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Molecular Studies of in-vitro Propagated Three *Mentha species* on "KFA+"Media

Kakoli Biswas[1], Harsha Rohira[1] and Rajesh Biswas[2*]

[1] Department of Biotechnology, DAV College, Sector-10, Chandigarh, India
[2] Department of Zoology, Government Home Science College, Sector-10, Chandigarh, India
**Corresponding author's email ID:rajeshbiswas63@yahoo.co.in,*

Abstract

Mentha arvensis, *M. citrata* and *M. spicata* were propagated *in vitro* on KFA+ and MS medium and assessed for genetic homogeneity using ITS and *Cox2* RFLP. ITS and *Cox2* gene amplification gave amplicons of 680bp and 320bp respectively of MS and KFA+ propagated plants. The amplicons when digested with *Hinf*I and *Msp*1, of MS and KFA+ propagated plants were found to be homogenous. From these results it can be inferred that KFA propagated *M. spicata, M. arvensis* and *M. citrata* exhibited no variation at genic level, thereby maintaining genetic homogeneity and the true to type nature of the *in vitro* cultured plants as compared to plants propagated in Murashige and Skoog (MS) Medium. To reduce the cost, MS medium can be substituted by the fly ash based KFA+ as a plant tissue culture medium.

Keywords: KFA+, Murashige and Skoog (MS) Media, PCR, ITS, RFLP.

INTRODUCTION

Menthas or mints are exclusively cultivated for their oils and terpenoid contents. Spearmint (*Mentha spicata*) oil benefits all respiratory problems, is refreshing to muscles, nervous and glandular systems. *M. citrata* has diaphoretic and vasodilator properties. The juice of leaves of *M. arvensis* is an effective gargle in ailments of oral cavity. It is also used as an expectorant, uterine tonic, in the diseases of liver and

spleen, asthma and for joint pains. In modern times, people are increasingly turning to medicinal herbs as a source of alternative therapy. The ever increasing demand for these herbal plants necessitate to higher production for these plants using tissue culture. However, plantlets derived from tissue culture are known to exhibit somaclonal variations which are generally due to genetic changes and are inheritable[1]. Since micropropagation refers to clonal propagations, therefore genetic changes are unlikely to occur in these plants. Any variations in genetic structure can be assessed with the help of various genetic markers like Random Amplified Polymorphic DNA (RAPD), microsatellite polymorphism, Internal Transcribed Spacer Region amplification Restriction Fragment Length Polymorphism (ITS-RFLP)[2-4]. ITS is the small region between the 18S and 28S gene of the rDNA. Many a times, single base pair variation in the ITS region causes an altered restriction site leading to Single Nucleotide Polymorphism (SNP) thereby generating different DNA pattern when digested with specific restriction enzyme for which the site has been altered, can be used for the assessment of intra generic variation. RFLP patterns of the amplified ITS can be also useful for assessing inter generic variation[5-11]. *Cox2* and other mitochondrial genes are known to be of prokaryotic origin and are well conserved and therefore can be used for RFLP studies. *Cox2* is the second sub unit of the cytochrome oxidase, a housekeeping gene and is known to accumulate mutations through evolution, and might be useful in studying phylogenetic relationship among species[12]. They are useful in generating phylogenetic trees for exploring relationships especially at sub generic or lower levels of various taxa[6,12]. In the present study three Mentha species, *M. arvensis, M. citrata, M. spicata* were cultured on Murashige and Skoog (MS)[13] and on very low cost novel KFA+ media developed by Biswas and Biswas[14] and tested for their genetic homogeneity using ITS-RFLP and *Cox2*-RFLP, to demonstrate KFA+ as a potential cost effective medium.

MATERIALS AND METHODS:

Plant Material: *M. arvensis, M. spicata* and *M. citrata* were cultured on MS medium[13] and on novel KFA+ medium[14,15]. MS medium propagated plants were taken as control.

Isolation of plant DNA: Approximately 300mg tissue from each plant was used for extraction of DNA using modified CTAB method as described by Biswas and Biswas[16]. The extracted DNA was subjected to ITS amplification and *Cox2* amplification by PCR.

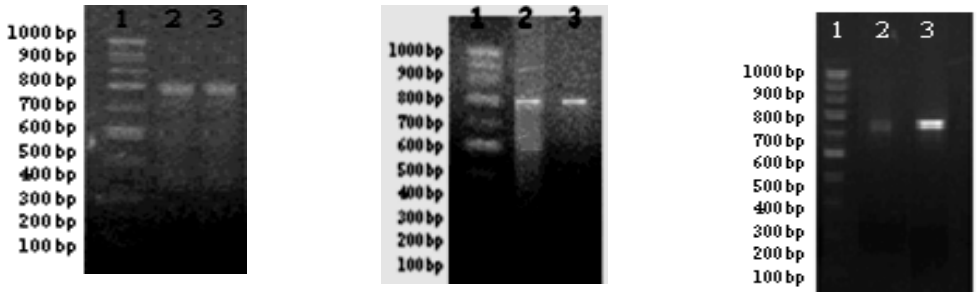
PCR procedure: ITS coding regions were amplified by using primer pairs for ITS-4(5’ TCC TCC GCT TAT TGA TAT GC 3’), ITS-1F(5’ AAG TCG TAA CAA GGT TTC CGT AG 3’);*Cox2* in 32(5’ TCC GAT ACC ATT GAT GTC C 3’),*Cox2* in 51(5’ GAT GCI GCI GAA CCI TGG CA 3’ *)(* I= Inosine**)** in an Eppendorf

Mastercycler Personal Thermal Cycler. For ITS, PCR was carried out for 40 cycles using the following program: Initial denaturation 94°C (5 min), denaturation- 94°C (1 min), Primer annealing- 55°C (1 min), Primer extension-72°C (2 min), Final extension- 72°C (7 min) with 2mM $MgCl_2$, 0.2mM dNTPs, 0.4µM each primer, 1mg/ml BSA and 0.05U/µl Taq polymerase in a 50µl reaction mixture, and for *Cox2*, PCR was carried out for 36 cycles using the following program: Initial denaturation 94°C (4 min), Final denaturation94°C (40 sec.), Primer annealing55°C (40 sec.), Primer extension72°C (1 min), Final extension72°C (4 min)with 3.5mM $MgCl_2$, 0.2mM dNTPs, 1.0µM each primer and 0.16U/µl Taq polymerase in a 50µl reaction mixture.

RFLP of the amplified ITS region and *Cox2* region: Approximately 1 µg each of amplified DNA was digested using *Msp*I and *Hinf*I (Fermentas), according to the manufacturer's instructions. The mixtures were placed in the water bath at 37^0C for 16-20 hours according to the time required by the enzymes for digestion. The mixtures were then placed in water bath at 65^0C for 20 minutes to inactivate the enzymes. DNA fragments were separated by electrophoresis on 2% agarose gel having ethidium bromide at 65V for 90 minutes to check the RFLP patterns obtained by restriction digestion.

RESULTS:

Amplification of ITS of *Mentha* species: High quality DNA obtained from all the plant samples grown in both MS and KFA+ media, were subjected to ITS amplification. Single amplicon of 680bp was observed in *M. arvensis* and *M. spicata* and *M. citrata* in both media (Fig.1)



Mentha arvensis *Mentha spicata* *Mentha citrata*

Fig. 1 Amplified ITS bands of three *Mentha* species: lane 1- 100bp DNA marker, lane 2- grown in MS, lane 3- grown in KFA+

Restriction Fragment Length Polymorphism (RFLP) of ITS amplified products by *Msp*I and *Hinf*I: With *Hinf*I, 2 bands were observed in MS and KFA+ grown *M. spicata*, *M. citrata* and *M. arvensis* (Table 1, Fig. 2). On digestion with *Msp*I, 3 bands were observed in MS grown *M. spicata* and 4 bands were observed in MS and KFA+ grown *M. citrata* and *M. arvensis* (Table 1, Fig.2).

Table1. ITS-RFLP band size in *Mentha* sp. digested with *Hinf*I and *Msp*I		
Mentha species	Band size when digested with *Hinf*I	Band size when digested with *Msp*I
MS *M. spicata*	390bp + 290bp	280bp + 210bp + 120bp + 70bp
MS *M. citrata*	390bp + 290bp	280bp + 210bp + 120bp + 70bp
MS *M. arvensis*	390bp + 290bp	280bp + 210bp + 120bp + 70bp
KFA+ *M. spicata*	390bp + 290bp	280bp + 210bp + 120bp + 70bp
KFA+ *M. citrata*	390bp + 290bp	280bp + 210bp + 120bp + 70bp
KFA+ *M. arvensis*	390bp + 290bp	280bp + 210bp + 120bp + 70bp

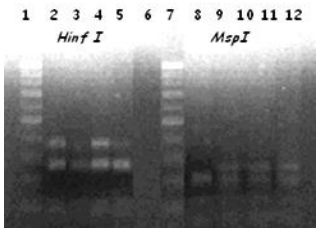


Fig.2: ITS-RFLP patterns digested with *Hinf*I and *Msp*I: Lane 1,7- 100bp DNA marker, Lane 2,8 - MS *M. spicata*, Lane 3,9- MS *M. citrata*, Lane 4,11- KFA+*M. citrata* and Lane 5,12- KFA+ *M. arvensis*

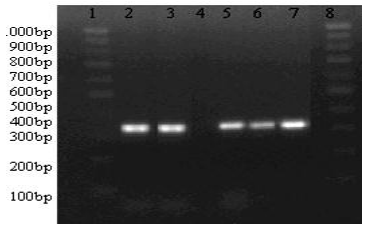


Fig.3: *Cox2* amplicons: Lane 1-100bp DNA marker, Lane 2,3- MS and KFA+ *M. arvensis*, Lane 4,5 - MS and KFA+ *M. spicata*, Lane 6,7- MS and KFA+ *M. citrata*, Lane 8- 100bp DNA marker

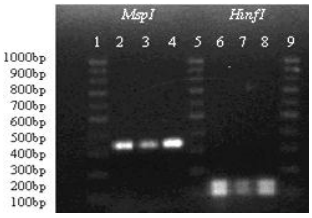


Fig. 4: Cox*2*-RFLP patterns digested with *Msp*I and *Hinf*I: Lane 1,5,9 - 100bp DNA marker, Lane 2,6 - KFA+ *M. arvensis*, Lane 3,7- KFA+ *M. spicata* and Lane 4,8- KFA+ *M. citrata.*

Amplification of *Cox2* region of *Mentha* species: An amplicon size of approximately 320bp for Cox*2* gene was observed in all *Mentha* species grown in both the media.

Restriction Fragment Length Polymorphism (RFLP) of *Cox2* amplified products by *Msp*I and *Hinf*I: RFLP patterns for MS and KFA+ grown *M. arvensis, M. spicata* and *M. citrata* were same when digested with *Msp*I and *Hinf*I. On digestion with *Msp*I, only one band corresponding to the original size of *Cox2* gene was observed whereas digestion with *Hinf*I gave 2 bands corresponding to size approximately 100bp and 170bp.

Table 2. RFLP band size of Cox*2* amplified region in *Mentha* species digested with *Msp*I and *Hinf*I

Mentha species	Band size digested with *Hinf*I	Band size digested with *Msp*I
KFA+*M. arvensis*	100bp + 170bp +50bp*	320bp
KFA+*M. spicata*	100bp + 170bp +50bp*	320bp
KFA+*M. citrata*	100bp + 170bp +50bp*	320bp

*** Band undetected.**

DISCUSSIONS:

ITS is the small conserved region within the rRNA gene. *Cox2* is another small conserved region in mitochondrial gene. Because of the conserved nature and small size, any change in the restriction site present in this region can be detected through RFLP patterns. Both ITS and *Cox2* were amplified and subjected to RFLP for assessing genetic homogeneity.

ITS amplification of MS and KFA+ grown *M. arvensis, M. citrata* and *M. spicata* gave a single amplicon of 680bp. Restriction digestion of ITS products with *Hinf*I, in all three species gave two bands of 390bp, 290bp. This confirmed that the homogeneity of the plants was maintained when grown in KFA+ medium. Earlier intra specific variations and genetic diversity on the basis of polymorphism in ITS have been reported in plants and fungi[2-4]. When digested with *Msp*I, all the three species grown in KFA+ and MS media, gave 4 bands of 280bp, 210bp, 120bp and 70bp. Differentiation among 3 *Mentha* species (*M. cordifolia, M. arvensis and M. spicata)* was carried out on the basis of PCR-RFLP of ITS by Sitthithaworn *et al*[5]. Amplification of *Cox2* gene of MS and KFA+ grown *M. arvensis, M. citrata* and *M. spicata* resulted in to a single band of approximately 320bp. No variation was observed in any of the species grown on KFA+ and MS medium in the present study. RFLP patterns of *Cox2* gene digested with *Msp*I and *Hinf*I were the same for all three species grown in KFA+ and MS media. An undigested single band of the amplicon size with enzyme *Msp*I, may be attributed to the fact that either the restriction site is methylated or there was no restriction site for this particular enzyme. However, two

bands were observed when digested with *Hinf*I and one band of approximately 50bp remained undetected. These observations reveals that *Msp*I and *Hinf*I are unable to distinguish between these species, so further studies with other restriction enzymes have to be carried out. No previous studies regarding *Cox2* RFLP have been reported in *Mentha* species. *Cox2* gene RFLP has been widely used for phylogenetic studies[12]. Earlier homogeneity studies based on microsatellite fingerprinting was reported on in vitro grown *M. arvensis* and *M. spicata* on KFA+ medium[17].

Based on the above results, it can be inferred that fly ash do not contribute to the change in genetic homogeneity and also maintains the true to type of the *in vitro* cultured plants. To reduce the cost, the fly ash based KFA+ can substitute MS as a plant tissue culture medium.

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