Minia J. of Agric. Res. & Develop.
Vol.(27) No.5 pp 987 - 1005, 2007

RAPD AND ISOZYME STUDIES OF *Mesembryanthemum* SPECIES IN EGYPTIAN FLORA

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Received 1 Sept. 2007 Accepted 30 Oct. 2007

ABSTRACT

Genus *Mesembryanthemum* L. belongs to family Aizoaceae. This genus in Flora of Egypt comprises three succulent wild species: *M. crystallinum* L., *M. forsskaolii* Hochst. ex Boiss., and *M. nodiflorum* L. These species has different values. RAPD markers and isozyme studies were carried out on green leaves. To clarify the genetic diversity among the three cogeneric wild species and their relationships between these species, fifteen RAPD markers are used. The results showed a notable polymorphic in ten RAPD markers (OPJ01, OPK05, OPK09, OPN01, OPN03, OPN12. OPN14, OPQ14, OPLBand OPCa). The RAPD markers showed a little variation in the genetic divergence between the studied species. However, the esterase zymogram showed a little genetic divergence between the studied species. While the genetic divergence is few between *M. nodiflorum* and the other studied species using peroxidase isozyme test.

INTRODUCTION

Genus *Mesembryanthemum* L. (Aizoaceae.) is succulent grown species. It includes some 300 and more species nearly all of which are South African (Bailey 1947). The family Aizoaceae consists of 143 genra. It comprises from 2300 to2500 species. Genus *Mesembryanthemum* is not divided into the hundred or more genra that some modern authors recognize. Perhaps 50 of these segregates from *Mesembryanthemum* can be recognized by their aspect (Cronquist 1981; Jones & Luchsinger 1987). The genus comprises three wild

species in Egypt: *M. crystallinum* L., *M. forsskaolii* Hochst.ex Boiss., and *M. nodiflorum* L. These species are distributed in Mediterranean Coastal, desert, Sinai and the Oases (Täckholm, 1974 and Boulos, 1999). The species of *M. crystallinum* L., is used for feeding (Wiersema & Leon 1999). The species of *M. crystallinum* L., is growing in Southern California. The leaves have caused it to be used as a fresh table vegetable for summer use in warm, dry countries. The seed of *M. forsskaolii* Hochst. ex Boiss., is used in making bread in North Africa however is not eaten by other Arab (Hedrick, 1972).

RAPD markers were used for species differentiation, definition of phylogenetic and genetic relationships between the different species as well as species variation (Schmit *et al*., 1993; Briand *et al*., 1998; Parra *et al*., 1998; Yonghe *et al*., 1998; and Zouhair *et al*., 2000). The isozyme technique is based on the principle of detecting the presence of enzyme heterogeneitly among individuals (Scandalios 1969; Costa 1974; Alfenas *et al*., 1991; and Ramirez *et a*l., 1991). Isozyme analysis revealed that genetic variability within and between landraces exist in some species (Alvarez *et al*., 1998).

Several studies have been conducted to study genotype differentiation in different plant tissues, growth stages and isozyme analysis of germplasm bank collection (Montarroyos *et al*., 2003).This work aims were: 1] to study of RAPD and isozyme fingerprint of the *Mesembryanthemum* species and 2] defining the genetic divergence among these three species. This study may also draw the attention to the forage working, future development and conservation of these species.

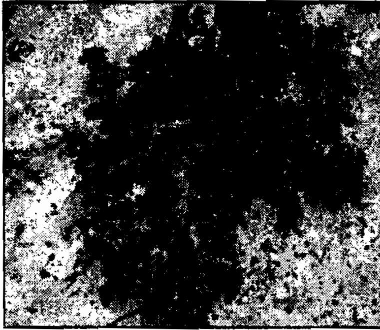
MATERIALS AND METHODS

Species Description:

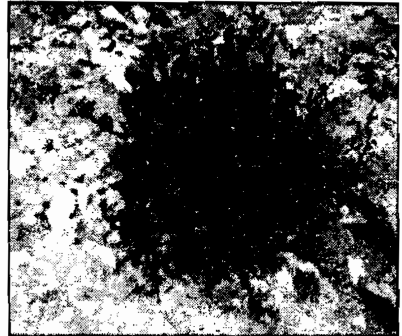
Täckholm, (1974), Crnquist, (1981), Jones & Luchsinger (1987) and Boulos, (1999) were consulted to identify the specimens collected through the different trips during 2006. Thereafter authentication procedure of studied specimens were carried out by means of comparison with preserved specimensin Herbarium of Flora & Phyto-Taxonomy Dept.,(CAIM).

Plant Material:

Genus *Mesembryanthemum* is represented in Egypt by three wild species *M. crystallinum* L., *M. forsskaolii* Hochst.ex Boiss., and *M. nodiflorum* L. Seeds of *M. crystallinum* and *M. nodiflorum* (Fig 1) were collected from the Mediterranean Coast near El- Hammam [(1) N 30° 56′ 53.5″ and E 29° 31′ 12.8″] while *M. forsskaolii* was collected from Ismailia Road kilo 15 Cairo Ismailia road [(2) N 30° 10′ 29.5″ and E 31° 33′ 52.5″] in June 2006 locality was defined using GPS48 (Fig 2). Seeds were grown in green house under normal conditions. Juvenile leaves were harvest and kept at -20° C for RAPD and isozyme studies.



A



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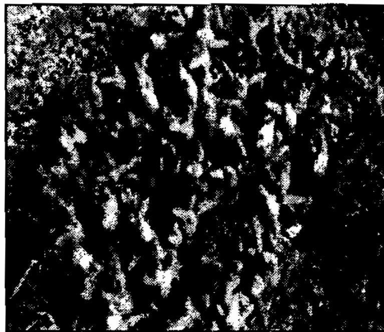

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Fig. 1: Photographs of *Mesembryanthemum* species (A= *M. crystallinum*, B= *M. nodiflorum* and C= *M. forsskaolii*).

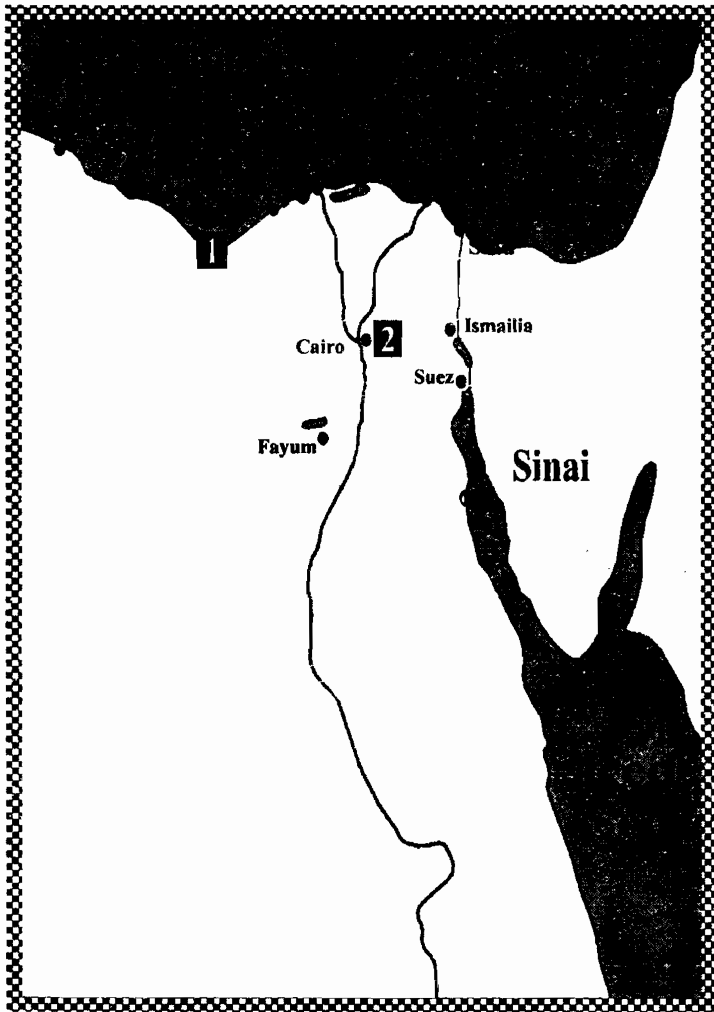

Fig. 2: Map showing collection areas of *Mesembryanthemum* species in present study (1= *M. crystallinum* and *M. nodiflorum*, 2= *M. forsskaolii*).

DNA Isolation:

DNA samples were extracted from 50 mg of Juvenile leaf tissues in each sample according to Porebsky *et al.* (1997). The modified CTAB protocol was followed. The coprecipitated RNA was eliminated by adding 0.7 units of RNAase to each sample. DNA was dissolved in 20µl sterilized bi-distilled water. Polymorphism of DNA in each sample was detected using standard DNA primers (fifteen primers were obtained from the Information Company Operon Technologies Inc., Almeda, California). Sequences of the used primers are outlined in Table 1.

Polymerase Chain Reaction (PCR):

The DNA fraction purified from the previous protocol was submitted to enzymatic amplification using an automatic thermal cycler. PCRs were conducted in total volume of 50µl in a Perkin Elmer Cetus DNA Thermal Cycler. The agarose fragment containing the nucleic acid band was melted and 1µl of the agarose –DNA suspension directly added to PCR mixture. The reaction mixture (50µl) contained 16.6mM-$(NH_4)_2SO_4$, 67mM Tris-HCl pH8.8 (at25°C), 6.7mM-$MgCL_2$, 10mM-β-mercaptoethanol, 200mM each of dATP, dCTP, dGTP and dTTP, 0.05% (v/v) detergent W-1 (Bethesda Research Laboratories, Githersburg, MD), 0.3µg each of amplification primers, and 2.5 units Taq polymarease (Bethesda, Research, Laboratories, Githersburg, MD). The amplification reaction was preformed in 500 ml Eppendorf type vials using a Perkin Elmer. Cetus (Thermal Cycler, Perkin Elmer, Norwalk, USA).

The amplification conditions were set at 95°C for 1min (DNA denaturation), 55°C for 20Sec (oligonucleotide aneling) and 72°C for 15Sec (primer elongation) for 30 cycles. The amplification is done with random synthethic-16-oligonucleotide primers of 10 and 20 bases each (Table 1). The developed bands stained by ethidium bromide, visualized under UV- light and photographed.

Table 1 Sequences of the used primers.

Primer Code	Sequences
OPA01	CAGGCCCTTC
OPB01	GTTTCGCTCC
OPC01	TTCGAGCCAG
OPC02	GTGAGGCGTC
OPC03	GGGGGCGTTT
OPJ01	CCCGGCATAA
OPK05	TCTGTCGAGG
OPK09	CCCTACCGAC
OPN01	CTCACGTTGG
OPN03	GGTACTCCCC
OPN12	CACAGACACC
OPN14	TCGTGCGGGT
OPQ14	GGACGCTTCA
OPLB	AATCCCGTATTGTACCAGCG
OPCa	GTCCGCTGTATATTCAGGTG

Isozyme Analysis:

Esterase (EST) and Peroxidase (PRX) Isozymes were assayed using 100 mg freshly harvested leaves of the studied species according to the method of Stegemann *et al.*, (1983). A polyacrylamide standard gel was prepared by dissolving 8.55 g acrylamide and 0.45 g bisacrylamide in 150 ml Tris borate buffer 0.125M Tris, pH8.9. After filtration, 145 ml of this monomer solution were used to prepare the gel by adding 50 mg sodium sulphate, 0.1 ml TEMED, and 2.8 ml ammonium persulphate. PAGE electrophoresis was preformed in a Biometra apparatus using Slab gels (11x12cm). After sample application, electrophoresis was carried out at 20 mA and 120 Volt for 20 minutes and then at 40 mA and 200 Volt for 4 hours.

Esterase was detected by incubating the gel for an hour at room temperature, in 200 ml phosphate buffer (0.15 M pH 7.2) containing α-naphthyl acetate (40 mg) and fast blue RPSalt (100 mg). Peroxidase was incubated for 5 minutes in a mixture of 15 ml benzidine (10%), 85ml ethanol (95%), 100mM K-acetate pH 4.67 and 0.2 ml H_2O_2 (30%) . Only anodic bands were analyzed, Bands were characterized

by their rates of migration and, in the case of esterase also by color; α-esterase dark brownish, β-estrase light to dark reddish, and α /β intermediate (Lang and Schifino-Wittman, 2000). The rate of migration is generally calculated for each band by dividing the distance migrated by the band by the distance migrated by the front line.

RESULTS

Key of the studied species:

1. Leaves flattened, obovate to spathulate, the basal petiolate............2. *M .crystallinum*+Leaves thick terete or liner, all sessile

2. Leaves 0.5–2 cm thick; staminodes longer than the calyx..................3. *M. forsskaolii* +Leaves 2-4 mm thick; staminodes shorter than or equaling the calyx....1. *M. nodiflorum*

Species Description (based on Boulos 1999):

1- *Mesembryanthemum nodiflorum* L.

Annual succulent herb, minutely papillose; stems 10-20 cm, branched, ascending or procumbent, leaves 1.2 – 4 x 0.2 -0.4 cm, the lower opposite the upper alternate, sessile, terete to liner, flowers axillary, subsessiles; calyx 1cm; lobes linear, unequal; staminodes white or cream . Shorter than or equalling the calyx; stigmas 5; capsule 0.8 – 1 cm.

2- M. crystallinum L.

Annual succulent herb, covered by crystalline vesicles; stems 25 – 80 cm, procumbent, branched,often making mats; leaves 3 -8 x 2-5 cm; radical leaves opposite, spathulate, petiolate; the cauline alternate, obovate, undulate, shortly petiolate or sessile; flowers 1 cm diam; axillary, subsessile; calyx – lobes broadly – ovate; staminodes numerous, longer than the calyx; capsule 1 cm.

3- M. forsskaolii Hochst.ex Boiss.,

Annual succulent papillose herb; stems 10 -25 cm, erect or ascending, simple or branched from the base; leaves 2 – 5 x 1 -2 cm, opposite, sessile, teret-conical; flowers axillary; calyx – lobes unequal; staminodes whitish – cream, longer than the calyx; capsule 1.2 -1.5 cm .

RAPD Analysis:

The RAPD analysis of *M. crystallinum* L., *M. forsskaolii* Hochst. Ex Boiss., and *M. nodiflorum* L. are shown in Figs (3a & b and Table 2. Ten random primers namely: OPJ1, OPK5, OPK9, OPN1, OPN3, OPN12, OPN14, OPQ14, OPCa, and OPLB from preselected 15 gave the more stable bands showed polymorphic patterns the bands Figure (1 a & b). Polymorphic can be summarized as follows. The ten markers showed close affinity among the three studied species concerning the bands similarity in many bands among them these band at 1 and 0.75 Kbp. On the other hand, the OPK9, OPN14, and OPLB gave more stable bands at 2 Kbp, alternatively OPK9 and OPN14 gave more stable bands at 0.16 Kbp as the OPN1 and OPN3 gave bands at 1.5 Kbp than the OPK9, OPN12, OPN14 and OPLB gave the bands at 1.4 Kbp while the OPJ1, OPK5, OPK9, OPN12 and OPN14 gave the bands at 0.85 and 0.6 Kbp. In OPCa and OPLB gave the bands at 0.5Kbp. However in OPJ1, OPK5, OPN1, OPN3 and OPQ14 gave the polymorphism bands at 0.3 Kbp than OPJ1, OPK5, OPK9, OPN12 and OPN14 gave the more stable bands at 0.2 Kbp. These results were recorded in all the studied species so the polymorphism bands were disappeared at 2.6 and 1.7 Kbp respectively.

Isozyme Analysis:

Peroxidase (PRX) and Esterase (EST) results are shown in Fig 4 and Table 3.

Peroxidase Analysis:

A total of 14 bands, three bands in all samples.Two similar bands in case of *M. crystallinum* and *M. nodiflorum* at R_f 0.08 and 0.15. While *M. nodiflorum* and *M .forsskaolii* showed bands R_f 0.42. The developed isozymes bands showed polymorphism between *M. crystallinum* and *M. forsskaolii* than both of them with. The three species showed close affinity in case of bands 0.04 and 0.27.

Esterase analysis:

A total of 15 bands of samples four of them at R_f 0.08, 0.16, 0.23 and 0.54 in all the studied species. The α esterase analysis were detected showed a little polymorphism in the studied species, in case of bands Rf 0.38 and 0.43(Table3).

DISCUSSION

RAPD analysis:

The genetic correlation between the three wild species: *M. crystallineum M. nodiflorum* and *M .forsskaolii*. The development of DNA Markers has opened a new perspective on the study of genetic relationships in plant.RAPD has generated a large number of polymorphic markers and it is the most common method used (Rafaiski & Tingley 1993; and Powell *et al.* 1996). However, RAPDs have been widely criticized especially for its lack of reproducibility (Ellsworth *et al.*, 1993; Meunier & Grimont 1993; Skroch *et al.*, 1995; Pan *et al.*, 1996; Perez *et al.*, 1998 and Rabouam *et al.*, 1999). In this study RAPD analysis for these samples cleared the presence of 198 polymorphic bands in ten primers, 8 primers of short sequence and 2 primers of long sequence (OPJ1, OPK5, OPK9, OPN1, OPN3, OPN12, OPN14, OPQ14, OPCa and OPLB) [The polymorphisms percentages were calculated to 81%]. The studied species in this work gave more stable bands and showed polymorphic patterns from 2.6 to 0.15 kbp (Table 2 and Fig 3a & b).

Isozyme analysis:

The peroxidase analysis of the studied species revealed the presence of 14 bands: 4 bands in *M. crystallinum*, 3 bands in *M. forsskaolii* and 7 bands in *M. nodiflorum*. The peroxidase analysis detected a little genetic diversity between *M. crystallinum* and *M. forsskaolii* while the genetic diversity is few between the species of *M. nodiflorum* alternatively the species of *M. crystallinum* and *M. forsskaolii* (Table 3).

The esterase analysis was detected of 15 bands α esterase in the studied species. 4 bands in *M. crystallinum*, 5 bands in *M. forsskaolii* and 6 bands in *M. nodiflorum*. The variability of the genetic diversity is little in all studied species. The isozymes bands can explain the variation in genetic factors of these results (Wagih 1991). Since the studied species are grown in a homogenous environment so the variability in isozymes results are induced by the genetic variabilities between the species. It is a naive that the present work resembles a bench mark for the future development of these invader species in the Egyptian flora. Generally *M. crystallinum* and *M. forsskaolii* are

Table 2: RAPD polymorphic bands developed in the studied *Mesembryanthemum* species.

Primer bands Kbp	*M. crystallinum*	*M. nodiflorum*	*M forsskaolii*
OPJ1			
0.85	+	+	-
0.75	-	+	-
0.60	+	+	+
0.40	+	+	+
0.30	+	+	+
0.20	+	+	+
OPK5			
0.85	+	+	+
0.75	+	+	+
0.60	+	+	+
0.40	+	+	+
0.30	+	+	-
0.25	+	-	-
0.24	+	-	-
0.20	+	-	+
0.15	+	-	-
OPN1			
1.50	+	+	+
1.00	+	+	+
0.75	+	+	+
0.50	+	+	+
0.30	+	+	+
0.24	+	-	-
OPN3			
2.60	-	-	+
2.50	-	-	+
2.00	-	-	+
1.50	+	+	+
1.00	+	+	+
0.75	+	+	+
0.50	+	+	+
0.30	+	+	+
0.24	-	-	+
OPQ14			
1.00	-	+	+
0.75	+	+	+
0.50	+	+	+
0.30	+	+	+
0.24	-	+	+

(Cont.)

Table 2: Cont.

Primer bands Kbp	*M. crystallinum*	*M. nodiflorum*	*M. forsskaolii*
OPK9			
2.50	-	+	-
2.00	+	+	+
1.60	+	+	+
1.40	+	+	+
1.00	+	+	+
0.85	+	+	+
0.75	-	+	-
0.60	-	+	+
0.20	+	-	+
0.15	-	+	+
OPN12			
1.40	+	+	+
1.00	+	+	+
0.85	+	+	+
0.75	+	+	+
0.60	+	+	+
0.20	+	+	+
OPN14			
2.50	+	+	+
2.00	+	+	+
1.60	+	+	+
1.40	+	+	+
1.20	+	+	+
0.85	+	+	+
0.75	+	+	+
0.60	+	+	+
0.20	+	+	+
0.15	+	-	+
OPCa			
1.20	+	+	+
1.75	+	+	+
0.65	+	+	+
0.50	+	+	+
0.35	+	-	+
0.25	+	-	+
OPLB			
2.00	+	+	+
1.70	-	+	+
1.50	+	+	+
1.40	+	+	-
1.20	+	+	+
1.00	+	+	+
0.75	+	+	+
0.65	+	+	+
0.50	+	+	+
0.35	+	+	+

+ = Present, - = Absent.

similar to each other and their genetic relevance to *M. nodiflorum* is similar in both species(Table 3). It is worthy to note that the key constructed to identify studied species mainly considered the morphological aspects of the leaves and stamens in addition to the plant habit. However the RAPD and isozyme studies added more identification criteria to differentiation among the studied species isozyme data observes that *M..crystallium* and *M. forsskaolii* are genetically more related than *M. nodiflorum*. The ten RAPD markers showed close affinity among the three studied species.

Table 3: Isozyme polymorphism of *Mesembryanthemum* species (1= *M. crystallinum*, 2= *M. nodiflorum* and 3= *M. forsskaolii*)

R_f	Peroxidase			R_f	α Esterase		
	1	2	3		1	2	3
0.04	+	+	+	0.08	+	+	+
0.08	+	+	-	0.16	+	+	+
0.15	+	+	-	0.23	+	+	+
0.27	+	+	+	0.38	-	+	-
0.42	-	+	+	0.43	-	+	+
0.56	-	+	-	0.54	+	+	+
0.62	-	+	-				

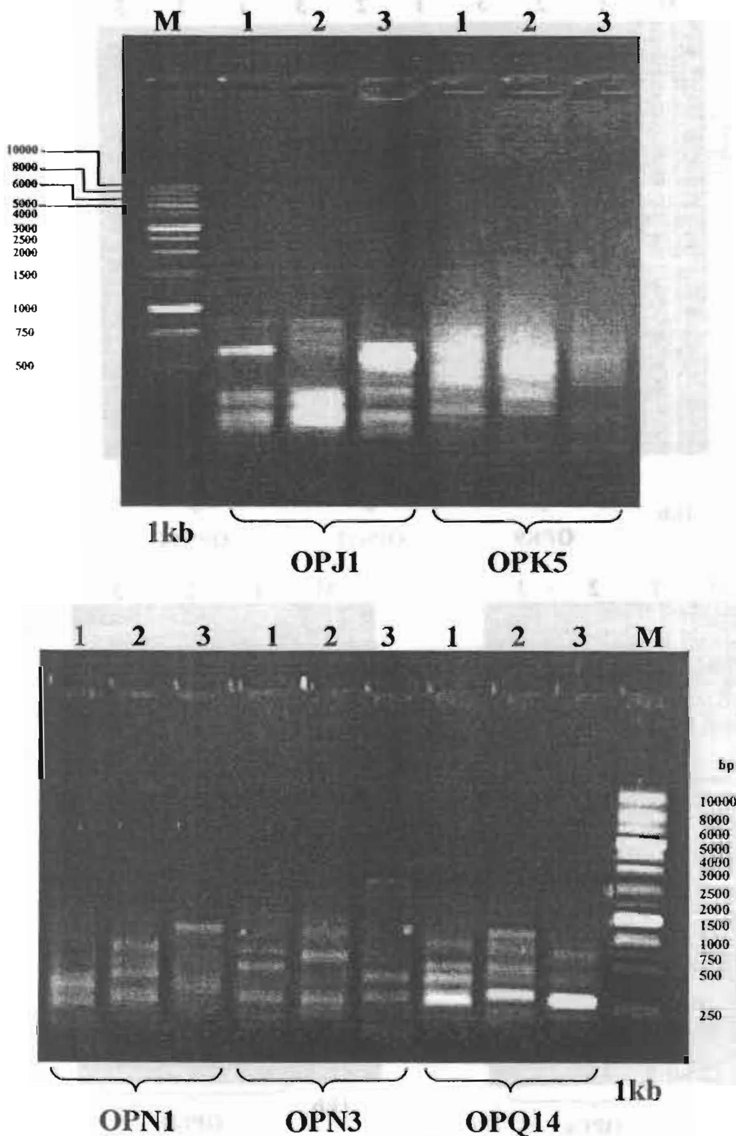

Fig. 3a: Genomic DNA polymorphism of the studied *Mesembryanthemum* species using ten primers (1= *M. crystallinum*, 2= *M. nodiflorum*, 3= *M. forsskaolii* and M= Marker).

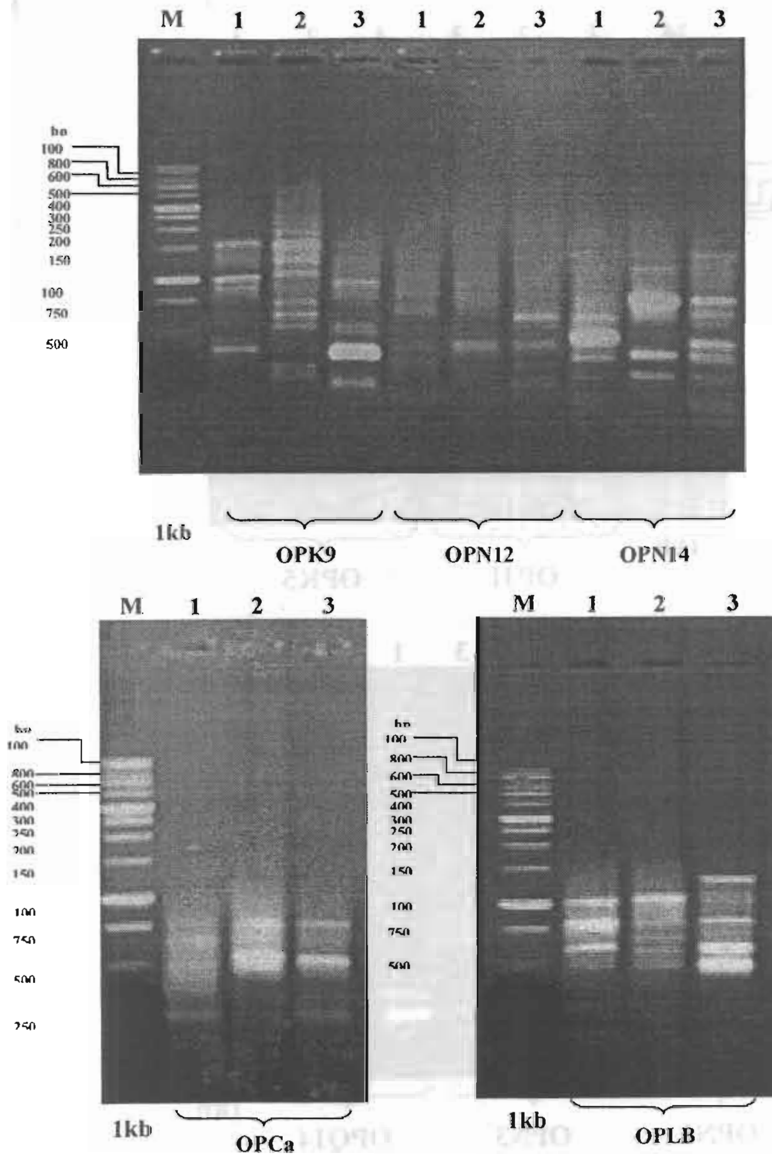

Fig. 3b: Genomic DNA polymorphism of the studied *Mesembryanthemum* species using ten primers (1= *M. crystallinum*, 2= *M. nodiflorum*, 3= *M. forsskaolii* and M= Marker).

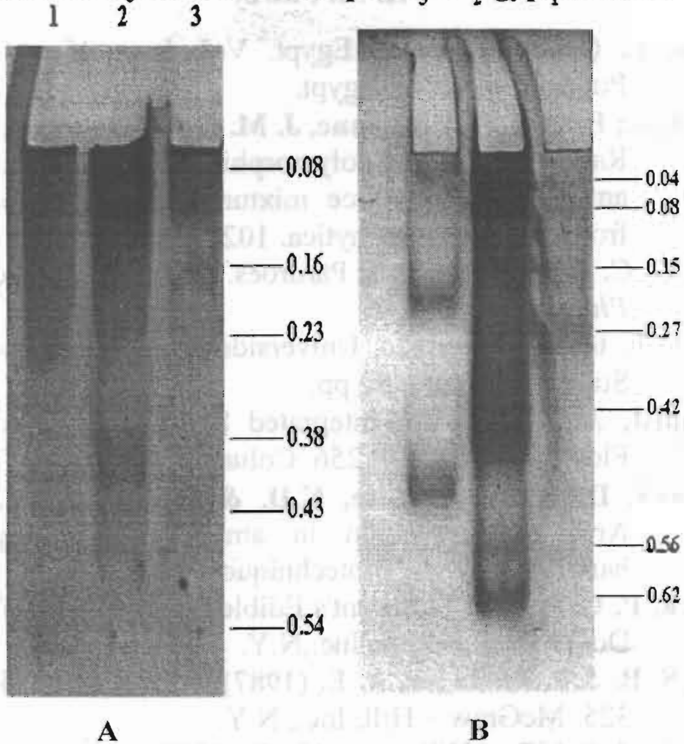

Fig. 4: Zymograms of estrase (A) and peroxidese (B) isozymes of the studied *Mesembryanthemum* species (1= *M. crystallinum*, 2= *M. nodiflorum* and 3= *M. forsskaolii*).

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RAPD and isozyme studies of species in Egyptian flora

البصمة الوراثية لأنواع الغاسول فى الفلورة المصريه.

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يتبع جنس الغاسول الفصيله الأيزويه وهو نباتات عصيريه. ويوجد من هذا الجنس في مصر ثلاثة أنواع برية هى على الترتيب الكريستالينيم؛ النوديفلورم ؛ الفورسكولياى. هذه الأنواع لها استخدامات مختلفه فى مناطق نموها. فى هذه الدراسة تم إستخدام تقنية التضاعف العشوائى متعدد المظاهر للماده الوراثيه وإثنين من مشابهات الإنزيمات المتناظرة على أوراق الأنواع موضع الدراسة. وقد تم إستخدام١٥ بادئ لتحديد مدى الاختلاف والتقارب الوراثى بين هذه الأنواع وقد اوضحت النتائج أن:

١٠ بادئات هي على الترتيب:

(OPJ1, OPk5, Opk9, OpN1, OPN3, OPN12, OPN14, OPQ14, OPCa and OPLB)

هن اللاتي أعطين تباينا واضحا للعلاقة الوراثية وقد وجد إختلاف بسيط بين الأنواع موضع الدراسة. وكذلك عند إجراء بصمة الإنزيمات المتناظرة بإستخدام إنزيمى الاستريز والبيروكسيديز وجد إختلاف بسيط بين الأنواع موضع الدراسة عند إجراء إختبار إنزيم الاستريز وعند إستخدام إنزيم البيروكسيديز وجد تباينا واضحا بين النوع نوديفلورم والنوعين الآخرين موضعى الدراسة .