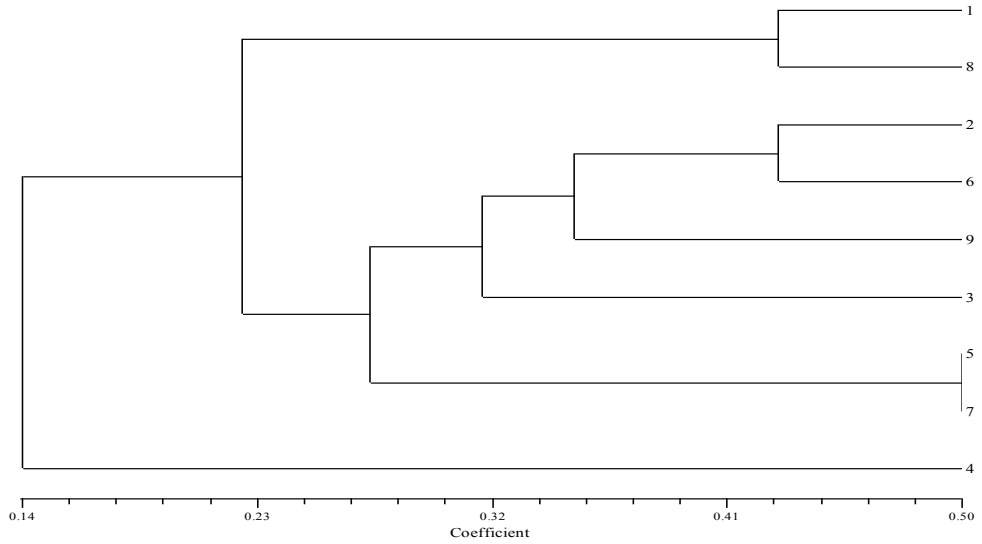

Fig. 2. Dendrogram obtained using UPGMA cluster analysis based on Jaccard's similarity index. 1-9 are the DNA samples of calluses from MS media with different hormonal concentration.

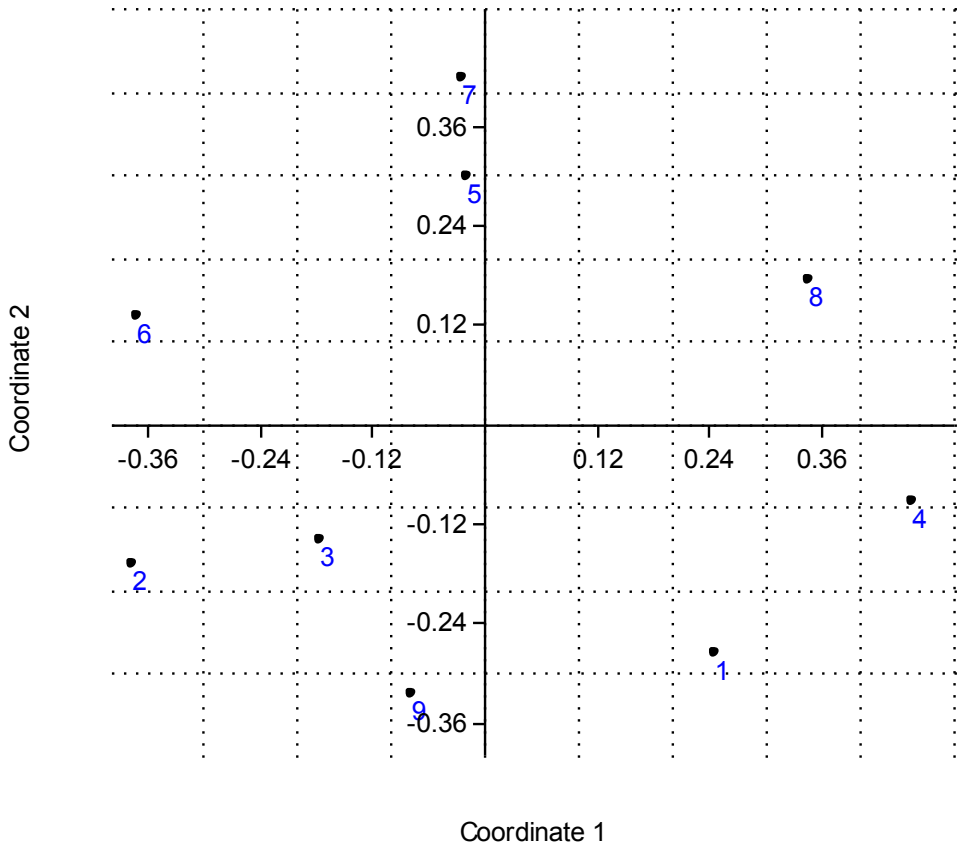

Fig. 3. Principal coordinate analysis (PCA) of the nine samples based on genetic distances calculated with AFLP markers.

Conclusions

AFLP technique is known as the methylation-sensitive amplification polymorphism. In our current study AFLP analysis of selected plants was performed using two different digestion systems. Digested DNA fragments were ligated to adapters and amplification was done using suitable primers. Using two different digestion systems, considerable polymorphism was observed. Considering obtained polymorphism and differences of used enzymes to methylation, it can be concluded that observed variations in regenerated plants are resulted from genome methylation. Additionally, the applied method has the potential to be used for assessment of somaclonal variations for *in vitro* regenerated plant.

Acknowledgment

We would like to acknowledge the institute of medicinal plants (ACECR), Karaj, Iran for supporting this study.

References

1. Bennici A, Anzidei M, Vendramin GG – 2004 - Genetic stability and uniformity of Foeniculum vulgar Mill. Regenerated plants though organogenesis and somatic emberyogenesis. Plant Science. 166, 221-227.
2. Dellaporta SL, Wood J, Hicks JB – 1983 - A plant DNA minipreparation: version II. Plant Mol. Biol. Rep. 1: 19.
3. Gaafar, R.M, Saker, M.M., 2006. Monitoring of cultivars identity and genetic stability in strawberry varieties grown in Egypt. World J Agric Sci 2(1):29-36.
4. Gaj, M.D., 2004. Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to Arabidopsis thaliana (L.) Heynh. Plant Growt Regul. 43: 27-47.
5. Janssen, A.M., Scheffer, J.J., Baerheim, Svendsen, A., Aynechchi,Y., 1984. The essential oil of Ducrosia anethifolia (DC.) Boiss. Chemical composition and antimicrobial activity. Pharm. Weekbl Sci. 6(4):157-160.
6. Kaeppler, SM., Kaeppler, H.F., Rhee, Y., 2000. Epigenetic aspects of somaclonal variation in plants. Plant Mol Biol 43:179-88.
7. Kumar-Sharma, S., Bryan, G.J., Winfield, M.O., Millam, S., 2007. Stability of potato (Solanum tuberosum L.) plants regenerated via somatic embryos, axillary bud proliferated shoots, microtu-bers and true potato seeds: a comparative phenotypic, cytogenetic and molecular assessment. Planta. 226:1449-1458.
8. Labra, M., Vannini, C., Grassi, F., Bracale, M., Balsemin, M., Basso, B., Sala, F., 2004. Genomic stability in Arabidopsis thaliana transgenic plants obtained by floral dip. Theoretical Appl Genet 109(7):1512-8.
9. Lambe, P., Mutambel, H., Fouche, J., Deltour, R., Foidart, J., Gaspar, T., 1997. DNA methylation as a key process in regulation of or-ganogenic totipotency and plant neoplastic progression. In Vitro Cell Dev. Biol. Plant. 33: 155–162.
10. Lei, C.P., Jiun, K.S., Choo, C.S., Singh, R., 2006. Analysis of tissue culture – derived regenerants using methylation sensitive AFLP. Asia Pacific J of Mol. Biol. Biothec. 14(2)47-55.
11. Lubberstedt, T., Melchinger, A.E., Duble, C., Vauylsteke, M., Kupie, M., 2000. Relationship among U.S. maize inbreds: IV Genetic diversity revealed with AFLP markers and comparison with RFLP, RAPD and pedigree data. Crop. Sci. 31: 893-899.
12. Lukens, L.N., Zhan, S., 2007. The plant genome's methylation status and response to stress: implications for plant improvement. Curr Opin Plant Biol. 10: 317-322.
13. Morcillo, F., Gagneur, C., Adam, H., Richaud, F., Singh, R., Cheah, S.C., Rival, A., Duval, Y., Tregear, J.W., 2006. Somaclonal variation in micropropagated oil palm. Characterization of two novel genes with enhanced expression in epigenetically abnormal cell lines and in response to auxin. Tree Physiol 26(5):585-94.
14. Mozaffarian, V., 1996. A Dictionary of Iranian Plant Names. Farhang Moaser Publishers, Tehran. 193.
15. Mozaffarian, V., 2003. Two New Genera of Iranian Umbelliferae. Bot. Zhurn. 88: 88–94.
16. Negezahayo, F., Dond, Y., Liu, B., 2007. Somaclonal variation at nucleotide sequence lenel in rice (Oryza sativa L.) at revealed by RAPD and ISSR markers, and by pairwise sequence analysis. Appl Genet. 48: 329-336.
17. Peraza-Echeverria, S.P., Valencia, V.A.H., Kay, A.J., 2001. Detection of DNA methylation changes in micropropagated banana plants using methylation sensitive amplification polymorphism (MSAP). Plant Sci. 161: 359-367.
18. Pontaroli, A.C., Camadro, E.L., 2005. Somaclonal variation in Asparagus officinalis plants regenerated by organogenesis from long term callus cultures. Gen. Mol. Biol. 28: 423-430.
19. Pourjabar, A., Mohammadi, S.A., Khosroshahli, M., Motalebi, A.R., Ziai, S.A., 2009. Optimization of in vitro culture of Milk Thistle (Silybum marianum) and analysis of somaclonal variation by RAPD markers. J. Plant Physiol. Breed 19(2): 95-106. (In Farsi).
20. Purohit, S.S., 2003. Agricultural Biotechnology. Agrobios, Jodhpur, India. 833-852.
21. Rohlf, F.J., 2000. NTSYSpc, Numerical Taxonomy and Multivariate Analysis System, Version 2.1 User Guide. Exeter Software, Setauket, New York, USA.
22. Schellenbaum, P., Mohler, V., Wenzel, G., Walter, B., 2008. Variation in DNA methylation patterns of grapevine somaclones (Vitis vinifera L.). Bmc. Plant Biol. 8(78): 1-10.

23. Skirvin, R.M., Norton, M., Mcpheeters, K.D., 1993. Somaclonal variation: has it proved useful for plant improvement? Acta Hort, v.336, p.333-340,
24. Sneath, P.H.A., Sokal, R.R., 1973. Numerical Taxonomy: The Principles and Practice of Numerical Classification. W.H. Freeman and company, San Francisco, USA, pp. 359.
25. Stavri, M., Mathew, K.T., Bucar, F., Gibbons, S., 2003. Pangelin, an antimycobacterial coumarin from Ducrosia anethifolia. Planta Med. 66(10): 856-959.
26. Vos, P., Hogers, R., Bleeker, M., Rijans, M., Van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M. 1995. AFLP: a new technique for DNA fingerprinting. Nuclecic Acids Res. 23: 4407-4414.