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Genetic Variation among *Hibiscus Rosa-sinensis (Malvaceae)* of Different Flower Colors Using Issr and Isozymes

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Abstract: Germplasm identification and characterization is an important link between the conservation and utilization of plant genetic resources. Traditionally, species or cultivars identification has relied on morphological characters like growth habit or floral morphology like flower color and other characteristics of the plant. Studies were undertaken for identification and determination of genetic variation between four cultivars of *Hibiscus rosa-sinensis* L. with different colors (red, pink, orange and white) through inter-simple sequence repeat (ISSR) and isozymes pattern. ISSR result produced scorable banding patterns with nine primers out of ten. A total number of 89 DNA fragments were amplified with different lengths over all the four cultivars with the nine primers. The result showed that 39 DNA amplified fragments were polymorphic in the four cultivars. Eighteen DNA amplified fragments were considered as cultivar- specific markers. Isozymes results indicated that Peroxidase and esterase isozymes revealed extensive polymorphism among four cultivars. PX6 were positive specific marker detected in the yellow flowers while EST3 was unique marker with pink flower color cultivar. The similarity indices and the consensus trees of the four cultivars were developed based on the banding that pink flower contrasting cultivars were distantly related cultivars.

Key words: Genetic Relationship, *Hibiscus* sp., ISSR Marker.

INTRODUCTION

Hibiscus (Rose mallow) is a large genus of about 200–220 species of flowering plants in the family *Malvaceae*, native to warm temperate, subtropical and tropical regions throughout the world. The genus includes both annual and perennial herbaceous plants, and woody shrubs and small trees. In Egypt, it also grows very well during whole year. The flowers are large (generally red in the original varieties), firm, but generally lack any scent. Numerous varieties, cultivars and hybrids are available, with flower colors ranging from white through yellow and orange to scarlet and shades of pink, with both single and double sets of petals (photo.1).

Photo. 1:

ISSR markers are inherited in Mendelian mode and segregated as dominant markers. This technique has been widely used in the studies of cultivar identification, genetic mapping , genetic diversity, evolution and molecular ecology (Zietziewicz *et al.*, 1994; Reddy and Nagaraju, 1999; Leroy *et al*., 2000and 2001; Wang, 2002; Bornet and Branchard, 2004; Li and XIA, 2005; Ripley and Roslinsky, 2005, Slotta and Portern (2006); Domaand Azzam, 2007 and Abdel-tawab *et al*., 2007) because of its advantages in overcoming limitations of allozymes and RAPD techniques (Wolfa *et al*., 1998; Ratnaparkhe *et al*.,1998; Esseman *et al*.,1999).

Despite the importance of *H. sinensis*, little is known about its genetic**s**. Its wide geographical distribution and varied habitats indicate that there is probably a large amount of genetic diversity. Amplified fragment length polymorphism (AFLP) (Zabeau and Vos, 1993; Vos *et al*., 1995) is a DNA fingerprinting technique that

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approaches the ideal as a marker system for resolving genetic diversity among individuals, populations and species (Mueller and Wolfenbarer, 1999). This technique is highly reproducible and can be used to survey overall genetic differences in the nuclear genome in a single assay without any prior sequence knowledge (Jones *et al*., 1997). As a consequence of these features, AFLP has been used to investigate genetic variation in a wide variety of micro-organisms, plants and animals (Janssen *et al*., 1996; Albertson *et al*., 1999; Muluvi *et al*., 1999; Ajmone-Marsan *et al*., 2001;Terefework *et al*., 2001; Maguire *et al*., 2002).

Kudoh and Whigham (1997) reported that microgeographic genetic variation in populations of a wetland macrophyte, *Hibiscus moscheutos L*. (Malvaceae), was investigated using allozyme polymorphism. Klips (1995). Using isozymes for genetic affinity of the rare eastern Texas endemic Hibiscus dasycalyx (Malvaceae), Hiron *et al.*, (2006) also used isozymes specially (phosphatase and peroxidase isozymes) for differentiation between two species of *Hibiscus* (*H. cannabinus* and *H. sabdariffa*) and electrophoresis pattern indicates that both species were close each other. A dendrogram based on Nei (1978) unbiased genetic distances and the unweighted pair-group method with arithmetic averages (UPGMA) was constructed using the TFPGA program version 1.3 (Miller, 1997). Marker frequencies were estimated based on Lynch and Milligans (1994) frequency correction for dominant markers. Bootstrapping over loci was also performed with TFPGA with 1000 permutations.

In the current study, ISSR and isozymes techniques were used to reveal the extent and distribution of genetic variation in four natural populations of *H. sinensis* from the gardens of 6 of October city, Egypt as a first step towards gaining a better knowledge of genome diversity in *Hibiscus rosa-sinensis*. The objective of this study was to develop polymorphic ISSR and Isozyme markers that may help in distinguishing *H. rosa-sinensis* cv. 'Bhlliant Red' from other cultivars with different colors; pink, orange and white.

MATERIAL AND METHODS

Young and fresh leave samples were collected separately from four *hibiscus* cultivar with different colors (red, pink, orange and white) were obtained from garden in 6 of October city, Giza, Egypt.

DNA extract & ISSR Techniques:

DNA was extracted from fresh leaves collected from nursery garden raised plants of different species/varieties by the CTAB method (Doyle and Doyle 1990). Approx. 200 mg of fresh leaves was ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to a 50 ml falcon tube with 10 ml of cetyltrimethyl ammonium bromide (CTAB) buffer [2% (w/v) CTAB, 1.4 M Nacl, 20mM EDTA, 100mM Tris (tris(hydroxymethyl)aminomethane)-HCl, pH 8.0, and 0.2% (v/v) b -mercaptoethanol]. The homogenate was incubated at 60°C for 2h, extracted with an equal volume of chloroform/isoamyl alcohol (24 : 1 v/v) and centrifuged at 10,000g for 20 min (Kubota KR-2000 C, Rotor-RA-3R, Tokyo, Japan). DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol. After centrifugation at 10,000g for 10 min, the DNA pellet was washed with 70% (v/v) ethanol, air-dried and resuspended in TE (10mM Tris-HCl, pH 8.0, and 0.1mM EDTA) buffer. DNA quantifications were performed by visualizing under UV light, after electrophoresis on 0.8% (w/v) agarose gel. The resuspended DNA was then diluted in TE buffer to 5m g/m l concentration for use in polymerase chain reaction.

Forty 10-mer primers Table (1), corresponding to kits A, B, D, and N from Operon Technologies (Alameda, California, USA) clearly resolved and polymorphic amplified products between cultivars of *Hibiscus rosa-sinensis.* To ensure reproducibility, the primers generating no, weak, or complex patterns were discarded. For ISSR study, the initial optimization of PCR was conducted including concentration of template DNA primer, MgCl2, number of PCR cycle and annealing temperature. The PCR reaction had a total volume of 25m l containing 20 ng templates DNA, 100 mM each dNTPs, 20 ng of oligonucleotides synthesized primer (M/S Bangalore Genei, Bangalore, India), 2.5mM MgCl2, 1_Taq buffer (10mM Tris-HCl [pH 9.0], 50 mM KCl, and 0.01% gelatin) and 0.5U Taq DNA polymerase. DNA amplification was performed in a PTC_100 thermal cycler (M J Research Inc., Watertown, MA, USA) programmed for a preliminary 5min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 20 s., annealing temperature depending on the synthesize primer (ranged from 32–56°C) for 30 s. and extension at 72°C for 45 s., finally at 72°C for 5 min. Amplification products were separated alongside a molecular weight marker (1.0 Kb plus ladder, M/S Bangalore Genei, Bangalore, India) by 1.2% agarose gel electrophoresis in 1_ TAE (Tris Acetate EDTA) buffer stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System and evaluated using the software Quantity one (BioRad, California, USA). Data were recorded as presence (1) or absence (0) of band products from the examination of photographic. Each amplification

fragment was named by the source of the primer, the kit letter or number, the primer number and its approximate size in base pairs. Bands with similar mobility to those detected in the negative control, if any, were not scored. Similarity index was estimated using the Dice coefficient of similarity (Nei and Li, 1979). The average of similarity matrices was used to generate a tree by UPGMA (Unweighted Pair-Group Method Arithmetic Average) using NTSYS-PC, version 2.0 (Rohlf, 1998).

Table 1: ISSR primer name and their sequence.

Name	Sequence	Name	sequence
HB1	(CT)8TG	HB12	(CAG)3GC
98B	(CA)3 (TA)3 GT	HB15	(GTC)3GC
HB08	(GA)6GG	844A	(CT)8GC
HB09	(GT)6 GG	44B	(CT)8 GC
HB10	((GA)6 CC	49B	(CA)3 (TA)3 GG

Isozymes techniques:

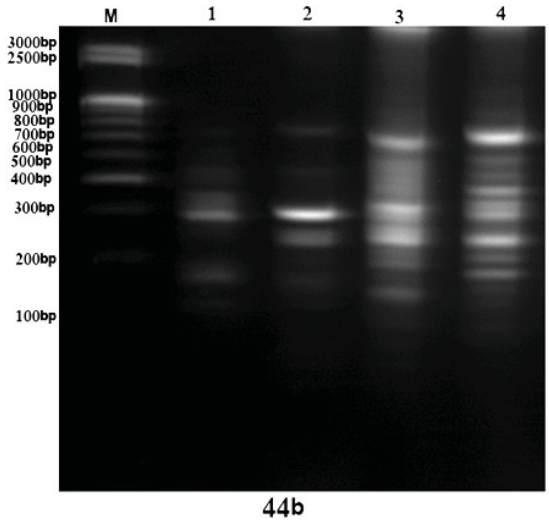
Four enzymes systems were analyses: peroxidase (PER).esterase (EST), Poly phenyl- oxidase (PPO) and Alkaohol- Dehydrogenase (ADH). All enzymes were studied in leaf, small pieces of leaf was homogenized in a cool mortar without buffer. Strips of filter paper were used to absorb the cell homogenate and were placed on the origin line of the get plates. The plates were stored at 4^{o}C for 30 minutes, and the filter papers were removed before electrophoresis. Agar- starch- polyvinylpyrolidine gel electrophoresis was used (Shaw and Kaen, 1997). For ACP, AKP, GOT and PER, the gel buffer was Tris citrate-NaOH, pH 8.6 and the electrode buffer was Sodium borate, pH 8.0 (Poulik, 1957). The staining procedures were modified from Bernie May (1998) for ACP, AKP and PER. Esterase and GOT staining was done according to the methods of Jonathan and Norman (1989). Isozyme patterns were scored visually. Levels of marker polymorphism according to isozyme banding patterens were calculated. Asimilarity dendrogram was produced using NTSYS (Numerical Taxonomic and Multivariate/analysis System) software package, version 2.1, Applied Biostatistics Inc. (Rohlf, 2000).

RESULT AND DISCUSSION

ISSR result is shown in Table (2) and Figs. (1 to 7), where seven primers out of a total of ten produced scorable banding patterns. A total number of 87 DNA fragments were amplified with different lengths overall the flour cultivars under investigation. The result showed that 18 DNA amplified fragments were monomorphic in the twelve cultivars and 39 amplified were fragments were polymorphic. Fig. (2) shows some typical ISSR banding patterns. Eighteen DNA amplified fragments were considered as cultivar- specific markers .Primer 44B produced two negative cultivar- specific marker with length 200 bp and 260bp in cultivar (red flower) and cultivar (pink flower) respectively. Another negative cultivar- specific marker with length of 80bp was produced in cultivar (red flower) using primer 49A. Three positive cultivar- specific markers were produced with lengths 620bp, 280bp and 160bp by using primer 98B in cultivar (pink flower). While red flower cultivar has two specific markers; one is positive marker with length 350 bp and other negative with length790 bp with the same primer. White flower cultivar have four cultivar specific markers; two negative with length 500bp and 750 bp and other positive cultivar-specific markers with length 490 and 60 bps using primer HB10. Yellow cultivar is characterize from all other cultivars by two negative specific markers with length 560 bp and 90 bp using HB09. Slotta and Portern (2006) support these result with two species of Malvaceae. Yidong *et al*. (2006) and Fu Rong Gui *et al*. (2007)) support these result with other different species. Isozyme patterns of PER, EST, were examined in order to characterize and investigate variations among the four hibiscus cultivars with different flower colors (red, pink, orange and white) as well as between plants.

Table 2: TAF (Total Amplified Fragment), PB (Polymorphic Band), AF (Amplified Fragment), SM (Specific Marker), TSM 'Total Specific Marker + positive marker – negative marker).

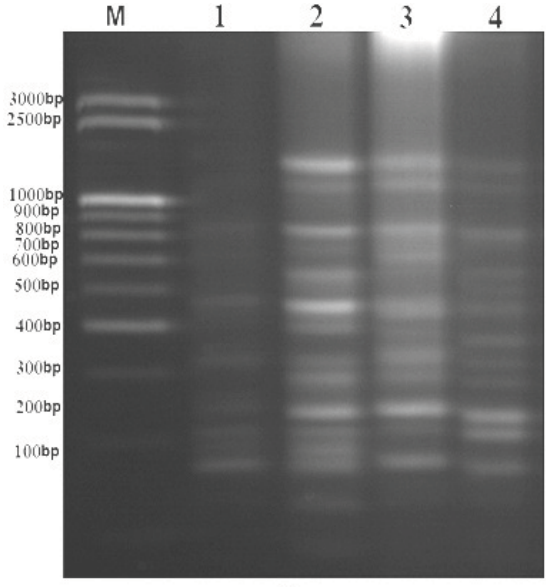
Primer No.	TAF	Pb	1		2		3		4		TSM
			AF	SM	AF	SM	AF	SM	AF	SM	
44b	9	4	6	-1	6	-1	9	0	9	0	2
49a	14	7	9	-1	12	0	12	0	11	-1	2
98b	15	9	12	-1+1	12	-1+1	9	0	9	0	4
Hb08	11	2	10	0	11	0	9	0	10	0	0
HB090	14	8	8	-1	11	0	12	-2+2	10	0	5
HB10	10	5	7	0	8	0	10	0	7	-2	2
HB12	14	4	13	-1	13	-1	13	0	12	-1	3
Total	89	39	65	6	63	4	74	4	48	4	18



44b

BandNo.	M.W Bp	Cultivars			
		1	2	3	4
1	590.0	1	1	1	1
2	420.0	0	0	1	1
3	350.0	1	1	1	1
4	260.0	1	0	1	1
5	220.0	1	1	1	1
6	200.0	0	1	1	1
7	170.0	0	0	1	1
8	150.0	1	1	1	1
9	130.0	1	1	1	1
Total		6	6	9	9

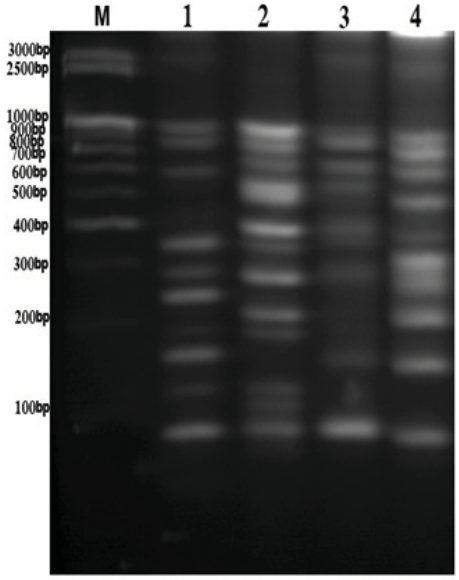
Fig. 1: Banding patterns for four *Hibiscus* cultivars with some ISSR primers (M. Marker 1- Red flower 2- pink flower 3- orange flower 4- white flower) generated by primer 44b



49 a

Band No.	M.W bp	Cultivars			
		1	2	3	4
1	1530.0	0	0	1	1
2	1480.0	0	0	1	1
3	1150.0	1	1	1	1
4	800.0	1	1	1	0
5	620.0	0	1	0	1
6	540.0	1	1	1	1
7	480.0	0	1	1	0
8	460.0	1	1	1	1
9	420.0	1	1	1	1
10	320.0	1	1	1	1
11	280.0	1	1	0	0
12	195.0	1	1	1	1
13	145.0	1	1	1	1
14	80.0	0	1	1	1
Total		9	12	12	11

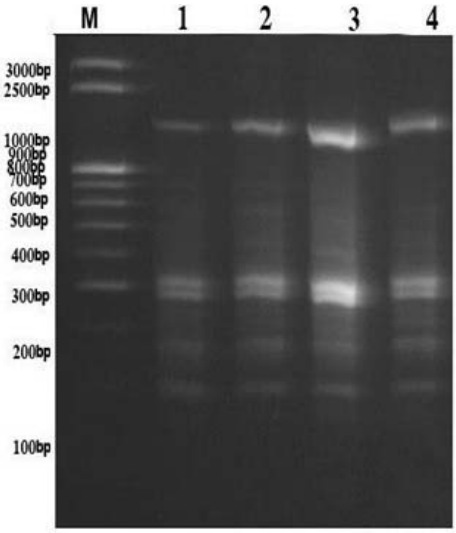
Fig. 2: Banding patterns for four *Hibiscus* cultivars with some ISSR primers (M. Marker 1- Red flower 2- pink flower 3- orange flower 4- white flower) generated by primer 49a.



98 b

Band No.	M.W bp	Cultivars			
		1	2	3	4
1	950.0	1	1	1	1
2	860.0	1	1	1	1
3	820.0	1	1	1	1
4	790.0	0	1	1	1
5	620.0	0	1	0	0
6	530.0	1	0	1	0
7	450.0	1	1	1	1
8	400.0	1	1	1	1
9	350.0	1	0	0	0
10	280.0	0	1	0	1
11	280.0	1	1	0	0
12	240.0	1	0	1	1
13	180.0	1	1	0	0
14	160.0	0	1	0	0
15	85.0	1	1	1	1
Total		12	12	9	9

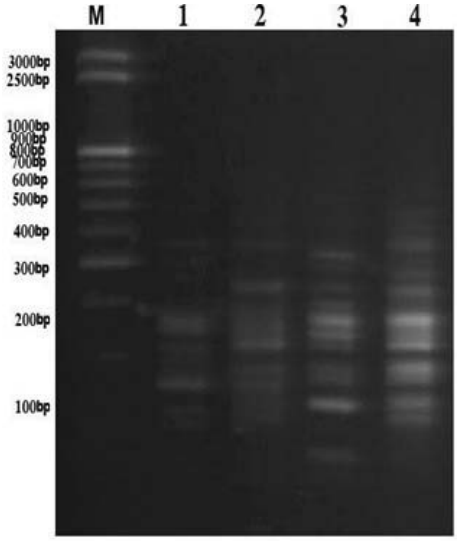
Fig. 3: Banding patterns for four *Hibiscus* cultivars with some ISSR primers (M. Marker 1- Red flower 2- pink flower 3- orange flower 4- white flower) generated by primer 98b.



HB 08

Band No.		M.W bp	Cultivars		
		1	2	3	4
1	950.0	1	1	1	1
2	785.0	1	1	0	0
3	960.0	0	1	0	1
4	580.0	1	1	1	1
5	560.0	1	1	1	1
6	390.0	1	1	1	1
7	320.0	1	1	1	1
8	245.0	1	1	1	1
9	225.0	1	1	1	1
10	210.0	1	1	1	1
11	90.0	1	1	1	1
Total		10	11	9	10

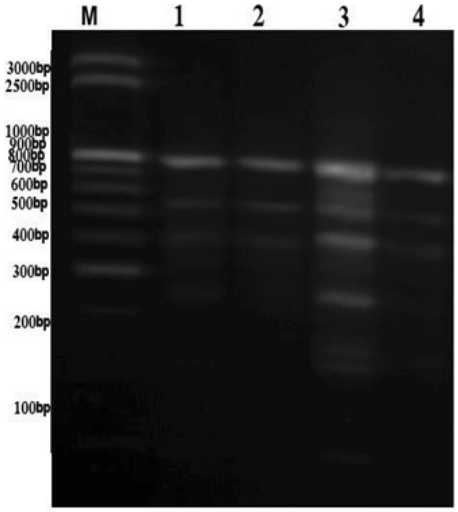
Fig. 4: Banding patterns for four *Hibiscus* cultivars with some ISSR primers (M. Marker 1- Red flower 2- pink flower 3- orange flower 4- white flower) generated by primer HB-08.



HB 10

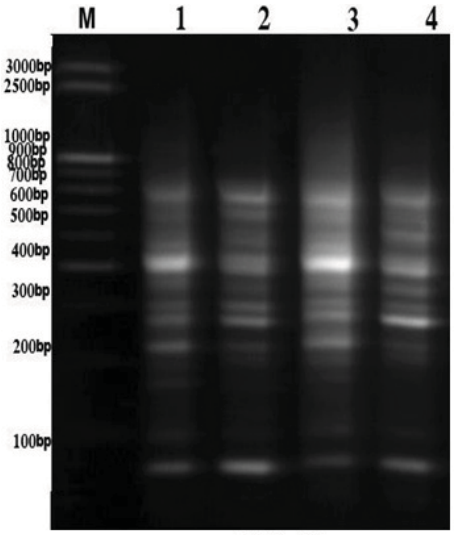
Band No.	M.W bp	Cultivars			
		1	2	3	4
1	700.0	0	1	1	0
2	650.0	1	1	1	1
3	500.0	1	1	0	1
4	490.0	0	0	1	0
5	335.0	0	0	1	1
6	260.0	0	1	1	0
7	230.0	1	1	1	1
8	215.0	1	1	1	1
9	180.0	1	1	1	1
10	160.0	0	1	1	1
11	140.0	1	1	1	1
12	115.0	1	1	1	1
13	75.0	1	1	0	1
14	60.0	0	0	1	0
Total		8	11	12	10

Fig. 5: Banding patterns for four *Hibiscus* cultivars with some ISSR primers (M. Marker 1- Red flower 2- pink flower 3- orange flower 4- white flower) generated by primer HB-10.



Band No.	M.W bp	Cultivars			
		1	2	3	4
1	950.0	1	1	1	1
2	780.0	0	0	1	1
3	720.0	1	1	1	1
4	685.0	1	1	1	1
5	580.0	1	1	1	1
6	560.0	1	1	1	0
7	385.0	0	1	1	0
8	325.0	0	0	1	1
9	220.0	1	1	1	1
10	90.0	1	1	1	0
Total		7	8	10	7

Fig. 6: Banding patterns for four *Hibiscus* cultivars with some ISSR primers (M. Marker 1- Red flower 2- pink flower 3- orange flower 4- white flower) generated by primer HB-09.



HB- 12

Band No.	M.W bp	Cultivars			
		1	2	3	4
1	755.0	1	1	1	1
2	735.0	1	1	1	1
3	670.0	0	1	1	1
4	540.0	1	0	1	1
5	515.0	1	1	1	1
6	465.0	1	1	1	1
7	390.0	1	1	1	1
8	375.0	1	1	0	0
9	315.0	1	1	1	1
10	280.0	1	1	1	0
11	240.0	1	1	1	1
12	200.0	1	1	1	1
13	95.0	1	1	1	1
14	60.0	1	1	1	1
Total		13	13	13	12

Fig. 7: Banding patterns for four *Hibiscus* cultivars with some ISSR primers (M. Marker 1- Red flower 2- pink flower 3- orange flower 4- white flower) generated by primer HB-12.

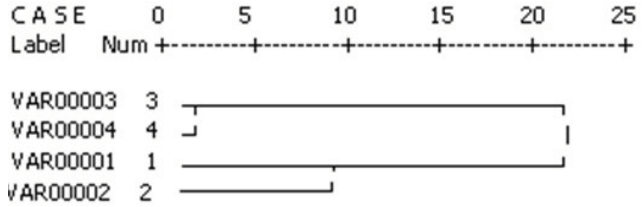


Fig. 8: Dendogram analysis of four cultivars of Hibiscus by using ISSR analysis.

Table 3: Similarity index of four cultivars of Hibiscus by using ISSR analysis Isozymes analysis.

	1	2	3
1			
2	0.30		
3	0.89	0.67	
4	1.00	0.76	0.0

Peroxidase Isozymes:

The electrophoretic patterns of peroxidase isozymes of the plants for the four cultivars in Fig. (9). Four plants, the number of isozyme bands ranged from six bands in one cultivar (White) to seven band in red flower plant and eight bands in other two cultivars. Twenty nine bands of peroxidase (PER) isozyme with Rm 0.1 to 0.85 were observed. Peroxidase were present in all cultivars whereas PX7 and PX10 were observed in all cultivars except with red and yellow flower color cultivars; this negative specific marker, it could be useful in distinguishing cultivars. On additional bands PX6 was positive specific mark detected in the yellow flower color cultivar rather than other cultivars. Hiron *et al.* (2006) and Naznin *et al*. (2006) reported similar results using starch gel electrophoresis to study enzymes from two species of *Hibiscus.*

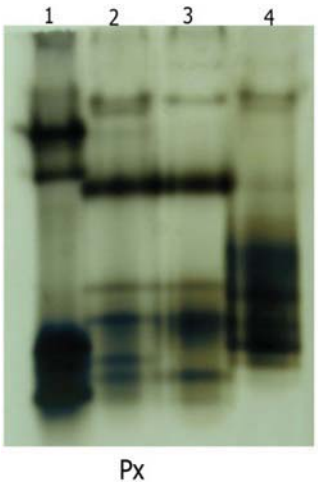
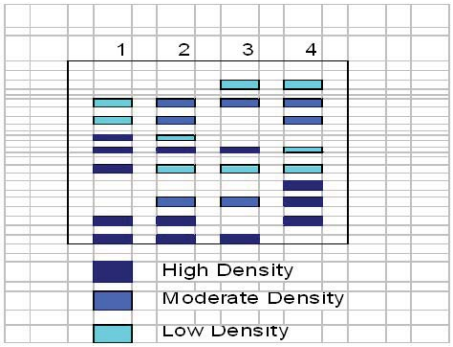


Fig. 9: Zymogram of isozyme peroxidase patterns in the Hibiscus cultivars having different flower colors, lane (1) red flower, lane (2) pink flower color, lane (3) orange flower color, lane (4) white flower color.



Peroxidase groups	Rm	Cultivars			
		1	2	3	4
Px1	0.1	0	0	1^{-}	1^{-}
Px2	0.2	1^{-}	1^{+}	1^{+}	1^{+}
Px3	0.3	1^{-}	1^{+}	0	1^{+}
Px4	0.45	1^{++}	1^{-}	0	0
Px5	0.5	1^{++}	1^{++}	1^{++}	1^{-}
Px6	0.6	0	0	0	1^{++}
Px7	0.65	0	1^{+}	1^{+}	1^{++}
Px8	0.7	1^{-}	1^{+}	1^{+}	1^{+}
Px9	0.8	1^{++}	1^{++}	0	1^{++}
Px10	0.85	1^{++}	1^{++}	1^{++}	0

1^{++}, positive specific marker; 0, Negative specific marker

Fig. 10: Ideogram of peroxidase isozyme patterns in four cultivars of *Hibiscus rosa-sinensis.*

Esterase isozymes:

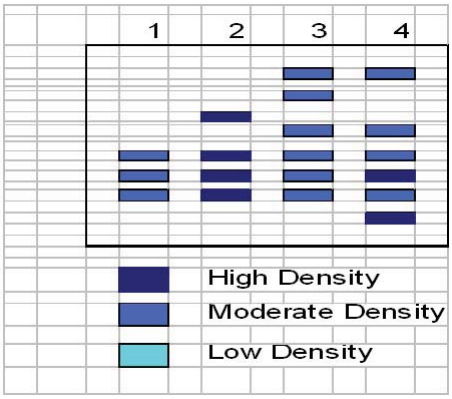
The electrophoretic pattern of Esterase isozymes and the ideograms of the plantlets for four cultivars are shown in Fig. (11). The number of isozymes bands ranged from three bands in the red flower color to six bands in the white and yellow flower color cultivars.

A total of nineteen electrophretic bands were detected for EST and were grouped into nine isozyme phenotypes designated as EST1 to EST 9.

EST2 was unique to orange flower color cultivars, EST 3 was unique to pink flower colors cultivars, both with positive specific markers whereas EST4 was unique to red flower color cultivar. There are three bands EST 7, 8, 9 (Rm=0.8-0.9) were present in all the genotype (cultivars). Previous investigations used esterase isozymes patterns as biochemical markers, to identify different species of *Hibiscus* (Lee, S. 1994, Robert, 1995 and Hiron *et al*., 2006). Isozyme banding patterns have been used to investigate genetic distances between cultivars of Hibiscus dasycalyx (Robert, 1995; Lee., 1994 and Hiron *et al*. 2006). Optimal and Spatial Analysis of Hormones, Degrading Enzymes and Isozyme Profiles in Tomato Pedicel Explants during Ethylene-Induced Abscission

Mostly results published in this respecte emphasize the genetic nature of isozymes of *Hibiscus.* Several isozymes loci have been used repeated to detect genetic differences between hibiscus genotypes and cultivars as well as linking such differences to traits of economic value (Miroslav, 2000).

Fig. 11: Zymogram of isozyme Esterase patterns in the Hibiscus cultivars having different flower colors, lane (1) red flower, lane (2) pink flower color, lane (3) white flower color, lane (4) yellow flower color.



Esterase groups	Rm	Cultivars			
		Red	Pink	Orange	white
Est1	0.2	0	0	1^{+}	1^{+}
Est2	0.25	0	0	1^{+}	0
Est3	0.3	0	1^{++}	0	0
Est4	0.4	0	1^{+}	1^{+}	1^{+}
Est5	0.6	0	0	1^{+}	1^{+}
Est6	0.7	0	0	1^{+}	1^{+}
Est7	0.8	1^{+}	1^{++}	1^{+}	1^{+}
Est8	0.85	1^{+}	1^{++}	1^{+}	1^{++}
Est9	0.9	1^{+}	1^{++}	1^{+}	1^{+}

1^{++}, positive specific marker; 0, Negative specific marker

Fig. 12: Ideogram of Esterase isozyme isozyme patterns in four cultivars of *Hibiscus rosa-sinensis*.

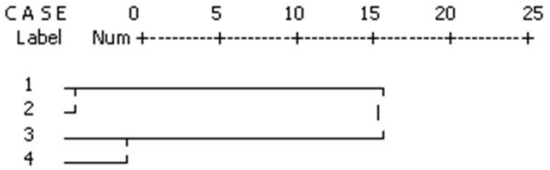


Fig. 13: Dendrogram analysis of four cultivar of Hibiscus by using two isozymes systems.

Table 3: Similarity index of four cultivar of Hibiscus by using two isozymes systems.

	1	2	3
1			
2	0.0		
3	1.0	0.7	
4	0.8	0.5	0.14

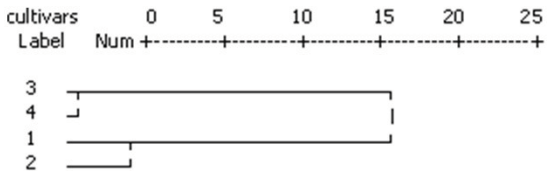


Fig. 14: Dendrogram analysis of four cultivar of Hibiscus by using ISSR analysis and two isozymes systems.

Table 4: Similarity index of four cultivar of Hibiscus by using ISSR analysis and two isozymes systems.

	1	2	3
1			
2	0.14		
3	0.99	0.72	
4	1.00	0.72	0.0

U8uuuuuu8u8u8

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