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GENETIC DIVERGENCE STUDY OF SAFFLOWER (*CARTHAMUS TINCTORIUS* L.) USING MOLECULAR MARKERS

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ABSTRACT

A study was carried out at PMAS Arid Agricultural University, Rawalpindi, Pakistan during the year 2016 to assess genetic divergence among 36 local and 30 exotic safflower (*Carthamus tinctorius* L.) accessions utilizing random amplified polymorphic DNA (RAPD) markers. The results revealed that , out of 15 RAPD primers, seven were polymorphic. Further the amplification product yielded 64 number of total loci with 6.4 as an average number of alleles for each loci in local whereas 72 number of total loci with 7.2 as an average number of alleles for each loci in exotic germplasm. The values of similarity co-efficient for local and exotic germplasm ranged from 0.32 (32 percent), to 1.00 (100 percent) and 0.40 (40 percent) to 1.00 (100 percent), respectively. Cluster analysis based on the data from RAPD markers, divided local germplasm into two distinct groups, which revealed two unique genotypes L6 and L21. Similarly, in exotic germplasm, cluster analysis dividedexotic germplasm into two distinct groups and showed three unique genotypes E3, E5 and E28 which were found to be the most diverse in the germplasm.

KEYWORDS: Carthamus tinctoriust; safflower; Genotypes; RAPD: molecular markers; germplasm: genetic divergence; Pakistan.

INTRODUCTION

Safflower is technically known as *Carthamus tinctorius,*, a diploid with 2*n* = 24, self pollinated and annual herbaceous crop (Pherson *et al.,* 2004) that come into flower in hot and dry climatic condition . It is a well acclimatized plant to aridareas , pertaining to its ability to tolerate drought conditions quite efficiently (Omidi *et al.,* 2009); due to its long root system, it can reach deep down in the soil profile (Yau and Ryan, 2010). Safflower has received a lot of advertising in recent times, not so much for its colourful petal but for the reason that it is one of the most essential source of vegetable oils (Camas *et al.,* 2007). Safflower oil has fewer amounts of saturated fats similar to that in canola and olive oils, and is nutritionally comparable to olive oil (Sangam *et al.,* 2005). Its oil can be used for human consumption or utilized in the industries (Killi and Altunbay, 2005). The estimation of genetic divergence in various crop plants is of attention for preservation of genetic wealth, expansion of the genetic base (Amini *et al.,* 2008), and its proper use are key components of crop improvement programs.

Agriculture in Pakistan accounts for 20.9 percent of the GDP and is a vital source of living about 43.5 percent of rural population. Edible oil is one of the major components of our diet. Pakistan has been facing a continual shortage of locally produced edible oils and as a consequence large quantities of edible oil are being imported yearly to compensate the production and consumption gap. During 2014-15, total edible oil demand in the country remained 2.335 million tonnes against local production of only 0.546 million tonnes. A massive gap of 1.789 million tonnes between availability and demand was covered by import of edible oil worth Rs: 139.344 billion. (GOP, 2015). Such gigantic burden on national economy can be curtailed by increasing the area and productivity of oilseed crops.

There are a number of molecular methods which are accessible to study the genetic divergence. These include Isozymes, amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), random amplified microsatellite polymorphisms (RAMP), simple sequence repeats (SSRs), and inter simple sequence repeats (ISSR). These markers have been effectively used to study the genetic divergence in crop plants. The selection of suitable marker system depends on the aims, technical considerations, accessibility of laboratory services and costs. The RAPD analysis procedure can work with unknown genomic markers and requires only minute quantity of DNA (Coetno *et al.,* 1991). The RAPD markers are extensively used in genetic mapping, diversity studies, and in identifying markers that are linked with required traits. The RAPD markers, due

to its technical ease and speed, have been used for diversity studies in several crops. However, their use is quite limited due to low levels of polymorphism and environmentally highly influenced isozyme expression. The RAPD markers can be efficiently utilized to identify useful polymorphisms (Ali *et al.,* 2014; Azam *et al.,* 2013; Jahangir *et al.,* 2014; Ko *et al.,* 1998; Ottai *et al.,* 2012; Shan *et al.,* 2015; Zameer *et al.,* 2015).

Although genetic markers have been utilized to assess germplasm diversity in crops yet, unlike major oilseed crops, the use of such markers in safflower is limited and research has not been carried out to develop genetic markers that are molecularly attached to the traits of interest for further improvements in safflower through molecular breeding.

Therefore, in the present study, efforts were made to ascertain genetic divergence in local as well as exotic safflower genotypes by using molecular markers.

MATERIALS AND METHODS

The present experiment was conducted at PMAS Arid Agriculture University, Rawalpindi, Pakistan during the year 2016.The experimental material comprised of 36 local and 30 exotic genotypes of safflower and seeds of these genotypes were grown in pots in glass house. Fresh leaf samples (8-10) from potted safflower plants were taken 4-6 weeks after germination and DNA from these samples was isolated following a modified version of CTAB protocol.

DNA isolation

Newly emerged fresh leaves of 4-6 weeks old safflower plants were used for DNA isolation. Two leaves of each genotype were grinded with the help of a pre-autoclaved pestle and mortar to a fine paste. A volume of 2-3 ml CTAB buffer was used during grinding procedure. The homogenate was then transferred to eppendorf tubes (1.5 ml) for incubation at 65 ºC for a period of 45 minutes in water bath. After this, chloroform: isoamylalcohol (24:1) was added in volume equal to that of the homogenate and inverted gently. The homogenate was then centrifuged at separation speed of 12000 rpm for 20 minutes at room temperature. The upper layer formed in the tubes due to precipitation of debris was then transferred to fresh tubes and an equal volume of ice chilled isopropanol was added and mixed gently inverting tubes 4-5 times. Tube was placed at 4ºC for 20 minutes. Then DNA was recovered as a pellet through precipitation at a speed of 12000 rpm for 10 minutes at 4ºC. The freshly isolated DNA pellet was then washed with 75 % ethanol and dried at room temperature. Finally, the DNA pellets were re-suspended by adding 100 µl of freshly prepared TE buffer. The re-suspended DNA was then treated with 1 µl of RNase and incubated at 37ºC for 30 minutes to remove any traces of RNA. After RNase treatment, the DNA samples were resolved on 1.5% of agarose gel for quality checks. The isolated DNA samples were stored at -20ºC. For PCR, DNA dilutions of 1:1 were made in ddH_2O

Random amplified polymorphic DNA analysis

The selection of RAPD primers was made by keeping in view the clarity and repeatability of polymorphic bands. For this purpose a set 15 decamer primers (Gene Link., USA) from A and B series were selected for amplification through polymerase chain reaction (PCR) of isolated DNA samples of 66 safflower genotypes. The final volume of reaction mixture was 20µl, that included ; 20ng template DNA; 0.2µl Taq DNA polymerase; 1.6µl $MgCl_2$ (25mM); 2µl primer; 0.4µl of dNTPs and 2µl of 10X reaction buffer. The PCR was performed in a thermo cycler programmed for an preliminary denaturation for 5 minutes at 94°C, then 40 cycles of 1 minutes at 94°C, 1 min at specific annealing temperature (36°C) for RAPD markers and 2 minutes at 72°C, and in the end a final extension for 10 minutes at 72°C.

PCR amplified products were resolved by electrophoresis in 1.5% agaros gels in TBE buffer. Ethidiumbromide (0.5 lg/ml) was used to help in visualizing the DNA banding patterns under UV radiations through gel documentation system.

Data analysis

Statistical analysis of RAPD was computed following Nei and Li's (1979). The scoring of amplified DNA fragments was done as presence '1' or absence '0' for each safflower genotype. The resultant dendrogram was developed using (UPGMA) clustering algorithm.

$$GD_{xy} = 1 - d_{xy}/d_x + d_y - d_{xy}$$

Where

GD_{xy} = Genetic distance between two genotypes
D_{xy} = Total number of common loci (bands) in two genotypes
D_x = Total number of loci (bands) in genotype 1
D_y = Total number of loci (bands) in genotypes 2

RESULTS AND DISCUSSION

RAPD analysis of local genotypes

Fifteen PCR based random amplified polymorphic DNA decamer primers from A series were utilized to amplify RAPD fragments on 36 local genotypes. Complete nucleotide sequences of these primers are given in Table 1.

Table 1. RAPD molecular markers used for genetic study.

S. No.	Primer Names	Sequences
1.	OPA01	CAGGCCCTTC
2.	OPA02	TGCCGAGCTG
3.	OPA03	Agtcagcca
4.	OPA04	AATCGGGCTG
5.	OPA05	AGGGGTCTTG
6.	OPA06	GGTCCCTGAC
7.	OPA07	GAAACGGGTG
8.	OPA08	GTGACGTAGG
9.	OPA09	GGGTAACGCC
10.	OPA10	GTGATCGCAG
11.	OPA11	CAATCGCCGT
12.	OPA12	TCGGCGATAG
13.	OPA13	CAGCACCCAC
14.	OPA14	TCTGTGCTGG
15.	OPA15	TTCCGAACCC

Only clear and prominent bands were scored, whereas, faint bands were not considered for population genetic analysis. Each band was rendered as a single locus/ allele and loci were scored as 1 for present and 0 for absent. Out of total 15 primers used, only 7 primers had given clear and polymorphic bands amplification pattern. A total number of 64 loci were traced by these primers with an average number of 6.4 alleles per locus. The molecular size of the amplification product ranged from 50bp (all primers) to 1000bp (A10, A12). RAPD pattern produced by using the OPA10, OPA11 and OPA12 primer are presented in Fig. 1 and Fig. 2.

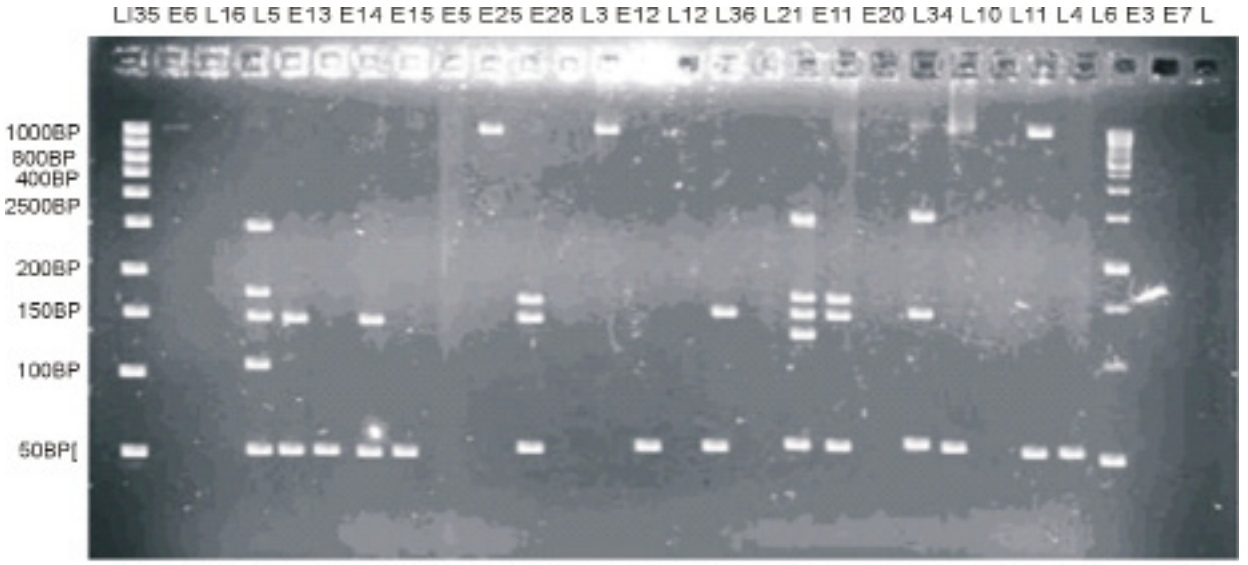

Fig. 1. Agarose gel amplification profile of primer A10 from DNA of local and exotic germplasm of safflower. Lane L is ladder while remaining lanes represent the local and exotic genotypes.

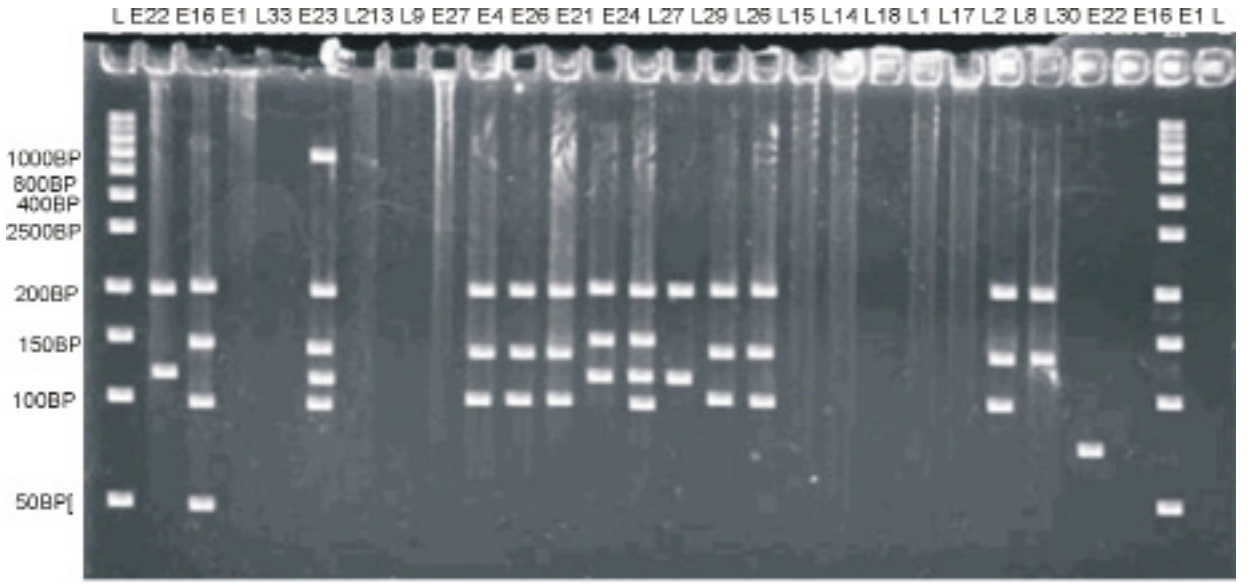

Fig. 2. Agarose gel amplification profile of primer A11 and A12 from DNA of locala nd exotic germplasm of safflower. Lane L is ladder while remaining lanes represent the local and exotic genotypes.

The primer A12 gave highest number of scorable bands, whereas, no scorable band was obtained with primer A3, A4 and A6. Maximum genotypes (i.e. 11) were amplified by primer A12 and A11 while minimum genotypes (i.e. 2) by primer A8. Various primers used in the experiment showed variation in detecting polymorphism. Primer A12 showed higher polymorphism while primer A2 illustrated lowest level of polymorphism according to population genetics analyses.

A comparison matrix was generated to estimate genetic divergence and relatedness amongst 30 local genotypes from RAPD amplification data. The lowest genetic relatedness was observed between L6 and L34 (32%) and the highest value of similarity was shown by remaining genotypes (100%).

The genetic distances for 36 local genotypes were used to develop a dendrogram for determine grouping pattern based on relatedness and differences. Similar genotypes are placed in small group recognized as cluster. Grouping the genotypes at 0.56 (56%) genetic distance revealed that there are two main clusters which can be named as A and B.

Cluster A comprised of two genotypes which are L21 and L6 showing a genetic distance of 0.59 (59 percent), be the most diverse genotypes in this dendrogram (Fig.3). Cluster B can be further subdivided into smaller clusters named as subcluster B1, B2 and B3. B1 subcluster comprised of 11 genotypes, which were, L35, L34, L30, L33, L8, L10, L16, L11, L5, L36 and L12 (sequence of genotype is according to dendrogram). Six genotypes were present in sub cluster B2 which were L23, L9, L29, L27, L26 and L15.In sub cluster B3, 17 genotypes were there, which were, L4, L32, L31, L28, L25, L24, L22, L20, L19, L13, L7, L3, L2, L17, L18, L14 and L1.

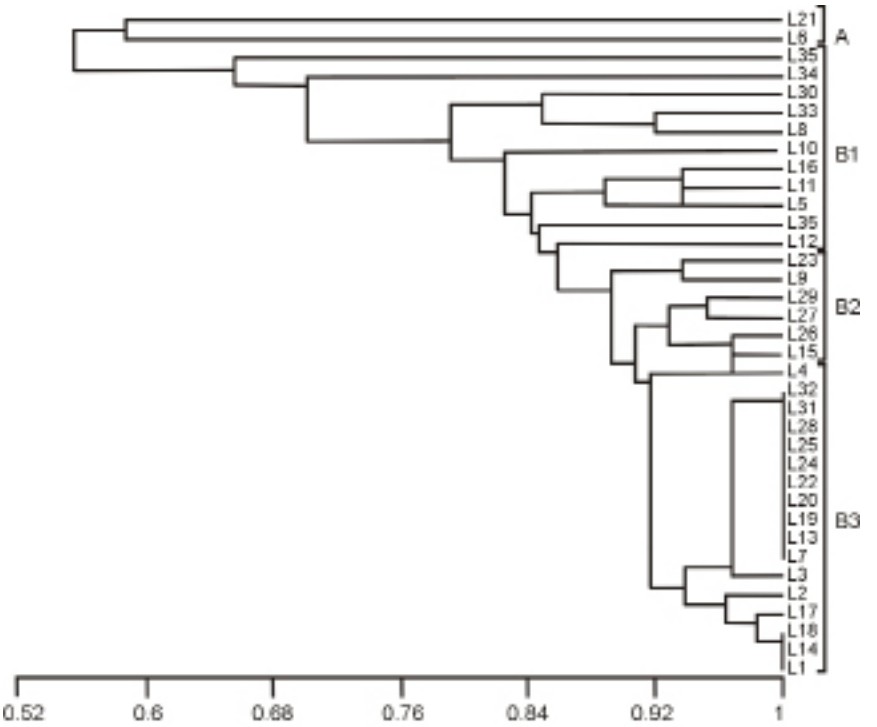

Fig. 3. RAPD based cluster analysis of local genotypes of safflower.

RAPD analysis of exotic genotypes

A total of 15 PCR based random amplified polymorphic DNA decamer primers from A series were used to amplify RAPD fragments on 30 exotic genotypes. A clear and prominent bands were used in scoring, whereas, faint bands were avoided to be considered for population genetic analysis. Each band was regarded as a single allele and loci were scored as 1 for presence and 0 for absence.

In terms of band number, out of 15 primers used, seven primers gave bright and polymorphic amplification pattern. More over, these primers were used to explore exotic genotypes and total number of 72 loci were traced by these primers through population genetic analysis with an average number of 7.2 alleles per locus. The molecular size of amplification product ranged from 50bp (primer A2, A9, A10, A11 and A12) to 1000bp (primer A12).

Sufficient variation was observed in the ability of a primer to detect polymorphism. Primer A12 showed

higher level of polymorphism while primer A2 showed the lowest. The highest number of scorable bands (14) was obtained with primer A2 and A11. Primer A3, A4 and A6 could not amplify any exotic genotype. Genotype E5 and E15 were amplified by maximum number of primers (6) while genotypes E3, E22, E17. E18, E19, E29, and E30 were not amplified by any single primer. Similar results in safflower were also observed by Rehman *et al*. (19).

A similarity matrix was generated to estimate genetic divergence and similarities among 30 exotic genotypes from RAPD amplification data. The genetic distances among 30 exotic genotypes were used to construct a dendrogram for determining grouping among these genotypes on the basis of similarities and differences.

For convenience in interpretation, dendrogram is divided into clusters. Similar genotypes are placed in small group known as cluster. Grouping the genotypes revealed that there are two main clusters which are named as A and B.

Cluster A comprised of three genotypes i.e. E5, E28 and E3 (Fig. 4).This cluster can be further divided in three smaller subclusters named as subcluster B1, subcluster B2 and subcluster B3.In subcluster B1, six genotypes are present E6, E22, E13, E12, E15 and E14. There are eight genotypes present in this subcluster B2 which are E11, E24, E16, E9,E26, E23, E21 and E4. B3 subcluster was comprised of 13 genotypes, which are, E25, E20, E7, E30, E29, E19, E18, E17, E10, E8, E2, E27 and E1.

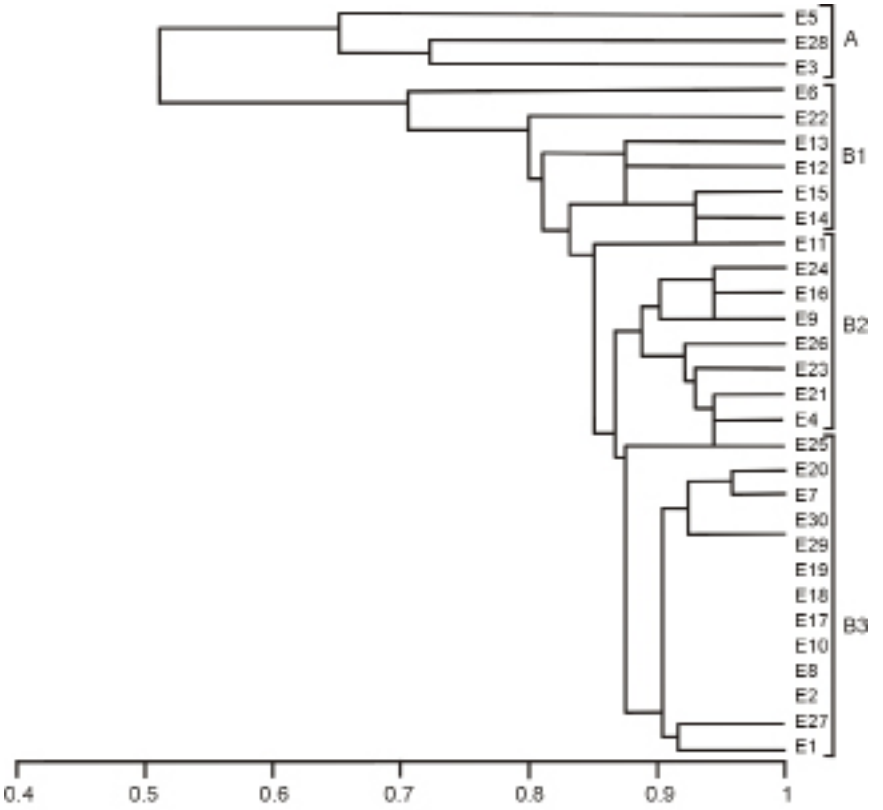

Fig. 4. RAPD based cluster analysis of exotic genotypes of safflower.

The RAPD markers revealed a high degree of polymorphism among 36 local and 30 exotic safflower genotypes. The cluster analysis also depicted that all genotypes of safflower could be studied for variation through RAPD markers. In molecular case, genetic diversity showed no relationship towards geographical origin of the genotypes. This was deduced from the random projection of genotypes of unknown origin over the whole dendrogram. Since a small number of RAPD markers was used in the present study that amplified a good number of polymorphic bands thus indicating a good level of genetic diversity present in studied germplasm. RAPDs detect polymorphism in coding as well as in non-coding regions of the genome (17). RAPD primers showed a good level of polymorphism and a greater number of clearly amplified bands among accessions in our study. These results are supported by Fu *et al*. and Žiarovská*et al*., (2012) where they reported that a huge amount of genetic divergence exists in the world collection of linseed. High genetic variation for polymorphism at DNA level in safflower was also observed by Galkar *et al*. (2011).

CONCLUSION

Morphological and molecular evaluation of safflower germplasm suggested that L1, L10, L12, E9 and E13 are the best genotypes and can be used in breeding programs for the betterment of traits and development of high yielding safflower cultivars. The findings of high genetic variation for polymorphism at DNA level revealed that the genetic variability present in these genotypes can be used in different breeding programs for the development of new genetic stock.

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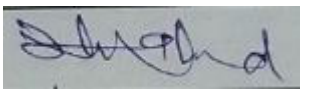
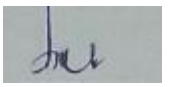
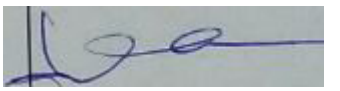
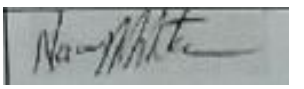
2.	Talat Mahmood	Conceived idea and planned experiment	
3.	Ghulam Shabbir	Helped in review of experiment	
4.	Zahid Akram	Critically checked the draft	
5.	Naeem Akhtar	Helped in data analysis	
6.	Ijaz Tabasum	Provided technical assistance