USE OF RAPD AND ISSR MARKERS FOR THE IDENTIFICATION OF EGYPTIAN HENBANE (*Hyoscyamus muticus* L.) GENOTYPES

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Medicinal plants have acquired increasing significance over the last few years, not only because their healing ability, but also due to their minor side effects comparing to the chemical medicine. Worldwide, 35000 plant species are used for medicinal purposes but only 90 species are considered in the most important industrial medicines. In developing countries traditional medicine is spread because natural remedies are cheaper than chemical medicine and are often the only medicine available in the remote rural area. Besides serving medicinal functions, medicinal plants in developing countries have an important economic role because they have 90% of the earth genetic diversity exists in the developing countries.

Family *Solanaceae* is one of the most intriguing plant families in the world not only because it is one of the largest families in plant kingdom with more than 3,000 species. Also, because it includes species which are essential for life such as *Hyoscyamus muticus* (an important medicinal plant of the Solanaceous family).

The Solanaceae is known for possessing a diverse range of alkaloidal glucosides, or simply alkaloids. One of the most important groups of these compounds is called the tropane alkaloids. *Hyoscyamus* plants have been known from ancient time as a remedy for various diseases, and serve today as a source of their pharmaceutically active constituents; the tropane alkaloids. The medicinal importance of scopolamine, hyoscyamine and atropine is illustrated by their presence in the list of the ten substances of plant origin most used as drugs in the USA in the 1973.

Random amplified polymorphic DNA (RAPD) markers and inter - simple sequence repeats (ISSR) markers are two molecular approaches that have been used to detect variation among plants. Each method has been used extensively to identify and determine relationships at the species and cultivar levels (Rajaseger *et al*., 1997; Raina *et al*., 2001; Martins *et al*., 2003). These methods are widely applicable because they are rapid, inexpensive, simple to perform, do not require prior knowledge of DNA sequence

and require very little starting DNA template (Esselman *et al*., 1999). The ISSR method has been reported to produce more complex marker patterns than the RAPD approach (Parsons *et al*., 1997). In addition, ISSR markers are more reproducible than RAPD markers, because ISSR primers, designed to anneal to a micro-satellite sequence, are longer than RAPD primers, allowing higher annealing temperatures to be used (Goulao and Oliveira, 2001).

The present work aimed to identify some genotypes of Egyptian henbane (*Hyoscyamus muticus* L.) using (RAPD and ISSR) techniques as molecular genetic fingerprints. Also the result of the combination between the RAPD and ISSR techniques analysis revealing the genetic distance and the dendrogram of the henbane genotypes under study.

MATERIALS AND METHODS

A. Plant materials

Three Egyptian henbane collections (*Hyoscyamus muticus* L.), (Table 1), were obtained from three different locations; Wadi Sudr, El Maghara and Shalateen, Egypt.

B. Methods

1. DNA isolation

Total DNA was isolated from young leaves of the Egyptian henbane genotypes (*Hyoscyamus muticus* L.) according to Dellaporta *et al*. (1983).

2. RAPD analysis

Reaction conditions for amplification were optimized according to Williams *et al*. (1990) using seven arbitrary primers synthesized by Operon Technology, Inc. (USA). The amplification was performed for 42 cycles, using a Coy thermocycler programmed, as follows; initial denaturation at 94°C for 3 min, one cycle, denaturating at 94°C for 45 sec., annealing at 37°C for 30 sec., extension at 72°C for 2 min., (40 cycles) and final extension at 72°C for 12 min., (one cycle).

The product was resolved on agarose gel (1.2%) in TAE buffer and stained with ethidium bromide. Two different DNA markers were used; a 100 bp ladder mix and a 1 kb DNA ladder mix (Pharmacia Biotech). The seven arbitrary primers used for RAPD amplification, their names and sequences are found in Table (2). The run was performed for about 1 h at 80 V in a mini gel agarose electrophoresis apparatus (Bio-Rad). The products were visualized by UV light. PCR products were photographed by gel electrophoresis system (Gel Doc. BIORAD 2000) under transilluminator.

3. Inter simple sequence repeats (ISSRs) analysis

The five used primers in PCR amplification of inter simple sequence repeat regions (Table 3) were synthesized

on an ABI 392 DNA/RNA synthesizer (Applied Biosystems) at AGERI, ARC. The amplification was performed as RAPD but with 55°C for annealing instead of 37°C.

4. Genetic distance relationships

The banding patterns of the seven RAPD and the five ISSR primers were scored. Data were feed to the PC computer as 1 and 0 for the presence and absence of bands, respectively. Pair-wise comparisons of genotypes, based on the presence or absence of unique and shared polymorphic products, were used to regenerate similarity coefficients, according to Jaccard (1908). The similarity coefficients were then used to construct dendrograms, using the unweighted pair group method with arithmetic averages (UPGAMA) employing the SHAN (Sequantial, Agglomerative, Hierachical, and Nested clustering) from the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System), version 2.1 Program (Rohlf, 2000).

RESULTS AND DISCUSSION

In the present study, the genetic variability and relationships of the natural medicinal plant (*Hyoscyamus muticus* L.) which collected from three different locations have been studied. These were done based on molecular markers (RAPD and ISSR) techniques. The results obtained could be summarized as follows:

1. Randomly amplified polymorphic DNA (RAPD) analysis

The banding patterns of RAPD fragments using the seven arbitrary primers with the three henbane genotypes (Fig. 1) showed 57 amplified fragments; 30 of them were polymorphic (52.63%). The total number of amplified and polymorphic fragments obtained with each primer is found in Table (4). Results revealed the presence of 21 positive RAPD molecular markers in addition to 9 negative molecular markers. The developing of RAPD approach (Williams *et al*., 1990) has allowed simple, easy and less time consuming genome analysis at DNA level compared with RFLP (Restriction Fragment Length Polymorphism). Similar results were obtained in wheat (Abdel-Tawab *et al*., 2003), in maize (Abdel-Tawab *et al*., 2002), in sorghum (Abdel-Tawab *et al*., 1998).

2. Inter simple sequence repeats (ISSR) analysis

The banding patterns of ISSR fragments using the five specific primers with the three henbane genotypes (Fig. 2) revealed 41 amplified fragments; 15 of them were polymorphic (36.59%). The total number of amplified and polymorphic fragments obtained with each primer is found in Table (4). ISSR data revealed 13 positive and 2 negative molecular markers for the three henbane genotypes. Ratnaparkhe *et al*. (1998), Reddy *et al*. (2002) and Fahmy *et al*.

(2008) showed that ISSR markers could be used as highly informative markers for genomic mapping and gene tagging because the evolutionary rate of change within microsatellites is considerably higher more than many other types of DNA markers.

3. Genetic relationships of the three henbane genotypes based on RAPD and ISSR molecular markers:

The identification of the wild type germplasm is necessary for varietal improvement. Recently, RAPD and ISSR techniques are used to measure the degree of similarity/dissimilarity among genotypes and also to estimate the genetic distance between these germplasm. The obtained results of the banding patterns of RAPD and ISSR were used to study the genetic relationships of the three henbane genotypes at the levels of the markers and in combination between the two techniques.

RAPD amplification revealed different degrees of polymorphisms between the henbane genotypes. The obtained 57 bands were treated with Ntsyspc2 software to release the similarity matrices (Table 5) and the dendrogram of the genetic distances (Fig. 3). The genetic similarity matrices depended on all possible pairs of genotypes ranged from 10.00 % to 66.70%. The highest genetic similarity indices were found between henbane genotypes from El Maghara and Shalateen (66.7%). The lowest genetic similarity indices were found between henbane genotypes from Wadi Sudr and El Maghara and Shalateen (10.0%). Meanwhile, the genetic similarity indices between henbane genotypes from Wadi Sudr and Shalateen were (23.3%). The obtained results revealed broad genetic diversity within the three henbane genotypes, especially between genotypes from Wadi Sudr and Shalateen. As found in Fig. (3), the dendrogram based on RAPD similarity indices separated into one cluster including the two genotypes from El Maghara and Shalateen and Wadi Sudr genotype is alone. Moreover Votava *et al.* (2005) used random amplified polymorphic DNA (RAPD) molecular markers to compare the relative genetic diversity of native chili (*Capsicum Annuum* Var. *Annuum* L.) landraces from Northwestern Mexico. The results indicated that the molecular analysis of these accessions provided a powerful means by which their genetic structures were characterized. The same results was obtained by Singh *et al*. (2006) who used Random amplified polymorphic DNA (RAPD) technique as a tool for assessing genetic diversity and species relationships among 28 accessions of *eggplant* from different parts of the country. The results indicates presence of high level of genetic diversity in *eggplant* and a dendrogram shows that *Solanum incanum* is closest to *Solanum melongena* followed by *Solanum nigrum.*

The ISSR data revealed that the genetic similarity indices ranged from 6.7% to 73.3% (Table 6 and Fig. 4). The

nearest relationship was detected between the henbane genotypes from El Maghara and Shalateen (73.3%). On the other hand, the farest relationship of similarity matrices was detected between the henbane genotypes from Wadi Sudr and El Maghara (6.7%), followed by Wadi Sudr and Shalateen (20.0%). As indicated by the Fig. 4, the dendrogram based on the similarity matrices of ISSR-PCR banding patterns separated the three henbane genotypes into one cluster (including El Maghara and Shalateen genotypes) and Wadi Sudr alone. ISSR amplification does not require genome sequence information but produce highly polymorphic patterns (Zietkiewicz *et al.,* 1994; Nagaoka and Ogihara, 1997; Prevost and Wilkinson, 1999). It was applied to the study of genetic relationships and phylogenetic analysis of various crop plants (Joshi *et al*., 2000; Martin and Yelemo, 2000; Iruela *et al.,* 2002).

The results indicated that it was possible to discriminate between *H. muticus* L. accessions which collected from three different locations, since each genotype banding pattern was not similar to the others. Based on ISSR analysis, the same results were obtained by Kochieva *et al.* (2002) who used Inter-simple sequence repeats (ISSR) analysis to study the genetic diversity and phylogenetic relationships in 54 wild accessions and cultivars of the genus *Lycopersicon*.

Depending on the combination between the banding patterns of RAPD-PCR and ISSR-PCR data through using NTsyspc2 software, the similarity indices were 68.9% between the henbane genotype from El Maghara and Shalateen regions, followed by 22.2% between the henbane genotype from Wadi Sudr and Shalateen. The lowest similarity indices were detected between the henbane genotype from Wadi Sudr and El Maghara (8.9%). The dendrogram resulted from the combination of the two techniques (Fig. 5) was found also in one cluster (including the henbane genotype from El Maghara and Shalateen regions) while the henbane genotype from Wadi Sudr found alone. Petros *et al.* (2007) studied the genetic diversity among the Niger populations grown in different regions of Ethiopia using ISSR markers in an effort to provide some information for future research that might be aimed for improving some of the agronomic traits of Niger for efficient utilization.

SUMMARY

Three Egyptian henbane genotypes from three different locations (Wadi Sudr, El Maghara and Shalateen) were screened using two different molecular Techniques; RAPD and ISSR. Both of these techniques were used to detect some molecular markers associated with the genotype identification. RAPD results, from using seven arbitrary primers, revealed 57 amplified fragments, 30 of them were polymorphic (52.63%). The results revealed the presence of 21 positive and 9 negative molecular markers for the genotypic identification. ISSR

results with five specific primers revealed 41 amplified fragments, 15 of them were polymorphic (36.59%). The results revealed the presence of 13 positive and 2 negative molecular markers for the genotypic identification. The Similarity indices and dendrogram for the genetic distances of the combination between the two techniques revealed that the highest similarity was 68.9% between the henbane genotypes from El Maghara and Shalateen regions. Meanwhile, the lowest similarity index was between the henbane genotypes from El Maghara and Wadi Sudr regions (8.9%). The dendrogram resulting from the combination of the two techniques separated the three henbane genotypes into one cluster (including El Maghara and Shalateen genotypes) with the Wadi Sudr genotype alone.

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Table (1): Name and origin of the three henbane genotypes collected from the three locations.

Number	Genotype name	Origin	Family
1	Henbane	Wadi Sudr	Solanaceae
2	Henbane	El Maghara	Solanaceae
3	Henbane	Shalateen	Solanaceae

Table (2): RAPD primers code and their sequences used for detection of banding patterns in the three henbane genotypes.

Primer code	Sequence (5 → 3)	Primer Code	Sequence (5 → 3)
OP A10	CTGCTGGGAC	OP D07	ACCGCGAAGC
OP A19	CAATCGCCGT	OP I15	AAGAGAGGGG
OP B12	CCTTGACGCA	OP L20	TGGTGGACCA
OP A16	TGCGCGATAG		

Table (3): ISSR primers code and their sequences used for detection of banding patterns in the three henbane genotypes.

Primer code	Sequence (5 → 3)	Primer Code	Sequence (5 → 3)
A 98	$(CA)_6$ GT	HB 11	$(GT)_6$ CC
HB 09	$(GT)_6$ GG	HB 13	$(GA)_6$ GC
HB 10	$(GA)_6$ CC		

Table (4): The total number of amplified and polymorphic fragments, percentage of polymorphism and specific markers in the three henbane genotypes using both of RAPD and ISSR data.

Primer Number	Primer code	TAF	PF	Polymorphism %	SM
RAPD					
1	OP A10	8	5	62.50	+3 and -2
2	OP A19	8	3	37.50	+3
3	OP B12	6	3	50.00	+3
4	OP A16	6	3	50.00	+2 and -1
5	OP D07	9	5	55.56	+2 and -3
6	OP I15	9	4	44.44	+2 and -2
7	OP L20	11	7	63.63	+6 and -1
		57	30	52.63	+21 and -9
ISSR					
1	A 98	8	5	62.50	+3 and -2
2	HB 09	9	2	22.22	-2
3	HB 10	8	2	25.00	+2
4	HB 11	8	2	25.00	+2
5	HB 13	8	4	50.00	+4
		41	15	36.59	+13 and -2

Table (5): Similarity index (Pair wise comparison) among the three henbane genotypes based on RAPD data.

Genotype	Wadi Sudr	El Maghara
El Maghara	0.100	
Shalateen	0.233	0.667

Table (6): Similarity index (Pair wise comparison) among the three henbane genotypes based on ISSR data.

Genotype	Wadi Sudr	El Maghara
El Maghara	0.067	
Shalateen	0.200	0.733

Table (7): Similarity index (Pair wise comparison) among the three henbane genotypes based on RAPD and ISSR data.

Genotype	Wadi Sudr	El Maghara
El Maghara	0.089	
Shalateen	0.222	0.689

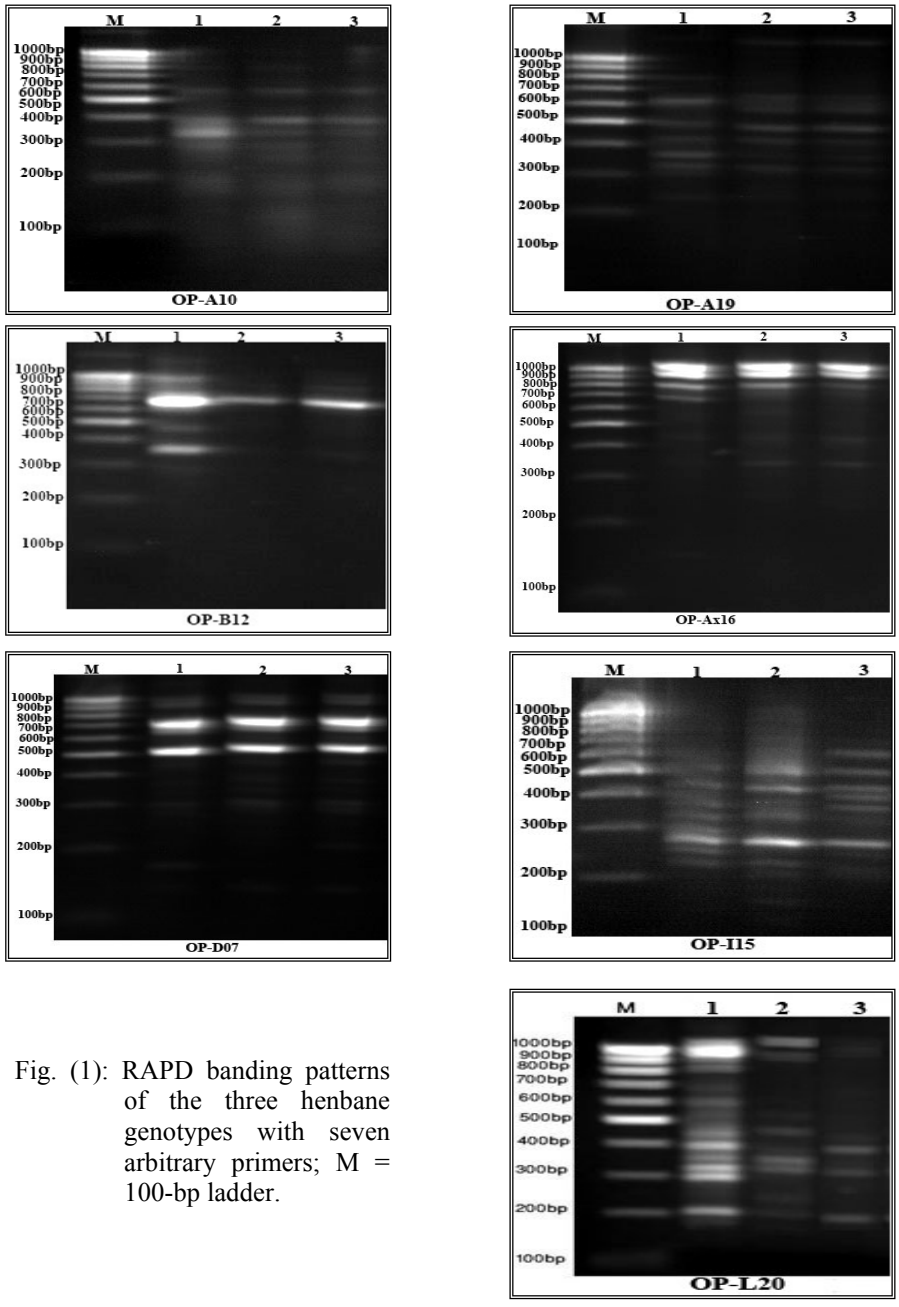
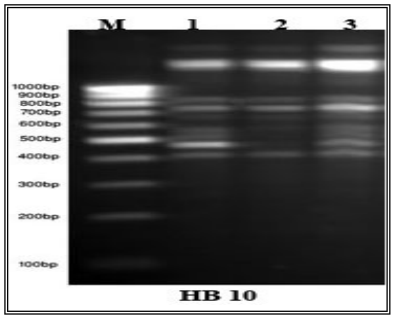
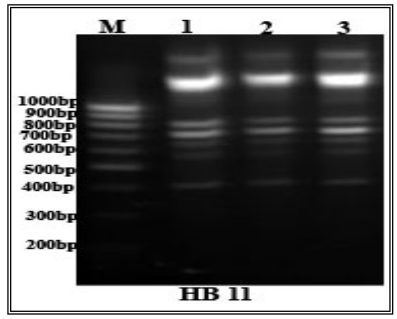
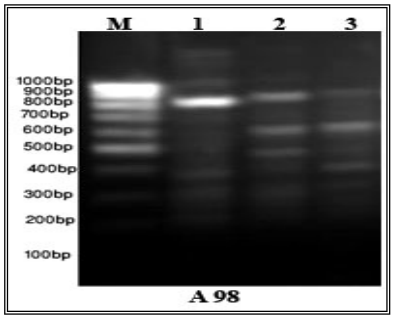


Fig. (1): RAPD banding patterns of the three henbane genotypes with seven arbitrary primers; M = 100-bp ladder.







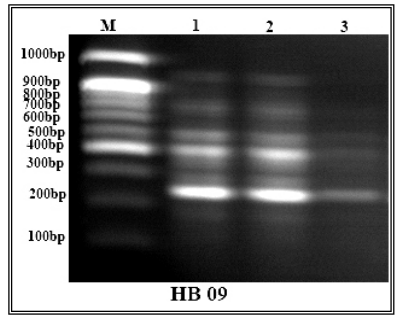
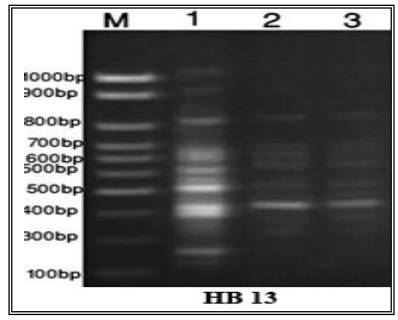


Fig. (2): ISSR banding patterns of the three henbane genotypes amplified with the five ISSR primers; M= 100 bp ladder.



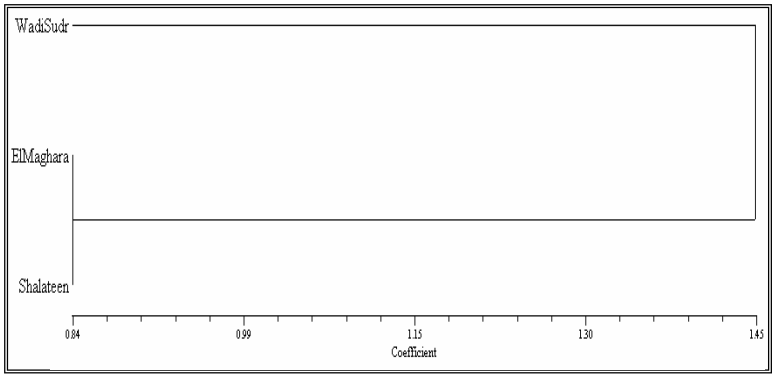


Fig. (3): Dendrogram for the genetic relationships among the three henbane genotypes based on RAPD-PCR data.

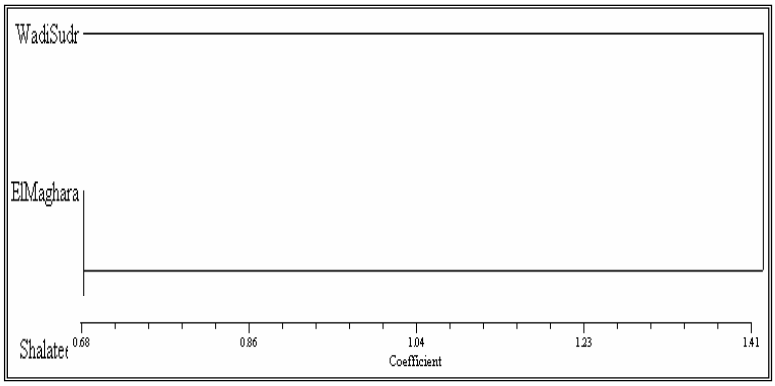


Fig. (4): A Dendrogram revealing the genetic distance among the three henbane genotypes using ISSR data.

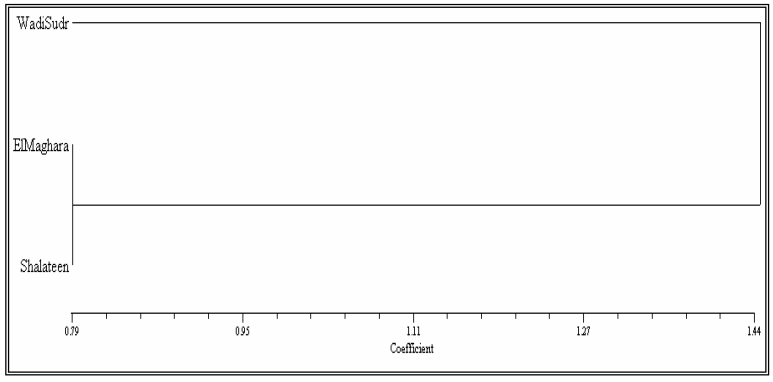


Fig. (5): Dendrogram for the genetic relationships among the three henbane genotypes based on RAPD-PCR and ISSR data.