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MOLECULAR MARKERS ASSISTED GENETIC CHARACTERIZATION OF DIFFERENT SALT TOLERANT PLANT SPECIES

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ABSTRACT

Genetic diversity of thirteen salt tolerant plant species namely *Pergularia tomentosa*, *Salvadora oleoides*, *Malcolmia africana*, *Peganum hermala*, *Capparis spinosa*, *Tamarix aphylla*, *Prosopis juliflora*, *Rhazya stricta*, *Cynodon dactylon*, *Aerva persica*, *Cymbopogon jwarancusa*, *Calotropis procera* and *Salavadora persica* was determined by using RAPD markers. In total ten primers from the OPC series were used, out of which nine primers gave reproducible amplification profiles. Out of the nine all except OPC-1, produced polymorphic bands and Numerical Taxonomy and Multivariate Analysis System (NTSYS) revealed 56% similarity among the selected species. Monomorphic bands produced by OPC-1 may be related to some genetic elements involved in managing the life under high salt conditions. There is a need to further find out the relationship of these monomorphic bands with genes involved in salt tolerance. Such type of genetic resemblance among halophytes should be further studied for the utilization of rich gene pool for crop improvement.

Key words: Genetic Diversity, RAPD, Salt tolerance

INTRODUCTION

Plants are usually exposed to different types of stresses which adversely affect their growth and productivity. The most severe type of stresses that the plant my face include salinity and drought stress (Bohnert *et al*., 1999). More than 20 % of all the cultivated lands around the world are affected by salinity (Flowers and Yeo, 1995). Salinity causes reduction in the ability of the plants to take up water from the soil leading to reductions in the growth rate, premature senescence and reduction in the photosynthetic leaf area of the plant (Munns, 2002). Salt stress may also cause stomata closure causing reduction in the availability of carbon dioxide (Parida and Das, 2005) and generation of reactive oxygen species (ROS) causing oxidative stresses (Parvaiz and Satyawati, 2008). Most of the plants cannot be grown on a salt affected land (Glenn and Brown, 1999) but some of the plants have the ability to grow under salinity due to the presence of different mechanisms in them for salt tolerance such plants are known as salt resistant plants, salt tolerant plants or halophytes (Flowers *et al*., 1986). These salt tolerating plants represent only 2 % of terrestrial plant species but they represent a wide diversity of plant forms (Glenn and Brown, 1999). The largest number of halophytes is included in Chenopodiaceae and it consists of about 550 halophyte species (Aronson, 1989). Halophytes can be divided into two main types on the basis of habitat. These two types are hydro halophytes which include those plants that can be grown in aquatic soils and in wet conditions and xerohalophytes representing those plants that can be grown in the soil where the water content of the soil is less due to evaporation but the soil is saline (Youssef, 2009).

Plants generally respond against salinity at two levels i.e. physiological level and molecular level (Munns and Tester, 2008). According to Sabovljevic and Sabovljevic (2007), the mechanisms for the salt resistance in halophytes generally fall into two main categories i.e. salt tolerance and salt avoidance. Salt tolerance involves certain physiological or biochemical adaptations in the plants, which help the plant to maintain protoplasmic viability (Sabovljevic and Sabovljevic, 2007). Salt avoidance may involve certain physiological and structural adaptations so as to minimize the salt concentrations of the cell or physiological exclusion by root membranes, which may involve passive exclusion of ions by means of a permeable membrane, the active expelling of ions by means of a pump or dilution of ions in the tissues of the plants (Allen *et al*., 1994). Molecular mechanisms against salt stress involve the regulation of certain genes due to salt tress (Xiong and Zhu, 2002). These genes encode LEA proteins (late embryogenesis proteins), enzymes (involved in biosynthesis of osmolytes, hormones, detoxification and general metabolism), transporters (ions transporters, ABC i.e. ATP-binding cassette transporters) and regulatory molecules (protein kinases etc).

DNA based molecular markers are a versatile tool and have applications in various fields like taxonomy, physiology, embryology, genetic engineering etc. (Joshi *et al*., 1999). Polymerase chain reaction (PCR) based techniques have been used for the identification of

molecular markers associated with various traits of agricultural importance (Raman *et al*., 2002; Masood *et al*., 2005). Among PCR based molecular markers randomly amplified polymorphic DNA (RAPD) is a widely used technique in different plants (Mahmood *et al*., 2010 a,b; Kayani *et al*., 2011; Mahmood *et al*., 2011 a,b,c; Nazar and Mahmood, 2011; Shinwari *et al*. 2011). Earlier, the association between RAPD markers and salt tolerance was investigated by Wang *et al*. (1997) and later on by Zhang *et al*. (1999) out of the total hundred and forty-eight polymorphic bands Zhang *et al*. (1999) found six bands to be significantly associated with salt tolerance. In another study, RAPD markers associated with salt tolerance in tomato were identified (Foolad and Chen, 1997). The present study employed the use of RAPD markers for the identification of genetic elements that might be involved in salt tolerance as well as the evolutionary relationships among thirteen salt tolerant plant species were also determined.

MATERIAL AND METHODS

Salt tolerant plant specimens were collected from Khewra (District Jehlum, Punjab, Pakistan) located at a distance of 160 Km from the city of Islamabad and is about 288 meters (945 feet) above the sea level. Khewra mines are situated at the foot hills of salt range between longitudes 07300, 26.9 E and latitudes 3239, 03.4 N (Naz *et al*., 2009). The plant collection was made in the month of April from a region near to the Khewra salt mine. A total of thirteen plant samples were selected (Table 1).

Determination of electrical conductivity (EC) and pH of soil sample collected from Khewra: Ten cm deep soil sample was collected from the same region, from where plants were collected. Sample was air dried, sieved out, subjected to grinding and was preserved at 4 ºC. Soil sample (2 g) was dissolved in distilled water (20 ml) and EC meter (Milwaukee instruments, SM802) was used for the determination of electrical conductivity and pH of the soil.

Extraction of the genomic DNA: Different protocols were tried for the isolation of genomic DNA but finally CTAB method (Richards, 1997) was adapted. The extracted DNA was preserved at -20 ºC. The quality of the isolated DNA was checked by running it on 1 % agarose gel prepared in 0.5 X TAE (Tris –acetate EDTA) buffer.

Screening of the RAPD primers: The amplification reactions were performed by random primers from OPC series (OPC-1 to OPC-10, Table 2).

Polymerase chain reaction: A total of 25 µl reaction mixture containing 1 µl primer (25 pmol), 12.5 µl PCR master mix (Fermentas), 10.5 µl of nuclease free water (Fermentas) and 1 µl of the DNA template (25-50 ng) was prepared. The amplification was conducted in a thermal cycler (Labnet multi gene). After several experiments for optimizing the best suitable conditions, a program for PCR reaction was standardized with following settings; initial denaturation step at 94 ºC for 1 minute, followed by a run of 44 cycles, each starting with denaturation at 94 ºC for 30 seconds, annealing at 40 ºC for 1 minute and ended by extension at 72 ºC for 2 minutes. Final extension was carried out at 72 ºC for 10 minute, and then the temperature was set at 4 ºC till the removal of the PCR tubes. The PCR products and 100 bp plus DNA ladder (Fermentas) were subjected to electrophoresis using 1.5 % agarose gel in 0.5 X TAE buffer, followed by staining with ethidium bromide. The gel was finally visualized in a gel documentation system (Wealtech, Dolphin Doc Plus).

Scoring of bands and data analysis: All the gels were analyzed carefully and the data scoring was performed by marking the presence of a specific band as 1 and its absence as 0. A data matrix was developed in this way for the estimation of similarities and dissimilarities in salt tolerant plants. Cluster analysis was then performed based on similarity coefficient among the samples, based on molecular data by using Numerical Taxonomy and Multivariate Analysis System (NTSYS) Pc version 2.01 (Rohlf, 2002).

RESULTS AND DISCUSSION

Isolation of genomic DNA and EC/pH determination of the soil sample: High quality DNA was observed after running the extracted DNA on 1 % agarose gel. EC value and pH of the soil was found to be 4.3 dS/m and 8.64 respectively. Usually EC value for the non saline soil ranges from 0 – 2 dS/m, whereas the soils having EC value > 4 dS/m are considered to be saline.

RAPD analysis: Out of the total 10 decamer primers of the OPC series, 8 have shown polymorphic banding patterns, one (OPC-7) has shown no results, while OPC-1 has shown monomorphic banding patterns. The amplified band produced by OPC-1 (550 bp) may be significantly associated with those genetic elements that are regulated by salt stress as the existence of monomorphic band produced by OPC-1 in all the salt tolerant plant species collected from Khewra. All these salt tolerant species are important for plant breeding as plants growing under saline conditions have a wide range of adaptations. Moreover, they are useful candidate to study mechanisms promoting the salt tolerance in plants. None of the other primer produced monomorphic banding pattern, but some of the primers produced polymorphic bands that were present in majority of the species, while absent in two or three species such as OPC-4 produced one DNA fragment with a molecular weight of 550 bp which was

found in all the species except *Tamarix aphylla* (6) and *Aerva persica* (10). Similarly, OPC-5 produced a band with molecular weight of 350 bp that was present in all the species except *Tamarix aphylla* (6). Such bands may also be related to those traits which are involved in salt tolerance due to their existence in majority of the salt resisting species.

The species which produced maximum number of bands was *Capparis spinosa* (5) which produced a total of 16 bands while the species which produced least number of bands was *Calotropis procera* (12) which displayed only 8 amplified fragments. It was observed that OPC-2 has produced maximum number of fragments i.e. thirty three while the least number of bands (4) were produced by OPC-9 (Table 3). Moreover, the highest value of percentage polymorphism (50 %) was revealed by OPC-9, while OPC-5 showed the least (Table 4). The bands produced by all the primers were found to be having molecular weight in the range of 200-700 bp, while 4 unique and 3 rare bands were observed (Table 4), indicating the significance of these primers to distinguish the genotypes with useful traits. *Salvadora persica* (13) displayed three unique bands with the molecular weight of 300, 700 and 600 bp while, 1 unique band was displayed by *Capparis spinosa* (5) with the molecular weight of 400bp.

In total nine primers amplified a total of 148 bands (Table 3), revealing a polymorphism of 91.2 % and a monomorphism of 8.8 %. Although the species under study were very diverse and belonged to different families and genera but the existence of a monomorphism of 8.8 % indicates the common genetic elements among salt tolerant plant species which might be related to salt tolerance.

Cluster analysis based on amplified products by 8 primers: Un weighted pair group method with arithmetic mean (UPGMA) cluster analysis based on 8 primers revealed that all the salt tolerant plant species under study had a similarity between 55-90 % (Fig. 1). Genetic similarity coefficient of thirteen salt tolerant plant species based on Euclidean distances is given in Table 5. The high values of genetic similarity coefficients indicated close genetic relationships between the compared samples and the low values indicated remote relationships among the species (Hammad and Qari, 2010). The highest similarity value (0.90) was recorded between the species *Salvadora oeloides* (true halophytes) and *Rhazya stricta* (not reported as halophyte) while the second highest similarity value (0.85) was found between the species *Pergularia tomentosa* and *Aerva persica*, which are classified as true halophytes (Khan and Qaiser, 2006). On the other hand, the lowest similarity value (0.33) was found between the species namely *Peganum hermala* and *Salvadora persica* indicating that these species are distantly related to each other.

In this study there are collections with high similarity index although they belong to different species. Such high similarity indices suggest that true halophytes are genetically closely related to each other (0.85) however, during the course of evolution the non halophytic plants growing under saline conditions tend to develop genetic similarities with true halophytes, similarity value of 0.90 indicates this fact. By employing molecular markers the present study has helped to resolve the genetic relationship among the taxonomically diverse salt tolerant plant species. The genetic diversity is believed to be the result of ecological interactions particularly the competition within small habitat. The increasing competition between halophytes and their associates, as evidenced by their deep and wide intrusion into halophytic habitats, may ultimately lead to the evolution of new species which is better adapted to the ecological conditions. Such as genetic associations found between *Salvadora oleoides* and *Rhazya stricta* which seems to be a positive indication towards this fact. The competition for survival on salt affected land caused a great deal of genetic modification in *Rhazya stricta* (8), and hence in order to sustain itself in the saline habitat this species may have developed characters similar to true halophyte i.e. *Salvadora oleoides* (2). Further, elaborating the study on the phylogeny of halophytes using molecular markers may help to understand the evolution of these specialized taxa, and the evolutionary pathways by which the highly specialized and adaptive characteristics of halophytes to the saline soil has been achieved.

Dendrogram constructed using neighbor joining method of cluster analysis separated all the thirteen species of salt tolerant plants into 2 main cluster (C 1 and C 2). C 1 consisted of five species. Out of the 5, 4 species namely (*Pergularia tomentosa*, *Aerva persica*, *Tamarix aphylla* and *Salvadora persica*) are true halophytes (Khan and Qaiser, 2006) indicating that these halophytes are genetically related to each other. The remaining species (*Capparis spnosa*) diverged into a separate lineage at 57 % similarity level and remained parallel to the other lineages of C 1. In the list of halophytes from Pakistan reported by Khan and Qaiser (2006) none of the member of Capparaceae family is included, but the present study revealed that *Capparis spinosa* (5) showed a close genetic relationship with the true halophytes reported by Khan and Qaiser (2006). In order to survive on salt affected land along with the true halophytes, *Capparis spinosa* has developed some genetic characters similar to the true halophytes which enable it to compete with them in saline environment; such results highlight the trends towards adaptation and evolution. However, *Capparis spinosa* (5) is mentioned along with other salt tolerant plants of coastal subkhas of Pakistan by Khan and Gul (2002).

C 2 consisted of 8 species namely *Salvadora oleoides* (2), *Rhazya stricta* (8), *Cynodon dactylon* (9),

Malcolmia africana (3), *Pergularia tomentosa* (4), *Prosopis juliflora* (7), *Cymbopogon jwarancusa* (11) and *Calotropis procera* (12). At 60 % similarity level C 2 was diverted into two sub clusters (Sb 2 and Sb 3). Such diversion indicates evolutionary processes which occur due to the modification in DNA with the passage of time. The lineage which appeared at 69 % similarity level consisted of 2 species namely *Malcolmia africana* (3) and *Peganum hermala* (4). These two species formed separate lineages at 76 % similarity level which remained parallel to each other. Both of the species have not been reported as true halophytes however their existence with other halophytes on salt effected land (EC= 4.3dS/m and pH=8.64) indicates their genetic resemblance with them based on the RAPD primers used. The remaining 3 species which are true halophytes namely *Prosopis juliflora* (7), *Cymbopogon jwarancusa* (11) and *Calotropis procera* (12) constituted Sb 3 indicating their common ancestor and genetic resemblance.

Table -1. List of investigated salt tolerant plant species.

S.No.	Botanical names	Family
1.	*Pergularia tomentosa*	Asclepiadaceae
2.	*Salvadora oleoides*	Salvadoraceae
3.	*Malcolmia Africana*	Brassicaceae
4.	*Peganum hermala*	Nitrariaceae
5.	*Capparis spinosa*	Capparaceae
6.	*Tamarix aphylla*	Tamaricaceae
7.	*Prosopis juliflora*	Fabaceae
8.	*Rhazya stricta*	Apocynaceae
9.	*Cynodon dactylon*	Poaceae
10.	*Aerva persica*	Amaranthaceae
11.	*Cymbopogon jwarancusa*	Poaceae
12.	*Calotropis procera*	Asclepiadaceae
13.	*Salvadora persica*	Salvadoraceae

Table -2. Sequence of primers used from OPC series (OPC-1 to OPC-10)

Sr. #	Primer Code	Primer Sequence	Nucleotide Length (bp)
1.	OPC1	TTCGAGCCAG	10
2.	OPC2	GTGAGGCGTC	10
3.	OPC3	GGGGGTCTTT	10
4.	OPC4	CCGCATCTAC	10
5.	OPC5	GATGACCGCC	10
6.	OPC6	GAACGGACTC	10
7.	OPC7	GTCCCGACGA	10
8.	OPC8	TGGACCGGTG	10
9.	OPC9	CTCACCCTCC	10
10.	OPC10	TGTCTGGGTG	10

Table -3. Total bands produced by each primer from thirteen salt tolerant plant species

Species	Primers									Total
	OPC1	OPC 2	OPC 3	OPC 4	OPC 5	OPC 6	OPC 8	OPC 9	OPC10	bands
Pergularia tomentosa	1	3	0	1	1	1	2	1	1	11
Salvadora oleoides	1	3	0	1	2	0	4	0	0	11
Malcolmia Africana	1	3	0	3	2	1	3	0	0	13
Peganum hermala	1	4	1	3	2	0	2	0	1	14
Capparis spinosa	1	4	2	0	2	1	4	1	1	16
Tamarix aphylla	1	1	0	1	0	1	4	0	1	9
Prosopis juliflora	1	3	0	3	2	1	1	1	0	12
Rhazya Stricta	1	3	0	2	2	0	2	0	0	10
Cynodon dactylon	1	3	2	0	2	1	3	0	0	12
Aerva Persica	1	3	0	1	2	1	3	0	1	12
Cymbopogon jwarancusa	1	2	2	3	1	1	0	0	0	10
Calotropis procera	1	0	2	2	2	1	0	0	0	8
Salvadora persica	1	1	1	1	2	1	2	1	0	10
Total bands	13	33	10	21	22	10	30	4	5	148

Table- 4. Total number of monomorphic and polymorphic bands that were produced by nine RAPD primers from thirteen salt tolerant plant species

S.No.	Primers	Total bands	Monomorphic bands	Polymorphic bands	Rare bands	Unique bands	% polymorphism
1	OPC-1	13	1	0	0	0	0
2	OPC-2	33	0	4	1	0	12.1
3	OPC-3	10	0	3	0	1	30
4	OPC-4	21	0	3	2	0	14.2
5	OPC-5	22	0	3	0	1	13.6
6	OPC-6	10	0	1	0	0	10
7	OPC-8	30	0	5	0	1	16.6
8	OPC-9	4	0	2	0	1	50
9	OPC-10	5	0	1	0	0	20

Table -5: Genetic similarity coefficient of thirteen salt tolerant plant species based on Euclidean distances

	1	2	3	4	5	6	7	8	9	10	11	12	13
1	1.00												
2	0.61	1.00											
3	0.71	0.71	1.00										
4	0.57	0.66	0.76	1.00									
5	0.66	0.67	0.47	0.52	1.00								
6	0.71	0.71	0.61	0.47	0.47	1.00							
7	0.57	0.66	0.66	0.61	0.52	0.47	1.00						
8	0.61	0.90	0.71	0.76	0.47	0.61	0.66	1.00					
9	0.57	0.76	0.66	0.61	0.71	0.57	0.52	0.76	1.00				
10	0.85	0.76	0.76	0.61	0.71	0.76	0.61	0.66	0.61	1.00			
11	0.52	0.52	0.61	0.66	0.47	0.42	0.76	0.61	0.66	0.47	1.00		
12	0.47	0.47	0.57	0.52	0.42	0.47	0.61	0.57	0.61	0.52	0.76	1.00	
13	0.66	0.57	0.47	0.33	0.42	0.66	0.61	0.57	0.42	0.61	0.47	0.52	1.00

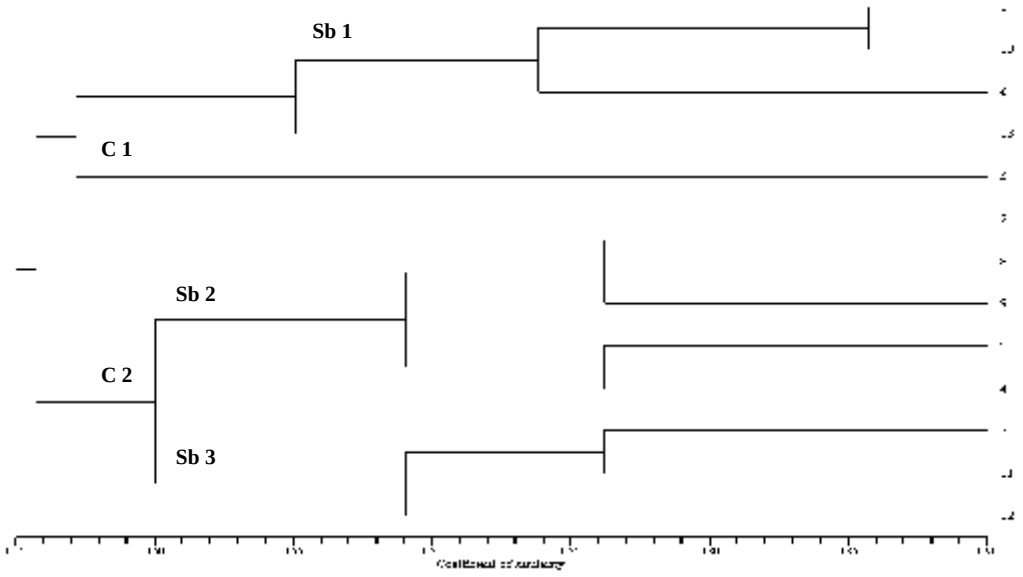

Figure1. Dendrogram representation of the genetic relationship among thirteen salt tolerant plants using NTSYS cluster analysis generated from eight RAPD primer.

Key to Fig. 1. 1: *Pergularia tomentosa*, 2: *Salvadora oleoides*, 3: *Malcolmia africana*, 4: *Peganum hermala*, 5: *Capparis spinosa*, 6: *Tamarix aphylla*, 7: *Prosopis juliflora*, 8: *Rhazya stricta*, 9: *Cynodon dactylon*, 10: *Aerva persica*, 11: *Cymbopogon jwarancusa*, 12: *Calotropis procera*, 13: *Salvadora persica*

Conclusion: The present study highlights that RAPD markers can be used to resolve the genetic relationships among taxonomically diverse salt tolerant plant species. These markers can also be used to find out common DNA fragments among the salt tolerant plants and relating the amplified bands with those genetic elements that may have some role in salt tolerance. Furthermore, the present study has revealed to find out the genetic associations among halophytes and their associates.

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