"EFFECT OF LIMITED IRRIGATION ON QUALITY OF DURUM WHEAT (*Triticum durum* Desf.)"

A

THESIS

SUBMITTED TO

ANAND AGRICULTURAL UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF DEGREE

OF

Master of Science

IN

PLANT MOLECULAR BIOLOGY & BIOTECHNOLOGY

BY

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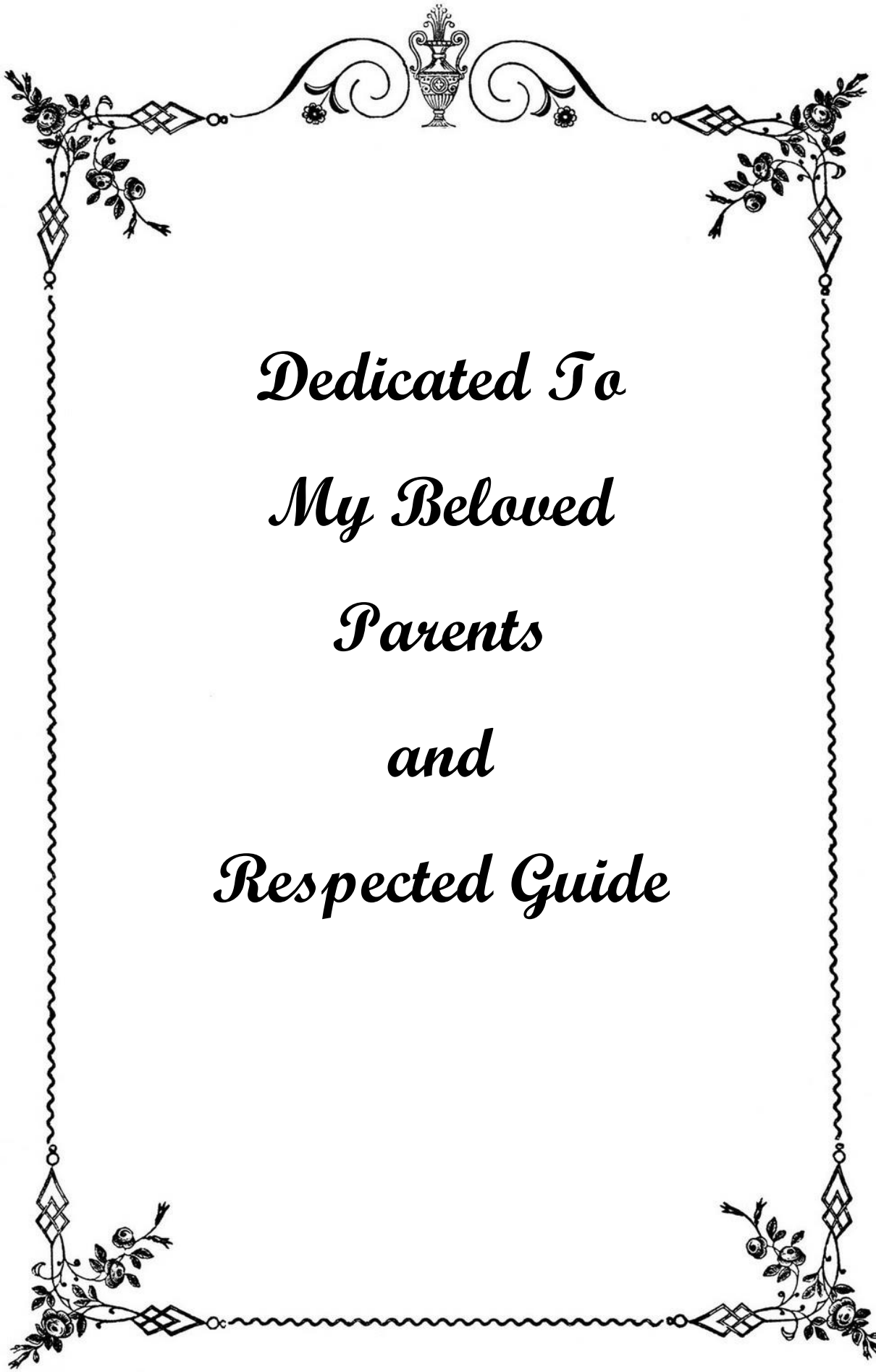
2016

Registration No. 04-2094-2013



Dedicated To

My Beloved

Parents

and

Respected Guide

Abstract

"EFFECT OF LIMITED IRRIGATION ON QUALITY OF DURUM WHEAT (*Triticum durum* Desf.)"

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ABSTRACT

Durum wheat (*Triticum durum* Desf.) is the only tetraploid species of wheat which is cultivated worldwide. Durum wheat contains high protein, as well as its strength and structures and interactions of the grain storage proteins gliadin and glutenin make durum good for special uses, the most well-known being pasta, spaghetti and macaroni, extensively in bread making.

Environmental factors, such as temperature, water, and nitrogen nutrition also influence the wheat quality characteristics, ratio between soluble and insoluble proteins, affects bread-making quality, which is a function of protein composition that is genetically controlled. Irrigation and varietal improvement are two major ways of increasing and stabilizing durum wheat (*Triticum durum* Desf.).

Quality of three durum wheat varieties (GW1, A-206, A-9-30-1) with respect to irrigation treatments were tested using qualitative tests like, moisture, ash, total soluble sugar, starch, total protein, lysine, tryptophan, oil, mineral content.

Irrigation treatments showed significant differences in proximate composition of durum varieties. Protein, lysine and tryptophan content recorded significantly higher in varieties with one irrigation treatment. Two

irrigation (T_2) treatment showed significantly higher oil content, total soluble sugar and ash content among all the varieties. GW 1 variety showed significantly increased protein, potassium, calcium and sodium content in irrigation treatments compare to A-206 and A-9-30-1 varieties.

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) of seed proteins showed variability and could be effectively used for the effect of irrigation on different varieties on the basis of variations in banding pattern and intensity of bands. Profile of protein fractions albumin, globulin, glutenin and gliadin has been analyzed through SDS-PAGE. Albumin and glutenin showed difference in banding pattern in terms of absence/presence of bands. However, both fractions gave some idea about the identification of the effect of limited irrigation.

Relative expression profiling of the glutenin gene subunit (*LMW* and *HMW*) was done by the cDNA, synthesized from the total RNA extracted from the caryopsis of all the three varieties in different treatments along with the control sample.

Among the endogenous genes 26S rRNA was observed to have highest stability with least deviation in C_T values and specific product amplification with a single peak in the melt curve analysis among all the tested genes (*RLI*, *18sRNA, TA50503, ACTIN*). The expression pattern of *LMW 6* and *HMW 10*, two subunits of glutenin gene were analyzed in caryopsis from different durum wheat varieties under both the treatments and control against 26S rRNA expressions.

A-206 and GW1 showed significantly higher protein content in one irrigation (12.77 % and 13.77 % respectively) as compared to the control. These results were confirmed by the expression profiling of glutenin gene was up regulated for one irrigation in both the varieties as compared to the control.

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CERTIFICATE

This is to certify that the thesis entitled **"Effect of Limited Irrigation on Quality of Durum Wheat (*Triticum durum* Desf.)"** submitted by **Arpita Jayeshbhai Dalwadi, Reg. No. 04-2094-2013** in partial fulfillment of the requirements for the degree of **Masters Of Science** in **Plant Molecular Biology and Biotechnology** of the Anand Agricultural University is a record of bonafide research work carried out by her under my guidance and supervision. The thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

Place: Anand **(J. G. Talati)**

Date: /07/2016 **Major Advisor**

DECLARATION

This is to declare that the whole of research work reported here in the thesis for partial fulfillment of the requirements for the degree of **Master Of Science** in the subject of **Plant Molecular Biology And Biotechnology** by the undersigned is a result of investigation done by me under direct guidance and supervision of **Dr. J. G. Talati** , Research Scientist and Head, Department of Biochemistry, Anand Agricultural University, Anand– 388 110 and no part of work had been submitted for any other degree so far.

Place: Anand **(Arpita J. Dalwadi)**

Date: /07/2016

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ACKNOWLEDGEMENT

Towards the end of my endeavour, it's the right moment to extend my profound gratitude to all those who have directly or indirectly helped me in obtaining my M. Sc programme. This thesis has been kept on track and been seen through to completion with the support and encouragement of numerous people. At the end of my thesis, it is a pleasant opportunity to express my thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for me.

I owe my deepest gratitude and admiration to my Major Advisor, ***Dr. J. G. Talti****, Research Scientist and Head, Department of Biochemistry, B. A. College of Agriculture, AAU, Anand, for his guidance. He has made available his support in a number of ways during my Masters.*

I am highly thankful to Dr. N. C. Patel, Vice-Chancellor of AAU, Dr. K.P. Patel, Principal and Dean, B. A. College of Agriculture and Dr. K. B. Kathiria, director of research, B. A. College of Agriculture, AAU, Anand for providing me with all the required facilities to complete my research.

I express my hearty sense of regards towards members of my committee, Dr. N. J. Patel, Associate Professor, Department of Biochemistry, Mr. Amar Sakure Assistant Professor, Department of Agricultural Biotechnology, AAU, Anand, and Dr. V. B. Darji , Associate Prof. of Statistics, AAU, Anand.

I am equally thankful to ***Dr. R. S. Fougat****, Unit Head, Department of Agricultural Biotechnology,* ***Dr. Y. M. Shukla****, Principal and Unit Officer of College of Agriculture, AAU, Vaso,* ***Dr. Shushil Kumar****, Assistant professor, Department of Agricultural Biotechnology, AAU, Anand for their valuable suggestions, co-operation and help during the course of study.*

My special word of thanks should also go to ***Shri. R. N. Pavashiya,*** *Agriculture officer, AAU, Dhandhuka, for conducting field trial and giving his whole hearted support for the collection of samples.*

I gracefully acknowledge the facilities and assistance provided by R. S. Rathwa, Jignesh Patel, Jayesh patel, Hina ben and Balvant Kaka, Kanu kaka and Raman kaka.

I express my sincere thanks to my seniors, Adinath, Amit kumar, Arpan, Divyraj, Husen, Jalpesh, Kuldeep, Kelvin, Kashyup, Nitesh, Navin, Rutul, Ravikiran, Vijay, Vyomesh, Dipal, Dimpal, Saleha, Rutika, Swati, Pooja, Ankita, Kalyani, Jincy, Geeta, Chandani, Heli, Poonam, Ruchi for their benevolent help, valuable advice, co-operation and providing me necessary facilities during my research

work. I feel delighted in acknowledge my juniors Anila, Kalyani, Dhaniya, Gareema, Priya, Nilam, Maitry, Saiyad.

Though thanks is taboo in friendship, my conscience does not permit me, to retain myself from expressing my heartfelt feelings towards my friends ***Hemangini*** *for her exuberant nature and support research work, Kinjal for her timely suggestions and indefatigarable encouragement, Janki and Riddhi. My special thanks to the craziest friends Monika and* ***Dhruvi*** *(Besteeii). From the bottom of my heart I would like to thank, my dearest friends* ***Virali Antala*** *(viru) and* ***Krupali Ramanuj*** *(Sunbae) for their constant support, caring nature, happiness, better understanding, craziness and being my booster energy during my M. Sc phase. Thank you so much for making my M. Sc memorable.*

I owe bundle of gratitude to my senior ***Dhara Savsani****, who showed me the way of education. She was the driving force, whose encouragement, support as well as criticism, which made this thesis a reality.*

A special mention to my Grand Father Balubhai Dalwadi and Grand Mother Shardaben Dalwadi for providing their constant moral support and unconditional love.

I express my heartfelt gratitude to the people who mean the world to me, my father ***Jayeshbhai Dalwadi*** *and mother* ***Pannaben Dalwadi*** *whose blessings and sacrifices anabled me to materialize my goals. I am heartily thankful to my brother* ***Krunal Dalwadi*** *and bhabhi* ***Jinal Dalwadi*** *for your endless love and support. I express my deepest gratitude to my beloved Fiance* ***Kalpit Dalwadi,*** *for his moral support, patience and sacrifice of his own happiness for cooperation.*

I ardently pray my curtsey to the "Almighty" with whose grace and blessings I could accomplish this endeavor. From the core of my heart, I bow my head in extreme regards to the almighty (Ghanshyam Maharaj) whose blessings enabled me to reach this destination.

Place: Anand
Date: / /2016 *(Arpita Dalwadi)*

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ABBREVIATIONS

%	Percentage
µg	microgram
µl	microlitre
^{0}C	Degree Celsius
AOAC	Association of Official Analytical Chemists
APS	Ammonium per-sulphate
2-βME	2- beta mercaptoethanol
BPB	Bromophenol Blue
bp	Base pair
C.D	Critical Difference
CRD	Completely randomized design
cDNA	Complementary deoxyribose nucleic acid
C.V.	Coefficient of Variation
C_T	Threshold cycle
DDW	Double Distilled Water
dNTPs	Deoxyribose Nucleotide Triphosphate
DMSO	Dimethy sulfoxide
DW	Distilled Water
EDTA	Ethylene Diamine Tetra Acetate
e.g.	Example gratia
EtBr	Ethidium Bromide
etc.	etcetera
FAO	Food and Agriculture Organization
Fig.	Figure
g	gram
h	Hours
ha	Hectare
HCl	Hydroxy chloride
kDa	Kilo Dalton

Kb	Kilo base
kg	Kilogram
M	Molar
m	meter
mg	milligram
min	minute
ml	mililitre
mm	milimeter
mM	milimolar
mRNA	messenger RNA
ng/µl	nanogram per microlitre
Mr	Molecular weight
nm	nanometer
N	Normal
NaCl	Sodium Chloride
ng	Nanogram
No.	Number
OD	Optical density
NTC	Non Template Control
PCR	Polymerase Chain Reaction
pmol	picomole
pH	Reciprocal of The Hydrogen Ion Concentration
qRT PCR	Qualitative Real Time Polymerase Chain Reaction
rpm	Revolution per minute
RNA	Ribonucleic acid
RT	Real time
RLI	RNase L inhibitor protein
s	second
SDS-PAGE	Sodium Dodecyl Sulphate– polyacrylamide gel electrophoresis

Sr.	Serial
SEm	Standard error of mean
T	Temperature
ΔT	Annealing Temperature
TBE	Tris Borate EDTA
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
TSS	Total soluble sugar
USDA	United state department of agriculture
Tm	Melting Temperature
U.V.	Ultra Violet
V	Volt
Viz.	Namely

Introduction

I. INTRODUCTION

Wheat is (*Triticum spp.*), a universally grown and the most important of all food grains, it is an ancient grain and believed to have originated in South Western Asia. It is a self pollinated cereal crop, belonging to the family Poaceae (grasses). It is estimated that one-third of the world's population depend on wheat for their nutritional requirements.

Wheat is commercially produced in Russia, USA, China, India, France and Canada. India holds the second position (after China) in total world wheat production which was grown over 25 million hectares of land. Major wheat growing states in India are Uttar Pradesh, Madhya Pradesh, Punjab, Rajasthan, Haryana, Bihar, Gujarat and Maharashtra. Wheat is one of the major cereal grains of Gujarat and contributes 2% in total area and production with a productivity of 2.5 t/ha (Singh, 2010). North Gujarat and North Saurashtra regions of the state contribute the most in wheat production. Wheat is grown both under rainfed and irrigated conditions (Chakraborty, 1992).

The world total area, production and productivity of wheat was 219.03 million/ha, 730.83 million metric ton and 3.33 metric ton respectively in the year 2016-2017. Total area, production and productivity of coarse grains during 2016-2017 are 322.02 million/hector, 1,303 million metric tons, 4.05 million ton. India is the second largest wheat producer after China. The total area, production and productivity of wheat in India was 29.80 million/ha, 88.0 million metric ton and 2.95 million ton respectively during 2016-2017. (Anonymous, 2016).

Common wheat or bread wheat is a hexaploid species that is most widely cultivated in the world and Durum is the only tetraploid form of wheat, which is the second most widely cultivated. In India, three types of wheat are grown *Triticum aestivum* L. (Common / Bread wheat) with 90%

area, *Triticum durum* L. (Durum wheat) and *Triticum dicoccum* L. having 2n=6x=42 and 2n=4x=28 chromosomes, respectively (Sramkova *et al*., 2009).

Durum wheat or macaroni wheat is the only tetraploid species of wheat which is commercially important and is widely cultivated worldwide. Durum wheat contains high protein, as well as its strength, make it good for special uses, the most well-known being pasta, spaghetti, macaroni and extensively in bread making. Durum wheat owing to its unique properties such as high carotenoid pigments content, high vitreousness and hardness as well as gluten proteins composition is used by pasta industry (Rachon 2002, Obuchowski 1999). Most of the wheat production is used after processing, mainly by the pasta industry in the case of durum wheat which requires specific functional properties. These properties largely depend on structures and interactions of the grain storage proteins gliadin and glutenin. (Gras *et al.,* 2001, Shewry and Halford, 2002).

Wheat consumption is increasing worldwide as a result of higher income levels, urbanization, and substitution with other cereals. Therefore, the nutritional quality of the wheat whole meal has a significant impact on human health and well-being especially in the developing world (Peleg *et al*., 2008). The wheat quality characteristics are usually influenced by genotype, environmental factors, and interactions between genotype and environment (Jiang *et al*., 2009). Environmental factors, such as temperature, water, and nitrogen nutrition also influence the ratio between soluble and insoluble proteins, affect bread-making quality, which is a function of protein composition that is genetically controlled (Seligman et *al*., 1995). Water deficit and temperature being the important environmental condition influencing the amount, composition, and/or polymerization of wheat storage proteins (Ozturk and Aydin, 2004). Drought had negative impact on wheat production. Drought stress, which is the most serious environmental problem limiting crop production in rain-fed agriculture (Bahieldin *et al.,* 2005), can

severely impact plant growth and development, limit plant production and the crop performance (Shao *et al.,* 2009). A high evaporation demand around heading stage affect seriously grain yield and biomass production through reduction of ear number, spike fertility and individual grain weight (Rasmussen *et al.,* 2003, Zhang *et al.,* 1998).Therefore, irrigation is necessary to meet crop evapotraspiration requirement.

Irrigation and varietal improvement are two major ways of increasing and stabilizing durum wheat. Water availability belongs to strongest factor determining uptake and effectiveness of N use, yield, and grain quality (Haberle and Svoboda *et al*., 2007). Depending on the water avaibility, small amount of irrigation water applied at strategic period could achieve substantial increase yield and water use efficiency of rainfed wheat (Zhang and Oweis, 1999). Timely irrigation at proper growth and development stage is a viable reason to improve production and wheat cultivation (Muhammad, *et al*., 1997). Limited irrigation applied at sensitive crop stages increased yield of rainfed crops. A significant increased wheat grain yield, after the application of reduced irrigation at jointing (Kang *et al.,* 2002). Irrigation levels influenced glutenin biosynthesis and glutenin particle formation. Irrigation level significantly increases yield and yield components such as spikes weight, plant height, number of grains per spike, 1000 grain weight, biological and grain yield. In irrigation condition grain yield depends on equally on the components NE (numbers of ears), NGE (numbers of grain per ear) and TGW (total grain weight). Irrigation increases the entire three yield component, with a consistent effect of TGW (Garcia *et al.,* 2005).

Storage proteins in seeds and baking quality highly depend on genetic background and environmental factors, especially influence of drought and heat stress, during the grain filling period and nitrogen availability (Altenbach *et al.,* 2002; Luo *et al.,* 2000; Ottman *et al.,* 2000; Rharrabti *et al.,* 2001). Storage protein is a method to investigate genetic variation and to

classify plant varieties (Iqbal *et al.,* 2005). Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is most economical simple and extensively used biochemical technique for analysis of genetic structure of germplasm. Accumulation of specific proteins and other compounds for nutrient storage to high levels is one of the characteristic events during seed development (Singh *et al.,* 1993).

In Wheat Glutenin subunits occupies 10% high molecular weight glutenin subunits (HMW-GS) and 30% low molecular weight glutenin subunits (LMW-GS) (Branlard *et al.*, 2003). Environmental factors, such as temperature, water and nitrogen nutrition also influence the ratio between soluble and insoluble protein, affect quality, which is a function of protein composition that is genetically controlled.

Grain development is associated with massive changes in gene expression and any comparisons between genotypes or environments therefore needs to place the results in a developmental context. Functional genomics studies the function of gene of an organism and focuses on the dynamic process such as transcription, translation, interaction of gene and how they are related to different phenotypes (Torres *et al.,* 2009).

Gene expression analysis is increasingly important in many fields of biological research. Several methods are currently being employed to analyze the expression profiles of gene expression in plants (Rabbani *et al*., 2003). Most commonly used techniques are cDNA-AFLP, SAGE (Serial Analysis of Gene Expression), MPSS (Massively Parallel Signature Sequencing), Real-time PCR and Microarrays (Torres *et al.,* 2009). Analyzing gene expression patterns requires tools enabling sensitive, precise, and reproducible quantification of specific mRNAs. Quantitative real-time polymerase chain reaction (qPCR) is currently the technique of choice for this purpose (Petit *et al.,* 2012). Besides being extremely powerful technique, real-time PCR suffers from certain pitfalls, most important being the normalization with a reference or

housekeeping genes. The expression of reference gene used for normalization in real-time PCR analysis should remain constant between the cells of different tissues and under different experimental conditions, or else it can lead to erroneous results (Jain *et al.,* 2006).

Durum wheat (*Triticum turgidum* L. var. *durum*) cultivated in Bhal Region- Geographical Indication of Gujarat, due to long grain wheat variety and some specific end use quality related to proteins are present. Productivity of durum wheat in Bhal region 650-700 kg/ha. One or Two irrigation increases total yield of durum wheat. Since last two years farmers are applying this practices to get higher yield. No information is available on the effect of the limited irrigation on protein profiling. Thus this study has been planned with following objectives:

Objective:

1) To study the effect of limited irrigation application on nutritional Proximate composition of wheat (*Triticum durum* Desf.).
2) To study the variations in protein fractions and protein profile (HMW and LMW) for end use quality.
3) Real time expression pattern for glutenin gene in caryopsis at maturity Stage.

Review of Literature

II. REVIEW OF LITERATURE

Wheat (*Triticum durum* L.) is the most important cereal crop for the majority of world's population. It is the most important staple food of about two billion people (36% of the world population). Worldwide, wheat provides nearly 55% of the carbohydrates and 20% of the food calories consumed globally. Wheat belongs to family Poaceae (Gramineae) which includes major crop plants such as wheat (*Triticum* spp. L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), rye (*Secale cereale* L.), maize (*Zea mays* L.) and rice (*Oryza sativa* L.). *Triticeae* is one of the tribes containing more than 15 genera and 300 species including wheat and barley. Genome size of wheat is 10.8 GB.

Wheat is the main source of world's food energy and nutrition; these are principally concerned with providing the characteristic substance 'gluten', which is very essential for bakers. Large number of end use products such as Chapatti, bread, biscuit, pasta, noodles, macaroni, spaghetti, cakes pizzas and doughnuts etc. The ability of wheat flour to be processed into different foods is largely determined by the proteins. These proteins are usually classified into two major groups: gliadins and glutenins. The ability of wheat flour to be processed into different food products is largely determined by the gluten proteins (Weegls *et al.*, 1996). Mature wheat grains contain 8-16% protein. The gluten proteins constitute up to 80-85% of total flour protein, out of 80% gluten protein comprises of 40% gliadins (α, β, γ, ω type), 40% glutenin subunits occupies 10% high molecular weight glutenin subunits (HMW-GS) and 30% low molecular weight glutenin subunits (LMW-GS) (Branlard *et al.*, 2003).

Environmental factors, such as temperature, water and nitrogen nutrition also influence the ratio between soluble and insoluble protein, affect quality, which is a function of protein composition that is genetically controlled. Literature survey showed very limited references or no references are available on effect of limited irrigation on quality of durum wheat. The reviews papers are not presented in relation to particular parameters such as

SDS-PAGE of protein fractions and expression analysis of glutenin protein. Looking in to the different aspects of quality and their bearing on produce, an effort has been made in this chapter to review the available literature related to the present investigation.

2.1. Biochemical composition

2.1.1 Proximate analysis

2.2 Protein electrophoresis (SDS-PAGE)

2.3 Protein fractionation (SDS-PAGE)

2.4 Relative gene expression studies (q RT- PCR)

2.1. Biochemical composition

The composition of wheat varies markedly depending upon variety, environment, season, manuring and other agricultural practices. The range of variation in the different biochemical constituents in wheat is depicted in table 2.1 (Gooding and Davis, 1997). Wheat grain precisely known as caryopsis consists of the pericarp and the true seed. In the endosperm of the seed, about 72% of the protein is stored which forms 8-15% of total protein per grain weight. Wheat grains are also rich in pantothenic acid, riboflavin and some minerals. The bran which consists of pericarp testa and aleurone layer is also a dietary source for fiber, potassium, phosphorus, magnesium, calcium, and niacin in small quantities.

Table 2.1 Chemical composition and nutritive value

Parameter	Grain %	Flour %
Moisture	9-18	13-15.5
Starch	60-68	65-70
Protein	8-15	8-13
Cellulose	2-2.5	Trace
Fat	1.5-2	0.8-1.5
Sugars	2-3	1.5-2
Mineral matters	1.5-2	3-6
Crude fiber	2-2.5	-
Ash	1.5-2.0	-

2.1.1 Proximate analysis

2.1.1.1 Moisture and ash

It is one of the most important factors for determination of wheat grain quality. Moisture content is inversely related to the dry matter of grain and has more effect on keeping quality of wheat. Dry and sound wheat that can be kept for years when it is stored properly but wheat with high moisture may deteriorate faster in few days. Seed or flour with high moisture content (greater than 14.5 percent) attracts mold, bacteria, and insects, all of which cause deterioration during storage. Moisture content can be an indicator of profitability in milling.

Ash is defined as the residue left following the controlled combustion of the test material. Higher the ash content, higher is the bran content, which affects the appearance and shear value (Prabhasankar *et al*., 2002). Ash determination is an important analytical tool in management of the extraction rate of wheat (*T. aestivum*) during the milling process (Fjell *et al*., 1994).

Danilton *et al.,* (2013) examined moisture content in Seeds of wheat *(Triticum aestivum* L.) of IPR 118 cultivar in conventional sprinkler irrigation (Irrigated Treatment) and the other without irrigation (Non-irrigated Treatment). The grain moisture collected in both treatments showed no significant difference. Moisture content in both treatments around 14%. This value is considered the lower limit suitable for the wheat harvesting, being values of 16 to 18% more recommended because they provide better flour quality and longer storage time.

2.1.1.2 Total soluble sugar (TSS)

Chakraborty, (1992) studied some commercial varieties of wheat growing in Gujarat for TSS and found a range of 2.0-4.0%. He recommended at least 2.5% of total sugar for good chapati making.

2.1.1.3 Starch

Starch has two important roles in the bread making process, one as a substrate for yeast, through its breakdown by amylolytic enzymes to maltose, and secondly by its contribution to the physical structure of the finished product. Overall, it was apparent that damaged starch was more important than any other chemical characteristic in determining the quality of chapati. Damaged starch, indicating that higher hectoliter weight and it yields softer chapati (Prabhasankar *et al*., 2002).

Kousar *et al.,* (2012) investigated effect of post-anthesis drought stresses on starch grain content in different drought tolerant wheat varieties viz. RSP-566, RSP-561, PBW-396, HD-2687, C-306, PBW-175, RSP-81, PBW-550, DBW-17 and WH-542. Grain starch content varied inter genotypically among the wheat cultivars under normal irrigated condition (control) and also under all stages (10, 17, and 24 days) of moisture stress conditions with maximum effect at 24 days of stress. HD-2687 recorded highest values in grain starch (74.30 %) under irrigated condition. RSP-561and RSP-566 which had low starch contents (74.15 and 72.33%) under irrigated condition.

Zhao *et al.,* (2009) examined starch content in wheat by using single different water conditions at post-anthesis stage. Starch contents of the strong-gluten winter in drought (w1) and more water supplies can decrease starch contents. 65.5% of the final starch weight in wheat grains was accumulated during the later growth period of wheat imposed to w2 treatment (65% SWC), and only 61.1% (treatment w1) to 63.2% (treatment w3) for grains in the same period.

2.1.1.4 Oil content

Zhao *et al.,* (2009) examined lipid contents of the wheat samples by using single different water conditions at post-anthesis stage. Soil water content (ranged from 45% to 85%) can have a significant effect on oil contents (0.88– 1.21%) of the grain samples at post-anthesis stage. Water deficit during grain filling can result in a decrease in lipid contents in wheat grains. The

results indicated that wheat grains study had higher lipid contents from wheat exposed to high SWC at post-anthesis stage.

2.1.1.5 Protein

Protein content varies from 8-16% depending on variety and class of wheat with environmental conditions during the growth. Wheat having high protein content get high premium price in the market as they are useful in blending with low protein wheat flours for bread production. Flours of low protein content wheat are useful in making other different products like cakes, cookies and biscuits.

Gupta *et al*., (2002) reported protein content of *T. aestivum* varieties LOK-1, GW-273 and GW-322 are 12.24, 12.05 and 11.47%. Kundu *et al.,* (2009) reported protein content of *T. durum* varieties GW-1139, NP-404, RAJ-911 and JAY are 12.75, 14.35, 14.82 and 14.49%.

Danilton *et al.,* (2013) examined protein content in seeds of wheat *(Triticum aestivum* L.) of IPR 118 cultivar in conventional sprinkler irrigation (Irrigated treatment) and the other without irrigation (Non-irrigated treatment). Higher values of protein in the Non-irrigated treatment (18.38%) compared to the Irrigated treatment (15.87%). Guttieri *et al.*, (2005) also observed a lower concentration of proteins in irrigated wheat compared with those grown in conditions of low water availability.

Zhao *et al.,* (2009) studied effects of soil water environment on protein content in wheat by using single different water conditions at post-anthesis stage. Protein contents of the strong-gluten winter varied from 14.8% to 16.7%. Wheat grain samples from 45% SWC (w1) and 85% SWC (w3) had the highest (16.7%) and lowest (14.8%) protein contents, respectively, which showed over high SWC was not benefit to the accumulation of protein in wheat grain at post-anthesis stage, water deficit (65% SWC) was benefit to protein formation of wheat grain at later growth stage.

Parvaneh *et al.,* (2014) studied the effect of different levels of irrigation (Full irrigation, drought stress at stem elongation, flowering and grain

formation stages) on Grain Protein Content of three durum wheat cultivars (Yavaros, Seimareh and Karkheh). Protein content was significant affected by drought stress. The highest protein content (12.9%) was obtained from drought stress at grain formation stage. In general, full irrigation and drought stress at grain formation stage had the lowest (11.3%) and highest protein content (12.9%), respectively. Pierre *et al. (*2008*)* reported that drought stress at grain formation stage increases the means of protein content in 9 genotypes of wheat.

2.1.1.6 Lysine and tryptophan

Lysine and tryptophan are limiting amino acids in wheat. Brandt *et al*., (2000) have shown that protein content is inversely related to lysine content in wheat. Rharrabti *et al*., (2001) studied relationship between lysine and protein content and they found highly negative correlation (r = -0.864***) between lysine content and protein content. Lysine content was mainly influenced by environmental growing conditions. Faqir *et al*., (2005) found that the decline in lysine content ranged from 1.11% to 23.58% during chapati baking (12.4% protein basis).

Chakraborty, (1992) studied some commercial varieties of wheat growing in Gujarat ranges lysine and tryptophan between 1.7 to 3.6 and 0.7 to 1.5 (% protein), respectively. Yadav *et al*., (2000) investigate the percent distribution of different protein fractions during grain development indicated a decrease in albumin + globulin fraction and an increase in gliadin and glutenin. Amino acid composition analyzed by HPLC did not show much difference amongst the varieties but in the gliadin fraction, lysine was very low whereas it was higher in glutenin fraction.

2.1.1.7 Mineral

Fubara *et al*., (2011) analysed the mineral contents of raw and processed (boiled, dried, roasted and fried) maize by atomic absorption spectrophotometric (AAS) method. The results were showed that the raw maize had the highest values for all the minerals analyzed. The Ca, K and Na content in all the raw and processed (boiled, dried, roasted and fried) maize was ranged from 0.009 to 0.04 6 mg/kg , 0.013 to 0.006 mg/kg and 0.002 to 0.061 mg/kg respectively.

Kousar *et al.,* (2012) studied effect of post-anthesis drought stresses on micronutrients (Calcium, phosphorus, iron, zinc) in different drought tolerant wheat varieties viz. RSP-566, RSP-561, PBW-396, HD-2687, C-306, PBW-175, RSP-81, PBW-550, DBW-17 and WH-542. With increasing trend was observed in calcium composition of wheat grains up to 17days of stress but afterwards a downfall was registered in all the wheat cultivars till 24 days of stress. PBW-396 maintained highest calcium level all along under moisture stress conditions (80.25, 82.75 and 78.50 mg/g) followed by RSP-561. However, highest percent enhancement of calcium was found in PBW-175 (25.59%) and C-306 (26.95%) at 17 days and even after downfall at 24 days of stress.

Mohammad and Razzaghi (2015) examined the effects of water supply (normal irrigation and post anthesis drought stress condition) on macronutrients (P, K, Ca and Mg) uptakes in five winter wheat cultivars (Zarrin, Peshgam, Orum, Zareh and Mihan). Grain P, K, Ca and Mg macronutrients uptake were significantly affected by irrigation treatments ($P \leq 0.01$). The interaction of irrigation × cultivars for grain P, K, Ca and Mg uptake was also significant ($P \leq 0.01$). P, K, Ca and Mg uptake decreased under water deficit condition. Water limitation led to decreased grain K, P, Ca and Mg uptake by an average of 51%, 41%, 67% and 60%, respectively. Zareh cultivar has the most Ca and Mg uptake under well-irrigation, but under water deficit condition Mihan has the highest grain P, K, and Mg uptake.

Zhao et al., (2009) assessed effects of different water availability at post-anthesis stage on mineral composition of grain in strong-gluten winter

wheat. Calcium (Ca) and Potassium (K) were identified in ash samples from the experiments. Calcium content in grain samples were linearly correlated with the increasing in SWC (ranged from 45% to 85%), their contents increased, except that K of all the samples was enhanced in response to SWC (ranged from 45% to 85%) increasing and decreased by too high SWC which indicated mineral contents in the grains of winter wheat were greatly affected by soil water conditions in the field. Whereas water deficit (low SWC) was benefit to mineral accumulation in wheat grain at post-anthesis stage, serious water shortage can lead to high decreasing in grain yield during the stage. Proper water conditions at the later growth stage of wheat can be favourable to more mineral concentrations in wheat grain.

2.2 Protein electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) provides a versatile, gentle, high resolution method for fractionation and physical-chemical characterization of molecules on the basis of size, conformation and net charge. The polymerization reaction can be vigorously controlled to provide uniform gels of reproducible, measurable pore size over a wide range. This makes it possible to obtain reproducible relative mobility (Rm) values as physical-chemical constants. (Chrambach and Rodbard, 1971).

Sharma *et al.,* (2015) assessed genetic diversity of 12 Indian wheat genotype (six genotypes were resistant to salinity and others are susceptible) on the basis of seed storage protein profiling. Genetic diversity was calculated (UPGMA) cluster analysis and Jaccard's similarity coefficients between these varieties. Seed storage protein analyzed by 15 subunits ranging from 14.3-97.4 kDa protein molecular weight marker (PMW-M). No unique band found in both gel. The polypeptides having molecular weight 49 kDa & 29 kDa are appeared in all genotypes in 10% and 15% gel respectively. A band of 71 kDa is appeared only in G7 and G12 and absent in other.

2.3 Protein fractionation (SDS-PAGE)

Mariana *et al.,* (2013) evaluated 16 wheat genotypes by correlating both glutenin subunits of high and low molecular weight and gliadin subunits with the physicochemical characteristics of the grain. The glutenin and gliadin subunits were separated using polyacrylamide gel in the presence of sodium dodecyl sulfate. The IPR 85, IPR Catuara TM, T 091015, and T 091069 genotypes stood out from the others, which indicate their possibly superior grain quality and possessed the subunits 1 (Glu-A1), 5 + 10 (Glu-D1), c (Glu-A3), and b (Glu-B3), with exception of T 091069 genotype that possessed the g allele instead of b in the Glu-B3.

Nadeem *et al.,* (2016) studied the glutenin and gliadin fractions in Six spring wheat (*Triticum aestivum*) (Sehar-06; Shafaq-06; FSD-08; Lassani-08; Aas-10; and AARI-10) varieties produced during two different cropping years (2010-11 and 2011-12) through SDS-PAGE .The SDS-PAGE patterns of molecular weight of glutenin subunits of different wheat varieties showed the presence of glutenin subunits in the range of 28.23 to 110.89 kDa and 28.29 to 113.51 kDa and gliadin ranged from 31.03 to 89.61 kDa and 32.91 to 92.22 kDa during the cropping years 2010-11 and 2011-12, respectively. The highest molecular weight glutenin subunit (110.89 kDa) and (113.51 kDa) were observed in wheat variety Lassani-08, during the crop year 2010-11 and 2011-2012 respectively. The maximum numbers of total gliadin electropherograms ranged from 31.03 to 89.61 kDa and 32.91 to 92.22 kDa, were found in Lassani-08 during 2010-11 and 2011-12, respectively.

Sondeep *et al.,* (2012) investigated the influence of drought and sowing time on protein composition, of wheat whole meal of two (*T. aestivum* L.) genotypes, PBW343 (high yielding, drought susceptible) and C 306 (drought tolerant), differing in their water requirements. Albumin-globulin and gliadin proteins ranged from 9 to 13% and 21 to 32% of total protein, respectively, under irrigated conditions. Albumin-globulin proteins were enhanced under irrigated LS (Late sowing) as well as under RF (rainfed) conditions while lower accumulation of gliadin. C 306 had lower gliadin percentage and higher

percentage of glutenin as compared to the respective grains of PBW 343 in rainfed TS and rainfed ES condition. Late sown conditions offered higher protein content accompanied by increased albumin-globulin but decreased glutenin content.

Zhao *et al*., (2009) studied effects of soil water environment on protein content and composition in wheat by using single different water conditions at post-anthesis stage. The protein fractions (globulin, gliadin, glutenin, and glutenin/gliadin ratio) in grain samples were down-regulated in response to SWC increasing at post-anthesis except albumin, which showed that the accumulation of protein components was sensitive to water stress at the later stage. The results of protein fractions also revealed that good management of water in wheat field at post-anthesis stage was helpful both to improving protein content and composition in wheat grain and to yield formation.

Enver *et al.,* (2014) evaluated ten durum wheat (commonly cultivated in the region) samples for high molecular weight (HGMW-GS) and low molecular weight glutenin subunit (LGMW-GS) composition using SDS-PAGE with Kyle cultivar as a reference. In terms of LMW, all cultivar had LMW-2 similar to Kyle cultivar, except Sarıçanak 98. In gliadin fraction it was found that 9 cultivar contained γ-45, While Sarıçanak 98 had γ-42 and in terms of HMW, all cultivar were similar in four locations except Guneyyildizi. Cultivars with glutenin allele LMW-2 (or gliadin band γ-45) generally give stronger gluten than cultivars with allele LMW-1 (or gliadin band γ-42). Sariçanak 98 showed weak gluten, relating with pasta cooking quality, other varieties showed strong gluten, relating with quality pasta cooking.

Slađana *et al.,* (2011) determine the classical osborne wheat protein fractions (albumins, globulins, gliadins, and glutenins) in the grain of five bread (*T. aestivum* L.) and five durum wheat (*T. durum* Desf.) genotypes. No significant differences in the mean of AG (albumin + globulin), as well as gliadin content were observed between bread and durum wheat. Gliadins and glutenins comprise from 58.17% to 65.27% and 56.25% to 64.48% of total proteins and as such account for both quantity and quality of the bread and

durum wheat grain proteins, respectively. The ratio of gliadin/total glutenin varied from 0.49 to 1.01 and 0.57 to 1.06 among the bread and durum genotypes, respectively.

2.4 Gene expression profiling/analysis to study Glutenin gene

Functional genomics studies the function of gene of an organism and focuses on the dynamic process such as transcription, translation, interaction of gene and how they are related to different phenotypes (Torres *et al.,* 2009). Gene expression analysis is increasingly important in many fields of biological research. Understanding patterns of expressed genes is expected to provide insight into complex regulatory networks and will most probably lead to the identification of gene relevant to new biological processes. The gene expression profiling is an important tool to investigate how an organism responds to environment changes (Rabbani *et al.,* 2003).

Real-time PCR include two methods for the quantification of a target nucleic acid sequence: absolute and relative quantification. In absolute quantification (AQ), the exact level of a sequence in a sample is directly inferred from a standard curve prepared from a dilution series of control template of known concentrations. On the other hand, relative quantification is mainly used in gene expression studies to determine the up- or down-regulation of a gene of interest relative to a calibrator.

2.4.1 Converting an RNA population into cDNA

The most important consideration in generating useful data with real time RT-PCR is the quality of isolated RNA (Farrell, 1998). The mRNA is converted to cDNA in a reaction catalyzed by an RNA dependent DNA polymerase enzyme called reverse transcriptase enzyme.

Nicot *et al.,* (2005) suggested that plant stress studies are more and more based on gene expression. The analysis of gene expression requires sensitive, precise, and reproducible measurement for specific mRNA

sequence. Real time RT-PCR is at the most sensitive method for the detection of low abundance mRNA.

2.4.2 PCR Reaction for Real Time Analysis

A single stranded cDNA template is converted to double stranded DNA in the PCR reaction using forward and reverse primers that is specific to mRNA.

2.4.3 Detection of Amplicon in the Real Time Thermal Cycler

Detecting the PCR product in the real time involves the use of fluorescent dye. These can be either nonspecific dye, such as florescent DNA binding dyes (SYBR Green I) or strand specific probes (*Taq*man or molecular Beacons). SYBR Green dye is most commonly used, because they are economical and easier to optimize. The principle behind the SYBR Green dye is that they undergo a 20 to 100 fold increase in their fluorescence upon binding double stranded DNA increases in the reaction mixture there will be corresponding increase in the fluorescent signal.

The specificity of the amplified product in the real time PCR is verified by the melt curve analysis. Melting point analysis is performed for the occurrence of specific amplification peaks and the absence of primer dimer formation. Different ampicons can generate different peaks centered on different temperature and, fortunately, PCR artifact has a lower melting temperature as compare to target amplicon.

2.4.4 Real time PCR quantification

Relative quantification measures the changes in a genes expression in response to different treatments or state of tissues. The lack of confidences in qRT-PCR in due to disappointments arising from the conflicting results sometimes obtained using this technique due to the failure in applying a robust normalization strategy. Making the systematic validation of reference

genes obligatory for reported analysis would greatly improve the accuracy and consistency of RT-PCR analysis in plants (Guenin *et al.,* 2009).

2.4.5 Validation of constitutively expressed reference genes

Several variables need to be controlled for gene expression analysis, such as the amount of starting material, enzymatic efficiencies and differences between tissues or cells in overall transcriptional activity. Various strategies have been applied to normalize these variations. An ideal reference or endogenous control should be constantly transcribed in all types of cells or tissues for all the treatments and RNA transcription should not be regulated by internal or external influences, at least not more than general variation of m-RNA synthesis. The accurate quantification of a true reference gene allow the of differences in the amount of amplifiable RNA or cDNA in individual sample generated by (i) Different amount of starting material, (ii) The quality of starting material and (iii) Differences in RNA preparation and cDNA synthesis, since the reference gene is exposed to the same preparation step as gene of interest (Livak, 1997, 2001; Morse et al., 2005). Multiple possibilities are obvious to compare a gene of interest mRNA expression to one of the following parameters. A gene expression can be relative to:

- An endogenous control, e.g. a constant expressed reference gene.
- An exogenous control, e.g. a universal and/or artificial control RNA or DNA.
- A reference gene index, e.g. consisting of multiple averaged endogenous control.

Jain et al., (2006) studied the gene expression of 10 frequently used endogenous genes, including 18S rRNA, 25S rRNA, UBC, UBQ5, UBQ10, ACT11, GAPDH, eEF-1a, eIF-4a and β-TUB, in a diverse set of 25 rice samples through RT- PCR. Their expression varied considerably in different tissue samples analyzed. For drought stress treatments, 7-day-old light-grown rice seedlings were transferred to 400 mM mannitol for 3 h. The expression of

UBQ5 and eEF-1a was most stable across all the tissue samples examined. However, 18S and 25S rRNA exhibited most stable expression in plants grown under various environmental stress conditions. Also, a set of two genes was found to be better as control for normalization of real-time PCR results for gene expression studies in a wide variety of samples in rice.

Paolacci *et al.,* (2009) studied 20 novel candidate reference genes and 12 commonly used HKGs suitable for gene expression normalization in wheat. The expression stability of the 32 selected genes was assessed by qRT-PCR in 24 different plant samples, which included different tissues, developmental stages where the plants were exposed to temperature stresses. The computer programs GeNorm and NormFinder were used to compare the expression patterns of the 32 candidate genes and to identify the best reference genes, which were not the same. The results indicated that many of the new identified reference genes outperform the traditional HKGs in terms of expression stability under all the tested conditions. Ta54227 (Cell division control protein, AAA-superfamily of ATPases), Ta2291 (ADP-ribosylation factor) and Ta2776 (RNase L inhibitor-like protein) were found as the best and most stable reference genes to normalize gene expression in different tissue and development stages of wheat and they outperformed all traditional HKGs.

Anna *et al*., (2009) identify and validate reference genes for quantitative RT-PCR normalization in wheat. The expression stability of 32 genes was assessed by q RT-PCR using set of cDNA from 24 different plant samples, which included different tissues, developmental stages and temperature stresses. Ta54227 (Cell division control prot.), Ta2291 (ADP-ribosyltion factor) and Ta2776 (RNase L inhibitor-like protein) resulted best and more stable reference gene to normalize gene expression in different tissue and development stages of wheat.

Caldana *et al*., (2007) evaluated the expression stability of seven reference genes (*actin* (*ACT*), *actin1* (*ACT1*), β-*tubulin* (*TUB*), *cyclophilin* (*CYC*), *elongation factor* 1α (*EF-1*α), *expressed protein* (*EP*) and *TIP41-like protein*

(*TIP41*)) using different rice cultivars, tissues and physiological conditions. Their expression stability was measured by qRT-PCR in a set of 11 different cDNA samples, and calculated using the gene expression stability measure (M) implemented in the geNorm software. Considering all the experimental categories, *ACT1* and *EF-1*α were found the most stable reference genes. Additionally, *EP*, similar to its *Arabidopsis* orthologue also showed good stability in all experiments.

Barbara *et al.,* (2014) assessed several candidate reference genes for the expression studies with brassinosteroids in *Lolium perenne* and *Triticum aestivum*. UBQ, EF1A, ACT, GAPDH, Tub4, CYP18-2, ADP used in wheat (*Triticum aestivum*) and UBI, eEF1A, ACT1, GAPDH, Tub2, TBP-1, H3.3, 25S rRNA in *Lolium perenne*. Different statistical approaches (qBasePLUS, BestKeeper, NormFinder) were used to prepare rankings of expression stability in two species of an economic importance: common wheat (*Triticum aestivum*) and perennial ryegrass (*Lolium perenne*). The maximum stability values indicated that the expression stability was higher in *T. aestivum.* TBP-1 and UBI in ryegrass and ACT, ADP and EF1A in wheat expressed more stable than the other that should be used as reference genes.

2.4.6 Relative expression patterns of genes related to Glutenin Gene

Relative quantification or comparative quantification measures the relative change in mRNA expression levels. It determines the changes in steady state mRNA levels of a gene across multiple samples and expresses it relative to the levels of RNA. Relative quantification does not require a calibration curve or standards with known concentrations and the reference can be any transcript, as long as its sequence is known (Bustin, 2002). The units used to express relative quantities are irrelevant, and the relative quantities can be compared across multiple real-time RT-PCR experiments (Vandesompele *et al.,* 2002). It is the adequate tool to investigate small physiological changes in gene expression levels.

Yongfang *et al.,* (2008**)** studied the transcriptome analysis of developing caryopses from hexaploid wheat (*Triticum aestivum*, cv. Hereward) using Affymetrix wheat GeneChip® oligonucleotide arrays which have probes for 55,052 transcripts. 14,550 showed significant differential regulation in the period between 6 and 42 days after anthesis (daa). Large changes in transcript abundance were observed which were categorised into distinct phases of differentiation (6–10 daa), grain fill (12–21 daa) and desiccation/maturation (28–42 daa) and were associated with specific tissues and processes.

Dong *et al.,* (2016) investigated quality properties and the transcriptional expression profiles of eight protein disulfide isomerase (PDI) genes (PDI1-1, PDI3-1, PDI4-1, PDI5-1, PDI6-1, PDI7-1, PDI7-2 and PDI8-1) during grain development in three wheat glutenin near isogenic lines (NILs) CB037A, CB037B and CB037C.

Bravo *et al.,* (2013) evaluated transcript accumulation of HMW-GS genes in endosperm during grain development across 41 days (day 56 to 97) using qRT-PCR in wheat cultivar Cojitambo, cv. Carnavalero, and cv. Sibambe. Expression profile in cv. Cojitambo showed a coordinated pattern of induction of glutenin genes with significant higher levels at a period between 82 and 86 days (data not shown). Up-regulation of most HMW-GS genes began at day 77 and was undetectable at day 97. Genes 1Bx7, 1Dy10, and 1Dx5 were significantly induced during day 82 and 84 in all wheat cultivars. Transcript of 1Bx7, 1Dy10, and 1Dx5 in wheat cv. Carnavalero and cv. Cojitambo showed significantly more accumulation that wheat cv. Sibambe.

Chope *et al.,* (2014) studied gene expression analysis of HMW and LMW related genes in Five U.K. bread making wheat cultivars (Cordiale, Hereward, Malacca, Marksman, and Xi19) and one feed wheat cultivar (Istabraq) with three levels of nitrogen (100, 200, and 350 kg/ha in total, split in up to three applications) before anthesis. The relative levels of hybridization to the probe sets suggested that Marksman had the highest level of HMW gene expression. Cordiale, Malacca, Marksman, and Xi19 showed the highest level of LMW GS gene expression while Hereward had by

far the lowest level. There was a significant variety by N (Nitrogen) interaction for gliadin gene expression, which appeared to be lowest for Istabraq and Malacca.

2.4.7 Analysis of expression stability of reference genes.

Increasing concerns about normalization using ideal reference genes have led to the development of several mathematical algorithms aimed at determining the stability of reference genes (Vandesomple, 2009).

GeNorm software developed by Vandesomple et al., (2002) addressed the ciritical issues of reference gene validation and ranks candidate reference gene according to their expression stability using raw, non normalized expression levels.

Pfaffl *et al., (*2004*)* developed the software BestKeeper that takes into account C_T values of candidate reference gene instead of relative quantities. This software employe a statistical algorithm wherein the Pearson correlation coefficient for each reference gene pair is calculated along with the probability of correlation significance of the pair.

Andersen *et al.,* (2004) used a model based evaluation strategy which ranks candidate reference genes with minimal inter and intra-group variations and developed the software NormFinder.

2.4.8 Relative quantification through Bio-Rad CFX Manager 3.1 Software.

To determine the level of expression, by using the differences (Δ) between the threshold cycles. (Morse *et al.,* (2005), Livak *et al., (*2001) and Schmittgen, (2000)). To calculate the expression of a target gene in relation to an adequate reference gene various mathematical models are established. Calculations are based on the comparison of the distinct cycle determined by various methods (Pfaffl, 2004). Relative quantification was performing using comparative C_T ($\Delta\Delta C_T$) method by Bio-Rad CFX Manager 3.1 Software.

Materials and Methods

III. MATERIALS AND METHODS

The present investigation entitled **"Effect of Limited Irrigation on Quality of Durum Wheat (*Triticum durum* Desf.)"** carried out at Department of Biochemistry, in collaboration with Center of Excellence in Biotechnology and B.A. College of Agriculture, Anand Agricultural University, Anand.

3.1 Experimental Materials

3.1.1 Sample collection:

1. Wheat Samples were collected from Agriculture Research Station, AAU, Dhandhuka, AAU.

Table 3.1. List of Varieties:

Sr. No.	*T. durum* Varieties
1	GW-1
2	A 206
3	A-9-30-1

Table 3.2. Details of treatments employed in the field

Sr. No	Treatment	Details
1	T_0	**No Irrigation** (Rainfed) Sowing in moisture conserved soil.
2	T_1	**One Irrigation** at 30 days after date of sowing.
3	T_2	**Two Irrigation** First Irrigation at 30 days after date of sowing. Second Irrigation at 60 days after date of Sowing.

Table 3.3. Field experiment

1	Crop	*Triticum durum* Desf.
2	Year	2015-2016
3	Design	FCRD (Factorial Complete Randomized Design)
4	Replication	3 (Three)
5	Plot size	Gross: 2.76 x 6.0 m
6	Spacing	60 X 30 cm
7	Manures and fertilizers	N: P: K @ 00: 00: 00 kg/ha.
8	Date of sowing	23 - 10 – 2015
9	Date First Irrigation	24 – 11 – 2015
10	Date Second Irrigation	23 – 12 – 2015

3.2 Materials

3.2.1 Chemicals, Buffers and Reagents

All the chemicals and fine reagents used in the experiments were of molecular and analytical grade obtained from standard manufacturers, like Applied Biosystems, Sigma, Amresco, Fermentas, Himidia,Sigma-Aldrich, Merck and Qualigenes.

3.2.2. Glass-wares and Reagents

Properly cleaned and neutral glassware (Borosil grade) were used. The glass-wares were sterilized in oven before use. Plastic-wares used for the experiment were compatible with molecular biology work. All the plastic-wares like micropipette tips, PCR tubes, centrifuge tubes and eppendorf tubes were autoclaved before use.

3.3 Equipments and Instruments used

Instruments/Equipments Make/Manufacturer

Thermal Cycler	: Bio-RAD, Eppendorf
Distillation unit	: Millipore
Flame Photometer	: ELICD CL 361
Hot Air Oven	: NSW India
Ice Machine	: ICETRONIC
Microwave oven	: Kenstar, India
Micropipettes	: Finnpipette Lab. System, Finland, Japan
UV Spectrophotometer	: Shimadzu UV1700Pharma Spec
Oil extraction unit	: Socs Plus, SCS 6 AS, PELICAN Equipments, Chennai
Stirring Machine	: SPI NOT
Real time PCR	: Bio-rad
Vertical slab gel	: Bangalore Genei Ltd., India
Weighing balance	: Bp 210D, Sartorius, Germany
Water bath	: Yorko YSI- 413, York Scientific Ind. Ltd.
Autoclave	: Mediquip
Micro Kjeldahl Unit	: Kelplus
Cyclo mixer	: Remi
Electrophoresis unit	: Hoefer
Freezer (-20 ^{o}C)	: Vestfrost
Gel documentation system	: Alpha Innotech
pH meter	: Eutech
Nanodrop	: Nanodrop® ND-1000, Nanodrop Technologies, INC. Wilmin.gton, USA.

3.4 Biochemical characterization

Following Biochemical characterization were carried out from durum wheat seed powder.

3.4.1 Proximate compositions from seed powder

3.4.1.1 Moisture content

3.4.1.2 Ash content

3.4.1.3 Total soluble sugar

3.4.1.4 Starch content

3.4.1.5 Oil content

3.4.1.6 Total protein content

3.4.1.7Amino acids

3.4.1.8.1 Lysine

3.4.1.8.2 Tryptophan

3.4.1.8 Minerals content

3.4.1.9.1 Calcium

3.4.1.9.2 Sodium

3.4.1.9.3 Potassium

3.4.2 Protein characterization through SDS-PAGE

3.4.3 Protein fractions on the basis of their solubility

3.4.1 Proximate compositions

3.4.1.1 Moisture content

Moisture content from wheat flour was determined using the method described by AOAC, (1995).

Five grams of seeds of each genotype were kept in the small aluminium boxes. The weight of each box with seeds was prerecorded then the boxes were kept in the hot air oven at 100 ^{0}C for 5 hrs. All boxes were taken out of the oven and allowed to cool for 15 min. All the boxes were weighed to measure the moisture loss from the seeds. The amount of total moisture was calculated as-

$$\text{Moisture \%} = \frac{\text{Wt. of sample} - \text{Wt. after 6 hrs oven drying}}{\text{Wt. of sample}}$$

3.4.1.2 Ash

Ash content was determined using the method described by AOAC, (1965). The samples were ignited at 600^0C to burn off all organic material. The inorganic material which does not volatilize at that temperature was called as ash. Two grams of flour of each sample was weighed and filled in crucibles. These crucibles were placed in a muffle furnace after weighing and the temperature adjusted at 600^0C for 6 hrs. Crucibles were transferred to a desiccator and cool to room temperature. All crucibles were weighed as quickly as possible to prevent moisture absorption. Ash content was calculated as,

$$\text{Ash \% on partial dry basis} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

3.4.1.3 Total soluble sugars (TSS)

Total soluble sugars were determined using Phenol-sulphuric acid method described by Dubois *et al.*, (1956).

A soluble sugar was extracted from 200 mg of flour in 80% ethanol. One milliliter (ml) of extract was evaporated to dryness and dissolved in 10 ml of hot distilled water. One ml of sample was pipetted in 30 ml test tube. In a similar way 0.2, 0.4, 0.6, 0.8 and 1.0 ml of the working standard glucose solution (0-100 µg) was pipetted into a series of tubes and volume were make up to 1ml by distilled water. 1ml of 5% phenol solution and 5 ml of sulphuric acid were added to each tube and shaken well. Again after 10 min the contents in the tube were shaken and placed in an ice bath for 20 min. The absorbance was read at 490 nm.

The amount of total soluble sugar was calculated as-

TSS % = Sample O.D × Dilution Factor × Graph Factor × 100

3.4.1.4 Starch content

Starch content was estimated using the method described by MacCready (1950).

Two hundred mg of sample with 5 ml of distilled water and 25 ml if 80% ethyl alcohol was taken in 50 ml centrifuge tube. This was centrifuged at 8000 rpm for 6 min supernatant was discarded, to the pellet 30 ml of 80% ethyl alcohol was added and centrifuged again, supernatant was discarded, to the residue 20 ml of distilled water plus 6.5 ml of perchloric acid was added and centrifuged. Repeated it twice and then transferred the aqueous phase to 100 ml volumetric flask and final volume made up to 100mL. One ml of filtrate was taken and diluted to 100 ml with distilled water. Again 5 ml from this was taken and 10 ml of freshly prepared 10 % anthrone reagent was added and boiled them for 7.5 min on boiling water bath. The tubes were allowed to cool down at room temperature and read at 630 nm in spectrophotometer.

Starch content was calculated as per the following formula:

% Glucose = O.D. X Graph factor

% Starch = % Glucose X 0.9

3.4.1.5 Oil content

Total oil from the seeds was extracted by Soxhlet extraction method using hexane (Bhatnagar *et al.,* 2007). Oil percentage was calculated using the following formula:

$$\% \text{ Oil} = \frac{(\text{Weight of oil + flask}) - (\text{Weight of flask}) \times 100}{\text{Weight of sample}}$$

3.4.1.6 Total protein (Microkjeldahl method)

Total nitrogen content from grains was determined using the standard Kjeldhal method by AOAC, (1965). The grain-protein percentage was calculated after multiplying Kjeldhal nitrogen by a conversion factor of 5.7 and expressed on a dry weight basis. The method of AOAC, (1965) was used for determination of protein content.

40 mg of flour were taken in the glass digestion tubes. 2 ml of concentrated H_2SO_4 and the digestion mixture (1:3 of $CuSO_4$ and K_2SO_4) was added to the tubes. Then the tubes were transferred on electric burner for 2 hrs to carry out the digestion. After digestion the content of the tube became colourless which is the indication of complete digestion. Then 10-15 ml of double distilled water followed by 10 ml of 40 % NaOH was added to the digestion tube as a result of which the whole solution turned black. In a 250 ml conical flask 10 ml of 4 % boric acid was taken and 2 drops of mixed indicator was added to it. Then the digestion tube containing the solution was steam distilled in the Kelplus machine and the ammonia was trapped in the boric acid as a result of which the solution in the conical flask turned blue. This solution was titrated with 0.02 N H_2SO_4.

The amount of total protein was calculated as-

$$\%N = \frac{\text{(Vol. of } H_2SO_4 \text{ in determination – blank) X 0.02 X 14.007}}{\text{Sample weight (mg)}}$$

Total protein % = % N X 5.7

Where, N = Nitrogen

3.4.1.7 Amino acids

3.4.1.7.1 Lysine

Lysine was determined using method described by Bhatnagar *et al*., (2007). Defatted flour (100 mg) taken in vial and papain solution (4 mg/ml in 0.03 M phosphate buffer, pH 7.4, 5 ml) was added, shaken for few minutes and kept in oven overnight at 65 ^{o}C.A blank with papain solution without sample was also run simultaneously. Hydrolyzed samples were removed, mixed well and allowed to cool to room temperature. Clear supernatant (1 ml) was transferred to centrifuge tube containing 0.6 M carbonate buffer (pH-9, 0.5 ml). Copper phosphate suspension [A: $CuCl_2.12H_2O$ {2.8 g} in distilled water (100 ml), B: $Na_3PO4.\ 12H_2O$ {13.6 g} in double distilled water (200 ml)]. Solution A and B were mixed prior to use {0.5 ml}) was added to each tube to block α-amino acids. The mixture was mixed well and centrifuged to

precipitate the excess copper phosphate. Supernatant (1 ml) was added in a large test tube and into that 2-chloro, 3-5 dinitropyridine (40 mg/ml of methanol, 0.1ml) solution was added. The content of tubes was shaken well. The mixture was allowed to stand for 2 hours at room temperature with shaking at every 30 mins to form p- dinitropyridyl derivative of lysine. 1 N HCl (5 ml) was added in each tube to acidify the reaction and shaken well. Ethyl acetate (5 ml) was added to remove unbound 2-chloro, 3-5 dinitropyridine and shaken well. Upper layer (solvent layer) was removed with the help of syringe and this step was performed twice (washing with ethyl acetate). Optical density of the aqueous phase was measured at 390 nm with the help of colorimeter against the blank. Lysine content of sample was determined by comparing it with standard curve.

Standard curve was prepared in the range of 0 to 200 µg of lysine per ml from stock solution of lysine (2500 µg/ml). Stock solution was prepared by dissolving lysine (50 mg) in carbonate buffer (20 ml). The stock solution was diluted with carbonate buffer to get the following concentrations of lysine 0, 250, 500, 750 and 1000 (µg/ml). Aliquots (1 ml) from each dilution were taken in oak ridge tube and papain (5 mg/ml, 4 ml) solution was added. Respective concentration of lysine became 0,50,100,150, and 200 µg/ml. Solution were centrifuged and supernatant (1ml) was transferred in centrifuge tube and 0.5 ml of the amino acid mixture solution (Cysteine 20 mg, Phenyl alanine 40mg, Methionine 20 mg, Valine 40 mg, Histidine 30 mg, Arginine 50mg, Alanine 30 mg, Serine 50 mg, Isoleucine 30 mg, Aspartic acid 60 mg, Threonine 30 mg, Glutamic acid 300 mg, Threonine 30 mg, Leucine 80 mg, Glycine 40 mg, Proline 80 mg. Amino acid mixture (100 mg) was weighed and dissolved in carbonate buffer {10 ml}) and copper phosphate suspension (0.5 ml) was added. The detailed procedure described above was then applied and standard curve was derived.

Lysine % = Sample optical density × graph factor × Dilution factor

$$\text{Lysine per protein \%} = \frac{\text{\% lysine in sample}}{\text{\% protein in sample}} \times 100$$

3.4.1.7.2 Tryptophan

Tryptophan content was determined using method described by Nurit *et al*., (2009). Defatted flour (80 mg) taken in a 15 ml falcon tube and 3 ml of papain solution (1mg/ml) was added to each tube. A blank with papain solution without sample was also run simultaneously. The tubes were shaken thoroughly and kept in oven for overnight at 64 ^{0}C. Samples were allowed to cool at room temperature and centrifuged at 3600g for 5 min. One milliliter of hydrolysate (supernatant) was carefully transferred to a glass tube and 3 ml of freshly prepared reagent D (20 ml of reagent A: 1.8 mM $FeCl_3$-$6H_2O$ + 20ml of reagent C: 30 N H_2SO_4) was added. Samples were thoroughly vortexed and then incubated for 30 min at 65 ^{0}C. The samples were allowed to cool to room temperature before reading their optical density (OD) at 560 nm. Tryptophan content of samples was determined by comparing it with standard curve.

Standard curve was prepared in the range of 0 to 30 µg/ml from stock solution of tryptophan (100 µg/ml). The stock solution was diluted with 0.1 M sodium acetate buffer (pH 7) to get concentration of 0, 10, 20, 25, and 30 µg/ml. Aliquots (1 ml) from each dilution taken in a tube and 3 ml of freshly prepared reagent D was added. The detailed procedure described above for samples was then applied and standard curve was derived.

$$\text{Tryptophan \%} = \text{Sample opitical density} \times \text{Graph factor} \times \text{Dilution factor}$$

$$\text{Tryptophan per protein \%} = \frac{\text{\% Tryptophan in sample}}{\text{\% protein in sample}} \times 100$$

3.4.1.8 Mineral content

Preparation of Acid extract:

Hundred mg of sample was taken in 250 ml conical flask. Ten ml of nitric acid was added to the flask and kept overnight. Next day the flask containing sample and 10 ml nitric acid was put on hot plate and content was vaporized till 1-2 ml solution remains. 10 ml of di-acid mixture (2:1 ratio of nitric acid: perchloric acid) was added to the solution and again it was vaporized till 1-2 ml. Finally the solution was made upto 100 ml and acid extract was ready for analysis of following minerals (Ca, Na and K).

3.4.1.8.1 Calcium

Ten ml aliquot of acid extract, a pinch of mureoxide powder and few drops of 4% NaOH were taken in 100mL conical flask and titrated against 0.01M Ethylene Diamine Tetra acetic Acid (EDTA) till pink coloured complex was formed. Amount of calcium was calculated using following formula.

% of calcium = Reading X Normality of EDTA X Original volume X 32

Weight of sample X Aliquot taken X 1000

3.4.1.8.2 Sodium

The amount of sodium from the seeds was extracted as per the method given by Bhatnagar *et al.,* (2007).

For the determination of Na, 10 ml solution from acid extract prepared for calcium determination was used. Na content in the sample was measured by using flame photometer as against blank set.

The amount of sodium can be calculated as photometer

$$\text{Na (mg/100g)} = \frac{100/0.1 \text{ X Reading obtained through Flame photometer}}{10}$$

3.4.1.8.3 Potassium

Potassium was also determined with the help of Flame photometer as shown above method (3.4.1.8.2).

3.4.2 Protein characterization through SDS- PAGE

3.4.2.1 Preparation of stock solutions

The reagents and buffers for PAGE and SDS-PAGE analysis were prepared as per the procedure described by Sambrook *et al.,* (1989). The composition and procedure for preparation of various stock solutions and buffers are given in the table 3.4 and 3.5.

Table 3.4. Preparation of stock solutions

Sr. No	Solution	Method of preparation
1	Acrylamide/ Bis-acrylamide solution (30%), 100ml	29.2 g of Acrylamide and 0.8 g of Bis-acrylamide was dissolved in 50 ml deionised water. Final volume was adjusted to 100 ml. Solution were heated to 37˚C to dissolve the chemicals, filtered and stored in amber colored bottle at room temperature.
2	Tris-HCL buffer, 1.5M (pH 8.8), 100ml	Tris-base 18.17 g was dissolved in 50 ml Millipore water. pH was adjusted to 8.8 with concentrated HCl and final volume was made to 100 ml. Dispensed in to reagent bottle and stored at 4 ^{0}C
3	Tris-HCL buffer, 0.5M (pH 6.8), 100ml	Tris-base 6.0579 g was dissolved in 50 ml Millipore water. pH was adjusted to 6.8 with concentrated HCl and final volume was made to 100 ml. Dispensed in to reagent bottle and stored at 4˚C
4	SDS (Sodium Dodecyl/ Lauryl sulphate) 10%, 100ml	10 g of SDS was dissolved in 50 ml Millipore water. Final volume was made to 100 ml. Dispensed in to reagent bottle and stored at room temperature.
5	10% Ammonium per sulphate (APS)	100 mg APS was dissolved in 1 ml of distilled water. Prepared fresh at the time of gel casting.
6	TEMED	TEMED (Electrophoresis grade) was used directly
7	Electrode buffer (pH 8.3)	3 g 0.025 M Tris base and 14.4 g 0.192 M glycine was dissolved in distilled water and finally adjusted to 1000 ml. For SDS-PAGE 10 ml of 10% SDS was added and finally volume made up to 1000 ml.
8	Gel loading dye (1X)	For native-PAGE TrisCl (pH 6.8) 50 mM β- mercaptoethanol 100 mM 0.1% Bromophenol blue 10% Glycerol β- mercaptoethanol was added freshly
9	Commassie Brilliant Blue staining solution	0.25 % Commassie brilliant blue G-250 40 % Methanol 10 % Glacial acetic acid 50 % Distilled water Methanol, acetic acid solution was prepared. Commassie brilliant blue was dissolved in it and filtered with Whatman filter No. 1
10	Destaining solution	40 % Methanol 10 % Glacial acetic acid 50 % Distilled water

Table 3.5. Composition of Resolving and Stacking Gels

Resolving gel (8%)			Stacking gel (5%)		
	Components	20 ml		Components	4 ml
1	H2O	9.3 ml	1	H2O	2.700 ml
2	30% ACRYLAMIDE	5.3 ml	2	30 % ACRYLAMIDE	0.670 ml
3	1.5 M TRIS (pH 8.8)	5.0 ml	3	1 M TRIS (pH 6.8)	0.500 ml
4	10 % SDS	0.200 ml	4	10 % SDS	0.040 ml
5	10 % APS	0.200 ml	5	10 % APS	0.040 ml
6	TEMED	0.012 ml	6	TEMED	0.004 ml

3.4.2.2 Protein extraction:

Exactly, 300 mg of seed powder was taken and homogenized with 1ml of protein extraction buffer and vortexed the sample in 2 ml centrifuge tubes. These samples were centrifuged at 13,000 rpm for 10 minutes. Clear supernatant was collected and used for analysis as per the method described by Bhatnagar *et al.,* 2007.

Proteins were separated on 12% SDS polyacrylamide gel. The gel was washed in double distilled water to remove excess of SDS and stained for 4 hrs. Staining solution prepared by mixing 0.1 gm commassie brilliant blue R-250 in methanol: acetic acid and double distilled water (40:10:50). The gel was destained by using methanol: acetic acid and millipore water without dye.

3.4.3 Protein fractions on the basis of their solubility

Protein fractions were extracted by the method Bhatnagar *et al.* (2007). 100 mg of wheat flour was homogenized in a suitable solvent, time and temperature showed in Table 3.6 and then centrifuged at 6000 g for 10 mins. Filtrate was used for further analysis. Further estimation was done by the method described by Bhatnagar *et al.* (2007).

Table 3.6. Suitable temperature, time and solvent used for different protein fractions.

Fraction	Temp & Time	Solvent used
Albumin	4 °C, 4hrs	Distilled water
Globulin	4 °C, 4hrs	5% NaCl solution
Prolamine	20 °C 4hrs	70 % ethanol

3.4.3.1 SDS-PAGE Electrophoresis

Electrophoresis was conducted on vertical slab gel PAGE unit (Banglore Genie-Weltec) at 30 mA for 2hrs. The concentration of resolving gel was 8% and that for stacking gel was 5%. Each lane was loaded with 20 µl of protein extract and 6 µl protein molecular weight marker (29 kDa to 200 kDa). After the electrophoresis gels were washed to remove excess of SDS and stained with 0.1% commassie brilliant blue-G250 in a mixture of methanol: acetic acid : distilled water in the ratio 40: 10: 50. The gels were destained by using a mixture of methanol: acetic acid: distilled water in the ratio 40: 10: 50 without dye (Sadasivam and Manickam, 1992).

3.4.3.2Extraction of glutenin protein

Glutenin protein was extracted using protocol described by Pfluger *et al*., (2001) with some modifications; glutenin precipitate was dissolved in sample dilution buffer given by Singh *et al*., (1991).

20 mg of finely grinded flour was taken in a 2 ml eppendorf centrifuge tube and 100 µl of 1.5 M dimethyl formamide (DMF) was added. The solvent volume to seed weight ratio was 5:1 (µl: mg). The solution was centrifuged for 10 min at 14,000 g and the supernatant was stored at -20^0 C for subsequent gliadins analysis.

The pellet remaining after gliadin extraction was treated with 1 ml of 0.08 M Tris-HCL buffer (pH 8.5) containing 1% SDS for 30 min with intermittent vortexing followed by centrifugation 8 min at 14000 g. This procedure was

repeated twice discarding the supernatant. The pellet was used to extract glutenin subunits, using 0.08 M Tris-HCL buffer (pH 8.5) containing dithiotreitol (DTT) (1.5% w/v) and SDS (1% w/v). The extraction was carried out for 30 min at 60^0C with occasional vortexing followed by centrifugation for 10 min at 14,000g. 200µl of each supernatant were used for alkylation, employed 2.8 µl of 4-vinyl pyridine. Samples were incubated at 60^0C for 30 min with occasional vortexing. 1 ml Acetone was added in each sample to precipitate glutenins. Samples were maintained at -20^0C and then centrifuged for 10 min at 14,000g; discard supernatant and pellet were dissolved in dilution buffer (2% SDS, 40% Glycerol, 0.02% Bromophenol Blue and 0.08 M Tris HCL pH-8.0). After brief vortexing the samples were kept at 65^0C for 15 min and centrifuged at 10,000 rpm for 2 min and store samples at -20^0C.

3.5. Relative gene expression studies

3.5.1. Materials

3.5.1.1. Reagents, Glass-wares and Plastic wares

All the chemicals and reagents used in the present investigation were of extrapure or molecular biology grade quality obtained from various companies in India or abroad. The glass-wares and plastic-wares used were from Schott Duran, Germany and Axygen or Eppendorf, respectively. All the glass-wares used in the study were cleaned by using lab wash or detergent (Fine Chem. Pvt. Ltd., Biosar), washed with tap water and finally rinsed with distilled water. Glass-wares were dried in oven before use. All the plastic-wares like micropipette tips, PCR tubes, centrifuge tubes and eppendorf tubes were chloroform treated and double autoclaved before use.

3.5.1.2. Sample collection

Caryopses were collected from control and treated sample during maturity stage. Stored in RNA *later* at -80ċ for RNA isolation Chang *et al*. (1993).Three samples were collected from a plant and samples were pooled.

Three biological replicates (independent plants) were collected for quantitative PCR studies.

Table 3.7. List of durum Varieties with different irrigation treatment for the relative gene expression studies.

Sr. No.	***T. durum* Varieties**	**Treatment**
1	GW 1	Zero irrigation (T_0)
2	GW 1	One irrigation (T_1)
3	GW 1	Two irrigation (T_2)
4	A 206	Zero irrigation (T_0)
5	A 206	One irrigation (T_1)
6	A 206	Two irrigation (T_2)
7	A-9-30-1	Zero irrigation (T_0)
8	A-9-30-1	One irrigation (T_1)
9	A-9-30-1	Two irrigation(T_2)

3.5.2. Total RNA isolation

Total RNA was extracted from all the samples using modified CTAB method as described by Chang *et al*. (1993) with minor modifications.

The composition and procedure for preparation of various stock solutions and buffers are given in table Table 3.8 and 3.9.

Table 3.8. Preparation of stock solutions for RNA extraction and electrophoresis

Sr. No	Solution	Method of preparation
1	1M TrisHCl (pH 8.0),100 ml	In 80 ml distilled water 12.11 g of Tris base (Himedia) was dissolved and the pH was adjusted to 8.0 by adding concentrated HCl and the total volume was adjusted to 100 ml. It was dispensed into reagent bottle and sterilized by autoclaving.
2	0.5M EDTA (pH 8.0), 100 ml	In 80 ml distilled water 18.60 g of EDTA di-Sodium salt (Himedia) was dissolved and the pH was adjusted to 8.0 by adding NaOH pellets. The total volume was adjusted to 100 ml and dispensed into a reagent bottle and sterilized by autoclaving.
3	5M NaCl, 100 ml	In 50 ml of distilled water 29.22 g of NaCl (Himedia) was added and mixed well. When the salts were completely dissolved, the final volume was adjusted to 100 ml. It was dispensed into a reagent bottle and sterilized by autoclaving.
4	Chloroform: Isoamyl alcohol (24:1), 100 ml	Ninety six millilitre of chloroform (Fisher Scientific) and 4 ml of Isoamyl alcohol (Fisher Scientific) were measured, mixed well and stored in a amber bottle at room temperature.
5	70% Ethanol	For 1 ml, 700µl of ethanol mixed with 300µl of distilled water. Dispensed in to reagent bottle and store at 4^0C.
6	Ethidium Bromide (10 mg/ml), 1 ml	Ten milligram ethidium bromide (Himedia) was added to 1.0 ml of distilled water and it was dissolved completely. It was dispensed into a Sorenson Eppendorf tube and stored at 4 ^{o}C.
7	Chloroform	Ready to use, biotechnological grade.

8	Nuclease free water	Ready to use from FERMENTAS.
9	1 % SDS (Sodium Dodecyl/ Lauryl sulphate)	1g of SDS was dissolved in 50ml Millipore water. Final volume was made to 100ml. Dispensed in to reagent bottle and stored at room temperature.
10	8 M LiCl	33.912 g LiCl was dissolved in 50ml Millipore water. Final volume was made to 100ml. Dispensed in to reagent bottle and stored at room temperature.

Table 3.9. Preparation of buffers for RNA extraction

Sr. No.	Buffer	Method of preparation
1	CTAB (Cetyltrimethyl ammonium bromide)-Extraction buffer (5%), 10 ml	1 ml of 1 M TrisHCl (pH 8.0), 2.8 ml of 5 M NaCl, and 1 ml of 0.5 M EDTA (pH 8.0) were measured and mixed with about 5.2 ml of hot distilled water, add 0.5 g (W/V) CTAB (Himedia) and 0.2 g (W/V) PVP (Himedia), 1% SDS. This was dispensed to a reagent bottle. Just before use, 150 µl (1.5%) β-mercaptoethanol was added.
2	TE buffer (0.1 mM), 100 ml. 10 mMTrisHCl (pH 8.0) 0.1 mM EDTA(pH 8.0)	Hundred millilitre of TE buffer was prepared by adding 1 ml of TrisHCl (1M), 200 µl of EDTA (0.5M) to 99 ml of sterile distilled water in a reagent bottle. It was mixed thoroughly, autoclaved and stored at room temperature.
3	5X TBE buffer (1 liter)	Weighed 54 g of Tris base, in which 27.5 g of boric acid, 20 ml of 0.5M EDTA (pH 8.0) and around 450 ml distilled water were added. The salts were dissolved and the volume was adjusted to 1 litre.

3.5.2.1 Protocol for RNA isolation

RNA was extracted from the caryopsis by Cetyltrimethyl ammonium bromide (CTAB) method Chang *et al*. (1993) with minor modifications as follows:

1) Approximately 1.5 g of whole caryopsis were weighed, ground into clean mortar-pestle with liquid N_2 (-196 ^oC) and transferred the fine frozen powder to the centrifuge tube.
2) Eight hundred microliters of preheated CTAB extraction buffer was added to each sample and mixed well by inversion.
3) The sample was incubated for 1 hr at 65 ^oC in water bath (Allowed it to cool down).
4) Extract two times with an equal volume of chloroform:isoamyl alcohol, separating phases at room temperature by centrifugation for 15 min at 14,000 x g. Centrifuge longer if phases are not well separated.
5) Add 1/4 volume 8 M LiCl to the supernatant and mix. The RNA is precipitated overnight at 4℃ and harvested by centrifugation at 12,000 x g for 20 min.
6) After centrifugation, supernatant discarded and wash pellet with 70% ethanol by centrifugation at 13,000 x g for 15 min.
7) Ethanol was carefully drained and the pellet was air dried.
8) The pellet was dissolved into 100/200 µl nuclease free water.
9) RNA concentration was assessed through nanodrop and stored at -80^{0}C.

3.5.2.2 Qualitative and quantitative assessment of total RNA

To estimate the quantity and quality (in terms of protein and DNA contamination) of isolated RNA, 1µl RNA sample was loaded into the well of Nanodrop spectrophotometer (Thermo Scientific, U. S. A.) and the concentration and purity of the total RNA extracted was determined as the 260/280 nm and 260/230 nm ratio automatically calculated by the software.

The U. V. absorbance was checked at 260 and 280 nm for determination of sample concentration and purity.

3.5.3. cDNA synthesis

A two step quantitative RT-PCR protocol was carried out where reverse transcription and PCR mediated cDNA amplification were carried out in subsequent steps in separate tubes. Two step protocol was preferred when SYBR green was used as the detection dye because it diminishes the unwanted primer-dimer formation (Wong and Medrano, 2005). cDNA was synthesized from 3µg of total RNA using the first strand cDNA synthesis kit (Fermentas) using manufacturer's instruction. Reverse transcription reaction was performed with Oligo-(dT) primers because of the presence of the poly (A) tail present at the 3'-end of eukaryotic mRNA and reverse transcriptase enzyme.

3.5.3.1 Protocol

1. The following reagents were added into a sterile, nuclease-free tube on ice in the indicated order:

Template RNA	total RNA	0.1- 5 µg
Primer	oligo (dT)18 primer	1 µl
Nuclease-Free Water	_	Up To 11 µl
	Total Volume	11 µl

2. The RNA template which was GC-rich or contained secondary structures, mixed gently, centrifuged and incubated at 65°C for 5 min. After Chilling on ice it was spin down and the vial was placed back on ice.

3. The following components were added in the indicated order:

5X Reaction Buffer	4 µl
RiboLockRNase Inhibitor (20 µ/ µl)	1 µl
10 mMdNTP Mix	2 µl
M-MuLV Reverse Transcriptase (20 µ/ µl)	2 µl
Total volume	20 µl

4. Mix gently and centrifuge.

5. For oligo (dT)18 primed cDNA synthesis, incubation for 60 min at 37°C was practiced.

6. Terminated the reaction by heating at 70°C for 5 min.

The 20 µl cDNA samples synthesized were diluted to a final volume of 100µl by adding 80 µl nuclease free water and aliquoted into 5 tubes containing 20 µl each and stored at -20^0 C until downstream processing.

3.5.3.2 Standardization of newly designed glutenin gene specific and endogenous primers with cDNA through PCR

3.5.3.2.1 Primer design and synthesis:

The primers of Glutenin gene (Table 3.11) and endogenous control gene (Table 3.10) were mined from NCBI database and designed from the Primer BLAST a bioinformatic tool. Gene specific primers were designed from the accession numbers. For further conformation about primer designing IDT was used to check hairpin structure, GC content, melting temperature of primers. Primers stock was prepared in 100 pmol concentrations using 0.3 X TE buffer (Himedia) and it further diluted into 10 pmol concentrations for primer screening and RT-PCR work.

Table 3.10. Primers of endogenous genes for real time PCR:

1	*18SrRNA*	F	GACTCAACACGGGGAAACT
		R	AGACAAATCGCTCCACCAAC
2	*Actin*	F	GAATCCATGAGACCACCTAC
		R	AATCCAGACACTGTACTTCC
3	*26S rRNA*	F	CCGGTTGTTATGCCAATAGCA
		R	GCGGCGCAGCAGTTCT
4	*RLI*	F	TTGAGCAACTCATGGACCAG
		R	GCTTTCCAAGGCACAAACAT
5	*Ta50503*	F	GCACCTTGGCGGACTACAACATTC
		R	GACACCGAAGACGAGACTTGTGAACC

Table 3.11. Primers of glutenin genes for real time PCR:

1	LMW 6	F	AAATGTGGCAGCAGAGCAGT
		R	GGATGATGGAGTAGGTGATGG
2	LMW 8	F	ATGTGGCAGCAGAGCAGTT
		R	GGATGATGGAGTAGGTGATGG
3	HMW 10	F	TCGAGGCATACCAACAGGT
		R	GCACCACAGGTTGCTGCT
4	HMW 12	F	GGGCACGAGACAATACGAG
		R	AATATCATTTGTTGGAGTTGCTG

3.5.4. Screening of primers through PCR:

The screening of designed primers for identification of suitable internal control/endogenous genes for Glutenin gene, was carried out by thermal cycling conditions (Fig.3.1.) in all durum wheat varieties. Each primer pair was amplified with PCR reaction component (Table 3.12) in all genotypes and also a NTC (Non Template Control) was kept to decipher the intensity of primer dimers. The primer pairs that showed specific and unique band of expected size in gel analysis were further selected for qRT-PCR reactions.

Table: 3.12. PCR reaction mixture for screening of Primers

Sr. No	PCR Components	Volume
1	Master mix	7.5 µl
2	Forward primer	5.5µl
3	Reverse primer	0.5µl
4	cDNA	0.5µl
5	Nuclease free water	5.5µl
Total volume		**20.0 µl**

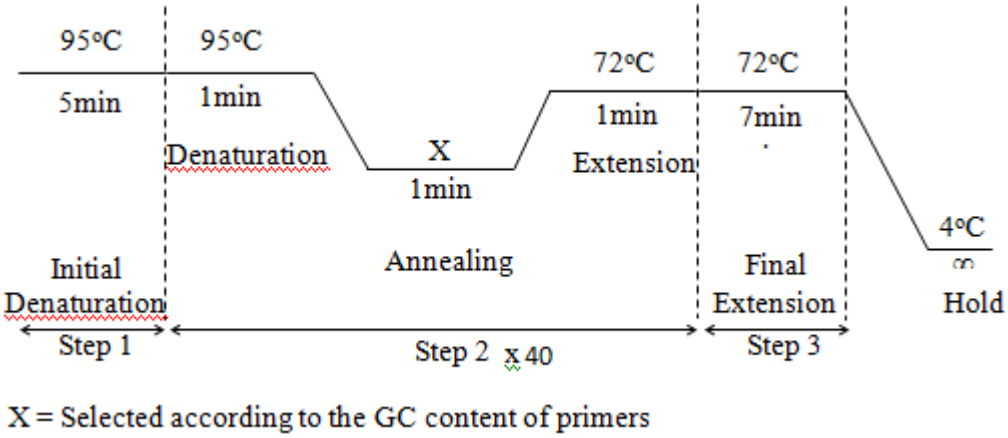


Fig.3.1. Thermal cycling conditions for screening of Primers

3.5.4.1 Electrophoresis.

Reagents / chemicals:

(a) Agarose (low EEO type; Bangalore Genei, India)

(b) 1 X Tris Borate EDTA (TBE), pH 8.0 (Amresco)

(c) 6X Gel loading dye (Bangalore Genei, India)

(d) Ethidium bromide (1 mg/ml)

(e) 100 bp DNA ladder (Bangalore Genei)

(f) 50 bp DNA ladder (Bangalore Genei)

Table 3.13. Preparations of buffers and solutions for gel electrophoresis

Ethidium bromide (10mg/ml)	1 g of ethidium bromide was added to 100ml of double distilled water and was dissolved properly. The container was wrapped in aluminum foil or transferred the solution to a dark bottle and stored at room temperature or 4^0C.
TBE buffer 5x (1 liter) pH 8.0	54.5 g of tris base, 27.5 g of boric acid (biogene) were taken, 20 ml of 0.5 M EDTA (pH 8.0) was added. The final volume of 1 liter was adjusted by adding distilled water and the pH was adjusted to 8.0.

All the PCR products were run on 1.5% agarose gel containing 4 µl of ethidium bromide (1 mg/ml). PCR product of 10 µl was mixed with 2 µl of 6x gel loading dye and loaded onto the well. The gel was run at 80 V current (constant) to separate the amplified bands. The 100 bp standard DNA marker (Bangalore Genei) was also run along with the samples. The separated bands were scored and documented under UV transilluminator using a gel documentation system (Bio-Rad, California).

3.5.5. Quantitative Real-time polymerase chain reaction (qRT- PCR)

3.5.5.1. Validation of endogenous genes and Relative quantification through qRT-PCR

3.5.5.1.1. Real-Time PCR Assay

PCR reactions were performed in 96-well plates (Bio-Rad, California) using SYBR Green detection chemistry to detect dsDNA synthesis. The amplification was carried out in a final reaction volume of 20 µl containing 1X Fast Power SYBR Green PCR master mix, 10 pmol of each gene specific forward and reverse primer and 2µl of cDNAtemplate. The PCR protocol was designed for 40 cycles. Fluorescence signals were measured once in each cycle at the end of the extension step. Three biological replicates for each sample

were used for real time PCR analysis and three technical replicates were analyzed for each biological replicates.

For each gene of interest, negative and positive controls were used. Reverse transcribed RNA was used as positive control. Negative control (NTC) were the samples in which cDNA was not added. For each sample a melt curve was generated after completion of amplification and analyzed in comparison to negative and positive controls, to determine the specificity of PCR reaction.

3.5.5.1.2 Components used for Real Time PCR

1) SYBR GREEN MASTER MIX (2X) Takara
2) Nuclease free water (Fermentas)
3) Primers (10pmoles) MWG
4) cDNA Template

Table 3.14. RT-PCR reaction mixture

Sr. No	PCR Components	Volume.
1	SYBR GREEN MASTER MIX	10µl
2	Forward Primer (10 pmoles/µl)	0.20µl
3	Reverse Primer (10 pmoles/µl)	0.20µl
4	cDNA	2.0µl
5	Nuclease free water	7.60µl
Total volume		**20.0 µl**

Since the quantitation work was carried out with non-specific SYBR green dye, it may result in false positives. Therefore detection of specific amplicon and primer dimers in each well has to be undertaken (Ririe *et al*., 1997). Therefore after 40 cycles, a melting-curve analysis (68°C to 95°C, one fluorescence read every 0.3°C) was performed to check the specificity of the amplifications.

Reactions were run in Bio-Rad PCR machine using the following cycling parameters as shown in Table 3.15.

Table 3.15. RT-PCR thermal cycling conditions

Sr. No.	Step	Temperature(°C)	Time
1	Holding	94	5 seconds
2	Cycling	94	10 seconds
3	Annealing	60	30 seconds
4	Extension	72	15 seconds
	Repeat step 2 to 4 for 40 cycles		
5	Final extension	60	35 seconds

3.5.6 Data analysis

Gene expression was evaluated by the $2^{-\Delta\Delta CT}$ method (Livak, 2001) values data analyzed by Data Assist software V 3.0 designed to perform relative quantification using comparative C_T ($\Delta\Delta C_T$) method by Applied Biosystem for relative gene expression of target genes.

Gene quantification was achieved using the C_q (Cycle Threshold) comparative method, and is expressed as ''n-fold up or down regulation of transcription'' in relation to a calibrator which is represented by the smallest signal detectable for that specific gene. For relative quantification by the comparative C_q method, values were expressed relative to a reference sample, called the calibrator. The expression of selected genes was calibrated by that of the reference gene, at each time point and converted to the relative expression ratio (fold of expression),

$$\text{Fold of Expression} = 2^{-\Delta\Delta C_q}$$

Where,

$\Delta\Delta C_q$ = Average dC_T of target– Average dC_q of calibrator

ΔC_q = Average C_T of target- Average C_T of endogenous control

Results and Discussion

IV. RESULT AND DISCUSSION

The results of the research experiment titled **"Effect of Limited Irrigation on Quality of Durum Wheat (*Triticum durum* Desf.)"** conducted at Department of Biochemistry, in collaboration with Center of Excellence in Biotechnology and B. A. College of Agriculture, Anand Agricultural University, Anand during 2013-2016 are being presented and discuss here by comparing to recent literature pertaining to different experiments in the following sub heading.

4.1 Biochemical characterization

4.1.1 Qualitative parameters /proximate composition

Different quality parameters affecting the quality of wheat in response to three irrigation treatments have been studied for durum wheat varieties. Mean data are presented in table 4.1.

4.1.1.1 Moisture content

Nasir *et al*., (2003) studied effect of moisture on the shelf life of wheat flour and found that moisture has significant effect on crude protein, crude fat, mould growth and insect infestation. Changes in ash and fiber were non-significant with regard to treatments and storage period. He concluded that 9 and 10% moisture content is suitable for storage stability and longer shelf life of wheat flour.

Moisture content of durum wheat varieties with irrigation treatment ranged from 10.68 to 12.63% (Table 4.2 (A)). These results revealed that

Table 4.1 Proximate composition

Variety	Moisture (%)	Ash (%)	TSS (%)	Starch (%)	Oil (%)	Total protein (%)	Lys/ Protein (%)	Tryp/ Protein (%)	Calcium (ppm)	Sodium (ppm)	Potassium (ppm)
GW 1	11.59	1.47	1.81	62.90	1.82	13.03	1.22	0.94	49.44	2.31	42.90
A-206	12.36	1.24	2.06	62.36	1.86	12.14	1.81	1.41	45.22	1.87	35.82
A-9-30-1	11.22	1.44	2.17	63.38	1.86	12.17	1.87	1.07	44.44	1.75	38.00
Sem±	0.153	0.009	0.028	0.051	0.010	0.064	0.019	0.005	0.525	0.003	0.019
CD at 5 %	0.454	0.025	0.083	0.152	0.030	0.189	0.055	0.016	1.554	0.010	0.056
Irrigation Treatment											
T_0	11.91	1.44	1.69	64.97	1.81	11.67	1.52	1.04	46.44	1.81	37.16
T_1	11.70	1.36	2.05	62.34	1.85	13.30	1.87	1.30	46.11	2.11	41.48
T_2	11.56	1.36	2.29	61.33	1.88	12.36	1.50	1.09	46.55	2.02	38.08
Sem±	0.153	0.009	0.028	0.051	0.010	0.064	0.019	0.005	0.525	0.003	0.019
CD at 5 %	NS	0.025	0.0.83	0.152	0.030	0.189	0.055	0.016	NS	0.010	0.056
V X I	NS	0.044	0.143	0.264	0.053	0.327	0.032	0.009	0.909	0.006	0.033
CV %	3.92	1.85	4.16	0.25	1.67	1.54	3.41	1.38	3.40	0.51	0.51

Abbreviations:

TSS-Total soluble sugar, **Lys**-Lysine, **Trp**-Tryptophan.

T_0 – No irrigation, **T_1** – One irrigation, **T_2**.Two irrigation.

Table 4.2 Proximate composition

Moisture (%)			
Varieties	T_0	T_1	T_2
GW 1	11.53	11.86	11.39
A-206	12.41	12.03	12.62
A-9-30-1	11.77	11.21	10.68
Sem±	0.266		
CD %	NS		
C.V %	3.92		

(A)

Ash (%)			
Varieties	T_0	T_1	T_2
GW 1	1.52	1.35	1.55
A-206	1.28	1.33	1.12
A-9-30-1	1.53	1.40	1.41
Sem±	0.015		
CD %	0.044		
C.V %	1.85		

(B)

TSS (%)			
Varieties	T_0	T_1	T_2
GW 1	1.80	1.90	1.73
A-206	1.44	2.06	2.14
A-9-30-1	1.84	1.66	3.02
Sem±	0.048		
CD %	0.143		
C.V %	4.16		

(C)

Starch (%)			
Varieties	T_0	T_1	T_2
GW 1	65.01	62.39	61.31
A-206	65.01	61.69	60.37
A-9-30-1	64.89	62.93	62.33
Sem±	0.089		
CD %	0.264		
C.V %	0.25		

(D)

Oil (%)			
Varieties	T_0	T_1	T_2
GW 1	1.82	1.82	1.82
A-206	1.80	1.87	1.93
A-9-30-1	1.81	1.87	1.91
Sem±	0.018		
CD %	0.053		
C.V %	1.67		

(E)

Total Protein (%)			
Varieties	T_0	T_1	T_2
GW 1	12.97	13.77	12.35
A-206	11.48	12.77	12.18
A-9-30-1	10.58	13.37	12.57
Sem±	0.110		
CD %	0.327		
C.V %	1.54		

(F)

Lys/ protein (%)			
Varieties	T_0	T_1	T_2
GW 1	1.09	1.48	1.10
A-206	1.70	2.15	1.58
A-9-30-1	1.79	2.00	1.82
Sem±	0.032		
CD %	0.095		
C.V %	3.41		

(G)

Tryp / protein (%)			
Varieties	T_0	T_1	T_2
GW 1	0.95	1.00	0.88
A-206	1.18	1.75	1.31
A-9-30-1	0.98	1.15	1.07
Sem±	0.009		
CD %	0.027		
C.V %	1.38		

(H)

Abbreviations:
TSS-Total soluble sugar, **Lys**-Lysine, **Trp**-Tryptophan.
T_0 – No irrigation, **T_1** – One irrigation, **T_2** -Two irrigation

Table 4.2 Proximate composition

Calcium (ppm)			
Varieties	T_0	T_1	T_2
GW 1	52.33	49.66	46.33
A-206	45.66	43.00	47.00
A-9-30-1	41.33	45.66	46.33
Sem±	0.909		
CD %	2.692		
C.V %	3.40		

(I)

Sodium (ppm)			
Varieties	T_0	T_1	T_2
GW 1	2.23	2.14	2.58
A-206	1.93	2.07	1.63
A-9-30-1	1.29	2.11	1.85
Sem±	0.006		
CD %	0.017		
C.V %	0.51		

(J)

Potassium (ppm)			
Varieties	T_0	T_1	T_2
GW 1	40.03	42.75	45.93
A-206	32.01	40.06	35.38
A-9-30-1	39.45	41.62	32.93
Sem±	0.033		
CD %	0.097		
C.V %	0.15		

(K)

Abbreviations:
TSS-Total soluble sugar, **Lys**-Lysine, **Trp**-Tryptophan.
T_0 – No irrigation, **T_1 –** One irrigation, **T_2** -Two irrigation

irrigation treatment did not play significant role in moisture content of durum wheat grains.

The study correlated with Danilton *et al.,* (2013) in which the grain moisture of three Durum wheat varieties showed no significance difference in non irrigated or irrigated treatments.

4.1.1.2 Ash content

Ash is primarily concentrated in the bran and is an indicator of flour yield. Higher bran content affects the appearance and shear value yields and is responsible for softer chapati (Prabhasankar *et al*., 2002).

Ash content of three wheat varieties with different irrigation treatment was ranged from 1.12 % to 1.55 %. GW 1 in two irrigation (T_2) registered highly significant ash content (1.55 %), which is at par with GW 1 (1.52 %) and A-9-30-1 (1.53 %) variety with no irrigation (T_0) (Table 4.2 (B)). Results were within the range (1.4 % to 2.1 %) by Prabhasankar *et al*., (2002).

4.1.1.3 Total soluble sugars (TSS)

TSS content in flour where ranged from 1.44 % to 3.02 % (Table 4.2 (C)). A-9-30-1 showed significantly higher Total soluble sugar (3.02 %) followed by A-206 (2.14 %) with two irrigation (T_2). Gooding and Davis (1997) reported total soluble sugars ranges from 1.5 to 2%.

4.1.1.4 Starch

Starch content was ranged from 61.31 % to 65.02 % with irrigation treatments in different wheat varieties. GW 1 and A-206 with no irrigation (T_0) showed significantly higher starch content (65.0167 %), at par with A-9-30-1 with no irrigation (T_0) (64.89 %) (Table 4.2 (D)). In the present study results were similar with Kousar *et al.,* (2012), Zhao *et al.,* (2009) and Gooding and Davis (1997).

4.1.1.5 Oil content

The oil content of different treatments varied from 1.77 to 1.88% in durum wheat varieties (Table 4.2 (E)). Significantly higher oil content was observed in A-206 with two irrigation (T_2), which at par with A-206 and A-9-30-1 with one irrigation (T_1). Zhao *et al.,* (2009) also reported oil contents in ranged 0.88 % – 1.21 % of the grain samples of wheat.

4.1.1.6 Total protein content

Protein content of three durum wheat varieties varied from 10.58 % to 13.77 %. Irrigation treatment showed significant effect on protein content. Significantly higher content of protein (13.77 %) registered in GW 1 with one irrigation treatment (T_1) (Table 4.2 (F)). The present finding was comparable with Danilton *et al.,* (2013) observed that higher values of protein in non-irrigated treatment (18.38 %) as compared to irrigated treatment (15.87 %). Guttieri *et al.,* (2005) also observed a lower concentration of proteins in irrigated wheat varieties as compared with those grown in conditions of low water availability.

4.1.1.7 Lysine and Tryptophan

The lysine content in studied durum wheat varieties varied from 1.09 % to 2.15 %, whereas tryptophan content varies from 0.88 % to 1.31 % (Table 4.2 (G)).

A-9-30-1 variety showed significantly higher lysine content (2.00 %) with one irrigation (T_1) whereas A-206 showed significant higher tryptophan content (1.75 %) in one irrigation (T_1). These results revealed that irrigation treatment did not show significant difference in lysine and tryptophan content of durum wheat grains. Faqir *et al.,* (2005) also found that lysine content ranged from 1.11% to 23.58% during chapati baking (12.4% protein basis).

4.1.1.8 Mineral content

Different mineral constituent are directly or indirectly related with the quality parameters, physico-chemical properties and enzyme activity of the plant. Three important mineral constituents have been studied and the results were tabulated in the Table 4.2.

4.1.1.8.1 Calcium content

The total amount of calcium present in durum wheat varieties with different irrigation treatments ranged from 41.33 – 55 ppm (Table 4.2 (I)). GW 1 showed significantly higher calcium content (52.33 %) with no irrigation (T_0), followed by GW 1 with one irrigation treatment (T_1). Similar range of calcium content (45 % to 85 %) was found by Zhao *et al.*, (2009).

4.1.1.8.2 Potassium content

Potassium content of three durum wheat varieties ranged from 32.01 to 45.93 ppm (Table 4.2 (J)). GW 1 variety showed significantly higher potassium content (45.93 %) in two irrigation (T_2) whereas A-206 reported significantly lower potassium content (32.01 %) in no irrigation (T_0) followed by A-9-30-1 (32.93 %) in two irrigation treatment (T_2). Earlier studied by Mohammad *et al.,* (2015) also analyzed that water limitation led to decrease in grain P uptake.

4.1.1.3 Sodium content

Sodium content of three durum wheat varieties with irrigation treatment ranged from 1.63 to 2.58 ppm (Table 4.2 (K)). Significantly higher sodium content (2.58) was recorded in GW 1 with two irrigation treatment while A-9-30-1 variety showed lower sodium content (1.29 %) in no irrigation (T_0).

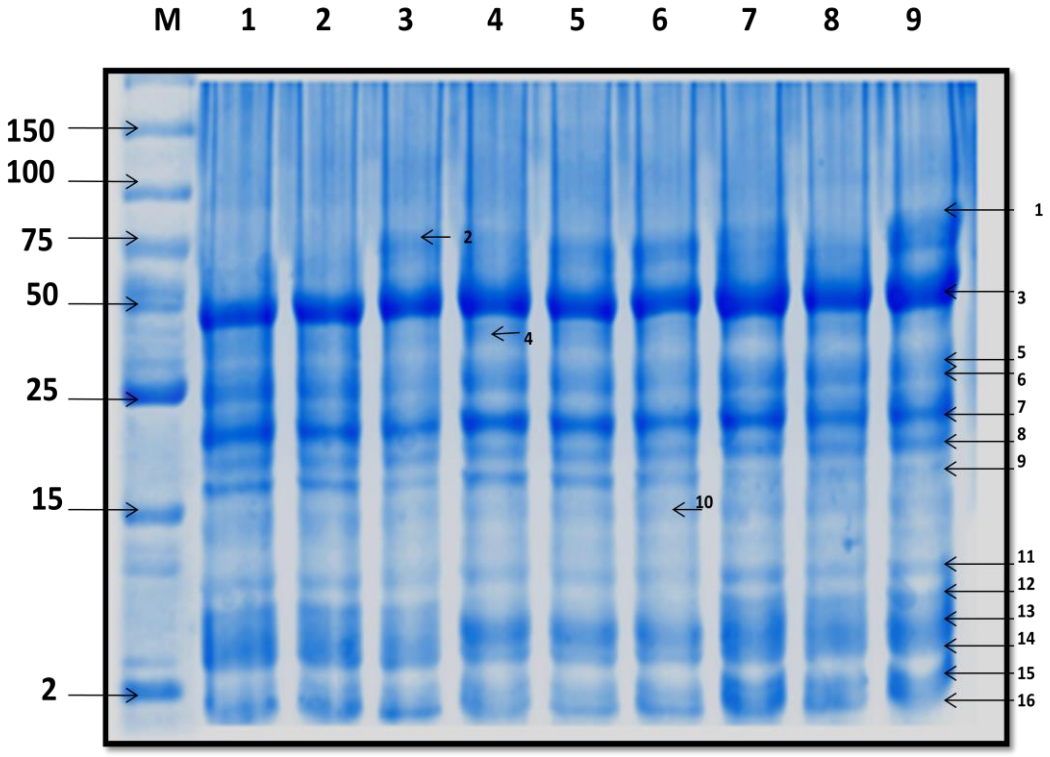


Fig. 4.2.1 Protein profile of Total protein from durum wheat varieties under different irrigation treatment.

M: Protein marker, 1: GW 1 (T_0), 2: GW 1 (T_1), 3: GW 1 (T_2), 4: A-206 (T_0), 5: A-206 (T_1), 6: A-206 (T_2), 7: A-9-30-1 (T_0), 8: A-9-30-1 (T_1), 9: A-9-30-1 (T_2)

*** T_0: No Irrigation, T_1: One Irrigation, T_2: Two Irrigation.**

Table 4.2.1 Banding pattern of Total proteins from durum wheat varieties by SDS-PAGE.

Band No.	Rm value	GW 1 (T_0)	GW 1 (T_1)	GW 1 (T_2)	A-206 (T_0)	A-206 (T_1)	A-206 (T_2)	A-9-30-1 (T_0)	A-9-30-1 (T_1)	A-9-30-1 (T_2)
1	0.21	+	+	+	-	-	-	-	-	+
2	0.25	-	-	+	-	+	+	+	+	+
3	0.33	+	+	+	+	+	+	+	+	+
4	0.37	-	-	-	+	-	-	+	+	-
5	0.41	+	+	-	+	-	-	+	+	+
6	0.44	+	+	-	+	+	+	+	+	+
7	0.49	+	+	+	+	+	+	+	+	+
8	0.54	+	+	+	+	+	+	+	+	+
9	0.57	+	+	+	+	+	+	+	+	+
10	0.63	+	+	-	+	+	+	+	+	-
11	0.72	+	+	+	+	+	+	+	+	+
12	0.76	-	-	-	-	-	-	+	+	+
13	0.78	-	-	-	+	+	+	+	-	-
14	0.82	+	+	+	+	+	+	+	+	+
15	0.87	-	-	-	+	+	+	+	+	+
16	0.91	+	+	+	+	+	+	+	+	+

Table 4.2.2 Similarity Index of total protein in durum wheat varieties with different treatment of irrigation.

	GW1 (T0)	GW1 (T1)	GW1 (T2)	A-206 (T0)	A-206 (T1)	A-206 (T2)	A-9301 (T0)	A-9301 (T1)	A-9301 (T2)
GW1(T0)	1.00								
GW1(T1)	**1.00**	1.00							
GW1(T2)	0.71	0.71	1.00						
A206(T0)	0.80	0.80	0.67	1.00					
A-206(T1)	0.73	0.73	0.71	0.93	1.00				
A-206(T2)	0.67	0.67	0.77	0.86	0.92	1.00			
A9301(T0)	0.73	0.73	0.60	0.93	0.86	0.79	1.00		
A9301(T1)	0.73	0.73	0.60	0.93	0.86	0.79	1.00	1.00	
A9301(T2)	**0.50**	0.50	0.57	0.67	0.71	0.64	0.71	0.71	1.00

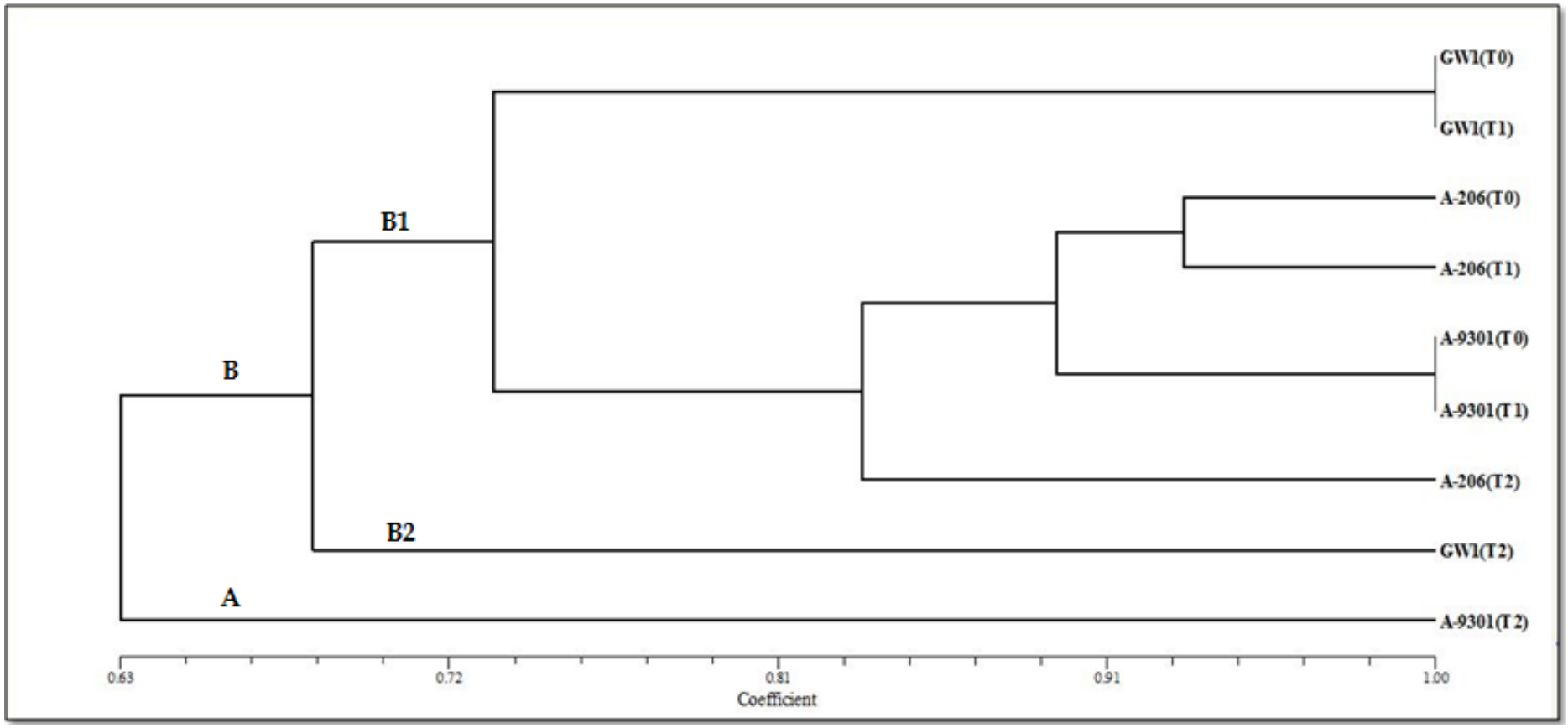


Fig 4.2.1 Dendogram of SDS-PAGE Profile of Grain protein in durum wheat variteies with irrigation treatments

4.2 Protein characterization through SDS-PAGE

Grain protein has been analyzed using 12 % resolving gel through SDS-PAGE. Analysis of total protein profile by SDS-PAGE revealed the presence of 16 bands of diverse molecular weight ranging from 2 to 150 kDa (Fig 4.2.1), in which 7 were monomorphic bands (Table 4.2.1). Though the varieties differed for the number of polypeptides, polymorphism was observed mostly for the molecular weight in the region of 15 to 75 kDa.

Varieties were identified on the basis of presence and absence of bands. For example, band no.1 (Rm=0.21) was only present in variety A-9-30-1 (T_2). Band no. 2 (Rm=0.25) was present in all the samples except GW 1 (T_0) and GW 1 (T_1). Band no. 3 (Rm= 0.33) were present all samples. Band no. 4 (Rm= 0.37) was present in GW 1(T_0), GW1 (T_1), A-206 (T_0), A-9-30-1 (T_0) and A-9-30-1 (T_1). Band no. 5 (Rm=0.41) was present in all samples. Band no. 6 (Rm=0.44) was present in all samples except GW 1 (T_2) and A-206 (T_2). Band no. 7 (Rm=0.49), (Rm=0.54) and 11(Rm=0.72) were present in all samples. Band no.9 (Rm=0.57) was absent in A-9-30-1 (T_0), A-9-30-1 (T_1) and A-9-30-1 (T_2). Band no. 10 (Rm=0.63) was present in all samples except GW 1 (T_2) and A-9-30-1 (T_2). Band no. 12 (Rm=0.76) was present in A-9-30-1 (T_0), A-9-30-1 (T_1) and A-9-30-1 (T_2). Band no. 13 (Rm=0.78) was peresnt in A-206 (T0), A-206 (T_1), A-206 (T_2), A-9-30-1 (T_0) and A-9-30-1 (T_2). Band no. 15(Rm=0.87) was present in all the samples except GW 1(T_0), GW 1(T_1) and GW 1(T_2). Band no.16 (Rm=0.91) was present in all the samples.

The Jaccard's similarity index (SI) was recorded in order to evaluate the degree of closeness among Varieties with different treatments. A perusal of the results has been presented in the Table 4.4.2, which indicated that, SI value was in the range of 0.5-1.0000. The maximum similarity value 1.00 was observed between GW1 (T_0) and GW (T_1) whereas the minimum value 0.5 was observed between GW1 (T_0) and A-9-30-1 (T_2), GW1 (T_1) and A-9-30-1 (T_2), A-9-30-1 (T_0) and A-9-30-1 (T_1). The high SI value indicated closeness may be either due to the effect of Irrigation.

Dendogram was obtained by Unweighted Pair Group Method of Arithmetic Means (UPGMA) clustering of the similarity matrix. Dendogram revealed the formation of two main clusters depicted as A and B. Main cluster A compromised only one variety A-9-30-1 (T_2). Main cluster B was divided into the sub-clusters B1 and B2. In Sub-cluster B1 varieties GW 1 (T_0) and GW 1 (T_1), A-9-30-1 (T_0) and A-9-30-1 (T_1) were closely related while variety A-206 (T_0) and A-206 (T_1) distantly related. Sub-cluster B2 includes only one variety GW 1 (T_2). T_2 treatment (Two irrigation) in all varieties were distantly related as compare to T0 (no irrigation) and T_1 (one irrigation) treatments.

Sharma *et al.*, (2015) studied diversity in 12 Indian wheat genotype on the basis of seed storage protein profiling by SDS-PAGE in which 15 subunits ranging from 14.3-97.4 kDa protein molecular weight marker (PMW-M).

4.3 Protein fractions on the basis of their solubility

4.3.1 Protein fraction of Albumin

Albumin protein profile by SDS-PAGE revealed the presence of 12 bands of diverse molecular weight ranging from 2 to 150 kDa (Fig 4.3.1) in which 2 were monomorphic bands (Table 4.3.1). Though the varieties differed for the number of polypeptides, polymorphism was observed mostly for the molecular weight in the region of 2 to 75 kDa.

Varieties were identified on the basis of presence and absence of bands. For example, band no.1 (Rm= 0.35) and 2 (Rm= 0.38) were present in all variety except GW 1 (T_2), A-9-30-1 (T_1) and A-9-30-1 (T_2). Band no. 3 (Rm= 0.41) was present only in A-206 (T_0) and A-206 (T_1) while Band no.4 (Rm= 0.44) present in A-206 (T_0), A-206 (T_1) and A-206 (T_2). Band no. 5 (Rm=0.49) was present in A-206(T_2) and A-9-30-1 (T_0). Band no. 6 (Rm=0.56) was present in A-206 (T_0), A-206 (T_1) and A-9-30-1 (T_2). Band no. 7 (Rm=0.62) was present A-206 (T_1), A-206 (T_2) and A-9-30-1 (T_0). Band no.8 (Rm=0.66) was in all samples except A-9-30-1 (T_1). Band no. 9 (Rm=0.70) and 10 (Rm=0.77) were present in all samples. Band no. 11(Rm=0.85) was GW 1 (T_0), GW 1 (T_1) and

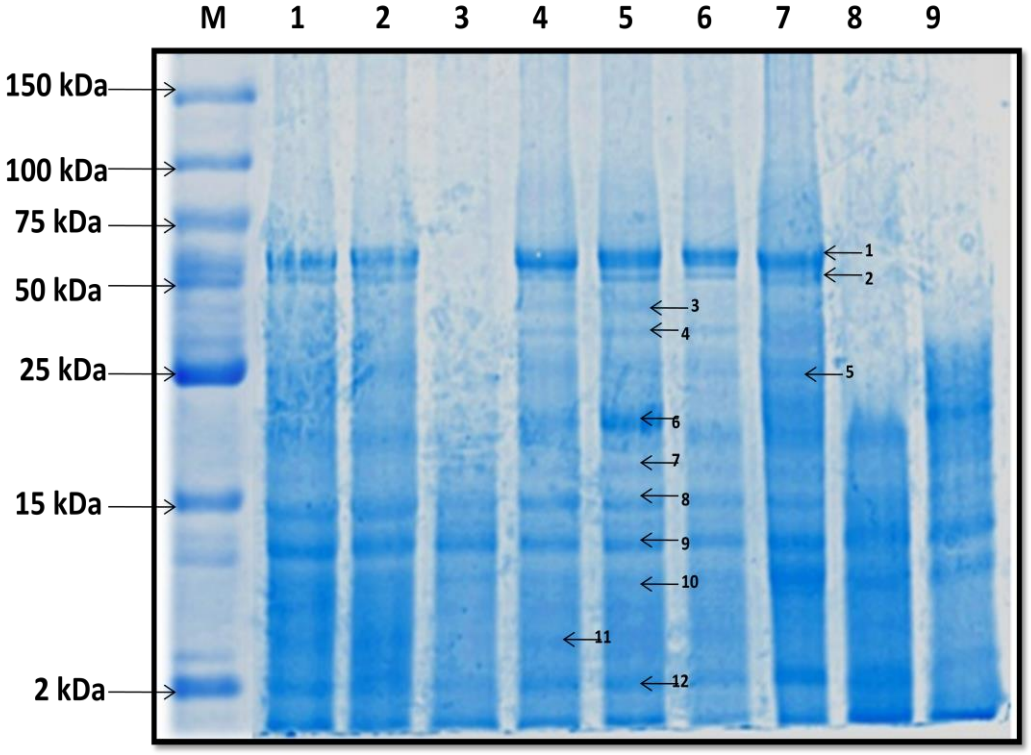


Fig. 4.3.1 Protein profile of Albumin from durum wheat varieties under different irrigation treatments

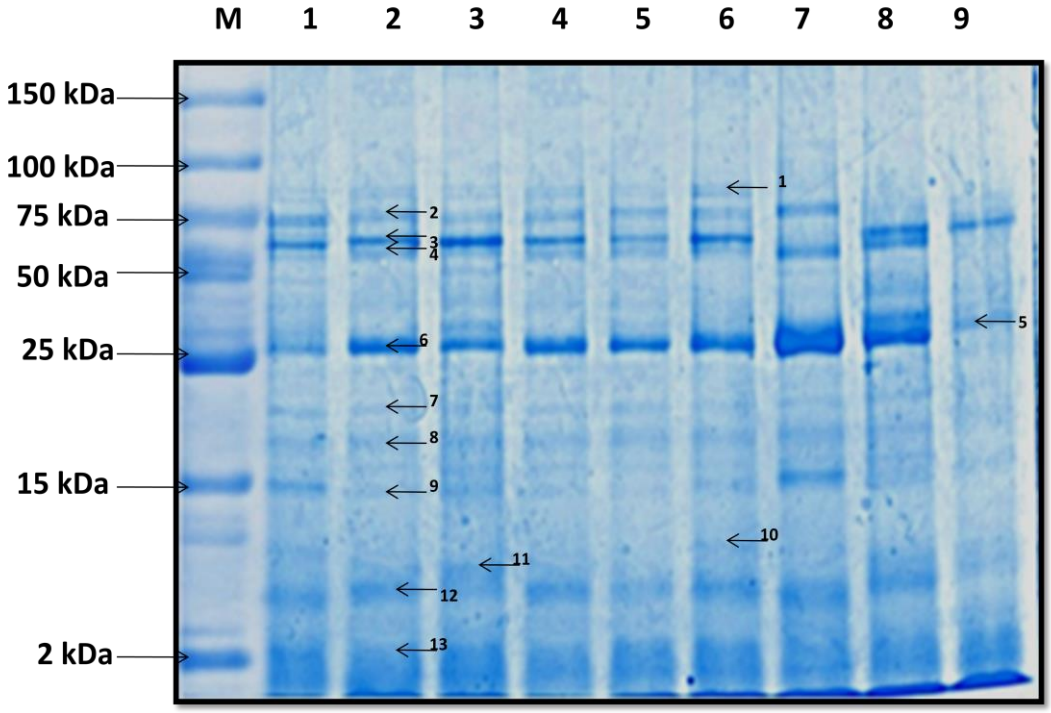


Fig. 4.3.3 Protein profile of Globulin from durum wheat varieties under different irrigation treatment.

M: Protein marker, 1: GW 1 (T_0), 2: GW 1 (T_1), 3: GW 1 (T_2), 4: A-206 (T_0), 5: A-206 (T_1), 6: A-206 (T_2), 7: A-9-30-1 (T_0), 8: A-9-30-1 (T_1), 9: A-9-30-1 (T_2)

T_0: No Irrigation, T_1: One Irrigation, T_2: Two Irrigation.

Table 4.3.1 Banding pattern of Albumin protein from durum wheat varieties by SDS-PAGE.

Band No.	Rm value	GW1 (T_0)	GW 1 (T_1)	GW 1 (T_2)	A-206 (T_0)	A-206 (T_1)	A-206 (T_2)	A-9-30-1 (T_0)	A-9-30-1 (T_1)	A-9-30-1 (T_2)
1	0.35	+	+	-	+	+	+	+	-	-
2	0.38	+	+	-	+	+	+	+	-	-
3	0.42	-	-	-	+	+	-	-	-	-
4	0.45	-	-	-	+	+	+	-	-	-
5	0.49	-	-	-	-	-	-	+	-	-
6	0.56	+	+	-	+	+	-	-	-	+
7	0.61	-	-	-	+	+	+	-	-	-
8	0.67	+	+	+	+	+	+	+	-	+
9	0.72	+	+	+	+	+	+	+	+	+
10	0.77	+	+	+	+	+	+	+	+	+
11	0.84	+	+	-	+	-	-	-	-	-
12	0.90	+	+	+	+	+	+	+	+	-

Table 4.3.2 Similarity Index of Albumin protein in durum wheat varieties with irrigation treatments of irrigation.

	GW1 (T0)	GW1 (T1)	GW1 (T2)	A-206 (T0)	A-206 (T1)	A-206 (T2)	A-9301 (T0)	A-9301 (T1)	A-9301 (T2)
GW1(T0)	1.00								
GW1(T1)	**1.00**	1.00							
GW1(T2)	0.57	0.57	1.00						
A206(T0)	0.64	0.64	0.36	1.00					
A-206(T1)	0.55	0.55	0.40	0.91	1.00				
A-206(T2)	0.60	0.60	0.44	0.67	0.73	1.00			
A-9301(T0)	0.67	0.67	0.50	0.58	0.64	0.89	1.00		
A-9301 (T1)	0.43	0.43	0.75	**0.27**	0.30	0.33	0.38	1.00	
A-9301 (T2)	0.38	0.38	0.60	0.36	0.40	0.30	0.33	0.40	1.00

Table 4.3.4 Similarity Index of Globulin protein in durum wheat varieties with irrigation treatments

	GW1 (T0)	GW1 (T1)	GW1 (T2)	A-206 (T0)	A-206 (T1)	A-206 (T2)	A-9301 (T0)	A-9301 (T1)	A-9301 (T2)
GW1(T0)	1.00								
GW1(T1)	**1.00**	1.00							
GW1(T2)	0.82	0.82	1.00						
A206(T0)	1.00	1.00	0.82	1.00					
A-206(T1)	0.90	0.90	0.73	0.90	1.00				
A-206(T2)	0.91	0.91	0.75	0.91	0.82	1.00			
A-9301(T0)	0.80	0.80	0.64	0.80	0.89	0.73	1.00		
A-9301(T1)	0.73	0.73	0.58	0.73	0.80	0.67	0.70	1.00	
A-9301(T2)	0.25	0.25	0.36	0.25	0.27	**0.23**	0.18	0.27	1.00

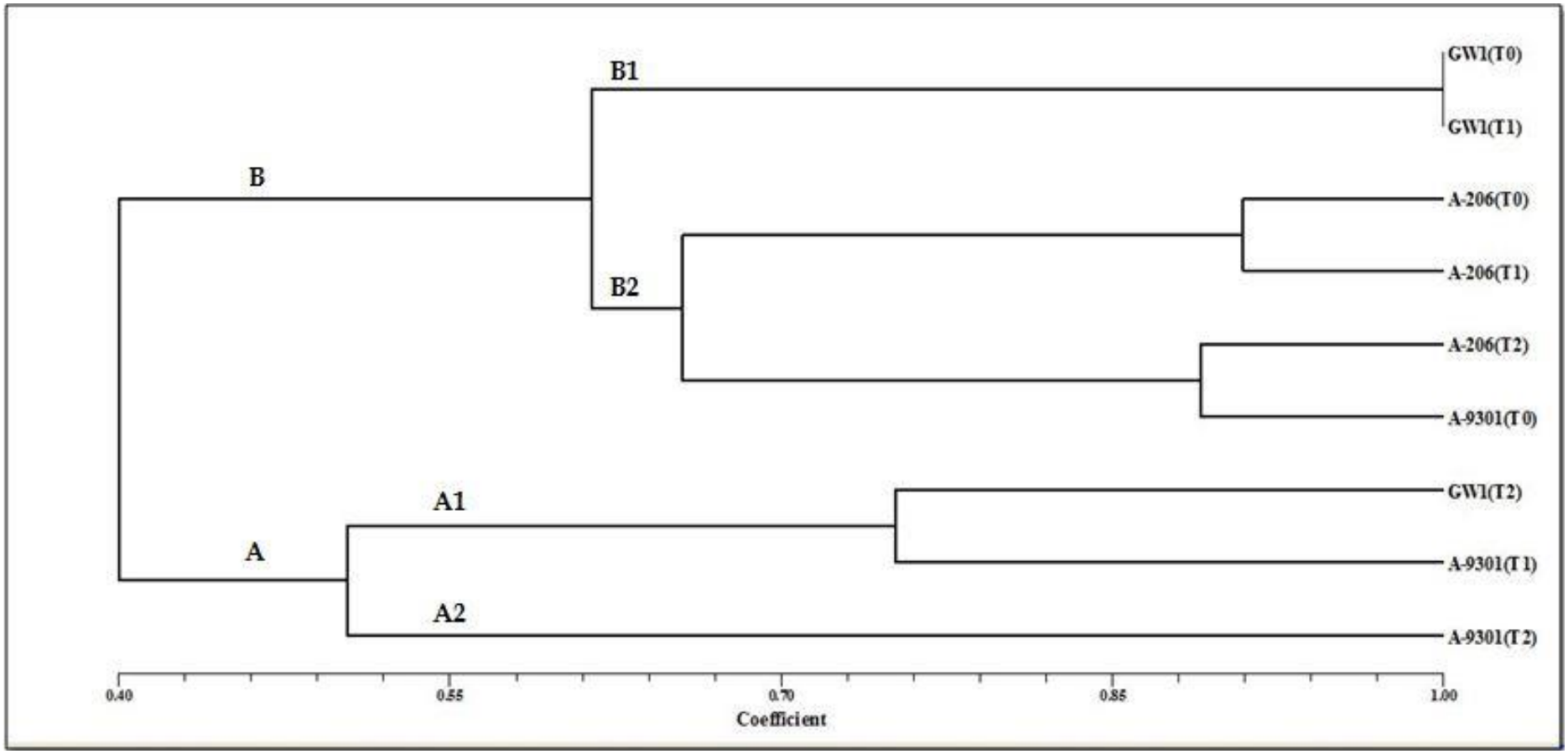


Fig. 4.3.2 Dendogram of SDS-PAGE Profile of Albumin protein in durum wheat variteies with irrigation Treatments.

A-206 (T_0). Band no. 12 (Rm=0.90) was peresnt in all variety except A-9-30-1 (T_2).

The Jaccard's similarity index (SI) was recorded in order to evaluate the degree of closeness among varieties with different treatments. A perusal of the results has been presented in the Table 4.3.2, which indicated that, SI value was in the range of 0.27-1.00. The maximum similarity value 1.00 was observed between GW1 (T_0) and GW (T_1) whereas the minimum value 0.27 was observed between A-206 (T_0) and A-9-30-1 (T_1). The high SI value indicated closeness may be either due to the effect of Irrigation.

Clustering of albumin on the basis of similarity matrix was obtained by Unweighted Pair Group Method of Arithmetic Means (UPGMA). Dendogram revealed the formation of two main clusters depicted as A and B. Main cluster A was divided in sub cluster A1 and A2. Cluster A1 include GW 1 (T_2) and A-9-30-1 (T_1) while Cluster A2 include A-9-30-1 (T_2). Cluster B was divided in sub cluster B1 and B2.Sub-cluster B1 includes variety GW 1 (T_1) and GW 1 (T_1) closely related. In Sub-cluster B2 variety A-206 (T_0) and A-206 (T_1), A-206 (T_1) and A-9-30-1 (T_0) distantly related (Figure 4.3.2). In clustering pattern of albumin variety A-9-30-1 with two irrigation treatment showed distantly related as compare to GW 1 and A- 206 with control, one and two irrigation treatments.

Due to effect of irrigation all varieties were differentiate on the basis of albumin banding pattern. GW 1 (T_2) variety and A-9-30-1 (T_1) and A-9-30-1 (T_2) showed some diffused banding pattern while all other variety with irrigation treatment comprised these diffused bands. So Albumin banding pattern found to be useful to differentiate with irrigation treatments.

4.3.2 Protein fraction of Globulin

All varieties showed 13 bands of globulin protein by SDS-PAGE with molecular weight ranging from 2 to 150 kDa (Fig 4.3.3), in which 1 was monomorphic bands (Table 4.3.3). Though the varieties differed for the

Table 4.3.3 Banding pattern of Globulin proteins from durum wheat varieties by SDS-PAGE.

Band No.	Rm value	GW 1 (T_0)	GW 1 (T_1)	GW 1 (T_2)	A-260 (T_0)	A-260 (T_1)	A-260 (T_2)	A-9-30-1 (T_0)	A-9-30-1 (T_1)	A-9-30-1 (T_2)
1	0.20	+	+	-	+	-	+	-	-	-
2	0.24	+	+	+	+	+	+	+	-	-
3	0.27	+	+	+	+	+	+	-	+	+
4	0.29	+	+	+	+	+	+	+	+	-
5	0.40	-	-	-	-	-	-	-	+	+
6	0.43	+	+	+	+	+	+	+	+	-
7	0.53	+	+	-	+	+	+	+	+	-
8	0.57	+	+	+	+	+	+	+	+	-
9	0.65	+	+	-	+	+	+	+	-	-
10	0.72	+	-	-	-	-	+	-	-	-
11	0.76	-	-	+	-	-	-	-	-	-
12	0.80	+	+	+	+	+	+	+	+	+
13	0.89	+	+	+	+	+	+	+	+	-

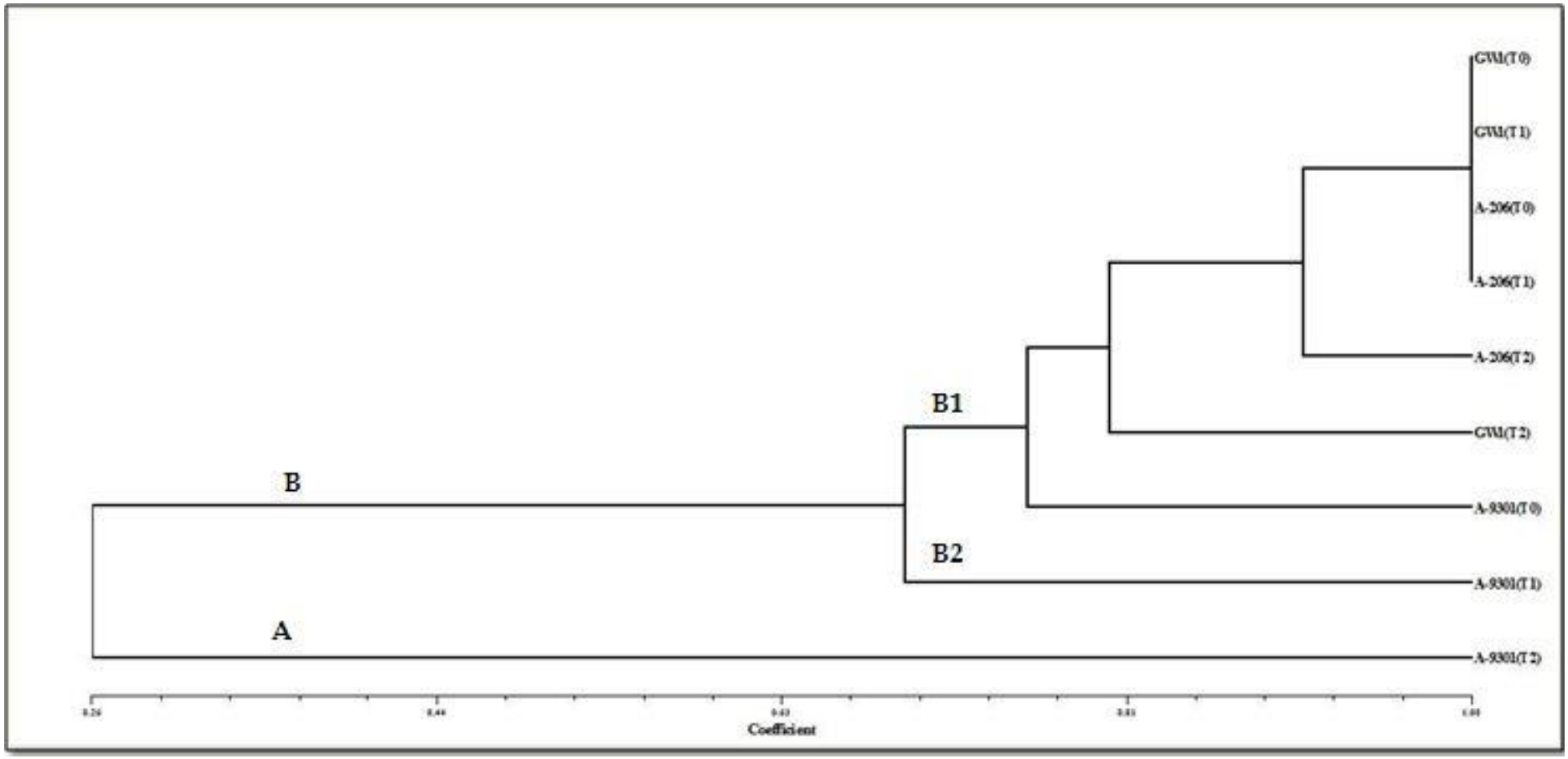


Fig 4.3.4 Dendogram of SDS-PAGE Profile of Globulin protein in durum wheat variteies with irrigation Treatments.

number of polypeptides, polymorphism was observed mostly for the molecular weight in the region of 15 to 100 kDa.

Varieties were identified on the basis of presence and absence of bands. For example, band no.1 (0.20) was present in all samples except variety A-9-30-1 (T_0), A-9-30-1 (T_1) and A-9-30-1 (T_2). Band no. 2 (Rm=0.24) was present in all the samples except A-9-30-1 (T_1). Band no. 3 (Rm= 0.27) was in present all sample except A-9-30-1 (T_0). Band no. 4 (Rm= 0.29) was only absent in A-9-30-1 (T_2). Band no. 5 (Rm=0.40) was present in A-9-30-1 (T_1) and A-9-30-1 (T_2). Band no. 6 (Rm=0.43) was present in all samples except A-9-30-1 (T_2). Band no. 7 (Rm=0.53) was present in all samples except in GW 1(2) and A-9-30-1 (T_2). Band no. 8 (Rm=0.57), 9 (Rm=0.64), 12 (Rm=0.80) were present in all samples except A-9-30-1 (T_2). Band no.10 (Rm=0.72) was present only in A-206 (2). Band no. 11 (Rm=0.76) was present in GW 1(T_2) and A-9-30-1 (T_2). Band no. 13(Rm=0.89) was peresnt all varieties with different treatments.

The Jaccard's similarity index (SI) was recorded in order to evaluate the degree of closeness among varieties with different treatments. A perusal of the results has been presented in the Table 4.3.4, which indicated that, SI value was in the range of 0.18-1.0000. The maximum similarity value 1.00 was observed between GW1 (T_0) and GW (T_1) whereas the minimum value 0.18 was observed between A-9-30-1 (T_0) and A-9-30-1 (T_1).

Dendogram generated by Globulin protein formed two main clusters depicted as A and B. Main cluster A compromised only one variety A-9-30-1 (T_2). Main cluster B was divided into the sub-clusters B1 and B2. In Sub-cluster B1 varieties GW 1 (T_0), GW 1 (T_1), GW 1 (T_2), A-206 (T_0), A-206 (T_1) were closely related while GW1 (T_2) A-206 (T_1) distantly related. Sub-cluster B2 includes only one variety A-9-30-1 (T_1) (Figure 4.3.4).

4.3.3 Protein fraction of Glutenin

Glutenin protein showed 20 bands of diverse molecular weight ranging from 14.4 to 150 kDa (Fig 4.3.5), in which 9 were monomorphic bands

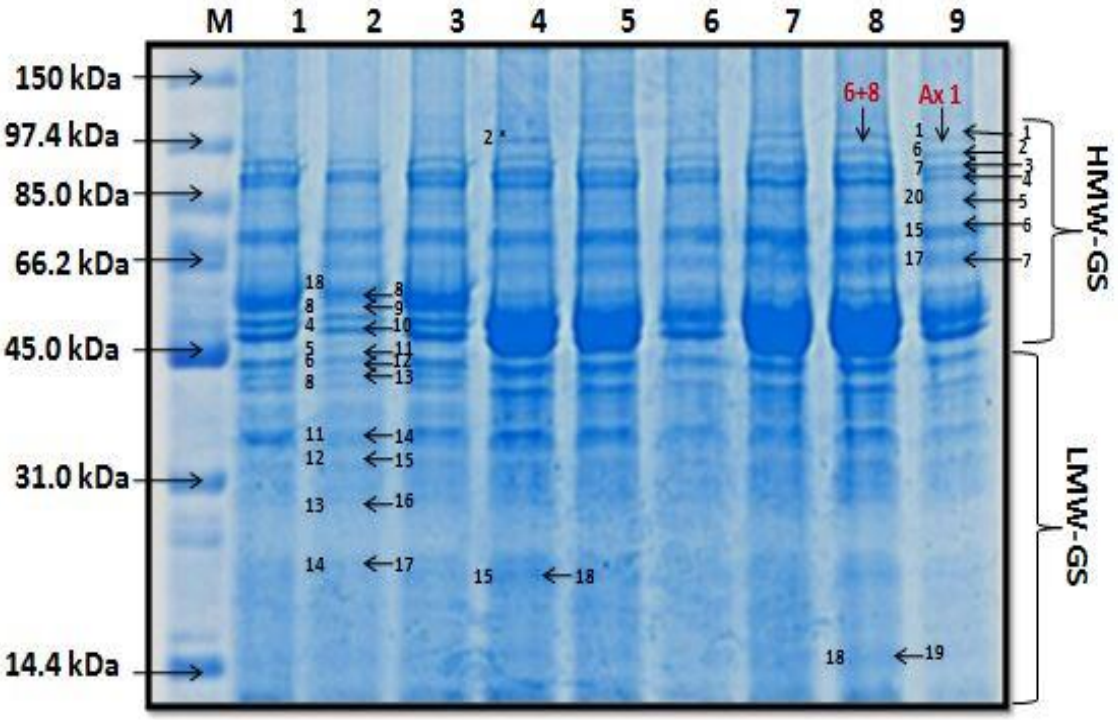


Fig. 4.3.5 Protein profile of Glutenin from durum wheat varieties under different irrigation treatment.

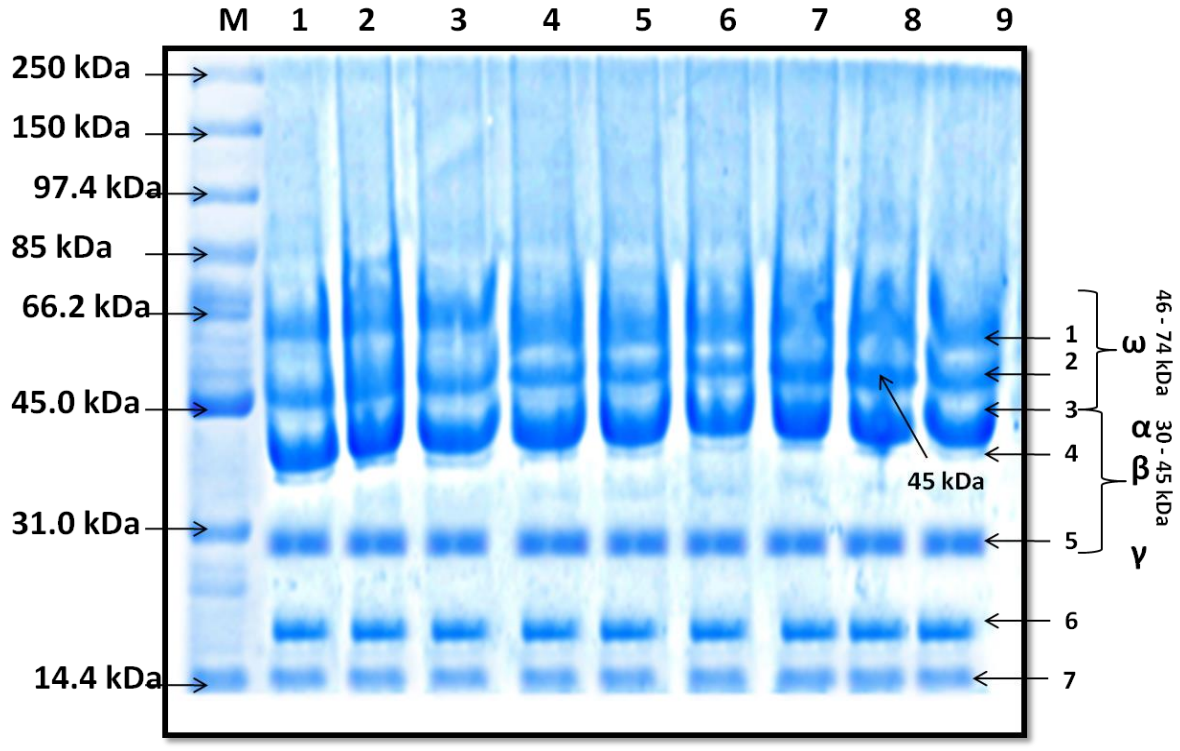


Fig.4.3.7 Protein profile of Gliadin from durum wheat varieties under different irrigation treatment.

M: Protein marker, 1: GW 1 (T_0), 2: GW 1 (T_1), 3: GW 1 (T_2), 4: A-206 (T_0), 5: A-206 (T_1), 6: A-206 (T_2), 7: A-9-30-1 (T_0), 8: A-9-30-1 (T_1), 9: A-9-30-1 (T_2)

T_0: No Irrigation, T_1: One Irrigation, T_2: Two Irrigation.

Table 4.3.5 Banding pattern of Glutenin protein from durum wheat varieties by SDS-PAGE.

Band No.	Rm value	GW 1 (T_0)	GW 1 (T_1)	GW 1 (T_2)	A-206 (T_0)	A-206 (T_1)	A-206 (T_2)	A-9-30-1 (T_0)	A-9-30-1 (T_1)	A-9-30-1 (T_2)
1	0.33	-	-	-	+	+	+	+	+	+
2	0.36	-	-	+	+	+	+	+	+	+
3	0.37	+	+	+	+	+	+	+	+	+
4	0.39	+	+	+	+	+	+	+	+	+
5	0.41	-	-	+	-	-	-	+	+	+
6	0.45	+	+	+	+	+	+	+	+	+
7	0.48	+	+	+	+	+	+	+	+	+
8	0.51	+	+	+	+	+	-	+	+	+
9	1.52	+	+	+	+	+	+	+	+	+
10	1.54	+	+	+	+	+	+	+	+	+
11	1.58	-	+	-	-	-	-	-	-	-
12	1.60	+	+	+	+	+	+	+	+	+
13	1.62	+	+	+	+	+	+	+	+	+
14	1.68	+	+	+	+	+	+	+	+	+
15	1.70	+	+	-	-	-	-	+	+	-
16	1.74	+	+	-	-	-	-	+	+	-
17	1.80	+	+	+	-	+	-	+	+	-
18	1.82	-	-	-	+	-	-	-	-	-
19	1.92	-	-	-	-	-	-	+	+	-

Table 4.3.6 Similarity Index of Glutenin protein in wheat varieties with irrigation treatments

	GW1 (T0)	GW1 (T1)	GW1 (T2)	A-206 (T0)	A-206 (T1)	A-206 (T2)	A-9301 (T0)	A-9301 (T1)	A-9301 (T2)
GW1(T0)	1.00								
GW1(T1)	**0.93**	1.00							
GW1(T2)	0.73	0.69	1.00						
A206(T0)	0.63	0.59	0.73	1.00					
A-206(T1)	0.73	0.69	0.86	0.86	1.00				
A-206(T2)	0.60	**0.56**	0.71	0.85	0.85	1.00			
A-9301(T0)	0.76	0.72	0.76	0.67	0.76	0.65	1.00		
A-9301(T1)	0.76	0.72	0.76	0.67	0.76	0.65	1.00	1.00	
A-9301(T2)	0.63	0.59	0.86	0.86	0.86	0.85	0.76	0.76	1.00

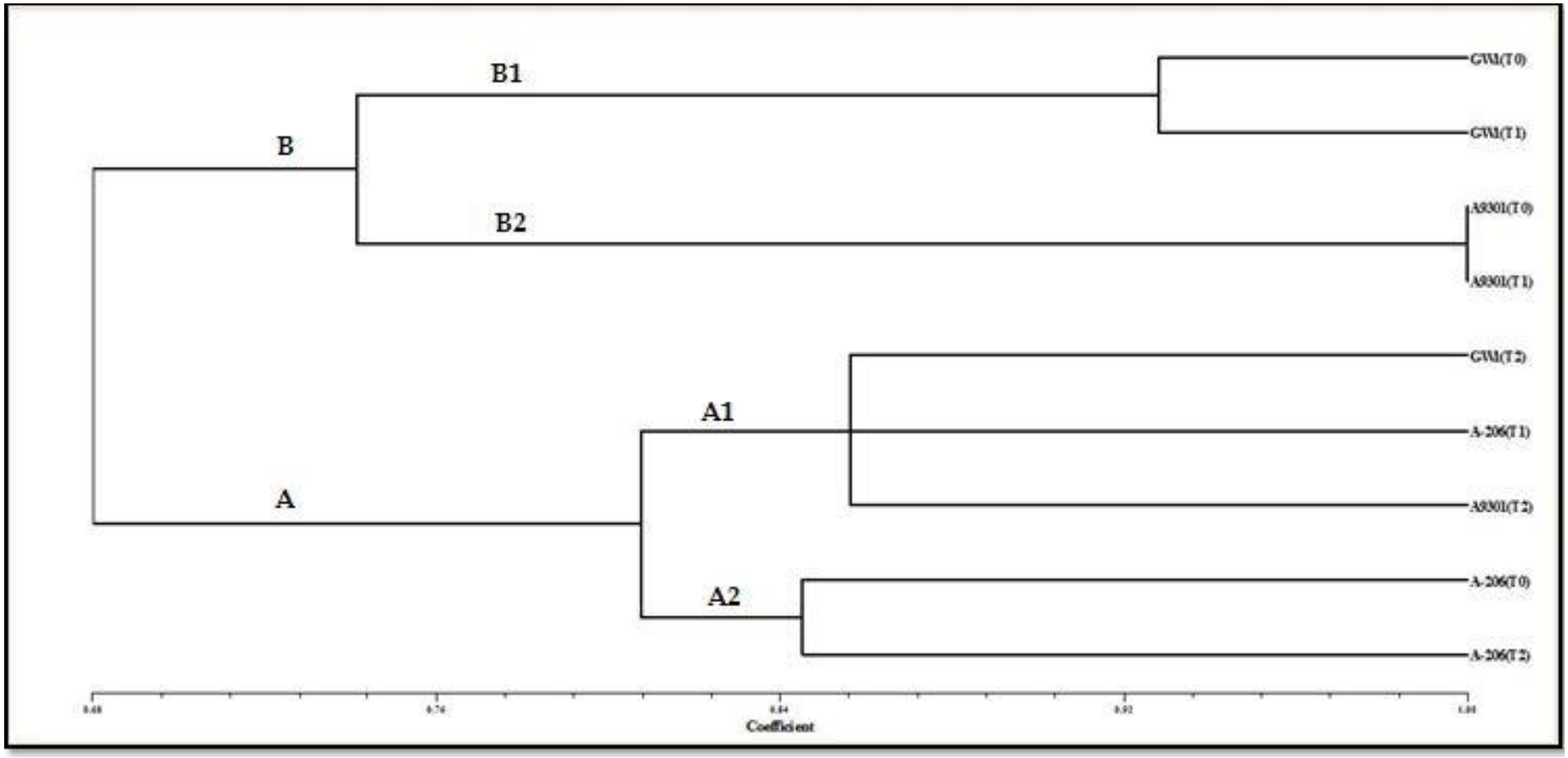


Fig 4.3.6 Dendogram of SDS-PAGE Profile of Glutenin protein in durum wheat variteies with irrigation Treatments.

(Table 4.3.5). Though the varieties differed for the number of polypeptides, polymorphism was observed mostly for the molecular weight in the region of 14.4 to 66.2 kDa.

Varieties were identified on the basis of presence and absence of bands. For example, band no.1 (0.33) was present in all samples except variety GW 1(T_0), GW 1(T_1) and GW 1(T_2). Band no. 2 (Rm=0.36) was absent in GW 1(T_0), GW 1(T_1). Band no. 3 (Rm=0.37), 4 (Rm=0.39), 6 (Rm=0.45), 7 (Rm=0.48), 9 (Rm=0.52), 10 (Rm=0.54), 12 (Rm=0.60), 13 (Rm=0.62), and 14 (Rm= 0.68) were present all samples. Band no. 5 (Rm= 0.41) was present in GW 1(T2), A-9-30-1 (T_0) and A-9-30-1 (T_1) and A-9-30-1 (T_2). Band no. 8 (Rm=0.51) was absent only in A-206 (T_2). Band no. 11 (Rm=0.58) was present only in GW 1 (T_1). Band no. 15 (Rm=0.70) and 16 (Rm=0.74) were present in GW 1 (T0), GW 1 (T_1), A-9-30-1(T_0) and A-9-30-1 (T_1). Band no.17 (Rm=0.81) was present in A-206 (T_0), A-206 (T_2) and A-9-30-1 (T_2). Band no. 18 was absent only in A-206 (T_0). Band no. 19 (Rm=0.92) was present in A-9-30-1 (T_0) and A-9-30-1 (T_1).

The Jaccard's similarity index (SI) was recorded in order to evaluate the degree of closeness among varieties with different treatments. A perusal of the results has been presented in the Table 4.3.6, which indicated that, SI value was in the range of 0.59-0.93. The maximum similarity value 0.93 was observed between GW1 (T_0) and GW (T_1) whereas the minimum value 0.59 was observed between GW1 (T_1) and A-206 (T_0). The high SI value indicated closeness may be either due to the effect of Irrigation.

Glutenin protein comprised two main clusters depicted as A and B. Main cluster A was subdivided into sub-cluster A1 and A2. Sub-cluster A1 compromised GW 1 (T_2), A-206 (T_1) and A-9-30-1 (T_2).Sub-cluster A2 includes A-206 (T_0) and A-206 (T_2). Main cluster B was divided into the sub-clusters B1 and B2. Sub-cluster B1 includes GW 1(T_0) and GW 1 (T_1). In Sub-cluster B2 varieties A-9-30-1 (T_0) and A-9-30-1 (T_1) were closely related (Figure 4.3.6).

Glutenin protein was separated in two major groups on one-dimensional discontinuous SDS-PAGE. First group was HMW-GS (45-112

kDa) and second group was LMW-GS (14-43 kDa). In durum varieties. Ax-1 allele was present in A-9-30-1 with all treatments of irrigation whereas Ax-2* allele present in all varieties of durum wheat except GW-1, which were encoded by Glu-A1 locus, responsible for good bread making quality. These two allele Ax-1 and Ax-2* was absent in GW 1 revealed suitability for other end use product. Allele 6+8 and 20+8 were present in all durum varieties that encoded by Glu-B1 locus.

4.3.1 Protein fraction of Gliadin

Gliadin protein profile generated 7 bands of diverse molecular weight ranging from 14.4 to 85 kDa by SDS PAGE (Fig 4.3.7), in which 7 monomorphic bands.

The Gliadin protein was separated in α, β, γ and ω subunits ranging from 30-80 kDa on SDS-PAGE. Varieties were identified on the basis of presence and absence of bands. All bands were present in all variety with different treatment of irrigation. In durum wheat gliadin γ-42 and γ-45 serve as marker for poor and good gluten strength, respectively (Payne *et al.*, 1983). The γ-45 was present in all durum wheat varieties with irrigation treatments, which shows some specific characteristic in relation to end use quality.

4.4 Gene expression profiling.

4.4.1. Qualitative and Quantitative analysis of RNA.

The quality and quantity of total RNA isolated from control as well as treatment samples (T_0, T_1, and T_2) by CTAB method were tested on nanodrop spectrophotometer (Table 4.4.1.). It is mandatory that RNA extracted should be free of contaminants such as proteins, DNA, cellular material and reagents associated with RNA extraction (phenol, ethanol and salts). Impurities in the RNA sample may lead to inhibition of the RT-PCR reaction, yielding biased data.

The quality of total RNA extracted ranged from 2.1 to 2.17 at an absorbance ratio of A260/A280 and 2.00 to 2.20 at an A260/A230. The concentration of RNA ranged from 807.8 to 1516.0 ng/µl. Importance of similar good quality and quantity of RNA for Real Time PCR was highlighted by Chomczynski and Mackey, (1995); Gallup, (2011). The quality of RNA obtained was also desirable for downstream process. The consistency in both purity and quality across all RNA samples ensure reduction in variability between biological replicates as reported by Imbeaud *et al*., (2005).

Table 4.4.1. Quantification of RNA from Durum wheat Varieties.

Name of the sample	Treatment	Concentration of RNA (ng/µL)	260/280 ratio	260/230 ratio
GW 1	T_0	1432.4	2.15	2.28
	T_1	807.8	2.16	2.21
	T_2	952.9	2.15	2.20
A-206	T_0	967.8	2.16	2.16
	T_1	1103.7	2.15	2.16
	T_2	1516.0	2.18	2.22
A-9-30-1	T_0	1189.1	2.1	2.15
	T_1	1094.1	2.17	2.16
	T_2	1451.5	2.15	2.14

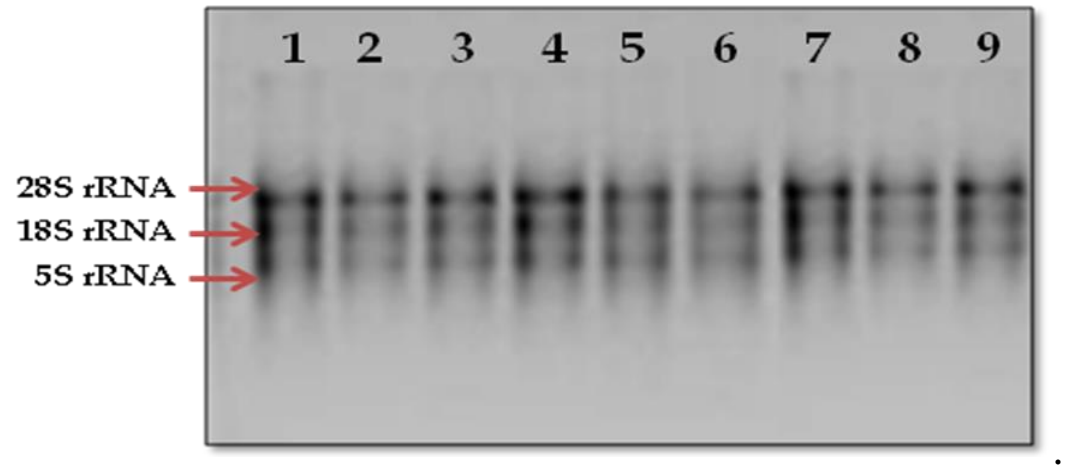


Fig. 4.4.1 RNA of durum wheat samples on 1.5 % agarose gel electrophoresis

1. **GW 1 (T_0)**
2. **GW 1 (T_1)**
3. **GW 1 (T_2)**
4. **A-206 (T_0)**
5. **A-206 (T_1)**
6. **A-206 (T_2)**
7. **A-9-30-1 (T_0)**
8. **A-9-30-1 (T_1)**
9. **A-9-30-1 (T_2)**

4.4.2. Qualitative analysis of cDNA

To ensure same amount of starting sample for all cDNA synthesis, all RNA samples prepared were diluted to a final concentration of 2000 ng/µl for three treatments (T_0, T_1 and T_2) which in turn minimize the variability. cDNA was prepared from Oligo-(dT) primers using RNA as template. The integrity of the cDNA samples prepared was checked on 1.5 % agarose gel, which was further used for RT-PCR.

4.4.3. Validation of constitutively expressed genes

For each sample cDNA was used for the screening of the five designed primers from housekeeping genes, through PCR generated single amplicon of expected size to check the specificity of the primers (Fig 4.4.2. to 4.4.6).

In the present investigation quantification was done using non-specific SYBR Green dye. Since it binds to any type of double stranded DNA, which can be the actual amplicon or primer dimer, it is necessary to differentiate the presence of population in the amplification reaction. The amplification plot for all the five primers shown in the figures (4.4.7 to 4.4.11). Melt curve analysis was initiated after forty normal cycles of amplification. The melt curve peaks positioned at the higher melting temperature in relation to its

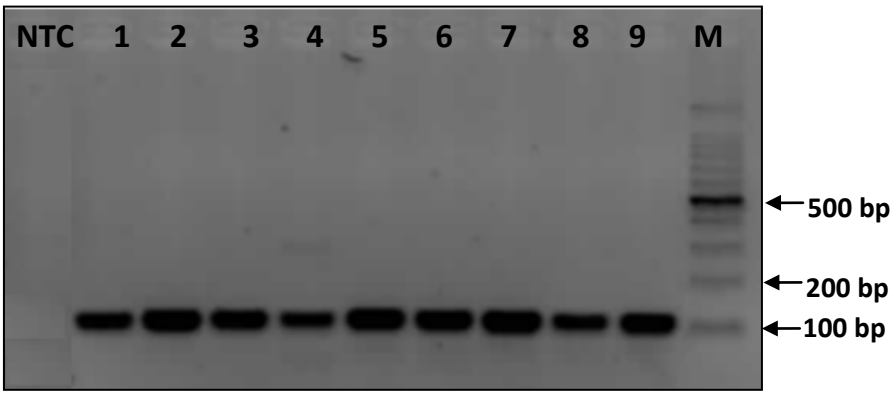


Fig. 4.4.2 Banding pattern of *26S rRNA* in durum wheat varieties

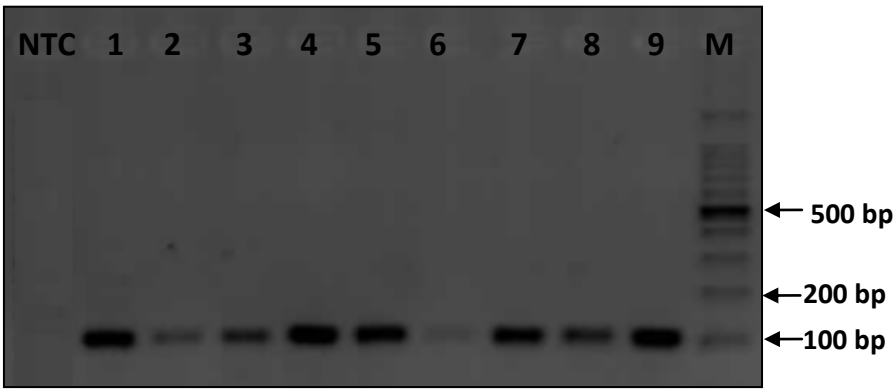


Fig. 4.4.3 Banding pattern of *18S rRNA* in .durum wheat varieties

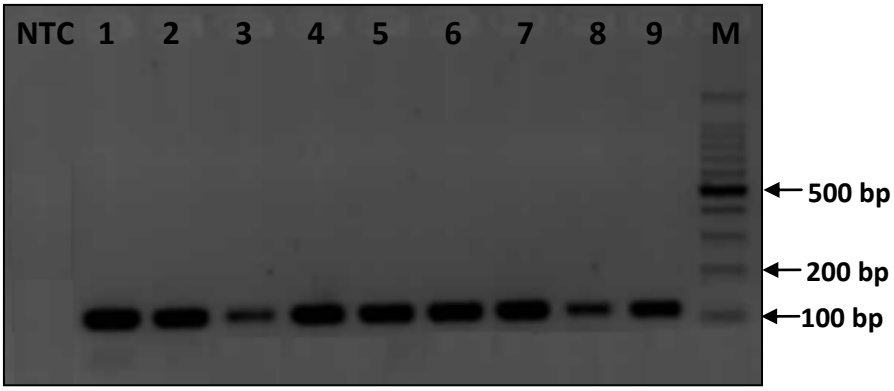


Fig. 4.4.4 Banding pattern of *RLI* in durum wheat varieties

M: 100bp;

1. GW 1 (T_0)	**4. A-206 (T_0)**	**7. A-9-30-1 (T_0)**
2. GW 1 (T_1)	**5. A-206 (T_1)**	**8. A-9-30-1 (T_1)**
3. GW 1 (T_2)	**6. A-206 (T_2)**	**9. A-9-30-1 (T_2)**

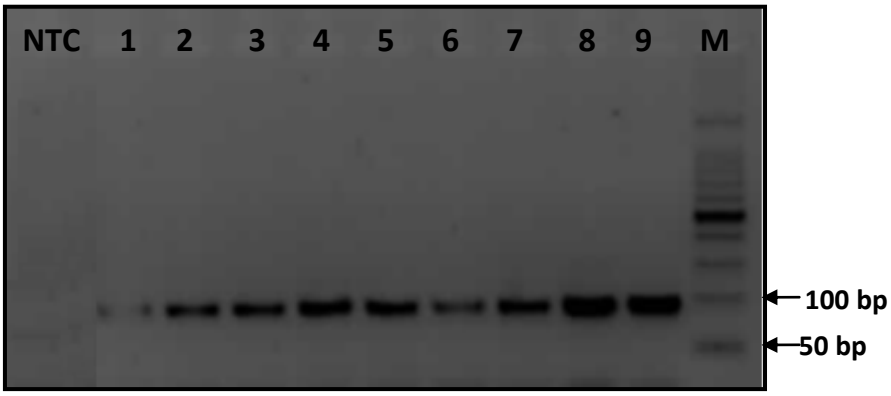


Fig. 4.4.5 Banding pattern of *Ta50503* in durum wheat varieties

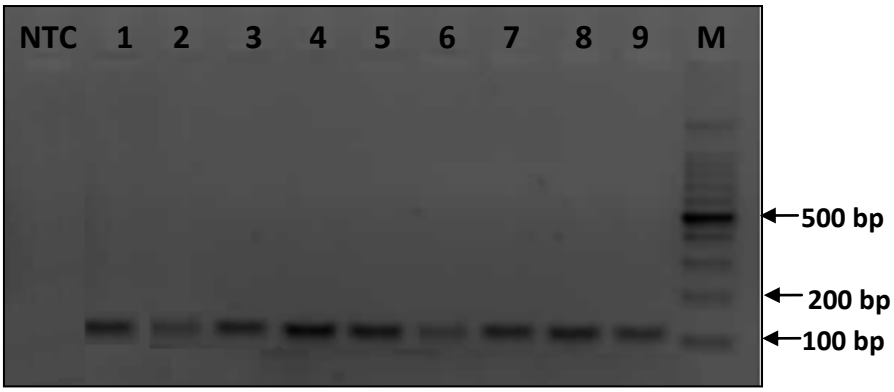
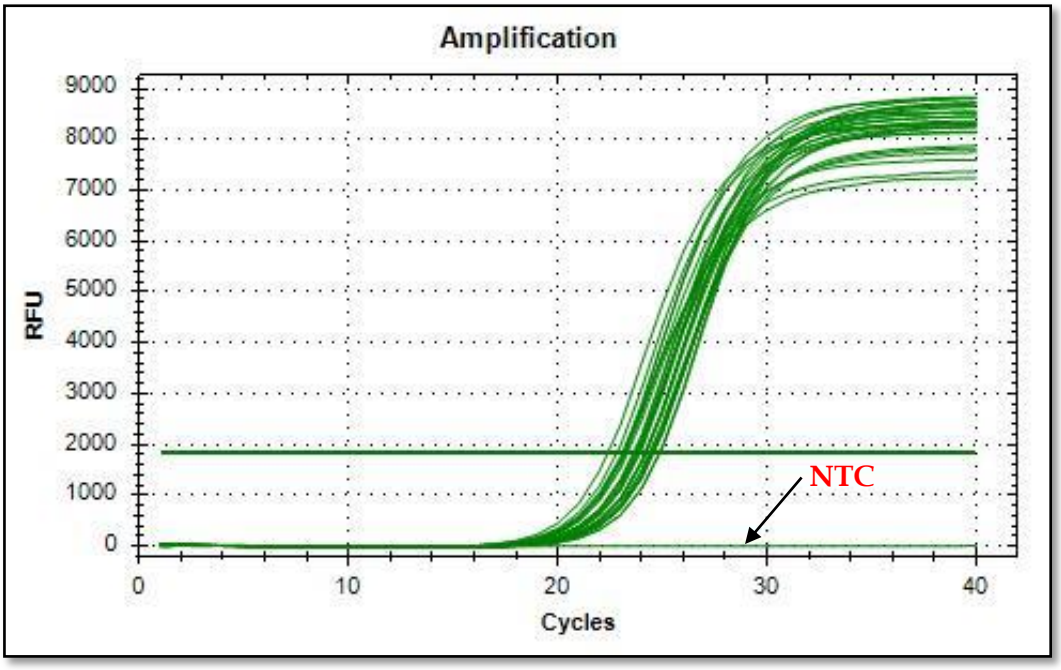


Fig. 4.4.6 Banding pattern of *ACTIN* in durum wheat varieties

M: 100bp; M: 50 bp;

1. **GW 1 (T_0)**
2. **GW 1 (T_1)**
3. **GW 1 (T_2)**
4. **A-206 (T_0)**
5. **A-206 (T_1)**
6. **A-206 (T_2)**
7. **A-9-30-1 (T_0)**
8. **A-9-30-1 (T_1)**
9. **A-9-30-1 (T_2)**



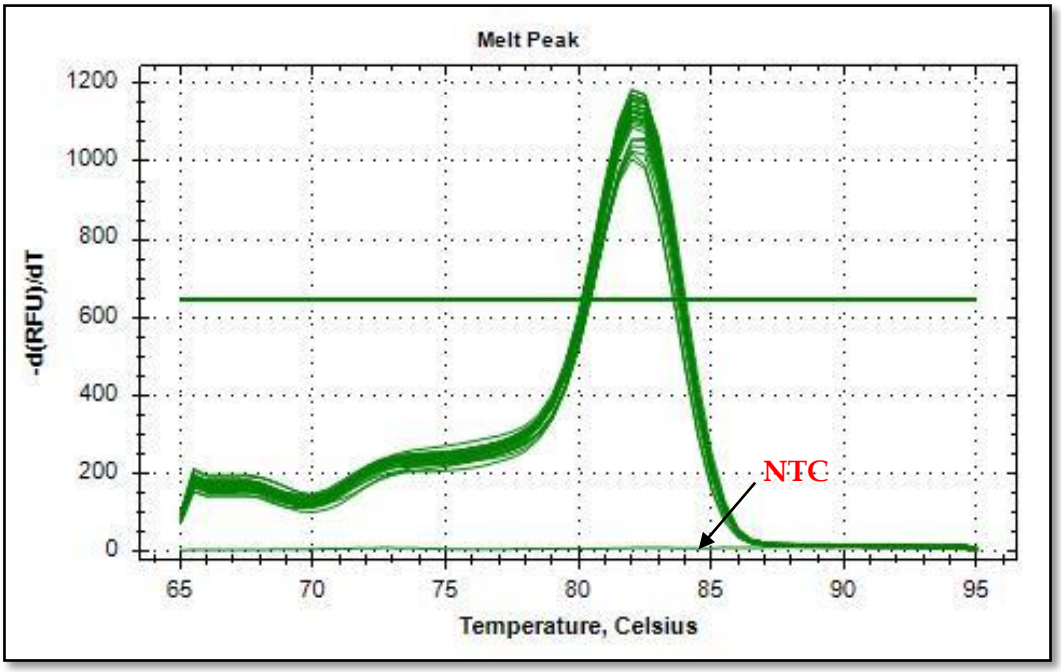


Fig. 4.4.7 Amplification plot and Melt curve of *26S rRNA* in durum wheat varieties under different treatments of irrigation

*** NTC – No Template Control**

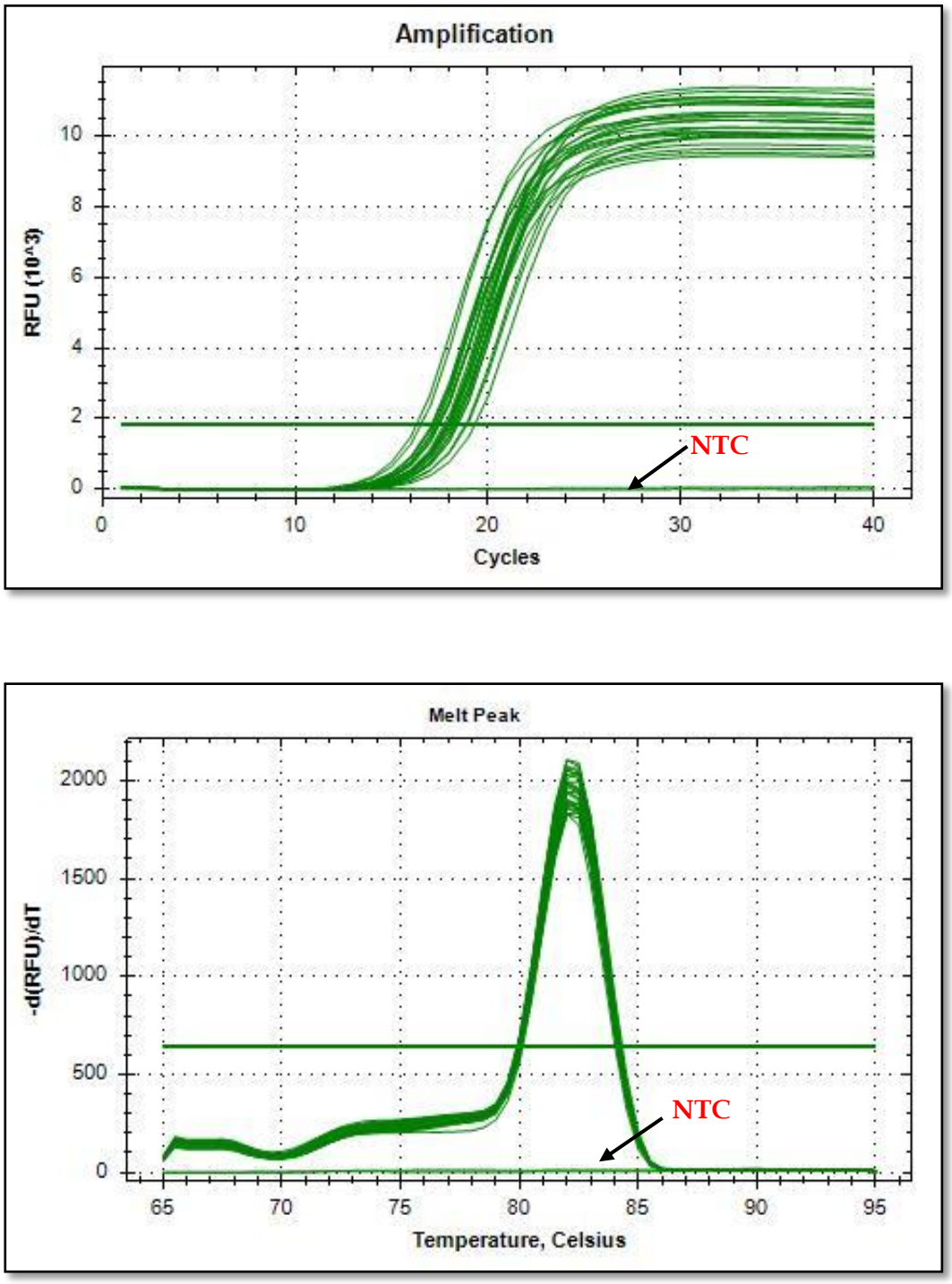


Fig. 4.4.8 Amplification plot and Melt curve of *18S rRNA* in durum wheat varieties with different treatments of irrigation.

*** NTC – No Template Control**

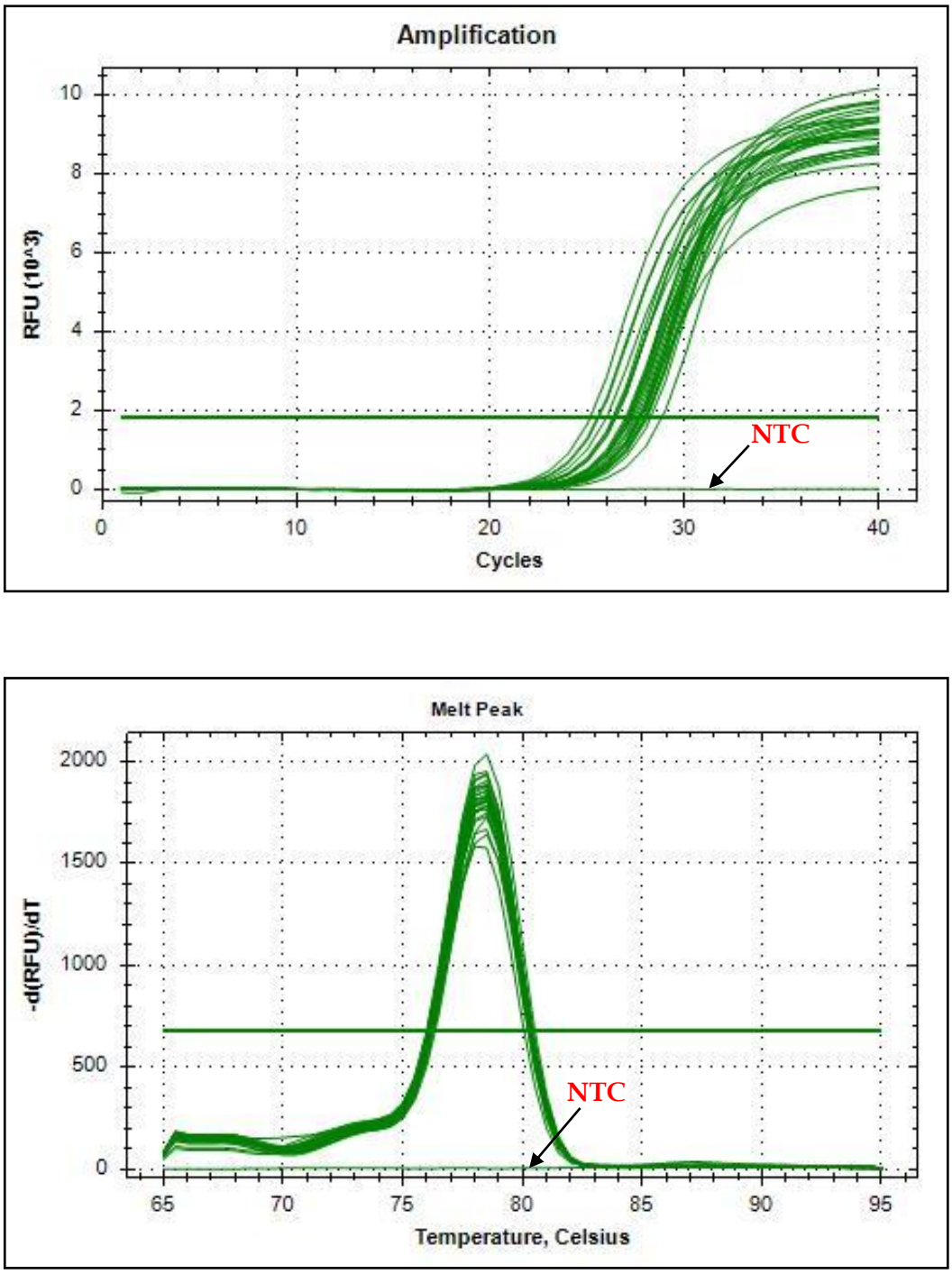


Fig 4.4.9. Amplification plot and Melt curve of *RLI* in durum wheat varieties with different treatments of irrigation.

*** NTC – No Template Control**

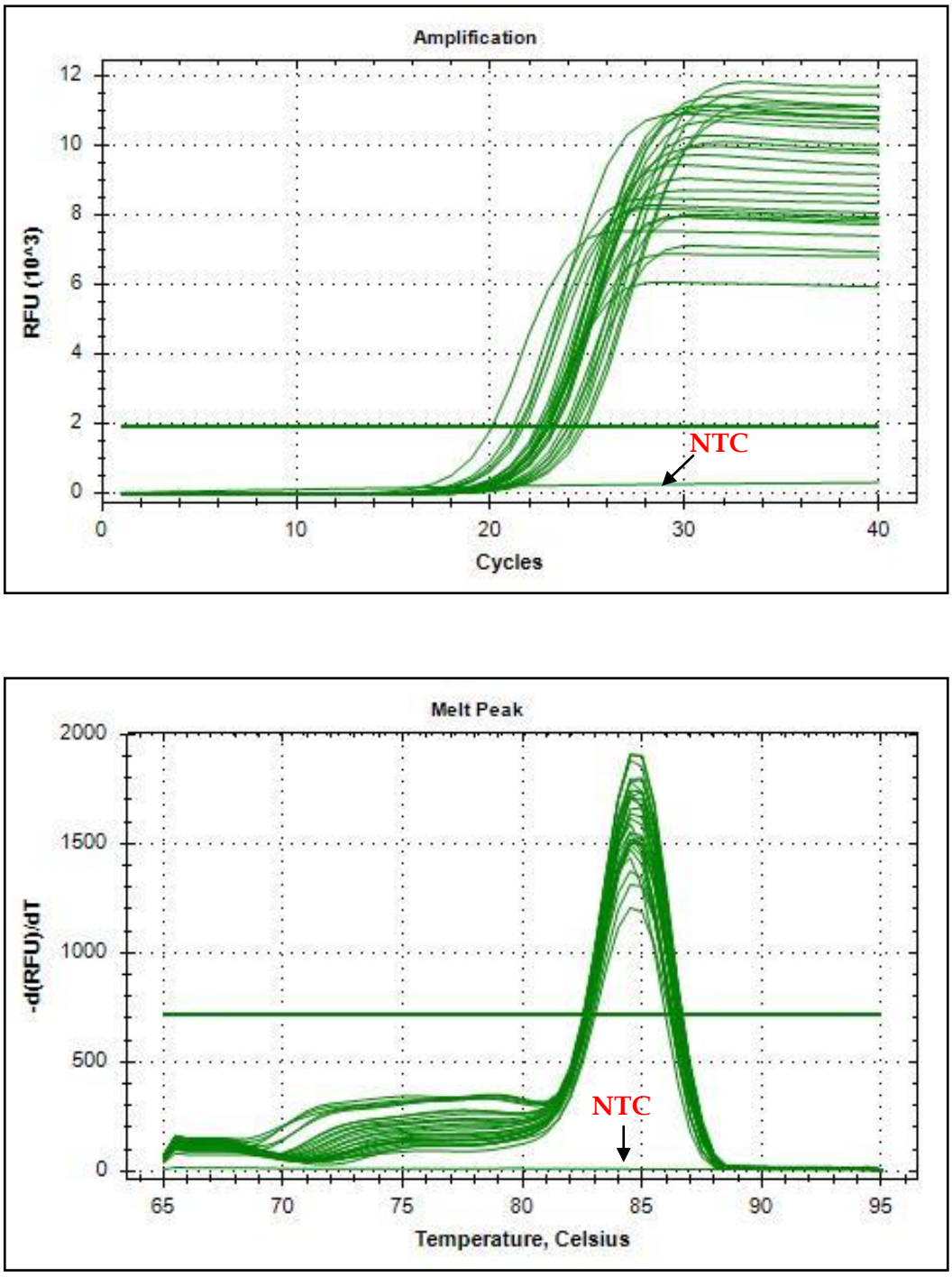
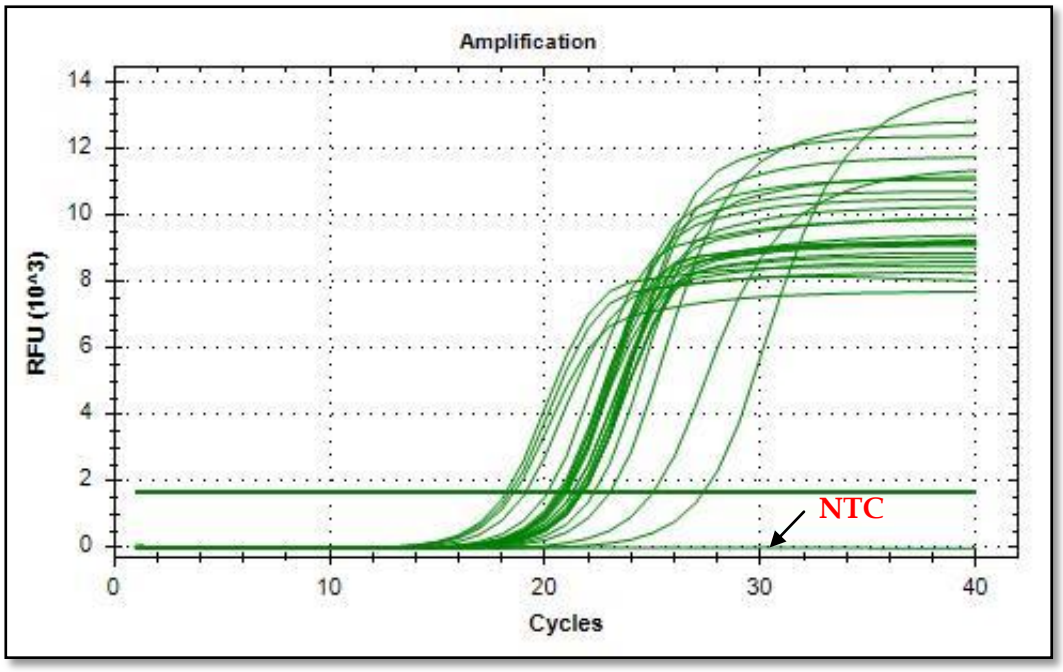


Fig 4.4.10 Amplification plot and Melt curve of *Ta50503* in durum wheat varieties with different treatments of irrigation.

*** NTC – No Template Control**



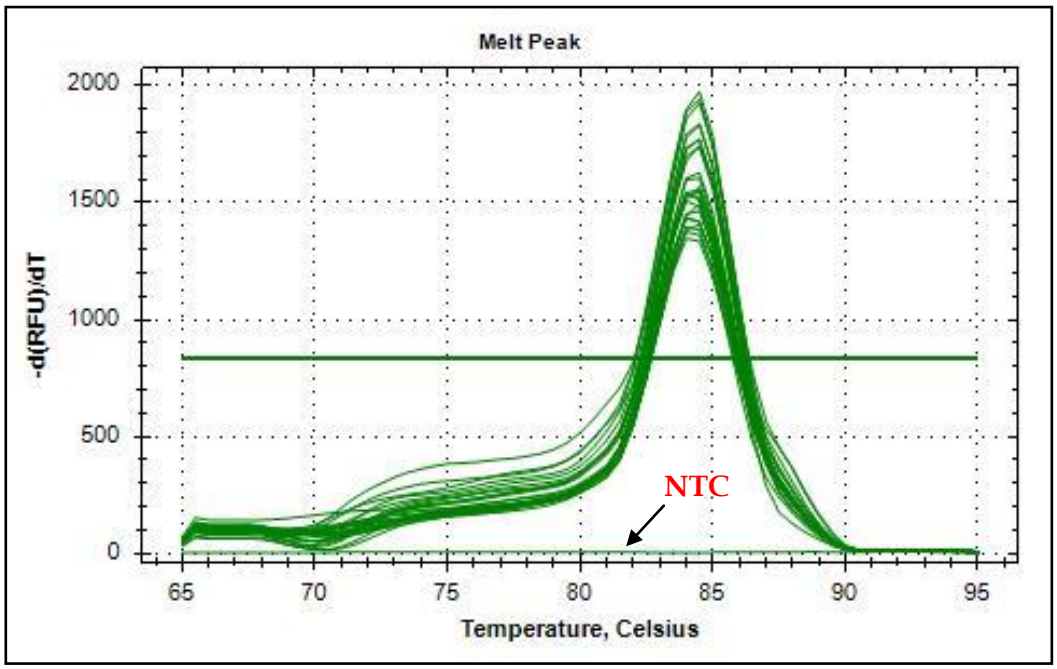


Fig 4.4.11 Amplification plot and Melt curve of *ACTIN* in durum wheat varieties under different treatments of irrigation.

*** NTC – No Template Control**

NTC (No Template Control), confirmed that all the primers were designed specifically to detect its respective endogenous genes.

Primers were validated on the basis of melt curve peak and not the agarose gel electrophoresis because these primers were ultimately used in the real time PCR and they were optimized for the instrumental conditions. The results obtained in the endogenous control validation for glutenin gene using five reference genes are given in Table 4.4.2.

Table 4.4.2. Average Cq value of selected endogenous under different treatments and durum wheat varieties.

Name of the sample	Treatment	Average Cq value				
		26S rRNA	***RLI***	***18S rRNA***	***Ta50503***	***ACTIN***
GW 1	T_0	24.07	27.61	18.05	21.88	21.86
	T_1	23.28	26.34	17.01	24.49	20.73
	T_2	23.17	28.34	17.31	23.68	21.51
A-206	T_0	24.22	27.35	16.94	23.95	21.29
	T_1	24.03	27.72	17.85	22.89	20.74
	T_2	24.75	26.45	18.29	23.05	18.39
A-9-30-1	T_0	24.01	26.54	18.02	22.91	20.55
	T_1	24.19	26.97	18.99	22.84	27.99
	T_2	23.54	27.29	17.68	21.22	28.64

Maria *et al.,* (2011) assessed the expression stabilities of eight housekeeping genes by real time PCR in plants of ten different species belonging to the *Triticeae*. All genes tested, were stable but their ranking in terms of stability differed among subsets of samples. Ammg the tested genes,

CDC (cell division control protein, AAA-superfamily of ATPases, Ta54227) and *RLI* (68 kDa protein HP68 similar to Arabidopsis thaliana RNase L inhibitor protein, Ta2776) were the three most stable genes.

4.4.3.1. Selection of stable endogenous gene.

Out of the five tested reference genes, in the treatments and among the durum wheat varieties *26S rRNA* showed the least deviation in C_q value (Table 4.4.2.) so it was selected as the endogenous control. (Fig. 4.4.7). Dissociation curve analysis was performed for *26S rRNA* and there was no amplification in NTC (Non-template control) (Fig.4.4.7).

The expression stability of the selected reference gene *26S rRNA* among all the samples with different treatments cross validated using three popular algorithms: GeNorm , NormFinder and BestKeeper.

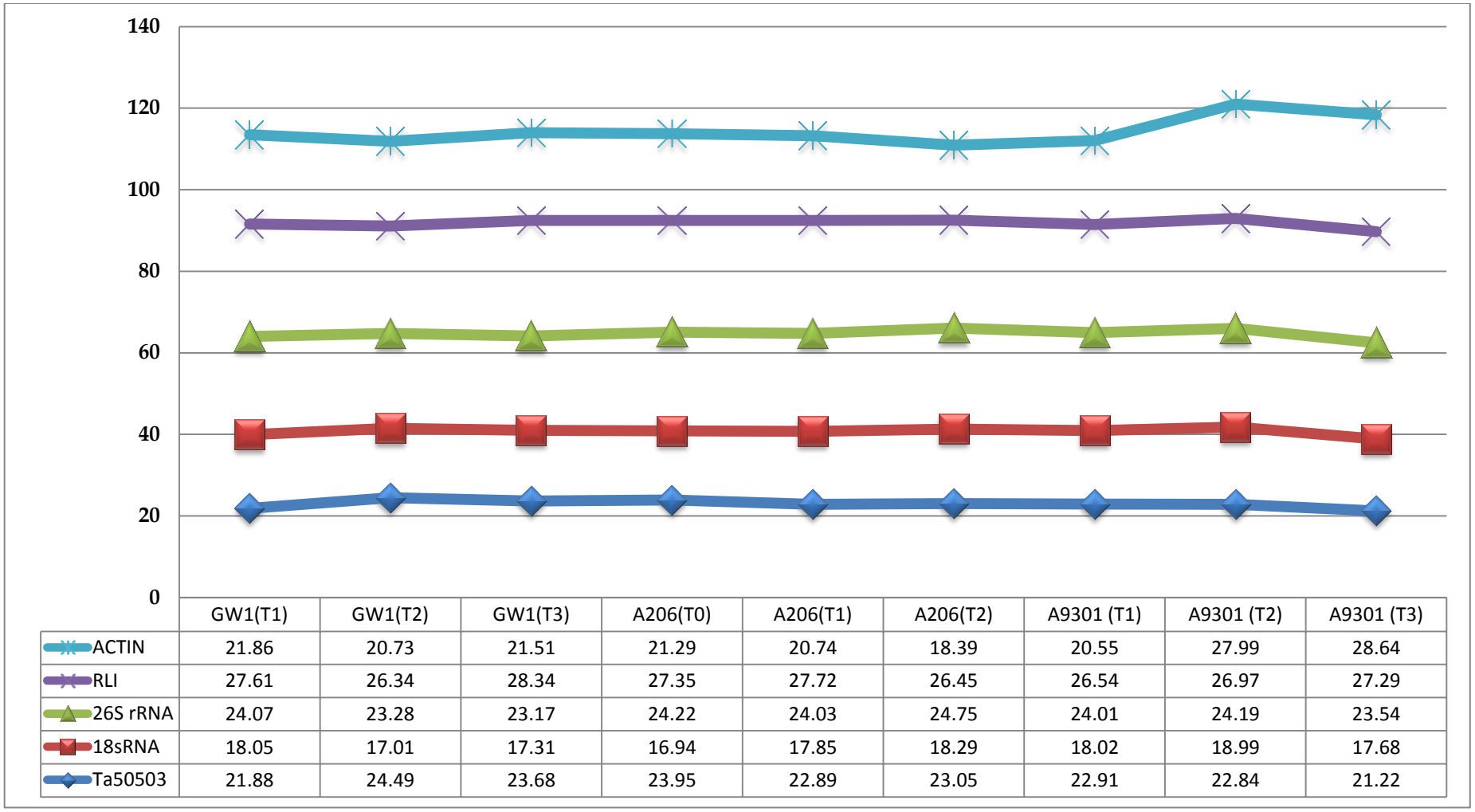
1) **GeNorm Analysis**

Investigation of raw non-normalized data of 9 tissue samples allowed sorting of genes ranked on the basis of their expression stability (M) from least stable to most stable (*26S rRNA, RLI, 18S rRNA, Ta50503, ACTIN*). The respective individual M values compared to the other candidate genes were 0.579, 0.579, 0.700, 0.940, and 1.942. Elimination of the least stable genes based on the highest M values led to the identification of *26S rRNA* as most stable reference gene.

2) **NormFinder Analysis**

Analysis of the gene expression of reference genes in 9 tissue samples, found *26S rRNA* (0.289) with lowest stability value as compare to other reference genes, *Ta50503* (0.457), *RLI* (0.920), *18S rRNA* (1.281), *ACTIN* (3.407).

3) **BestKeeper Analysis**



	GW1(T1)	GW1(T2)	GW1(T3)	A206(T0)	A206(T1)	A206(T2)	A9301 (T1)	A9301 (T2)	A9301 (T3)
ACTIN	21.86	20.73	21.51	21.29	20.74	18.39	20.55	27.99	28.64
RLI	27.61	26.34	28.34	27.35	27.72	26.45	26.54	26.97	27.29
26S rRNA	24.07	23.28	23.17	24.22	24.03	24.75	24.01	24.19	23.54
18sRNA	18.05	17.01	17.31	16.94	17.85	18.29	18.02	18.99	17.68
Ta50503	21.88	24.49	23.68	23.95	22.89	23.05	22.91	22.84	21.22

Fig. 4.4.12 Score graph of selected five endogenous genes in *T. durum* wheat varieties under irrigation treatments.

*** T_0: No Irrigation, T_1: One Irrigation, T_2: Two Irrigation.**

Unlike GeNorm and NormFinder, input data for analysis by Bestkeeper was raw Cq values of each gene. Initial analysis of the data, calculated variations SD and CV for all the reference genes in all the samples. Further data processing using pair wise correlation and regression analysis assessed the inter-gene relation and 26S rRNA was selected as the stable gene with least r value.

In all the three tested algorithms, *26S rRNA* was selected as consistent gene in all samples of Durum wheat with different treatments of irrigation.

Table 4.4.3. Comparison of selected reference gene by all three software.

Sr. No.	Name of Gene	GeNorm Expression stability (M)	NormFinder stability (p)	BestKeeper Coefficient of correlation (r)
1	*26S rRNA*	0.579	0.289	0.630
2	*RLI*	0.579	0.920	0.139
3	*18S rRNA*	0.700	01.281	0.001
4	*Ta50503*	0.940	0.457	0.894
5	*ACTIN*	1.942	3.401	0.872

4.4.4. Relative expression profiling.

The relative quantification was performed for glutenin genes (One LMW glutenin sub unit and One HMW glutenin sub unit) under different Irrigation treatments in three Durum wheat varieties in the real time PCR. Amplification plot and melt curve were generated for two primers. The single peak in melt curve shows that only the specific product was generated.

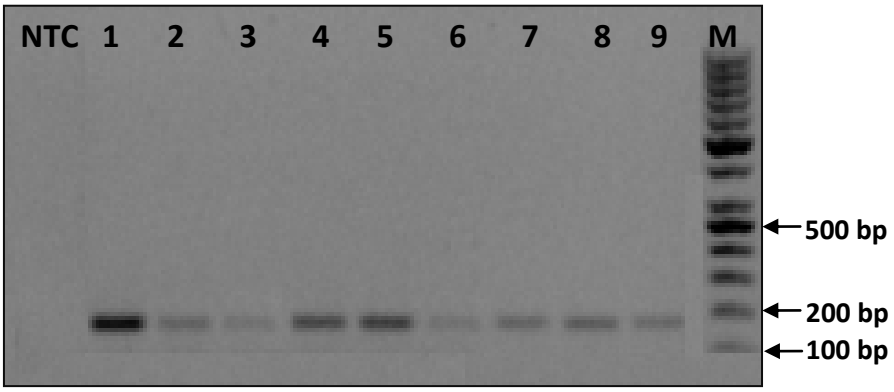


Fig.4.4.13 Banding pattern of *LMW 6* in durum wheat varieties

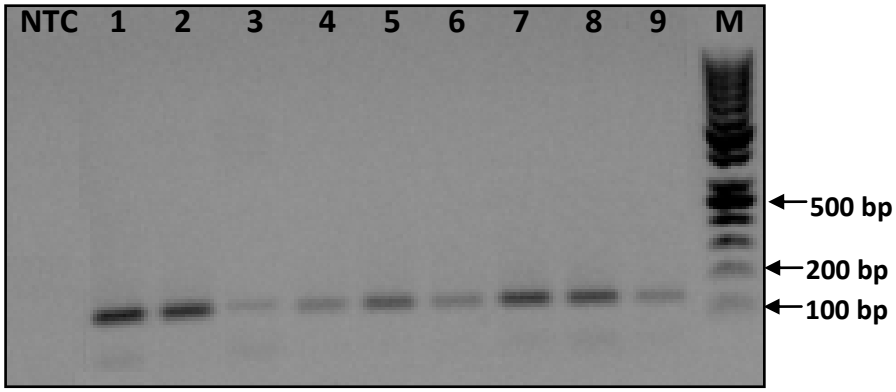


Fig.4.4.14 Banding pattern of *HMW 10* in durum wheat varieties

M: 100bp;

1. **GW 1 (T_0)**
2. **GW 1 (T_1)**
3. **GW 1 (T_2)**
4. **A-206 (T_0)**
5. **A-206 (T_1)**
6. **A-206 (T_2)**
7. **A-9-30-1 (T_0)**
8. **A-9-30-1 (T_1)**
9. **A-9-30-1 (T_2)**

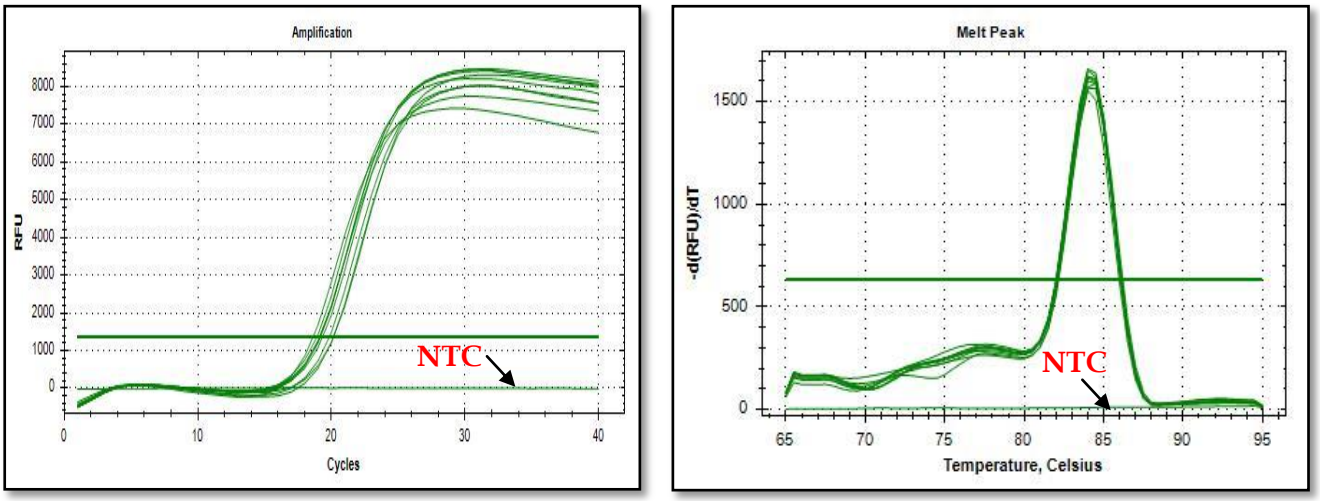


Fig 4.4.15 Amplification plot and melt curve of *LMW 6* in GW 1 variety

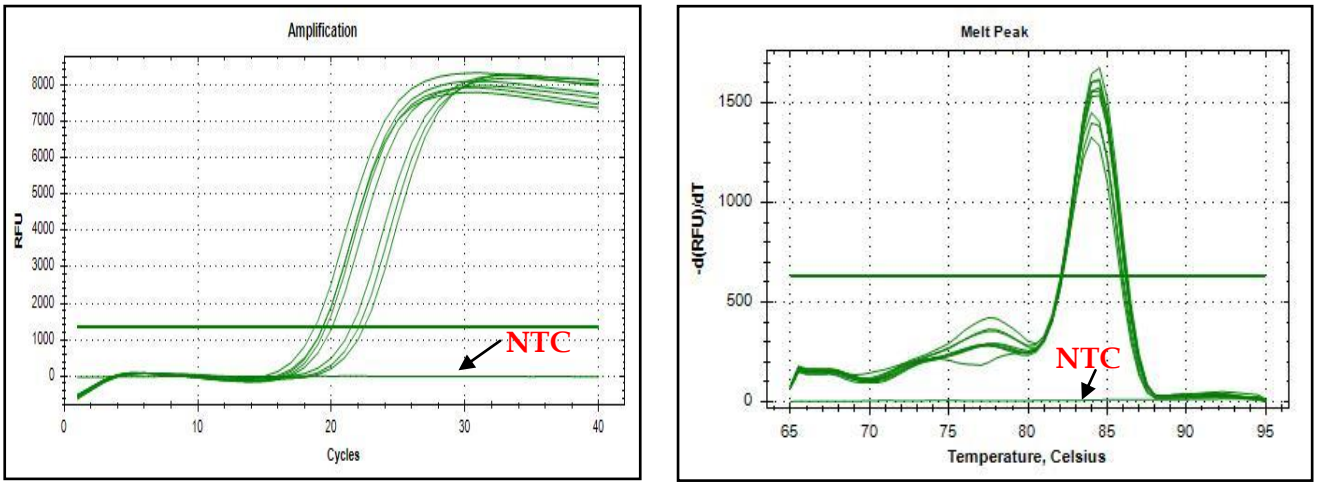


Fig. 4.4.16 Amplification plot and melt curve of *LMW 6* in A-206 variety

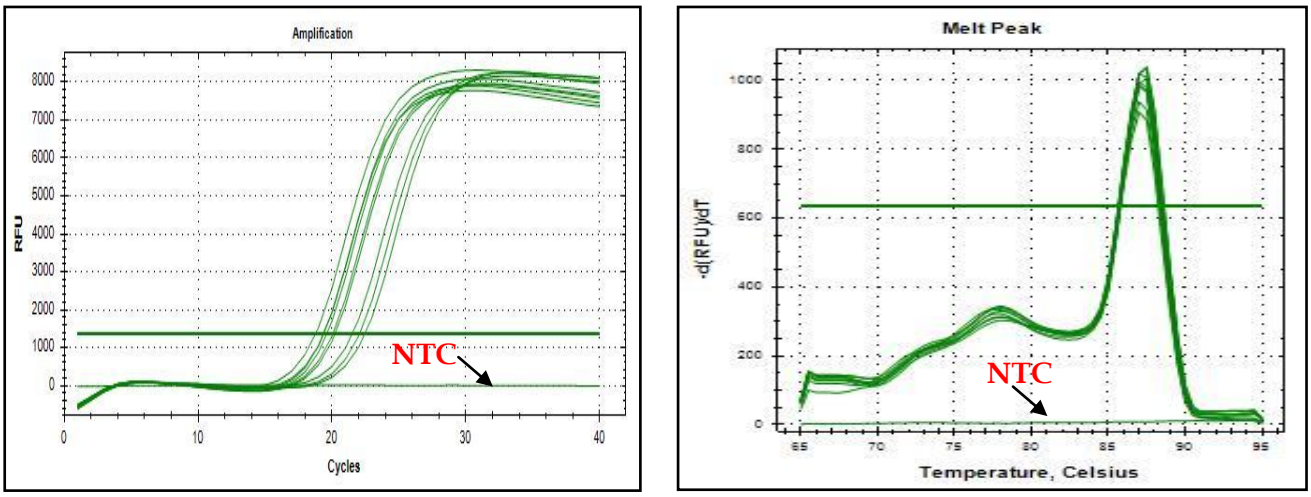


Fig 4.4.17 Amplification plot and melt curve of *LMW 6* in A-9-30-1 variety

*** NTC – No Template Control**

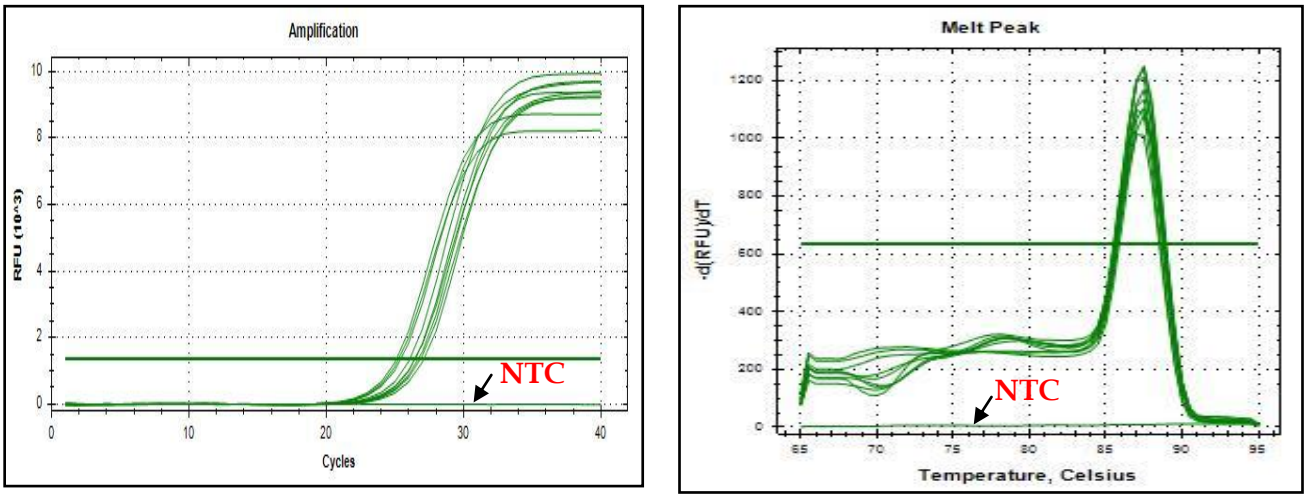


Fig 4.4.18 Amplification plot and melt curve of *HMW 10* in GW 1 variety

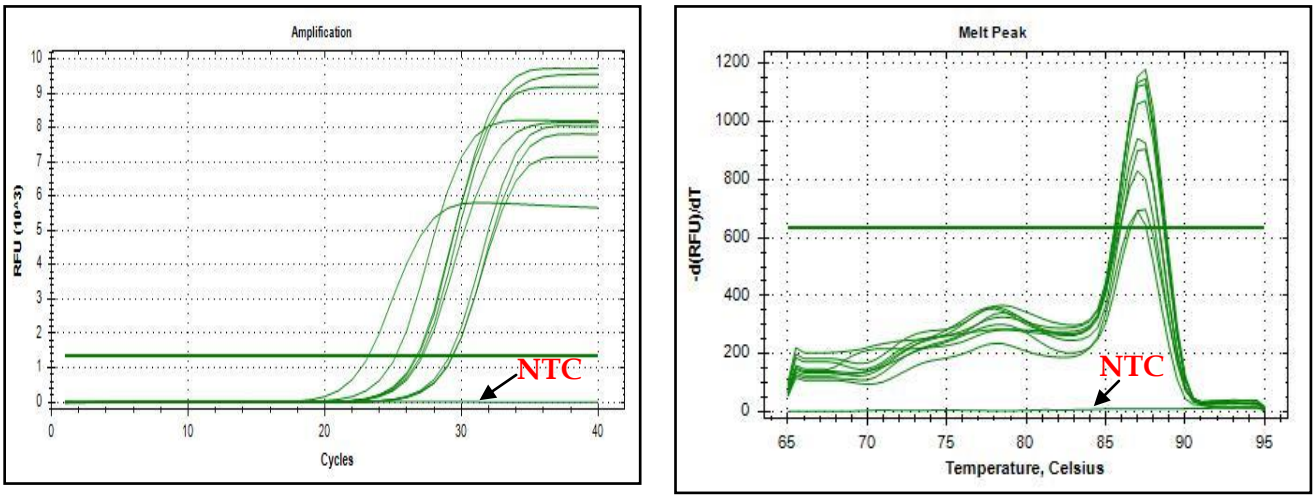


Fig. 4.4.19 Amplification plot and melt curve of *HMW 10* in A-206 variety

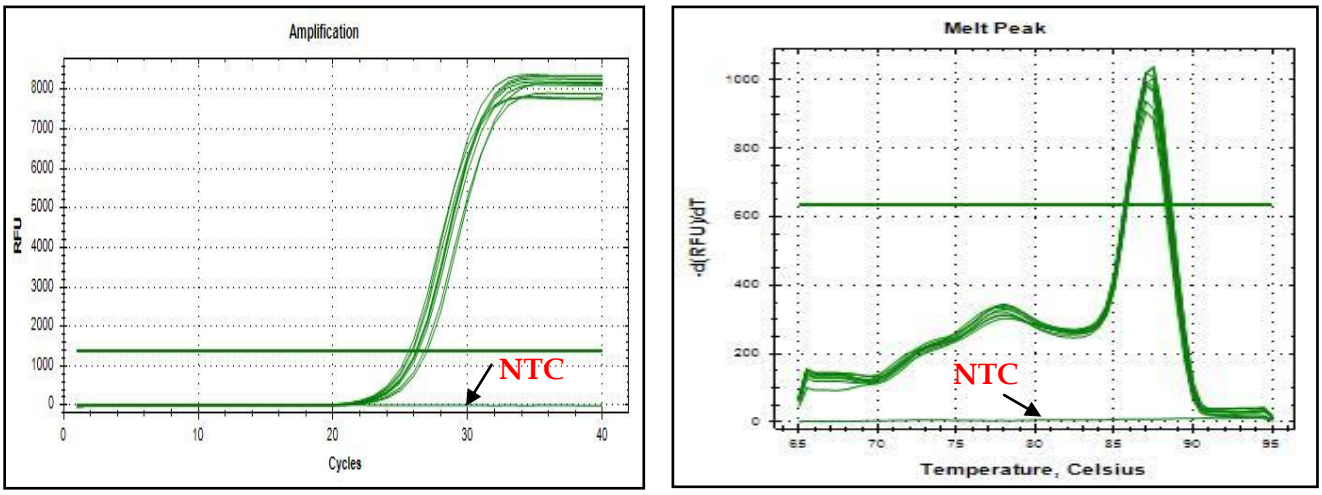


Fig 4.4.20 Amplification plot and melt curve of *HMW 10* in A-9-30-1 variety

*** NTC – No Template Control**

Relative quantification of genes as fold changes in gene expression, compared to a control sample (CC), using *26S rRNA* as endogenous control was analyzed by $2^{-\Delta\Delta C_t}$ method. Detailed study for glutenin related gene with respect to *26S rRNA* endogenous gene has been described here.

4.4.4.1 Data obtained from real time PCR for the Glutenin related gene

The data on mean Ct value and the melting temperature (Tm) of two glutenin related genes in three Durum wheat varieties with different treatment are presented in Table 4.4.4 and Table 4.4.5. The mean C_t value of the two primers for different samples obtained from the qRT-PCR varied between 18.96 and 29.29. The lowest C_t value was obtained for *LMW 6* gene and thus it is identified as the highly expressed gene. Melting temperature was highest for *HMW 10* gene (87.50 ^oC) and lowest for *LMW 6* (84.00 ^oC).

Table 4.4.4. Mean C_q values of Glutenin related gene in various durum wheat varieties with different treatment of irrigation.

Sr. No.	Sample Name	Threshold cycle (C_q) Value	
		LMW 6	***HMW 10***
1	GW 1 (T_0)	18.96	26.23
2	GW 1 (T_1)	19.46	25.48
3	GW 1 (T_2)	19.70	26.74
4	A-206 (T_0)	22.00	29.29
5	A-206 (T_1)	19.39	26.91
6	A-206 (T_2)	19.72	25.15
7	A-9-30-1 (T_0)	20.85	25.96
8	A-9-30-1 (T_1)	20.05	26.10
9	A-9-30-1 (T_2)	19.62	26.23

Table 4.4.5 Tm values of Glutenin related gene

Sr. No.	Gene	Melting Temperature (Tm) in °C
1	*LMW 6*	84.00
2	*HMW 10*	87.50

4.4.4.2 Real time PCR analysis of *LMW 6* gene

PCR analysis was performed for the presence of single amplicon of 117 bp in 1.5 agarose gel. The mean C_t values of *LMW 6* gene in all Durum wheat varieties with three treatment of irrigation studied ranged from 18.96 to 20.85 which showed that the gene is highly expressive in all samples. The real time PCR amplification plot of *LMW 6* gene is given in Fig. 4.4.14. Melt curve of *LMW 6* gene showed a single peak at Tm 84.00 °C that indicates specific amplified product (Fig.4.4.14). NTC showed no amplification indicating absence of primer dimer formation and only amplification of specific product of target gene.

LMW 6 gene showed the treatment specific variation in the level of expression for three Durum wheat varieties. In GW 1 variety, the RQ values for *LMW 6* revealed that there was decrease in the expression of *LMW 6* in sample GW 1 (T_1) (RQ=0.7087) and GW 1 (T_2) (RQ=0.5988) as compared to control sample GW 1(T0). It indicated that the expression of *LMW 6* in GW 1 variety was down regulated. Expression of *LMW 6* gene was up regulated in A-206 variety and A-9-30-1 variety with increasing RQ value as compare to their control sample A-206 (T_0) and A-9-30-1(T_0) respectively. A-206 (T_1) shows higher expression of LMW 6 gene with high RQ value (6.0871) compare to A-206 (T_2) (RQ=4.8609). In A-9-30-1 variety, up regulation of *LMW 6* was reported in A-9-30-1 (T_2) with RQ value 2.3463 compared to A-9-30-1 (T_1) (RQ=1.7387).

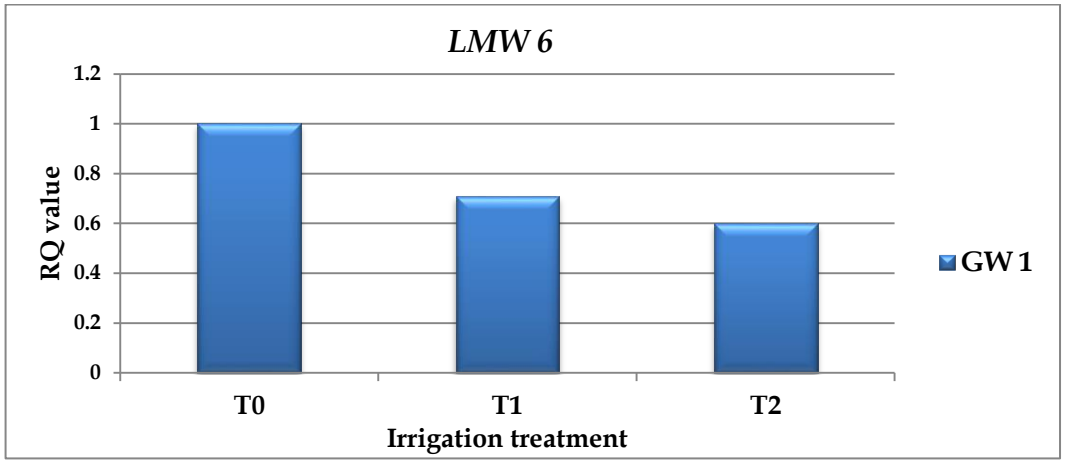


Fig 4.4.21 Relative expression (RQ plot) for *LMW 6* in with *26S rRNA* as reference gene and GW 1(T_0) as a control.

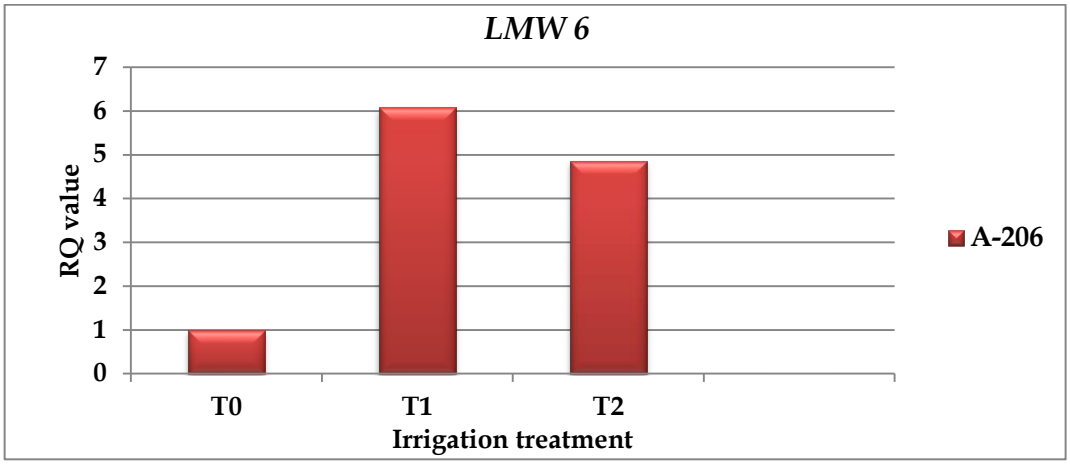


Fig 4.4.22 Relative expression (RQ plot) for *LMW 6* in with *26S rRNA* as reference gene and A-206 (T_0) as a control.

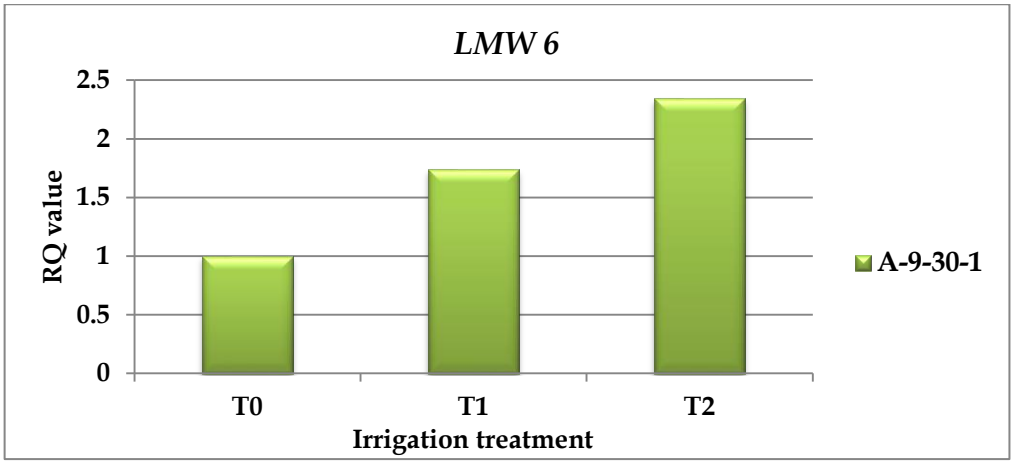


Fig 4.4.23 Relative expression (RQ plot) for *LMW 6* in with *26S rRNA* as reference gene and A-9-30-1 (T_0) as a control.

*** T_0: No Irrigation, T_1: One Irrigation, T_2: Two Irrigation.**

Higher transcript accumulation was observed for A-206 in both the treatments as compared to the control suggesting it to be the highly responsive variety for improving the grain glutenin content.

Higher expression of LMW 6 in A-206 the first irrigation was higher as compared to control and decrease in the subsequent second irrigation. The later was confirmed by the banding pattern on the PAGE, in which the higher intense band was observed in first irrigation while the intensity of the band decreased in the second irrigation, suggesting the role of the LMW in the synthesis and accumulation of the glutenin protein in the developing grain of wheat (Susan *et al* 2002).

4.4.4.3 Real time PCR analysis of *HMW 10* gene

PCR analysis detected a single amplicon of 111 bp. The mean C_t values of *HMW 10* gene in all Durum wheat varieties with three treatment of irrigation studied ranged from 26.10 to 29.29 which showed that the gene is relatively less expressive as compared to *LMW6* in all samples. The real time PCR amplification plot of *HMW 10* gene is given in Figure 4.4.15. Melt curve of *HMW 10* gene showed a single peak at Tm 87.00 ^{o}C that indicates specific amplified product (Figure 4.4.18 to 4.4.20). NTC showed zero amplification indicating absence of primer dimer formation and only amplification of specific product of target gene.

HMW 10 gene showed the treatment specific variation in the level of expression for three Durum wheat varieties. The expression pattern of *HMW10* was significantly higher in GW1 at T_1 treatment and was reduced at T_2 as compared to the endogenous 26sRNA. These results were affirmed by the banding pattern of the *HMW 10* gene in PAGE. The RQ values revealed that there was slight increase in the expression of *HMW 10* in GW 1 (T_1) (RQ=1.6817) and decrease in the expression of GW 1 (T_2) (RQ=0.7074) as compare to control sample GW 1(T_0) (Figure 4.4.24). It indicates that the

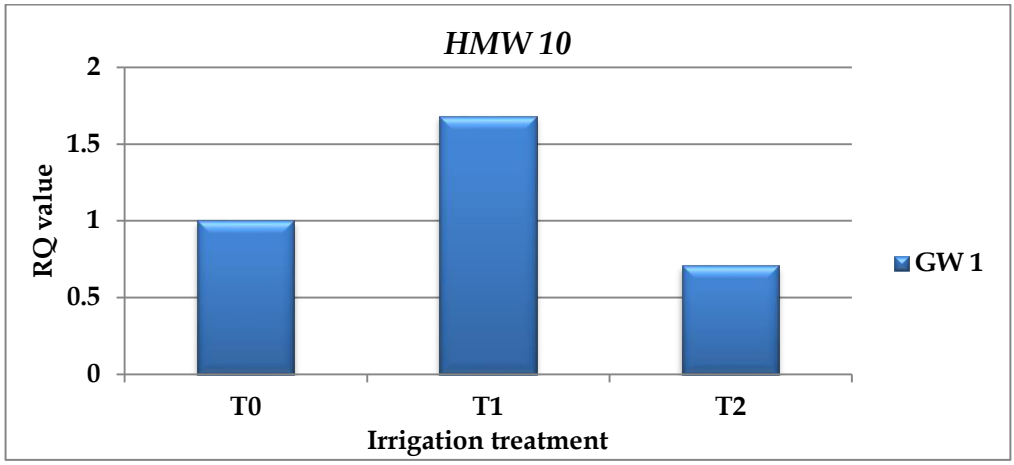


Fig 4.4.24 Relative expression (RQ plot) for *HMW 10* in with 26S rRNA as reference gene and GW 1(T0) as a control.

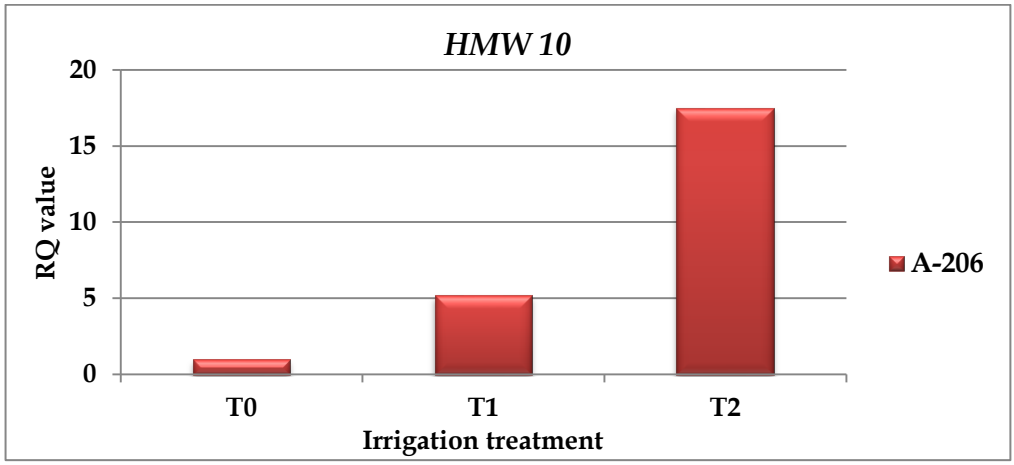


Fig 4.4.25 Relative expression (RQ plot) for *HMW 10* in with *26S rRNA* as reference gene and A-206(T_0) as a control.

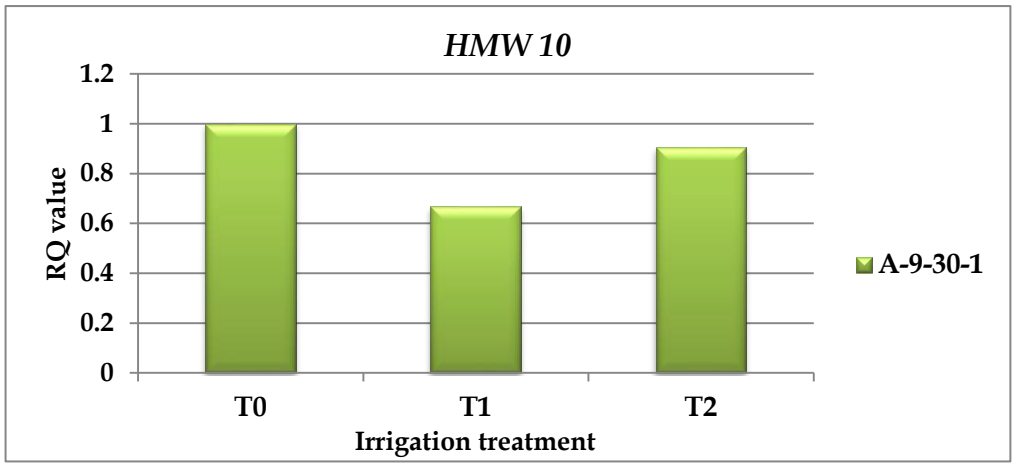


Fig 4.4.26 Relative expression (RQ plot) for *HMW 10* in with *26S rRNA* as reference gene and A-9-30-1(T_0) as a control.

*** T_0: No Irrigation, T_1: One Irrigation, T_2: Two Irrigation.**

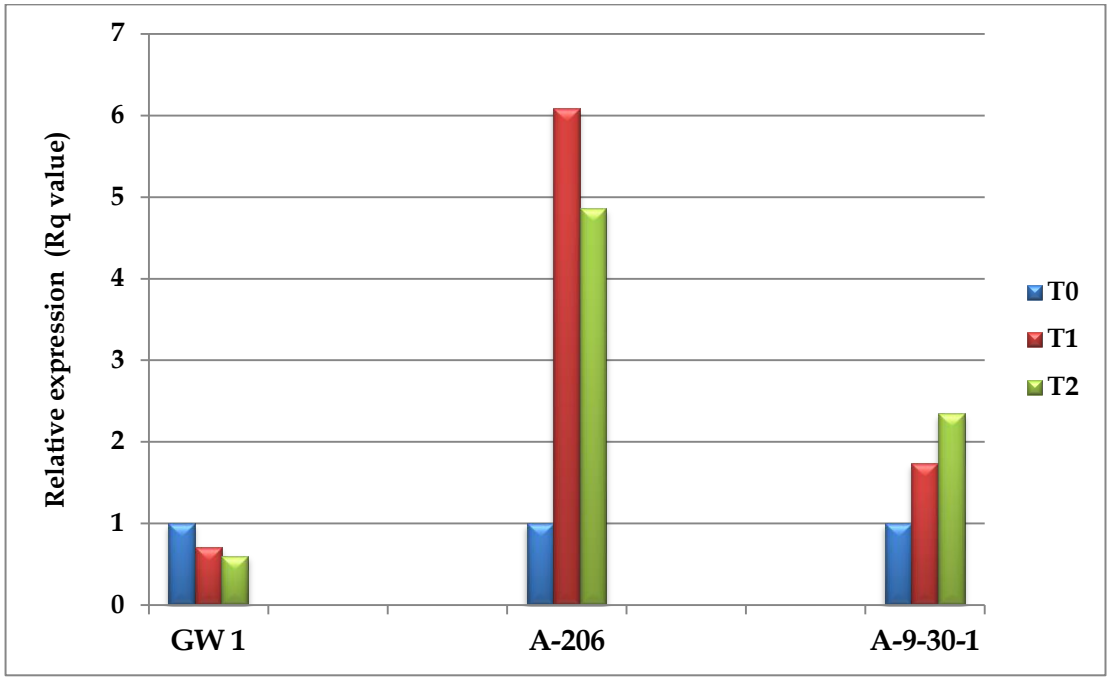


Fig 4.4.27 Relative expression (RQ plot) for *LMW 6* gene with *26S rRNA* as reference gene with Different Irrigation treatment in durum varieties.

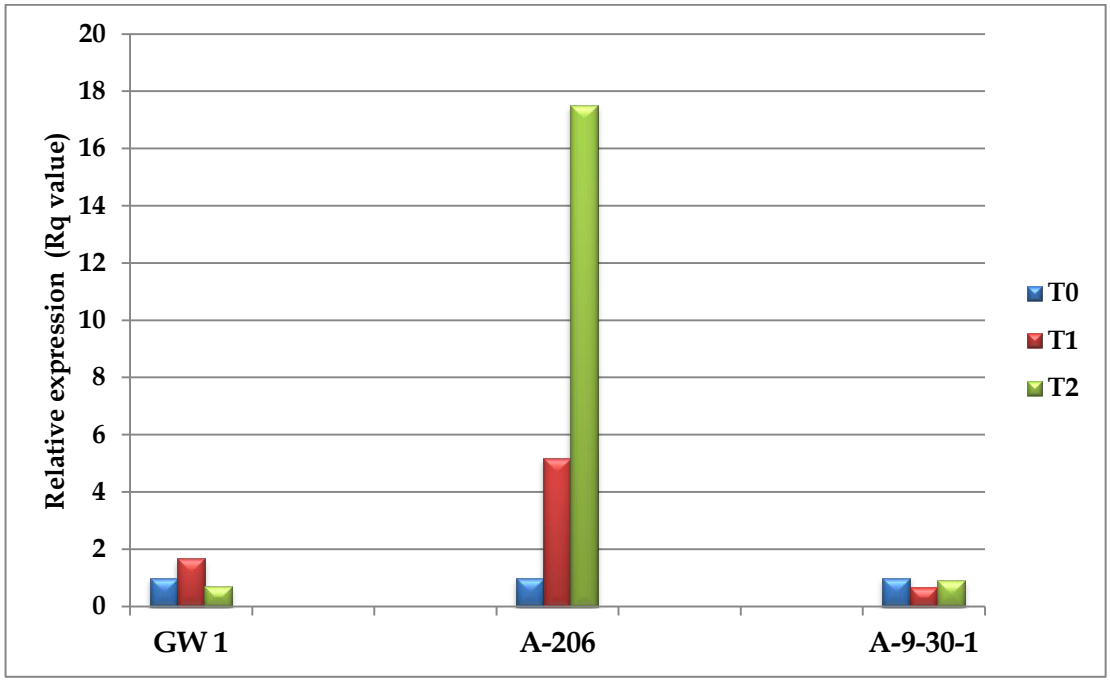


Fig 4.4.28 Relative expression (RQ plot) for *HMW 10* gene with *26S rRNA* as reference gene with Different Irrigation treatment in durum varieties.

*** T_0: No Irrigation, T_1: One Irrigation, T_2: Two Irrigation.**

expression of *HMW 10* in GW 1 variety was up regulated with (T_1) Treatment. Expression of *HMW 10* gene was up regulated in A-206 variety with increasing RQ value as compare to their control sample A-206 (T_0) (Figure 4.4.25). A-206 (T_2) shows high expression of *HMW 10* gene with high RQ value (17.5259) among the A-206 (T_1) Treatment (RQ=5.1986) as compared to control A-206 (T0). The expression level of *HMW 10* gene in A-9-30-1 variety was down regulated in both the treatments, T_1 (RQ=0.6685) and T_2 (RQ=0.9049) respectively as compared to control A-9-30-1 (T_0).

The results suggested that A-206 variety showed high expression of *HMW 10* in A-206 (T_2) treatment as compared to A-206 (T_1) and control A-206 (T_0).

The overall results suggested that the expression pattern of *LMW 6* was higher in all the tested varieties (A-206 and A-9-30-1) among all the treatments as compared to control. LMW 6 exhibited varietal specific variation in all the treatments during relative expression studies and was also confirmed by the banding pattern of the protein fraction on SDS – PAGE.

Summary and Conclusion

IV. SUMMARY AND CONCLUSION

Durum wheat (*Triticum durum* Desf.) is the only tetraploid species of wheat which is cultivated worldwide. Durum wheat contains high protein, as well as its strength and structures and interactions of the grain storage proteins *viz.* gliadin and glutenin make durum good for special uses like pasta, spaghetti, macaroni and in bread making.

Environmental factors, such as temperature, water, and nitrogen also influence the wheat quality characteristics, ratio between soluble and insoluble proteins, affects bread-making quality, which is a function of protein composition that is genetically controlled.

Irrigation and varietal improvement are two major ways of increasing yield and stabilizing durum wheat. Timely irrigation at proper growth and developmental stages is a viable reason to improve production. Limited irrigation applied at sensitive crop stages increased yield of rainfed crops. Irrigation levels influenced glutenin biosynthesis and glutenin particle formation.

Durum wheat (*Triticum durum* Desf.) cultivated in Bhal Region-Geographical Indication of Gujarat, due to long grain wheat variety and some specific end use quality related to proteins are present. One or Two irrigation increases total yield of durum wheat. Since last two years farmers are applying this practice to get higher yield. No information is available on the effect of the limited irrigation on protein profiling. Therefore detail study related to effect of irrigation on quality of wheat was selected with following objectives,

1) To study the effect of limited irrigation application on nutritional Proximate composition of wheat (*Triticum durum* Desf.).
2) To study the variations in protein fractions and protein profile (HMW and LMW) for end use quality.
3) Real time expression pattern for glutenin gene in caryopsis at maturity Stage.

Quality of three durum wheat varieties with respect to irrigation treatments were tested using qualitative tests like moisture, ash, total soluble sugar, starch, total protein, lysine, tryptophan, oil and mineral content.

- Irrigation treatments did not show significant role in moisture content in all varieties. GW 1 with two irrigation (T_2) registered highly significant ash content (1.55 %).
- A-9-30-1 showed significantly higher total soluble sugar (3.02 %) followed by A-206 (2.14 %) with two irrigation (T_2).
- GW 1 and A-206 with no irrigation (T_0) showed significantly higher starch content (65.0167 %).
- Significantly higher oil content was observed in two irrigation (T_2) among all varieties.
- Significantly higher protein content (13.77 %) registered in GW 1 with one irrigation treatment (T_1).
- Lysine and tryptophan content showed significance differences in two irrigation treatment in A-9-30-1 and A-206 variety respectively.
- GW 1 variety showed significant increased potassium, calcium and sodium content in irrigation treatments.

5.2 Protein characterization of total protein and protein fractions through SDS-PAGE

Grain protein of durum wheat varieties have been analyzed through SDS-PAGE. Variation for the protein banding pattern and band intensity were observed in durum wheat under irrigation treatments. Studies indicated that application of different irrigation obtained total 16 bands, in which only 7 were monomorphic. Two irrigation treatments showed absence of 6 bands in GW 1, 4 bands in A-206 and 3 bands in A-9-30-1 variety. These data showed the total protein profiling could not provide any conclusive idea about the effect of irrigation on wheat.

Protein fractions profile of albumin, globulin, glutenin and gliadin have been analyzed through SDS- PAGE. When comparing different protein fraction on PAGE, no difference was observed in banding pattern of gliadin and globulin in durum wheat varieties with irrigation treatments while Albumin and glutenin showed difference in banding pattern in terms of absence/presence of bands with irrigation treatments. However, both fractions gave idea about the effect of irrigations on protein profiling.

5.3 Gene expression profile of glutenin gene

Relative gene expression profile was performed for glutenin gene with two subunits (*LMW 6* and *HMW 10*) under different irrigation treatments in three durum wheat varieties through real time PCR.

- On the basis of RQ value, the data generated for *LMW 6* gene was highly upregulated in one irrigation treatment as compared to two irrigation treatment and control.
- Expression pattern of *HMW 10* was up regulated (RQ= 17.52) in A-206 with two irrigation treatment in comparison with to one irrigation treatment and control.
- Among all the varieties, A-206 revealed significantly higher accumulation of transcripts (*LMW 6* and *HMW 10*) in both the treatments as compared to the control. The same was confirmed by the protein content which was higher in one irrigation (12.77 %) and two irrigation (12.18 %) as compared to the control (11.48 %).
- A-206 variety showed higher protein content was also revealed by expression studies. It is inferred that this variety can be exploited further for enhancing the protein content with respect to limited irrigation.

- Significantly higher protein content was observed in GW1 for one irrigation (13.77 %) as compared to control (12.97 %) which was akin by transcript accumulation in real time studies for HMW 10 for one irrigation (RQ=1.68) in comparision to the control (RQ=1).

References

VI. REFERENCES

A.O.A.C. (1965). Official Methods of Analysis, of the Association of Official Analytical Chemists, 10th ed., Washington, D.C.

A.O.A.C. (1995). Official Methods of Analysis, of the Association of Official Analytical Chemists, 16th ed., Washington, D.C.

Altenbach, S. B., Kothari, K. M., and Lieu, D. (2002). Environmental conditions during wheat grain development alters temporal regulation of major gluten protein genes. *Cereal Chem.,* **79**:279 – 285.

Andersen, C., Jensen, J. and Orntoft, T. (2004). Normalization of Real time quantitative reverse transcription PCR data: a model based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*., **64**: 5250-5245.

Anna, R. P., Oronzo, A., Tanzarella, Enrico, Porceddu and Mario Ciaffi. (2009). Identification and validation of reference genes for quantification of RT-PCR normalization in wheat. *BMC Mol. Biol.,* **10:** 1-27.

Anonymous (2016). World Agriculture Production, Department of Agriculture, United State.

Bahieldin, A., Mahfouz, H. T., Eissa, H. F., Saleh, O. M., Ramadan, A. M., Ahmed, I. A. andMadkour, M. A. (2005). Field evaluation of transgenic wheat plants stably expressing the HVA1 gene for drought tolerance. *Physiol Plant.,* **123:** 421-427.

Barbara, J., Ewa, P., Anna, J., Robert, P. and Marcin, R. (2014). Assessment of candidate reference genes for the expression studies with brassinosteroids in Loliumperenne and *Triticum aestivum*. *J Plant Physiol.,* **171**: 1541–1544.

Bhatnagar, R., Shukla, Y. M. and Talati, J. G. (2007). Biochemical methods for agricultural sciences, Department of Biochemistry. A.A.U., Anand, pp 51-52.

Brandt, D. A., Brand, T. S. and Cruywagen, C. W. (2000). The use of crude protein content to predict concentrations of lysine and methionine in grain harvested from selected cultivars of wheat, barley and triticale grown in the Western Cape region of South Africa. *S Afr J Anim Sci.,* **30**(1): 22-25.

Branlard, G., Dardevet, M., Amiour, N. and Igrejas, G. (2003). Allelic diversity of HMW and LMW glutenin subunits and omega gliadins in French bread wheat (*T. aestivum* L.), *Genet Res and Crop Evol.,* **50**: 669–679.

Bravo, A., Ponce, L., Parraga, P., Oliva, R. and Proano, K. (2013). Expression of high molecular weight glutenin subunit genes correlated with gluten content in common wheat varieties from the highland of ecuador, *Cereal Res Commun., doi: 10.1556/CRC.2013.0028.*

Bustin (2002). Quantification of mRNA using real time reverse transcription polymerase chain reaction assays. *J mol Endocrinol.,* **25**: 169-193.

Caldana, C., Scheiblel, W., Mueller, B. and Ruzicicl, S. (2007). A quantitative RT-PCR plateform for high-throughput expression profiling of 2500 rice transcription factors. *Plant Methods.,* **3**(7).

Chang, S., Puryear, J., Cairney, J. (1993) A Simple and Efficient Method for Isolating RNA from Pine Trees. *Plant Molecular Biology Reporter.,* **11:** 113-116.

Chakraborty, M. K. (1992). Grain quality in Gujarat, National Agricultural Research Project, Gujarat Agricultural University, Anand-388110.

Chomczynski, P. and Mackey, K. (1995). Short technical reports.Modification of the TRI Reagent procedure for isolation of RNA from polysaccharide and proteoglycan rich sources. *Biotechniques.,* **19**: 942–945.

Chope, G., Wan, Y., Penson, S., Bhandari, D., Powers, S., Shewry, P. and Hawkesford, M. (2014). Effects of Genotype, Season, and Nitrogen Nutrition on Gene Expression and Protein Accumulation in Wheat Grain, *J Agric Food Chem.*, **62:** 4399−4407.

Chrambach, A. and Rodbard, D. (1971). Polyacrylamide gel electrophoresis. *Science,* **172** (3982): 440-451.

Danilton, L., Lena, B., Faria, R., Scholz, M. and Medina, C. (2013). Influence of irrigation on wheat crop, *Eng Agric., J aboticabal,* **33(1)**:75-88.

Dong, L., Ning, L., Xiaobing, L., Prodanovic, S., Xu, Y., Zhang, W. and Yan, Y. (2016). Quality properties and expression profiling of protein disulfide isomerase genes during grain development of three spring wheat near isogenic lines, *Genetika.*, **48**(1): 249-269.

Dubios, M., Gilles, K.A, Hamilton, J. K., Rebers, P. A. and Smith, F. (1956). Colorimetric methods of determination of sugars and related substances. *Anal Chem.*, **26**:350.

Enver, K. (2014). Characterization of durum wheat (*Triticum durum* Desf.) cultivars in terms of gliadin and glutenin protein composition, *Int J Env Sci.*, **4**(3): 174-176.

Eric, W., Storlie, Ihry, R., Baehr, L., Tieszen, K., Engbers, J. (2009). Genomic regions influencing gene expression of the HMW glutenins in wheat, *TheorAppl Genet.*, **118**:295–303.

Faqir, A., Khan, M., Ahmad, D., Saeed, M., Pasha, I. and Arshad, A. (2007). Wheat gluten: high molecular weight glutenin subunits structure, genetics, and relation to dough elasticity. *J Food Sci.,* **72**:56-63.

Faqir, M. A., Ijaz, A., Masood, S.B., Sheikh, M. A. and Imran, P. (2005). Abstract: Amino acid composition of spring wheat and losses of lysine during chapati baking, *J Food Comp Anal.,* **18**(6): 523-532.

Farrell, R. E. (1998). RNA methodologies: A Laboratory Guide for Isolation and Characterization. 2nd ed. Academic Press. San Diego, Calif.

Fjell, K. M., Seibel, W. and Gerstenkorn, P. (1994). Method for ash determination by conductivity. *Cereal Chem.,* **73**(4):510-511.

Fubara, E. P., Bassey, O. E and Ozioma, A. E. (2011). Evaluation of the effects of processing on the mineral contents of maize (*Zea mays*). *Libyan Agric Res Cent J Int.,* **2(3):** 133-137.

Gallup, J. M. (2011). qPCR inhibition and amplification of difficult templates Norfolk, UK: Caister Academic Pres, 101-121.

Garcia, L. F., Rharrabti, Y., Elhani, S., Martos, S. and Royo, C. (2005). Yield formation in Mediterranean Durum wheat under two contrasting water regime based on path coefficient analysis. *Euphytica.,* **146:** 203-212.

Gimenez, M., Piston, F. and Atienza S. (2010). Identification of suitable reference genes for normalization of qPCR data in comparative transcriptomics analyses in the Triticeae, *Planta.,* **233**(**1**):163-73

Gooding, M. J. and Davis, W. P. (1997). Wheat production and utilization, systems, quality and the environment. Book CAB International.

Gras, P. W., Anderssen, R. S., Keentok, M., Bekes, F.andAppels, R. (2001). Gluten protein functionality in wheat flour processing: a review. *Aust J Agric Res.,* **52:** 1311–1323.

Guenin, S., Mauriat, M., Pelloux, J., van Wuytswinkle, O., Bellini, C. and Gutierrez, L. (2009). Normalization of qRT-PCR data: the necessity of adopting a systematic, experimental conditions-specific, validation of references. *J Exp Bot*., **60**: 487-493.

Gupta, R. K., Sewa R. and Chauhan, D. S. (2002). All India coordinated wheat and barley improvement project, Directorate of Wheat Research Progress Report., **4**:19-20.

Guttieri, M., Mclean, R., Stark, J. C. and Souza, E. (2005). Managing irrigation and nitrogen fertility of hard spring wheat for optimum bread and noodle quality. *Crop Sci.*, Madison, **45**:2049-2059

Haberle, J. and Svobada, P. (2007). The effect of water supply during grain growth on the utilization of soil mineral nitrogen by winter wheat. *Sci Agric Biochem.,* **38:** 105-110.

Imbeaud, S., Graudens, E., Boulanger, V., Barlet, X., Zaborski, P., Eveno, E., Mueller, O., Schroeder, A. and Auffray, C. (2005). Towards standardization of RNA quality assessment using user-independent classifiers of microcapillary electrophoresis traces. *Nucleic Acids Res.,* **33**: 235-247.

Iqbal, S. H., Ghafoor, A. and Ayub, N. (2005). Relationship between SDS-PAGE markers and Ascochyta blight in chickpea. *Pak J Bot.,* **37**: 87-96.

Jain M. N. A., Tyagi, A. K. and Khurana, J. P. (2006).Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-ime PCR. *Biochem Biophys Res Commun.*, **345**: 646-651.

Jiang, D., Yue, H. and Wollenweber, B. (2009). Effects of post anthesis drought and water logging on accumulation of high molecular-weight glutenin subunits and glutenin macro polymers content in wheat grain. *J Agron Crop Sci.,* **195**: 89–97.

Kang, S. L., Zhang, Y., Ling, X., Hu, H. Lai and Gu, B. (2002). Effect of limited irrigation on yield and water use efficiency of winter wheat in the loess planteau of china. *Agric water manag.,* **55:** 203-216.

Kousar, A., Mallick, S., Gupta, M., Mondal, A. (2012). Drought Stress Responses of Wheat Genotypes with respect to Nutritional Quality, *Indian J Agric Biochem.,* **25** (2), 94-99.

Livak W. (1997, 2001). ABI Prism 7700 sequence detection system, User bulletin - 2.Relative quantification of gene expression.ABI company publication.

Luo, C., Branlard, G., Griffin, W. B. and McNeil, D. L. (2000). The effect of nitrogen and sulphur fertilization and their interaction with genotype on wheat glutenins and quality parameters. *J Cereal Sci.,* **31**: 185–194.

MacCready, R. M., Jack, G., Vernon, S and Owens, H. S. (1950). Determination of starch and amylase in vegetables-Application in Pea. *Anal. Chem.,* **22:**1156.

Mariana, C., Scholz, B. and Franco, C. (2013). Effect of high and low molecular weight glutenin subunits, and subunits of gliadin on physicochemical parameters of different wheat genotypes**,** Ciênc. Tecnol. Aliment., Campinas, **33**(1): 163-170.

Mohammad, R. and Razzaghi, S. (2015). Effect of drought stress on grain yield and p, k, ca and mg uptake of wheat cultivars, *Journal of Science.,* **5**(6): 414-417.

Morse, D. L., Caroll D., Weberg, Borgstrom, M. C., Ranger- Moore, J. and Gillies R. J. (2005). Determining suitable internal standard for mRNA quantification of increasing cancer progression in human breast cells by real time PCR. *Anal Biochem.,* **342 (1)**: 69- 77.

Muhammad, A. D., Haji, K. A. and Muhammad, S. (1997). Performance of different wheat (*Triticum aestivum* L.) varieties under the agro-climatic conditions of Dera Ismail Khan.Sarhad. *J Agric.,* **13**: 527-528.

Nadeem, M., Anjum, F., Khan, M., Sajjad M., Hussain S. and Arshad, M. (2016). Electrophoretic characteristics of gluten proteins as influenced by crop year and variety, *Int J Food Prop.*, **19**:897–910.

Nasir, M., Butt, M. S., Anjum, F. M., Sharif, K. and Minhas, R. (2003). Effect of moisture on the shelf life of wheat flour. *Int J Agril Biol.,* **5**:458–459.

Nicot, N., Hausman, J., Hoffmann, L. and Evers, D. (2005). Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *J of Exp Botany.*, **56(421)**: 2907–2914.

Nurit, E., Tiessen, A., Pixley, K. and Palclos-rojas, N. (2009). Reliable and inexpensive colorimetric method for determining protein-bound tryptophan in maize kernels. *J Agric Food Chem.,* **57** (16):7233-7238.

Obuchowski, W. (1999). Quality characteristics of durum wheat and the effect on past features. *Przegl.Zbo_.-Młyn.*, **1**: 33-34.

Ottman, M. J., Doerge, T. A. and Martin, E. (2000). Durum grain quality as affected by nitrogen fertilization near anthesis and irrigation during grain fill. *Agron J.,* **92**: 1035–1041.

Ozturk, A. and Aydin, F. (2004). Effect of Water Stress at Various Growth Stages on Some Quality Characteristics of Winter Wheat. *J Agron Crop Sci.,* **190**:93-99.

Paolacci, A., Oronzo, A., Tanzarella, E. and Ciaffi, M. (2009). Identification and validation of reference genes for quantification of RT-PCR normalization in wheat. *BMC Mol Biol.*, **10**: 1-27.

Parvaneh, V., Naseri, R., Moradi, M. (2014). The Effect of Drought Stress on Grain Yield, Yield Components and Protein Content of Durum Wheat Cultivars in Ilam Province, Iran, *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering*., **8**(6): 631-636.

Payne, P. I. and Lawrence, G. J. (1983). Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1 and Glu-D1 which code for HMW-GS of glutenin in hexaploid wheat. *Cereal Res Commun.,* **11**:29-35.

Peleg, Z. Y., Saranga, A., Yazici, T., Fahima, L.,Ozturk and I. Cakmak. (2008). Grain zinc, iron and protein concentrations and zinc-efficiency in wild emmer wheat under contrasting irrigation regimes. *Plant Soil*, **306**: 57–67.

Petit, C., Pernin, F., Hevdel, J. and Delve, C. (2012). Validation of a set of reference genes to study response to herbicide stress in grasses. *BMC Res Notes*., **5**:18.

Pfaffl, M.W. (2004). Quantification strategies in real-time PCR. In: Bustin SA (ed), La Jolla, CA: IUL Biotechnology Series, International University Line. *A–Z of Quantitative PCR*., 87–120.

Pfluger, L. A., D'Ovidio, R. D., Margiotta, B., Pena, R., Kazi, A. and Lafiandra, D. (2001). Characterization of high and low molecular weight glutenin subunits associated to the D genome of *Aegilopstauschii. TheorAppl Genet.,* **103:**1239-1301.

Pierre, C. S., Petersona, J., Rossa, A., Ohma, J., Verhoevena, M., Larsona, M. and Hoefera, B. (2008). White wheat grain quality changes with genotype, nitrogen fertilization, and drought stress, *Agron. J.,* **100**: 414-420.

Prabhasankar, P., Manohar, R. S. and Gowda, L. R. (2002). Physicochemical and biochemical characterization of selected wheat cultivars and their correlation to chapati making quality. *Eur Food Res Technol.,* **214**:131–137.

Rabbani, M, A., Maruyama, K., Abe, H., Khan, A., Katsura, K., Ito, Y.,Yoshiwara, K., Seki, M., Shinozaki, K. and Shinozaki, K. I. (2003).

Monitoring Expression Profiles of Rice Genes under Cold, Drought, and High-Salinity Stresses and Abscisic Acid Application Using cDNA Microarray and RNA Gel-Blot Analyses. *Plant Physiol.,* **1331**: 755–1767.

Rachon, L. (2002). Estimation of usefulness of domestic and foreign lines and varieties hard spring wheat (*Triticum durum* Desf.) for pasta production. *Biul Inst HodAklim Rosl.,* **231**: 129-137.

Rasmussen, C. C., Payne W. A., Smiley, R. SW. and Stolz, M. A. (2003). Yield and water use efficiency of eight wheat genotypes planted on seven dates in North Eastern Orregon. *Agron J.,* **95:** 836-843.

Rharrabti, Y., Elhani, S., Martos, V., Luis, F. and Moral, G. (2001). Protein and lysine content, grain yield, and other technological traits in durum wheat under Mediterranean conditions. *J Agric Food Chem.,* **49**:3802-3807.

Rharrabti, Y., Villegas, D., Garcia, M., Aparicio, N., Elhani, S. and Royo, C. (2001). Environmental and genetic determination of protein content and grain yield in durum wheat under Mediterranean conditions. *Plant Breed.*, **120**: 381–388.

Ririe, K. M., Rasmussen, R. P. and Wittwer, C. T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Anal Biochem.,* **245(2)**: 154-160.

Sadasivam, S. and Manickam, A. (1992). Biochemical methods. New Age International (P) Limited, Publishers.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual. Ed. 2, Cold Spring Harbor Laboratory, Cold Spring, Harbor, New York

Schmittgen, T. D. and Zakrajsek, B. A. **(**2000). Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods.,* **46**: 69-81.

Seligman, N. G. and Sinclair, T. R. (1995). Global environment change and simulated forage quality of wheat. II. Water and nitrogen stress. *Field Crops Res*., **40**: 29–37.

Shao, H., Chu, L. J., Manivannan, P., Panneerselvam, R. and Shao, M. (2009). Understanding water deficit stress-induced changes in the basic metabolism of higher plants biotechnologically and sustainably improving agriculture and the eco environment in arid regions of the globe. *Crit RevBiotechnol.,* **29:** 131-151.

Sharma, D., Saharan, V., Joshi, A. and Jain, D. (2015). Biochemical Characterization of Bread Wheat (*Triticum aestivum* L.) Genotypes Based on SDS-PAGE, *Triticeae genomics and genetics.,* **6**(2): 1-7.

Shewry, P. R. and Halford, N. G. (2002). Cereal seed storage proteins: structures, properties and role in grain utilization. *J Exp Bot.,* **53**: 947–958.

Singh, A., Chaudhry, R. and Shaema, R. (1993). Effect of inoculation and fertilizer level on yield, nutrients uptake and economics of summer pulses. *Ind J Potassium Res*., **9**: 176-178.

Singh, N. K., Shepherd, K. W. and Cornish, G. B. (1991). A simplified SDS-PAGE procedure for separating LMW- subunits of glutenin. *J of Cereal Res.,* **14**:203-208.

Singh, S. S. (2010). Wheat production in India and future prospects 8th International Wheat Conference.

Sladana, Z., Miroljub, B., Mirjana, L., Dejan D. and Drugana I. (2011). Characterization of protein from grain of different bread and durum wheat genotype. *Int J Mol Sci.,* **12:** 5878-5894.

Sondeep, S., Gupta, A., Kaur, N. (2012). Influence of Drought and Sowing Time on Protein Composition, Antinutrients, and Mineral Contents of Wheat, *Scientific World J.,* doi:10.1100/2012/485751.

Sramkova, Z., Gregova, E. and Sturdik, E. (2009). Genetic improvement of wheat- a review. *Nova Biotechnologica*. **9 (1)**: 27-52.

Torres, E. P., Paredes, M., Polanco, V. and Becerra, V. B. (2009). Expression analysis: A way to study tolerance to abiotic stress in crop species. *Chilean J Agri Res*., **69(2)**: 260-269.

Vandesomple, J., De Pretu, K. and Pattyn, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal controls gene. Genome Biology.

Vandesomple, J., Kubista, M., Pfaffl, M. W. (2009). Reference Gene Validation Software Improved Normalization. In Real-time PCR: current technology and application. Vol.47. Edited by Logan J, Edwards R, Saunders N. Norfolk UK: Caister Academic Press: 64.

Weegels, P. L., Pijpekamp, A. M., Graveland, A., Hamer, R. J. and Schofield, J. D. (1996). Depolymerisation and re-polymerization of wheat glutenin during dough processing. Relationships between glutenin macro polymer content and quality parameters. *J Cereal Sci.,* **23:**103–11.

Wong, M. L., and Medrano, J. F. (2005). Real Time PCR m-RNA quantitation. *Biotechiniques*., **39**: 75-85.

Yadav, S., Jolly, M., Sachdev, A. and Johari, R. (2000). Protein fractions and amino acid composition in developing grains of wheat. *Indian J of Agril Biochem.,* **13:**25-98.

Yongfang, W., Poole, R., Huttly, A., Toscano-Underwood, C., Feeney, K., Welham, S., Gooding, M., Mills, C., Edwards, K., Shewry, P. and Mitchell, R. (2008). Transcriptome analysis of grain development in hexaploid wheat, *BMC Genomics*., **9**:121

Zhang, H. P and Oweis, T. Y. (1999). Water-yield relations and optimal irrigation scheduling of wheat in the Mediterranean region. *Agric Water Manage.,* **38**: 195–211.

Zhang, J., Sui, X., Li, B., Su, B., Li J. and Zhou, D. (1998). An improved water use efficiency for winter wheat grown under reduced irrigation. *Field Crop Res.,* **59:** 91-98.

Zhao, Chang-Xing. Z., Ming-Rong, H., Zhen-Lin W., Yue-Fu, W. and Qi, L. (2009). Effects of different water availability at post-anthesis stage on grain nutrition and quality in strong-gluten winter wheat, *C. R. Biologies.,* **332:** 759–764.

Appendix

APPENDIX

Appendix 1 Production data of durum wheat varieties under irrigation treatments

Grain Yield Data (kg/ha)

Variety	T_0 (No irrigation)	T_1 (One irrigation)	T_2 (Two irrigation)
GW 1	1500	1628.80	2234.86
A-206	1325	1477.28	2325.77
A-9-30-1	886.37	1416.68	2136.38