Influence of specific abiotic stress factors on durum wheat gluten proteins and their relation with pasta quality

By

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SUMMARY

In recent years, breeding for high quality durum wheat has become an important research focus due to an increase in demand for good quality products. Wheat storage proteins, termed gluten, have a major influence on dough quality characteristics, such as visco-elasticity. The gluten proteins are strongly influenced by genotype, environment and genotype by environment interaction. This may consequently cause variation in quality. Limited information is available on the impact of genotype, environment and their interaction on durum wheat gluten proteins. Most research has focused on the effects of environmental conditions on bread wheat quality. In order to improve durum wheat quality, it is important to understand the impact of environmental conditions on durum wheat gluten proteins. In this study, six durum wheat cultivars were planted in Mexico under six environmental conditions: optimum, flood irrigation, moderate heat, severe heat, moderate drought and severe drought. The aim of this study was to determine the effect of heat and drought stress on protein quantity and quality, using size exclusion-high performance liquid chromatography (SE-HPLC), reversed phase-high performance liquid chromatography (RP-HPLC) and proteomics techniques. Flour protein content was significantly increased by all stress treatments. High flour protein content was observed under severe heat and drought stress conditions. There were significant differences between genotypes for protein fractions separated by SE-HPLC. All stress treatments caused a significant reduction in extractable and unextractable albumin/globulin protein fractions, as well as in extractable and unextractable low molecular weight (LMW) gluten proteins. Severe drought stress caused an increase in large SDS-unextractable polymeric proteins. Moderate and severe heat stress significantly increased SDS-extractable gliadin. Genotype effect was large for extractable LMW for all environments except for severe drought. The SDS-unextractable LMW correlated significantly with alveograph strength (alvW) and mixograph peak time (MPT).

RP-HPLC indicated a significant reduction in LMW glutenins and γ-gliadin, and an increase in α-gliadins for all stress treatments. Genotype affected α-gliadins more than environment and genotype by environment interaction, indicating a strong genetic influence on the expression of these gliadins. The γ-gliadins showed positive significant correlations with alvW, alvP and MPT for most treatments. The ω- and α-gliadins had a negative relationship with alveograph parameters.

Proteomic analysis revealed 330 protein spots differentially expressed in cultivar AtilC2000, with the largest number of spots (14.24%) up-regulated by all stress conditions. In Mexicali75, 205

spots were differentially expressed and most protein spots (35.12%) increased due to moderate drought stress conditions. Highly up-regulated protein spots were analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) followed by database searching. Of these, some were HMW-GS, gliadins, serpins, and β-amylase involved in carbohydrate metabolism. Drought and heat significantly altered the gluten protein composition, by up-regulating HMW and gliadin proteins in the two cultivars. Generally, cultivars differed in terms of their reaction to stress conditions. This suggests that the effects of stress cannot be generalised in durum wheat.

Key words: Abiotic stress, durum wheat quality, glutenin, size exclusion-high performance liquid chromatography, reversed phase-performance liquid chromatography, proteomics

DECLARATION

I declare that the thesis hereby submitted by me for the degree of Philosophiae Doctor in Plant Breeding at the University of the Free State (UFS) is my own independent work and has not previously been submitted by me at another university/faculty.

I further cede copyright of the thesis in favour of the UFS.

Keneuoe Phakela K Phakela January 2021

DEDICATION

To my late daughter Reithabetse Phakela. Life has to go on without you, I have to hide my heartache and will always remember the laughter we shared throughout six weeks.

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ABBREVIATIONS

µl	Microliter
α	Alpha
β	Beta
ϒ	Gamma
ω	Omega
^{o}C	Degrees Celsius
1 DE	One dimensional gel electrophoresis
2 DE	Two dimensional gel electrophoresis
a	Redness
AACC	American Association of Cereal Chemists
AG	Albumin-globulins
alvL	Alveograph extensibility
alvP	Alveograph tenacity
alvP/L	Alveograph tenacity to extensibility ratio
alvW	Alveograph strength
b	Yellowness
cm	Centimetre
cm^3	Cubic centimetre
CHAPS	Cholamidopropyl dimethylammonio propanesulfonate
CIMMYT	International Maize and Wheat Improvement Center
CV	Coefficient of variation
Da	Daltons
DDT	Dough development time
DTT	Dithiothreitol
DNA	Deoxyribonucleic acid
E	Environment
exAG	Extractable globulins-albumins
EDTA	Ethylenediaminetetraacetic acid
exGLI	Extractable gliadins
exHMW	Extractable high molecular weight
exLMW	Extractable low molecular weight
ESI	Electrospray ionisation
FAO	Food and Agriculture Organization
FPC	Flour protein content
FTICR	Fourier transform ion cyclotron resonance
FY	Flour yield
g	Gram
G	Genotype
GxE	Genotype by environment
GLI	Gliadins
GLU/GLI	Glutenin to gliadin ratio
g/L	Gram per litre
GQQ	Tripeptides

GS	Glutenin subunits
GV	Genetic variance
GYYPTLSQQ	Nanopeptides
HCl	Hydrochloric acid
HMW	High molecular weight
HMW-GS	High molecular weight glutenin subunits
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IPG	Immobilized potential hydrogen gradient
IGC	International Grain Council
IPO	International Pasta Organisation
IT	Ion trap
L	Brightness
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography tandem mass-spectrometry
LMW	Low molecular weight
LMW-GS	Low molecular weight glutenin subunits
LMW-i	Low molecular weight-isoleucine
LMW-m	Low molecular weight-methionine
LMW-s	Low molecular weight-serine
LSD	Least significant difference
LUPP	Large unextractable polymeric proteins
LV	Loaf volume
M	Meter
MALDI	Matrix assisted laser desorption
MALDI-TOF	Matrix assisted laser desorption-time of light
MDT	Mixograph development time
mg	Milligram
mm	Millimetre
min	Minutes
ml/L	Millilitre per litre
MPT	Mixograph peak time
MS	Mass spectrometry
mRNA	Messenger-ribonucleic acid
nano-RP-HPLC-ESI-IT-MS/MS	Nano-reversed phase-high performance liquid chromatography-electrospray ionization-ion trap tandem mass-spectrometry
NBC	Narrow bore column
NIR	Near infrared
Nm	Nanometre
PGQGQQ	Hexapeptides
PDA	Photometric diode array
pH	Potential hydrogen
pI	Isoelectric point
PMT	Post translational modification
RCBD	Randomised complete block design

RP-HPLC	Reversed phase-high performance liquid chromatography
RP-HPLC/nESI-MS/MS	Reversed phase-high performance liquid chromatography-electrospray ionization tandem mass-spectrometry
rpm	Revolutions per minute
RNA	Ribonucleic acid
SAGL	South African Grain Laboratory
sec	Seconds
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SDSVOL	Sodium dodecyl sulphate sedimentation volume
SE-HPLC	Size exclusion-high performance liquid chromatography
S-S	Disulphide bond
SSA	Sub-Saharan Africa
TCEP	Triscarboxylethyl phosphine
TFA	Trifluoroacetic acid
TKW	Thousand kernel weight
TOF	Time of flight
UFS	University of the Free State
unAG	Unextractable albumins-globulins
unGLI	Unextractable gliadins
unHMW	Unextractable high molecular weight
unLMW	Unextractable low molecular weight
UPP	Unextractable polymeric proteins
UV	Ultraviolent
VK	Vitreous kernels
v/v	Volume per volume
v/w	Volume per weight

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CHAPTER 1

Introduction

Durum wheat is an economically important cereal crop grown throughout the world, with an estimated annual production of 36 million ton (Sall et al. 2019). Durum grain is used as a staple food crop in a number of countries. It is an important source of energy and protein, and supplies a range of minerals (such as calcium, magnesium, iron, zinc, copper, manganese, molybdenum, boron) essential for the human diet. Durum wheat is used for the preparation of food products, such as bread, couscous, freekeh, bulgur and most importantly, pasta (Pompa et al. 2013; Guzmán et al. 2016).

The importance of wheat, compared to other cereals, lies in its unique physiochemical properties. When wheat flour is mixed with water, proteins bind to form a gluten mass. Gluten gives dough its ability to trap gas and stretch, both aspects necessary to produce wheat based products (Barak et al. 2015). In durum wheat, gluten gives the dough the necessary cohesiveness to be extruded to form different pasta shapes when dried. Gluten is the primary element of protein in wheat grain, and consists of monomeric gliadins and polymeric glutenins and makes up about 80-85% of total wheat protein (Wieser 2007; Barak et al 2015). Glutenin proteins are further divided into low molecular weight (LMW) and high molecular weight (HMW) glutenins. Gliadins are another important constituent of the gluten complex and is the most abundant protein contained in the wheat seed. Gliadins are divided into α-, γ-, and ω-subunits. Both glutenins and gliadins contribute towards durum wheat quality, and confer dough visco-elastic properties (Yang et al 2011; Labuschagne et al. 2016). Considering this, these proteins can be used to assess quality of durum wheat (Li Vigni et al. 2013). The contribution made by different types of protein in determining the rheological properties of the wheat dough is caused by the different structural properties of the polypeptides (size and the number as well as position of cysteine groups) (Wieser 2007; Malik et al. 2013).

Temperature and drought are implicated in causing considerable changes in the accumulation of gluten proteins during the grain-filling period, which affect dough rheological characteristics, resulting in variations in durum wheat quality (Lindon et al. 2014). Higher temperatures were reported to cause an increase in HMW-GS, α-gliadins, and a decrease in LMW-GS in bread wheat (DuPont et al. 2006). This also affects the market value of durum wheat. However, the industry

requires durum wheat semolina with consistent quality to produce a good end-product (Pinheiro et al. 2014; Guzmán et al. 2016). A better understanding of the influence of environment on gluten protein composition could shed light on fluctuations in pasta making quality. This could increase the possibilities for breeding and growing wheat cultivars with good, consistent and uniform quality. It is important to determine and quantify the extent to which factors like the environment, genotype and the interaction between the two, contribute to variations in durum wheat quality parameters (Aghagholizadeh et al. 2017).

Unlike in common wheat, where several studies on environmental stress conditions on wheat proteins have been done, less information is available on the effect of environmental stresses on storage proteins in durum wheat. Much focus has been on metabolic proteins, especially in durum wheat. Limited information is available on the effects of excess or lack of water, on durum wheat quality (Li et al. 2013).

Aim of the study

This study aimed to determine the effects of environmental stress conditions (different levels of heat and drought stress) on gluten proteins of durum wheat by a gluten protein analysis approach. The individual effects of heat and drought stress on the gluten proteins in the flour of mature wheat grain, were compared, and consequently related to the primary quality indicators of durum wheat quality.

Specific objectives

- To determine the influence of different levels of heat and drought stress, compared to optimal conditions and flood irrigation, in two consecutive years, on gluten protein fractions separated by size exclusion-high performance liquid chromatography (SE-HPLC) and reversed phase-high performance liquid chromatography (RP-HPLC)
- To correlate protein fractions separated by SE-HPLC and RP-HPLC with pasta making quality characteristics under different growing conditions
- To separate protein fractions using two dimensional gel electrophoresis (2-DE) followed by liquid chromatography tandem mass-spectrometry (LC-MS/MS) and identify differentially expressed proteins under different stress conditions
- To do peptide sequencing and protein identification through library matching for differentially expressed proteins

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CHAPTER 2

Literature review

2.1 Classification of wheat

Wheat belongs to the family Gramineae (*Poaceae*), genus *Triticum* (Shewry 2009). Wheat is believed to have originated in South Eastern Turkey from wild grasses. Wheat was first grown as a crop about 10 000 years ago, during the Neolithic revolution, from hunting-gathering to settled agriculture (Feldman 2001; Shewry 2009). Both einkorn and emmer were the earliest forms of cultivated wheat.

The genus *Triticum* encompasses species that are polyploid, with the basic chromosome number of 7 (x = 7) (Feldman 2001; Peng et al. 2011). Wheat is divided into three major groups based on their chromosome number, diploids (2n = 2x =14), tetraploids (2n = 4x =28) and hexaploids (2n = 6x = 42). The wild forms of einkorn (diploid) are the source of the A genome found in both tetraploid and hexaploid wheat. *Aegilops speltoides* Tausch or closely related species is the source of the B genome found in tetraploid and hexaploid wheat. The D genome found in hexaploid wheat is related to *T. tauschi* as indicated in Figure 2.1 (Feldman 2001; Shewry 2009; Peng et al. 2011). Durum wheat, *T. turgidum* spp *durum* (Desf.) Hasn. evolved from domesticated emmer wheat, *T. turgidum* spp *dicoccum* (Schrank ex. Schübl) Thell. (Maccaferri et al. 2019).

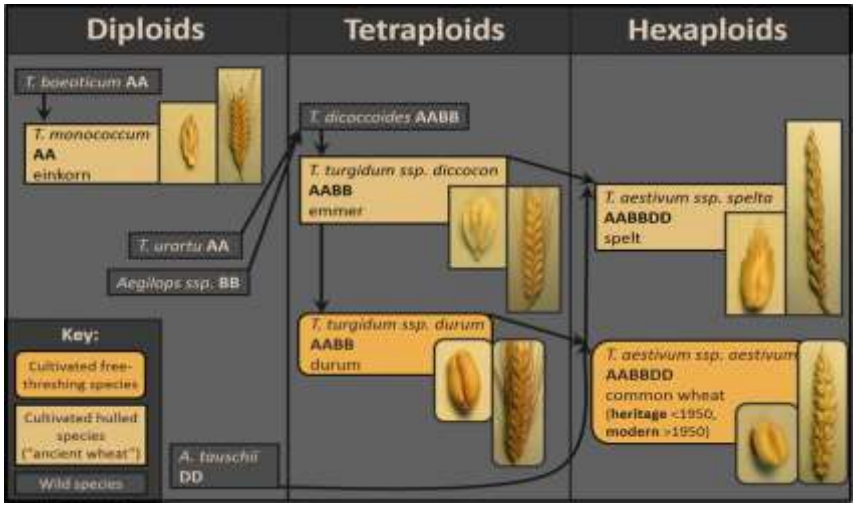

Figure 2.1 Schematic presentation of the evolution of wheat

2.2 Importance of durum wheat

As a world crop, wheat is an economically important cereal in terms of production and use (Halford et al. 2014). Among the cultivated wheat species, bread (*T. aestivum*) and durum (*T. durum*) are the most widely grown. The estimated global durum wheat production is estimated around 38 million tons, grown on about 13 million hectares in the 2017/2018 season. The largest world durum wheat producing countries in the 2016/2017 season were the European Union (9.5 million ton), Canada (7.8 million ton) and Italy (5.4 million ton) (Mutwali et al. 2016; International Grain Council (IGC) 2017). Globally, durum wheat accounts for about 5% of the total wheat production (IGC 2020). In the Mediterranean region durum is very important, where it is consumed as part of a staple diet, and its production covers between 50-90% of the total area used for wheat production (Guzmán et al. 2016). In Africa, northern Africa is the major durum wheat producer (IGC 2017). In the sub-Saharan African countries (SSA), Ethiopia is the largest durum wheat producer on approximately 0.6 million hectares (Sall et al. 2019). In South Africa durum wheat is produced on a very small scale in the Western Cape in the Fish River Valley, but most durum wheat is imported (De Kock 2012).

Durum wheat is used to make a wide variety of food such as freekeh, couscous, bulgur, bread and pasta (Sissons 2008; Guzmán et al. 2016). It is best suited for making pasta because of its hardness, golden amber colour and high protein content (Bechere et al. 2002; Sissons 2008). Pasta is a common name for end-products such as spaghetti, macaroni, tagliatelle and noodles (Ficco et al. 2014). In the Mediterranean countries, Middle East and North Africa, durum is usually used for bread-making (Boggini et al. 1995; Licciardello et al. 2013). Pasta is preferred by many people because it is nutritious (rich in protein, minerals and vitamins), easy to transport, has a good taste and can be stored for a long period (Tudorica et al. 2002; Kumar et al. 2011). Pasta has gradually gained popularity in current years, therefore the demand for durum wheat is likely to increase (Guzmán et al. 2016).

The demand for durum wheat as a raw material for pasta has increased due to an increase in the world population (Mutwali et al. 2016; IGC 2017). According to the International Pasta Organization (IPO 2013), the three major world leading pasta producing countries include Italy, the United States of America and Turkey. The highest pasta consumption is in Italy, followed by Venezuela and Tunisia.

2.3 Wheat storage proteins

Wheat proteins are important components of the endosperm, found in the seed (Shewry 2009). Wheat proteins affect ability of the flour to be used for a particular product (Žilić et al. 2011; Pompa et al. 2013). Wheat contains four types of proteins, depending on their solubility in various solvents. These include albumins soluble in water, globulins soluble in dilute saline solutions, gliadins soluble in alcohol mixtures and glutenins soluble in basic solutions (Arena et al. 2017). The albumins and globulins form about 20-25% of the wheat grain protein (Žilić et al. 2011). Gliadins and glutenins constitute the major part of wheat protein, approximately 80% (Pompa et al. 2013). Gliadin and glutenin together make gluten, which is regarded as a major factor governing pasta quality. They affect visco-elastic properties of dough (Giuliani et al. 2015). The glutenins are composed of HMW and LMW subunits (Shewry et al. 2002; Wieser 2007).

2.3.1. High molecular weight-glutenin subunits

High molecular weight-glutenin subunits (HMW-GS) are important components of the glutenin polymer and make up about 5-10% of grain protein (Shewry and Halford 2002). The size and the structure of HMW-GS are related to dough rheological characteristics. The HMW-GS molecular weights range between 80 000-120 000 Daltons (Da) (Gianibelli et al. 2001; Gao et al. 2010). The HMW-GS are divided into x-type and y-type based on their electrophoretic mobility in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The x-type has slower mobility than the y-type. The HMW-GS have high amounts of glutamine (37-39%), glycine (14-19%) and proline (12-14%) (Shewry and Tatham 1990; Santagati et al. 2016). The amino acid sequences of HMW-GS consist of three structural parts, the central part, which is repetitive, flanked by non-repetitive N and C terminal parts. The repetitive part is made up of hexapeptides (PGQGQQ) and nanopeptides (GYYPTSLQQ) in the y-type subunits and tripeptide repeats (GQQ) only found in the x-type subunits. The N terminal and central parts are comprised of high amounts of cysteine residues, 80-105 and 480-700 for the N terminal and central terminal, respectively. The C domain has 42 cysteine residues that are non-variable (Shewry and Tatham 1990; Wieser 2007). The cysteine residues play an important role in structure and functionality of glutenin proteins. The cysteine amino acid group has the potential to form disulfide bonds that make proteins stable and allow them to fold correctly. The differences in the structural domain are responsible for variations in functionality of glutenins (Shewry et al. 2002; Wieser 2007).

In durum wheat, HMW-GS are controlled by genes found on the long arm of group 1 chromosomes of the A and B genomes. These genes are located at the *Glu-A1* and *Glu-B1* loci (Payne and

Lawrence 1983; Babay et al. 2015). Each locus comprises of two genes linked to x- and y-type HMW-GS. *Glu-A1* is silent in the majority of durum wheat genotypes (Branlard et al. 1989). Certain HMW-GS have been indicated to affect pasta quality. HMW-GS *Glu-B1* 20 has been related to poor pasta making properties (Carrillo et al. 2000; Racit et al. 2003; Sissons et al. 2005; Nazco et al. 2014). Subunits 7+8 of *Glu-B1* are associated with good gluten strength in durum wheat (Sissons et al. 2005; Pompa et al. 2013; Giuliani et al. 2015). Pirozi et al. (2008) indicated that high gluten strength observed in lines with subunit 7 might be associated with a large number of cysteine residues, which participate in the formation of intermolecular disulphide bonds, thus extending the glutenin polymer (chain extender). Lines with subunit 7 had four cysteine residues, while lines with subunit 20 had two cysteine residues in bread wheat (Pirozi et al. 2008) and durum wheat (Santagati et al. 2016). Brites and Carrillo (2001) reported that cultivars with HMW-GS *Glu-B1* 14 + 15 had higher SDS-sedimentation volume (SDSVOL) and mixograph development time (MDT). Contrary to this, Edwards et al. (2007) reported that genotypes with subunits 14 + 15 had weaker dough strength. Subunits 2* and 6 + 8 were found to have greater gluten strength as measured by the alveograph, compared to 2* and 20. They further indicated that subunits expressed at *Glu-B1* have a larger effect on gluten strength than *Glu-A1*.

2.3.2 Low molecular weight-glutenin subunits

The low molecular weight-glutenin subunits (LMW-GS) are one of the main components of wheat proteins. The LMW-GS comprise about one third of the total seed protein and represent about 60% of the total glutenin fraction (Gianibelli et al. 2001; Peng et al. 2011). Although they form a large component of glutenins, they have received less attention compared to HMW-GS because they are difficult to differentiate and characterise (D'Ovidio and Masci 2004; Zheng et al. 2009; Zhang et al. 2012). The LMW-GS show poor resolution on SDS-PAGE, since they share similar electrophoretic mobilities with some gliadins. The LMW-GS are controlled by many genes, making it difficult to characterise (Masci et al. 1998; Zhang et al. 2012). Highly skilled personnel are required to separate and identify individual alleles on SDS-PAGE. This makes them less amenable for use in early generations of breeding programmes (Zhang et al. 2012; Xia et al. 2014). The contribution made by individual LMW-GS in pasta quality is not yet fully established (Tosi et al. 2005; Mamone et al. 2009). Recent developments in 2-DE and mapping approaches enlightened researchers on molecular, structure and functional roles of LMW-GS (Vensel et al. 2005; Zhang et al. 2012). The LMW characteristics such as distribution of cysteine residues which participate in disulphide bonds (Shewry and Tatham 1997), and amino acid composition that take

part in noncovalent bonds, play a key role in determining dough rheological properties (Pomeranz 1988). The number of cysteine residues available for disulphide bonds can influence functionality of LMW-GS. They can either serve as chain extenders or chain terminators of glutenin polymers (Masci et al. 1998; Tosi et al. 2005).

The LMW-GS can be classified into three groups (B, C and D) based on their isoelectric point (pI) and mobility in 2-DE. The D group has mobilities which fall between group B and C (Muccilli et al. 2011; Rasheed et al. 2014). The B group is regarded as the major LMW and is alkaline. The D group is more acidic and associated with modified ω-gliadins, which have gained a cysteine residue that is not present in ω-gliadins. The additional cysteine residue found in the D group makes it an integral part of the glutenin subunits (Masci et al. 2000; D'Ovidio and Masci 2004). The C group has N-terminal sequences similar to α-, β- and γ-gliadins (Jackson et al. 1983; Masci et al. 2000; D'Ovidio and Masci 2004). The gliadin like sequences found among the glutenin subunits might be due to mutations that influence the position and number of cysteine residues (Masci et al. 1995; Masci et al. 2002).

The amino acid sequence of LMW-GS is made up of four regions; signal peptide, N-terminal, repetitive domain and C terminus (non-repetitive) region (Cassidy and Dvorak 1991; Cassidy et al. 1998). The signal region is made up of 20 amino acids while the N-terminal region consists of 13 amino acids with a cysteine residue at position five. The N-terminus is followed by a repetitive domain high in glutamine and proline, but lacks a cysteine residue. The C terminal region is long and characterised by low proline content and high cysteine residue. This C-terminal is sub-divided into three regions, A, B and C. The A region contains five cysteine residues, the B region is rich in glutamine and consist of one cysteine residue. Region C is conserved and has one cysteine residue (Cassidy and Dvorak. 1991; Cassidy et al. 1998; Wang et al. 2016). The complete sequence of the LMW-GS structure comprises eight cysteine residues (Cassidy and Dvorak 1991).

The B group is further classified into three subgroups on the basis of N-terminal amino acid sequences; LMW-m, LMW-s and LMW-i. The m, s and i correspond to methionine, serine and isoleucine, respectively, the first amino acid residue of protein (Wang et al. 2016; De Santis et al. 2017). By N-terminal amino acid sequencing, LMW-s type subunits begin with SHIPGL. The LMW-m starts with METSHIPGL-, METSRIPGL-, and METSCIPGL in their N-terminus and show a large variation. The LMW-i type does not have a typical N-terminal sequence. After the signal peptide is the repetitive domain, which starts with ISQQQQ (Lew et al. 1992; Rasheed et

al. 2014; Wang et al. 2016). Wang et al. (2011) identified a new LMW-l type in the *Aegilops* species, which starts with amino acid residue leucine.

In durum wheat, genes controlling LMW are found on the short arm of group 1A and 1B chromosome at the *Glu-A3* and *Glu-B3* loci. Some genes controlling LMW-GS are found on *Glu-B2* and *Glu-B4* (Ruiz and Carrillo, 1993; Rodriguez-Quijano et al. 2010). The C- and D-LMW-GS resemble gliadins and are controlled by group 6 chromosomes (Masci et al. 2000; D'Ovidio and Masci 2004).

Previous studies have shown the influence of LMW-GS on pasta making quality (D'Ovidio and Masci 2004; Babay et al. 2015; De Santis et al. 2017). The LMW-GS affecting pasta making quality are classified into LMW-1 and LMW-2. The LMW-1 is related to γ-42 gliadin and LMW-2 to γ-45 gliadin. There is a close genetic linkage between *Glu-3* loci coding for LMW-1 and LMW-2 and *Gli-1* loci controlling γ-42 gliadin and γ-45 gliadin. The LMW-1 has been associated with poor pasta properties and LMW-2 with good pasta making quality (Bechere et al. 2002; Edwards et al. 2007; De Santis et al. 2017). Various studies indicated that gluten strength is affected by allelic differences in LMW-GS. The LMW-GS found on *Glu-B3c* and *Glu-B3j* had higher SDSVOL and MDT. The *Glu-B3a* and *Glu-B3c* had a larger effect on alveograph strength than *Glu-B3b* and *Glu-B3k* alleles. This might be due to their association with LMW-2 and LMW-1 models, respectively (Brites and Carillo 2001). Nazco et al. (2014) reported that cultivars with LMW-2 (aaa) had increased dough strength.

2.3.3 Gliadins

Gliadins are monomeric proteins linked by intra chain disulphide bonds (α-, β-, γ-gliadins) or non-disulphide bonds (ω-gliadins) (Mamone et al. 2009; Pompa et al. 2013). Gliadins are soluble in alcohol and can further be classified as ω5-, ω1-, ω2-, α- and γ-gliadins, according to their primary structures (Wieser 2007; Barak et al 2015). The α-, β-, and γ-gliadins have a similar amino acid composition. The ω-gliadins comprise amino acids which differ from other gliadin groups. They do not have the sulphur rich cysteine amino acid (Mamone et al. 2009). Gliadins in durum wheat are controlled by genes found on the short arms of group 1 and 6 chromosomes of the A and B genomes (Pompa et al. 2013). The α- and β-gliadins are controlled by genes clustered at loci *Gli-A2* and *Gli-B2* on the short arm of group 6 chromosomes, whereas the γ- and ω- gliadins are coded at loci *Gli-A1* and *Gli-B1* on the short arms of chromosomes 1A and 1B. The *Gli-B1* loci coding

for γ- and ω-gliadins are closely linked with *Glu-B3* loci encoding for LMW. Gliadins affect viscosity and extensibility properties of dough (Dukić et al. 2008; Pompa et al. 2013). A strong correlation exists between certain gliadin components and gluten strength and pasta cooking quality (Yildirim et al. 2011). Gliadin γ-45 has been associated with pasta with superior quality and has been used for selection of good pasta making quality. Gliadin γ-42 is related to poor pasta making properties. Durum wheat cultivars with the γ-45 gliadin produce pasta which is firm with good viscoelastic properties (Yildirim et al. 2013). Gliadin γ-45 is related to ω-35 gliadin and LMW-2 subunits, while gliadin γ-42 is related to ω-33, 35, 38 gliadin and LMW-1 subunits. Many durum wheat breeding programmes have fixed the LMW-2/γ-45 because of their contribution to good pasta making quality. Nevertheless, variation in pasta quality still exists in genotypes having these proteins; this indicates that other factors contribute towards pasta quality (Galterio et al. 1993; Sissons et al. 2012; Pompa et al. 2013).

2.3.4 Albumins and globulins

Albumins and globulins (AG) are regarded as non-prolamin proteins, which are soluble in water and salt, respectively (Hasniza et al. 2014; Arena et al. 2017). Albumins exist in greater amounts than globulins. Large amounts of AG are found in the aleurone layer and embryo, with lower quantities in the starchy endosperm. The main components of AG are enzymes, which take part in metabolic or structural activities. Other components include hydrolytic enzymes necessary for germination, and enzyme inhibitors (Garcia Del Moral et al. 2007; Arena et al. 2017). Albumins are mostly enzymes such as carbohydrases, including α-amylase and β-amylase. Albumins are mainly comprised of serpins and make up about 4% of the endosperm protein. The globulins are characterised as storage proteins (triticin) (Hasniza et al. 2014). In comparison with gluten proteins, AG have higher nutritional value because of high amounts of amino acids lysine and methionine. However, because of their occurrence in minor quantities in starchy endosperm, they cannot overcome the lack of lysine in wheat flour. The HMW AG have a storage function; however, they have been reported to have little effect on flour quality as most of these proteins are concentrated in the aleurone layer and embryo, which are removed during the milling process. The AG have been reported to accumulate earlier during growth and development of the plant (Triboï et al. 2003; Arena et al. 2017), and high temperatures applied during the early stages tend to increase the AG (Hasniza et al. 2014). It is believed that an increase in temperature may lead to overexpression of globulin variants. The occurrence of globulin variants may negatively affect nutritional quality. Globulin-2 and globulin-3 are associated with health problems such as allergens

and diabetes, respectively (Hasniza et al. 2014). Globulin-3 and triticin are comprised of six cysteine residues which resemble some of the y-type HMW-GS, and as a result, may influence dough quality because of their involvement in gluten polymerisation (Veraverbeke and Delcour 2002; Hasniza et al. 2014). Serpins are high in glutamine motifs, similar to those present in prolamin storage protein and form intermolecular disulphide bridges, and as such may affect functional flour quality properties (Roberts and Hejgaard 2008). Laino et al. (2010) used a classical proteome approach based on 2-DE to analyse the effect of heat stress on metabolic proteins in durum wheat, where 132 protein spots were differentially expressed. In the study, 47 spots were identified which encompass heat shock proteins, proteins involved in the glycolysis and carbohydrate metabolism, as well as stress-related proteins. Heat induces polypeptides that have been reported to cause allergy in some people.

2.4 Pasta making quality

Durum wheat kernels are the hardest of all wheat and this is related to a lack of the starch granule puroindoline on the surface of starch granules (Samaan et al. 2006). When durum wheat is milled, it gives a coarse particle product called semolina. Semolina is mainly used for pasta, because of its yellow colour, flour and cooking quality. The quality of durum wheat can be described in terms of its physical, chemical, rheological and processing properties. The physical characteristics are defined in terms of thousand kernel weight (TKW), test weight, vitreous kernels and damaged kernels. The physical quality characteristics are used in wheat grading and determine the market price of wheat (Sissons 2008; Guzmán et al. 2016). TKW is an important quality characteristic associated with grain yield and potential amount of flour within the grain (Protic et al. 2007; Li et al. 2015). For durum wheat, TKW is related to test weight and semolina. Kernels of a larger size are expected to give a higher milling yield, because the proportion of endosperm to non-endosperm should be higher and a TKW value of 35-40 g is acceptable (Dziki and Lawskowski, 2005). The environment has a large influence on TKW and kernel composition (DuPont and Altenbach 2003) with heritability estimates between 0.37 and 0.69 (Jochum et al. 2001; Collaku and Harrison 2005). Matsuo and Dexter (1980) observed a high correlation between grain size and milling yield in durum wheat. Test weight is a physical trait mostly used for wheat grading and classification. It measures kernel density. Kernels that are shrivelled tend to have a low test weight. Test weight is determined by genetic and environment factors with heritability values between 0.44 and 0.83. Dexter et al. (1987) reported a strong correlation between semolina yield and test weight for durum wheat; Khattak et al. (2005) reported a negative correlation between test weight and protein content.

Durum wheat chemical properties include yellow colour, protein content and quality, and ash content. Bright yellow to amber colour is favoured by consumers compared to a brown or cream colour (Pasqualone et al. 2015). The yellow colour found in durum wheat is associated with the carotenoid pigment content found in the endosperm, mainly lutein, that is a xanthophyll, which amounts to 86-94% of the total carotenoids (Hentschel et al. 2002; Ficco et al. 2014). These carotenoids also have nutritional value. Semolina colour is highly heritable and dependent on the genotype, however, Clarke et al. (2006), indicated that yellow pigment content increased under cool and wet environmental conditions. Starch damage, α-amylase activity, temperature and oxidative degradation by lipoxygenase during processing also affects pasta colour. Starch damage and α-amylase activity enhance production of reducing sugars during mixing and extrusion. This leads to formation of Maillard reaction products during drying under high temperatures, thus increasing the red components of pasta (Sissons et al. 2012). The colour in semolina and pasta can be assessed using a Minolta colour meter device with L (brightness), a (redness) and b (yellowness) indications, where high L values indicate a lighter colour.

The ability of semolina to be processed into pasta of good quality is due to the protein content and gluten quality (Dexter and Matsuo 1980; Guzmán et al. 2016). These two factors affect rheological properties of dough (Novaro et al. 1993). Semolina, which is high in protein, produces pasta that is firm, less sticky and maintains good texture, even when overcooked. Durum wheat with low protein content is less preferred in the market and tends to have a lower value because it has limited use outside the pasta industry (Dexter and Matsuo 1980; Guler et al. 2002). High protein content is related to high gluten strength. However, an increase in protein content is not a good indicator of gluten strength, as it does not provide assurance of good gluten strength. The gluten proteins are hydrophobic, this inhibits water from penetrating pasta during cooking, thus reducing stickiness, swelling and surface breakdown (Feillet 1988; Shewry and Halford 2002). The relationship between protein content and pasta firmness is well established in Canadian durum wheat cultivars. It is common practice for durum wheat buyers to demand a certain minimum protein content as guarantee for good pasta quality. Grain protein content of 13%, which corresponds to 12% flour protein content, is necessary to meet textural needs (Sissons et al. 2012). Petrova (2007) indicated that a protein content between 12% and 16% percent is acceptable.

The contribution made by protein content and gluten quality towards pasta quality depends on the drying technology used (Guler et al. 2002; Bruneel et al. 2010). Protein content is essential when high temperature drying technology is used to improve colour and cooking quality of pasta. High

temperatures affect the physicochemical properties of gluten. At low temperature drying, protein content and gluten quality are both required to produce pasta of good quality (Guler et al. 2002; Bruneel et al. 2010). The gluten quality is defined in terms of viscosity and elasticity. Dough that is weak and elastic, tends to produce pasta with poor cooking quality. The visco-elastic properties of the gluten are associated with the balance between gliadins and glutenin. Durum wheat with high glutenin to gliadin ratio produces pasta with good cooking quality (Feillet 1988).

2.5 High performance liquid chromatography techniques

HPLC is one of the most important techniques used in analytical chemistry. It separates, identifies and quantitates the compounds that are present in a sample, which can be dissolved in a liquid (Moreno-Arribas and Polo 2003; Naeem and Sapirstein 2007). The separation involves two major phases; the mobile phase and the stationary phase. The mobile phase is made of liquid, which flows through the stationery phase and it carries the components of a mixture with it. The HPLC system is made up of various components. Thorough knowledge of components of the system is essential to optimize the performance of the system, as this will result in better protein separations (Marchylo et al. 1989). The pump is an important part of the system. It drives the eluting solvent through the HPLC system at a constant flow rate and pressure (Bietz and Kruger 1994; Moreno-Arribas and Polo 2003).

2.5.1 Size exclusion-high performance liquid chromatography

SE-HPLC, also known as a molecular sieving technique, has been widely used for analysing cereal proteins. Proteins are separated and assessed according to characteristics associated with molecular weight distribution (Goyon et al. 2017). The stationary phase is made of a solid bound to a medium support material. The stationary phase is a sieving medium made of a porous gel matrix. Proteins larger than the pore size of the sieving medium are not retained, therefore come out first. Proteins of a smaller size are able to penetrate the pores and pass through the column slowly. SE-HPLC is used to determine the relative amounts of different components in a sample of interest. The technique is accurate, sensitive, reproducible and easily automated. The idea of separating molecules according to their size was first devised by Synge and Tiselius (1950). They noticed that molecules of a smaller size are omitted from small pores of zeolites due to their molecular size. Zeolites are comprised of a three dimensional crystal structure made of elements such as aluminium, oxygen and silicon with alkali metals (such as sodium, potassium and magnesium) and water molecules trapped in the fissures between them (Hong et al. 2012). Later zeolites were named molecular sieves by J.W. McBain, which illustrates the characteristics of zeolites and

formed the basis of size exclusion chromatography. Studies on the separation of biomolecules using improved SE-HPLC were first reported by Lindqvist and Storgårds (1955) as well as Lathe and Ruthven (1956) using columns packed with maize or potato starch. These columns demonstrated low adsorption of proteins (Hong et al. 2012). The major drawback associated with columns packed with starch was low mechanical strength, with reduced speed and it could not withstand high pressure. Later on, dextrans cross-linked with epichlorohydrin were developed, which overcame major drawbacks related to columns packed with starch. The material resulted in limited interaction with proteins and improved mechanical strength (Porath et al. 1960; Hong et al. 2012). Porous silica material was developed in the 1970‘s with high mechanical strength, non-swelling and higher stability with increased performance, and as such became the major chromatographic stationary phase media. However, the presence of silanol groups found on the surface of silica porous material increased the ionic strength (Chongying 1992). The development of porous hybrid and inorganic particles reduced activity of silanol. This allowed the use of little quantities of salt additives, which decreased ionic interactions with proteins, leading to improved chromatographic separations (Hong et al. 2012)

SE-HPLC separates proteins into three major groups; glutenins, gliadins and AG (Larroque and Békés 2000). The technique has been used in wheat studies to evaluate bread and pasta making quality. Hailu et al. (2016) used SE-HPLC to assess the quality of durum wheat varieties from Ethiopia and Spain. High percentages of unextractable polymeric proteins (UPP) were observed in Ethiopian varieties, which indicated high gluten strength. Edwards et al. (2007) indicated that UPP correlated with alveograph parameters desirable for pasta making quality. They further indicated that UPP fractions could be used in early generation selection in the breeding programmes as a small flour sample is required. Labuschagne et al. (2016) used SE-HPLC to study the effect of abiotic stress on bread wheat, where heat stress caused a reduction in SDS soluble large monomeric proteins. Both heat and drought caused an increase in insoluble large polymeric proteins. The protein fractions correlated with bread-making quality characteristics. Application of SE-HPLC by Labuschagne et al. (2009) indicated that both heat and cold caused a reduction in small polymeric proteins, while monomeric proteins increased. The results further indicated that durum wheat had less polymeric proteins and more monomeric proteins than bread wheat. The correlations between protein fractions and quality characteristics can be used to predict quality in bread and durum wheat.

2.5.2 Reversed phase-high performance liquid chromatography

RP-HPLC is a separation tool used for qualitative and quantitative analysis of proteins (Burnouf and Bietz 1989; Naeem and Sapirstein 2007). These proteins are separated according to their hydrophobic properties instead of molecular size. Therefore, it supplements other methods, which fractionate proteins based on molecular weight and charge. Variations in the hydrophobic properties are due to dissimilarities in amino acid sequences and conformation. RP-HPLC is most applicable in cereal chemistry due to good resolution obtained for amino acids with similar sequences, as well as molecules with distinctive structures. The technique is fast, highly reproducible and can be automated. It is also sensitive; a small fraction of flour is required which is thus less disruptive of valuable germplasm (Burnouf and Bietz 1989).

The application of RP-HPLC was less successful until the development of wide pore 'end capped' spherical columns in the 1980s, which reduced adsorption of proteins by the stationary phase. Relatively small pore (80-100Å) columns were used before, however, were not able to separate HMW protein fractions correctly, due to protein binding resulting in poor recovery (Bietz 1983). The development of wide pore (300-500Å) columns led to improved separations of HMW proteins (Bietz 1983; Bietz and Kruger 1994). The use of RP-HPLC in cereal wheat proteins was first published by Bietz (1983) using a wide pore (300Å) column. High resolution and reproducible results were observed for gluten proteins. The use of RP-HPLC for varietal identification is well documented (Marchylo et al. 1988). However, pre-fractionation of gluten proteins is required, since different groups of proteins can elute as one peak. Burnouf and Bietz (1984) used RP-HPLC to distinguish between durum wheat varieties with strong and weak gluten. Yan et al. (2009) used RP-HPLC to characterise HMW-GS from Italian durum wheat. Schalk et al. (2017) characterised gluten proteins from wheat, rye, oats and barley and, using the analysis of gliadin and glutenin proteins by RP-HPLC, predicted wheat quality. However, the changes in relative amounts of proteins due to the environment could affect identification or quality prediction procedures (Huebner and Bietz 1985; Marchylo et al. 1990). In a study conducted to assess the influence of environment on RP-HPLC elution profiles using cultivar Neepawa, it was observed that the environment did not qualitatively influence protein elution profiles (Kruger and Marchylo 1985). However, differences in height of peaks were reported in samples grown under different environmental conditions. This suggests that the environment could affect relative amounts of proteins. The peaks obtained from RP-HPLC were found to be correlated with bread-making and in a study by Sutton et al. (1989) two HMW-GS peaks correlated with loaf volume. However,

Hailu et al. (2016) did not find significant correlations between protein fractions separated by RP-HPLC in a study on durum wheat quality parameters.

2.6 Effect of environment on durum wheat quality

The amount of proteins and composition play an important role in final use and quality of durum wheat (Giuliani et al. 2015). However, these factors are largely affected by environmental conditions, genotype and their interaction. This may cause fluctuations in durum wheat quality (Halford et al. 2014; Giuliani et al. 2015). The extent to which growing conditions affect quality is a major concern, and has received much attention recently. It is necessary to develop high yielding varieties with uniform end-use quality. The growing conditions before anthesis affect germination percentage, photosynthesis, tiller formation as well as inflorescence growth. This has an impact on the number of grains produced. After flowering, the environmental stresses impact kernel size and composition. The environmental conditions after flowering influence kernel size and composition (DuPont and Altenbach 2003).

Research has been done to assess quality characteristics of wheat as influenced by abiotic factors such as temperature, drought and cold. The environmental factors affect the duration of grain fill and influence the rate of accumulation of proteins in the grain. The assembly of proteins differs among genotypes. Cooler temperatures extend the period of grain fill and leads to high starch accumulation. Temperatures between 15^{o}C and 20^{o}C are suitable for wheat production, giving higher yields (DuPont and Altenbach 2003). DuPont et al. (2006) indicated that an increase in temperatures caused an increase in HMW-GS and α-gliadins, while the LMW-GS decreased under high temperature (37/28^{o}C day/night) in hexaploid wheat. Labuschagne et al. (2016) subjected two bread wheat cultivars to heat stress (32/15^{o}C day/night) and observed an increase in SDS-soluble large monomeric proteins (gliadins). However, the SDS-insoluble small polymeric proteins (LMW) were decreased by heat stress. The SDS-insoluble polymeric proteins were increased by both drought and heat stress in one cultivar. Corbellini et al. (1997) indicated that high temperatures (35-40^{o}C) during middle to late grain filling stage caused an increase in soluble polymeric proteins and LMW-gliadins while the insoluble polymeric proteins were reduced, both in durum and bread wheat. High temperatures applied late in grain filling have dough weakening effects. This may reduce the commercial value of the product. High temperatures have been observed to reduce the molecular weight distributions by affecting the normal process of disulfide bond formation, resulting into weaker doughs. High temperatures applied at dehydration stage may

negatively impact on the grain quality, as glutenin polymerisation continues rapidly at this stage (Naeem et al. 2012).

In durum wheat, Ferreira et al. (2012) observed that high temperatures (up to 27.5ºC) applied throughout the developmental stages showed an increase in γ--gliadins at the expense of glutenin. The amount of total polymers decreased, and the SDS-insoluble glutenin fraction increased. However, the proportion of SDS-insoluble glutenin polymers remained the same under high temperature at grain fill up to physiological maturity. Flagella et al. (2010) indicated a strong correlation between gluten index and number of days, with temperature regimes between 30-35ºC at grain fill. An increase in the proportion of HMW/LMW was observed when the plants were subjected to water stress throughout the growing season. Water stress applied at grain fill also increased the glutenin polymers.

High temperatures have been observed to cause a reduction in the duration of grain fill. Ferreira et al. (2012) observed that the grain fill period decreased by 4 days under high temperatures as opposed to lower temperatures in durum wheats. Hurkman et al. (2009) showed that the grain took 44 days after anthesis to reach maturity under moderate temperature compared to 28 and 33 days after anthesis under high temperature applied at 10-20 days after anthesis in bread wheat cultivar 'Butte 86'.

The environment had a larger effect on characteristics related to protein content and yield components in durum wheat compared to genotype and interaction between genotype and environment. High temperatures and drought decreased yields and caused an increase in protein content (Flagella et al. 2010; Li et al. 2013; Pinheiro et al. 2013), while Garcia Del Moral et al. (2007) and Sissons et al. (2014) observed that irrigation increased grain yield. Reduction in grain yield components may be associated with changes and accumulation during starch synthesis (Tashiro and Wardlaw 1989). Starch is a major component affecting grain yield and accounts for 70% of dry weight of the wheat grain. Starch synthesis decreases under high temperatures. The starch synthase enzyme activity responsible for conversion of sucrose to starch is inactivated (Corbellini et al. 1997; DuPont and Altenbach 2003). Majoul et al. (2004) reported that a temperature regime of 34/10ºC day/night resulted in a down-regulation of some enzymes involved in starch synthesis, such as ground bound starch synthase enzyme (granule bound SSE) and glucose-1-phosphate adenyltransferase, in bread wheat. Heat stress reduces grain weight by decreasing grain growth duration (Stone and Nicolas 1996) and grain growth rate (Tashiro and

Wardlaw 1990). The accumulation of nitrogen is less influenced by high temperatures and more nitrogen is absorbed prior to flowering. This causes an increase in protein content at the expense of grain yield (Corbellini et al. 1997). Sulphur is another important nutrient element that plays a major role in storage protein accumulation. Bread wheat produced in soils which lack sulphur, accumulated storage proteins poor in sulphur (Halford et al. 2014). The alterations that occur in accumulation of various types of proteins in response to nutrition can be caused by changes in gene expression.

The environment (E), genotype (G) and genotype by environment (GxE) interaction have been found to affect pasta making quality (Taghouti et al. 2010; Li et al. 2013; Sissons et al. 2014). However, the effect of G x E was smaller compared to effects of genotype and environment separately. Pasta making quality characteristics are genotype dependent (Rharrabti et al. 2003a; Li et al. 2013). Flour or semolina yellowness is an important characteristic in durum wheat quality. The genotype was observed to exert a large influence on flour yellowness (Taghouti et al. 2010; Pinheiro et al. 2013; Magallanes-Lopez et al. 2017). This indicates that flour yellowness is primarily genetically controlled (Rharrabti et al. 2003a; b). High temperatures were found to increase flour yellowness of semolina (Taghouti et al. 2010; Li et al. 2013). However, Guzmán et al. (2016) observed a decrease in flour yellowness under severe heat stress, which agrees with Pinheiro et al. (2013) who observed lower pigment content under high temperatures at grain fill. Li et al. (2013) observed an increase in flour yellowness under drought stress. Rharrabti et al. (2003b) indicated no change in pigment content both under irrigated and rainfed conditions.

Various studies on durum wheat indicated that SDSVOL increased under water shortage conditions. It was further indicated that genotype contributed more to the variation in SDSVOL than environment and GxE (Flagella et al. 2010; Guzmán et al. 2016; Magallanes-Lopez et al. 2017) in durum wheat cultivars. Li et al. (2013) found that heat stress also caused an increase in SDSVOL. Labuschagne et al. (2009) reported that low temperatures at grain fill reduced SDSVOL, while heat increased kernel hardness in durum wheat. Other durum wheat traits for processing have been reported to be influenced by genotype, traits such as mixograph peak time (MPT) and alveograph tenacity to extensibility ratio (AlvP/L) (Li et al. 2013; Guzmán et al. 2016). Guzmán et al. (2016) and Magallanes-Lopez et al. (2017) indicated high AlvP/L values under irrigated and drought environments. MPT values were high under irrigated conditions (Magallanes-Lopez et al. 2017). Li et al. (2013) observed increased MPT and dough strength under drought, while the heat stress condition caused a reduction in these parameters.

2.7 Proteomics and its applications in durum wheat quality

Proteomics is the discipline dedicated to the study of proteins, including the identification, structural and functional properties of proteins (Jorrin-Novo et al. 2009; Gallardo et al. 2013). It also allows quantitative analysis of proteins and identification of modifications as well as localisation of proteins (Carbonaro et al. 2004; Ortea et al. 2016). The proteome is defined as a set of proteins expressed by a genome (genes) at a particular time, under certain environmental conditions (Chen and Harmon 2006; Ortea et al. 2016).

Proteomics is progressively becoming an important tool in protein research. DNA is relatively constant and gives information on how the cell might utilise its proteins. However, the life of a cell is a constantly changing process, in which it is continually reacting to internal and external factors (Graves and Haystead 2002; Kosová et al. 2011). In responding to internal or external changes, proteins can be altered by posttranslational modifications (PMT), translocated within the cell, or be increased or decreased. In order to determine gene expression in an organism usually requires examination of mRNA or proteins. However, the characterisation of gene expression through mRNA has been less successful. There is often poor or no significant association between mRNA and protein content (Graves and Haystead 2002; Kosová et al. 2011; Ribeiro et al. 2013). Considering alternate splicing, protein proteolysis and PTM, one gene can produce many protein isoforms (Han and Wang 2008). It is possible that the genome of an organism can produce a large number of proteins (Graves and Haystead 2002). The active form of protein is difficult to predict from gene sequences and RNA expressions as proteins are subjected to further modifications. Proteomics can be used to fill in the gap between DNA sequences and cellular behaviour (Labuschagne and Igrejas 2020).

The gluten proteins have received significant attention because these proteins determine the functional end-use quality of wheat and they are used in cultivar discrimination (Ribeiro et al. 2013; Giuliani et al. 2015). Therefore, the composition, structure and function of gluten proteins related to good and poor quality characteristics have been in the spotlight. Gluten proteins are complex. Certain subgroups are structurally or chemically similar, which makes their analysis quite difficult (Ribeiro et al. 2013). Various methods have been used for assessment, characterisation and selection of proteins related to good quality properties. These methods can be divided into two groups: gel based; one dimensional gel electrophoresis (1-DE) and 2-DE as well as gel free methods, such as chromatography, RP-HPLC and SE-HPLC (Dworschak et al 1998; Zhang et al 2008). Although these methods have mostly been used to analyse gluten proteins, they

fail to discriminate between protein subunits with similar physicochemical properties (the same molecular weights and hydrophobicity) (Zhang et al. 2008; Ribeiro et al. 2013).

In recent years, 2-DE combined with advanced mass spectrometry (MS) has been the most important tool in proteomics (Newton et al. 2004; Pompa et al. 2013). The combination of the two techniques has enabled rapid identification and characterisation of proteins. The 2-DE is a gel based approach which separates proteins in two steps. First the proteins are separated according to their pI and then on the basis of molecular weight distribution (Agrawal et al. 2013; Aslam et al. 2017). The pI is defined as the pH value at which proteins carry a net charge of zero. Proteins can either have a positive, negative or zero net charge, based on the existing pH. The 2-DE is simple, reproducible and robust, and can give high-resolution images. It can also distinguish between proteins with similar molecular weights but different pI (Lilley and Dupree 2006; Rabilloud and Lelong 2011). Two-DE can show proteins which are up and down regulated. However, the technique cannot be automated and depends on the skills of the scientist (Agrawal et al. 2013; Aslam et al. 2017). The outcome is an image with many spots (Figure 2.2), which appears after staining of the gel, where each spot signifies a protein (Chandrasekhar et al. 2014). The 2-DE gels are subjected to computer software to assess spot patterns and intensities, in order to examine the differences in proteins expressed among the samples (Agrawal et al. 2013; Chandrasekhar et al. 2014; Aslam et al. 2017).

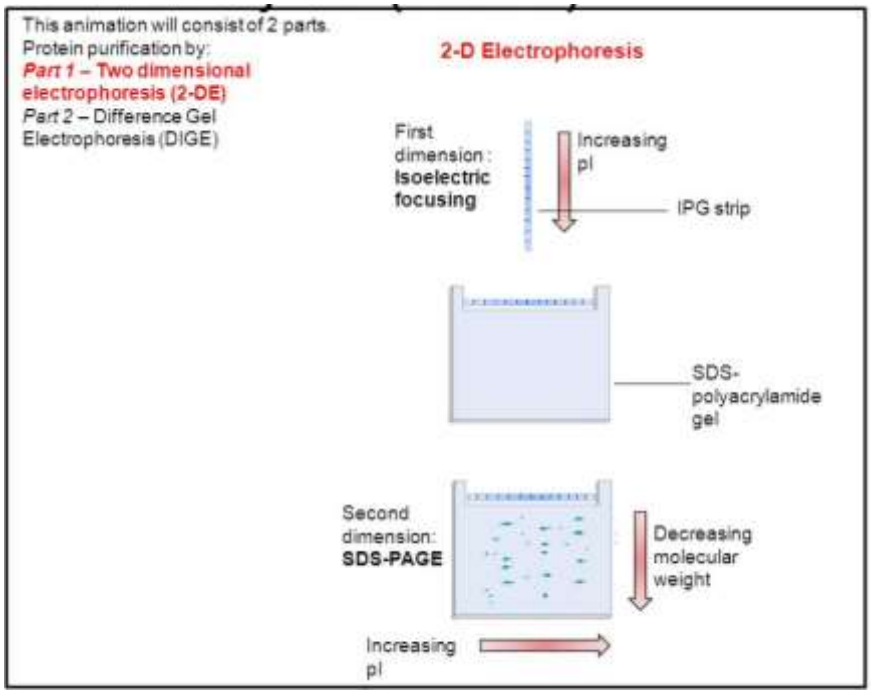

Figure 2.2 Schematic presentation of 2-dimensional gel electrophoresis (adapted from Nelson and Cox 2004)

Usually the spots of interest are digested using enzymes and subjected to analysis by MS for identification and characterisation (Cunsolo et al. 2012). MS measures mass to charge ratio and relative abundance of peptides. The MS has four essential components. The sample is introduced into the MS through the inlet system and then ionised by the source and separated according to the basis of mass to charge ratio by the analyser. The detector measures the relative abundance of ions and a mass spectrum (Olshina and Sharon 2016) is then generated as indicated in Figure 2.3. There are two ionization techniques used in MS: electrospray ionisation (ESI) and matrix assisted laser desorption (MALDI). In MALDI the sample is mixed with an organic matrix. The solution is placed on a MALDI plate. The matrix must solidify before analyses. The laser is used to heat the matrix, providing the matrix with energy. The matrix absorbs energy and transfers charge to the sample (Cunsolo et al. 2012; Chandrasekhar et al. 2014). In ESI, high pressure is applied to the sample coated with liquid solvent for ionization (Chandrasekhar et al. 2014; Olshina and Sharon 2016). The development of the two ionization techniques resulted in improved sensitivity and resolution. Use of ion sources in combination with mass analysers such as time of flight (TOF), quadrupole and Fourier transform ion cyclotron resonance (FTICR), ion trap (IT) and Orbitrap, have led to a major breakthrough in proteomics.

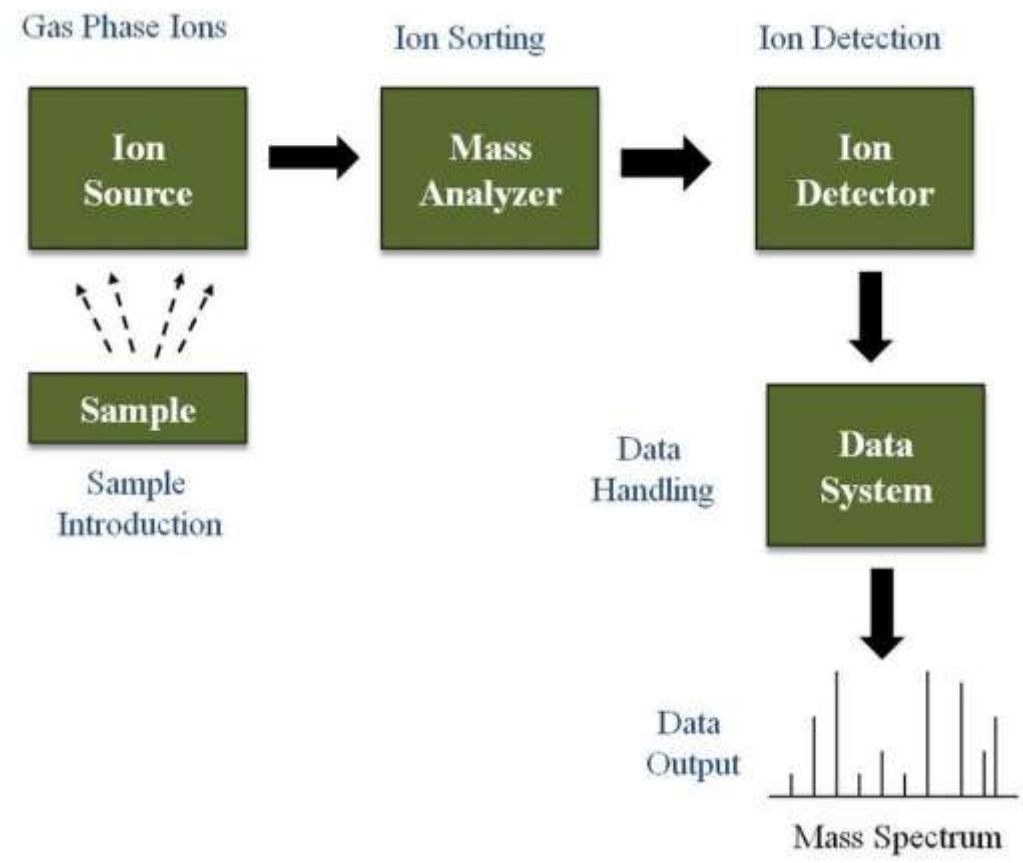

Figure 2.3 Components of mass spectrometry (adapted from Rauniyar et al. 2007)

Two-DE and MS have been successfully used for comparative proteomics, which include assessment of minor differences between treatments and controls. The two techniques have been used in various studies to determine changes that occur in gluten proteins due to drought, high temperature, cold and fertilizer with environmental cues. Dupont et al. (2006) reported that HMW-GS and α-gliadins increased under high temperatures, while the LMW-GS decreased under high temperatures in bread wheat. Similar results were also observed by Pompa et al. (2013) in durum wheat. They further indicated that high temperatures result in down regulation of γ-gliadins. Giuliani et al. (2015) studied the expression of gluten proteins using two Italian durum cultivars under water stress conditions. The results indicated that HMW-GS increased both under water stress and optimum conditions, while the LMW-GS decreased under water stress in both cultivars.

The 2-DE and MS have been applied to assess the accumulation of gluten proteins. Hurkman et al. (2013) observed that HMW-GS and α-gliadins reached a maximum at 18 days after anthesis and remained the same until 37 days after anthesis under moderate temperatures in bread wheat. The LMW-GS reached a maximum at 28 days after anthesis and declined slightly at 37 days after anthesis. Naeem et al. (2012) indicated that gliadins accumulated early (10 days after anthesis), while both HMW-GS and LMW-GS accumulated at 13 days after anthesis until maturity in bread wheat. However, Mazzeo et al. (2017) reported that α- and γ- gliadins accumulated at 3 days after anthesis while HMW Bx7 accumulated at 11 days after anthesis in durum wheat.

Proteomics has been successfully used for various identification and characterisation studies of cultivars. Muccilli et al. (2010) characterised LMW-GS of durum wheat in order to get in depth knowledge regarding their structural features. Using two durum wheat cultivar; Svevo and a line carrying the 1BL.RS translocation, proteomic results using 2-DE and MS indicated the presence of LMW-s, LMW-m and LMW-i types, while the cultivar carrying the translocation only had the LMW-i type. Gliadin proteins α- and γ- were also identified, which was not expected, due to washing away of gliadin proteins during extraction. It was assumed that proteins identified as gliadins might be LMW-C type subunits corresponding to α- and γ-gliadins modified in the number of cysteine residues (Masci et al. 2002).

Mamone et al. (2009) used 2-DE coupled with MALDI-TOF and nano LC-MS/MS to characterise glutenin proteins in durum wheat. The 2-DE reduced the complexity of glutenin proteins. The MALDI-TOF analysis allowed detection of large tryptic peptides. By using MALDI-TOF and nano LC-MS/MS simultaneously, high sequence coverage of 97.2% was obtained for HMW Bx7

and By8. In addition, truncated By8, which was never reported before, was observed. The LMW-GS B types were identified with reasonable coverage. The LMW-GS-C and LMW-GS-D identification was limited by lack of a complete DNA database. Other proteins such as serpins and β-amylase were identified by nano LC-MS/MS. Peptides with large molecular weight (5000 Da) were difficult to identify using nano LC-MS/MS. Non-gluten proteins such as serpins were also found. The presence of these proteins might be due to the protocol used for glutenin extraction, however it must also be noted that serpins can be incorporated into the gluten network by formation of inter-chain disulfide bonds (Mamone et al. 2009; Vensel et al. 2014). These studies represented the first detailed analysis of durum storage proteins using a proteomic approach.

Muccilli et al. (2011) used MALDI-TOF and RP-HPLC/nESI-MS/MS for comparative analysis of HMW-GS found in old durum wheat landraces and the most popular cultivars. Cultivar Simeto was used to represent popular cultivars with documented HMW-GS, while cultivar Tamila was used to represent landraces. The results indicated differences in molecular weight for both HMW-GS 1Bx and 1By for both cultivars, which were revealed by differences in amino acid sequences. Subunit Bx in Tamila showed 96% amino acid similarities with Simeto. HMW-GS By showed 10 amino acid substitutions among the two cultivars.

Pompa et al. (2013) analysed gluten proteins in three cultivars with differing technological differences using 2-DE and nano-HPLC-EST-IT MS/MS. Significant differences were observed and the cultivars which conferred poor technological parameters showed reduced expression of LMW-GS, and lacked LMW-2 and LMW-m type subunits associated with good rheological characteristics. With regard to year effect, for HMW, α-gliadin and globulin, three proteins were up-regulated while LMW and γ-gliadin were down-regulated in the warmer season. Cultivars with intermediate and poor quality parameters showed marked variation in the two seasons for certain, and differentially expressed spots. A cultivar with good quality parameters exhibited consistency in spot patterns across the two seasons.

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CHAPTER 3

Durum wheat gluten proteins separated by SE-HPLC and their association with pasta making quality

Abstract

Protein content and composition play an important role in pasta quality of durum wheat. However, these factors are largely affected by environmental conditions, genotype and the interaction between these. The aim of this study was to determine the effect of heat and drought stress on protein quantity and quality using size exclusion-high performance liquid chromatography (SE-HPLC) in six durum wheat cultivars with the same high (HMW) and low molecular weight (LMW) glutenin subunit composition as determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The experiments were conducted in Mexico under six different environmental conditions; optimum, flood irrigation, moderate drought stress, severe drought stress, moderate heat stress and severe heat stress conditions. High flour protein content (FPC) was observed under severe heat and drought stress conditions. Environment had a large influence on FPC across all environments. Large SDS-unextractable polymeric proteins (LUPP) were much higher under severe drought stress and flood irrigation conditions than under the other conditions. The SDS-extractable HMW proteins were significantly increased by all stress conditions while SDS-unextractable HMW proteins were significantly reduced by all stress conditions. Moderate heat stress and severe heat stress caused a significant increase in SDS-extractable gliadin proteins. The SDS-extractable and unextractable LMW glutenins were significantly reduced by stress conditions. For all environments, LUPP were more influenced by the environment than was the case in other protein fractions. The LUPP and UPP correlated significantly with alveograph strength and mixograph peak time in all conditions. The genotype ranking was inconsistent under different treatments, which indicated genotype by environment interaction. There was a large variation between cultivars in terms of reaction to stress. This suggests that the effects of stress cannot be generalised for all cultivars, and that although the HMW-GS and LMW-GS composition of the six cultivars were the same, there were still significant differences in the protein fractions as measured by SE-HPLC. This indicates that factors other than the HMW-GS and LMW-GS are at play in causing differences in protein fractions. Differences in the gliadin and albumin/globulin proteins between cultivars may have been a factor.

3.1 Introduction

Durum wheat is a staple food and is of great importance in the Mediterranean region, where it represents more than 50% of global durum wheat production (Guzmán. et al. 2016; De Santis et al. 2017). Durum wheat is used for making food products like freekeh, couscous, bulgur and bread, but its primary use is for pasta. Durum wheat end-products (pasta, semolina, couscous and bulgur) are currently trading at a higher price (10-20%) than bread wheat products (Sall et al. 2019). The properties of the end-products depend on the grain quality parameters, which have received much attention because of their impact on the nutritional and economic value.

A part of the protein found in the grain of wheat will form the viscoelastic protein network called gluten when the wheat flour/semolina is mixed with water. The gluten properties greatly influence end-use quality of durum wheat (Sissons et al. 2012; De Santis et al 2017). Gluten proteins are divided into two main groups: polymeric (glutenins) and monomeric (gliadins). The glutenin proteins are further classified into two groups, HMW and LMW proteins, depending on their mobility in SDS-PAGE. The monomeric proteins are divided into gliadins (ω, γ, α and β) and AG (Wieser. 2007; Barak et al. 2015). These protein fractions affect dough rheological properties of durum wheat, such as elasticity and extensibility. Durum wheat semolina with strong gluten is necessary for preparation of high quality pasta. Gluten strength is used as selection criteria in breeding programmes. High gluten strength reduces breakage loss of pasta during processing, and makes pasta firm and tolerant to overcooking (Sissons et al. 2012; Barak et al. 2015).

The amount and quality of protein fractions are influenced by the genotype, environment and how they interact (DuPont et al. 2006; Giuliani et al. 2015). It has been found that higher temperatures alter flour and dough quality, which has been associated with increase in gliadin/glutenin ratios. Temperatures above 35^{o}C caused a reduction in larger polymeric proteins and had a dough weakening effect (Flagella et al. 2010; Naeem et al. 2012). Contrary to this, DuPont (2006) showed an increase in HMW glutenins at 37^{o}C. Temperature affects the rate of biochemical processes and the timing of developmental processes within grain. Temperature and water stress applied at grain filling was reported to increase the glutenin fractions (Flagella et al. 2010).

SE-HPLC is a technique widely used in the separation of wheat proteins. The technique separates proteins according to molecular weight distribution (Goyon et al. 2017). Various studies using SE-HPLC indicated that quantity and molecular weight distribution are important in dough strength and pasta making quality. Edwards et al. (2007), Flagella et al. (2010) and Labuschagne et al.

(2016) used SE-HPLC to separate and quantify gluten protein as influenced by drought and heat. Heat stress significantly increased monomeric proteins, while the insoluble polymeric proteins were increased by drought in bread wheat flour. Flagella et al. (2010) reported an increase in HMW-GS under water deficit conditions in durum wheat flour. Good quality in durum wheat is necessary for acceptance by the industry and consumers (Guzmán et al. 2016). In order to pursue good wheat quality, it is important to understand environmental effects on the grain protein composition.

The objectives of this study were to:

- Investigate the influence of different levels of heat and drought stress on protein fractions separated by SE-HPLC
- To determine the relationship between these protein fractions and durum quality characteristics under the different stress conditions.

3.2 Materials and methods

3.2.1 Plant materials

Six durum wheat cultivars (MexicaliC75, YavarosC79, AltarC84, AtilC2000, JupareC2001 and CirnoC2008) from the International Maize and Wheat Improvement Center (CIMMYT) durum wheat breeding programme were used.

Table 3.1 List of genotypes with their high- and low molecular weight glutenin subunit composition as determined by SDS-PAGE

Genotypes	HMW-GS	LWM-GS		
	Glu-B1	***Glu-A3***	***Glu-B2***	***Glu-B3***
MexicaliC75	7+8	6	12	2+4+15+19
YavarosC79	7+8	6	12	2+4+15+19
AltarC84	7+8	6	12	2+4+15+19
AtilC2000	7+8	6	12	2+4+15+19
JupareC2001	7+8	6	12	2+4+15+19
CirnoC2008	7+8	6	12	2+4+15+19

HMW-GS = high molecular weight-glutenin subunits, LMW-GS = low molecular weight-glutenin subunits

3.2.2 Trial designs and treatments

The trial was laid out as a randomized complete block design, with three replications. The trial was conducted in six different growing conditions: full drip irrigation (optimum conditions), full basin irrigation, reduced irrigation or moderate drought stress, severe drought stress, moderate heat

stress and severe heat stress. The genotypes were sown at Ciudad Obregon Sonora, in Northwest Mexico in two consecutive growing seasons (data given in Appendix 1). The seeds were sown in November for all the treatments except for moderate heat stress (planted in January) and severe heat stress (planted in February). During grain filling, the maximum temperatures varied between 31ºC and 32ºC in March and April for all the treatments, excluding genotypes under severe heat stress, where temperatures were around 35-36ºC in May. All the trials were grown under full irrigation (>500 mm) except for moderate drought stress (300 mm) and severe drought stress (180 mm). The data obtained from Ciudad Obregon meteorological station showed an almost total lack of rainfall during the wheat growing season. Under moderate and severe drought stress, the plants were subjected to drought stress from stem elongation until physiological maturity. Under heat stress conditions, the plants experienced higher temperatures from shoot elongation until seed ripening. At sowing, all the trials received nitrogen (N) application of 50 kg/ha and 150 additional units of N at tillering in all treatments, except for severe drought stress, which received only 50 additional units of N. Required agronomic practices were applied and weeds were removed. When the plants reached physiological maturity, the whole plot was harvested and 1 kg of seed obtained from two replications of each genotype was used for quality analysis. Grain samples were conditioned to 16% moisture content, milled into flour using a Brabender Quadrumat Jr. (C.W. Brabender OHG, Germany).

3.2.3 Quality measurements

Flour protein content

The protein and moisture content of flour was determined using near-infrared spectroscopy (NIR Systems 6500, Foss Denmark) according to the American Association of Cereal Chemists (AACC) methods 39–10 and 46–11A (AACC 2010). FPC values were expressed based on 14% moisture basis.

Alveograph

The alveograph parameters were determined according to the alveograph manufacturer instructions (Chopin, France), using 60 g of flour to determine extensibility (alvL), tenacity (alvP), tenacity/extensibility ratio (alvP/L) and elasticity or strength (alvW) at constant water absorption (55%). Higher water absorption than in the official methodology (50%) was used to compensate for the typically higher water absorption caused by high levels of starch damage occurring during milling of the very hard durum wheat grain (Dexter et al. 1994; Peña et al. 1994; Ammar et al. 2000).

Mixograph peak time

Flour samples of 35 g were analysed on a mixograph (National Mfg. Co.) to obtain optimum dough mixing time and %Torque × min according to AACC method 54–40A (AACC 2010).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Glutenin subunit composition was identified by SDS-PAGE, according to the methodology of Peña et al. (2004), following the nomenclature of Payne and Lawrence (1983) for HMW-GS and Nieto-Taladriz et al. (1997) for LMW-GS. All the cultivars had the same HMW-GS and LMW-GS composition (Figure 3.1) which allowed accurate comparison of the effects of stress conditions on the measured quality characteristics, without variation in these proteins.

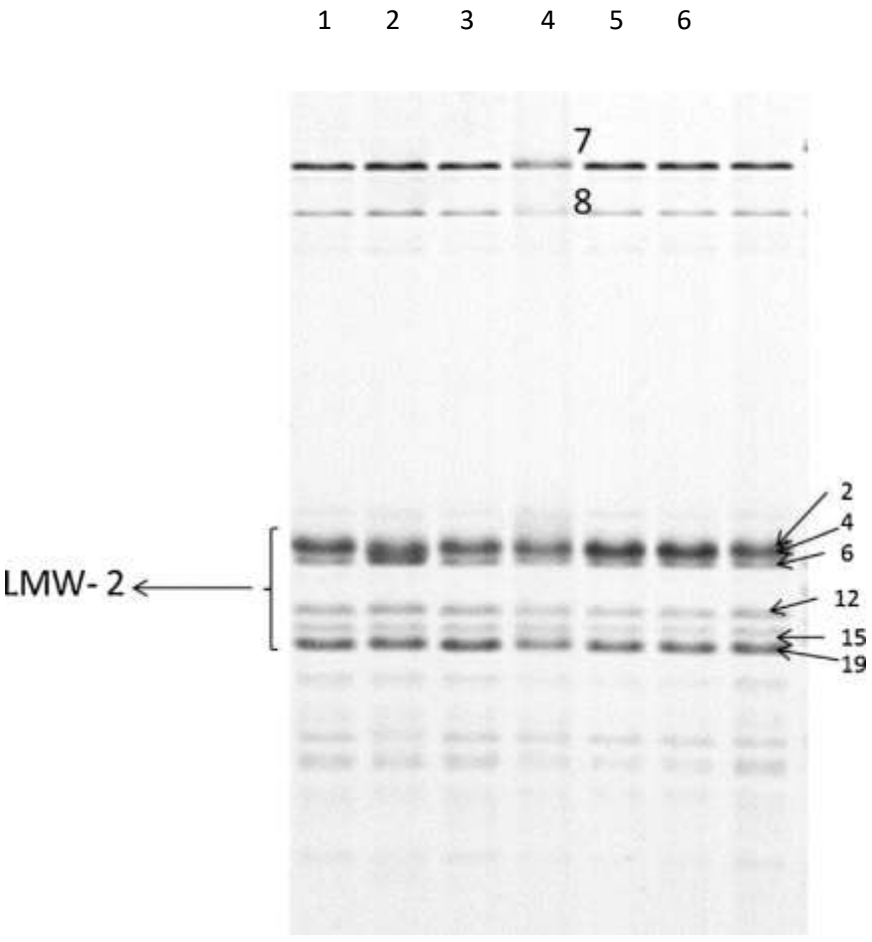

Figure 3.1 SDS-PAGE of HMW and LMW glutenin-subunits of durum wheat samples

Lanes are as follow: 1 = MexicaliC75, 2 = YavarosC79, 3 = AltarC84, 4 = AtilC2000, 5 = JupareC2001, 6 = CirnoC2008.

Size exclusion high performance liquid chromatography

Proteins from wheat flour were extracted using a two-step procedure according to Gupta et al. (1993). The first step involves extraction of proteins soluble in SDS buffer while the second step involves sonication. SE-HPLC analysis was done on the Shimadzu HPLC system equipped with a PDA detector and using a Phenomenox BIOSEP-SEC 4000 column (300 x 4.6 mm). The protein fractions (Figure 3.1) were calculated based on percentage of the respective peak areas relative to the total area using CLASS VP ™ software.

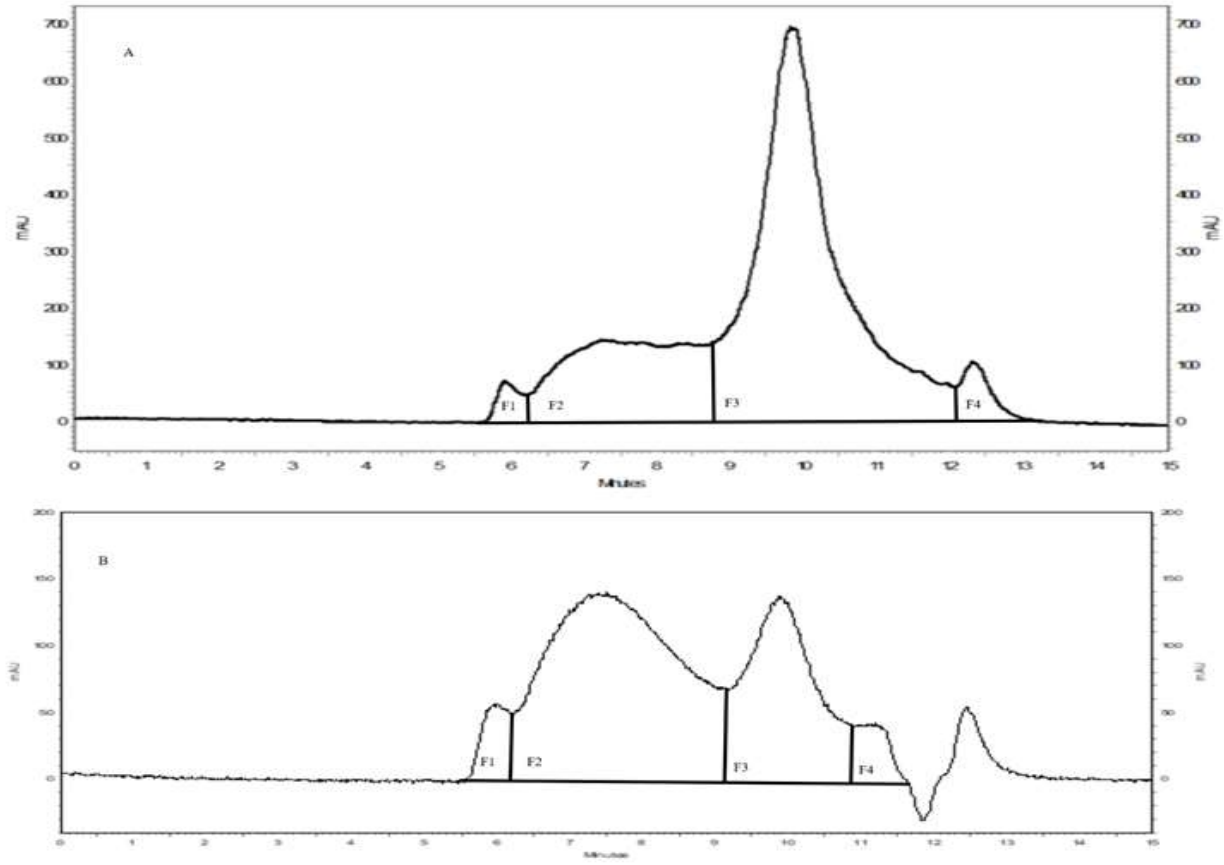

Figure 3.1 Example of SE-HPLC profiles for SDS-soluble (A) and SDS-insoluble (B) fractions. Fraction 1 = high molecular weight polymeric proteins, Fraction 2 = low molecular weight polymeric protein fractions, Fraction 3 = gliadins, Fractions 4 = albumins and globulins.

3.3 Protein fraction calculations

The protein fractions were calculated as follow:

LUPP	(F1 unextractable)/(F1 extractable + F1 unextractable)*100
UPP	(F1 unextractable + F2 unextractable)/(F1 extractable + F2 extractable + F1 unextractable + F2 unextractable)*100
exHMW	(F1 extractable)/[(F1 extractable – F4 extractable) + (F1 unextractable – F4 unextractable)]*100
unHMW	(F1 unextractable)/[(F1 extractable – F4 extractable) + (F1 unextractable – F4 unextractable)]*100
exLMW	(F2 extractable)/[(F1 extractable – F4 extractable) + (F1 unextractable – F4 unextractable)]*100
unLMW	(F2 unextractable)/[(F1 extractable – F4 extractable) + (F1 unextractable – F4 unextractable)]*100
exGLI	(F3 extractable)/[(F1 extractable – F4 extractable) + (F1 unextractable – F4 unextractable)]*100
unGLI	(F3 unextractable)/[(F1 extractable – F4 extractable) + (F1 unextractable – F4 unextractable)]*100
exAG	(F3 extractable)/[(F1 extractable – F4 extractable) + (F1 unextractable – F4 unextractable)]*100
unAG	(F3 unextractable)/[(F1 extractable – F4 extractable) + (F1 unextractable – F4 unextractable)]*100

Where LUPP = large unextractable polymeric proteins, UPP = unextractable polymeric proteins, exHMW = extractable high molecular weight, unHMW = unextractable high molecular weight, exLMW = extractable low molecular weight, unLMW = unextractable low molecular weight, exGLI = extractable gliadins, unGLI = unextractable gliadins, exAG = extractable albumins-globulins, unAG = unextractable albumins-globulins, F1 = fraction 1, F2 = fraction 2, F3 = fraction 3, F4 = fraction 4 (see Figure 3.1 for fractions).

3.4 Statistical analysis

Analysis of variance was done on measured and derived protein fractions using Agrobase (2019) software. Each treatment was analysed separately followed by analysis across two seasons. Correlations between proteins and selected quality characteristics were also determined.

3.5 Results

3.5.1 Analysis of variance for SDS-extractable protein fractions for year 1 and year 2

Flour protein content

In year 1, the effect of genotype was significant for all treatments with the exception of moderate heat stress (Table 3.2). In year 2, genotype effect was significant in all environments except for optimum conditions and flood irrigations (Table 3.3). Severe drought stress caused the highest FPC value of 16.95% in AtilC2000, which ranked the highest for FPC in year 1 (Table 3.4). In year 2, severe drought stress caused the highest FPC of 16.18% (CirnoC2008) (Table 3.5). The lowest FPC values were found under flood irrigation in both years, in MexicaliC75 (10.19%) and CirnoC2008 (10.01%) for year 1 and 2 respectively (Tables 3.4 and 3.5). Severe drought stress showed the highest average FPC values for both years in all genotypes, followed by the moderate heat conditions.

High molecular weight protein fractions

The genotype effect for exHMW was significant for all stress environments in year 1 except for severe heat stress conditions (Table 3.2). In year 2, genotype effect was significant for optimum, severe heat and severe drought stress conditions (Table 3.3). The highest exHMW values were obtained under moderate heat stress conditions at 8.20% (AtilC2000) and lowest under optimum conditions for CirnoC2008 at 2.72% in year 1 (Table 3.4). In year 2, severe heat stress caused the highest exHMW values in MexicaliC75 (8.22%) and the lowest value was found in MexicaliC75 under optimum conditions (2.97%) (Table 3.5). The exHMW values were higher in year 2 than in year 1 in all environments.

Low molecular weight protein fractions

Genotype effect for exLMW was significant for flood irrigation, moderate and severe drought stress conditions in year 1 (Table 3.2). In year 2, genotype effect was only significant for moderate and severe heat stress conditions (Table 3.3). In year 1, the highest exLMW value (YavarosC79, 24.03%) was obtained under severe drought stress (Table 3.4) and in year 2 under moderate heat conditions (JupareC2001, 24.28%) (Table 3.5). The lowest exLMW values were found under flood irrigation (JupareC2001, 13.66%) and severe heat conditions (MexicaliC75, 12.22%) for year 1 and 2 respectively (Tables 3.4 and 3.5).

Table 3.2 Mean squares from analysis of variance for flour protein content and protein fractions for year 1

Environments	FPC	exHMW	exLMW	exGLI	exAG	LUPP	UPP	unHMW	unLMW	unGLI	unAG
Optimum conditions	0.62***	2.14**	14.73	48.16*	1.91*	149.31***	53.58**	2.01***	25.50*	3.36*	0.96*
Flood irrigation	2.10**	5.50**	7.62**	101.23	0.35	157.79*	123.88*	7.49*	22.78**	2.53	0.21
Moderate heat stress	0.19	5.11*	11.14	74.57**	1.74	277.68*	75.71	3.61*	26.64*	2.84	0.04
Severe heat stress	1.44***	3.75	10.98	107.03**	3.21*	111.64	75.98	5.17*	7.00**	0.94**	0.08
Moderate drought stress	1.31 **	2.22*	9.31*	73.96***	0.59*	120.64*	82.18**	2.42*	16.11**	2.12*	0.07
Severe drought stress	3.66**	2.33**	20.90**	59.91*	1.22	140.71**	66.22*	4.05**	11.85*	0.75	0.02

FPC=flour protein content, exHMW=extractable high molecular weight, exLMW=extractable low molecular weight, exGLI= extractable gliadins, exAG=extractable albumins-globulins, LUPP=large unextractable polymeric proteins, UPP=large unextractable polymeric proteins, unHMW=unextractable high molecular weight, unLMW=unextractable low molecular weight, unGLI=unextractable gliadins, unAG= unextractable albumins-globulins *p≤0.05, **p≤0.01, ***p≤0.001

Table 3.3 Mean squares from analysis of variance for flour protein content and protein fractions for year 2

Environments	FPC	exHMW	exLMW	exGLI	exAG	LUPP	UPP	unHMW	unLMW	unGLI	unAG
Optimum conditions	0.28	4.92*	15.09	43.51	4.69**	259.52*	140.49	4.95*	27.53**	2.45	0.20*
Flood irrigation	0.42	1.62	7.79	56.68	2.63	278.38**	265.34*	7.64**	48.87*	2.06	0.22
Moderate heat stress	1.33*	1.97	17.34**	92.69***	0.77	275.87	156.92*	10.70*	11.85	2.14*	0.07
Severe heat stress	1.70***	100.03**	4.23***	0.03	0.98	8.39	12.09	2.24*	0.51	0.07	128.72*
Moderate drought stress	0.71**	1.01	4.76	74.61	2.76	4.76	37.45*	0.78	2.95*	0.38	114.22*
Severe drought stress	1.09**	1.80**	2.98	57.59**	2.40*	51.46	161.98**	3.19**	37.07	1.80*	0.05

FPC=flour protein content, exHMW=extractable high molecular weight, exLMW=extractable low molecular weight, exGLI= extractable gliadins, exAG=extractable albumins-globulins, LUPP=large unextractable polymeric proteins, UPP=large unextractable polymeric proteins, unHMW=unextractable high molecular weight, unLMW=unextractable low molecular weight, unGLI=unextractable gliadins, unAG= unextractable albumins-globulins *p≤0.05, **p≤0.01, ***p≤0.001

Table 3.4 Mean values for flour protein content and SDS-extractable proteins for year 1 for six durum wheat cultivars

Environments	Protein fractions	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Mean	LSD	CV
Optimum conditions	**FPC**	11.68	11.49	12.39	11.02	11.26	10.84	11.44	0.22	0.97
	exHMW	4.30	5.95	4.00	4.02	4.08	2.72	4.18	0.72	8.50
	exLMW	23.78	17.26	18.36	18.13	17.80	15.89	18.53	5.27	14.10
	exGLI	33.70	36.04	41.70	47.38	39.27	42.69	40.13	4.76	5.89
	exAG	8.55	7.80	7.64	7.69	7.45	5.62	7.46	0.64	4.23
Flood irrigation	**FPC**	10.19	11.55	10.93	13.25	11.36	11.85	11.52	3.49	0.81
	exHMW	4.91	8.05	4.60	4.63	3.19	4.04	4.90	1.17	11.85
	exLMW	19.10	16.93	15.86	16.43	13.66	18.50	16.75	1.25	3.69
	exGLI	32.94	27.77	35.42	46.07	30.32	26.51	33.17	13.23	19.79
	exAG	8.26	7.33	7.22	8.12	7.70	7.60	7.70	7.70	15.02
Moderate heat stress	**FPC**	11.22	11.63	11.35	12.02	11.41	11.19	11.47	0.49	2.11
	exHMW	4.88	4.81	5.44	8.20	3.72	4.07	5.18	1.95	18.69
	exLMW	15.35	22.01	18.16	17.20	17.99	15.91	17.77	3.45	9.77
	exGLI	45.49	43.92	45.90	50.49	32.86	39.53	43.03	5.24	6.04
	exAG	7.82	7.66	7.06	8.34	5.63	7.34	7.31	1.29	8.74
Severe heat stress	**FPC**	14.34	14.56	13.69	15.71	14.31	15.83	14.74	1.45	1.39
	exHMW	5.64	4.97	7.16	3.94	7.48	6.64	5.97	2.17	18.06
	exLMW	15.61	22.87	20.06	18.95	18.66	19.13	17.77	3.50	9.77
	exGLI	43.39	44.28	40.93	55.77	43.73	33.03	43.52	4.35	4.96
	exAG	6.88	7.19	5.49	6.89	4.91	4.09	5.91	1.10	9.26
Moderate drought stress	**FPC**	11.60	12.70	11.88	13.78	12.86	13.18	12.66	0.53	2.08
	exHMW	3.71	4.64	4.10	3.69	6.52	4.36	4.51	0.91	9.98
	exLMW	15.11	21.17	18.24	15.90	16.94	18.37	17.61	1.91	5.39
	exGLI	30.05	41.47	42.02	48.36	43.63	42.94	41.41	3.21	3.85
	exAG	8.06	7.29	6.40	7.18	7.07	7.52	7.50	0.68	4.68
Severe drought stress	**FPC**	13.14	15.82	14.09	16.95	15.51	15.52	15.17	0.77	2.50
	exHMW	3.42	4.27	2.81	5.15	4.38	5.75	4.29	0.70	8.13
	exLMW	14.43	24.03	17.30	16.46	18.35	18.60	18.19	2.87	7.83
	exGLI	46.50	46.12	35.58	52.50	45.65	44.02	45.06	6.19	6.82
	exAG	6.54	6.87	6.43	6.56	8.44	7.50	7.05	1.29	9.11

FPC=flour protein content, exHMW=SDS-extractable high molecular weight, exLMW=SDS-extractable low molecular weight, exGLI= SDS-extractable gliadins, exAG=SDS-extractable albumins-globulins, LSD=least significant differences, CV=coefficient of variation.

Table 3.5 Mean values for flour protein content and SDS-extractable proteins for year 2 for six durum wheat cultivars

Environment	Protein fractions	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Mean	LSD	CV
Optimum conditions	**FPC**	10.65	10.75	10.34	11.17	10.48	10.07	10.57	0.88	4.11
	exHMW	2.97	7.51	5.625	5.57	5.65	3.99	5.22	1.47	14.01
	exLMW	14.96	20.78	23.31	18.94	18.72	19.70	19.40	5.99	15.33
	exGLI	32.41	37.90	34.10	44.79	40.92	41.05	38.53	10.20	13.13
	exAG	7.83	9.80	5.31	6.65	6.39	6.97	7.16	1.10	7.67
Flood irrigation	**FPC**	10.22	10.46	10.17	11.29	10.29	10.01	10.40	0.20	0.94
	exHMW	5.10	4.49	6.47	6.24	6.58	4.94	5.64	3.11	27.41
	exLMW	20.08	18.22	19.09	21.16	15.91	16.83	18.55	4.87	13.03
	exGLI	30.38	31.49	27.39	41.07	38.71	31.00	33.34	8.72	12.98
	exAG	4.47	7.31	6.68	5.84	7.41	7.22	6.94	3.00	22.69
Moderate heat stress	**FPC**	11.07	11.47	11.41	13.03	12.52	12.72	12.04	0.73	3.01
	exHMW	4.69	6.855	7.14	7.25	5.68	6.55	6.36	1.91	14.91
	exLMW	18.94	21.15	20.65	17.76	24.28	15.82	19.76	1.95	4.89
	exGLI	32.97	36.38	37.78	40.67	48.32	50.05	41.03	2.65	3.20
	exAG	7.07	7.52	7.88	7.64	8.33	8.82	7.87	1.75	11.05
Severe heat stress	**FPC**	13.94	13.75	13.43	14.79	12.73	15.27	13.98	0.21	0.74
	exHMW	8.22	6.55	4.11	5.20	7.21	6.17	6.24	0.52	4.15
	exLMW	12.22	16.44	14.71	17.19	17.96	15.78	15.71	4.54	14.33
	exGLI	55.25	50.15	44.77	44.77	33.98	46.19	45.85	3.93	4.25
	exAG	8.48	7.645	6.8	6.79	6.725	6.95	7.23	1.33	9.12
Moderate drought stress	**FPC**	11.32	11.89	11.72	13.06	11.78	11.69	11.91	0.45	1.88
	exHMW	6.21	6.37	7.87	7.07	7.08	7.88	7.09	2.42	16.97
	exLMW	18.94	18.15	15.81	17.07	17.16	20.17	17.88	5.61	15.59
	exGLI	28.06	34.01	37.57	39.54	42.52	45.01	37.78	7.22	9.49
	exAG	7.05	8.28	7.84	6.24	6.07	5.15	6.77	2.12	15.54
Severe drought stress	**FPC**	14.33	15.60	14.72	15.93	15.83	16.18	15.43	0.64	2.07
	exHMW	6.035	5.335	5.43	7.49	6.22	4.75	5.88	1.20	10.09
	exLMW	18.05	15.86	17.8	16.98	16.85	19.42	17.50	1.73	4.91
	exGLI	45.06	32.53	42.75	41.47	40.91	48.55	41.88	4.16	4.94
	exAG	6.49	5.20	6.30	6.09	5.53	8.33	6.32	1.09	8.62

FPC=flour protein content, exHMW=extractable high molecular weight, exLMW=extractable low molecular weight, exGLI= extractable gliadins, exAG=extractable albumins-globulins, LSD=least significant differences, CV=coefficient of variation.

Gliadins

Genotype effect was significant for all the environments except for flood irrigation in year 1 for exGLI. There were significant genotypic effects for moderate heat and severe drought in year 2 but not for other treatments (Tables 3.2 and 3.3). The highest exGLI was found under severe heat in both years in AtilC2000 (55.77%) and MexicaliC75 (55.25%) for year 1 and 2 respectively. Flood irrigation caused the lowest exGLI values for both years in CirnoC2008 (26.51%) and AltarC84 (27.39%) (Tables 3.4 and 3.5).

Albumins and globulins

There were significant genotype effects for optimum, severe heat and moderate drought stress in year 1, but not for the other treatments (Table 3.2). In year 2, genotype effect was significant for optimum and severe drought stress (Table 3.3). In year 1, optimum conditions showed the highest exAG content (MexicaliC75, 8.55%) and the lowest value was obtained under severe heat stress (CirnoC2008, 4.09%) (Table 3.4). In year 2, the highest value was found in YavarosC79 (9.80%) under optimum and the lowest value under flood irrigation in MexicaliC75 (4.47%) (Table 3.5).

3.5.2 Analysis of variance for SDS-unextractable protein fractions for year 1 and year 2

Large unextractable polymeric proteins

The genotype effects on LUPP were significant ($p \leq 0.05$) in year 1 in all environments except for severe heat (Table 3.2). In year 2, genotype effect was significant for optimum conditions and flood irrigation (Table 3.3). The highest LUPP values were found under optimum and flood irrigation for year 1 and 2 respectively. YavarosC79 had the highest LUPP values of 68.76% for year 1 and CirnoC2008 (69.44%) ranked the highest in year 2 (Tables 3.6 and 3.7). The lowest LUPP values were found under severe heat stress (AtilC2000, 38.06%) and optimum conditions (MexicaliC75, 31.80%) (Tables 3.6 and 3.7).

SDS-unextractable polymeric proteins

Significant differences between values were observed in all environments except for moderate heat and severe heat in year 1. In year 2, genotype effect was significant for flood irrigation and severe heat stress stress conditions (Tables 3.2 and 3.3). The highest UPP value was obtained under flood irrigation in AtilC2000 (68.93%) in year 1 and in CirnoC2008 (66.88%) for year 2 under severe drought stress conditions (Tables 3.6 and 3.7). The lowest UPP was observed under severe heat

stress conditions in both years in YavarosC79 (41.44%) and lowest in MexicaliC75 (35.14%) year 1 and 2 respectively.

High molecular weight proteins

The maximum unHMW values were 9.87% in year 1 and 9.59% in year 2. YavarosC79 showed the highest unHMW for both years. The highest unHMW values were found under flood irrigation for both years. The lowest values were 3.47% (MexicaliC75) in year 1 and 3.17% (JupareC2001) in year 2 (Tables 3.6 and 3.7). Moderate heat and optimum conditions caused the lowest unHMW values for year 1 and 2 respectively. Genotype effect was significant for all environments in year1. In year 2, genotype effect was significant in all environments except moderate drought stress conditions (Tables 3.2 and 3.3).

Low molecular weight proteins

There were significant differences between genotypes in all environments in year 1. In year 2 genotype effect was significant for optimum, flood and moderate drought conditions (Tables 3.2 and 3.3). The highest unLMW values were found in YavarosC79 in both years, 27.71% and 27.39% for year 1 and 2, respectively. The highest unLMW values were found under flood irrigation. The lowest values were 12.82% (AltarC84) in year 1 and 13.08% (CirnoC2008) in year 2 (Tables 3.6 and 3.7). Moderate heat stress conditions caused the lowest unLMW values.

Gliadins

In year 1, genotype effect was significant for all environments except for flood irrigation, moderate heat and severe drought stress (Table 3.2). In year 2, genotype effect was significant only for moderate heat and severe drought stress conditions (Table 3.2). Flood irrigation presented the highest unGLI value for both years. CirnoC2008 ranked the highest in both years with unGLI values of 5.96% and 5.93% for year 1 and year 2, respectively (Table 3.7). The lowest unGLI values were 1.73% (JupareC2001) in year 1 and 1.27% in year 2 (Tables 3.6 and 3.7). Optimum conditions showed the lowest unGLI for year 1 and severe heat stress for year 2.

Table 3.6 Means for measured SDS-unextractable proteins for year 1 for six durum wheat cultivars under different environmental conditions

Environment	Protein fractions	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Mean	LSD	CV
Optimum Conditions	LUPP	54.08	68.76	58.82	58.49	56.14	42.09	56.39	4.26	3.75
	UPP	62.15	58.55	55.53	46.58	55.60	56.74	55.86	4.54	4.03
	unHMW	5.03	7.69	5.71	5.67	5.03	6.38	5.92	0.42	3.56
	unLMW	22.54	25.38	19.88	14.72	22.18	21.34	21.01	2.60	6.15
	unGLI	3.40	5.77	3.48	3.54	1.73	3.17	3.51	0.99	13.97
	unAG	0.84	1.02	0.89	0.73	2.50	0.66	1.10	0.70	31.52
Flood Irrigation	LUPP	42.30	61.30	63.03	54.78	54.27	67.55	57.20	7.94	6.89
	UPP	44.36	56.86	58.83	68.93	55.00	57.61	56.93	9.61	8.37
	unHMW	5.10	9.87	7.64	5.61	5.02	7.86	6.85	2.26	16.36
	unLMW	24.21	27.71	23.42	20.63	20.45	18.21	22.44	2.06	4.55
	unGLI	4.59	2.53	3.95	3.95	4.62	5.96	4.27	1.67	19.45
	unAG	0.80	1.32	1.41	1.07	1.76	1.33	1.28	1.45	56.31
Moderate heat stress	LUPP	56.63	51.35	47.72	36.51	66.80	67.11	54.35	11.51	10.51
	UPP	53.70	42.00	46.00	59.32	53.57	51.32	50.98	8.04	7.83
	unHMW	3.47	5.05	4.92	6.01	6.43	7.31	5.53	1.58	14.17
	unLMW	18.15	15.68	12.82	23.45	19.32	19.78	18.20	3.23	8.81
	unGLI	1.75	2.53	2.84	3.06	3.35	5.30	3.14	1.59	25.16
	unAG	0.48	0.54	0.50	0.73	0.72	0.78	0.62	0.73	56.99
Severe heat stress	LUPP	51.40	46.50	43.84	38.06	41.55	58.85	46.70	15.80	16.79
	UPP	57.26	41.44	43.00	42.96	43.00	49.94	46.26	12.62	13.54
	unHMW	5.72	4.40	5.56	4.04	3.98	8.22	5.32	1.77	16.55
	unLMW	19.01	15.06	16.26	13.44	15.19	15.03	15.66	1.00	3.16
	unGLI	3.48	3.07	3.76	3.15	1.77	2.96	3.03	0.56	9.19
	unAG	0.80	0.69	0.80	0.41	0.66	1.00	0.72	0.57	38.91
Moderate drought stress	LUPP	43.01	52.46	55.83	57.10	60.54	65.94	55.81	4.35	7.20
	UPP	54.06	45.59	52.49	55.07	60.23	42.88	51.72	7.79	3.76
	unHMW	7.64	4.55	5.46	5.29	5.06	4.92	5.48	1.28	12.03
	unLMW	20.59	17.08	19.21	23.79	19.27	15.62	19.26	4.34	3.97
	unGLI	5.66	3.01	3.74	3.87	3.31	2.81	3.73	1.08	12.14
	unAG	1.20	0.81	0.84	0.85	0.73	0.67	0.85	1.38	20.57
Severe drought stress	LUPP	55.13	50.41	62.12	55.04	67.79	44.05	55.75	6.02	5.36
	UPP	46.9	41.6	52.3	58.1	46.6	46.2	48.61	7.37	7.52
	unHMW	3.52	4.82	4.96	7.84	5.52	4.98	5.27	1.25	11.82
	unLMW	20.68	14.04	17.12	14.19	15.93	16.03	16.33	2.55	7.76
	unGLI	3.52	2.36	3.62	3.63	2.67	2.44	3.04	2.03	33.19
	unAG	0.75	0.51	0.66	0.64	0.59	0.53	0.61	0.34	27.50

LUPP=large unextractable polymeric proteins, UPP=large unextractable polymeric proteins, unHMW=unextractable high molecular weight, unLMW= unextractable low molecular weight, unGLI=unextractable gliadins, unAG= unextractable albumins-globulins, LSD=least significant difference, CV=coefficient of variation.

Table 3.7 Means for SDS-unextractable proteins for year 2 for six durum wheat cultivars under different environmental conditions

Environment	Protein fractions	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Mean	LSD	CV
Optimum conditions	**LUPP**	31.80	66.44	50.18	42.92	44.77	50.36	47.74	11.42	11.87
	UPP	61.78	54.92	37.46	45.30	46.63	48.31	49.06	22.48	22.75
	unHMW	7.985	5.08	5.875	4.82	3.17	5.185	5.35	2.02	18.74
	unLMW	17.23	25.22	23.38	15.29	19.50	20.52	20.19	2.41	5.91
	unGLI	5.26	2.21	2.73	2.42	3.44	3.125	3.20	1.80	27.94
	unAG	1.18	0.74	1.39	0.54	0.77	0.79	0.90	0.37	20.31
Flood irrigation	**LUPP**	52.46	52.13	47.75	41.09	35.00	69.41	49.64	8.56	8.56
	UPP	53.61	57.97	55.70	38.64	36.44	65.90	51.38	11.16	10.78
	unHMW	8.04	9.59	7.78	5.07	4.68	8.42	7.26	1.25	8.56
	unLMW	22.32	27.39	24.36	13.3	23.84	25.47	22.78	5.00	10.90
	unGLI	4.51	3.445	4.23	3.00	4.00	5.93	4.19	2.10	24.79
	unAG	1.11	0.97	1.01	0.87	0.58	1.58	1.09	0.69	33.48
Moderate heat stress	**LUPP**	56.03	41.04	39.63	45.24	65.28	33.54	46.79	17.51	18.57
	UPP	55.68	42.08	39.79	57.85	37.87	39.71	44.5	7.68	8.38
	unHMW	3.31	3.49	5.44	5.57	7.25	9.39	5.74	2.20	19.02
	unLMW	19.62	15.57	13.61	16.56	17.33	13.08	15.96	7.56	23.50
	unGLI	4.29	2.56	2.26	2.24	1.75	1.27	2.39	1.03	21.74
	unAG	0.76	0.75	0.55	0.66	0.34	0.36	0.57	0.55	48.30
Severe heat stress	**LUPP**	60.92	45.84	44.68	43.24	36.53	45.57	46.13	9.44	10.16
	UPP	35.14	44.37	48.10	48.80	46.56	45.66	44.77	11.83	13.12
	unHMW	3.87	4.53	4.15	5.25	4.91	4.72	457	1.49	16.16
	unLMW	22.56	17.43	20.22	16.45	16.16	18.59	18.57	5.39	14.41
	unGLI	1.745	2.88	3.57	2.49	2.84	4.85	3.06	0.94	15.27
	unAG	0.31	0.68	0.69	0.61	0.87	0.68	0.64	0.32	25.14
Moderate drought stress	**LUPP**	52.45	40.96	34.05	39.21	47.92	33.53	43.35	4.35	5.23
	UPP	57.14	50.21	38.87	46.81	51.17	46.78	48.52	7.88	7.97
	unHMW	4.37	4.44	4.56	5.57	6.35	7.34	5.44	1.28	11.71
	unLMW	14.12	25.70	23.00	22.8	20.29	16.72	20.44	3.44	10.55
	unGLI	4.22	3.08	2.57	2.90	3.25	2.50	3.09	1.08	17.40
	unAG	0.81	0.875	0.88	1.88	0.67	1.09	1.03	1.37	66.09
Severe drought stress	**LUPP**	56.90	47.74	52.16	50.08	47.99	60.27	52.52	10.81	10.22
	UPP	39.96	52.21	48.09	46.38	51.01	66.88	50.75	5.84	5.71
	unHMW	5.57	4.94	6.35	5.66	7.89	7.94	6.39	1.03	8.02
	unLMW	14.60	25.39	18.47	15.91	17.94	13.22	17.59	6.74	19.01
	unGLI	1.93	3.44	4.40	2.22	3.69	3.64	3.22	1.16	17.91
	unAG	1.03	0.71	0.73	0.55	0.74	0.64	0.73	0.31	20.80

LUPP=large unextractable polymeric proteins, UPP=large unextractable polymeric proteins, unHMW=unextractable high molecular weight, unLMW= unextractable low molecular weight, unGLI=unextractable gliadins, unAG= unextractable albumins-globulins, LSD=least significant difference, CV=coefficient of variation.

Albumins and globulins

In year 1, the highest unAG content was found under optimum conditions (2.50%) in JupareC2001 (Table 3.6). In year 2, the highest value was found in AtilC2000 (1.88%) under moderate drought stress conditions (Table 3.7). The lowest unGLI values were 0.41% (AtilC2000) in year 1 and 0.31% (MexicaliC75) in year 2 (Tables 3.6 and 3.7). Severe heat stress conditions had the lowest unGLI value in both years. Most environments showed non-significant differences between genotypes with exception of optimum conditions in year 1 (Table 3.2). In year 2, genotype effect was only significant for flood, moderate drought and severe heat stress conditions (Table 3.3).

3.5.3 Analysis of variance for flour protein content and SDS-extractable protein fractions combined for two years

Flour protein content

Analysis of variance revealed significant genotype effects in all environments. Environment effect was significant in all environments except for severe drought stress. The GxE interaction effect was significant in all treatments except for optimum conditions and severe heat stress (Table 3.8). The lowest FPC value was observed under flood irrigation at 10.20% (MexicaliC75) and severe drought showed the highest FPC value (16.44% for CirnoC2008) (Table 3.9). Environment was the main contributor to variation in FPC except under severe drought stress, where genotype made a large contribution to variation (Table 3.8). The severe drought stress caused the highest FPC percentage, followed by severe heat stress conditions (Table 3.9).

High molecular weight protein fractions

Genotype effect was significant for exHMW for all the environments (Table 3.8) except for moderate and severe drought stress. Environment effect was significant for exHMW for all environments except for flood irrigation and severe heat stress. The GxE effects were only significant for optimum conditions, severe heat and severe drought stress conditions. Moderate heat stress showed the highest percentage of exHMW 7.72% (AtilC2000) and optimum conditions the lowest 3.35% (CirnoC2008) (Table 3.9).

Low molecular protein fractions

The genotype effect was only significant for flood and moderate heat stress. Environment effect was significant for flood and moderate heat stress conditions. GxE effect was significant in environments except under optimum conditions, severe heat stress and moderate drought stress

(Table 3.8). The highest amount of exLMW was obtained under moderate heat stress (21.58%) for YavarosC79. The lowest value was found under severe heat stress conditions (15.67%) for CirnoC2008 (Table 3.9).

Gliadins

Genotype effect for gliadins was significant in all environments except for flood irrigation. Environment effect was significant for moderate heat and severe heat stress conditions as well as for severe drought stress. GxE contributed significantly to variation in moderate and severe heat stress and severe drought stress environments (Table 3.8). The moderate drought conditions showed the lowest exGLI percentage of 29.05% (MexicaliC75) and severe heat the highest of 55.51% (AtilC2000) (Table 3.9).

Albumins and globulins

There was significant genotype effect on exAG in all environments except for moderate heat stress and moderate drought stress. Environment effect was only significant for severe heat stress and severe drought stress conditions. GxE effects for exAG were significant in all environments except for moderate and severe heat stress (Table 3.8). The lowest exAG value was observed under severe heat stress at 5.52% (CirnoC2008) and optimum conditions showed the highest exAG value of 8.80% (YavarosC79) (Table 3.9).

Table 3.8 Mean squares for flour protein and SDS-extractable and unextractable proteins for two years combined

Environment	Sources of variation	FPC	exHMW	exLMW	exGLI	exAG	LUPP	UPP	unHMW	unLMW	unGLI	unAG
Optimum conditions	**Genotype**	0.64**	5.6***	4.58	79.75*	3.91***	295.30***	150.84	3.11**	44.11***	1.76*	0.52**
	Environment	4.54***	6.50***	4.49	15.41	0.54	448.85***	276.76	1.91	3.99	0.60	0.25
	GxE	0.263	1.37*	25.24	11.91	2.69***	113.52**	43.24	3.85**	8.92**	4.05**	0.65**
Flood irrigation	**Genotype**	1.98***	4.750*	100.16**	2.71	8.74*	0.743	32.93	7.91	0.13	1.21	247.55**
	Environment	7.47***	3.249	343.30**	0.04	0.71	8.89	0.168	19.458*	0.41	1.012	185.09*
	GxE	0.54**	2.362	336.01***	1.88	62.91***	2.238	124.98*	7.497	0.30	13.91***	141.66**
Moderate heat stress	**Genotype**	0.99**	5.14**	21.30***	124.69***	0.64	346.63**	183.61***	4.18*	24.67	1.07	0.02
	Environment	1.93**	8.27*	23.92	24.10*	1.93	342.77*	180.68**	0.27	30.06	3.32*	0.02
	GxE	0.54**	1.95	7.18*	42.56***	1.87	206.92*	49.02	10.13***	13.83	3.91*	0.09
Severe heat stress	**Genotype**	2.92**	4.04**	8.22	133.32***	3.10**	80.87	43.77	1.73	6.42	1.90***	0.04
	Environment	3.42***	0.44	73.36**	32.57*	10.53***	1.94	13.38	3.37	50.63**	0.01	0.04
	GxE	0.22	3.94**	11.15	73.74***	1.09	159.49*	81.89	3.95**	12.67*	1.27*	0.11
Moderate drought stress	**Genotype**	1.78***	1.67	7.30	142.08***	1.31	173.723***	91.59**	1.39*	28.50***	2.50**	0.21
	Environment	3.42***	39.76***	0.41	79.03	1.38	1254.84***	62.31*	0.01	8.34	2.50	0.21
	GxE	0.25*	1.54	6.78	6.50	2.04*	61.13**	63.87**	3.97**	25.07**	0.397	0.24
Severe drought stress	**Genotype**	4.14***	0.49	8.73	71.70**	1.87*	51.00	88.96*	4.16***	37.44	1.33	0.03
	Environment	0.40	15.03***	2.95	60.71*	3.23*	62.79	27.61	7.55***	9.44	0.20	0.09
	GxE	0.61*	3.64***	15.15***	45.80**	1.79*	141.17**	139.24***	3.08**	11.48	1.23	0.04

LUPP=large unextractable polymeric proteins, UPP=large unextractable polymeric proteins, exHMW=extractable high molecular weight, unHMW=unextractable high molecular weight, unLMW= unextractable low molecular weight, unGLI=unextractable gliadins, unAG= unextractable albumins-globulins, CV=coefficient of variation, LSD=least significant differences FPC=flour protein content, exHMW=extractable high molecular weight, exLMW=extractable low molecular weight, exGLI= extractable gliadins, unGLI=unextractable gliadins, exAG=extractable albumins-globulins, GxE=genotype by environment, *p≤0.05,**p≤0.01, ***p≤0.001

Table 3.9 Means for flour protein content and SDS-extractable proteins for two years combined for six durum wheat cultivars under different environmental conditions

Environment	Protein Fractions	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Mean	LSD	CV
Optimum conditions	**FPC**	10.74	11.00	10.68	11.78	10.98	10.88	11.01	0.41	2.88
	exHMW	3.63	6.73	4.81	4.79	4.86	3.35	4.70	0.74	12.24
	exLMW	19.37	19.02	20.84	18.53	18.26	17.79	18.97	3.59	14.76
	exGLI	33.05	36.97	37.90	46.09	40.09	41.87	39.33	5.06	10.04
	exAG	8.19	8.80	6.47	7.17	6.92	6.30	7.31	0.57	6.13
Flood irrigation	**FPC**	10.20	11.00	10.55	12.27	10.82	10.93	10.96	0.37	2.67
	exHMW	5.00	7.14	5.53	4.79	3.84	5.31	5.27	1.50	22.15
	exLMW	19.59	19.04	17.48	16.63	15.94	17.20	17.65	2.26	10.00
	exGLI	31.66	34.42	31.40	38.53	30.90	32.61	33.25	7.13	16.72
	exAG	6.37	7.32	6.95	6.98	7.56	7.41	7.10	1.70	18.66
Moderate heat stress	**FPC**	11.14	11.55	11.38	12.52	11.96	11.95	11.75	0.39	2.63
	exHMW	4.78	5.83	6.29	7.72	4.70	5.31	5.77	1.23	16.61
	exLMW	17.14	21.58	19.40	17.48	21.13	15.87	18.77	1.80	7.49
	exGLI	40.93	42.29	41.84	50.27	32.91	43.92	42.03	2.64	4.90
	exAG	7.45	7.59	7.47	7.99	6.98	8.08	7.59	0.98	10.06
Severe heat stress	**FPC**	14.14	14.15	13.56	15.25	13.52	15.55	14.36	0.21	1.13
	exHMW	6.42	5.08	5.64	5.05	7.01	7.43	6.10	1.00	12.84
	exLMW	16.78	20.03	17.38	17.37	17.55	15.67	17.46	2.69	12.01
	exGLI	38.68	45.23	42.85	55.51	44.25	41.59	44.68	2.64	4.60
	exAG	7.68	7.42	6.15	6.84	5.82	5.52	6.57	0.78	9.22
Moderate drought stress	**FPC**	11.46	12.30	11.80	13.42	12.32	12.43	12.88	0.31	1.99
	exHMW	4.96	5.50	5.98	5.38	6.80	6.12	5.79	1.16	15.66
	exLMW	17.02	19.66	17.02	16.48	17.05	19.27	17.75	2.67	11.73
	exGLI	29.05	39.52	38.02	46.68	41.58	42.73	39.60	3.56	7.01
	exAG	7.55	7.78	7.12	6.71	6.57	6.34	7.01	1.00	11.15
Severe drought stress	**FPC**	13.73	14.40	15.67	15.71	15.85	16.44	15.30	0.45	2.29
	exHMW	4.38	5.24	5.15	5.29	5.21	5.25	5.09	0.62	9.57
	exLMW	16.11	20.44	18.36	17.25	17.10	17.79	17.84	1.51	6.59
	exGLI	43.71	39.32	39.17	50.52	45.35	42.74	43.47	3.36	6.03
	exAG	6.51	6.03	6.36	6.32	6.98	7.91	6.69	0.76	8.91

FPC=flour protein content, exHMW=extractable high molecular weight, exLMW=extractable low molecular weight, exGLI= extractable gliadins, unGLI=unextractable gliadins, exAG=extractable albumins-globulins, CV=coefficient of variation, LSD=least significant difference

3.5.4 Analysis of variance for measured SDS-unextractable protein fractions for two years combined

Large unextractable polymeric proteins

Genotype effect for LUPP was significant for optimum conditions, moderate heat stress and moderate drought stress conditions. The environment effect was significant for all environments except for optimum conditions, moderate heat and moderate drought stress conditions. The GxE effect was significant for all environments except for flood irrigation (Table 3.8). The highest LUPP value was under optimum conditions (67.60%) in YavarosC79. The lowest value was obtained under severe heat conditions in AtilC2000 (40.65%) (Table 3.10).

Unextractable polymeric proteins

Analysis across two years showed significant genotype effect for UPP under moderate heat, moderate drought and severe drought stress. Environment effect on UPP was only significant for moderate heat and moderate drought stress conditions. GxE effect was only significant for flood irrigation, moderate drought stress and severe drought stress conditions (Table 3.8). Flood irrigation caused the highest UPP value of 67.41% (AtilC2000) and moderate heat the lowest value of 42.04% (YavarosC79) (Table 3.10).

High molecular weight proteins

Highly significant genotype effects were evident for unHMW for all environments except for flood irrigation and severe heat stress. Environment effect was only significant for flood irrigation and severe drought conditions. The GxE effect was highly significant for all environments except flood irrigation (Table 3.8). The highest unHMW (7.71%) was observed in AltarC84 under flood irrigation conditions and the lowest (4.10%) in JupareC2001 under optimum conditions (Table 3.10).

Low molecular weight proteins

Genotype effect was only significant for optimum and moderate drought stress conditions. Environment effect was significant for unLMW only in severe heat stress conditions. The GxE effect was non-significant for unLMW for flood irrigation, moderate heat stress and severe drought stress. (Table 3.8). The lowest unLMW value was observed under moderate heat stress condition in AltarC84 (13.22%) and highest in YavarosC79 (25.30%) under optimum conditions (Table 3.10).

Gliadins

The effect of genotype on unGLI was significant in all conditions except for flood irrigation, moderate heat and severe drought stress. The effect of environment was only significant for moderate heat stress. GxE for unGLI was significant for all conditions except for moderate drought and severe drought stress (Table 3.8). The highest amount of unGLI was obtained under flood irrigation (4.98%) for CirnoC2008. The lowest value was found under moderate heat stress (2.00%) for MexicaliC75) (Table 3.10).

Albumins-globulins

Genotype, environment and GxE effect was non-significant for unAG for all conditions except for flood irrigation and optimum conditions (Table 3.8). Environment effect was non-significant for optimum conditions. Optimum conditions had the highest unAG value of 1.63% (JupareC2001) and moderate heat the lowest of 0.52% (AltarC84) (Table 3.10).

Table 3.10 Mean values for SDS-unextractable proteins for two years combined for six durum wheat cultivars under different environmental conditions

Environment	Genotypes	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Mean	LSD	CV
Optimum conditions	**LUPP**	42.94	67.60	54.50	50.71	50.45	46.22	52.07	5.48	8.21
	UPP	61.96	56.73	46.49	45.94	51.12	52.52	52.46	10.32	15.35
	unHMW	6.51	6.39	5.79	5.24	4.10	5.78	5.63	0.93	12.86
	unLMW	19.88	25.30	21.63	15.01	20.84	20.93	20.60	1.59	6.04
	unGLI	4.33	3.99	3.11	2.98	2.58	3.15	3.36	0.92	21.48
	unAG	1.01	0.88	1.14	0.63	1.63	0.72	1.00	0.36	27.75
Flood irrigation	**LUPP**	47.38	51.19	55.39	62.09	53.20	51.27	53.42	5.25	7.67
	UPP	48.98	47.75	57.26	67.41	56.48	47.03	54.15	6.62	9.54
	unHMW	6.57	7.47	7.71	7.02	7.31	6.27	7.06	1.16	12.84
	unLMW	23.27	20.50	23.89	23.05	23.92	21.02	22.61	2.43	8.39
	unGLI	4.55	2.76	4.09	4.94	4.03	4.98	4.23	1.20	22.23
	unAG	0.95	1.10	1.21	1.33	1.37	0.96	1.15	0.72	49.06
Moderate heat stress	**LUPP**	56.33	46.19	43.67	40.87	66.04	50.32	50.57	9.42	14.54
	UPP	54.69	42.04	42.90	58.59	45.72	45.51	48.24	5.00	8.09
	unHMW	5.36	5.25	5.24	7.70	4.96	5.31	5.63	1.22	16.86
	unLMW	18.88	15.63	13.22	20.01	18.33	16.43	17.08	3.70	16.89
	unGLI	2.00	2.55	2.66	2.70	3.28	3.41	2.76	0.85	24.08
	unAG	0.62	0.65	0.52	0.69	0.53	0.57	0.60	0.41	53.27
Severe heat stress	**LUPP**	48.04	53.71	44.70	40.65	43.69	47.69	46.41	8.28	13.92
	UPP	51.91	45.12	45.55	44.31	43.68	42.54	45.52	7.78	13.34
	unHMW	5.31	4.82	4.85	4.38	4.25	6.04	4.94	1.04	16.43
	unLMW	17.58	15.75	18.24	16.01	16.31	18.79	17.11	2.47	11.24
	unGLI	3.16	2.78	3.66	4.00	2.32	2.35	3.05	0.49	12.63
	unAG	0.83	0.65	0.74	0.55	0.67	0.65	0.68	0.29	33.66
Moderate drought stress	**LUPP**	56.49	41.98	45.57	47.52	56.93	42.99	48.58	4.14	6.64
	UPP	55.60	47.90	45.68	50.94	55.70	44.83	50.11	3.92	6.11
	unHMW	6.04	5.94	5.90	4.92	5.32	4.64	5.46	0.83	11.87
	unLMW	17.35	21.39	21.11	23.30	19.78	16.17	19.85	2.07	8.15
	unGLI	4.94	3.04	3.16	3.38	3.28	2.65	3.41	0.64	14.57
	unAG	0.70	0.84	0.86	0.88	1.01	1.37	0.94	0.64	53.00
Severe drought stress	**LUPP**	51.56	50.24	54.93	55.97	59.97	52.16	54.14	5.57	8.02
	UPP	49.58	46.29	49.34	53.08	43.27	56.53	49.68	4.23	6.64
	unHMW	4.23	6.35	5.31	7.10	5.54	6.46	5.83	0.73	9.78
	unLMW	23.04	15.99	16.52	16.33	15.26	14.62	16.96	3.24	14.91
	unGLI	3.48	3.03	2.92	4.02	2.30	3.04	3.13	1.05	26.26
	unAG	0.73	0.62	0.60	0.68	0.81	0.58	0.67	0.20	23.88

LUPP=large unextractable polymeric proteins, UPP=large unextractable polymeric proteins, unHMW=unextractable high molecular weight, unLMW= unextractable low molecular weight, unGLI=unextractable gliadins, unAG= unextractable albumins-globulins, LSD=least significant differences, CV=coefficient of variation.

3.5.5 Combined analysis of variance across environments and years for flour protein content and SDS-extractable proteins

Analysis of variance (Table 3.11) showed a highly significant genotype effect for FPC, exLMW and exGLI and significant genotype effect for exHMW and exAG. Environment effect was highly significant for FPC, exHMW, exGLI and exAG, and significant for exLMW. Year effect was significant for FPC and SDS-extractable proteins except for exLMW and exAG. Significant interactions of genotype with environment, environment with stress conditions, and interactions of all three these variables were also observed for all SDS-extractable proteins and FPC (Table 3.11).

Environmental means (Table 3.12) for FPC ranged between 10.96% (optimum conditions) and 15.30% (severe drought stress). The environmental means for exHMW and exLMW ranged between 4.70% (optimum conditions) and 6.10% (severe heat stress), 17.46% (severe heat stress) and 18.97% (optimum conditions), respectively. Environmental means for exGLI and exAG were 33.25% (flood irrigation) and 44.68% (severe heat stress), and 6.57% (severe heat stress) and 7.59% (moderate heat stress) respectively (Table 3.12). The FPC, exHMW, exLMW, exGLI and exAG means for genotypes ranged between 11.90-13.61%, 4.86-5.92%, 17.27-19.96%, 36.18-47.93%, and 6.75-7.49%, respectively (Table 3.13). Optimum environmental conditions resulted in the highest exLMW value. Severe heat stress showed the highest exHMW and exGLI values and severe drought stress showed the highest FPC values and moderate heat stress had the highest exAG value (Table 3.12).

The FPC was significantly increased by all stress treatments compared to the optimum conditions. Significant increases occurred in exHMW under all stress treatments except for severe drought stress, which caused a slight increase compared to optimum conditions (but not significant). All stress treatments caused a decrease in exLMW in the genotypes means and optimum conditions had the highest exLMW value. The exGLI showed an increase under all stress treatments, except for flood irrigation, that was signficiantly lower than the other treatments. The exAG protein fraction was sensitive to stress. All stress conditions caused a significant reduction in exAG with exception of moderate heat stress which caused a slight, but non-significant increase (Table 3.12).

On average (Table 3.13), AtilC2000 ranked the highest for FPC and exGLI, with values significantly higher than that of other cultivars. YavarosC79 ranked significantly higher for exHMW and exLMW values than the rest of the cultivars. The highest exAG values was also

obtained in YavarosC79, significantly higher than for most other genotypes except for MexicaliC75. AltarC84 and MexicaliC75 had the lowest exAG and exGLI values, respectively. CirnoC2008 ranked lowest in exLMW and MexicaliC75 ranked lowest in exHMW and FPC.

3.5.6 Combined analysis of variance across environments and years for SDS-unextractable proteins

There was a significant genotype effect for all measured SDS-unextractable proteins except for unLMW and unAG (Table 3.11). Highly significant differences between environments were observed for all the unextractable protein fractions. Year effect was significant except for unHMW, unLMW and unAG. Highly significant GxE was evident for all proteins except for unAG. The genotype by year effect was only significant for LUPP, UPP, unHMW and unGLI proteins. Environment by year effect was significant for most measured proteins, except for unGLI and unAG. Genotype with environment and year interaction effects were highly significant for all proteins except for unGLI and unAG (Table 3.11).

Table 3.11 Mean squares for flour protein content, and SDS-extractable and unextractable proteins across environments and years

	Protein fractions	G	E	Y	GxE	GxY	ExY	GxExY
SDS-extractable proteins	**FPC**	9.19***	78.78***	7.12***	0.65***	0.56***	2.81***	0.37***
	exHMW	2.82**	6.63***	54.45***	3.80***	1.05	3.76***	2.75***
	exLMW	24.80***	9.56*	0.53	6.65*	7.25	24.81***	13.15***
	exGLI	385.92***	399.77***	62.71*	39.71***	91.97***	29.85*	42.71***
	exAG	2.00**	3.48***	0.70	1.91***	2.20**	5.16***	1.90***
SDS-unextractable proteins	**LUPP**	130.06***	210.31***	1767.15***	183.53***	148.62***	137.47***	173.93***
	UPP	190.63***	223.74***	416.33***	123.14***	68.73*	65.90**	90.04***
	unHMW	2.11**	11.88***	0.15	2.73***	5.75***	2.80***	6.63***
	unLMW	9.76	132.92***	6.91	28.02***	9.52	19.25**	25.07***
	unGLI	3.82***	6.00***	2.47*	1.49***	1.96**	0.84	2.16
	unAG	0.14	1.19***	0.09	0.16	0.34	0.19	0.22

FPC=flour protein content, exHMW=extractable high molecular weight, exLMW=extractable low molecular weight, exGLI= extractable gliadins, exAG=extractable albumins-globulins, LUPP=large unextractable polymeric proteins, UPP=unextractable polymeric proteins, unHMW=unextractable high molecular weight, unLMW= unextractable low molecular weight, unGLI=unextractable gliadins, unAG=unextractable albumins-globulins, G=genotype, E=environment, Y=year, GxE=genotype by environment, GxY=genotype by year, ExY=environment by year, GxExY=genotype by environment by year, *p≤0.05,** p≤0.01, ***p≤0.001

Environmental means (Table 3.12) varied between 46.41% (severe heat stress) and 54.14% (severe drought stress) for LUPP. The UPP varied between 45.52% (severe heat stress) and 54.15% (flood irrigation). The environmental means for unHMW and unLMW ranged between 4.94% (severe heat stress) and 7.06% (flood irrigation), and 16.96% (severe drought stress) and 22.61% (flood irrigation). Environmental means for unGLI and unAG were between 2.76% (moderate heat stress) and 4.23% (flood irrigation), and 0.60% (moderate heat stress) and 1.15% (flood irrigation) respectively (Table 3.12). The flood irrigation showed the highest values for all unextractable proteins except for LUPP. Severe heat stress had the lowest LUPP, UPP and unHMW. The LUPP, UPP, unHMW and unLMW, unGLI and unAG means for genotypes varied between 48.44-55.05%, 47.64-53.79%, 5.24-6.06% and 17.99-20.00%, 2.84-3.98%, and 0.73-0.95%, respectively (Table 3.13).

Severe drought stress and flood irrigation caused significant increases in LUPP and unHMW. Under moderate drought and heat stress conditions, LUPP and unHMW were significantly reduced. Unextractable polymeric proteins and unLMW and unAG were significantly decreased by all stress conditions with the exception of flood irrigation which caused a significant increase. Moderate drought caused a slight decrease in unAG and unLMW, and UPP (non-significant). Severe drought stress and heat stress conditions caused a significant reduction in UPP, unLMW and unAG. For unGLI, flood irrigation caused a significant increase, while moderate drought caused a slight, but non-significant increase. Moderate heat stress caused a significant reduction, and severe drought and severe heat stress caused a smaller but non-significant reduction in unGLI. For the unAG, significant decreases occurred under all stress treatments, as well as flood irrigation (Table 3.12).

The overall performance of genotypes showed that JupareC2001 had significantly higher LUPP than other cultivars and ranked the lowest in unGLI and unHMW (Table 3.13). JupareC2001 also ranked highest in unAG, however, JapareC2001 was only significantly different from CirnoC2008 in unAG. MexicaliC75 had the highest UPP and unGLI values and ranked lowest for unLMW. MexicaliC75 was significantly different from other cultivars except AtilC2000 for UPP and significantly different from other cultivars for unGLI. AtilC2000 ranked the highest in unHMW and was only significantly different from JupareC2001. CirnoC2008 ranked the highest for unLMW and was significantly different from MexicaliC75 and YavarosC79. YavarosC79 ranked lowest for UPP and CirnoC2008 ranked lowest for LUPP and unAG (Table 3.13).

Table 3.12 Environmental means for flour protein content, and SDS-extractable and unextractable proteins combined for years

	Protein fractions	Optimum conditions	Flood irrigation	Moderate heat	Severe heat	Moderate drought	Severe drought	LSD
SDS-	**FPC**	10.96	11.01	11.75	14.36	12.29	15.30	0.15
extractable	**exHMW**	4.70	5.27	5.77	6.10	5.79	5.09	0.38
proteins	**exLMW**	18.97	17.65	18.77	17.46	17.75	17.84	0.90
	exGLI	39.33	33.25	43.47	44.68	39.60	42.03	1.59
	exAG	7.31	7.10	7.59	6.57	7.01	6.69	0.37
SDS-	**LUPP**	52.07	53.42	50.57	46.41	48.58	54.14	2.49
unextractable	**UPP**	52.46	54.15	48.24	45.52	50.11	49.68	2.40
proteins	**unHMW**	5.63	7.06	5.63	4.94	5.46	5.83	0.37
	unLMW	20.60	22.61	17.08	17.11	19.85	16.96	1.00
	unGLI	3.35	4.23	2.76	3.04	3.41	3.13	0.83
	unAG	1.00	1.15	0.60	0.68	0.94	0.67	0.33

FPC=flour protein content, exHMW=extractable high molecular weight, exLMW=extractable low molecular weight, exGLI= extractable gliadins, exAG=extractable albumins-globulins, LUPP=large unextractable polymeric proteins, UPP=unextractable polymeric proteins, unHMW=unextractable high molecular weight, unLMW= unextractable low molecular weight, unGLI=unextractable gliadins, unAG= unextractable albumins-globulins, LSD=least significant differences

Table 3.13 Mean performance of genotypes for flour protein content, SDS-extractable and unextractable proteins combined for environments and years

	Protein fractions	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Grand mean	LSD	CV
SDS-	**FPC**	11.90	12.62	12.06	13.61	12.55	12.93	12.61	0.15	0.15
extractable	**exHMW**	4.86	5.92	5.57	5.50	5.40	5.46	5.45	0.38	14.62
proteins	**exLMW**	17.67	19.96	18.41	17.29	17.84	17.27	18.07	0.9	10.33
	exGLI	36.18	39.63	38.53	47.93	39.18	40.91	40.39	1.59	8.19
	exAG	7.29	7.49	6.75	7.00	6.80	6.92	7.04	0.37	10.82
SDS-	**LUPP**	50.45	51.82	49.79	49.63	55.05	48.44	50.86	2.49	10.18
unextractable	**UPP**	53.79	47.64	47.87	53.38	49.33	48.16	50.03	2.4	9.96
proteins	**unHMW**	5.67	6.04	5.80	6.06	5.24	5.75	5.76	0.37	13.48
	unLMW	17.99	18.95	19.07	19.09	19.10	20.00	19.03	1.00	10.95
	unGLI	3.98	3.05	3.26	3.55	2.84	3.24	3.32	0.33	20.7
	unAG	0.86	0.79	0.84	0.87	0.95	0.73	0.84	0.18	45.05

FPC=flour protein content, exHMW=extractable high molecular weight, exLMW=extractable low molecular weight, exGLI= extractable gliadins, exAG=extractable albumins-globulins, LUPP=large unextractable polymeric proteins, UPP=unextractable polymeric proteins, unHMW=unextractable high molecular weight, unLMW= unextractable low molecular weight, unGLI=unextractable gliadins, unAG= unextractable albumins-globulins, LSD=least significant differences CV=coefficient of variation.

3.5.7 Significant correlations between measured protein fractions and quality characteristics

All measured quality characteristics averaged for two years are given in Appendix 2. The UPP correlated significantly with alvW for the flood irrigation conditions, moderate drought stress and across environments. The UPP significantly correlated with alvL for flood irrigation and moderate heat stress, and negatively correlated with alvP and alvP/L for moderate heat stress. The LUPP was significantly correlated with alvW for moderate drought conditions and across environments. FPC correlated highly significantly with alvW under optimum conditions (r=0.66***), moderate heat stress (r=0.69***) and to a lesser extent across the environments. The FPC significantly correlated with alvL in all environments except for optimum conditions and severe drought stress. Flood irrigation showed the highest correlations between FPC and alvL (r=0.74***). FPC correlated negatively with alvP/L for moderate drought, severe drought and across all environments (Table 3.14 and Table 3.15).

Flood irrigation and across all environments (Table 3.14 and Table 3.15) showed negative correlations between exHMW and alvW. There was also significant negative correlation between exHMW and MPT for flood irrigation and across environments. The unHMW correlated highly positively with alvP (r=0.71***) under moderate drought stress conditions but correlated negatively with alvP under moderate heat stress. The unHMW negatively correlated with alvP/L and positively with alvL under moderate heat stress conditions. The exHMW showed significant negative correlations with alvL and positive correlations with alvP/L for optimum conditions. The exLMW positively correlated with alvP/L for optimum condotions, flood irrigation, moderate heat stress and across environments. Moderate heat stress conditions and across environments showed significant negative correlations between exLMW and alvL. Severe drought stress and across environments presented negative correlations between exLMW and alvW. Both alvP and alvP/L positively correlated with unLMW under optimum conditions but unLMW negatively correlated with alvP and alvP/L under moderate drought stress conditions. Moderate heat stress and moderate drought stress conditions showed significant correlations between unLMW and alvL but optimum conditions showed negative correlations between unLMW and alvL.

The unGLI positively correlated with alvP and alvP/L under moderate drought conditions, and negatively with alvP/L for flood irrigation. The exGLI, correlated positively with alvW under moderate drought and severe heat stress conditions and positive with alvP under moderate drought and negative with alvP/L under optimum conditions and across environments.

Table 3.14 Significant correlations between protein fractions and quality characteristics combined for two years, for flood irrigation and moderate and severe heat stress

Optimum conditions			Flood irrigation			Moderate heat stress			Severe heat stress		
Protein fractions	**Quality characteristics**	**Correlations**	**Protein fractions**	**Quality characteristics**	**Correlations**	**Protein fractions**	**Quality characteristics**	**Correlations**	**Protein fractions**	**Quality characteristics**	**Correlations**
exHMW	alvL	-0.48*	LUPP	MPT	0.47*	UPP	alvP	-0.48*	LUPP	alvL	-0.51*
	alvW	-0.46*	UPP	alvL	0.50*		alvL	0.61**		alvP	0.43*
	alvP	0.59*		alvW	0.54*		alvP/L	-0.59**	exGLI	alvW	0.42*
exLMW	alvP/L	0.43*		MPT	0.55**		MPT	-0.46*	exAG	alvP	0.72***
unLMW	alvP	0.48*	exHMW	alvW	-0.67***	unHMW	alvP	-0.48*		alvW	0.43*
	alvL	-0.66***		MPT	-0.64**		alvL	0.57**		alvP/L	0.43*
	alvP/L	0.66***	exLMW	alvP/L	0.43*		alvP/L	-0.58**	FPC	alvP	-0.44*
exGLI	alvL	0.59*	exGLI	MPT	-0.43*	exLMW	alvL	-0.41*			
	alvP/L	-0.47*	unGLI	alvP/L	-0.48*		alvP/L	0.48*			
exAG		-0.49*		MPT	0.44*	unLMW	alvL	0.43*			
unAG	alvP	0.4612*	unAG	MPT	0.45*		MPT	-0.44*			
	MPT	0.55**	FPC	MPT	-0.53*	FPC	alvL	0.48*			
FPC	alvW	0.66***		alvL	0.74***		alvW	0.69***			

LUPP=large unextractable polymeric proteins, UPP=unextractable polymeric proteins, exHMW=extractable high molecular weight, unHMW=unextractable high molecular weight, exLMW=extractable low molecular weight, unLMW= unextractable low molecular weight, exGLI= extractable gliadins, unGLI=unextractable gliadins, exAG=extractable albumins-globulins, unAG= unextractable albumins-globulins, FPC=flour protein content, *p≤0.05, **p≤0.01, ***p≤0.001

Table 3.15 Significant correlations between measured protein fractions and quality characteristic combined for two years for moderate and severe drought stress and combined for all environments

Moderate drought stress			Severe drought stress			Across environments and years		
Protein Fractions	**Quality characteristics**	**Correlation**	**Protein Fractions**	**Quality characteristics**	**Correlation**	**Protein fractions**	**Quality characteristics**	**Correlation**
LUPP	alvP	0.43*	exLMW	alvW	-0.45*	LUPP	alvP	0.23**
	alvW	0.57**	unHMW	MPT	-0.48*		alvL	-0.16*
UPP	alvW	0.45**	exGLI	alvP	-0.48*		alvW	0.17*
unHMW	alvP	0.71***		alvL	0.47*		alvP/L	0.20*
exLMW	alvW	-0.56**		alvP/L	-0.49*	UPP	alvW	0.17*
unLMW	alvP	-0.42*	FPC	alvP	-0.69***	exHMW	alvP	-0.18*
	alvL	0.52*		alvP/L	-0.54**		alvW	-0.17*
	alvP/L	-0.52*					MPT	-0.19*
exGLI	alvP	0.52*				exLMW	alvL	-0.32***
	alvW	0.36					alvW	-0.18*
unGLI	alvP	0.47*					alvP/L	0.29***
	alvP/L	0.53*				exGLI	alvP	-0.20**
FPC	alvL	0.54**					alvP	0.40***
	alvP/L	-0.48*				exGLI	alvP/L	-0.28***
	MPT	-0.67***					MPT	-0.33***
						unAG	MPT	0.29***
						FPC	alvL	0.38***
							alvW	0.26**
							alvP/L	-0.23**
							MPT	-0.40***

LUPP=large unextractable polymeric proteins, UPP=unextractable polymeric proteins, exHMW=extractable high molecular weight, unHMW=unextractable high molecular weight, exLMW=extractable low molecular weight, unLMW= unextractable low molecular weight, exGLI= extractable gliadins, unGLI=unextractable gliadins, exAG=extractable albumins-globulins, unAG= unextractable albumins-globulins, FPC=flour protein content, *p≤0.05, **p≤0.01, ***p≤0.001

Severe heat stress showed significant positive correlations between exAG and alveograph parameters (alvP, alvW alvP/L), and optimum conditions presented significant positive correlations between unAG and alvP (Table 3.14). Moderate drought, flood irrigation conditions and across environments conditions (Table 3.15) presented negative correlations between FPC and MPT. The unHMW significantly negatively correlated with MPT under severe drought stress. The unLMW correlated negatively with MPT under moderate heat stress. The exGLI negatively correlated with MPT for flood irrigation and across environments and unGLI significantly correlated with MPT for flood irrigation. The unAG positively correlated with MPT under flood and optimum conditions and across environments (Tables 3.14 and 3.15).

3.6 Discussion

When selecting genotypes adapted to changing climatic conditions, it is important to consider their tolerance to high temperatures and drought, as well as the effects of these factors on quantity and quality of grain protein and its components (Balla et al. 2010). The genotype effect was non-significant for unAG in all environments except for flood irrigation and optimum conditions. This indicates that variability between cutivars for unAG in these environments was limited. FPC showed significant genotypic effect in all environments. The exGLI showed significant genotypic effects in all environments except for flood irrigation. Significant genotypic effect for measured protein fractions indicate genetic variability among genotypes.

There was large variation in FPC, and SDS-extractable and unextractable proteins fractions for all the environments. The analysis of variance showed significant genotype, environment, and GxE interaction effects for measured traits. Environment is known to have a large influence on FPC (Taghouti et al. 2010; Hasniza et al. 2014). This was also observed in this study, especially under severe heat and drought stress. There were large genetic effects for UPP and unAG under moderate drought stress and flood irrigation respectively. Large variances associated with genotype compared to environment and GxE effects showed that these traits were largely genetically determined. The exHMW varied significantly between cultivars under optimum conditions, LUPP under optimum conditions and moderate heat stress, and unHMW under severe drought stress conditions. Across year analysis indicated that environment was the largest contributor to variation in exGLI.

High FPC was observed under severe heat and drought stress in both years. Li et al. (2013), who studied the influence of heat on wheat under field conditions, and Labuschagne et al. (2016) who

investigated the influence of drought and heat in wheat in greenhouse trials, found an increase in FPC due to heat stress. Growth and development of plants as well as quality characteristics are affected by heat, with grain fill stage being mostly affected (Halford et al. 2014). Elevated protein quantities are associated with increased rates of nitrogen deposition and decreased carbohydrate synthesis under high temperature regimes. Higher temperatures increase FPC at the expense of grain yield. This happens due to a denaturing of enzymes responsible for starch granule biosynthesis, which causes a decrease in grain yield (Flagella et al. 2010; Park et al. 2014). Their studies also confirmed the findings in the current study that lower FPC values were found under optimum conditions and flood irrigation than under heat and drought stress conditions.

FPC is a primary grading factor that determines the price of wheat and is an indicator of potential end-use quality (Sissons et al. 2012). Petrova et al. (2007) and Sissons et al. (2012) stated that a FPC between 12 and 16% is essential for acceptance of durum wheat varieties by the industry. Most genotypes had FPC within an acceptable range under severe heat stress and both drought stress conditions. This suggest that these stress conditions had a positive influence in FPC treatments. Most genotypes under moderate heat stress conditions showed FPC below 12% with the exception of AtilC2000 which showed a FPC of 12.02%.

Lower LUPP, and UPP fractions for two years combined were found under severe heat stress conditions. This was also observed across the environments where significant reduction of LUPP and UPP occurred under severe heat stress conditions. Higher values for LUPP were observed under severe drought stress, indicating an increased expression of these proteins under severe drought stress condition. Labuschagne et al. (2016) reported increases in insoluble polymeric proteins due to drought. The results reported by these authors are in agreement with what was found in this study. Contrary to this, significant reduction in LUPP and UPP under severe heat stress conditions is in agreement with findings of Corbellini et al. (1997) who reported a decrease in insoluble polymeric under heat stress conditions.

Higher amounts of exHMW protein fractions were found under heat stress than other environmental conditions. This trait varied across years. Cornibelli et al. (1997) found an increase in extractable polymeric proteins due to high temperatures (37-40^{o}C) in both durum and bread wheat genotypes. Majoul-Haddad et al. (2013) exposed winter bread wheat at gain fill stage to four hours of heat stress at 38^{o}C for four successive days, and the glutenin protein fractions increased at 8 days after anthesis after heat treatment.

The highest unHMW and unLMW values were obtained under flood irrigation across two years and across environments, indicating that stress conditions compared to flood irrigation, significantly reduced these protein fractions. Both exLMW and unLMW proteins were sensitive to heat and drought stress conditions, where they were reduced. DuPont at al. (2006) and Labuschagne et al. (2016) reported significant reduction of LMW protein fractions under high temperatures, which corroborates what was observed in this study

The genotypes varied across environments per year and across years. This points to the occurrence of GxE interaction, which indicates that genotype rankings changed under different growing conditions. Therefore, it is necessary to assess genotypes for GxE interaction within different environments. Labuschagne et al. (2016) reported GxE interaction for insoluble large polymeric proteins and soluble small polymeric proteins, which agrees with results obtained in this study. Environment effects were significant for all protein fractions and FPC for combined analysis, confirming the large effect of growing season on protein fractions.

Significant correlations obtained between UPP and alvW (seen for flood irriation, moderate drought stress and across all environments in this study) were also reported by Gupta et al. (1993) in different wheat sets and by Edwards et al. (2007) in durum wheat cultivars. This indicated that UPP had a large influence on alvW, and can therefore be used as a good predictor of dough strength under moderate drought stress and flood irrigation conditions. UPP testing requires small flour samples and can thus be a useful tool in breeding programmes for early generation screening of lines with good rheological characteristics (Edwards et al. 2007). The exHMW correlated negatively with alvW (under flood irrigation and optimum conditions). This is contradictory to data reported by Dachkevitch and Autran (1989), Morel et al. (2000) and Wentzel (2017) who reported significant correlations between HMW and alvW. The exLMW (optimum conditions, flood irrigation, moderate heat and across environments) and unLMW (optimum conditions) correlated mostly significantly posively with alvP and alvP/L. Contrary to this, unLMW correlated negatively with alveograph parameters under moderate drought conditions. Significant correlations between unLMW and exLMW with alveograph characteristics were in agreement with findings of Zhang et al. (2009) and Wentzel (2010). These results indicated that unLMW and exLMW play an important role in alveograph parameters. In a study done by Waski (1998) in determining the relationship between protein composition of durum wheat and pasta quality and effects on processing and cooking quality, reported flours with high insoluble proteins gave firmer spaghetti with better cooking quality, these indicated importance of insoluble proteins in pasta

quality. The polymeric proteins are reported to affect dough strength and elasticity (Pompa et al. 2013), this was found in this study where polymeric proteins significantly correlated with dough strength and elasticity as measured by alvP and alvW.

The LMW-GS C- and D-types are classified as forms of α-/γ - and ω-gliadins, which has additional cysteine residues due to mutation. These types of LMW proteins are similar to gliadins in terms of structure but perform functions similar to glutenin proteins (Rustgi et al. 2019; Labuschagne et al. 2020). This may explain the significant correlations observed in this study, where both exGLI and unGLI showed significant positive correlation with alvW in some of the environments. Wang et al. (2008) reported that ω-gliadins had an influence on dough strength. Metakovsky et al. (1997) observed positive relationships between gliadin alleles and dough strength in French and Italian wheat cultivars. Significant positive correlation between the unAG and MPT, alvW and alvP and negative relationship with MPT justifies further research, as these proteins were reported to have no effect on dough functionality (Chaudhary et al. 2016).

3.7 Conclusions

There was wide variation in terms of response of genotypes to stress conditions. It is worth noting that all genotypes had the same HMW- and LMW-GS, although reaction to stress conditions differed among genotypes, indicating that other factors than the glutenins were at play. The effect of flood irrigation was more pronounced on unHMW and unLMW fractions across two years and across environments. Flood irrigation across two years and across all environments analysis presented the highest unHMW and unLMW values and caused significant increases in those fractions. Flood irrigation caused reductions in the other fractions, indicating flood irrigation to have a negative effect on these protein fractions. Severe drought stress caused increases in LUPP and unHMW. The results indicated that severe drought stress had a larger effect on unextractable protein fractions than heat stress conditions. The LMW and unAG proteins were sensitive to all stress conditions, as they were reduced by all heat and drought conditions.

Heat stress and severe drought stress conditions caused a significant increase in unGLI and moderate heat stress caused an increase in unAG. Severe drought stress, severe heat stress and flood irrigation presented little differences between genotypes for measured protein fractions, while optimum conditions showed more significant differences between genotypes for protein fractions. Inconsistency in genotype ranking observed in this study indicates occurrence of GxE

interaction. The reaction of genotypes to stress treatments differed, which indicates that the effects of stress conditions on wheat cultivars cannot be generalised.

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CHAPTER 4

Environmental effects on durum gluten protein composition measured by reversed phase-high performance liquid chromatography, and their impact on pasta quality

Abstract

Reversed phase-high performance liquid chromatography was used to assess the effect of environmental stress conditions (drought and heat) on quantitative and qualitative variation of wheat storage proteins in six durum wheat cultivars with the same high molecular weight and low molecular weight-glutenin subunit compositions. The trial was conducted by CIMMYT in Mexico under six different environmental conditions; optimum conditions, flood irrigation, moderate drought stress, severe drought stress, moderate heat stress and severe heat stress conditions. The low molecular weight proteins and γ-gliadin were significantly reduced by all stress treatments. All stress treatments caused an increase in α-gliadins. Genotype showed a stronger influence on α-gliadins than environment and the interaction between genotype and environment, indicating that these gliadins were largely genetically determined. High molecular weight proteins were significantly and positively correlated with dough strength under severe heat stress conditions. The γ-gliadins showed positive significant correlations with dough strength, alveograph tenacity and mixograph peak time for most treatments. The ω- and α- gliadins had a negative influence on alveograph parameters, irrespective of the conditions the wheat was grown under.

4.1 Introduction

The development of wheat varieties that will provide end-products of good quality is one of the primary objectives of wheat breeding programmes (Jang et al. 2017). Durum wheat quality is determined by gluten protein quantity and quality, which are major factors in determining elasticity and viscosity of wheat dough. The protein quality and quantity are determined by the wheat genotype, growing conditions as well as interaction between genotype and growing conditions (Sissons et al. 2005; Lagrain et al. 2013). Traditionally, gluten proteins can be classified into three major classes, each consisting of two or three different groups of protein, namely glutenin, gliadin and AG. Glutenins are classified into two main groups, HMW and LMW, based on their molecular weights. The gliadins are heterogeneous complex mixtures of proteins made up of α-, β-, γ-gliadins held by intra chain disulphide bonds, and ω-gliadins linked by non-disulphide bonds (Lagrain et al. 2013; Pompa et al. 2013).

Glutenins are essential for pasta quality, as they appear to influence strength and elasticity. Gliadins are responsible for extensibility and stickiness. The wheat proteins are directly involved in physical properties of pasta (Mailhot and Patton 1988). Quality of pasta has been observed to be correlated with specific allelic variants of HMW-GS. Subunits 7+8 coded by *GluB1* are linked with good gluten strength in durum wheat cultivars (Sissons et al. 2005; Giuliani et al. 2015). Various studies also indicated the contribution of LMW to durum gluten strength. Studies showed LMW-1 and LMW-2 fractions to be useful predictors of dough strength. The LMW-2 protein fractions have been reported to be major contributing factors to dough strength as measured by the alveograph. The LMW-1 is associated with poor gluten strength, resulting in sticky pasta during cooking. Two gliadin subgroups, gliadin 42 and 45, are associated with poor and good pasta cooking quality, respectively. The LMW-1 is closely linked to γ-42 gliadin and LMW-2 is linked to γ-45 gliadin (Edwards et al. 2003; De Santis et al. 2017).

In the developing grain, the accumulation process of certain protein fractions is highly ordered and asynchronous (Daniel and Triboï 2000). Gliadins are the first proteins to accumulate in large amounts and are rapidly produced during the middle growth period of the wheat kernel. The glutenin proteins are available in smaller amounts until the last half of the grain filling cycle and are measurable about 20 days after anthesis (Gupta et al. 1996; Panozzo and Eagles 2000; DuPont and Altenbach 2003). Based on these findings, the proportion of gliadins and glutenins has been reported to increase during the first half of the grain fill cycle and decline afterwards towards the end of the grain filling period, when there is a sharp decrease in kernel moisture content. Growing conditions during the grain filling phase affects protein quality. For instance, a reduction in the length of the grain filling phase because of high temperature has been shown to decrease the period of glutenin production, thus reducing dough strength (Stone and Nicolas 1996; Corbellini et al. 1997; Stone et al. 1997; Ferreira et al. 2012).

RP-HPLC has been widely used for allelic analysis in wheat (Jang 2107). Protein subunits are separated according to their hydrophobic properties on a linear gradient of organic solvent. RP-HPLC is precise, fully automated and highly reproducible for identification and quantification of proteins (Burnouf and Bietz 1984; Huebner and Bietz 1999; Jang et al. 2017). RP-HPLC has been successfully applied in cereal protein studies for protein fractionation for varietal identification, genetic studies and quality analysis (Burnouf and Bietz 1984; Naeem and Sapirstein 2007; Kaisoon et al. 2008; Yan 2009). Protein fractions identified from RP-HPLC have been found to correlate significantly with wheat quality parameters (Wieser and Kieffer 2001; Edwards et al. 2003). This

led to a better understanding of protein structure and conformation and how it relates to functionality. RP-HPLC can be a useful tool in wheat breeding programmes, as it opens possibilities to discover a wealth of data encoded in proteins of wheat grain. This may help in the development of varieties with desirable characteristics (Bean et al. 1998).

4.2 Materials and methods

4.2.1 Plant materials

Six durum wheat cultivars were planted under six different stress treatments. The complete description is given in Chapter 3 (Section 3.2.1). All the cultivars had the same HMW-GS and LMW-GS composition (Figure 3.1), in order to accurately compare the effects of stress conditions on the measured quality characteristics, without variation in these proteins.

4.2.2 Trial designs and treatments

Trail designs and treatments were carried as outlined in Chapter 3 (Section 3.2.2).

4.2.3 Quality measurements

The quality measurements were carried out as outlined in Chapter 3 (Section 3.2.3).

4.2.4 Reversedd-phase high-performance liquid chromatography

Gliadin extraction

Proteins were extracted following a sequential method described by Marchylo et al. (1989). One hundred milligram of white flour sample was weighed into a 2 ml reaction tube and 1 ml of 70% ethanol (v/v) was added into tubes. The samples were vortexed for 2 min at room temperature until the pellet was suspended. Samples were shaken for 30 min and centrifuged for 5 min at 14000 rpm. The supernatant was filtered through a 0.45 μm HT Tuffryn Acrodisc® syringe filter into a glass vial for injection. The extractions were done in duplicate.

Glutenin extraction

The remaining pellet from the gliadin extraction was washed twice with 1 ml 50% propan-1-ol (v/v). Glutenins were extracted from the pellet using RP-extraction buffer (1 ml) (containing 50% v/v propan-1-ol, 2 M urea, 0.2 M Tris pH 6.6) and freshly prepared 1% dithiothreitol (DTT) was added. Samples were placed in a water bath at 60°C for 60 min, with intermittent vortexing every 20 min. After this, 10 μl 4-vinylpyridine was added to each sample, mixed and placed in a water

bath at 60°C for 15 min. Samples were centrifuged for 5 min at 14000 rpm. Supernatants were filtered through a 0.45 µm HT Tuffryn Acrodisc® syringe filter into a glass vial for auto-sampler injection. The extractions were done in duplicate.

Protein separation

Protein separation was performed on a Shimadzu (Japan) 20A HPLC system with a photodiode array (PDA) detector using a Jupiter® 5 µm C18 300 Å, LC Column 250 x 4.6 mm from Phenomenex®. Runs were done at a column temperature of 50°C the injection volume was 25 µl. Protein fractions were detected at a wavelength of 210 nm. The elution system consisted of: A) 95% acetonitrile containing 5% water and 0.1% (v/v) trifluoroacetic acid (TFA); B) 95% water containing 5% (v/v) acetonitrile and 0.1% (v/v) TFA.

The linear gradient for gliadin analysis consisted of: 0 – 2 min 0% A; 2 – 15 min 20 - 26% A; 15 – 30 min 26 - 46% A; 30 – 35 min 46 - 60% A; 35 – 40 min 60 - 90% A; 40 – 45 min 90% A; 45 – 55 min 90 - 20% A; 55 – 60 min 20% A and a flow rate of 0.8 ml/min. The linear gradient for glutenin analysis consisted of: 0 – 40 min 20 - 40% A; 40 – 42 min 40 -56% A; 42 – 60 min 56 - 90% A; 70 – 80 min 90 - 20% A. The flow rate was 0.5 ml/min.

The identification of gliadin fractions were done according to Wieser et al. (1998) and Schalk et al. (2017). Glutenins were identified according to Sissons et al. (2007).

4.3 Statistical analyses

Statistical analyses were done with Agrobase Generation II SQL Software (Agrobase 2019), and included analysis of variance for separate and combined treatments, per year and combined for years. The two HMW-GS were grouped together as HMW proteins and the LMW-GS subunits were grouped together as LMW proteins in order to establish the effect of stress conditions on these two classes of proteins, rather than on individual protein subunits, as the HMW-GS and LMW-GS of all the cultivars were the same.

4.4 Results

4.4.1 Analysis of variance for measured proteins for year 1 and 2

High molecular weight proteins

There were non-significant differences in HMW proteins in different environments in year 1 (Table 4.1). In year 2, significant difference for HMW proteins under severe heat stress and

moderate drought and severe drought stress occurred (Table 4.2). In year 1, HMW proteins ranged between 2.23-4.83%, 3.44-4.34%, 3.76-5.02%, 3.09-4.77%, 2.68-3.76%, and 2.80-3.36% for optimum conditions, flood irrigation, moderate heat and severe heat stress, moderate drought and severe drought stress conditions, respectively (Table 4.3). The highest HMW protein values were obtained under moderate heat stress at 5.02% (AtilC2000) and the lowest was obtained under optimum conditions at 2.23% (CirnoC2008) in year 1. In year 2, the highest HMW content was obtained under moderate drought stress at 5.18% (YavarosC79). Contrary to this, the lowest value was found under severe drought stress at 3.22% in AltarC84 and under severe heat stress for CirnoC2008 with the same HMW percentage. The HMW protein values ranged between 4.61-4.96%, 3.29-4.73%, 3.92-4.43%, 3.22-4.90%, 3.36-5.18% and 3.22-4.54% under optimum conditions, flood irrigation, moderate heat, severe heat, moderate drought and severe drought stress conditions, respectively (Table 4.4).

The low molecular weight proteins

In year 1, there were significant differences in LMW proteins between genotypes for all treatments, except for flood irrigation and moderate heat stress conditions (Table 4.1). In year 2, genotype effect was only significant for moderate heat and severe heat stress conditions (Table 4.2). Moderate heat stress conditions showed the highest LMW value of 86.21% (CironoC2008) in year 1. Contrary to this, severe heat stress caused the lowest value of 75.05% (CirnoC2008). In year 2, the highest LMW protein content was observed under optimum conditions at 84.70% (AtilC2000), while moderate heat stress showed the lowest value of 70.99% (AtilC2000). In year 1 LMW values ranged between 77.97-84.02%, 78.03-83.81%, 76.38-86.21%, 75.05-83.46%, 76.04-81.53% and 76.80-82.12% for optimum conditions, flood irrigation, moderate heat stress, severe heat stress, moderate drought and severe drought stress conditions, respectively (Table 4.3). In year 2, LMW protein content varied between 78.44-84.70%, 77.42-81.91%, 70.99-78.40%, 74.00-80.37%, 80.02-80.92%, and 77.05-77.88% for optimum conditions, flood irrigation, moderate heat and severe heat stress, moderate drought and severe drought stress conditions, respectively (Table 4.4). There was lower variation in LMW protein values for moderate and severe drought stress conditions.

Omega-gliadins

Optimum conditions caused the highest ω-gliadin value in both years, of 7.53% (CirnoC2008) and 8.69% (MexicaliC75) for year 1 and 2 respectively (Table 4.3 and 4.4). The lowest values were obtained under flood irrigation (4.22%) in AltarC84 and moderate drought stress (5.32%) in

AtilC2000 for year 1 and 2 respectively (Tables 4.3 and 4.4). The ω-gliadin varied between 4.71-7.53%, 4.22-6.50, 5.41-7.36%, 6.46-7.31%, 4.86-7.46% and 4.55-7.50% for optimum conditions, flood irrigation, moderate heat and severe heat stress, moderate drought and severe drought stress conditions respectively in year 1 (Table 4.3). Heat stress conditions showed higher ω-gliadin values compared to other environments. In year 2, ω-gliadin ranged between 5.78-8.69%, 5.76-7.52%, 6.90-8.28%, 5.67-8.16%, 5.32-7.77% and 6.22-8.18% for optimum conditions, flood irrigation, moderate heat and severe heat stress, moderate drought and severe drought stress conditions, respectively, in year 2 (Table 4.4). Year 2 showed higher ω-gliadin values than year 1. In year 1, genotype effect was significant for all environments except for severe heat stress and moderate drought stress conditions (Table 4.1). Genotype effect was non-significant for all environments, except for severe heat stress and moderate drought stress in year 2 (Table 4.2).

Alpha-gliadins

In year 1, genotypes performed significantly different in all environments for α-gliadins, with the exception of flood irrigation and moderate heat stress conditions (Table 4.1). In year 2, genotype effects were only significant for severe heat stress and moderate drought stress conditions (Table 4.2). The α-gliadins varied between 46.45-55.44%, 49.41-54.99%, 54.15-57.65%, 52.09-58.06%, 36.52-41.53% and 52.45-58.32% in year 1 for optimum conditions, flood irrigation, moderate and severe heat stress, and moderate and severe drought stress conditions, respectively (Table 4.3). In year 2, α-gliadins ranged from 52.24-58.16%, 53.39-57.48%, 50.43-54.61%, 53.57-57.92%, 50.32-56.14%, 51.99-57.69% for optimum conditions, flood irrigation, moderate heat and severe heat stress, moderate drought and severe drought stress conditions, respectively (Table 4.4). Year 2 showed higher α-gliadin values than year 1. The lowest α-gliadin values were obtained under moderate drought stress for both years in YavarosC79 (36.52%) in year 1 and JupareC2001 (50.32%) in year 2 (Tables 4.3 and 4.4). The highest value was obtained under severe drought conditions (58.32%) in AtilC2000 in year 1. In year 2, optimum conditions presented the highest α-gliadin value of 58.16% in AtilC2000. Year 2 showed higher α-gliadin values than year 1.

Gamma-gliadins

Moderate drought stress conditions showed the highest γ-gliadin values in both years, of 57.82% (AtilC2000) and 42.84% (JupareC2001) for year 1 and 2 respectively (Table 4.3 and 4.4). The lowest values were obtained for cultivar CirnoC2008 under severe heat stress (35.38%) and flood irrigation (35.41%) for year 1 and 2 respectively (Table 4.3 and 4.4). The γ-gliadin varied between

40.40-44.88%, 38.51-45.35%, 37.16-38.59%, 35.38-40.60%, 52.79-57.82% and 35.90-39.98% for optimum conditions, flood irrigation, moderate and severe heat stress, and moderate and severe drought stress conditions, respectively, in year 1 (Table 4.3). In year 2, γ-gliadin ranged between 36.03-40.00%, 35.41-39.45%, 38.19-42.74%, 36.27-40.18%, 36.84-42.84% and 36.16-39.38% for optimum conditions, flood irrigation, moderate and severe heat stress, and moderate and severe drought stress conditions, respectively, in year 2 (Table 4.4). In year 1, genotype effect was significant for all environments, except for moderate heat and severe heat stress conditions (Table 4.1). In year 2, genotype effect was significant for severe heat stress, moderate drought and severe drought stress conditions (Table 4.2).

Table 4.1 Mean squares from analysis of variance for protein fractions for year 1

Environments	HMW	LMW	ω-gliadin	α-gliadin	γ-gliadin
Optimum	1.70	9.70*	2.34*	19.41*	6.89*
Flood	0.23	1.12	1.44**	8.21	10.23**
Moderate heat	0.39	30.26	1.03**	3.01	0.56
Severe heat	0.75	5.58*	0.23	9.58**	7.91
Moderate drought	0.45	0.41*	2.18	6.84**	7.29*
Severe drought	0.08	8.92*	2.10**	10.05***	6.37**

HMW=high molecular weight. LMW=low molecular weight. *p≤0.05, **p≤0.01, ***p≤0.001

Table 4.2 Mean squares from analysis of variance for protein fractions for year 2

Environments	HMW	LMW	ω-gliadin	α-gliadin	γ-gliadin
Optimum	0.037	10.01	1.91	10.14	4.38
Flood	0.67	5.60	1.17	4.01	4.74
Moderate heat	0.12	26.62***	0.58	5.06	6.06
Severe heat	0.84**	10.56**	1.68*	7.06*	5.52**
Moderate drought	1.04*	0.23	1.66**	9.84**	9.69*
Severe drought	0.47 *	0.23	1.220	6.79	2.49*

HMW=high molecular weight. LMW=low molecular weight. *p≤0.05, **p≤0.01, ***p≤0.001

Table 4.3 Mean values for protein fractions of six durum wheat cultivars under different environmental conditions for year 1

Environments	Proteins	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Grand mean	CV	LSD
Optimum conditions	**HMW**	3.01	4.10	4.83	2.97	3.31	2.23	3.41	1.80	2.05
	LMW	83.30	84.02	81.06	77.97	80.05	80.92	81.22	3.12	1.50
	ω-gliadin	5.59	4.71	6.04	6.17	4.59	7.53	5.77	1.39	0.94
	α-gliadin	50.95	54.08	51.00	55.44	46.45	50.96	51.48	3.72	2.80
	γ-gliadin	43.13	40.43	40.40	40.44	44.88	41.25	41.75	2.23	2.10
Flood irrigation	**HMW**	3.95	4.34	4.31	3.98	3.44	4.26	4.04	12.70	1.04
	LMW	83.81	81.24	81.01	82.08	82.85	78.03	81.05	8.13	3.90
	ω-gliadin	6.50	5.27	4.22	4.25	4.88	4.66	4.96	5.57	0.56
	α-gliadin	54.99	54.01	53.53	54.60	49.41	52.70	53.20	1.01	1.09
	γ-gliadin	38.51	40.76	41.98	41.09	45.35	42.54	41.70	1.52	1.28
Moderate heat stress	**HMW**	4.03	4.03	3.76	5.02	4.35	4.01	4.20	17.20	1.45
	LMW	79.92	78.01	76.38	83.92	85.82	86.21	81.71	8.62	4.10
	ω-gliadin	7.02	7.36	7.24	5.41	6.95	7.04	6.84	3.59	0.49
	α-gliadin	54.15	55.75	54.59	57.65	55.05	55.38	55.43	2.54	2.84
	γ-gliadin	38.16	37.56	38.59	37.16	37.78	37.37	37.77	2.55	1.94
Severe heat stress	**HMW**	3.85	4.06	3.56	4.77	3.25	3.09	3.76	10.52	0.80
	LMW	79.81	83.46	76.09	78.13	75.81	75.05	78.06	3.56	1.80
	ω-gliadin	7.17	7.09	6.96	7.31	7.41	6.46	7.06	11.77	1.68
	α-gliadin	52.09	56.67	56.96	57.72	57.13	58.06	56.43	1.37	1.56
	γ-gliadin	40.60	36.50	36.02	35.82	35.45	35.38	36.63	3.65	2.69
Moderate drought stress	**HMW**	2.87	3.75	3.76	3.07	2.68	2.85	3.16	0.11	0.69
	LMW	79.46	79.55	76.50	81.53	81.38	76.04	79.07	3.20	1.60
	ω-gliadin	7.11	7.46	5.96	4.86	5.08	6.15	6.10	0.18	2.15
	α-gliadin	40.22	36.52	38.27	37.01	41.53	38.35	38.65	0.03	2.03
	γ-gliadin	52.79	55.77	55.63	57.82	53.21	55.31	55.09	0.01	1.17
Severe drought stress	**HMW**	2.80	3.05	2.94	3.36	3.11	2.93	3.03	10.30	0.63
	LMW	76.80	80.33	79.22	80.59	82.12	77.09	79.36	2.57	1.30
	ω-gliadin	7.50	6.61	6.89	4.55	6.67	6.98	6.53	5.58	0.73
	α-gliadin	52.45	56.58	56.62	58.32	53.22	54.83	55.34	0.74	0.82
	γ-gliadin	39.88	36.72	36.38	35.90	39.98	38.06	37.82	1.88	1.43

HMW=high molecular weight. LMW=low molecular weight, ω-omega, α-alpha or beta, γ-gamma, CV=coefficient of variation, LSD=least significant difference

Table 4.4 Mean values for protein fractions of six durum wheat cultivars under different environmental conditions for year 2

Environments	Protein	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Grand mean	CV	LSD
Optimum conditions	**HMW**	4.71	4.96	4.61	4.80	4.92	4.86	4.81	14.66	1.42
	LMW	80.04	78.44	81.90	84.70	80.69	83.08	81.47	2.98	4.90
	ω-gliadin	8.69	7.15	7.23	5.78	7.55	8.00	7.40	16.87	2.51
	α-gliadin	54.28	55.65	55.22	58.16	52.24	52.24	54.63	1.07	1.17
	γ-gliadin	36.90	37.29	37.54	36.03	40.00	39.15	37.82	5.02	3.83
Flood irrigation	**HMW**	4.07	4.26	3.30	4.73	4.35	3.29	4.00	14.18	1.14
	LMW	80.60	81.88	79.98	77.42	81.05	81.91	80.47	2.63	4.26
	ω-gliadin	6.64	5.79	7.27	5.76	7.19	7.52	6.69	9.69	1.31
	α-gliadin	54.17	55.21	54.33	55.36	53.39	57.48	54.99	1.64	1.82
	γ-gliadin	39.45	39.25	38.83	37.75	39.12	35.41	38.30	3.02	2.33
Moderate heat stress	**HMW**	4.43	4.20	4.43	4.39	3.92	3.92	4.22	5.38	0.46
	LMW	78.38	71.68	78.38	70.99	78.40	78.40	76.04	0.85	1.31
	ω-gliadin	8.28	7.29	7.73	7.32	6.90	8.16	7.61	10.94	1.68
	α-gliadin	52.71	54.61	54.25	52.17	50.43	54.07	53.04	1.44	1.54
	γ-gliadin	39.73	38.19	38.32	40.02	42.74	38.38	39.56	3.35	2.67
Severe heat stress	**HMW**	4.44	3.70	4.44	4.90	4.74	3.22	4.24	7.02	0.60
	LMW	77.04	80.37	74.00	74.44	76.53	77.34	76.62	0.87	1.34
	ω-gliadin	8.16	5.82	6.72	5.67	6.16	6.89	6.57	8.92	1.18
	α-gliadin	53.57	57.92	56.40	57.48	53.78	56.89	56.00	1.64	1.85
	γ-gliadin	38.88	36.29	36.35	36.86	40.18	36.27	37.47	1.89	1.42
Moderate drought stress	**HMW**	4.45	5.18	3.36	3.77	3.43	4.55	4.12	10.73	0.89
	LMW	80.33	80.02	80.08	80.92	80.11	80.51	80.33	2.42	3.92
	ω-gliadin	7.61	7.77	7.29	5.32	6.82	6.41	6.87	5.13	0.71
	α-gliadin	55.98	54.43	53.09	56.14	50.32	52.62	53.76	1.44	1.56
	γ-gliadin	36.84	37.83	39.57	38.52	42.84	41.01	39.43	2.45	1.94
Severe drought stress	**HMW**	4.54	3.64	3.22	3.41	3.79	3.31	3.65	6.44	0.47
	LMW	77.05	77.33	77.39	77.75	77.87	77.88	77.54	0.75	1.17
	ω-gliadin	8.18	7.72	6.63	6.22	6.87	7.86	7.24	9.22	1.35
	α-gliadin	51.99	54.19	57.69	55.06	55.33	55.03	54.88	0.84	0.93
	γ-gliadin	39.38	38.09	36.16	38.84	37.95	37.54	37.99	1.85	1.42

HMW=high molecular weight. LMW=low molecular weight, ω-omega, α-alpha or beta, γ-gamma, CV=coefficient of variation, LSD=least significant difference

4.4.2 Combined analysis of variance for measured protein fractions across environments

High molecular weight proteins

Genotype effects for HMW proteins were only significant for severe heat stress and moderate drought stress. Environment effect was highly significant except for flood irrigation and moderate heat stress. The GxE interaction effect was only significant for severe heat and moderate drought conditions (Table 4.5). The highest HMW value (4.84%) was obtained in AtilC2000 under severe heat stress conditions and the lowest (3.05%) in JupareC2001 under moderate drought conditions (Table 4.6).

Low molecular weight proteins

Genotype effect for LMW was significant for moderate heat and severe heat stress conditions. The environment effect was highly significant for moderate heat and severe heat stress, and severe drought conditions. The GxE effect was significant for optimum conditions as well as for moderate heat and severe heat stress conditions (Table 4.5). The highest LMW value was under moderate heat stress (82.30%) in cultivar CirnoC2008. Contrary to this, the lowest value was also observed under moderate heat stress in YavarosC79 (74.85%) (Table 4.6). Although the highest value was obtained under moderate heat stress, optimum conditions showed higher values on average, followed by flood irrigation.

Omega-gliadins

There were significant differences among genotypes for ω-gliadins for flood irrigation, moderate drought and severe drought stress conditions. Environment effect was significant for all environments except for severe heat stress. The GxE effect was only significant for flood irrigation (Table 4.5). Severe drought stress conditions showed the highest value of 7.84% (MexicaliC75) and floor irrigation caused the lowest value of 5.01% (AtilC2000) (Table 4.6).

Alpha-gliadins

Genotype effect for α-gliadin was significant for all conditions except moderate heat stress and severe drought stress. Environment effect was significant for all conditions except for severe heat stress. The GxE interaction was significant for all stress conditions and optimum conditions was not significant (Table 4.5). Severe heat stress conditions lead to the highest value α-gliadin of 57.60% (AtilC2000) and optimum conditions the lowest of 50.60% (JupareC2001) (Table 4.6)

Gamma-gliadins

Genotype effect was significant in all environments for γ-gliadins, except for moderate and severe heat stress conditions. Environment effect was highly significant for optimum conditions and flood irrigation and significant for moderate heat stress. The effect of GxE interaction was significant for flood irrigation, moderate and severe drought stress conditions (Table 4.5). The lowest γ-gliadin value was observed under severe heat stress conditions (35.83% for CirnoC2008). Optimum conditions showed the highest γ-gliadin value of 42.44% (JupareC2001) but flood irrigation showed the highest grand mean. (Table 4.6).

Table 4.5 Mean squares from analysis of variance for protein fractions for two years combined

Environments	Sources of variation	HMW	LMW	ω-gliadin	α-gliadin	γ-gliadin
Optimum	**Genotype (G)**	0.29	1.21	1.92	16.94***	8.96*
	Environment (E)	12.60**	0.340	19.26**	43.04***	92.98***
	GxE	0.40	18.50*	0.80	1.283	2.30
Flood irrigation	**Genotype**	0.28	4.45	1.14*	7.64***	6.08*
	Environment (E)	0.00	6.38	17.98***	19.08***	69.43***
	GxE	0.62	9.10	1.47**	4.58**	8.90**
Moderate heat	**Genotype**	0.12	34.22**	0.99	3.61	3.13
stress	**Environment (E)**	0.73	192.95***	3.62**	34.30***	19.28**
	GxE	0.21	27.31*	0.62	4.46*	3.49
Severe heat	**Genotype**	1.16**	24.94***	0.78	13.53***	8.81
stress	**Environment (E)**	1.56**	12.49**	1.48	1.10	4.26
	GxE	0.45*	5.71**	1.12	3.11*	4.61
Moderate drought	**Genotype**	0.87**	3.91	3.46**	11.42***	12.54***
stress	**Environment (E)**	5.50***	7.42	3.55*	10.53***	3.71
	GxE	0.63**	3.40	0.38	5.26***	4.43*
Severe drought	**Genotype**	0.26	3.49	2.85***	12.654	5.86***
stress	**Environment (E)**	2.11***	28.34*	3.05**	1.26*	0.18
	GxE	0.26	1.91	0.46	4.19***	3.00**

HMW=high molecular weight. LMW=low molecular weight, ω-omega, α-alpha or beta, γ-gamma, *p≤0.05, **p≤0.01, ***p≤0.001

Table 4.6 Cultivar means for protein fractions for different environments for two years combined

Environments	Protein fractions	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Grand mean	CV	LSD
Optimum conditions	**HMW**	3.86	4.33	4.47	3.88	4.11	3.85	4.08	21.27	1.11
	LMW	81.67	81.23	81.48	81.33	80.37	82.00	81.35	2.38	2.48
	ω-gliadin	5.47	5.93	6.57	6.63	7.14	7.26	6.50	18.66	1.55
	α-gliadin	52.62	54.87	54.01	56.05	50.60	51.60	53.29	2.05	5.33
	γ-gliadin	40.02	38.86	38.97	38.23	42.44	40.20	39.78	3.71	1.89
Flood irrigation	**HMW**	4.01	4.30	3.73	4.35	3.89	3.78	4.01	14.36	0.74
	LMW	82.21	81.56	80.49	79.75	81.95	79.97	80.99	3.35	3.47
	ω-gliadin	6.57	5.53	5.74	5.01	6.03	6.09	5.83	8.56	0.64
	α-gliadin	54.58	54.61	53.93	54.98	51.40	55.09	54.10	1.37	0.95
	γ-gliadin	38.98	40.00	40.40	39.42	42.24	38.97	40.00	2.33	1.19
Moderate heat stress	**HMW**	4.33	4.36	4.34	4.70	4.38	4.21	4.39	9.79	0.55
	LMW	79.15	74.85	77.38	77.46	82.11	82.30	78.87	3.06	3.09
	ω-gliadin	7.65	7.32	7.48	6.36	6.93	7.60	7.22	8.50	0.79
	α-gliadin	53.43	55.18	54.42	54.91	52.74	54.72	54.23	2.09	1.45
	γ-gliadin	38.95	37.87	38.46	38.59	40.26	37.88	38.67	3.00	1.49
Severe heat stress	**HMW**	4.04	3.88	4.00	4.84	3.99	3.15	3.98	8.78	0.45
	LMW	78.43	81.92	75.05	76.29	76.17	76.19	77.34	1.41	1.40
	ω-gliadin	7.66	6.46	6.84	6.49	6.78	6.67	6.82	10.55	0.92
	α-gliadin	52.83	57.29	56.68	57.60	55.45	57.47	56.22	1.51	1.09
	γ-gliadin	39.74	36.39	36.18	36.34	37.81	35.83	37.05	2.89	1.37
Moderate drought stress	**HMW**	3.66	4.46	3.56	3.42	3.05	3.70	3.64	10.89	0.51
	LMW	78.28	79.21	79.78	79.90	80.25	81.22	79.77	2.16	2.21
	ω-gliadin	7.36	7.61	6.62	5.09	5.95	6.28	6.49	12.27	1.02
	α-gliadin	54.38	55.10	54.36	56.98	51.77	53.96	54.42	1.26	0.88
	γ-gliadin	38.53	37.18	38.92	37.77	42.19	39.68	39.04	2.53	1.26
Severe drought stress	**HMW**	3.77	3.34	3.08	3.39	3.45	3.10	3.35	8.80	0.38
	LMW	77.50	78.83	78.49	79.24	80.00	77.74	78.63	1.58	1.60
	ω-gliadin	7.84	7.16	6.76	5.38	6.77	7.42	6.89	7.81	0.69
	α-gliadin	52.22	55.38	57.15	56.69	54.27	54.93	55.11	0.79	0.56
	γ-gliadin	39.63	37.40	36.27	37.37	38.96	37.80	37.90	1.87	0.91

HMW=high molecular weight. LMW=low molecular weight, ω-omega, α-alpha or beta, γ-gamma CV=coefficient of variation, LSD=least significant difference

4.4.3 Combined analysis of variance across environments and two years

There was a highly significant genotype effect ($p \leq 0.001$) for α-, ω-, and γ-gliadins and significant ($p \leq 0.05$) genotype effects for HMW proteins. Highly significant differences ($p \leq 0.001$) between the different environments were observed for all the protein fractions. Highly significant GxE interactions ($p \leq 0.001$) were evident for all proteins except for HMW (Table 4.7). The genotype by year effect was only significant for LMW and α-gliadin. Environment by year effect was highly significant ($p \leq 0.001$) for all measured proteins, except for ω-gliadins, indicating that genotype rankings changed between year 1 and 2. The GxExY effect was significant for all proteins except for HMW protein (Table 4.7).

Table 4.7 Mean squares from analysis of variance for protein fractions across environments and years

Source of variation	HMW	LMW	ω-gliadin	α-gliadin	γ-gliadin
Genotype (G)	0.80*	6.23	7.81***	41.37***	26.98***
Environment (E)	3.16***	55.24***	5.45***	24.02***	30.42***
Year (Y)	9.86***	80.09***	27.98***	0.02	14.01***
GxE	0.43	13.20***	0.67***	4.89***	3.68***
GxY	0.60	13.75*	0.36	2.16**	2.02
ExY	2.53***	33.58***	4.20	21.86***	35.17***
GxExY	0.39	10.43**	0.90***	4.14***	4.94***

HMW=high molecular weight. LMW=low molecular weight, ω-omega, α-alpha or beta, γ-gamma, *p≤0.05, **p≤0.01, ***p≤0.001

Genotype means varied between 3.68-4.11%, 78.68-80.14%, 5.63-7.37%, 52.70%-56.20% and 37.95%-40.65% for HMW and LMW proteins, and ω-, α- and γ-gliadins, respectively (Table 4.8). Across environments and years, AtilC2000 had significantly higher α-gliadin values than the other cultivars, but had the lowest ω- and γ-gliadin values. JupareC2001 showed significantly higher LMW protein and γ-gliadin content than other cultivars, but had low α-gliadin values. YavarosC79 had significantly higher HMW protein values than JupareC2001 and CirnoC2008. MexicaliC75 had significantly higher ω-gliadin values than the other cultivars. The lowest HMW and LMW protein values were observed in CirnoC2008 and AltarC84, respectively.

Table 4.8 Mean performance of genotypes for protein fractions across environments and two years

Protein	MexicaliC75	YavarosC79	AltarC84	AtilC2000	JupareC2001	CirnoC2008	Grand mean	CV	LSD
HMW	3.94	4.11	3.86	4.09	3.81	3.63	3.91	14.80	0.28
LMW	79.81	79.69	78.68	79.21	80.14	79.41	79.49	2.69	1.03
ω-gliadin	7.37	6.67	6.68	5.63	6.50	6.89	6.62	11.31	0.36
α-gliadin	53.34	55.40	55.09	56.20	52.70	54.63	54.56	1.53	0.40
γ-gliadin	39.31	37.95	38.20	37.95	40.65	38.39	38.74	2.73	0.51

HMW=high molecular weight. LMW=low molecular weight, ω-omega, α-alpha or beta, γ-gamma, CV=coefficient of variation, LSD=least significant differences

The minimum and maximum environmental means were 3.35% (severe drought stress), 4.39% (moderate heat stress); 77.34% (severe heat stress), 81.35% (optimum conditions); 5.83% (flood irrigation), 7.22% (moderate heat stress); 53.29% (optimum conditions), 56.22% (severe heat stress) and 37.05% (severe heat stress) 40.00% (flood irrigation), for HMW, LMW, ω-, α- and γ-gliadins respectively (Table 4.9).

Table 4.9 Environmental means for protein fractions across two years

Proteins	Optimum	Flood	Moderate heat	Severe heat	Moderate drought	Severe drought	LSD
HMW	4.08	4.01	4.39	3.98	3.64	3.35	0.28
LMW	81.35	80.99	78.87	77.34	79.77	78.63	1.03
ω-gliadin	6.50	5.83	7.22	6.82	6.48	6.89	0.36
α-gliadin	53.29	54.10	54.23	56.22	54.42	55.11	0.40
γ-gliadin	39.78	40.00	38.67	37.05	39.04	37.90	0.51

HMW=high molecular weight. LMW=low molecular weight, ω-omega, α-alpha or beta, γ-gamma, LSD=least significant differences

Moderate heat stress caused the highest HMW protein value, significantly higher than all other treatments. It also caused the highest ω-gliadin value, significantly higher than all except the value for severe drought stress (Table 4.9). In contrast to this, moderate and severe drought stress caused a significant reduction in these proteins compared to the optimal conditions. The LMW proteins were significantly reduced by all heat and drought stress treatments. Optimum conditions showed the highest LMW protein value. The ω-gliadins were increased by all stress treatments, significantly for moderate heat and severe drought. The ω-gliadins were significantly decreased under flood irrigation. The α-gliadins were significantly increased by all treatments compared to the optimal conditions, with the highest value obtained under severe heat stress conditions. The γ-

gliadins were significantly reduced by all stress treatments compared to the optimal conditions, but the value under flood irrigation was similar to optimal conditions. The flood irrigation caused a slight increase in γ-gliadins (non-significant).

4.4.4 Correlations between protein fractions and quality characteristics

Most protein fractions correlated negatively with quality characteristics (Table 4.10). The HMW proteins correlated negatively with alvW in all the environments except for severe heat stress, where the correlation was positive. Under moderate heat stress and in combined analysis across the treatments over the two years, there were significant negative correlations between HMW and alvP. The α-gliadins showed negative correlations with alvW under flood irrigation, and moderate and severe heat conditions, and combined for all treatments, with a higher correlation for the moderate heat conditions (r=-0.70***). Flood irrigation, moderate heat, severe heat, moderate drought and combined analysis across all treatments over two years presented significant negative correlations between α-gliadins and MPT, with higher negative correlations for the flood irrigation (r=-0.76*). Moderate drought conditions and combined analysis for all treatments showed significant correlations between α-gliadins and alvP. The LMW proteins negatively correlated with alvL for severe heat conditions and combined analysis for all treatments, however, they were positively correlated with alvP/L under severe heat stress conditions (Tables 4.10 and 4.11)

The γ-gliadins correlated positively and significantly with alveograph parameters for most environments. The γ-gliadins significantly correlated with alvW for all environments except for severe drought, with the highest correlation obtained under flood irrigation (r=0.67***). Flood irrigation, heat stress, moderate drought stress and combined analysis across treatments over two years showed significant positive correlations between γ-gliadins and MPT, with a higher correlation coefficient under moderate drought (r=0.75*). The ω-gliadins correlated negatively with alvW under flood irrigation and moderate drought stress conditions. Moderate drought conditions caused positive correlations between ω-gliadins and alvL and alvP/L, but they were negatively correlated with AlvL under severe drought stress conditions (Table 4.10).

Table 4.10 Significant correlations between protein fractions and quality characteristics for optimum, flood, moderate and severe heat environments for two years

Optimum			Flood			Moderate heat			Severe heat		
Protein fraction	**Quality characteristic**	**Correlation**	**Protein fraction**	**Quality characteristic**	**Correlation**	**Protein fraction**	**Quality characteristic**	**Correlation**	**Protein fraction**	**Quality characteristic**	**Correlation**
HMW	alvW	-0.60*	ω-gliadin	alvP	-0.44*	HMW	alvP	-0.53*	HMW	alvW	0.63**
γ-gliadin	alvW	0.49*		alvW	-0.44*		alvW	-0.43*	LMW	alvL	-0.45*
			α/β-gliadin	alvW	-0.68*		MPT	-0.59*		alvP/L	0.62**
				MPT	-0.76*	αβ-gliadin	alvP	-0.60**	αβ-gliadin	alvW	-0.54*
			γ-gliadin	alvW	0.67***		alvW	-0.70***		MPT	-0.64**
				MPT	0.64**		MPT	-0.66***	γ-gliadin	alvW	0.62*
						γ-gliadin	alvP	0.51*		MPT	0.60**
							alvW	0.63**			
							MPT	0.65**			

HMW=high molecular weight. LMW=low molecular weight, MPT=moxograph peak time, alvW=alveograph strength, alvP=alveograph stability, alvL=alveograph extensibility, alvP/L=alveograph stability to extensibility ratio, *p≤0.05, **p≤0.01, ***p≤0.001

Table 4.11 Significant correlations between protein fractions and quality characteristics for moderate and severe drought and combined environments for two years

Moderate drought			Severe drought			Across environments and two years		
Protein Fractions	**Quality characteristics**	**Correlations**	**Protein fractions**	**Quality characteristics**	**Correlations**	**Protein fractions**	**Quality characteristics**	**Correlations**
HMW	alvW	-0.51*	ω-gliadin	alvL	-0.45	HMW	alvP	-0.26**
ω-gliadin	alvW	-0.44*					alvW	-0.20*
	alvL	-0.64**				LMW	alvL	-0.22*
	alvP/L	0.49*				α-gliadin	alvP	-0.38***
αβ-gliadin	alvP	-0.44*					alvW	-0.39***
	MPT	-0.63**					alvP/L	-0.20*
γ-gliadin	alvW	0.59*					MPT	-0.56***
	MPT	0.75*				γ-gliadin	alvP	0.31***
							alvW	0.40***
							MPT	0.53***

HMW=high molecular weight. LMW=low molecular weight, ω-omega, α-alpha or beta, γ-gamma, MPT=mixograph peak time, alvW=alveograph strength, alvP=alveograph stability, alvL=alveograph extensibility, alvP/L=alveograph stability to extensibility ratio, *p≤0.05, **p≤0.01, ***p≤0.001

4.5 Discussion

Drought and increased temperatures are major environmental factors affecting wheat yield and quality. Maintaining quality standards is of particular importance, as annual product quality fluctuations are not acceptable, especially for pasta production (Gagliardi et al. 2020). In this study, the six cultivars had exactly the same HMW and LMW protein composition, although the gliadin composition was not measured prior to the study, as the glutenins are generally seen as the most important factor influencing dough characteristics. This means that differences in dough characteristics between cultivars were due to other factors than glutenin composition.

Genotype effect was significant for α-gliadin and γ-gliadin for all environments, except moderate heat and drought stress conditions for α-gliadin and γ-gliadin, respectively. Analysis across environments over the two years showed significant genotype effect for all proteins, except for LMW. Significant genotype effects for measured characteristics indicate that these characteristics are under genetic control, and in this study it was clear that this remained the fact even under heat and drought stress conditions. High heritability or genetic control is also an indication that selection can be done for the presence of required protein fractions for specific end-use quality within cultivars.

Both LMW proteins and γ-gliadin were significantly reduced under all stress treatments, suggesting that these protein fractions were sensitive to stress. Contrary to this, α-gliadins were significantly increased by all stress treatments. Daniel and Triboï (2000) observed a significant increase in α-gliadin and reduction of γ-gliadin with increase in temperature, which is in agreement with results observed in this study where heat stress conditions caused significant increase in α-gliadin and decrease in γ-gliadin. Ferreira et al. (2012) reported an increase in γ-, α-gliadin under high temperatures.

The HMW proteins were sensitive to drought stress specifically, which caused a significant reduction in these proteins. This was also seen with combined analysis for two years, where drought stress conditions showed lower HMW protein values, but interestingly this was not the case for moderate heat stress conditions, which indicated that stress severity plays a large role. Dupont et al. (2006) observed an increase in HMW proteins under high temperatures, which corroborates the results obtained in this study. This suggests that moderate heat stress favours HMW proteins production. Contrary to this, Cornibelli et al. (1997) observed a reduction in HMW

proteins due to high temperatures. In previous studies (Jiang et al. 2009; Zhang et al. 2013; Dai et al. 2016) it was shown that drought reduced HMW proteins. The level of stress applied probably has a large effect on the amount of HMW proteins, where different biochemical processes are probably initiated at different levels of stress severity.

Based on the results from numerous studies, certain HMW subunits (7+8) are associated with dough strength in durum wheat (Sissons et al. 2005; Edwards et al. 2007 Pompa et al. 2013; Giuliani et al. 2015). In the current study all the entries had subunits 7+8, indicating that they were selected for good end-use quality. Edwards et al. (2003), in a study determining the relative contribution of HMW-GS to durum wheat viscoelastic properties, reported that addition of HMW-GS to semolina increased overall dough strength. However, in the current study, only subunits 7+8 were present, and these HMW proteins negatively correlated with alvW under optimum conditions, moderate heat stress, and moderate drought stress conditions, and combined for all the environments. This indicated that in these specific cultivars, subunits 7+8 did not contribute to dough strength. This may be due to the specific genetic backgrounds in which the subunits were expressed, and the outcome may be different in a different set of cultivars. The outcome may also have been different if an additional subunit (1 or 2*) was expressed on the A genome.

There is evidence that certain γ-45 gliadin types are correlated with good viscoelastic properties of dough and these were reported as genetic markers for durum wheat quality. Qi et al. (2006), Sissons et al. (2012) and Pompa et al. (2013) reported significant correlations between γ-gliadins and gluten strength as measured by the Zeleny sedimentation test in bread wheat studies. In this study, γ-gliadins showed positive significant correlations with alvW, alvP and MPT for most environments. The γ-gliadins are reported to have an extra cysteine residue that could contribute to glutenin polymer formation, contributing to good flour quality characteristics (Gianebelli et al. 2001; Qi et al. 2006). The ω-gliadins are characterised by lack of sulphur-rich amino acid cysteine residues for intermolecular disulphide-bonds which are related to good viscoelastic properties (Marins et al. 2011), and the influence on viscoelastic properties could be less effective (Barak et al. 2015). This may explain negative correlations obtained between ω-gliadins and alveograph properties, alvW, alvP and alvP/L, and MPT, for most environments. Fido et al. (1997) and Khatkar et al. (2002) indicated that gliadin fractions had a negative effect on mixing times, but a positive effect on alvL. The significant negative correlations found between ω-gliadins and alvL in the current study is contradictory to what was found by these authors. Branlard and Dardevet (1985) reported negative correlations of ω- and α-gliadins with dough strength. Sissons et al.

(2007) in a study analysing the effects of gluten on durum wheat dough and spaghetti cooking quality, observed decreases in dough strength with addition of gliadins. The data obtained in this study indicated that ω- and α-gliadins had a negative influence on alveograph parameters. The LMW proteins positively correlated with alvP/L under severe heat stress conditions, and negatively correlated with alvL under severe heat conditions and across all the environments. This indicates that a higher proportion of LMW had a positive influence on alvP under severe heat conditions, while an increased quantity of LMW had a negative effect on alvL under severe heat stress conditions and across the environments. Severe drought stress and optimum conditions presented few correlations between protein fractions and quality characteristics. Significant correlations obtained between proteins and quality characteristics will make it easier to determine and evaluate basic quality of wheat in breeding programmes.

4.6 Conclusions

Wheat quality is a complicated concept, governed largely by gluten proteins. The expression of gluten proteins is affected by genotype response to environmental conditions. Consistency in flour quality is essential regardless of environmental changes. In this study, the HMW proteins were significantly reduced by severe drought stress conditions. Both LMW and γ-gliadin were sensitive to all stress treatments. The α-gliadins were significantly increased by all stress treatments. Optimum conditions and flood irrigation (which also creates optimal conditions) tend to favour expression of LMW. Both ω- and α-gliadins negatively correlated with alveograph characteristics for most treatments. The γ-gliadin had a positive effect on alveograph characteristics for most environments. This indicates that γ-gliadin can be used to predict durum wheat quality in these environments. The genotype performance varied across environments, even though the cultivars had the same glutenin composition, indicating that glutenin alone was not the only determining factor in dough characteristics. This indicated that genetic background in which the glutenins are expressed also contributes to quality characteristics. The gliadin composition of the cultivars was different, which may have contributed to some of the observed differences in measured characteristics. Previous RP-HPLC studies were mostly done on bread wheat proteins rather than durum wheat gluten. The results obtained from this study may form the future basis of understanding the environmental effects on gluten proteins in durum wheat using RP-PHLC.

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CHAPTER 5

Proteomic analysis of durum glutenin proteins under heat and drought stress

Abstract

Flour quality used for pasta making is mainly influenced by the gluten proteins, a complex mixture of proteins made of high molecular weight-glutenin subunits (HMW-GS), low molecular weight-glutenin subunits (LMW-GS), and α-, γ-, and ω-gliadins. Two durum wheat (*Triticum durum* Desf.*)* cultivars (AtilC2000 and MexicaliC75) with contrasting quality characteristics, were grown in field trials in Mexico. The trial was conducted under five different growing conditions: optimum, moderate drought stress, severe drought stress, moderate heat stress and severe heat stress. The aim of the study was to evaluate the influence of abiotic stress on gluten protein composition as measured by proteomics analysis. Selected samples were analysed by two-dimensional gel electrophoresis (2-DE). Proteins were identified by liquid chromatography - tandem mass spectrometry (LC-MS/MS) of peptides. A total of 330 spots were differentially expressed at $p \leq 0.05$. Approximately 305 spots were up-regulated, of which 7.5% were induced by moderate drought stress, 10.61% by severe drought stress, 8.79% by moderate heat stress, 7.27% by severe heat stress and 65.88% due to a combination of stress conditions in AtilC2000. In MexicaliC75, 205 spots were differentially expressed and were upregulated. About 35.12% of the protein spot increases were induced by moderate drought, 4.39% by severe drought, 1.46% by moderate heat and 2.95% by severe heat stress. Sixteen spots were down-regulated. Highly up-regulated spots were selected and analysed by LC-MS/MS followed by data base searching. Of these, some were HMW-GS, gliadins, serpins and β-amylase involved in carbohydrate metabolism. AtilC2000 showed the least changes due to stress treatments.

5.1 Introduction

Increases in global temperatures have been observed since the beginning of the century. More severe changes in the climate are likely to occur in the future than previously predicted (Yang et al. 2011; Zhang et al. 2017). Wheat is one of the most important food sources for humans, and is cultivated worldwide. Durum wheat contributes about 8% of total global wheat production, with more than 50% produced in the Mediterranean region (Guzmán et al. 2016; Mazzeo et al. 2017). Wheat endosperm usually comprises of starch, non-starch polysaccharides and a heterogeneous mixture of proteins. Gluten proteins are responsible for pasta making quality properties, conferring

the visco-elastic properties of dough (Barak et al. 2015). Gluten proteins are classified into two groups, monomeric gliadins (α, γ and ω) and polymeric glutenin (HMW-GS and LMW-GS) stabilised by inter and intra molecular disulphide bonds (Pompa et al. 2013; Barak et al. 2015). The influence of gluten protein composition on dough making properties is well established. High gluten strength in bread wheat is ascribed to HMW-GS such as 5+10 and 7+8. HMW-GS Bx20 resulted in weak gluten strength (Nazco et al. 2014; Santagati et al. 2016). LMW-GS were also reported to have a significant influence on dough properties of durum wheat cultivars. Durum wheat cultivars with the specific group of LMW-2 have better dough properties compared to cultivars possessing allelic group LMW-1. In addition, gliadins γ-42 and γ-45 were found to be associated with weak and strong gluten, respectively (Yildrim et al. 2011; De Santis et al. 2017). Correlations of HMW-GS and LMW-GS with technological quality parameters is an indication that glutenin subunits can be used as protein markers in wheat breeding programmes for selecting superior cultivars (Pompa et al. 2013).

The quantity and composition of gluten protein determine dough making properties and are influenced by genotype and environment. Drought and heat are key environmental constraints affecting yield and quality of wheat (Laino et al. 2010; Halford et al. 2014). The proteome of a plant is dynamic, and may change in response to external and internal environmental conditions (Kamal et al. 2010). Identification of durum wheat gluten proteins that change in response to environmental conditions is necessary to understand gene-function relationships, effects of environmental conditions on wheat quality and to predict functional end-use quality of durum wheat cultivars (Pompa et al. 2013; Mazzeo et al. 2017). Several authors used proteomic approaches to assess environmental effects on gluten proteins (Majoul et al. 2004; Kamal et al. 2010; Laino et al. 2010; Giuliani et al. 2015; Labuschagne et al. 2020). The results obtained revealed changes in spots ranging from as few as 3 to as many as 107 spots in 2-DE gels. However, more focus was placed on metabolic proteins in the past, than on gluten (storage) proteins, especially in durum wheat cultivars. Furthermore, most of the studies conducted have focused on the effect of high temperature rather than water deficit on proteins. High temperature and water deficit reduce the grain filling stage and time taken to reach maturity (Giuliani et al. 2015). Gliadins were found to increase under water stress conditions (Yang et al. 2011). This study also observed increased amounts of both gliadins and glutenins under higher temperatures, contrary to some studies that indicated that gliadins accumulate at the expense of glutenins at maturity under high temperatures (Triboi et al. 2000). It is of great importance to understand the environmental effects on timing of grain development and the process of storage protein accumulation (Giuliani et al.

2015). The aim of this study was to assess the effect of different levels of heat and drought stress on gluten proteins using a proteomic approach, in durum wheat cultivars. Two durum wheat cultivars with contrasting quality characteristics were selected for this study.

5.2 Materials and methods

5.2.1 Cultivars used and field trials

Two durum wheat cultivars (MexicaliC75 and AtilC2000) from the International Maize and Wheat Improvement Centre (CIMMYT, Mexico) with contrasting quality characteristics were used. They were selected based on their alveopgraph values under optimal conditions (MexicaliC75 had low alvL and high alvP values with a high alvP/L ratio, while AtilC2000 was the opposite, with high alvL and low alvP, and consequently a low alvP/L ratio).

5.2.2 Trial design and experiments.

Trail designs and treatments were carried as outlined in Chapter 3 (Section 3.2.2).

5.2.3 Proteomic analysis

Protein extraction and quantification

Grain samples were conditioned to 16% moisture content after which they were milled into flour using a Brabender Quadrumat Jr. (C.W. Brabender OHG, Germany). Hundred milligram of flour sample from optimum conditions, moderate and severe drought, and moderate and severe heat stress was used. The HMW and LMW proteins were extracted by washing the durum wheat semolina three times with 1 ml of 50% propanol to remove gliadins and AG. Every step was done by centrifugation at 13 000 rpm for 15 min followed by removal of the supernatant. The glutenin proteins in the pellet were extracted using a 1:10 solution of 50% 1-propanol Tris-HCl (pH 8.8), 1% DTT, 1 mM EDTA, 10 mM iodoacetamide at 70^{o}C for 1 h, after which the samples were centrifuged (13 000 rpms for 15 min). The supernatant was recovered and precipitated with four volumes of acetone overnight at -20^{o}C. Protein was recovered by further rinsing the supernatant with cold acetone and centrifugation (13 000 rpms for 15 min). The supernatant was dried in a vacuum concentrator. Dried proteins were resuspended in 50% acetonitrile and 0.1% TFA for protein quantification. Protein concentration was determined using a QuantiPro™ BCA protein assay kit (Sigma-Aldrich).

Two dimensional gel electrophoresis

Samples containing 450 µg protein were dried and solubilised in 350 ml rehydration buffer [7 M urea, 4% CHAPS, 2 M thiourea, 1.2% (v/v), destreak reagent and 0.5% IPG buffer pH 3-10], by vortexing and then incubated for 90 min at room temperature. Of the sample 350 µg was used for passive rehydration overnight at room temperature on 17 cm IEF linear IPG gel strips, pH 3-10 (GE Healthcare). Proteins were isofocused using the following voltage gradient: 500 V for 3 hr, 1000 V for 1 h, 3 hr at 10 000 V, and 2 hr at 10 000 V at 20^{o}C using the IPGphor^{TM}Isoelectric Focusing System (GE Healthcare) with the manifold support. Each gel strip was equilibrated for 15 min in 6 ml of an equilibration buffer [6 M urea, 0.05 mM Tris-HCl, (pH 8.8), 30% (w/v) glycerol and 2% (w/v) SDS with 2% DTT and a trace of bromophenol blue], followed by 15 min in 6 ml of equilibration solution containing 6 M urea, 0.05 mM Tris-HCl, (pH 8.8), 30% (w/v) glycerol and 2% (w/v) SDS with 2.5% iodoacetamide.

For the second dimension, the strips were placed on 18 x 20 cm polyacrylamide gels (T=12%, C=2.67%) of 1 mm thickness (Protean Plus multi-casting chamber, Bio-Rad; Protean Plus Dodeca Cell, Bio-Rad) and run at 30 mA per gel for 5-6 h at 10°C until the dye front left the gel.

A total of 20 gels were run conforming to two biological and two technical replicates. Proteins in gels were fixed in a solution followed by overnight staining according to Neuhoff et al. (1988) with Coomassie Brilliant Blue G-250 (Sigma Aldrich). The gels were destained using distilled water for 6 hr and scanned at 300 dots per inch and 16-bit grey scale pixel depth using Gel Doc™ XR of Bio-Rad.

Gel analysis

SameSpots Progenesis software (version 5, Nonlinear Dynamics, UK) was used to analyse gels. Spots which were statistically significantly ($p \leq 0.05$) different from the control treatment and a fold value equal or greater than 3 different from the control, were considered differentially expressed.

Protein identification by mass spectrometry

Ten highly significantly up-regulated glutenin protein spots were identified for each of the two cultivars (Table 5.1). The selected protein spots were manually cut out and placed in 1.5 ml Eppendorf tubes. These gel slices were dehydrated with acetonitrile for 3 min and dried in a speed vacuum centrifuge for 30 min. These spots were analysed using nano liquid chromatography

tandem mass spectrometry (LC-MS/MS) at the University of Stellenbosch Central Analytical Facility.

In-gel digest of protein spots

The chemicals used for making buffers or solutions were of analytical grade or equivalent. Gel pieces excised from 2-DE were washed with a bleaching solution [200 mM NH_4HCO_3 and acetonitrile (50:50, V/V)] from Sigma-Aldrich, in 1.5 ml Eppendorf tubes until colourless. After liquid removal, samples were dehydrated and desiccated before reduction for 15 min at room temperature with 2 mM triscarboxyethyl phosphine (TCEP; Fluka) in 25 mM NH_4HCO_3 with gentle agitation. Extra TCEP was removed and the gel pieces again dehydrated. Cysteine residues were thiomethylated with 20 mM S-Methyl methanethiosulfonate (Sigma) in 25 mM NH_4HCO_3 for 30 min at room temperature. After thiomethylation, the gel pieces were dehydrated and washed with 25 mM NH_4HCO_3 followed by another dehydration step. Proteins in gel slices were digested with trypsin (Pierce) solution (20 ng/µl) at 37°C for overnight. After trypsin digestion, peptides from the gel slices were extracted in 50 µl water and 50% acetonitrile (Rommil). The samples were dried and 30 µl of 2% acetonitrile in water containing 0.1% formic acid was added.

Residual digest reagents were removed using an in-house manufactured C18 stage tip (Empore Octadecyl C18 extraction discs; Supelco). The samples were loaded onto the stage tip after activating the C18 membrane with 30 µl methanol (Sigma) and equilibration with 30 µl 2% acetonitrile:water; 0.05% TFA. The bound sample was washed with 30 µl 2% acetonitrile:water; 0.1% TFA before elution with 30 µl 50% acetonitrile:water 0.05% TFA. The eluate was evaporated to dry. The dried peptides were dissolved in 2% acetonitrile:water; 0.1% TFA for LC-MS analysis.

Liquid chromatograph separations

Nano liquid chromatography (LC) was performed on a Thermo Scientific Ultimate 3000 RSLC for separation of digested peptides. The peptide solution was directly loaded onto a C18-trap column (5 mm x 300 µm, Dionex, Thermo Scientific), equilibrated with 2% acetonitrile in water with 0.1% TFA at a flow rate of 10 µl/min for 5 min from a temperature controlled auto-sampler set at 7^{o}C for sample purification. Separation of proteins was carried out using a C18 analytical column (Thermo Scientific EASY, 10 cm length x 75 µm i.d 3 µm particle size) (Thermo Scientific). Sample elution to the analytical column was performed at a flow of 450 nl/min and the gradient generated as follows: 5.0-35% B over 60 min using Chromeleon non-linear gradient 6;

35-50% B from 60-70 min. Chromatographic separations were done at 45°C and samples were introduced into the mass spectrometer through a stainless steel nano-bore emitter.

The mobile phase comprised of loading solvent in water, 0.1% Solvent A: 2% acetonitrile and 0.1% FA in water and solvent B 100% ACN in water. The samples were injected onto the trap column using loading solvent at a flow rate of 10 µL/min from a temperature controlled autosampler set at 7^{o}C.

Mass spectrometry analysis

Mass spectrometry analysis was done using a Thermo Scientific Fusion mass spectrometer equipped with a Nanospray Flex ionization source operating in the positive ion mode. The samples were injected through a stainless steel emitter. The MS instrument was operated with following settings; spray voltage 1.8 kV and ion transfer capillary temperature 280°C. Spectra were internally calibrated using polysiloxane ions at m/z = 445.12003 and 371.10024. Mass spectrometer was operated in data-dependent mode, and all MS1 spectra were operated with an m/z scan range of 350-1650 using an Orbitrap detector with resolving power 120 000. An automatic gain control (AGC) target of 3 E5 and a maximum ion injection time (IT) of 40 ms were set to generate precursor spectra. Data was obtained in profile mode. MS2 scans were acquired using monoisotopic precursor selection, ions with charges +2 to +7 with error tolerance set to +/- 10 ppm were selected. Dynamic exclusion of 60 s was done in order to eliminate repeated fragmentation of most abundant ions. Generated precursor ions were selected for fragmentation in HCD mode using the quadrupole mass analyser operating at 30% HCD energy. For detection of ions, orbitrap mass analyser with resolving power of 30 000 was used. The AGC target was set to 5E4 and the maximum IT of 80ms was set. The data was acquired in centroid mode.

5.3 Data analysis

For protein identification, nano LC-MS/MS data were imported into Proteome Discoverer v1.4 (Thermo Scientific) and processed using the Sequest algorithm. Database interrogation was performed against a concatenated database created using the customer supplied sequence concatenated with the cRAP contaminant database (https://www.thegpm.org/crap/) as well as the Uniprot (www.uniprot.org) wheat database concatenated with the cRAP contaminant database. Mass tolerance values of 10 ppm and 0.02 Da for precursor and fragment masses respectively, with missed cleavage tolerance of 2. Deamidation (NQ), oxidation (M) and acetylation of protein

N-terminal was allowed as dynamic modifications and thiomethyl of C as static modification. Peptide validation was performed using the Target-Decoy PSM validator node. The search results were imported into Scaffold Q+ for further validation (www.proteomesoftware.com).

5.4 Results

AtilC2000

A total of 330 spots were differentially expressed at $p \leq 0.05$ in AtilC2000. Approximately 305 spots were up-regulated, of which 7.57% were induced by moderate drought, 10.61% by severe drought stress, 8.79% by moderate heat stress, and 7.27% by severe heat stress (see Appendix 3 for data). Several protein spots were also observed to be up-regulated by two or more stress conditions, 3.03% were increased under moderate drought and heat, 10% by moderate drought and severe heat, 4.84% by moderate and severe drought, 4.55% by severe drought and moderate heat, 5.15% by both severe drought and heat, 2.54% by both moderate and severe heat. Approximately 2.42% of total protein spots were up-regulated by moderate drought, moderate heat and severe heat, 8.79% by moderate drought, severe drought and moderate heat, and 2.73% by severe drought, moderate heat and severe heat. A total of 14.24% of the total protein spots increased under all stress treatments, while 7.56% was down-regulated under all stress treatments. Although some spots were upregulated by more than one condition, the magnitude of changes in spot volume differed with stress treatment. Under moderate drought, no protein spots were up-regulated by more than 100%, ranging between 14.52-72.20% in AtilC2000. The spots that were up-regulated by moderate heat increased by more than 72.20%. A similar pattern was observed for spots up-regulated by both moderate drought and severe heat, where moderate drought contributed less than severe heat to increase in volume of most protein spots. In the spots that were up-regulated by moderate and severe drought and moderate heat, severe drought contributed the least to the increase in spot volume in all spots with the exception of spots 37, 600 and 428 where moderate drought was the lowest contributor to increase in spot volume. For the spots that were up regulated by both severe drought and heat, severe heat made the lowest contribution to total spot volume changes in most spots.

MexicaliC75

In MexicaliC75, a total of 205 spots were differentially expressed (Appendix 4). A total of 197 spots were up-regulated, 34.47% were up-regulated by moderate drought stress only, 3.88% by severe drought, 0.97% by moderate heat and 2.91% by severe heat stress. Similar to AtilC2000,

some protein spots in MexicaliC75 were found to be up-regulated by more than one stress condition. A total of 4.36% of up-regulated spots were induced by moderate heat and moderate drought stress, 7.28% by moderate and severe drought, and 0.49% by moderate drought and severe heat, and 5.82% by moderate and severe heat. Severe drought and severe heat were responsible for 3.39% while moderate heat and severe heat stress conditions accounted for 3.39% of up-regulation of spots. About 4.36% of total protein spots were up-regulated by moderate drought, moderate heat and severe heat, 1.94% by moderate drought, severe drought and moderate heat, and 19.90% by severe drought, medium heat and severe heat stress.

The effects of stress treatments on protein spots that were up-regulated by more than one stress condition differed for the two cultivars. For spots that were up-regulated by both severe drought and moderate heat, moderate heat made the lowest contribution towards increase in spot volume, as spots were increased by not more than 50%. For the spots that increased under both moderate and severe drought, only three spots increased by more than 50% under severe drought (6, 147, 541). It also accounted for the lowest contributions in spot volume increase compared to moderate drought. Only four spots were up-regulated by all stress conditions, the highest spot volume was obtained under severe drought in three spots, while severe heat made the highest contribution to the other spot.

Comparison of AtilC2000 and MexicaliC75

A total of 91 spots were common in both durum wheat cultivars with different expression patterns. Only one spot (150) showed similar expression in both cultivars. Spot 150 was up-regulated by moderate drought stress in both cultivars and showed greater levels of expression in AtilC2000 than in MexicaliC75. Significantly increased spots might contribute to variations in pasta quality, therefore to fully understand their role in pasta quality, identification of certain spots was done using LC-MS/MS

LC-MS/MS

Accession numbers and names of proteins corresponding to spots analysed by LC-MS/MS are shown in Tables 5.2 and 5.3. The results showed heterogeneity of protein components. Some of the spots contained more than one protein. The total number of proteins spots excised from the 2-DE gel was 20, 10 spots from each cultivar. Of the 20 spots selected, 18 proteins were identified by the database with 82.3% identification match (Tables 5.2 and 5.3). In AtilC2000 (Table 5.2) 19 spots matched HMW-GS (Ax2, Dx5, and Dx2) by Gluprot, while according to the Uniprot

database they matched HMW-GS (Dx2, Bx23). Spot 85 was identified as HMW-GS Dy10, Dy1, By8, By15 and By16 by Gluprot, and according to Uniprot spot 85 matched HMW-GS By8, Dy10, Dy11 and y-type) and α gliadins. Protein spot 86 matched HMW-GS By8, Dy12, By15 and D1-2 according to Gluprot, while Uniprot characterised proteins as HMW-GS Dy11 and Dy10, α-gliadins and uncharacterised protein. Spot 87 matched HMW-GS Dy10 and By16 and ω-gliadins by Gluprot database. The Uniprot database matched spot 87 as HMW-GS Dy10. Spot 221 was identified as HMW-GS and γ-gliadins by Gluprot while the Uniprot database did not reveal any match. Spot number 361 was identified as α-gliadins by Gluprot, while Uniprot database matched spot 361 to α-gliadins and globulins. Spots 538 and 611 were identified as gliadins by both databases. Spots 622 and 669 were identified as γ-gliadins.

In MexicaliC75 (Table 5.3) only spot 83 was matched to HMW-GS by the two databases. Spot 83 matched HMW-GS Ax2, Dx5, Dy12 By8 and α-gliadins according to the Gluprot database. Uniprot data matched spot 83 to HMW-GS Bx17, Dx5, By9 Dy12, serpins and β-amylase. Spots 178 and 189 were not identified by the two database. Spots 366, 381 and 532 matched serpins according to the Uniprot database. Spots 394, 515 and 642 matched α and γ gliadins according to both databases. Spot 679 was identified as α- and γ-gliadins by Gluprot and Uniprot respectively.

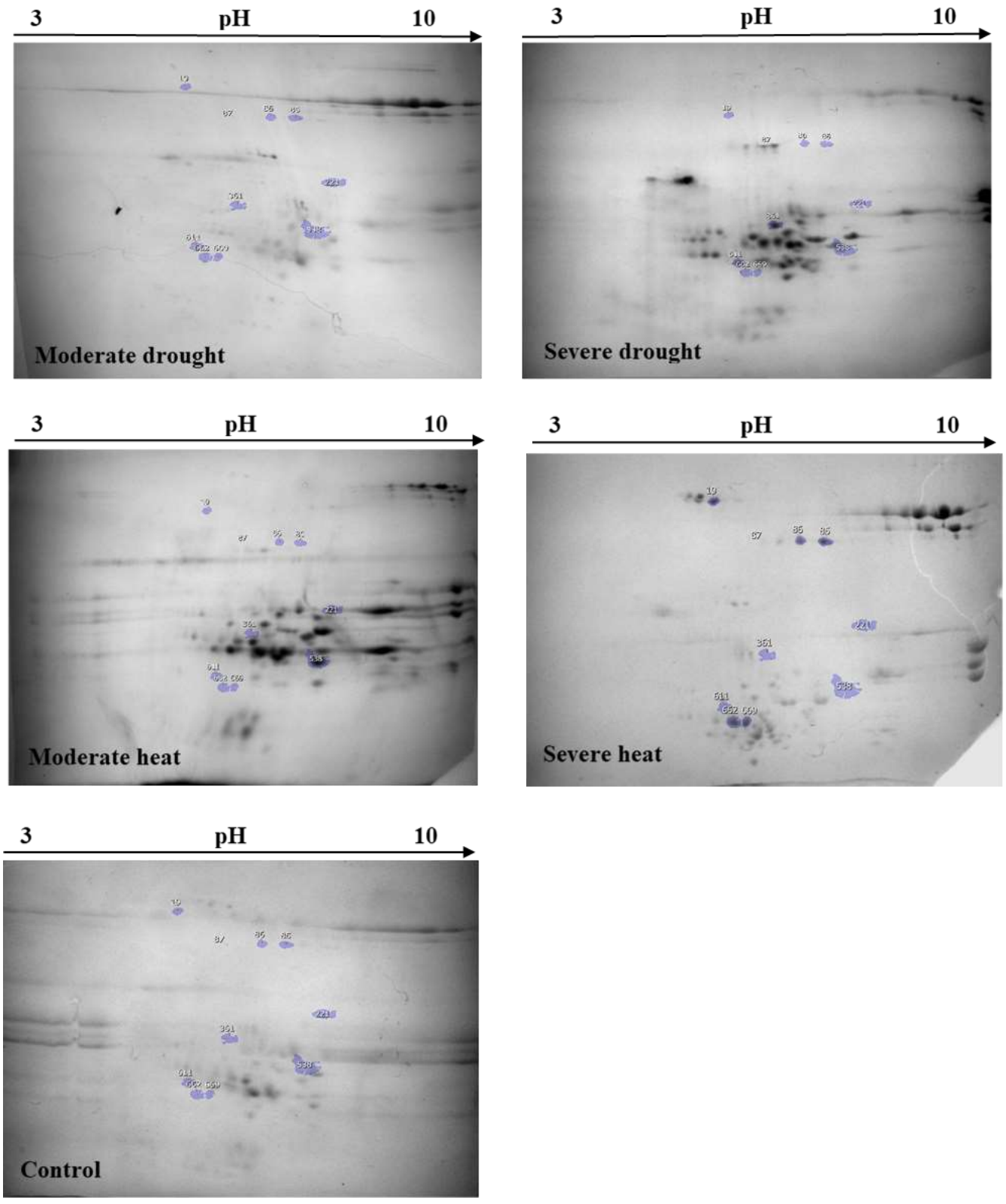

Figure 5.1 Two dimensional gel electrophoresis gels of AtilC2000 under different treatments

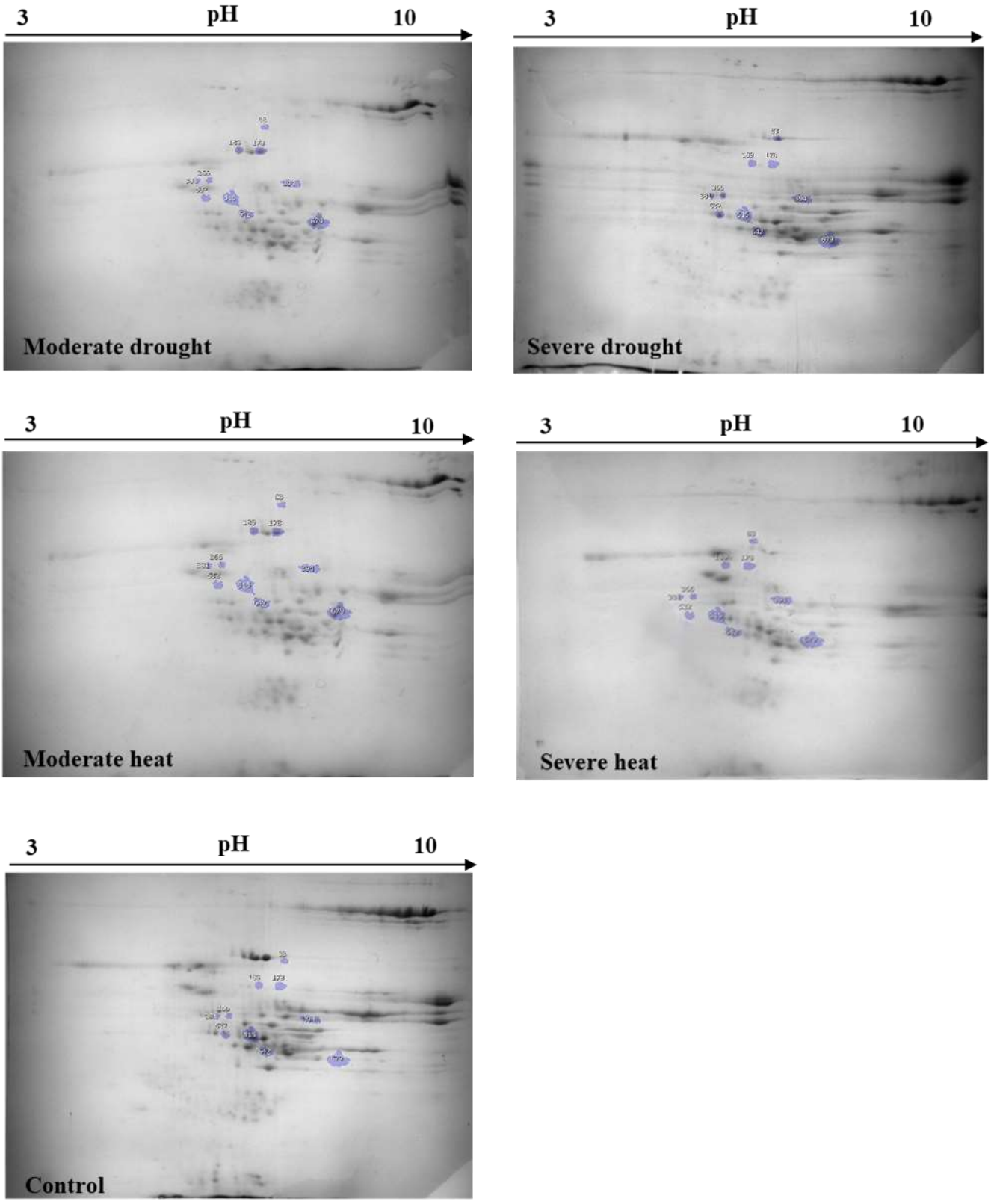

Figure 5.2 Two dimensional gel electrophoresis gels of MexicaliC75 under different treatments

Table 5.1 Volume and fold increase of 20 spots used for LC-MS/MS analysis of two cultivars in various environments

Genotype	Spot number	P-value	Fold	Optimum	Moderate drought	Severe drought	Moderate heat	Severe heat
AtilC2000	19	0.001	1.7	30290	33340	21380	11590	193200
	85	0.001	12.8	55880	45640	31070	18960	243300
	86	0.001	8.5	27880	19650	18030	15090	128900
	87	0.001	16.2	16	7.56	122.46	11.298	14.88
	221	0.001	5.2	85250	58120	49580	258800	122600
	361	0.001	7.6	81150	38960	295200	78430	73350
	538	0.001	6.5	220700	270200	343000	855800	131100
	611	0.001	5.1	72940	40230	154500	30500	57560
	622	0.001	6.1	208	92.77	187.54	33.89	107.11
	669	0.001	15.1	36570	30440	39990	9587.81	144800
MexicaliC75	83	0.001	6.7	188000	30680	35370	206100	27910
	178	0.001	7.2	159.18	253.72	112.46	349	810.11
	189	0.001	5.6	1610000	1049000	694900	1685000	3883000
	366	0.001	8.8	115900	13220	20300	411220	29020
	381	0.001	5.1	77.29	261.63	258.83	51	71.45
	394	0.001	5.2	296.16	181.13	245.45	296	942.80
	515	0.001	6.2	65640	150400	177400	91590	409500
	532	0.001	10.6	108800	13610	249300	21610	50070
	642	0.001	8.7	123890	417500	677500	138900	78060
	679	0.001	8.3	119000	1021100	142600	105300	47450

Table 5.2 Protein identification of spots from 2D maps of AtilC2000 by LC-MS/MS analysis using two databases

Spot Number	Data base	Accession	Description	Score	Coverage	# Unique Peptides	# PSMs	MW [kDa]	calc. pI
19	GluPro	Q41553	HMW glutenin subunit Ax2 OS=Triticum aestivum PE=4	116.33	74.36	7	807	88.4	6.55
		A9YSK4	High molecular weight glutenin subunit OS=Triticum aestivum GN=Glu-D1-1 PE=4	434.56	53.28	15	933	89.3	6.89
		D7REK2	HMW glutenin subunit 1Dx5' OS=Triticum aestivum PE=4	280.62	62.55	4	762	88.8	6.90
		Q6R2V1	High-molecular-weight glutenin subunit 1Dx2.1 OS=Triticum aestivum PE=4	300.15	45.69	3	492	88.9	6.19
	Uniprot	A0A060MZP1	High molecular weight glutenin subunit (Fragment) OS=Triticum aestivum GN=glu PE=4	138.60	58.44	14	164	86.3	5.68
		Q7M1M7	High-molecular-weight glutenin OS=Triticum aestivum PE=4 SV=1 - [Q7M1M7_WHEAT]	243.29	80.75	5	126	17.9	5.49
		S4U1S7	High molecular weight glutenin subunit 1Dx OS=Triticum spelta GN=Glu-D1-1 PE=4	386.17	47.17	2	390	90.4	7.30
		Q6R2V1	High-molecular-weight glutenin subunit 1Dx2.1 OS=Triticum aestivum PE=4 SV=2	300.15	24.16	2	198	88.9	6.19
		W8Q5H7	X-type high molecular weight glutenin subunit 1Bx23 OS=Triticum turgidum GN=1Bx23* PE=4	24.18	45.50	2	66	85.1	8.95
85	GluPro	A9ZMG8	High-molecular-weight glutenin subunit y10 OS=Triticum aestivum GN=Glu1Dy10 PE=4	815.77	80.09	60	1385	69.7	7.55
		D2CPI7	HMW glutenin subunit OS=Triticum aestivum GN=1Dy11 PE=4	390.16	79.28	1	807	68.3	7.97
		Q52JL3	HMW glutenin subunit OS=Triticum aestivum PE=4	361.10	70.36	1	658	70.8	8.46
		Q670Q5	High molecular weight glutenin subunit 1Dy10.1 OS=Triticum aestivum PE=4	334.00	72.37	1	704	70.3	7.77
		Q0Q5D8	High-molecular-weight glutenin By8 OS=Triticum aestivum GN=Glu PE=4	208.07	77.64	1	515	77.3	8.43
		Q4JHY1	High molecular weight glutenin subunit 1By15 OS=Triticum aestivum PE=2	197.57	80.91	1	624	77.8	8.43
		A5HMG2	HMW glutenin subunit 1By16 OS=Triticum aestivum PE=4	151.05	79.27	1	509	79.4	8.54
	Uniprot	Q0Q5D8	High-molecular-weight glutenin By8 OS=Triticum aestivum GN=Glu PE=4	208.07	27.36	4	131	77.3	8.43
		A0A165B8S1	High molecular weight glutenin subunit Dy10-m328SF OS=Triticum aestivum PE=4	815.77	73.46	4	872	69.6	7.77
		K7X0N8	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	13.85	17.65	3	8	33.4	8.37
		D2CPI7	HMW glutenin subunit OS=Triticum aestivum GN=1Dy11 PE=4	390.43	59.03	2	326	68.3	7.97
		J3S5N1	Y-type high-molecular-weight glutenin subunit 1Sshy2.3 OS=Aegilops sharonensis PE=4	227.58	29.99	2	143	82.4	7.44
86	GluPro	A9YSK3	High molecular weight glutenin subunit OS=Triticum aestivum GN=Glu-D1-2 PE=4	835.05	88.12	67	1514	69.7	8.15
		Q0Q5D8	High-molecular-weight glutenin By8 OS=Triticum aestivum GN=Glu PE=4	191.58	83.47	5	597	77.3	8.43
		A9QUS3	High molecular weight glutenin subunit OS=Triticum aestivum GN=1Dy12* PE=2 [A9QUS3_WHEAT]	413.52	81.28	2	902	72.3	7.78
		Q4JHY1	High molecular weight glutenin subunit 1By15 OS=Triticum aestivum PE=2	186.11	82.99	2	679	77.8	8.43
	Uniprot	J3S5N1	Y-type high-molecular-weight glutenin subunit 1Sshy2.3 OS=Aegilops sharonensis PE=4	204.94	26.86	3	139	82.4	7.44
		V9TRL3	High molecular weight glutenin subunit 1Dy protein OS=Triticum aestivum GN=Glu-D1-2 PE=4	883.25	72.60	2	940	69.5	8.15
		D2CPI7	HMW glutenin subunit OS=Triticum aestivum GN=1Dy11 PE=4	372.13	59.97	2	359	68.3	7.97
		A0A165B8S1	High molecular weight glutenin subunit Dy10-m328SF OS=Triticum aestivum PE=4	900.75	73.15	2	992	69.6	7.77
86	Uniprot	A0A165B8S1	High molecular weight glutenin subunit Dy10-m328SF OS=Triticum aestivum PE=4 SV=1	463.15	68.67	33	546	69.6	7.77

Table 5.2 Protein identification of spots from 2D maps of AtilC2000 by LC-MS/MS analysis using two databases (continued)

Spot Number	Data base	Accession	Description	Score	Coverage	# Unique Peptides	# PSMs	MW [kDa]	calc. pI
221	GluPro	R9XUS6	Gamma-gliadin OS=Triticum aestivum PE=4	45.63	32.49	6	95	40.8	7.93
		G3FLC5	High-molecular-weight glutenin subunit OS=Triticum aestivum GN=Glu PE=4	15.50	77.23	6	279	85.4	8.56
221	Uniprot	N/D							
361	GluPro	I0IT57	Alpha/beta-gliadin OS=Triticum aestivum PE=4	101.36	58.28	6	238	33.4	7.71
		I0IT55	Alpha/beta-gliadin OS=Triticum aestivum PE=4	90.19	51.20	2	250	33.5	7.71
		K7XR61	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	46.51	77.74	2	228	32.4	8.03
		K7X1J5	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	33.61	66.24	4	151	36.5	7.36
	Uniprot	I0IT58	Alpha/beta-gliadin OS=Triticum aestivum PE=4	119.58	29.37	5	125	30.9	7.74
		A7LHC2	Alpha gliadin OS=Elymus elongatus GN=gli-A-62 PE=4	73.07	24.21	0	93	32.4	7.71
		M7ZK46	12S seed storage globulin 1 OS=Triticum urartu GN=TRIUR3_30124 PE=4	38.23	21.16	2	36	63.8	7.11
538	GluPro	A5JSB6	Alpha-gliadin OS=Triticum aestivum GN=Gli-Z6 PE=2	78.73	51.55	3	217	33.5	7.75
		Q41528	Alpha-gliadin OS=Triticum aestivum PE=4	71.40	62.37	2	252	33.2	8.27
		R9XSV7	Alpha-gliadin OS=Triticum aestivum	75.09	48.11	2	183	33.5	7.75
		K7X0N8	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	121.01	59.17	2	278	33.4	8.37
		I0IT63	Alpha/beta-gliadin OS=Triticum aestivum PE=4	141.20	60.98	1	281	33.1	7.72
	Uniprot	Q0GK30	Omega-gliadin OS=Triticum timopheevii PE=4	136.47	21.75	3	136	32.8	8.03
		K7X0N8	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	121.01	19.72	2	137	33.4	8.37
611	GluPro	I0IT62	Alpha/beta-gliadin OS=Triticum aestivum PE=4	52.69	50.15	2	162	38.4	8.02
		K7X0N8	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	165.31	69.55	2	381	33.4	8.37
		A7LHB3	Alpha gliadin OS=Triticum aestivum GN=gli-w24 PE=4	32.54	63.52	2	181	35.4	8.02
	Uniprot	B8XU36	Alfa gliadin OS=Triticum monococcum PE=2	93.72	38.91	2	161	33.8	7.72
		K7X0N8	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	165.31	33.56	2	178	33.4	8.37
622	GluPro	B6UKM5	Gamma-gliadin OS=Triticum aestivum GN=ll903 PE=4	328.14	90.73	4	719	34.5	7.90
		R9XWA1	Gamma-gliadin OS=Triticum aestivum PE=4	325.19	73.84	2	429	34.3	7.37
		I0IT55	Alpha/beta-gliadin OS=Triticum aestivum PE=4	60.47	67.01	2	171	33.5	7.71
		Q41528	Alpha-gliadin OS=Triticum aestivum PE=4	37.56	70.38	2	197	33.2	8.27
	Uniprot	Q84M19	Gamma gliadin (Fragment) OS=Triticum turgidum subsp. durum PE=4	330.89	48.58	18	219	32.2	6.83
		I0IT55	Alpha/beta-gliadin OS=Triticum aestivum PE=4	60.47	32.30	3	77	33.5	7.71
		Q3YFI1	Alpha-type gliadin OS=Triticum dicoccoides GN=Gliw-4 PE=4	66.64	24.48	2	77	32.9	7.40
669	GluPro	B6UKM5	Gamma-gliadin OS=Triticum aestivum GN=ll903 PE=4	336.78	89.07	2	972	34.5	7.90
		R9XU85	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	32.88	82.87	2	363	32.9	8.05
	Uniprot	B6DQC6	Gamma-gliadin (Fragment) OS=Elymus elongatus PE=4]	79.03	21.94	2	282	31.9	7.93

Table 5.3 Protein identification of spots from 2D maps of MexicaliC75 by LC-MS/MS analysis using two databases

Spot Number	Data base	Accession	Description	Score	Coverage	# Unique Peptides	# PSMs	MW [kDa]	calc. pI
83	Glupro	G3FLC5	High-molecular-weight glutenin subunit OS=Triticum aestivum GN=Glu PE=4	53.39	77.36	9	325	85.4	8.56
		Q41553	HMW glutenin subunit Ax2 OS=Triticum aestivum PE=4	45.73	65.40	12	361	88.4	6.55
		D7REK2	HMW glutenin subunit 1Dx5' OS=Triticum aestivum PE=4	40.74	58.22	7	296	88.8	6.90
		E4W506	HMW glutenin subunit OS=Triticum aestivum GN=1Dy12.2* PE=4	40.02	72.19	9	319	70.6	7.75
		Q0Q5D8	High-molecular-weight glutenin By8 OS=Triticum aestivum GN=Glu PE=4	38.42	72.92	5	318	77.3	8.43
		K7X0N8	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	30.82	62.98	2	135	33.4	8.37
83	Uniprot	M8AQX5	Beta-amylase OS=Aegilops tauschii GN=F775_28031 PE=3	67.40	33.46	7	29	59.9	5.22
		Q18MZ6	High-molecular-weight glutenin subunit Bx17 OS=Triticum aestivum GN=Glu-B1 PE=4	53.39	41.90	2	63	80.0	8.70
		M8B5G5	Beta-amylase OS=Triticum urartu GN=TRIUR3_08670 PE=3	50.95	26.86	2	24	58.7	5.59
		Q41593	Serpin-Z1A OS=Triticum aestivum GN=WZCI PE=1	47.64	45.73	2	21	43.1	5.90
		Q9ST58	Serpin-Z1C OS=Triticum aestivum PE=1	42.98	41.46	5	19	42.9	5.97
		Q03871	HMW glutenin subunit 1By9 OS=Triticum aestivum GN=Glu-1By9 PE=4	38.42	26.81	5	40	75.7	8.43
		E4W506	HMW glutenin subunit OS=Triticum aestivum GN=1Dy12.2* PE=4 S	37.47	27.96	6	33	70.6	7.75
		M8CT91	12S seed storage globulin 1 OS=Aegilops tauschii GN=F775_30249 PE=4	33.20	29.37	4	19	62.5	6.92
		Q9ST57	Serpin-Z2A OS=Triticum aestivum PE=1	30.66	27.39	2	15	43.3	5.71
178		N/D							
189		N/D							
366	GluPro	N/D							
	Uniprot	Q9ST58	Serpin-Z1C OS=Triticum aestivum PE=1 SV=1 - [SPZ1C_WHEAT]	374.10	68.59	22	214	42.9	5.97
		Q41593	Serpin-Z1A OS=Triticum aestivum GN=WZCI PE=1 SV=1 - [SPZ1A_WHEAT]	206.23	47.74	5	140	43.1	5.90
		Q9ST57	Serpin-Z2A OS=Triticum aestivum PE=1 SV=1 - [SPZ2A_WHEAT]	89.55	53.77	9	69	43.3	5.71
381	GluPro	N/D							
	Uniprot	Q9ST57	Serpin-Z2A OS=Triticum aestivum PE=1 SV=1 - [SPZ2A_WHEAT]	323.53	65.33	25	178	43.3	5.71
		W5FZ62	Uncharacterised protein OS=Triticum aestivum PE=3 SV=1 - [W5FZ62_WHEAT]	142.75	60.90	8	103	43.0	5.69
		Q9ST58	Serpin-Z1C OS=Triticum aestivum PE=1 SV=1 - [SPZ1C_WHEAT]	156.93	59.05	6	98	42.9	5.97
394	GluPro	R9XUS6	Gamma-gliadin OS=Triticum aestivum PE=4	76.81	40.06	3	129	40.8	7.93
	Uniprot	R9XUS6	Gamma-gliadin OS=Triticum aestivum PE=4	74.88	14.01	2	52	40.8	7.93

Table 5.3 Protein identification of spots from 2D maps of MexicaliC75 by LC-MS/MS analysis using two databasis (continued)

Spot number	Data base	Accession	Description	Score	Coverage	# Unique Peptides	# PSMs	MW [kDa]	calc. pI
515	GluPro	B6UKM5	Gamma-gliadin OS=Triticum aestivum GN=ll903 PE=4	104.67	73.51	14	209	34.5	7.90
		I0IT63	Alpha/beta-gliadin OS=Triticum aestivum PE=4	33.24	58.54	3	167	33.1	7.72
		I0IT55	Alpha/beta-gliadin OS=Triticum aestivum PE=4	31.95	43.99	3	91	33.5	7.71
	Uniprot	Q84M19	Gamma gliadin (Fragment) OS=Triticum turgidum subsp. durum PE=4	104.67	19.50	5	75	32.2	6.83
		I0IT55	Alpha/beta-gliadin OS=Triticum aestivum PE=4	31.95	8.25	3	21	33.5	7.71
		B6UKL1	Gamma-gliadin OS=Triticum dicoccoides GN=ll819 PE=4	80.49	14.05	3	67	27.2	7.69
532	GluPro	N/D							
	Unipro	M7Z1Z4	Serpin-Z2B OS=Triticum urartu GN=TRIUR3_06337 PE=3	255.12	54.70	10	169	45.1	6.46
		Q41593	Serpin-Z1A OS=Triticum aestivum GN=WZCI PE=1	616.64	66.83	9	324	43.1	5.90
		Q9ST58	Serpin-Z1C OS=Triticum aestivum PE=1	222.87	51.51	4	124	42.9	5.97
		Q9ST57	Serpin-Z2A OS=Triticum aestivum PE=1	162.55	53.27	3	96	43.3	5.71
		M1MQ64	Serpin 3 (Fragment) OS=Triticum aestivum GN=SER3 PE=2	267.94	65.98	3	151	21.1	4.93
642	GluPro	K7X0N8	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	84.57	53.98	2	221	33.4	8.37
		A7LHB5	Alpha gliadin OS=Triticum aestivum GN=gli-wE12 PE=4	75.21	62.02	2	199	33.0	8.27
		Q41528	Alpha-gliadin OS=Triticum aestivum PE=4	58.82	54.01	2	229	33.2	8.27
		H6VLP7	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	50.89	58.16	2	207	32.3	7.75
	Uniprot	A0A0E3Z536	Alpha-gliadin (Fragment) OS=Triticum urartu PE=4	77.59	29.14	3	93	35.0	8.10
		H6VLP7	Alpha-gliadin OS=Triticum aestivum GN=gli-2	50.89	20.21	2	77	32.3	7.75
		B6UKS0	Gamma-gliadin OS=Triticum urartu GN=qp715 PE=4	16.87	14.67	2	19	34.4	8.13
679	GluPro	K7X0N8	Alpha-gliadin OS=Triticum aestivum GN=gli-2 PE=4	82.59	55.02	2	242	33.4	8.37
		Q41528	Alpha-gliadin OS=Triticum aestivum PE=4	55.72	57.49	2	230	33.2	8.27
		A5JSA7	Alpha-gliadin OS=Triticum aestivum GN=Gli-G4 PE=2	34.65	49.83	2	138	33.2	8.48
	Uniprot	Q0GK30	Omega-gliadin OS=Triticum timopheevii PE=4	58.02	20.70	2	79	32.8	8.03

5.5 Discussion

Storage proteins of wheat are important components governing processing quality. Drought and heat affect the protein composition and quality of wheat grains (Halford et al. 2014). In this study, five types of proteins were identified by LC-MS/MS in two durum wheat cultivars, that were influenced by heat and drought stress, which were serpins, gliadins, HMW glutenins, β-amylase and globulins. Most of these proteins are closely related to processing quality. Serpins form minor components of the grain, and make up about 4% of wheat grain endosperm proteins. The exact role of serpins is not known, but their activity suggests inhibition of enzyme proteinases (Laino et al. 2013; Vensel et al. 2005). Serpins are also believed to have an influence on grain quality parameters due to the presence of amino acid motifs that match those of prolamin proteins and form intermolecular disulphide bridges, therefore the up-regulation of serpins might improve flour quality (Vensel et al. 2005; Roberts and Hejgaard 2008). The presence of serpins was not expected due to the glutenin extraction method used. The presence of serpins in durum wheat flour was also reported with the same extraction protocol previously in a study by Masci et al. (2002). Three protein spots (361, 538 and 611) in AtilC2000 were identified as α-gliadins, and in MexicaliC75 spots 622 and 669 were identified as γ-gliadins. The presence of gliadin proteins was not expected due to washing out of gliadins during extraction, however these might be LMW-C and LMW-D type due to close genetic linkage between LMW proteins and some gliadins. It has been indicated that LMW proteins comprise of gliadin-like sequences caused by mutation in the cysteine residues in LMW (Masci et al. 2002; Muccilli et al. 2010; Barak et al. 2015). The presence of gliadin proteins was also reported previously (Mamone et al. 2009; Zhang et al. 2017) where gliadin proteins were expressed despite using an extraction protocol that removed the gliadins. However, these authors did not conclude whether the presence of gliadins might be due to the extraction protocol or whether they are LMW proteins matching α- and γ-gliadins. Gliadin and LMW have an influence on dough rheological properties such as dough strength (Pompa et al. 2013; De Saintis et al. 2017). Other proteins that were identified were HMW-GS 7 and 8, which are functional proteins which affect semolina quality. Subunits 7 and 8 are related with good gluten strength in durum wheat flours (Pompa et al. 2013) and both the cultivars had these subunits, as identified by SDS-PAGE (Figure 4.1). Peptide sequences similar to protein subunits controlled by the D genome was detected. The durum wheat cultivars used had only one pair of HMW coded by the B genome, which were 1Bx7 and 1By8. Accurate determination of HMW-GS is limited due to lack of suitable reference standards in this mass range and close amino acid similarities in their primary structures (Cunsolo et al. 2012; Mazzeo et al. 2017).

This suggests that subunits controlled by D-genome detected in this study might be 1Bx7 or 1By8. The identification of non-gluten proteins such as beta-amylase and globulin is noteworthy. The detection of these proteins may improve understanding of their role in gluten functionality. Beta-amylase plays an important role in the starch synthesis pathway, and globulins have structural and metabolic roles (Majoul et al. 2004; Arena et al. 2017). High temperatures may result in up-regulation of globulins. (Hasniza et al. 2014). In a study on the effect of drought stress on wheat gluten proteins via 2-DE and MALDI-TOF-TOF, results indicated significant increases of AG under drought stress conditions (Labuschagne et al. 2020). Some of the globulin variants have six cysteine residues similar to y-type HMW-GS, and are therefore likely to influence dough properties because of their contribution to gluten polymerization (Veraverbeke and Delcour 2002; Hasniza et al. 2014).

Durum wheat quality is largely affected by environmental conditions, with heat and drought being major factors which influence protein content, metabolism of cells and carbohydrates, as well as enzymes, thus disturbing grain development. In a study on the effects of heat and cold wheat proteins using 2-DE followed by LC-MS/MS, HMW and α-gliadins increased in abundance (Labuschagne et al. 2020). In a study to assess the influence of high temperature and water deficit using 2-DE and MALDI-TOF-TOF, high temperatures induced major changes in gluten proteins accumulation at grain fill period. Glutenin proteins increased in abundance under high temperatures. However, gliadins and AG increased under water deficit. The type and period of stresses affected the production of specific protein fractions.

The results observed showed various responses of the glutenin proteins in the two cultivars to drought and heat stress. In AtilC2000, the largest number of spots were up-regulated under all stress conditions combined (14.24%) followed by moderate drought stress. This suggests that AtilC2000 can be considered to be more adapted to a wide range of environments. Although moderate drought stress caused an up-regulation in quite a number of spots, for most protein spots it contributed the least to increases in volume of protein spots. More protein spots were resolved in AtilC2000 compared to MexicaliC75. In MexicaliC75, most spots were increased by moderate drought stress conditions (35.12%) followed by severe drought stress conditions. This indicated that moderate drought had a large impact on the up-regulation of proteins in MexicaliC75. In terms of magnitude of effects of various stress conditions on proteins, moderate heat made the lowest contribution to increase in spot volume in MexicaliC75. In AtilC2000, moderate drought caused the least increase in spot volume. Certain protein spots

were up-regulated by more than one stress condition, while other protein spots showed a decrease due to certain stress treatments.

5.6 Conclusions

Drought and heat affects the synthesis of gluten proteins, by up-regulating HMW and gliadin proteins as identified by LC-MS/MS in the two cultivars. In MexicaliC75, a higher percentage of protein spots (35.12%) increased significantly under moderate drought stress. In AtilC2000, the largest number of spots were up-regulated under all stress conditions (14.24%). Increase in volume of protein spots by a particular stress condition suggests such proteins were more responsive to that particular stress conditions.

The formation of proteins in grain is a complicated process undergoing spatial and temporal regulation, influenced by abiotic and biotic factors. The use of 2-DE coupled with LC-MS/MS allowed the identification of glutenin proteins up-regulated by various stress conditions. Identification of gluten protein in this study would contribute to an improved understanding of the influence of environmental effects on gluten proteins and clarification of biochemical aspects of gene-function relationship. Genome sequencing of durum wheat for future studies can be done to assess genetic control of wheat gluten proteins under different environmental stress conditions. This will complement and supplement 2-DE and mass spectrometry analysis.

It should be noted that for this study, LC-MS/MS was done on limited number of proteins spots and did not cover all up-regulated spots in the gel, due to cost implications.

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CHAPTER 6

Conclusions and recommendations

The suitability of a wheat cultivar for specific end-use quality is largely determined by qualitative and quantitative differences in gluten protein composition. It is the task of the wheat breeder to identify flour quality traits to satisfy processing requirements. Gluten proteins are complicated and are influenced by genetic make-up of the cultivar and the environment in which it is grown. The overall objective of this study was to increase the understanding of environmental effects on durum gluten proteins, in order to make it possible to improve the production of wheat with desired quality for different end-uses. Six durum wheat cultivars with the same HMW-GS and LMW-GS composition, as measured by SDS-PAGE, were grown under six different environmentatl conditions. SE-HPLC, RP-HPLC and proteomics were applied to assess the effects of different levels of drought and heat stress on durum wheat gluten proteins. All stress conditions caused significant increases in FPC. High FPC was seen particularly under severe heat and drought stress conditions. Significant G, E and GxE were found for protein fractions separated by SE-HPLC. High values for both LUPP and UPP were observed under moderate heat stress and severe drought stress. Severe drought and moderate heat stress caused significant increases in LUPP. Unextractable polymeric proteins and unHMW were significantly increased by all stress treatments and severe heat stress caused a significant increase in unGLI. The exGLI showed an increase under all stress treatments. The flood irrigation treatment expressed significantly lower values for exGLI, unGLI, unHMW and UPP proteins than the values under stress conditions. The exAG, exLMW and unLMW proteins were sensitive to all stress, showing significant reduction under all stress treatments.

RP-HPLC results indicated significant genotype effects for all measured proteins except for LMW. Environment effect was also highly significant for all protein fractions. This indicates that these protein fractions were influenced by both the genotype and growing conditions. The GxE effect was significant for all proteins except for HMW proteins, indicating that ranking of cultivars changed under different growing conditions. Moderate heat caused significant increases in HMW proteins. The α-gliadins were significantly increased by all stress treatments. LMW proteins were reduced by all stress treatments, with severe heat and severe drought causing the largest reduction.

The γ-gliadins showed consistent correlations with alveograph parameters. The HMW proteins showed significant correlations with alvW. LMW proteins as analysed by both SE-PLC and RP-HPLC showed a significant reduction under all stress treatments, indicating their sensitivity to environmental stress conditions.

With proteomics, four types of proteins were identified that were severely influenced by heat and drought stress, and these were serpins, gliadins, HMW glutenins and globulins. Most of these proteins are closely related to processing quality. This indicated various responses of gluten proteins to drought and heat stress by the two tested cultivars. In AtilC2000, the largest number of spots increased under all stress conditions (14.24%), followed by moderate drought stress alone. Even though moderate drought stress caused an increase in quite a number of spots, the increases in volume of protein spots was not that high. More protein spots were up-regulated in AtilC2000 compared to MexicaliC75. In MexicaliC75, most spots were increased by moderate drought stress conditions (35.12%) followed by severe drought stress conditions. This indicated that moderate drought had a large impact on the up-regulation of proteins in MexicaliC75. In terms of magnitude of effect of various stress conditions on proteins, moderate heat made the least contribution to increases in spot volume in MexicaliC75. In AtilC2000, moderate drought caused the least increases in spot volume. Certain protein spots were up-regulated by more than one stress condition, while other protein spots showed a decrease due to certain stress treatments. Increase in volume of protein spots by a particular stress condition suggests such proteins were more responsive to that particular stress condition.

The genotype ranking for measured proteins was inconsistent under different treatments, which indicated GxE interaction. This suggests that the effects of stress cannot be generalised for all cultivars, and that cultivars reacted differently to stress conditions. Effective selection by wheat breeders is affected if there is inconsistency in the ranking of genotypes among environments for quality characteristics. For the industry, consistency of wheat quality is very important. The ideal is to select cultivars that perform consistently under all conditions, and that is what breeders usually strive to do. Genome sequencing of durum wheat for future studies can be done to assess genetic control of wheat gluten proteins under different environmental stress conditions.

LIST OF APPENDICES

Appendix 1. Temperature data (°C) in Campo Experimental Norman E. Borlaug, CIMMYT, Cd. Obregon, Mexico, for two cropping seasons

	Year 1			Year 2		
Months	**Maximum temperature**	**Average temperature**	**Minimum temperature**	**Maximum temperature**	**Average temperature**	**Minimum temperature**
November	27.80	20.50	14.70	28.50	19.80	13.80
December	25.40	16.60	9.80	25.60	16.70	9.90
January	26.90	16.50	8.50	23,6	14.10	6.60
February	23.80	14.10	6.00	29.10	18.50	9.50
March	28.60	18.90	11.00	31.10	20.60	11.00
April	31.10	20.60	11.00	35.30	25.30	15.00
May	36.50	25.90	15.10	36.70	29.90	21.50
June	36.70	28.90	21.50	38.70	31.40	21.50

Appendix 2. Quality data measured acrosse two years for six different treatments

Quality trait	Environment	MexicaliC75	YavarosC79	AltarC84	AtilC2000	Jupare C2001	Cirno C2008	Mean	LSD
AlvL	Optimum	74.75	40.25	62.00	112.25	74.25	73.00	72.75	6.78
	Flood	42.00	35.75	42.00	80.00	69.50	55.75	54.17	5.14
	Moderate heat	55.50	40.25	41.25	92.25	53.00	65.25	57.92	6.52
	Severe heat	56.25	40.00	43.50	65.75	58.25	50.50	52.38	1.79
	Moderate drought	50.50	36.50	47.50	102.00	58.00	76.25	61.79	4.97
	Severe drought	77.25	33.75	58.50	82.00	65.25	72.75	64.92	10.11
	Combined								
AlvP	Optimum	155.75	135.75	145.50	115.25	153.25	99.75	134.21	16.46
	Flood	256.25	167.00	216.50	167.25	195.25	199.75	200.33	11.79
	Moderate heat	160.25	173.75	182.00	132.50	206.75	134.25	164.92	11.18
	Severe heat	189.25	228.50	234.25	155.00	191.50	177.25	195.96	10.98
	Moderate drought	180.50	144.00	175.75	106.50	175.25	123.50	150.92	15.82
	Severe drought	133.50	166.50	159.25	130.00	158.00	112.25	143.25	16.57
	Combined								
AlvP/L	Optimum	3.06	3.51	3.27	1.10	3.16	1.46	2.59	0.69
	Flood	6.10	4.78	5.17	2.10	2.83	3.68	4.11	0.50
	Moderate heat	3.07	4.31	4.43	1.45	3.91	2.04	3.20	0.45
	Severe heat	3.34	5.70	5.49	2.58	3.32	3.58	4.00	0.33
	Moderate drought	3.77	3.97	3.75	1.04	3.08	1.65	2.87	0.42
	Severe drought	2.26	5.59	4.42	1.82	2.24	1.76	3.18	0.70
	Combined								
AlvW	Optimum	351.25	205.75	273.75	396.50	333.75	229.50	298.42	34.11
	Flood	460.25	238.00	386.75	478.75	508.25	444.00	419.33	35.20
	Moderate heat	316.50	280.25	303.00	404.00	450.00	314.75	344.75	28.52
	Severe heat	412.75	381.25	421.00	361.25	433.00	354.75	394.00	22.10
	Moderate drought	336.25	211.50	332.75	354.00	401.25	323.25	326.50	38.73
	Severe drought	313.50	216.75	261.50	347.25	343.00	235.75	286.29	15.30
	Combined								
MDT	Optimum	2.71	2.48	2.72	2.58	3.07	2.44	2.66	0.32
	Flood	2.68	1.67	2.75	2.15	2.43	2.43	2.35	0.20
	Moderate heat	2.77	2.48	2.88	2.74	3.16	2.71	2.79	0.18
	Severe heat	2.52	1.95	2.60	2.27	2.69	2.49	2.42	0.16
	Moderate drought	2.61	2.36	2.90	2.56	2.92	2.86	2.70	0.20
	Severe drought	2.95	1.88	2.58	2.46	2.82	2.51	2.53	0.27
	Combined								

Appedix 3 Significantly up-regulated spots under different stress environments compared to the control for cultivar AtilC2000

Spot number	P-value	Fold	Control	Moderate drought	Severe drought	Moderate heat	Severe heat	Moderate drought	Severe drought	Moderate heat	Severe heat
57	p≤0.05	22.40	28.00	303.04	73.95	39.07	626.79	-982.29	-164.11	-39.54	-2138.55
66	p≤0.05	9.20	11.00	101.70	30.17	24.01	19.84	-824.55	-174.29	-118.25	-80.32
80	p≤0.05	6.10	35.00	138.81	49.10	68.26	214.22	-296.59	-40.30	-95.03	-512.06
83	p≤0.05	7.70	54560.00	135600.00	97030.00	69630.00	421400.00	-148.53	-77.84	-27.62	-644.74
88	p≤0.05	5.60	18.00	41.92	100.57	23.54	22.81	-132.87	-458.74	-30.77	-26.73
94	p≤0.05	9.30	28.00	162.17	83.42	41.90	259.84	-479.19	-197.92	-49.63	-828.01
97	p≤0.05	13.30	5.00	35.73	66.26	19.77	42.65	-614.66	-1225.22	-295.44	-752.92
114	p≤0.05	7.50	31.00	232.95	83.42	104.51	68.43	-651.45	-169.09	-237.12	-120.75
128	p≤0.05	10.70	15.00	160.11	126.01	76.73	101.16	-967.40	-740.09	-411.55	-574.40
130	p≤0.05	5.40	109.00	278.30	589.25	151.58	227.11	-155.32	-440.59	-39.07	-108.36
132	p≤0.05	5.30	32.00	168.36	40.82	44.72	52.56	-426.11	-27.57	-39.75	-64.26
164	p≤0.05	29.80	3.00	37.79	89.33	39.54	45.62	-1159.80	-2877.80	-1218.10	-1420.70
169	p≤0.05	22.40	7.00	76.28	79.28	47.08	156.70	-989.66	-1032.51	-572.50	-2138.54
183	p≤0.05	14.10	29.00	190.35	69.22	410.03	267.78	-556.37	-138.69	-1313.88	-823.37
210	p≤0.05	6.20	69.00	96.89	102.94	426.03	83.31	-40.42	-49.19	-517.44	-20.74
266	p≤0.05	6.30	24.00	114.07	24.26	150.64	130.91	-375.29	-1.07	-527.67	-445.47
270	p≤0.05	5.40	19.00	67.34	35.50	101.68	46.61	-254.43	-86.83	-435.17	-145.33
291	p≤0.05	10.30	30.00	201.34	208.25	310.23	184.47	-571.13	-594.16	-934.09	-514.89
296	p≤0.05	16.90	4.00	42.60	25.44	22.60	67.44	-965.10	-535.98	-464.90	-1586.00
352	p≤0.05	5.20	135.00	216.46	699.29	159.59	255.88	-60.34	-417.99	-18.21	-89.54
353	p≤0.05	6.20	2775.00	3108.40	6884.02	2825.45	4432.18	-12.01	-148.07	-1.82	-59.72
364	p≤0.05	11.90	123.00	230.20	1461.88	902.90	371.91	-87.16	-1088.52	-634.07	-202.37
366	p≤0.05	37.00	17.00	59.10	223.63	628.93	185.46	-247.62	-1215.47	-3599.56	-990.94
377	p≤0.05	6.40	24.00	90.71	152.64	34.37	40.66	-277.94	-535.98	-43.19	-69.43
383	p≤0.05	6.20	781.00	2463.50	4864.84	1457.92	1768.31	-215.43	-522.90	-86.67	-126.42
390	p≤0.05	5.30	955.00	2299.27	988.59	5089.77	1895.26	-140.76	-3.52	-432.96	-98.46
419	p≤0.05	8.30	2438.00	5245.16	20270.00	15160.00	2931.65	-115.14	-731.42	-521.82	-20.25
424	p≤0.05	9.50	107.00	397.18	168.61	1019.18	246.95	-271.20	-57.58	-852.50	-130.79
431	p≤0.05	5.80	344400.00	536900.00	2011000.00	1424000.00	442300.00	-55.89	-483.91	-313.47	-28.43
434	p≤0.05	8.10	943.00	2591.31	7663.77	1416.96	1255.57	-174.79	-712.70	-50.26	-33.15
450	p≤0.05	15.80	7.00	30.92	110.63	12.71	20.83	-341.76	-1480.46	-81.57	-197.53
459	p≤0.05	7.70	103.00	415.05	459.68	796.04	232.07	-302.96	-346.30	-672.86	-125.31
462	p≤0.05	6.70	54.00	75.59	359.70	145.46	99.18	-39.98	-566.11	-169.37	-83.66
464	p≤0.05	5.80	34.00	71.47	83.42	197.72	137.86	-110.19	-145.35	-481.52	-305.46
494	p≤0.05	11.90	90.00	364.89	811.70	1068.61	163.64	-305.43	-801.88	-1087.34	-81.82
524	p≤0.05	9.30	12.00	82.46	111.22	34.84	27.77	-587.17	-826.86	-190.30	-131.41
532	p≤0.05	8.40	90.00	97.58	759.63	209.01	249.92	-8.42	-744.04	-132.24	-177.69

535	p≤0.05	17.40	3.00	19.24	27.81	52.25	15.87	-541.37	-826.87	-1641.77	-428.93
540	p≤0.05	7.60	33.00	96.89	90.52	252.32	224.14	-193.61	-174.29	-664.62	-579.21
571	p≤0.05	5.10	32.00	34.36	162.10	71.55	77.36	-7.37	-406.57	-123.61	-141.74
584	p≤0.05	6.10	34.00	106.51	144.35	205.72	185.46	-213.27	-324.57	-505.06	-445.47
589	p≤0.05	5.60	100.00	222.64	408.21	72.97	307.45	-122.64	-308.21	27.03	-207.45
598	p≤0.05	6.50	71.00	249.44	460.87	116.28	251.91	-251.33	-549.11	-63.77	-254.80
603	p≤0.05	13.30	9.00	71.47	119.51	48.49	39.67	-694.06	-1227.84	-438.74	-340.78
659	p≤0.05	5.40	17.00	56.35	49.10	83.32	92.23	-231.46	-188.85	-390.14	-442.55
677	p≤0.05	8.90	79.00	338.77	706.39	174.65	542.49	-328.83	-794.16	-121.07	-586.70
716	p≤0.05	7.00	10.00	36.42	21.89	11.30	70.42	-264.20	-118.90	-12.98	-604.15
1	p≤0.05	15.60	31.00	13.74	12.42	4.71	1.98	55.67	59.92	84.81	93.60
14	p≤0.05	9.70	2786.00	1297.37	507.01	287.16	641.67	53.43	81.80	89.69	76.97
24	p≤0.05	6.80	94.00	54.97	78.69	43.31	294.55	41.52	16.29	53.93	-213.35
84	p≤0.05	10.60	65.00	33.67	21.89	6.12	34.71	48.20	66.32	90.58	46.60
181	p≤0.05	7.60	206.00	117.51	27.21	104.04	144.80	42.96	86.79	49.50	29.71
335	p≤0.05	7.20	462.00	308.54	363.25	64.49	292.57	33.22	21.37	86.04	36.67
437	p≤0.05	9.00	314.00	61.85	34.91	118.63	103.14	80.30	88.88	62.22	67.15
525	p≤0.05	5.60	246.00	96.89	195.82	94.15	43.64	60.61	20.40	61.73	82.26
579	p≤0.05	5.50	4093.00	1683.56	808.15	1474.87	750.76	58.87	80.26	63.97	81.66
586	p≤0.05	8.00	111700.00	69980.00	21810.00	14040.00	16340.00	37.35	80.47	87.43	85.37
588	p≤0.05	22.80	890.00	191.72	106.49	39.07	320.34	78.46	88.03	95.61	64.01
622	p≤0.05	6.10	208.00	92.77	187.54	33.89	107.11	1156.50	704.55	935.46	1043.14
640	p≤0.05	6.50	113.00	100.33	93.48	17.42	41.65	101.98	18.10	154.08	89.28
643	p≤0.05	5.90	961.00	410.93	551.38	162.88	608.94	1.32	2.03	9.95	7.42
644	p≤0.05	11.80	552.00	155.99	317.11	46.60	242.98	99.65	74.21	144.59	63.78
645	p≤0.05	5.80	3184.00	910.50	1737.57	552.19	1437.06	12.44	7.38	15.87	9.71
652	p≤0.05	19.50	251.00	206.15	122.46	133.22	12.89	905.78	576.27	1048.53	695.99
682	p≤0.05	6.00	5923.00	1847.11	1560.68	989.52	3007.02	0.76	2.17	1.99	4.02
683	p≤0.05	8.00	1303.00	289.30	289.30	163.35	356.04	312.81	334.79	378.62	223.79
684	p≤0.05	8.70	1241.00	399.93	281.02	143.11	179.51	81.68	81.68	91.83	76.31
686	p≤0.05	6.30	166.00	103.08	73.95	26.36	40.66	506.67	578.30	661.38	639.45
707	p≤0.05	11.10	17940.00	1979.73	2229.20	1615.62	3390.83	0.35	0.51	0.78	0.70
708	p≤0.05	5.10	160.00	41.23	55.61	31.07	62.48	9975.17	9819.25	10202.74	9093.23
712	p≤0.05	5.80	21600.00	6205.13	8774.82	3718.00	7509.62	0.55	0.48	0.60	0.45
610	p≤0.05	5.20	2980.00	574.47	1514.53	1034.24	810.27	516.61	430.38	600.07	472.83
34	p≤0.05	5.30	47900.00	56710.00	20520.00	10620.00	24780.00	-18.39	57.16	77.83	48.27
36	p≤0.05	11.30	21.00	42.60	18.93	3.77	16.86	-102.88	9.85	82.07	19.71
47	p≤0.05	5.10	168.00	205.46	98.80	40.49	152.73	-22.30	41.19	75.90	9.09
58	p≤0.05	8.20	155.00	374.51	78.09	45.66	78.35	-141.62	49.62	70.54	49.45
60	p≤0.05	10.80	293.00	835.60	123.65	77.20	96.20	-185.19	57.80	73.65	67.17
62	p≤0.05	5.90	145.00	172.48	39.05	29.19	66.45	-18.95	73.07	79.87	54.17
73	p≤0.05	6.40	75240.00	211600.00	33460.00	32900.00	58580.00	-181.23	55.53	56.27	22.14
76	p≤0.05	6.30	11540.00	18990.00	4632.34	3032.59	9907.70	-64.56	59.86	73.72	14.14
133	p≤0.05	9.80	91.00	617.08	81.05	63.08	71.41	-578.11	10.93	30.68	21.53
134	p≤0.05	5.60	186.00	614.33	110.04	184.06	179.51	-230.28	40.84	1.04	3.49
136	p≤0.05	22.70	69.00	480.33	42.60	21.18	37.69	-596.13	38.27	69.30	45.38
139	p≤0.05	6.40	147.00	228.14	68.04	35.78	72.40	-55.20	53.72	75.66	50.75
140	p≤0.05	7.50	32130.00	112200.00	14870.00	18770.00	25910.00	-249.21	53.72	41.58	19.36
145	p≤0.05	8.50	188.00	291.36	140.80	34.37	87.28	-54.98	25.10	81.72	53.58
150	p≤0.05	16.30	25010.00	144600.00	11060.00	8849.67	13410.00	-478.17	55.78	64.62	46.38
394	p≤0.05	7.10	268.00	359.39	213.57	50.84	153.72	-34.10	20.31	81.03	42.64

400	p≤0.05	26.90	3239.00	38730.00	1440.58	1492.28	2343.53	-1095.74	55.52	53.93	27.65
404	p≤0.05	17.10	16670.00	175600.00	10290.00	11630.00	12800.00	-953.39	38.27	30.23	23.22
415	p≤0.05	20.80	37380.00	176300.00	9959.23	8471.66	14990.00	-371.64	73.36	77.34	59.90
417	p≤0.05	98.90	268.00	5025.95	62.12	50.84	220.17	-1775.35	76.82	81.03	17.85
423	p≤0.05	7.20	110.00	135.37	53.84	92.74	18.84	-23.07	51.06	15.69	82.87
658	p≤0.05	6.00	1506.00	1673.94	1260.14	839.82	276.70	-11.15	16.33	44.23	81.63
690	p≤0.05	7.30	146900.00	539300.00	117500.00	73910.00	130500.00	-267.12	20.01	49.69	11.16
691	p≤0.05	14.60	175.00	651.44	101.17	48.49	44.63	-272.25	42.19	72.29	74.50
696	p≤0.05	8.80	194.00	585.47	82.83	66.38	161.66	-201.79	57.31	65.79	16.67
186	p≤0.05	9.70	63.00	106.51	45.55	221.72	22.81	-69.07	27.69	-251.94	63.79
212	p≤0.05	6.20	136.00	193.09	59.75	318.70	51.57	-41.98	56.06	-134.34	62.08
337	p≤0.05	7.60	185000.00	251700.00	151700.00	1158000.00	189200.00	-36.05	18.00	-525.95	-2.27
386	p≤0.05	9.20	391500.00	594000.00	79820.00	733500.00	174900.00	-51.72	79.61	-87.36	55.33
465	p≤0.05	7.10	527.00	813.61	159.14	1137.34	451.25	-54.38	69.80	-115.81	14.37
468	p≤0.05	7.70	378.00	571.72	116.55	900.55	354.06	-51.25	69.17	-138.24	6.33
477	p≤0.05	6.60	57.00	65.28	49.70	249.03	37.69	-14.53	12.81	-336.89	33.88
488	p≤0.05	9.20	7985.00	9863.61	3726.58	34170.00	6949.28	-23.53	53.33	-327.93	12.97
636	p≤0.05	5.70	168.00	289.30	110.63	432.62	75.37	-72.20	34.15	-157.51	55.13
663	p≤0.05	6.40	84.00	139.50	21.89	100.74	56.53	-66.07	73.94	-19.93	32.70
69	p≤0.05	8.20	5873.00	40980.00	5024.58	6848.50	13490.00	-597.77	14.45	-16.61	-129.70
193	p≤0.05	6.70	451.00	742.83	207.07	1386.37	975.89	-64.71	54.09	-207.40	-116.38
200	p≤0.05	7.30	117.00	121.63	82.23	596.91	170.58	-3.96	29.71	-410.18	-45.80
225	p≤0.05	9.50	66.00	76.28	18.34	173.71	94.22	-15.57	72.21	-163.19	-42.75
346	p≤0.05	8.20	119.00	155.30	53.84	441.57	132.90	-30.50	54.76	-271.06	-11.68
393	p≤0.05	7.50	50.00	50.85	15.97	81.91	120.00	-1.70	68.05	-63.82	-140.01
541	p≤0.05	5.60	246.00	329.84	71.59	275.86	403.65	-34.08	70.90	-12.14	-64.08
544	p≤0.05	8.50	82.00	205.46	34.91	295.16	129.92	-150.56	57.43	-259.95	-58.44
3	p≤0.05	6.90	1893.00	3560.91	7261.47	1054.01	1853.60	-88.11	-283.60	44.32	2.08
13	p≤0.05	11.00	58.00	62.53	73.95	12.24	134.88	-7.81	-27.50	78.90	-132.55
25	p≤0.05	13.80	10.00	23.36	34.31	9.42	129.92	-133.64	-243.14	5.85	-1199.21
41	p≤0.05	6.20	3042.00	7665.36	4252.52	1229.61	2736.27	-151.98	-39.79	59.58	10.05
67	p≤0.05	12.70	32.00	265.25	125.42	71.55	20.83	-728.90	-291.94	-123.61	34.92
310	p≤0.05	6.00	578.00	1236.90	2458.16	410.97	541.50	-114.00	-325.29	28.90	6.31
316	p≤0.05	8.10	27380.00	32720.00	82190.00	10100.00	20000.00	-19.50	-200.18	63.11	26.95
412	p≤0.05	10.40	286.00	324.34	340.77	80.03	32.73	-13.41	-19.15	72.02	88.56
426	p≤0.05	12.90	1040.00	1100.84	5035.23	389.31	798.37	-5.85	-384.16	62.57	23.23
449	p≤0.05	7.30	304.00	338.09	592.21	81.44	238.02	-11.21	-94.80	73.21	21.70
538	p≤0.05	6.50	220700.00	270200.00	343000.00	855800.00	131100.00	-22.43	-55.41	-287.77	40.60
593	p≤0.05	5.70	13.00	24.74	39.64	7.06	6.94	-90.29	-204.91	45.68	46.60
612	p≤0.05	5.20	14.00	17.18	34.31	6.59	10.91	-22.71	-145.10	52.92	22.08
651	p≤0.05	5.10	161.00	241.20	323.61	64.02	99.18	-49.81	-101.00	60.23	38.40
695	p≤0.05	8.50	123.00	637.69	128.97	89.91	75.37	-418.45	-4.86	26.90	38.72
201	p≤0.05	8.00	79.00	227.45	146.72	494.76	61.49	-187.92	-85.72	-526.28	22.17
220	p≤0.05	5.60	289.00	428.11	298.77	799.34	141.82	-48.13	-3.38	-176.59	50.93
262	p≤0.05	9.90	81.00	123.00	224.81	224.55	22.81	-51.86	-177.55	-177.22	71.84

278	p≤0.05	9.10	37.00	116.13	90.52	188.77	20.83	-213.87	-144.64	-410.19	43.71
360	p≤0.05	7.20	211.00	458.34	1486.14	339.41	206.29	-117.22	-604.33	-60.86	2.23
367	p≤0.05	65.00	64.00	206.84	91.11	257.97	3.97	-223.18	-42.36	-303.08	93.80
413	p≤0.05	16.10	52.00	54.97	175.71	80.50	10.91	-5.72	-237.90	-54.81	79.02
495	p≤0.05	8.10	265500.00	340900.00	801200.00	1315000.00	161600.00	-28.40	-201.77	-395.29	39.13
507	p≤0.05	10.00	271.00	364.20	960.19	95.56	370.92	-34.39	-254.31	64.74	-36.87
556	p≤0.05	6.40	29.00	87.27	120.10	120.98	18.84	-200.93	-314.13	-317.18	35.02
581	p≤0.05	7.90	1089.00	1737.85	1294.45	7870.51	998.70	-59.58	-18.87	-622.73	8.29
16	p≤0.05	16.60	169.00	224.02	236.65	72.97	1208.96	-32.55	-40.03	56.82	-615.36
37	p≤0.05	9.20	91.00	147.74	185.77	48.49	446.29	-62.35	-104.14	46.72	-390.43
40	p≤0.05	15.40	1299.00	2644.91	1325.22	538.54	8307.99	-103.61	-2.02	58.54	-539.57
44	p≤0.05	8.60	235.00	676.17	310.01	209.96	1815.92	-187.73	-31.92	10.66	-672.73
45	p≤0.05	6.90	134.00	188.28	152.64	86.15	592.08	-40.51	-13.91	35.71	-341.85
55	p≤0.05	9.30	34.00	210.96	81.64	22.60	43.64	-520.47	-140.13	33.54	-28.35
65	p≤0.05	5.50	38.00	144.99	48.51	26.36	69.42	-281.56	-27.66	30.63	-82.69
81	p≤0.05	10.80	138.00	1071.30	140.80	99.33	244.97	-676.30	-2.03	28.02	-77.51
135	p≤0.05	8.40	83.00	544.92	85.19	64.96	133.89	-556.53	-2.64	21.73	-61.31
143	p≤0.05	11.30	181.00	604.02	202.92	53.67	231.08	-233.71	-12.11	70.35	-27.67
168	p≤0.05	7.20	6949.00	16840.00	7717.02	2349.05	8683.87	-142.34	-11.05	66.20	-24.97
428	p≤0.05	5.30	60.00	109.95	217.71	41.43	60.50	-83.25	-262.86	30.96	-0.83
573	p≤0.05	9.60	124000.00	142600.00	575400.00	60120.00	148500.00	-15.00	-364.03	51.52	-19.76
595	p≤0.05	5.70	1779.00	2665.53	4798.58	840.29	2205.68	-49.83	-169.73	52.77	-23.98
597	p≤0.05	8.60	43280.00	96800.00	154000.00	17970.00	105400.00	-123.66	-255.82	58.48	-143.53
600	p≤0.05	5.40	95.00	102.39	150.27	27.77	118.02	-7.78	-58.18	70.76	-24.23
616	p≤0.05	7.60	99.00	226.77	523.58	70.61	536.54	-129.06	-428.87	28.67	-441.96
673	p≤0.05	15.80	111.00	190.35	152.64	35.78	564.31	-71.48	-37.51	67.77	-408.39
2	p≤0.05	15.50	28810.00	30290.00	24400.00	18230.00	282400.00	-5.14	15.31	36.72	-880.22
4	p≤0.05	6.00	56.00	75.59	41.41	26.83	161.66	-34.98	26.05	52.08	-188.67
6	p≤0.05	6.30	46.00	62.53	34.91	21.18	132.90	-35.94	24.12	53.95	-188.90
18	p≤0.05	5.30	30.00	32.98	24.26	12.71	67.44	-9.95	19.15	57.63	-124.80
19	p≤0.05	16.70	30290.00	33340.00	21380.00	11590.00	193200.00	-10.07	29.42	61.74	-537.83
30	p≤0.05	7.30	1871.00	2359.74	942.44	889.72	6461.33	-26.12	49.63	52.45	-245.34
32	p≤0.05	6.10	75.00	101.70	35.50	42.84	217.20	-35.60	52.67	42.88	-189.59
33	p≤0.05	8.30	142.00	153.93	70.40	44.25	365.96	-8.40	50.42	68.84	-157.72
38	p≤0.05	17.80	49060.00	166500.00	34820.00	36760.00	619100.00	-239.38	29.03	25.07	-1161.92
39	p≤0.05	10.90	51.00	90.71	42.01	18.83	205.30	-77.85	17.64	63.08	-302.54
43	p≤0.05	10.40	52160.00	56110.00	28370.00	18770.00	194800.00	-7.57	45.61	64.01	-273.47
48	p≤0.05	5.90	1194.00	1394.26	412.95	490.52	2424.86	-16.77	65.41	58.92	-103.09
49	p≤0.05	7.40	1652000.00	3175000.00	695000.00	428000.00	1790000.00	-92.19	57.93	74.09	-8.35
50	p≤0.05	5.30	37.00	69.40	30.76	25.42	133.89	-87.58	16.85	31.29	-261.86
53	p≤0.05	10.60	450.00	2185.88	442.53	207.13	479.02	-385.75	1.66	53.97	-6.45
54	p≤0.05	16.10	18210.00	48340.00	9360.52	6006.80	96830.00	-165.46	48.60	67.01	-431.74
56	p≤0.05	20.50	845.00	953.10	147.31	537.60	3017.93	-12.79	82.57	36.38	-257.15
59	p≤0.05	7.30	179.00	255.63	97.62	53.67	393.73	-42.81	45.47	70.02	-119.96
64	p≤0.05	12.60	214.00	1170.94	128.38	92.74	292.57	-447.17	40.01	56.66	-36.71

70	p≤0.05	10.20	2827.00	12300.00	2044.03	1207.48	5316.84	-335.09	27.70	57.29	-88.07
71	p≤0.05	26.20	2299.00	3687.35	779.16	1402.37	20430.00	-60.39	66.11	39.00	-788.65
74	p≤0.05	6.20	52350.00	146300.00	23480.00	25370.00	84230.00	-179.47	55.15	51.54	-60.90
75	p≤0.05	5.50	6333.00	13500.00	2719.06	2438.03	9314.63	-113.17	57.07	61.50	-47.08
82	p≤0.05	8.20	85.00	311.97	71.59	38.13	104.14	-267.03	15.78	55.14	-22.51
96	p≤0.05	6.50	50.00	112.01	32.54	22.60	145.79	-124.02	34.92	54.81	-191.58
147	p≤0.05	5.40	82960.00	163600.00	42100.00	30320.00	86730.00	-97.20	49.25	63.45	-4.54
662	p≤0.05	12.90	59540.00	64870.00	35440.00	21640.00	279700.00	-8.95	40.48	63.65	-369.77
666	p≤0.05	9.90	20.00	39.17	13.61	8.94	88.27	-95.85	31.97	55.28	-341.34
667	p≤0.05	6.50	652.00	755.89	233.10	272.10	1511.45	-15.93	64.25	58.27	-131.82
676	p≤0.05	9.70	130.00	168.36	74.54	62.14	603.98	-29.50	42.66	52.20	-364.60
678	p≤0.05	6.60	581.00	641.13	215.35	371.89	1411.28	-10.35	62.93	35.99	-142.90
697	p≤0.05	5.70	360.00	391.00	212.98	187.83	1079.04	-8.61	40.84	47.83	-199.73
699	p≤0.05	7.10	137.00	503.69	91.11	72.97	519.68	-267.66	33.50	46.74	-279.33
701	p≤0.05	11.00	5623.00	8591.67	5346.42	2797.21	30810.00	-52.80	4.92	50.25	-447.93
188	p≤0.05	11.10	4089.00	2357.67	3932.46	26240.00	3790.51	42.34	3.83	-541.72	7.30
189	p≤0.05	7.60	463.00	323.66	185.77	742.38	97.19	30.10	59.88	-60.34	79.01
194	p≤0.05	9.30	1130.00	915.31	669.12	6241.70	775.56	19.00	40.79	-452.36	31.37
196	p≤0.05	5.90	766.00	441.85	301.13	1773.32	470.10	42.32	60.69	-131.50	38.63
208	p≤0.05	15.80	1252.00	1032.13	486.31	7686.44	913.41	17.56	61.16	-513.93	27.04
228	p≤0.05	8.10	191.00	93.46	138.44	416.15	51.57	51.07	27.52	-117.88	73.00
231	p≤0.05	9.90	857.00	407.49	697.51	4031.99	736.88	52.45	18.61	-370.48	14.02
232	p≤0.05	5.50	596.00	141.56	483.35	778.62	473.07	76.25	18.90	-30.64	20.63
323	p≤0.05	5.50	37.00	24.74	7.69	42.37	34.71	33.14	79.21	-14.51	6.18
327	p≤0.05	5.60	250.00	214.40	78.09	436.39	160.67	14.24	68.76	-74.55	35.73
328	p≤0.05	11.70	38.00	30.24	16.57	69.67	5.95	20.43	56.41	-83.34	84.34
342	p≤0.05	5.40	290.00	160.11	147.90	660.47	122.98	44.79	49.00	-127.75	57.59
345	p≤0.05	7.00	282.00	105.82	134.30	318.23	45.62	62.47	52.38	-12.85	83.82
349	p≤0.05	6.90	673.00	600.59	201.15	1382.13	463.15	10.76	70.11	-105.37	31.18
369	p≤0.05	11.50	507.00	377.94	410.58	1295.98	113.06	25.46	19.02	-155.62	77.70
370	p≤0.05	5.60	244.00	160.11	157.37	641.16	115.04	34.38	35.50	-162.77	52.85
445	p≤0.05	12.50	3540.00	1711.74	912.86	11400.00	2491.31	51.65	74.21	-222.03	29.62
446	p≤0.05	10.50	5907.00	2978.87	1484.36	15600.00	2779.91	49.57	74.87	-164.09	52.94
460	p≤0.05	12.20	109.00	27.49	15.38	188.30	65.46	74.78	85.89	-72.75	39.95
469	p≤0.05	12.30	1729.00	1552.31	720.59	8880.27	894.57	10.22	58.32	-413.61	48.26
472	p≤0.05	13.30	253.00	251.50	60.94	289.51	21.82	0.59	75.91	-14.43	91.38
473	p≤0.05	10.00	1001.00	658.99	762.59	6508.62	653.57	34.17	23.82	-550.21	34.71
490	p≤0.05	7.80	64.00	49.48	28.99	226.43	32.73	22.69	54.70	-253.80	48.86
499	p≤0.05	8.60	27200.00	13590.00	9459.32	81550.00	13080.00	50.04	65.22	-199.82	51.91
537	p≤0.05	8.30	585.00	412.30	502.87	1634.45	196.37	29.52	14.04	-179.39	66.43
547	p≤0.05	8.70	25.00	8.93	14.79	78.15	14.88	64.27	40.84	-212.58	40.50
559	p≤0.05	5.70	485.00	171.79	160.33	906.67	244.97	64.58	66.94	-86.94	49.49
561	p≤0.05	6.30	192.00	149.80	92.88	582.32	144.80	21.98	51.62	-203.29	24.58
674	p≤0.05	5.10	20.00	15.12	4.73	24.01	19.84	24.41	76.34	-20.04	0.82
187	p≤0.05	5.60	397.00	281.74	115.37	644.93	639.69	29.03	70.94	-62.45	-61.13

219	p≤0.05	7.20	597.00	123.69	409.40	811.58	890.60	79.28	31.42	-35.94	-49.18
221	p≤0.05	5.20	85250.00	58120.00	49580.00	258800.00	122600.00	31.82	41.84	-203.58	-43.81
237	p≤0.05	6.90	1887.00	1856.04	1352.43	9286.53	1925.01	1.64	28.33	-392.13	-2.01
280	p≤0.05	6.10	218.00	52.23	70.40	316.82	219.18	76.04	67.71	-45.33	-0.54
452	p≤0.05	8.70	183.00	107.20	47.92	417.09	257.86	41.42	73.81	-127.92	-40.91
482	p≤0.05	5.40	118.00	98.95	57.39	308.81	130.91	16.14	51.37	-161.71	-10.94
715	p≤0.05	7.10	155.00	131.25	134.89	212.78	938.21	15.32	12.98	-37.28	-505.29
87	p≤0.05	16.20	16.00	7.56	122.46	11.30	14.88	52.76	-665.40	29.39	7.03
95	p≤0.05	7.70	44670.00	38020.00	178500.00	23200.00	33940.00	14.89	-299.60	48.06	24.02
98	p≤0.05	7.90	19.00	4.12	32.54	13.65	10.91	78.30	-71.26	28.15	42.58
127	p≤0.05	10.10	247200.00	200800.00	1422000.00	141000.00	187000.00	18.77	-475.24	42.96	24.35
155	p≤0.05	5.00	2958.00	2516.41	7687.43	1533.24	2325.68	14.93	-159.89	48.17	21.38
285	p≤0.05	18.00	64.00	52.23	124.83	61.67	6.94	18.40	-95.05	3.64	89.15
311	p≤0.05	5.40	861.00	755.89	2274.17	417.56	489.93	12.21	-164.13	51.50	43.10
321	p≤0.05	20.00	828.00	666.55	1886.66	458.51	94.22	19.50	-127.86	44.62	88.62
357	p≤0.05	8.10	406.00	153.93	1251.86	394.96	287.61	62.09	-208.34	2.72	29.16
359	p≤0.05	7.20	195.00	51.54	371.53	93.68	120.00	73.57	-90.53	51.96	38.46
361	p≤0.05	7.60	81150.00	38960.00	295200.00	78430.00	73350.00	51.99	-263.77	3.35	9.61
380	p≤0.05	5.90	322.00	114.76	675.62	208.07	253.89	64.36	-109.82	35.38	21.15
385	p≤0.05	5.30	1026.00	450.78	2401.95	503.71	530.59	56.06	-134.11	50.91	48.29
405	p≤0.05	6.30	223.00	44.67	281.61	213.72	170.58	79.97	-26.28	4.16	23.51
406	p≤0.05	9.00	112.00	22.68	204.70	47.08	42.65	79.75	-82.77	57.97	61.92
407	p≤0.05	5.40	1041.00	461.09	2471.17	713.19	533.57	55.71	-137.38	31.49	48.74
409	p≤0.05	6.20	70.00	28.17	116.55	18.83	19.84	59.75	-66.50	73.10	71.66
411	p≤0.05	5.80	24920.00	15050.00	56310.00	9647.12	13770.00	39.61	-125.96	61.29	44.74
421	p≤0.05	11.00	3552.00	3109.44	12810.00	1166.99	1999.39	12.46	-260.64	67.15	43.71
441	p≤0.05	12.30	40.00	27.49	150.86	12.24	34.71	31.28	-277.16	69.40	13.22
478	p≤0.05	5.40	3509.00	1584.61	8537.58	1853.82	1767.32	54.84	-143.31	47.17	49.63
549	p≤0.05	9.30	3154.00	2385.85	10640.00	1148.16	2644.04	24.35	-237.35	63.60	16.17
570	p≤0.05	9.70	5807.00	2385.85	9679.99	1002.70	1985.51	58.91	-66.70	82.73	65.81
585	p≤0.05	6.10	729.00	643.88	781.52	420.38	127.94	11.68	-7.20	42.33	82.45
590	p≤0.05	6.10	2552.00	1384.64	5687.19	939.15	1609.63	45.74	-122.85	63.20	36.93
594	p≤0.05	5.60	6479.00	2165.95	9814.29	1759.67	5394.19	66.57	-51.48	72.84	16.74
606	p≤0.05	11.00	68.00	57.04	76.32	48.49	6.94	16.13	-12.23	28.70	89.79
609	p≤0.05	6.70	185500.00	116900.00	316400.00	47050.00	155000.00	36.98	-70.57	74.64	16.44
611	p≤0.05	5.10	72940.00	40230.00	154500.00	30500.00	57560.00	44.85	-111.82	58.18	21.09
620	p≤0.05	5.90	488.00	266.62	500.51	84.74	160.67	45.36	-2.56	82.64	67.08
630	p≤0.05	8.90	158.00	38.48	213.57	24.01	81.32	75.64	-35.17	84.81	48.53
632	p≤0.05	6.60	1565.00	935.24	3159.81	478.76	1108.79	40.24	-101.90	69.41	29.15
637	p≤0.05	6.20	163.00	124.38	195.82	31.54	131.90	23.70	-20.14	80.65	19.08
654	p≤0.05	6.80	1140.00	240.51	1645.87	323.88	555.39	78.90	-44.37	71.59	51.28
680	p≤0.05	5.70	412.00	397.18	573.87	100.74	230.09	3.60	-39.29	75.55	44.15
91	p≤0.05	5.30	45600.00	40580.00	216800.00	59650.00	43140.00	11.01	-375.44	-30.81	5.39
199	p≤0.05	10.50	47.00	39.86	56.20	332.82	31.74	15.20	-19.58	-608.13	32.48
251	p≤0.05	16.10	5.00	4.12	7.69	16.01	0.99	17.54	-53.82	-220.12	80.16

277	p≤0.05	5.50	67.00	51.54	92.88	115.33	20.83	23.08	-38.63	-72.14	68.91
322	p≤0.05	6.00	973.00	611.58	3670.97	1268.68	949.12	37.14	-277.28	-30.39	2.45
378	p≤0.05	5.80	97380.00	42670.00	247700.00	115100.00	93020.00	56.18	-154.36	-18.20	4.48
408	p≤0.05	8.30	79190.00	41450.00	96330.00	343900.00	52760.00	47.66	-21.64	-334.27	33.38
410	p≤0.05	9.30	1236.00	754.51	1552.40	6615.95	711.09	38.96	-25.60	-435.27	42.47
440	p≤0.05	8.30	2521.00	677.55	3528.39	5608.07	2205.68	73.12	-39.96	-122.45	12.51
456	p≤0.05	6.20	571.00	475.52	1594.99	1443.80	256.87	16.72	-179.33	-152.85	55.01
475	p≤0.05	5.30	622.00	583.41	3072.25	1022.47	601.01	6.20	-393.93	-64.38	3.37
484	p≤0.05	21.80	1500.00	950.35	2283.04	2309.04	106.12	36.64	-52.20	-53.94	92.93
486	p≤0.05	5.10	285200.00	199300.00	740500.00	796400.00	156900.00	30.12	-159.64	-179.24	44.99
500	p≤0.05	9.50	5997.00	3652.30	27560.00	34680.00	4537.31	39.10	-359.56	-478.29	24.34
514	p≤0.05	6.00	356.00	318.16	377.45	624.22	104.14	10.63	-6.03	-75.34	70.75
246	p≤0.05	5.80	621.00	483.77	1490.87	2801.92	1323.01	22.10	-140.08	-351.19	-113.05
252	p≤0.05	8.80	15530.00	10890.00	29910.00	96370.00	19460.00	29.88	-92.59	-520.54	-25.31
374	p≤0.05	6.70	186.00	165.61	1108.69	461.81	312.41	10.96	-496.07	-148.28	-67.96
397	p≤0.05	6.60	700.00	568.98	969.06	3763.66	969.94	18.72	-38.44	-437.67	-38.56
427	p≤0.05	6.00	76.00	39.17	234.28	81.91	107.11	48.46	-208.26	-7.78	-40.93
467	p≤0.05	5.60	426.00	303.73	965.51	1709.77	650.60	28.70	-126.65	-301.35	-52.72
481	p≤0.05	5.90	52.00	37.11	105.90	220.31	78.35	28.64	-103.65	-323.68	-50.67
604	p≤0.05	10.60	69.00	68.72	205.29	19.30	121.00	0.41	-197.52	72.03	-75.36
713	p≤0.05	9.40	585.00	344.27	676.22	590.79	3242.07	41.15	-15.59	-0.99	-454.20
15	p≤0.05	5.30	19.00	13.74	32.54	16.01	72.40	27.67	-71.26	15.76	-281.05
77	p≤0.05	14.40	21.00	4.81	31.95	16.95	69.42	77.10	-52.13	19.30	-230.59
138	p≤0.05	5.80	132.00	96.20	310.01	53.67	140.83	27.12	-134.85	59.34	-6.69
144	p≤0.05	6.30	696.00	577.91	1670.72	266.45	701.18	16.97	-140.05	61.72	-0.74
149	p≤0.05	7.50	261.00	149.80	557.30	74.38	265.79	42.60	-113.52	71.50	-1.84
162	p≤0.05	8.50	330.00	155.99	546.06	64.49	434.39	52.73	-65.47	80.46	-31.63
340	p≤0.05	6.20	57.00	39.17	163.29	26.36	86.28	31.28	-186.46	53.75	-51.37
355	p≤0.05	5.20	156.00	136.75	714.67	144.52	189.43	12.34	-358.12	7.36	-21.43
528	p≤0.05	5.40	17.00	15.12	68.04	12.71	21.82	11.07	-300.21	25.24	-28.35
626	p≤0.05	6.90	476.00	174.54	972.02	140.28	654.56	63.33	-104.21	70.53	-37.51
629	p≤0.05	5.00	535.00	303.04	1333.50	264.56	690.27	43.36	-149.25	50.55	-29.02
669	p≤0.05	15.10	36570.00	30440.00	39990.00	9587.81	144800.00	16.76	-9.35	73.78	-295.95
685	p≤0.05	7.30	196300.00	105100.00	372800.00	51000.00	197800.00	46.46	-89.91	74.02	-0.76
703	p≤0.05	6.10	244.00	195.16	633.03	104.51	296.54	20.02	-159.44	57.17	-21.53
704	p≤0.05	7.60	299.00	296.17	948.95	124.28	387.78	0.95	-217.37	58.44	-29.69
705	p≤0.05	5.50	937.00	751.76	1308.06	268.80	1487.64	19.77	-39.60	71.31	-58.77
709	p≤0.05	10.70	40390.00	36780.00	41570.00	20060.00	214000.00	8.94	-2.92	50.33	-429.83
10	p≤0.05	6.50	10130.00	9364.04	8134.69	6941.71	45070.00	7.56	19.70	31.47	-344.92
20	p≤0.05	6.70	189.00	64.59	113.00	55.55	371.91	65.82	40.21	70.61	-96.78
26	p≤0.05	20.20	1220.00	540.80	390.47	330.47	6664.64	55.67	67.99	72.91	-446.28
27	p≤0.05	14.20	305.00	288.61	219.49	203.37	2895.94	5.37	28.04	33.32	-849.49
28	p≤0.05	9.70	1489.00	1430.68	1328.17	914.20	8887.18	3.92	10.80	38.60	-496.86
29	p≤0.05	7.60	145.00	99.64	122.46	136.05	757.71	31.28	15.54	6.17	-422.56
31	p≤0.05	6.00	1059.00	773.75	720.59	789.45	4344.91	26.94	31.96	25.45	-310.28

68	p≤0.05	7.50	354.00	265.93	134.89	62.61	468.11	24.88	61.90	82.31	-32.24
85	p≤0.05	12.80	55880.00	45640.00	31070.00	18960.00	243300.00	18.32	44.40	66.07	-335.40
86	p≤0.05	8.50	27880.00	19650.00	18030.00	15090.00	128900.00	29.52	35.33	45.88	-362.34
172	p≤0.05	5.90	13010.00	7012.55	8221.07	5057.76	29900.00	46.10	36.81	61.12	-129.82
372	p≤0.05	5.60	657.00	292.73	199.37	572.44	1116.72	55.44	69.65	12.87	-69.97
660	p≤0.05	17.60	212.00	124.38	44.37	21.18	373.89	41.33	79.07	90.01	-76.37
661	p≤0.05	7.80	446.00	386.88	131.93	122.40	952.09	13.26	70.42	72.56	-113.47
689	p≤0.05	6.60	492.00	307.16	227.18	131.81	864.82	37.57	53.83	73.21	-75.78
692	p≤0.05	9.00	143.00	503.69	56.20	59.32	65.46	-252.23	60.70	58.52	54.23
693	p≤0.05	5.10	185.00	179.35	152.64	89.91	462.16	3.05	17.49	51.40	-149.82
706	p≤0.05	5.50	2406.00	2023.02	1778.98	1110.98	6055.70	15.92	26.06	53.82	-151.69
710	p≤0.05	11.70	58.00	54.29	28.99	44.25	338.19	6.40	50.02	23.71	-483.09
711	p≤0.05	8.60	9080.00	5536.52	5641.04	3672.34	31430.00	39.03	37.87	59.56	-246.15
714	p≤0.05	6.80	198.00	138.81	100.57	112.98	681.34	29.89	49.21	42.94	-244.11
717	p≤0.05	17.60	874.00	359.39	121.28	252.32	2136.26	58.88	86.12	71.13	-144.42
718	p≤0.05	5.60	13340.00	9964.63	9152.27	9441.40	51340.00	25.30	31.39	29.22	-284.86
719	p≤0.05	14.40	278.00	240.51	232.50	70.14	1008.62	13.49	16.37	74.77	-262.81

Appendix 4. Significantly up-regulated spots under different stress environments compared to the control for cultivar MexicaliC75

Spot number	P-value	Fold	Spot volume				Percentage protein increase				
			Control	Moderate drought	Severe drought	Moderate heat	Severe heat	Moderate drought	Severe drought	Moderate heat	Severe heat
48	p≤0.05	13.8	90.30	299.72	712.24	673.00	1242.53	-231.91	-688.73	-645.28	-1275.97
61	p≤0.05	10.7	43.62	98.47	468.58	94.00	147.55	-125.75	-974.23	-115.50	-238.25
64	p≤0.05	8.2	81.88	241.50	672.97	110.00	155.90	-194.93	-721.85	-34.34	-90.39
103	p≤0.05	13.7	52.04	217.07	185.65	173.00	712.67	-317.13	-256.75	-232.45	-1269.52
107	p≤0.05	7.5	417.84	1172.30	775.61	1191.00	3141.13	-180.56	-85.62	-185.04	-651.76
110	p≤0.05	5.3	261.72	525.41	297.21	1001.00	1375.23	-100.75	-13.56	-282.47	-425.46
113	p≤0.05	5.6	672.67	3751.92	834.51	1282.00	1620.21	-457.77	-24.06	-90.58	-140.86
140	p≤0.05	8.9	111.73	991.17	290.07	364.00	185.59	-787.12	-159.62	-225.79	-66.11
142	p≤0.05	5.4	211.21	530.44	309.71	515.00	1138.60	-151.14	-46.63	-143.83	-439.07
143	p≤0.05	13.2	9.95	28.03	54.44	49.00	130.84	-181.79	-447.29	-392.56	-1215.26
144	p≤0.05	5.4	166.06	697.91	254.37	319.00	901.97	-320.27	-53.18	-92.10	-443.15
146	p≤0.05	19.6	5.36	37.38	41.06	40.00	104.86	-597.69	-666.40	-646.69	-1857.42
150	p≤0.05	12.3	53.57	413.29	263.30	197.00	659.78	-671.50	-391.51	-267.75	-1131.64
151	p≤0.05	12.5	39.03	332.79	325.77	265.00	489.03	-752.66	-734.69	-578.98	-1153.00
153	p≤0.05	8.5	2.30	10.06	9.82	8.00	19.49	-338.28	-327.61	-248.43	-748.74
155	p≤0.05	7.9	700.22	5536.60	1416.44	1425.00	1391.93	-690.70	-102.29	-103.51	-98.79
159	p≤0.05	9.1	189.79	1732.21	352.55	220.00	378.61	-812.72	-85.76	-15.92	-99.49
162	p≤0.05	17.7	22.19	392.44	132.09	88.00	148.47	-1668.31	-495.21	-296.52	-569.01
163	p≤0.05	5.4	216.57	1161.51	387.36	404.00	484.39	-436.32	-78.86	-86.54	-123.66
164	p≤0.05	5.5	9.18	50.31	13.39	24.00	10.21	-447.89	-45.79	-161.35	-11.16
166	p≤0.05	5.7	39.79	227.13	142.80	90.00	53.82	-470.76	-258.86	-126.16	-35.25
169	p≤0.05	11.7	64.28	749.67	108.89	126.00	152.19	-1066.21	-69.39	-96.01	-136.75
173	p≤0.05	6.4	299.22	1786.83	797.03	794.00	1907.88	-497.17	-166.37	-165.36	-537.62
183	p≤0.05	5.6	339.78	561.35	356.12	495.00	1892.10	-65.21	-4.81	-45.68	-456.86
239	p≤0.05	7.5	96.42	721.63	272.22	352.00	271.89	-648.40	-182.32	-265.05	-181.97
246	p≤0.05	11.6	53.57	305.47	443.59	211.00	620.80	-470.24	-728.06	-293.88	-1058.88
330	p≤0.05	6.2	47.45	112.13	98.18	196.00	293.23	-136.32	-106.92	-313.09	-518.02
346	p≤0.05	5.6	70.41	124.35	392.71	162.00	123.42	-76.61	-457.79	-130.10	-75.30
354	p≤0.05	6	163.00	975.36	398.07	388.00	167.96	-498.37	-144.21	-138.03	-3.04
355	p≤0.05	31.6	3.83	120.75	32.13	48.00	57.53	-3056.06	-739.81	-1154.57	-1403.74
358	p≤0.05	126.1	5.36	675.63	126.74	138.00	152.19	-12512.15	-2265.86	-2476.07	-2740.86
370	p≤0.05	6.9	104.84	235.75	249.91	162.00	727.52	-124.87	-138.37	-54.52	-593.92
374	p≤0.05	6.7	94.89	638.98	500.71	452.00	266.32	-573.36	-427.66	-376.33	-180.66
377	p≤0.05	14.1	12.24	172.50	89.25	20.00	33.41	-1308.87	-628.95	-63.35	-172.84
379	p≤0.05	6.8	633.64	4305.36	1254.00	2679.00	1068.08	-579.46	-97.90	-322.79	-68.56
392	p≤0.05	18.1	114.79	234.32	412.35	2072.00	668.13	-104.12	-259.22	-1705.04	-482.04
409	p≤0.05	12.1	1868.02	4866.00	2532.99	22600.00	4350.25	-160.49	-35.60	-1109.84	-132.88
411	p≤0.05	9.4	1221.37	1995.27	2138.49	11430.00	1516.28	-63.36	-75.09	-835.84	-24.15
424	p≤0.05	5.6	400100.00	705700.00	855100.00	645800.00	2244000.00	-76.38	-113.72	-61.41	-460.86

435	p≤0.05	5.2	49.74	258.75	50.87	98.00	127.13	-420.19	-2.28	-97.02	-155.58
440	p≤0.05	12.8	159.94	940.86	628.34	721.00	2048.00	-488.25	-292.86	-350.79	-1180.47
442	p≤0.05	16.8	2565.18	6489.67	8330.85	5226.00	43030.00	-152.99	-224.77	-103.73	-1577.47
452	p≤0.05	8.4	32.14	60.38	83.90	39.00	269.11	-87.85	-161.03	-21.34	-737.27
466	p≤0.05	7.9	1679.76	3727.48	3509.42	4579.00	13270.00	-121.91	-108.92	-172.60	-689.99
471	p≤0.05	6.7	931.33	2954.82	1657.42	1466.00	6195.96	-217.27	-77.96	-57.41	-565.28
478	p≤0.05	6	18.37	46.00	41.95	19.00	109.50	-150.47	-128.41	-3.45	-496.20
497	p≤0.05	6.6	19300.00	23200.00	22340.00	28570.00	126700.00	-20.21	-15.75	-48.03	-556.48
501	p≤0.05	13.9	87.24	1134.20	939.83	1211.00	697.82	-1200.09	-977.29	-1288.12	-699.89
506	p≤0.05	8.6	52.80	456.41	124.95	291.00	247.76	-764.37	-136.64	-451.11	-369.22
515	p≤0.05	6.2	65640.00	150400.00	177400.00	91590.00	409500.00	-129.13	-170.26	-39.53	-523.86
523	p≤0.05	8.8	27640.00	42620.00	38350.00	38400.00	244500.00	-54.20	-38.75	-38.93	-784.59
536	p≤0.05	6.6	564.00	631.07	3750.40	831.00	573.48	-11.89	-564.96	-47.34	-1.68
542	p≤0.05	6	1549.67	9222.39	3303.24	1929.00	5152.94	-495.12	-113.16	-24.48	-232.52
549	p≤0.05	14.5	29.85	127.22	276.68	432.00	134.55	-326.27	-827.07	-1347.48	-350.84
570	p≤0.05	5.1	24.49	124.35	49.09	53.00	56.61	-407.76	-100.45	-116.42	-131.14
617	p≤0.05	5.3	6914.19	24020.00	36690.00	10270.00	12740.00	-247.40	-430.65	-48.54	-84.26
621	p≤0.05	7.7	2343.25	3502.51	4781.27	3816.00	18050.00	-49.47	-104.04	-62.85	-670.30
626	p≤0.05	9.1	17.60	160.28	99.96	63.00	53.82	-810.65	-467.94	-257.93	-205.78
627	p≤0.05	7.9	12.24	97.03	66.05	43.00	16.70	-692.49	-439.42	-251.19	-36.42
633	p≤0.05	5.7	16.07	38.09	21.42	91.00	57.53	-137.04	-33.29	-466.24	-257.99
638	p≤0.05	9.2	136.98	229.28	386.46	412.00	1262.95	-67.38	-182.13	-200.77	-821.97
640	p≤0.05	5.1	152.29	429.82	329.34	493.00	777.63	-182.24	-116.26	-223.73	-410.63
673	p≤0.05	10.1	7.65	77.63	72.30	50.00	39.90	-914.32	-844.66	-553.34	-421.39
678	p≤0.05	9.5	118.62	181.13	232.06	482.00	1125.61	-52.70	-95.64	-306.35	-848.95
692	p≤0.05	6.5	3082.50	8193.13	7793.54	10350.00	20150.00	-165.80	-152.83	-235.77	-553.69
696	p≤0.05	6.3	5144.89	8332.57	9008.27	10180.00	32210.00	-61.96	-75.09	-97.87	-526.06
2	p≤0.05	5.3	685.68	132.97	159.76	129.00	586.47	80.61	76.70	81.19	14.47
4	p≤0.05	6.2	781.34	196.22	125.85	177.00	655.14	74.89	83.89	77.35	16.15
5	p≤0.05	7.8	1173.92	474.38	150.84	315.00	164.25	59.59	87.15	73.17	86.01
7	p≤0.05	9.2	59.69	18.69	16.96	22.00	6.50	68.69	71.59	63.14	89.12
16	p≤0.05	5.1	3533.24	698.63	1652.07	2424.00	2384.85	80.23	53.24	31.39	32.50
21	p≤0.05	6.7	28560.00	4254.33	10450.00	20860.00	5895.30	85.10	63.41	26.96	79.36
22	p≤0.05	5.8	10380.00	1796.90	2982.82	6618.00	2577.86	82.69	71.26	36.24	75.17
23	p≤0.05	8.8	179600.00	20400.00	60430.00	95000.00	52610.00	88.64	66.35	47.10	70.71
26	p≤0.05	12.5	626500.00	50160.00	228300.00	362400.00	60040.00	91.99	63.56	42.15	90.42
27	p≤0.05	5.4	876.23	296.85	447.16	372.00	163.32	66.12	48.97	57.55	81.36
29	p≤0.05	10.1	288700.00	31150.00	122600.00	121400.00	28520.00	89.21	57.53	57.95	90.12
31	p≤0.05	11.6	9379.88	810.04	2727.56	3522.00	1058.80	91.36	70.92	62.45	88.71
34	p≤0.05	8.4	279800.00	33300.00	77950.00	176100.00	56690.00	88.10	72.14	37.06	79.74
42	p≤0.05	5.7	260.96	46.00	110.67	101.00	153.11	82.37	57.59	61.30	41.33
43	p≤0.05	16.8	9109.74	543.38	1830.57	4993.00	1078.28	94.04	79.91	45.19	88.16
46	p≤0.05	13	10880.00	835.92	3762.00	3543.00	921.46	92.32	65.42	67.44	91.53
49	p≤0.05	9.5	449.21	47.44	77.65	48.00	64.96	89.44	82.71	89.31	85.54
53	p≤0.05	8.7	136.98	15.81	20.53	19.00	22.27	88.46	85.01	86.13	83.74

54	p≤0.05	13.1	4030.66	383.10	1395.02	1537.00	307.15	90.50	65.39	61.87	92.38
65	p≤0.05	13	776900.00	59740.00	214500.00	484500.00	132200.00	92.31	72.39	37.64	82.98
79	p≤0.05	7.4	5105.86	690.73	3785.21	2060.00	1944.99	86.47	25.87	59.65	61.91
108	p≤0.05	5.5	293.86	53.19	128.52	227.00	148.47	81.90	56.26	22.75	49.48
174	p≤0.05	6.2	850.98	631.79	351.66	445.00	138.27	25.76	58.68	47.71	83.75
177	p≤0.05	6.4	1029.28	160.28	235.63	616.00	165.18	84.43	77.11	40.15	83.95
182	p≤0.05	5.5	1683.59	513.19	306.14	607.00	694.11	69.52	81.82	63.95	58.77
209	p≤0.05	6.3	208.92	88.41	35.70	88.00	33.41	57.68	82.91	57.88	84.01
249	p≤0.05	5.6	2720.53	1062.33	628.34	487.00	1262.95	60.95	76.90	82.10	53.58
266	p≤0.05	5.2	414.01	79.78	155.30	157.00	184.66	80.73	62.49	62.08	55.40
273	p≤0.05	5.3	947.40	312.66	385.57	471.00	179.10	67.00	59.30	50.29	81.10
304	p≤0.05	8.7	871.64	791.35	99.96	544.00	339.63	9.21	88.53	37.59	61.04
308	p≤0.05	5.1	8337.59	5016.93	1927.86	1627.00	4274.16	39.83	76.88	80.49	48.74
362	p≤0.05	5.9	13390.00	5200.22	11370.00	2267.00	6502.18	61.16	15.09	83.07	51.44
476	p≤0.05	5.2	116300.00	22450.00	78500.00	36600.00	85290.00	80.70	32.50	68.53	26.66
509	p≤0.05	5.3	1630.78	678.51	600.67	1001.00	305.30	58.39	63.17	38.62	81.28
543	p≤0.05	5.2	2956.99	2036.96	1744.89	2425.00	572.55	31.11	40.99	17.99	80.64
577	p≤0.05	5.6	331300.00	201300.00	246700.00	144000.00	59640.00	39.24	25.54	56.53	82.00
590	p≤0.05	5.7	4217.39	1081.01	4069.92	3913.00	739.58	74.37	3.50	7.22	82.46
601	p≤0.05	7.5	305.34	144.47	258.83	82.00	40.83	52.69	15.23	73.14	86.63
655	p≤0.05	5.2	1040.00	436.29	641.73	567.00	199.51	58.05	38.30	45.48	80.82
662	p≤0.05	6.4	1423.40	264.50	638.16	222.00	366.54	81.42	55.17	84.40	74.25
703	p≤0.05	5	29900.00	5948.45	26860.00	25370.00	12530.00	80.11	10.17	15.15	58.09
263	p≤0.05	6.6	243.36	434.13	227.59	163.00	65.89	-78.39	6.48	33.02	72.93
339	p≤0.05	5.7	61.99	168.91	29.45	43.00	51.97	-172.49	52.49	30.63	16.17
437	p≤0.05	5.1	10570.00	11240.00	8683.39	7099.00	2207.61	-6.34	17.85	32.84	79.11
628	p≤0.05	5	24120.00	30970.00	6171.82	20370.00	11990.00	-28.40	74.41	15.55	50.29
160	p≤0.05	7.7	38.26	116.44	15.17	16.00	33.41	-204.31	60.35	58.18	12.69
234	p≤0.05	5.1	54.33	206.28	45.52	104.00	40.83	-279.66	16.22	-91.41	24.85
361	p≤0.05	7.1	115.56	126.50	105.32	572.00	744.22	-9.47	8.86	-395.00	-544.04
541	p≤0.05	11.8	1600.17	2619.88	332.91	3919.00	1914.37	-63.72	79.20	-144.91	-19.64
591	p≤0.05	6.3	78.82	79.06	49.98	316.00	102.08	-0.30	36.59	-300.90	-29.50
630	p≤0.05	6.3	47140.00	67830.00	32170.00	48450.00	201800.00	-43.89	31.76	-2.78	-328.09
6	p≤0.05	8.4	4735.47	10960.00	1717.22	7986.00	14470.00	-131.44	63.74	-68.64	-205.57
112	p≤0.05	7.3	94.13	213.47	72.30	98.00	525.22	-126.79	23.20	-4.11	-457.99
139	p≤0.05	7.1	48.98	97.03	32.13	131.00	227.35	-98.12	34.40	-167.47	-364.20
147	p≤0.05	9	10180.00	43070.00	4782.16	12890.00	10610.00	-323.08	53.02	-26.62	-4.22
178	p≤0.05	7.2	159.18	253.72	112.46	349.00	810.11	-59.40	29.35	-119.25	-408.94
196	p≤0.05	7.6	268.61	362.25	157.09	332.00	1196.13	-34.86	41.52	-23.60	-345.31
236	p≤0.05	6.9	261.72	1111.20	161.55	474.00	519.66	-324.57	38.28	-81.11	-98.55
499	p≤0.05	7.3	8711.80	13850.00	7665.02	12630.00	56020.00	-58.98	12.02	-44.98	-543.04
400	p≤0.05	9.2	89.54	132.25	85.68	785.00	170.74	-47.71	4.30	-776.74	-90.70
214	p≤0.05	14.8	29.85	108.53	25.88	383.00	90.94	-263.66	13.28	-1183.30	-204.71
383	p≤0.05	5.6	4451.56	14580.00	2601.72	8665.00	8057.44	-227.53	41.55	-94.65	-81.00
505	p≤0.05	8.5	55.10	339.97	40.16	144.00	242.20	-517.02	27.11	-161.35	-339.57

192	p≤0.05	5.3	780.57	936.54	644.40	837.00	3405.60	-19.98	17.44	-7.23	-336.29
257	p≤0.05	5.1	335.95	843.10	481.96	164.00	209.72	-150.96	-43.46	51.18	37.58
360	p≤0.05	5.1	77.29	189.75	106.21	37.00	54.75	-145.50	-37.42	52.13	29.17
369	p≤0.05	6	115.56	408.97	194.57	68.00	76.09	-253.92	-68.38	41.15	34.15
381	p≤0.05	5.1	77.29	261.63	258.83	51.00	71.45	-238.49	-234.88	34.02	7.55
465	p≤0.05	8.2	206.62	266.66	689.92	84.00	132.70	-29.06	-233.91	59.35	35.78
468	p≤0.05	5.3	64.28	85.53	197.25	37.00	44.54	-33.06	-206.85	42.44	30.71
531	p≤0.05	5.1	2005.77	4263.68	4705.40	1544.00	920.53	-112.57	-134.59	23.02	54.11
580	p≤0.05	7.4	9444.93	10720.00	11450.00	6756.00	1547.83	-13.50	-21.23	28.47	83.61
602	p≤0.05	5.3	153.82	415.44	165.12	79.00	139.19	-170.08	-7.34	48.64	9.51
405	p≤0.05	5.5	4092.65	21890.00	6694.84	12430.00	3952.16	-434.86	-63.58	-203.72	3.43
550	p≤0.05	7.6	175.25	646.16	507.85	209.00	85.37	-268.72	-189.79	-19.26	51.28
522	p≤0.05	7.3	450.74	1333.30	970.18	513.00	183.74	-195.80	-115.24	-13.81	59.24
564	p≤0.05	5.2	81.88	150.22	111.57	268.00	51.97	-83.46	-36.25	-227.29	36.54
642	p≤0.05		123890.00	417500.00	677500.00	138900.00	78060.00	-236.99	-446.86	-12.12	36.99
679	p≤0.05		119000.00	1021100.00	142600.00	105300.00	47450.00	-758.07	-19.83	-19.83	60.13
544	p≤0.05	6	3175.09	12370.00	4078.85	2047.00	5668.88	-289.59	-28.46	35.53	-78.54
328	p≤0.05	10.3	77.29	171.78	605.13	59.00	342.42	-122.25	-682.92	23.67	-343.01
469	p≤0.05	6.9	90.30	120.75	400.75	58.00	95.58	-33.72	-343.78	35.77	-5.84
516	p≤0.05	8.4	71030.00	94810.00	86750.00	53930.00	453300.00	-33.48	-22.13	24.07	-538.18
579	p≤0.05	5.4	497.42	1366.36	991.60	254.00	512.23	-174.69	-99.35	48.94	-2.98
643	p≤0.05	6.8	35.20	39.53	75.87	33.00	223.64	-12.30	-115.51	6.26	-535.30
387	p≤0.05	6.2	5089.79	9589.68	1541.39	2170.00	5215.11	-88.41	69.72	57.37	-2.46
138	p≤0.05	5.5	85920.00	311100.00	56230.00	58100.00	205400.00	-262.08	34.56	32.38	-139.06
154	p≤0.05	5.9	29.85	58.22	16.96	13.00	77.02	-95.07	43.18	56.44	-158.07
446	p≤0.05	7.4	92.60	96.31	42.84	87.00	315.51	-4.01	53.73	6.04	-240.73
195	p≤0.05	6.5	9702.82	4441.93	9516.12	28680.00	7936.80	54.22	1.92	-195.58	18.20
221	p≤0.05	11.2	3089.38	2104.53	1269.17	5827.00	521.51	31.88	58.92	-88.61	83.12
225	p≤0.05	7.7	56.63	43.84	8.03	62.00	32.48	22.58	85.81	-9.48	42.65
268	p≤0.05	5.5	531.10	186.88	224.02	981.00	179.10	64.81	57.82	-84.71	66.28
366	p≤0.05		115900.00	13220.00	20300.00	411220.00	29020.00	88.59	82.48	-254.81	74.96
488	p≤0.05	6	323.71	193.35	274.01	476.00	79.80	40.27	15.35	-47.05	75.35
491	p≤0.05	5.4	235.70	96.31	141.91	271.00	50.11	59.14	39.79	-14.98	78.74
595	p≤0.05	9.4	1140.25	518.94	664.04	4892.00	725.66	54.49	41.76	-329.03	36.36
190	p≤0.05	5.6	332.13	123.63	151.73	352.00	687.62	62.78	54.32	-5.98	-107.03
184	p≤0.05	9.9	640.53	610.23	244.55	647.00	2416.40	4.73	61.82	-1.01	-277.25
189	p≤0.05	5.6	1610000.00	1049000.00	694900.00	1685000.00	3883000.00	34.84	56.84	-4.66	-141.18
210	p≤0.05	7.6	277.79	127.22	251.69	967.00	280.24	54.20	9.40	-248.10	-0.88
391	p≤0.05	6.7	208.15	140.16	83.01	240.00	553.06	32.67	60.12	-15.30	-165.70
402	p≤0.05	13.4	1203.77	945.17	849.69	11380.00	1341.82	21.48	29.41	-845.37	-11.47
532	p≤0.05		108800.00	13610.00	249300.00	21610.00	50070.00	87.49	-129.14	80.14	53.98
35	p≤0.05	6.2	356.61	205.57	1279.88	321.00	310.87	42.36	-258.90	9.99	12.83
62	p≤0.05	7.2	230.35	158.85	788.10	109.00	173.53	31.04	-242.14	52.68	24.67
63	p≤0.05	5.7	200.50	151.66	587.28	151.00	103.93	24.36	-192.91	24.69	48.16
68	p≤0.05	10	11410.00	1677.58	16850.00	4472.00	5803.43	85.30	-47.68	60.81	49.14

199	p≤0.05	6.1	102.55	92.00	267.76	99.00	43.61	10.28	-161.11	3.46	57.47
278	p≤0.05	5.5	807.36	250.85	991.60	741.00	179.10	68.93	-22.82	8.22	77.82
450	p≤0.05	6.3	35930.00	33320.00	75460.00	11910.00	16710.00	7.26	-110.02	66.85	53.49
475	p≤0.05	10.6	15.31	10.78	68.73	10.00	6.50	29.56	-349.04	34.66	57.56
479	p≤0.05	7.7	294.63	71.16	550.69	179.00	115.07	75.85	-86.91	39.25	60.95
584	p≤0.05	9.2	342.07	66.85	399.85	246.00	43.61	80.46	-16.89	28.09	87.25
623	p≤0.05	7.1	6640.99	1266.45	9045.76	2046.00	3722.03	80.93	-36.21	69.19	43.95
560	p≤0.05	5.2	306000.00	299700.00	330700.00	313700.00	63640.00	2.06	-8.07	-2.52	79.20
481	p≤0.05	8.6	265.55	207.72	744.37	388.00	86.30	21.78	-180.31	-46.11	67.50
483	p≤0.05	9.1	18.37	11.50	50.87	20.00	5.57	37.38	-177.00	-8.90	69.68
71	p≤0.05	6.8	133.16	86.25	431.98	223.00	63.10	35.23	-224.42	-67.47	52.61
38	p≤0.05	5.4	59.69	38.09	189.22	66.00	35.26	36.18	-216.99	-10.57	40.93
500	p≤0.05	6.3	44.39	31.63	199.93	132.00	128.06	28.75	-350.44	-197.40	-188.52
518	p≤0.05	5.3	25970.00	25590.00	26960.00	109500.00	135300.00	1.46	-3.81	-321.64	-420.99
528	p≤0.05	5.3	73.47	71.16	159.76	242.00	375.82	3.14	-117.46	-229.40	-411.56
530	p≤0.05	8.7	78.82	72.60	130.31	271.00	628.23	7.90	-65.32	-243.81	-697.01
619	p≤0.05	5.6	5180.09	3088.51	7762.31	5311.00	1389.15	40.38	-49.85	-2.53	73.18
32	p≤0.05	9.7	38600.00	30840.00	300000.00	53960.00	95650.00	20.10	-677.20	-39.79	-147.80
652	p≤0.05	7.6	45.92	33.06	55.34	132.00	250.55	27.99	-20.52	-187.48	-445.67
77	p≤0.05	5	524.97	166.75	838.98	542.00	678.34	68.24	-59.81	-3.24	-29.21
24	p≤0.05	9.6	149.99	110.69	1060.32	223.00	171.67	26.20	-606.92	-48.67	-14.45
47	p≤0.05	6.1	97.19	90.56	297.21	49.00	116.92	6.82	-205.81	49.58	-20.30
80	p≤0.05	6.4	912.20	219.94	1409.30	602.00	1054.16	75.89	-54.49	34.01	-15.56
338	p≤0.05	6.2	8.42	7.91	37.49	6.00	10.21	6.08	-345.31	28.72	-21.26
226	p≤0.05	6.6	239.53	135.85	109.78	199.00	720.09	43.29	54.17	16.92	-200.63
52	p≤0.05	5.5	40.56	9.34	21.42	22.00	51.04	76.96	47.19	45.76	-25.84
83	p≤0.05		188000.00	30680.00	35370.00	206100.00	27910.00	83.68	81.19	-9.63	85.15
216	p≤0.05	6.2	237.23	86.97	215.99	145.00	538.21	63.34	8.95	38.88	-126.87
394	p≤0.05	5.2	296.16	181.13	245.45	296.00	942.80	38.84	17.12	0.05	-218.34
399	p≤0.05	7.6	252.54	82.66	63.37	148.00	482.54	67.27	74.91	41.39	-91.08