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ISSR-PCR OF THE MICROPROPAGATED *Ajuga iva* PLANTS

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ABSTRACT

In general, the genetic relationships among Ajuga iva samples derived from tissue culture exhibited high similarity together with the mother plant when were compared by using ISSR. No. somaclonal variations were observed by using shoot tip through tissue culture and the plants were true to type .

Key words :***Ajuga iva , Genetic Similarity, ISSR, Micropropagation*** .

INTRODUCTION

Ajuga iva L. is one of the most common species in the halophytic vegetation of the saline deserts in Egypt. It is known locally as Shandakora and used by Bedouins as a source of active substances which can be used for medicinal diabetes. Tissue culture techniques have become one of the most important way of reproducing crops that are difficult to propagate by conventional methods such as seeds or cuttings. Micropropagation allows the production of alarge number of plants in a relatively small growing area and in a relatively shorter time. The tissue culture technology has been accelerated by its commercialization for many crops (Nizar, 2001).

The micropropagated plants should be genetically stable to their origin. The genetic similarity can be determined by using RAPD, and other techniques Shasany *et al* (1998). Shoyama *et al* (1997) reported that the analysis of the RAPD profiles of 100 regenerated plantlets each of Himalaya and Kalka showed homogeneity with respect to the parents. Ajith *et al* (1997) stated that comparison of RAPD fingerprints of 20 micropropagated plants and the mother plant with 10 decamer primers indicated polymorphism. Gilbert *et al* (1999) used ISSR-PCR to reveal genetic variability within and between accessions held in a collection of lupin germplasm. The results showed that pooling of DNA from individuals within accessions was found to be the most appropriate strategy for assessing large quantities of plant material.Ajibade *et al* (2000) investigated the utility of ISSR marker polymorphisms to distinguish taxa within the genus *Vigna*. In contrast, ISSR analysis was not able to clearly differentiate subgeneric divisions within *Vigna*. Arnaud *et al* (2000) used ISSR analysis for the identification of strawberry varieties. Huang and Sun (2000) successfully used ISSR to determine the closest native species related to the hexaploid *Ipomoea batatas*'. King and Ferris (2000) successfully used ISSR

to determine the level of genetic variation between sympatric species of *Almis* (*Betulaceae*) in Italy. Zavodna *et al* (2000) used inter-simple sequence repeats (ISSRs) to distinguish between commercial lentil cultivars (*Lens culinaris*) that are closely related or identical judged from their pedigree and by using agronomical and morphological traits. The DNA marker systems used generated polymorphic DNA banding patterns and the lentil cultivars examined could be differentiated from each others. Alexander (2002) successfully used ISSR to determine the levels and distribution of genetic differentiation within and among populations of *Astragalus oniciformis*. Ahmed (2005) tested five individual plants from caper and arghel species,which were collected from their natural habitat . RAPD and ISSR markers were used to study the genetic variations among these five individual plants from each species. The results indicated that it was possible to differentiate between individual plants of the same species, since each individual plant banding pattern was not similar to the others within the five individual plants of each species. Moreover, RAPD and ISSR markers are useful tools to assess he genetic variations. Guo *et al,* (2005) used ISSR to test one hundred and ninety individuals from ten populations sampled from the entire distribution area of *Mentha chinense*. Hussein (2005) used 21 RAPD and ten ISSR primers to differentiate among four *Mentha* and three *Ocimum* species. He reported that the detection of the phylogenetic relationships based on these techniques indicated that these techniques succeeded in separating the seven species into main clusters of *Mentha* and *Ocimum* genera. Pharmawati *et al.* (2005) evaluated the genetic variation and relationships among 30 *Leucadendron* cultivars using 64 ISSR primers. They confirmed that ISSR profiling is a powerful method for identification and molecular classification of *Leucadendron* cultivars. Fahmy (2006) suggested that RAPD and ISSR markers are the best choice for the evaluation of diversity and assessing the genetic relationships between *P. tortuosus* and fennel genotypes with high accuracy. The aim of this study is to study the genetic similarity for the micropropagated plants and the *Ajuga iva* mother plants.

MATERIALS AND METHODS

This work was carried out in the plant tissue culture laboratory, North Sinai Research Station (El-Sheikh ZuwAyed), Desert Research Center (DRC), Mataria, Cairo, Egypt during the period from 2006 to 2008.

. Inter Simple Sequence Repeats (ISSRs) has only recently been developed as an anonymous, RAPDs-like approach that assesses variation in numerous microsatellite regions dispersed throughout the various genomes (particularly the nuclear genome). Microsatellites are very short (usually 10-20 base-pair) stretches of DNA that are "hyper variable", expressed as

different variants within populations and among different species. Shot tips of *Ajuga iva* plants were excised from the wild plants and were disinfected by using sodium hypochlorite solution and were washed several times with sterilized water and stored until culture. The shot tips were cultured on Murashig and Skoog basal salt medium which were supplemented with different concentration of benzyl adenine (0.0,0.5 and 1.0mg/l) mentioned Ms0, Ms1 and Ms2 . These shot tips were developed to shoots wich were successfully transferred to rooting stage .Samples of the tissue culture derived plants and the mother plant were used for this purpose. Samples of these plants were used to extract DNA. The DNA was extracted according to CTAB method (Dellaporta *et al* 1983). DNA concentration and quality was determined using a spectrophotometer at wave length 260nm and 280nm. ISSR– PCR reactions were conducted using 5 specific primers, as presented in the reaction optimized conditions and mixtures:-

dNTPs (8 mM mix)	2.5 µl
Taq DNA polymerase (5 U/ µl)	0.3 µl
10 X buffer with 15 mM MgCl2	3.0 µl
Primer (10mM)	2.0 µl
Template DNA (50 ng/ µl)	2.0 µl
H_2O (dd)	up to 25 µl

Amplification was carried out in Stratgene Robocycler Gradient 96 which was programmed for 45 cycles as follows: Denaturation (one cycle) 94°C for 2 minutes, followed by 30 cycles: as follows 94 °C for30 second, 44°C for 45 secs, 72°C for 1 minute and 30 secs, and finally one cycle extension at 72°C for 2.0 min, and 4°C (infinitive) for 15 min.

Volumes (15 µl) of PCR- products were resolved in 1.5 % Nusieve GTG agarose gel electrophoresis with 1x TAE running buffer. The run was performed at 80 V for 180 min and the gel was stained with ethidium bromide .A marker of 1 Kb plus DNA Ladder 1µg /µl (Invitrogen) that contains a total of twenty bands ranging from: 12000,to 100 bp was used.

Bands were detected on UV- transilluminator and photographed by Gel documentation system Biometra Bio Doc Analyze 2000. Names and the sequences of the ISSR primers were described as follows:

Primer name	Sequence	Primer name	Sequence
17898A	(CA)6 AC	17899A	(CA)6 AG
17898B	(CA)6 GT	17899B	(CA)6 GG
HB15	(GTG)3 GC		

RESULTS AND DISCUSSION

The ISSR analysis was performed on the four DNA samples representing the *Ajuga iva* using five primers composed of short tandom repeat sequences with or without anchor. Table (1) illustrates the ISSR pofile of four *Ajuga* samples. A total of 28 amplicons – amplified fragments – (ranging from 260 to 1534 bP) were generated by the tested primers with an average number of 5.6 amplicons per primer. ISSR primer 17899B exhibited the highest number of fragments as eight amplicones, followed by primers 17899A, 17898A, 17898B and HB15 which generated five amplicons for each. Table (2) revealed that the total number of polymorphic bands was 14 with an average of 0.5 polymorphic amplicons per primer. This represents a level of polymorphism of about 50 %, and the number of monomorphic bands was 14 with an average of 0.5 monomorphic amplicons per primer. This exhibited 50 % monomorphism. The number of polymorphic markers varied among the different primers. Primer 17899B generated five polymorphic bands (62.5%) with a fragment size of 1534, 870, 900, 771, 530 and 260 bp. While primer 17898A generated 3 polymorphic bands of about 60% with fragment sizes of 1102, 730 and 530 bp. However, primers HB15, 17899A and 17898B showed the lowest level of polymorphism as 2 amplicons of about 40% for each primer with a fragment sizes of 360 and 286 bp for primer HB15 and with a fragment size of 600 and 450 bp for primer 17899A and with a fragment size of 1028 and 370 bp for primer 17898B. Table (2) showed that the number of monomorphic level marker varied among the different primers. Primers 17899A, 17898B, HB15 and 17899B generated 3 monomorophic bands of about 60% for each primer with a fragment size of 1023,900 and 670 bp for primer 17899A, 880, 700 and 500 bp for primer 17898B and 850, 690 and 446 bp for primer HB15 and with 612, 440 and350 bp for primer 17899B. However, primer 17898A generated 2 fragment sizes of 900 and 500 bp monomorphic bands with 40 % monomorphism. The similarity matrix was developed by SPSS computer package (Table 3). The analysis was based on the number of markers that were different between any given pair of samples. The closest relationship was scored between two pairs of plant samples produced on the treatments MS0&MS1 and MS1&MS2, respectively (similarity of 0.94).

The Dendrogram (Fig. 1) classified the four samples the *in vitro* treatments, and the wild plant. The first cluster was separated into two sub-clusters and the first sub-cluster included treatments of MS0 and MS2 while the second sub-cluster comprised MS1 treatments. In general, the relationships among *Ajuga iva* samples derived from tissue culture exhibited high similarity together and no somaclonal variations were observed by tissue culture and plants were true to type .

Table 1. Banding patterns of ISSR profiles of the four *Ajuga iva* sample between *in vitro* and wild plant using 5primers.

Primer	primer size (bp)	Band no	Wild plant	MS free	MS+ 0.5BA	MS+1.0 BA
17899A	450-1032	5	4	5	3	5
HB15	286-850	5	٣	٥	٥	٥
17898B	370-1028	5	٣	5	5	5
17898A	350-1102	5	2	5	5	5
17899B	260-1534	8	4	8	7	8
Total		28	16	28	25	28
%			57	100	89.9	100

Table 2. ISSR analysis from the DNAs of *Ajuga iva* by using five primers

Primer code	Total amplified fragments	Fragment Length range (bp)	Polymorphic bands	Polymorphism (%)	Monom-orphic bands	Monom-orphism (%)
17899A	5	450-1032	2	40	3	60
HB15	5	286-850	2	40	3	60
17898B	5	370-1028	2	40	3	60
17898A	5	350-1102	3	60	2	40
17899B	8	260-1534	5	62.5	3	37.5
Total	28		14		14	

Table 3. Similarity matrix percentage for the four *Ajuga iva* plants based on ISSR-PCR analysis.

	Wild	MS0	MS1	Ms2
MS0	0.727	-	-	-
MS1	0.683	0.943	-	-
MS2	0.727	0.99	0.943	-

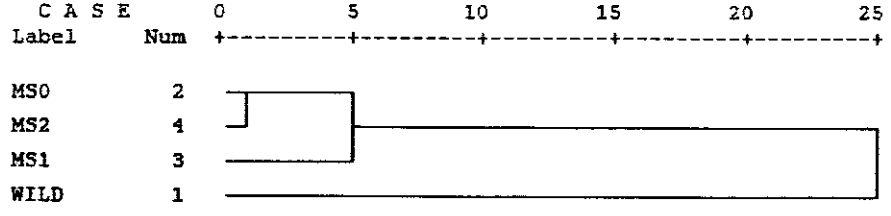
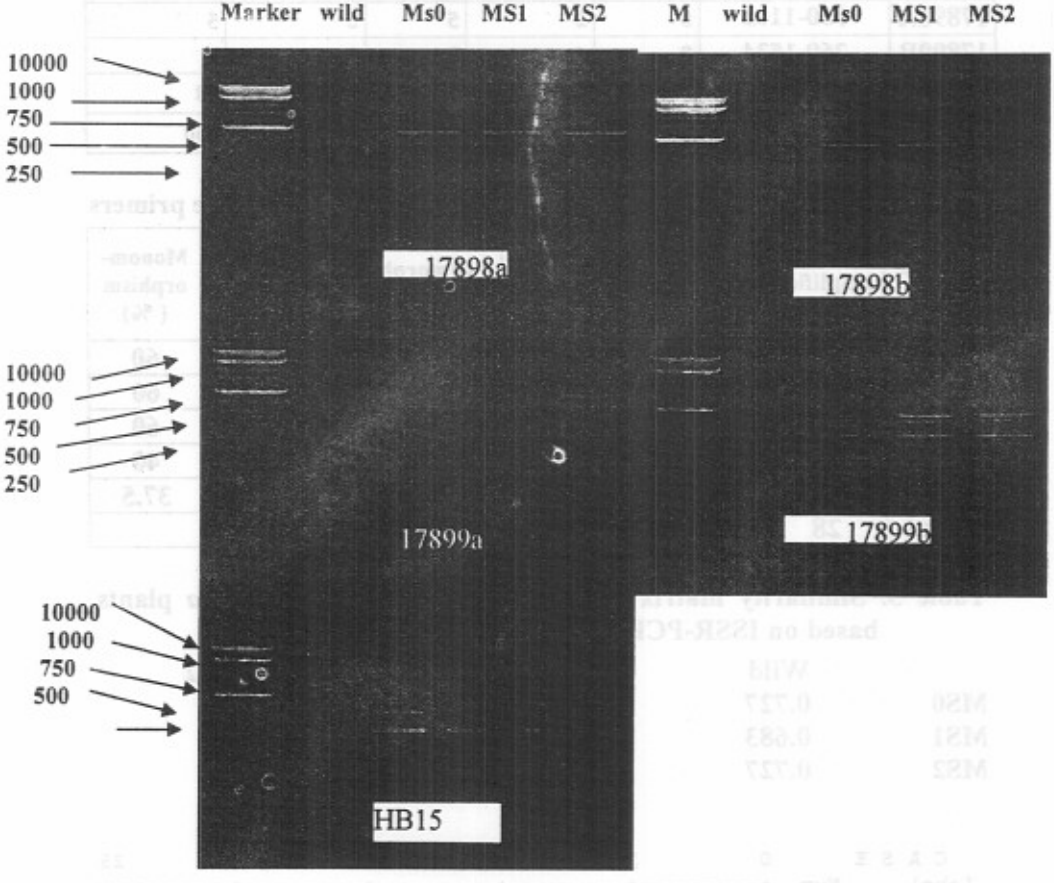


Figure (1). Dendrogram reveals the relationship among the four *Ajuga iva* samples based on ISSR-PCR.

The similarity within the tissue culture plants was more than 94.3% that indicated that these plants were high similar together and no variation was observed through tissue culture process. But it was observed that between the tissue culture plants and the mother plant there were low similarity (68.3and 72.7) that due to the fent band of the mother plant with the primers as it obvious in figure(2) which were undetected by the documentation program.



Figure(2). ISSR-PCR of DNAs of *Ajuga iva* (1-20) using 5 primers. M:1 Kb DNA laddder (strtagene®).

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تكرارات التتابعات البسيطة للحمض النووي المتخصصة المتكررة (ISSR-PCR) على نباتات الشندقورة الناتجة من الاكثارالدقيق

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١. مركزبحوث الصحراء بالمطرية القاهرة- زراعة الانسجة
٢. كلية الزراعة والعلوم البيئية بالعريش

اظهرت دراسات التماثل الوراثي على حمض النووي للنباتات الناتجة من الاكثار المعملي لنبات الشندقورة باستخدام تقنية تكرارات التتابعات البسيطة البينية المكبرة بالتفاعل المتسلسل أن هناك درجة عالية من التماثل بينها بنسبة من ٩٩:٩٤% وإنها مطابقة للأصل الذي نشات منه ولا توجد اختلافات جسمية بينها باستخدام القمم النامية في زراعة الانسجه.

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