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Identification of genetic basis associated with agronomic traits in a global safflower panel using genome-wide association study

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Abstract: Safflower is an underutilized and minor oilseed crop that received less attention from the scientific community compared to other oilseed crops like soybean and sunflower. Exploring the genetic basis associated with agronomic traits is crucial for marker-assisted breeding of safflower. A genome-wide association study was conducted using a total of 12,232 DArTseq markers to identify the marker-trait association for important agronomic traits in an international safflower panel derived from 26 different geographical countries of the world. Statistically significant genotypic effects ($p < 0.05$) were observed across mean data of both locations (Pakistan and Turkey). Moderate to high heritability estimates were observed for the studied traits. Studied material showed higher performance for all traits except seeds per capitulum in Pakistan compared to Turkey. Phenotypic diversity for important agronomic traits, such as plant height (60.08 to 121.48 cm), capitula per plant (8.7 to 80.4), seeds per capitulum (15 to 42.05), and seed yield per plant (4.85 to 51.02 g), was illustrated. Seed yield per plant showed a highly significant and positive correlation with capitula per plant (0.4985^{***}). Constellation plot analysis resulted in four groups, i.e. A, B, C, and D. Genotyping by sequencing resulted in 12,232 DArTseq markers being used for the investigation of marker-trait association through mixed linear model (Q + K) approach. DArT-38077549 showed significant association with capitula per plant, while two markers (DArT-22763576, DArT-22763253) were associated with plant height. A total of two markers (DArT-38079422, DArT-100043360) were associated with seeds per capitulum. A total of five DArTseq markers showed significant association with seed yield per plant and maximum variation was resulted by DArT-100004992. The results of this study provide a new insight to understand the genetic basis associated with agronomic traits in safflower. We envisage that significant markers identified through this investigation may be applicable in future safflower marker-assisted breeding programs.

Key words: Oilseed, *Carthamus tinctorius*, DArTseq, agronomic traits, marker-assisted breeding

1. Introduction

Safflower (*Carthamus tinctorius* L.) is a self-pollinated crop and belongs to *Compositae* family having a haploid genome size of about 1.4 GB (Kumari et al., 2017). It is planted as a key industrial crop for a variety of applications, including edible oil extraction, dye manufacture, and it has uses in the pharmaceutical industry (Marinova and Riehl, 2009; Chapman et al., 2010; Ali et al., 2019). Safflower also gained importance because it has a stronger ability to adjust the stresses such as salinity and drought, and it also posseses the capability of bio-fuel production (Ali et al., 2020a). Despite having a high content of polyunsaturated fatty acids and being drought resistant, safflower has not yet established itself as a major oilseed crop. Low seed production, low oil content, susceptibility to numerous biotic and abiotic stresses, and spininess are the main issues that have impeded its cultivation on large scale (Nimbkar, 2008). Therefore, the enhanced acceptability and utilization of safflower as an oilseed crop require genetic improvement for the traits of interest.

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Very few research groups are working on the different aspects of safflower and access to the advanced biotechnological tools in terms of information, equipment, and various techniques are insufficient compared to the cereals and legumes. Keen interest has been witnessed from the industrial sector in safflower production for different purposes. However, safflower breeding programs experience inadequate complementation with advance biotechnological tools (Sujatha et al., 2008). In the current era of advanced modern sequencing technologies, the genome sequencing of safflower is yet to be performed.

Genotyping by sequencing (GBS) and multiplex sequencing are the next-generation sequencing technologies that aid in the creation of enormous genomic data for a variety of applications (Raman et al., 2011). Use of current polymerase chain reaction (PCR)-based marker technologies aimed at whole-genome analysis for association studies, development of genetic maps, assessment of gathered germplasm for large-scale molecular evaluation, and genome-wide selection of preferred alleles is not feasible due to consumable and labor costs (Raman et al., 2011).

Mining the genetic basis of important agronomic traits is crucial for sustainable crop production. Identification of the genetic basis of important agronomic traits is regarded as a key challenge for plant breeders in the 21st century (Losos et al., 2013). Approaches, i.e. quantitative trait locus (QTL) mapping and genome-wide association studies (GWAS) have been developed for the identification of the genetic basis of complex plant traits (Korte and Farlow, 2013). QTL mapping, also known as bi-parental mapping, is primarily used to investigate QTLs linked to traits of interest. However, in addition to its benefits, QTL mapping has a number of disadvantages, including limited recombination, being time-consuming, and the population specificity of the identified QTLs (Nadeem et al., 2018). GWAS overcomes all the drawbacks of QTL mapping, and the identified markers through this approach can be used for any population (Korte and Farlow, 2013). GWAS are implemented as a common approach to dissect the genetic basis of complex plant traits (Risch and Merikangas, 2007). It has been successfully utilized for unraveling and mining of genomic regions controlling agronomic plant traits in many crop species (Wang et al., 2012; Monostori et al., 2017; Hazzouri et al., 2018; Nadeem et al., 2021). However, there are many genomic regions/genes for these traits yet to be identified using diverse germplasm and new genome sequencing information. Very recently, Ali et al. (2020a) used DArTseq markers for the identification of marker-trait association for 100 seed weight in world safflower germplasm. Ambreen et al. (2018) used SSR markers to investigate marker-trait association for oil and agronomic traits. Similarly, Ebrahimi et al. (2017) used AFLP markers for the identification of marker-trait association for some oil and agronomic traits. As the identification of the genetic basis of important agronomic traits is regarded as a key challenge for plant breeders in the 21st century (Losos et al., 2013), current study was conducted to evaluate marker-trait association for important agronomic traits in an international safflower panel using 12,232 DArTseq markers.

2. Materials and methods

2.1. Plant material, field experiments and phenotypic evaluation

A total of 92 safflower accessions and two check cultivars (Thori-78 from Pakistan and Dinçer from Turkey) were utilized as plant material. Seeds were provided by the United States Department of Agriculture (USDA). A detailed information about studied germplasm and field experimentation can be found from our previous study (Ali et al. 2020a). Recommended agronomic cultural practices were maintained during the whole crop season at both locations. All safflower accessions were harvested at the time of maturity, i.e. when 95% of the capitulum changed the color from green to yellow, and the crop was ready to harvest. Data was recorded on important agronomic traits, i.e. plant height, capitula per plant, seeds per capitulum, and seed yield per plant at their proper time. Plant height was recorded on ten randomly selected plants using meter rod in unit centimeter (cm). As the matter of fact, height is the length of space from ground to the peak of the plant where main capitulum is present. Total number of capitulum produced by the individual plant was counted manually. Same selected plants were used to calculate capitula per plant, and the sum was then averaged. To determine number of seeds per capitulum, main capitulum of each selected plant was used from each accession. The selected capitulum was threshed separately and the number of seeds was counted and recorded. To determine seed yield per plant, each plant was harvested and dried. Seeds were cleaned from impurities and dust and weighed in grams with the help of an electronic balance.

2.2. DNA extraction and genotyping for DArTseq markers

DNA extraction was performed following CTAB protocol (Doyle and Doyle, 1990) and specific protocol described by Diversity Arrays Technology (https://www.diversityarrays.com/orderinstructions/plant-dna-extraction protocol for-dart/). The purity of each extracted safflower DNA sample was checked with agarose gel electrophoresis of 0.8%, while quantification was determined with DS-11 FX series spectrophotometer/fluorometer (Denovix, Wilmington, DE, USA). The final concentration of each DNA sample

was maintained at 50 ng μL^{-1}. All DNA samples were prepared and processed to Diversity Array Technology Pty, Ltd., Bruce, Australia for genotyping by sequencing purpose (http://www.diversityarrays.com/). Genotyping by sequencing analysis for DArTseq marker was achieved according to previous studies (Kilian et al., 2012; Li et al., 2015b).

2.3. Statistical analysis

2.3.1. Phenotypic data analysis

Statistical inferences of the augmented block design were performed through online software developed by Rathore et al. (2004). Mean phenotypic data recorded on plant height, capitula per plant, seeds per capitulum, and seed yield per plant across both locations (Pakistan and Turkey) were averaged, and their means were executed for various analyses. Analysis of variance was computed implementing SAS software 9.3 version. Appropriate variance components were extracted from the linear mixed model equation and utilized to estimate heritability (Pereira et al., 2017). Mean phenotypic data was executed to calculate summary statistics, i.e. maximum, minimum, mean, standard deviation, the Pearson correlation coefficient and frequency distribution via statistical software XLSTAT (Addinsoft, 2018) (www.xlstat.com). The cluster constellation plot was constructed with the help of JMP 14.1.0 statistical software (2018, SAS Institute Inc., Cary, NC, USA).

2.3.2. Population structure, genome-wide association analysis, and putative gene identification

Population structure was undertaken as described by Ali et al. (2020a). Identification of marker-trait association was practiced in TASSEL 5.0.5 implementing the Mixed linear model (MLM, Q + K) approach (Bradbury et al., 2007). Q-metrics (Q) and kinship (K) were applied to correct the population and family structure. Kinship matrix was detected by the descent method utilizing the scaled identity in TASSEL 5.0.5 (Bradbury et al., 2007). Relatedness between associated trait and their identified marker is represented by p value, while the proportion of phenotypic variation revealed by the identified marker is denoted by R^2 during the process of association analysis (Jin et al., 2011). Both FDR and Bonferroni thresholds were used and markers having FDR and Bonferroni p = 0.01 thresholds were considered associated with trait. The qq-man R Package in the R 4.0.0 statistical software was used to construct a Pseudo-Manhattan plot (Turner, 2014). The sequences of identified DArTseq markers were used to BLAST in National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/), and comprehensive information about identified putative genes was collected from TAIR database (http://www.arabidopsis.org).

3. Results

3.1. Agronomic traits evaluation

Significant variation among safflower accessions and locations was observed for the studied traits calculated across mean data of both locations as revealed from analysis of variance (Table 1). Accession × Location interaction was only significant for seed yield per plant (Table 1). The studied traits revealed moderate to high heritability for capitula per plant (0.36), plant height (0.32), seeds per capitulum (0.31), and seed yield per plant (0.70), respectively (Table 1). The evaluated safflower accessions exhibited superior mean performance for plant height, capitula per plant, and seed yield per plant in Pakistan, while better mean performance for seeds per capitulum was observed in Turkey (Table 2). Mean data across both locations (Pakistan and Turkey) showed minimum and maximum plant height of 60.08 and 121.48 cm with mean value of 92.63 cm, respectively. Safflower accessions exhibited minimum of 8.7 and maximum of 80.4 capitula per plant with a mean value of 28.94. Seeds per capitulum observed minimum and maximum values of 15 and 42.05 with a mean of 25.29, respectively. Seed yield per plant ranged from 4.86 to 51.02 g with a mean value of 15.95 g, respectively (Table 3).

Frequency distribution reflected normal distribution for studied traits using mean data of both locations (Figure 1). Correlation analysis revealed highly significant and positive association between seed yield per plant and capitula per plant (0.4985^{***}) (Table 4). Constellation plot analysis separated the 94 accessions into four groups, i.e. A, B, C, and D based on plant height, seed yield per plant, seeds per capitulum, and capitula per plant (Figure 2).

3.2. Marker-trait association

A total of 12,232 highly informative DArTseq markers were used for GWAS analysis. At Pakistan location, 13 markers showed significant association with the studied traits, including one marker each for plant height and capitula per plant, two markers for seeds per capitulum, and nine markers for seed yield per plant (Table 5). A total of two markers were found significantly associated with the studied traits, including one each for plant height and seeds per capitulum at Turkey location (Table 5). Combined data from both locations (Pakistan and Turkey) identified a total of 10 significantly associated markers for the studied traits. A total of two markers (DArT-22763576 and DArT-22763253) showed significant association with plant height (Table 5, Figure 3). DArT-38077549 was found as the only marker having significant association with capitula per plant (Table 5, Figure 4). A total of two markers were associated with seeds per capitulum, and maximum variation was resulted by DArT-38079422 (Table 5, Figure 5). A total of five markers were significantly associated with seed yield per plant and DArT-100004992

Table 1. Analysis of variance and heritability estimates of four agronomic traits across mean data of two locations (Pakistan and Turkey).

Trait	Source of Variation	F Value	Heritability (broad sense)
Plant Height	Accessions	1.71*	0.32
	Locations	443.16***	
	Accession × Location	0.76	
	Error	-	
Capitula Per Plant	Accessions	1.91*	0.36
	Locations	106.46***	
	Accession × Location	1.63	
	Error	-	
Seeds Per Capitulum	Accessions	1.15*	0.31
	Locations	25.09***	
	Accession × Location	0.94	
	Error	-	
Seed Yield Per Plant	Accessions	5.6***	0.70
	Locations	289.39***	
	Accession × Location	4.99***	
	Error	-	

marker accounted for maximum phenotypic variation for this trait (Table 5, Figure 6). BLAST search in NCBI for DArT-22763253 revealed AT2G01150.1 as a putative gene for plant height (Table 5). A search using the sequence of DArT-38077549 in NCBI resulted into AT1G04780.1 as a putative gene for capitula per plant. A search against the query markers DArT-38079422 and DArT-100043360 resulted in the retrieval of AT3G18770.1 and AT5G17310.2 genes, respectively for seeds per capitulum. A total of four putative genes were identified for seed yield per plant (Table 6).

4. Discussion

4.1. Agronomic traits evaluation

Agronomic traits viz., capitula per plant, seeds per capitulum, plant height, and seed yield per plant were reported as key factors for the improvement of safflower production (Ali et al., 2020b). Therefore, identification of genetic basis related to these important agronomic traits could be helpful to develop elite safflower cultivars with increased production and better adaptation under diverse climatic conditions. Keeping in view these agronomic traits, an international safflower panel comprised of 94 accessions was used to identify the genetic basis revealing significant association with these agronomic traits. Analysis of variance (ANOVA) revealed statistically significant variation for the studied traits that might be helpful for breeding purposes (Table 1). El-Lattief et al. (2012) found statistically significant genotypic effects for plant height, capitula per plant, and seed weight per plant in safflower accessions. Similarly, Mahasi et al. (2006) also supported our results as they observed significant genotypic effects among the 36 safflower accessions evaluated at different locations in Kenya. Furthermore, significant genetic variation for seed yield was observed among 18 safflower accessions evaluated at three different locations (Jamshidmoghaddam et al., 2012). Amini et al. (2008) tested 16 lines along with 4 exotic safflower accessions in different locations of Iran and observed significant genotypic effects for plant height, capitula per plant, seeds per capitulum, and seed yield per plant. These findings clearly supported our results about the presence of significant genotypic effects of safflower material evaluated in different environments. Accession × Location interaction was not significant for the studied traits except seed yield per plant, proposing that selections made in one location can be usefully exploited in the other location, which can expedite the process of cultivar development and cut the costs associated with this activity. The significant Accession × Location interaction for seed yield per plant was in accordance with the results of Tahernezhad et al. (2018), but it contrasted with their findings for plant height, capitula per plant, and seeds per capitulum. A significant Accession × Location interaction

Table 2. Mean data of 94 safflower accessions evaluated during current study for important agronomic traits across two locations (Pakistan and Turkey).

Trait	Plant Height			Capitula Per Plant			Seeds Per Capitulum			Seed Yield Per Plant		
Accession	Pakistan	Turkey	Mean	Pakistan	Turkey	Mean	Pakistan	Turkey	Mean	Pakistan	Turkey	Mean
Israel-1	110.62	72.2	91.41	17.2	9.6	13.4	16.3	23.8	20.05	6.25	9.85	8.05
Romania-1	123.44	80.2	101.82	34	25.6	29.8	21.7	25	23.35	10.39	15.15	12.77
Morocco-1	122.43	79.4	100.92	34.2	31.6	32.9	26.8	24.6	25.7	10.14	11.35	10.74
Egypt-1	109.73	64.4	87.06	24.4	13.2	18.8	16.7	26.4	21.55	8.99	4.43	6.71
Pakistan-1	120.32	78.6	99.46	33.6	26.8	30.2	25.9	23	24.45	27	6.71	16.85
Pakistan-2	88.90	61	74.95	33.2	25.2	29.2	24.2	41.2	32.7	19.29	15.21	17.25
Pakistan-3	82.35	56.4	69.37	30.2	28	29.1	20.3	23.4	21.85	17.96	7.16	12.56
Pakistan-4	75.96	44.2	60.08	17.2	23.4	20.3	27	42.4	34.7	9.87	5.57	7.72
Pakistan-5	110.74	71	90.87	18.6	25.6	22.1	30.4	29	29.7	5.39	8.49	6.94
Pakistan-6	108.18	67.8	87.99	36	30.4	33.2	23.4	40.4	31.9	19.71	15.76	17.73
Pakistan-7	103.12	73	88.06	52.6	27.2	39.9	28.1	38.4	33.25	66.55	20.08	43.31
Egypt-2	132.97	63.6	98.28	43.6	14.6	29.1	21.3	17	19.15	17.87	3.01	10.44
Egypt-3	124.46	71.4	97.93	50.4	22.4	36.4	15.8	28.4	22.1	62.73	8.99	35.86
Egypt-4	107.04	75.2	91.12	33.2	7.2	20.2	19.3	26.2	22.75	26.98	7.68	17.33
India-1	90.36	71.4	80.88	32.4	15.8	24.1	29.7	20.8	25.25	18.45	9.55	14
Egypt-5	125.98	83.2	104.59	36.6	15.4	26	27.2	12	19.6	59.66	5.98	32.82
Egypt-6	106.05	79.8	92.92	29.4	18	23.7	23	27	25	19.55	6.04	12.79
Iran-1	94.11	74.8	84.45	53.8	18.4	36.1	32.5	28	30.25	49.72	9.19	29.45
Jordan-1	109.19	82.4	95.79	58.6	19.2	38.9	26	34.6	30.3	52.81	10.22	31.51
Jordan-2	100.79	80.6	90.69	73.2	19	46.1	16.4	20.6	18.5	71.1	7.27	39.18
Israel-2	104.14	74.6	89.37	12.6	18.2	15.4	21.9	26.8	24.35	9.11	10.42	9.76
Spain-1	110.74	71.2	90.97	23	23.6	23.3	30.7	31.4	31.05	25.81	14.26	20.03
Spain-2	117.89	85.4	101.65	18.6	27.8	23.2	23.7	20.8	22.25	10.64	10.23	10.43
Spain-3	112.53	89.6	101.07	20.8	23	21.9	16.6	32.2	24.4	14.73	13.34	14.03
Spain-4	107.69	81.8	94.74	43.4	23.8	33.6	15.7	15.6	15.65	25.14	1.78	13.46
Portugal-1	112.42	87.6	100.01	41.2	29	35.1	15.5	31.8	23.65	18.61	13.56	16.08
Portugal-2	124.08	89.6	106.84	41	22	31.5	27.9	38.2	33.05	32.39	6.94	19.66
Morocco-2	114.30	88	101.15	52	34.6	43.3	23.4	27	25.2	16.52	4.35	10.43
Portugal-3	146.06	81.6	113.83	21.6	26	23.8	18.8	29	23.9	24.84	9.17	17.00
Portugal-4	126.49	66.8	96.64	20.4	9	14.7	24.1	27.8	25.95	37	4.63	20.81
Portugal-5	124.48	72.6	98.54	33	17	25	25.4	43.4	34.4	16.03	9.65	12.84
Iraq-1	131.46	81.2	106.33	31.2	18.8	25	19.4	28.8	24.1	13.47	5.67	9.57
Iraq-2	104.52	87.6	96.06	47.75	8.8	28.275	30.5	24.2	27.35	25.65	3.56	14.60
Afghanistan-1	110.64	84.4	97.52	26.8	22.6	24.7	29.5	23.8	26.65	8.16	3.63	5.89
Israel-3	126.49	76.2	101.35	35	28	31.5	25.4	24.4	24.9	26.7	6.08	16.39
Syria-1	119.30	83.6	101.45	35.8	23	29.4	12.5	30.4	21.45	6.22	14.28	10.25
Syria-2	121.41	82.8	102.11	12.6	17.8	15.2	14.2	27.6	20.9	1.72	10.43	6.07
Portugal-6	119.89	81.4	100.65	44.8	15.2	30	27.7	31.6	29.65	23.57	6.19	14.88
Uzbekistan-1	94.09	73.8	83.94	33.6	14.8	24.2	26.2	18.2	22.2	15.398	2.64	9.01
China-1	110.24	86.4	98.32	54.8	17.6	36.2	27.4	37.4	32.4	36.8	12.15	24.47

Table 2. (Continued).

China-2	113.93	74.8	94.36	45.4	11	28.2	18.4	31.4	24.9	13.5	6.59	10.04
Iran-2	123.44	75	99.22	62	19	40.5	30.9	41.6	36.25	20.52	6.6	13.56
Iran-3	113.76	78.2	95.98	59.4	20	39.7	19.2	29.4	24.3	33.47	2.18	17.82
Turkey-1	117.99	83.2	100.6	45	16.6	30.8	22.4	30.8	26.6	26.14	10.58	18.36
Turkey-2	87.88	71.4	79.64	33	19.2	26.1	11.3	20.8	16.05	23.07	8.35	15.71
Turkey-3	99.06	59.8	79.43	54.6	18.6	36.6	22.8	27.8	25.3	27.35	5.99	16.67
Turkey-4	97.41	77	87.20	46.4	13.6	30	24.8	32	28.4	50.47	10.45	30.46
Afghanistan-2	133.60	81	107.3	22.4	9.2	15.8	31.5	14.6	23.05	7.68	2.45	5.06
India-1	100.58	54.6	77.59	33.2	35	34.1	21.2	16.2	18.7	7.93	9.9	8.91
Russia-1	130.65	74.8	102.73	11.8	28.4	20.1	28	26.8	27.4	4.92	8.82	6.87
India-2	102.05	72.8	87.42	16.6	18.6	17.6	18.5	21.2	19.85	9.73	3.72	6.72
India-3	89.03	75.2	82.11	17.2	19	18.1	22.9	9.4	16.15	9.78	5.21	7.49
India-4	88.12	65.8	76.96	22.8	17.6	20.2	19.8	10.2	15	7.27	3.36	5.31
Kazakhstan-1	109.73	65.6	87.66	15.6	15.2	15.4	43.5	28	35.75	3.04	9.38	6.21
Turkey-5	136.14	73.2	104.67	31.6	31.8	31.7	20	33	26.5	6.41	16.92	11.66
Argentina-1	93.47	65.2	79.33	25.4	18.8	22.1	20.7	43.2	31.95	15.45	11.36	13.40
Uzbekistan-2	109.15	71	90.07	31.25	28.8	30.025	19.6	28.8	24.2	9	14.53	11.76
Uzbekistan-3	88.14	66.6	77.37	19.6	25.2	22.4	17.1	19.4	18.25	4.1	15.49	9.79
Syria-3	95.11	82.2	88.65	23.4	20.8	22.1	19.6	31	25.3	7.2	12.52	9.86
Thailand-1	101.58	80.2	90.89	21.5	18.4	19.95	12.5	34.2	23.35	7.32	8.19	7.75
Iran-4	149.35	93.6	121.48	31.4	24.6	28	24.4	28.2	26.3	3.87	11.56	7.71
Iran-5	120.41	76	98.20	35.6	18.6	27.1	33.1	23.8	28.45	5.49	4.28	4.88
Bangladesh-1	111.46	76.6	94.03	19.4	15.8	17.6	14.1	23.2	18.65	21.2	5.94	13.57
Bangladesh-2	102.00	71.2	86.6	28	28.4	28.2	12.5	33.8	23.15	13.754	14.65	14.20
Bangladesh-3	105.13	87.8	96.46	26	16.2	21.1	15.5	36.2	25.85	15.65	6.01	10.83
India-5	105.53	75.4	90.46	40.2	21.2	30.7	12.3	24	18.15	13.99	4.87	9.43
Afghanistan-3	97.85	80.6	89.22	73.2	21.6	47.4	18.3	28.4	23.35	25.61	6.93	16.27
Australia	108.20	64.2	86.2	61.4	22.6	42	23.4	28.6	26	18.96	9.26	14.11
Turkey-6	128.62	76.6	102.61	59	17.4	38.2	31	23	27	14.97	7.16	11.06
Pakistan-8	95.50	58	76.75	132	28.8	80.4	28.9	24.2	26.55	60.51	6.64	33.57
Pakistan-9	98.55	64	81.27	71.6	27.6	49.6	23.8	31.8	27.8	44.39	7.99	26.19
Iran-6	128.02	87	107.51	53.8	21	37.4	27.1	40.4	33.75	26.29	9.75	18.02
Jordan-3	103.20	77.8	90.5	54.4	20.8	37.6	22.3	27.4	24.85	35.75	10.76	23.25
Jordan-4	94.20	70	82.1	65.8	23.2	44.5	21	28.8	24.9	30.65	10.12	20.38
Jordan-5	102.11	71.2	86.65	88.6	22.6	55.6	19.4	28	23.7	42.41	14.03	28.22
Israel-4	122.94	73.8	98.37	40.8	23.2	32	17.6	22.8	20.2	28.35	17.01	22.68
Turkey-7	137.16	85	111.08	74	23.8	48.9	13.8	27.8	20.8	24.29	10.68	17.48
Turkey-8	129.43	74.6	102.02	29.2	19	24.1	26.9	23.6	25.25	12.08	17.47	14.77
Austria-1	108.20	77.2	92.7	36.2	24	30.1	23.2	29.2	26.2	14.65	10.13	12.39
Hungary-1	124.09	62.6	93.34	67.8	21.8	44.8	22.8	19.8	21.3	49.48	13.63	31.55
Libya-1	115.17	74.8	94.98	29.8	22.2	26	22.2	26.4	24.3	8.34	5.69	7.01
Bangladesh-4	77.95	52.2	65.07	15.4	28.6	22	17.7	13.2	15.45	17.73	6.43	12.08
Iran-7	113.46	71.8	92.63	47.8	18.6	33.2	41.1	43	42.05	21.38	7.25	14.31
Turkey-9	121.58	79.2	100.39	46.2	17.8	32	18.8	43.2	31	30.02	13.07	21.54

Table 2. (Continued).

Pakistan-10	112.55	73	92.77	33.8	16.8	25.3	24	32.4	28.2	18.09	12.75	15.42
China-3	96.10	74.4	85.25	23.4	19.4	21.4	33	37.2	35.1	90.67	11.37	51.02
China-4	108.84	81.4	95.12	28.8	20	24.4	28.6	15.4	22	60.15	6.08	33.11
China-5	95.90	95.4	95.65	21.2	31.2	26.2	15.3	33.4	24.35	54.65	17.14	35.89
China-6	121.18	69	95.09	45.2	14.2	29.7	11.6	28.8	20.2	29.16	6.53	17.84
China-7	81.06	78.6	79.83	8.2	14	11.1	22.4	27.6	25	7.99	7.55	7.77
France-1	95.70	68.4	82.05	11.4	12.4	11.9	25.5	21.4	23.45	5.93	3.78	4.85
Austria-2	110.15	57.8	83.97	38.2	16.8	27.5	24.8	18.6	21.7	34.45	5.08	19.76
Pakistan-11 (Thori-78)	120.90	66.4	93.65	22.53	16.65	19.59	39.8	15.375	27.588	11.78	4.143	7.961
Turkey-10 (Dinçer)	117.88	86.6	102.24	5.6	11.8	8.7	18.2	46.2	32.2	8.82	4.39	6.60

Table 3. Minimum, maximum, mean, and standard deviation (StD) of the mean data across both locations.

Traits	Minimum	Maximum	Mean	Std. deviation
Plant height	60.08	121.48	92.63	10.32
Capitula per plant	8.7	80.40	28.94	10.70
Seeds per capitulum	15	42.05	25.29	5.19
Seed yield per plant	4.86	51.02	15.95	9.31

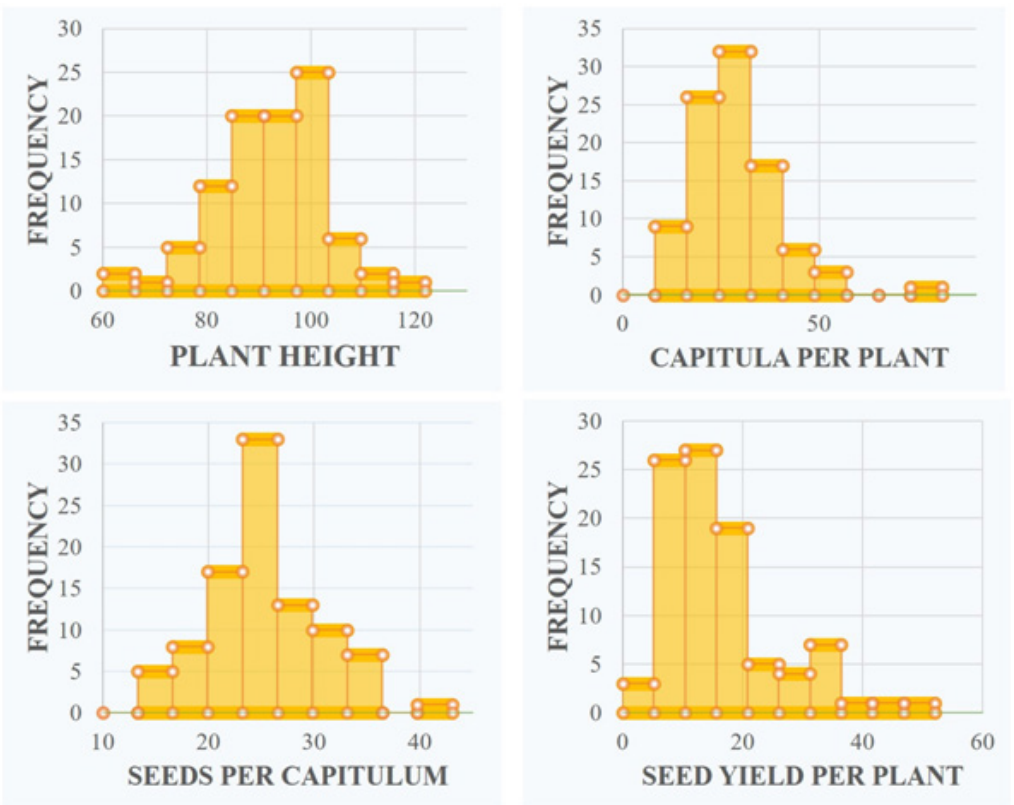

Figure 1. Frequency distribution for the studied agronomic traits in safflower germplasm.

Table 4. Correlation coefficients among four agronomic traits in international safflower panel.

Traits	Plant height	Capitula per plant	Seeds per capitulum	Seed yield per plant
Plant height	1			
Capitula per plant	–0.0022	1		
Seeds per capitulum	0.0960	0.0772	1	
Seed yield per plant	–0.0304	**0.4985*****	0.1585	1

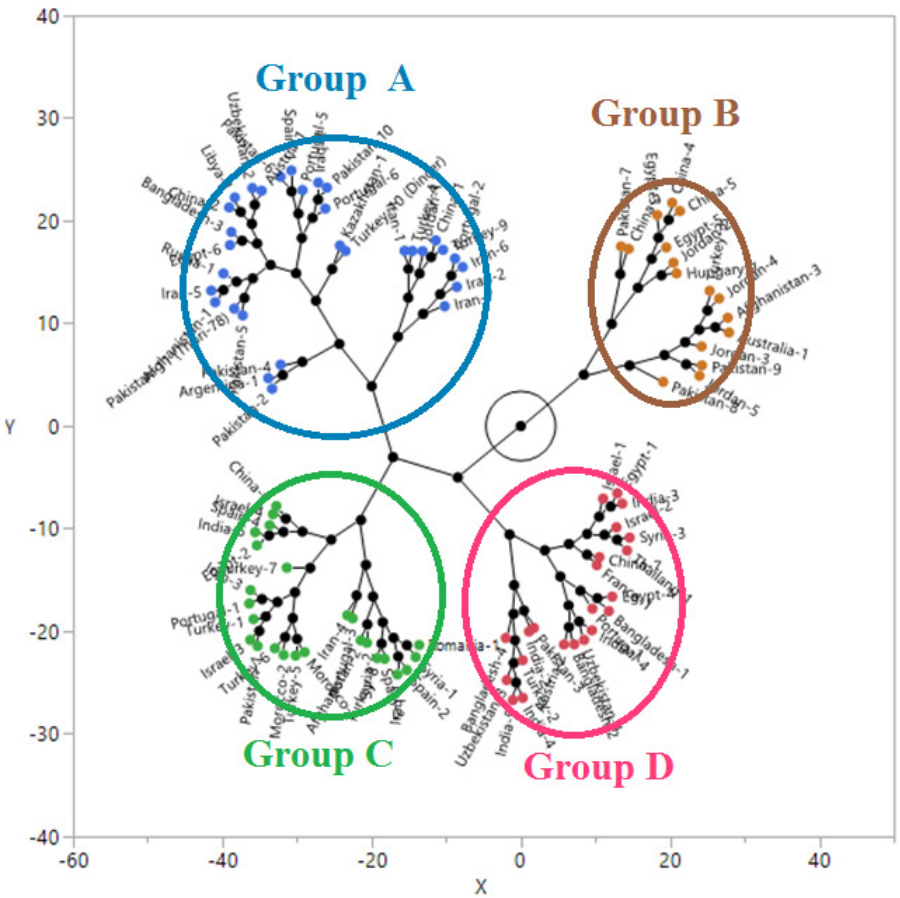

Figure 2. Constellation plot analysis in international safflower panel.

for the studied traits shows that the tested safflower accessions had dissimilar behavior in different locations and vice versa. The estimation of heritability aids in the selection of best performing accessions from a genetically diverse population. The efficiency of selection for a specific trait can be improved when estimated heritability is found to be high. Heritability can be grouped as low (below 30%), moderate (30%–60%), and high (above 60%) (Reddy et al., 2013). The current study revealed moderate heritability for capitula per plant, plant height, and seeds per capitulum, while high heritability was observed for seed yield per plant, respectively. The quantity of genetic diversity determines the magnitude of heritability, and higher heritability leads to lower environmental effects on trait under study (Nadeem et al., 2020). Analysis of variance revealed significant genotypic variances within and across locations, thus, responsible for observing moderate to high heritability estimates. Tahernezhad et al. (2018) estimated moderate heritability for capitula per plant and supported our results. Reddy et al. (2004) and Mohammadi and Pourdad (2009) strengthen our finding as they reported moderate heritability for plant height. This study estimated moderate heritability for seeds per capitulum, but it contrasted with the findings of Camas and Esendal (2006), Arslan (2007a), and Sirisha (2009) as they all resulted high heritability for this trait. Previous research

Table 5. Marker-trait associations of the four agronomic traits with its associated markers in international safflower panel.

Location	Trait	Marker	*p*-value	R^2 (%)
Pakistan	Plant height	DArT-45483093	2.56E-04	15.7
	Capitula per plant	DArT-38077549	1.34E-04	17.4
	Seeds per capitulum	DArT-45485351	6.00E-05	20.7
		DArT-15673104	1.07E-04	18.3
	Seed yield per plant	DArT-100004992	1.66E-05	22.9
		DArT-100004976	4.19E-05	19.9
		DArT-100019720	4.19E-05	19.9
		DArT-45482287	6.30E-05	19.4
		DArT-100004975	6.30E-05	19.2
		DArT-100039734	7.77E-05	18.5
		DArT-100045083	9.46E-05	17.9
		DArT-45483620	9.53E-05	19.5
		DArT-100005068	9.74E-05	17.9
Turkey	Plant height	DArT-22763576	9.86E-05	20.3
	Seeds per capitulum	DArT-17813868	6.19E-04	13.5
Mean data (Pakistan and Turkey)	Capitula per plant	DArT-38077549	2.56E-04	15.7
	Plant height	DArT-22763576	1.94E-04	18.5
		DArT-22763253	1.44E-04	17.5
	Seeds per capitulum	DArT-38079422	2.00E-04	18.1
		DArT-100043360	3.35E-04	15
	Seed yield per plant	DArT-100004992	3.99E-05	20.5
		DArT-100004976	4.99E-05	12.7
		DArT-100004975	9.21E-05	18.3
		DArT-100039734	1.07E-04	17.7
		DArT-100045083	1.23E-04	17.3

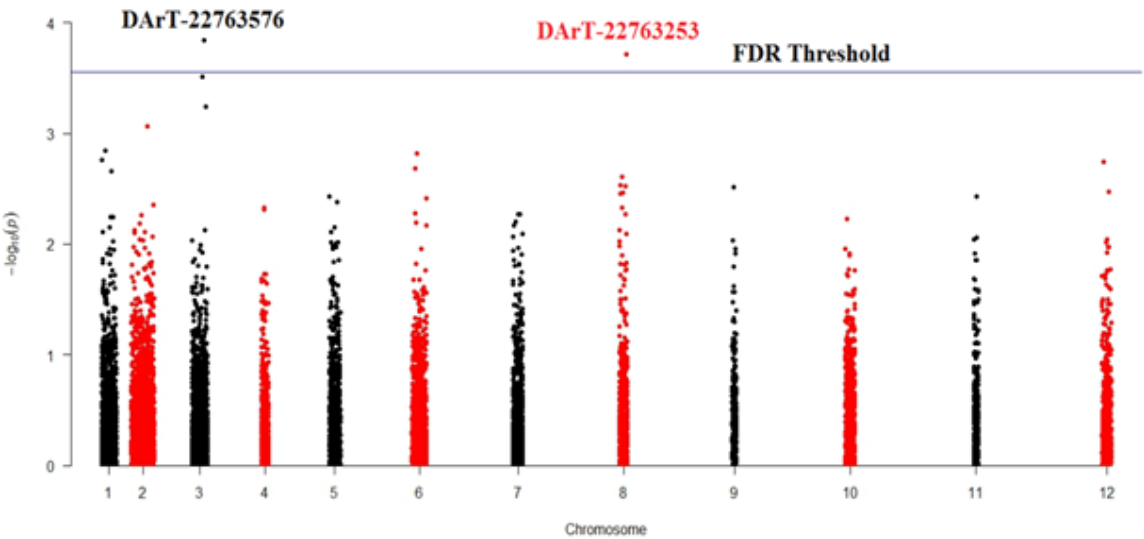

Figure 3. Pseudo manhattan plot for plant height in world safflower panel.

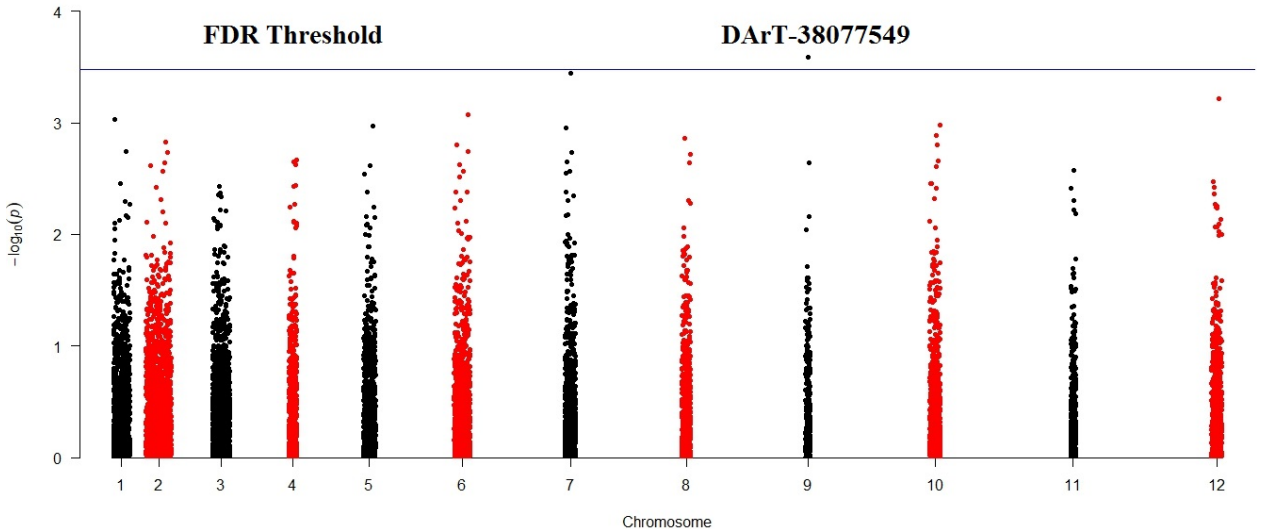

Figure 4. Pseudo manhattan plot for capitula per plant in world safflower panel.

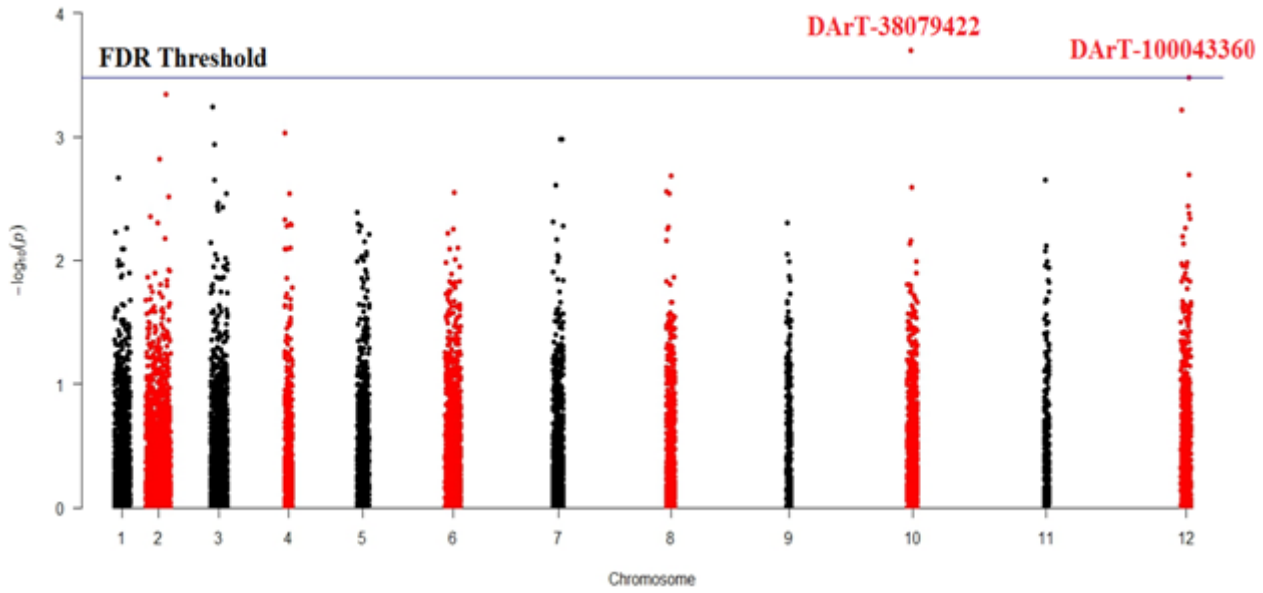

Figure 5. Pseudo Manhattan plot for seeds per capitulum in world safflower panel.

work exhibited moderate to high heritability for seed yield per plant (Mohammadi and Pourdad, 2009; Baydar and Erbas, 2014) and confirmed our results. These data strongly suggested that traits with moderate to high heritability are largely determined by genetics, with little influence from environmental factors under multilocations.

During this study, superior mean performance was observed for traits viz., plant height, capitula per plant, and seed yield per plant in Pakistan, while safflower accessions recorded better performance for seeds per capitulum in Turkey (Table 2). Amini et al. (2008) supported our results as they recorded plant height in the range of 57.1–135.8 cm. Kiran et al. (2015) reported plant height in the range from 51 to 98 cm and from 66.8 to 100 cm for two consecutive years evaluating 148 safflower accessions. Omidi et al. (2009) tested 100 safflower accessions and supported our results as they observed similar performance for seeds per capitulum (17 to 43 seeds per capitulum). Alizadeh (2005) also observed similar pattern for seeds per capitulum (10.16 to 40.17) while evaluating the performance of 100 safflower accessions. Observing similarity in the mean performance of the plant genetic material during various investigations can be explained as either kinship of the evaluated accessions or similarity in the environmental conditions where experiments were performed. Hassani et al. (2020) strengthen our finding as they recorded

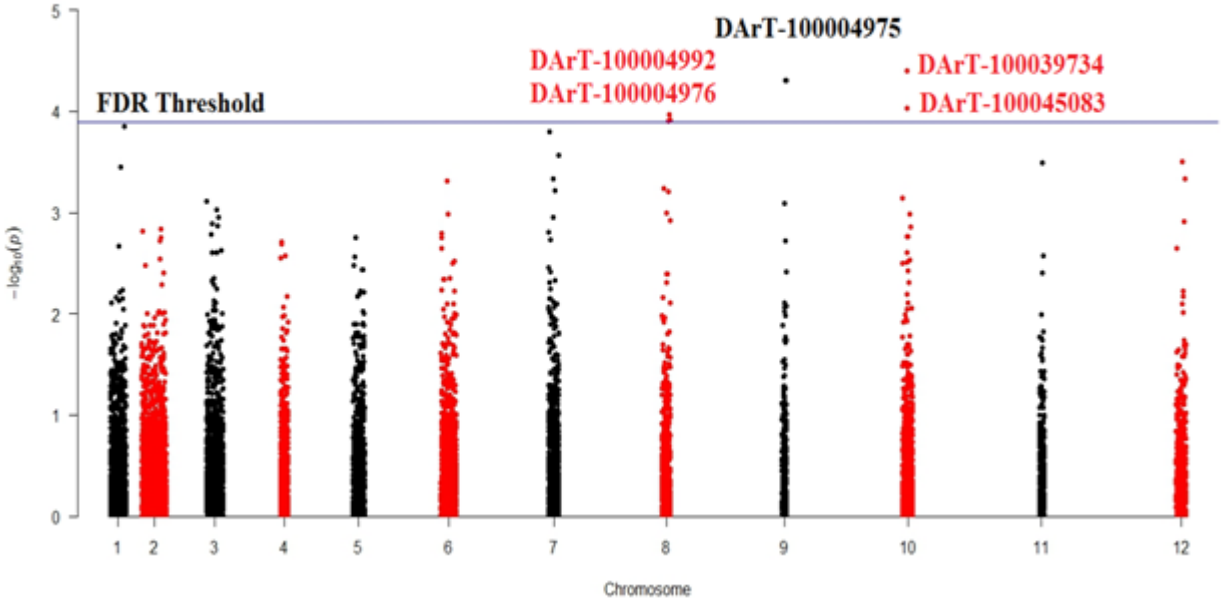

Figure 6. Pseudo Manhattan plot for seed yield per plant in world safflower panel.

seed yield per plant in a similar range (2.64 to 63.06 g). Shinwari et al. (2014) also observed a similar pattern for seed yield per plant (3.0 to 38.1g). These results confirmed the presence of a sufficient amount of phenotypic variation for seed yield per plant among the studied safflower accessions. Numerous studies reported capitula per plant as an important trait for the improvement of safflower production (Mozaffari and Asadi, 2006) as it showed significantly positive correlation with seed yield (Bagawan and Ravikumar, 2001). The obtained number of capitula per plant during this investigation ranged from 8.7 to 80.4 with mean value of 28.94. Amini et al. (2008) recorded lower capitula per plant (ranged from 10.5 to 32.2 with mean value of 21.5) as compared to our current results. Kiran et al. (2015) evaluated 148 safflower accessions originating from 15 countries of the world for two consecutive years. They recorded capitula per plant in the range of 10.4 to 54 and 9.8 to 51, respectively. These results suggested the presence of higher mean performance of the studied safflower accessions during current investigation and can be utilized as parental genotypes in future safflower breeding activities. Frequency distribution was implemented to deeply understand the nature of the data and results of this study exhibited normal distribution for the studied traits (Figure 1). Pearl et al. (2014) strengthen our results as they observed normal distribution for the studied agronomic traits in safflower. Dwivedi et al. (2005) performed frequency distribution analysis for 12 agronomic traits evaluating 5515 safflower accessions and revealed normal distribution. These results indicated the presence of homogeneity distribution for the studied traits among the tested safflower material.

Correlation analysis was performed to understand the relationship between various traits. The obtained information can be useful in crop improvement via selecting traits effecting yield (Karakoy et al., 2014). Current investigation observed highly significant and positive correlation between capitula per plant and seed yield per plant (0.4985***) (Table 4). Previous research work also reported capitula per plant and seed yield per plant as important yield contributing traits, as they revealed significantly positive correlation between each other (Mahasi et al., 2006; Arslan, 2007). Thus, it is very clear that safflower accessions with highest number of capitula per plant and seed yield per plant will eventually enhance safflower production.

Constellation plot analysis resulted into four groups, i.e. A, B, C, and D with a total number of 31, 16, 25, and 22 safflower accessions, respectively (Figure 2). Constellation plot analysis was based on plant height, seed yield per plant, seeds per capitulum, and capitula per plant. Safflower accessions having maximum seed yield per plant (14.11 to 51.05 g) were present in group B, while group D observed safflower accessions exposing low seed yield per plant (4.85 to 20.81 g). Accessions with maximum capitula per plant (21.4 to 80.4) were present in group B, while group C comprised of safflower accessions having intermediate number of capitula per plant (15.2 to 48.9). Safflower accessions with maximum number of seeds per capitulum (21.7 to 42.05) were present in group A. Group C clustered safflower accessions observing maximum plant height (90.46 to 121.48 cm). These findings clearly proposed the clustering of safflower accessions into different groups based on various traits that can be utilized for their improvement in safflower breeding programs.

4.2. Marker-trait associations

Very few studies have been conducted to identify markers/loci associated with agronomic traits in safflower (Hamdan et al., 2008, 2012; Mayerhofer et al., 2010; García-Moreno et al., 2011; Pearl et al., 2014; Ebrahimi et al., 2017, Ambreen et al., 2018; Ali et al., 2020a). The current investigation involved association analysis for the identification of genetic basis associated with four agronomic traits, including capitula per plant, plant height, seeds per capitulum, and seed yield per plant (Table 5). This study revealed a total of 15 DArTseq markers significantly associated with the studied traits from two individual locations (Table 5). A total of 13 DArTseq markers were obtained from Pakistan location, while two markers showed significant association with the studied traits from Turkey location. Furthermore, the identified markers in Pakistan location were not found to be significantly associated with the studied traits in Turkey location. Nadeem et al. (2021) and Kamfwa et al. (2015) supported our findings, as they also identified significantly associated markers in one location, which were not detected in other location. Mean data from both locations (Pakistan and Turkey) identified a total of 10 significantly associated DArTseq markers with the studied traits (Table 5). Among the 10 identified DArTseq markers from mean data of both locations (Pakistan and Turkey), seven were also identified either in Pakistan or Turkey location. Like, DArT-22763576 identified from mean data of both locations was present in Turkey location and significantly associated with plant height. Similarly, the six significantly identified markers from mean data of both locations were also found to be associated with the studied traits in Pakistan location. Five of these markers (DArT-100004992, DArT-100004976, DArT-100004975, DArT-100039734, and DArT-100045083) were found associated with seed yield per plant, while one marker (DArT-38077549) was associated with capitula per plant. Therefore, it is suggested to utilize the markers identified from mean data of both locations and also present in individual location for marker-assisted breeding of safflower. Plant height determines plant architecture and also influences crop yield. Our current analysis observed two DArTseq markers (DArT-22763576 and DArT-22763253) significantly associated (*p-value*; 1.94E-04 and 1.44E-04) with plant height. Various loci/markers have been associated to plant height as identified in previous research. Ambreen et al. (2018) reported two loci (NGSaf_156 and NGSaf_296) associated with plant height utilizing SSR markers. Mirzahashemi et al. (2015) identified two markers (qPh6_1 and qPh6_2) associated with plant height. Capitula per plant is known as one of the important yield related traits in safflower. Our current analysis observed one DArTseq marker (DArT-38077549) significantly associated (*p-value*; 2.56E-04) with capitula per plant. Ambreen et al. (2018) reported one locus (NGSaf_279) associated with capitula per plant utilizing SSR markers. Mirzahashemi et al. (2015) identified one marker (qCpno2) associated with capitula per plant. Pearl et al. (2014) proposed one marker (H76) linked with capitula per plant using ESTs. Our current analysis observed two DArTseq markers (DArT-38079422 and DArT-100043360) significantly associated (*p-value*; 2.00E-04 and 3.35E-04) with seeds per capitulum. This is very first report claiming genetic basis having association with seeds per capitulum in safflower. Association analysis for seed yield per plant exhibited five DArTseq markers. Mirzahashemi et al. (2015) obtained two molecular markers (qSyp2 and qSyp9) associated with seed yield per plant in safflower. As there are few studies claiming the identification of genetic basis associated with agronomic traits, there is a need to validate previously and currently identified markers for the marker-assisted breeding of safflower.

The fact that the blast search against DArT-22763253 marker has an association with plant height resulted in a putative gene, *AT2G01150.1*. This gene encodes a RING-H2 finger protein that was expressed in vascular tissue, root tips, embryos, and pistils. RING finger protein is a large family that exists widely in eukaryotes. Earlier studies confirmed that this protein has significant role in the regulation of various physiological and biochemical processes like plant growth and development, stress resistance, and hormone signaling responses (Li et al., 2013). The RING-finger protein also contributes to leaf and height development (Sun et al., 2019). Overexpression of this protein related gene (CaRZFP1) resulted in rapid growth, size with increased number of leaves and more fresh vegetation in tobacco (Zeba et al., 2009).

AT1G04780.1 was found putative gene against DArT-38077549 that showed association with capitula per plant. *AT1G04780.1* gene encodes the Ankyrin (ANK) repeat family protein. This protein is largely distributed in plants and significantly contributes in various physiological and developmental processes like plant growth and development, hormone response, and response to biotic and abiotic stresses (Lopez-Ortiz et al., 2020). Previous studies also explored the role of ANK protein in pollen germination and polarized pollen tube growth (Huang et al., 2006; Huang et al., 2009).

The BLAST search against DArT-38079422 marker having association with seeds per capitulum resulted into *AT3G18770.1* as a putative gene that encodes autophagy-related protein. Autophagy is an important catabolic pathway required for the growth and development. In plants, it is activated due to environmental factors and

developmental process and contributes to the regulation of fitness, longevity, and fecundity, underpinning plant tolerance to various biotic and abiotic stresses (Minina et al., 2018). It was also reported that autophagy-related (*ATG*) genes manage nutrient resources in source leaves and play fundamental role in seed formation and seed filling (Guiboileau et al., 2012; Li et al., 2015a). Minina et al. (2018) reported that overexpression of *ATG* genes in Arabidopsis resulted in higher seed yield and fatty acid contents.

DArT-100043360 was the 2nd identified marker having association with seeds per capitulum, and BLAST search against this marker revealed *AT5G17310.2* as a putative gene. This gene encodes UDP-glucose pyrophosphorylase (UGPase). Previous studies documented the role of UDP-Glc in plant growth and development and acts as a signaling and energy transport/storage molecule (Li and Sheen, 2016; Ciereszko, 2018). Kleczkowski and Decker (2015) reported UDP-Glc as a precursor for the formation of various cell wall polymers like cellulose, callose, and hemicellulose.

Seed yield per plant is an important key targeted trait in oilseed crops. A total of five DArTseq markers showed significant association with this trait. However, DArT-100004976 did not reflected putative gene during functional analysis. BLAST search against DArT-100004992 marker revealed *AT5G05140.1* as putative gene. This gene encodes transcription elongation factor (TFIIS) family protein. Gene transcription is very complex process in eukaryotes and governed by three specialized RNA polymerases (Pol I, II, and III). Transcription elongation factor stimulate efficient transcription by making a binary complex with RNA Polymerase II (Grasser et al. 2009). Oh et al. (2004) revealed the role of RNA Pol II transcript elongation factor (TFIIS) in the developmental stages of Arabidopsis. Grasser et al. (2009) comprehensively explored the role of TFIIS in the regulation of seed dormancy in Arabidopsis. Miao et al. (2019) stated that seed dormancy delays the germination stage to make plants avoid unfavorable climatic conditions for growth. Shu et al. (2015) stated that dormancy results in pre-harvest sprouting (PHS) that significantly decreases the crop yield. By keeping in view, DArT-100004992 can be suggested for future safflower breeding programs. *AT1G18710.1* was found as putative gene for DArT-100004975 marker. This gene is a member of the R2R3-MYB gene family. This gene promotes seed longevity in Arabidopsis. The R2R3-MYB protein contains one of the most families of transcription factors in plants. The R2R3-MYB protein contributes to multiple biological processes like secondary metabolism, plant development, hormone signal transduction, and disease resistance (Allan et al., 2008; Cominelli et al., 2009). Recently, Erfatpour and Pauls (2020) reported that R2R3-MYB gene-based marker has an association with seed coat trait in common bean.

The blast search against DArT-100039734 marker resulted in *AT3G59790.1* as a putative gene. This gene belongs to MAP Kinase family. Mitogen-activated protein kinase (MAPK) are very critical by playing a role of mediators in intracellular signal transduction. Similarly, MAPK has significant contribution in plant growth, development, and resistance to various stresses (Neupane et al. 2019; Mohanta et al. 2015). Xing et al. (2008 and 2009) explored the role of MAPK in seed germination in Arabidopsis. *AT2G01150.1* was found putative gene for DArT-100045083 marker. This gene encodes a RING-H2 finger protein that is expressed in vascular tissue, root tips, embryos, and pistils. DArT-22763253 and DArT-100045083 showed association with plant height and seed yield per plant, respectively and BLAST search of these markers resulted in *AT2G01150.1* as a putative gene. The AT2G01150.1 gene encodes a RING-H2 finger protein and previous studies confirmed that this protein has significant role in the regulation of various physiological and biochemical processes like plant growth and development, stress resistance, and hormone signaling responses (Li et al., 2013). Therefore, these markers (DArT-22763253 and DArT-100045083) can be used for future safflower breeding programs.

5. Conclusion

A good level of phenotypic diversity was observed for the studied agronomic traits in an international safflower panel. Analysis of variance reflected significant genotypic effects for the studied traits across both locations. Accession × Location interaction was nonsignificant for the studied traits except seed yield per plant. Seed yield per plant exhibited highest heritability among the studied traits. The studied traits revealed superior performance at Pakistan except seeds per capitulum, which showed better performance in Turkey. A total of 10 markers from mean data of both locations were identified for all the studied traits. DArT-22763253 and DArT-100045083 showed association with plant height and seed yield per plant, respectively and BLAST search of these marker resulted in *AT2G01150.1* as a putative gene. We are confident that identified markers during current investigation will be very helpful in the marker-assisted breeding of safflower regarding agronomic traits.

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