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Antioxidant Enzymes and Molecular Markers Associated with Salinity Tolerance of *Halocnemum strobilaceum* (Pall.) Bieb

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Abstract: Soil salinity is one of the major abiotic stresses impairs reactive oxygen species detoxification systems and causes oxidative damage to plants. Soil and plant samples of *Halocnemum strobilaceum* were collected from five different regions of Egypt, Siwa Oasis (L1), Burg El-Arab (L2), Balouza (L3), Wadi Gharandal (L4) and Wadi Halazeen (L5). The specific activities of peroxidase (POD), polyphenol oxidase (PPO), catalase (CAT) and ascorbate peroxidase (APX) were investigated in plant samples. In addition, total soluble proteins and inter simple sequence repeats (ISSR) were estimated. The highest soil electrical conductivity, POD and CAT specific activities were recorded in L5, while the highest APX and PPO specific activities were recorded in L1 and L2, respectively. Nine protein polymorphic bands and four unique bands were detected among the five *H. strobilaceum* genotypes by SDS-PAGE produced (62%) polymorphism. Peroxidase and polyphenol oxidase produced twelve total bands, nine polymorphic bands (75% of polymorphism). *H. strobilaceum* of L5 and L2 produced the highest number of bands for POD and PPO, respectively. Twelve polymorphic bands (35% of polymorphism) and nine unique bands were produced by five ISSR primers. The dendrogram based on overall analysis, separated the five *H. strobilaceum* genotypes into two main clusters. Moreover, *H. strobilaceum* from L3 and L2 were separated in the first cluster, while, L1, L4 and L5 were separated in the second cluster. Biochemical, molecular markers and enzyme activity could be used to choose the most suitable genotypes for stress tolerance.

Key words: *Halocnemum strobilaceum* · Antioxidant enzymes · Genetic variation · Salinity

INTRODUCTION

Halophytes are remarkable plants which have the ability to complete their life cycle in a substrate rich in NaCl that normally found toxic to other species and destroy almost 99% of their population [1]. In response to salt and oxidative stresses, the ecophysiology of halophytes has been reviewed occasionally [2-5]. Under stresses, plants possess several antioxidant enzymes like catalase (CAT), peroxidase (POD), superoxide dismutase (SOD) and ascorbate peroxidase (APX) to protect their cell from the negative effects of active oxygen species [6]. SOD catalyzes the first step of the enzymatic defense mechanism, the conversion of superoxide radicals to O_2 and H_2O_2. The hydrogen peroxide produced is then scavenged by CAT into water and molecular oxygen whereas POD decomposes H_2O_2 by oxidation of co-substrates [7]. The APX had a much higher affinity for H_2O_2 than CAT [8]. *Halocnemum strobilaceum* (Chenopodiaceae) is a halophytic plant which can grow under highly saline conditions [9], glabrous shrubs; old stems woody; young stems jointed, succulent, with numerous opposite bud-like branches, leaves opposite, succulent, decussate, leaves blade reduced to fleshy cups, connate at the base [10]. SDS-PAGE reflects the genetic variability, it is the direct end products of genes under stress and can help in exploring important tolerant genotypes, highly useful for solving various problems of plant taxonomy, genetic diversity and phylogenetic systematic at intragenic and specific levels. In addition, it is the cheapest and simplest traditional method that offer sufficient information and serve as a starting point for

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DNA-based techniques [11]. Molecular markers had been used in Chenopodiaceae for detecting genetic diversity, genotype identification and phylogenetic relationships [12]. Basel [13] investigated the efficiency of RAPD and ISSR techniques to estimate the genetic diversity among *Arthrocnemum macrostachyum* (Chenopodiaceae) genotypes from different locations in Syria. ISSR primers generated 88 bands and (90.91% of polymorphism) and proved that the use ISSR fingerprints could be a powerful tool to assess the genetic diversity.

The objectives of this study were to establish which of the antioxidant enzymes play a major role in protection and defense of *Halocnemum strobilaceum* against oxidative stress. Estimation of the biochemical and molecular variations that occurred under salinity stress to differentiate among the five genotypes of *H. strobilaceum*.

MATERIALS AND METHODS

Samples of new vegetative parts of shoot system of *Halocnemum strobilaceum* and soil supporting (0-30cm depth) were collected from salt marshes of five different areas of Egypt desert as illustrated in Table 1 during March, 2014.

Physical and Chemical Soil Analysis: Samples of soil were air-dried to a constant mass up to 65°C, homogenized and sieved through 2 mm mesh. The electrical conductivity (EC) of soil-water extract (1:1) expressed as dSm^{-1} of soil was measured by conductivity meter. The soil acidity (pH) was determined in distilled water at soil solution ratio of 1:1 with a potentiometric glass electrode using pH-meter. Moisture content was determined according to Rowell [14]. Mechanical analysis was carried out by pipette method according to Kilmer and Alexander [15].

Enzymes Assay

Preparation of Enzymes Crude Extracts: A new vegetative parts of shoot (0.5 g) was ground to a fine powder in 4 ml of 50 mM potassium phosphate buffer (pH 7.0), 1 mM ethylenediamine tetraacetic acid (EDTA), 1% (w/v) polyvinylpyrrolidone (PVP) and 0.05% (w/v) Triton X-100 using a chilled pestle and mortar. The homogenate was centrifuged at 10,000 g for 20 min at 4°C and the supernatants thus collected was used for the assays of Peroxidase (POD), poly phenol oxidase (PPO), catalase (CAT) and ascorbate peroxidase (APX). Protein concentrations in the enzyme extract were determined by the method of Bradford [16] using bovine serum albumin as a standard.

POD Assay: The activity of POD was assayed according to Hammerschmidt *et al*. [17] with some modifications. The reaction mixture (3 ml) consisted of 2.9 ml 0.25% guaiacol in 10 mM sodium phosphate buffer, pH 6.0 containing 10 mM H_2O_2 followed by addition of 100µl enzyme extract to initiate the reaction. The changes of optical density at 470nm were recorded in a spectrophotometer. Unit of enzyme (1U) equal 0.01 $\Delta OD\ min^{-1}$.

PPO Assay: PPO activity was determined according to Mayer *et al*. [18]. The reaction mixture consisted of 200µl of the enzyme extract, 1.5 ml of 0.1 M sodium phosphate buffer, pH 6.5, 200µl of 0.01 M catechol and the change in OD was recorded at 30s interval up to 3 min at 495 nm. Unit of enzyme (1U) equal 0.001 ÄOD min^{-1}.

CAT Assay: CAT activity was measured according to Chandlee and Scandalios [19], with modification. The assay mixture contained 2.5 ml of 50 mM potassium phosphate buffer, pH 7, 0.4 ml of 15 mM H_2O_2 and 100µl of enzyme extract. The decomposition of H_2O_2 was followed by the decrease in absorbance at 240 nm and calculated using the extinction coefficient (40 mM cm^{-1}).

APX Assay: APX was assayed by the method as described by Nakano and Asada [20]. The reaction mixture (3 ml) consisted of 2.9 ml of 50 mM potassium phosphate buffer, pH 7.0 containing 0.1 mM EDTA, 0.5 mM ascorbic acid and 0.1 mM H_2O_2 and 100µl enzyme extract. The decrease in absorbance at 290 nm for 1 min was recorded and the amount of APX was calculated using the extinction coefficient 2.8 $mM^{-1}cm^{-1}$. The specific activities were expressed as (U. mg^{-1} protein).

Biochemical and Molecular Genetic Markers

SDS-Polyacrylamide Gel Electrophoresis: Bulked new vegetative shoot sample (0.5g) of each genotype was ground with liquid nitrogen and mixed with extraction buffer pH 7.5 (50 mM Tris, 5% glycerol and 14 mM β-mercaptoethanol) in a mortar with pestle, left overnight then vortexed for 15 sec and centrifuged at 12,000 rpm at 4°C for 10 min. The supernatants were transferred to new eppendorf tubes and kept at -20°C until use for (SDS-PAGE) analysis according to Laemmli [21]. The gel was photographed scanned and analyzed by Biometra Bio Doc Analyze 2005.

Table 1: Geographical position (GPS) of *H. strobilaceum* from studied regions

No.	Areas	The GPS Reading
L1	Siwa Oasis	29° 13' 11.8° N and 25° 25' 36.1° E
L2	Burg El-Arab (West Alexandria)	30° 55' 28.2°N and 29° 31' 30.7° E
L3	Balouza (North Sinai)	31° 01' 52.4° N and 32° 34' 52.6°E
L4	Wadi Gharandal (Southwest Sinai)	29° 26' 11.6° N and 32° 47' 1° E
L5	Wadi Halazeen (West Matruh)	31°27' 41.7° N and 26° 51' 99° E

Table 2: List of the primer names and their nucleotide sequences used in the study

No.	Name	Sequence
1	814	5`CTC TCT CTC TCT CTC TTG 3`
2	44B	5 CTC TCT CTC TCT CTC TGC 3
3	HB-10	5` GAGAGAGAGAGACC 3`
4	HB-11	5` GTGTGTGTGTGTCC 3`
5	HB-12	5 CAC CAC CAC GC 3

Table 3: Sequence and annealing temperature of PCR primers used in amplification of BADH gene

Specific primer	Primer Nucleotide Sequence	Annealing temperature	Product gene analogs sizes
BADH	F 5- TCCTCTCGTCTCCAGTCCAC -3	60	300 bp
	R-5- AATGCAGACTAACAACCCATGA-3		200bp
			700bp

Isozymes Patterns: Native-polyacrylamide gel electrophoresis (Native-PAGE): 1gm of new vegetative shoot samples were homogenized in 2ml distilled water as extraction buffer using a mortar and pestle. The extract was then transferred into clean Eppendorf tubes and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was transferred to new Eppendorf tubes and kept at –20°C until use for electrophoretic analysis according to Stegemann *et al.* [22]. After electrophoresis, the gels were stained according to their enzyme system with the appropriate substrate and chemical solution. Then, incubated at 37°C in a dark room for complete staining. The staining gels were carried out according to Heldt [23] for POD and PPO. Gels were washed two or three times with tap water, fixed in ethanol: 20% glacial acetic acid (9:11 v/v) for 24 hours and photographed by Biometra Bio Doc Analyze 2005.

DNA Preparation: Genomic DNA was extracted from new vegetative aerial parts of ten plants chosen randomly for each population by DNA isolation Thermo scientific kit Gene JET Plant Genomic DNA Purification Mini Kit. DNA was quantitated by gel electrophoresis for one hour at 100 volt in Biometra submarine (40 x 20 cm). Bands were detected on UV- transilluminator and photographed by Biometra Bio Doc Analyze 2005.

ISSR Analysis for DNA Amplification: ISSR-PCR reactions were conducted according to Sharma *et al.* [24] using five preselected primers which were synthesized by (Metabion GmbH Germany); 814, 44B, HB10, HB11and HB12 (Table 2). The reaction conditions were optimized and mixtures contained in 25µl total volumes as the following: 1.0µl dNTPs (8 mM), 1.0µl Taq DNA polymerase (1 U/ 1µl), 2.5µl 10 X buffer, 3 µl MgCl2 (15 mM), 1.0µl Primer (10 mM), 1.0µl Template DNA(10-50 ng/µl) and 15.5µl H_2O up to 25µl. Amplification was carried out in a Strategene Robocycler Greadient 96 Robocycler device programmed for 30 cycles as follows: denaturation, (one cycle) 94°C for 4 minutes, annealing, 36°C for 1 minute and 30 seconds and finally extension, (one cycle) 72°C for 7 minutes. Agarose gel electrophoresis (1.5%) was used for resolving the PCR amplification products according to Sambrook *et al*. [25]. The run was performed for one hour at 100 V in Biometra submarine (40 x 20 cm). Bands were detected on UV-transilluminator and photographed by Biometra Bio Doc Analyze 2005.

Detection of Salinity using Specific Stress Gene: Genomic DNA based analysis of five *Halocnemum strobilaceum* genotypes were amplified by one primer of BADH salinity tolerance gene (Table 3). This primer was designed as degenerate primer based on gene conserved sequences and depending on the data bases of NCBI (National Center for Biotechnology Information).

Polymerase Chain Reaction (PCR): PCR reaction was accomplished by adding 3.00µl 10X buffer, 3.00µl dNTPs ((2.5 mM), 0.20µl Taq DNA polymerase, 2.00µl of each primer (forward and reverse), 2.00µl DNA and RNase free water up to 50µl and H_2O (d.w.) 16.80µl.

The DNA amplifications were performed in an automated thermal cycle (model Techno 512) programmed for one cycle at 94°C for 4 min followed by 35 cycles of 1 min at 94°C, 1 min at (52-58°C) and 1min at 72°C. The reaction was finally left at 72°C for 10 min. 20µl of PCR reaction was subjected to electrophoresis in 1.5 % agarose gel containing ethidium bromide (5µl), subjected to 100 V for 1hr and then photographed using UV gel documentation system and photographed by Biometra Bio Doc Analyze 2005.

Statistical Analysis: Analysis of variance was conducted for studying specific activities of POD, PPO, CAT and APX and total soluble protein according to Steel and Torrie [26] through computer program MSTATC and graph by Excel software then comparison of means were conducted for evaluated traits by least significant difference (LSD) at 5% probability level. Similarity matrix was developed by the statistical package for social science programme SPSS based on combined analysis of overall molecular and biochemical markers.

RESULTS

Some Physical and Chemical Properties of Soil Supporting *H. strobilaceum* at the Studied Regions: Data in Table 4 indicated that, soil texture were sandy clay loam in the four regions; L2, L3, L4 and L5 and loamy sand in L1. Moisture content was high, ranged from 26.76% at L2 to 12.29% at L3. Soil pH varied from moderate alkaline at L2, L3, L4 and L5 to high alkaline (9.02) at L1. Soil salinity could be classified to two orders; moderate salinity at L4 and high salinity at the others where, the salinity varied among them and L5 recorded the highest salinity.

Specific Activity of Enzymes of *H. strobilaceum* from Different Regions: Four enzymes POD, PPO, APX and CAT were investigated for specific activity as illustrated in Fig. 1. The highest and the lowest specific activities of POD were recorded in *H. strobilaceum* of Wadi Halazeen and Wadi Gharandal, respectively. While, those of Burg El-Arab and Wadi Halazeen had the highest and lowest PPO specific activities. With regard to APX, *H. strobilaceum* of Siwa Oasis had the highest specific activity followed by those of Burg El-Arab with non-significant change between them, Wadi Halazeen and Balouza plants without significant change between them, whereas *H. strobilaceum* of Wadi Gharandal attained the lowest specific activity. Specific activity of CAT can be ordered descendicaly among *H. strobilaceum* under the five different regions as Wadi Halazeen, Siwa Oasis, Burg El-Arab, Wadi Gharandal and Balouza plants. On the other hand, soluble protein recorded the highest accumulation in *H. strobilaceum* of Wadi Gharandal followed by those of Siwa Oasis, while *H. strobilaceum* of Wadi Halazeen recorded the lowest accumulation.

Biochemical Markers

Protein Marker: Protein analysis was carried out on five *H. strobilaceum* genotypes as illustrated in Fig. 2 and Table 5. Twenty one protein bands were observed among them included eight common bands with high density, nine polymorphic bands and four unique bands produced 62% of polymorphism. The four unique bands were distributed as two for *H. strobilaceum* from Wadi Gharandal with molecular weights (97.8 and 29.9 kDa), one for *H. strobilaceum* from Wadi Halazeen with molecular weight (27.7 kDa) and one for *H. strobilaceum* from Siwa Oasis with molecular weight (6.5 kDa) as marker proteins.

Isozyme Analysis: Two isozyme systems including POD and PPO were used to investigate genetic variations of five *H. strobilaceum* genotypes as shown in Fig. 3 and Table 6. A total of twelve bands, nine polymorphic bands (75% of polymorphism) and three monomorphic bands were observed. *H. strobilaceum* from Wadi Halazeen produced the highest number of bands (6) bands for POD followed by Burg El-Arab (5) bands, Siwa Oasis, Balouza (4) bands and Wadi Gharandal that produced the lowest number (3) bands. *H. strobilaceum* from Wadi Halazeen revealed one unique band with (Rf, 0.805) for POD. While, *H. strobilaceum* from Burg El-Arab produced the highest number of bands, (5) bands for PPO followed by Siwa Oasis, Balouza that produced (4) bands and finally, W. Gharandal that produced the lowest number of bands, (3) bands. *H. strobilaceum* from Burg El-Arab revealed one unique band with (Rf, 0. 0.064) for PPO. Additionally, PPO and POD produced (6) bands for each, PPO produced (100%) polymorphism and POD produced (50%) polymorphism.

Genetic Similarity and Cluster Analysis Based on Isozymes: Based on isozyme markers, similarity matrix was developed by SPSS computer package system as presented in Table 7 and Fig 4. The highest relationship (88%) was scored between *H. strobilaceum* from Burg El-Arab and Balouza. While, the lowest relationship (16%)

Table 4: Some physical and chemical properties of soil supporting *H. strobilaceum* taken from different regions

Studied regions	Silt %	Clay %	Sand %	Texture	Moisture %	pH	EC (dSm^{-1})
L1 (Siwa Oasis)	17.44	0.50	82.06	Loamy sand	20.87	9.02	36.2
L2 (Burg El-Arab)	23.57	26.52	49.91	Sandy clay loam	26.76	8.40	26.5
L3 (Balouza)	5.22	23.47	71.31	Sandy clay loam	12.29	8.33	35.6
L4 (Wadi Gharandal)	13.31	29.24	56.75	Sandy clay loam	24.29	7.97	3.2
L5 (Wadi Halazeen)	5.69	25.58	68.73	Sandy clay loam	15.66	8.57	47.4

Table 5: Number, types and polymorphism percentage of soluble protein of five *H. strobilaceum* genotypes

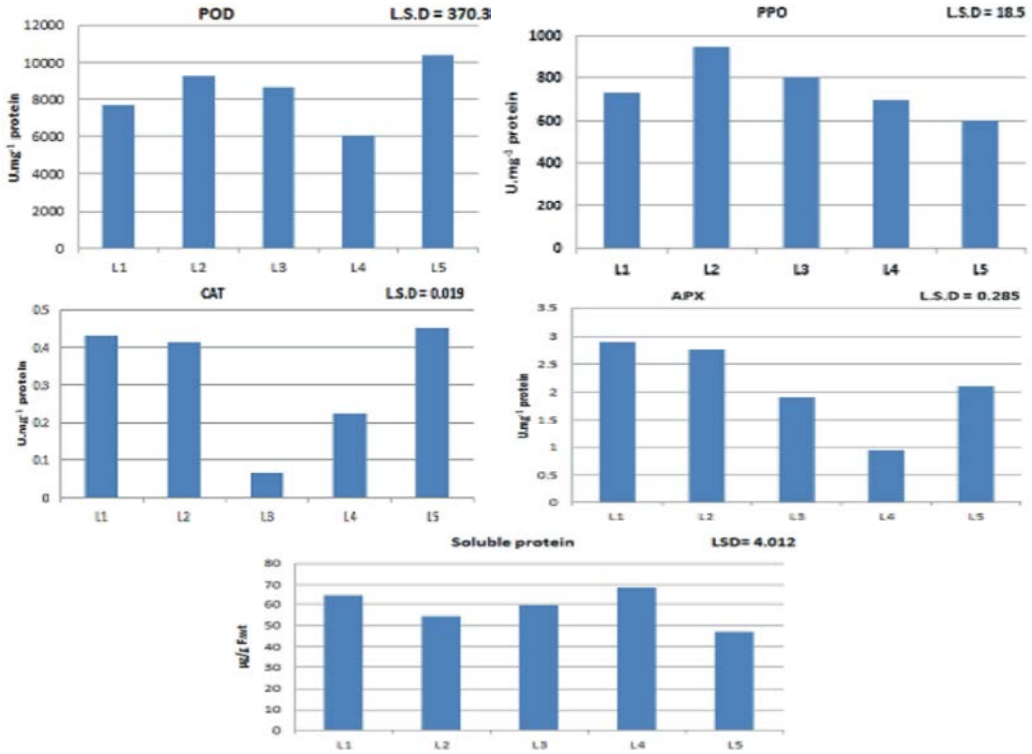
	Polymorphic bands			
Monomorphic bands	Non-unique bands	Unique bands	Total bands	Polymorphism %
8	9	4	21	62

Table 6: Polymorphism percentages generated by the two isozyme systems of five *H. strobilaceum* genotypes

Isozyme types	Mono-morphic bands	Polymorphic bands	Unique bands	Total bands	Polymorphism %
POD	3	2	1	6	50
PPO	0	5	1	6	100
Total	3	7	2	12	75

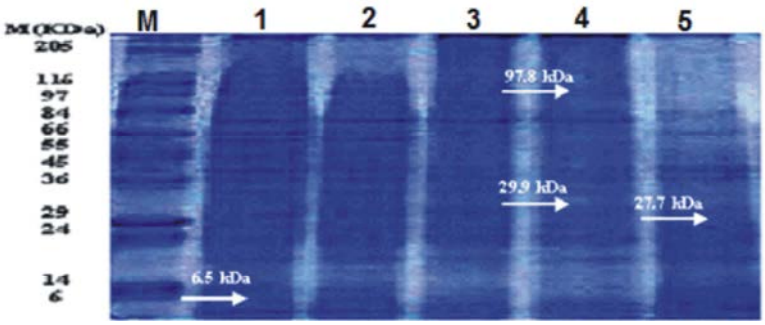
Table 7: Similarity matrix based on isozymes of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and Wadi Halazeen

Genotypes	1	2	3	4	5
1	100				
2	22	100			
3	20	88	100		
4	47	16	21	100	
5	87	58	40	46	100



L1= Siwa Oasis, L2 = Burg El-Arab, L3 = Balouza, L4 =Wadi Gharandal and L5 = Wadi Halazeen

Fig. 1: Specific activity of peroxidase (POD), polyphenol oxidase (PPO), catalase (CAT) and ascorbate peroxidase (APX) and soluble protein of *H. strobilaceum* from studied regions using LSD at 5%



* (M) = Marker

Fig. 2: SDS-PAGE profile of total soluble proteins of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and Wadi Halazeen

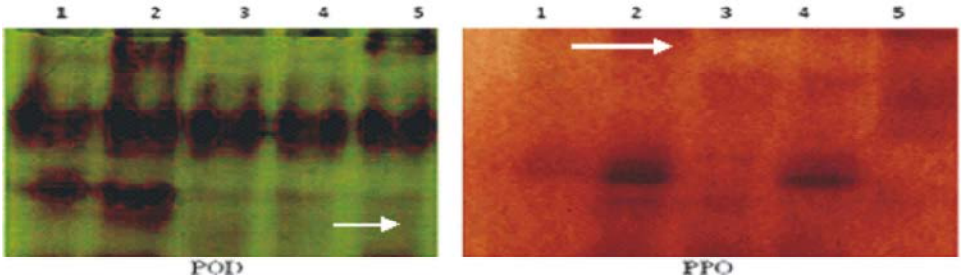


Fig. 3: POD and PPO isozyme banding patterns of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and Wadi Halazeen

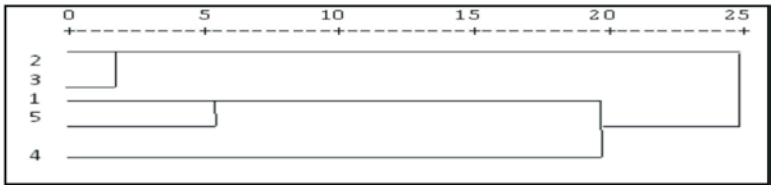


Fig. 4: Dendrogram based on isozymes markers of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and Wadi Halazeen

was scored between *H. strobilaceum* from Burg El-Arab and Wadi Gharandal. The dendrogram based on isozymes, separated the five *H. strobilaceum* genotypes into two main clusters. Moreover, *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza and Wadi Halazeen were separated in the first cluster. While, *H. strobilaceum* from and Wadi Gharandal were separated in the second cluster.

Molecular Markers

ISSR Analysis: Five ISSR primers were used in the present investigation to study the genetic diversity and relationships of five *H. strobilaceum* genotypes as shown in Fig. 5 and Table 8. Thirty four total bands, twenty two monomorphic bands and twelve polymorphic bands (35%) polymorphism were revealed. Nine bands were unique and could be considered as specific bands for each of the genotypes. *H. strobilaceum* from Wadi Halazeen produced (29) total bands, five unique bands of the molecular weights; 674, 475.9, 453.7, 400.2 and 300.5 bp and one negative marker of the molecular weight; 251.9bp. Also, *H. strobilaceum* from Burg El-Arab produced (29) total bands, four unique bands of the molecular weights; 606.3, 500.6, 223.5 and 213.5bp. Finally, *H. strobilaceum* from Siwa Oasis produced one negative marker of the molecular weight; 589.9 bp.

Genetic Similarity and Cluster Analysis Based on ISSR: Based on ISSR marker, similarity matrix was developed by SPSS computer package system as presented in Table 9 and Fig. 6. The highest relationship (90%) was scored between *H. strobilaceum* from Burg El-Arab and Balouza. While, the lowest relationship (26%) was scored between *H. strobilaceum* from Siwa Oasis and Wadi Halazeen.

Table 8: Primer codes, sequences, length range (bp), monomorphic bands, polymorphic bands, total amplified bands and polymorphism percentages of the five ISSR primers

Primer code	Length range (pb)	Mono-morphic bands	Poly-morphic bands	Unique bands	Total amplified bands	Polymorphism (%)
814	214-606	3	3	6	12	75
44B	223-614	5	0	1	6	16
HB10	199-400	4	0	1	5	20
HB11	150-500	4	0	0	4	0
HB12	123-674	6	0	1	7	14
Total	--	22	3	9	34	35

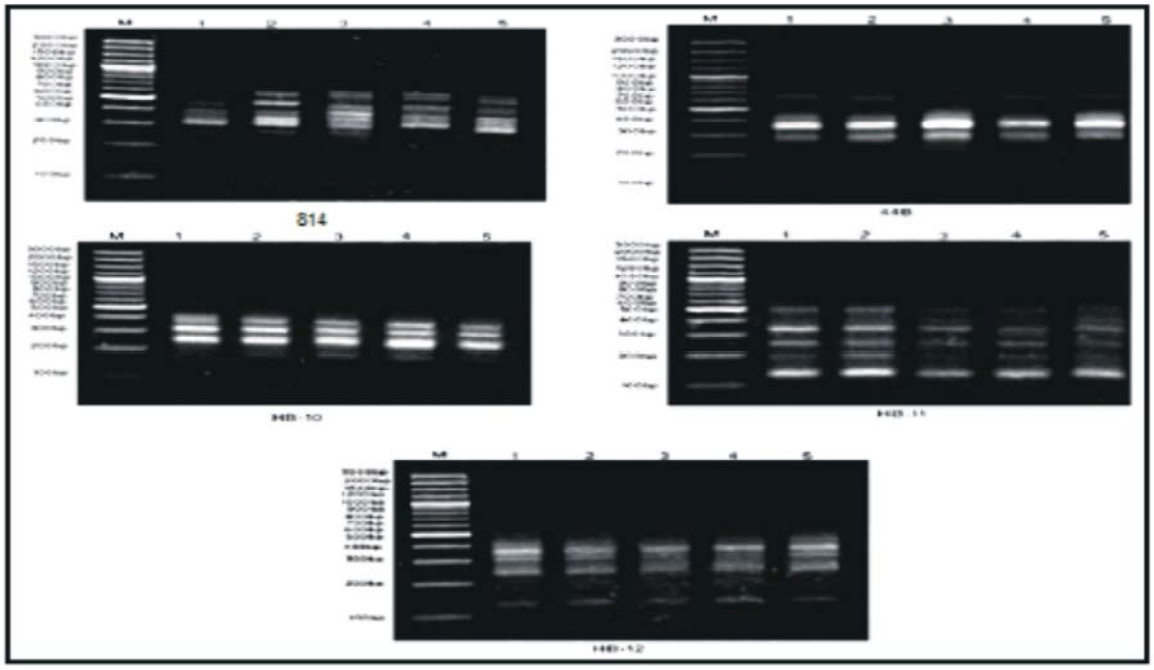


Fig. 5: ISSR primers of 814, 44B, HB10, HB11and HB12 of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and Wadi Halazeen

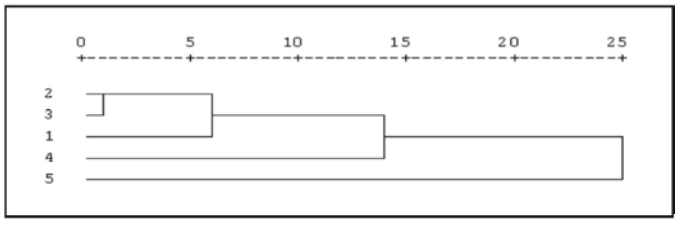


Fig. 6: Dendrogram based on ISSR marker of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and Wadi Halazeen

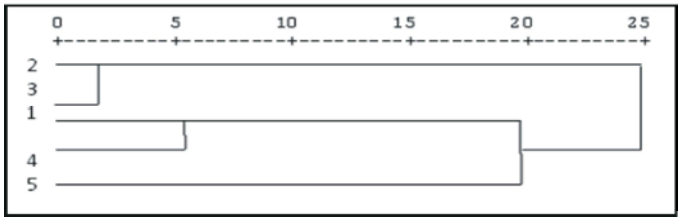


Fig. 7: Dendrogram based total analysis (protein, isozymes and ISSR) markers of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and Wadi Halazeen

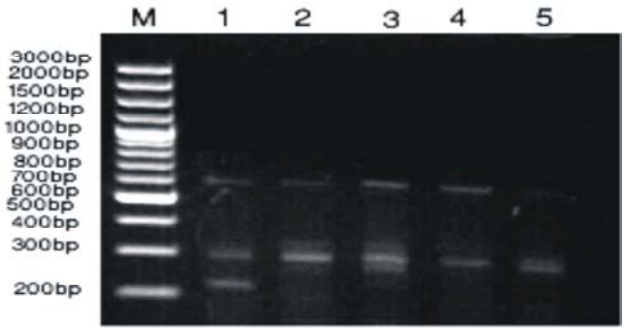


Fig. 8: The PCR product using specific primer of BADH gene paraloges indicated that appearance of three bands with fragment size 200,300 and 700bp

Table 9: Similarity matrix based on ISSR of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and W. Halazeen

Genotypes	1	2	3	4	5
1	100				
2	82	100			
3	62	90	100		
4	40	57	74	100	
5	26	45	40	37	100

Table 10: Similarity matrix of total analysis (protein, isozymes and ISSR) markers of five *H. strobilaceum* from Siwa Oasis, Burg El-Arab, Balouza, Wadi Gharandal and Wadi Halazeen

Genotypes	1	2	3	4	5
1	100				
2	68	100			
3	64	89	100		
4	85	48	78	100	
5	41	86	47	17	100

Combined Analysis Based on (Protein, Isozymes and ISSR): Based on total analysis (protein, isozymes and ISSR), similarity matrix was developed by SPSS computer package system as in Table 10 and shown in Fig. 7. The closest relationship was scored between *H. strobilaceum* from Burg El-Arab and Balouza with similarity (89%) and the lowest similarity (35%) was between *H. strobilaceum* from Wadi Gharandal and Wadi Halazeen. The dendrogram based on overall analysis, separated the five *H. strobilaceum* genotypes into two main clusters. Moreover, *H. strobilaceum* from Balouza and Burg El-Arab were separated in the first cluster. While, *H. strobilaceum* from Siwa Oasis, Wadi Gharandal and Wadi Halazeen were separated in the second cluster.

Detection of Salinity Gene in *H. strobilaceum*: The PCR product using specific primer of BADH gene indicated the appearance of three bands with fragment size 200, 300 and 700 bp as shown in Fig. 8.

DISCUSSION

The induction of antioxidant enzymes such as POD and CAT with high specific activity in *H. strobilaceum* inhabiting Wadi Halazeen associated with the highest soil salinity and high total band numbers in POD isozyme, which could be considered as one mechanism of salinity tolerance in *H. strobilaceum*. These enzymes are involved in eliminating H_2O_2 from the salt stressed plants [27]. Similar results were obtained by Youssef [3], who found that catalase tended to increase in halophytic plants collected from higher salinity site. Also, the decrease of POD in *H. strobilaceum* of Wadi Gharandal which was associated with the lowest soil salinity and high moisture content indicated that POD play an important role in the defense against oxidative stress resulted from salinity. In addition, the presence of a unique band of POD in *H. strobilaceum* from Wadi Halazeen could be considered as specific marker for salinity tolerance. These results agreed with Arafet *et al*. [28], who indicated the correlation between the increases in peroxidase expression in relation to salinity in *Lepidium sativum*. Furthermore, Tayefi *et al*. [29] found that the increase of catalase expression in *Carthamus tinctorius* L. was in relation to salinity tolerance as a defense system against ROS. Regarding PPO, data indicated that *H. strobilaceum* from Burg El-Arab produced the highest specific activity, bands number and a unique band with Rf 0.064. While *H. strobilaceum* from Wadi Halazeen which is supported by the highest salinity contained the lowest activity. In this trend, Silambarasan and Natarajan [30] concluded that *Clerodendron inerme* plant treated by NaCl enhanced the activity of CAT and PPO up to optimum level of salinity and at higher salinity these activities declined steadily. APX was correlated positively with pH, recorded the highest activity in Siwa Oasis plants (high alkaline soil) and the lowest in those of

Wadi Gharandal (moderate alkaline). APX increased in *H. strobilaceum* under high salinity till optimum value in Siwa Oasis and Burg El-Arab, then decreased in Wadi Halazeen (the highest salinity) and attained the lowest activity in Wadi Gharandal supported by the lowest salinity. Boughalleb *et al*. [31] reported that, the antioxidative defense capacity of *Zygophyllum album* L. plants might be achieved by the increasing activities of SOD, POD and APX which showed to participate efficiently in restriction of oxidative damages caused by the H_2O_2 generation under salt stress. The APX keeps the plasticity of cell wall by reducing hydrogen peroxide. The ascorbate reversibly inhibits the activity of apoplast APX, prevents the conversion and secretion of free radicals into the apoplast and regulates the lignification of plant cell wall [32].

In the present study, the enzyme specific activities of POD and PPO could be used as markers depending on the highest enzymes activity recorded. The high percentage of polymorphism 50% of POD and 100% of PPO were in agreement with those obtained by Haddioui and Baaziz [33], who used seven isozyme systems to investigate genetic diversity in *Atriplex halimus* L. and detected high percentage of polymorphism among populations from different locations in Morocco. Additionally, Abd El-Maboud and Khalil [5], used eight isozymes to detect genetic diversity and relationships in some species of the genus *Suaeda* from different sites in Egypt along the Mediterranean Sea coast, produced twenty one total bands with (76%) of polymorphism. Finally, they indicated that antioxidant defense mechanism by different isozymes protected the cellular structures from the harmful effects of free radicals. Twenty one protein bands were observed among the five genotypes of *H. strobilaceum*, nine polymorphic bands produced (62%) polymorphism. Furthermore, four unique bands were detected, two for *H. strobilaceum* from Wadi Gharandal, one for *H. strobilaceum* from Wadi Halazeen and one for *H. strobilaceum* from Siwa Oasis as specific markers for each. On the other hand, *H. strobilaceum* from Wadi Gharandal produced the highest number of bands followed by Siwa Oasis, Burg El-Arab, Balouza and Halazeen which was in accordance with the soluble protein accumulation results. A decrease in soluble protein under the highest salinity in *H. strobilaceum* of Wadi Halazeen could be a typical symptom of oxidative stress. These results agreed with Bavei *et al*. [34], who found that soluble protein content were decreased in leaves and roots of the three *Sorghum bicolor* L. varieties; Payam, Kimia and Jambo with increasing salinity. Protein content in the tissues of many plants declined under drought or salinity stress, because of proteolysis and decreased protein synthesis [35]. The five studied ISSR primers produced 35% polymorphism. This result agreed with that obtained by Tuiray and Roy [12], who differentiated Chenopodiaceae species from Amaranthaceae of India. A total of 293 RAPD and 177 ISSR fragments were generated with 15 (out of 50) arbitrary primers and 11 (out of 30) ISSR primers, respectively, produced 53% and 55% of polymorphism. In addition, De Britto *et al*. [36] evaluated the genetic diversity of *Nothapodytes nimmoniana* landraces collected from different populations of western and south India by ISSR where 12 primers produced a total of 108 bands with 87% polymorphism. Analysis of ISSR can be accomplished for studying the genetic relationships among and within genotypes facilitate the conservation and utilization of such species in breeding programs. Recently, ISSR primers produced 99% of polymorphism in some species of the genus *Suaeda* (Chenopodiaceae) from different sites in Egypt along the Mediterranean Sea coast [5]. The PCR product using specific primer of BADH gene indicated that the appearance of three bands with fragment size 200, 300 and 700 bp. These results agreed with Shrestha [37], who characterized BADH gene in hexaploid wheat with three paralogs of the gene. Spinach from Chenopodiaceae had two BADH paralogs; BADH1 and BADH2 [38]. The dendrogram based on overall analysis, separated the five *H. strobilaceum* genotypes into two main clusters. Moreover, *H. strobilaceum* from Balouza, Wadi Gharandal and Burg El-Arab were separated in the first cluster. While, *H. strobilaceum* from Siwa Oasis and W. Halazeen were separated in the second cluster.

CONCLUSION

The results of the study, focused on estimating the polymorphism of five *H. strobilaceum* genotypes from different regions, using biochemical markers *viz*; protein and isozymes and each were realistic in comparing the gene expression variation of the five *H. strobilaceum* genotypes with a considerable level of polymorphism that reflected the different adaptive responses to different environmental conditions by increasing the expression and activity of antioxidant enzymes and showing that POD was the most promising antioxidant defense system and proved to be the effective tool to evaluate genetic diversity. In addition, ISSR revealed the lowest polymorphism because of the close relationship among

the five genotypes represent the same plant and as stable marker. The gain of this investigation may help to assess national conservation programs in Egypt and breeding programs to get benefit from valuable stress tolerance genes for the improvement of other crops.

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