Morpho-physiological and expression analysis of drought tolerant gene in maize

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AUTHOR'S DECLARATION

I would like to declare that the data presented in this thesis **"Morpho-physiological and expression analysis of drought tolerant gene in maize"** is generated myself from original research work in under the supervision of **Dr. Shaukat Ali,** at National Institute of Genomics and Advanced Biotechnology (NIGAB), PARC Institute of Advance studies in Agriculture (PIASA), NARC, Islamabad, Pakistan. The results and material used in this thesis never presented anywhere else earlier.

_____

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Words	Abbreviations
µl	Microliter
µg	Microgram
µM	Miro molar
mol/g	Moles per gram
Mg/g	Milligram per gram
mg	Milligram
ml	Millimetre
pm	Pico mole
pH	Power of Hydrogen Ion
cm	Centimetre
rpm	Revolution per minute
m ha	Million hacters
Ct	Cycle Threshold
NARC	National Agricultural Research Center
NIGAB	National Institute of Genomics and Advanced Biotechnology
HN	Haq Nawaz
CP	CIMMYT PAK
DNA	Deoxyribonucleic Acid
dNTPs	deoxyribonucleotide triphosphate
bps	Base Pairs
cDNA	Complementary deoxyribonucleic acid

PCR	Polymerase Chain Reaction
TAE	Tris-acetate-EDTA
qRT-PCR	Quantitative reverse transcriptase PCR
RNA	Ribonucleic Acid
Gln-2	Glutamine synthetase 2
BLAST	Basic Local Alignment Search Tool
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Centre for Biotechnology Information
GSDS	Gene Structure Display Server
CDD NCBI	Conserved Domain Database NCBI
UV	Ultraviolet
MSI	Membrane stability index
N	Nitrogen
P	Phosphorus
K	Potassium
LSD	Least Significant Difference
ANOVA	Analysis of variance
GRAVY	Grand Average of Hydropathicity
aa	Amino acid
PI	Isoelectric point
MW	Molecular weight

ABSTRACT

Drought is a major limitation to maize (*Zea mays*) production in the world. The present study was designed to investigate drought effects on morpho-physiological growth, changes in the biochemical (N, P, K) levels, identification of drought responsive gene orthologs and expression level of glutamine synthetase 2 gene in two maize cultivars i.e. Haq Nawaz (drought tolerant) and CIMMYT Pak (drought sensitive) under various developmental stages. Results revealed that drought stress significantly reduced plant height, root length, leaf length, leaf width, cob-diameter, silk length, ear length, cob length, fresh and dry biomass of maize at seedling, flowering and grain filling developmental stages and drought effect were more severe in C. Pak as compared to H. Nawaz cultivar. Chlorophyll a & b levels were more affected under drought stress condition at grain filling stage and the increase was more in C. Pak than H. Nawaz in comparison to the control conditions. Physiological parameters like proline, membrane stability index and soluble sugar contents were increased in response to drought as 23.34%, 2.67% and 18.24% in H. Nawaz and 24.09%, 9.05%, and 22.97% in C. Pak, respectively. Under drought stress the decrease in nitrogen and phosphorus content was 5.88% and 6.19% in H. Nawaz and 6.29% & 19.34% in C. Pak, respectively. Under drought stress increased level of potassium content was 5.72% & 6.77% in H. Nawaz and Cimmyt Pak, respectively. Drought responsive GS2 gene (*OsGS2*; LOC_Os04g56400) of rice was aligned against eight species of Poacea family to find out their orthologs. Based on computational analysis, 25 glutamine synthetase family genes were selected and their phylogeny, gene structural analysis, conserved domain and motifs analysis demonstrated that all the glutamine synthetase genes showed a conserved pattern. Furthermore, glutamine synthetase 2 (Gln2) gene was identified in maize as a drought responsive gene. Gln2 gene expression was performed through quantitative Real-Time PCR to confirm up-regulation of Gln2 under drought stress condition. The expression analysis of drought responsive gene (Gln2) was found more in H. Nawaz than C. Pak maize cultivar under drought stress. H. Nawaz cultivar has the potential to tolerate mild to moderate drought stress. It has been concluded from our findings that the screening of drought responsive parameters and expression analysis of drought responsive gene in drought tolerant genotype could be a better source to improve tolerance in maize under drought prone regions.

Key words: Drought, *Zea mays* Glutamine Synthetase, Expression Analysis

CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1 General introduction of maize

Maize belongs to the grass family *Poaceae* and a member of genus *Zea*, (Greek word name for a food grass). There are four species of genus *Zea* and among them *Zea mays* is most economically important (Tenaillon *et al*., 2011; Murdia, *et al*., 2016). *Zea mays* L. is the third important cereal crop after wheat and rice. It is a leading cash crop occupied a significant position in all other cultivated crops in the world, able to fulfil the 50 to 60% of calories requirements (Thirunavukkarasu *et al*., 2017). It is considered as the symbolic of green revolution for their vital role in fulfillment of the world nutrients and food requirements (Muqadas *et al*., 2020). The maize genome size is ranging from 2.3 to 2.7 Gb, consisting of 10 number of chromosomes and genetically is diploid (Schnable *et al*., 2009).

1.2 Nutritional importance

Cereal crops are the sources to fulfill the food demand of increasing population. Cereal grains are consisting of carbohydrates, substantial amount of lipids, proteins, minerals, and other vitamins (Q. Ali *et al*., 2013). Maize is one of the most planted cereal crop in the world and has incredible value for forage, pharmaceuticals, food, biofuels, and for other industrial products (Shengxue Liu *et al*., 2013). It contributes up to the 19.5% of global caloric intake (Waqas *et al*., 2021) and has the highest protein content among all other food crop species and plants (Muqadas *et al*., 2020). Maize also have a significant number of tocopherols, carotenoids and oil as compared to other major nutritionally important food crops like wheat and rice. Maize is mostly cultivated for carbohydrates production but in the past few years it has gained significant importance in food industries to produce vegetable oil (Murdia, *et al*., 2016). Among other edible oils, maize oil has significant benefits as it consists of large amount of unsaturated fatty acids like linoleic and oleic acid in the range from 65% to 85% depending upon the environmental conditions and type of cultivar. There are a lot of secondary metabolic antioxidant compounds, are present in maize oil such as carotenoids, flavonoids, tocopherols, and phenolics, which play a chief role in oil oxidative stability. They have multiple beneficial effects to human health like anti-allergic, anti-inflammatory, antimicrobial, anti-atherogenic, cardioprotective and

antithrombotic (Q. Ali *et al*., 2013). It is widely used to produce animal feed, starch, syrups, cooking oil, ethanol and for many other valuable products. Nutritionally analysis of maize show that it consists of 18% protein, 24% carbohydrates and 7% fats. It is also consisting of other valuable biomolecules like Vitamin A, C, E, B1, B2, B6, manganese, magnesium, copper, zinc, iron, phosphorus, pantothenic acid, folate and niacin. It is a good model plant species because of its nutritional, agronomic, and industrial importance (Shengxue Liu *et al*., 2013; Tiwari *et al*., 2019).

It is a C4 specie, so it utilizes sunlight and moisture efficacy to produce a high yield plant (Muqadas *et al*., 2020). During the past century, it has been studied more for genetic studies as compared to other cereal crops. It is considered as a keystone specie for genomics and cytogenetic studies because of its vast range of characteristics, large heterochromatic chromosomes, an enormous collection of mutant stocks, within related species genetic co-linearity and wide-ranging nucleotide diversity. All these features made the maize a good choice to identify its potential in different climatic condition (Tiwari *et al*., 2019).

1.3 Area under cultivation and geographical distribution of maize

Globally maize is a leading cultivated crop (Muqadas, *et al*., 2020) on an area of 177 million hectares worldwide (FICCI 2014) with the production of 1067.21 million tons during 2016-17. *Zea mays* is considered as a multipurpose evolving C4 crop because of its broader adaptability to different climatic conditions across the world. Worldwide, it is also known as a queen of cereals because of its high potential of genetic yield. Globally, the highest producing region of maize is United States with yield of 377.5 million tonnes as per the 2014 FAOSTAT data. India with 42.3 million tonnes is the fourth largest maize producing country (Tiwari *et al*., 2019).

Pakistan is an agricultural country and agriculture is a second most important sector for economic of country after manufacturing and textile industries (Akhter Ali, *et al*., 2020). In Pakistan, maize is a fourth chief cereal crop after rice, wheat and cotton. In Pakistan it is sown in two seasons such as autumn and spring (Rehman *et al*., 2015; Muqadas, *et al*., 2020). In Pakistan, the maize area under cultivation was 1413 thousand hectares with increase of 2.9% over last year's 1374 thousand hectares. It contributes

0.6% to Pakistan GDP and 2.9% to agriculture value. As compared to last year, its production increase by 6.0% as from 6.826 million tonnes to 7.236 million tonnes. Maize production increased due to availability of improved varieties, increase in area and economic returns of Pakistan (Pakistan Economic Agriculture Survey 2019-20).

It can be grown in the areas with 250-300 mm range of rain fall and at sea level as below as up to 4000 m of altitude. Since, 1960 the maize grain availability is increasing from 79 g capita-1day-1 up to as higher as 185 g capita-1day-1, which is significant to compete with increasing population in the world (Muqadas *et al*., 2020). Almost 30% of the maize growing land is used for the maize hybrids production and other 70% is used to produce open pollinated varieties (OPVs). In Pakistan, maize is widely used in several sectors as in poultry feed sector 60% of maize is consumed, 25% in wet milling and remaining for nourishment of animals and human. Multi-uses of maize stimulating the farmers to invest more for maize production with high yield (Akhter Ali *et al*., 2020).

1.4 Yield loses in maize:

There are various abiotic (heat, salinity, drought, cold, etc) and biotic factors (weeds, pathogens, herbivores etc) which effecting the production of important cereal crops worldwide by limiting their growth, production, and yield (Zeng *et al*., 2019). In maize heat stress leads to 1.0-1.7% yield loss per day for every increase in temperature above 30°C. Soil acidity causes the maize yield losses up to 69% (Liliane *et al*., 2020). Different environmental stresses cause damage to the cell membranes by accumulation of reactive oxygen species (ROS) and production of toxic chemical within different maize plant tissues. Various growth regulators could be used to make the maize plant resistance to all these environmental stresses (Muqadas *et al*., 2020).

Among all these factors, drought is a sole important abiotic factor effecting the production of agricultural crops approximately with 70% of yield losses in the world (Zeng *et al*., 2019). Drought stress is a critical threat to the sustainable growth of maize crop (G. Ghahfarokhi *et al*., 2015). It effects the yield of crops by effecting the certain physiological and biochemical pathways of plants (Songtao Liu *et al*., 2019). Globally, maize suffers approximately 15 to 20% of grain yield losses due to the drought stress. Losses are increasing because of the water limitations due to the urbanization, climate changes, and industrialization (Zeng *et al*., 2019). Drought stress effect the plant cell

membranes which cause abnormality to the cell growth and different developmental stages of plant (Muqadas *et al*., 2020).

It is forecasted to increase in the intensity, duration, and occurrence of drought events because of global climate changes, most probably in the semi-arid and arid region of the world, which could lead to the drastic decrease in the maize production. So, to food security it poses a serious challenge to combat with increasing world human population which is by the year of 2050 expected to reach the 9 billion people (Songtao Liu *et al*., 2019). Global warming and erratic rain fall pattern largely affected the maize production at global scale. Certain progress has made to improve the production of maize but the problem is to overcome the maize sensitivity to drought. So, it is proposed that by improving the maize drought tolerance mechanisms rather than focusing on the primary productivity, can be helpful to attain high yielding maize varieties. Therefore, the use of genetic improvement techniques to enhance the maize drought tolerance has become a priority (Mao *et al*., 2015).

1.5 Drought stress and their effect on maize developmental stages

Water shortage is a global issue and a serious threat to the sustainable agriculture sector. C4 plants including maize require enough water to complete their life cycle. Water scarcity at developmental stages interfere with physiological processes in maize plant like photosynthesis, which leads to decline in the overall crop yield per unit area. Complete understanding of maize drought tolerant mechanisms are mandatory for further studies (G. Ghahfarokhi *et al*., 2015).

Maize growth stages are divided into seedling stage, vegetative stage, Flowering and fertilization stage and the grain filling stage followed by maturity (Farooq *et al*., 2009; Ciampitti e al., 2011). Drought stress has different effects to plant growth at different developmental stages (Liliane *et al*., 2020). Under drought stress vegetative growth period of plant prolonged, leads to change in the carbohydrate distribution in plant and cause decrease in the plant growth rate. It is reported that during plant vegetative growth phase short time water deficient conditions of maize plant leads to losses of dry weight up to 28-32% and during ear formation and tasseling phase it leads to 66-93% losses of dry weight (Cakir, 2004). Seedling stage of maize is sensitive to the drought stress as at early establishment growth phase it influences the plant

adaptation to drought stress. After planting, within 4-9 days maize seedling emerge depending on intrinsic factors like temperature, moisture, etc. Maize plants are very sensitive to drought stress at this stage, as severe conditions can lead to entire plant damage (A. Badr *et al*., 2020).

Long term drought stress at maize pre-flowering stage has showed to reduce the final size of internodes and leaves, delaying in the silk emergence and tasseling leads to the decrease in the grain yield from 15 to 25%. During vegetative stage the maize plant begin to grow rapid with increase in the dry weight and nutrients accumulation leads to the reproductive growth stage (B. Wang *et al*., 2019). Drought stress of five days at the pre-pollination and post-pollination stages also showed decrease in the kernel set mostly in the ear apical regions (Setter, *et al*., 2001). Maize ear leaf is important to the accumulation of biomass as most of the photosynthate for kernel yield is produced by the five or six leaves near to the ear (Subedi & Ma, 2005). Due to the drought stress, the photosynthetic rate is decreased resulting to decline in the sources to plant which hamper the growth and development of plant (T. Liu *et al*., 2015). Under drought stress the reduction in total biomass accumulation is 34% at grain filling stage, 37% at silking stage and 21% at maturity period (Mugo e al., 2012; Kamara *et al*., 2003). Under drought conditions maize final grain yield can decrease up to 63-85% (Liliane, *et al*., 2020).

1.6 Morphological responses of maize under drought stress

Drought stress adversely affect certain morphological traits including decrease in stem growth, leaf size, root proliferation (Farooq *et al*., 2009), number of leaves and biomass production (Ghatak, *et al*., 2017). It delayed silking, cause leaf rolling and stomatal closure (Zenda *et al*., 2019). It causes damage to the cell membrane and slow down the activity of certain enzymes, reduced the CO_2 assimilations by leaves due to stomatal closure, which leads to decrease in the photosynthetic rate (Min *et al*., 2016). Over the years, different morphological characteristics of maize like root weight, root volume, stomatal behavior, and dry matter production have been studied for their drought responsive characteristics in certain maize cultivars under limited supply of water (T. Ge *et al*., 2012). Proteomic analysis of maize roots related to the drought tolerance shown that among different maize varieties roots display different drought responsive characteristics. Therefore, such drought responsive roots characteristics

could be used as an important indicator for water stress on plant. Thus, the molecular mechanism of maize roots related to the drought response is critical to explicate (Zeng *et al*., 2019).

Different transcriptomics analysis of maize leaves, tassels, roots, and ears at developmental stages have shown that drought stress cause more changes in the roots and leaves of maize (He *et al*., 2020; Zenda *et al*., 2019). Studies have shown that root tolerance of maize plants to drought stress depend upon its ability to maintain cell wall protein composition, osmotic potential, carbohydrates metabolism and other metabolic pathways which involved in oxidative stress responses (Zeng *et al*., 2019). To deal with drought stress plants have advanced comprising morphological mechanisms (Miao *et al*., 2017) including the reduction in the leaf size to decrease the water transpirational loss. Other drought impacts on plant morphology are the reduction in the plant height, number of leaves per plants, leaf area and reduction in the maize fresh and dry biomass (Hasibuzzaman *et al*., 2021). Leaf rolling and leaf senescence also resulted from severe drought stress on maize plants (Manivannan *et al*., 2007).

1.7 Physiological responses of maize under drought stress

To deal with drought stress plants induce several physiological mechanisms in different plant organs. Different drought tolerant strategies involved shortage of life cycle and developmental plasticity, enhanced uptake of water and reduce its loss by desiccation tolerance, antioxidant capacity and osmotic adjustment (X. Wang *et al*., 2016).

Different physiochemical changes take place in the plants under drought stress. An instantaneous response involves the closure of stomatal cells to alter different metabolic pathways by reducing the CO2 and other nutrients uptake. During stress condition due to photo-oxidation and chlorophyll degradation the total chlorophyll content is decreased effecting the plant photosynthesis system (Anjum *et al*., 2011). In maize plants the chlorophyll a and b work as photoreceptors in photosynthetic system (Khaleghi *et al*., 2012). Different changes in the thylakoid membrane structure takes place which leads to the deficiency in the chlorophyll synthesis. Photosynthesis inhibition and imbalance between the capturing of light and its utilization leads to oxidative stress.

In plants proline work as osmotic adjustment during water stress conditions (G. Ghahfarokhi *et al*., 2015). Adjustment of different metabolites like buildup of osmotically active solutes such as polyamines, betaine, sugar, and accumulation of different amino acids like glycine and proline help the plant to harbor the drought stress condition and maintain the physiological activity of plant cells. Defense system by antioxidant scavenging and osmo-protection are main drought stress responsive strategies in plant cells. In response to water deficient conditions proline accumulated in large quantities, work for osmotic adjustment. It works to stabilize the biological membranes, sub-cellular organelles structures, protein content and remove the free radicals. It also works as an important osmolyte to buffer the cellular redox potential and contributes to the cytoplasmic osmotic adjustment (Q. Ali *et al*., 2013). Studies have shown that during stress condition osmolytes accumulated in plants at higher concentration to alleviating the enzymes inactivation and membrane integrity losses. Sucrose accumulation, a soluble sugar work as an osmo-protectant during drought condition by maintaining the cell turgor and membrane structure (Valentovic, *et al*. 2006).

Drought adverse effects on plant radiation use efficiency, photosynthetic potential, reproductive activities, and plants growth leads to decrease in the maize yield. So, there is a need to explore the physiochemical responses of maize cultivars to the drought stress to discriminate the drought tolerant commercialized maize cultivars, so that they can be recommend for cultivating at the drought hit areas than other drought sensitive areas to attain high grain yield (Shafiq *et al*., 2019). Maize drought tolerance based on variation in different seedling and germination traits under controlled and drought stress conditions has been used to recognize the maize varieties with drought tolerant genotypes. This can be done by identification of the genotypes having different responses to the drought stress index (SI), stress tolerance index (STI), and Drought susceptibility index (DSI) (Badr *et al*., 2020; A. Badr *et al*., 2020).

1.8 Nutritional responses of maize under drought stress

Drought stress interfere with certain biochemical pathways through the accumulation of certain antioxidant, and reactive oxygen species (ROS) (Jogaiah *et al*., 2013). The growth of the maize is highly affected by the nutrient's deficiency. In subtropical and tropical regions of the world the maize growth, development and grain

yield is highly affected by the long-term drought conditions, even could be leads towards the famine state in those maize growing areas. Maize productivity is depending upon different factors especially the mineral salt nutrition, which most importantly involves the potassium (K), phosphorus (P) and nitrogen (N) nutrients (Muqadas *et al*., 2020).

Maize requirement for essential nutrients is high, as it is a fast growing crop. Therefore, the deficiency of any nutrient significantly affect the plant growth and yield (Bender *et al*., 2013). Drought stress cause the deficient nitrogen uptake by maize plants (C. Zörb *et al*., 2014). Nitrogen play important role in metabolism of lipid peroxidation and in the antioxidant defense enzymatic process (Saneoka *et al*., 2004). Studies revealed that nitrogen application improve the drought tolerance of maize plants and enhanced the crop yield (Z. Xu *et al*., 2005). N has positive effects to drought resistance of crops as it promote the root growth, increase the soil space to absorb more nutrients and water, increase the plant transpiration efficiency and decrease the evaporation rate (Li, 2007; M. Haghjoo *et al*., 2015). Under water deficient conditions nitrogen application significantly increased the activities of antioxidant enzymes and help the plant to harbor the water deficient conditions (Zhang *et al*., 2007).

Among drought induced disruptions nutritional imbalance is a major drawback. P is a macronutrient which is responsible for energy balance in higher plants. P deficiency under drought stress decrease the maize grain yield. P deficiency not only cause the hindrance in the P uptake by plant but also affect the uptake of many other nutrients, mainly potassium and magnesium (Saleque *et al*., 2001). Under drought stress phosphorus deficiency is induced by drying of soil. Maize plants are particular prone to P scarcity, which ultimately effect the plant growth and yield parameters (Ramos *et al*., 2018). P element is involved in a number of plant key reactions like photosynthetic oxidation-reduction and energy transfer reactions. It is also a part of different important biochemical compounds including structural proteins, enzymes and nucleic acid (Pandey *et al*., 2015). As P is linked with ATP formation and energy storage mechanisms in plants, therefore under drought stress its deficiency impair the membrane transport mechanisms and affect the plant growth (Kaya *et al*., 2020).

Under P deficient conditions, plants develop certain strategies to combat it like the alteration of root structure, increased acid phosphatase activity and enhanced the organic acid efflux. All these mechanisms help the plant to increase P intake under Deficient conditions (Pandey *et al*.) Therefore to attain best yield an optimum amount of nutrients and water is essential for plants (Chotchutima *et al*., 2016).

Potassium plays an important role in plant different processes including protein synthesis, photosynthesis, stomatal regulation, ionic balance control, photosynthates translocation, enzymes activation, water use and in many others (Reddy *et al*., 2004). For plant growth and development potassium is an essential macronutrient, also work as a primary osmoticum to maintain low level of water potential in plant tissues. Therefore, during drought condition in maize plant accumulation of potassium ions may be helpful for water uptake. K+ accumulation in maize plant is mostly occur in response to the soil water deficient condition. Stomatal guard cells control the release and accumulation of K+ to change the stomatal turgor which leads to control the closing and opening of stomata. During drought conditions the increased level of abscisic acid (ABA) stimulate the K+ release from stomatal guard cells and giving rise to the closure of stomatal cells to limit the water transpiration activity. Many studies have shown that potassium fertilizers could be used to lower the adverse effects of drought stress on maize plant. High level of potassium leads to increase plant drought resistance capacity by osmoregulation, charge balance, stomatal regulation, homeostatic, and protein synthesis. To cope with drought, stress the K+ accumulation is more beneficial than any other organic solute production because osmotic adjustment through K+ uptake is more energy efficient. Fusheing (2006) has revealed that K+ accumulation leads to lower the water losses by stomatal regulation and mesophyll cells osmotic potential (K. Zare *et al*., 2014).

To get a better under understanding of K and P distribution in maize plant cells throughout the plant development stages, it is essential to conduct the field-based experiments to collect the data related to the K and P uptake dynamics to predict the changes occur during the plant different growth and developmental stages in response to the drought stress depending upon its duration and intensity (Ge *et al*., 2012).

1.9 Role of glutamine synthetase in maize

At molecular level, different drought responsive signaling pathways, transcription factor, drought responsive proteins and many other strategies are involved to cope with drought stress condition in maize plant. Different molecular responses, such as biosynthesis and accumulation of drought defensive proteins, chaperons, antioxidant defensive enzymes, aquaporins, and late embryogenesis abundant (LEA) work under plant drought protective mechanisms. These molecular mechanisms help the maize plant to withstand with drought condition via regulation of different drought responsive genes (Songtao Liu *et al*., 2019).

Glutamine synthetase is an imperative enzyme in plants that catalyze the incorporation of ammonium ions into glutamine to form glutamate in an ATP dependent manner (Bernard & Habash, 2009). GS involved in the assimilation of ammonia, a reactive and cytotoxic metabolite which produced from nitrate or direct uptake of ammonia from soil and from the atmospheric N fixation (Hirel & Lea, 2001). Glutamine synthetase is also responsible for re-assimilation of ammonia, produced during different cellular metabolic processes including, protein degradation and photorespiration during stress conditions (Bernard & Habash, 2009). Plant glutamine synthetase enzyme is octamer with subunits of approximately 40 KD. Most of the higher plants have one or more cytosolic (GS1) and one chloroplast (GS2) isoforms (James *et al*., 2018a). In maize all these isoforms are encoded by a total of six nuclear genes, five for the GS1 cytosolic isoforms, named as GS1-1 to the GS1-5 and one for the GS2 isoform. Glutamine synthetase is a primarily important enzyme expressed in green leaves of maize plant, where it involves in the reassimilation of the photorespiratory ammonia and assimilation of nitrate in leaves and roots. GS1-3 and GS1-4 are the main cytosolic isoforms which expressed constitutively throughout the plant and others GS1-1 and GS1-5 are maize root isoforms. The *GLN2* is an important gene encoding glutamine synthetase enzyme, which during grain developmental filling stage of maize involved in the nitrogen remobilization. GSII gene type is a most studied gene type among all glutamine synthetase genes (Swarbreck *et al*., 2011). GS2 isoform is encoded by a single chloroplast located active gene, however some level of its activity also shown in the mitochondria (Taira *et al*., 2004). Under water deficient conditions the expression of *GLN2* gene improve the grain yield by post-anthesis N uptake and remobilization of whole plant (Gallais *et al*., 2004). GS also takes part in the GS/GOCAT cycle pathway, which is a focal point for N metabolism in higher plants. Glutamate and glutamine amino acids produced during this pathway are used to synthetize different organic

nitrogen compounds including chlorophyll, nucleotides, proline and other amino acids. Buildup of high concentration of ammonia can cause severe damage to plant tissues and eventually death, so proper functioning of GS is crucial to plants functioning under drought stress (Bernard & Habash, 2009; Brian & Lea, 2007).

1.10 Expression analysis under drought stress condition

Over the years, there are different molecular biological techniques have been developed to study plants responses to abiotic stresses (Songtao Liu *et al*., 2019). Different studies have been done to explain the drought responsive mechanism of maize crop at genomics and molecular levels, and at transcriptional level many drought responsive genes have been identified (Thirunavukkarasu *et al*., 2017). RNA sequencing and microarray hybridization-based experiments have been used to monitor the global gene expression profiles of maize tissues in response to drought condition. Maize drought stress responses are tissue specific and depend upon the duration and level of stress (B. Wang *et al*., 2019).

Different studies have postulated the role of GS enzymes to abiotic factors. In rice a comparative study on the expression and activity of different GS isoform under water deficient conditions inferred that maintained OsGS2 *(GLN2)* activity and its overexpression enhanced the plant tolerance to drought condition (James *et al*., 2018b). Yousfi ., 2015 showed that genetic expression of durum wheat genotypes under drought condition revealed the high expression level of GS1 and GS2 isoforms as compared to controlled conditions (Yousfi *et al*., 2016).

The current study focusses on the morphological, physiological, elemental, and expression analysis of maize in response to the drought stress at different developmental stages. This study focuses on the identification of drought responsive gene and its expression in both roots and leaves of a maize inbred line. Maize cultivars used in this research were Haq. Nawaz and CIMMYT PAK. This work lays the foundations for the evolutionary relationship and functionally analysis of GS genes in addition to explore biological and molecular mechanisms to understand maize biology under drought stress.

The main objectives of the study were;

1. Evaluation of morphological and physiological data in response to drought stress condition
2. Evaluation of nutritional changes in maize cultivars in response to drought condition
3. Identification of a drought responsive gene in maize by using bioinformatics tools.
4. Expression analysis of drought tolerant gene in maize.

CHAPTER 2

MATERIAL AND METHOD

MATERIAL AND METHOD

2.1. Experimental site and design

This study was conducted at National Institute for Genomic and Advanced Biotechnology (NIGAB), National Agriculture Research Centre (NARC), Islamabad, Pakistan. To determine the effect of drought stress on two maize cultivars i.e. drought tolerant Haq Nawaz and drought sensitive CIMMYT PAK with respect to morphological, physiological, elemental and gene expression, experiments were carried out under glass house condition of National Institute for Genomics and Advanced Biotechnology (NIGAB), NARC during 2020-21. The seeds of two maize cultivars of contrasting responses to drought tolerance were collected from maize program of Crop Sciences Institute (CSI), NARC. Their seeds were sown in pots under glasshouse conditions of NIGAB in triplicates by applying CRD design. Germinated plantlets in pots were watered as per daily requirements for some days. After 15 days, the germinated plantlets in pots were divided into two sets. One set was watered daily (control) while on other set water was stopped for 10±3 days to achieve drought stress.

2.2. Morphological characterization under drought stress treatments

In this study, three drought stress treatments were applied at three different developmental stages of maize cultivars. The first drought stress was applied at seedling stage and various parameters were taken under stress as compared to control. Second drought stress treatment was applied at flowering stage while third stress was given to both cultivars at grain filling stage. Under drought stress at each developmental stages, morphological data was recorded. Within different time intervals morphological data of two contrasting varieties under drought and control conditions were taken. The studied morphological characters include plant height, root length, leaf length, leaf width, plant fresh biomass, plant dry biomass, silk length, ear length, cob length and cob diameter.

After 10±3 days of drought stress at seedling stage then these plants were irrigated. After plants recovery period, drought stress was applied to plants for the next 10±3 days at flowering stage and the same morphological data was recorded as compared to control. After the recovery of drought stressed plants, again stress was applied at grain filling stage wherein morphological data for two cultivars were

recorded. Silk length, Ear Length, Cob diameter, and Cob length of both cultivars was measured at Kernel Blister stage (R2) and Kernel Milk stage (R3) under drought and control conditions.

2.3. Application of drought stress at grain filling developmental stage

For further evaluation of maize cultivars, drought stress was applied at grain filling stage and various parameters like physiological, elemental and gene expression were studied only at this stage. The upper suspended second leaf of two maize cultivars i.e. Haq Nawaz and CIMMYT PAK was taken from control and drought stress plants at grain filling developmental stage for physiological, elemental and expression analysis. Furthermore, for gene expression analysis besides leaf, roots were also taken from stress and control plants for comparison. Well-watered plants samples were taken as control in this experiment.

2.4. Physiological responses of maize cultivars under drought stress

Various physiological parameters of maize were studied at grain filling stage under drought stress condition as compared to control. The studied physiological parameters include in this study were chlorophyll a & b content of leaves, total soluble sugar contents, membrane stability index (MSI) of leaves and proline contents respectively.

2.4.1. Chlorophyll contents

Chlorophyll a and chl. b contents of H. Nawaz and C. PAK were measured by using (Arnon, 1949) method. For this purpose, 2 grams of each sample was taken into 10 ml of 80% ethanol in test tube. Capped the tube and for extraction purpose put all tubes in water bath at 80°C for 10 minutes. Optical density was measured at 645 nm and 663 nm by using a UV spectrophotometer. Then, chlorophyll a and b contents were measured by using the following formula.

$$\text{Chl a} = [(12.7 \times 663) - (2.69 \times 645)]\ V/W/1000$$

$$\text{Chl b} = [(22.91 \times 645) - (4.68 \times 663)]\ V/W/1000$$

2.4.2. Total soluble sugar contents

Total soluble sugar content of both maize varieties were measured by using colorimetric method of (Johnson *et al*., 1966), which is a modified form of (Dubois *et al*., 1956)**.** Leaf sample (0.2 g) was taken in 2 ml of distilled water. Homogenized the samples in the solution by using clean pestle and mortar. Centrifuged the contents of all test tubes at 3000 rpm for 15 minutes. Then, 0.1 ml of suspension was taken out into 1 ml of 80% phenol. Incubated all samples for 1 hour at room temperature and then, 5 ml of concentrated sulfuric acid was added. Optical density of each sample was measured at 420 nm by using UV IMPLEN Nanophotometer.

2.4.3. Membrane stability index

Membrane stability index (MSI) of Haq Nawaz and C. PAK leaves were measured by using (Sairam, 1994) protocol. Each leaf sample was cut down into uniform size discs. Then, 0.1 g of each sample were taken into 10 ml of doubled distilled water in test tube. Capped the tubes and put in water bath at 40°C for 30 minutes. Conductivity was recorded by using the Conductivity Meter to record C1. Then, all tubes were put in water bath at 100°C for 15 minutes. Conductivity was again recorded of each sample by using Conductivity meter to record C2. Values obtained for C1 and C2 were used to calculate the membrane stability index (MSI) by using the following formula:

$$MSI = (1\text{-}C1 / C2) \times 100$$

2.4.4. Proline contents

Proline content from maize leaves was measured by using (Bates *et al*., 1973). Leaf sample 0.2 g was blended in 3% aqueous sulphosalicylic acid. Homogenate of each sample was filtered by using the Whatman#2 filter paper. Then 2 ml of acid ninhydrin reagent for each 12 samples was prepared by using 0.75 g of ninhydrin crystals, 12 ml of glacial acetic acid, 4.2 ml of phosphoric acid and 7.8 ml of distilled water in a flask to make total 24 ml acid ninhydrin solution. Then 2 ml of filtrate of each sample was taken in test tubes to which 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid was already added. All test tubes were put in water bath at 100°C

for 1 hour. Then, 4ml of toluene was mixed to each sample to terminate the reaction. Vortex the all samples for 15-20s to separate the pink color organic phase. The organic phase (chromophore region) was collected, and optical density of each sample was measured at 520 nm by using UV spectrophotometer to calculate the proline content. The standard curve was prepared by weighing known concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 mg following above protocol.

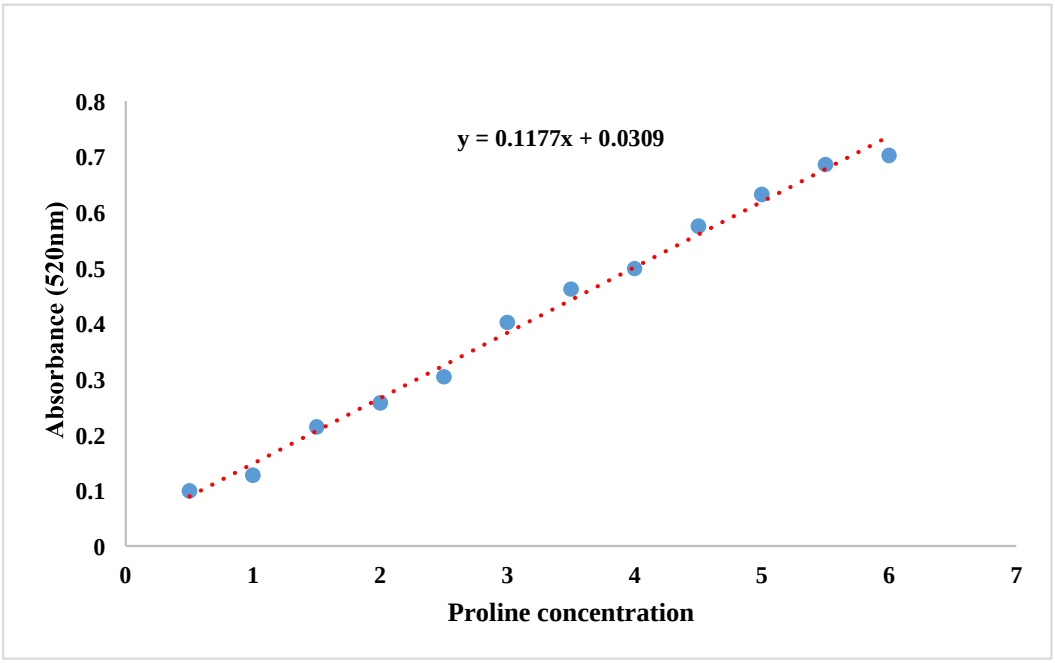

Figure: 2.1 Standard curve for proline concentration

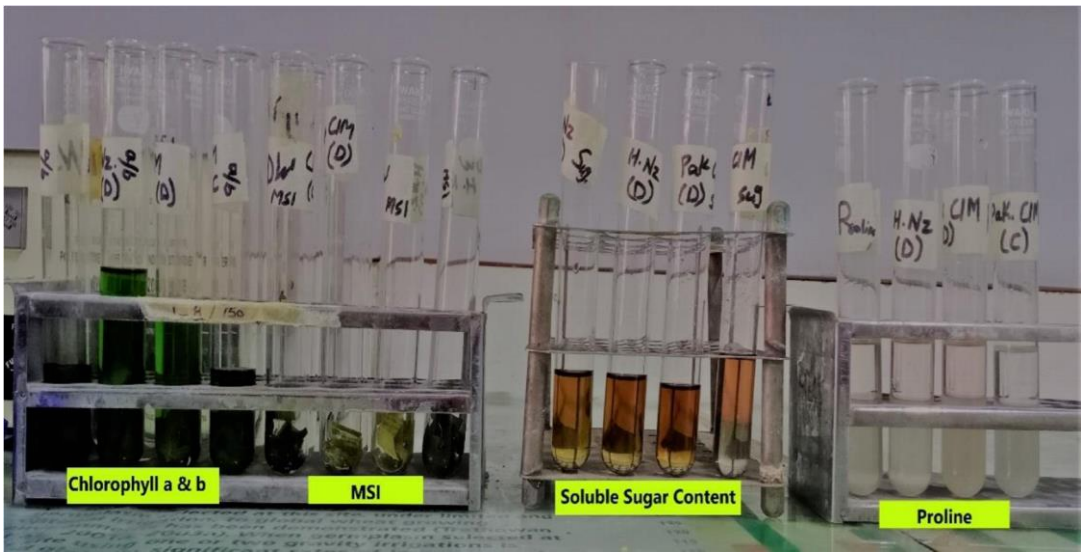

Figure: 2.2 Physiological analysis of Haq Nawaz and C. PAK maize cultivars under drought stress in comparison to the control conditions

2.5. Effect of drought stress on nutritional elements

2.5.1. Nitrogen determination

Plant sample was taken and grounded. 1g of finally grounded sample was taken into a plastic vial and dry it for overnight in an oven at 60°C. Then, cool it in a desiccator. 0.25 g of dried plant material was taken in 100 ml digestion tube and add few pumice boiling granules. Then, add 3 g of catalyst mixture of K_2SO_4-Se and 10 mL of concentrated H_2SO_4 by using a dispenser. Stir the material by using vortex to get a homogenized mixture. Then, placed all the tubes in block-digester (VELP SCIENTIFICA DK-20 heating digester) for 20 minutes at 100°C. Agitate the tubes content and place them back into the block digester for 2 hours at 380°C. After digestion, tubes were removed from digester and let them to cool down. Then, the material was diluted with distilled water in 100 ml flask. Along the treated sample, 1 blank sample with no plant sample, and one standard sample with 0.1 g of EDTA and as internal reference one standard plant sample was also run. First distillation unit (VELP SCIENTIFICA UDK 159 Automatic Distillation and Titration system) is steamed out for at least 10 minutes at the rate of 7-8 ml distillate per minute. In a Pyrex evaporating dish dispense 1 mL of saturated H_3BO_3 solution and 1ml of distilled water and place it into the distillation unit under the condenser tip, as condenser touching the surface of solution. 10 ml of aliquot is pipetted out into the 100 ml of distillation flask. Then, add 10 ml of N NaOH solution into the distillation flask. Flasks were connected to the distillation unit and distillation begins. After completion of distillation 35 ml of distillate was taken out in a collecting dish. By using standardized 0.01 N H_2SO_4 titrate the distillate to PH 5 by using an auto-titrator and resulted in Nitrogen value (Wolf *et al*., 1991).

2.5.2 Procedure of wet digestion for phosphorus and potassium analysis

Plant sample was taken and finally grounded it until it looks uniforms. 1g of finally grounded sample was taken into a plastic vial and dry it for overnight in an oven at 60°C. 0.25 g of dry grounded maize plant sample was taken and transferred into 100 mL conical flask quantitatively. Then, 10 ml of nitric acid-perchloric acid mixture with 2:1 ratio was added and let it to stand for overnight until a vigorous reaction phase is over. To reflux the acid small and short-stemmed funnels were placed into the tubes.
When the preliminary digestion was done, all the tubes were placed into a cold blockdigester and raised the temperature to 150°C for 1 hour. To exit volatile vapors

from funnel U-shaped glass rods were placed under each funnel. Then, raised the temperature slowly for disappearance of all traces of HNO_3. Then, all U-shaped glass rods were removed, and temperature was raised to 235°C until in tubes the dense white fumes of $HClO_4$ were appeared. For 30 minutes more continued the digestion and then, tubes rack was removed from the block digester. Tubes were cool down for few minutes and few drops of distilled water was added through the funnel carefully. After condensation of vapors, few drops of distilled water were added and solution in the tubes was mixed. Then, the mixture was leaved for few hours undisturbed. One blank reagent with no plant sample was also run as reference.

2.5.2.1 Phosphorus

Phosphorus contents of H. Nawaz and C. PAK were measured by using (Olsen *et al*., 1982) method. 5 ml of clear filter was taken from wet digestion into test tube and 5ml of ammonium-vanadomolybdate reagent was added. Then, a standard curve is prepared by pipetting out 1, 2, 3, 4, and 5ml of standard stock solution and same as for the sample. A blank solution was prepared with 5 mL ammonium-vanadomolybdate reagent and proceed as for the samples. After 30 minutes read the absorbance of standard, blank and samples on the spectrophotometer at the wavelength of 410nm. A calibration curve was prepared for standards and absorbance was plotted against the respective P concentrations. Then, from calibration curve the P concentration of samples was measured by using this formula:

P (%) = ppm P (from Calibration Curve) × V1/Wt × 100/V2 × 1/10000

Where:

V1 = Total volume of the plant digest (mL)
V2 = Volume of plant digest used for measurement (mL) Wt=
Weight of dry plant (g)

2.5.2.2 Potassium

Potassium contents of H. Nawaz and C. PAK were measured by using (chapman *et al*., 1961). 1 ml of clear filter was taken from wet digestion out into test tubes and 5 ml lithium chloride solution and 4 ml De-ionized water was added. A blank solution

was prepared and proceed as for the samples. Read the absorbance of standard, blank and samples on the Flame Photometer (Sherwood Model 420 Flame Photometer) and K concentration of samples was measured by using formula:

K = ppm K (from calibration curve) × V / Wt Where:

V = Total volume of the plant digest (mL) Wt
= Weight of dry plant (g)

2.6 Genome wide analysis of glutamine synthetase genes

2.6.1 Identification of drought responsive gene in maize

A drought responsive gene *glutamine synthetase 2* was identified in Oryza sativa according to (James *et al*., 2018). The amino acid sequences of gene rice chloroplastic GS2 (*OsGS2*; LOC_Os04g56400) were downloaded from (https:// plants. ensembl. org/Oryza_sativa/Info/Index) and (http://www.ncbi.nlm.nih.gov/) and used to blast against the *Zea mays, Triticum aestivum, Sorghum bicolor, Hordeum volgari, Saccharum spontaneum, Oryza sativa, Arabidopsis thaliana,* and *Setaria italica* genome database by using BLASTP program (http://plants.ensembl.org/index.htm). Genes selection parameters threshold were set as following E-value < e-10, and 75% of percent identity. Genomics, Coding, and protein sequences of glutamine synthetase family genes of 8 Poaceae family species following the set threshold were downloaded. Redundant sequences were removed, and SMART web server were used to examine the conserved Gln-synt_C domain.

2.6.2 Phylogenetic analysis of glutamine synthetase family genes in maize

Multiple sequence alignment of protein sequences of Glutamine synthetase genes of *Zea mays, Triticum aestivum, Sorghum bicolor, Hordeum volgari, Saccharum spontaneum, Oryza sativa, Arabidopsis thaliana,* and *Setaria italica* were conducted by Clustal X software with defaulted parameters. Phylogenetic analysis was performed with Neighbor-Joining method using Clustal X tool in conjunction with MEGA X software (Kumar *et al*., 2018). For reliability of clades bootstrap with 1000 replicates was used.

2.6.3 Gene structure analysis of Glutamine synthetase family genes

The information of Glutamine synthetase genes, including accession number, chromosomal location, CDS, genomic sequences retrieved from plant ensemble database. Full length cDNA, protein, and genomics sequences of Glutamine synthetase genes were obtained from the plant ensemble database. Structures of Glutamine synthetase genes were showed by Gene Structure Display Server (GSDS) tool (http://gsds.cbi.pku.edu.cn/) showing the number of exons, introns, upstream and downstream regions (Hu *et al*., 2015).

2.6.4 Motifs display of Glutamine Synthetase family proteins

MEME software was used to display motifs of glutamine synthetase proteins from maize, rice, wheat, Barley, sugarcane, millet, and barley (http://meme.nbcr.net/ meme4_1/cgi-bin/meme.cgi). Parameters were set as followings: the occurrences of a single motif—zero or one per sequence, maximum number of motifs to find—10, other parameters were defaulted (Bailey *et al*., 2009).

2.6.5 Domain Display of Glutamine synthetase family genes

Domain architecture analysis of identified GS genes was performed by using full length protein sequences to CDD NCBI software. (https:// www. ncbi. nlm. nih. gov/ Structure/ cdd/wrpsb.cgi), and downloaded hit data subjected to TB Tool for domains visualization (Lu *et al*., 2020) (Marchler-Bauer *et al*., 2015).

2.6.6 Physicochemical properties

Physiochemical properties of all 25 glutamine synthetase sequences were identifies using ExpasyProtparam tool (https://web.expasy.org/protparam/) and Subcellular localization were identified using WoLF PSORT Prediction tool (https://www.genscript.com/wolf-psort.html). Chromosomal location were retrieved from ensemble plant (https://plants.ensembl.org/index.html).

2.7 Expression analysis of *glutamine synthetase 2* (*GLN-2*) gene in maize under drought stress

At grain filling stage, drought stress was applied to maize plants and from stressed plants leaves and roots were taken for expression analysis of GLN-2 gene. Expression of drought responsive gene was also checked in control plants of maize cultivars. For this purpose, total RNA was extracted and then c-DNA and RT-PCR was performed. Their complete details are given as follow.

2.7.1 RNA Extraction

Total RNA of leaf and root samples was extracted from two cultivars of maize by using kit Invitrogen by Thermo Fisher Scientific. Harvested 1 g of frozen leaf and root tissue samples of maize were ground to fine powder by using a mortar and pestle in liquid nitrogen to prevent ribonuclease activity. Then, almost 100 mg of powder were transferred to the 1.5 ml eppendorf tubes and 1ml of lysis buffer was added. Vortex the samples to get a homogenized mixture and centrifuge at 14000 rpm for 2 minutes. 70% ethanol was added to each tube and the ratio of ethanol: g tissue was 1:1. Then, vortex the homogenate and take out 700 µL of sample to the spin cartridge with collection tube. Centrifuge the homogenate at 12000 rpm for 30 s. Discarded the flow through and 700 µL of wash buffer 1. Centrifuged at 12000 rpm for 30 s. Discarded the flow through including collection tube and put the spin cartridge to a new collection tube. 500µL of wash buffer ll was added with ethanol. Centrifuged at 12000 rpm for 30 s and discarded the flow through. Again, centrifuge the spin cartridges at 12000 rpm for 2 minutes and discard the flow through along with collection tube. Insert the spin cartridge to a recovery tube and add 50 µL of RNase free water. Incubate at room temperature for 1 minute and centrifuge at 12000 rpm for 2 minutes. RNA eluted from the membrane into the recovery tube. RNA was extracted by using a protocol given with kit Invitrogen by Thermo Fisher Scientific.

2.7.2 RNA integrity and quantity

RNA quantification was done by using Nanodrop1000 (Thermo Scientific product, USA). RNA integrity and quantity were measured using agarose gel, as 5 µL

of total RNA from each sample a long with 1µL loading dye was run on a 1% agarose gel. 2 µL of 1kb and 100 bp Ladder was used as standard.

2.7.3 DNase treatment

For the removal of genomic DNA contamination from total RNA preparations, DNase treatment was done. 1 µg of RNA was taken into RNase free tube. 10X reaction buffer was added a long with 1 µL of $MgCl_2$. 1 µl of DNase 1 was added. Then, 1µL of nuclease free water was added incubate at 37ºC for 30 minutes. 1 µL of 50 mM EDTA was added to avoid RNA hydrolysis with divalent cations. Then, samples were incubated at 65ºC for 10 minutes. RNA free of genomic DNA contamination was obtained. DNase treatment was performed according to the protocol given by Thermo Scientific along with RevertAid first strand cDNA synthesis kit.

2.7.4 cDNA synthesis:

cDNA preparation Thermo Scientific RevertAid first strand cDNA synthesis kit is used to synthesize the cDNAs from extracted RNA of all maize samples. 5µg of template RNA was taken into tubes into which 1 µL of random hexamer primer was added and then, nuclease free water was added to make the solution final volume up to 12 µL. Incubated at 65ºC for 5 minutes. After incubation period chill the samples on ice, spin for short time and placed them back to ice. 4 µL of 5X reaction buffer, 1µL of ribolock RNase inhibitor, 2 µL of 10 mM dNTPs mixture and 1 µL of Revert Aid MulVRT was added, respectively. All samples were mixed gently and centrifuge them for a short period. Then, incubated at 25ºC for 5 minutes followed by incubation at 42ºC for 60 minutes. Then, reaction was terminated by heating at 70ºC for 5 minutes. Prepared cDNA was stored at -20ºC for further conventional PCR and qPCR experiments. cDNA was prepared according to the protocol given with Thermo Scientific RevertAid first strand cDNA synthesis kit.

2.7.5 Primer designing:

Primers for both *GLN2* and housekeeping gene (tubulin) were designed through primer 3 software (Rozen et al., 2000). The primers for drought responsive *GLN2* gene and for internal control are given in (Table# 1)

Table 1: List of rt-PCR primers

Sr. #	Primer	Sequence
1	**Gln-2-Forward**	ATCAGCTGACGGAATGATCC
	Gln-2-Reverse	TTGATGCCACTGATGTCGAT
2	**β-TUB _Forward**	CTACCTCACGGCATCTGCTATGT
	β-TUB _Reverse	GTCACACACACTCGACTTCACG

2.7.6 Quantitative Real-Time PCR:

Quantification of two maize cultivars under drought and control conditions was measured by using BioSpec-nano Spectrophotometer of Life Science and template dilution was done for 50ng/ml. qPCR was carried out on the AB Applied Biosystems by using thermo-scientific Maxima SYBR Green/ROX qPCR Master Mix (2X). The qPCR was carried out by using 10 µL reaction volume with 1µL of diluted cDNA sample, 0.1 µL of forward and reverse primers (100µM), 5 µL of thermo-scientific Maxima SYBR Green/ROX qPCR Master Mix (2X) and 3.8 µL of double distill water. Reaction without cDNA template, only water was used as an internal control. The qPCR conditions maintained were as follows: holding stage at 95 °C for 10 minutes, cycling stage step 1 maintained at 95 °C for 30s, step 2 at 52 °C for 30s, step 3 and 4 maintained at 72 °C for 30s, then melt curve stage step 1 is maintained at 95 °C for 15 s, step 2 at 60 °C for 1 minute and step 3 at 95 °C for 15s. 35 number of cycles were set and three biological repeats for each sample. Tubulin gene was used as internal control. In the excel software the calculation of relative gene expression of genes was done by using $2^{-\Delta\Delta Ct}$ method (Livak *et al*., 2001).

2.8 Statistical Analysis

Analysis of variance (ANOVA) was used for testing of means regarding impact of drought on Haq Nawaz and CIMMYT PAK morphological, physiological, elemental and expression analysis. With 5% probability level, the obtained data of three replicates were further subjected to least significant difference (LSD) test.

CHAPTER 3

RESULTS

RESULTS

3.1 Morphological characterization of maize under drought stress

Based on overall results of morphological data, Haq Nawaz is comparatively more drought tolerant than C. PAK. The details confirmation of results, the data of morphological traits were recorded under drought stress and control conditions for comparison at three different maize developmental stages such as seedling stage, flowering stage and grain filling stage respectively. Comprehensive trait wise morphological data of maize cultivars at three different developmental stages under drought stress is given as follow.

3.1.1 Effect of drought stress on maize plant height (cm) at different growth stages

Under fully drought stress condition, plant height was measured from both maize cultivars (Haq. Nawaz and C. PAK) at seedling, flowering, and grain filling developmental stages in comparison to the control conditions. Based on plant height results at three distinct developmental stages, the highest plant height was reported in control plants of H. Nawaz at grain filling stage followed by flowering and seedling stages. Decrease in plant height at seedling, flowering and grain filling developmental stages in H. Nawaz was reported 26.09%, 8.46% and 31.86% while in C. PAK 34.22%, 10.21% and 42.70%, respectively. Analysis of variance (ANOVA) was used to demonstrate the variation in the plant height of both cultivars such as H. Nawaz and C. PAK under drought and control conditions at seedling, flowering, and grain filling stages. ANOVA results for plant height illustrated the significant decrease in length under drought stress as compared to the control conditions at all developmental stages of maize. Variety*variety interaction showed significant decrease in the plant height of C. PAK as compared to H. Nawaz with $P<0.05$. The Variety*Treatment interactions demonstrated that significant ($P<0.05$) differences in the mean plant height at seedling developmental stage was observed whereas, Variety*Treatment showed non-significant results at flowering and grain filling developmental stages as well as shown in figure 3.1.

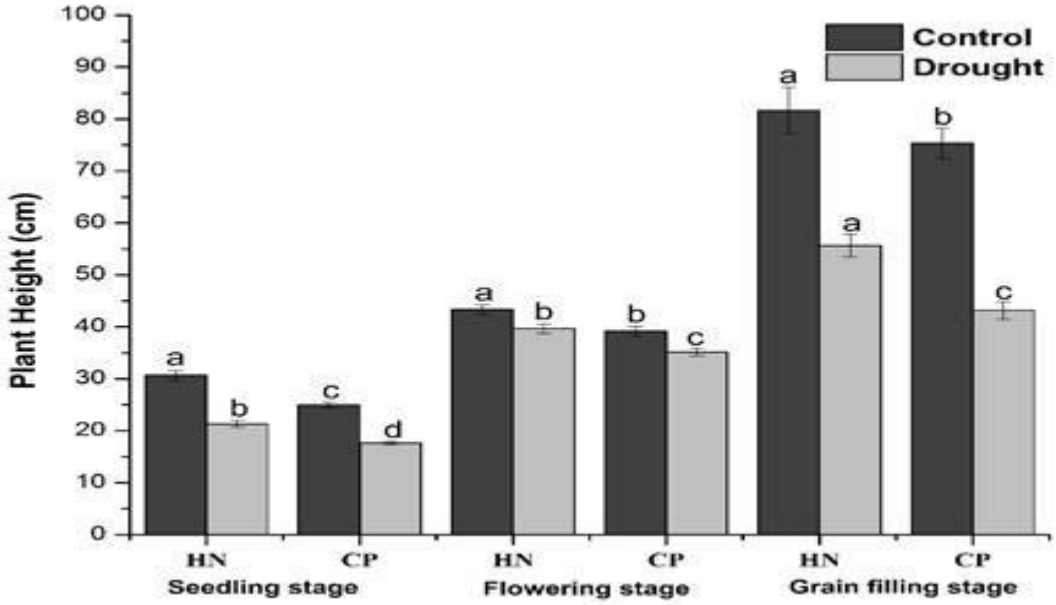

Figure: 3.1 Comparison of plant height of H. Nawaz and C. PAK at seedling, flowering, and grain filling developmental stages. Mean values having standard error of mean (SEM) bar followed by different letters demonstrate the significant difference of varieties from each other at (P<0.05%). HN and CP indicated the Haq Nawaz and CIMMYT PAK maize cultivars

3.1.2 Effect of drought stress on root length (cm)

Root length of H. Nawaz and C. PAK cultivars was measured at seedling, flowering and grain filling developmental stages. Results illustrated the decrease in root length at seedling, flowering, and grain filling developmental stages in H. Nawaz was reported 10%, 20.7% and 7.47% while in C. PAK 23.81%, 34.2%, and 21.9%, respectively. Analysis of variance (ANOVA) resulted the significant decrease in the root length of both maize cultivars under drought stress in comparison to the control conditions at all developmental stages. Variety*variety interaction showed significant decrease in the root length of C. PAK as compared to H. Nawaz with $P<0.05$ and thus, H. Nawaz showed improved response to drought stress in terms of root length as compared to C. PAK. The interaction of Variety*Treatment showed non-significant results for ANOVA at maize seedling and grain filling stage, whereas showed significant results at maize flowering developmental stage.

Results revealed that Haq Nawaz had greater growth performance at seedling, flowering and grain filling stages than at CIMMYT PAK under drought-stress conditions as shown in figure 3.2.

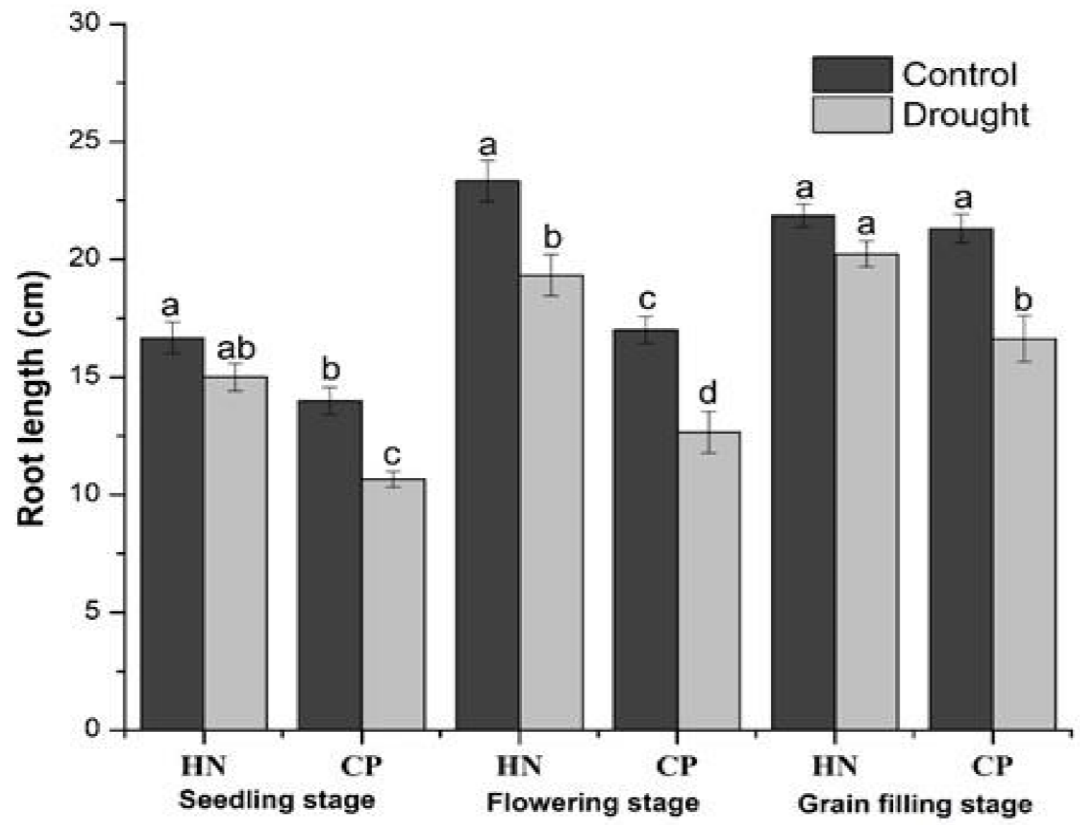

Figure: 3.2 Effect of drought stress on root length of H. Nawaz and C. PAK at different developmental stages. Mean values having standard error of mean (SEM) bar followed by different letters demonstrate the significance of ANOVA results. HN and CP indicated the Haq Nawaz and CIMMYT PAK maize cultivars, respectively.

3.1.3 Drought effect on leaf length (cm)

Leaf length of H. Nawaz and C. PAK was measured at seedling, flowering and grain filling developmental stages to determine drought effects on both cultivars in comparisons to the control conditions. Results illustrated that leaf length of both maize varieties were affected under drought stress in comparison to the control conditions (Fig. 3.3). Under drought stress, reduction in plants leaf length at seedling, flowering, and grain filling developmental stages was reported 18.18%, 11.16% and 18.34% in H. Nawaz, while 25.6%, 12.3%, and 19.09%, in C. PAK, respectively. Based on leaf length results at three distinct developmental stages, the highest leaf length was reported in control plants of H. Nawaz at grain filling stage followed by flowering and seedling stages.

ANOVA results for leaf length illustrated the significant decrease in length under drought stress as compared to the control conditions at seedling, flowering, and grain filling developmental stages of maize. Variety*variety interaction showed significant ($P<0.05$) decrease in the leaf length of C. PAK as compare to the H. Nawaz at all developmental stages. All-Pairwise Comparisons Test of Plant for Treatment*Variety resulted non-significant results at seedling and flowering development stage, whereas showed significant results for maize grain filling developmental stage.

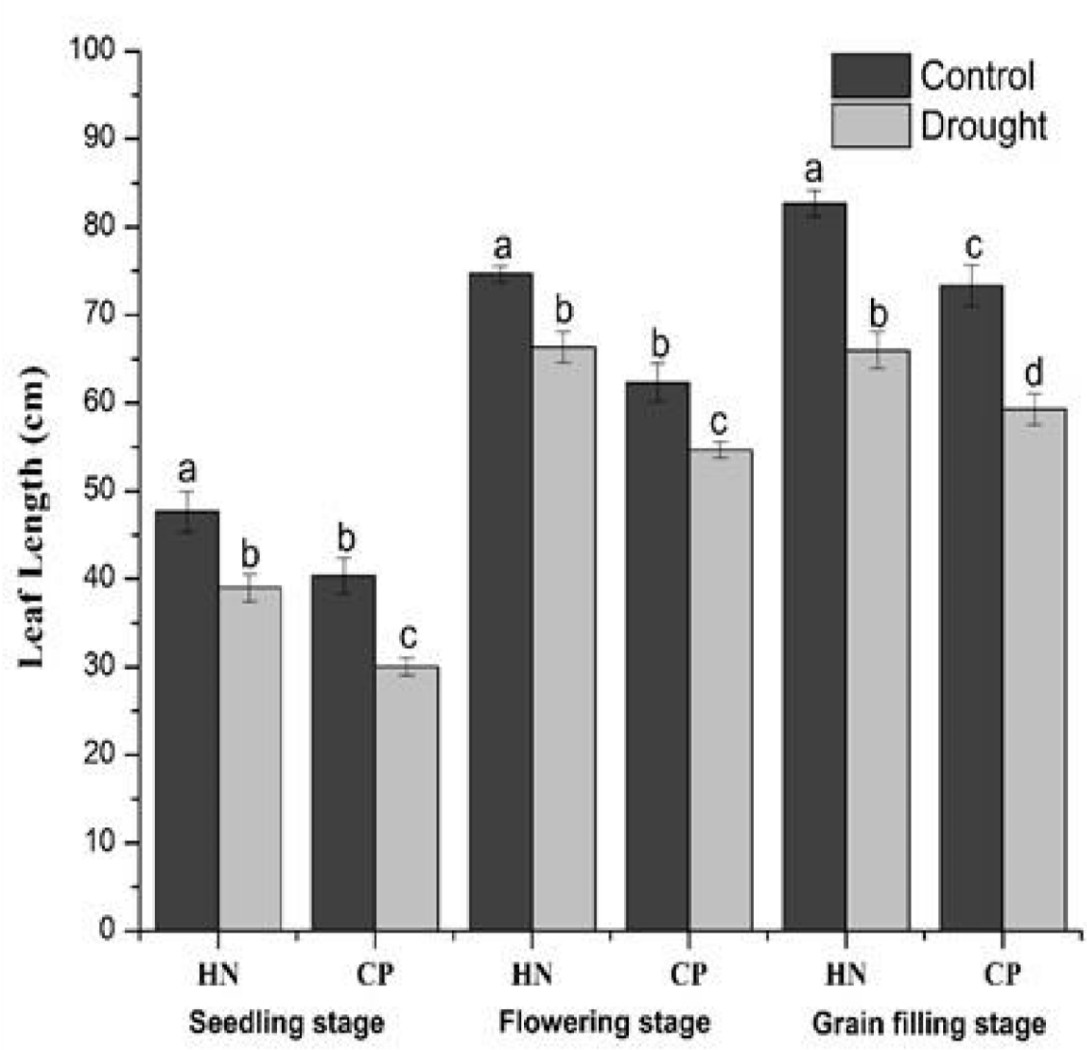

Figure: 3.3 Drought effect on leaf length of H. Nawaz and C. PAK in comparison to the control conditions. Mean values having standard error of mean (SEM) bar followed by different letters are significantly different from each other at (P<0.05%). HN and CP indicated the Haq Nawaz and C. PAK maize cultivars, respectively

3.1.4 Drought effect on leaf width (cm)

Leaf width data collected at different maize developmental stages illustrated that under drought stress the reduction of leaf width was 14.28%, 5.82% and 37.5% in H. Nawaz, while 17.27%, 8.03%, and 40.22% in CIMMYT PAK at seedling, flowering and grain filling developmental stages, respectively. ANOVA treatments used for leaf width at seedling, flowering, and grain filling developmental stage demonstrate the significant ($p<0.05$) difference in the means of leaf width of H. Nawaz and C. PAK for seedling and grain filling stages, whereas non-significant at flowering developmental stage. Results illustrated that under drought stress the means of leaf width of H. Nawaz and C. PAK was more effected at seedling and grain filling stages than flowering developmental stage. The treatment*variety interaction for leaf width resulted in non-significant results at all developmental stages of maize under study.

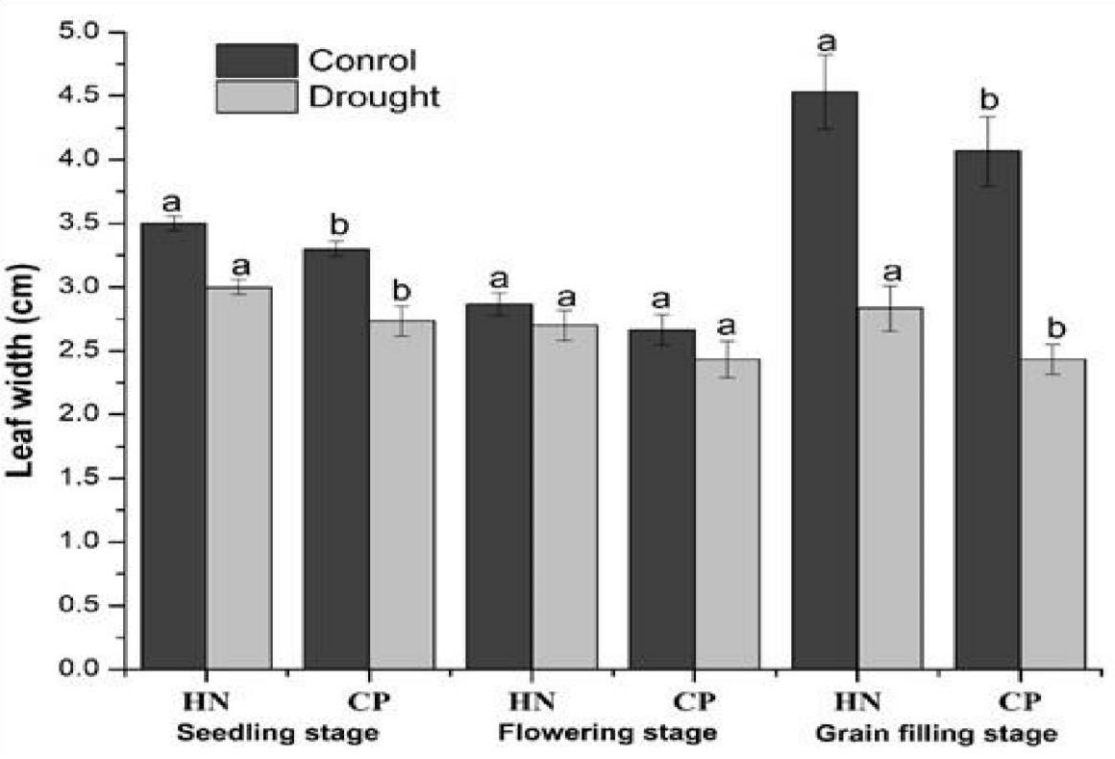

Figure: 3.4 Effect of water stress on maize leaf width during maize different development stages. Bars represent standard errors. Treatments with different letters represent the significance of results at the 0.05 level of probability. HN and CP indicated the Haq Nawaz and CIMMYT PAK maize cultivars, respectively.

3.1.5 Drought effect on plant fresh biomass (g)

Fresh weight of H. Nawaz and C. PAK cultivars was determined at maize seedling, flowering, and grain filling developmental stages. Under drought stress, reduction in plants fresh biomass at seedling, flowering, and grain filling developmental stages was reported 28.79%, 13.47% and 30.86% in H. Nawaz, while 38.9%, 27.28%, and 34.5%, in C. PAK, respectively. Plant fresh weights of H. Nawaz and C. PAK cultivars decreased under drought stress (Fig 3.5). Analysis of variance (ANOVA) results illustrated that water stress induced a significant decrease ($P<0.05$) in fresh weight of H. Nawaz and C. PAK cultivars in comparison to the control conditions at seedling, flowering, and grain filling developmental stages. Variety*variety interaction showed significant ($P<0.05\%$) decrease in the fresh weight of C. PAK as compare to the H. Nawaz at all developmental stages of maize. All-Pairwise Comparisons test of both cultivars for Treatment*Variety resulted non-significant results for seedling and flowering stage, whereas significant results for grain filling developmental stage.

H. Nawaz showed improved response in terms of plant fresh biomass as compared to C. PAK. Up to some extent H. Nawaz has the potential to tolerate mild to moderate drought stress at different developmental stages.

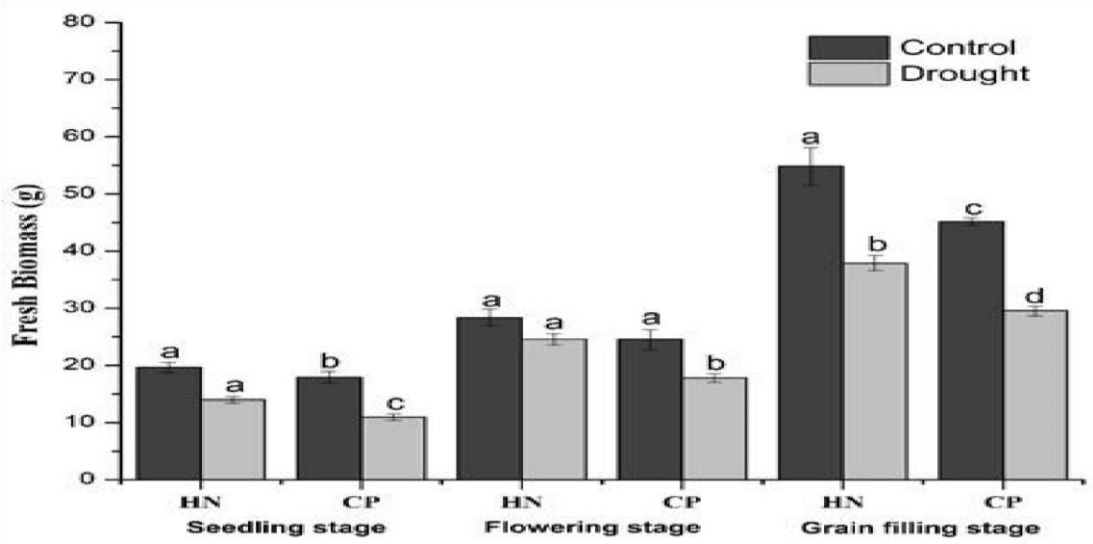

Figure: 3.5 Effects of water stress on plants fresh biomass of Haq Nawaz (HN) and CIMMYT PAK (CP) at seedling, flowering and grain filling developmental stages. Each value represents mean ± SEM. Bars represent the standard errors. HN and CP indicated the Haq Nawaz and CIMMYT PAK maize cultivars, respectively.

3.1.6 Drought effect on plant dry biomass (cm)

After oven drying dry weight of H. Nawaz and C. PAK cultivars was determined at maize seedling, flowering, and grain filling developmental stages. Under drought stress, reduction in plant dry biomass at seedling, flowering, and grain filling developmental stages was 30.96%, 26.53% and 30.24% in H. Nawaz, while 32.63%, 33.93%, and 30.77%, in C. PAK, respectively.

ANOVA treatments used for dry weight demonstrated the significant (P<0.05%) difference in the means of dry weight of H. Nawaz and C. PAK under water stress in comparisons to the control conditions. Variety*variety interaction implies that there is a significant difference among the H. Nawaz and C. PAK drought response at seedling, flowering and grain filling developmental stages. H. Nawaz appeared tolerant in facing drought stress conditions as compared to C. PAK with respect to maize plants dry weights at all developmental stage. ANOVA illustrated the significant results for Variety*Treatment interaction at seedling developmental stage, whereas give non-significant results at maize flowering, and grain filling developmental stages.

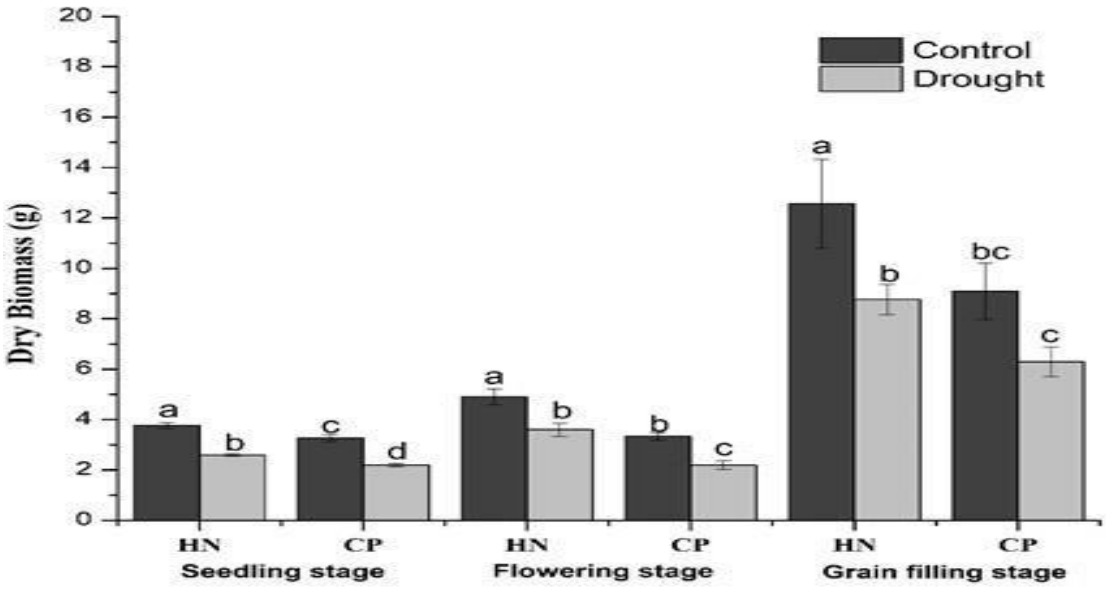

Figure: 3.6 Effect of prolonged drought stress on dry biomass of maize plants at different developmental stages. Each value represents the mean of three different experiments. Bars represent the standard error. Bars followed by different letters representing the significant difference at (P<0.05%). HN and CP indicated the Haq Nawaz and CIMMYT PAK maize cultivars, respectively.

3.1.7 Drought effect on silk length (cm)

Silk length of H. Nawaz and C. PAK cultivars was determined at maize kernel blister (R2) and kernel milk (R3) stages of grain filling developmental stages. Drought produced significant reduction in silk length of both maize cultivars as compared to well-irrigated conditions. Under drought stress, reduction in silk length at kernel blister (R2) and kernel milk (R3) stages was 18.42%, and 37.7% in H. Nawaz, while 20.07%, and 47.44%, in C. PAK, respectively. Analysis of variance (ANOVA) results illustrated that water stress induced a significant (P<0.05%) decrease in silk length of H. Nawaz and C. PAK cultivars in comparison to the control conditions at both developmental stages, under study. Results for variety*variety interaction resulted the significant difference (P<0.05%) in means of all three replications of silk length of H. Nawaz and C. PAK under water stress at kernel blister (R2) and kernel milk (R3) stages. AllPairwise Comparisons test of both cultivars for Treatment*Variety resulted nonsignificant results at maize kernel blister (R2) and kernel milk (R3) stages. Based on results, it is concluded that the decrease in silk length in H. Nawaz was slightly low as compared to the C. PAK. Effects of water stress on silk length of H. Nawaz and C. PAK at kernel blister (R2) and kernel milk (R3) developmental stage are depicted in figure 3.7.

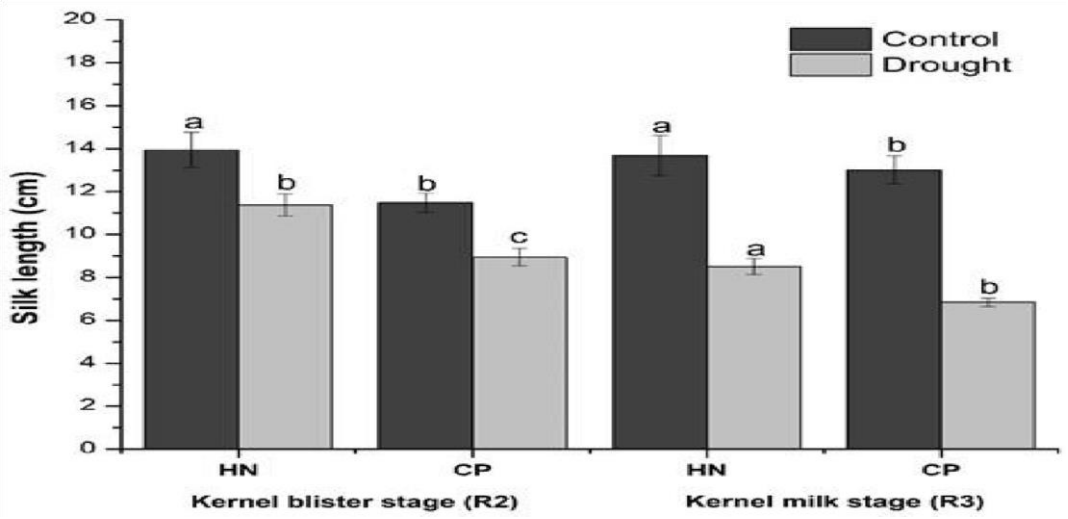

Figure: 3.7 Silk length of two maize cultivars as influenced by drought stress. Vertical bars above mean indicate standard error of three replicates. Mean value for each treatment followed by different letters indicate significance of results compared with control according to least significant difference (LSD) test ($p \leq 0.05$).

3.1.8 Drought effect on ear length (cm)

Ear length was measured at kernel blister stage (R2) and kernel milk stage (R3) of H. Nawaz and C. PAK cultivars. Result illustrated that the reduction in silk length at kernel blister (R2) and kernel milk (R3) stages was 23.51%, and 26.21% in H. Nawaz, while 26.74%, and 30.78%, in C. PAK, respectively.

Analysis of variance (ANOVA) resulted the significant decrease in the ear length of both maize cultivars under drought stress in comparison to the control conditions at kernel blister (R2) and kernel milk (R3) maize developmental stages. Variety*variety interaction showed significant decrease in the ear length of C. PAK as compare to the H. Nawaz with P<0.05 at both developmental stages, which indicated the improved responses of H. Nawaz showed to drought stress than C. PAK. The interaction of Variety*Treatment showed non-significant results at maize kernel blister (R2), whereas significant results was showed at kernel milk (R3) developmental stage.

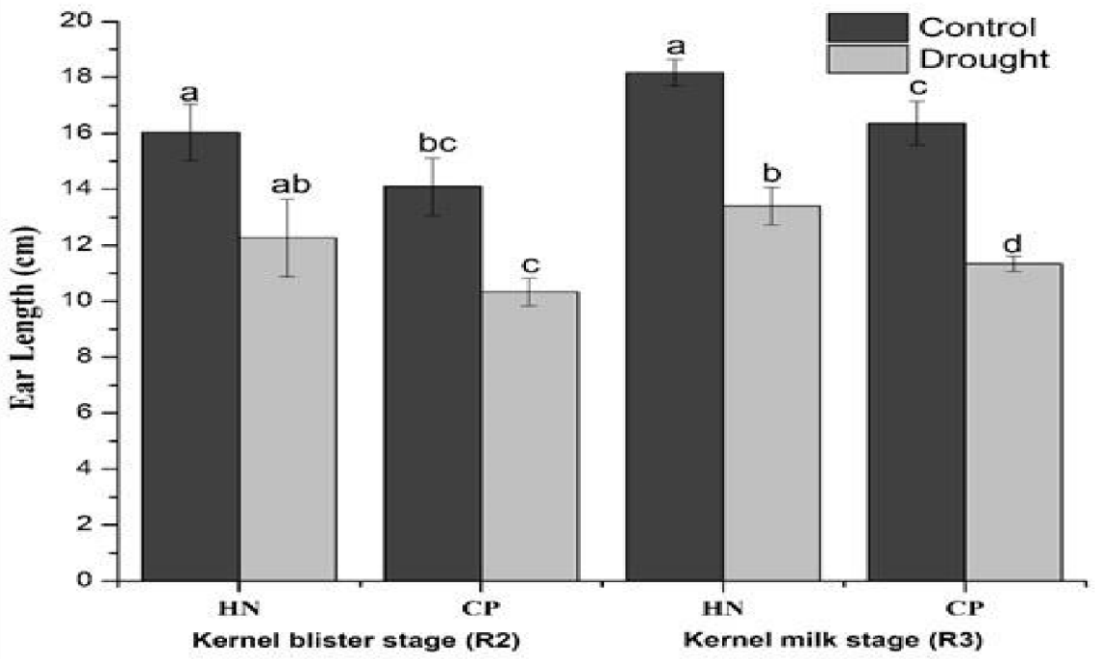

Figure: 3.8 Ear length of H. Nawaz and C. PAK at kernel blister (R2) and kernel milk (R3) stages in comparison to the control conditions. Mean values having standard error of mean (SEM) bar followed by different letters demonstrate the significance of results at (P<0.05%). HN and CP representing the Haq Nawaz and CIMMYT PAK maize cultivars.

3.1.9 Drought effect on cob diameter (cm)

Cob diameter of H. Nawaz and C. PAK cultivars was determined at maize kernel blister (R2) and kernel milk (R3) stages of grain filling developmental stage. Results illustrated the decrease in cob length at maize kernel blister (R2) and kernel milk (R3) stages in H. Nawaz was 29.24%, and 47.52%, while in C. PAK 39%, and 48.39%, respectively.

Analysis of variance (ANOVA) resulted the significant ($P<0.05$) decrease in the cob diameter of both maize cultivars under drought stress in comparison to the control conditions at kernel blister (R2) and kernel milk (R3) stages of maize. Variety*variety interaction showed non-significant decrease in the cob diameter of C. PAK and H. Nawaz at both developmental stages. All-Pairwise Comparisons Test of Plant for Treatment*Variety resulted that all the means are not significantly different from one another at kernel blister (R2) and kernel milk (R3) stages, as depicted in figure 3.9.

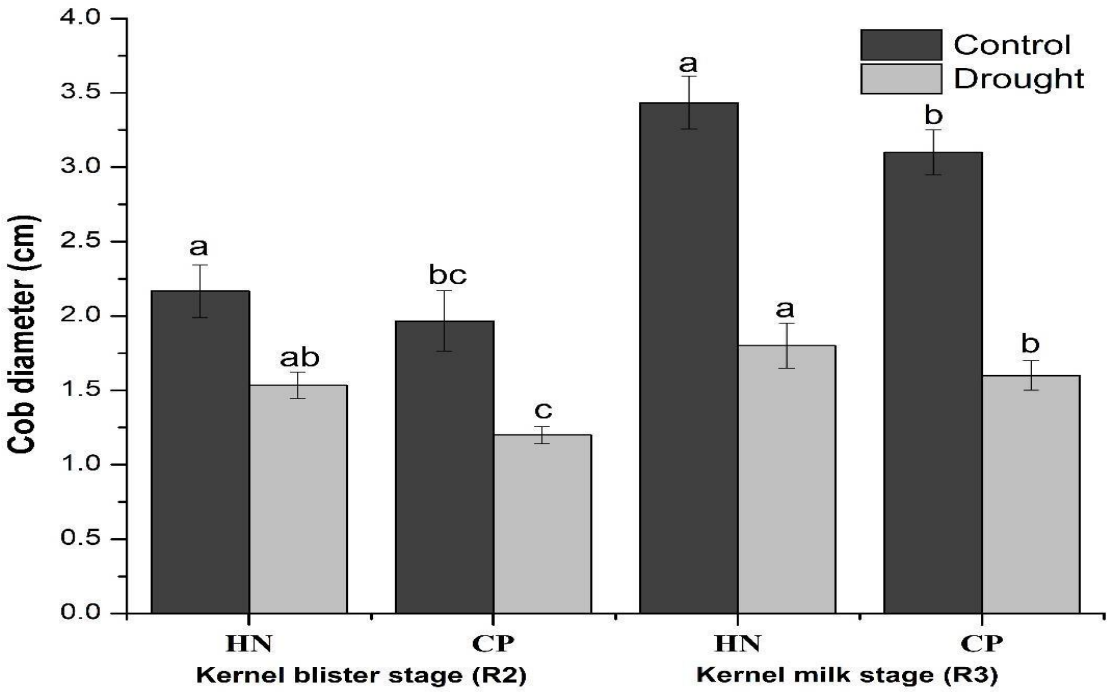

Figure: 3.9 Changes in the cob diameter of H. Nawaz and C. PAK at different reproductive developmental stages. Bars represent the standard error of mean (SEM) of three replicates, followed by different letters are representing the significance of results compared with control according to least significant difference (LSD) test ($p \leq 0.05$).

3.1.10 Drought effect on cob length (cm)

Cob length of H. Nawaz and C. PAK cultivars was determined at maize kernel blister (R2) and kernel milk (R3) stages of grain filling developmental stages. Drought produced significant reduction in silk length of both maize cultivars as compared to well-irrigated conditions. Under drought stress, reduction in cob length at kernel blister (R2) and kernel milk (R3) stages was 21.5%, and 37.84% in H. Nawaz, while 28.4%, and 44.1%, in C. PAK, respectively.

ANOVA results for cob length illustrated the non-significant decrease in length under drought stress as compared to the control conditions at kernel blister (R2) stage whereas significant results was showed at kernel milk (R3) stage of maize. Variety*variety interaction showed significant decrease in the cob length of C. PAK as compare to the H. Nawaz at both developmental stages of maize and thus, H. Nawaz showed improved responses to drought stress in comparisons to the C. PAK. All pairwise comparisons Test for Treatment*Variety resulted that all the means are not significantly different from one another at kernel blister (R2) and kernel milk (R3) stages.

Reduction of cob length of H. Nawaz and C. PAK maize lines under drought conditions, in comparisons to the control conditions illustrated the effects of drought stress on maize cob diameter at Kernel blister (R2) and Kernel milk (R3) Stages as depicted in figure 3.10.

3.1.11 Heat Maps for Haq Nawaz and CIMMYT PAK morphological data

Heat map illustrated the significant difference in morphological traits of H. Nawaz and C. PAK under drought conditions, in comparisons to the control conditions.

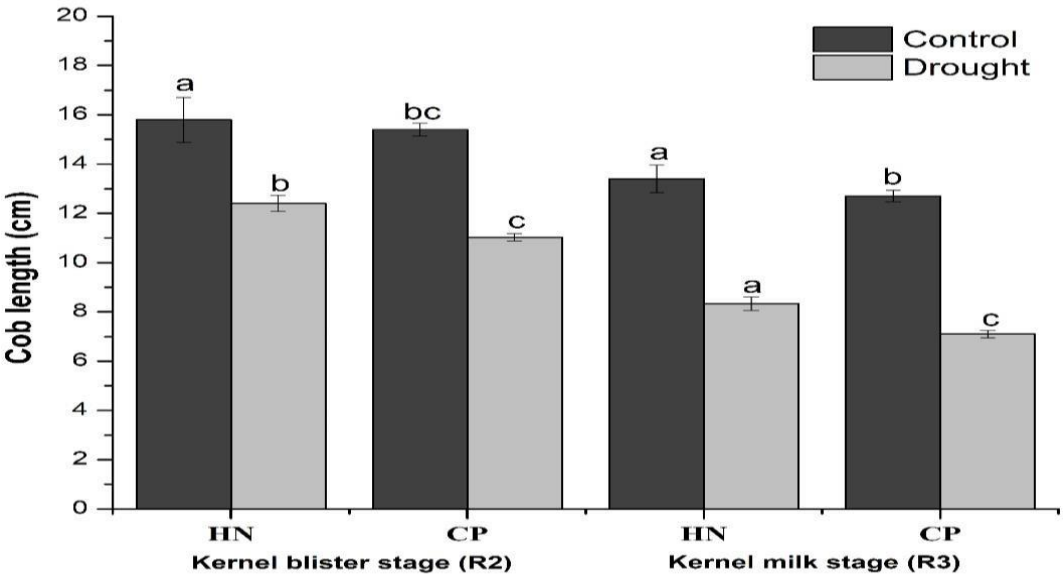

Figure: 3.10 Changes in the cob length of H. Nawaz and C. PAK at different reproductive developmental stages. Bars represent the standard error of mean (SEM) of three replicates, followed by different letters are representing the significance of results at (P<0.05%).

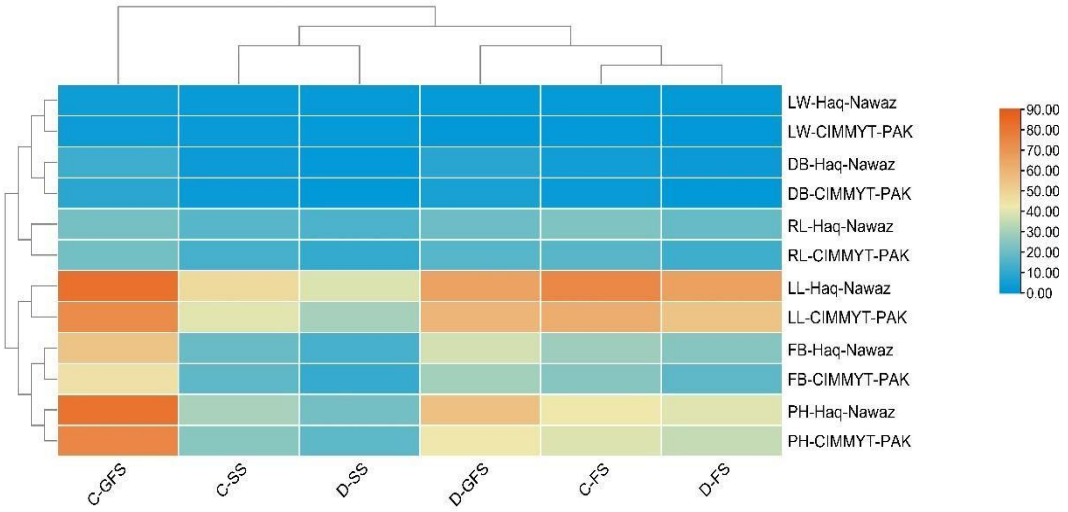

Figure: 3.11 Heatmap for Haq Nawaz and CIMMYT PAK morphological traits. C-SS, D-SS, C-FS, D-FS, C-GFS and D-GFS representing the seedling stage under control and drought conditions, flowering stage under control and drought conditions and grain filling stage under control and drought conditions, respectively. LW, DB, RL, LL, FB and PH representing leaf width, dry biomass, root length, leaf length, fresh biomass and plant height, respectively.

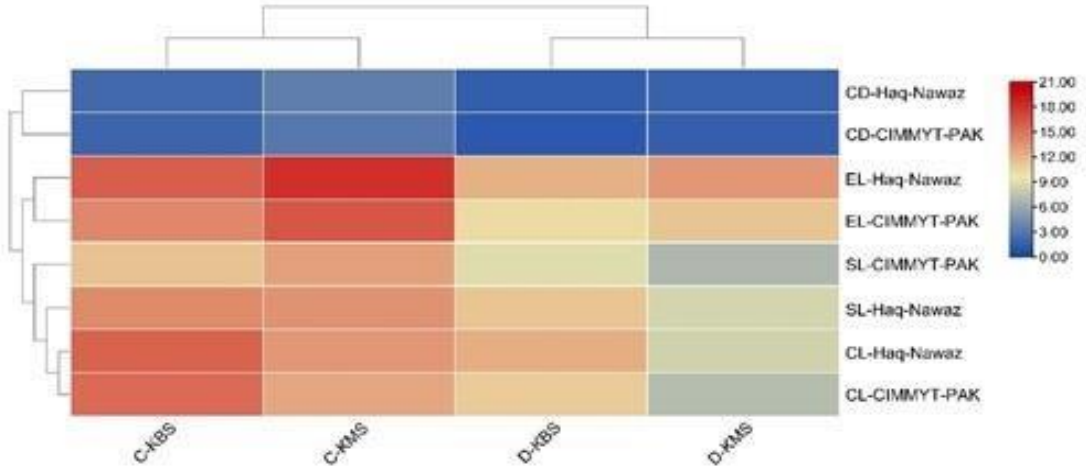

Figure: 3.12 Heatmap for Haq Nawaz and CIMMYT PAK morphological traits at kernel blister stage (KBS) and kernel milk stage (KMS) under control and drought conditions. CB, EL, SL AND CL representing the cob diameter, ear length, silk length, and cob length, respectively.

Figure: 3.13 Comparison of different morphological parameters of Haq Nawaz and CIMMYT PAK at seedling developmental stage of maize. HN-C, HN-D, CPC, and CP-D representing the Haq Nawaz under control, Haq Nawaz under drought, CIMMYT PAK under control, and CIMMYT PAK under drought, respectively

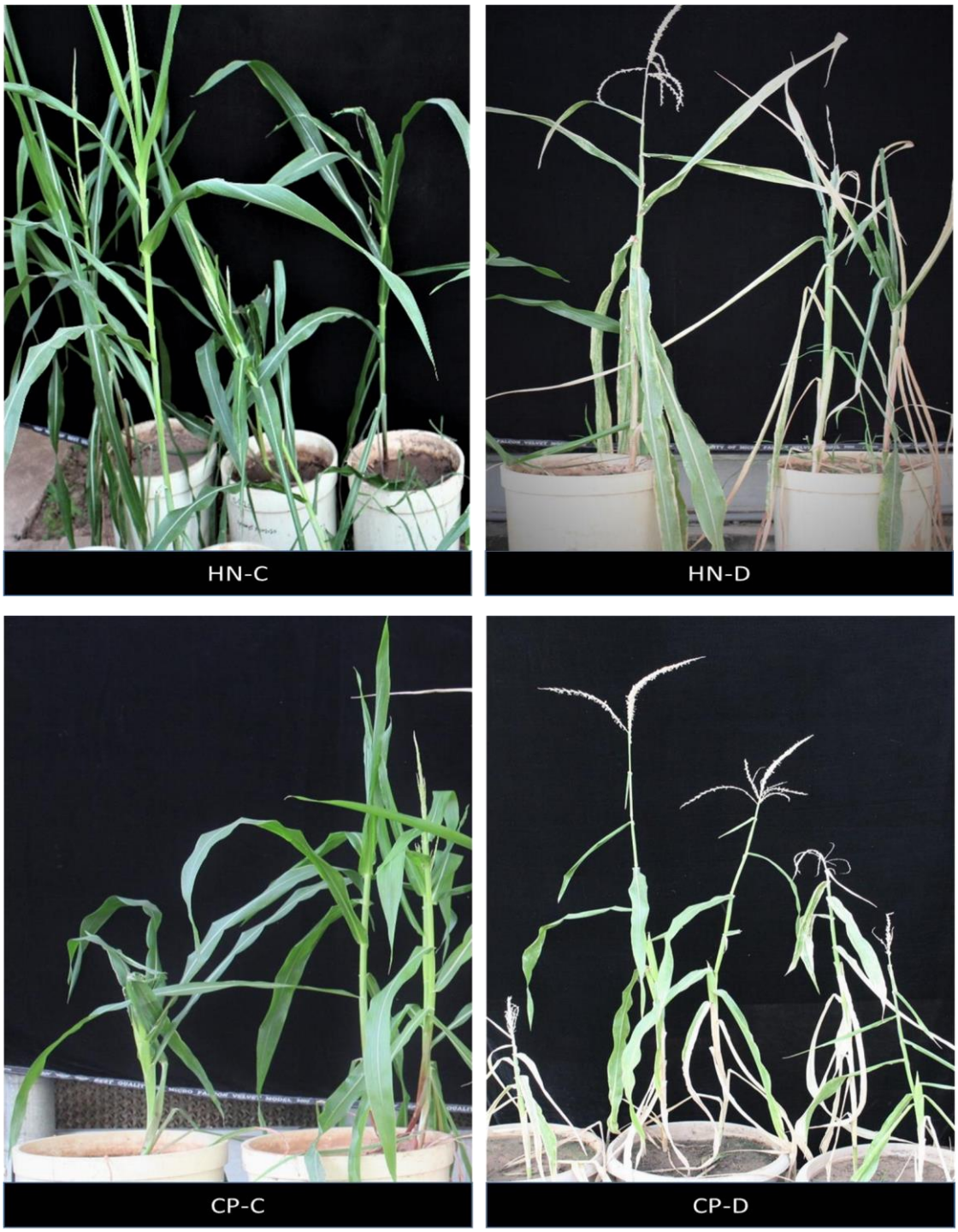

Figure 3.14 Comparison of different morphological parameters of Haq Nawaz and CIMMYT PAK at flowering developmental stage of maize. HN-C, HN-D, CP-C, and CP-D representing the Haq Nawaz under control, Haq Nawaz under drought, CIMMYT PAK under control, and CIMMYT PAK under drought, respectively.

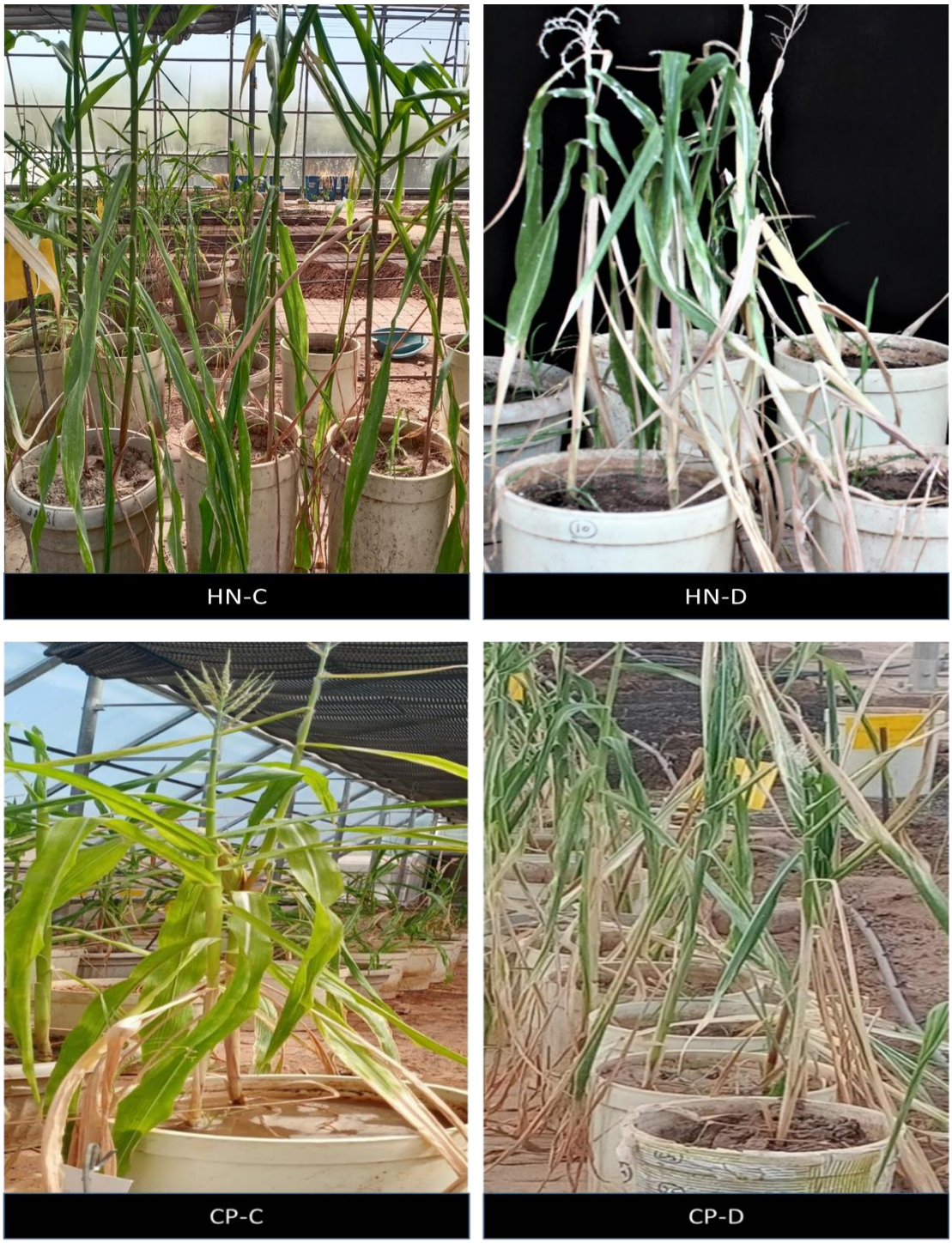

Figure: 3.15 Comparison of different morphological parameters of Haq Nawaz and CIMMYT PAK at grain filling developmental stage of maize. HN-C, HN-D, CP-C, and CP-D representing the Haq Nawaz under control, Haq Nawaz under drought, CIMMYT PAK under control, and CIMMYT PAK under drought, respectively.

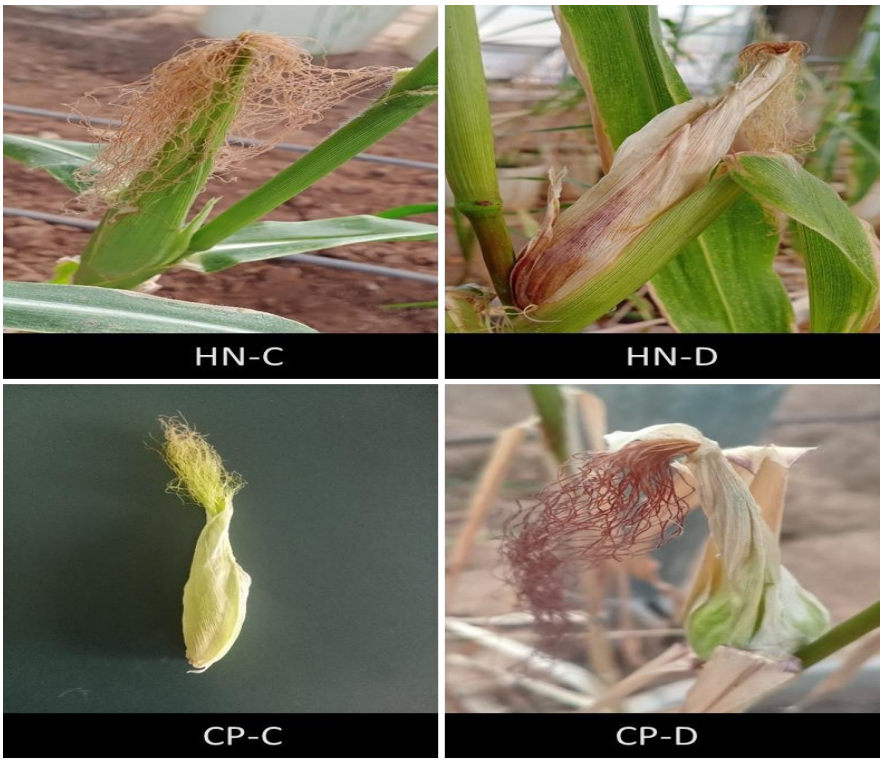

Figure: 3.16 Comparison of silk length, cob length and cob diameter of Haq Nawaz and CIMMYT PAK at kernel blister and kernel milk grain filling stage of maize. HN-C, HN-D, CP-C, and CP-D representing the H. Nawaz under control conditions, H. Nawaz under drought condition, C. PAK under control conditions, and C. PAK under drought conditions, respectively.

3.2 Maize physiological responses under drought stress

Two cultivars of maize (Haq. Nawaz and CIM) plants when grown to grain filling stage, we have applied drought stress (stop H2O/irrigation 10±3 days) until symptoms appear on plants as compared to control. Under fully drought stress condition, Chl. 'a' & 'b', membrane stability index, soluble sugar content, and proline content was measured from both maize cultivars at grain filling stage.

3.2.1 Chlorophyll a content (mg/g)

Results illustrated that C. PAK cultivar Chl. a content was lower than Haq Nawaz. Under drought stress reduction of Chl., a content was 34.18% & 46.46% in H. Nawaz and C. PAK, respectively.

ANOVA results illustrated that water stress induced a significant decrease ($P<0.05$) in Chl. a content of H. Nawaz and C. PAK cultivars in comparison to the control conditions. ANOVA results for variety*variety interaction resulted that H. Nawaz showed significant improved response in terms of Chl. a content as compare to C. PAK under water stress with $P<0.05$. Based on results, it is concluded that as compare to C. PAK, H. Nawaz has the potential to tolerate mild to moderate drought stress at grain filling stage.

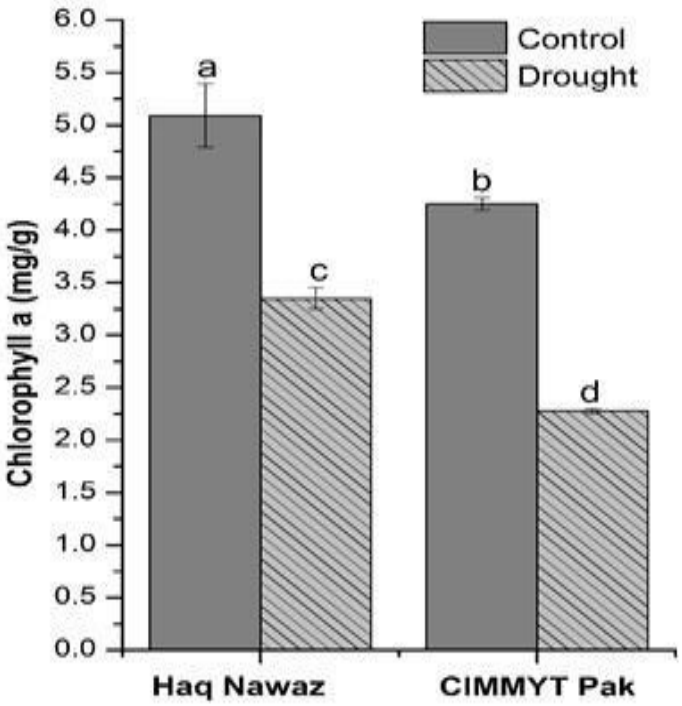

Figure: 3.17 Chlorophyll a content of H. Nawaz and C. PAK at grain filling stage. Mean values having standard error of mean (SEM) bar followed by different letters are significantly different from each other at (P<0.05%)

3.2.2 Chlorophyll b content (mg/g)

Chl b content was measured in comparison to the control conditions and results illustrated that Chl b content was reduced under drought stress as compare to control plants. Under drought stress reduction of chlorophyll b content was 4.09% and 18.04% in H. Nawaz and CIMMYT PAK, respectively.

Analysis of variance (ANOVA) results illustrated that water stress treatments significantly affected the Chl. b content of both maize cultivars under drought stress. Figure 3.18 depicted that increase in the severity of water stress decrease the Chl. b synthesis. ANOVA for all pairwise comparison tests of Chl. b for treatment*variety resulted that all four means are significantly different from each other with P<0.05%. H. Nawaz maize cultivar showed improved response in term of Chl. b content as compare to C. PAK, which shows that H. Nawaz has the potential to tolerate drought stress.

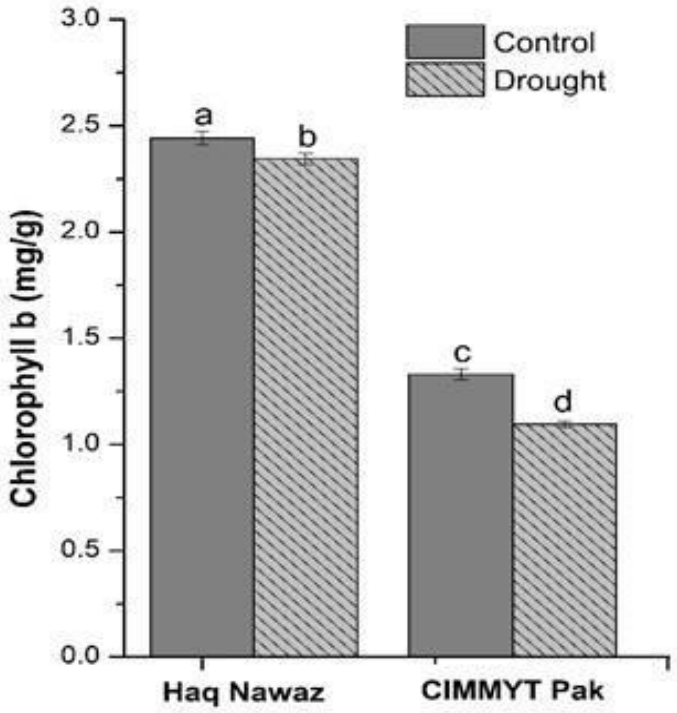

Figure: 3.18 Changes in the Chl. b content of H. Nawaz and C. PAK at grain developmental stage. Bars represent the standard error of mean (SEM) of three replicates, followed by different letters are significantly different from each other at (P<0.05%)

3.2.3 Proline content (µmol/g)

Proline content of H. Nawaz and C. PAK maize cultivars were measured and results illustrated that proline content of both varieties were elevated linearly with increase of drought stress. Leaf proline content increased 23.34% in H. Nawaz and 24.09% in C. PAK, as compare to the control plants.

Statistically significant were recorded for all pairwise treatment*variety interaction. ANOVA results for proline content illustrated the significant increase in the proline content as compare to the control conditions at maize grain filling developmental stage. The means difference of proline content in H. Nawaz and C. PAK were significant at P<0.05%. All-Pairwise Comparisons Test of Proline for Treatment*variety resulted that all means are significantly different from one another as depicted in figure 3.19. Increase of proline content in C. PAK was higher than N. Nawaz illustrated that H. Nawaz had higher tolerance to water deficient conditions than C. PAK.

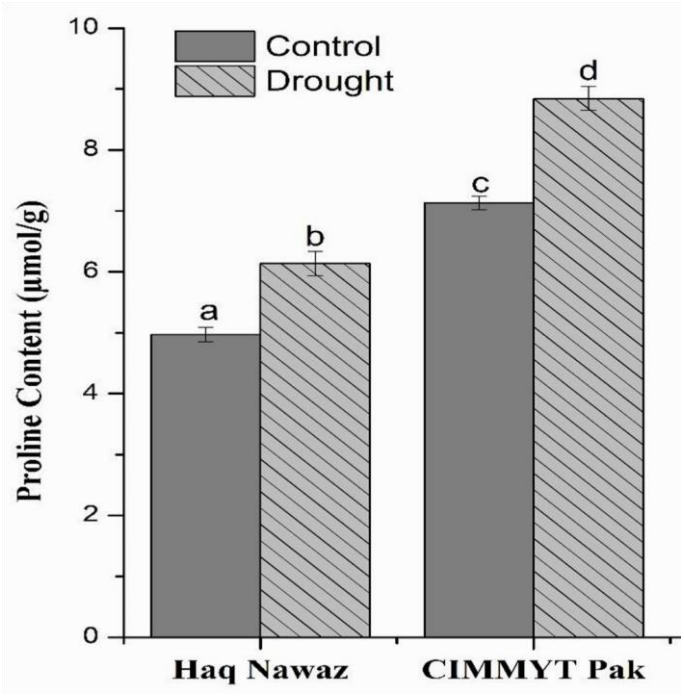

Figure: 3.19 Effects of drought stress on proline content in the leaves of two maize cultivars at grain filling stage. Results are shown as mean±standard error (p<0.05) bar followed by different letters are not significantly different from each other

3.2.4 Membrane Stability Index (%)

Results illustrated that membrane stability index was increased under drought stress as compare to control plants. Haq Nawaz cultivar showed improved stability index as compare to C. PAK. Under water deficient conditions increase in membrane stability index was 2.67% & 9.05% in H. Nawaz and C. PAK respectively.

Analysis of variance (ANOVA) was used to demonstrate the variation in the membrane stability index of both cultivars such as H. Nawaz and C. PAK under drought

and control conditions at grain filling developmental stage. ANOVA results for membrane stability illustrated the significant increase in the membrane stability under drought stress as compared to the control conditions at grain filling developmental stage of maize. The treatments used for this study demonstrate the significant difference in the means of membrane stability index at P<0.05. Varieties*variety interaction showed significant difference in plants membrane stability at P<0.05, whereas All pairwise comparisons test for treatment*variety resulted with non-significant results.

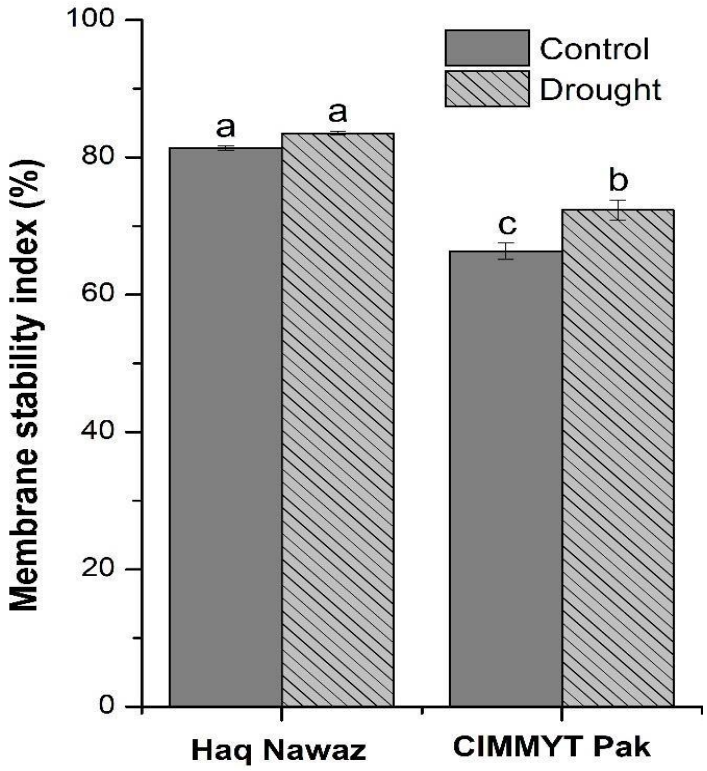

Figure: 3.20 Effect of drought stress on membrane stability index in the leaves of H. Nawaz and C. PAK. Mean values having standard error of mean (SEM) bar followed by different letters are significantly different from each other at (P<0.05%).

3.2.5 Soluble sugar content (mg/g)

Soluble sugar content was measured from leaves of H. Nawaz and C. PAK cultivars. Results illustrated that soluble sugar content was increased linearly in both varieties under drought stress as compare to control plants. CIMMYT-PAK cultivar soluble sugar content was higher than Haq Nawaz cultivar. Under drought stress the

increase of soluble sugar content was 18.24% & 22.97% in H. Nawaz and CIMMYT PAK, respectively.

Water stress induced a significant increase (P<0.05) in soluble sugar content of H. Nawaz and C. PAK cultivars in comparison to the control conditions. ANOVA results for variety*variety interaction resulted the significant difference (P<0.05%) in means of all three replications of soluble sugar content of H. Nawaz and C. PAK under water stress. The interaction of Treatment*variety showed significant result for ANOVA at P<0.05. Based on results, it is concluded that as compare to C. PAK, H. Nawaz has the potential to tolerate drought stress at grain filling stage. Effects of water stress on H. Nawaz and C. PAK soluble sugar content at grain filling developmental stage are depicted in figure 3.21.

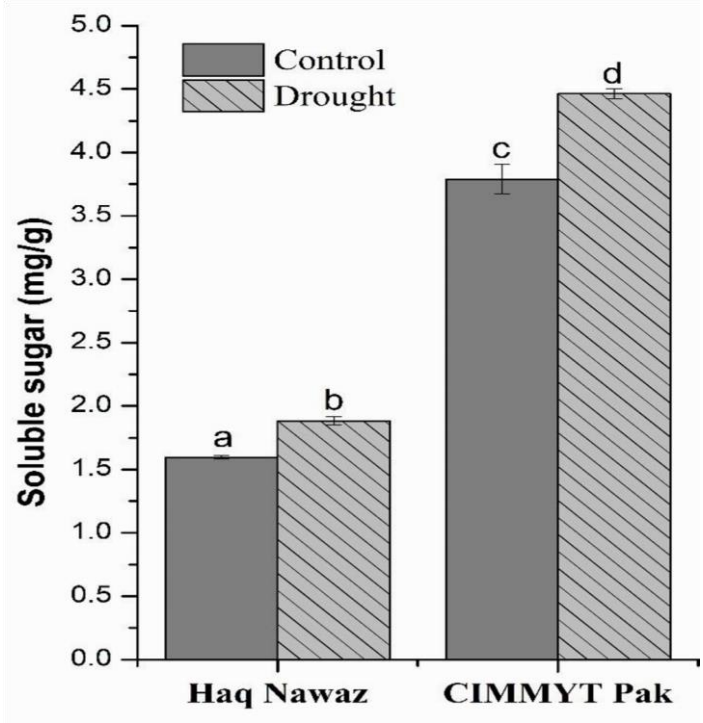

Figure 3.21 Effect of drought stress on soluble sugar content in the leaves of H. Nawaz and C. PAK. Mean values having standard error of mean (SEM) bar followed by different letters are significantly different from each other at (P<0.05%)

3.3 Maize elemental responses under drought stress

3.3.1 Nitrogen content (%)

Nitrogen content of H. Nawaz and C. PAK was determined at maize grain filling developmental stage under drought and control conditions. Results illustrated that under drought stress reduction of nitrogen content was 5.88% & 6.29% in H. Nawaz and CIMMYT PAK, respectively. Nitrogen content was decreased under drought stress as compare to control plants. CIMMYT-PAK cultivar nitrogen content was lower than Haq Nawaz.

Analysis of variance (ANOVA) was used to demonstrate the variation in the nitrogen content of both cultivars such as H. Nawaz and C. PAK under drought and control conditions at grain filling developmental stage. ANOVA results for nitrogen content illustrated the significant decrease under drought stress as compared to the control conditions. The treatments used for this study demonstrate the significant difference in the means of nitrogen content of both maize cultivars at $P<0.05$. Whereas the interaction of Treatment*variety showed non-significant result for ANOVA at $P<0.05$, which demonstrate that all means are not significantly different from each other as depicted in figure 3.22.

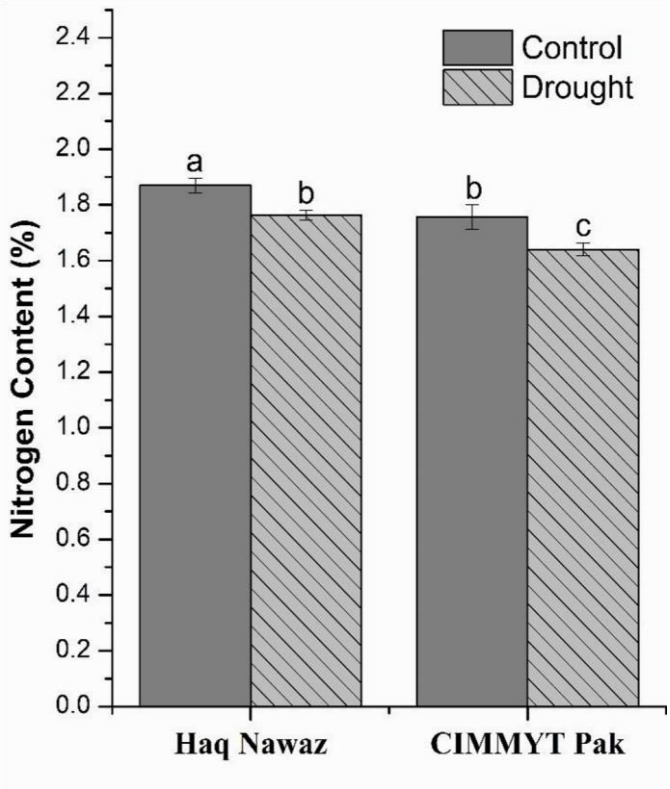

Figure: 3.22 Nitrogen concentrations of two maize cultivars at grain filling developmental stage under controlled and water deficient conditions

3.3.2 Potassium content (%)

Potassium content of H. Nawaz and C. PAK maize cultivars was measured in comparisons to control Plants. Results illustrated that potassium content was increased under drought stress as compare to control plants. CIMMYT PAK cultivar potassium content was higher than Haq Nawaz. Under drought stress increased level of potassium content was 5.72% & 6.77% in H. Nawaz and CIMMYT PAK, respectively. Water stress treatments significantly affected the potassium content of both maize cultivars. Figure 3.23 depicted the variations in the potassium content with increase in the severity of water stress.

Statistically significant differences were recorded while G × E interaction. Water stress induced a significant increase ($P<0.05$) in potassium content of H. Nawaz and C. PAK cultivars in comparison to the control conditions. ANOVA for all pairwise comparison tests of potassium content for treatment*variety resulted that all four means are not significantly different from one other with $P<0.05\%$. Low level of potassium content in H. Nawaz under drought stress in comparison to C. PAK, indicated the potential of H. Nawaz tolerance to mild to moderate drought stress at grain filling stage.

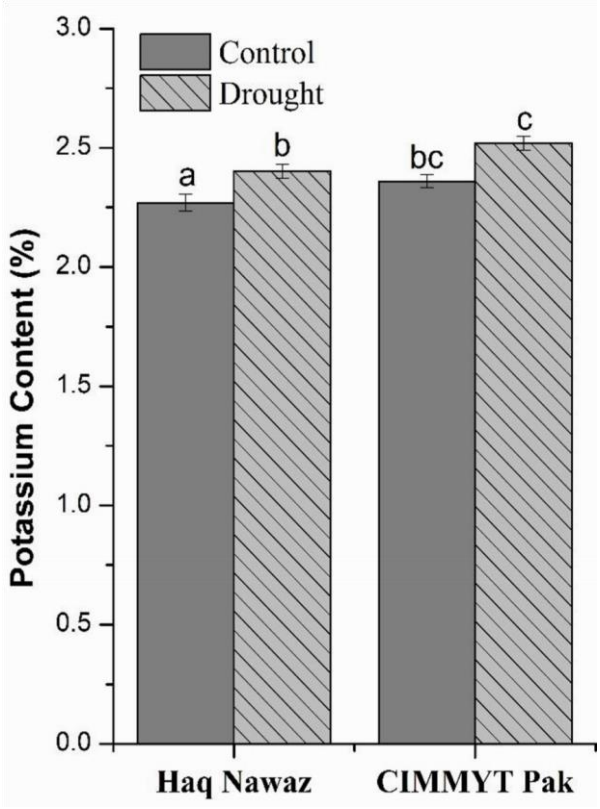

Figure: 3.23 Potassium content of H. Nawaz and C. PAK at grain filling stage under well-water and water deficient conditions. Mean values having standard error of mean (SEM) bar followed by different letters are significantly different from each other at (P<0.05%)

3.3.3 Phosphorus content (%)

Phosphorus content was measured at grain filling stage and results indicated that phosphorus content was decreased under drought stress as compare to control plants. Under drought stress reduction of phosphorus was 6.19% & 19.34% in H. Nawaz and CIMMYT PAK, respectively. Analysis of variance (ANOVA) was used to demonstrate the variation in the phosphorus content of both cultivars such as H. Nawaz and C. PAK under drought and control conditions at grain filling stage. ANOVA results for phosphorus content illustrated the significant decrease under drought stress as compared to the control conditions at grain filling developmental stage of maize. The treatments used for this study demonstrate the non-significant difference in the means of phosphorus content at P<0.05, which illustrated the non-significant difference of phosphorus content under drought and control conditions. Whereas variety*variety interaction showed significant difference in the phosphorus content of both maize cultivars at P<0.05%. All-Pairwise Comparisons Test of Plant for Treatment*Variety resulted that all 4 means are non-significantly different from one another. H. Nawaz

showed improved response in terms of phosphorus content as compare to C. PAK. So, up to some extent H. Nawaz has the potential to tolerate mild to moderate drought stress at grain filling stage.

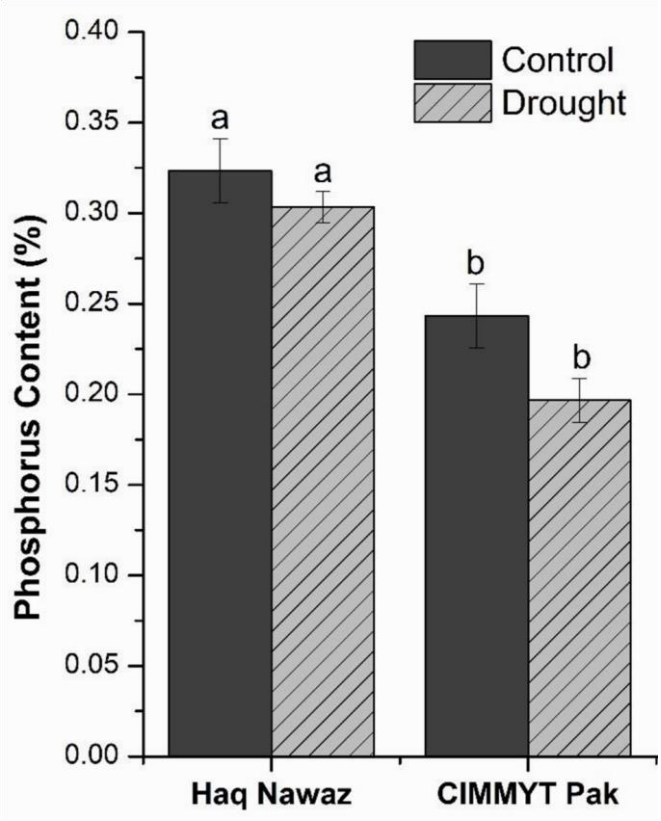

Figure: 3.24 Effect of drought stress on phosphorus content in the leaves of H. Nawaz and C. PAK. Mean values having standard error of mean (SEM) bar followed by different letters demonstrate the significant difference of H. Nawaz and C. PAK from each other at (P<0.05%).

3.3.4 Heatmap for maize physiological and nutritional values

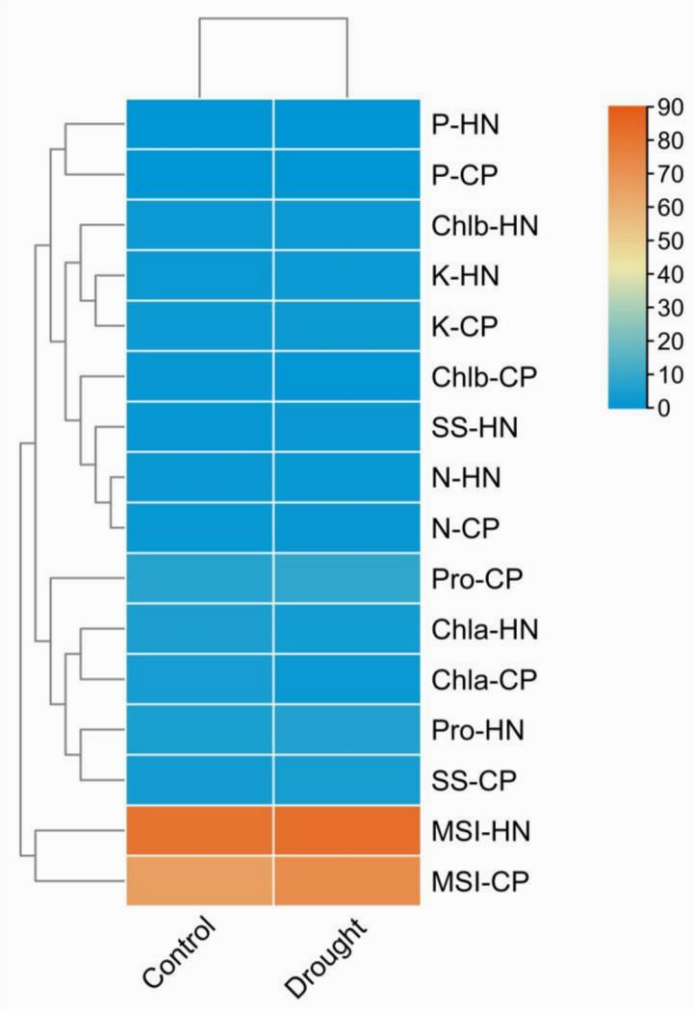

Figure: 3.25 Heatmap for Haq Nawaz (HN) and CIMMYT PAK (CP) physiological and nutritional traits at grain filling stage under control and drought conditions. Chla, Chlb, Pro, SS, MSI, N, P and K representing the chlorophyll a, chlorophyll b, proline, soluble sugar, membrane stability index, nitrogen, phosphorus and potassium content, respectively.

3.4 Bioinformatics analysis

3.4.1 Genome_wide identification of Glutamine synthetase family genes

In rice drought responsive GS2 (*OsGS2*; LOC_Os04g56400) was identified and used as query sequence to BLAST against eight Poacea family species. Total of 27 genes of glutamine synthetase family; 6 genes of *Zea mays*, 4 of *Triticum aestivum*, 2 of *Sorghum bicolor*, 4 of *Hordeum volgari*, 6 of *Saccharum spontaneum*, 2 of *Oryza sativa*, 2 of *Arabidopsis thaliana*, and 1 of *Setaria italica* was selected with the threshold of E-value < e-10, and 75% of percent identity and after deletion of duplicates. Protein Gln-

synt_C domain of all these genes is confirmed by SMART and remaining 25 sequences were selected for further analysis.

3.4.2 Evolutionary analysis of selected GS genes

The phylogenetic tree was generated for 25 GS genes of *Zea mays, Triticum aestivum, Sorghum bicolor, Hordeum volgari, Saccharum spontaneum, Oryza sativa, Arabidopsis thaliana,* and *Setaria italica* using the neighbour joining method. The evolutionary relationship was determined using the protein sequences. To analyze the evolutionary observation, the bootstrap method and entire deletion strategy were used. Phylogenetic analysis group all of the GS proteins into 3 clades depending upon their sequence homologs, while the *Arabidopsis thaliana* gene At5g16570 is resulted as out group. Every individual GS gene organized in a distinct clade with different number. Each clade defining the level of divergence by length of their branches and number of genes due to the whole genome duplication. Clade 3 contains greater number of genes than others. Different genes like Ssp-GS-2B and Ssp-GS-1A, Traes-GS2-2A and Traes*GLN2*B, Traes-GS1-6B and Traes-GS1-6B revealed in more than one duplicated form because of the different chromosome number within the same species. Bootstrap values of all these genes clearly indicate that all GS genes under study have clear evolutionary relationships. *Zea mays GLN2* gene present in clade 1, closely distant to the *Saccharum spontaneum* Ss-GS-2D gene.

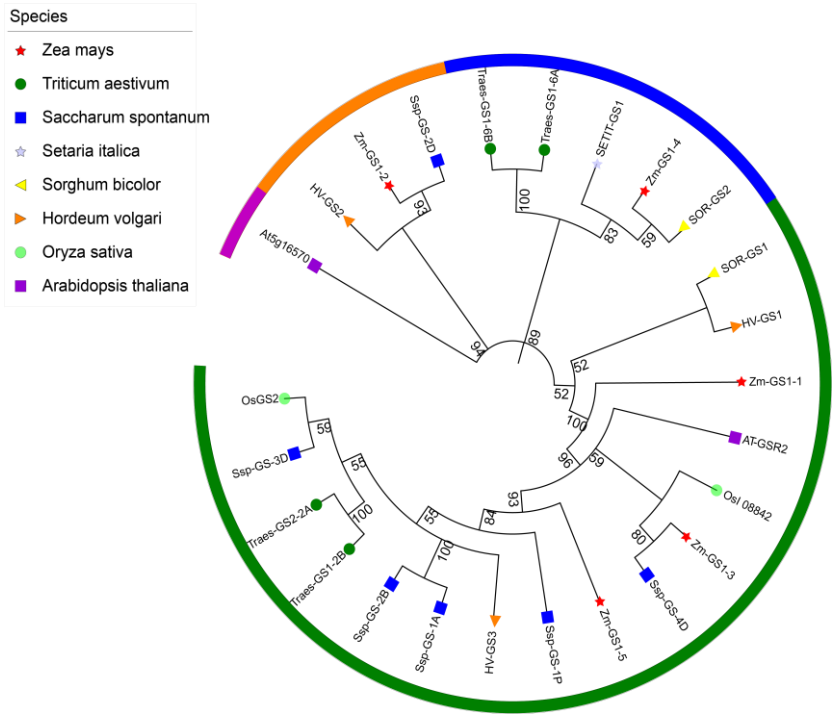

Figure: 3.26 Neighbour joining evolutionary tree of GS genes from distinct 8 crops. Prefixes such as Zm, Traes, SOR, HV, Ssp, Os, At, and SETTT were used for *Zea mays*, Triticum aestivum, Sorghum bicolor, Hordeum volgari, Saccharum spontaneum, Oryza sativa, Arabidopsis thaliana, and Setaria italica, respectively. Near the nodes of each branch bootstrap values were also mentioned. Mustard, blue and green outlines representing the Clade 1, 2 and 3, respectively

3.4.3 Gene structural analysis

It has been reported that the distribution pattern of exons/introns in a gene is related to the gene biological functions (Qanmber *et al*., 2019). We found that all of the glutamine synthetase genes showed a conserved pattern of exons and intron distribution including their up-stream and downstream regions. *Zea mays GLN2* showed a conserved pattern of axons, introns to all other sequences under study, with 13 number of axons and 12 number of intron regions.

3.4.4 Analysis of conserved domains of glutamine synthetase genes

Specific domain of glutamine synthetase C (Gln-Syn_C) was identified in all identifies 25 protein sequences of 8 crops. It's concluded that all of the glutamine synthetase genes including *Zea mays GLN2* showed a conserved distribution patterns in all identified genes in different crops. All studied sequences have Gln-synt_C domain.

3.4.5 Analysis of conserved motifs in GS genes of 8 different crops

MEME (Multiple Em for motif Elicitation) motif search tool was used to identify the 10 conserved motifs of 25 protein sequences of 8 crop species. Each of the following 25 sequences has an *E*-value less than 10. The motif matches shown have a position *p*-value less than 0.0001. Distribution pattern of glutamine synthetase protein motifs revealed that the similar motifs had conserved distribution patterns in all identifies 25 protein sequences of 8 crops. *Zea mays GLN2* showed a conserved distribution of 10 number of motifs to all other sequences, as depicted in figure 2.27. Identified 10 motifs are represented in distinct colors and name of all motifs display in the right side of the figure. On specific protein sequence the order of motif correlate to its specific position on that sequence.

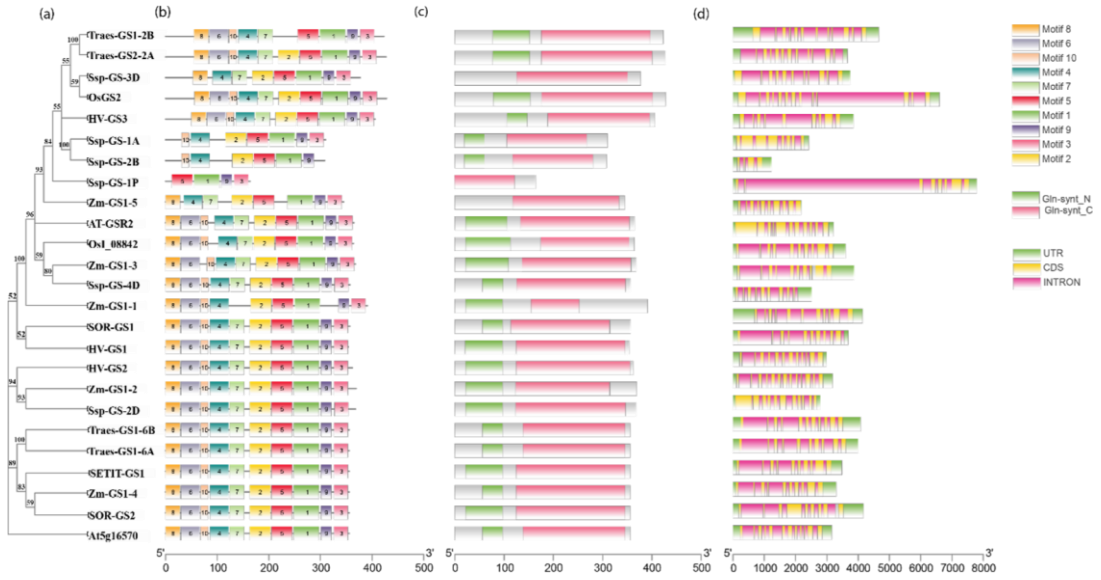

Figure: 3.27 Advanced gene structural view of gutamine synthetase gene. (a) Phylogeny relationship of glutamine synthetase genes of different crops. (b) Schematic representation of motifs identified in 25 protein sequences of GS genes (c) Representation of conserved domains of glutamine synthetase genes. (d) Schematic representation of selected GS genes structural analysis. A conserved pattern of exons and introns were shown including upstream and downstream regions.

3.4.6 Physicochemical properties

The physicochemical properties of glutamine synthetase proteins are listed in table 3.1. Glutamine synthetase proteins range from 164 (Ssp-GS-1P) – 428 (OsGS2) amino acids in size and average molecular weight of 39425.3632 kDa. The pI lies in the range of 5.12 – 7.97. The rest of parameters including sub-cellular localization, negatively charged residues, positively charged residues aliphatic index and Grand average of hydropathicity index (GRAVY) could be seen from the table 3.1.

Table. 3.1 In silico study of Glutamine synthetase proteins and sequence feature.

Gene Name	Gene ID	Chr. No.	Sub-cellular localization	Protein Length	MW (kDa)	PI	Negatively charged residues	Positively charged residues	Aliphatic index	GRAVY
Zm-GS1-2	Zm00001d033747	1	Cytoplasmic	367	40202.2	5.81	47	41	72.71	-0.453
Zm-GS1-5	Zm00001d048050	9	Cytoplasmic	345	37995.86	5.4	45	37	77.77	-0.322
Zm-GS1-3	Zm00001d017958	5	Cytoplasmic	368	40559.53	5.43	45	37	71.06	-0.472
Zm-GS1-4	Zm00001d051804	4	Cytoplasmic	356	39239.21	5.34	44	36	73.99	-0.415
Zm-GS1-1	Zm00001d028260	1	Cytoplasmic	391	43261.02	5.59	50	42	80.08	-0.309
Traes-GS1-6B	TraesCS6B02G327500.1	6B	Cytoplasmic	356	39213.32	5.41	44	37	75.87	-0.394
Traes-GS1-6A	TraesCS6A02G298100.2	6A	Cytoplasmic	356	39197.32	5.41	44	37	76.15	-0.387
Traes-GS1-2B	TraesCS2B02G528300.1	2B	Chloroplast	423	46082.17	5.89	48	43	79.57	-0.32
Traes-GS2-2A	TraesCS2A02G500400.1	2A	Chloroplast	427	46702.83	5.75	49	43	78.83	-0.338
SOR-GS1	OQU92979	1	Cytoplasmic	357	39240.1	5.3	46	37	75.71	-0.44

SOR-GS2	KXG30808	4	Cytoplasmic	356	39183.04	5.51	43	36	73.46	-0.452
SETIT-GS1	KQL31407	1	Cytoplasmic	356	39158.05	5.51	43	36	72.61	-0.452
Ssp-GS-1A	Sspon.01G0007760-1A	1A	Cytoplasmic	310	34293.44	5.87	41	36	70.84	-0.577
Ssp-GS-1P	Sspon.01G0007760-1P	1B	Cytoplasmic	164	18258.56	7.97	22	23	71.4	-0.633
Ssp-GS-2B	Sspon.01G0007760-2B	1B	Cytoplasmic	308	33953.18	6.36	37	35	69.71	-0.5
Ssp-GS-2D	Sspon.01G0026870-2D	1D	Cytoplasmic	368	40181.09	5.44	49	39	74.51	-0.434
Ssp-GS-3D	Sspon.05G0021990-3D	5D	Chloroplast	377	41221.83	6.41	42	44	80.74	-0.305
Ssp-GS-4D	Sspon.04G0004050-4D	4D	Cytoplasmic	357	39167.01	6.34	40	38	71.06	-0.476
OsGS2	Os04t0659100-01	4	Cytoplasmic	428	46642.74	5.96	48	44	78.62	-0.313
OsI_08842	BGIOSGA005667-TA	2	Cytoplasmic	364	40213.22	5.69	44	37	73.96	-0.456
HV-GS1	HORVU4Hr1G066860.1	4H	Cytoplasmic	354	38774.74	5.71	42	36	78.84	-0.364
HV-GS2	HORVU4Hr1G007610.1	4H	Cytoplasmic	362	39709.65	5.96	45	41	73.07	-0.462
HV-GS3	HORVU6Hr1G074030.2	6H	Cytoplasmic	406	44430.45	6.15	44	41	75.44	-0.381
AT-GSR2	AT1G66200.3	1	Cytoplasmic	360	39766.71	5.14	47	35	77.25	-0.394
At5g16570	AT5G16570.1	5	Cytoplasmic	356	38986.81	5.12	46	35	76.21	-0.406

3.5 Expression analysis of *Glutamine synthetase 2* gene in maize

Computational analysis revealed the presence of *Glutamine synthetase 2* gene in different crops of Poaceae family. In rice, experimental studies revealed that under drought stress maintained Os*GLN2* (*GLN2*) activity and its overexpression enhanced the plant tolerance to drought condition. Conserved pattern of *Zea mays glutamine synthetase 2 (GLN2)* gene was showed by phylogenetic evolutionary relationship, gene structure analysis, analysis of conserved domains and motifs. That's why we used this gene to check their function in maize under drought stress.

The expression profile of drought responsive gene, glutamine synhetase 2 in Haq Nawaz and C. PAK using Real-Time PCR analysis at maize grain filling stage. The tissues selected for the expression of *GLN2* gene were leaves and roots. Results of total RNA extracted from leaves and roots tissues depicted in figure 3.30 & 3.31. cDNA quantification results and dilutions used for real time PCR master mix are depicted in appendix 1. Threshold cycle (ΔCT) value give the information of drought responsive gene copy number at the time of amplification in Haq Nawaz and C. PAK maize varieties. The expression data of *GLN2* gene comparison with reference gene i.e. tubulin (Tub) proposed that this gene transcript is induced under drought stress. Furthermore, the expression profile revealed that *GLN2* gene was differentially expressed in Haq Nawaz and C. PAK maize cultivars. Results illustrated that drought responsive *glutamine synthetase 2 (GLN2*) gene expression was found more in H. Nawaz than C. PAK in both leaves and roots tissues. Higher expression level in H. Nawaz than C. PAK illustrated that Haq Nawaz is more tolerant to the drought stress than C. PAK. However, in the root tissues of both maize cultivars the expression was high in comparison to the leaves. This correlation of *GLN2* gene to drought stress is completely noticeable in the evidence that drought responsive genes play a major role in drought tolerant pathways.

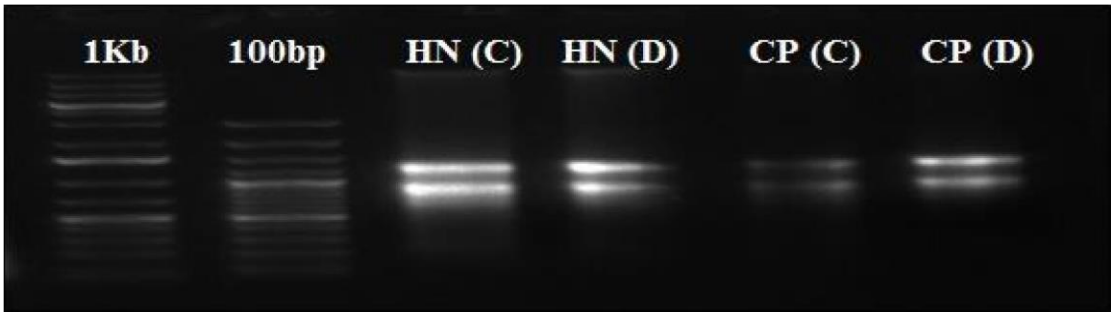

Figure: 3.30 RNA extraction from leaves sample of H. Nawaz (HN) and C. PAK (CP) maize cultivars

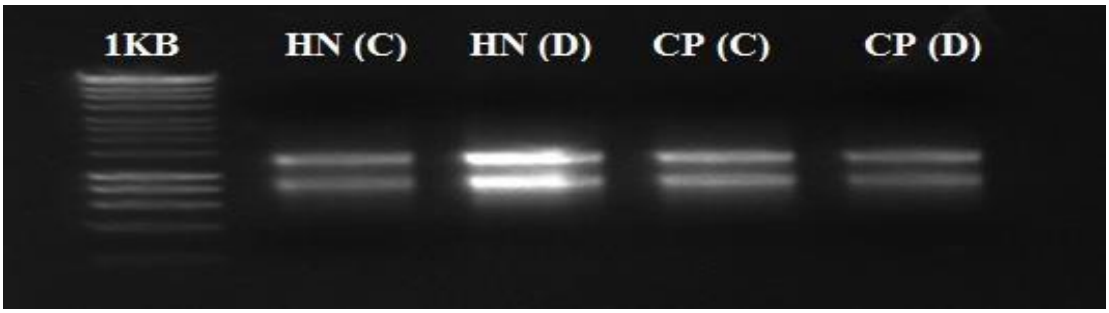

Figure: 3.31 RNA extraction from root sample of Haq Nawaz (HN) and CIMMYT PAK (CP) maize cultivars

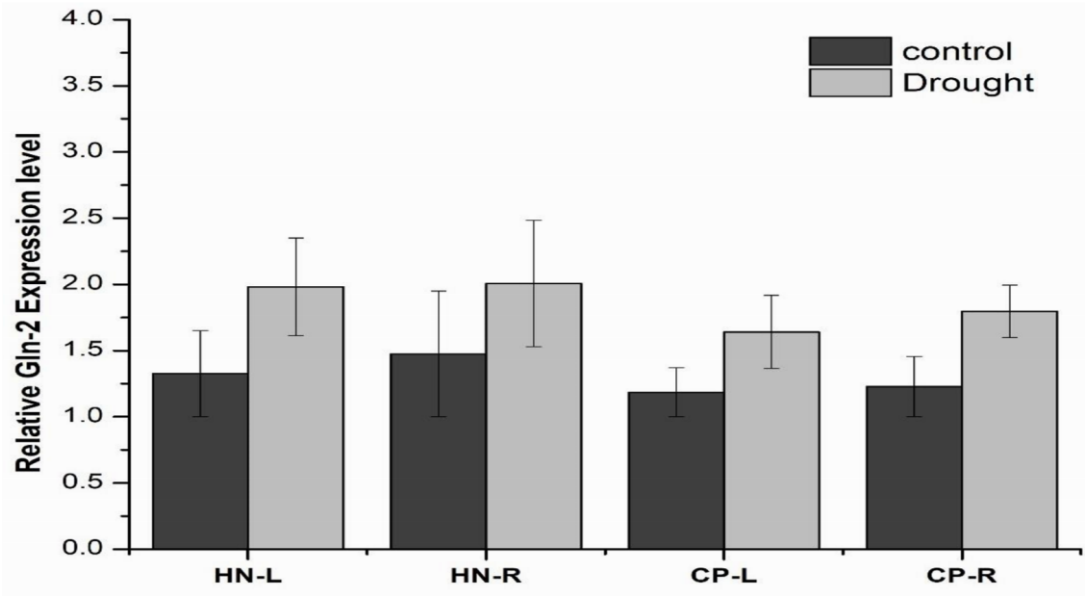

Figure: 3.32 Relative expression analysis of Glutamine Synthetase-2 (*GLN2*) gene under drought stress in Haq Nawaz and CIMMYT PAK maize cultivars. HN-L, HN-R, CP-L, and CP-R represent Haq Nawaz leaf, Haq Nawaz root, CIMMYT PAK leaf and CIMMYT PAK root tissues respectively.

CHAPTER 4

DISCUSSION

DISCUSSION

During growth and development maize is subjected to various abiotic stress factors. Drought stress is a major abiotic factor that leads yield losses to maize every year. The grain filling stage of maize is most effected by drought stress. (Badr *et al*., 2020). The screening of the drought tolerant maize varieties in Pakistan in relation to their drought responsive mechanisms at morphological, physiological, elemental and genetic level can have a significant contribution for improvement of maize yield.

In this study morphological data of drought tolerant maize cultivars Haq Nawaz and drought sensitive maize C. PAK were recorded at maize seedling, flowering and grain filling stages. Similarly, physiological, elemental and gene expression analysis were performed at grain filling stage only for both maize cultivars. In maize, genotypic differences in response to drought-stress, with respect to physiological and phenotypic traits, have been identified (Zhao *et al*., 2016; Thirunavukkarasu *et al*., 2017). Here, our experimental observations on both morphological and physiological, traits showed that maize H. Nawaz and C. PAK lines performed differently under drought stress conditions. Reduction in maize plant growth rate under water deficient conditions has been reported (Anjum *et al*., 2011; Khodarahmpour *et al*., 2011). As compare to the controlled conditions, the drought stressed varieties show retarded growth of maize height, root length, shoot length, leaf length, leaf width, plant fresh biomass, plant dry biomass, silk length, ear length, cob diameter, and cob length. However, these growth traits, were less affected by drought stress in H. Nawaz than in C. PAK.

Imposing of water deficient conditions cause a significant reduction in the plant height (Anser Ali *et al*., 2018). The results obtained are congruent with the previous reports, which demonstrate that increase in water stress leads to decrease in plant height, leaf length, leaf area, and plant biomass (T. Ge *et al*., 2012). After a critical level of drought stress phenotypic expression is critically suppressed at flowering and grain filling stages. Most prominent phenotypic effects are reduction in green-leaf duration, plant height, ear length, number of leafs per plant and early leaf senescence (Sah *et al*., 2020).

Under drought stress, our study showed decline of shoot and root length. Retardation of shoot and root length under drought stress conditions was also reported by other researchers (Kolarovič *et al*., 2006). Maize root structure plays important role in uptake of nutrients, water, lodging, and for survival of plant under soil unfavorable conditions (Sah *et al*., 2020). Studies showed a significant decrease in the leaf length and leaf width under drought stress (Badr *et al*., 2020b; Hussain *et al*., 2020). Haq Nawaz and CIMMYT PAK maize cultivars showed a reduction in fresh and dry weight of plants. Results also supported by other researchers (Aslam, 2014). Reduction in fresh and dry weight of both cultivars is due to dehydration, production of reactive oxygen species, denaturation of proteins, which leads to plant biomass reduction (Ge *et al*., 2012b). Maize ear height helps to receive many pollen grains for fertilization and reduces the damages (Sah *et al*., 2020). Reduction in ear length was lower in Haq Nawaz in contrast to CIMMYT PAK and control plants. Reduction in ear length and cob diameter was not significant under drought stress, also supported by other results (Zamaninejad *et al*., 2013).

Chlorophyll synthesis in maize plant is decreased with increase in the drought severity (Hussain *et al*., 2020; M. Haghjoo *et al*., 2015). The reduction in the H. Nawaz (34.17%) and CIMMYT PAK (46.44%) chlorophyll content attributed to the reduction in water supply and leaf water content which leads to decline the photosynthetic pigments synthesis (Hussain *et al*., 2020). The chlorophyll a and b content of Haq Nawaz and CIMMYT PAK is significantly ($p \leq 0.05$) decreased with increasing the drought stress at maize grain filling stage. The decline in chlorophyll a and b content in the sensitive line CIMMYT PAK was evidently than the tolerant line Haq Nawaz under water stress conditions (Fig 3.17 & 3.18). Chlorophyll content significantly decreased when plants subjected to the drought conditions (Anser Ali *et al*., 2018).

Statistical analysis shown that proline, total soluble sugar content, and membrane stability index significantly ($p \leq 0.05$) increased in Haq Nawaz and CIMMYT PAK under drought stress at grain filling stage of maize. The increase in proline, total soluble sugar content, and membrane stability index was 23.34% & 24.09%, 18.24% & 22.9%, and 2.67% & 9.05% in H. Nawaz and C. PAK, respectively. Comparatively, tolerant line Haq Nawaz maintained little bit increase in values of proline, total soluble sugar content, and membrane stability index than the sensitive line CIMMYT PAK (Fig 3.19, 3.20 & 3.21). Studies show that there is a direct correlation

between the intensity of drought stress and proline accumulation in the leaves of maize plant (IBARRA-CABALLERO *et al*., 1988). Membrane stability index increased during water deficient conditions in maize cultivars (Anser Ali *et al*., 2018) (T. Wang *et al*., 2013). Soluble sugar analysis of H. Nawaz and C. PAK showed increased level of sugar content during drought stress. During drought condition plants tolerance mechanism associated with the accumulation of osmo-protectants like soluble sugar. So, studies show that there is direct correlation between total soluble sugar content and maize plant drought severity (Mohammadkhani *et al*., 2008). Soluble sugar involves in plant metabolism work as a substrate in biosynthesis processes, product of hydrolytic processes, and in energy production. Work as osmoprotectant to maintain cell turgor and to stabilize cellular membranes (Mohammadkhani *et al*., 2008).

Under drought stress the uptake of essential nutrients decreases and causes a variety of morphological and biochemical modifications (Aqaei *et al*., 2020). In the current study, the decrease in the nitrogen content for H. Nawaz and C. PAK was 5.88% and 6.29%, respectively. The decrease in nitrogen content was greater in sensitive line CIMMYT PAK and low in tolerant line Haq Nawaz under drought-stress conditions. Lack of moisture content cause reduction in ammonium and nitrate transfer to the surface of roots, resulting in less uptake of nitrogen content (C. Zörb *et al*., 2014). During water stress conditions in roots nutrients-uptake kinetics per unit in decreased, which effect the enzymes activity for nutrients assimilations and reduce the nutrients uptake. Studies show that under stress condition phosphorus content decreased in both maize cultivars in comparisons to the control conditions. Results illustrated that decrease in phosphorus content was 6.19% in Haq Nawaz and 19.34% in C. PAK at maize grain filling stage. The lower phosphorus content changes in Haq Nawaz imparted improved drought-stress tolerance in Haq Nawaz in comparisons to the CIMMYT PAK. Phosphorus is necessary for plant water use efficiency and stomatal control (Aqaei *et al*., 2020; Robredo *et al*., 2011). Results illustrated the significantly ($p<0.05$) increase of potassium content in H. Nawaz and C. PAK in comparisons to the control conditions. The increase in potassium content was 5.72% and 6.77% in C. PAK and H. Nawaz, respectively. During water deficient conditions potassium work as an osmoticum, to maintain plant potential under drought stress (K. Zare *et al*., 2014).

Different studies have been conducted to check the expression level of Glutamine synthetase gene in different crops (James *et al*., 2018). In this study expression level of glutamine synthetase drought responsive gene was analyzed in two Pakistan local cultivars, Haq Nawaz and CIMMYT PAK. A comprehensive analysis of glutamine synthetase gene in different crops *Zea mays, Oryza sativa, Triticum aestivum, Hordeum volgari, Saccharum spontanum, Sorghum bicolor, Setaria italica*, and *Arabidopsis thaliana* was performed to explore the roles of GS gene family, that can be helpful for future studies. GWAS helpful for identification of candidate genes involved in biological pathways during drought stress in different crops. (Korte *et al*., 2013). Bioinformatics analysis were performed to identify the evolutionary relationship of GS genes among species. Genome wide association studies (GWAS) applied to identify the drought associated variation of traits (J. Xu *et al*., 2014). In identified drought responsive genes, a pattern of conservation was also found. Phylogenetic tree, Protein motif distribution and gene structure of all GS genes, demonstrate that GS genes were highly conserved during evolutionary period of various plant species. *Zea mays glutamine synthetase 2 (GLN2*) gene show a conserved pattern to other reported drought responsive glutamine synthetase genes of other crops. In our analysis, under drought stress *GLN2* gene expression was more in Haq Nawaz and CIMMYT PAK in leaves and root tissues, in comparisons to the control conditions. Under drought stress the upregulation of *GLN2* gene was also supported by other researchers at Expression Atlas (https://www.ebi.ac.uk/gxa/home) (Opitz *et al*., 2014; Zheng *et al*., 2010). Results based on ΔCT value illustrated that *GLN2* expression was more in Haq Nawaz than CIMMYT PAK, indicating that H. Nawaz has the potential to tolerate drought stress at grain filling stage. Higher expression of *GLN2* in Haq Nawaz suggested that it consequently helping the maize plant to endure drought stress and this gene is highly involved in drought response pathways to make the plants tolerant to water deficient conditions.

Findings of this research demonstrates the importance of Zm00001d033747 gene during the drought stress. Further studies should be carried out to investigate the mechanisms of the Zm00001d033747 function in drought stress, and to provide new insights into the drought resistance.

FUTURE PROSPECTIVE

Future Prospective

Drought is a major limitation to maize (*Zea mays*) production worldwide. The present study was designed to investigate the effects of drought stress under different developmental stages on morpho-physiological, biochemical, and expression analysis of *glutamine synthetase 2* gene in two maize cultivars as compared to control. Meanwhile, identification of drought responsive gene orthologs were also confirmed through computational tools. Based on results, maize cultivar Haq Nawaz performed well in all the studied traits as compared to CIMMYT PAK cultivar. Haq Nawaz cultivar showed drought tolerance (mild to moderate) response while CIMMYT PAK exhibited drought sensitive behavior in the studied traits. Further molecular analysis are required to confirm the mechanism of action of *GLN2* gene under drought stress condition. The effect of drought stress under open field conditions for Haq Nawaz cultivar are also required to validate drought tolerance at maize growing areas in the country. Beside these two cultivars, the response of other maize high yielding varieties need to be tested under drought condition. Moreover, *GLN2* gene transformation into model plant is required for functional characterization in the future.

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APPENDICES

Appendix 1: ANOVA for the plant height of Haq Nawaz and CIMMYT PAK at seedling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	6.292	3.1458		
Treatment	1	44.083	44.0833	23.25	0.0029
Variety	1	56.333	56.3333	29.71	0.0016
Treatment*Variety	1	0.083	0.0833	0.04	0.8409
Error	6	11.375	1.8958		
Total	11	118.167			

Appendix 2: ANOVA for the plant height of Haq Nawaz and CIMMYT PAK at flowering developmental stage.

Source	DF	SS	MS	F	P
Reps	2	10.500	5.2500		
Treatment	1	24.083	24.0833	51.00	0.0004
Variety	1	60.750	60.7500	128.65	0.0000
Treatment*Variety	1	2.083	2.0833	4.41	0.0804
Error	6	2.833	0.4722		
Total	11	100.250			

Appendix 3: ANOVA for the plant height of Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	86.54	43.27		
Treatment	1	266.02	266.02	12.51	0.0123
Variety	1	2537.52	2537.52	119.30	0.0000
Treatment*Variety	1	28.52	28.52	1.34	0.2909
Error	6	127.63	21.27		
Total	11	3046.23			

Appendix 4: ANOVA for the root length of Haq Nawaz and CIMMYT PAK at seedling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	2.6667	1.3333		
Treatment	1	18.7500	18.7500	24.11	0.0027
Variety	1	36.7500	36.7500	47.25	0.0005
Treatment*Variety	1	2.0833	2.0833	2.68	0.1528
Error	6	4.6667	0.7778		
Total	11	64.9167			

Appendix 5: ANOVA for the root length of Haq Nawaz and CIMMYT PAK at flowering developmental stage.

Source	DF	SS	MS	F	P
Reps	2	13.167	6.583		
Treatment	1	52.083	52.083	110.29	0.0000
Variety	1	126.750	126.750	268.41	0.0000
Treatment*Variety	1	0.083	0.083	0.18	0.6891
Error	6	2.833	0.472		
Total	11	194.917			

Appendix 6: ANOVA for the root length of Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	5.6517	2.8258		
Treatment	1	29.7675	29.7675	32.19	0.0013
Variety	1	13.0208	13.0208	14.08	0.0095
Treatment*Variety	1	6.9008	6.9008	7.46	0.0341
Error	6	5.5483	0.9247		
Total	11	60.8892			

Appendix 7: ANOVA for the leaf length of Haq Nawaz and CIMMYT PAK at seedling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	14.000	7.000		
Treatment	1	270.750	270.750	25.65	0.0023
Variety	1	200.083	200.083	18.96	0.0048
Treatment*Variety	1	2.083	2.083	0.20	0.6724
Error	6	63.333	10.556		
Total	11	550.250			

Appendix 8: ANOVA for the leaf length of Haq Nawaz and CIMMYT PAK at flowering developmental stage.

Source	DF	SS	MS	F	P
Reps	2	3.500	1.750		
Treatment	1	192.000	192.000	21.67	0.0035
Variety	1	432.000	432.000	48.75	0.0004
Treatment*Variety	1	0.333	0.333	0.04	0.8526
Error	6	53.167	8.861		
Total	11	681.000			

Appendix 9: ANOVA for the leaf length of Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	78.167	39.083		
Treatment	1	705.333	705.333	357.63	0.0000

Variety	1	192.000	192.000	97.35	0.0001
Treatment*Variety	1	5.333	5.333	2.70	0.1512
Error	6	11.833	1.972		
Total	11	992.667			

Appendix 10: ANOVA for the leaf width of Haq Nawaz and CIMMYT PAK at seedling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.02167	0.01083		
Treatment	1	0.85333	0.85333	40.96	0.0007
Variety	1	0.16333	0.16333	7.84	0.0312
Treatment*Variety	1	0.00333	0.00333	0.16	0.7030
Error	6	0.12500	0.02083		
Total	11	1.16667			

Appendix 11: ANOVA for the leaf width of Haq Nawaz and CIMMYT PAK at flowering developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.03167	0.01583		
Treatment	1	0.12000	0.12000	2.34	0.1773
Variety	1	0.16333	0.16333	3.18	0.1249
Treatment*Variety	1	0.00333	0.00333	0.06	0.8075
Error	6	0.30833	0.05139		
Total	11	0.62667			

Appendix 12: ANOVA for the leaf width of Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.6867	0.34333		
Treatment	1	8.3333	8.33333	92.59	0.0001

Variety	1	0.5633	0.56333	6.26	0.0464
Treatment*Variety	1	0.5633	0.00333	0.04	0.8537
Error	6	0.5400	0.09000		
Total	11	10.1267			

Appendix 13: ANOVA for the fresh biomass (g) of Haq Nawaz and CIMMYT PAK at seedling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	1.167	0.583		
Treatment	1	120.333	120.333	53.48	0.0003
Variety	1	16.333	16.333	7.26	0.0358
Treatment*Variety	1	1.333	1.333	0.59	0.4706
Error	6	13.500	2.250		
Total	11	152.667			

Appendix 14: ANOVA for the fresh biomass (g) of Haq Nawaz and CIMMYT PAK at flowering developmental stage.

Source	DF	SS	MS	F	P
Reps	2	4.362	2.1808		
Treatment	1	83.213	83.2133	14.63	0.0087
Variety	1	84.270	84.2700	14.82	0.0085
Treatment*Variety	1	6.163	6.1633	1.08	0.3380
Error	6	34.118	5.6864		
Total	11	212.127			

Appendix 15: ANOVA for the fresh biomass (g) of Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	41.78	20.890		
Treatment	1	792.19	792.188	116.25	0.0000
Variety	1	245.71	245.708	36.06	0.0010

Treatment*Variety	1	1.40	1.401	0.21	0.6662
Error	6	40.89	6.814		
Total	11	1121.96			

Appendix 16: ANOVA for the dry biomass (g) of Haq Nawaz and CIMMYT PAK at seedling developmental stage.

Source	DF	SS	MS	F	P
Reps	2				
Treatment	1	3.74083	3.74083	130.75	0.0000
Variety	1	0.60750	0.60750	21.23	0.0037
Treatment*Variety	1	0.00750	0.00750	0.26	0.6269
Error	6	0.17167	0.02861		
Total	11	4.60917			

Appendix 17: ANOVA for the dry biomass (g) of Haq Nawaz and CIMMYT PAK at flowering developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.1217	0.06083		
Treatment	1	4.4408	4.44083	21.75	0.0035
Variety	1	6.6008	6.60083	32.33	0.0013
Treatment*Variety	1	0.0208	0.02083	0.10	0.7602
Error	6	1.2250	0.20417		
Total	11	12.4092			

Appendix 18: ANOVA for the dry biomass (g) of Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	19.7117	9.8558		
Treatment	1	32.6700	32.6700	18.77	0.0049
Variety	1	26.4033	26.4033	15.17	0.0080
Treatment*Variety	1	0.7500	0.7500	0.43	0.5359
Error	6	10.4417	1.7403		
Total	11	89.9767			

Appendix 19: ANOVA for ear length of Haq Nawaz and CIMMYT PAK at Kernel Blister Developmental stage (R2).

Source	DF	SS	MS	F	P
Reps	2	15.0467	7.52333		
Treatment	1	42.5633	42.5633	25.30	0.0024
Variety	1	11.2133	11.2133	6.67	0.0417
Treatment*Variety	1	2.027	2.027E-30	0.00	1.0000
Error	6	10.0933	1.68222		
Total	11	78.9167			

Appendix 20: ANOVA for ear length of Haq Nawaz and CIMMYT PAK at Kernel Milk Developmental stage (R3).

Source	DF	SS	MS	F	P
Reps	2	6.2467	3.1233		
Treatment	1	72.0300	72.0300	230.70	0.0000

Variety	1	11.2133	11.2133	35.91	0.0010
Treatment*Variety	1	0.0533	0.0533	0.17	0.6938
Error	6	1.8733	0.3122		
Total	11	91.4167			

Appendix 21: ANOVA for silk length of Haq Nawaz and Commit at Kernel Blister Developmental stage (R2).

Source	DF	SS	MS	F	P
Reps	2	7.0350	3.5175		
Treatment	1	19.5075	19.5075	174.26	0.0000
Variety	1	18.0075	18.0075	160.86	0.0000
Treatment*Variety	1	0.0008	0.0008	0.01	0.9341
Error	6	0.6717	0.1119		
Total	11	45.2225			

Appendix 22: ANOVA for silk length of Haq Nawaz and CIMMYT PAK at Kernel Milk Developmental stage (R3).

Source	DF	SS	MS	F	P
Reps	2	3.980	1.9900		
Treatment	1	96.333	96.3333	123.15	0.0000
Variety	1	4.083	4.0833	5.22	0.0624
Treatment*Variety	1	0.750	0.7500	0.96	0.3653
Error	6	4.693	0.7822		
Total	11	109.840			

Appendix 23: ANOVA for cob diameter of Haq Nawaz and CIMMYT PAK at Kernel Blister Developmental stage (R2).

Source	DF	SS	MS	F	P
Reps	2	0.21167	0.10583		
Treatment	1	1.47000	1.47000	30.59	0.0015
Variety	1	0.21333	0.21333	4.44	0.0797
Treatment*Variety	1	0.01333	0.01333	0.28	0.6173
Error	6	0.28833	0.04806		
Total	11	2.19667			

Appendix 24: ANOVA for cob diameter of Haq Nawaz and CIMMYT PAK at Kernel Milk Developmental stage (R3).

Source	DF	SS	MS	F	P
Reps	2	0.15167	0.07583		
Treatment	1	7.36333	7.36333	117.81	0.0000
Variety	1	0.21333	0.21333	3.41	0.1142
Treatment*Variety	1	0.01333	0.01333	0.21	0.6604
Error	6	0.37500	0.06250		
Total	11	8.11667			

Appendix 25: ANOVA for cob length of Haq Nawaz and CIMMYT PAK at Kernel Blister Developmental stage (R2).

Source	DF	SS	MS	F	P
Reps	2	2.3817	1.1908		
Treatment	1	2.2533	2.2533	3.63	0.1054
Variety	1	14.9633	14.9633	24.10	0.0027
Treatment*Variety	1	19.2533	19.2533	31.01	0.0014
Error	6	3.7250	0.6208		
Total	11	42.5767			

Appendix 26: ANOVA for cob length of Haq Nawaz and CIMMYT PAK at Kernel Milk Developmental stage (R2).

Source	DF	SS	MS	F	P
Reps	2	1.2117	0.6058		
Treatment	1	85.3333	85.3333	337.95	0.0000
Variety	1	2.8033	2.8033	11.10	0.0158
Treatment*Variety	1	0.2133	0.2133	0.84	0.3935
Error	6	1.5150	0.2525		
Total	11	91.0767			

Appendix 27: ANOVA for the Chlorophyll a content in Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.2236	0.1118		
Treatment	1	10.3305	10.3305	153.81	0.0000
Variety	1	2.7629	2.7629	41.14	0.0007
Treatment*Variety	1	0.0406	0.0406	0.60	0.4664
Error	6	0.4030	0.0672		
Total	11	13.7606			

Appendix 28: ANOVA for the Chlorophyll b content in Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.00730	0.00365		
Treatment	1	0.08265	0.00365	56.52	0.0003
Variety	1	4.17897	4.17897	2857.71	0.0000
Treatment*Variety	1	0.01373	0.01373	9.39	0.0221
Error	6	0.00877	0.00146		
Total	11	4.29143			

Appendix 29: ANOVA for the membrane stability index in Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	2.375	1.187		

Treatment	1	50.021	50.021	14.91	0.0083
Variety	1	513.521	513.521	153.10	0.0000
Treatment*Variety	1	11.021	11.021	3.29	0.1198
Error	6	20.125	3.354		
Total	11	597.063			

Appendix 30: ANOVA for the praline content in Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.0092	0.0046		
Treatment	1	6.2352	6.2352	60.40	0.0002
Variety	1	17.7390	17.7390	171.84	0.0000
Treatment*Variety	1	0.2269	0.2269	2.20	0.1887
Error	6	0.6194	0.1032		
Total	11	24.8297			

Appendix 31: ANOVA for the total soluble sugar content in Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.0557	0.0278		
Treatment	1	0.6922	0.6922	87.00	0.0001
Variety	1	17.0981	17.0981	2149.00	0.0000
Treatment*Variety	1	0.1117	0.1117	14.05	0.0095
Error	6	0.0477	0.0080		
Total	11	18.0054			

Appendix 32: ANOVA for the nitrogen content in Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.00455	0.00228		
Treatment	1	0.03741	0.03741	13.53	0.0103
Variety	1	0.04201	0.04201	15.20	0.0080
Treatment*Variety	1	0.00008	0.00008	0.03	0.8746
Error	6	0.01658	0.00276		
Total	11	0.10063			

Appendix 33: ANOVA for the potassium content in Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.00362	0.00181		
Treatment	1	0.06453	0.06453	20.33	0.0041
Variety	1	0.03203	0.03203	10.09	0.0192
Treatment*Variety	1	0.00053	0.00053	0.17	0.6961
Error	6	0.01905	0.00317		
Total	11	0.11977			

Appendix 34: ANOVA for the phosphorus content in Haq Nawaz and CIMMYT PAK at grain filling developmental stage.

Source	DF	SS	MS	F	P
Reps	2	0.00062	0.00031		
Treatment	1	0.00333	0.00333	4.49	0.0783
Variety	1	0.02613	0.02613	35.24	0.0010
Treatment*Variety	1	0.00053	0.00053	0.72	0.4290
Error	6	0.00445	0.00074		
Total	11	0.03507			

Appendix 35: Gene Ids of 25 glutamine synthetase genes from poacea family.

Gene Name	Gene Ids
Zm-GS1-2	Zm00001d033747
Zm-GS1-5	Zm00001d048050
Zm-GS1-3	Zm00001d017958
Zm-GS1-4	Zm00001d051804
Zm-GS1-1	Zm00001d028260
Traes-GS1-6B	TraesCS6B02G327500.1
Traes-GS1-6A	TraesCS6A02G298100.2
Traes-GS1-2B	TraesCS2B02G528300.1
Traes-GS2-2A	TraesCS2A02G500400.1
SOR-GS1	OQU92979
SOR-GS2	KXG30808
SETIT-GS1	KQL31407
Ssp-GS-1A	Sspon.01G0007760-1A
Ssp-GS-1P	Sspon.01G0007760-1P
Ssp-GS-2B	Sspon.01G0007760-2B
Ssp-GS-2D	Sspon.01G0026870-2B
Ssp-GS-3D	Sspon.05G0021990-3D
Ssp-GS-4D	Sspon.04G0004050-4D
OsGS2	Os04t0659100-01
OsI_08842	BGIOSGA005667-TA
HV-GS1	HORVU4Hr1G066860.1
HV-GS2	HORVU4Hr1G007610.1
HV-GS3	HORVU6Hr1G074030.2
AT-GSR2	AT1G66200.3
At5g16570	AT5G16570.1

Appendix 36: RNA dilution table for cDNA preparation

Sr. #	Varieties	Nucleic Acid Conc. (ng/µl)	RNA Dilution	
			RNA + H_2O	
1	Naq Nawaz LC	754.95	300/754.95× 20	7.94 µl + 4.06 µl
2	Naq Nawaz LD	576.64	300/576.64× 20	10.40 µl + 1.6 µl
3	CIMMYT PAK LC	929.87	300/929.87× 20	6.45 µl + 5.55µl
4	CIMMYT PAK LD	925.46	300/925.46× 20	6.48 µl + 5.52 µl
5	Naq Nawaz RC	375.63	300/375.63× 20	15.97µl

6	**Naq Nawaz RD**	556.82	300/556.82× 20	10.77µl + 1.23 µl
7	**CIMMYT PAK RC**	501.82	300/501.82× 20	11.95 µl
8	**CIMMYT PAK RD**	785.66	300/785.66× 20	7.636 µl + 4.364 µl

Appendix 37: cDNA sample dilution for rt-PCR

Sr. #	**Varieties**	**Nucleic Acid Conc. (ng/µl)**	**cDNA Dilution**	
			cDNA + H_2O	
1	**Naq Nawaz LC**	4053	50/4053× 50	0.6168 + 49.38
2	**Naq Nawaz LD**	4053	50/4053× 50	0.6168 + 49.38
3	**CIMMYT PAK LC**	3916.99	50/3916.99× 50	0.63824 + 49.36
4	**CIMMYT PAK LD**	3754.86	50/3754.86× 50	0.66580 + 49.33
5	**Naq Nawaz RC**	3759.83	50/3759.83× 50	0.66492 + 49.34
6	**Naq Nawaz RD**	3458.73	50/3458.73× 50	0.7228 + 49.28
7	**CIMMYT PAK RC**	3675.03	50/3675.03× 50	0.68026 + 49.32
8	**CIMMYT PAK RD**	3814.23	50/3814.23× 50	0.65544 + 49.34