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Genetic Relationship among Wild Medicinal Genotypes of *Ziziphora canescens* Benth. and *Ziziphora tenuior* L. and Detection of Genetic Variations Resulted From Tissue Culture, Salinity and pH Media

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Abstract: *Ziziphora tenuior* L. and *Ziziphora canescens* Benth. are two of plants which using in folk medicine in the Kalamoon Mountains areas of Syria for cough, stomachache and dysentery. Samples of *Ziziphora* genotypes were collected from four different locations (Assal Al-Ward, Yabroud, Qarah and Maaraba), 20 RAPD and 12 ISSR primers were used to assess the genetic diversity of 10 genotypes. Amplified fragments were polymorphic with percentage 93.4% and 100% when RAPD and ISSR markers were used respectively. The first cluster depending on RAPD data formed by the grouping of all *Ziziphora tenuior* L. and the second cluster formed by grouping of all *Ziziphora canescens* Benth. genotypes. While the first cluster based on ISSR data formed by two genotypes *Ziziphora tenuior* L. (Maaraba), the second cluster formed by three genotypes *Ziziphora tenuior* L. and five genotypes *Ziziphora canescens* Benth. (Assal Al-Ward, Yabroud and Qarah). ISSR markers were recorded a high degree of biodiversity among *Ziziphora tenuior* L. genotype collected from Maaraba and other genotypes. Genetic stability of *Ziziphora tenuior* L. and *Ziziphora canescens* Benth. was confirmed among mother plant and shoots that underwent one to nine cycles of *in vitro* subculturing by RAPD markers that produced monomorphic bands, while ISSR bands were polymorphic especially in 7,8 and 9 subcultures. Also Genetic variations of *Ziziphora canescens* Benth. that resulted from salinity (1,2,3,4 and 5 g/L) and pH (7,8 and 9) media were detected, both of RAPD and ISSR bands were polymorphic compared with control except some RAPD primers produced monomorphic bands. Callus from *Ziziphora tenuior* L. that induction on different media (MS + 1.5 mg/L NAA + 0.5 mg/L Kin) or (MS + 2 mg/L IBA + 0.5 mg/L Kin) showed high variations compare with micropropagated plants from apical mirestem on media (MS + 1 mg/L Kin + 0.1 mg/L NAA).

Keywords: RAPD, ISSR, *Ziziphora tenuior* L., *Ziziphora canescens* Benth., Syria

Introduction

Many wild plant species among the flora of Syria play an important role in traditional medicine, *Ziziphora* sp. are one of plants which using in folk medicine in the Kalamoon Mountains areas of Syria for cough, stomachache and dysentery. *Ziziphora* species are used for aperitif, carminative and antiseptic effects in treatment of various diseases (Ozturk and Ercisli, 2007). *Z. teniour* L. is used to treat fever and dysentery (Talebi *et al*., 2012), gut inflammation, cough (Safa *et al*., 2012), expectorant, bladder stone and painful menstruation (Naghibi *et al*., 2005). *Ziziphora clinopodioides* L. (*Z. canescens* Benth) is widely used in Iranian traditional medicine for treatment of common cold, gastrointestinal disorders and inflammations (Naghibi *et al*., 2005).

Nowadays, molecular markers have been used to define the species relatives and their taxonomy, Several molecular markers have been developed and applied since 1990, three widely used Polymerase Chain

Reaction (PCR)-based markers are random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990), Inter Simple Sequence Repeats (ISSR) (Zietkiewicz *et al*., 1994) and Amplified Fragment Length Polymorphisms (AFLP) (Vos *et al*., 1995). These molecular markers have been widely used in various fields for the assessment of genetic diversity, genotype fingerprinting and molecular breeding (Yang *et al*., 2013) and the choice of a molecular marker technique depends on its simplicity and reproducibility. RAPD technique did not require knowledge of the DNA sequence for the targeted genome (Russell *et al*., 1993), costs are low and the possibility of using a small amount of DNA (Asemota *et al*., 1996). RAPD markers were shown to be sensitive for detecting variations among individuals between and within species (Carlson *et al*., 1991; Roy *et al*., 1992). So RAPD analysis was used in many of ancient and modern studies for molecular characterization and identification of genetic relationship among species. RAPD have been used successfully to assess the genetic relationship among species of *Mentha* sp. (Khanuja *et al*., 2000), molecular characterization of some selected wild olive Ecotypes (*Olea oleaster* L.) Grown in Turkey (Özkaya *et al*., 2009) also (Al-Rawashdeh, 2011) studied the molecular taxonomy and the genetic relationships among two species of *Mentha* namely M. *spicata*, *M. longifolia* and *Ziziphora tenuior L.*, (Giri *et al*., 2012) showed the extent of diversity between different populations of *Acorus calamus* L. and (Rajani *et al*., 2013) used RAPD analysis to assess the genetic diversity in ten selected cultivars of rice (*Oryza sativa* L.). ISSR has several advantages, particularly in reproducibility and higher informative nature (Nagaoka and Ogihara, 1997). Also ISSR markers were shown to be specific and sensitive for detecting variations between and within species (Bornet and Branchard, 2001) and highly reproducible due to their primer length and to the high stringency achieved by the annealing temperature (Gürcan *et al*., 2009). Muthusamy *et al*. (2008) were used RAPD and ISSR markers in accessing genetic variation of *Vigna umbellate*. Also (Singh *et al*., 2014) were studied genetic diversity among 35 *Vigna* genotype using SSR, ISSR and RAPD markers.

Several strategies can be used to assess the genetic fidelity of *in vitro* derived clones, but most have limitations, for example: Karyological analysis cannot reveal alterations in specific genes or small chromosomal rearrangements (Isabel *et al*., 1993). Isozyme markers provide a convenient method for detecting genetic changes, but are subject to ontogenic variations, they are also limited in number and only DNA regions coding for soluble proteins can be sampled (Rout *et al*., 1998). Molecular markers suitable for generating DNA profiles have proved to be an effective tool in assessing the genetic stability of regenerated plants (Chandrika *et al*., 2008).

Screening the tissue culture derived plants using molecular markers will assist in reintroducing true-totype plants (Heinze and Schimidt, 1995) and protecting their genetic integrity. RAPD markers have been used successfully to assess genetic stability among somatic embryos in spruce species (Isabel *et al*., 1993; 1996). Panda *et al*. (2007) evaluated the genetic stability of micropropagated clones of *Curcuma longa* L. over 26 months in culture by random amplified polymorphic DNA (RAPD) analysis, Also Genetic stability of thyme was confirmed in the *in vitro*-germinated mother plant as well as the shoots that underwent two, four, six, eight and ten cycles of *in vitro* subculturing by RAPD analysis (Ozudogru *et al*., 2011). The ISSR markers have also been used successfully to assess genetic stability among micropropagated plants (Joshi and Dhawan, 2007). Chandrika *et al*. (2008) were studied of genetic stability of *in vitro* grown *Dictyospermum ovalifolium* using ISSR markers.

The present study was undertaken to study the genetic relationship among five genotype of *Ziziphora canescens* Benth. (perennial) and five genotype of *Ziziphora tenuior* L. (annual) that grown in Syria and study of genetic stability of *in vitro* plants that were propagated by (Dakah *et al*., 2014a; 2014b; 2014c) and compare it with wild plants, because morphological, cytological and molecular variations may be generated *in vitro* (Larkin *et al*., 1989).

Materials and Methods

This research was carried out in the Plant Tissue Culture and Molecular Biology Laboratory in Damascus University, Faculty of Science and Department of Plant Biology.

Genetic Relationship between Wild Plants

Genomic DNA of wild plants *Ziziphora canescens* Benth. were collected from two sites: Assal Al-Ward (4 genotypes) and Yabroud (1 genotype). *Ziziphora tenuior* L. were collected from three sites: Assal Al-Ward (2 genotypes), Qarah (1 genotypes) and Maaraba (2 genotypes) and authenticated by Dr. Imad Alkadi at the Department of Plant Biology, Damascus University.

Detection of Genetic Stability

Genomic DNA of micropropagated plants of *Z. canescen* and *Z. tenuior* L. by (Dakah *et al*., 2014a; 2014b; 2014c) were collected from *in vitro* of Plant Tissue Culture Laboratory in Damascus University, Department of Plant Biology.

Effect of Number of Subculture on Genetic Stability

Genomic DNA of *in vitro* propagated shoots obtained after one, two and three to nine subculturing. Where medium MS (Murashige and Skoog, 1962) supplemented with 0.1 mg\L of Kin plus 0.1 mg/L of

IBA used to propagate *Ziziphora canescen*. and medium MS supplemented with 1 mg/L of Kin plus 0.1 mg/L of NAA used to propagate *Ziziphora tenuior* L..

Effect of Salinity and pH Media on Genetic Stability of Ziziphora canescen

Genomic DNA of *in vitro* propagated shoots obtained after one month. Five treatments were prepared to test the effect of salinity on genetic stability, where NaCl was added to medium WPM (Lloyd and McCown, 1981) with different concentration (1 - 2 - 3 - 4 - 5 g/L). Also different pH levels 7, 8 and 9 were tested.

Effect of Culture Media on Genetic Stability of Ziziphora tenuior L.

Genomic DNA of *in vitro* propagated shoots obtained after one month. Two ways were used to propagated *Ziziphora tenuior* L. The first was by callus induction from leaf parts on media MS supplemented with 1.5 mg/L of NAA plus 0.5 mg/L Kin or media MS supplemented with 2 mg/L of IBA plus 0.5 mg/L Kin. The second way was by culture apical mirestem on media MS supplemented with 1 mg/L of Kin and 0.1 mg/L of NAA.

DNA Isolation

DNA was extracted from fresh leaves of *in vitro* propagated plants and wild grown plants by the Sodium Dodecyl Sulphate (SDS) in combination with phenol: chloroform: Isoamlyalcohol (Nalini *et al*., 2004) with minor modifications.

Purity and Quantitation of DNA Concentration

The purity of the extracted genomic DNA was confirmed through its OD_{260}/OD_{280} ratio (1.8 to 2). The DNA was also observed on 1.2% agarose gel by electrophoresis using 1X TAE buffer. The DNA fragments were stained with ethidium bromide and viewed under UV light and photographed. The DNA concentration was calculated using the following equation: OD_{260} * 50 ng/µL * dilution factor

PCR Amplification

RAPD Analysis

About 20 primers were tested for PCR amplification. Polymerase Chain Reactions (PCR) for amplification of DNA preparations were carried out in a final volume 25 µL consisting of 50 ng of total genomic DNA from each sample, 0.25 mM dNTPs, 0.4 µM of primers, 1.5 mM MgCl2, 1X PCR buffer (10 mM Tris- HCl pH 8.3, 50 mM KCl) and 1.25U of Taq DNA polymerase. Amplification was achieved in a thermal cycler programmed for one cycle of 1 min at 94°C followed by 40 cycles, each consisting of a denaturation step for 1min at 94°C, followed by a primer annealing for 1min at 36°C and an extension step for 2 min at 72°C and final extension step for 10 min at 72°C. After the final cycle the samples were cooled to 4°C.

ISSR Analysis

About 12 primers were tested for PCR amplification. Polymerase Chain Reactions (PCR) for amplification of DNA preparations were carried out in a final volume 25 µL consisting of 50 ng of total genomic DNA from each sample, 0.4 µM of primers, 12.5 µL of Taq 2X Master Mix. Amplification was achieved in a thermal cycler programmed for one cycle of 1 min at 94°C followed by 40 cycles, each consisting of a denaturation step for 1min at 94°C, followed by a primer annealing for 1min at 50-58°C (depending on the GC content of the primer) and an extension step for 2 min at 72°C and final extension step for 10 min at 72°C. After the final cycle the samples were cooled to 4°C.

Visualisation of Amplification Products

About 8 µL of the 25 µL products were loaded in a 1.5% agarose gel containing 5 µL of ethidium bromide in 1X TAE buffer. A 100-bp DNA ladder was used to estimate the approximate molecular weight of DNA bands for each PCR product. Electrophoresis was performed at 100 V for 1 h the amplified products were detected under UV light and photographed. RAPD and ISSR analyses were repeated twice for all samples and only clear bands produced in both replicates were scored as mentioned below.

Data Analysis

Only clear and repeatable amplification products were manually scored as present bands (1) or absent ones (0). The similarity coefficient values between the species were derived based on the Jaccard's (1908) similarity coefficient matrices. The dendrogram was constructed between selected species by using a distance matrix using the Unweighed Pair-Group Method with Arithmetic average (UPGMA) by PAST software. Polymorphism percentage was calculated by dividing the number of polymorphic bands over the total number of bands * 100.

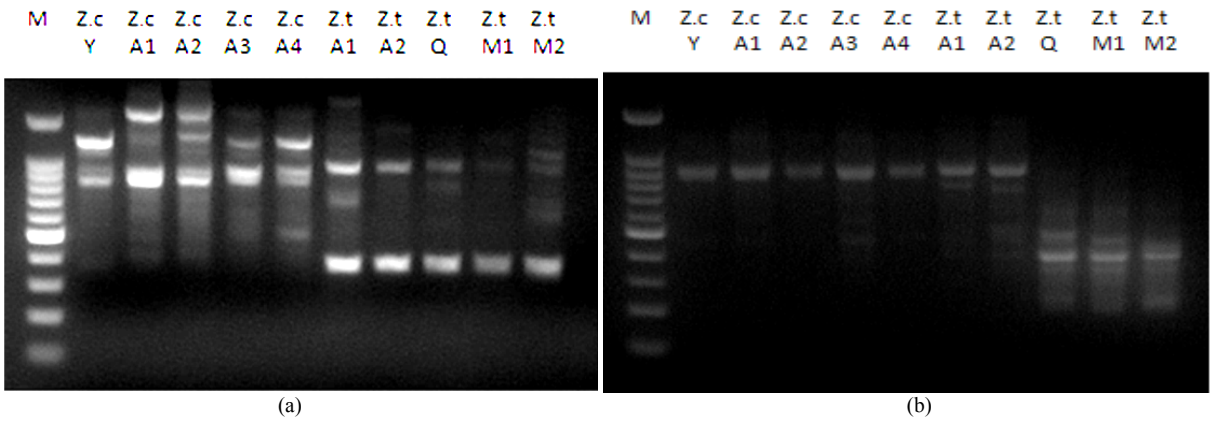
Results

Genetic Relationship

About 20 RAPD and 12 ISSR primers were examined. The total number of markers and polymorphic markers were 160 and 154, respectively, with percentage of polymorphism 96.25% (Table 1). Where RAPD produced a total of 91 bands, ISSR produced a total of 69 bands. The number of amplification products produced by a primer ranged from 2 to a maximum 9 with an average of 5.15 bands per primer. All of ISSR primers and 14 RAPD primers showed high percentages of polymorphism (100%).

Table 1. Number of polymorphic bands per primer obtained by PCR amplification of DNA of wild plants Z. *canescens* and Z. *tenuior* L. using RAPD and ISSR primers

Primer name	Sequence (5`- 3`)	Total number of Bands	Number of polymorphic bands	Percentage of polymorphism
Oligo 1	GGACGACCGT	6.00	5.00	83.300
Oligo 2	CTCGGATGTC	6.00	6.00	100.000
Oligo 3	GAAGGCTGGG	4.00	4.00	100.000
Oligo 4	TTCGGCCGAC	3.00	3.00	100.000
Oligo 5	CTTCGGTGTG	5.00	5.00	100.000
Oligo 6	TTTTGCCCCC	6.00	5.00	83.300
Oligo 7	CCTCCACCAG	2.00	2.00	100.000
Oligo 8	TCACTCGCTC	5.00	5.00	100.000
Oligo 9	GTGCGGAGAG	5.00	4.00	80.000
Oligo 10	CTCCACGACT	4.00	4.00	100.000
Oligo 11	CTGGCGTGTC	3.00	3.00	100.000
Oligo 12	GTCCCGACGA	5.00	4.00	80.000
Oligo 13	GTCGCCGTCA	6.00	6.00	100.000
Oligo 14	CTACTGCCGT	6.00	5.00	83.300
Oligo 15	GTGCAACGTG	5.00	4.00	80.000
Oligo 16	GTCCGTACTG	4.00	4.00	100.000
Oligo 17	GTTTCGCTCC	3.00	3.00	100.000
Oligo 18	GGACTGGAGT	5.00	5.00	100.000
Oligo 19	TGCGCCCTTC	4.00	4.00	100.000
Oligo 20	GTCCACACGG	4.00	4.00	100.000
ISSR1	AGAGAGAGAGAGAGAGG	3.00	3.00	100.000
ISSR2	GAGAGAGAGAGAGAGAA	8.00	8.00	100.000
ISSR3	ACACACACACACACACC	9.00	9.00	100.000
ISSR4	CACACACACACACACAA	9.00	9.00	100.000
ISSR5	ATATATATATATATATT	4.00	4.00	100.000
ISSR6	TATATATATATATATAA	3.00	3.00	100.000
ISSR7	GTGTGTGTGTGTGTGTT	5.00	5.00	100.000
ISSR8	TGTGTGTGTGTGTGTGG	5.00	5.00	100.000
ISSR9	CGCGCGCGCGCGCGCGG	8.00	8.00	100.000
ISSR10	GCGCGCGCGCGCGCGCC	7.00	7.00	100.000
ISSR11	CTCTCTCTCTCTCTCTT	5.00	5.00	100.000
ISSR12	TCTCTCTCTCTCTCTCC	3.00	3.00	100.000
	Total (RAPD primer)	91.00	85.00	93.400
	Mean per primer	4.55	4.25	94.450
	Total (ISSR primer)	69.00	69.00	100.000
	Mean per primer	5.75	5.75	100.000
	Total (RAPD+ISSR primer)	160.00	154.00	96.250
	Mean per primer	5.15	5.00	97.225



(a) (b)

Fig. 1. RAPD and ISSR marker profile (a) RAPD profile obtained with primer Oligo 1. (b) ISSR profile obtained with primer ISSR 1. Lan 1 to 5 *Ziziphora canescence* and lan 6 to 10 *Ziziphora tenuior* L.

Table 2. Genetic similarity among collected wild Ziziphora genotypes based on Jaccard coefficient by RAPD marker data

	Ziziphora canescens				*Ziziphora tenuior L.*					
	Yabroud	Assal Al-Ward				Assal Al-Ward		Qarah	Maaraba	
	Z.c-Y	Z.c-A1	Z.c-A2	Z.c-A3	Z.c-A4	Z.t-A1	Z.t-A2	Z.t-Q	Z.t-M1	Z.t-M2
Z.c-Y	1.00000									
Z.c-A1	0.80000	1.00000								
Z.c-A2	0.90000	0.90000	1.00000							
Z.c-A3	0.88889	0.70000	0.80000	1.00000						
Z.c-A4	0.80000	0.63636	0.72727	0.88889	1.00000					
Z.t-A1	0.28571	0.28571	0.26667	0.30769	0.28571	1.00000				
Z.t-A2	0.30769	0.30769	0.28571	0.33333	0.30769	0.88889	1.0000			
Z.t-Q	0.23077	0.23077	0.21429	0.25000	0.23077	0.77778	0.8750	1.00000		
Z.t-M1	0.25000	0.25000	0.23077	0.27273	0.25000	0.66667	0.7500	0.62500	1.00000	
Z.t-M2	0.23077	0.23077	0.21429	0.25000	0.23077	0.60000	0.66667	0.55556	0.85714	1.0000

Table 3. Genetic similarity among collected wild Ziziphora genotypes based on Jaccard coefficient by ISSR marker data

	Ziziphora canescens				*Ziziphora tenuior* L.					
	Yabroud		Assal Al-Ward			Assal Al-Ward		Qarah	Maaraba	
	Z.c-Y	Z.c-A1	Z.c-A2	Z.c-A3	Z.c-A4	Z.t-A1	Z.t-A2	Z.t-Q	Z.t-M1	Z.t-M2
Z.c-Y	1.00000									
Z.c-A1	0.17647	1.00000								
Z.c-A2	0.20000	0.75000	1.00000							
Z.c-A3	0.26667	0.61111	0.68750	1.00000						
Z.c-A4	0.33333	0.52941	0.50000	0.66667	1.00000					
Z.t-A1	0.11111	0.25000	0.21739	0.26087	0.36842	1.0000				
Z.t-A2	0.11111	0.25000	0.21739	0.26087	0.36842	0.76471	1.000000			
Z.t-Q	0.00000	0.16667	0.13043	0.17391	0.26316	0.33333	0.473680	1.00000		
Z.t-M1	0.00000	0.10000	0.00000	0.05000	0.12500	0.10000	0.157890	0.42857	1.00000	
Z.t-M2	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.058824	0.23077	0.42857	1.0000

Table 4. Classification of Ziziphora genotypes on the basis of RAPD and ISSR data

Cluster	Subcluster	Genotypes	Number of genotypes
RAPD			
I	Ia	Z.t-A1, Z.t-A2 and Z.t-Q	3
	Ib	Z.t-M1 and Z.t-M2	2
II	IIa	Z.c-A3, Z.c-A4	2
	IIb	Z.c-A1, Z.c-A2 and Z.c-Y.	3
ISSR			
I	-	Z.t-M1 and Z.t-M2	2
II	Ia	Z.t-A1, Z.t-A2 and Z.t-Q	3
	Ib	Z.c-A1, Z.c-A2, Z.c-A3, Z.c-A4 and Z.c-Y.	5

Table 5. RAPD and ISSR banding of micropropagated plants (nine subculture) and wild plant of Z. *canescens* and Z. *tenuior* L.

Primer name	Total number of Bands of *Z. canescens* (wild plant)	Number of polymorphic bands of *Z. canes cens* (*in vitro* plant)	Total number bands of *Z. tenuior L* (wild plant)	Number of polymorphic bands *Z.tenuior* of *L* (*in vitro* plant)
Oligo 1	2	0	2	0
Oligo 9	3	0	4	0
Oligo 10	3	0	3	0
Oligo 12	3	0	2	0
Oligo 15	2	0	4	0
ISSR1	6	6	4	4
ISSR2	4	3	5	5
ISSR3	5	5	7	6
ISSR4	2	2	4	3
ISSR8	1	0	3	3

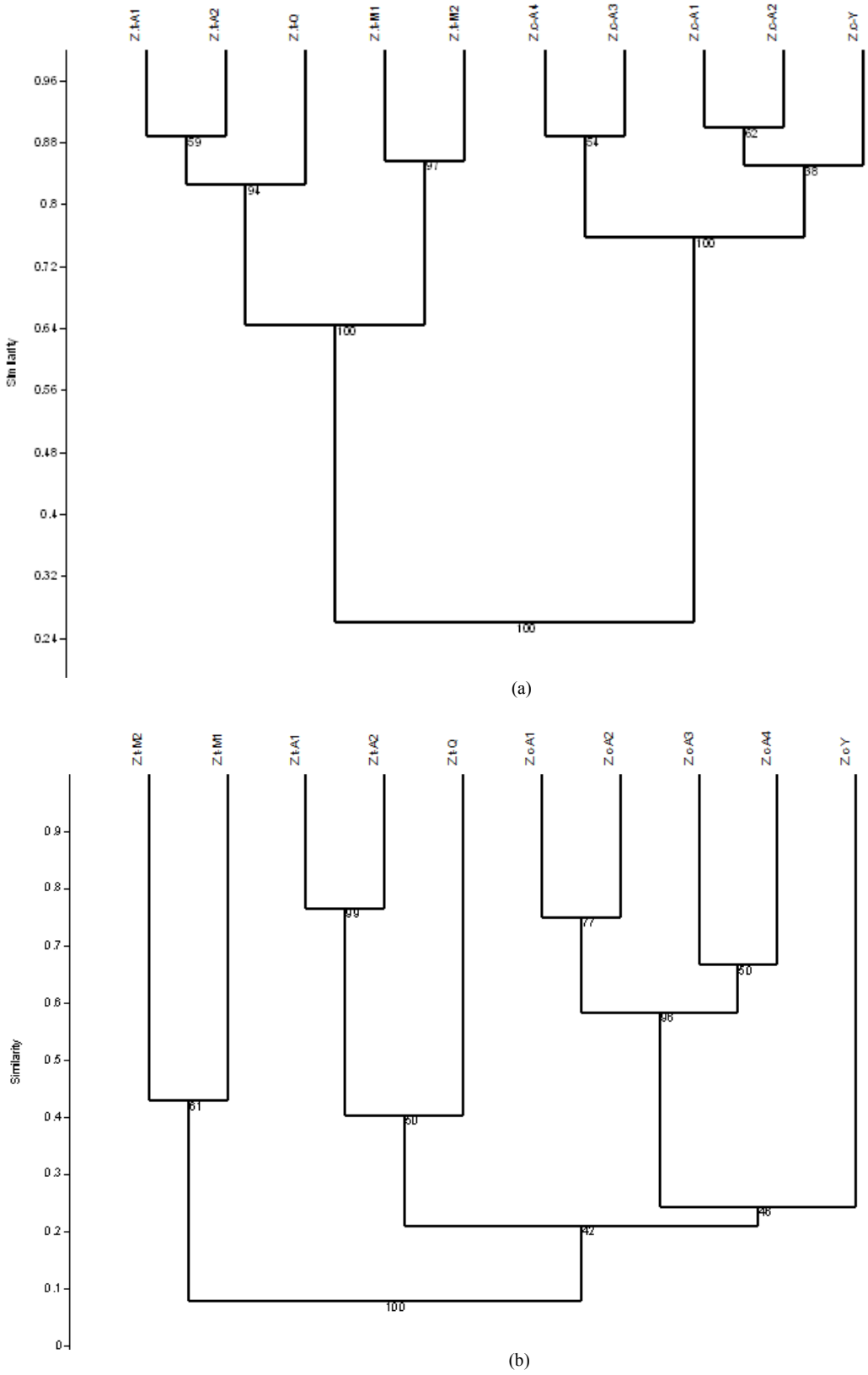
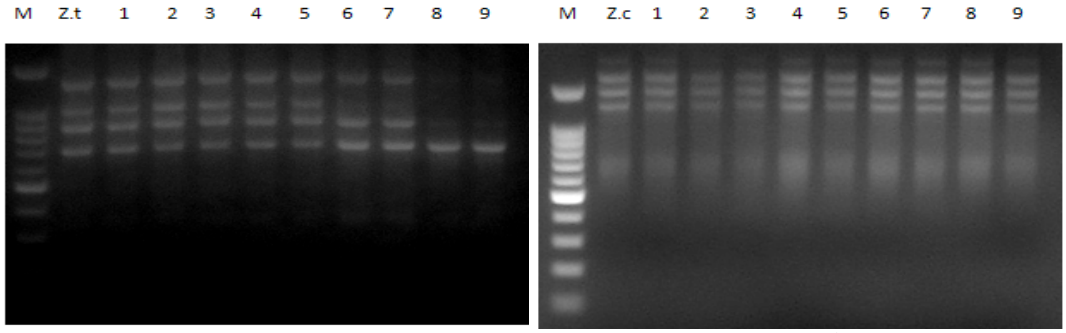
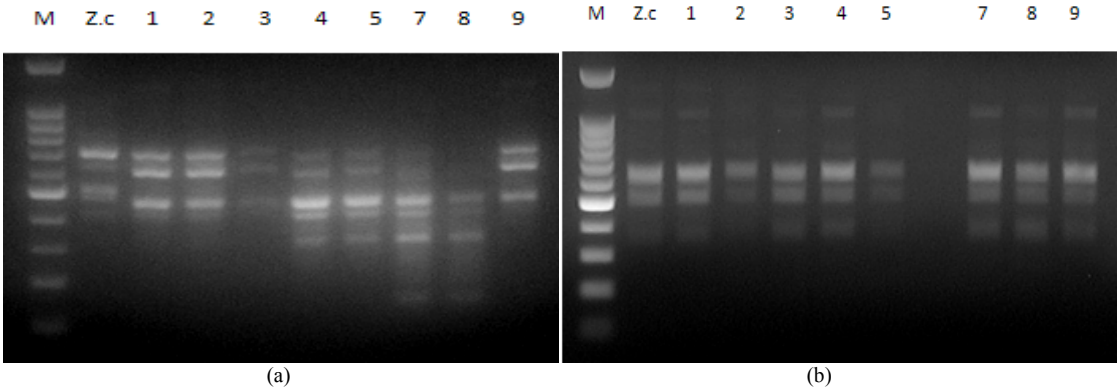


Fig. 2. Dendrogram generated using UPGMA analysis, showing relationships between five genotypes of *Ziziphora Canescens* and five genotypes of *Ziziphora tenuior* L. using (a) RAPD (b) ISSR



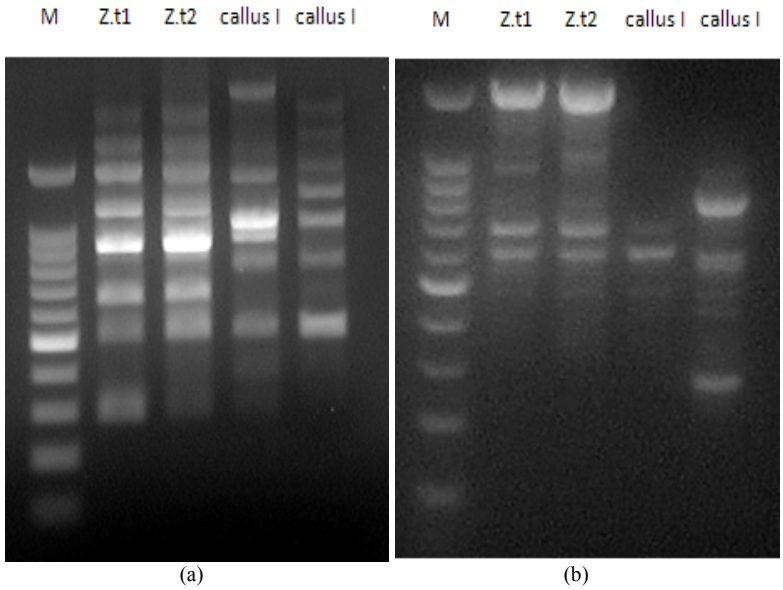
(a) (b)

Fig. 3. RAPD and ISSR banding of micropropagated plants for 9 subcultures and wild plant (a) ISSR profiles of Z. *tenuior* L. generated by primer ISSR4 (b) RAPD profiles of Z. *canescens* Benth. generated by primer Oligo 9. (lane 1 wild plant and lane 2 to 9 micropropagated plants)



(a) (b)

Fig. 4. RAPD and ISSR banding of micropropagated plants of Z. *canescens* Benth. on different media (Z.c control plant on WPM medium, 1to 5 contain 1,2,3,4 and 5 g/L NaCl. 7, 8 and 9 refer to pH media 7, 8 and 9) (a) ISSR profiles of Z. *canescens* Benth. Generated by primer ISSR2 (b) RAPD profiles of Z. *canescens* Benth. generated by primer Oligo 20



(a) (b)

Fig. 5. RAPD and ISSR banding of wild plant Z. *tenuior* L. (Z.t1) and compare with micropropagated plants on different media. Z.t2 (MS+1 mg/L Kin + 0.1 mg/L NAA), callusI (MS+1.5 mg/L NAA + 0.5 mg/L Kin) and callusII (MS+ 2 mg/L IBA + 0.5 mg/L Kin). (a) ISSR profiles of Z. *tenuior* L. generated by primer ISSR3 (b) RAPD profiles of Z. *tenuior* L. generated by primer Oligo 20

Table 6. RAPD and ISSR banding of micropropagated plants on salinity and pH media of Z. *canescens*

Primer name	Total number of bands of micropropagated plants on WPM media WPM (control)	Number of polymorphic bands of micropropagated plants on different WPM media							
		Different concentration of NaCl (g/L)						pH media	
		1	2	3	4	5	7	8	9
Oligo 1	2	0	No bands	0	0	No bands	1	1	1
Oligo 2	2	0	1	0	No bands	No bands	0	0	0
Oligo 3	5	0	4	2	3	3	2	2	4
Oligo 9	5	0	0	0	2	No bands	1	4	No bands
Oligo 15	2	0	1	1	No bands	2	3	2	No bands
Oligo 16	2	0	0	0	0	0	0	0	0
Oligo 17	3	0	0	0	0	0	0	0	0
Oligo 20	3	0	0	0	0	0	0	0	0
ISSR1	5	1	4	2	3	2	2	2	3
ISSR2	2	2	2	2	3	3	3	2	2
ISSR3	3	2	2	2	1	2	1	No bands	2
ISSR4	1	1	1	1	1	1	3	3	1
ISSR8	3	1	1	2	2	2	No bands	No bands	1

Table 7. RAPD and ISSR banding of wild plant, micropropagated plants from apical mirestem and callus on different culture media of *Ziziphora tenuior* L.

Primer name	Total number of bands of wild plant	Number of polymorphic bands of micropropagated plants on different media		
		micropropagated plants from apical mirestem on MS+1Kin+0.1NAA	Callus II on MS+1.5NAA+0.5Kin	Callus I on MS+2IBA+0.5Kin
Oligo 1	7	1	No bands	6
Oligo 2	2	1	No bands	1
Oligo 3	3	0	2	2
Oligo 9	4	1	3	No bands
Oligo 15	3	0	No bands	2
Oligo 16	3	0	4	No bands
Oligo 17	3	0	No bands	No bands
Oligo 20	8	1	4	4
ISSR1	2	0	1	1
ISSR2	3	0	1	2
ISSR3	4	0	2	3
ISSR4	4	0	3	0
ISSR8	2	0	No bands	No bands

RAPD and ISSR marker profile produced by the primer Oligo 1 and ISSR 1 respectively are shown (Fig. 1).

According to the RAPD marker results (Table 2), genetic similarity ranged from 0.21 (low similarity) between Z.c-A2 and Z.t-Q, Z.t-M2 up to 0.9 (high similarity) between Z.c-A2 and Z.c-A1, Z.c-Y. while ISSR marker data (Table 3) didn't show any similarity (0) between Z.t-M2 and Z.c-Y, Z.c-A1, Z.c-A2, Z.c-A3, Z.c-A4, Z.t-A1. Also between Z.t-M1 and Z.c-Y, Z.c-A2. Also between Z.t-Q and Z.c-Y. And showed high similarity (0.76) between Z.t-A1 and Z.t-A2.

Dendrograms obtained using the UPGMA method based on RAPD and ISSR data (Fig. 2) clearly distinguished the genotypes of species. The genotypes were divided into two main clusters in two dendrograms. The first cluster depending on RAPD data formed by the grouping of all *Ziziphora tenuior* L. and within this cluster Z.t-A1, Z.t-A2 and Z.t-Q grouped together (subcluster I) and Z.t-M1 and Z.t-M2 grouped together (subcluster II). The second cluster formed by grouping of all *Ziziphora canescens* Benth. and within this cluster Z.c-A3 and Z.c-A4 grouped together (subcluster I) and Z.c-A1, Z.c-A2 and Z.c-Y grouped together (subcluster II). While the first cluster based on ISSR data formed by Z.t-M1 and Z.t-M2. The second cluster was divided into two subclusters. The first subcluster formed Z.t-A1, Z.t-A2 and Z.t-Q and second subcluster formed Z.c-A1, Z.c-A2, Z.c-A3, Z.c-A4 and Z.c-Y (Table 4).

Effect of Subculture on Genetic Stability

About 5 RAPD and 5 ISSR primers were used to determinate genetic stability of *Ziziphora canescens* Benth and *Ziziphora tenuior L.* The results were scored as bands obtained from wild plant and compared with *in*

vitro micropropagated plants. All RAPD profiles from micropropagated plants for nine subcultures were monomorphic and similar to those of wild grown mother plants. No RAPD polymorphism or variation was detected within the micropropagated plants. While ISSR primers products were polymorphic especially on subculture 7, 8 and 9 except ISSR 8 produced monomorphic bands of *Ziziphora canescens* Benth (Table 5). RAPD and ISSR Profile obtained by Oligo 9 and ISSR4 respectively are shown (Fig. 3).

Effect of Salinity and pH Media on Genetic Stability of Ziziphora canescen

About 8 RAPD and 5 ISSR primers were used to determinate effect of salinity and pH media on genetic stability of *Ziziphora canescens* Benth.. Oligo 16, Oligo 17 and Oligo 20 showed monomorphic bands and no variation was detected, while Oligo 1, Oligo 2, Oligo 3, Oligo 9, Oligo 15 and all ISSR primers showed polymorphic bands. Also some primers didn't show any band in some media like media contain 5 g/L NaCl or pH media 9 (Table 6). RAPD and ISSR Profile obtained by Oligo 20 and ISSR2 respectively, are shown (Fig. 4).

Effect of Callus Culture on Genetic Stability of Ziziphora tenuior L.

About 8 RAPD and 5 ISSR primers were used to determinate differences between wild plant and micropropagated plant from apical mirestem or callus. Most of primers showed monomorphic bands and not much variation were detected between wild plant and micropropagated plant from apical mirestem. Oligo 20, 9, 2 and Oligo 1 showed one variation between them while all primers products were polymorphism between wild plant and callus. Also some primers didn't show any band in callus I or callus II (Table 7). RAPD and ISSR Profile obtained by Oligo 20 and ISSR3 respectively are shown (Fig. 5).

Discussion

Genetic Relationship

Molecular markers allow the direct selection for genotypes, thereby providing a more efficient means of selection. Molecular markers provide an opportunity to identify and isolate the gene relating to character by map-based cloning (Ajit *et al*., 2013).

This research comprises the first study about genetic variations among genotypes of two *Ziziphora* species (*Z. canescens* Benth. and *Z. tenuior* L.) from growing in Kalamoon Mountains. The used primers showed high polymorphism, *Z. canescens* Benth. and *Z. tenuior L.* formed the two different clusters using RAPD primers indicating that each species has a unique DNA sequence, the first cluster contain *Z. tenuior* L. genotypes. Where Z.t-A1 was closer to Z.t-A2 (Assal Al-Ward), Z.t-M1 was closer to Z.t-M2 (Maaraba), while Z.t-Q (Qarah) was closer to Z.t-A1 and Z.t-A2. A possible reason Qarah near to Assal Al-Ward. The second cluster contain *Z. canescens* Benth. genotypes. Where Z.c-A1 was closer to Z.c-A2 and Z.c-A3 was closer to Z.c-A4, while Z.c-Y closer to Z.c-A1 and Z.c-A2. Al-Rawashdeh (2011) confirmed that *Mentha* species are genetically different from *Ziziphora tenuior* L. and a genetic variation was found between and within the species by RAPD analysis, also the genetic variability found among the species genotypes, could be due to out-breeding and the wide dispersal of seeds and pollen grains. ISSR dendrogram showed more separated genotypes according to their geographic distribute, where Z.t-M1 was closer to Z.t-M2 in the first closter and eight genotypes formed the second closter, Z.c-Y was closer to Z.c-A1, Z.c-A2, Z.c-A3 and Z.c-A4 to form subcluster while Z.t-Q was closer to Z.t-A1 and Z.t-A2 to form another subcluster. This close relationship between species from different geographic region was supported by (Abd El-Hady *et al*., 2010) they reported the two Egyptian genotypes of *Vigna* Species tended to cluster together with high degree of genetic similarity regardless the type of molecular marker and German genotypes tended to cluster together into two separated groups. Also (Rahimmalek, 2012) showed that the collected genotypes of *Achillea tenuifolia* indicated a considerable variation based on their geographical regions.

The difference of results between RAPD and ISSR may be that two marker techniques target different regions of the genome (Souframanien and Gopalakrishn, 2004) and the number of potential ISSR markers depends on the frequency of microsatellites, which changes with species (Depeiges *et al*., 1995). Also (Galvan *et al*., 2003) concluded that ISSR would be a better tool than RAPD for phylogenetic studies.

Effect of Subculture on Genetic Stability

Molecular analysis is being commonly used for monitoring genetic fidelity of *in vitro* plants and provide an effective procedure to determine tissue culture induced variations (Peredo *et al*., 2009; Samarfard *et al*., 2013). The RAPD and ISSR techniques were applied with the purpose of assessing the genetic stability of plants regenerated. According to RAPD markers, true-to-type plants could be produced because no differences were detected between the wild plant and *in vitro* regenerated plants through 9 subcultures. All of markers were monomorphic. This result are in agreement with Peyvandi *et al*. (2013) who reported all of the mentioned fragments were monomorphic, confirming the genetic stability of the micropropagated plants of *Olea europaea*. Also agree with Ozudogru *et al*. (2011) who reported all of bands were monomorphic through 10 subculture of *Thymus vulgaris* and *T. longicaulis*. In

opposite of, ISSR markers showed high polymorphic bands especially in subcultures 7, 8 and 9. These variations during *in vitro* culture of plants can happen due to different reasons such as modifications in DNA methylation, gene amplification, chromosomal abnormality and point mutation (Saker *et al*., 2000). ISSR analysis of *Dictyospermum ovalifolium* showed low variations among 14 individuals in vitro regenerants plants (Chandrika *et al*., 2008). Our results depends on ISSR markers supports that plants derived from organized meristem collected may not always true-to-type. These differences between ISSR and RAPD due to ISSR has been proven as more useful molecular marker for genotyping and for studying genetic diversity in plants compared with widely used RAPD marker (Guo *et al*., 2006) and ISSR depends on the frequency of microsatellites.

Effect of Salinity and pH Media on Genetic Stability of Ziziphora canescen

Salinity is one of the most environmental stresses that affect on growth and productivity of agricultural crops (Al-Karaki, 2000; Lopez *et al*., 2002). Also pH of medium is one of important environmental chemical and physical factors during the evolution of plant tissue in the conditions of *in vitro* culture (Williams *et al*., 1990). Genetically diverse provide enough opportunity to create a favorable gene and probability of producing a unique genotype differs of parents and *In vitro* screening with marker can facilitate the selection and isolation of useful tolerant lines. Our results showed RAPD and ISSR technique was very sensitive for detecting genetic variations. Some RAPD primers (3 primers) bands were monomorphic while all of ISSR bands were polymorphic, that means no genetic stability and all *in vitro* plants on these media not true-to-type. Because saline and pH stress may cause alterations in DNA. Werker *et al*. (1983), Katsuhara and Kawasaki (1996) reported that salinity stress causes nuclear deformation and subsequent nuclear degradation. Rasheed *et al*. (2001) analyzed genetic variability in response to salt stress of sugarcane using RAPD technique and two primers enabled the identification of polymorphism. Also Balkrishna and Shankarrao (2013) reported that seven RAPD primers showed unique bands associated with salt tolerance in maize. In this study, the unique bands obtained from *in vitro* plants in salt and pH tolerant media will be used as a RAPD and ISSR marker for developing salt and pH tolerant genotypes and present study support RAPD and ISSR technique as the fast method to selected salinity and pH tolerant in *in vitro* plants.

Effect of Callus Culture on Genetic Stability of Ziziphora tenuior L.

The treatment used in tissue culture may increase the variant numbers (Bairu *et al*., 2006). In the present study, callus I and II showed genetic variations compare with wild plant and micropropagated plant from apical mirestem. Genetic changes occur during the introduction into *in vitro* culture and continuous culturing due to genome rearrangement during early culturing, when cell is under stress to survive in the new environment. Jose *et al*. (2012) used RAPD marker to detect the variation in leaf callus cultures of *Jatropha curcas* and reported 37 RAPD bands were polymorphic (91.12%). Also Vasconcelos *et al*. (2008) found higher level of variation in maize callus culture.

Conclusion

In this study we successfully assessment of genetic diversity of five *Ziziphora canescens* Benth. genotypes (Assal Al-Ward and Yabroud) and five *Ziziphora tenuior L.* genotypes (Assal Al-Ward, Qarah and Maaraba) by RAPD and ISSR analysis. ISSR markers provided more information than RAPD markers about geographic distribution. A high degree of biodiversity was recorded among *Ziziphora tenuior L.* genotype collected from Maaraba and other genotypes. Also our results support that plant tissue culture media, pH and salinity lead to higher polymorphism especially callus media.

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Author's Contributions

Abdulkarim Dakah: The main researcher, design and work of the experiments, analyzed and interpreted data, wrote the manuscript.

Mohamad Suleiman: Contributions to design experiments, analysis and interpretation of data.

Salim Zaid: Contributions to design experiments, analysis and interpretation of data.

Ethics

The authors wish to state that this article conforms to the ethical standards specified by the American Journal of Agricultural and Biological Sciences.

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