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EVALUATION OF BIOCHEMICAL AND MOLECULAR DIVERSITY IN *EXCOECARIA AGALLOCHA* LINN. GROWING AT VARYING HABITATS

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The study focuses the changes in potein and DNA banding patterns coupled with biochemical parameters in *Excoecaria agallocha* L. collected from different sites of Kollam, Kerala. The methodologies employed include- Random amplified polymorphic DNA (RAPD), Sodium dodecyl sulphate polyacrylamide gel elecrophoresis (SDS-PAGE) : biochemical and analytical. Notably proline concentratin sowed a remarkable variation in the plants collected from different sites. Higher concentrations of proline might be the reason of salinity tolerance in the species. The photosynthetic pigments, carbohydrates and crude protein also show varied response in the plants growing at different mangrove habitats. Total phenols exhibited significant variation whithin the population collected from the different habitats. This in turn suggests the possibility of variation in the magnitude of secondary metabolic pathway in these plants in tune to the habitat. From three varying habitats of study, 62 DNA bands were detected in total notwithstanding these bands were not found commonly to all. The protein finger printing revealed a maximum of 12 bands. Some bands are specific to the population of their habitats. These observations suggest the possible involvement of these polypeptides as stress proteins in the plants. Dendrogram of *E. agallocha* prepared from the RAPD data reveals three different clusters.

Keywords : Arabian Sea coast; *DNA; Excoecaria agallocha;* Habitat; Mangrove; Protein; Salinity.

Introduction

Mangrove ecosystem possesses great ecological and commercial value. This unique flora is fastly disappearing from their natural habitat due to various reasons. The plants belongs to diverse communities distributed along the inter-tidal zones of tropical and subtropical regions which have been widely loaded with sediments of effluents and solid wastes discharged. So they have large capacity in retaining heavy metals. The mangrove *Excoecaria* and *Xylocarpus* have the ability to dump excess salt in senescent leaves for new fruiting and growing season[1]. Only a few sutdies are available on mangroves, physiological and biochemical mechanisms under multiple heavy metal stress. In fact, mangrove plants are today growing in complicated environments that include multiple heavy metals and salinity. Hence, it is necessary to study the correlation of mangroves at different sites for the purpose of improving the conservation of mangrove ecosystem.

Excoecaria belongs to Euphorbiaceae, comprising about 40 species. The genus is native of the old tropical world. *Excoecaria agallocha* L. is an evergreen tree common to the estuarine banks, canals, creeks tidal forest and mangrove swamps. It is distributed in India, Sri Lanka, Burma, tropical South East Asia, North Australia and New Caledonia Southern Japan and Pacific Islands. The milky sap or latex that exudes from broken leaves, bark and twigs is poisonous. So it is commonly known as Blind-your-eye mangrove. Various parts of this plant have been used in the traditional medicine for the treatment of various ailments as well as the leaf exract showed antimicrobial property also[2]. Previous reports on mangroves of Kerala were restricted to the taxonomic aspects at gross level. A thorough knowledge of the physiological and biochemical features that are exclusively adapted to the growth of mangroves for their existence in the original habitat has not been elucidated so far. Based on this lacuna the present study has been done on *E. agallocha* Linn. collected from ecologically three distinct habitats under sub heading : analytical, molecular and biochemical variation.

Material and Methods

Plant materials- Three populations of *E. agallocha* Linn. were collected from their natural habitats from diverse regions of Kollam *i.e.* Ayiramthengu, Dhalavapuram and Maruthadi. The morphological, phenological and floral

observations of the species under the natural conditions do not show much difference at the vegetative stage.

Estimation of total phenols : Total phenol content of *Excoecaria* leaf was estimated and the absorbance was recorded at 650nm[3].

Estimation of soluble proteins : The soluble proteins was estimated with the absorbance read at 670nm after 30 min using a proper blank[4].

Estimation of proline : Proline was estimated colorimetrically and the absorbance was noted at 520 nm, using toluene as the blank[5].

Estimation of sugars : Total sugars in the leaves of *Excoecaria* were isolated and estimated. The OD was measured at 620nm[6].

Polyacrylamide gel electrophoresis (PAGE) : Protein banding pattern was studied by SDS-PAGE[7,8].

Molecular-Random Amplification of Polymorphic DNA (RAPD) : Genomic DNA was extracted and purified by CTAB[9] with some modifications. Fifteen Oligo nucleotide 10mer primers of Operon (OPD series, Operon Technologies, USA) were used for the random amplification of the genomic DNA (Table 1). Critical factors, which influence the optimization of the DNA amplification during PCR reaction, include the quality and quantity of the genomic DNA, annealing temperature, concentrations of $MgCl_2$, dNTPs and Taq polymerase and the number of cycles during PCR amplification (Table 2). Standardization of these factors is crucial for the elimination of defects associated with PCR amplification such as smear on running gel, nonspecific bands and false amplification. The reactions were carried out in a DNA thermocycler (MJ Research Inc. USA) using 20 μL reaction mixture. Reactions without DNA were used as negative control. DNA banding pattern was analyzed by agarose gel using standard molecular weight from the GeneRuler™ DNA ladder mix[10]. The polymorphic DNA bands that showed consistency in repeated experiments were screened according to their presence ('1') or absence ('0') in each of the genotypes. Percentage of genetic distance between the genotypes was estimated by the pair wise comparison[11]. After calculation of all pair wise similarities between varieties, the relationships among them were expressed by performing cluster analysis using the software GENSTAT. It is then graphically represented as a dendrogram[12].

Results and Discussion

RAPD Analysis : Development of molecular marker technology and consequent identification of marker loci linked to important agronomic taits has created exciting new opportunities for plant breeders. DNA marker techniques offer powerful tool for the characterization of genetic variability, genotypic identification, genetic analysis and selection and breeding programmes in plants. Important advantages of molecular markers include lack of sensitivity of changes in environmental conditions, as well as a nearly unlimited potential number of markers and speed of the marker assays as compared with field tests. In many species, random amplification of polymorphic DNA has proven useful for revealing polymorphism among genotypes[13]. They can provide additional information such as the amount of genetic divergence between cultivars and other genotypes and the amount of genetic variability between seedling replicates of genotypes. So this part of the study has been done to reveal the genetic polymorphism of *E. agallocha* plants adapted to different habitats using RAPD markers. An attempt was also made to correlate the genetic difference between the genotypes. The RAPD data was subjected to cluster analysis for detecting the genetic polymorphism of the species collected from the different habitats and their phylogenetic status was also detected from the dendrogram.

The genomic DNA was isolated and purified from the leaf samples of *E. agallocha* plants collected from three different regions of Kerala *i.e.* from Ayrimathengu, Dhalavapuram, and Maruthadi of Kollam. Each sample was properly identified from the habitat. For the convenience of the study, the genotypes were numbered as '1' genotype from Ayrimathengu; '2' from Dhalavapuram; '3' from Maruthadi. The DNA polymorphism at the level of genetic variability in *Excoecaria* genotypes was analyzed by RAPD method. Of the 15 RAPD primers P1 to P15 screened, 13 produced distinct reproducible polymorphic bands within the three genotypes of *Excoecaria* (Table 1). Reproducibility of the amplification pattern was checked by repeating the reactions in minimum five members of each genotype. Even though diagnostic bands were observed, most of them are faint or not repeatedly formed in all the representative individuals of the three genotypes. Thus a large number of potentially genotype specific bands were eliminated from consideration. Figure 1 represents the amplification pattern obtained with the 13 primers for the three genotypes of the categories : from 1, 2, 3 plants. The RAPD profile shows a total number of 62 bands with appropriate band size range of 100 to 4000 bp. PCR reactions were optimized at annealing temperature 36^{0}C for 40 cycles. All the 13 primers in the three genotypes were considered as a single reaction.

Identification of Excoecaria genotypes based on specificity of primers : Based on the screening of the RAPD profile of the three genotypes of the plants, it is possible to categorize the plants into three groups. Table 1 demonstrates the list of primers identified from the 15 ones for detecting the three genotypes together. The primer P1,2,4,5,6,7,8,9,11,12,13,14 and 15 showed amplification in all the three genotypes so that the band profile can be used to identify them from one another (Fig.1). It is obvious from the figure that the primer P1 expressed specificity in the amplification pattern in genotype '3' with specific individual bands 3500 bp. Similarly, P4 showed unique bands at 2500, 3000 bp and p15 at 3400 bp. Population '2' showed unique bands at P4 (1000bp), P5 (1250 bp), P6 (1000 and 850 bp), P12 (2000 bp), P13 (800 bp), P14 (2250 bp) and P15 (2500, 2000, 1750 bp). Population '1' was identified by RAPD bands at P2 (1500 bp), P4 (1900 and 1500 bp), P9 (4000 bp), P12 (1400 bp) and p15 (2750 bp). Figure 2 demonstrates the RAPD profile of genomic DNA amplified by the primers P1 with the sequence AACCGCGGG. It could be visible from the figure that the primers showed their ability to detect the genotypes developed from the leaves. Meanwhile, the primers P2, 4, 5 and 6 GGGGGTCGTT, CCAGACCCTG, AAGCTCCCCG, TACCACCCCG express their bands for the genotypes of the plants (Fig.2). P7, 8, 9 and 11 showed the DNA profile of the plants collected from the varied habitats (GGCGGACTGT, GTCACTCCCC, ACCGCGAAGG, GTCGCCGTCA). Similarly, the RAPD profile of genomic DNA amplified by the primers P12, 13, 14 and 15 with the sequence TCTGGTGAGG, TGAGCGGACA, ACCTGAACGG and ACCTGAACGG respectively produced unieque and common bands among the populations 1, 2 and 3. Thus the RAPD data of the primers indicates the discriminatory power in amplification and it can be successfully appllied to reveal the genetic diversity between genotypes from different habitats.

Dendrogram of Excoecaria genotypes : The RAPD data of the three genotypes was extended further for statistical analysis in order to measure the genetic distances among them. The bands were secored according to their presence ('1') or absence ('0') and were aranged as per the molecular size. A pair wise genetic distance among the three genotypes was analysed by statically[11]. Table 3 demonstrates the pair wise gentic distance among the three genotypes estimated based on RAPD data. It is evident from the percentage of genetic distance that genetic dissimilarity was found in the categories of plants of Ayiramthengu, Dhalavapuram and Maruthadi regions. RAPD data was further extended for statistical analysis in order to measure the genetic distances among the genotypes. From the genetic distance calculated from the RAPD data, a dendrogram was prepared by GENSTAT cluster analysis software. The cluster tree grouped basically into three clusters. The first cluster contains the population from 'Ayiramthengu'. 'Dhalavapuram' formed another cluster. 'Maruthadi' is found to be positioned apart from other cluster (Fig.2). The dendrogram preparred from the RAPD data reflects a unique grouping of genotype in the way of the adaptation of the particular habitat. Thus the RAPD profile of *Excoecaria* provides sufficient insight to categorize the genotypes based on its gentic relatedness.

Protein electrophoresis profiles : Polyacrylamide gel electrophoresis provides good marker for the identification and charecterization of species growing at different habitats. Electrophoretic analysis of protein is assumed to provide information concerning the type and biosynthesis of different protein fractions. The studied three populations were fingerprinted by SDS-PAGE of total proteins. They exhibited a maximum number of 12 bands, which were not necessarily present in all populations (Fig.3). Protein band number 1, 2, 3, 4 and 5 were found in populations from 1, 2 and 3 while, band number 6 was found only in populations from 2. It can be concluded that the latter bands can be considered as specific for these populations. Similarly, the protein band 3 of mass 28, 38, 46, 50, 59 and 64 kDa are shared in the populations collected from different habitats. 96 kDa protein band was present only in population 1. These may include dehydrins (25-60 kD) or aquaporins (25-30 kD) which are adaptive to desiccation tolerance mechanism. These results suggest that production of these stress proteins enables mangroves to withstand the harmful effect of salinity. Some of the induced proteins might be a group of membrane-bound proteins that regulate trans-membrane transport between the cytoplasm and the vacuole.

Generally, the three studied populations have band each with molecular weight of 30 and 32 KDa was specific in population 2 whereas, band 96 kDa was characteristic of population 1 which could be considered as a positive marker for the populations. The former results were quietly lined with those obtained from similar studies on *Acacia spp*[14], *Zygophyllum spp*[15] and some halophytic plants[16]. Therefore, it can be interpreted that the genetic behaviour of *E. agallocha* populations was varied by environmental conditions.

Table 1. Arbitrary primers of P1-P15 and their sequences used for Random Amplified Polymorphic DNA (RAPD) analysis of *Excoecaria agallocha* genotypes.

Primer	Sequence
P1	AACCGACGGG
P2	GGGGGTCGTT
P3	TGCCCTGCCT
P4	CCAGACCCTG
P5	AAGCTCCCCG
P6	TACCACCCCG
P7	GGCGGACTGT
P8	GTCACTCCCC
P9	ACCGCGAAGG
P10	GGACCCAACC
P11	GTCGCCGTCA
P12	TCTGGTGAGG
P13	TGAGCGGACA
P14	ACCTGAACGG
P15	TTGGCACGGG

Table 2. Ingredients in PCR reaction mixture and Thermocycler program for the reaction.

Parameter	Tested Range
Primer	100ng
dNTP	100µM
$MgCl_2$	2mM
10X PCR buffer	2.5 µl
Taq polymerase	2U
DNA	50 ng
ddH_2O	Xµl
Total	25µl

Programme	Temperature	Time	Cycles
Initial Denaturation	96°C	2 min	
Denaturation	96°C	10 sec	25 cycles
Annealing	50°C	5 sec	25 cycles
Extension	60°C	4 min	25 cycles
Termination	60°C	1 min	

Table 3. Percentage of genetic distance between *Excoecaria agallocha* genotypes Sample 1-Ayiramthengu, Sample 2-Dhalavapuram and Sample 3-Maruthadi.

Percentage of genetic distance between	
sample 1 and 2	72.8%
sample 1 and 3	62.1%
sample 2 and 3	62.5%

Table 4. Pigment composition in *Excoecaria agallocha.*

	Sample 1 (Ayiramthengu) (mg/g tissue)	Sample 2 (Dhalavapuram) (mg/g tissue)	Sample 3 (Maruthadi) (mg/g tissue)
Chlorophyll a	1.702	1.022	0.709
Chlorophyll b	0.765	0.764	1.2511
Total chlorophyll	2.467	1.753	1.966
Carotenoid	53.95	32.26	22.09

Table 5. Total phenol, carbohydrates, proline and protein content in *Excoecaria agallocha* growing at varied habitats.

	Sample 1 (Ayiramthengu) (mg/g tissue)	Sample 2 (Dhalavapuram) (mg/g tissue)	Sample 3 (Maruthadi) (mg/g tissue)
Phenol	8.665	12.688	19.785
Carbohydrate	70.192	14.736	38.025
Proline	9.462	7.761	5.68
Protein	1.696	2.250	0.895
Sugar	70.2	38.1	35.6

Photosynthetic pigments : Plants employ chlorophyll *a* and *b* and a variety of carotenoids to capture light for photosynthesis. Chlorophyll *b* is mainly involved in light harvesting and thus is predominantly found in the chlorophyll *a/b* antenna proteins, whereas chlorophyll *a* is closely associated with the reaction centre complexes[17]. Carotenoids are associated with both antenna and reaction centre proteins, and have multiple functions in photosynthesis. Carotenoids play a vital role by masking the chloroplast from photo-oxidative damage caused by high intensity of light. Concentrations and ratios of photosynthetic pigments, (*i.e.*, chlorophyll *a* and *b* and carotenoids) are correlated to the radiance experienced by plants in their natural habitat.

The three populations showed varied response on the chlorophyll and carotenoid content as shown in Table 4. Chlorophyll *a* content was higher in the population 1 followed by population 2 but its reduction was more obvious in population 3. Meanwhile chlorophyll b was higher in population 3. The carotenoid content was also higher in population 1 followed by population 2. Carotenoids protect plants against photo-oxidation, by effectively quenching the excited triplet state of chlorophyll and singlet oxygen. Protection of the photosynthetic apparatus from excess light absorption requires carotenoids (oxygenated)[18].

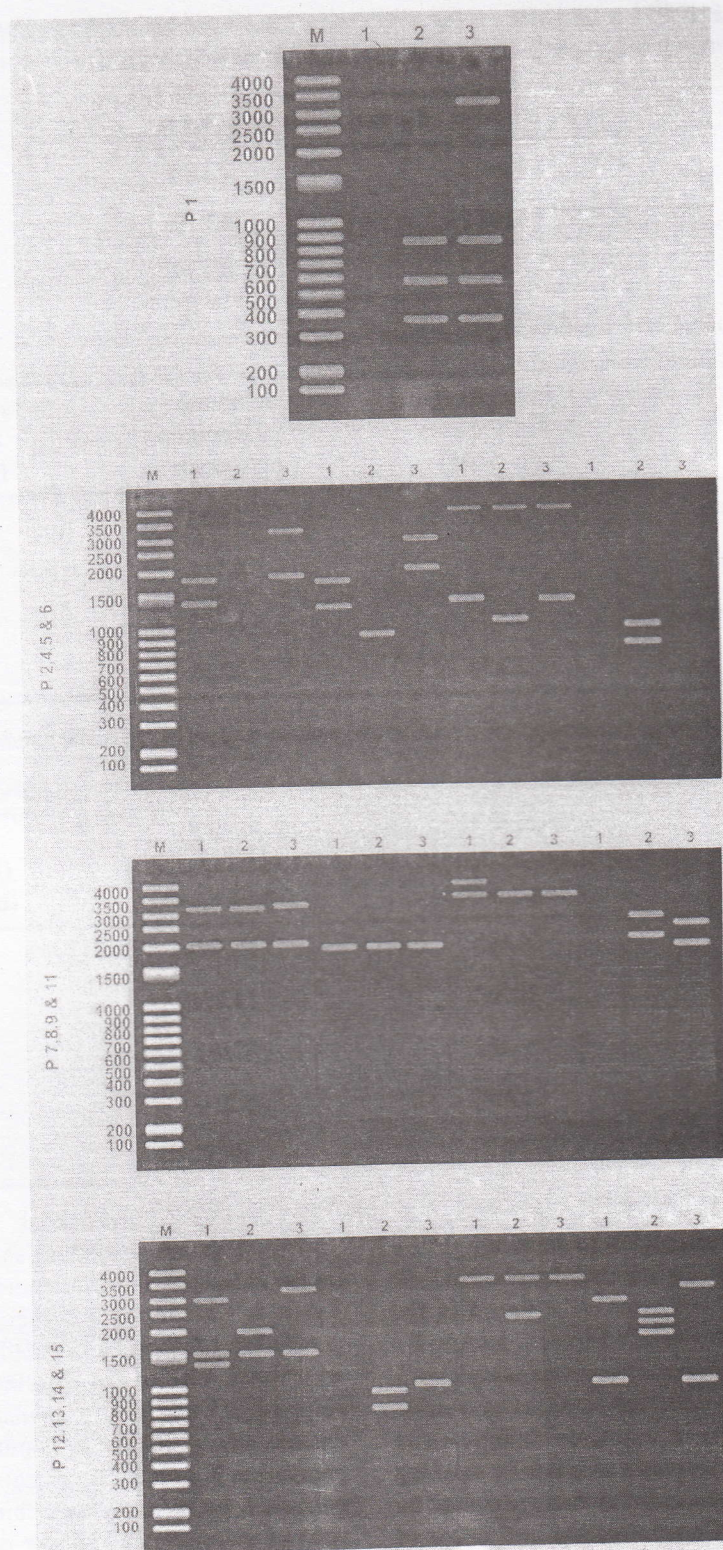

Fig.1. RAPD profile of *Excoecaria agallocha* Linn. from different habitats using arbitrary primers. 1-Ayiramthengu; 2-Dhalavapuram; 3-Maruthadi

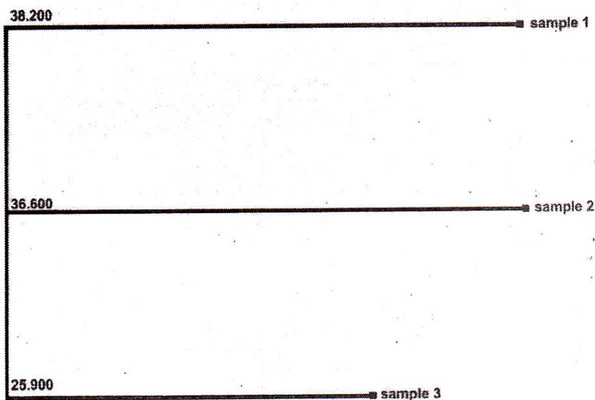

Fig.2. Dendrogram of *Excoecaria agallocha* Linn. analyzed by RAPD.

Excoecario taxa studied here have acclimated to the levels of light available, within their habitat. Plants from habitats with low radiation inputs had higher concentrations of photosynthetic pigments (*i.e.,* chlorophyll and carotenoids) and higher cholorphyll to carotenoid ratios, than from sunny environments. Plants from shaded environments usually modify chlorophyll *a/b* protein complexes to increase light harvesting, illustrated by a low chlorophyll *a* to *b* ratio[19, 20].

Proline content : Proline also show a varied response with the highest value of 9.46 mg/g in population 1 followed by population 2. The population 3 has less proline content (5.68 mg/g) suggesting the importance of the stress amino acid related with drought tolerance. Since the accumulation of proline is linked to the physiological water stress in plants, the present data clearly suggest that the high content of proline observed in *E. agallocha* can be interpreted to the affinity of the taxa towards terrestrial habitat. Proline provides protection against water deficit as a hydrophilic protectant for enzymes and cellular structures. However, some researchers consider the higher proline level merely as a stress effect rather than a cause of stress tolerance[21]. Under water stress in transgenic tobacco, the increased level of proline was reported to be due to the enhanced activity of proline biosynthetic enzyme and the suppressed activity of proline catabolizing enzymes. Thus the present analytical data clearly indicate the physiological interaction of proline with the water deficit, which is an event comparable to the samples collected from the different habitats.

Total protein : Protein content showed variation from 0.895 to 2.3 mg/g tissue. Protein is an importnat nitrogen containing biomolecule that intermediates and also acts as osmotically active ingredients in plant metabolism or

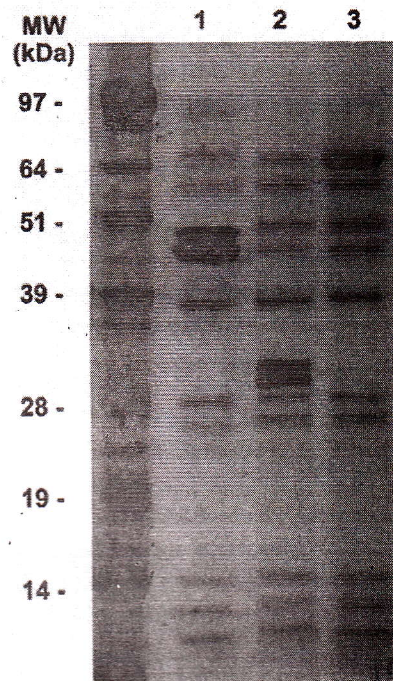

Fig.3. Protein banding pattern in *Excoecaria agallocha* Linn. 1-Ayiramthengu; 2-Dhalavapuram; 3-Maruthadi

as stress proteins in their response to environmental stressess[22] (Table 5).

Total phenol contents : All *Excoecario* members collected from different habitats exhibited a remarkable total phenol contants (Table 5). Population 1 showed the lowest level (8.7 mg/g), while, population 3 the highest (19.8 mg/g tissue). The overall higher profile of total phenol suggests the active phase of secondary metabolism and inturn the resistance of the plants to pest and pathogens. The waxing and waning pattern of phenols in sample requires further investigation related with the fractionation of the phenolic acids.

Total sugar : Total sugar showed much diversity among the population collected from the different habitats. Population 1 has high sugar content *i.e.,* 70.2 mg/g followed by population 2 (38.1 mg/g tissue) (Table 5). Plants can enhance tolerance by gradual exposure to temperature extremes known as acclimation. Soluble sugars have confirmed to play an important role during the process. This is based on the fact they are most commonly detected in various species of terrestrial plants that have undergone seasonal cold or arid acclimation. Soluble sugars exert their positive effects to protect plant cells from damage caused by cold or high temperature stress through many wasys that including serving as osmoprotectants, nutrients as well as interacting with the

lipid bilayers. In addition to these functions, more focus today is on their important homone-like functions as primary messengers in signal transduction. On the other hand, higher sugar concentrations can trigger leaf senescence, which indicates that the accumulation of soluble sugars during cold acclimation might impact on plants negatively. Further study can be done to investigate the mechanisms by which soluble sugars are involved in the plants, response to the environmental signal by advanced molecular biology methodology to examine sugar-regulated gene expression under extreme conditon[23].

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