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Study of genetic variability of local Oregano (*Origanum vulgare*) through RAPD markers

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Abstract

In the present study *Origanum vulgare* seeds were exposed to chemical mutagens at different concentration of Ethyl methane sulphonate and Sodium azide. Mutants and control (without mutagen treatment) of *Origanum vulgare* seeds were than culture *in-vitro* on MS media (supplemented with hormones) for regeneration and callus induction. DNA was isolated from all treated and control *Origanum vulgare* and polymorphism of isolated DNA was studied using six RAPD markers. In this study six RAPD primers were used as markers to study the genetic differences in mutagenized Oregano plants and callus cultures. The DNA samples of plants and callus cultures showed common bands. As common amplified bands in RAPD separated in agarose gels also showed variable intensity of their bands. This type of polymorphism may be due to different copy numbers of corresponding DNA loci. RAPD showed variations in mutagenized Oregano plants and callus cultures. The callus cultures can be used for several studies like for mass propagation, production of secondary compounds and gene transfer studies. RAPD has frequently used as molecular marker for genetic variability studies in plants due to its simplicity, rapidity and lack of prior genetic information about the plant.

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Introduction

Tissue culture is the method through which genetic exploitation studies can be done fruitfully (Parrott *et al.* 1994). Molecular marker practices, for example random amplified polymorphic DNA (RAPD) analysis, have been used to evaluate somaclonal changes (Al-Zahim *et al*., 1999; Hashmi *et al*., (1997), Rind *et al*., (2016) Rout and Das, (2002) and Zucchi *et al*., (2002) in addition to assess the effects of growth regulators (Mangolin *et al.,* (2002) and chemical mutagens (Teparkum and Veilleux, 1998) in various discrete loci inside a genome (Samal *et al.,* (2003). As primers customized by even a single nucleotide create diverse banding profiles, the RAPD technique can produce polymorphisms among very closely-related genotypes (Deng *et al., (*1995). The RAPD assay is well-organized for selection of nucleotide sequence polymorphism between individuals as each primer (on average) will straight to the amplification of numerous discrete loci inside a genomeSamal *et al*., (2003).

Origanum onites and *Origanum vulgare* taxa can be known by the most flawlessly using RAPD markers. RAPD has also been used to differentiate extraordinary plant material under the name Mediterranean oregano for class control (Marieschi *et al*.(2009). Currently, many special objectives have been effectively recognized by the concerned of molecular markers, on different oregano species for example RAPD in *Origanum majorana* (Klocke *et al.,* (2002). and in *Origanum* subspecies (Katsiotis *et al*., (2009), and simple sequence repeat (SSR) in *Origanum vulgare* (Novak *et al*., (2008). Though, it is not yet finely available about the connection among genetic diversity and chemical compounds of essential oils of *Origanum onites*. RAPD is still one of the most successfully and frequently used molecular techniques because of its simplicity, low cost and high speed. Thus, genetic diversity among diverse genotypes of many crops can be easily and rapidly evaluate by victoriously using RAPD markers (Rafalski and Tingey, (1993), Ragot and Hoisington, (1993), Williams *et al.,* (1990). Universally, RAPD can present valuable data for the assessment of population, genetic structure as well as genetic diversity within and among populations, population sections and degree of inbreeding and individual relation (Ragot and Hoisington, (1993). In case of herbaceous plants, RAPD markers have been hardly applying for genetic diversity studies, e.g. in *Origanum majorana* L. (Klocke *et al.,* (2002), *Cunila galioides* Benth (Fracaro *et al.,* (2005), *Curcuma zedoaria* (Christm) Rosc (Islam *et al*., (2007), Syamkumar and Sasikumar, (2007), *Matricaria chamomilla* L. (Solouki *et al.,* (2008), *Satureja hortensis* L. Solouki *et al.,* (2008) and *Foeniculum vulgare* Mill Zahid *et al*., (2009). RAPD-PCR is a quick attempt in which no complex technology and no previous sequence information is necessary, it also allows high-quality appearance levels when the DNA to be probed in its low quantities. Marieschi *et al*., (2009) have used RAPD-PCR method to evaluate its competence as correspondingly, quick and steady assay to probe the attendance of existing contamination and speed pharmacognostic examination of large batches of samples.

In recent study, six different arbitrary primers were used as RAPD markers to assess the genetic diversity among mutagen treated plants and callus cultures. *Origanum vulgare* germplasm collected from the local market of Hyderabad, Pakistan.

The seeds were treated with various concentrations of EMS and Na_2N grown in the field and under *in vitro* conditions, mutant plantlets and four to six month old calli were used to study the genetic variability created through chemical mutagens in plants and callus cultures through the use of Random amplified polymorphic DNA.

Materıal and method

Collection and Mutagenic treatment of Plant materials

The seeds of *Origanum vulgare* were cultivated at Institute of Biotechnology and Genetic Engineering University green house were exposed to chemical mutagens at different concentration of Ethyl methane sulphonate and Sodium azide direct plant source then

cultivated in field as shown in fig-1 A and*Origanum vulgare*inoculated for callus induction as shown in fig-1 B. and then callus was treated different concentrations of NaN3 and EMS are shown in table.1.

Table 1. Different concentrations of Sodium Azide and EMS mutagens.

A	0.3%	Ethyl methane sulfonate	Callus
B	0.3%	Sodium azide	Plant
C	0.3%	Sodium azide	Callus
D	0.25%	Ethyl methane sulfonate	Callus
E	0.2%	Ethyl methane sulfonate	Callus
F	0.2%	Ethyl methane sulfonate	Plant
G	0.1%	Ethyl methane sulfonate	Plant
H	0.75%	Ethyl methane sulfonate	Plant
I	0.5%	Sodium azide	Callus
J	0.5%	Ethyl methane sulfonate	Callus

Fig. 1. Origanum vulgare leaf samples A and proliferated callus B.

Dna Isolatıon

For study of genetic polymorphism through PCR based RAPD technique in Oregano plants (*Origanum vulgare*), Genomic plant DNA was isolated from fresh leaves of Oregano plants previously treated with ethyl methane sulfonate and Sodium azide. DNA was also isolated from four to six months old calli raised from EMS and Na_2N treated shoots and leaves of Oregano. The samples were prepared in following manner. The DNA was isolated using the CTAB method with slight modification in Murray and Thampson, method Murray and Thompson, (1980).

Table 2. List of selected RAPD primers for polymorphism studies in mutant *Lagenaria siceraria* and *cucurbita pepo* plants.

Primers	Primer Sequence 5´ to 3´
OPA-15	5`-TTCCGAACCC-3`
OPA-17	5`-GACCGCTTGT-3`
OPAG-02	5`-CTGAGGTCCT-3`
OPAG-04	5`-GGAGCGTACT-3`
OPAE-10	5´-CTGAAGCGCA-3´
OPB-08	5`-GTCCACACGG-3`

Total six RAPD primers were selected that have already reported to be used for Oregano plants in RAPD analysis (Eurofins MWG Operon) as shown in table-2. All six primers were used in RAPD-PCR and then amplified DNA was separated on 1.4% agarose gel the visualized under Gel documentation.

Data Analysis

Unweighted pair group method of arithmetic analysis (UPGMA) a method of cluster analysis and genetic relationship was used. The data collected after scoring the RAPD profiles the number of bands /DNA fragments present or absent was used to study the similarity index on the basis of number of shared amplification products through Nei and Li formula Nei and Li, (1979)

Results and discussion

The genomic DNA isolated from ten treated plants and calli of *Oregano vulgaris* were screened for polymorphic DNA fragments six RAPD primers including OPA15, OPA17, OPAE10, OPAG02, OPAG04 and OPB08. These primers produced DNA bands for the mutagen treated ten Oregano plantlets and calli separately. RAPD profiles using all treated plant and calli samples simultaneously generated the amplicons ranging 50 – 3000bp as presented in Fig. 2 and 3. The genetic relationship among the selected ten oregano

plants and their calli was generated on the basis of polymorphism generated by six primers (OPA15, OPA17, OPAE10, OPAG02, OPAG04 and OPB08).

For genetic polymorphism studies, DNA of 10 mutagen treated callus cultures and plants were used. In RAPD PCR, primer OPA15 (5`-TTCCGAACCC-3`) produced total 13 amplified bands. Out of total amplified bands, 12 were common bands or monomorphic among different samples while one amplified band polymorphic in callus cultures treated with 0.5% Sodium azide. Similarly two samples B (0.3% sodium azide treated plant) and J (0.5% EMS treated callus) showed (0) bands or no amplification. The average number of bands per primer was 2.87. Out of 201 amplified bands, 176 were common in majority of samples while 24 were polymorphic as shown in table-3. Primer OPA17 (5`-GACCGCTTGT-3`) produced 15 amplificons, out of which 13 bands were common while 2 bands showed polymorphism. The polymorphic bands were from sample E DNA (0.2% EMS treated callus cultures).

Primer OPAE-10 (5`-CTGAAGCGCA-3`) produced 39 amplified bands, which were in the range between 100bp to 1000bp size (Fig. 2). Out of amplified products 35 were common bands produced in different samples while 4 bands were uncommon and were polymorphic bands. The polymorphic bands were obtained one from sample F (0.2% EMS treated plants), one from sample G (0.1% EMS treated plants), one from sample I (0.5% sodium azide treated callus) and one from sample J (0.5% EMS treated callus). Primer OPAG02 (5`-CTGAGGTCCT-3`) produced total 54 amplions of various sizes in the range of 100bp to about 2500bp in length (Fig. 3), From the total number of amplified bands produced, 49 were common bands among various samples while 5 amplified bands were uncommon and said to be polymorphic. Out of these polymorphic bands 2 were from sample E (0.2% EMS treated callus), one sample D (0.25% EMS treated callus) and one from each sample I (0.5% Sodium azide treated callus) and J (0.5% EMS treated callus) polymorphic and were not common among all callus and plantlets of Oregano. Primer OPAG-4 (5`-GGAGCGTACT-3`) produced total 37 amplified products of varying sizes ranges from 200bp to 2500bp. From total amplified products, 32 bands were common among various samples while 5 were uncommon and polymorphic. Sample J (0.5% EMS treated callus) showed three polymorphic bands. Similarly primer OPB-08 (5`-GTCCACACGG-3`) also produced total 14 amplified bands ranging from 300bp to 2000bp. Out of 14 bands, 11 were common among various samples and 3 were polymorphic in nature.

Table 3. Different primers produced amplified common and uncommon bands with didderent % polymorphism.

Primer	Primer Sequence	No of total bands	Monomorphic bands	Polymorphic bands	% polymorphism
OPA-15	5`-TTCCGAACCC-3`	13	12	1	7.67
OPA-17	5`-GACCGCTTGT-3`	15	13	2	13.33
OPAG02	5`-CTGAGGTCCT-3`	39	35	4	10.25
OPAG04	5`-GGAGCGTACT-3`	32	27	5	15.62
OPAG10	5`-CTGAAGCGCA-3`	39	35	4	11.42
OPB-08	5`-GTCCACACGG-3`	14	11	3	21.4
OPAG-02	5`-CTGAGGTCCT-3`	49	43	5	10.20
Total bands		201	176	24	11.94%

Total number of bands amplified from primers is presented in Table 3. Polymerase chain reaction with RAPD primers OPA15, OPA17, OPAE-10, OPAG02, OPAG4 and OPB8 showed polymorphic as well as monomorphic bands in all DNA samples isolated from mutagenized Oregano plants and calli samples. Using Nei's and Li's coefficient analysis was done. Jaccard's coefficient based similarity matrix for the ten mutagenized Oregano plant and calli samples as an out group by an RAPD pattern generated by primers OPA15, OPA17, OPAE-10, OPAG02, OPAG4 and OPB8 is given in table-4. Highest similarity index 0.7 was noted having lowest diversity between the samples of A and D followed by D and H similarity

index was 0.64 Oregano DNA samples. Lowest similarity index was noted 0.1 between the samples of G and B as shown in table-4. Comparative genomic DNA of origano with different treatments molecular study has been done with the use of RAPD-PCR method produced fragments ranged from 100 to 2000 kbs. Common DNA bands almostly ranged at 450 kbs as hsown in fig-2 with OPAG02 RAPD primer. Clear and reproducible bands are shown in fig-3. With OPAE-10 Marker.

Table 4. Jaccard's coefficient based similarity matrix for the ten mutagenized Oregano plant and calli samples as an out group by an RAPD pattern generated by primers.

DNA Samples	A	B	C	D	E	F	G	H	I	J
A	1.00									
B	0.23	1.00								
C	0.48	0.11	1.00							
D	0.7	0.25	0.55	1.00						
E	0.36	0.1	0.32	0.46	1.00					
F	0.2	0.21	0.095	0.262	0.235	1.00				
G	0.6	0.1	0.34	0.561	0.457	0.275	1.00			
H	0.47	0.13	0.418	0.614	0.45	0.3	0.608	1.00		
I	0.31	0.16	0.32	0.326	0.321	0.204	0.349	0.271	1.00	
J	0.38	0.16	0.375	0.46	0.43	0.162	0.532	0.476	0.278	1.00

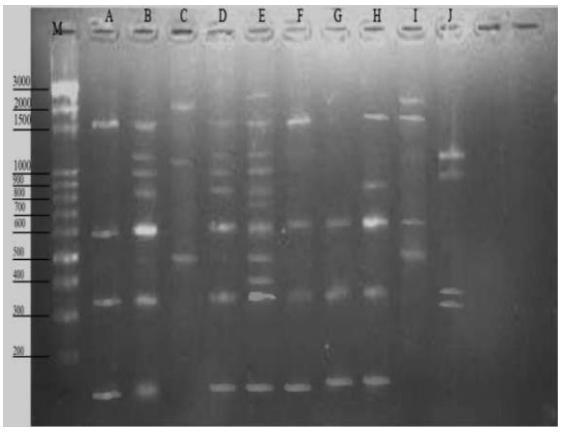

Fig. 2. Agarose gel electrophoresis of RAPD PCR of DNA isolated from mutagenized oregano plants and calli using primer OPAG02.

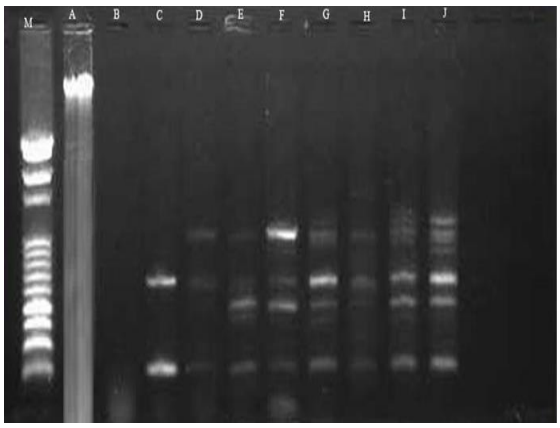

Fig. 3. Agarose gel electrophoresis of RAPD PCR of DNA isolated from mutagenized oregano plants and calli using primer OPAE-10.

For establishment of callus cultures, MS medium was used supplemented with different concentrations of different plant growth regulators. Various concentrations of sodium azide and ethyl methane sulfonate were used for mutation induction in growing plants and proliferation calli. Plants leaves, shoots and seeds were used for callus proliferation. The use of vegetative parts of plants especially leaves are desirable explants for in vitro cultures. Regeneration from leaf explant would preserve the genetic identity of parent plant Sridhar and Naidu, (2011). Similarly proliferation of callus cultures is prerequisite for commercial production of secondary compounds as well as for improvement of crop through gene transfer. Addition of auxin and cytokinin in media affect the texture of callus (Martin, 2002) increase the somaclonal variations ultimately cause the genetic variability during in callus cultures and regenerated plants.Khan *et al.*, (2008) reported the treatment of callus cultures with phytohormone induced genetic variability in regenerated plants of *Saccharum officinarum*. Similarly regenerated plantlets from leaf derived callus have shown genetic variation in *Curcuma longa* Salvi *et al.,* (2001), Tyagi *et al.*, (2007). Somaclonal variations can be detected at early stage in callus cultures through various methods such as cytological assessment, analysis of secondary metabolites and isozyme patterns but may be difficult

to observe chromosomes in many species in cytological assessment or limited to their sensitivity as secondary metabolites and isozyme pattern is used Morell *et al*., (1995). The use of molecular markers is an acceptable approach for genetic polymorphism studies in regenerates Bogani *et al.* (2001).

In present study, 0.25% and 0.5% ethyl methane sulfonate and sodium azide treated callus cultures inoculated on MS medium supplemented with growth regulators showed more genetic variations as compared to compared to EMS and NaN_2 treated plants. The stimulation of unorganized growth in callus cultures is also important source of genetic variations. As long term callus cultures have more deletion, addition, substitution mutations and chromosomal aberrations as compared to fresh callus cultures. Similarly directly regenerated plantlets from leaf and shoot explants showed more stability in genomes compared with regenerated plantlets from callus cultures. Present study not only showed morphological variations in mutagenized Oregano plants but also showed variations in biochemical attributes like in protein and sugar contents, phenolics and antioxidants.

Callus cultures treated with different concentrations of EMS and NaN_2 also showed variations in biochemical attributes. These variations can also be comparable with mutagenized Oregano plants.

In our study, six RAPD primers were used as markers to study the genetic differences in mutagenized Oregano plants and callus cultures. The DNA samples of plants and callus cultures showed common bands. As common amplified bands in RAPD separated in agarose gels also showed variable intensity of their bands. This type of variations in amplified bands intensity was also reported by Kozyrenko *et al.*(2001).

In the samples under study, this type of polymorphism may be due to different copy numbers of corresponding DNA loci Stegniĭ *et al*, (2000). RAPD showed variations in mutagenized Oregano plants and callus cultures. The callus cultures can be used for several studies like for mass propagation, production of secondary compounds and gene transfer studies. RAPD has frequently used as molecular marker for genetic variability studies in plants due to its simplicity, rapidity and lack of prior genetic information about the plant.

Conclusion

According to result highest polymorphism was noted 11.94% among treated plants and callus. Total 24 polymorphic bands were generated out of 201 total bands. Highest Jacquards similarity index was 0.7 noted between the samples of A (0.3% EMS treated Oragino Callus) and D (0.3% NaN_3 treated Oragino plants) followed by D and H 0.614 similarity index was noted.

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