PLANT BREEDING AND SEED SCIENCE
Volume 68 2013

DOI: 10.2478/v10129-011-0082-2

Omid Sofalian*, Somayyeh Azimy, Sodabeh Jahanbakhsh,
Saeid Khomari, Sara Dezhsetan

Department of Agronomy and Plant Breeding, Faculty of Agricultural Science, University of Mohaghegh Ardabili, Ardabil 179,
*Corresponding author: sofalian@gmail.com

EVALUATING GENETIC DIVERSITY OF CHILLING STRESS IN COTTON GENOTYPES

ABSTRACT

In order to study genetic diversity and some physiological features related to chilling stress using molecular markers, an experiment was conducted at University of Mohaghegh Ardabili. Treatments were set in a factorial experiment based on randomized complete block design with 3 replications and 3 stress levels (25, 15 and 5°C) between 20 cotton genotypes. The results showed that chilling stress influenced on some physiological features such as the activity of catalase, proline content, soluble carbohydrates and proteins. Cluster analysis carried out using WARD method in physiological features showed that genotypes located in three groups in the acclimation level and after acclimation, respectively. Nazilli, Ciakra, Avangard and B-557 were in the better group in studied levels. Also based on the results Avangard, Chegurava, Tashkand and Shirpan 603 were the most tolerant genotypes. In the ISSR marker analysis using of 12 primers produced 96 polymorphic bands. The mean of PIC, MI and EMR were 0.283, 1.065 and 3 respectively, for all primers. Some of markers had promising results that confirmed ISSR markers as powerful tool in any marker assisted program for plant breeders.

Key words: chilling, cotton, ISSR marker

INTRODUCTION

Cotton cultivars (diploid and tetraploid cultivars) have sownin 17 out of 30provinces in Iran. Based on report published by Agriculture ministry (Iran cotton report to 68th plenary meeting of ICAO, Cape Town, South Africa, 2009).Cotton (*Gossypium hirsutum* L.) is sensitive to chilling condi-

Communicated by Grzegorz Żurek

tions during germination and establishment (Lauterbach *et al.*, 1999; Bolek, 2010). The chilling effect is exhibited by physiological agitation, a phenomenon known as chilling injury (Yan *et al.*, 2010). Following this process many biochemical and physiological changes occurred including: increase levels of carbohydrates, antioxidant enzymes, proline, gene expression and membrane lipid composition changes (Xin and browse, 2000), the appearance of new isoforms of protein (Heidarvand and Maali amiri, 2010) that all of which lead to cold acclimation. Inter Simple Sequence Repeat or ISSR (Zietkiewicz *et al.* 1994) markers are more and more in demand, because they are known to be abundant, very reproducible, highly polymorphic, highly informative and quick to use (Zietkiewicz *et al.* 1994, Bornet and Branchard 2001, Sofalian *et al.* 2009). ISSR uses the presence throughout the genome of Simple Sequence Repeats (SSR) which is ubiquitous, abundant and highly polymorphic tandem repeat motifs composed of 2 to 5 nucleotides (Sofalian *et al.*, 2009). Liu and Wendel (2001) declared ISSR as an easy and efficient genetic marker system in cotton for display both inter and intraspecific variations.

The present study focused on the characterization of genetic diversity among cotton genotypes, belonging to *G. hirsutum* using ISSR DNA markers and its relationship to physiological features influenced chilling tolerance.

MATERIALS AND METHODS

Experiment was conducted during the summer season of 2012 at the research greenhouse of the University of Mohaghegh Ardabili. Treatment were set in a factorial experiment based on randomize complete block design (RCBD) with 3 replication per treatment. The seeds of 20genotypes (Table 1) belonging to the Gossypium hirsutum species were used in this study. Uniformly germinated seeds were conveyed to plastic pot in the greenhouse under a controlled environment at 25±1°C and 16 h light/8 h dark photoperiod. Then, plants at 4 and 5 leaf stages of each genotype were divided into two groups. One group of each species was maintained in the same chamber with 16 h light / 8 h dark photoperiod and at 25±1°C as the control. The other group of each genotype was transferred in a chamber for cold acclimation with: 16 h light/8 h dark photoperiod and 15°C for 1 day as well as 5°C for 1 day. 48 and 72 h after treatment, seedlings were randomly taken from chilling-treated seedlings of each genotype (Rapacz, 2002). Leaves were ground with mortar and pestle using liquid nitrogen and kept at -70°C for further analyses.

Table 1

Name and origin of studdied cotton genotypes

Cultivar Number	Cultivar Name	Origin	Cultivar Number	Cultivar Name	Origin
1	Chegurava	Turkey	11	Nazil'i	Turkey
2	Sahel	Iran Trade	12	Bakhtegan	Iran Trade
3	Beliisovar	Turkey	13	Varamin 349	Iran Trade
4	4.S.4	Greece	14	43347	Greece
5	4325	Greece	15	Khordad	Iran Trade
6	Tashkand	Uzbekistan	16	Opal	America
7	No-228	Greece	17	Ciakra	Iran Trade
8	Syndose	Greece	18	Avangard	Bulgaria
9	No-200	Greece	19	Oltan	Iran Trade
10	Shirpan 603	Bulgaria	20	B557	Pakistan

Extraction Physiological Features

Proline content was measured using the acid ninhydrin by method of (Bates *et al.*, 1973). In order to measure the total soluble proteins from leaves utilized Guy *et al.* (1992) procedure. Leaves were homogenized in 50 mM Tris-HCL, pH 7.5; 0.04 % (v/v) 2-mercaptoethanol and 2 mM EDTA. Then centrifuged at 11500 rpm and 4°C for 21 min. supernatant stored at -20°C for analysis. Protein concentrations were assayed with bovine serum albumin (BSA) as standard protein according to the Bradford (1976) method. Soluble sugar content was analyzed using the enthrone method described by Irigoyen *et al.* (1992). Absorbency of the resulting solution was read at 625 nm and a calibration curve with D-glucose was performing as a standard. The activity of catalase as well as poly phenol oxidase enzymes was determined using the Tris (50 mM; pH=7) and hydrogen peroxide (3%) (Kar and Mishra, 1976). Electrolyte leakage was estimated as reported by Fry *et al.*, (1991).

DNA extraction, PCR methods and gel electrophoresis

Genomic DNA was extracted from young leaf tissue using the CTAB extraction method according to Saghai-maroof *et al.*, (1984) with some minor modifications. Quantity and quality of the isolated DNA was measured by spectrophotometer and visualized using 0.8% agarose gel electrophoresis. Amplification reaction was carried out in a total volume 20 µl with Tris-HCl, $MgCl_2$, dNTPs, Taq DNA polymerase, corresponding primer and

30 ng DNA templates using thermal cycler. After initial denaturation at 94°C for 5 min, 40 cycles of PCR were conducted where each cycle consist of 30 sec denaturation at 92°C, 40 sec annealing (annealing temperature optimized for each primer according to theoretical Tm°C for hybridization, 45-54) and 1min extension at 94°C with a final extension five min at 72°C. 12 ISSR primers were selected based on polymorphism and robustness of the bands obtained. All the generated patterns were repeated twice in order to verify reproducibility. Products of PCR reaction were mixed with 4 µl of 6X loading dye, separated on 1.5% agarose gel in 1X TBE buffer (Tris 0.89 M, EDTA 2 mM, Boric Acid 0.89 M, pH= 8.3), stained with ethidium bromide and visualized under U.V light.

ISSR and physiological data analysis

ISSR products were scored as the presence (1) or absence (0) of bands and a binary matrix was constructed. Only ISSR fragments that could be scored unambiguously were included in the analysis. Cluster analysis was performed for the molecular data based on Unweighted Pair Group Method Analysis UPGMA and using NTSYS-pc. GenALEx6.3 was used to calculate Shannon Diversity Index, Marker Index (MI) and Polymorphism Information Content (PIC) and to perform Mantel's test. ISSR data were also subjected to genetic analysis using POPGENE 1.32 software. Physiological Data analysis was done by SPSS and MSTATC.

RESULTS AND DISCUSSION

Cluster analysis based on Physiological features was carried out using WARD method according to the Euclidean distance on standardized data. The genotypes were divided into three clusters at acclimation level (Figs 1, 2). Group one includes seven genotypes, with higher mean for soluble protein, catalase and poly phenol oxidase activity. Considering the features, this group can be considered as tolerant genotypes based on the investigated features under chilling stress. Second group, which includes two genotypes with higher mean for electrolyte leakage and average proline concentration. Group three includes Remained genotypes. This group has lower mean of carbohydrate, electrolyte leakage, and catalase and poly phenol oxidase activity. Also three clusters identified after acclimation level (Figs 3, 4)which among them group 1 and 3 demonstrated the highest and lowest the deviations from the total mean respectively. Group one comprised eleven genotypes with higher mean for soluble protein and middle mean for catalase and poly phenol oxidase activity. Tertiary Group includes six genotypes. This Group has higher mean of carbohydrate.Considering the cluster analy-

sis, Nazilli, Ciakra, Avangard and B-557 were been in the better group in studied levels.

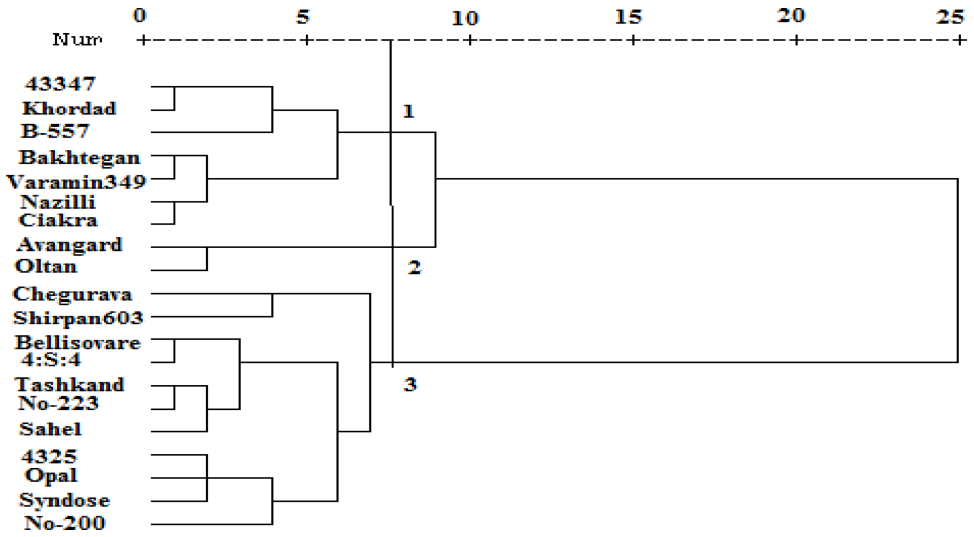

Fig.1. Cluster analysis dendrogram of 20 cotton commercial cultivars based on physiological features at acclimation level

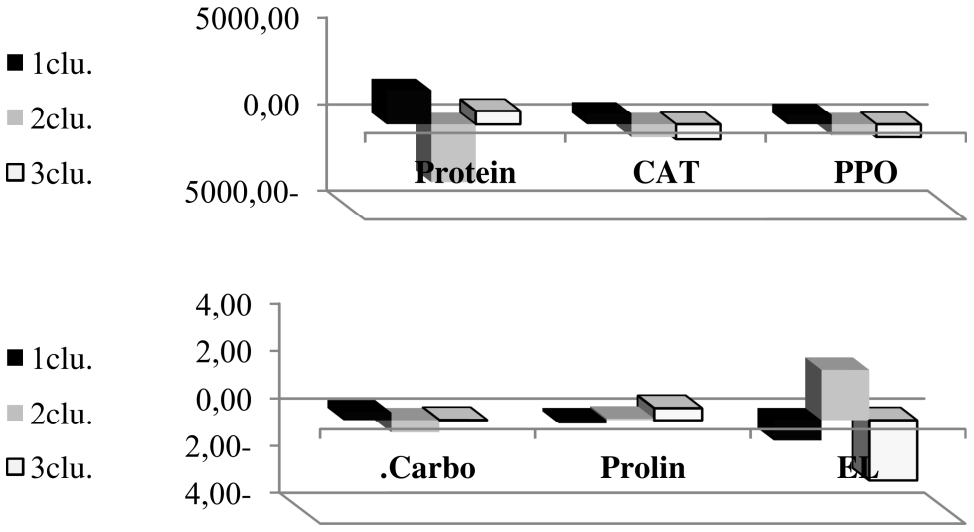

Fig.2. The average deviation of the mean cluster at acclimation level

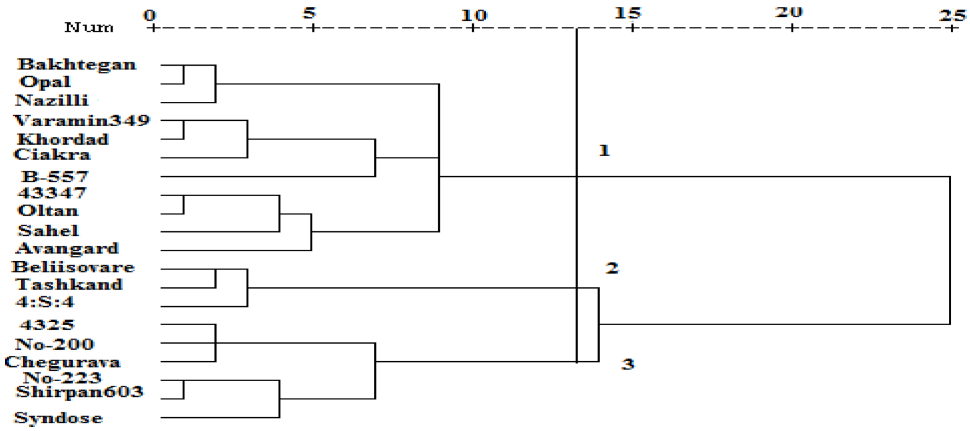

Fig.3.Cluster analysis dendrogram of 20 cotton commercial cultivars based on physiological features after acclimation level

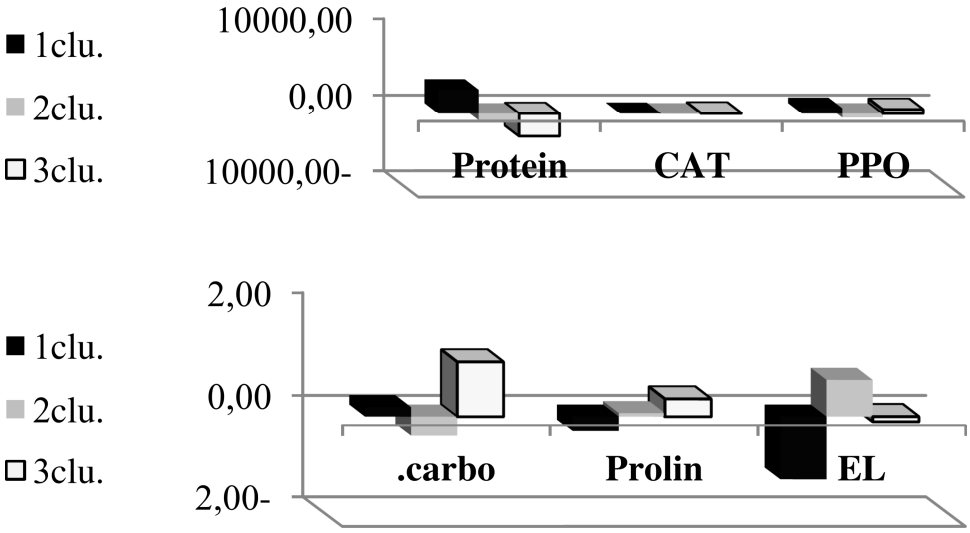

Fig.4. The average deviation of the mean cluster after acclimation

ISSR Data Analysis

At the beginning 34 ISSR primers were screened, of these, 12 primers produced 69 reliable bands (Fig. 5), out of which 48 were polymorphic (69.57%).Average number of polymorphic loci amplified per primer was 4. Primers ISSR-1and ISSR-14were polymorphic in all loci (100% polymorphic). The mean PIC value of the polymorphic loci was 0.283. These values can range from 0.0 for monomorphic markers to 0.5 for markers that are present in 50% of accessions and absent in the

other 50% (Gomes *et al.*, 2009). The MI values ranged between 0.27 for primer ISSR-2 and 2.72 for primer ISSR-14 with average 1.065. The primers that showed higher polymorphism had higher EMR values. This trait was observed from 0.33 to 8 with a mean value of 3 per primer (Table 2)

Table 2

Total number of bands, polymorphic bands, amplified by eleven ISSR primers in Cotton genotypes and Marker parameters calculated for each ISSR primer.

1	2	3	4	5	6	7	8	9	10
ISSR-1	5/ AGAC AGACGC 3/	3	3	100	0.220	0.66	3	0.226	0.383
ISSR-2	5/ GACAGACAGACA GACA 3/	5	3	60	0.095	0.27	1.8	0.125	0.119
ISSR-3	5/ AGAGAGAGAGAGAGAGC 3/	8	4	50	0.116	0.44	2	0.158	0.115
ISSR-9	5/ TCTCTCTCTCTCTCTCC3/	6	4	66	0.362	1.44	2.64	0.242	0.361
ISSR-14	5/ CACACACACACAGT 3/	8	8	100	0.349	2.72	8	0.353	0.527
ISSR-15	5/ ACGACGACGACGAAC 3/	6	3	50	0.326	0.96	1.5	0.165	0.253
ISSR-16	5/ CACACACACACAAG 3/	5	4	80	0.245	0.96	3.2	0.297	0.314
ISSR-19	5/ AGAGAGAGAGAGAGAGT 3/	8	6	75	0.361	2.16	4.5	0.274	0.412
ISSR-22	5/ ATGATGATGATGATGATG 3/	3	1	33	0.355	0.37	0.33	0.2578	0.187
ISSR-24	5/ GACAGACAGACAGACA 3/	6	5	83	0.339	1.65	4.15	0.284	0.426
ISSR-31	5/ GAGGAGGAGGC 3/	6	5	83	0.294	1.45	4.15	0.247	0.387
ISSR-32	5/ AGAGAGAGAGAGAGAC 3/	5	2	40	0.331	0.66	0.8	0.132	0.119
	Mean	6	4	69.57	0.283	1.065	3	0.231	0.306

1 - Primers
2 - Primer sequences
3 - Number of amplified bands
4 - Number of polymorphic bands
5 - Polymorphic / amplified bands [%]
6 - PIC
7 - MI
8 - EMR
9 - Nei's gene diversity
10- Shannon information index

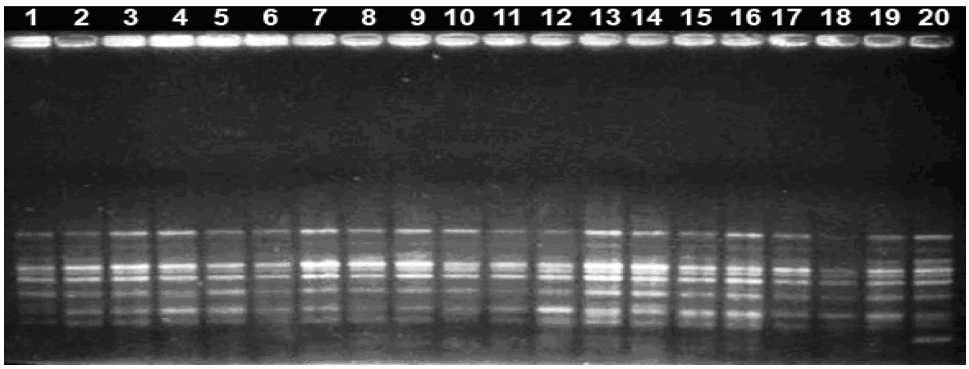

Fig.5. Example of electrophoresis gel of DNA obtained by amplification of accessions using the ISSR-16 primer

Estimation of genetic diversity

Nei's gene diversity index (Nei, 1973) ranged from 0.125 for primer 2 to 0.353 for primer 14, with an average 0.25 and the Shannon information index observed between 0.115 for primer 3 to 0.527 for primer 14 with an average 0.30. In this regards, Sheidai *et al.*, (2010) reported the average 0.22 and 0.34 by the two indices, respectively among thirteen cultivars of *G. hirsutum* using RAPD markers.

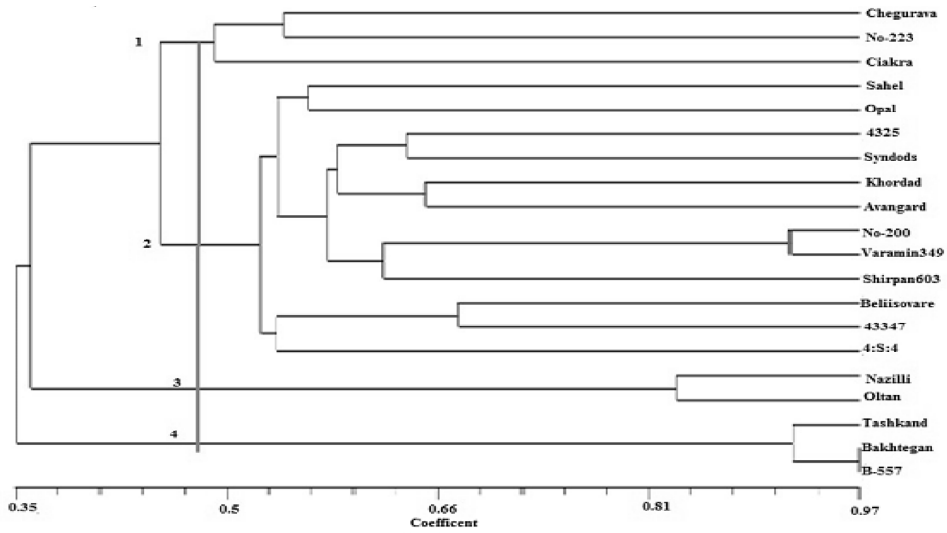

Fig.6. Dendrogram based on UPGMA cluster analysis of ISSR data

Cluster analysis based on molecular data was performed to develop a dendrogram by unweighted pair group method with arithmetic mean

(UPGMA) according to Jaccard's similarity coefficients (Fig. 6). The cophenetic correlation was high (r=0.94, α=1%) indicating a very good fit of the cluster analysis performed. Therefore, the 20 genotypes were divided into four groups. Group one includes two genotypes (4: S: 4, Varamin 349, No-200, Avangard, Shirpan 603, Cindose, Beliisovar, Khordad, Opal, 4325, Sahel, 43347). Second Group includes ('Chegurava 15:18, Ciacra, 'No-223). Group three includes (Nazil'i, Oltan) and Bakhtegan, B557, Tashkand placed in Group four. Rana and Bhat (2005) studied genetic similarity among 41G. hirsutum cultivars of cotton using RAPD marker and the UPGMA cluster analysis placed all the tetraploid cultivars within their respective known taxonomic groups.

Identification of informative markers

Table 3

Regression coefficients and adjusted R square in the multiple regressi on between the physiological features and location ISSR gene in acclimation

	Protein	PPO	CAT	Carbo	Proline	EL
Intercept	12699.472	41.501	34.109	4.299	2.592	32.842
P5n8					0.119	
P9n1					0.425	
P9n4					0.714	
P9n6	0.757				-0.932	
P14n2						0.514
P14n8				-0.498		-0.743
P15n4	0.313					
P15n5			-0.461	-0.946		
P15n6	0.542					0.348
P16n5		-0.563				
P16n1						0.704
P19n3				-0.605		
P28n1					0.262	-0.565
P30n4						-0.450
R2	0.691	0.500	0.168	0.566	0.818	0.929

Phenotypic selection has limitations especially when interest is focused on more complex physiological features. A more accurate way of selection would be at the genetic level where markers linked to the gene(s) or quantitative trait loci (QTLs) underlying the trait can be screened for. A prerequisite for genotypic selection is the establishment of associations between features of interest and genetic markers. QTL mapping of physiological features will provide crop breeders with a better understanding of

the basis for the genetic correlation between economically important traits. This has potential to facilitate a more efficient incremental improvement of specific individual target traits (Graham et al, 2008). Information on QTL analysis has accumulated quickly, and will eventually help the manipulation of complex features in cotton breeding (Tanksley, 1993; Preetha and Raveendren, 2008). The results of stepwise regression analysis revealed a significant correlation/association between the physiological features and some of the studied ISSR loci. One or more informative markers were identified for almost all of the studied features. The important informative markers in acclimation level are listed below. MarkersP9n6, P15n4and P15n6accounted for 69% of total variability of protein feature.P14n8, P15n5 and P19n3, were responsible for carbohydrate feature with 52%, P5n8 ،P9n1, P9n4P9n6, andP28n1for proline feature with 81% of the total variability. Markers which responsible for electrolyte leakage explained 92%of variation (Table 3). After cold acclimation three markers were associated with soluble protein, tow markers for electrolyte leakage and one marker with catalase, carbohydrate and proline concentration features. Markers in association with soluble protein explained 67% of variation whereas amount of explained variance by electrolyte leakage were 75% (Table 4).

Table 4

Regression coefficients and adjusted R square in the multiple regression between the physiological features and location ISSR gene after acclimation.

	Protein	PPO	CAT	Carbo	Proline	EL
Intercept	21501.457	52.327	33.518	3.651	3.844	31.354
P9n1						0.268
P9n6	0.624					
P15n3					-0.455	
P15n4	0.427					
P15n5	0.410		-0.451	-0.678		
P16n2						0.432
P19n5		-0.499				
R2	0.677	0.185	0.164	0.430	0.163	0.758

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