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Impact of heat and drought stresses on grain nutrient content in chickpea: Genome-wide marker-trait associations for protein, Fe and Zn

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ABSTRACT

Chickpea is a cheap source of protein and micronutrients to the poor and vegetarian population living in south-Asia and sub-Saharan Africa. Due to changes in climatic conditions and cropping systems, the crop is being exposed to severe drought and heat stress during its reproductive period, which leads to significant yield losses and fluctuations in grain nutrient accumulation. The study was conducted with 140 diverse genotypes under non-stress, drought, and heat stress conditions to estimate their effects on grain nutrient (protein, Fe and Zn) contents and identify the marker-trait associations. Analysis of variance revealed highly significant differences among genotypes for nutrient content under respective planting conditions. The seed yield was negatively associated with the grain Fe (r = −0.37, −0.25, 0.11) and Zn (r = −0.49, −0.35, −0.72) under respective planting conditions. The PCA indicated that PC1 was positively associated with grain Fe in non-stress and heat, while Zn in all planting conditions, whereas PC2 was positively influenced by protein content followed by grain yield. Cluster analysis identified eight clusters, of which cluster VI showed maximum cluster means for Fe (73 mg kg-1) and Zn (48.1 mg kg-1). The Genome-wide association study revealed, a total of 181 MTAs for grain Fe, Zn, and protein content in all three growing conditions. A total of 4, 2, and 48 SNPs for grain Fe and Zn content, whereas, 66, 46, 15 SNPs for grain protein content have shown significant association under non-stress, drought, and heat stress, respectively. One SNP each on chr1 (S1_35622241; $P \leq 3.47 \times 10^{-6}$) and chr4 (S4_44607232; $P \leq 1.35 \times 10^{-5}$) was co-associated under drought and non-stress conditions for protein and Fe, respectively. The identified robust MTAs will be validated and used in marker-assisted selection towards the rapid development of nutrient-rich varieties.

1. Introduction

Food and nutritional security under changing climate and population explosion is the greatest challenge ahead in agriculture. Being rich in grain protein and micronutrients, legumes play a vital role in reducing hunger and malnutrition (Ritchie et al., 2018). Among them, chickpea (*Cicer arietinum* L.) is a cheap source of quality protein with essential amino acids and minerals to millions of families in Asia and Africa (Jukanti et al., 2012).

Changing climate is predicted to increase earth's average temperature from 2° to 4.5°C, (Masson-Delmotte et al., 2018; Raza et al., 2019) and it was estimated that about 815 million people will be affected by malnutrition worldwide, especially in South Asia and sub-Saharan African regions (Abberton, 2010; Food and Agriculture Organization FAO, 2015; Kumar et al., 2019) where major chickpea area is present. Water scarcity, frequent drought spells, abrupt heavy rainfall, and increased fluctuations in day and night temperatures are going to be a common phenomenon in the years ahead. The major chickpea area falls under arid and semi-arid tropics (SAT) regions, where the crop is grown under receding soil moisture conditions under rainfed conditions. Thus, soil moisture deficit during reproductive stages (called terminal drought) became the common abiotic stress that affecting over two-thirds of the global chickpea area (Gaur et al., 2019; Rani et al., 2020). On the other hand, due to changing cropping systems and area expansion to warmer

Abbreviations: ANOVA, Analysis of variance; GWAS, Genome wide association; MTA, Marker trait association; PCA, Principal component analysis; LD, Linkage disequilibrium.

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regions the crop is being exposed to high-temperatures (>35 ^{o}C) during reproductive stage affecting pod filling. As a result, significant yield losses in chickpea have been evidenced due to individual and combined drought and heat stresses (Devasirvatham and Tan, 2018; Rani et al., 2020).

Mineral nutrients accumulation in the grain/edible portion is a complex mechanism involving many genes and highly influenced by environmental conditions. A study conducted with different legumes showed that chickpea is more sensitive to high-temperatures compared to groundnut, soybean and pigeonpea, in terms of membrane stability and photo system (PS II) functions (Srinivasan et al., 1996). It was also observed that heat stress reduced the duration and amount of protein accumulation in crop plants (Stone et al., 1997) and deteriorated the protein quality in pea (Mession et al., 2013), wheat (Stone et al., 1997), soybean (IWABUCHI and YAMAUCHI, 1984) and lentil (Sita et al. 2018). The preliminary studies showed that a significant reduction in accumulation of grain protein (36%) during late pod-filling stages in chickpea due to high-temperature (Kaur et al., 2008). The cultivars with improved adaptation to drought and heat stress regions associated with enhanced nutrient contents are in high demand for predicted changing climate.

To breed varieties with stable grain nutrient content under drought and heat stress conditions, a deep insight into the genetic control of nutrient traits and its association with grain yield is necessary. Genome-wide association studies (GWAS) based on linkage disequilibrium (LD) is an effective tactic for mapping quality traits in panel and has been efficiently applied in a range of plant species (Huang and Han, 2014; Gupta et al., 2005). It provided an advantage over genetic and linkage mapping in terms of greater mapping resolution to identify markers nearby causal genes because of more recombination events in diverse set of genotypes (Liu et al., 2016; Alqudah et al., 2020). Genotyping-by-sequencing (GBS) approach (Elshire et al., 2011) provides cost effective genotyping that can deal with enormous number of samples, and provides a large number of SNPs suitable for LD-based mapping (Huang and Han, 2014). Through screening large and diverse collections with ample genetic marker density, GWAS can detect causal loci underlying natural phenotypic variation. In chickpea, genome wide studies were conducted for phenology, seed related and abiotic tolerance traits (Diapari et al., 2014, Thudi et al., 2014; Bajaj et al., 2015) and very limited information available on grain nutrient traits (Upadhyaya et al. 2016a, b; Sab et al., 2020). It is therefore, the present study involves the genotyping of association panel with high quality accurate genome wide SNP markers. The identified significant association with SNPs for grain Fe, Zn and protein content could be used in marker-assisted selection (MAS), in order to improve the grain mineral and nutrition content in chickpea. To date, the range of grain iron content (2.4–11 mg/100 g) in chickpea was discussed by various authors (USDA Basic Report, 16056; Meiners et al., 1976; Wood and Grusak, 2007; Tan, et al., 2017) who suggested a huge scope for improvement in the nutrient content.

Narrow genetic base and limited understanding about the association between nutrient traits and grain yield has put forth a major bottleneck for chickpea improvement. In this context, the present study was conducted to (1) assess the genetic variation for grain Fe, Zn and protein content in the association panel of chickpea under different growing conditions; (2) determine the population structure of diverse germplasm using genome wide high density GBS based SNP markers; and (3) identify the candidate markers linked to the grain Fe, Zn and protein content through genome wide association under different planting conditions.

2. Material and Methods

2.1. Plant material

A set of 140 chickpea genotypes (Table S1) as association mapping (AM) panel including 83 genotypes collected from different growing regions in India; 35 from other countries, 20 lines from improved breeding lines developed at ICRISAT, Patancheru, India and two genotypes from unknown source maintained at ICRISAT Gene Bank was used in the study.

2.2. Phenotyping of AM panel for grain nutrients

To investigate environmental impact on nutrient traits, the panel was phenotyped under non-stress (irrigated), drought stress (rainfed) and heat stress (late or summer) condition during 2018–19 at ICRISAT (17°30′ N; 78°16′ E; altitude 549 m). The drought and non-stress trials were conducted in post-rainy season and heat screening trial was conducted in summer season. During post-rainy and summer seasons, the average rainfall, minimum and maximum temperatures recorded were 18 mm and 13 mm, 16 ^{0}C and 20 ^{0}C, 31 ^{0}C and 36 ^{0}C, respectively. The average relative humidity (69%) was higher during post-rainy than the summer. Each genotype was grown in an area of 1.2 m^{2} plot with inter- and intra-row spacing of 60 × 10 cm. The experiment was conducted on vertisols in an alpha-lattice design with three replications in all the environments. The planting for non-stress and drought was done during the first week of October 2018, while the heat stress trial was planted in second week of January 2019 to expose the reproductive stage of the crop to critical temperature (>35 ^{o}C). Single irrigation was provided for the rainfed trial to obtain proper germination and no further irrigation was provided. The non-stress experiment was provided with three irrigations at planting, 20 and 50 days after planting. In the summer trial four irrigations were provided at sowing, 20, 40 and 60 days after sowing to avoid the confounding effect of moisture stress during the crop season. The standard agronomic practises were followed for successful crop establishment in each environment. The genotypes were harvested at maturity when all the plant parts were dried completely. Precautions were taken to avoid any metallic or dust contamination of grains while harvesting. Agronomic observations such as flowering time, seed yield, and seed size (100 seed weight) were recorded in each plot.

2.3. Grain Fe, Zn and protein content estimation procedure

The grain samples were washed with distilled water for one minute in petri plates to remove any surface contamination and dried in a hot air oven at 50 °C for 24 hours. For Fe and Zn analyses, the grounded samples (0.5 g) along with operational blanks and standard solution of known concentrations were digested in 5 ml of distilled nitric acid (Analytical Reagent Grade, Merck) at 140 °C for 45 min in a Microwave Digestion System (Perkin Elmer) to obtain clear digests. Following digestion, the volume of each sample was made up to 25 ml using Milli-Q water and elemental determination was performed by ICP-OES. For calculating the grain micronutrient concentration, the mean value of element specific blank concentration was subtracted from each data point. The data were then multiplied by initial sample volume, divided by initial weight of grains, and mesaured as mg element g^{-1} dry grain material and expressed as mg kg^{-1} (Khokhar et al., 2018).

Similarly, for estimation of grain protein content the grounded seed (~10 g) samples were analyzed using Kjeldahl method (Kjeldahl, 1883). The samples were manually digested with sulfuric acid, anhydrous sodium sulfate and catalysts (copper, selenium, titanium, or mercury) that releases ammonia in the form of ammonium ion (NH^{4+}) which binds to the sulfate ion (SO_4^{2-}). The digested form was neutralized by adding sodium hydroxide, which converts the ammonium sulfate into ammonia gas, followed by titration to remove excess acids. The final hydrogen ions concentration is equivalent to the concentration of nitrogen. The determined nitrogen was converted into total protein (%) using the conversion factor of 6.25 (equivalent to 0.16 g nitrogen per gram of protein).

2.4. Statistical analysis

The best linear unbiased predictors (BLUPs) were estimated for nutritional and agronomic traits. Analysis of variance was performed using Generalized Linear Model procedures following a random-effects model (GENSTAT v19 software). Broad sense heritability was estimated as a ratio of genotypic variance to the phenotypic variance and expressed in percentage (Hanson et al., 1956). Using Microsoft Excel v 2013, the two-tailed student *t*-tests were performed for testing the level of significance for the variables between treatment means, at a 95% confidence interval (Okoth et al., 2017).

The Pearson's correlation coefficients among the nutritional traits (Fe, Zn and protein) and hundred seed weight (in grams), grain yield (yield in kg/ha) traits were calculated using R version 3.5.1 (R Project for Statistical Computing (R package Performance Analytics v 2.0.4; https://CRAN.R-project.org/package=PerformanceAnalytics). Trait associations were determined by principal component analysis (PCA) (Hatcher and O'Rourke, 1994) using R version 3.5.1 (R Project for Statistical Computing, (https://www.r-project.org). The Ward's distance (Murtagh and Legendre, 2011) approach was used to plot hierarchical clustering of genotypes using the statistical package "dendextend" and "circlize" for R version 3.5.1. The number of clusters was fixed at 92% similarity, and the significance between the clusters was tested using Student-Newman-Keul's test (Abdi and Williams, 2010).

2.5. Genotyping of association panel

The genomic DNA was extracted from young leaf tissues using the QIAGEN DNeasy 96 plant kit and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). A total of 136 genotypes were used for GBS sequencing (4 genotypes were excluded due to poor quality DNA). The DNA samples were diluted in 20 ng/μl using 1 × TE buffer, pH 8.0. The 136 samples were genotyped using the GBS platform under standard experimental conditions as described by Elshire et al. (2011). The resulted DNA libraries were quantified using a Bioanalyzer (Agilent Technologies) and the 59-plex libraries were sequenced on a single lane of Illumina HiSeq™ 2500 platform (Illumina® Inc., San Diego, CA, USA).

2.6. SNP calling and filter for GWAS study

The resulting reads from the sequencer were assigned to 136 genotypes based on the 4–6 bp adapters ligated to each DNA of accession using in house program scripts. The adopters were removed from the sequence reads and high-quality reads were trimmed by Trimmomatic-0.33. The higher quality filtered reads were used for determined SNP polymorphisms, mapped to chickpea (*C. arietinum*) reference genome ASM33114v1 assembly (Varshney et al., 2013; https://www.ncbi.nlm.nih.gov/assembly/GCF_000331145.1). SNP markers were LD pruned and filters were used for minor allele frequency (MAF> 0.01) and maximum missing sites per SNP< 20%. A total of 3,44,345 SNPs were selected genome wide on eight chromosomes of chickpea and used to determine the population structure and marker-trait associations in the study.

2.7. Analysis of population structure and linkage disequilibrium

To investigate the subpopulation structure, ADMIXTURE v1.22 was used (Alexander et al., 2009). It is a model-based clustering algorithm to identify genetic clusters in the form of K (sub-population) value. The analysis was performed in multiple runs arranging successive values of K from 2 to 12. The optimum K value was determined based on the lowest cross-validation error value (CV error) (Pritchard et al., 2000). The output file was used to make cluster visualization by Cluster Markov Packager A cross K (CULMPAK, http://clumpak.tau.ac.il/index.html, Mayrose Lab, Aviv University) (Kopelman et al., 2015). An unweighted neighbor-joining (NJ) tree was constructed using a shared SNP index based on a dissimilarity matrix (DM) estimated from the SNP dataset (Perrier and Flori, 2003). The genome wide Linkage Disequilibrium (LD) was generated by plotting average r^2 (correlation frequency among SNPs) values against eight chromosomes (at 50 kb uniform physical intervals) across the chickpea genome using the GAPIT3 package (https://www.r-project.org).

2.8. Association mapping-GWAS

Marker trait associations (MTA) were studied in drought and non-stress treatments as these are the common chickpea growing conditions globally. The MTA results under heat stress conditions were also provided for relative comparison. Mixed linear model (MLM) was used to evaluate the marker-trait associations using GAPIT3 package (Lipka et al., 2012) with K values and principal coordinate values as covariates. The relative distribution of observed and expected –log10(P)-value in each trait-associated genomic locus was compared based on quantile-quantile (Q-Q) plot. The accuracy and robustness of the SNP marker-trait association was determined based on False Discovery Rate (FDR). In the present study the P-value threshold for significant markers for multiple comparisons were performed at FDR cut-off $\leq$ 0.05. Significant-traits-SNPs associations were selected based on an arbitrary but high threshold cut-off P value (threshold $P < 1 \times 10^{-4}$ & 1×10^{-6}) (Suwarno et al., 2015; Gowda et al., 2015; Longmei et al., 2021). The Q-Q plots of the observed and expected P values were plotted at $-\log_{10}(P)$ values to assess the adequacy of a fitted normal straight line to the markers. Phenotypic variance (R^2) and marker effects (+/-) were extracted from GWAS output file. The Manhattan plots and QQ plots were visualized using R version 3.5.1 (R package CMPlot https://CRAN.R-project.org).

3. Results

3.1. Analysis of variance and correlations

The analysis of variance (ANOVA) revealed a significant difference among genotypes (Table 1). A wide range of variation for grain Fe (47.8–83.0, 49.4–86.2, 41.4–77.6 mg kg^{-1}), Zn (29.5–55.0, 28.1–63.1, 29.7–55.4 mg kg^{-1}), protein content (11.6–24.8, 15.7–26.2, 15.9–24.7%) and grain yield (418–5114, 484–3472, 268–2470 Kg/ha) were observed under non-stress, drought and heat stress conditions, respectively. T-test indicated a significant difference ($P < 0.05$; $t \geq 1.96$) among the mean values of grain Fe (65.44, 69.20 and 55.27 mg kg^{-1}), Zn (41.35, 45.69 and 40.52 mg kg^{-1}), protein (18.16%, 19.37% and 18.51%) and yield (2878, 1857 and 1413 Kg/ha) in non-stress, drought and heat stress conditions, respectively. Heat stress caused more reduction in the grain nutrient contents compared to drought. In the current experiment a threshold of ± 10% change in the nutrient content was not considered as the treatment effect. Under heat stress, 91% and 57% of the genotypes showed 10–39% and 10–31% of reduction in grain Fe and Zn contents, respectively. The protein content showed a negative trend in 90% of the genotypes, and 6.4% genotypes showed a reduction of 10–13.7% under heat stress. The details of highly and least affected genotypes due to heat stress are presented (Table S2). Under different planting conditions, broad sense heritability was observed in the range of 60–97% for the nutrient traits.

All the planting conditions exhibited significant correlations (r) between grain nutrients and seed yield, while frequency distributions showed normal, except for seed weight (Fig. 1). Grain Fe ($r = -0.25$; $p < 0.01$, $r = -0.37$; $p < 0.001$) and Zn ($r = -0.35$; $p < 0.001$, $r = -0.72$; $p < 0.001$, $r = -0.49$; $p < 0.001$) recorded significantly negative correlation with seed yield under all growing conditions except for grain Fe ($r = 0.11$) in heat stress. Similarly, 100-seed weight recorded a significant and positive correlation with seed yield under drought, however, it was

Table 1
Analysis of variance for grain nutrients and yield of AM panel evaluated in three planting conditions.

Traits	Non stress				drought				Heat stress			
	Fe (mg kg^{-1})	Zn (mg kg^{-1})	Protein (%)	Yield (kg/ha)	Fe (mg kg^{-1})	Zn (mg kg^{-1})	Protein (%)	Yield (kg/ha)	Fe (mg kg^{-1})	Zn (mg kg^{-1})	Protein (%)	Yield (kg/ha)
Mean	65.44	41.35	18.16	2877.76	69.20	45.69	19.37	1856.64	55.27	40.52	18.51	1412.55
Range	47.8–83.0	29.5–55.0	11.6–24.8	418.1–5113.8	49.4–86.2	28.1–63.1	15.7–26.2	484.1–3472.1	41.4–77.6	29.7–55.4	15.9–24.7	268.0–2470.2
CV	5.15	7.48	18.87	21.97	6.13	8.87	4.54	15.74	5.01	6.36	3.47	10.02
LSD (5%)	5.42	4.97	5.51	1017.16	6.82	6.52	1.41	471.23	4.46	4.15	1.03	228.23
H%	92	85.6	60.7	86.5	90.5	90.9	89.1	93.4	94.5	94.1	95.6	97.5

negatively correlated under other planting conditions. The association between grain protein and seed yield was found non-significant in all planting conditions. Whereas, the grain Fe exhibited a significant positive correlation ($r = 0.35$; $p < 0.001$, $r = 0.53$; $p < 0.001$) with grain Zn for Drought and non-stress conditions. A weak correlation ($r = 0.15$, 0.14, 0.056) was observed between grain protein and Fe under all the planting conditions. Similarly, a non-significant correlation ($r = 0.097$, 0.063, 0.012) between grain Zn and protein content were noticed in all the planting conditions (Fig. 1; A-C).

3.2. Association between grain nutrients and yield

As there was a significant change in nutrient content of AM panel across planting conditions, the panel was further analyzed to assess the relationship between grain nutrients and yield. PCA of the panel generated a total of four principal components (PCs), among them PC1 and PC2 (Eigen values ≥ 1) collectively explained 75.2 (non-stress), 66.7 (drought) and 73.4 (heat)% of the total variance under respective planting conditions (Table S3). The PC1 (x-axis), explained 48.2%, 42.5% and 44.9% of the total variance, under non-stress, drought and heat stress conditions, respectively. Grain Fe in non-stress (0.56) and heat (0.57) conditions and Zn (0.61, 0.57, and 0.66) under all the planting conditions contributed positively towards PC1. Similarly, PC2 (y-axis) accounted for 27%, 24.1% and 28.5% of the total variation under non-stress, drought and heat stress conditions, respectively. In non-stress condition, protein (0.92), Fe (0.25) and Zn (0.11) contents contributed positively with PC2. However, all the nutrient traits under drought and heat stress associated negatively towards PC2 except the seed yield under non-stress (0.29) and heat (0.11) conditions. The association of the nutrients and grain yield traits in all growing conditions was less than 90^0 (Fig. 2).

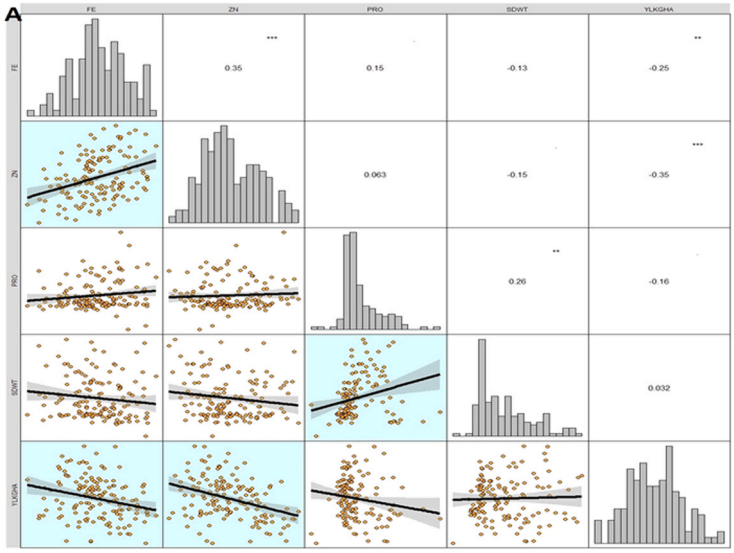
3.3. Genetic diversity for grain nutrients

Cluster analysis of AM panel under three planting conditions for grain nutrients revealed eight main clusters (Table S4). Under non-stress condition, each cluster was possessing 36, 20, 6, 19, 44, 4, 6, and 5 genotypes, respectively. Among them, cluster V was the largest followed by clusters I and II. Cluster VI recorded the maximum cluster mean for Fe (73.01 mg kg^{-1}) and Zn (48.09 mg kg^{-1}) while for protein content (19.88%) followed by cluster II, VII, Similarly, the cluster VIII showed the highest (4797 Kg/ha) cluster mean for grain yield followed by cluster III (4205.61 Kg ha-1). Under drought (Fig. 3), cluster VI contains the largest (28) number of genotypes followed by the clusters II (25) and IV (25), while cluster II was followed by clusters I and VII under heat stress. Each cluster under drought and heat exhibited variable cluster means for nutrient traits, under drought, the cluster VIII recorded the maximum protein content (20.11%), seed weight (23.67 g) and yield (3333.25 Kg/ha), while cluster VII and V for Fe (73.09 mg kg^{-1}) and Zn (51.36 mg kg^{-1}) respectively. Similarly, the cluster VII for seed weight (15.49 g) and yield (2186.28 Kg ha-1) while cluster VIII and III for Fe and Zn, respectively were the highest cluster means observed under heat stress condition.

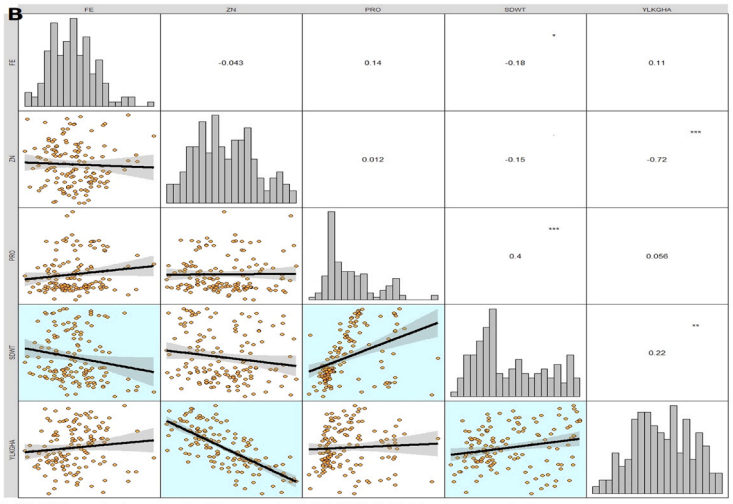
3.4. Population structure and linkage disequilibrium

The population structure of 136 genotypes was analyzed using 3,44,345 high quality genome wide SNPs. The density and distribution of SNPs were presented in Fig. 4. The lowest CV-error value (0.49497) was identified at K= 7 after 30 repetitions (Fig. 5a). The seven sub-populations (SP1 to SP7) generated from the entire set was used in the study (Fig. S1). Similar results were observed in the unweighted neighbor-joining (NJ) tree generated from the dissimilarity matrix (DM) which indicates the existence of large diversity in the AM panel (Fig. 5b).

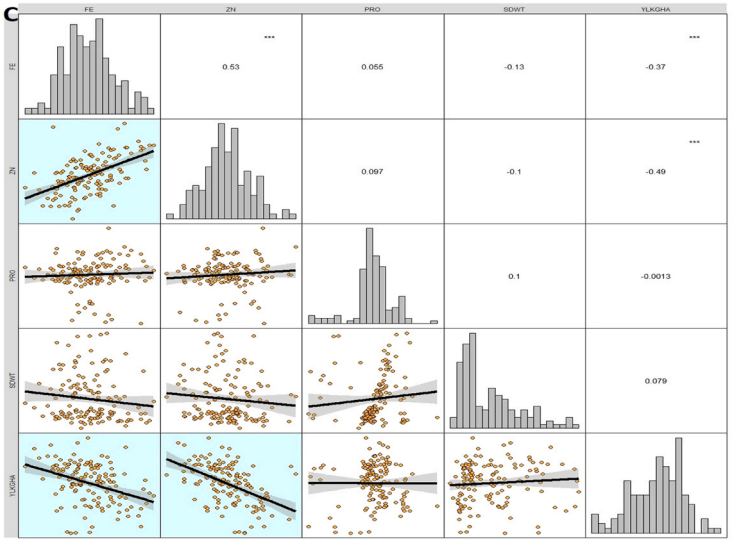
LD decay (r^2) based on SNP markers in complete genome was calculated. The r^2max90, and the LD1/2 max90 percentiles (the physical



*, **, ***correlations are significant at the 0.05, 0.01, 0.001 level, respectively



*, **, ***correlations are significant at the 0.05, 0.01, 0.001 level, respectively



*, **, ***correlations are significant at the 0.05, 0.01, 0.001 level, respectively

Fig. 1. : Phenotypic distribution and correlations for grain nutrients and yield under different growing environments (A: drought; B: heat stress; C: non-stress;).

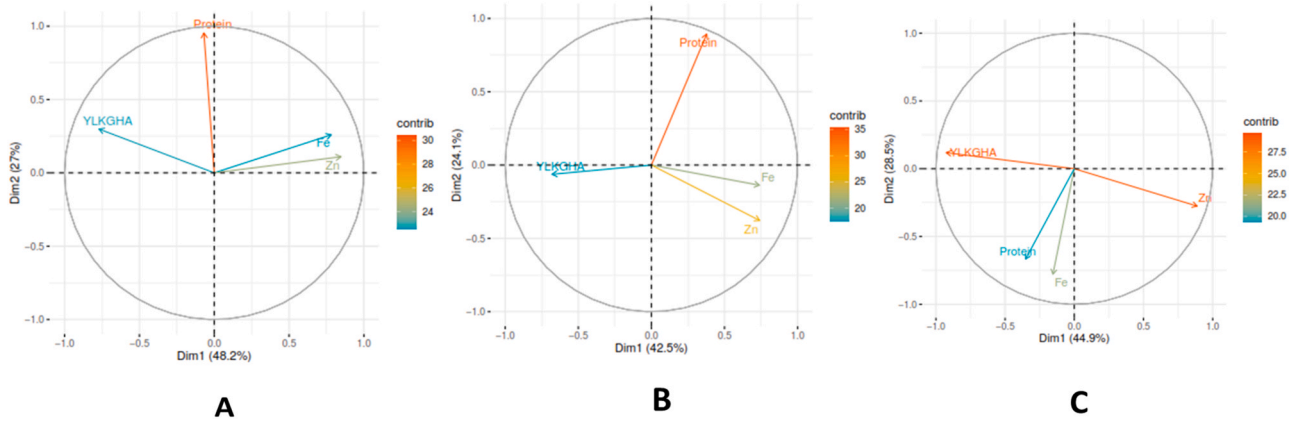


Fig. 2. : Estimated PCs for grain nutrients and yield at three planting environments (A: Non stress; B: Rainfed; C: Summer).

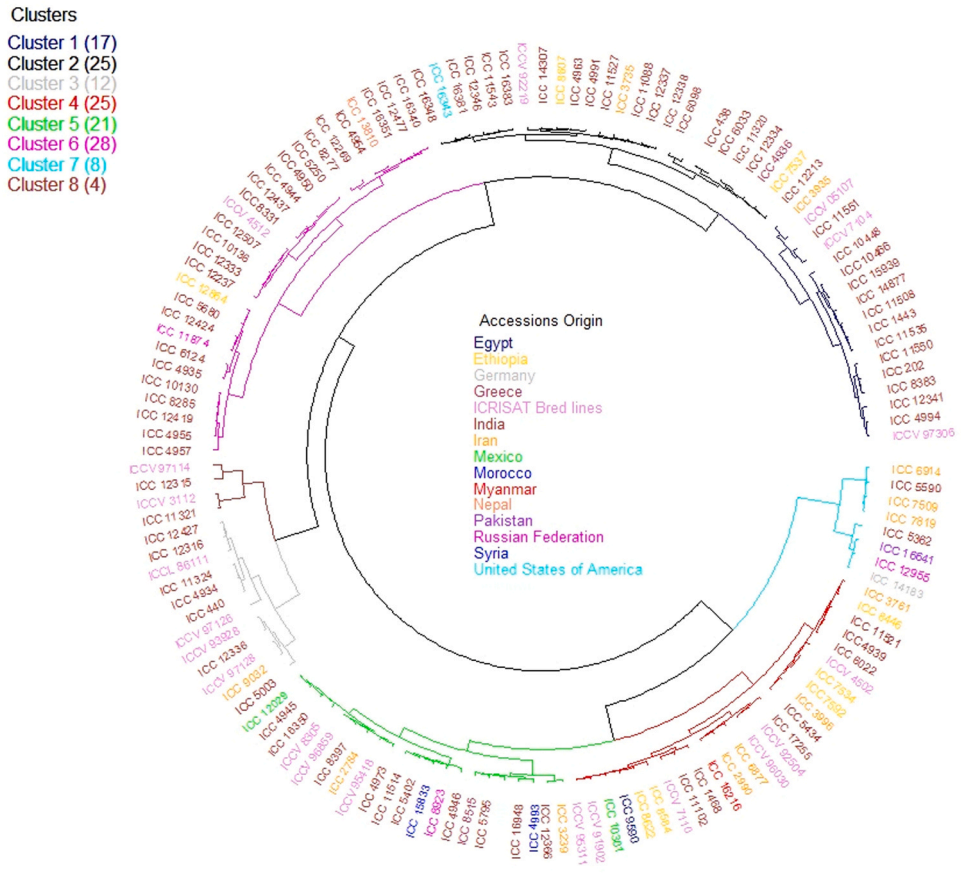


Fig. 3. : Clustering pattern studied for grain nutrients in 140 accessions of AM panel under Drought stress condition (Each cluster denoted by different color).

distance in bp at which LD has decayed) values were obtained at 0.4, 0.04 respectively. The average LD decay of the chickpea germplasm was relatively low and very few SNPs showed $r^2 \geq 0.8$ and 0.6 (Fig. 6a). A huge LD block was observed on linkage group 4 (Fig. 6b).

3.5. *Association mapping for grain nutrient traits*

A total of 181 marker-trait associations (MTAs) (Table S5) were identified based on significant p-values under three different growing conditions. In drought condition, a total of 48 markers showed significant association with grain Fe, Zn and protein content. A single marker S4_44607232 (S represents chromosome) explained 9% phenotypic variation (PV) was associated with grain Fe traits. On chr7 a single marker S7_10599675 showed 11% PV for Zn trait. For the grain protein content, a total of 46 markers were significantly associated (*P-value* ranged between $P \leq 7.55 \times 10^{-7}$ and $P \leq 9.34 \times 10^{-5}$). The marker S6_42913961 showed (Fig. 7a) the highest PV (18%) for protein content ($P \leq 7.55 \times 10^{-7}$), followed by S6_12788060 (PV 15%) and S4_39150978 (PV 13%).

The MTAs were also studied in non-stress and heat stress conditions, interestingly results were found highly significant. In non-stress condition, a total of 70 MTAs were observed. A single marker S4_44607232 on chr4 showed 11% of PV for grain Fe. Three markers S1_15267578, S7_11907729 and S4_9867593 showed a cumulative PV of 34% for Zn

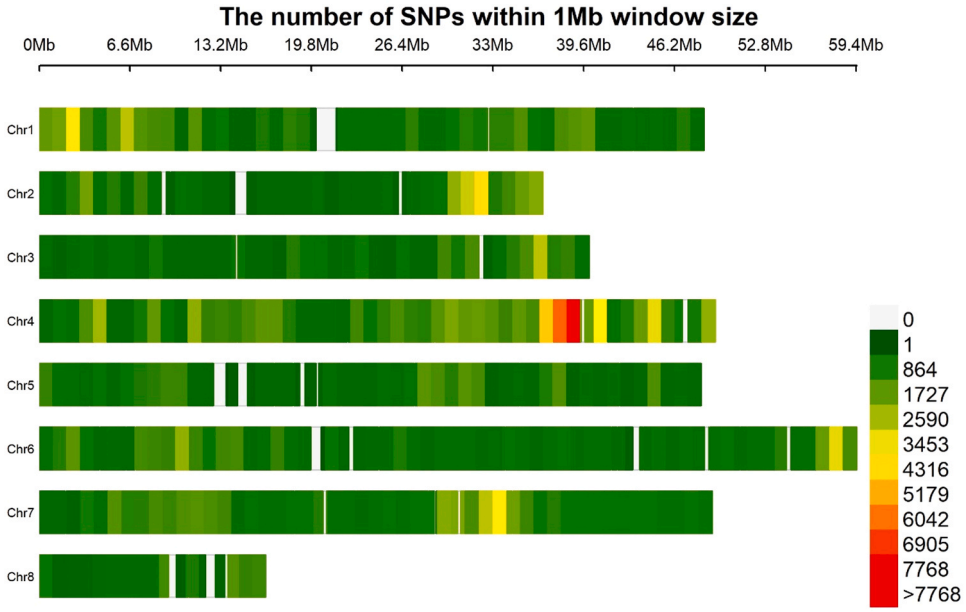


Fig. 4. SNP density and distribution on chickpea 8 chromosomes.

content. For protein content, a total of 66 MTAs (PV ranged from 1% to 28%) were associated across the genome. Maximum number of MTA were observed on chr5 (#17) and the marker S5_3793636 showed a PV of 14% at p value 2.43 × $10^{-6.}$ Similarly, highly significant markers were observed on chr3, (S3_28729262, S3_5346023; S3_5969219), chr4, (S4_38618901), chr6 (S6_46910162) and chr8 (S8_13109034) for grain protein content (Fig. 8a).

A total of 63 MTAs were identified for Fe (#43), Zn (#5) and protein (#15) traits under heat stress condition. Of the total 43 SNPs associated with grain Fe content the maximum PV (22%) was shown by S1_12185432 at P value 6.62 x 10^{-5} and the highly significant marker S3_37090540 recorded a PV of 6%. For grain Zn content three SNPs on chr2, (S2_2323804; S2_2370534; S2_2312104), one each on chr7 (S7_37159003) and chr4 (S4_32672776) were found significantly associated. Similarly, for grain protein 15 MTAs were found on chr1, 2, 3, 6 and 7 with their *P-value* ranging from $\leq 9.75 \times 10^{-5}$ *to* $\leq 1.52 \times 10^{-6}$. Two highly significant markers S3_10482045 and S6_59061568 showed a cumulative PV of 24% (Fig. 9a).

The significant MTAs were represented on 8 chromosomes for the studied seasons represented in Fig. S2 (a,b,c). The marker S4_44607232 ($P \leq 1.35 \times 10^{-5}$) co-associated with grain Fe under both non-stress and drought conditions (Fig. 10a). For grain protein content five MTAs (S1_1451316, S1_18239723, S1_812178, S1_35622241 and S6_12788060) were consistent under both drought and heat stress conditions (Fig. 10b). Significant MTAs were visualized in Manhattan plots (Fig. 7a and b for drought; 8a and b for non-stress and 9a and b for heat stress) on multiple chromosomes. The Q-Q plots indicated that observed –log10 (p) values of protein were higher than the expected values in all the three growing conditions, whereas such trend was not observed for grain Fe and Zn (Fig. 7c for drought; 8c for non-stress and 9c for heat stress).

4. Discussion

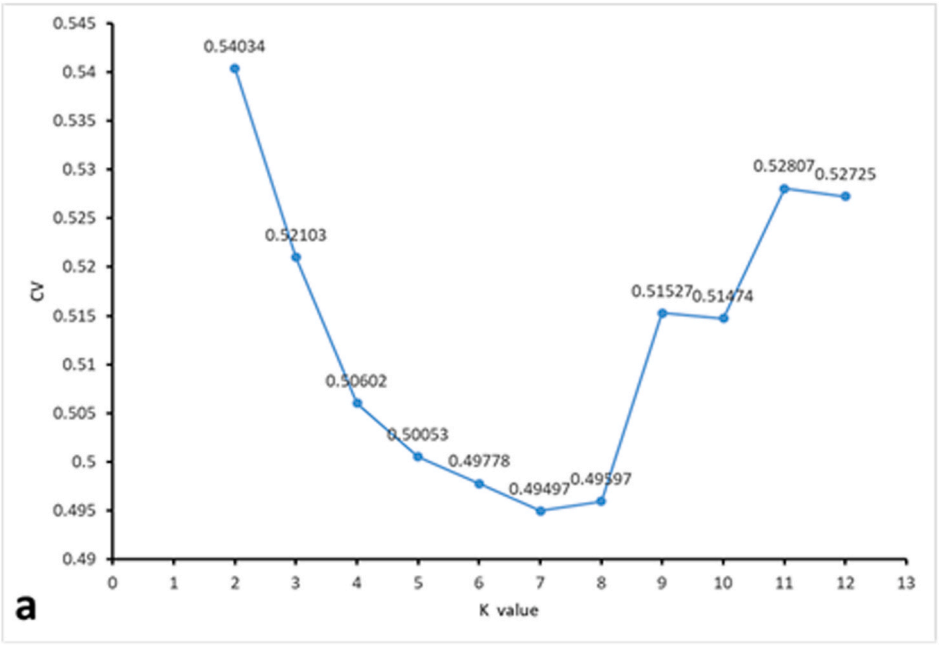
Development of improved cultivars for desired traits mainly depends on extent of genetic diversity available in the breeding material. Evaluation of AM panel under diverse environments will help understand the interaction of environments on the expression of quality traits and also to quantify the genetic diversity. Similarly, the pattern of marker trait association under different environments helps identify candidate markers for deploying in the breeding populations.

4.1. Genetic variability for grain nutrients

The ANOVA revealed highly significant variation for all traits studied in three planting conditions. Under drought conditions, mean grain Fe increased by 6% compared to non-stress conditions, whereas under heat, it was reduced by 20% (T value <= 2). Similarly, the mean grain Zn under drought was increased to the extent of 10%, while it was reduced by 11% under heat stress. Similarly, mean grain protein was increased by 6% under drought and reduced by 4% under heat compared to non-stress condition. Similar results were observed in other legume crops such as lentil, where the grain quality (minerals, iron and zinc contents) significantly affected under heat stress condition due to reduction of root nutrient uptake, with reduced root biomass and metabolic rate (Heckathorn, 2013) or by direct damage to roots (Huang et al., 2012). While the variation under drought was attributed to decreasing water availability under stress condition (Choukri et al., 2020). Conversely, the mean grain yield was reduced to the extent of 27% and 42% under drought and heat, respectively, compared to non-stress condition. The severe yield reduction under heat stress could have caused due to detrimental effects of high temperatures on flower and seed development processes and translocation of photosynthates during the reproductive period in chickpea (Kaushal et al., 2013), lentils (Bhandari et al., 2016) and Mung bean (Kaur et al., 2015). As a consequence, the *per se* performance of these nutrients was varied significantly under heat and drought conditions. Recent studies in lentil (Choukri et al., 2020) and common bean (Ghanbari et al., 2013), showed that heat stress caused reduction in grain Fe (16.5–18%), Zn (22%) and crude protein content (14%). High broad sense heritability was recorded for grain nutrients and yield in all the studied environments which indicate that it is possible to improve the traits by following simple selection and advancement and Paul et al., 2018).

4.2. Correlations among grain nutrients

The correlations between grain nutrients and seed yield varied from positive to negative in each planting condition. Among them, the grain Fe and Zn (Diapari et al., 2014) recorded significantly negative correlation with seed yield in all the growing conditions, except for grain Fe



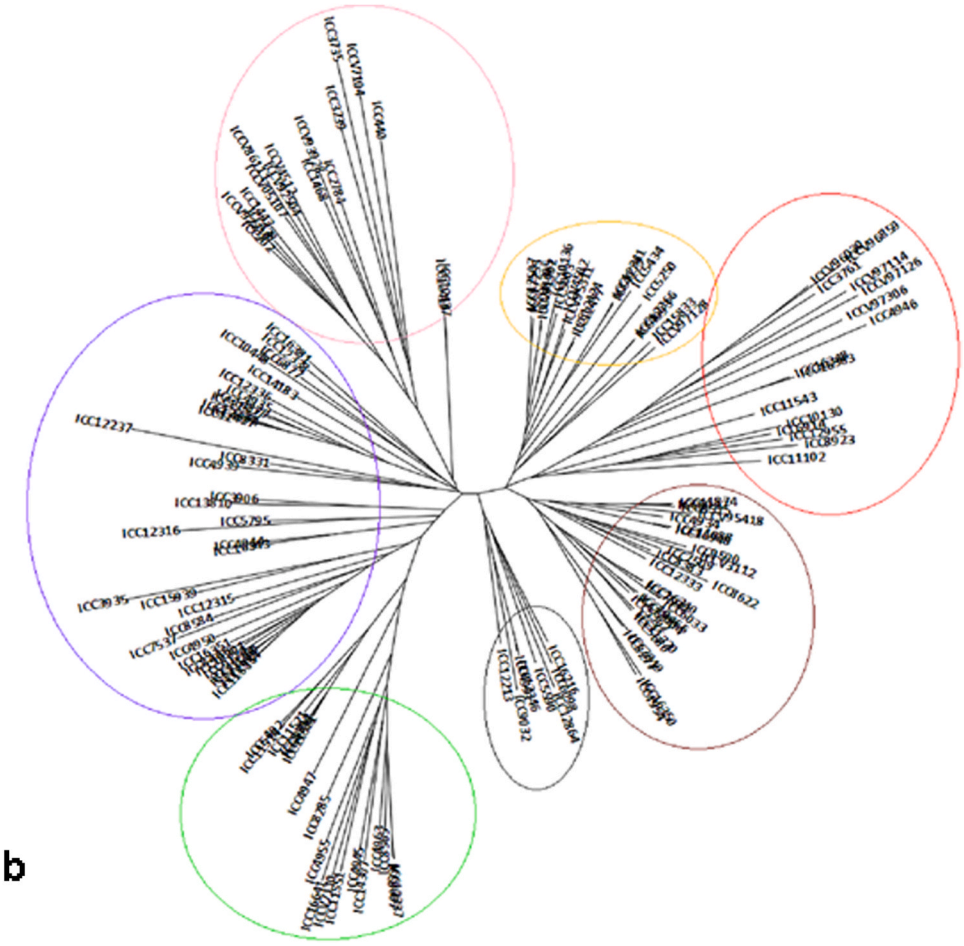


Fig. 5. a. **Population structure estimation of optimal population number K= 7. 5b. A unweighted neighbor-joining (NJ) tree depicting the genetic relationships among 136 chickpea accessions based on Nei's genetic distance using high quality GBS based** 3,44,345 genome wide SNPs**.**

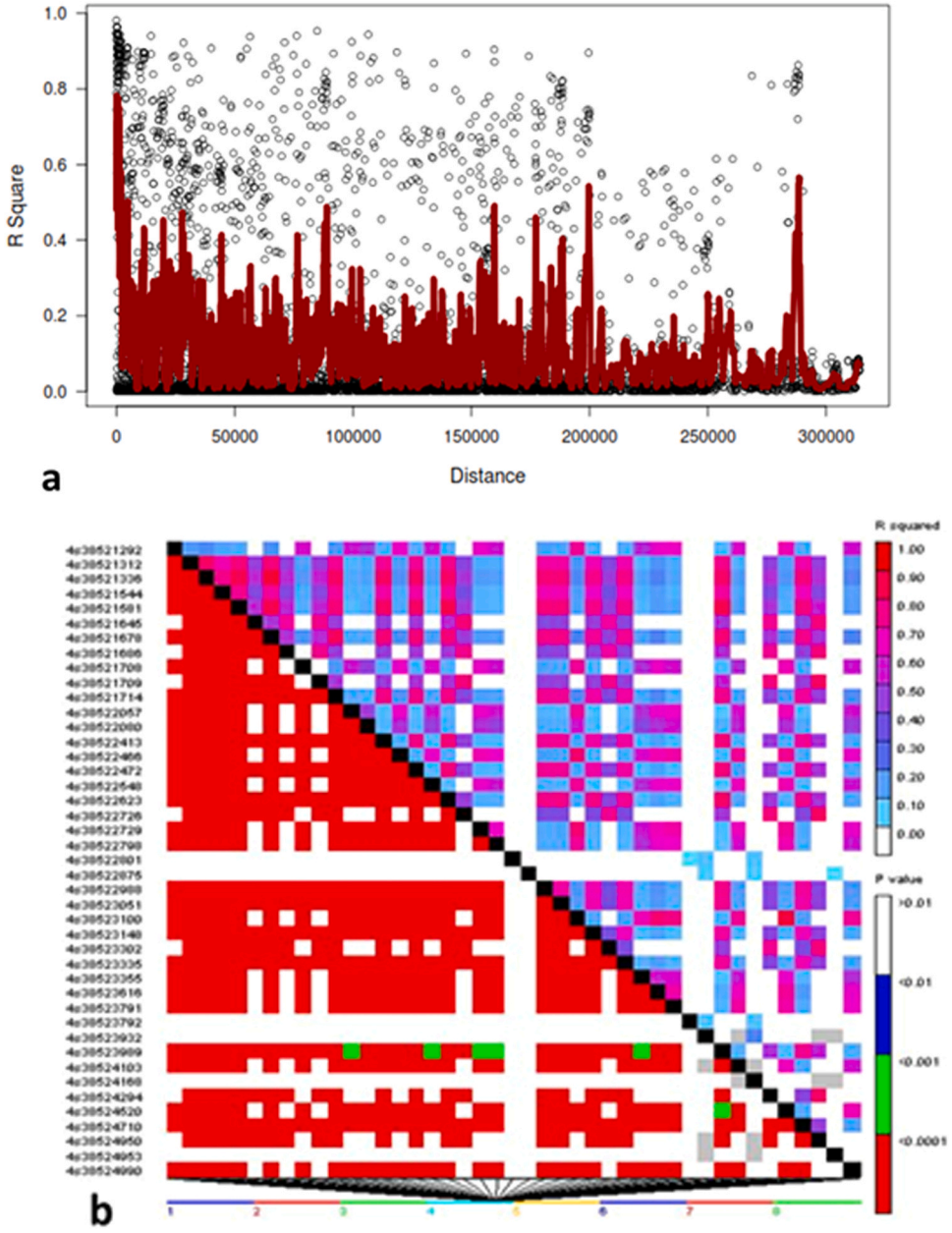


Fig. 6. a. Linkage disequilibrium (LD) decay of whole genome representing GBS based SNPs vs correlation coefficient (r^2) plotted. The arrow represented the start point of LD decay. 6b. LD decay pattern in matrix form representing all 8 chromosomes showing the squared correlation coefficient (r^2) each pair of the marker and their corresponding test in upper and lower triangle respectively. A huge LD decay observed on chr4.

under heat stress. contrarily, protein content had non-significant correlation with seed yield in all the planting conditions, which shows the possibility of developing high protein genotypes with superior yield. A strong positive correlation was found between grain Fe and Zn under non-stress and drought, which was also observed in earlier studies in chickpea (Tan et al., 2018), field pea (Diapari et al., 2015, Dissanayaka, 2019), lentil (Khazaei et al., 2017), and pea (Ma et al., 2017). Interestingly, in the current study a significant positive correlation was observed between protein content and 100-seed weight which indicates the possibility of developing high protein content genotypes with higher seed size (Panthee et al., 2005; Kulwal and Mhase, 2017). However, many studies in legumes and other crops reported a common phenomenon of negative correlation (Saxena et al., 1987; Afzal et al., 2003; Gaur et al., 2016). The normal frequency distribution (Fig. 1) of studied traits under respective environment indicates polygenic nature of the traits, such distribution was observed for protein content (Gaur et al., 2016), yield (Paul et al., 2018), and grain Fe and Zn (Diapari et al., 2014; Upadhyaya et al., 2016a) in chickpea. The correlations identified in the study help develop nutrient rich chickpea varieties suitable for different agro ecologies.

4.3. Principal component analysis

Utilizing the PCA information, the trait contributing maximum variability in the given material under the different growing conditions were identified. The Table 2 and 3 showed Eigen values with percent variance contribution of each PCs over the traits across the testing conditions. Based on Eigen values of respective PCs (PC1 and PC2), the grain Fe and yield were the most representative traits accounting for high variability (75.2% and 73.36%) in the total variance under non-stress and heat stress, respectively (Diapari et al., 2014). Using PCs scores, a similar maximum and consistent variability for yield under

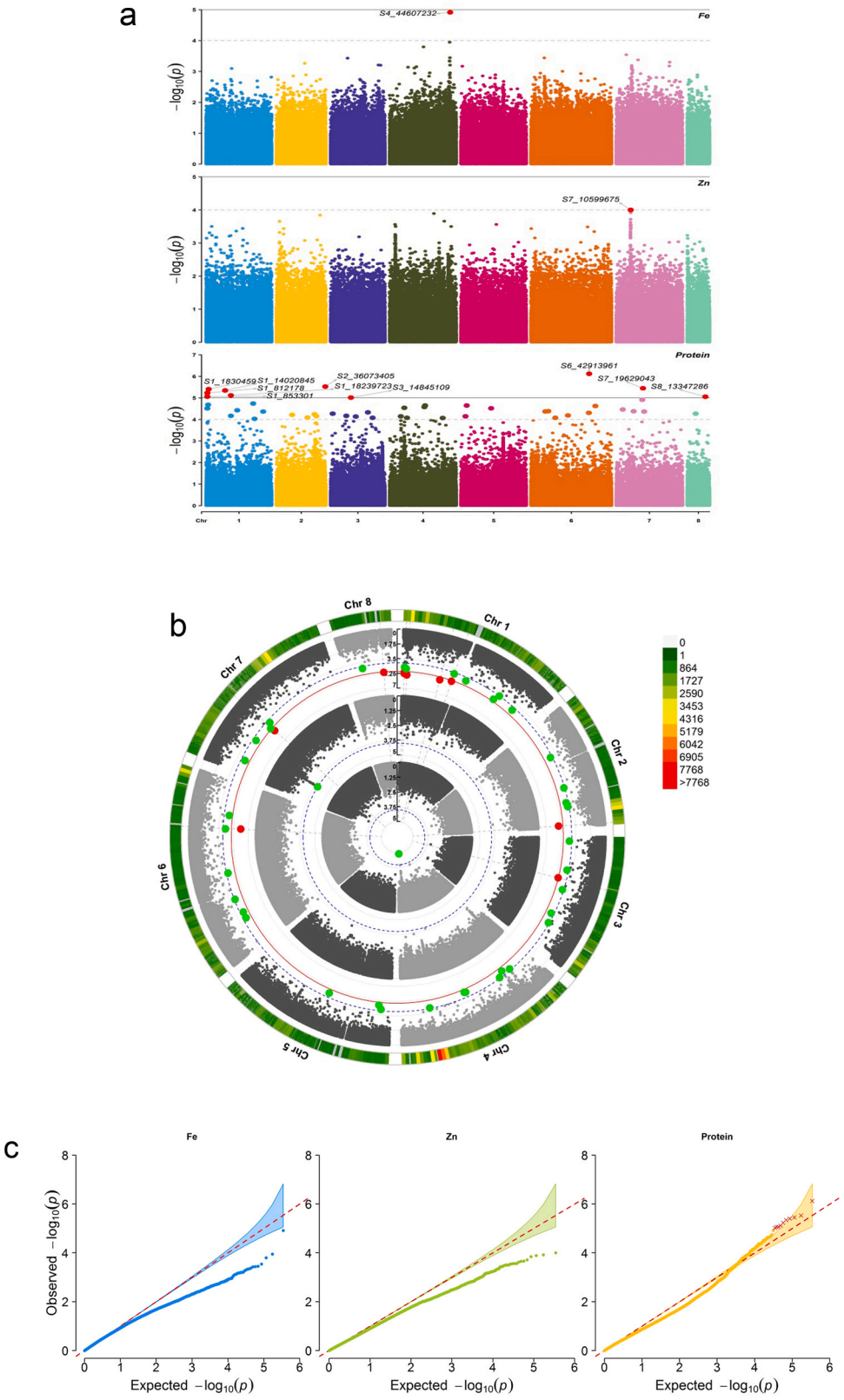
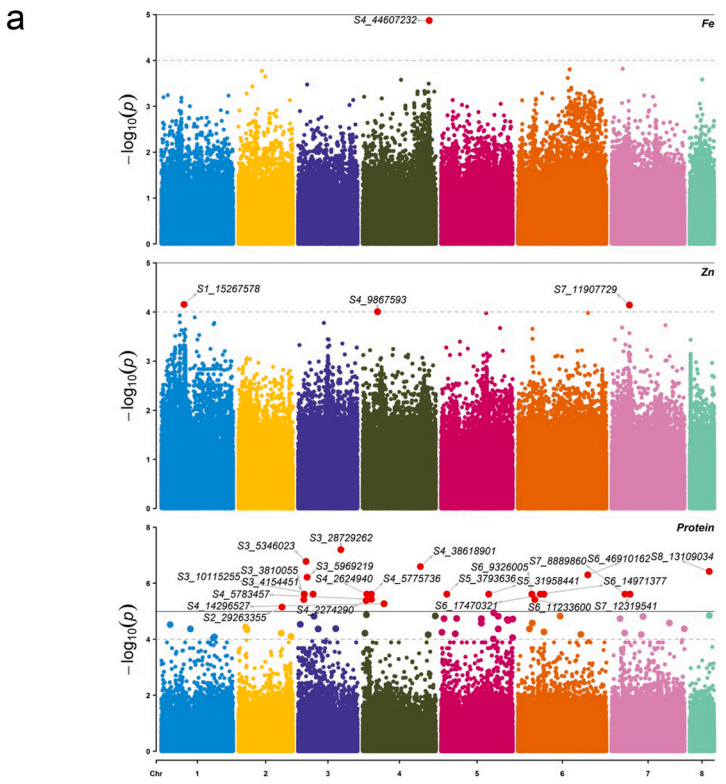


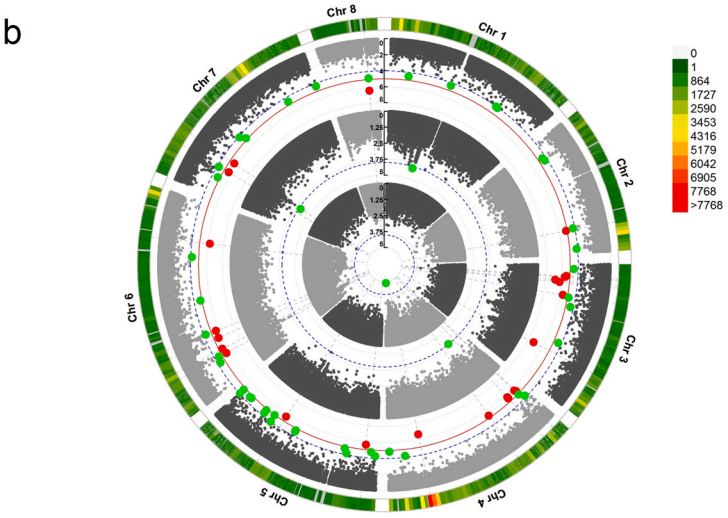
Fig. 7. a. Manhattan plots illustrated significant p-value (measured by MLM model) associated with grain Fe, Zn and protein content in chickpea under drought condition using high quality GBS based 3,44,345 genome wide SNPs. The relative density of SNP markers physically mapped on eight chromosomes on the x-axis. The –log10 (P)-value for significant association with Fe, Zn and protein trait denoted on the y-axis. The SNPs revealing significant association with grain Fe, Zn and protein content at cut-off P-value $\leq 1 \times 10 - 5$ are demarcated with red dots on thick lines. 7b. Circular Manhattan plot (drought condition) represented highly significant SNPs were in red color dots (from center to outside Fe, Zn and protein). 7c. Quantile-quantile plot (drought condition) measured by MLM model represented the comparison between expected and observed –log10 (P)-values at a FDR cut-off < 0.05 to scan the significant genomic SNP loci associated with grain Fe, Zn and protein content in chickpea.

stress environments has been reported in chickpea (Paul et al., 2018). Likewise, the contributions of PCs over the nutrients across the conditions denoted that PC1 was positively influenced by grain Fe in non-stress and heat, and Zn in all planting conditions, whereas PC2 was positively influenced by protein content followed by grain yield, which provides a unique opportunity to exploit desired alleles for grain nutrients in the population studied.

4.4. Genetic diversity analysis and cluster means

The cluster analysis of AM panel accommodated genotypes in eight clusters with a wide range of cluster means for nutrient traits. Most of





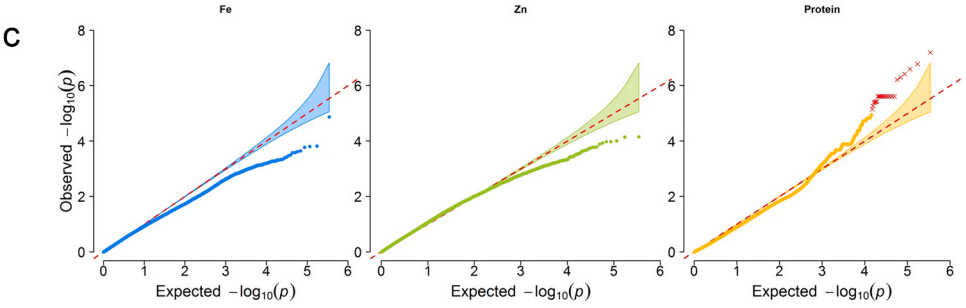


Fig. 8. a Manhattan plots illustrated significant p-value (measured by MLM model) associated with grain Fe, Zn and protein content under chickpea at irrigated (non-stress) condition using high quality GBS based 3,44,345 genome wide SNPs. The relative density of SNP markers physically mapped on eight chromosomes on the x-axis. The –log10 (P)-value for significant association with Fe, Zn and protein trait denoted on the y-axis. The SNPs revealing significant association with grain Fe, Zn and protein content at cut-off P-value $\leq 1 \times 10 - 5$ are demarcated with red dots on thick lines.8b. Circular Manhattan plot (irrigated condition) represented highly significant SNPs were in red color dots (from center to outside Fe, Zn and protein). 8c. Quantile-quantile plot (non-stress condition) measured by MLM model represented the comparison between expected and observed –log10 (P)-values at a FDR cut-off < 0.05 to scan the significant genomic SNP loci associated with grain Fe, Zn and protein content in chickpea.

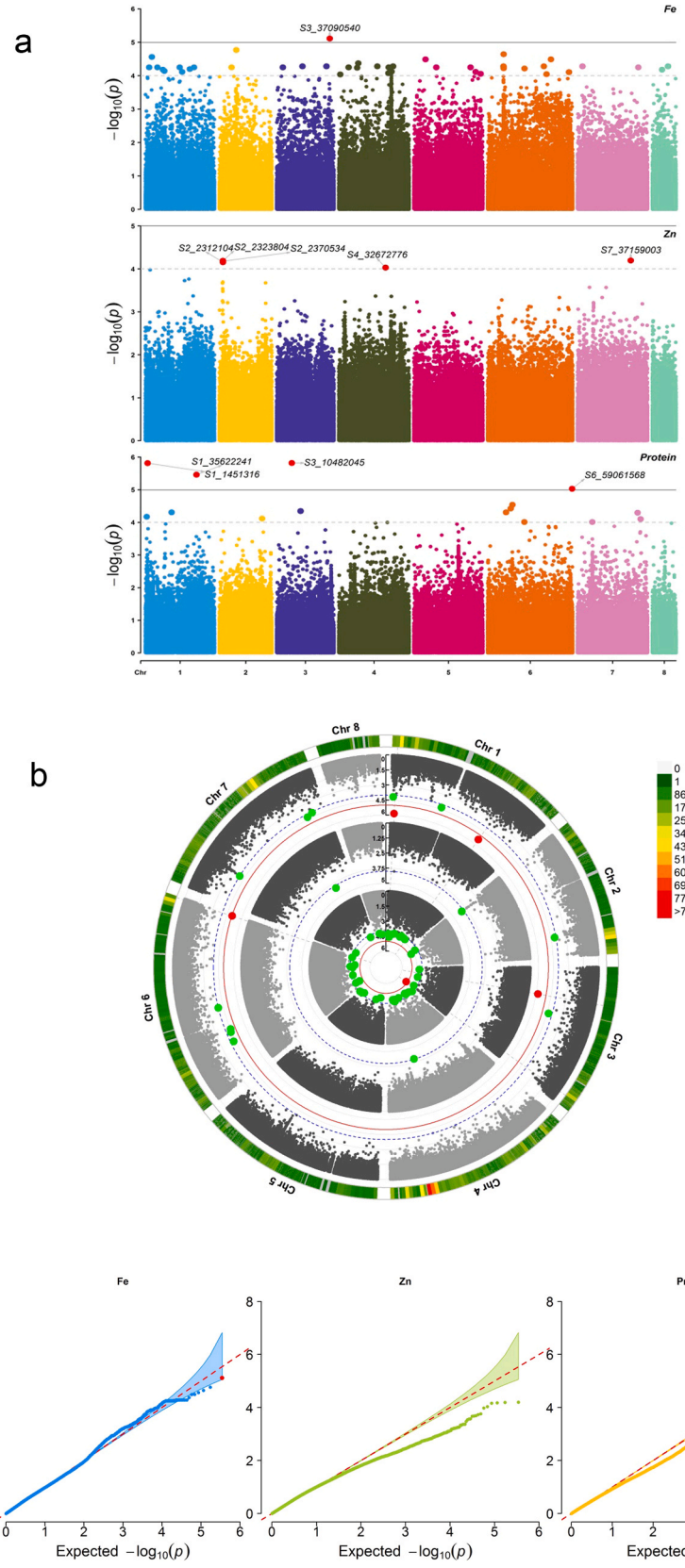


Fig. 9. a Manhattan plots illustrated significant p-value (measured by MLM model) associated with grain Fe, Zn and protein content in chickpea under heat stress (summer) condition using high quality GBS based 3,44,345 genome wide SNPs. The relative density of SNP markers physically mapped on eight chromosomes on the x-axis. The –log10 (P)-value for significant association with Fe, Zn and protein trait denoted on the y-axis. The SNPs revealing significant association with grain Fe, Zn and protein content at cut-off P-value $\leq 1 \times 10 - 5$ are demarcated with red dots on thick lines. 9b. Circular Manhattan plot (heat stress condition) represented highly significant SNPs were in red color dots (from center to outside Fe, Zn and protein). 9c. Quantile-quantile plot (heat stress condition) measured by MLM model represented the comparison between expected and observed –log10 (P)-values at a FDR cut-off < 0.05 to scan the significant genomic SNP loci associated with grain Fe, Zn and protein content in chickpea.

the accessions in drought and heat were captured by cluster VI, and II followed by cluster VII, IV, plus the differential response of the accessions revealed that these were genetically diverse with respect to nutrient content. The genotypes ICC 1664 for grain iron, ICC 5590 for grain zinc belonged to cluster VII, while ICC 95418 for grain protein associated to cluster IV observed higher nutrient contents under stress conditions. For improving the nutrient status, it is desirable to cross these genotypes from highly diverse clusters to bring the targeted trait

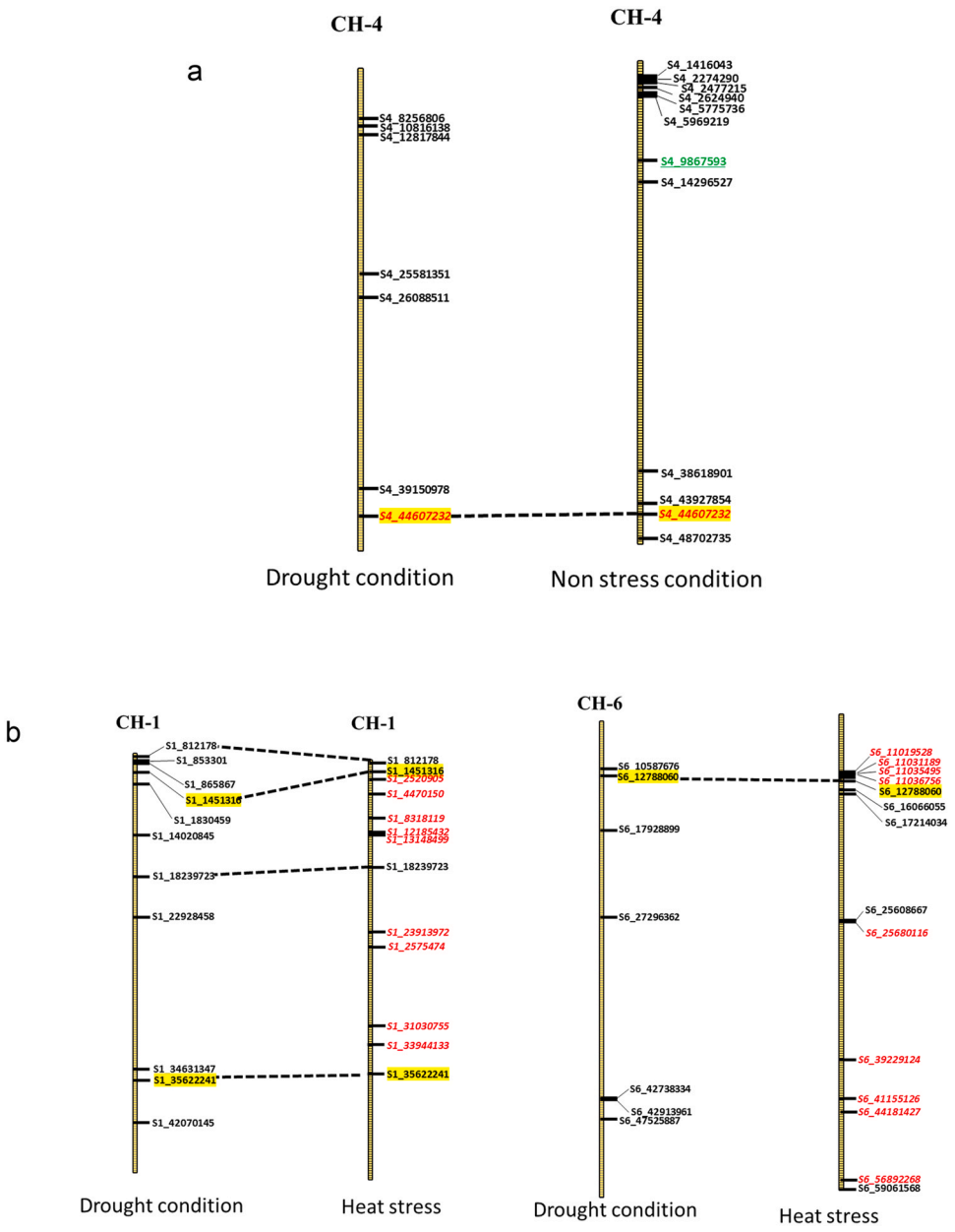


Fig. 10. a. The co-association for grain Fe was presented on chromosome 4 (CH-4) under drought and non-stress condition. Dotted lines were given for co-association. 10b. The co-association for grain protein was presented on CH-1 and CH-6 under drought and Heat stress condition. Dotted lines were given for co-association and the markers were highlighted.

Table 2
PCA estimation, Eigen value and their percent variance contribution for grain nutrients and yield of AM panel under three planting conditions.

Conditions	PCs	Yield	Fe	Zn	Protein	Eigen value	Variance %	Cumulative variance
Non stress	PC1	-0.55	0.56	0.61	-0.05	1.93	48.23	48.23
	PC2	0.29	0.25	0.11	0.92	1.08	27.00	75.23
	PC3	-0.66	-0.65	0.03	0.38	0.55	13.85	89.08
	PC4	0.42	-0.45	0.78	-0.10	0.44	10.92	100.00
Drought	PC1	-0.52	0.57	0.57	0.29	1.70	42.51	42.51
	PC2	-0.06	-0.14	-0.38	0.91	0.97	24.15	66.66
	PC3	0.81	0.55	0.10	0.18	0.75	18.85	85.51
	PC4	-0.27	0.60	-0.72	-0.23	0.58	14.49	100.00
Heat	PC1	-0.69	-0.12	0.66	-0.26	1.80	44.89	44.89
	PC2	0.11	-0.73	-0.26	-0.62	1.14	28.48	73.36
	PC3	-0.03	-0.67	0.14	0.72	0.82	20.39	93.75
	PC4	-0.71	0.03	-0.69	0.13	0.25	6.25	100.00

into desirable agronomic background, keeping in mind that these recorded low yields. Although, the cluster analysis in previous studies reported that the nutritional traits were independent of seed yield in chickpea (Gaur et al., 2016) and lentil (Filho, 2004). Further, (Jayalakshmi et al., 2018) has made the grouping of 54 chickpea germplasm lines into 5 clusters and reported 54% lines were grouped in cluster I having higher protein content and also higher micronutrients lines grouped into IV and V with 7% and 13%, respectively.(Jayalakshmi et al., 2018)

4.5. Population structure and linkage disequilibrium

LD patterns and decay are critical parameters in determining population structure and density of molecular markers suitable for GWAS (Gali et al., 2019; Mather et al., 2007). A total of 344,345 SNP markers were used to analyze the population structure and LD in this study. The higher LD was found in the populations due to low effective recombination rates (Huang and Han, 2014). The LD decay was found slow in highly self-pollinated crops compared to cross-pollinated crops (Niu et al., 2019). The average SNP call rate and reproducibility were very high in the current study (0.97 and 0.98, respectively). Similar results were observed in chickpea (Farahani et al., 2019) and common bean (Valdisser et al., 2017). The results from cluster analysis and population structure were in concordance with the discriminant analysis of principal components (DAPC). The analysis showed the intermix of the accession, some genotypes were comprised in different cluster groups and divided into seven subpopulation groups. In earlier studies the chickpea genotypes were sub populated into three (Kujur et al., 2015) and four (Farahani et al., 2019) groups.

4.6. Association mapping

Till date, very few studies have endeavored to uncover the genetic basis of nutrient content in chickpea (Diapari et al., 2014; Upadhyaya et al., 2016a, b; Sab et al., 2020). The conventional QTL mapping approaches to identify genomic region has been restricted to bi-parental mapping populations at less resolution (Ibrahim et al., 2020). Association studies reveal the natural diversity generated by multi-generational recombination events that occur in a population or germplasm panels (Deschamps et al., 2012). In the present investigation, the marker-trait associations identified for grain Fe, Zn and protein content, are valuable resources for chickpea biofortication programs globally. The MLM model of association analysis was able to minimize false-positive associations and found to be more robust as compared to GLM (Zhang et al., 2012).

The present study revealed 181 MTAs under all growing conditions. For grain Fe 43 out of 45 MTAs under heat stress were not correlated to any other planting conditions (Fig. 10a). For grain Zn a total of nine significant MTAs were identified and no common MTA observed among the three planting conditions. For grain protein, three were commonly found under both non stress and heat stress planting conditions of the 127 significant MTAs (Fig. 10b). This information gives us caution that the MTAs are highly influenced by type of growing conditions and specific to a particular population. The stable MTAs identified for protein content are valuable resources for improving protein levels in the new cultivars. Few earlier studies have reported such outcomes that the MTAs were varied from location to location. In chickpea (Diapari et al., 2014) and pea (Diapari et al., 2015) even though several MTAs were identified in each location there were no common MTAs identified between locations for grain Fe and Zn content. Whereas, in lentil (Khazaei et al., 2017) two MTAs for Fe and Zn were identified in two different locations out of nine and 12 MTAs, respectively. For grain protein content, 2 MTAs were commonly observed in two different locations out of 3 reported MTAs in pea (Gali et al., 2019).

Significant MTAs were identified for grain Fe and Zn contents on chr4, and chr4 and 7 with more than 10% PV, respectively. Recent studies indicated that, chr4 was harboring the genomic regions (Sab et al. 2020) and significant co-localized MTAs (Upadhyaya et al., 2016a; Diapari et al., 2014) for Fe (PV, 2–18.1) and Zn (PV, 7–18.2%) contents in chickpea. The tightly linked markers and significant MTA on chr 4 and 6 could be used for further validation in diverse populations and identification of candidate genes for early generation selections in the breeding pipeline. The MTA studies in other legume crops such as pea (Dissanayaka et al., 2020) and lentil (Khazaei et al., 2017) reported that the phenotypic variation was observed in the range of 4.7–14.5% for grain Fe and 7.6–13% for Zn contents. Interestingly, significant MTAs were observed on the same linkage groups in chickpea and other related legumes (Table S6).

For grain protein content, the major SNPs were observed on chr1 followed by chr4, 6 and 3. Previous reports have identified QTLs and MTA on different chromosomes or linkage groups. The major QTLs (PV, 44.8%) were identified on LG3 (Wang et al., 2019) whereas, Upadhyaya et al. (2016b) reported significant MTA on chr2, 4 and 7 with average PV of 10%, 14% and 16%, respectively, and (Jadhav, 2015) reported significant MTAs on LG5 followed by LG3 in chickpea. Majority of the studies could identify chr3 as common location in the genome for protein content in chickpea. In pea chr3 and 5 were found significant with average PV 5% and 6%, respectively, under different locations and over years (Gali et al., 2019). In soybean 31 SNPs observed on 12 of the 20 soybean chromosomes were associated with grain protein content (Li et al., 2019).

5. Conclusion

Large genetic variation was observed in the genotypes for grain Fe, Zn and protein content under three planting conditions. Heat stress caused more reduction in the grain nutrient contents compared to drought. Grain Fe and Zn contents reduced up to 39% and 31%, respectively compared to protein content (13.7%) under heat stress. Grain yield was negatively associated with Fe and Zn contents and no significant association with protein. The highly significant MTAs with large phenotypic variation identified under non-stress and drought conditions need to be further validated in diverse breeding populations for developing breeder-friendly marker for improving grain nutrient content in the chickpea breeding programs globally.

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Author contributions

SS and PMG designed and supervised the overall research and contributed to the preparation of the manuscript. NS, UC generate phenotypic data. AR provided technical guidance in analysis. MDM carried out statistical, GWAS and population structure analysis, MDM, AH prepared first draft. SS edited the manuscript for final submission. SS and MDM contributed to the article and approved the submitted version.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the corresponding authors, without undue reservation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2021.104688.

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