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GENETIC DIVERSITY OF INDIGENOUS FENNEL (*FOENICULUM VULGARE* MILL.) GERMPLASM IN PAKISTAN ASSESSED BY RAPD MARKERS

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Abstract

Fennel (*Foeniculum vulgare* Mill.) is an important, well-known aromatic and medicinal herb. Fifty accessions of fennel were collected from different parts of Pakistan and evaluated for important characteristics like seed germination percentage (ger %), days to initiation of flowering, plant height, stem girth, nodal distance, umbel diameter, days to 50% maturity, days to harvesting, seed yield per row, weight of 100 seeds, Harvest index (%). Genomic DNA of the accessions was extracted and subjected to RAPD analysis in order to ascertain their genetic diversity. Twenty-four out of 30 decimer primers generated 145 clear bands and 70 (48%) were polymorphic. Sixteen primers OPA-01, OPA-03, OPA-04, OPA-05, OPA-07, OPA-10, OPA-11, OPA-14, OPA-15, OPA-18, AC-11, AC-14, AC-15, AC-16, AC-18 and AC-20 gave polymorphism for different characters. About 66.6% of polymorphic primers generated the highest index to resolve genetic diversity even in small number of accessions. Seven accessions from Punjab, 3 from NWFP, one from Balochistan and one from Northern Areas of Pakistan had appeared with promising characters.

Introduction

Fennel (*Foeniculum vulgare* Mill.) belonging to the family Umbelliferae (Apiaceae) is a well-known aromatic and medicinal herb. It is carminative and commonly used to flavor liquors, bread, fish, salad, soups, cheese and in manufacturing of pickles, perfumes, soaps, cosmetics and cough drops (Tanira *et al.,* 1996; Beaux *et al*., 1997; Garcia-Jamenz *et al.,* 2000; Patra *et al*., 2002), while Indians and Egyptians knew it as culinary species (Farooqi *et al*., 1993). It can be an annual, biennial or perennial plant and is native to the Mediterranean areas (Piccaglia & Marotti, 2001). It is grown in the temperate and sub tropical areas of Pakistan up to an altitude of 2000 m and cultivated as an annual crop. Principal fennel producing countries include India, Argentina, China, Indonesia, Russia, Japan and Pakistan (Volak & Stodola, 1998).

Germplasm is a vital source in generating new plant types having desirable traits that help in increasing crop production with quality and thus improve the level of human nutrition. The genetic diversity is analyzed by using morphological as well as genetic based tools, DNA techniques (Bennici *et al.,* 2003) and advanced molecular methods etc. (Barazani *et al*., 2002; Shiran *et al*., 2007). The PCR based method for DNA profiling, random amplified polymorphic DNA (RAPD) techniques (Welsh & McClelland, 1990; Williams *et al*., 1990; Mir Ali & Nabulus, 2003, Fracaro *et al.,* 2005) has been extensively applied in assessment of genetic diversity of various plant species and is also quite helpful in detecting genetic variability within short time (Khan *et al.,* 2005). It serves as base for developing new high yielding varieties resistant to biotic and a-biotic stresses (Muehlbauer, 1991). In order to maintain, evaluate and utilize germplasm efficiently and effectively, it is important to investigate the extent of genetic diversity it contains (Smith & Smith, 1989; Gept, 1993; Weising *et al.,* 1995; Hills *et al.,* 1996) which is a pre-requisite for marking traits/genes to increase yields and for stabilizing production in the face of disease epidemics and environmental fluctuations. The present study was conducted for the first time to evaluate morphological as well as molecular characterization of fifty fennel accessions being cultivated in different parts of Pakistan.

Materials and Methods

Plant material and total genomic DNA extraction: A total of 50 accessions of fennel collected from different parts of Pakistan were raised in the experimental area at the Institute of Agri-Biotechnology and Genetic Resources (IABGR), National Agricultural Research Center, Islamabad (33.40 N and 73.07 E). The trial was planted in an augmented design with 37 accessions from Punjab, 9 from NWFP, 2 from Baluchistan and 2 from Northern Areas (NAs) of Pakistan. DNA was extracted from young leaves collected in January following the method described by Dellaporta *et al*., (1983) modified by Weising *et al.,* (1995). Approximately 1.5 gm of primary leaves were cut with disinfected scissor and washed with distilled water. After drying with blotting paper the leaves were crushed to a fine ground powder in liquid nitrogen and extracted with cetyltrimethylammonium bromide (CTAB), hot extraction buffer [100 mM Tris-HCl, pH = 8.0, 1.4 M NaCl, 20 mM EDTA, 2%CTAB, 1%w/v PVP (polyvinyl pyrrolidone) and 2% (v/v) ß-mercaptoethanol]. The mixture was incubated at 60°C for 1 hour, followed by two extractions with chloroform/isoamyl alcohol (24:1). Isopropanol was used to precipitate nucleic acids and the pellet obtained was dissolved in Tris-EDTA (TE) buffer (10 mM Tris-HCl, pH = 8.0 and 1 mM EDTA, pH = 8.0). Co-precipitated RNA was removed by digestion with RNaseA. Remaining impurities were extracted with processed phenol and chloroform. Total DNA was precipitated using Sodium acetate and cold ethanol. The precipitate was washed twice with 10 mM Ammonium acetate in 76 % ethanol and the pellet was dissolved in TE buffer. The purified total DNA was quantified by gel electrophoresis, and its quality verified by spectrophotometry. DNA sample were stored at 4°C. Two independent extractions were performed for each sample.

PCR amplification and product electrophoresis

RAPD reactions: Twenty arbitrary decimer primers of A series OPAI-OPA20 from Operon technologies Inc., Alameda, California, USA and 10 primers having 10 base pair from Oligo Series AC 11-20 were used to determine genetic diversity. Amplification reactions were performed in 15 µl volumes containing 1 µl primer 1µl DNA of fennel, 1.0 unit of Taq DNA polymerase, 6µl 10x PCR buffer and 6.5 µl distilled water. The reaction mixtures were overlaid with mineral oil prior to the amplification. DNA amplification reactions were carried out at the Perkins Elmer Thermocycler 480 programmed as followed: initial pre-denaturation step at 92°C for 3 min., followed by 45 cycles of 1 min., at 94°C, 1 min., at 65°C and 2 min., at 72°C followed by 5 min., incubation period at 72°C. The amplification products were stored at 4°C before analysis.

Agarose gel electrophoresis: The amplified fragments were separated on 1% agarose gel in 0.5 x Tris-borate-EDTA (TBE) buffer stained by Ethidium bromide (Sambrook, *et al.*, 1989) visualized and photographed by using the gel documentation system FAS 500 Epi-Ugent UV. All PCR reactions were repeated at least two times.

Data analysis: The photographs of gels were used to score data for RAPD markers. RAPDs behave as dominant markers (Shiran *et al.,* 2007) thus they tend to be bistate (Present-absent) type of scoring. Each DNA fragment amplified by a given primer was considered as a unit character and the RAPD fragments were scored as a binary variable (1) presence and (0) for absence of each of the primer accession combinations. Since DNA samples consisted of bulk sample of DNA extracted from individual plants, a low intensity of any particular fragment may be explained by the lesser representation of that specific sequence in the bulk sample of DNA. Therefore, the intensity of bands was not taken into

account and the fragments with the identical mobility were considered to be the identical fragments. Only major bands were scored and faint bands were not considered. The molecular size of the amplification products was measured with lambda DNA (λ Hind III/ EcoRI). The accessions were scored for the presence or absence of RAPD bands. The presence and absence of polymorphic and non-polymorphic bands was scored in a binary data matrix. The data was further analyzed by making dendrogram using method explained by Nei & Li (1979). The similarity coefficients were used to generate a graphic phenogram by means of Unweighed Pair Group Method with Arithmatic Means (UPGMA) cluster analysis as recommended by Sneath & Sokal (1973).

Results and Discussions

The analysis of the prescreening data using 50 selected fennel accessions and 30 RAPD primers showed that 24 primers generated bright and reproducible amplified products, which detected polymorphism among the accessions used. Using the described DNA extraction strategies and amplification condition with selected primers, good and clear patterns were obtained for various accessions. Results of amplification pattern obtained by RAPD in different accessions of fennel are shown in Fig. 1 and Fig. 2. In this study 10 primers having 10 base pair of A series from 20 Operon Technologies (OPA-1 to OPA-20) and 6 primers from Oligo series (AC-11 to AC-20) produced polymorphism and all accessions are distinguishable by unique RAPD profiles. These results agree with the reports by Verma *et al.,* (2004) who used RAPD primers and found genetic variation among 4 groups of land races of vegetatively propagated plants of belevine (*Piper behel*). These data indicated that RAPD markers were efficient for assessment of fennel genetic diversity.

While screening all the accessions, a total of 145 bands were revealed, out of which 70 (48%) fragments were polymorphic in one or other DNA amplified profiles of the 50 accessions. The percentage of polymorphism was within the range previously reported for other medicinal and aromatic species like *Lavandula angustifolia* (Echeverrigaray & Agostini, 2000) and *Ocimum gratissimum* (Vieira *et al.,* 2001). The rest of the 75 (52%) bands were monomorphic for the present fennel germplasm. Sixteen polymorphic primers generated a total of 111 reproducible and score-able amplification fragments across all the 50 accessions, out of which 70 (63.06%) fragments were polymorphic. Some of the primers generated several markers and were able to show a high level of genetic diversity, while others produced a single or few markers and detected a little variability. The number of RAPD bands detected by each primer depends on primer, sequence and the extent of variation in specific genotype (Shiran *et al.,* 2007). Therefore, the number of bands varied in different accessions. The number of bands generated varied from 4 (for OPA18) to 12 (for OPA11 and AC18), with an average of 9.3 bands per primer (Table 1). The primers OPA-4, OPA-15, AC-20, and OPA-5 gave maximum polymorphism with 75%, 75%, 75%, and 71.4% respectively. Hence these primers could be exploited for finding genetic variation of fennel germplasm. Four polymorphic fragments were observed in accession MP00164, followed by three polymorphic fragments in accession MP001085. Primers OPA-11 and AC- 18 were polymorphic in 12 accessions followed by primer OPA-18. Polymorphic fragments were the highest in MP00483 with AC-18 and followed by MP00318 with OPA 11 (Table 1). These were late maturing accessions that indicated nature of diversity in various accessions. The accessions with diverse pattern for RAPD are suggested to be used for further study and to select parents for inheritance or linkage groups (Eujayl *et al.*, 1998). High polymorphism revealed that RAPD could resolve genetic variation among crop germplasm, identification of cultivars and for estimating genetic relationship (Silva *et al*, 2005a; 2005b).

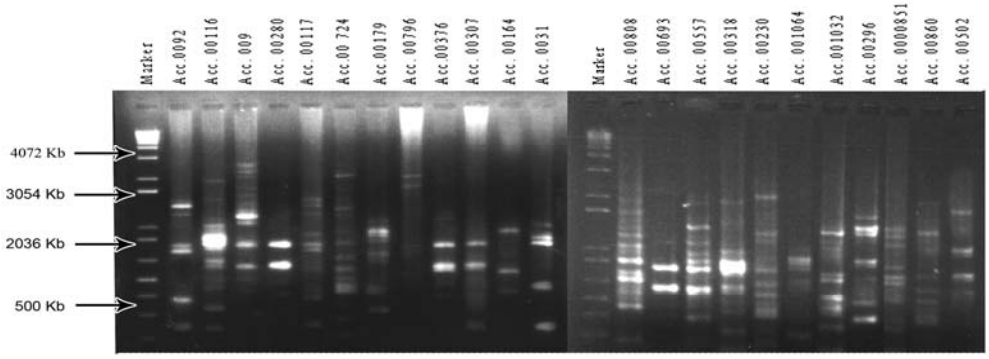

Fig. 1. RAPD pattern of fennel isolates obtained with OPA-15 the markers are indicated by arrows with molecular size (bp).

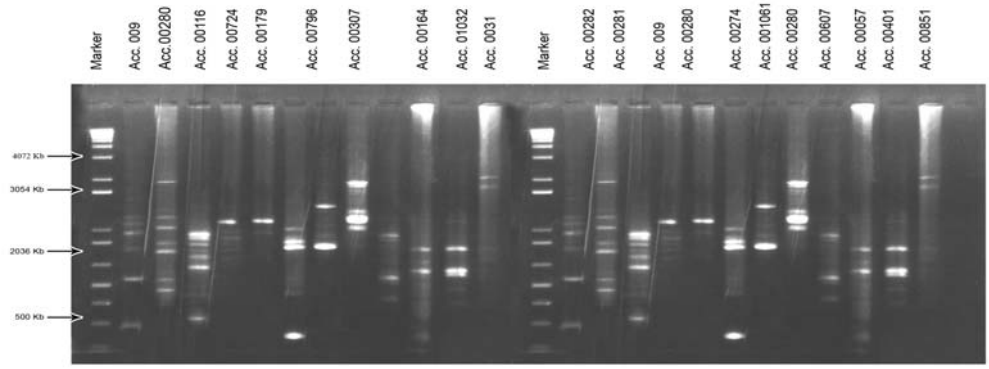

Fig. 2. RAPD pattern of fennel isolates obtained with AC-04 the markers are indicated by arrows with molecular size (bp).

Table 1. Degree of polymorphism and information content for RAPD primers along with their sequences, applied to 50 accessions of fennel.

S.#	Primers	Sequence	Polymorphic	Total (T) fragment	Polymorphic (POL) fragment	% Polymorphism (POL/T*100)
1	OPA-01	CAGGCCCTTC	Polymorphic	8	5	62.5
2	OPA-02	TGCCGAGCTG	Monomorphic	5		
3	OPA-03	AGTCAGCCAC	Polymorphic	10	7	70
4	OPA-04	AATCGGGCTG	Polymorphic	8	6	75
5	OPA-05	AGGGGTCTTG	Polymorphic	7	5	71.4
6	OPA-06	GGTCCCTGAC	Monomorphic	4		
7	OPA-07	GAAACGGGTG	polymorphic	6	4	66.6
8	OPA-08	GTGACGTAGG	Monomorphic	4		
9	OPA-09	GGGTAACGCC	Monomorphic	5		
10	OPA-10	GTGATCGCAG	Polymorphic	6	4	66.6
11	OPA-11	CAATCGCCGT	Polymorphic	12	7	58.3
12	OPA-14	TCTGTGCTGG	Polymorphic	4	2	50
13	OPA-15	TTCCGAACCC	Polymorphic	8	6	75
14	OPA-18	AGGTGCCGTT	Polymorphic	4	2	50
15	AC-11	CCTGGGTCAG	Polymorphic	4	2	50
16	AC-12	GGCGAGTGTG	Monomorphic	5		
17	AC-13	GACCCGATTG	Monomorphic	4		
18	AC-14	GTCGGTTGTC	Polymorphic	5	3	60
19	AC-15	TGCCGTGAGA	Polymorphic	7	3	42.85
20	AC-16	CCTCCTACGG	Polymorphic	6	3	50
21	AC-17	CCTGGACTT	Monomorphic	3		
22	AC-18	TTGGGGGGAA	Polymorphic	12	8	66.6
23	AC-19	AGTCCGCCTG	Monomorphic	4		
24	AC-20	ACGGAAGTGG	polymorphic	4	3	75
				145	70	48%

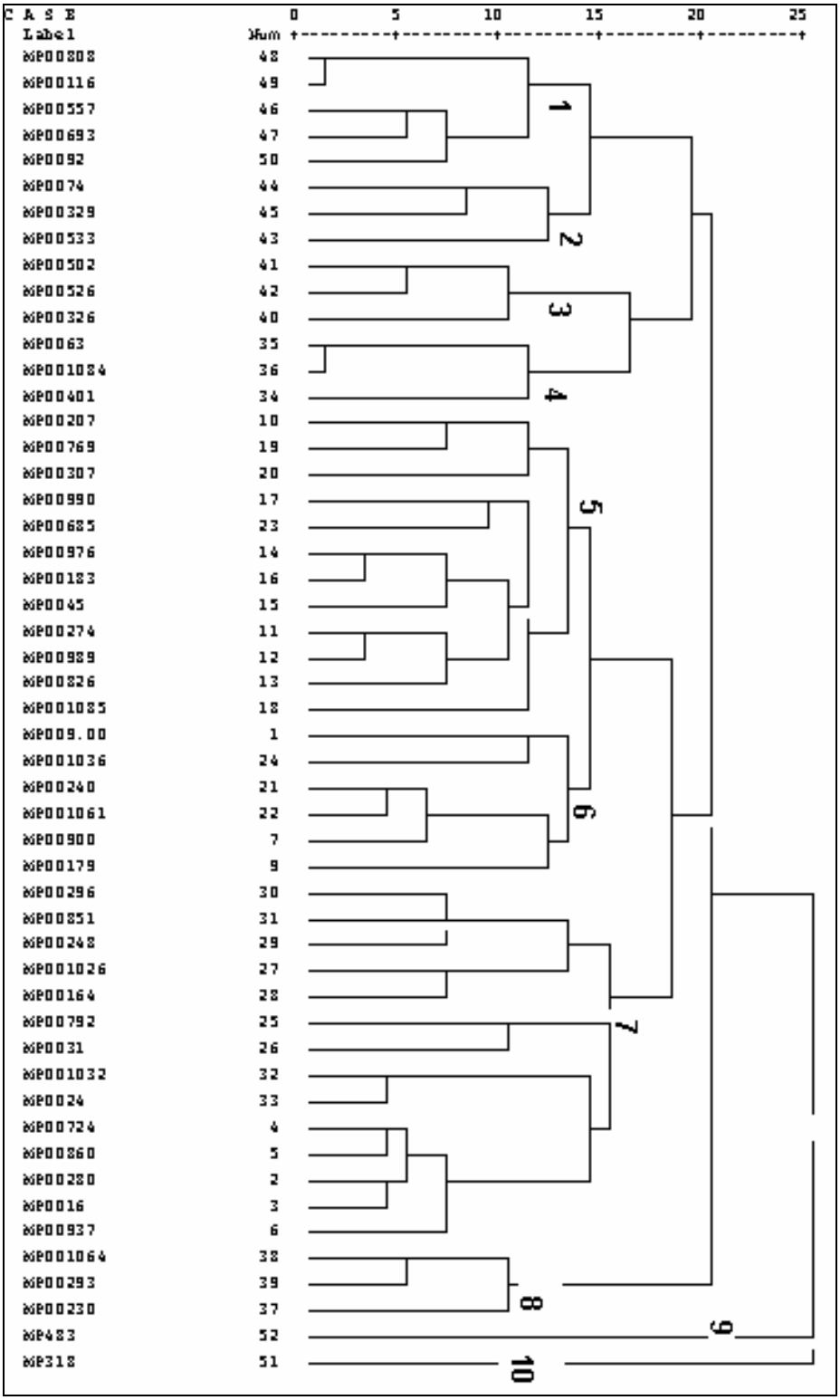

Fig. 3. Dendrogram of fifty accessions of fennel based on RAPD.

Genetic similarities and clustering of genotypes: In order to determine genetic diversity among the 50 accessions of indigenous fennel, cluster analysis was performed (Table 2). From dendrogram (Fig. 3) it was clear that all the accessions were divided into ten different clusters genetically at 70% distance. Cluster-1 was composed of 5 accessions. Out of these 5 accessions, two MP00808 and MP00116 were closer to each other and their mutual genetic distance is only 5%. In this cluster accession MP0092 is

entirely different from rest of 4 accessions at the genetically difference of 30%. At this distance this accession formed a solitary group. Cluster-3 was consisting of only 3 accessions, which is separated from each other at the distance of 50%. In this cluster accession MP00502 and MP00526 were closer as compared to accession MP00326. Three accessions are the part of cluster-4. In this cluster accession MP0063 and MP001084 were having 95% similarity. In cluster 5, the accessions MP00989, MP00274, MP00183, and MP00976 had minimum 10% genetically difference. Maximum seed yield/row (1620.01±102.4g) with highest harvesting index (32.96 ± 3.7) possessing 90% uniformity was recorded. This cluster can be utilized for getting high seed yield of fennel. Six accessions were the part of cluster 6. Cluster-7 is one of the largest clusters having fourteen accessions, within these 9 accessions depicted genetic diversity from 25% to 33% whole others 5 possessed the level of distinctness ranging from 3% to 6% only. In this cluster a group of 9 accessions have ample diversity level can be considered for a sound-breeding program

Cluster-8 contained 3 accessions MP001064, MP00293 and MP00230, which were genetically apart from each other at very low percentage of distance. Their further isolation is at 18-30% of distance. Last two clusters (9 and 10) are very different from rest of all clusters. These two clusters comprise of one accession each. These two accessions MP00483 and MP00318 are genetically so distinct from each other that they get separated from the rest of all accessions at the distance of 100%. To determine association of DNA marker with quantitative traits (days to flowering, plant height, number of umbels per plant, days to maturity, seed yield and harvest index), clusters were made on the basis of RAPD and average performance was calculated (Table 2). The results revealed that genotypes comprising cluster 9 and 10 consisted of single genotype in each case. These are late maturing accessions. Cluster 2, 3, 4 and 8 were characterized by three accessions in each cluster (Fig. 3). Cluster 1 having 5 accessions was early in days to 50% maturity (169.8±2.27), medium yielding (741.55±27.8). Cluster 10 comprising only one accession was categorized as late maturing (179±4.0 days) and low yielding (360±5.56g) along with medium harvest index (10.58±0.5%). Cluster 7 comprising of 14 genotypes required (176.6±1.9) days for maturity with average seed yield of (551.03±118.5 g) and harvest index (14.45±4.1%). Twelve genotypes comprising cluster 5 required medium period for maturity (171.16±1.49 days) with high seed yield (1620.10±102.4) and maximum harvest index (32.96±3.7 %). So this cluster could be categorized as the high yielding and medium maturity period while cluster 3 as early maturing (117 days) with long harvesting period and medium seed yield.

Cluster analysis on morphologically important characters revealed that accessions with promising characters were categorized mostly in cluster 1, 5, 7 and 10. It may be because of selection pressure for high yield potential and other related characters. This showed that only a portion of genetic diversity has been exploited and it is suggested to broaden the genetic base of cultivated fennel involving diverse parents in breeding program. High polymorphism among individual genotypes of fennel revealed that the RAPD technique is an effective technique in studying inter and intra specific variation in fennel. These results are in accordance with Fu *et al.,* (2003) who reported that out of 92 RAPD primers, 64 gave polymorphism which indicated that 51.2% of total diversity was among populations and 48.8 % within populations of *Changium smyrnioides* Wolff (Apiaceae) and results were also similar to findings of Khan *et al*., (2005) who found 40.7% of the total score-able bands were polymorphic in wheat germplasm revealed by RAPD markers.

Table 2. Cluster comparison of fennel accessions based on RAPD markers.

Clusters	Germination %	D. Init. Fl	D. 50% Fl	Pl. Ht. (cm)	st. gth.(cm)	Nodal dis (cm)	Umbel dia (cm)	No. Umbelper plant	D. 50% (Mat.)	D. Hav.	Biomass (kg)	Seed Yd/ Row (g)	Wt 100 sds (g)	H.I. (%)
Cluster-1	78	113.8	131.4	119.79	1.4214	14.8	8.86	38.84	169.6	205	4.62	741.55	0.61	13.618
	±8	± 2.27	±1.4	±7.87	±0.09	±0.54	±1.14	±6.87	±2.27	±0	±0.9	±27.8	±0.05	±3.5
Cluster-2	100	113	133	129.3	1.4195	16.65	11.375	39.3	170	205	3.95	529.4	0.585	12.2
	±0	± 0	±0	±6.9	±0.04	±1.15	±1.13	±1.1	±3.5	±0	±0.05	±46	±0.05	±1.18
Cluster-3	100	116.16	129.83	100.08	1.374	14.89	11.35	39	117.16	215.66	4.06	483.09	0.551	13.24
	±0	± 2.1	±1.16	±7.37	±0.09	±0.69	±1.40	±4.2	±1.07	±4.5	±0.23	±77.8	±0.03	±1.3
Cluster-4	81.81	119.27	131.90	106.70	1.36	13.78	9.28	30.94	173.09	219.36	4.30	815.82	0.58	19.14
	±5.52	±1.1	±051	±4.5	±0.05	±0.6	±0.42	±1.62	±1.00	±1.82	±0.17	±39.6	±0.01	±1.50
Cluster-5	81.66	119.6	133.5	89.06	1.46	12.54	9.04	36.26	171.16	215.66	3.86	1620.10	0.59	32.96
	±5.4	±1.2	±0.5	±2.7	±0.09	±1.36	±0.93	±7.7	±1.49	±4.45	±0.5	±102.4	±0.01	±3.7
Cluster-6	78	122	133.4	103.95	1.41	14.58	9.46	45.08	172.6	209.8	3.76	1270.96	0.608	18.23
	±8.6	±1.04	±1.12	±6.1	±0.1	±1.9	±0.7	±3.35	±1.4	±2.9	±0.28	±55.58	±0.05	±1.4
Cluster-7	65	122	131.25	79.96	1.35	13.48	8.80	30.85	176.6	219	4.05	551.03	0.53	14.45
	±2.8	±6	±1.18	±8.3	±0.1	±1.28	±1.7	±8.75	±1.9	±6.8	±0.35	±118.5	±0.03	±4.1
Cluster-8	90	122	129	109.88	1.627	13.11	11.375	37.7	176	211	2.25	495.45	0.62	22.265
	±10	±10	±1.2	±3.28	±0.21	±0.8	±0.37	±2.5	±3	±6	±0.25	±5.35	±0.05	±2.23
Cluster-9	100	113	129	99.8	1.341	11.52	10.2	79.2	179	211	4.9	477.65	0.65	13.65
	±10	±10	±1.4	±0.8	±0.34	±0.8	±0.3	±2.5	±4	±5	±0.9	±6.5	±0.05	±2.3
Cluster-10	100	116	131	69.2	1.311	15.8	10.4	31.4	179	205	3.4	360±	0.52	10.58
	±10	±2.1	±1.6	±0.2	±0.34	±0.8	±0.5	±0.4	±4	±6	±0.4	5.56	±0.05	±0.5

Germination percentage (ger %), Days to initiation of flowering (D.Init.Fl.), Plant height (Pl.ht), Stem girth (st.gth), Nodal distance (Nodal dis), Umbel diameter (Umbel dia), Days to 50% maturity (D. 50% Mat.), Days to harvesting (D. Harv), Seed yield per row (Seed yd/row), Weight of 100 seeds (Wt 100 sds) , Harvest index (%)(H.I) . Standard deviation

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