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Original scientific article

MOLECULAR CHARACTERIZATION OF MUTANT LINES OF AYVALIK OLIVE CULTIVAR OBTAINED BY CHEMICAL MUTATION

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This research was carried out with the aim to detect the mutagenic effect of three different doses (15, 20 and 25 mM) of Ethyl Methane Sulphonate on the Ayvalık olive cultivar (*Olea europaea* L.), to detect the variation and to identify whether individuals are genetically different from Ayvalık olive cultivar using molecular marker technologies. 23 mutant lines were screened using 28 SSR markers and a total of 138 alleles were detected and the number of alleles per primer ranged from 2 to 9 with an average of 4.9 alleles per primer. The polymorphism information content (PIC) value was changed between 0.4-0.98. The genetic similarity between Ayvalık olive cultivar and mutant lines was found between 5.5% and 78%. The mutant lines 15mM-12, 15mM-11 and 25mM-3 were found similar to Ayvalık cultivars at the rate of 78, 63 and 55%, respectively. Whereas, 20mM-5, 15mM-4 and 15mM-15 mutant lines were found distant to Ayvalık cultivar at the rate of 5.5, 5.6 and 8% genetic similarity, respectively. Ethyl Methane Sulphonate was successfully used in this study to extend the genetic diversity of the Ayvalık olive cultivar. Mutants might be screened for agricultural and quality traits and promising lines might be registered.

Key words: Chemical mutation, ethyl methane sulphonate, olive, olive tree, SSR marker

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INTRODUCTION

Olive (*Olea europaea* L.) is one of the fruit tree species that is grown in Mediterranean basins and has high socio-economic importance in these regions (HAGIDIMITRIOU *et al*., 2005). Olive production in the world is mostly done in northern hemisphere countries, including Turkey. Turkey ranks fourth in the World olive production after Spain, Greece and Italy, and olives are obtained from approximately 174 million olive trees in 846.000 hectares (FAOSTAT, 2017).

The enhancement of production in agricultural products is possible by increasing the cultivation areas or the yield obtained from the unit area. It is not possible to boost the cultivation areas too much in many countries such as Turkey, the only way is to improve the yield per unit area to achieve the desired production. Increasing the yield from the unit area might be achieved by eliminating the reasons that cause fluctuations in production. By developing high-yielding and high-quality varieties, growing these varieties with suitable agronomical practices may increase the productivity. Despite the hybridization technique is one of the principal breeding techniques to improve and develop new varieties, requires longer time, labor and funding.

The mutation breeding technique saves time to perform a planned study and developing new varieties in a short time (JAIN, 2010). Mutations are hereditary changes in the DNA sequence that are not of recombination origin, as a result of changing the physical and chemical structure of the heredity material (DNA and RNA) (VAN HARTEN, 1998). The rate of naturally occurring mutations is very low, so breeders create variations using physical and chemical mutagens to develop varieties with desired traits. Radiation, a physical mutagen, generally produces large-scale deletions on DNA and RNA, while chemical mutagens create point mutations on the inheritance material (SUPRASANNA *et al*., 2017). The primary purpose of mutation-based plant breeding studies is to create a high phenotypic variation with the appropriate dose of mutagen and to develop new varieties with better characteristics where several important traits were changed by positive selection (AHLOOWALIA *el al*., 2004).

Ethyl methane sulphonate (EMS) is a commonly used chemical mutagen to induce mutations (KRUPA-MAŁKIEWICZ *et al*., 2017) in plants such as eggplants (XIAO *et al*., 2019), tomatoes (SHIRASAWA *et al*., 2016). It is common to use DNA markers to characterize mutant lines at the molecular level. Simple sequence repeats (SSR), also known as microsatellites, can be used to detect genetic variations at the genome level. Microsatellites have some advantages such as high variability, co-dominant of inheritance, and high reproducibility. Also, these markers are abundant in the genome and show an even distribution over the genome (EFENDI *et al*., 2015). Simple sequence repeat markers are commonly used to detect genetic variation between olive germplasm at the molecular level (ABUZAYED *et al*., 2018).

This research was carried out with the aim to detect the mutagenic effect of three different doses (15, 20, and 25 mM) of ethyl methane sulphonate on the Ayvalık olive cultivar (*Olea europaea* L.) to detect the variation and to identify whether individuals are genetically different from Ayvalık olive cultivar using molecular marker technologies.

MATERIALS AND METHODS

Plant material

In this study, one-year old leafy cuttings belonging to the Ayvalik olive cultivar with an average of five buds were used as plant material. This study was carried out in the Agricultural Biotechnology Laboratory of the Faculty of Agriculture, Kahramanmaras Sutcu Imam University.

EMS induced mutagenesis assay

Two-hundred cuttings with 2 - 3 leaves, which are 15 - 20 cm length on average, were harvested from one tree and brought to the laboratory by wrapping them in a wet towel. Fifty of them were used as controls, and they were exposed to the same conditions as others, except EMS treatment. The remaining cuttings were divided into 3 groups, and 15, 20 and 25 mM doses of EMS were applied respectively. The cuttings were shaken in pure water for 4 h to activate the buds on the cuttings. Cuttings divided into 3 groups were treated separately by shaking (100 rpm) with the above-mentioned doses of EMS for 4 h. After the chemical treatment, the cuttings were rinsed with pure water 2 - 3 times and then shaken with pure water for 30 minutes. To increase the rooting performance of the cuttings, the bottom part of the cuttings was immersed into the 5000-ppm indole butyric acid (IBA) solution for five seconds (KURD *et al.*, 2010). All cuttings treated with the rooting hormone were transferred to pots containing perlite and irrigated daily. Also, they were subjected to daily spraying. All cuttings were kept in a climate cabinet set to 85% humidity, 2500 lux light, and 23°C for 16/8 h day/night. The cuttings, which started to root within 4 months, were transferred to the pots separately to adapt to the soil and continued to be maintained in these tubes until leaf samples were taken for DNA isolation. The stages of the EMS-induced mutagenesis assay are shown in Figure 1.

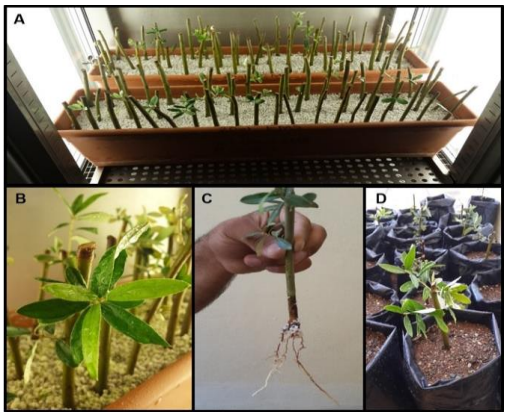

Figure 1. The stages of the EMS-induced mutagenesis assay (A: The cuttings in the climate cabinet after the EMS treatment (2 months later). B: New shoots after EMS treatment. C: Root structure of rooted cuttings before transfer to soil filled tubes. D: The cuttings just before the young leaf sample is taken for DNA isolation)

Extraction of genomic DNA and PCR amplification

Young leaf samples were taken from all new shoots consisting of buds on the cuttings and DNA isolation was made with some modifications in the cetyl Trimethyl Ammonium Bromide (CTAB) method (OLIVER *et al*., 2010). The quality of genomic DNA was determined using a Thermo Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Amplification was performed using polymerase chain reaction (PCR) technique using 28 microsatellite markers (Table 1). The amplification of DNA in PCR was conducted using the following conditions; 4 min at 94°C initial denaturation, then 40 cycles of 94°C (1 min), average 55°C (1 min), 72°C (2 min) and a final extension of 72°C (10 min), and PCR ended at 4°C. With QIAxcel Advanced System, PCR products were analyzed according to the band sizes using a Qiagen High-Resolution Kit.

Table 1. The list of microsatellite markers used in this study.

Marker	Primer sequences (*5′—3′*)	References
UDO-004	F-TTTGCCCTGGATTGGTACA	CIPRIANI *et al*. (2002)
	R-AGCTTGAGCATCATCTGTGAG	
UDO-009	F-TTGATTTCACATTGCTGACCA	
	R-CATAGGGAAGAGCTGCAAGG	
UDO-012	F-TCACCATTCTTAACTTCACACCA	
	R-TCAAGCAATTCCACGCTATG	
UDO-024	F-GGATTTATTAAAAGCAAAACATACAAA	
	R-CAATAACAAATGAGCATGATAAGACA	
UDO-026	F-AATTGACACCTACACACACACA	
	R-ACCTATTTCATGGTTTGCAC	
DCA1	F-CCTCTGAAAATCTACACTCACATCC	SEFC *et al*. (2000)
	R-ATGAACAGAAAGAAGTGAACAATGC	
DCA3	F-CCCAAGCGGAGGTGTATATTGTTAC	
	R-TGCTTTTGTCGTGTTTGAGATGTTG	
DCA7	F-GGACATAAAACATAGAGTGCTGGGG	
	R-AGGGTAGTCCAACTGCTAATAGACG	
DCA9	F-AATCAAAGTCTTCCTTCTCATTTCG	
	R-GATCCTTCCAAAAGTATAACCTCTC	
DCA10	F-CGTGACCACCTAAATCCGCCCC	
	R-CTGTCCAGAGCTAAAGGTTTCG	
DCA11	F-GATCAAACTACTGCACGAGAGAG	
	R-TTGTCTCAGTGAACCCTTAAACC	
DCA14	F-AATTTTTTAATGCACTATAATTTAC	
	R-TTGAGGTCTCTATATCTCCCAGGGG	
DCA15	F-GATCTTGTCTGTATATCCACAC	
	R-TATACCTTTTCCATCTTGACGC	
GAPU14	F-CACGCCAAGTCACTTTTCAA	
	R-CCCAGTAGCATGTTGTGAGC	

GAPU19	F-GATCAGTGTACTACGGTTC R-TCTGTCACAACTGCGGTA	
GAPU45	F-ATCGGGAGGGATGTGATGTA R-CATCGCATCGCCTGTAAATA	
GAPU59	F-CCCTGCTTTGGTCTTGCTAA R-CAAAGGTGCACTTTCTCTCG	
GAPU62	F-GATCACGAATCCCCAAATAA R-TGCGTTCCTGTATAATTGCATC	
GAPU72	F-GAGGCTTTTTAATCCGAGCA R-AAAAAGAGGGGAGGAGAGAG	
GAPU82	F-TGAATCAACCCGTCAATAAGG R-TGCTATTTGCACATCATTGTTT	CARRIERO *et al*. (2002)
GAPU89	F-GATCATTCCACACACGAGAG R-AACACATGCCCACAAACTGA	
GAPU90	F-GCTGAGCAGCGAAAAATGAT R-GCGACATATCTCTATGAGCAAGAA	
GAPU92	F-ATTGAGCGGCTCCTCAGTTA R-TGCAACAAGCTATAACGCAAA	
GAPU101	F-CATGAAAGGAGGGGGACATA R-GGCACTTGTTGTGCAGATTG	
GAPU108	F-GATCCTTAGAGGATTCAATGAGAA R-GCAAGTCCACCATCTTCAGAC	
GAPU71A	F-GATCATTTAAAATATTAGAGAGAGAGA R-TCCATCCATGCTGAACTT	
GAPU71B	F-GATCAAAGGAAGAAGGGGATAAA R-ACAACAAATCCGTACGCTTG	
GAPU103A	F-TGAATTTAACTTTAAACCCACACA R-GCATCGCTCGATTTTTATCC	

Statistical analysis

DNA bands of genotypes were scored as '0' (absence) or '1' (presence) and a binary data matrix was created. The genetic similarity between the mutant olive genotypes and control group was calculated using the Dice index (DICE, 1945) in Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc version, 2.20) (ROHLF, 2005). The polymorphism information contents (PIC) of each DNA marker used in molecular analysis were calculated in MS Excel software prepared by DUMLUPINAR *et al*., (2016) according to the $PIC=1-\sum Pi^2$ formula by WEIR, (1996). With the help of binary data matrix, a dendrogram showing the similarities of the mutant lines with the control was created using the UPGMA (unweighted pair group method with arithmetic average) method.

RESULTS AND DISCUSSION

A total number of 23 mutant lines were obtained from 14 rooted cuttings after the mutation treatment and the information about rooted cuttings and the shoots on them with related

EMS doses are given in Table 2. As the EMS dose rate was increased, the number of rooted cuttings decreased.

Table 2. The number of rooted cuttings and the number of new shoots on them.

EMS Dose	The number of rooted cuttings	The Number of new shoots
15 mM	7	15
20 mM	4	5
25 mM	3	3
Total	14	23

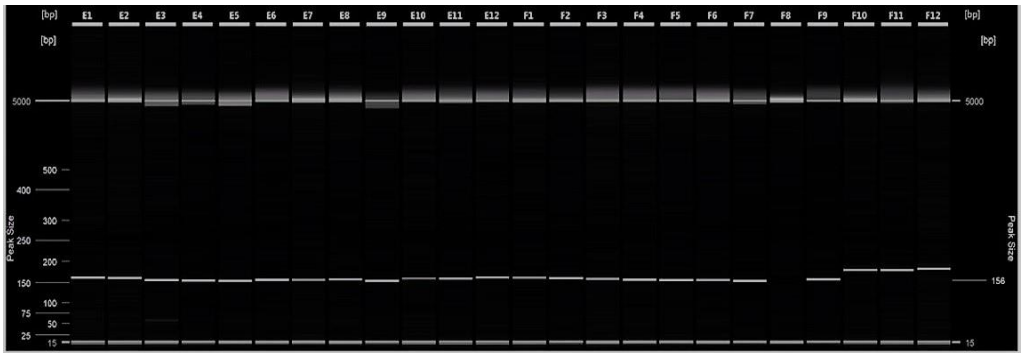

Figure 2. The banding profiles of Ayvalık olive cultivar and mutants using UDO26 SSR markers (Control in E1, 15 mM EMS applied samples between E2-F4, 20 mM EMS applied samples between F5-F9 and 25 mM EMS applied samples in the last 3 wells)

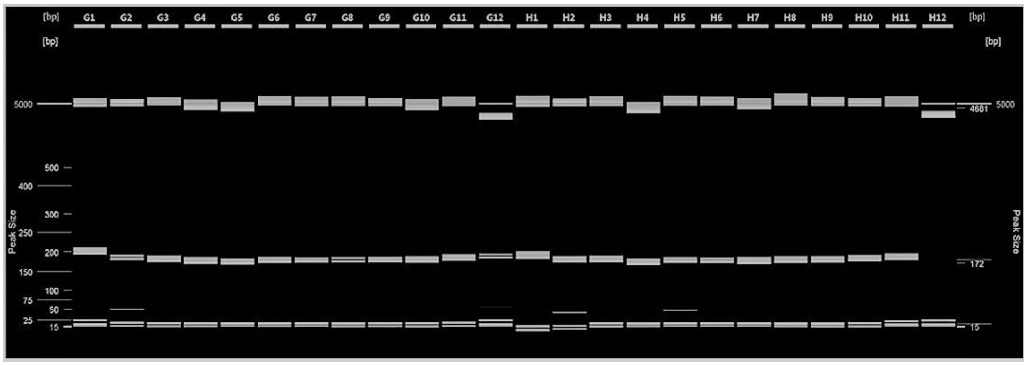

Figure 3. The banding profiles of Ayvalık olive cultivar and mutants using GAPU62 SSR markers (Control in G1, 15 mM EMS applied samples between G2-H4, 20 mM EMS applied samples between H5-H9 and 25 mM EMS applied samples in the last 3 wells)

The genetic similarity between Ayvalık olive cultivar and 23 mutant olive lines was determined using 28 SSR primers. In Figure 2 and Figure 3, the gel pictures are given which clearly show the genetic difference between the Ayvalik olive cultivar and the mutant lines. Twenty-eight SSR markers detected 138 polymorphic alleles in 24 olive genotypes, and the number of alleles identified by primers ranged from 2 to 9, with an average of 4.9 allele per primer. The most effective primers for separating among the analyzed genotypes were GAPU59 and GAPU62 primers with 9 alleles. The primers with lower polymorphic allele were DCA7, DCA10, GAPU71A, and GAPU90 with 2 alleles.

Table 3. Number of alleles, PIC value, and marker performance (Map: genetic mapping and Inf: informative markers) of 28 SSR markers used in the research

Marker	Performance of Marker	Number of Alleles	PIC value
UDO-004	Map.	7	0.81
UDO-009	Inf.	6	0.59
UDO-012	Inf.	3	0.63
UDO-024	Map.	6	0.77
UDO-026	Map.	5	0.98
DCA1	Map.	5	0.71
DCA3	Inf.	5	0.68
DCA7	Map.	2	0.96
DCA9	Map.	7	0.74
DCA10	Map.	2	0.87
DCA11	Map.	5	0.96
DCA14	Map.	4	0.81
DCA15	Map.	7	0.89
GAPU14	Inf.	3	0.57
GAPU19	Map.	8	0.97
GAPU45	Map.	4	0.85
GAPU59	Map.	9	0.92
GAPU62	Map.	9	0.98
GAPU72	Inf.	3	0.59
GAPU82	Map.	5	0.97
GAPU89	Inf.	5	0.67
GAPU90	Map.	2	0.93
GAPU92	-	3	0.40
GAPU101	Map.	5	0.90
GAPU108	Map.	6	0.97
GAPU71A	Inf.	2	0.66
GAPU71B	Map.	6	0.76
GAPU103A	Map.	4	0.82
Total average			0.80

The polymorphism information content (PIC) value of the 28 SSR markers ranged from 0.4 to 0.98. The average PIC value of the primers was determined to be 0.80. Among the 28 SSR primers, UDO-026, GAPU62 with 0.98, GAPU 82, GAPU108 and GAPU 19 with 0.97 had the highest PIC value. Primers with the lower PIC value were GAPU92, GAPU14, GAPU 72 and UDO-009 (0.40, 0.57, 0.59 and 0.59 respectively) (Table 3).

The PIC value varies from 0 to 1 and is used to estimate the discrimination capacity of a primer. A high PIC value (higher than 0.5 - close to 1) indicates that the polymorphism level of the markers is high (BOTSTEIN *et al*., 1980). The fact that PIC values calculated in this study are close to 1 indicates that our results were reliable in identifying mutant olive genotypes. In the current study, SSR markers were found to be a powerful tool to detect genetic diversity among olive genotypes. Many studies that previously attempted to determine genetic variation among olive varieties using SSR markers show similar findings mentioning SSR markers as a powerful tool for determining genetic diversity among olive varieties (ZHANG *et al*., 2015; ABDELHAMID *et al*., 2017).

Markers that have the PIC value higher than 0.7 are used for mapping studies, and markers with a PIC value higher than 0.5 are used as informative markers (HARBI *et al*., 2012). When the PIC values of the SSR markers were examined, it was concluded that the GAPU14, UDO-009, GAPU72, UDO-012, GAPU71A, GAPU89 and DCA3 markers can be used as informative markers, and the remaining markers except GAPU92 can be used in genetic mapping studies (Table 3).

The genetic similarity matrix was formed using a binary data matrix created by scoring SSR markers. Using this matrix, a dendrogram showing the genetic similarities among olive cultivars was prepared (Figure 4). According to the dendrogram, it is clearly seen that olive genotypes were divided into 2 main clusters. In the first cluster, there were genetically closer mutants to the Ayvalik olive cultivar, while in the second cluster, there were mutants that are genetically distant to the Ayvalik olive cultivar.

The genetic distance between Ayvalik olive cultivar and mutants was calculated (WEIR, 1996) as a percentage (%) and was found between 5.5% and 78% with an average of 23%. The mutants 15mM-12, 15mM-11 and 25mM-3 (78, 63 and 55% respectively) in the first cluster were found to be genetically closer to the Ayvalık cultivar. The most genetically distant mutants to the Ayvalik olive cultivar were 20mM-5, 15mM-4 and 15mM-15 (5.5%, 5.6% and 8% respectively) in the second cluster. The average genetic similarity of Ayvalık cultivar with mutants obtained applying 15 mM, 20 mM and 25 mM EMS treatment was 26.02, 10.78 and 33.9% respectively. It was clear that while the most effective EMS treatment increasing the genetic variation was 20 mM, the least was 25 mM. However, considering the number of mutants obtained from olive cuttings by applying 3 different EMS doses, it can be concluded that the most effective EMS dose was 15 mM. Because with this dose, a large number of mutants with high average genetic variation (26.02%) were obtained. SHEIKH and MORADNEJAD (2014), applied 5 different doses of sodium azide, a chemical mutagen, to the olive callus under in-vitro conditions and stated that the mutation occurred in all doses. MUZZALUPO *et al*., (2009), reported that olive genotypes with a genetic similarity rate higher than 94% were synonyms. According to the results of this study, it was concluded that 3 different doses of EMS created mutations in olives and increased genetic diversity.

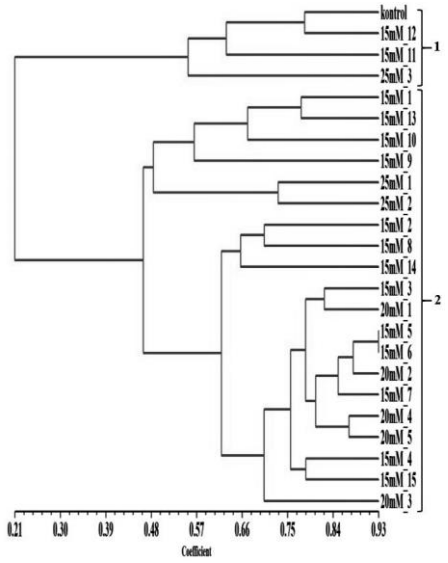

Figure 4. Genetic distance dendrogram of olive genotypes Ayvalık olive cultivar (kontrol: Control) and 23 mutant lines) prepared from similarity coefficients

CONCLUSION

In conclusion, the high genetic distance between Ayvalık olive cultivar and mutants (between 78% and 5.5%) showed that EMS was an effective chemical mutagen for creating genetic variation. The chemical mutation was successfully applied to olive cuttings and it was successfully proved by SSR markers used that these mutants were genetically different from the control Ayvalik olive cultivar. The mutants with high genetic variability resulting from chemical mutation of Ayvalik olive cultivar with EMS can be used in future breeding studies and suitable mutants may be selected by breeders and evaluated in breeding programs. In addition, reliable results have been obtained thanks to the high polymorphism rate of SSR markers. It was one more time confirmed by this study that SSR markers are a powerful tool for diversity studies and in this case distinguished Ayvalik olive cultivar and its mutant lines.

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MOLEKULARNA KARAKTERIZACIJA LINIJA SORTE MASLINE AJVALIK DOBIJENIH HEMIJSKOM MUTACIJOM

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Izvod

Ovo istraživanje je sprovedeno sa ciljem da se otkrije mutageno dejstvo tri različite doze (15, 20 i 25 mM) etil metan sulfonata na sortu masline Aivalık (*Olea europaea* L.), da se otkrije varijacija i da se identifikuje da li s individue genetski razlikuju od sorte masline Aivalık koristeći tehnologije molekularnih markera. 23 mutantne linije su pregledane korišćenjem 28 SSR markera i detektovano je ukupno 138 alela, a broj alela po prajmeru se kretao od 2 do 9 sa prosečno 4,9 alela po prajmeru.Vrednost sadržaja informacije o polimorfizmu (PIC) je bila između 0,4-0,98. Genetska sličnost između sorte masline Aivalık i mutantnih linija bila je između 5,5% i 78%. Pronađene su mutantne linije 15mM-12, 15mM-11 i 25mM-3 slične sortama Aivalık sa stopom sličnosti od 78, 63 i 55%, respektivno. Dok su mutantne linije 20mM-5, 15mM-4 i 15mM-15 bile udaljene od sorte Aivalık sa stopom od 5,5, 5,6 i 8% genetske sličnosti, respektivno. Etil metan sulfonat je uspešno korišćen u ovoj studiji za proširenje genetske raznolikosti sorte masline Aivalık. Mutanti bi se mogli oceniti na poljoprivredne i osobine kvaliteta, a obećavajuće linije bi se mogle registrovati.

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