UPLC-HRMS based Untargeted Metabolic Profiling Reveals Changes in Chickpea (*Cicer arietinum)* Metabolome following Long-Term Drought Stress

Running Title: UPLC-HRMS Based Metabolomic Profiling of Chickpea

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Importance of Work

Drought stress is one of the major problems in chickpea growing areas. Though drought stress changes biochemical mechanisms in plants, however, little is known about the complex metabolic regulation for genetic improvement in chickpea under drought stress environments. The present study was conducted to identify changes at different metabolites in two chickpea varieties contrasting for drought tolerance under drought and control conditions. This study also demonstrates the metabolic pathways potentially involved in drought tolerance mechanism in chickpea.

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ABSTRACT

Genetic improvement for drought tolerance in chickpea requires a solid understanding of biochemical processes involved with different physiological mechanisms. The objective of this study is to demonstrate genetic variations in altered metabolic levels in chickpea varieties (tolerant and sensitive) grown under contrasting water regimes through UPLC-HRMS based untargeted metabolomic profiling. Chickpea plants were exposed to drought stress at the three-leaf stage for 25 days and the leaves were harvested at 14 days and 25 days after the imposition of drought stress. Stress produced significant reduction in chlorophyll content, Fv/Fm, RWC, and shoots and root dry weight. Twenty known metabolites were identified as most important by two different methods including SAM and PLS-DA. The most pronounced increase in accumulation due to drought stress was demonstrated for allantoin, L-proline, L-arginine, L-histidine, L-isoleucine, and tryptophan. Metabolites which showed decreased level of accumulation under drought condition were choline, phenylalanine, GABA, alanine, phenylalanine, tyrosine, glucosamine, guanine, and aspartic acid. Aminoacyl-tRNA and plant secondary metabolite biosynthesis, and amino acid metabolism or synthesis pathways were involved in producing genetic variation under drought condition. Metabolic changes in light of drought conditions highlighted pools of metabolites that affect the metabolic and physiological adjustment in chickpea that reduced drought impacts.

Key words: UPLC-HRMS analysis, Drought, Chickpea, Metabolites.

INTRODUCTION

Chickpea (*Cicer arietinum*) is the fourth largest grown legume crop in the world, which is grown in an area of 10.2 million hectares with an average production of 11 million metric tons. Chickpea significantly contributes to world food security by providing calories and dietary protein for millions of people (Varshney et al. 2013). More than 80% of chickpea is

grown under rainfed condition where unpredictable rainfall pattern or drought stress is the most common limiting factor that affects its growth and productivity. Moreover, drought conditions also affect symbiotic association of nodulating bacteria that contribute 45% of nitrogen needed for agriculture (Babalola 2010). In addition, the climate change scenario, especially progressive drought stress induced by either declining rainfall or higher water stress allied with hot climate will cause significant damage to crop production in the future (Dai 2013; Lobell et al. 2014; Ruane et al. 2014). The major effects of drought include reduction in cell elongation, cell division, stem enlargement, and decrease in root growth (Li et al. 2009; Farooq et al. 2009). In addition, drought stress interrupts photosynthesis and increases translocation of carbohydrates, accelerates phasic development, and boosts senescence (Kobota et al. 1992; Yang et al. 2000; Yang et al. 2001). Genetic improvement is the most efficient and sustainable way to reduce drought stress effect and develop genetically superior germplasm that adapts to future climate change condition. However, genetic improvement requires a solid understanding of the biochemical mechanisms controlling different traits. The analyses of biologically important molecules are necessary to understand which molecules are influencing stress tolerance mechanism in chickpea plants.

Plants produce an array of biochemical compounds including hundreds of metabolites under stress condition. Plant metabolites play essential roles in growth, cell integrity, energy storage, cell signalling, membrane formation and scaffolding, cellular replenishment and whole-plant resource allocation, as well as roles in plant development and stress responses (Wen et al. 2015). Plants can modify their physiology to adapt to different conditions through metabolic changes (Khan et al. 2017; Kim et al. 2011). Plants have various metabolic adaptation mechanisms to defend against the negative effects of stress, which can play a crucial role in the adaptive mechanisms in plants. Changes in the metabolic level in an organism are likely to be correlated with the phenotype as metabolites are the end products of

the biological system. Empirical evidence suggests that metabolic components are linked to high temperature stress tolerance in corn (Chen et al. 2010) and in cool season grass (Su et al. 2009) as well as to cold and freezing tolerance in *Arabidopsis* (Welti et al. 2002). Additionally, recent studies have demonstrated altered level of metabolites between drought-stressed and well-watered plants (Hong-Bo et al. 2006; Bowne et al. 2012; Witt et al. 2012; Obata et al. 2015). The increased level of branched chain amino acids (leucine, isoleucine, and valine) was reported under drought condition in tolerant cultivars of wheat and barley (Bowne et al., 2012; Krugman et al. 2011; Rontein et al. 2002). Differing metabolite expression was associated with high temperature stress tolerance in maize (Chen et al. 2010) and in cool-season grasses (Su et al. 2009). Maize plants under high temperature stress showed increases in metabolites such as tryptophan, serine, threonine, beta-alanine, proline, glutamate, myo-inositol, and urea (Obata et al. 2015). In addition to this, certain metabolites showed a negative correlation (threonine, valine, trehalose, and glycerol) or a positive correlation (fumarate, succinate, and raffinose) with grain yield, and that change in metabolite level was consistent between greenhouse plants and field-grown plants (Obata et al. 2015). Approaches to profile metabolites are receiving increasing attention from the plant biologist community. Metabolic QTL identification revealed that heterosis is mainly under the control of epistasis in *Arabidopsis*, while in tomato, the underlying genetic variation was explained mainly by primary metabolites (Schauer et al. 2006; Lisec et al. 2009). Riedelsheimer et al. (2012) found a strong correlation between metabolites and agronomic traits in field-evaluated hybrid progeny in corn. They built whole-genome and metabolic prediction models with accuracies ranging from 0.72 to 0.81 for SNPs and from 0.60 to 0.80 for metabolites.

Little is known about the complex metabolic regulation for drought stress tolerance in chickpea. Thus, using the metabolic phenotype to study differential expression of contrasting varieties for complex traits might deepen our understanding of the metabolic pathways and expedite genetic gains in chickpea productivity. Metabolomics allows the parallel assessment of a large number of metabolites in a biological sample. Recent metabolomics studies have been of great value in phenotyping and diagnostics in crop breeding (Fernie & Schauer 2009). Recent progress in mass spectrometry with advanced data processing technology allows simultaneous measurement of hundreds of chemically different metabolite species in a single sample of plant tissue. These advances have made it possible to investigate more thoroughly the regulation of metabolic networks and to study their influence on complex traits. The applicability of this technology has been demonstrated in tomato, *Arabidopsis*, and potato through discovery of biomarkers associated with traits of interest (Schauer et al. 2006; Lisec et al. 2009; Rowe et al. 2008). Thus, metabolomics represents an important and exciting new addition for genetic improvement of plants. Metabolomics could be a powerful selection tool to establish the association between phenotype and genotype, leading to a better understanding of the genetic basis of plant responses to stress.

The objective of this study is to demonstrate the differential accumulation of metabolites in chickpea leaves under drought stress condition in different time periods, and the involvement of those metabolites in different pathways in relation to drought tolerance in chickpea. To achieve this objective, we employed a non-targeted global ultrahigh performance liquid chromatography-high resolution mass spectrometry (UPLC–HRMS) for the first time to identify metabolites from the leaf tissue of irrigated and drought-stressed chickpea plants. The UPLC-HRMS technique is sensitive and potentially more robust than Nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS) technology as it can

identify metabolites in low concentration and has better capacity to control the false discovery rate.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The experiment was conducted at the controlled greenhouse condition in the Department of Agronomy, University of Florida, in Gainesville, Florida, USA. Drought sensitive chickpea (*Cicer arietinum*) variety, Punjab Noor-2009 (G1), and drought tolerant variety, 93127 (G2), were used for the study (Khan & Bano 2016). The seeds of those varieties were obtained from Ayub Agricultural Research Institute (AARI) Faisalabad, Pakistan. Chickpea seeds were washed in 100% distilled water followed by surface sterilization with 95% ethanol for 2-3 min, and then soaked in 10% Clorox with concomitant shaking. The seeds were subsequently washed in autoclaved distilled water (Khan & Bano 2016). Seeds were grown in pots filled with 2000 g Metro-Mix® 360 soil mixture. The pots were well watered (typically 2 times/week) throughout until drought stress was applied and each pot contained five plants. Water was applied until the substrate was completely wet and the water started to seep out through the holes at the bottom of the pot. A teaspoon of osmocote (15N-9P-12K) was applied one time after germination. Experiment was laid out in a completely randomized block design with 6 (biological) replications. During the experiment, the greenhouse condition was maintained at 26/19±1 ^{o}C (day/night temperatures) with 70 ± 2% relative humidity and day/night lengths (11 hr/13 hr). Drought stress was imposed on 25-day old plants (3 leaf stage) by withholding water supply for 25 days until the soil water content reached 4%. A set of well-watered plants served as a control. Chlorophyll content, chlorophyll fluorescence (Fv/Fm ratio), shoot and root dry weight, and relative water content were estimated after 14 and 25 days of drought condition. Leaf tissues were harvested for metabolomics analysis also after 14 and 25 days of drought condition.

Physiological Characterization

Data were collected on physiological traits known to be affected by drought condition. Chlorophyll fluorescence (the ratio of variable, Fv, to maximum fluorescence, Fm) and SPAD chlorophyll content were used as an indirect method to assess PSII efficiency (Kadir et al., 2007; Ristic et al. 2007) and chlorophyll damages due to stress. Both traits were measured at 14 and 25 days after imposition of drought stress following the methods described by Talukder et al. (2014). Chlorophyll fluorescence was measured on intact leaves of the abaxial surface (third leaf) after 30 min of dark adaptation with a pulse modular fluorometer (Model OS5- FL, Opti-Sciences, Hudson, NH, USA) in both the control and drought stressed leaves. Estimates of leaf chlorophyll content were taken using a SPAD chlorophyll meter (Model 502, Spectrum Technologies, Plainfield, IL, USA) in both the control and drought leaves. Chlorophyll fluorescence and chlorophyll content were measured at three leaflets in each plant and five plants/pot (a total of 15 readings), and averaged. The average value of 15 readings was considered as a single replication, and six replicated values/variety were used for statistical analysis and comparison of treatment means, and significant testing at $P < 0.05$ level. The two chickpea varieties demonstrated differences from each other for different morphological and phenotypic traits under drought conditions (Khan and Bano, 2017).

Leaf relative water content (RWC) was determined with fully expanded leaves after 14 and 25 days of drought stress imposition using the method described in Barrs & Weatherley (1962). Leaves were clipped and weighed (fresh weight, FW) and placed in a Ziploc bag filled with distilled water to ensure both sides of leaves fully absorb water. They were soaked in water for 24 h at room temperature and then weighed immediately after excess moisture was removed with paper towels (turgid weight, TW). The leaves were then dried in an oven at 60 ^{o}C for 72 h to determine dry weight (DW). Leaf RWC was calculated as (FW–DW)/ (TW–DW) x 100.

Measures of Shoot and Root Dry Weights

Shoots of five plants/ replication were cut at the base and dried at 60 ^{o}C for 72 hours and dry weight was taken by using an electronic scale. The roots of the same plants with soil were isolated from the pots, washed carefully to separate roots, dried at 60 ^{o}C for 72 hours, and weighed using an electronic scale. The root and shoot dry weights were measured after 25 days of drought stress imposition.

Antioxidant Enzymes Extraction

Fresh leaves (0.5 g) were ground in 5 ml of 50 mM phosphate buffer and placed in an ice bath. The supernatant was taken after centrifuging the homogenate for 20 min at 13,000 rpm at 4 ^{0}C, which was used for assays of enzyme activities as outlined by Verma and Dubey (2003).

Leaf Tissue Collection and Sample Preparation

Leaf tissues were collected from control and drought plants at mid-day after 14 and 25 days of drought stress. Leaf blades were collected from six individual pots (biological replicates) (Bais et al. 2011), frozen in liquid nitrogen immediately after collection, and stored at -80^{o}C. Tissue samples were lyophilized for 72 hours and ground using a tissuelyser. Lyophilized powder (30 mg) was used for non-targeted global ultrahigh performance liquid chromatography-high resolution mass spectrometry (UPLC–HRMS) based metabolite profiling. Freeze-dried ground leaf tissues (30 mg) were spiked with a mixture of internal standards (20 µL) into a clean Eppendorf tube. Methanol (750 µL) and ammonium acetate (10 mM, 750 µL) were added to each sample and vortexed for 1 min at room temperature. Centrifugation (at 17,000 G for 10 min) of all samples was done after ultra-sonication for 20 minutes at room temperature. Supernatant (>1 mL) was transferred to 1.5 mL tube, followed by a 50 µL transfer of supernatant to an Eppendorf tube. The supernatant was dried down and

was reconstituted with 50 µL of injection standard solution. Samples were then vortexed (30 s) and put at 4°C for 10 min, centrifuged at 20,000 rpm for 10 min, and the supernatant was transferred into an LC-vial.

Ultrahigh Performance Liquid Chromatography-High Resolution Mass Spectrometry (UPLC-HRMS) Analysis

Untargeted metabolomics profiling was performed on an ultrahigh performance liquid chromatography-high resolution mass spectrometry (Model: Thermo Ultimate 3000 UPLC and Thermo QExactive mass spectrometer). All samples were analyzed in positive and negative heated electrospray ionization with a mass resolution of 70,000 at *m/z* 200 as separate injections. Chromatographic separation was attained on an ACE Excel 2 C18-PFP100 × 2.1 mm, A 2 µm particle size column with mobile phase A as 0.1% formic acid in water, and mobile phase B as acetonitrile, at a flow rate of 350 µL/min with a run time of 16.8 min, mass resolution of 35,000 @ *m/z* 200, and mass range of 70-1000 *m/z*. Injection volume was 4 µL for negative ion mode and 2 µL for positive ion mode. The total run time per sample was 20.5 minutes. Probe (HESI probe) temperature was maintained at 350°C for both positive and negative run with spray voltage of 3500 V and capillary temperature of 320 °C.

Data Analysis

The raw files were converted to .mzXML using MSConvert (ProteoWizard 3.0). MZmine 2.1 was used to identify features, deisotope, align features, and perform gap filling to fill in any features that may have been missed in the first alignment algorithm. All adducts and complexes were identified and removed from the data set. The data was searched against an internal retention time metabolite library. Data from positive and negative ion modes were

separately subjected to statistical analyses. Data tables with metabolite peaks (*mz*/*rt*) at two time points under both drought and control conditions for tolerant and sensitive varieties were formatted as comma separated values (.csv) files and uploaded to the MetaboAnalyst 3.0 server (http://www.metaboanalyst.ca) (Xia et al. 2012). To shrink any possible variance and to improve the performance for downstream statistical analysis, metabolite data generated by UPLC-HRMS were checked for data integrity and normalized using MetaboAnalyst's normalization protocols (selecting normalization by sum, log transformation, and auto-scaling) for statistical analysis.

We applied univariate analysis (t-test and one way ANOVA) to calculate the statistical significance and fold change (FC) of the metabolites between two group means (drought over control). As the multivariate methods take all the variables into consideration, we applied multivariate methods for comprehensive data analysis, for example, supervised method-Partial Least Squares Discriminant Analysis (PLS-DA) and unsupervised method-Hierarchical clustering with heat map. The supervised method, PLS-DA, was used to maximize the difference of metabolic profiles between control and drought groups to enable the detection of metabolites existing in the biological samples. The heat map was generated based on the Pearson distance measure and the Ward clustering algorithm, showing top 19 metabolites selected by PLS-DA VIP (variable importance in projection) score using a significance level of $P \leq 0.05$, and post-hoc analysis of Fisher's LSD. The samples were arranged according to their sampling time points in both control and drought groups. The important metabolites were identified by using two different methods separately: SAM (Significant Analysis of Metabolites) and PLS-DA.

The pathway analysis was performed using MetaboAnalyst for the identified important metabolites using *Arabidopsis thaliana* pathway libraries. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (http://www.genome.ad.jp/kegg/pathway.html) was also used for the metabolites that were not found in the rice and *Arabidopsis* pathway libraries.

For phenotypic data analysis, we used SAS version. 9.1. An ANOVA was performed to determine the effect of treatments and error associated with the experiment with replications and treatments as random effects. To identify significant differences among treatments, a mean comparison was carried out by using protected LSD ($P<0.05$) test where error mean square was used to estimate the standard error of differences between means.

RESULTS

Physiological Responses

Chlorophyll Content and Photochemical Efficiency (Fv/Fm)

In general, the chlorophyll content decreased significantly ($p < 0.01$) in both the sensitive variety (G1) and tolerant variety (G2) under drought condition as compared to control (Fig 1), and decreased with the increase in the duration of drought stress. However, the % decrease in the tolerant variety (G2) was less compared to the sensitive variety (G1). Maximum decrease (64%) in chlorophyll content was observed in the sensitive variety at 25 days under drought condition as compared to control. The drought tolerant variety, on the other hand, showed 7% and 16% decrease of chlorophyll compared to control after the 14 days and 25 days of stress. The photochemical efficiency of PSII (Fv/Fm) showed variability between varieties and at two time points (14 and 25 days) of stress treatments (Table 1). The Fv/Fm ratio was decreased for both varieties at both time points under drought condition but

the decrease was more pronounced (50% and 63%) for the sensitive variety after 14 and 25 days of stress imposition.

Relative Water Content (%)

Drought stress caused significant ($p < 0.01$) changes in relative water content in both the varieties (Fig 2). The relative water content of the tolerant variety (G2) decreased 27% and 33% under drought condition compared to the control condition at 14 and 25 days, respectively, but the decrease in relative water content was higher for the sensitive variety (G1) at both time points (40% and 65%, respectively) under drought condition. This result demonstrates that the drought sensitive variety was more affected due to drought stress.

Shoot and Root Dry Weights (g)

Shoot and root dry weights of both sensitive and tolerant varieties were reduced significantly ($p < 0.01$) due to drought stress compared to control after 25 days of drought stress imposition (Fig. 3). However, the reduction was more for the sensitive variety than the tolerant variety. Sensitive and tolerant variety showed 69% and 24% reduction in shoot dry weight under drought condition compared to control, respectively (Fig. 3). Similarly, 77% and 25% reduction in root dry weights were shown by the sensitive and the tolerant variety, respectively, under drought condition as compared to control.

Antioxidant Enzymes Activity

The antioxidant enzyme activities were significantly enhanced in plants under drought stress conditon (Fig. 4). Maximum increase was noted for superoxide dismutase (SOD) activities in the leaves of stressed plants as compared to controlled plants in the senstive (45%) and tolerant variety (62%), respectively. The tolerant vareity showed higher antioxidant activies than senstive variety. The increase was significant and 25% higher in tolerant variety as compared to senstive variety under stress condition. Simillarly the tolerant variety also

showed significant increase in catalase (24%), ascorbate peroxidase (APOX) (33%) and peroxidase (POD) (27%) activities as compared to sensitive variety under stress condition.

Metabolite Profiling

The non-biased UPLC-HRMS global metabolomics approach detected a total of 691 peaks, of which 175 were identified as known metabolites (178 including duplications) and the remaining peaks were unknown metabolites. Metabolites were highly reproducible among the six analyzed biological replications at the two different time points. The identified metabolites included amino acids, organic acids, sugars, polyamines, nitrogenous compounds, and polyphenols.

The Partial Least Squares-Discriminant Analysis (PLS-DA) was performed for drought (and control) conditions on both drought tolerant and sensitive varieties together at two different time points. The first PLS-component (PC1) explained 61.8% of total variation, while the second component (PC2) explained 8.9% variation across the data set (Fig. 5). The scores plot between PC1 and PC2 revealed two distinct groups associated with the drought and the control samples at two different sampling points (Fig. 5), suggesting a clear distinction in the metabolite accumulation under two conditions. The sensitive (G1) and the tolerant variety (G2) samples were separated from each other under both drought and control conditions, with overlapping at two time points, especially under drought condition.

The effect of water stress treatment on metabolic accumulation becomes evident from hierarchical clustering with heatmap (Fig. 6). Two major clusters were identified with distinct patterns of altered metabolite abundances. Metabolites which were highly accumulated in plants grown under drought condition compared to control condition formed the first cluster including amino acids (e.g., proline, tryptophan, histidine, and isoleucine) and organic acids. On the other hand, metabolites which showed decreased level of accumulation under drought

condition included putrescine, choline, phenylalanine, GABA, and alpha ketoglutaric acid forming the second cluster. Choline, phenylalanine, and putrescine were abundantly found in the sensitive variety, whereas tyrosine, GABA, and alpha ketoglutaric acid were abundantly present in the tolerant variety under control condition. The increased level of proline and tryptophan was evident in both the sensitive and the tolerant varieties when plants were exposed to drought condition. The patterns of metabolite clustering clearly indicate the metabolic changes under different water regimes.

To identify the important metabolites associated with drought condition, two statistical models, namely SAM and PLS-DA, were carried out (Table 2). The most significantly different compounds were identified by the SAM plot with the delta value of 1.4, FDR of 0.003 and with false positive of 0.4. Similarly, the PLS-DA method identified the most important metabolites based on the VIP score using the five-component model. The two methods showed quite a similar result, identifying the same metabolites. The top most important 20 metabolites (based on fold change values) were identified by two different methods and included different amino acids, sugars, organic acids/compounds, amines, fatty acids, and other compounds (Table 2).

Among different groups of metabolites, many amino acids were significantly accumulated in the leaves of plants grown in drought condition. Proline, L-arginine, L-histidine, L-isoleucine, allantoin, tyrosine, and tryptophan showed increased levels of accumulation in the leaves of drought plants (Table. 3). However, compounds like GABA, adenosine, alanine, alpha-ketoglutaric acid, phenylalanine, choline, glucosamine, guanine, and aspartic acid were highly accumulated in the leaves of plants grown under control condition, but decreased under drought condition. Higher accumulation of L-arginine occurred in the sensitive variety when plants were exposed to drought condition; however, the level of L-arginine was decreased in the tolerant variety. On the contrary, allantoin and tyrosine were highly

accumulated in the tolerant variety compared to the sensitive variety under drought condition. Isoleucine was accumulated under drought condition in the tolerant variety at both time points (14 and 25 days) but accumulation occurred only at 14 days after stress in the sensitive variety. The levels of isocitric acid and alpha-ketoglutaric acid were reduced in the tolerant variety at 25 days under drought condition (Table. 3).

It was evident from Table 2 that tryptophan was the most contributory of metabolites in the PLS-DA model with VIP score of 1.455 and a mean decrease accuracy of 0.005654, followed by choline and BOC-L-tyrosine (1.36), whereas N-methyl-D-aspartic acid had the lowest VIP score (0.52234) with a mean decrease accuracy of 0.003807, followed by 4-aminobutanoate (0.987) with a mean decrease accuracy of 0.00018. The results revealed that tryptophan, proline, and histidine were highly accumulated in the leaves of plants under drought condition at two time points and hence can be considered as responding to drought condition. Similarly, adenosine, alanine, and choline were significantly accumulated in the leaves of plants grown under control condition. The maximum increase (4.279) in fold change (FC) was recorded in 3,5-Di-tert-butylbenzaldehyde, followed by allantoin > N-methyl-L-glutamate > L-arginine > tryptophan (3.845, 3.613, 3.5674, and 3.395; Table 3), whereas, the maximum decrease in fold change was recorded in guanine followed by riboflavin <adenosine <N-methyl-D-aspartic acid <alanine<alpha-ketoglutaric acid (Table 3 and S1).

Figure 7 presents *mz*/*rt* peak values of the most important metabolites for sensitive and tolerant varieties at 14 and 25 days after drought stress initiated. In general, the peak values of choline, guanine, GABA, adenosine, phenylalanine, aspartic acid, and guanosine decreased due to drought stress in both varieties. This indicates that the accumulation of these metabolites was reduced over time due to drought stress. Glutathione disulphide accumulation was increased after drought stress imposition, but reduced due to a longer period of drought stress. Arginine also showed a similar trend for tolerant lines; however,

sensitive lines showed continuous accumulation in long-term drought stress (25 days of drought stress). The peak values of allantoin, histidine, L-proline, tyrosine, isoleucine, and tryptophan increased in both the tolerant and sensitive varieties. However, the peak values were consistently higher in the tolerant variety which indicates the higher accumulation of these metabolites in tolerant varieties compared to sensitive varieties under drought stress condition. The peak for isocitric acid showed an increase at both time points for the sensitive variety, while only at 25 days after drought stress for the tolerant variety. The mz/rt peak values were significantly different between tolerant and sensitive varieties under drought conditions for allantoin, histidine, L-proline, tyrosine, isoleucine, tryptophan, arginine, phenylalanine, glutamine, and glucosamine.

Metabolic Pathway Analysis

The Pathway Analysis of MetaboAnalyst3 was performed on significantly different known metabolites using *Arabidopsis thaliana* as the pathway libraries. Seventeen different pathways were identified where these metabolites are involved in different steps (Table 4), of which 5 are biosynthestic pathways (Aminoacyl-tRNA biosynthesis, Indole alkaloid biosynthesis, glucosinolate biosynthesis, Phenylalanine, tyrosine, and tryptophan biosynthesis and valine, leucine, and isoleucine biosynthesis), and the remaining are involved with primary metabolism.

DISCUSSION

Drought adversely affects plant morphology and physiology, and hence results in reduced growth. Drought causes reduction in photosynthesis and photochemical efficiency, which affects photosynthetic machinery, reduces leaf expansion, enhances senescence of new leaves, and results in decreased productivity (Farooq et al. 2009). To cope with the drought stress, the plant must develop a specific tolerance mechanism. Different varieties have shown

different abilities to maintain growth and productivity by acclimating to stress conditions through specific tolerance mechanisms. Genotypic variability in different attributes related to photosynthesis, relative water content, and root and shoot dry weight are good indicators of water stress or drought monitoring. Our results demonstrated that in the sensitive variety, even short-term exposure (14 days) to drought condition resulted in the significant decrease in the chlorophyll production but the tolerant variety sustained, and a lower decrease was recorded. The photochemical efficiency (Fv/Fm) of both the varieties was significantly affected by drought stress. However, the sensitive variety showed greater Fv/Fm reduction than the tolerant variety. Some other studies also demonstrated a significant reduction of photosynthetic capacity and chlorophyll content due to stress condition (Basu et al. 2016; Cielniak et al. 2006; Li et al. 2015; Lu et al. 2016; Ullah et al. 2017; Zhou et al. 2017). Our results are aligned with other reported studies and demonstrated that the experimental condition separated the expression of these traits between the tolerant and the sensitive varieties.

Drought-induced reduction in plant dry weight had also been studied previously in different crops (Boutraa et al. 2010; Munz 2002; Salma et al. 2016). We have found a significant reduction in chickpea dry weights when grown under drought condition in both the drought tolerant and the sensitive varieties. It is worth noting that the percent decrease in dry weights was significantly higher for the sensitive variety. Earlier studies demonstrated significant reduction in shoot dry weights when grown under drought conditions in chickpea (Pang et al. 2016) and in corn (Anjum et al. 2017)**.** Silvente et al (2012) attributed the decrease in dry weight under drought in the sensitive soybean variety to the depletion of sucrose in the leaves of this variety. However, Reddy et al. (2004) reported that water stress inhibited dry matter production due to limitation of photosynthesis. Our data also demonstrated a reduction of

photosynthetic capacity (demonstrated by reducing chlorophyll content and Fv/Fm values) which might have ultimately reduced shoot dry weight in our study.

Leaf relative water content (RWC) is an indicator of water status in plants under drought condition and reflects the balance between water supply to the leaf tissue and transpiration rate. RWC has been used to identify stress tolerant variety in different crops (Rampino et al. 2006; Soltys-Kalina et al. 2016). Significant correlations between RWC and relative growth rate, photosynthesis rate, chlorophyll, and proline contents have been reported in *Aeluropus lagopoide* (Mohsenzadeh et al. 2006). Though RWC was reduced due to drought stress in our study but reduction in the tolerant variety (93127) was significantly lower than in the sensitive variety (Punjab Noor-2009). In addition, the tolerant line showed less damage to chlorophyll content and photosynthetic capacity, and also reduced loss of dry weight, and increased proline level compared to the sensitive variety which is in agreement with the findings of Mohsenzadeh et al. (2006).

The increase of antioxidant enzyme activities is known to occur under water deficit, salinity, low temperature, heavy metal exposure and UV radiations (Sharma and Dietz 2006). These enzymes form an antioxidant defence system in plants to mitigate oxidative damage. Under drought stress, cells of plants are suffered from the accretion of reactive oxygen species (ROS), which is responsible for oxidative damage and cell death. The ROS molecules are found in various subcellular compartments. The stability among the creation and reclamation of ROS is influenced by enhanced antioxidant enzymes activities (Caverzan et al. 2016). The both verities showed increased level of ROS enzymes, however, the tolerant variety demonstrated higher level than sensitive variety. The observed increase in antioxidant enzyme activities in the tolerant variety enhanced ROS detoxification mechanism. Matsumura et al. (2002) reported increase in Cat gene expression under stress that improved tolerance to stress in transgenic rice by reducing the adverse effects of hydrogen peroxide

(H_2O_2). Significant increase in the activities of SOD, POD, APOX and CAT was also reported previously in other plant species under stress environments (Mafakheri et al. 2010; Pompelli et al. 2010; Wang et al. 2009).

In order to understand changes in metabolites in chickpea under drought condition, we carried out a metabolomic profiling analysis in two different chickpea varieties (drought sensitive and tolerant) as levels of metabolite accumulation during stress at different growth stages can provide more specific and accurate indication of stress tolerance. This is the first report on chickpea to demonstrate comprehensive differences in metabolic profiling in tolerant and sensitive chickpea varieties over an extended period of drought condition. For this purpose, we used a global UPLC-HRMS analysis to classify metabolites linked with drought tolerance in the chickpea crop. The study demonstrated variations in the metabolite levels in chickpea leaves of plants grown under drought and control conditions. Imposition of drought stress had considerably increased the accumulation of amino acids including proline, tryptophan, tyrosine, histidine, and isoleucine at both two time points. The increased accumulation of amino acids under drought stress was reported previously in wheat (Browne et al. 2012; Rahman et al. 2017), in soybean (Silvente et al. 2012), in grape vine (Hochberg et al. 2013), and in *Phaseolus vulgaris* (Sassi et al. 2010). The expanded level of amino acids is considered to enhance stress resilience in plants by influencing various physiological mechanisms, for example, adjustment to osmotic changes, reactive oxygen species detoxification, and regulation of the intracellular pH level (Krasensky and Jonak 2012). Regardless of the variety (tolerant or sensitive), drought stress conditions enhanced accumulation of those metabolites but their relative intensity was higher in the tolerant variety (Fig. 7) at different time points. These results suggest the biochemical pathways involving those metabolites potentially related to drought tolerance in chickpea. Drought stress leads to the reduction in protein synthesis and an increase in hydrolysis of proteins,

endorsing surge in soluble nitrogen compounds such as free amino acids (Farooq et al. 2009; Krasensky & Jonak, 2012). Proline, highly accumulated due to water stress, not only acts as an osmolyte during drought but also acts as a signalling moleculeand provides defence against oxidative damage (Hayat et al. 2012; Gagné-Bourque & Bertrand 2016; Cheng et al. 2015). In cell cytoplasm, it not only stabilizes protein structure but also helps in maintaining cell pH and redox status under severe stress condition by reducing the amount of singlet oxygen present, which causes lipid peroxidation of thylakoid membranes (Alia et al. 1997; Szabados and Savoure, 2010; Hayat et al. 2012; Hamad & Ali 2014). Like our study, proline was also highly accumulated in soybean under drought stress condition (Das et al. 2017) which clearly shows the potential for an advanced tolerance response provided by proline during adverse environmental conditions. Under drought condition, high accumulation of proline might function as a sink for excess products which are needed for the maintenance of photosynthetic and respiratory processes (Kishore et al. 2005). Tripathi et al. (2016) also found similar results for amino acid metabolism where metabolomic alterations during drought stress were monitored in soybean. Both varieties showed damage to photosynthetic capacity in the present study, however, the damage was significantly lower in the tolerant variety (16%) as compared to sensitive variety (64%). This could potentially be contributed by the strong accumulation of proline which helped in protecting the photosystem and maintaining redox balance in photosynthetic membranes.

Increased level of aromatic amino acids, such as, L-tryptophan and tyrosine was exhibited in the current study, which was also found in wheat (Browne et al. 2012; Rahman et al. 2017) and in soybean (Das et al. 2017) under drought conditions. The highly abundant aromatic amino acids potentially acted as an alternate source of energy supply and provided stress tolerance in chickpea. Under various stress conditions, the plant metabolic response is regulated by convergent signal transduction associated with energy production. These

aromatic amino acids are the precursors of different secondary metabolites including indole acetate, lipid precursor, and lignin in the shikimate pathway, which plays a vital role in stress tolerance (Maeda and Dudareva 2012; Suguiyama et al. 2014). In addition of acting as alternate source for energy under stress conditions, L-tryptophan has been demonstrated to play roles in transportation of ions, opening of stomata, reducing reactive oxygen species (ROS) and act as an osmolyte (Rai 2002; Ford et al. 2011). Tryptophan and reactive oxygen species (ROS) are synthesized in photosynthetic organs like chloroplast, and tryptophan is the target of oxidation. As tryptophan is a target for oxidation, free proteins may provide a buffer between ROS and protein (Browne et al. 2012). Ford et al. (2011) demonstrated a correlation between increased protein levels and their involvement in ROS scavenging and oxidative stress metabolism. Photosynthetic machinery generally generates different ROS, such as singlet oxygen, superoxide, and peroxide. However, under stress conditioning the production of ROS could be increased to such a level that can inhibit the production of D1 protein which is essential for the repair of damage in photosystem II (Nishiyama et al., 2001; Allakhverdiev and Murata, 2004). In addition, ROS can oxidase different protein including D1 protein (Silva et al., 2003). Higher tryptophan accumulation in our study has contributed to ROS scavenging mechanism, which is evidenced by higher ROS scavenging enzyme activities in drought stress compare to control, thus ultimately reduced the damage to the photosystem and the reduction in growth in chickpea plants. The tryptophan histidine biosynthesis pathway is integrated with a number of other metabolic pathways. In our study, drought tolerant variety showed very strong accumulation of histidine after 25 days of drought stress (based on mz/rt peak). Zemanova et al. (2014) demonstrated that *Noccaea caerulescens* (NC) accumulated 10-fold higher histidine than *Arabidopsis halleri* (AH) under cadmium stress. Their observations indicated that histidine may be involved in Cd resistance and accumulation by reducing oxidative damage. Histidine might have played a similar role in reducing oxidative

stress in our study which helped maintaining membrane structure and water status in the plant (demonstrated by RWC).

The increased level of tyrosin under drought stress and possible role in tolerance has been studied by different authors (Browne et al. 2012; Rahman et al. 2017; yao et al. 2013). Our study highlights the increase of amino acid tyrosine (mz/rt peak) in tolerant variety under drought condition. Since the last decade, extensive research has been carried out to study the role of protein tyrosine on plant physiology, and on biotic and abiotic stress tolerance (Mata-Pérez et al. 2016), and found it to be associated with drought tolerance in plants (Hammad & Ali 2014; Shankar et al. 2015). Witt et al. (2012) reported an increased level of tryptophan and phenylalanine in corn under drought condition. However, the phenylalanine level was decreased under drought compared to the control condition in our study. An increased level of branched amino acids (BCAAs) has been reported in wheat and *Arabidopsis* under drought condition (Bowne et al. 2012; Joshi et al. 2009; Rahman et al. 2017) and also has been regulated at the transcriptional level in *Arabidopsis* (Urano et al. 2009). Our study also demonstrated increased level of isoleucine in tolerant variety under drought condition, while the level of the same metabolite was decreased in the sensitive variety. Taylor et al. (2004) reported that branched chain amino acids are a source of alternate energy when *Arabidopsis* plants were under sugar starvation, which is a common phenomenon under drought stress conditions. This supports the increased level of isoleucine in the tolerant variety over different time points in our study.

This present study has demonstrated enhanced accumulation of arginine in the sensitive variety under drought condition. Arginine is a vital amino acid in plants that acts as a precursor in the synthesis of other amino acids including some polyamines and provides a medium for the transportation of nitrogen in plants under stressful environmental conditions (Flores et al. 2008*;* Brauc et al. 2012). It has been reported earlier that the expression of genes

involved in the biosynthesis of arginine increased while those enzymes involved in the breakdown of arginine decreased during stress. Besides this, enzyme arginine decarboxylase that is involved in arginine metabolism, is known for its synergistic effect on polyamines concentration and thus enhances plant tolerance to various abiotic stresses in *Arabidopsis*, rice, tomato, and tobacco (Wang et al. 2011; Wimalasekera et al. 2011). Peremarti et al. (2009) showed that over expression of arginine decarboxylase (ADC) in transgenic rice plants lead to increased tolerance to water stress. These results show that the variations in amino acid concentrations induced by abiotic stresses may be connected with the altered expression of genes that encode for enzymes intricate with plant metabolism (Krasensky & Jonak 2012).

For plant development and metabolism, nucleotide metabolism is one of the essential components which affect many metabolic pathways. Nucleotides are an essential building block for nucleic acid (NA). For the synthesis of carbohydrate, lipids, peptides, and secondary metabolites, NA provides nucleic acids which serve as an ultimate energy source (Stasolla et al. 2003). Enhanced accumulation of purine compounds such as allantoin was also recorded for the tolerant variety, but not for the sensitive variety in our study. Allantoin, an intermediate in purine catabolism, was reported to accumulate in plants during stress (Takagi et al. 2016). Silvente et al. (2012) separated sensitive and tolerant soybean genotypes on the basis of accumulation of allantoin, pinitol, and citric acid. Allantoin plays a vital role in plant survival under stress condition and has a significant role in plant metabolism, plant signalling, bioenergetics, and heredity. Allantoin may function in stress defence by reducing reactive oxygen species (ROS) accumulation and death of plant cells (Brychkova et al. 2008). Besides its role in scavenging ROS, allantoin may also trigger stress responses by increasing levels of the stress-induced hormone abscisic acid (ABA) (Watanabe et al. 2014). ABA has essential roles in developmental and adaptive responses to numerous environmental stresses,

because of its synergistic and antagonistic interactions with other plant hormones, notably with salicylic acid (SA), jasmonic acid (JA), and ethylene (De Vleesschauwer et al. 2013; Kohli et al. 2013). Takagi et al. (2016) confirmed an increased expression of ABA-related genes and also revealed altered expression of genes involved in jasmonic acid (JA) responses in an allantoin mutant in *Arabidopsis*. Their findings suggest a possible connection of purine catabolism with stress hormone homeostasis, and signals and highlights the potential importance of allantoin in these interactions. Our metabolic profiling indicates a down regulation of purine compounds, guanine and adenosine, under drought conditions compared to control in both drought tolerant and sensitive varieties which ultimately affects overall purine biosynthesis and nucleic acid biosynthesis. The key amino acids glutamate and aspartate for *de novo* biosynthesis of purine pathway were down regulated in both sensitive and tolerant chickpea variety. These results are the opposite of findings in soybean under drought conditions where purine nucleotides and those amino acids were up regulated (Das et al. 2017). However, the same authors reported down regulating those metabolites in soybean under high temperature stress conditions (Das et al. 2017).

The results of the present study did not show any enhanced accumulation of sugar derivatives. The accumulation of soluble sugars or sugar derivatives acts as osmoprotectant under water stress conditions (Sanchez et al. 1998). Lower accumulation of glucose and sucrose is potentially due to reduced photosynthetic capacity, as demonstrated by lower chlorophyll fluorescence and SPAD chlorophyll content of the drought stressed leaves at the grain filling stage (Ahmed et al. 2015; Akhkha et al. 2013). Our results were in agreement with soybean studied under drought conditions where most of the soluble sugar and sugar derivatives were down regulated under water stress condition (Silvente et al. 2012). The lower accumulation of sugars was further accompanied by reduced levels of organic acids or compounds including isocitric acid and α-ketoglutaric acid which are involved in Krebs cycle

in leaves of stressed plants. A decreased level of organic acids or compounds was reported in wheat (Browne et al. 2012) and soybean (Silvente et al. 2012) under drought stress in tolerant genotypes and in drought sensitive bean variety (Sassi et al. 2010).

We recorded decreased levels of choline and glutathione disulphide gamma-aminobutyric acid (GABA) due to drought stress in both tolerant and sensitive lines. GABA, a vital non-protein amino acid, exhibits many physiological roles and is involved in stress tolerance during environmental stresses by maintaining cytoplasmic pH, metabolism, and osmoregulation (Barbosa et al. 2010; Li et al. 2016). Earlier studies also verified that GABA levels of plants quickly increased when exposed to different environmental stresses (Kinnersley & Turano 2000). Zhou et al. (2016) reported the synergistic effects of GABA on the regulation of metabolomics pathway under stress condition.

We used MetaboAnalyst to determine the link between the metabolites and metabolic pathways with their biosynthetic and catabolic pathways using the KEGG database and *Arabidopsis* annotation project database. Our study demonstrated that biosynthesis of some amino acids such as arginine, phenylalanine, tyrosine and tryptophan, and isoleucine were elevated under drought conditions. These amino acids are produced through the shikimate pathway. Though regulation of the pathway is still not totally understood, the involvement of three enzymes, viz. chorismate mutase (phenylalanine and tyrosine synthesis), tryptophan synthase (tryptophan biosynthesis), and arogenate dehydratase (phenylalanine biosynthesis) have so far been experimentally evident in the regulation of their synthesis (Maeda & Dudareva 2012; Tzin & Galili 2010). Phenylalanine, tryptophane, and tyrosine are considered the three central molecules in plant metabolism (Galili and Hoefgen, 2002). They are synthesized from the precursor chorismate that originates from the shikimate pathway. The shikimate pathway plays a fundamental role in plant reproduction, development, pest-defense, and environmental stresses (Maeda and Dudareva 2012). In plants, the biosynthetic

pathway of these aromatic amino acids serves as precursor for the synthesis of plant hormones, viz. auxin and salicylates and for aromatic secondary metabolites including indoleacetate, lipid precursor, and lignin which plays a critical role is stress tolerance (Hermann and Weaver 1999; Vogt, 2010; Maeda and Dudareva 2012; Suguiyama et al., 2014). Drought stress reduced the level of glycine, serine, and threonine in our experiment. This pathway is very crucial for the synthesis of four key amino acids, namely lysine, threonine, methionine, and isoleucine (Azevedo et al. 2006; Hildebrandt et al. 2015). Arginine and proline metabolism is one of the unique pathways for the biosynthesis of amino acids and the regulatory mechanisms controlling proline metabolism, and intercellular and intracellular transport and connections of proline to other metabolic pathways which are associated with *in vivo* functions of proline metabolism (Verslues & Sharma 2010). Gene expression profiling for the pathway further suggest that NAGK, NAGS, NAOD, and NAOGAcT are regulated in coordination in reply to change in arginine demand during plant growth and developmental stages (Slocum, 2005). Less and Galili (2008) reported that for several amino acids, abiotic stress changed transcription of the catabolism genes but there was very little effect on the expression of those genes encoding for biosynthetic enzymes. Notably, exemptions to this were the linked pathways of proline and arginine metabolism, where abiotic stress triggered wide transcriptional regulation of the biosynthetic enzymes. Proline metabolism between various compartments of cell is of critical importance but not fully understood. The inter- and intracellular transport of proline, differences in proline metabolism within tissues, and the linkage of proline metabolism to other metabolic pathways are all significant for both to understand proline metabolism in a basic sense and to know how it may be beneficial for plant development in an applied sense. Upon exposure to stress, the proline metabolic pathway decreases mitochondrial oxidative phosphorylation and yields of ATP for repair of stress-induced damage (Hare & Cress 1997; Hare et al. 1998). The

up- and downregulated metabolomic pathways of this study in the leaves of two different chickpea varieties combined with their related main metabolic pathways and biosynthetic processes define a wide model of water stress responses in the chickpea plant. It has been reported earlier that glucosinolate biosynthesis pathway is controlled by two transcriptional factors (R2R3-Myb) which is responsible for the synthesis of glucosinolates in *Arabidopsis*. Myb28 and Myb29 are transcriptional factor genes that are commonly involved in the regulation of this pathway (Hirai et al. 2007). Pathways controlling threonine, methionine, isoleucine, and leucine metabolism are effectively interconnected in crop plants. It has been shown that threonine deaminase and methionine gamma lyase maintain the equilibrium of isoleucine in a variety of plants under different environmental conditions (Joshi et al. 2010). Valine, leucine, and isoleucine are co-ordinately regulated because all of them share four common enzymes in their biosynthetic pathway. Isoleucine belongs to aspartate derived pathway, whereas leucine and valine belong to pyruvate pathways. These amino acids are important in symbiotic N-filiation in leguminous plants and are essential for the development of bacteroids (Prell et al. 2009).

CONCLUSIONS

We used a non-targeted UPLC–HRMS global metabolomics method and multivariate analysis for identifying metabolites from the leaf tissue of the drought stressed and well-watered chickpea plants at two different time points. We have reported increased or decreased level of different metabolites in two different varieties at different time points and their involvement in different metabolic pathways and in drought tolerance mechanisms. Metabolites proline, L-arginine, L-histidine, L-isoleucine, and tryptophan showed increased level in tolerant line after drought stress induction, which was likely the indication of acclimation in responses to the drought stress. Increased accumulation of proline and tryptophan are common in other crops and were not surprising, but increased levels of L-

arginine, L-histidine, and L-isoleucine and decreased levels of choline, phenylalanine, and alpha ketoglutaric acid in response to drought stress have not been widely reported, and may serve as metabolite biomarkers for screening for greater drought stress tolerance in chickpea to develop climate resilient chickpea varieties. The most pronounced decrease in the accumulation level was demonstrated by gamma-aminobutyric acid (GABA), adenosine, alpha-ketoglutaric acid, choline, guanine, and aspartic acid. As these metabolites levels were constantly higher or lower in both sensitive and tolerant varieties and at different time points, this suggests their role in monitoring biochemical pathways related to drought tolerance. Although some of these newly identified metabolites are promising as biomarkers for improving drought stress tolerance in chickpea, their correlation to drought tolerance in chickpea and other pulses requires further investigation. Our results demonstrated that metabolites, allantoin, proline, tryptophan, histidine, tyrosine, isoleucine, and arginine were affected differentially in the varieties when long-term drought stress was exposed. These data provide information that may, with further investigation, help to understand the biochemical pathway underlying stress tolerance in chickpea.

AUTHOR CONTRIBUTION

NK carried out green house and lab experiments; AB and MAB designed project; MAR and NK performed data analysis; MAB design experiment; NK, MAR and MAB wrote manuscript; MAB and BR edited the manuscript.

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CONFLICT OF INTEREST

The authors declares no conflict of interest.

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Table 1: Chlorophyll florescence (Fv/Fm ratio) of two chickpea varieties under control and drought condition at 14 and 25 days after stress imposition.

Chickpea variety	Days after stress imposition	Chlorophyll florescence (Fv/Fm)	
		Control (Mean±SE)	Drought stress (Mean±SE)
Punjab Noor-2009 (Sensitive variety, G1)	14 days	0.946±0.015	0.472±0.02
93127 (Tolerant variety, G2)	14 days	0.976±0.008	0.794±0.023
Punjab Noor-2009 (Sensitive variety, G1)	25 days	0.927±0.012	0.336±0.019
93127 (Tolerant variety,G2)	25 days	0.910±0.007	0.681±0.021

Table 2: Important metabolites with their compound ID (KEGG ID/PubChem CID (*) and molecular formula, identified through partial least square discrepant analysis (PLS-DA), random forest (RF) and significant analysis of metabolites (SAM). (KEGG=Kyoto Encyclopedia of Genes and Genomes).

S.No.	Important Metabolites	Compound ID	Molecular Formula	SAM (d-value)	PLS-DA VIP score (variance For component 1)
1	Alanine	C00041	$C_3H_7NO_2$	7.4698	0.66574
2	N-Methyl-D-Aspartic acid	C00123	$C_5H_9NO_4$	-	0.52234
3	Choline	C00114	$C_5H_{13}NO$	4.4954	1.3675
4	BOC-L-Tyrosine	117439*	$C1_4H_{19}NO_5$	4.4658	1.3622
5	BOC-D-Phenylalanine	637610*	$C_{14}H_{19}NO_4$	4.405	1.3512
6	Adenosine	C00212	$C_{10}H_{13}N_5O_4$	3.9386	1.2596
7	4-Aminobutanoate	C00334	$C_4H_9NO_2$	-	0.98778
8	Guanine	C00242	$C_5H_5N_5O$	3.9403	1.2599
9	Allantoin	C01551	$C_4H_6N_4O_3$	-4.0799	1.2886
10	N-Methyl-L-Glutamate	C01046	$C_6H_{11}NO_4$	-4.79	1.4179
11	L-Arginine	C00062	$C_6H_{14}N_4O_2$	-	1.0363
12	L-Histidine	C00135	$C_6H_9N_3O_2$	-5.2754	1.4913
13	L-Proline	C00148	$C_5H_9NO_2$	-4.41	1.352
14	N-Acetylputrescine	C02714	$C_6H_{14}N_2O$	3.6282	1.1916
15	Glutathione Disulfide	C00127	$C_{20}H_{32}N_6O_{12}S_2$	-	0.67311
16	Alpha-Ketoglutaric Acid	C00026	$C_5H_6O_5$	4.2727	1.3264
17	L-Isoleucine	C00407	$C_6H_{13}NO_2$	-	1.22
18	Tryptophan	C00078	$C_{11}H_{12}N_2O_2$	-5.0264	1.455
19	Isocitric Acid	C00311	$C_6H_8O_7$	4.5211	1.3721
20	Glucosamine	C00329	$C_6H_{13}NO_5$	0.00098	0.64819

Table 3: Fold Changes of important metabolites identified by PLS-DA and SAM analysis.

S/No.	Compounds	Fold change	p-value
1	Alanine	0.36492	1.52E-19
2	N-Methyl-D-Aspartic acid	0.34825	0.020197
3	Choline	0.63339	4.46E-09
4	BOC-L-Tyrosine	1.55282	5.47E-09
5	BOC-D-Phenylalanine	0.60043	8.29E-09
6	Adenosine	0.33917	3.75E-08
7	4-Aminobutanoate (GABA)	0.59135	0.000123
8	Guanine	0.13727	0.042377
9	Allantoin	3.8455	7.30E-08
10	N-Methyl-L-Glutamate	3.613	5.61E-10
11	L-Arginine	3.564	0.0178
12	L-Histidine	2.3746	1.63E-11
13	L-Proline	2.1467	8.03E-09
14	N-Acetyl putrescine	0.40561	1.54E-09
15	Di-tert-butylbenzaldehyde	4.2729	4.40E-08
16	Alpha-Ketoglutaric acid	0.38964	1.80E-09
17	L-Isoleucine	2.0514	5.84E-07
18	Tryptophan	3.395	5.34E-10
19	Isocitric acid	0.40098	3.84E-10
20	Glucosamine	0.39251	0.000283

Table 4: Pathway names, total metabolites involved in that pathways, metabolites significantly accumulated in present study (hits), and false discovery rate (FDR).

Pathway name	Total	Hits	FDR
Aminoacyl-tRNA biosynthesis	67	6	0.0073
Glycine, serine and threonine metabolism	30	2	0.0032
Arginine and proline metabolism	38	2	0.0009
Indole alkaloid biosynthesis	7	1	0.0054
Glucosinolate biosynthesis	54	2	0.0093
Purine metabolism	61	2	0.0036
Histidine metabolism	16	1	0.0028
Selenoamino acid metabolism	19	1	0.0063
Phenylalanine, tyrosine and tryptophan biosynthesis	21	1	0.0079
Carbon fixation in photosynthetic organisms	21	1	0.0022
Alanine, aspartate and glutamate metabolism	22	1	0.0047
Glycerophospholipid metabolism	25	1	0.0024
Valine, leucine and isoleucine biosynthesis	26	1	0.0019
Gluthathione metabolism	26	1	0.0013
Tryptophan metabolism	27	1	0.0081
Valine, leucine and isoleucine degradation	34	1	0.0062
Amino sugar and nucleotide sugar metabolism	41	1	0.00921

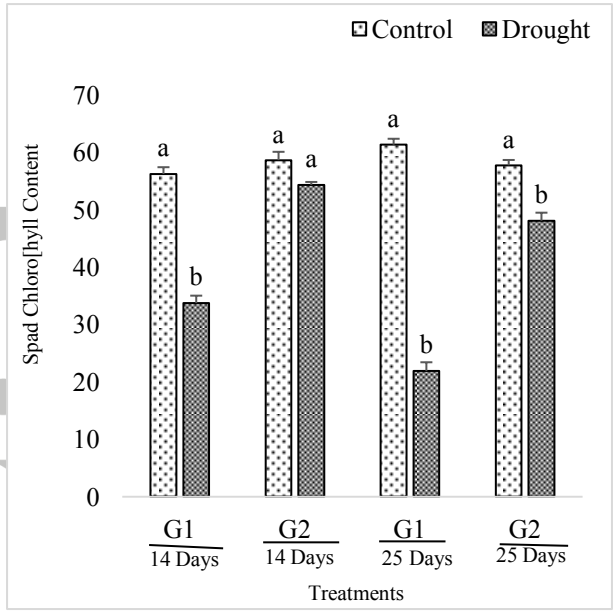


Figure 1: Chlorophyll content (±SE) in leaves of two chickpea varieties under drought and control conditions at 14 and 25 days after water treatment. G1-Drought sensitive variety (Punjab Noor-2009), G2-Drought tolerant variety (93127). Error bars represent standard errors of the mean (n= 6) at each time point Different letters are indicating significant differences ($P < 0.05$) among treatments (drought vs irrigation) for a genotype in a particular time point.

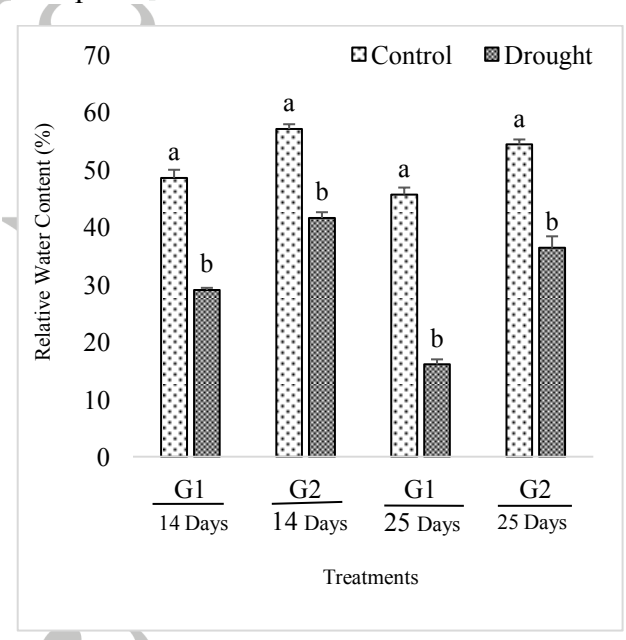


Figure 2: Relative water content (±SE) of chickpea under drought vs control condition. G1-Drought sensitive variety (Punjab Noor-2009), G2-Drought tolerant variety (93127). Error bars represent standard errors of the mean (n= 6) at each time point. Different letters are indicating significant differences ($P < 0.05$) among treatments (drought vs irrigation) for a genotype in a particular time point.

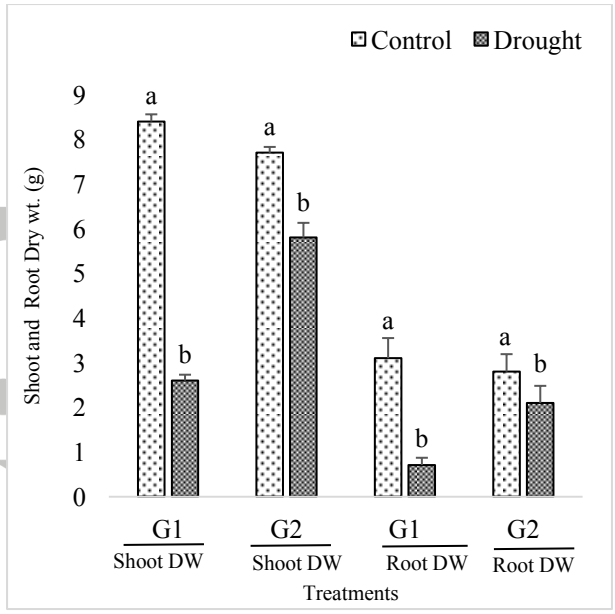


Figure 3: Shoot and Root dry weights (±SE) of chickpea under drought vs control condition after 25 days of drought stress imposition. G1-Drought sensitive variety (Punjab Noor-2009), G2-Drought tolerant variety (93127). Error bars represent standard errors of the mean (n= 6). Different letters are indicating significant differences ($P < 0.05$) among treatments (drought vs irrigation) for a genotype.

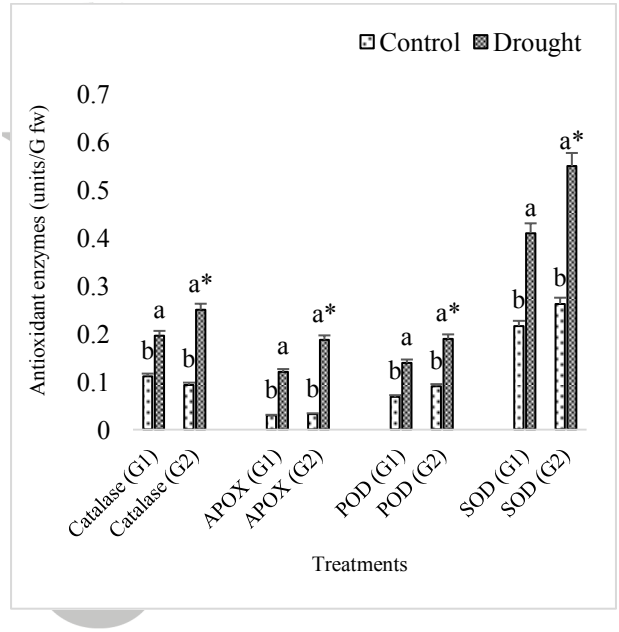


Figure 4: Antioxidant enzymes content (±SE) in the leaves of drought sensitive (G1) and tolerant variety (G2) after 25 days of drought treatment. Data are means of six replicates along with standard error bars. APOX-Ascorbate peroxidase, POD-Peroxidase, SOD-Superoxide dismutase. Different letters are indicating significant differences ($P < 0.05$) among treatments (drought vs irrigation) for a variety. *represents significant difference between sensitive (G1) and tolerant (G2) varieties under drought condition.

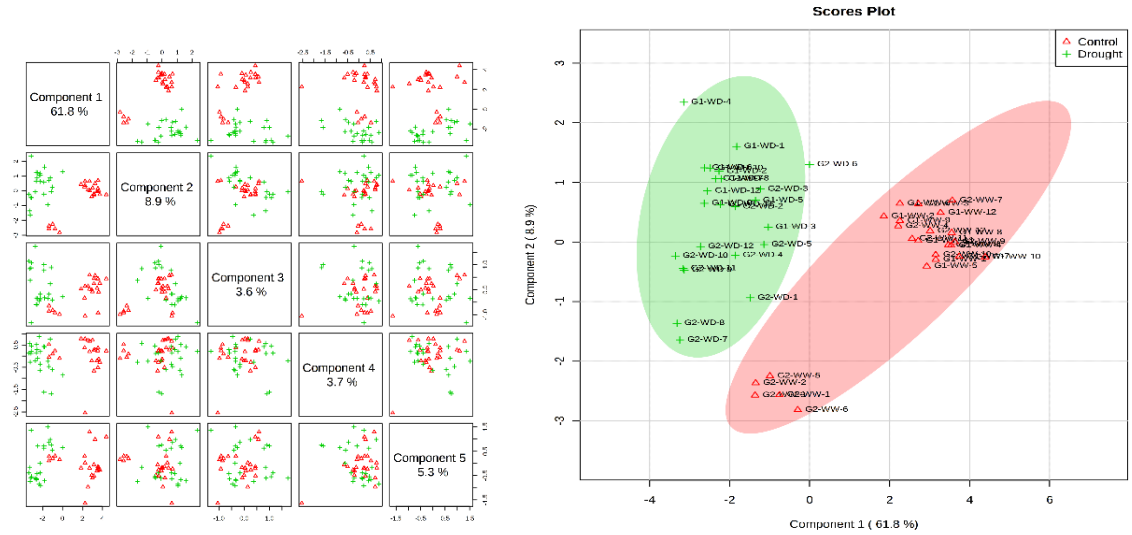


Figure 5: Partial least square discriminant analysis (PLS-DA) and 2D Scores loading plot for the Chickpea Punjab Noor-2009 (G1) and 93127 (G2) under control (well-watered) and drought treatments at 2 time points (14 and 25 days). Samples at control and drought treatments did not overlap with each other indicating an altered state of metabolite levels in the chickpea leaves. G1, sensitive variety; G2, tolerant variety.

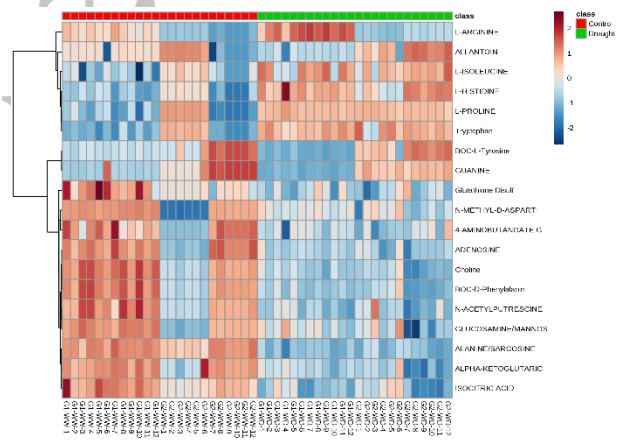
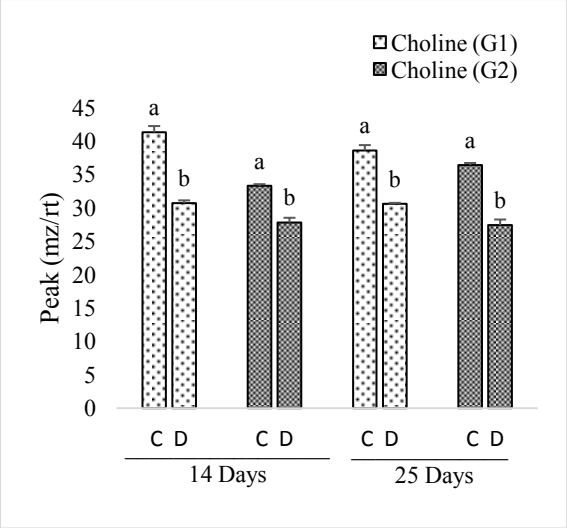
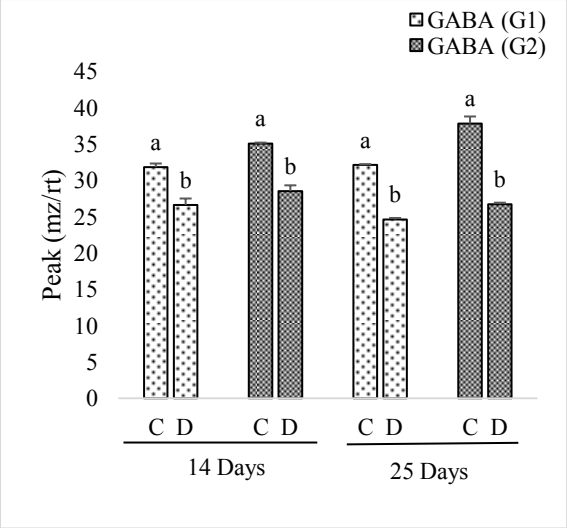
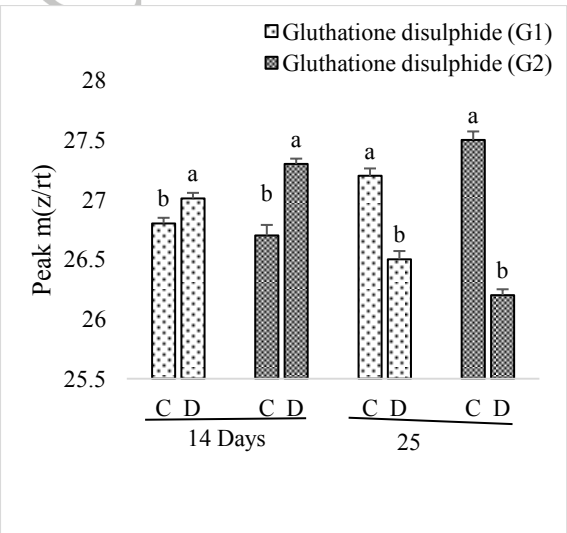
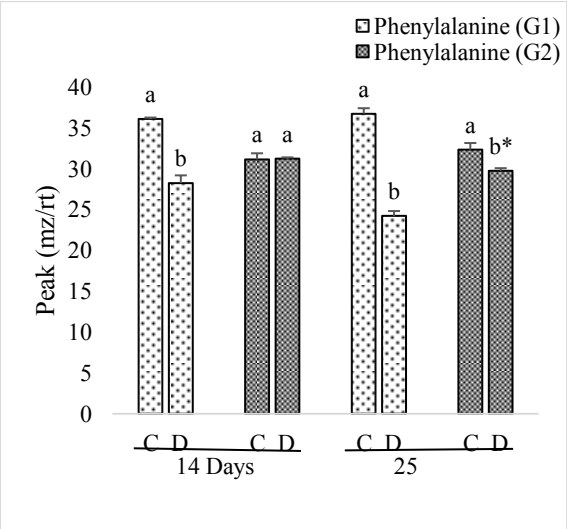


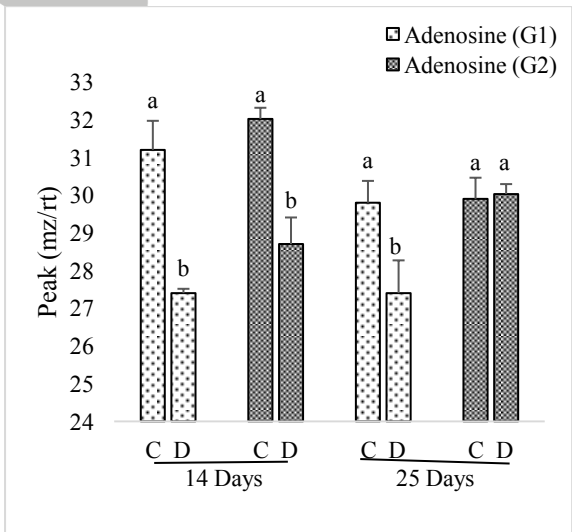
Figure 6: Heatmap illustrating levels of top metabolites in the leaves of two chickpea varieties according to the partial least square-discriminant analysis (PLS-DA) VIP scores under control (well-watered) and drought conditions at 2 time points (14 and 25 days). The heatmap was generated using 'Pearson' and 'Ward' for distance measure and clustering algorithm respectively. G1-Drought sensitive chickpea variety (Punjab Noor-2009) and G2-Drought tolerant chickpea variety (93127).

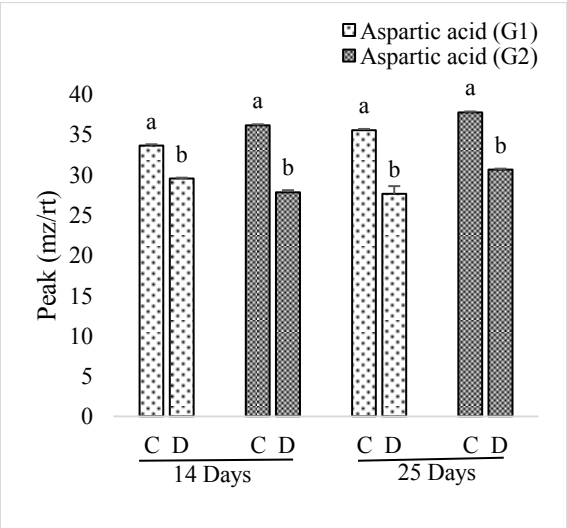


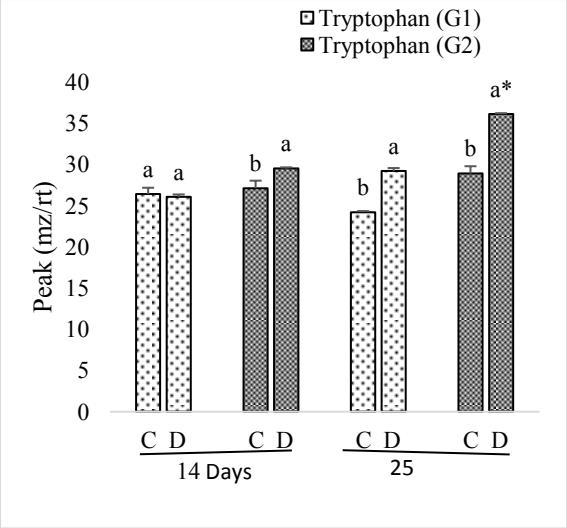


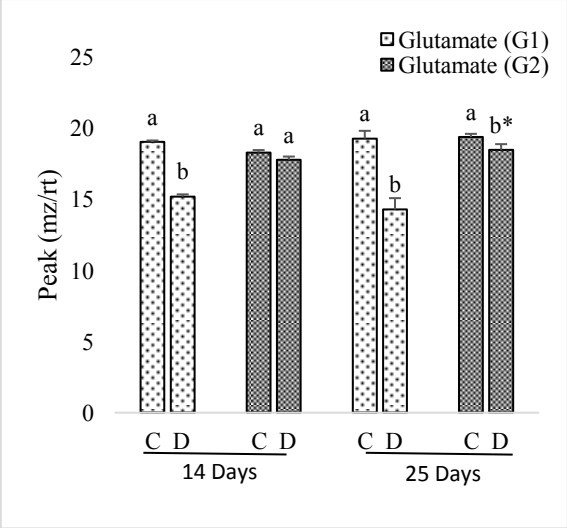


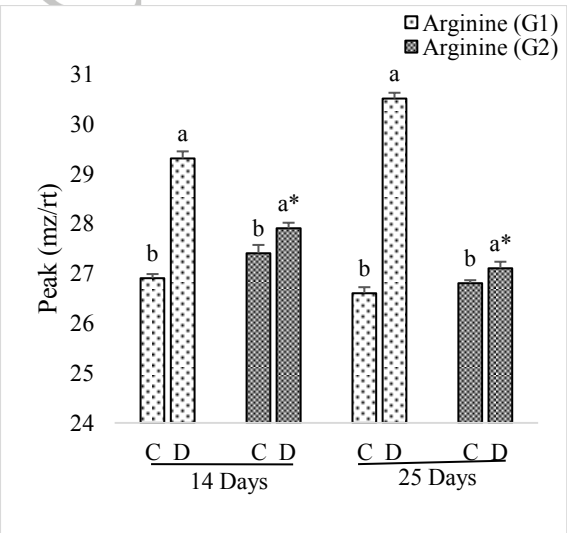


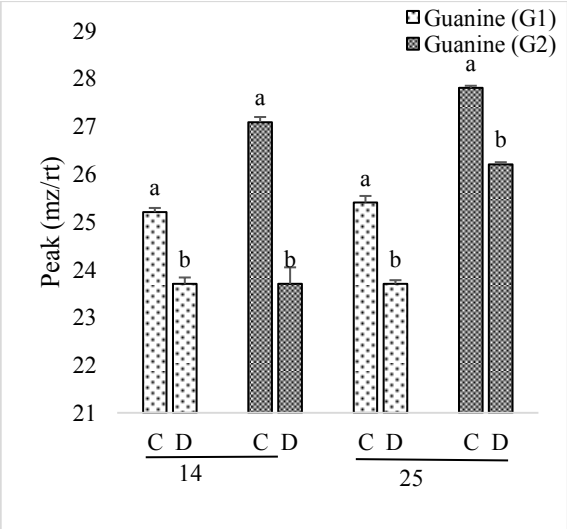


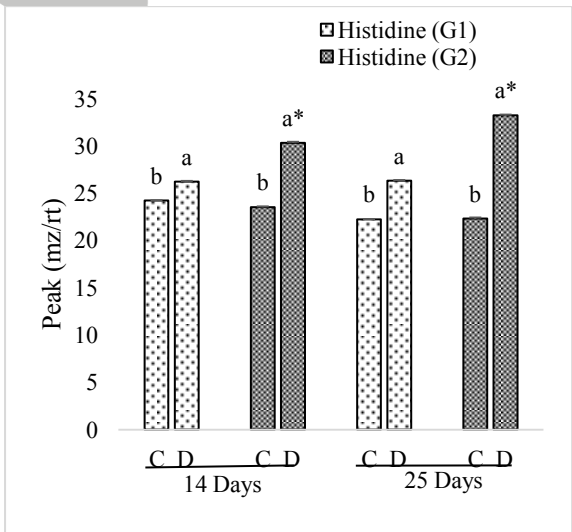


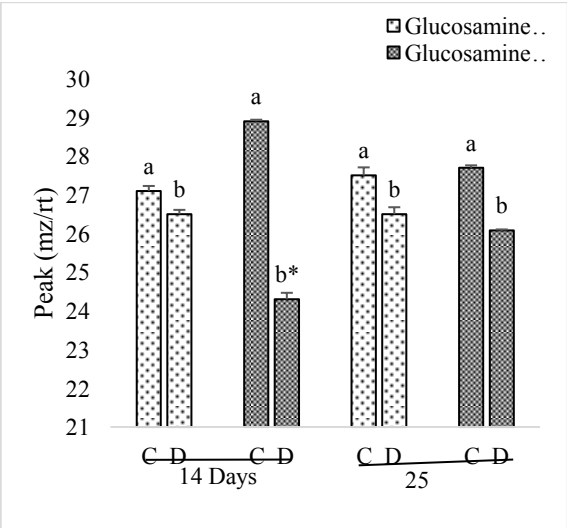












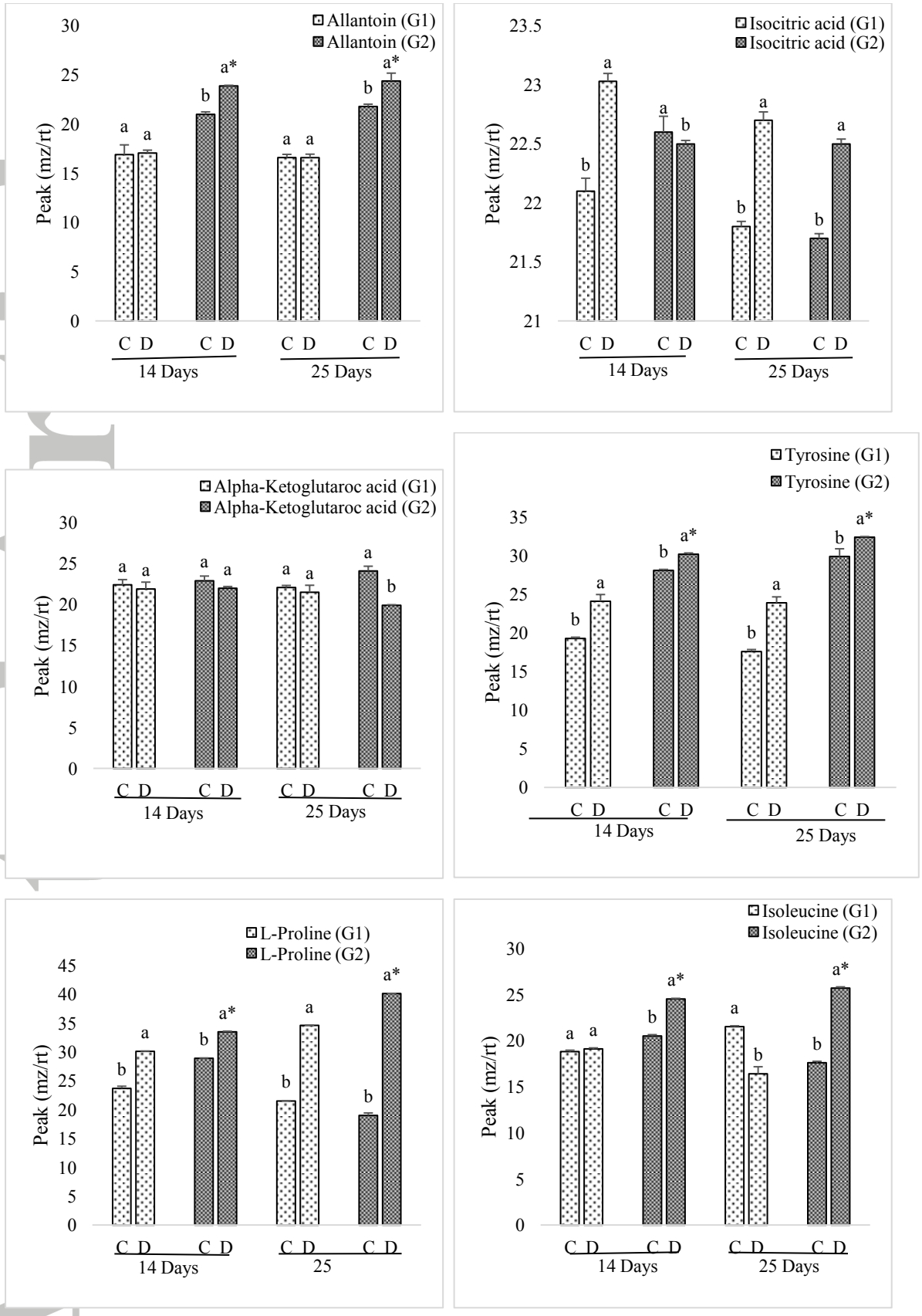


Figure 7: Significantly different levels of selected metabolites (ANOVA, $P \leq 0.05$, Tukey's HSD) in the leaves of 2 chickpea varieties under control and drought conditions at 2 time points (14 and 25 days). G1-Drought sensitive chickpea variety (Punjab Noor-2009) and G2-

Drought tolerant chickpea variety (93127). Error bars represent standard errors of the mean (n=6) at each time point. C-Control, D-Drought. Different letters are indicating significant differences ($P < 0.05$) among treatments (drought vs irrigation) for a genotype for mz/rt peak in a particular time point. *represents significant difference in mz/rt peak between sensitive (G1) and tolerant (G2) varieties under drought condition at a specific time point of sampling.