गेहूँ में सूखा-सहनशीलता हेतु इथलीन एंव पॉलीअमीन की पारस्परिक क्रिया संबंधी भूमिका

INTERACTIVE ROLE OF ETHYLENE AND POLYAMINES ON THE DROUGHT TOLERANCE IN WHEAT

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2014

INTERACTIVE ROLE OF ETHYLENE AND POLYAMINES ON THE DROUGHT TOLERANCE IN WHEAT

A thesis

By

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Submitted to the Faculty of Post-Graduate School
Indian Agricultural Research Institute, New Delhi
In partial fulfillment of requirements for the award of the degree of

DOCTOR OF PHILOSOPHY
IN
PLANT PHYSIOLOGY

NEW DELHI
2014

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This is to certify that the thesis entitled **"Interactive role of ethylene and polyamines on the drought tolerance in wheat"** submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Plant Physiology** embodies the results of bonafide research work carried out by **Ms. Pranjali H. Ghodke** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that any help or source of information that has been availed of in this connection has been duly acknowledged by her.

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ACKNOWLEDGEMENT

Words are bound and knowledge is limited to praise ALMIGHTY the Lord of the world, who gave me the requisite potential and diligence for the successful accomplishment of this task. It is time to remember all those who made this moment to happen in my life which I dreamt for.

I express my deep sense of gratitude with reverence to my Chairman, Dr. Ajay Arora, Principal Scientist, Division of Plant Physiology, I.A.R.I, New Delhi, for his sagacious guidance and intellectual stimulation throughout the present study and in preparation of this manuscript. His scholarly suggestions, sincere guidance, sustained interest, valuable help and affectionate behaviour have been a fountain of great inspiration to me. Without his sincere guidance, efforts and involvement, the proposed research couldn't be completed. He nurtured my capabilities and gave me ample freedom to work. His positive attitude and hardworking nature will always inspire me throughout my life.

It is a great privilege for me to express my esteem and profound sense of gratitude to my Co-chairman Dr. Viswanathan Chinnusamy, Head, Division of Plant Physiology, I.A.R.I, for his constant encouragement and valuable suggestions. My sincere gratitude towards the members of the advisory committee comprising of Dr. V.P. Singh, Principal Scientist, Division of Plant Physiology, Dr. Aruna Tyagi, Principal Scientist, Division of Biochemistry, Dr. R.C. Bhattacharya, Principal Scientist, NRCPB and Dr. Neera Singh, Senior Scientist, Division of Agricultural Chemicals who always helped me in sorting out problems which occurred during the course of investigation.

I sincerely acknowledge and thank Dr. R.K. Sairam, Dr. Bhupinder Singh, Dr. P.S. Deshmukh, Dr, Sangeeta Khetrapal, Dr. Pramod Kumar, Dr. Rakesh Pandey, Dr. Vijay Paul, Dr. Ashish, and Dr. G. P. Singh for their whole hearted support during the course of my work. I also thank all my teachers for helping me to cope up with my academic stint as a student and for enriching me with enlightening thoughts and ideas.

I sincerely acknowledge and thank for all the co-operation received from Dr. D.V. Singh and Dr. Khushwaha, Technical officer, Dr. Jyotsna, Divya mam, Santosh, Dr. Ashish, Division of Plant Physiology, Dr. Sangeeta, Praveen Sir, Dr. Khan,

Technical staff, Division of post harvest technology, for helping me in fulfilling the requirements for successful completion of research work. I express my deep sense of gratitude to other staffs members of Plant Physiology department, Rai Ji, Takur Ji, Baneerji sir, Chandeshwar Ji, Ashok Ji, Arvind Ji, Dayashankar Ji, Meena Ji and all others for their kind cooperation during the study.

It is beyond my words to express my heartful thanks to Krishna Kumar and Dr. Gitte, M. Sc. Chairman whose support was beyond words and really inevitable in the completion of the study. I do take memories to cherish during my Ph. D. Programme with my classmates Krishnapriya, Shivram krishnan, Prashant and Ramesh; memorable friends Tejashri, Nisha, Suhas, Prinsi, Shashank and all my friends, especially whose co-operation and help in various ways brought this task to completion. I do take memories to cherish during my Ph. D. Programme with my juniors and senior friends.

I express my deep sense of gratitude towards my family and more than a folks affiliate especially my parents, Uncle, Vaishali, Manisha, Shital, In-Laws, Atul for their fond of love and best wishes for the advancement of my career, love, affection prayers and blessings received from them cannot be expressed in words.

Finally, the financial assistance provided by the I.A.R.I. in the form of Rajiv Gandhi senior Research Fellowship during the tenure is gratefully acknowledged.

And once and all I express my deep gratitude to the Great Almighty…

Place: New Delhi

Date: *(Pranjali H. Ghodke)*

Norman Borlaug says…..

If you desire peace, cultivate justice, but at the same time cultivate the fields to produce more bread; otherwise there will be no Peace…

Dedicated To My

Beloved Family & Research Guides

CONTENTS

Abbreviations

Chl	Chlorophyll
SG	Stay-green
GI	Greenness index
SPAD	Soil and plant analyzer development
SCMR	SPAD chlorophyll meter reading
LSR	Leaf senescence rate
Pn	Net Photosynthesis
SC	Stomatal conductance
DTE	Drought tolerance efficiency
HI	Harvest index
MSI	Membrane stability index
RWC	Relative water content
ROS	Reactive oxygen species
LOX	Lipoxygenase
SOD	Superoxide dismutase
CAT	Catalase
GR	Glutathione reductase
POX	Peroxidase
APOX	Ascorbate peroxidase
TBARS	Thiobarbituric acid reactive substances
H_2O_2	Hydrogen peroxide
CHLase	Chlorophyllase
PaO	Pheophorbide a oxygenase
PEG	Polyethylene glycol
ACS	1-aminocylopropane-1-carboxylic acid synthase

ACO	1-aminocylopropane-1-carboxylic acid oxidase
ERS	Ethylene response sensor
ADC	Arginine decarboxylase
SAMDC	S-adenosyl methionine decarboxylase
SPDS	Spermidine synthase
AVG	Aminoethoxy vinylglycine
1-MCP	1-methyl cyclopropene
PCR	Polymerase chain reaction
cDNA	Complimentary DNA
RNA	Ribonucleic acid
RT	Reverse transcription
EDTA	Ethylene diamine tetra acetic acid
DNA	De-oxy ribonucleic acid
dNTP	De-oxy nucleotide tri phosphate
HPLC	High performance liquid chromatography
GC	Gas chromatography
M	Molar
mol	mole
µL	microlitre

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

Global climatic changes and human activities make our planet earth water-scarce for agriculture, and feeding more people with less water is a major challenge faced by humanity in the present world (Foley *et al*., 2011). We need a 'Blue Revolution' in agriculture that focuses on increasing productivity per unit of water with a slogan of "more crop per drop" (Pennisi, 2008). Water deficit stress has been defined by Zhu (2002) as the lack of adequate moisture necessary for a plant to grow normally and complete its life cycle. Drought is a worldwide problem, getting more serious with global climate change (Hameed and Iqbal, 2014). It continues to be an important challenge to agricultural research scientists and plant breeders and one of the most active field of research in plant science focusing on understanding of plant molecular, physiological and genetic responses to drought stress conditions and the development of approaches towards improving drought tolerance and acclimation (Cabello *et al*., 2014). It is assumed that by the year 2025, around 1.8 billion people will face absolute water shortage and 65 % of the world's population will live under water stressed environments (Nezhadahmadi *et al*., 2013). Many factors can affect plants' responses to drought stress such as genotype, growth stage, severity and duration of stress, functional aspects of growth and development (Niu *et al*., 2012; Ozfidan *et al*., 2013), respiration (Ribas-Carbo *et al*., 2005), photosynthesis machinery (Saeedipour *et al*., 2011; Chen *et al*., 2012) and gene expression (Kakumanu *et al*., 2012).

Wheat cultivation in India started 5000 years ago (Feldman, 2001) and now it is one of the most widely grown cereal crop in India, occupying 17 % of the total cultivated land of the world. Wheat is the staple food for 35 % of the world population, and provides more calories and protein in the human diet than any other crop (FAOSTAT, 2014). In India it is the second most important winter cereal crop after rice contributing substantially to the national food security serving as a protein source and providing around 20 % of calories for human consumption who mainly depend on it (USDA, 2014). Global annual wheat production is 710 million MT in 2013-14, which feeds about one-fifth of human population (DES-Department of Agriculture and Co-operation, Ministry of India, 2013). To ensure food for the rapidly growing world population, wheat production needs to be doubled by 2050 (Ray *et al*.,

2013). Further increases in wheat production depend on higher yields rather than an increase in cropping area (Araus *et al*., 2003). Declining water resources challenge this notion as water availability impacts heavily on crop yields. Globally, India is among the top wheat producing countries and it ranked second next to China with maximum area under wheat cultivation about 29.8 million ha area in 2012-13, while in terms of productivity it ranked thirteenth to world average (FAOSTAT, 2013). Production in 2012-13 is 92.46 million tonnes which fell down by 2.42 million tonnes in comparison to previous year's (2011-12) historic production of 94.88 million tonnes (DES-Department of Agriculture and Co-operation, Ministry of India, 2013). This reduction in production in 2012-13 is due to the decline in productivity by 58 kg/ ha (1.84 %) followed by marginal reduction in area by 0.22 million ha (0.73 %). In addition, weather parameters that favored last crop season didn't go well with this season bringing down the country's production in 2012-13. In India, state wise analysis indicates that Uttar Pradesh grows more wheat on 9.73 million ha area with 30.30 million tonnes production sharing 32.77 % of India's production, followed by Madhya Pradesh, Punjab, Haryana and Rajasthan (Ministry of Consumer Affairs-Food and Public Distribution; Government of India, 2013). In India, out of estimated 140 million ha net cultivated area, 79.44 million ha (57 %) is rainfed, contributing 44 % of the total food grain production and 82.6 million hectares as gross irrigated crop area which is the largest in the world. In India, nearly 80 % wheat is cultivated under irrigated conditions, 66 % of it receives only partial irrigations, and the remaining 20 % is grown under rainfed environments. Wheat yields have been reported to reduce by 50-90 % of their irrigated potential by drought in marginal rainfed environments (Sareen *et al*., 2014).

Drought stress is always threatening wheat productivity and in order to achieve a sustainable self-sufficiency in its production with higher yield, there is need to develop drought tolerant genotypes. In this regard, identification and production of drought tolerant wheat genotypes with high yield is of special place in research. Grain yield and its stability have been always employed as main selection criteria in screening and introducing genotypes in various regions of environmental stresses (Marti and Slafer, 2014). Drought can affect all growth stages of wheat plant, among which reproductive stage is considered to be the most sensitive phase to water deficit stress (Pradhan *et al*., 2012; Ahmed *et al*., 2013). Drought stress at reproductive stage

lowers the allocation of dry matter to grains thus decreasing the grain filling rate (Madani *et al*., 2010; Khakwani *et al*., 2012). Drought during grain filling could limit the rate and duration of grain filling processes, causing small grain size, earlier physiological maturity, reduced number of grains, lower grain weight and grain yield resulting in a marked decrease of harvest index in wheat (Zareian *et al*., 2014). Test weight is one of the main wheat grain yield components determined by grain filling rate and duration and is highly sensitive to drought (Kumari *et al*., 2014). Grain filling rate is controlled by many genes but grain filling duration is affected by drought stress (Bahari *et al*., 2014). Drought tolerance efficiency (DTE; %) given by Fischer and wood (1981), is regarded as a valuable tool for evaluating drought tolerant genotypes under field conditions (Puri *et al*., 2013). Yield improvement in crop plants may be possible by incorporating stable and ideal plant traits pertaining for drought tolerance among several crop species. The wheat productivity thus, could be improved by better understanding the drought tolerance mechanism and identifying the agronomical and physiological traits which directly contribute for yield enhancement for developing best drought tolerant wheat genotype for areas with limiter water supply.

Drought stress affects many physiological processes like decrease water potential of leaf, photosynthesis activity, stomatal conductance, development of oxidative stress, damage to cellular membranes and production of metabolites which are toxic and ultimately causing plants' death (Ashraf *et al*., 2013). There is a relationship between different physiological attributes that can be used to appraise drought tolerance in crop plants by improving plant water relations, gas exchange traits and oxidative defense mechanisms that significantly affect the plant biomass production and final grain yield (Ali and Ashraf, 2011). Drought stress leads to an increased production of toxic reactive oxygen species (ROS), which cause oxidative damage to biomolecules resulting in severe cellular impairment (Miller *et al*., 2010; Suzuki *et al*., 2012). To survive such constraints, plants have evolved a variety of mechanisms at their cellular and molecular level in order to trigger adaptive responses (Mittler, 2011). Consequently, one classical approach towards obtaining increased stress tolerance relies on boosting endogenous antioxidant defense system including enzymatic and non- enzymatic ROS scavenging mechanism (Gill and Tuteja, 2010; Uzilday *et al*., 2012). Recent progress in this field includes the simultaneous overexpression of several antioxidant enzymes *viz*. superoxide

dismutase, catalase, peroxidase, ascorbate peroxidase, dehydroascrobate reductase, gluthatione reductase, gluthatione-S-transferase (Le-Martret *et al*., 2011; Luo *et al*., 2013) or increased production of the osmoprotectants like sugars, proline, trehalose, sugar alcohols and glycine betaine by introducing their biosynthetic enzymes in important crop species under drought stress (Krasensky and Jonak, 2012).

Stay-green is an important drought adaptive trait in cereals, while chlorophyll content is an index for a stay green character of plant (Borrell *et al*., 2014; Thomas and Ougham, 2014). It is an important trait that allows plants to retain their leaves in an active photosynthetic state contributing to a stable yield, when the plants are subjected to drought stress (Vijayalakshmi *et al*., 2010; Kumari *et al*., 2013). Drought stress causes leaf senescence which results in degradation of chlorophyll and disorganization of the photosynthetic apparatus (Matile *et al*., 1999) resulting in lower crop yields. However, plant species maintaining stay green character are able to photosynthesize for longer times by a delay in senescence (Hortensteiner, 2009). Delayed leaf senescence was associated with increased grain yield in wheat under drought stress (Kipp *et al*., 2014). Since, there is a strong association between the stay-green traits and grain yield (Kumari *et al*., 2013), selection for stay-green is expected to have significant implications in terms of wheat productivity, particularly under water stress environments.

Plant development is regulated and coordinated through the action of several classes of small molecules (plant growth regulators/ phytohormones), which may act either proximal or remote from their sites of synthesis to mediate genetically programmed developmental changes or responses to environmental stimuli (Colebrook *et al*., 2014). Phytohormones are essential for the ability of plants to adapt to abiotic stresses by mediating a wide range of adaptive responses (Peleg and Blumwald, 2011). Through the action of these molecules, plants are able to modify their physiological and biochemical responses to changes in their environment, a critical requirement for their survival as sessile organisms (Skirycz and Inze, 2010). Various types of stresses promote ethylene production in different tissues of plant species (Narayana *et al*., 1991; Morgan and Drew, 1997). An overproduction of ethylene induced by drought has frequently been related to grain weight reduction in wheat (Yang *et al*., 2014), maize (Habben *et al*., 2014) and rice (Do *et al*., 2013). Application of ethylene inhibitors increase grain weight in wheat (Yang *et al*., 2006)

and improve dry matter partitioning and grain filling in rice (Chen *et al*., 2013), whereas application of ethephon/ ethrel, an ethylene-releasing agent, has an opposite impact. Polyamines and ethylene are endogenous plant growth regulators mediating many physiological processes such as cell division, morphogenesis, embryogenesis, fruit set, senescence and responses to environmental stresses (Jang *et al*., 2012; Torrigiani *et al*., 2012).

Polyamines are polycationic compounds of low molecular weight which are present in most living organisms (Igarashi and Kashiwagi, 2010). The major polyamines in higher plants are putrescine, spermidine, spermine, thermospermine and cadaverine are found in free and conjugated forms (Bitrian *et al*., 2012). Polyamines are endogenous plant growth regulators associated with responses of plants to environmental stresses, including mineral nutrient deficiencies, osmotic and drought stress, salinity, heat, chilling, hypoxia and environmental pollutants (Gill and Tuteja, 2010; Alcazar *et al*., 2011; Hussain *et al*., 2011; Todorova *et al*., 2012). In plants, the major polyamines involved in crop response to drought stress are putrescine, spermidine and spermine (Gill and Tuteja, 2010). Treatment with inhibitors of polyamine biosynthesis reduces stress tolerance whereas, addition of exogenous polyamines restores successful stress acclimation as reported in maize (Terzi *et al*., 2014). Polyamines and ethylene share a common biosynthetic precursor, S-adenosyl methionine (SAM) in their biosynthesis pathway (Maiale *et al*., 2004) and there is a competition for this precursor between these two growth regulators under stress conditions (Harpaz-Saad *et al*., 2012). There are few reports which showed that in comparison to ethylene, high levels of polyamines are closely associated with better grain development and yield under water limited conditions like in maize (Feng *et al*., 2011) and rice (Wang *et al*., 2012). Polyamines are thought to play an essential role in the drought stress tolerance mechanism in crop plants. Hence, a central role played by ethylene and polyamines in response to drought stress is becoming increasingly evident. Recent study in major cereal crops like maize (Xue *et al*., 2009), rice (Wang *et al*., 2012) and wheat (Yang *et al*., 2014) reported a positive role played by polyamines particularly, spermidine and spermine in response to water deficit stress.

Very little is known about how polyamines and ethylene are involved in regulating leaf senescence rate and drought tolerance mechanism in wheat crop under

water deficit stress condition. Keeping these facts in mind the experiment was planned with the following research objectives:

1. Physiological characterization of stay-green traits in wheat genotypes/ germplasms under drought stress.

2. To study the association between ethylene and polyamines (putrescine, spermidine and spermine) with stay-green traits under drought stress.

3. Expression analysis of polyamines and ethylene biosynthesis/ perception genes in contrasting set of wheat genotypes under drought stress.

2. BACKGROUND

Environmental stress such as drought, temperature and salinity affect plant growth and development, presently the most important threat to sustainable agriculture (Osakabe *et al*., 2014). This has become a hot issue due to concerns about the global climate changes on plant resources, biodiversity and global food security (Ahuja *et al*., 2010; Sharma and Mehrotra, 2014). To feed the several billion people living on this planet, the production of high-quality food must increase with reduced inputs and on same available land, but this accomplishment will be particularly challenging due to global environmental changes. Plants are continuously exposed to a broad range of biotic and abiotic stresses, which hamper plant growth and productivity and often cause a series of morphological, physiological and biochemical changes (Suzuki *et al*., 2014). They must regulate their growth and development in order to respond to numerous external stimuli and an ever-changing environment (Peleg and Blumwald, 2011). Though challenged by environmental cues, they are capable of surviving by means of adaptations at the molecular, cellular and chromatin level of organization (Kim *et al*., 2012). Amongst the various abiotic stresses, drought is the most significant environmental stress affecting agriculture production worldwide and improving yields in water-limited environments is a major goal of scientists all over the world. Achieving greater crop yield per unit of rainfall/ available soil moisture is one of the most important challenges in dryland environments and efficient use of limited water resources may be one means of achieving this goal (Deng, 2014). Drought stress tolerance is seen in almost all plants but its extent varies from species to species and even within species and the plant growth stage at which it drought occurs (Nouri *et al*., 2011; Pradhan *et al*., 2012).

Wheat (*Triticum aestivum* L.) is one of the major cereal crops for the majority of world population, which feeds about one-fifth of human population (FAOSTAT, 2014). To ensure food for the rapidly growing world population, wheat production needs to double by 2050 (Ray *et al*., 2013). It feeds about 40 % of the world's population and provides 20 % of the calorie and protein requirements in human nutrition globally (Curtis and Halford, 2014). It is grown over 200 mha area in a range of environments throughout the world with a predicted global annual production of 710 million MT in 2013-2014 (FAOSTAT, 2014). Wheat is grown in northern,

central and eastern India where, Uttar Pradesh, Haryana, Punjab, Rajasthan and Madhya Pradesh are the major wheat producing states and accounts for almost 80 % of total production in India (DES- Department of Agriculture and Co-operation, Ministry of India, 2013). In India, only 1/ 3 irrigated wheat receives desired irrigations and the remaining area gets limited irrigation only (Sareen *et al*., 2014). The study by Kumar *et al*. (2014) projects that climate change will reduce the wheat yield in India in the range of 6 to 23 % by 2050 and 15 to 25 % by 2080. Hence there is the need for further improvement in the yield potential of wheat in order to meet current and impending challenges of food security (Curtis and Halford, 2014). Developing promising high yielding wheat genotypes which are responsive to limited irrigation conditions in arid and semi-arid regions is a good solution to the current issue. To increase the wheat productivity under such conditions different physiological techniques need to be adopted, for improving water use efficiency and breeding wheat genotypes tolerant to water and heat stress (Jatoi *et al*., 2011). In order to obtain further insight into mechanism that affect yield under drought, there is a need to understand the hormonal regulation of drought tolerance mechanism in wheat under water deficit stress condition.

2.1 Yield attributes

Cereal crops production and its sustainability are largely influenced by environmental constraints, particularly drought stress, causing reduced crop yields (Jacobsen *et al*., 2012). Limited water supply is a major constraint on wheat production worldwide, particularly in arid and semiarid regions (Bennett *et al*., 2012). According to Rajaram (2001), about 45 % of the 120 million ha of land allocated in developing countries to wheat production is prone to drought stress. Although, drought impedes wheat performance at all growth stages, it is more critical during the reproductive stage resulting in substantial yield losses (Pradhan *et al*., 2012). The severity and duration of the stress determines the extent of the yield loss. The principal reasons for these yield losses are reduced rates of net photosynthesis owing to metabolic limitations, oxidative damage to chloroplasts and stomatal closure with poor grain set and development (Farooq *et al*., 2014). Thus, drought is the major responsible factor for yield losses and limitations of wheat growth in many parts of the world.

The most widely used criteria for selecting high yield performance are test weight, harvest index, grain and biomass yield and drought tolerance efficiency under drought-stressed and more favorable irrigated condition (Mohammadi *et al*., 2011). There are some reports which showed that high yield potential is advantageous under mild drought stress, while genotypes with low yielding potential and high drought tolerance may be useful when stress is severe (Yasir *et al*., 2013). Selection and breeding for grain yield is the ultimate way to produce stress tolerant crop plants but due to the low heritability and complexity of grain yield, and its high dependence on environmental conditions, other traits such as yield components associated with drought resistance and grain yield can be employed (Golabadi *et al*., 2011). Some indices to determine drought tolerance are drought susceptibility index (DSI; %) given by Fischer and Maurer (1978) and drought tolerance efficiency (DTE; %) given by Fischer and Wood (1981). These indices are yield stability parameters or yield components which are based on yield reduction under drought stress (Bahar and Yildirim, 2010). In chickpea minimum yield reduction was reported in genotypes which had the highest DTE % and the lowest DSI % (Parameshwarappa and Salimath, 2010). Comparable finding in wheat (Mohammadi and Amri, 2011) and rice (Puri *et al*., 2013) where the genotypes with the highest DTE % had maximum yield in drought stressed environments. Drought tolerance efficiency (DTE; %) thus can be used as efficient criteria for selecting drought tolerant wheat genotypes under water deficit stress condition.

2.2 Water deficit stress indicators

2.2.1 Plant water status

Relative water content (RWC; %) of leaves is an important indicator of plant water status than thermodynamic state variable like water potential, turgor potential and solute potential (Sinclair and Ludlow, 1985) and are directly linked with the soil moisture content (Sher *et al*., 2013) and thus, recommended to be the better measure under stress conditions. Wheat crop under drought stress showed decline in relative water content of their leaves (Loutfy *et al*., 2012). Genotypic variation for drought tolerance in relation to leaf water relations under water-deficit conditions has been reported in different crops such as wheat (Ashraf and Harris, 2013) and barley (Marok *et al*., 2013) and many more. These variations in RWC may be endorsed due to the

differences in their ability to absorb water from the soil or reduced transpiration rate or osmotic adjustment to maintain tissue turgor and hence physiological activities (Ashraf *et al*., 2013) and it can be positively and significantly correlated with gain yield under water deficit stress. In cereals it has been demonstrated that resistance to drought is a quantitative trait and that RWC is a relevant tool for screening drought tolerance (Ahmed *et al*., 2014). In fact, RWC gives an idea of water status at a specific point of time of the level of water deficit, and this has been used as first information about the response to drought. Thus, RWC may be recognized as a beneficial drought tolerance indicator and can be used as selection criteria in breeding program for screening drought tolerant wheat genotypes.

2.2.2 Membrane stability index

Stability of cell membrane under water deficit stress is an important mechanism to resist drought condition. Cell membrane stability index (MSI; %) indicates the functionality of plasma lemma and in turn the normal functioning of cellular machinery (Razzaq *et al*., 2013). Cellular membranes when subjected to drought stress with uncontrolled increase in free radicals, which cause lipid peroxidation which makes the membrane leaky (Smirnoff, 1993). The stress induced burst in free radicals could also be partially related to the activity of lipoxygenase, which convert C18:2 and C18:3 to the corresponding hydroxyl peroxides (Bell and Mullet, 1991). Further damage to fatty acid could then produce small hydrocarbon fragments including malondialdehyde (MDA) content and the rise in MDA content under stress conditions suggests that drought stress damaged membrane through induced lipid peroxidation by means of ROS (Hameed *et al*., 2014). Higher MSI certainly means ability of the plants to resist drought as postulated by Sairam *et al*. (2002). This physiological parameter definitely contributes towards growth and productivity of plants under water deficit conditions. MSI exhibited a positive correlation with osmotic potential, K^+ concentration, osmotic adjustment and relative water contents; the parameters that are equally affected by stress. However, depletion of water has usually been considered as one of the major causes of increased cell membrane permeability of plants growing under salt stress (Chaffei-Haouari *et al*., 2013). It has been reported that genotypes which are able to maintain their higher MSI with lower membrane damages are considered as tolerant genotypes as seen in rice

(Iseki *et al*., 2014) and wheat (Kocheva *et al*., 2014). Therefore, this may serve as a useful trait for screening drought tolerant wheat genotypes.

2.3 Oxidative stress indicators

2.3.1 Reactive oxygen species

Plants encounter a variety of biotic and abiotic constraints (Atkinson and Urwin, 2012) during which there is sudden burst of reactive oxygen species (ROS) (Heyno *et al.,* 2011, 2013). ROS includes free radicals such as superoxide anion ($O2^{\bullet-}$), hydroxyl radical (•OH), as well as non-radical molecules like hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and so forth (Choudhury *et al*., 2013). Stepwise reduction of molecular oxygen (O_2) by high-energy exposure or electron-transfer reactions leads to production of the highly reactive ROS. In plants, ROS are always formed due to leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as a byproduct of various metabolic pathways localized in different cellular compartments. Under drought conditions, limitation of gas exchange by stomatal closure causes a loss in the balance between the light reactions and the calvin cycle, electron carriers can be over-reduced in chloroplasts and mitochondria resulting in the production of ROS by the transfer of electrons to molecular oxygen (Goh *et al*., 2012). All ROS are extremely harmful to organisms at high concentrations. When the level of ROS exceeds the defense mechanisms, a cell is said to be in a state of "oxidative stress." Elevated ROS like hydroxyl radicals under drought induced oxidative stress can cause lipid peroxidation and consequently membrane injury, oxidation of proteins, damage to nucleic acids, enzyme inhibition, activation of programmed cell death pathway and ultimately cell death (Hameed *et al*., 2011). Apart from their destructive effects in cells, hydrogen peroxide (H_2O_2), a major kind of ROS plays an important role in signal transduction for abiotic stress tolerance and act as an secondary messenger in many biological processes such as stomatal closure, growth, development, and stress signaling (Hossain and Fujita, 2013). Some authors suggested that the application of H_2O_2 at low concentrations could improve plant tolerance to abiotic stresses such as drought (Du *et al*., 2013), heat and heavy metal stresses (Qiao and Fan, 2014). Due to this dual role of ROS, plants are able to fine-tune the concentrations between certain ROS and their scavenging mechanisms. ROS

homeostasis is disrupted under stress in favour of production, constitutive and induced enzymatic antioxidant defenses and is considered a crucial component of plant stress tolerance (Suzuki *et al*., 2014). Accordingly, this has been an intense area of research, but the role of antioxidant enzymes in abiotic stress tolerance has still not been fully elucidated. In particular, the signaling role of ROS in plants is still only in the very early stages of investigation and need to be further examined.

2.3.2 Lipid peroxidation

Drought stress lead to increase in ROS content and when their level exceed the capacity of the plant to scavenge, lipid peroxidation in biological membrane increases, thereby affecting the normal physiological processes of the cell. Malondialdehyde (MDA) is one of the final product of oxidative modification of lipids especially polyunsaturated fatty acid component of phospholipids and is responsible for cellular membrane damage including changes in membrane fluidity, ion transport, loss of enzyme activity and protein cross-linking all these cellular changes ultimately results in cell death (Sharma *et al*., 2011). Content of MDA, was determined using the thiobarbituric acid reaction (Uzilday *et al*., 2014). Lipid peroxidation is one of the most challenging and detrimental effect of water deficit stress in the cell membranes of all the cells exposed to varied degree of stress and TBARS content is one of the determinants which indicate the severity of stress experienced by any plant (Sekmen *et al*., 2014). Reports are available that increase in MDA content with increase in the degree of drought stress in many crops like soyabean (Anjum *et al*., 2011), maize (Ali and Ashraf, 2011) and wheat (Singh *et al*., 2012). In addition, Mohammadi *et al*. (2011) found that lipid peroxidation was lower in the leaves of drought-tolerant chickpea genotype than that in drought-sensitive one. Similar findings reported in wheat that drought tolerant genotype showed lower lipid peroxidation and higher MSI value than the susceptible ones which is favorable measure for drought tolerance (Chakraborty and Pradhan, 2012; Hameed *et al*., 2013). Thus, lipid peroxidation may serve as the sensitive and beneficial trait for screening drought tolerant wheat genotypes.

2.3.3 Protease activity

Proteins are involved in structural and functional aspects of plant life and the main players in responses to stress but at the same time, often attacked by reactive

oxygen species (ROS) induced by stress conditions, causing structural changes in proteins that disturbs its functions (Kidric *et al*., 2014). Such aberrant proteins are frequently misfolded and aggregated and can be degraded by plant proteolytic enzymes like protease (Simova-Stoilova *et al*., 2010). Proteases, together with the specific endogenous inhibitors that regulate their activities, are the main players in carrying out and regulating intracellular protein breakdown for the maintenance of protein homeostasis (Eason *et al*., 2014). Protease activity as well as the expression of genes coding for proteases are enhanced during drought stress which is necessary for reorganization of plant metabolism, remodeling of cell protein components, degradation of damaged or unnecessary proteins and nutrient remobilization (Hameed *et al*., 2013). Lower proteolytic activity and decreased expression of certain cysteine protease genes under water deficit stress could be regarded as an indicator for drought resistance of winter wheat genotypes (Simova-Stoilova *et al*., 2010). Overall, the genotype with lower protease activity can be identified as superior drought tolerant genotype and these trait can be consider as a valuable tool for screening wheat genotypes under drought stress.

2.3.4 Proline content

In many plants, free proline accumulates in response to a wide range of biotic and abiotic stresses and act as a potent non-enzymatic antioxidant, as a singlet oxygen quencher and scavenger of hydroxyl radicals when it accumulates it prevents oxidative damage caused by ROS (Signorelli *et al*., 2013; Ben-Rejeb *et al*., 2014). It helps in stabilizing DNA, membranes and protein complexes, and provides a source of carbon and nitrogen for growth after stress relief. Proline accumulation upon stress depends on both activation of its biosynthesis and inhibition of its degradation and (Ben-Rejeb *et al*., 2014). Proline metabolism is involved in the regulation of intracellular redox potential and the storage and transfer of energy and reducing power and these changes may be more beneficial for plant tolerance to various environmental stresses (Szabados and Savoure, 2010; Sharma *et al*., 2011). Housekeeping proline biosynthesis occurs in the cytosol of plant cells, but it is possible that production moves to chloroplasts upon stress. Several studies have indicated that proline content increases during drought stress, and proline accumulation is associated with improvement in drought tolerance in plants (Man *et al*., 2011). The transgenic plants accumulating proline and were able to cope with

water deficit stress by osmotic adjustment, lowering lipid peroxidation rate in comparison to controls (Surekha *et al*., 2014). Stress-inducible proline accumulation might therefore act as a component of an antioxidative defense system to counteract the deleterious effects of oxidative stress, either by directly scavenging free radicals or by activating antioxidant systems under stress conditions (De-Carvalho *et al*., 2013). The activities of antioxidant enzymes like SOD, CAT, APOX, GR and POX are increased during drought stress conditions in wheat genotypes and it can be positively correlated with enhanced cellular proline accumulation as per Yin *et al*. (2012). Accumulation of proline under stress can thus be correlated with stress tolerance mechanism in plant species, and its concentration has been known to be usually higher in stress tolerant than in stress-sensitive plants (Keyvan, 2010). In conclusion, selection and use of the genotypes with higher proline, content under drought stress may be a practical approach to improve drought tolerance in wheat crop.

2.4 Antioxidant activity

To scavenge damaging ROS, plants have developed detoxifying enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidases (POX), glutathione reductase (GR), ascorbate peroxidase (APX), and non-enzymatic antioxidants such as ascorbate, glutathione, carotene and tocopherols (Gill and Tuteja, 2010; Suzuki *et al*., 2014). The antioxidant protection in plant cells is complex and highly compartmentalized as shown in **Fig. 2.1.** The SOD is a family of enzymes catalyzing the dismutation of superoxide anion radical to hydrogen peroxide in organelles and in the cytosol. While CAT are localized in peroxisomes which remove the bulk of hydrogen peroxide generated in photorespiration, and peroxidases with broad specificities are located in vacuoles, cell walls and the cytosol which use hydrogen peroxide for substrate oxidation (Hameed *et al*., 2013). For instance, the antioxidant responses of sugarcane (Cia *et al*., 2012), wheat (Hameed *et al*., 2013; Kocheva *et al*., 2014), rice (Gusain *et al*., 2014) and barley (Marok *et al*., 2013) and have been evaluated under drought stress. Recent data indicated that the up-regulation of SOD and CAT activities under stress conditions appears to be one of the general mechanisms used for drought tolerant crops (Boaretto *et al*., 2014). Furthermore, a remarkable correlation between SOD and CAT activities and H_2O_2, MDA and proline contents with drought tolerance observed for some genotypes suggest that by

combining physiological, morphological, genetical and biochemical approaches specific traits may be further identified that are related to drought tolerance (Kaur *et al*., 2013).

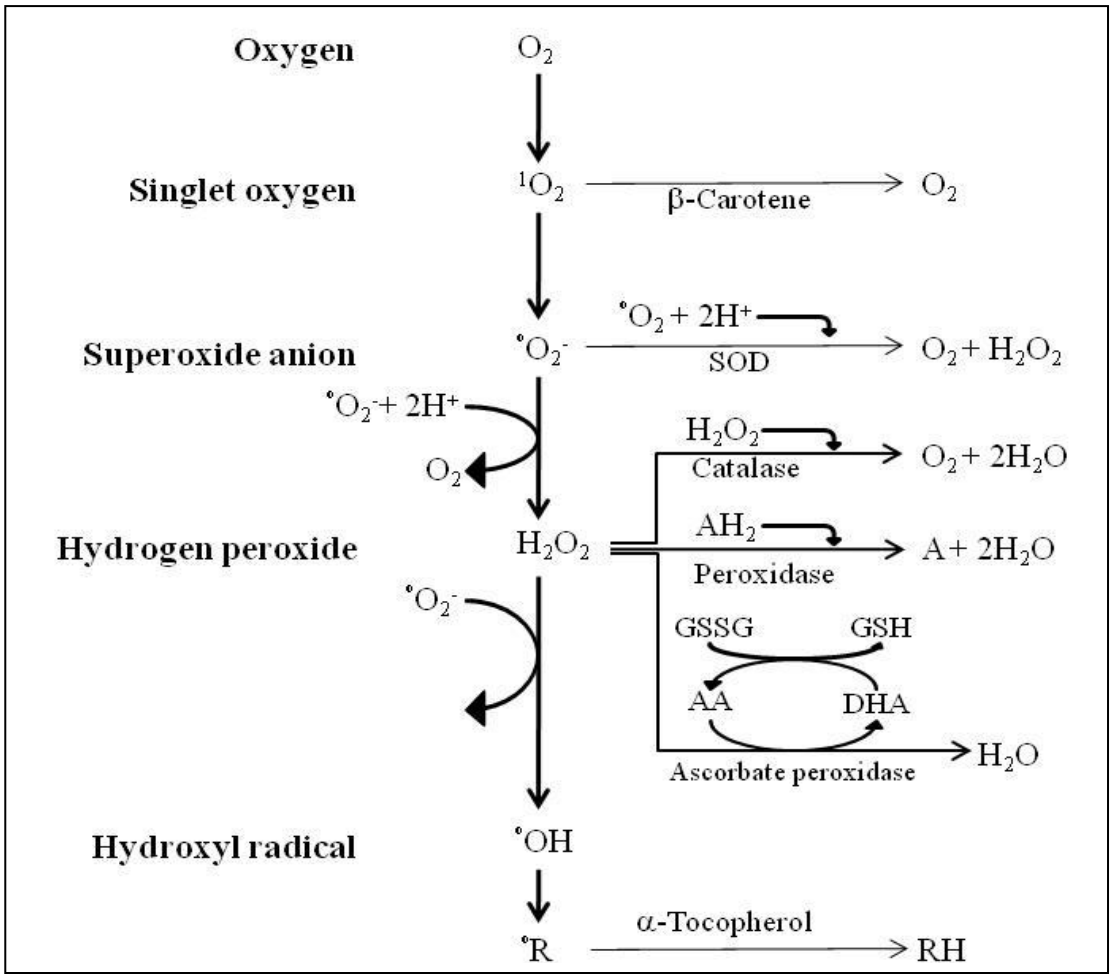


Fig. 2.1 Systematic representation of formation of reactive oxygen species (ROS) and its detoxifying scavenging antioxidant enzymes.

Abbreviations; AH_2: reduced substrate, AA: ascorbic acid, DHA: dehydro ascorbate, GSH: reduced glutathione, GSSG: oxidized glutathione, SOD: superoxide dismutase

2.5 Stay-green traits

Stay-green refers to the heritable delayed foliar senescence or chlorophyll catabolism character in crop plant species (Thomas and Ougham, 2014). It is an important trait that allows plants to retain their leaves in an active photosynthetic state when subjected to stress condition; it contributes to a long grain-filling period and stable yield even when the plant is under stressed condition (Vijayalakshmi *et al*., 2010). Stay-green and stress response traits are closely associated hence, genotypes

with stay-green traits would be believed to be better than the non stay-green one under drought and heat stress conditions (Kumari *et al*., 2013). The stay-green trait has been reported in different crops including wheat for drought resistance (De-Simone *et al*., 2014). Very few reports are available in wheat providing evidence of substantial variation for the stay-green trait (Kumari *et al*., 2013). In sorghum, genotypes with stay-green character have been selected successfully for their higher yield and yield stability index and now well thought-out as a promising selection tool for wheat under drought stress (Christopher *et al*., 2008). In wheat, stay-green genotypes has been associated with increased leaf area, duration of grain filling and photosynthetic rate all of which showed positive correlations with water use efficiency during the grain formation of wheat (Spano *et al*., 2003). Chen *et al*. (2010) physiologically characterized stay-green trait in wheat genotypes with higher photosynthetic competence during the grain filling stage under field conditions. Since there is a strong association between the duration of photosynthetically active leaf area and grain yield, stay-green trait, is therefore considered to be an important trait for improvement of yield under drought stress for cereals such as sorghum, maize, rice, wheat, etc. (Muchero *et al*., 2013). Many other scientists working on different crops have also reported this relationship. For example, in sorghum (Jordan *et al*., 2012; Borrell *et al*., 2014) and wheat (Lopes *et al*., 2012). Selection for stay-green wheat genotypes is thus expected to have significant implications in its productivity, particularly under water deficit stress conditions.

2.5.1 Pigment contents

Chlorophyll is the most abundant plant pigment on earth and responsible for light absorption during photosynthesis, a process upon which life on earth depends (Hortensteiner and Krautler, 2011). It composed of a tetrapyrrole ring with a central magnesium ion and a phytol chain and its metabolism can be divided into three phases; biosynthesis, chlorophyll cycle, and degradation (Hortensteiner, 2013). Chlorophyll a and b are the most important pigments active in the photosynthetic process while, the accessory pigment, carotenoids also have a very important role in photosynthesis (Marok *et al*., 2013). In plants, biosynthesis of carotenoids is a genetic characteristic, but environmental conditions also play a significant role in their synthesis. They plays a critical role as photoprotective compounds by quenching triplet chlorophyll and singlet oxygen derived from excess light energy, thus limiting

thylakoid membrane damage (Jahns and Holzwath, 2012). Plants with normal green colour leaf have higher chlorophyll content and normal chloroplasts which allow the plants to absorb more energy and efficient photosynthesis rate (Hamblin *et al*., 2014). Chlorophylls and carotenoids thus play an important role in photo assimilation and the synthesis of both molecules is coupled to chloroplast development. Leaf chlorophyll content (SPAD unit) was estimated nondestructively, using a SPAD-502 chlorophyll meter (Minolta Corp., Ramsey, New Jersey, USA). This index was selected preferentially, due to the close relationship between the readings of the portable chlorophyll meter and leaf chlorophyll content by analytical methods (Raymond and Daughtry, 2014). It has been used as a reliable tool for rapid screening of large number of genotypes for drought tolerance in field condition as it is a most reliable indication of leaf senescence. The SPAD values recorded with a portable chlorophyll meter provide an indication of the relative amount of total chlorophyll in the leaves, an indicator of foliar senescence. In Sorghum (Naus *et al*., 2010) found highly significant correlations between SPAD values and total chlorophyll content as well as between SPAD values and visual stay-green ratings.

One of the most affected compartments during abiotic stresses is the chloroplast. Chloroplastic pigments content are one of the most frequently used techniques to measure the degree of several abiotic stresses. In principle, another route to stay-green *via* pigment metabolism is the continued biosynthesis of chlorophyll in excess of the activity of the catabolic pathway. Plants engineered to overproduce chlorophyll by over expression of the gene encoding for chlorophyll biosynthesis (Kusaba *et al*., 2013). Whereas, the plants with disruption in chlorophyll biosynthesis showed reduced photosynthetic activity (Hortensteiner, 2009). Such chlorophyll deficient mutants have been used to study the function of photosynthetic components in many crop species like in rice (Morita *et al*., 2009) and wheat (Tian *et al*., 2012). High chlorophyll content is a desirable characteristic under drought stress conditions because it indicates a low degree of photo-inhibition of photosynthetic apparatus (Gregersen *et al*., 2013). Decrease in chlorophyll and carotenoid content was observed in wheat genotypes under drought stress conditions and reports showed that drought tolerant genotypes maintained their higher chlorophyll content as compared to susceptible one under water deficit stress (Abdipur *et al*., 2013).

Chlorophyll is a key component of photosynthesis but because of its light-absorbing properties it is a dangerous molecule and a potential cellular phototoxin (Hortensteiner, 2009). It is seen in situations where the photosynthetic apparatus of plants is overexcited, for example in high light conditions. Absorbed energy can then be transferred to oxygen, resulting in the production of reactive oxygen species (ROS). Likewise, inhibition of chlorophyll biosynthesis or degradation can lead to ROS production and cell death. Because of this, metabolism of chlorophyll is highly regulated during plant development (Hortensteiner and Krautler, 2011). Chlorophyll degradation occurring in response to biotic and abiotic stresses is not well understood yet. Drought induced leaf senescence is accompanied by a rapid breakdown of chlorophylls and a consequent loss of leaf green colour (Matile *et al*., 1999). A chlorophyll degradation pathway has been established based on the identification of key enzymes and catabolites in the pathway (**Fig. 2.2.**).

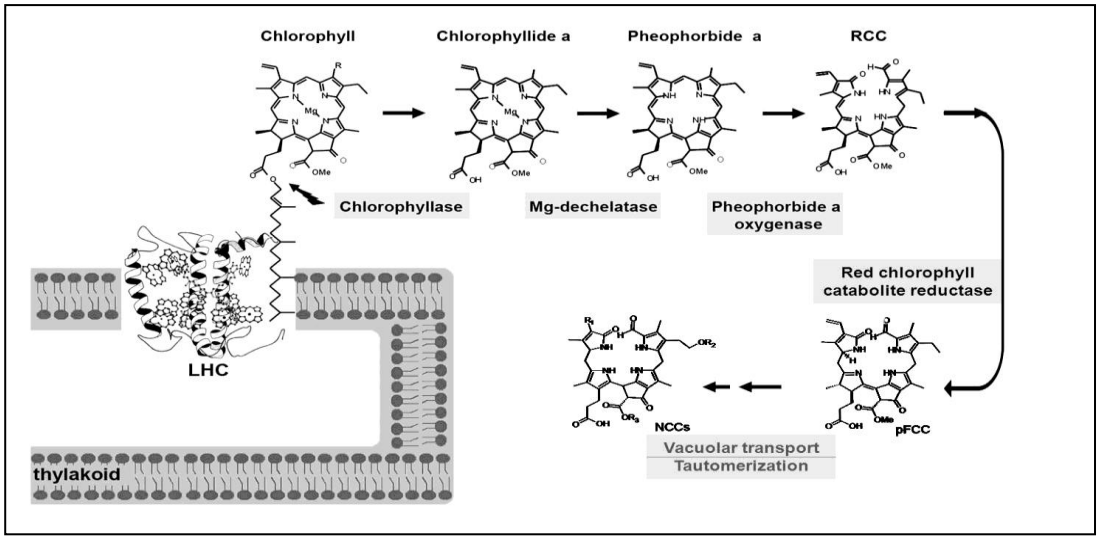


Fig. 2.2 Schematic representation of chlorophyll degradation pathway in higher plants as proposed by Pruzinska *et al*. (2005) and Shemer *et al*. (2008). the sequence of enzymes involved in pathway are chlorophyllase, Mg-dechelatase, pheophorbide a oxygenase (PaO) and red chlorophyll catabolite (RCC) reductase resulting in primary fluorescent chlorophyll catabolite (pFCC), exported to the vacuole and finally converted to non-fluorescent chlorophyll catabolites (NCCs), and stored indefinitely.

It takes place in chloroplast through the sequential action of enzymes starting with the dephytylation of chlorophyll, catalyzed by enzyme chlorophyllase, which

converts chlorophyll to phytol and chlorophyllide. Then, after the removal of the central Mg^{2+} from chlorophyllide by Mg-dechelatase, the porphyrin macrocycle of pheophorbide is oxygenolytically cleaved by the joint action of pheophorbide, an oxygenase (PAO) and Red chlorophyll catabolite reductase (RCCR). The product, a primary fluorescent catabolite (FCC), is further converted to the final non-fluorescent chlorophyll catabolites (NCCs) that are transported and stored within the vacuole. These sequential biochemical events were designated as PAO pathways due to the critical function of PAO enzymes, by which the pigments lose their green color (Christ and Hortensteiner, 2014). However, due to its complex biochemical structure, various aspects of chlorophyll metabolism are still poorly understood, and this is especially true for those associated with its degradation pathway (Hortensteiner, 2013).

2.5.2 Photosynthesis characteristics

Drought stress leads to a wide range of physiological responses in addition to the obvious reductions in photosynthesis, stomatal conductance, and leaf growth (Chen *et al*., 2011). Photosynthesis is one of the first physiological processes affected by drought stress (Signarbieux and Feller, 2011). Stomata are the site of CO_2 exchange for water in a leaf (Rebetzke *et al*., 2013). Variation in stomatal control offers promise in genetic improvement for photosynthesis, transpiration rate and stomatal conductance to improve wheat performance. Grain filling rate in wheat depends on current photosynthesis and redistribution of assimilates from reserve pools in vegetative tissues (Farooq *et al*., 2014). In wheat grain-filling rate under drought stress, decreases due to reduced photosynthesis, accelerated leaf senescence, and sink limitations (Madani *et al*., 2012). In wheat, the stay-green duration of the flag leaf and harvest index is positively correlated with water use efficiency during grain development (Bennett *et al*., 2012). Genotypes sustaining flag-leaf photosynthesis produce better yields as 30-50 % of the photosynthates needed during grain filling in wheat are contributed by flag-leaf photosynthesis. Drought affects carbon fixation through stomatal closure, which decreases CO_2 influx into mesophyll cells (Chaves *et al*., 2009). Reduced stomatal conductance is the primary cause of reduced photosynthesis during the drought stress (Cornic, 2000). There is good evidence that genotypic variation in leaf stomatal conductance (SC) has potential in selection for improved adaptation to a broad range of growing conditions. Stomatal conductance

affects the rates of diffusion of CO_2 into the leaf for photosynthesis and transpiration of water vapour out of the leaf (Rebetzke *et al*., 2013). Reductions in transpiration rate can slow water use and increase transpiration efficiency for wheat crops growing with limited water supply (Schoppach and Sadok, 2012). Alternatively, greater transpiration and photosynthetic rates may be associated with increased grain yield in irrigated environments where water is plentiful (Fischer *et al*., 1998). Separate genetic progress studies conducted under irrigated conditions have demonstrated strong, positive associations between stomatal conductance and wheat yield (Fischer *et al*., 1998). Similarly, increased stomatal conductance has contributed to greater photosynthesis capacity among wheat genotypes (Sadras and Lawson, 2011). Genotypic differences have been reported for stomatal conductance in wheat (Rebetzke *et al*., 2013). This variation is repeatable, indicating stomatal conductance may be targeted for improving adaptation of wheat crop to specific environments (Vassileva *et al*., 2011; Rebetzke *et al*., 2013). Hence, these gas exchange parameters *viz*. photosynthesis, transpiration rate and stomatal conductance plays vital role in maintaining yield and regulating drought tolerance mechanism in several crop species and there is a need to evaluate these traits, while screening the wheat genotypes for drought tolerance under water deficit stress condition.

2.5.3 Leaf senescence rate

Leaf senescence occurs at the final stage of leaf development in a genetically well-controlled manner (Penfold and Buchanan-Wollaston, 2014), and is considered the model for the study of plant senescence. It is one of the most remarkable developmental phenomena observed in plants in their natural habitat. It is the sequence of biochemical and physiological events comprising the final stage of leaf development from the mature, fully extended state, until death thereby, limiting the life span or longevity of a leaf (Jing and Nam, 2012). Although substantial progress toward understanding leaf senescence has been achieved in the past, we still know very little about the regulation of leaf senescence under drought stress. Leaf senescence represents a unique developmental process that is characterized by differential gene expression, active degeneration of cellular structures, and recycling of nutrients (Bleecker, 1998). Leaf senescence can impact crop production by either changing photosynthesis duration or by modifying the nutrient remobilization efficiency and harvest index (Wu *et al*., 2012). Loss of chlorophyll is the visible

symptom of leaf senescence. It is induced either by internal hormonal factors related to ageing or by external environmental factors such as high temperature and drought. Internal factors regulating senescence include age, developmental processes such as reproductive growth, and levels of plant hormones/ growth regulators (Jibran *et al*., 2013). Manipulation of many of these factors can thus alter the progression of leaf senescence. The plant hormones ethylene, abscisic acid, jasmonic acid, salicylic acid, auxin and brassinosteroids are believed to be inducers/ promoters of leaf senescence, while cytokinins and polyamines are antagonists of senescence (Gan and Hortensteiner, 2013; Jibran *et al*., 2013). In wheat, flag leaf senescence relates to the period of reallocating resources from the source to the sink during grain filling stage. Since, flag leaf photosynthesis in wheat contributes about 30-50 % of the assimilates for grain filling (Singh *et al*., 2012), the onset and rate of senescence are important factors for determining yield potential in wheat. Although many studies on the senescence of wheat have been reported (Gregersen *et al*., 2008), but now the research mainly focused on the correlation of leaf senescence with yield or drought tolerance in modern wheat genotypes. Visual rating of leaf senescence rate is quick to perform in the field on a plot basis using a 0–10 scale; as it represents an important tool used by plant breeders in screening large number of genotypes (Xu *et al*., 2000). It is practiced and confirmed as a valuable and efficient tool for evaluating the rate of leaf senescence in various crop species like wheat (Vijayalakshmi *et al*., 2010). Kipp *et al*. (2014) compared photosynthetic characteristics between two field grown wheat genotypes, and found that delayed flag leaf senescence is responsible for its higher grain yield. Among the traits evaluated for drought tolerance, visual scoring of leaf senescence rate and maintenance of leaf greenness were highly correlated with ability of plant to survive and recover from prolonged drought stress condition (Muchero *et al*., 2013). Earlier studies on senescence to assess genetic variation in wheat were mainly focused on induced leaf senescence or restricted to the flag leaf (Srivalli and Khanna-Chopra, 2009; Derkx *et al*., 2012; Naruoka *et al*., 2012). The fast senescing flag leaf mutants of wheat had low photosynthesis rate, photosynthate partitioning and yield (Derkx *et al*., 2012). Hence, leaf senescence rate is a valuable tool for screening drought tolerant wheat genotypes for any plant breeder with the objective of increasing yield under limited irrigation conditions. Furthermore, this understanding provides us with knowledge about molecular and biochemical pathways controlling leaf senescence under drought stress.

2.6 Plant growth regulators

Phytohormone are essential for the ability of plants to adapt to abiotic stresses by mediating a wide range of adaptive responses to changing environments, by mediating growth, development, nutrient allocation, and source/sink transitions (Do *et al*., 2014). The synergistic or antagonistic hormone action and the coordinated regulation of hormone biosynthetic pathways play crucial roles in the adaptation of plants to abiotic stress. They often rapidly alter gene expression by inducing or preventing the degradation of transcriptional regulators via the ubiquitin-proteasome system (Santner and Estelle, 2009). The overlap between hormone-regulated gene suites during the adaptive responses of plants to environmental stresses suggests the existence of a complex network with extensive cross-talk between the different hormone signaling pathways. The characterization of the molecular mechanisms regulating hormone synthesis, signaling, and action are facilitating the modification of hormone biosynthetic pathways for the generation of transgenic crop plants with enhanced abiotic stress tolerance (Peleg and Blumwald, 2011).

2.6.1 Ethylene

The phytohormone ethylene, the simplest alkene (C_2H_4), plays numerous roles in plant growth and developmental processes like seed germination, seedling growth, organ development, senescence, abscission and fruit ripening as well as plant responses to various environmental constraints like biotic and abiotic stresses (Iqbal *et al*., 2013; Lacey and Binder, 2014). It was the first gaseous biological signaling molecule discovered in 1901 when the Russian scientist Neljubov reported that ethylene was the active compound in illuminating gas that caused altered growth of pea seedlings (Neljubow *et al*., 1901). Three decades later Gane measured ethylene biosynthesis by apple fruits (Gane, 1934). Since these early observations, ethylene biosynthesis pathway has been observed and well elucidated in a large number of plant species. Yang and Hoffman (1984) brought the major discovery in ethylene biosynthesis by reporting the role of S-adenosyl L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) as the precursors of ethylene biosynthesis in plants. Ethylene is derived from the amino acid methionine which is converted to SAM (S-adenosyl L-methionine) by the enzyme SAM synthetase (**Fig. 2.3**). The SAM is then converted to ACC and 5′-deoxy-5′-methylthioadenosine

(MTA) by ACC-synthase (ACS). The conversion of SAM to ACC is the rate limiting step in the ethylene biosynthetic pathway. The ACC oxidase (ACO) catalyzes the conversion of ACC to ethylene. Thus, these two enzymes (ACS and ACO) are the most important in the formation and oxidation of the immediate precursor of ethylene *i.e.* ACC. The final conversion of ACC to ethylene is oxygen dependent (Kende, 1993) and yields CO_2 and cyanide as by-products. The conversion of ACC to ethylene catalyzed by ACO is oxygen dependent and under anaerobic conditions, ethylene formation is completely suppressed. In this reaction, Fe^{2+} and ascorbate are required as a cofactor and a co-substrate, respectively. The other reaction product, MTA must be recycled back into the methionine pathway to provide an adequate supply of methionine as substrate for the continuous ethylene production. The poisonous gas hydrogen cyanide (HCN) formed from the decomposition of ACC to ethylene is detoxified by β-cyanoalanine synthase (Kende, 1993). The ACS and ACO are thus the key enzymes in ethylene biosynthesis and are encoded by multigene families in response to internal stimuli and external stresses. Their activities are controlled at the transcriptional and post transcriptional level.

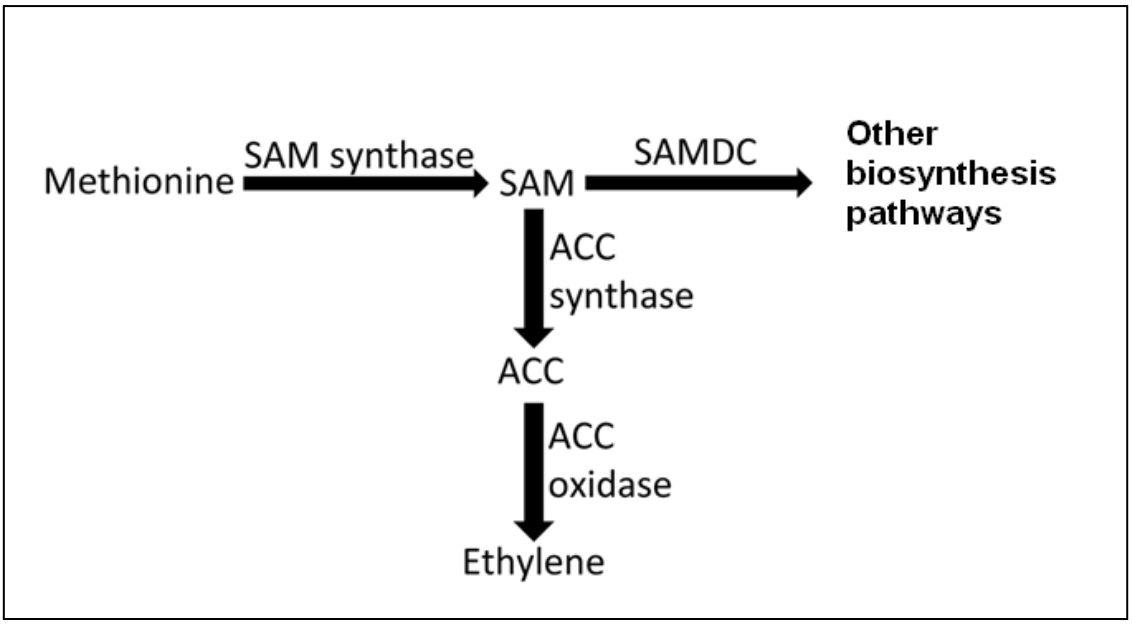


Fig. 2.3 Schematic representation of ethylene biosynthesis pathway in higher plants. ***Abbrevations***: ACC synthase, 1-amino-cyclopropane-1-carboxylic acid synthase; ACC oxidase, 1-amino-cyclopropane-1-carboxylic acid oxidase (Gill and Tuteja, 2010)

Plants exposed to environmental stresses speed up their ethylene production rate (Abeles *et al*., 1992). This occurs due to the regulation of ACS genes by environmental cues and the enhancement of its enzymatic action during stress

conditions. Therefore, if the ethylene production is modulated then the stress related injuries might be reduced. Under drought stress conditions plant accumulates ROS (reactive oxygen species) and results in oxidative burst inside the plant cells. This leads to activation of mitogen-activated protein kinases cascade (MAPK) in response to the oxidative burst, and induces the activation/ phosphorylation of ACC synthase enzyme (Liu *et al*., 2013). The phosphorylated ACC synthase becomes stabilized and subsequently enhances the production of ethylene. Ethylene interacts with nutrients uptake and controls plant responses under growth-limiting or stress conditions (Iqbal *et al*., 2013). The interaction between ethylene and drought stress has been a concern of many physiologists, but till date a collective study is lacking in relation to water deficit stress. Plant ethylene responses are mediated by a family of receptors, which have been studied in detail in *Arabidopsis* model plant. In *Arabidopsis thaliana* there are at least five ethylene receptor members (**Fig. 2.4**); ethylene receptor 1 (ETR1), ethylene receptor 2 (ETR2), ethylene response sensor 1 (ERS1), ethylene response sensor 2 (ERS2), and ethylene insensitive 4 (EIN4) (Hua *et al*., 1998; Hua and Meyerowitz, 1998; Bleecker and Kende, 2000). All the receptors are predicted to contain three transmembrane α-helices at the N-terminus that form the ethylene-binding domain. This is followed by a GAF (cGMP specific phosphodiesterases, adenylyl cyclases, and FhlA) domain, and a protein kinase domain. Three of the five *Arabidopsis* receptors (ETR1, ETR2 and EIN4) also contain a receiver domain at their C-termini that is similar to domains found in bacterial two-component receptors. Further, they all bind to ethylene and are related to two-component histidine kinase receptors (Hua *et al*., 1998, Kendrick and Chang, 2008) and are located at endoplasmic reticulum (ER) membranes (Dong *et al*., 2008). ERS homologues have been reported in a number of other plants including some monocots, such as rice (*Oryza sativa*), maize (*Zea mays*), and wheat (*Triticum aestivum*) (Chen *et al*., 2005).

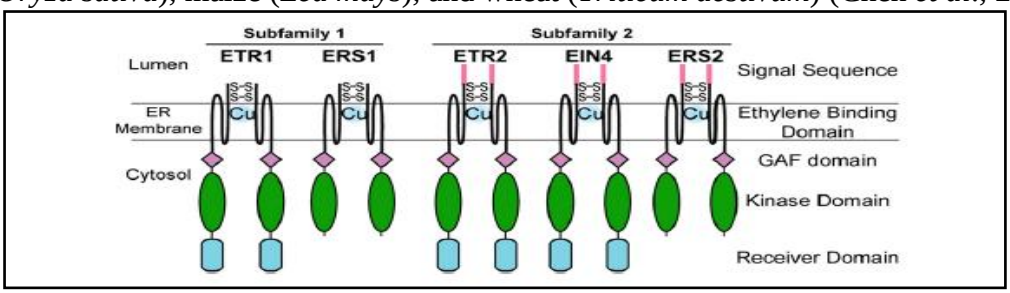


Fig. 2.4 Schematic representation of ethylene receptors *viz*. ETR1, ERS1, ETR2, EIN4 and ERS2 in *Arabidopsis* (Lacey and Binder, 2014).

Abbrevations: ETR1: Ethylene receptor; ERS: Ethylene response sensor; EIN: Ethylene insensitive

In field grown plants, ethylene production might be enhanced by exposure to environmental stresses, such as drought. It is recognized as one of the main factors that trigger the onset of leaf senescence (Abeles *et al*., 1992) under drought stress resulting in reduction in photosynthesis characteristics and ultimately yield in various economically important crop species (Wilkinson and Davies, 2010). Several types of evidence indicate that ethylene accelerates the leaf senescence rate in wheat genotypes during reproductive stage and hence reduces grain yield (Yang *et al*., 2014). Although the pattern of ethylene production differs between various wheat genotypes, there is clear evidence that the drought tolerant genotype reported with decreased ethylene production rate as compared to susceptible one under water deficit stress condition (Habben *et al*., 2014). In addition, exogenously sourced ethylene like ethephon or ethrel accelerated senescence, by the rapid chlorophyll degradation in the flag leaves and the vegetative parts of the ears (Beltrano *et al*., 1994), and reduced grain yield (Beltrano *et al*., 1994; Klassen and Bugbee, 2004). This decrease was completely reversed by inhibitors of ethylene biosynthesis like 1-aminopropyl vinylgylcine or 1-methyl cyclopropene (1-MCP), an antagonist of ethylene action (Labrana and Araus, 1991; Beltrano *et al*., 1994) resulting in increased grain weight and yield (Beltrano *et al*., 1994). Data concerning the effects of water stress on ethylene production in wheat are inconsistent and there is a need to evaluate the role played by ethylene in response to drought stress in wheat.

2.6.2 Polyamines

Polyamines (PAs) are small aliphatic amines that are ubiquitous in plants, animals and microorganisms (Takahashi and Kakehi, 2010). The major polyamines in plants, such as putrescine (Diamine; 1,4-diaminobutane), spermidine (Triamine; 1,8-diamino-4-azaoctane) and spermine (Tetramine; 1,2-diamino-4,9-diazadodecane), behave as positively charged cations at physiological pH and can interact with anionic macromolecules like DNA, RNA, acid phospholipids, proteins and cell wall components such as pectin. They can also act as a source of reactive oxygen species (ROS) but also as ROS scavengers and activators of key of antioxidant enzymes (Pottosin *et al*., 2014). The simplest polyamine, putrescine is formed either by the direct decarboxylation of L-ornithine through ornithine decarboxylase (ODC) or by decarboxylation from L-arginine to agmatine by arginine decarboxylase (ADC), arginine decarboxylase (ADC), agmatine iminohydrolase (AIH) and N-carbamoyl

putrescine amidohydrolase (CPA) and thereafter to putrescine. Putrescine is then converted to spermidine and spermine by successive action of the enzymes like spermidine synthase and spermine synthase with the use of decarboxylated S-adenosyl methionine (dcSAM) as an aminopropyl donor. The dcSAM is produced by S-adenosyl methionine decarboxylase enzyme (SAMDC) from SAM (Gill and Tuteja, 2010) as shown in **Fig. 2.5.** SAMDC is thus thought to be the major regulating enzyme in the spermidine and spermine biosynthesis. Polyamines are further metabolized by oxidation and conjugation with other molecules (Moschou *et al*., 2008).

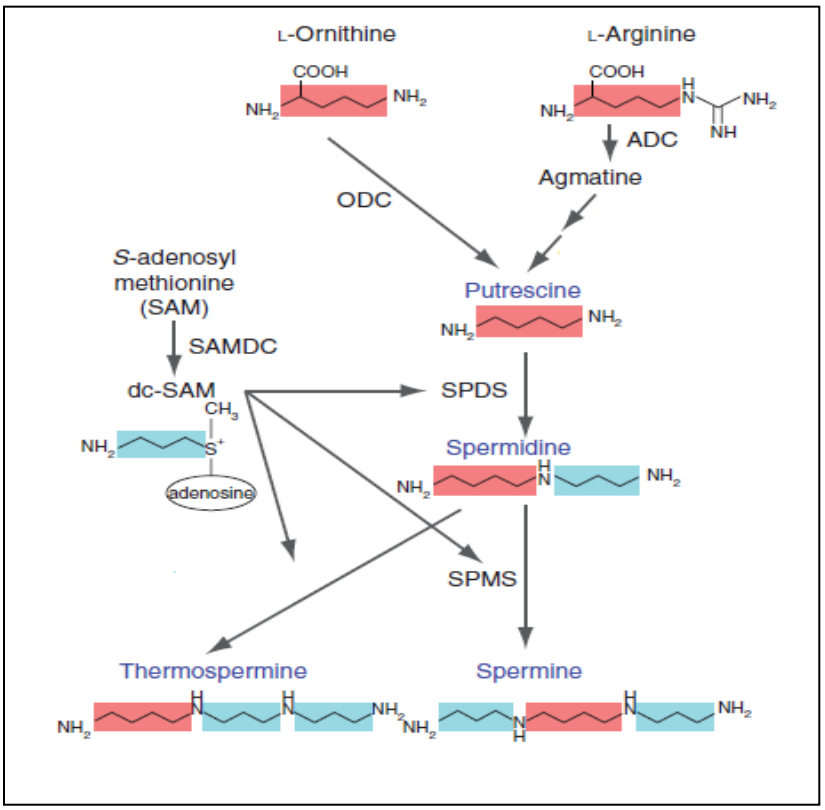


Fig. 2.5 Schematic representation of polyamine biosynthesis pathway in higher plants (Takahashi and Kakehi, 2010).

Abbrevations: ADC, arginine decarboxylase; ODC, ornithine decarboxylase; SAMDC, S-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase

Polyamines are involved in a wide range of biological processes, including growth, development (Moschou and Roubelakis- Angelakis, 2013; Do *et al*., 2014) as well as plant responses to various kind of environmental stresses like osmotic and

drought stress (Grzesiak *et al*., 2013, Yin *et al*., 2014), salinity and heavy metal stress (Gill and Tuteja, 2010; Hussain *et al*., 2011; Gupta *et al*., 2013). Many authors reported a possible involvement of polyamines in plant adaptation to several environmental stresses as treatment with polyamine biosynthesis inhibitors of MGBG, an inhibitor of SAMDC, significantly reduced, the grain yield in rice (Chen *et al*., 2013) whereas; addition of exogenous polyamines restores successful stress acclimation. Exogenous spermidine treatment can enhance tolerance of tomato plants to salinity-alkalinity stress (Hu *et al*., 2014) while, application of spermine would improve the plant reproductive health under osmotic stress condition in soybean (Radhakrishnan and Lee, 2013). In addition, exogenous spermine alleviates oxidative stress by regulating antioxidant systems in cucumber seedlings (Shu *et al*., 2013). Polyamines improved drought tolerance mainly by activation of antioxidant systems, both enzymatic and non-enzymatic (proline, anthocyanins and soluble phenolics) (Tiburcio *et al*., 2014). This reduces the amount of ROS produced and stabilizes membrane structures. Therefore, polyamines are thought to play an essential role in the environmental stress tolerance of plants. There are reports that high levels of PAs are closely associated with higher kernel set and better seed development in maize (Feng *et al*., 2011) and a greater endosperm cell division rate and grain-filling rate in rice (*Oryza sativa* L.) (Wang *et al*., 2012). Furthermore, it has been noted that genetic transformation with polyamine biosynthetic genes encoding ADC, SAMDC or SPDS improved environmental stress tolerance in various plant species. Polyamines have been modulated by the over-expression/ down-regulation of ADC/ODC/SAMDC genes (Minocha and Sun, 1997). Over-expression of polyamine biosynthetic genes like ADC in *Arabidopsis* (Wang *et al*., 2011) and SAMDC in rice (Peremarti *et al*., 2009) and SPDS (Kasukabe *et al*., 2006) has increased tolerance to various abiotic stresses. However, the question as to whether polyamines play a protective role against drought stress in wheat crop has received little attention in recent years due to changing environmental conditions and water shortage problem faced by large farmers population across the country.

2.6.3 Cross talk between ethylene and polyamines under water deficit stress

Polyamines and ethylene are endogenous plant growth regulators and play important roles in plant growth, development and responses to environmental stresses (Jang *et al*., 2012; Torrigiani *et al*., 2012). Little information was available describing

the synchronous changes of ethylene and polyamines levels, activities of enzymes involved in their synthesis, and expression levels of key genes encoding these enzymes in response to drought stress and relationships with grain yield. The biosynthesis pathway of polyamines is linked with that of ethylene *via.* S-adenosyl methionine (SAM) (**Fig. 2.6**) and it has been suggested that there is competition between ethylene and polyamine for SAM (Harpaz-Saad *et al*., 2012).

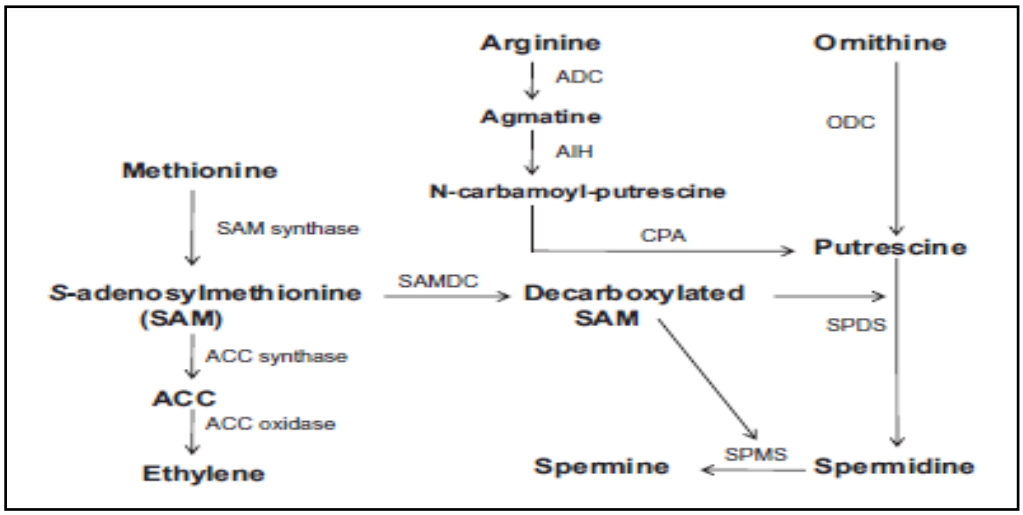


Fig. 2.6 Schematic representation of cross-talk between ethylene and polyamine biosynthesis pathways in plants (Wimalasekera *et al*., 2011).

Abbrevations: ADC, arginine decarboxylase; AIH, agmatine iminohydrolase; CPA, N-carbamoyl putrescine amidohydrolase ; ODC, ornithine decarboxylase; SAM synthase, S-adenosylmethionine synthase; SAMDC, S-adenosylmethionine decarboxylase; SPDS, spermidine synthase; SPMS, spermine synthase; ACC synthase, 1-amino-cyclopropane-1-carboxylic acid synthase; ACC oxidase, 1-amino-cyclopropane-1-carboxylic acid oxidase

In contrast to polyamines, high levels of ethylene or an overproduction of ethylene induced by drought have frequently been related to abortion in maize (Habben *et al*., 2014) and reduction in grain weight of rice (Wang *et al*., 2012) and wheat (Yang *et al*., 2014). These imply that there must a cross-talk between polyamines and ethylene in regulating plant response to drought stress as reported by Chen *et al*. (2013, 2014) in rice under drought stress. The increasing number of papers on this subject shows the importance of understanding polyamines and their interaction with ethylene in explaining the mechanism of plant resistance to drought stress. However, the exact molecular mechanism governing the cross talk between ethylene and polyamines in wheat under drought stress is not yet clear.

3. MATERIALS AND METHODS

The present study was conducted in the Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi (latitude 28 ^{0}N and longitude 77 ^{0}E, and about 250 m above mean sea level). The plants were grown in the pot culture, Division of Plant Physiology, in two *rabi* seasons during 2011-12 and 2012-13, with recommended cultural practices. The data on temperature and rainfall during the cropping seasons are presented in **Fig. 3.1** and **Fig. 3.2.** The experiments were also conducted in lab, by raising wheat seedlings for 10 days hydroponically in growth chamber; osmotic stress was imposed by polyethylene glycol (PEG-6000, 20 %).

3.1 Plant materials

In the first crop season (2011-12), thirty-five wheat genotypes were used for the experiment as suggested by the breeder to screen them on the basis of their stay-green and drought tolerance characteristics (**Table 3.1**). The genotypes were obtained from Division of Genetics, IARI, New Delhi.

Sowing was done in earthen pots (about 30 cm in diameter and 30 cm in depth) filled with clay loam soil and farmyard manure (FYM) in 3:1 ratio during *rabi* (winter) season. Nitrogen, phosphorus and potash fertilizers were applied at the rate of 60: 60: 60 kg per hectare, respectively in the form of urea, single super phosphate and muriate of potash at the time of sowing. Remaining 60 kg N ha^{-1} was given after 25 days of sowing.

In the second crop season (2012-13) further detailed studies were conducted with three selected wheat genotypes as follows by sowing in pot culture following recommended cultural practices as mentioned above.

1. HW 4022 (Drought tolerant and slow senescing)
2. HW 2078 (Relatively drought tolerant and intermediate slow senescing)
3. HW 4059 (Drought susceptible and fast senescing)

3.2 Experimental treatments

The genotypes were raised in pot culture of Division of Plant Physiology, for the first crop season, date of sowing was 25th November, 2011. Ten seeds per pot for all the thirty-five wheat genotypes were used at the time of sowing. On 15th December,

2011 thinning in each pot was done, keeping five plants per pot. At the time of 50 % anthesis stage water deficit stress treatment was imposed by with-holding irrigation for 10 days. Scheduled routine of irrigation was practiced for control plants. After screening the genotypes on the basis of stay-green and drought tolerance efficiency three wheat genotypes *viz*., HW 4022 as drought tolerant with slow senescing, HW 2078 as relatively drought tolerant with intermediate senescing and HW 4059 as drought susceptible and fast senescing was selected and sown in next growing season on 26th November, 2012. Recommended cultural practices and water deficit stress treatment were carried out as described above. Scheduled routine of irrigation was practiced for control plants and for treated plants when they were not subjected to water deficit stress, throughout the crop growth period. Each treatment was replicated thrice for all the genotypes studied.

For biochemical and molecular analysis, study was conducted in growth chamber under controlled growing conditions. Seeds of selected contrasting wheat genotypes (HW 4022, HW 2078 and HW 4059) were surface sterilized with 2.5 % sodium hypochlorite for 15 min and then washed 4-5 times with distilled water. These seeds were then germinated in petridishes with wetted germination paper at 25 ^{0}C under dark. After 48 h of incubation, uniformly germinated seeds were selected and raised in 50.0 mL test tubes containing half-strength Hoagland solution. The hydroponically raised seedlings were grown for 10 days in growth chamber at 25 ^{0}C day/ 20 ^{0}C night with light intensity of 1200 µmol $m^{-2}s^{-1}$ and 90 % relative humidity. For each genotype twenty seeds were sown in each test tube and twenty-four test tubes were used for each genotype in which three tubes will go for each treatment. Osmotic stress was imposed to 10 days old seedlings using 20 % polyethylene glycol (PEG-6000) solution giving osmotic potential of -4.91 MPa (Michael and Kaufman, 1972), where double distilled water (0.0 MPa) served as controls. They were treated with ethylene inducer (ethrel; 10 ppm), ethylene biosynthesis inhibitor (aminoethoxy vinylglycine; AVG, 2 ppm) and ethylene signalling inhibitor (1-methyl cyclopropene; 1-MCP, 10 ppm). The treatment combinations given to 10 days old seedlings were described in **Table 3.2.**

3.3 Sample collection

Plants were sampled and observations were recorded for growth, stay-green, physiological, biochemical and yield parameters. Tagging was done from the date of

Genotypes employed in the present study		
PBW 555	CBW 23	HUW 541
LOK 64	CBW 39	HW 2031
CBW 14	DBW 39	HW 2022
DBW 44	HD 2789	HW 2040
DBW 77	HD 2888	HW 2060
HD 2923	HW 2034	HW 2083
HI 1544	HW 2036	HW 4010
HW 2021	HW 2059	HW 4029
HW 4022	HW 2078	HW 4059
HW 4024	HW 4026	HW 5209
PBW 590	HW 4055	PBW 142
HW 4207	PBW 550	

Table 3.1 Indian and CIMMYT wheat genotypes employed in the present study

Treatment combinations		Duration
Osmotic stress		
T1	Control (Double distilled water)	6 h
T2	PEG 6000 (20 %)	6 h
T3	Control + Ethrel (10 ppm)	6 h
T4	PEG + Ethrel (10 ppm)	6 h
T5	Control + AVG (2 ppm)	6 h
T6	PEG + AVG (2 ppm)	6 h
T7	Control + 1-MCP (10 ppm)	24 h
T8	PEG + 1-MCP (10 ppm)	24 h
Drought stress		
T1	Normal Irrigation	
T2	Withholding Irrigation	10 days

Table 3.2 Treatment combinations given to wheat genotypes in 10 days old seedling/ reproductive stage

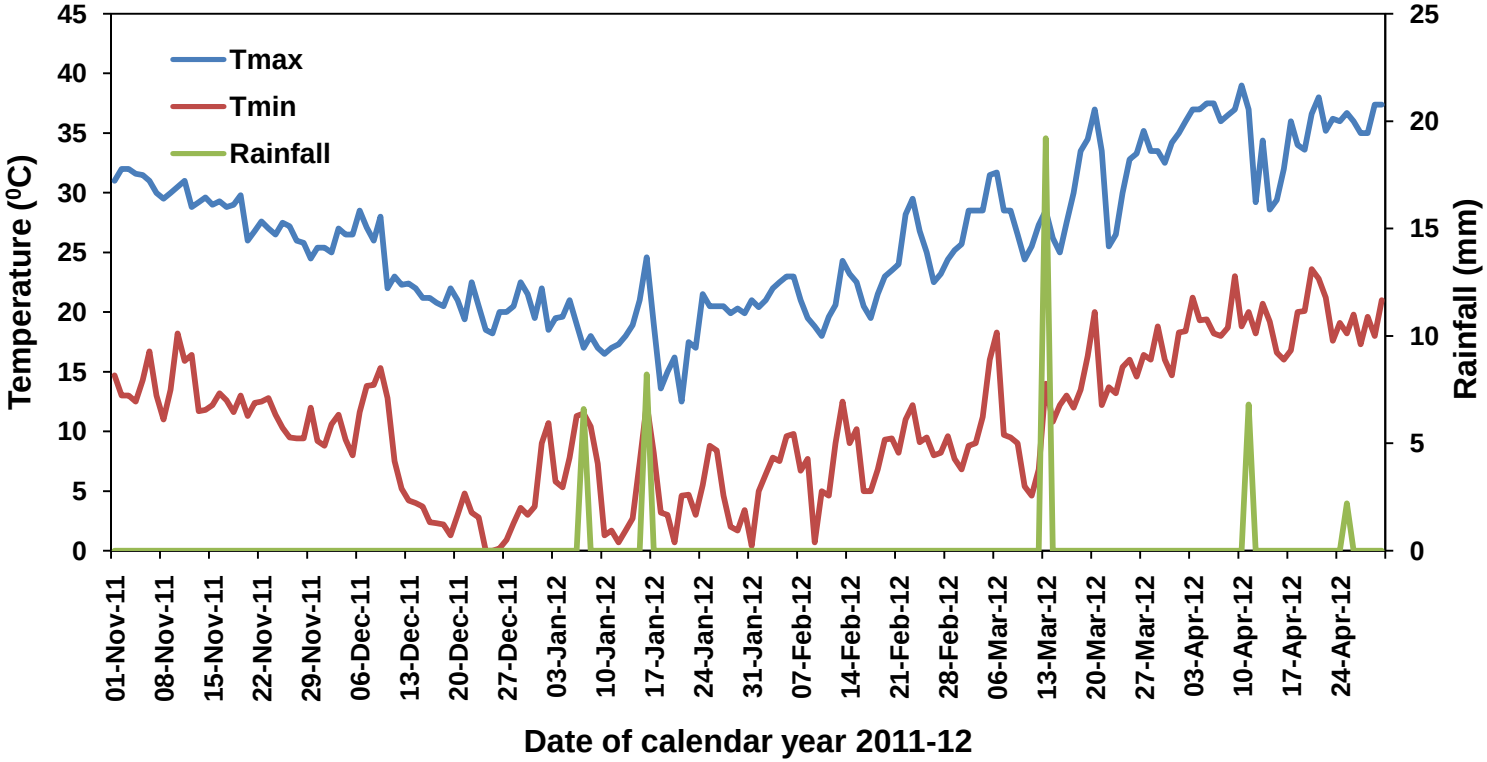


Fig. 3.1 Graphical representation of weather data during the wheat crop season 2011-12

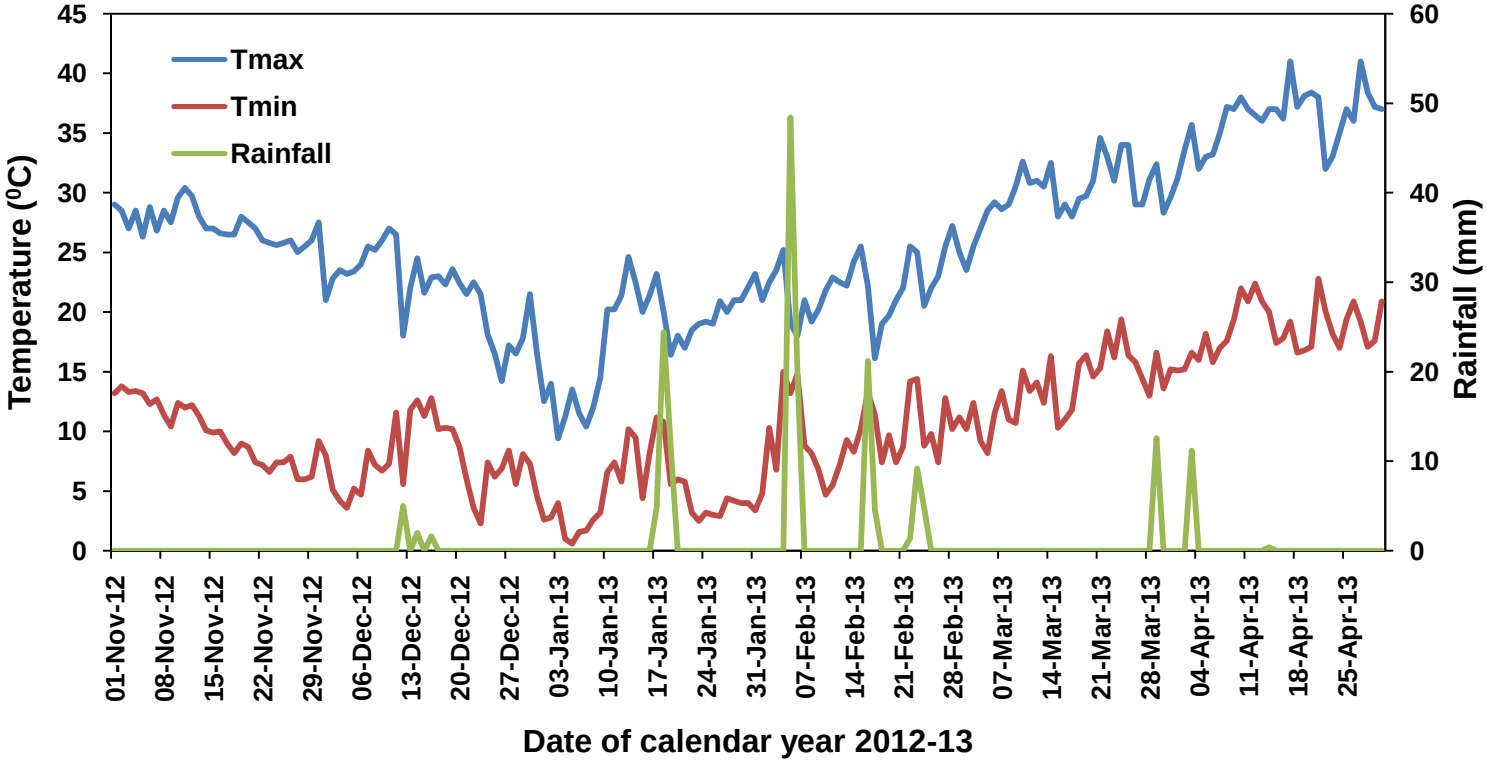


Fig. 3.2 Graphical representation of weather data during the wheat crop season 2012-13

50 % anthesis which is different for different genotypes. Uppermost fully expanded flag leaf was selected from control and water deficit stress plants for recording physiological observations and collected for molecular and biochemical studies. For molecular studies at seedling stage, 10 days old leaf samples from control and osmotic stress with different treatment combinations were used for total RNA extraction, which was done immediately after sample collection (**Table 3.2**).

3.4 Observations recorded

3.4.1 Physiological parameters		
3.4.1.1	Relative water content (RWC; %)	
3.4.1.2	Membrane stability index (MSI; %)	
3.4.1.3	Stay-green traits	
	3.4.1.3.1	SPAD chlorophyll meter reading (SCMR)
	3.4.1.3.2	Total chlorophyll and carotenoid content
	3.4.1.3.3	Photosynthesis rate
	3.4.1.3.4	Leaf senescence rate (LSR)
3.4.1.4	**Yield and yield related traits**	
	3.4.1.4.1	Test weight (1000 seed weight)
	3.4.1.4.2	Harvest index (HI; %)
	3.4.1.4.3	Drought tolerance efficiency (DTE; %)
3.4.2 Biochemical parameters		
3.4.2.1	**Oxidant production**	
	3.4.2.1.1	Hydrogen peroxide (H_2O_2) content
3.4.2.2	**Oxidant enzyme activity**	

	3.4.2.2.1	Lipoxygenase (LOX) activity
3.4.2.3	**Protease activity**	
3.4.2.4	**Lipid peroxidation**	
	3.4.2.4.1	Thiobarbituric acid reactive substances (TBARS) content
3.4.2.5	**Osmolyte accumulation**	
	3.4.2.5.1	Proline content
3.4.2.6	**Protein content**	
3.4.2.7	**Enzymatic antioxidant activity**	
	3.4.2.7.1	Superoxide dismutase (SOD)
	3.4.2.7.2	Catalase (CAT)
	3.4.2.7.3	Glutathione reductase (GR)
	3.4.2.7.4	Peroxidase (POX)
	3.4.2.7.5	Ascorbate peroxidase (APOX)
3.4.2.8	**Ethylene evolution rate**	
3.4.2.9	**Polyamine content**	
3.4.3 Gene expression study for selected enzymes/ proteins by RT-PCR		
	3.4.3.1	ACS1 (1-aminocyclopropane-1-carboxylic acid synthase 1)
	3.4.3.2	ACO2 (1-aminocyclopropane-1-carboxylic acid oxidase 2)
	3.4.3.3	ERS 1(Ethylene response sensor 1)
	3.4.3.4	ADC2 (Arginine decarboxylase 2)
	3.4.3.5	SAMDC (S-adenosyl-L-methionine decarboxylase)
	3.4.3.6	SPDS (Spermidine synthase)

	3.4.3.7	PaO (Pheophorbide a oxygenase)
	3.4.3.8	CHLase1 (Chlorophyllase 1)
	3.4.3.9	Actin (Control/ House keeping gene)
3.4.4 Cloning and *in-silico* analysis of candidate genes		

3.5 Details of methodology

3.5.1 Physiological parameters

3.5.1.1 Relative water content (RWC; %)

To evaluate the plant water status RWC was measured by Barrs and Weatherley (1962) method. Leaf RWC was estimated by recording the fresh weight (g) of leaf samples, thereafter immediately transferring in petridishes containing distilled water for 4 h to record turgid weight (g), followed by drying in hot air oven at 70 ^{0}C till constant dry weight (g) has reached.

RWC (%) = [(Fresh wt. – Dry wt.)/ (Turgid wt. – Dry wt.)] x 100

3.5.1.2 Membrane stability index (MSI; %)

Membrane stability index (MSI; %) was estimated by measuring the leakage of electrolyte (Conductivity) due to damaged cell membrane according to the method Premachandra *et al*. (1990). One gram of leaf material, in two sets was taken in test tubes containing 10.0 mL of double distilled water. One set was heated at 40 ^{0}C for 30 min in a metabolic water bath, and the electrical conductivity of the solution was recorded on a conductivity bridge (C1). Second set was boiled at 100 ^{0}C on a boiling water bath for 10 min, and its conductivity was measured on a conductivity bridge (C2) and it was calculated as:

MSI = [1 - (C1/C2)] x 100

3.5.1.3 Stay-green traits

3.5.1.3.1 SCMR (SPAD chlorophyll meter reading)

Soil and plant analyzer development (SPAD) values were measured in the middle part of flag leaves using portable Minolta SPAD-502 chlorophyll meter (Minolta camera Co. Ltd., Osaka, Japan) at the end of water deficit stress period for

10 days at 50 % anthesis stage from both control and treated plants. The average readings of 10 leaves per pot was recorded and used for analysis.

3.5.1.3.2 Total chlorophyll and carotenoid content

Chlorophyll and carotenoid content were estimated as per the method described by Hiscox and Israelstam (1979). The procedure for estimation of chlorophyll content in plants is based on the absorption of light by chlorophyll extracts prepared by incubating the leaf tissues in DMSO (dimethyl sulfoxide). DMSO renders plasmalemma permeable thereby, causing the leaching of the pigments (Hiscox and Israelstam, 1979). The absorbance of the known volume of solution containing known quantity of leaf tissue at two respective wavelengths (663 and 645 nm) was determined for chlorophyll content and at 470 nm for total carotenoid contents. Total chlorophyll content were estimated using the formula given by Arnon (1949) while carotenoid content was determined by following the formula given by Lichtenthaler and Welburn (1983). Fifty mg fresh leaf samples were added to the test tubes containing 4.0 mL DMSO. Tubes were kept in dark for 4 h at 65 ^{0}C. Then the samples were taken out cooled at room temperature and the absorbance was recorded at 663, 645 and 470 nm using DMSO as blank. The values thus obtained were in µg/ mL of extract (solvent). Values in mg/ g fresh weight were obtained by multiplying the above values with V/ W x 1000, where V is volume of extract and W is dry weight of sample.

Total chlorophyll content (mg gDW^{-1}) = $[20.2 \times A_{645} + 8.02 \times A_{663}] \times (V/W) \times 1000$

Total carotenoid content (mg gDW^{-1}) = $[A_{470} + (0.114 \times A_{663}) - (0.638\text{-}A_{645})] \times (V/W) \times 1000$

Where,

A_{470} = Absorbance values at 470 nm

A_{645} = Absorbance values at 645 nm

A_{663} = Absorbance values at 663 nm

3.5.1.3.3 Photosynthesis characteristics

Leaves were categorized into green and yellow/ dead leaves and the rate of photosynthesis, stomatal conductance and transpiration rate was measured using portable Infrared Gas Analyzer (IRGA), LI-6400XT Model (Li-COR Ltd., Lincoln, Nebraska, USA) by operating the IRGA in the closed mode between 10.00-11.00 a.m. when relative humidity, temperature, photosynthetic photon flux density and CO_2 conc ranged from 50-60 %, 30 to 35 ^{0}C, 1200 µmol $m^{-2}s^{-1}$ and 350 to 360 µmol mol^{-1},

respectively. Fifteen flag leaves were selected at random for the measurement of above parameters.

3.5.1.3.4 Leaf senescence rate (LSR)

Phenotyping for leaf senescence was done visually and LSR was scored using a scale from 0 to 10, dividing the percentage of estimate area that is dead by time duration in days as per Lu *et al*. (2011). A rating of 10 indicated essentially no leaf death, 5-6 indicated approximately 50 % mature leaf area dead, while 0 indicate 100 % leaf senescence.

10 = no leaf dead area; 9 = 10 % dead area; 8 = 20 % dead area; 7 = 30 % dead area; 6 = 40 % dead area; 5 =50 % dead area; 4 = 60 % dead area; 3 = 70 % dead area; 2 = 80 % dead area; 1 = 90 % dead area and 0 = 100 % dead area

After 10 days of water deficit stress at 50 % anthesis, LSR was scored 6 times after every 2 day interval.

$$\text{LSR} = \frac{\text{Change in the degree of senescence}}{\text{Total time duration (days)}}$$

3.5.1.4 Yield and yield related traits

3.5.1.4.1 Test weight (1000 seed weight)

Plants were collected at the time of harvest separately from control and water deficit stressed pots. Grains were separated and 1000 seeds were counted and their weight was recorded and expressed in grams.

3.5.1.4.2 Harvest index (HI; %)

The plants were harvested separately from control and water deficit stressed pots. Measurements on grain yield plant^{-1} were recorded in control and water deficit stressed plants as economic yield. The whole plant weight was measured as biological yield. The harvest index was calculated as the ratio of the economic yield to biological yield and was expressed as percentage (Gardner *et al*., 1985).

HI (%) = [Economic yield/ Biological yield] x 100

3.5.1.4.3 Drought tolerance efficiency (DTE; %)

It was calculated using the formula given by Fischer and Wood (1981) by recording yield at the time of final harvest from the plants under both water regimes.

DTE (%) = (Yield under water deficit stress/ Yield under irrigated condition) x 100

3.5.2 Biochemical parameters

3.5.2.1 Oxidant enzyme activity

3.5.2.1.1 Lipoxygenase (LOX) activity (ΔOD_{234} mg^{-1}protein min^{-1})

The lipoxygenase activity was estimated according to the method explained by Doderer *et al*. (1992).

Reagents

(i) Extraction buffer

Solution of potassium dihydrogen orthophosphate (0.1 mM) and dipotassium hydrogen orthophosphate (0.1 mM) were prepared by dissolving 13.6 g and 17.4 g separately in distilled water, respectively and volumes made upto one litre in each case. Solution of potassium dihydrogen phosphate and dipotassium hydrogen phosphate were mixed in a ratio of 16: 84 and the pH was adjusted to 7.5 with meter. To 100.0 mL of the solutions 0.186 g of ethylene diamine tetra acetic acid (EDTA) (0.5 mM) was added and used for enzyme extraction.

(ii) Preparation of substrate solution

The LOX activity was estimated according to the method explained by Doderer *et al.* (1992). For the measurement of this enzyme activity, the substrate solution was prepared by adding 35.0 µL linoleic acid to 5.0 mL distilled water containing 50.0 µL Tween 20. The solution was kept at pH 9.0 by adding 0.2 M NaOH until all the linoleic acid was dissolved and the pH remained stable. After adjusting the pH to 6.5 by adding 0.2 M HCL, 0.1 M phosphate buffer (pH 6.5) was added to a volume of 100.0 mL.

Enzyme assay

LOX activity was determined spectrophotometrically by adding 50 µL of sample to 2.95 mL substrate solution. Absorbance was read at 234 nm and the activity was expressed as change in OD per minute per mg protein in the leaves.

3.5.2.2 Protease activity

The protease activity was estimated as µM of amino acid produced per hour per gram per dry weight as per Nieri *et al*. (1998).

Reagents

(A) Extraction Medium

2.72 g of potassium phosphate (Mr. 136.1) in 400.0 mL of DDW (0.05 M)

280.0 µL mercaptoethanol

5.24 g of EDTA

Final volume made to 500.0 mL

(B) Assay Mixture

1.0 g casein in 100 mL of diphosphate buffer (pH = 7)

0.2 mL of phosphate buffer (pH=7)

1.0 mL of 20 % TCA

(C) Reaction Mixture

55.0 mL of 55 % glycerol in 45.0 mL of DDW

1.5 g of ninhydrin in 0.5 M of citrate buffer

(D) Citrate buffer

1.05 g of citric acid in 100.0 mL DDW

1.47 g of sodium citrate in 100.0 mL DDW (0.5 M)

13.7 mL of citric acid and 36.3 mL of sodium citrate (pH = 5.6)

(E) Diphosphate buffer

2.72 g of monobasic in 100.0 mL DDW

3.48 g of dibasic in 100.0 mL DDW

39 mL of monobasic in 100.0 mL DDW and 67.0 mL of dibasic in 100.0 mL DDW (pH=7)

Extraction

Leaf sample (1.0 g) was homogenized in 10.0 mL of extraction buffer. Crude homogenate was then passed through 4 layers of muslin cloth. Centrifuge the filtrate at 25000 g for 20 min at 4 ^{0}C. Take out the supernatant and use for the enzyme assay. To the 1.0 mL of the enzyme extract add 1.0 mL of 1 % casein in 0.2 M diphosphate

buffer (pH=7) and 1.0 mL of 0.2 M of phosphate buffer (pH=7). Incubate the solution at 40 ^{0}C for 90 minutes; stop the reaction after the end of incubation period by adding 1.0 mL of 20 % TCA. Keep the reaction vials in refrigerator for 30 min. Centrifuge the reaction mixture at 3000 g for 15 min. Take out the supernatant and use for the amino acid analysis. A control was also been run along with sample.

Estimation of protease activity

Take 0.5 mL of supernatant from the reaction mixture. Add 0.5 mL of 55 % glycerol, 0.5 mL of ninhydrin solution. Make the volume to 6.0 mL by adding double distilled water. Boil for 20 min. Record the spectrophotometer reading at 570 nm. To calculate amount of amino acid formed due to protease activity, a standard curve was prepared by using 0.1 to 1.0 µM of glycine. The protease activity expressed as µM of amino acid produced per hour per gram per dry weight.

3.5.2.3 Lipid peroxidation

3.5.2.3.1 Thiobarbituric acid reactive substances content

Lipid peroxidation was estimated as the thiobarbituric acid reactive substances, according to the method of Heath and Packer (1968).

Reagents

1. Trichloroacetic acid solution: Trichloroacetic acid (TCA) solution (0.1 %) was prepared by dissolving 0.1 g TCA in double distilled water and volume was made up to 100 mL with double distilled water.

2. Thiobarbituric acid reagent: Thiobarbituric acid (TBA) 0.5 g was dissolved in 20 % trichloroacetic acid (TCA) (prepared by dissolving 20.0 g TCA in 100 mL distilled water) and volume was made up to 100.0 mL with 20 % TCA solution.

Estimation

Leaf samples (0.5 g) were homogenized in 10.0 mL of 0.1 % trichloroacetic acid (TCA). The homogenate was centrifuged at 15000 g for 15 min. To 1.0 mL aliquot of the supernatant 4.0 mL of 0.5 % thiobarbituric acid (TBA) in 20 % TCA was added. The mixture was heated at 95 ^{0}C for 30 min in the water bath and then cooled under room temperature. After centrifugation at 10000 g for 10 min the absorbance of the supernatant was recorded at 532 nm. The TBARS content was calculated according to its extinction coefficient *i.e.*, 155 mM^{-1} cm^{-1}. The values for non-specific absorbance at 600 nm were subtracted.

3.5.2.4 Osmolyte accumulation

3.5.2.4.1 Proline content

Proline content was estimated as per the method described by Bates *et al*. (1973).

Reagents

1. Orthophosphoric acid (6 N): Required volume of orthophosphoric acid (38.1 mL) was taken and volume was made to 100.0 mL, using distilled water to get 6 N orthophosphoric acid.

2. Acid ninhydrin: Ninhydrin (1.25 g) was dissolved in a blend of 30.0 mL of glacial acetic acid and 20.0 mL of 6 N orthophosphoric acid.

3. Sulphosalicylic acid (3 %): Three gram of sulphosalicylic acid was dissolved in 100.0 mL of distilled water.

4. Toluene

5. Glacial acetic acid

Estimation

Leaf samples (0.5 g) were homogenized in 10.0 mL of 3 % sulphosalicylic acid and were filtered through Whatman no. 1 filter paper. Two mL of this filtrate was mixed with 2.0 mL of acid ninhydrin and 2.0 mL of glacial acetic acid in a test tube. The mixture was heated at 100 ^{0}C in a water bath for 1 h. The reaction was stopped by removing the tubes from hot water bath and placing them in ice bath. Toluene (4.0 mL) was added to the mixture and vortexed for 15-20 seconds. The chromophore was aspirated from the aqueous phase. Then the absorbance of toluene phase was measured at 520 nm. A blank was run and its absorbance was subtracted from the sample absorbance. The proline content was calculated from a standard curve using L-Proline as standard ($y = 0.0073x - 0.0003$; $R^2 = 0.9987$) and was expressed as µmol/ g dry weight.

3.5.2.5 Oxidant production

3.5.2.5.1 Hydrogen peroxide (H_2O_2) content

Hydrogen peroxide content was estimated as per the method described by Rao *et al*. (1997).

Reagents

1. Titanium reagent: One-gram titanium dioxide and 10.0 g potassium sulphate are digested in 150.0 mL conc. sulphuric acid over a hot plate for 4 h. The digested mixture is diluted to 500-600 mL and stirred with a magnetic stirrer cum heater at 70-80 ^{0}C till a clear transparent solution is obtained, diluted to 1.5 liter and store in dark brown bottle (Teranishi *et al*., 1974).

2. Acetone: Analytical grade reagent

3. Liquid ammonia: Analytical grade reagent

Estimation

One g leaf material was grinded to fine powder with the help of liquid nitrogen, followed by addition of 10.0 mL cooled acetone. Mixture is filtered with Whatman No. 1 filter paper followed by the addition of 4.0 mL titanium reagent and 5.0 mL liquid ammonia solution to precipitate the titanium-hydro peroxide complex. Reaction mixture is centrifuged at 10000 g for 10 min at 4 ^{0}C. Precipitate is dissolved in 10.0 mL of 2.0 M H_2SO_4 and than recentrifuged. Supernatant is read at 415 nm against reagent blank in UV-visible spectrophotometer. Concentration of hydrogen peroxide is computed by referring to a standard curve made from known concentrations of hydrogen peroxide (y = 0.124 x) and was expressed as µmol g^{-1} dry weight.

3.5.2.6 Estimation of total soluble proteins

Total soluble protein was estimated following the method of Lowry *et al.* (1951).

Reagents

1. Alkaline copper reagent

   Solution A: 2 % sodium carbonate solution was prepared in 0.1 N sodium hydroxide.

   Solution B: 0.5 % copper sulphate solution was prepared in 1 % potassium sodium tartrate.

   50.0 mL of solution A and 1.0 mL of solution B were mixed together thoroughly to make the reagent.

2. Folin-ciocalteau-phenol reagent

3. Phosphate buffer

4. Bovine serum albumin (for standard curve)

Extraction of protein

Leaf sample (1.0 g) was weighed and ground well in 10.0 mL of phosphate buffer with a pestle and mortar and was centrifuged at 20000 g for 15 min. The supernatant was taken for protein estimation and the pellets were discarded.

Estimation of protein

Take 0.1 mL of the above extract in a test tube and 1.0 mL of alkaline copper reagent was added to it. The mixture was stirred gently and allowed to stay at room temperature for 15 min. Then 0.2 mL of diluted 1:1 (v: v) folin-ciocalteau-phenol reagent was added to it and shaken well. It was kept for 30 min incubation at room temperature in dark, and blue colour was developed. The volume was made up to 5 mL with double distilled water. The absorbance was recorded at 500 nm wavelength. A blank was run, in which 0.2 mL of distilled water was added, instead of enzyme extract.

3.5.2.7 Enzymatic antioxidant activity

3.5.2.7.1 Estimation of superoxide dismutase (SOD) activity

The assay is based formation of blue coloured formazone by nitro-blue tetrazolium and $O_2^{\cdot -}$ radical, which absorbs at 560 nm, and the enzyme (SOD) decreases this absorbance due to reduction in the formation of $O_2^{\cdot -}$ radical by the enzyme (Dhindsa *et al*., 1981).

Reagents

1. Methionine (200.0 mM)
2. Nitroblue tetrazolium chloride (NBT) (2.25 mM)
3. EDTA (3.0 mM)
4. Riboflavin (60.0 μM)
5. Sodium carbonate (1.5 M)
6. Phosphate buffer (100.0 mM, pH 7.8)

Solution A: Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to 500.0 mL with double distilled water.

Solution B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500.0 mL with double distilled water.

8.5 mL of solution A and 91.5 mL of solution B was mixed and final pH was adjusted with the help of pH meter.

Grinding media

Phosphate buffer of 0.1 M, pH 7.5, containing 0.5 mM EDTA in case of SOD, CAT, GR and POX: EDTA 0.0186 g was dissolved in phosphate buffer 0.1 M, pH 7.5 (made by mixing 16.0 mL of solution A and 84.0 mL of solution B and final pH was adjusted with the help of pH meter) and volume was made to 100.0 mL with the buffer.

Preparation of enzyme extract

Enzyme extract for superoxide dismutase was prepared by first freezing the weighed amount of leaf samples (1.0 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 10.0 mL extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM. Homogenate was passed through 4 layers of cheesecloth and filtrate was centrifuged for 20 min at 15000 g and the supernatant was used as enzyme.

Enzyme assay

Superoxide dismutase activity was estimated by recording the decrease in optical density of formazone made by superoxide radical and nitro-blue tetrazolium dye by the enzyme. Three mL of the reaction mixture was prepared by mixing the following reagents:

1. 13.33 mM methionine (0.2 mL of 200.0 mM)
2. 75.0 µM nitroblue tetrazolium chloride (NBT) (0.1 mL of 2.25 mM)
3. 0.1 mM EDTA (0.1 mL of 3.0 mM)
4. 50.0 mM phosphate buffer (pH 7.8) (1.5 mL of 100.0 mM)
5. 50.0 mM sodium carbonate (0.1 mL of 1.5 M)
6. 0.1 mL enzyme
7. 0.9 to 0.95 mL of water (to make a final volume of 3.0 mL)

Reaction was started by adding 2.0 µM riboflavin (0.1 mL) and placing the tubes under two 15 W fluorescent lamps for 15 min. A complete reaction mixture without enzyme, which gave the maximal colour, served as control. Switching off the light and putting the tubes into dark stopped the reaction. The absorbency was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of

enzyme, which reduced the absorbency reading to 50 % in comparison with tubes lacking enzyme. The enzyme activity was expressed as units per mg protein per hour.

3.5.2.7.2 Estimation of catalase (CAT) activity

The total catalase activity was estimated following the method described by Aebi *et al*. (1984).

Reagents

1. Hydrogen peroxide (75.0 mM): Hydrogen peroxide (30 %, 775.0 µL) was dissolved in double distilled water and the volume was made upto 100.0 mL.
2. Phosphate buffer (100.0 mM, pH 7.0)

Preparation of enzyme extract

Leaf samples (1.0 g) were freezed in liquid nitrogen to prevent proteolytic activity and were homogenized with 10.0 mL extraction buffer containing 0.1 M phosphate buffer, pH 7.5 and 0.5 mM EDTA. Homogenate was passed through 4 layers of cheese cloth and the filtrate was centrifuged at 15000 g for 20 min and the supernatant was used as enzyme.

Enzyme assay

The reaction mixture was prepared with the following reagents

1. Potassium phosphate buffer 50.0 mM (1.5 mL of 100.0 mM buffer, pH 7.0)
2. Enzyme (50.0 µL)
3. Water (950.0 µL)

Then hydrogen peroxide (0.5 mL) was added to start the reaction and the decrease in absorbance was recorded for 1 min at 240 nm and enzyme activity was calculated as per extinction coefficient of hydrogen peroxide (36.5 $M^{-1}cm^{-1}$). Enzyme activity was expressed as µmol hydrogen peroxide reduced per minute per mg protein.

3.5.2.7.3 Estimation of glutathione reductase (GR) activity

The total glutathione reductase activity was estimated following the method described by Smith *et al*. (1988). Glutathione reductase catalyze the reduction of oxidized glutathione (glutathione disulphide) (GSSG) to reduced glutathione (GSH) by using reductant NADPH. The assay of the enzyme is based on the formation of a red coloured complex by reduced glutathione with 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), which absorbs at 412 nm (Smith *et al*., 1988).

Reagents

1. 5, 5-dithiobis-(2-nitrobenzoic acid) (DTNB) (3.0 mM) in phosphate buffer (10.0 mM, pH 7.5)

2. Oxidized glutathione (GSSG) (20.0 mM)

3. NADPH (2.0 mM)

4. Phosphate buffer (200.0 mM, pH 7.5)

Solution A: Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to 500.0 mL with double distilled water.

Solution B: Dipotassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500.0 mL with double distilled water.

Solution A of 15.0 mL and solution B of 85.0 mL were mixed and final pH was adjusted to 7.5 with the help of pH meter.

Preparation of enzyme extract

Leaf samples (1.0 g) were freezed in liquid nitrogen to prevent proteolytic activity and were homogenized with 10.0 mL extraction buffer containing 0.1 M phosphate buffer, pH 7.5 and 0.5 mM EDTA. Brie was passed through 4 layers of cheese cloth and the filtrate was centrifuged at 15000 g for 20 min and the supernatant was used as enzyme.

Enzyme assay

The reaction mixture was prepared with the following reagents

1. 66.67 mM potassium phosphate buffer (pH 7.5) and 0.33 mM EDTA (1.0 mL of 0.2 M buffer containing 1.0 mM EDTA)
2. 0.5 mM DTNB in 0.01 M potassium phosphate buffer (pH 7.5) (0.5 mL of 3.0 mM)
3. 66.67 µM NADPH (0.1 mL of 2.0 mM)
4. 666.67 µM GSSG (0.1 mL of 20.0 mM)
5. 0.1 mL enzyme extract

Distilled water to make up a final volume of 3.0 mL

Reaction was started by adding 0.1 mL of 20.0 mM GSSG (oxidized glutathione). The increase in absorbance at 412 nm was recorded spectro-photometrically and enzyme activity was calculated as per extinction coefficient of oxidised glutathione *i.e.* 6.2 $mM^{-1}cm^{-1}$. Enzyme activity was expressed as µmol oxidised glutathione formed per minute per mg protein.

3.5.2.7.4 Estimation of peroxidase (POX) activity

The total peroxidase activity was estimated following the method described by Castillo *et al*. (1984).

Reagents

1. Phosphate buffer (100.0 mM, pH 6.1)
2. Hydrogen Peroxide (12.0 mM): Hydrogen peroxide (30 %, 124.0 µL) was dissolved in double distilled water and the volume was made upto 100.0 mL.
3. Guaiacol (96.0 mM): Analytical grade guaiacol (1.07 mL) was dissolved in distilled water and the volume was made up to 100.0 mL.

Preparation of enzyme extract

Leaf samples (1.0 g) were freezed in liquid nitrogen to prevent proteolytic activity and were homogenized with 10 ml extraction buffer containing 0.1 M phosphate buffer, pH 7.5 and 0.5 mM EDTA. Homogenate was passed through 4 layers of cheese cloth and the filtrate was centrifuged at 15000 g for 20 min and the supernatant was used as enzyme.

Enzyme assay

The reaction mixture was prepared with the following reagents

1. Potassium phosphate buffer 50.0 mM (1.0 mL of 100.0 mM buffer, pH 6.1)
2. Guaiacol 16.0 mM (0.5 mL of 96.0 mM)
3. Hydrogen peroxide 2.0 mM (0.5 mL of 12.0 mM)
4. Enzyme (0.1 mL)
5. Water (0.4 mL) and the total volume were made up to 3.0 mL.

Absorbance due to the formation of tetra-guaiacol was recorded at 470 nm and enzyme activity was calculated as per extinction coefficient of its oxidation product, tetra-guaiacol, *i.e.* 26.6 mM^{-1} cm^{-1}. Enzyme activity was expressed as µmol tetra-guaiacol formed per minute per mg protein.

3.5.2.7.5 Estimation of ascorbate peroxidase (APOX) activity

The total ascorbate peroxidase activity was estimated following the method described by Nakano and Asada (1981).

Reagents

1. Ascorbic acid (3.0 mM): Ascorbic acid 0.0265 g was dissolved in water and the volume was made up to 50.0 mL with double distilled water.
2. EDTA (3.0 mM): EDTA 0.0558 g was dissolved in water and the volume was made up to 50.0 mL with double distilled water.
3. Hydrogen peroxide (1.5 mM): Sixteen µL of standard hydrogen peroxide (30 %) were dissolved in water and the volume was made up to 100.0 mL with double distilled water.
4. Phosphate buffer (100.0 mM, pH 7.0):

   Solution A: Potassium dihydrogen phosphate 6.80 g was dissolved in water and the volume was made up to 500.0 mL with double distilled water.

   Solution B: Di-potassium hydrogen phosphate 8.71 g was dissolved in water and the volume was made up to 500.0 mL with double distilled water.

   Buffer was prepared by mixing 39.0 mL of solution A and 61.0 mL of solution B, and final pH was adjusted with the help of pH meter.

Enzyme assay

Ascorbate peroxidase was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm. The 3.0 mL reaction mixture contained 50.0 mM potassium phosphate buffer (pH 7.0) (1.5 mL of 100 mM), 0.5 mM ascorbic acid (0.5 mL of 3.0 mM), 0.1 mM EDTA (0.1 mL of 3.0 mM), 0.1 mM H_2O_2 (0.2/ 0.6 mL of 1.5 mM), 0.1 mL enzyme and water 0.6 mL, to make a final volume of 3.0 mL. The reaction was started with the addition of 0.2 mL of hydrogen peroxide. Decrease in absorbance for a period of 30 sec. was measured at 290 nm in an UV-visible spectrophotometer (Model Specord Bio-200, AnalytikJena, Germany). Activity is expressed by calculating the decrease in ascorbic acid content by comparing with a standard curve drawn with known concentrations of ascorbic acid.

3.5.2.8 Ethylene evolution rate (nmol g^{-1}DW h^{-1})

For ethylene estimation, wheat seedlings were grown hydroponically in growth chamber for 10 days and were transferred to 50.0 mL test tubes containing above mentioned treatment combinations. Tubes' mouth was covered with rubber serum stopper and whole test tube set was covered with aluminium foil and finally with black cloth according to Beltrano *et al.* (1994) with little modifications. These test tubes were kept in dark for 24 h for ethylene evolution. After 24 h dark treatment, one mL gas sample was taken out from the respective tubes with the help of specialized syringe for ethylene assay using a gas chromatography (Model HP 5890, Hewlett Packard, USA) which was calibrated using standard ethylene gas (Laser Gases, New Delhi). The gas chromatography was equipped with Porapak-N (80-100 mesh) column and a flame ionization detector (FID). Nitrogen was used as the carrier gas at a flow rate of 30.0 mL min^{-1}, while hydrogen and air were fuel gases which had flow rate of 25 and 250.0 mL min^{-1}, respectively. The temperature of injector, column and detector were maintained at 110, 60 and 275 ^{0}C, respectively. The ethylene evolution rate was performed in triplicate and was expressed as nmol g^{-1} DW h^{-1}.

3.5.2.9 Polyamine content (µmol g^{-1}DW h^{-1})

Polyamines (putrescine, spermidine and spermine) were quantified by High Performance Liquid Chromatography (HPLC) using the method described by Flores and Galston (1982). Leaf sample were extracted in 5 % (v/ v) cold perchloric acid

($HClO_4$) at a ratio of about 100.0 mg tissues per mL $HClO_4$. After extraction for 1 h in ice bath, samples were pelleted at 48000 g for 20 min, and then supernatant phase containing the polyamine fraction was collected and stored at -20 ^{0}C in plastic vials (avoided glass vials since polyamines binds to glass). Standards and plant extracts were benzoylated according to Redmond and Tseng (1979). One mL of 2N NaOH was transferred to 250.0 µL of $HClO_4$ extract and polyamines standards (putrescine, spermidine and spermine). Then 10.0 µL of benzoyl chloride was added and vortexed for 10 s followed by incubation at room temperature for 20 min. Saturated NaCl, 2.0 mL was added with shaking and 2.0 mL diethyl ether was added. Centrifugation was done at 1500 g for 5 min, 1.0 mL of the upper ether phase was collected and allowed it to evaporate to dryness under a stream of warm air, and then redissolved in 100.0 µL methanol. These derivatized standards and samples were used for estimation of polyamine contents through HPLC (Model Prostar 210, Varian, Palo Alto, CA, USA) equipped with quaternary pump, UV detector and connected with rheodyne injection system using Lichrospher stainless steel 5 µm particle size reverse-phase (C_{18}) column (250 mm x 4 mm), acetonitrile: 0.1 % O-phosphoric acid (52:48) as mobile phase at a flow rate of 1.0 mL min^{-1} and wavelength 254 nm. Standard curve was prepared using concentration ranging between 0.625-5 ppm. Under these conditions, the retention time of putrescine, spermidine and spermine was 3.41, 4.18 and 5.11 min, respectively.

3.5.3 Gene expression study for selected enzymes/ proteins by semi-quantitative RT-PCR

3.5.3.1 Primer designing

Nucleotide sequences for candidate genes were obtained from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). The Basic Local Alignment Search Tool (Altschul *et al*., 1990, http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify the homologs of candidate genes. For RT-PCR expression analysis and cloning of cDNAs, the following oligonucleotide primers were designed manually, and oligo quality (to avoid primer dimmer, self dimer etc.), GC % and Tm were analyzed by using Oligoanalyzer 3.0 tool (http:// www.idtdna.com/ analyzer/ Applications/OligoAnalyzer/), Intergrated DNA Technologies, Coralville, IA 52241,

USA). Sequences of gene specific forward and reverse primers employed are described in **Table 3.3**.

3.5.3.2 RNA isolation and cDNA synthesis

Total RNA was extracted from leaf tissues of water deficit/ osmotic stress and control plants by using TRIzol® reagent (*Invitrogen*™, USA). About 100.0 mg sample was taken for isolating the RNA. The sample was finely ground using liquid nitrogen in a pre-chilled pestle and mortar. Decant tissue powder along-with a little liquid nitrogen into 2.0 mL eppendorf tube and then immediately add 1.0 mL of TRIzol reagent to the tubes. Cap each tube and lyse the cells by vortexing vigorously for 30 min. Incubate the samples for 10 min at room temperature. Then add 200.0 µL of chloroform to each tube and vortex vigorously for 1 min. Incubate samples for 1 min at room temperature. Centrifuge samples at 13000 rpm for 20 min at 4 ^{0}C for phase separation. There will be three phases as lower brown, middle 1-2 mm chloroform-phenol having DNA and upper aqueous phase having RNA. Pipette and transfer only the upper aqueous phase to a fresh tube. Add 600.0 µL of chilled isopropyl alcohol to precipitate RNA. Mix by inverting the tube up and down and incubate in -20 ^{0}C for 30 min. After incubation, centrifuge the samples for 10 min at 12000 g at 4 ^{0}C. Finally, the RNA will form a pellet on the side or bottom of the tube. Discard the supernatant and wash the pellet with 1.0 mL of 80 % ethanol twice. Centrifuge at 12000 g for 1 min at 4 ^{0}C. Air dry the pellet for 5-10 min avoid complete drying of pellet. Dissolve pellet in 50.0 to 80.0 µL RNase free water or autoclaved double distilled water and incubate for 3 min at 36 ^{0}C. RNase-free DNase I (Promega, USA) was applied to remove contaminating genomic DNA at 37 ^{0}C for 1 h. Quality and integrity of total RNA were then determined by running appropriate amount in a formamide denaturing gel and quantity of total RNA was determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized according to the instructions of the cDNA Synthesis Superscript® III First- Strand Synthesis System (*Invitrogen*™, USA). Resulting cDNA was stored at -20 ^{0}C and employed as template for two-step RT-PCR reactions following recommended conditions provided in user's manual. Every RT-PCR measurement was performed at least thrice. Expression of *TaActin* was used as an internal standard for normalization.

3.5.3.3 Agarose gel electrophoresis

Materials and reagents

1. Agarose
2. 5X TBE Buffer (pH 8.0)

Tris base	54.0 g
Boric acid	27.5 g
EDTA 0.5 M (pH 8.0)	20.0 mL
Final volume	1000.0 mL with distilled water

The buffer was autoclaved and stored at room temperature.

3. Gel loading dye (6X): 10.0 mM Tris-HCI (pH 7.6), 0.03 % bromophenol blue, 0.03 % xylene cyanol FF, 60 % glycerol and 60.0 mM EDTA
4. Ethidium bromide stock solution: 10.0 mg per mL in water
5. Mini gel apparatus and power supply
6. Gel documentation system: Alpha Imager

Procedure

Agarose gel (0.8 %) was prepared by heat dissolution of 0.40 g of agarose in 50.0 mL of 1X TBE buffer. Ethidium bromide from stock solution (10.0 mg mL^{-1}) was added to a final concentration of 0.5 mg $mL^{-1}$of gel. Gel was allowed to cool to almost 50-55 ^{0}C before pouring to the gel plate. Once the gel was ready, it was placed in the electrophoresis tank after removing the comb and tape. The electrophoresis tank was filled with 1X TAE buffer till the gel was completely submerged. The total RNA samples were mixed with appropriate volume of 6X loading dye before loading. The samples were loaded and run at 5 V/ cm, with the help of an electric supply. After one hour of electrophoresis, the agarose gel was viewed using gel documentation system.

3.5.3.4 RT-PCR expression analysis of target genes

DNA PCR using cDNA as template was used for expression analysis.

Reagents	**Quantity for 50.0 µL of reaction mixture**
Nuclease free water	21.0 µL
10X Taq DNA polymerase buffer	5.0 µL
$MgCl_2$ (50.0 mM)	3.0 µL
dNTP Mix (10.0 mM each)	2.0 µL
Forward + Reverse primers (10.0 µM)	2.0 µL
cDNA template (50.0 ng)	as per normalization
Taq polymerase enzyme (5 U/ µL	0.2 µL
Total volume	50.0 µL

The above reaction mixture was prepared in 0.2 mL PCR tubes and amplification carried out using *QB 96* thermal cycler (*Quanta Biotech*, Byfleet, *UK*), with the following program:

Hot Start 3 min 95 ^{0}C

3-step cycling

Denaturation 45 sec 94 ^{0}C

Annealing 1 min at standardized Tm for candidate genes

Extension n* min 72 ^{0}C

Number of cycles 27

Final extension 10 min 72 ^{0}C

Store ∞ min12 ^{0}C

* Extension period required is of the order of 1 min/ 1000 bp

To check the amplification, an aliquot of 5.0 µL of the reaction mixture was run on a 1 % (w/ v) agarose gel stained with 0.5 µg mL^{-1} ethidium bromide. 100 bp

plus DNA ladder (ThermoScientific, USA) was included as a marker for size comparison of the amplified products. 1X TAE buffer was used to prepare the gel as well as for running buffer and electrophoresis carried out at 5 Vcm^{-1} (Sambrook and Russel, 1989). 6X loading dye were used to load the samples. The stained DNA products were photographed using Gel Documentation System.

3.5.3.5 Purification of amplified cDNA

After fractionation on 1 % agarose gel electrophoresis, the entire amplified cDNA product was loaded and the bands of expected size were excised with a sharp sterile scalpel under UV light. The DNA from the gel piece was extracted using QIAquick Gel Extraction kit (QIAGEN) following the protocol given below:

Excise the DNA fragment from the agarose gel with a clean, sharp scalpel and transfer to 2.0 mL pre-weighed Eppendorf tube. To get the weight of gel, take the difference in weight of the tube from tube with gel. Add buffer QG (3 volumes) to 1 volume of gel (w/ v). For instance, if the gel weight is 100 mg, then add 300.0 µL QG buffer. Incubate at 50 ^{0}C for 15 min, with vortexing after every 3 min to get a fully dissolved solution. At this step, check whether the colors of the solution mix is yellow. If the color of the mixture is orange or violet, it means pH is too high and affects the DNA binding to column, as the column membrane will work efficiently only at pH ≤ 7.5. To avoid this, add 100.0 µL of 3.0 M sodium acetate, pH 5 and mix well, until the colour change back to yellow. Add 1 gel volume of isopropanol to the sample and mix. For instance, if the gel weight is 100.0 mg, then add 100.0 µL of isopropanol. To bind DNA, transfer the sample to the QIAquick Spin Column placed in a 2.0 mL collection tube and centrifuged at 8000 rpm for 60 sec. Discard the flow-through and QIAquick Spin Column was placed back in the same collection tube. To wash the column, buffer PE (750.0 µL) having ethanol was added to column and centrifuged for 60 sec. Discard the flow-through and place back the QIAquick Spin Column in the same collection tube and centrifuge for an additional 1 min at 10000 rpm. Discard the collection tube and place the QIAquick Spin column into a clean 1.5 mL Eppendorf tube. To elute DNA, add 25.0 µL Buffer EB (10.0 mM Tris HCI, pH 8.5) or RNase free water to the centre of the QIAquick Spin Column, keep for 60 sec at RT and centrifuge for 1 min. Repeat this step using the same eluted solution back to the column. The eluted DNA can be stored at -20 ^{0}C. The above mentioned genes (**Table 3.3**) were gel purified and the eluted DNA was sent for sequencing by

following dideoxychain termination method (Sanger *et al*., 1977) from Exelris Genomics, Ahmedabad-380054, India.

3.5.3.6 Cloning of cDNAs and transfomation of *E. coli*

3.5.3.6.1 Competent cell preparation

Competent cell of the *E. coli* strain XL1-Blue were prepared by calcium chloride method as described by Ausubel *et al*. (1999).

1. A single colony of *E. coli* (XL1-Blue) from a freshly streaked plate was inoculated into 10.0 mL LB medium (1 % Bacto-tryptone, 0.5 % yeast extract, 1 % NaCl, pH adjusted to 7.0) and allowed to grow overnight at 37 ^{0}C with constant shaking at 180 rpm in an incubator-shaker.
2. One ml of the overnight grown culture inoculated into 100.0 mL of LB medium (1:100 dilution v/ v) in a 1 L flask and grown at 37 ^{0}C with constant shaking (200 rpm) for 2.5 h to an OD_{590} of 0.6-0.8.
3. Aliquots of the culture were dispersed into sterile pre-chilled centrifuge tubes and kept on ice for 30 min.
4. The cells were pelleted by centrifugation at 4000 g for 10 min at 4 ^{0}C and the pellet was resuspended in 40 mL ice-cold $CaCI_2$ solution (80.0 mM $MgCI_2$ + 20.0 mM $CaCI_2$)
5. Keep the suspension in ice for 10 min and centrifuge at 4000 g, 10 min at 4 ^{0}C.
6. The pellet was then resuspended in 1-2 mL of ice-cold 100.0 mM $CaCI_2$ solution with 20 % glycerol and aliquots of 100.0 µL were dispersed into pre-chilled sterile microfuge tubes. These cells were quickly frozen by liquid nitrogen and stored at -80 ^{0}C for later use.

3.5.3.6.2 Ligation

TA cloning exploits the terminal transferase activity of some DNA polymerases such as Taq polymerase, which lack 3′-5′ proof reading activity and are capable of adding adenosine triphosphate residue to the 3′ ends of the double stranded PCR product in a template independent fashion. This makes it possible to clone this PCR product directly into a linearized cloning vector with single, 3′-T overhangs. The PCR products with dA overhang are mixed with this vector in high proportion. The

complementary overhangs of 'T' vector and PCR product will be ligated with the action of T4 DNA ligase. The cDNAs were cloned by using InsTAclone™ PCR Cloning Kit (Fermentas International Inc., Canada) following the manufacturer's protocol.

The purified PCR fragment was dissolved in 10.0-20.0 µL of TE buffer and the DNA concentration was determined by NanoDrop™. The following components were added into a 1.5 mL microcentrifuge tube:

Vector pTZ57R/T (0.165 µg, 0.18 pmol ends)	1.0 µL
Purified PCR fragment,	
(approx. 0.54 pmol ends)	3 times vector conc.
5X Ligation Buffer	4.0 µL
Nuclease free water	as per calculation
T4 DNA Ligase (5 Units)	1.0 µL
Total volume	**20.0** µL

The mixture was incubated at 4 ^{0}C overnight to obtain maximum yields of useful recombinants.

3.5.3.6.3 Transformation of *E. coli* and selection of recombinants

Reagents and media

1. Luria Broth

Bacto-tryptone	10.0 g
Bacto-yeast extract	5.0 g
NaCI	10.0 g

The above chemicals were dissolved in 800.0 mL of distilled water. The pH was adjusted to 7.0 and volume made up to 1000.0 mL with distilled water. The media was autoclaved for 20 min at 15 psi pressure and at a temperature of 121 ^{0}C.

2. Ampicillin stock solution

   100.0 mg ampicillin was dissolved in one mL of double distilled water and filter sterilized using a syringe filter (0.22 µm)

The sterilized ampicillin was stored in sterile microfuge tube (cover the tube with aluminium foil) at -20 ^{0}C.

3. X-Gal solution

X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) was dissolved in dimethyl formamide to a final concentration of 2 % and stored in a -20 ^{0}C freezer. The tubes were wrapped with foil since the X-gal is light sensitive.

4. IPTG solution

IPTG (isopropyl- β-D-thio-galactorpyranoside) was dissolved to a final concentration of 100.0 mM in sterile distilled water and stored in a -20 ^{0}C freezer.

5. Luria agar-ampicillin plates

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCL	10 g
Agar	15 g

The above chemicals were mixed in 800.0 mL of distilled water and pH was adjusted to 7.0. Volume was made up to 1000.0 mL with distilled water. The media was autoclaved for 20 minutes at 15 psi pressure and at a temperature of 121 ^{0}C. The medium was cooled to about 45 ^{0}C and ampicillin was added to a final concentration of 100.0 mg/ mL. About 30.0 mL of media was poured in each petri plate (8 cm diameter) and left in the Laminar Flow hood for solidification.

6. Luria agar-ampicillin IPTG/ X-Gal plates

i. The Luria-agar-ampilicillin plates were equilibrated to 37 ^{0}C.

ii. A mixture of 40.0 mL of 2 % X-gal and 10 ml of 100.0 mM IPTG was spreaded onto a Luria-agar-ampicillin plate; this was left to diffuse into the plate for at least 1 h.

3.5.3.6.4 Transformation

1. After completion of ligation reaction, the tubes were briefly spun to collect the ligation mix at the bottom of the tube.
2. Frozen XL1-Blue competent cells from -80 ^{0}C deep freezer was taken, placed in ice and allowed to thaw (about 5 min).
3. 10.0 µL of ligation reaction mix was added to each 100 µL of competent cell in sterile microcentrifuge tube on ice.
4. The cells were mixed with DNA by gently flicking the tube and placed in ice for 30 min.
5. Heat-shock to the cells was given for 45 seconds at 42 ^{0}C in water bath and immediately the cells were chilled in ice for 2 min.
6. 950.0 µL of LB medium was added to the tubes containing cells transformed with ligation reactions and incubated for 1.5 h at 37 ^{0}C with shaking (180 rpm).
7. 100.0 µL of each transformation culture were plated onto LA/ ampicillin/ IPTG/ X-Gal plates in replicates.
8. The plates were incubated overnight (16 to 24 h) at 37 ^{0}C.

Notes: Colonies containing β-galactosidase activity (generally non-recombinant) may grow poorly, relative to cells lacking this activity. After overnight growth, the blue colonies may be smaller than the white colonies, which are approximately one millimeter in diameter. Select white colonies for isolating recombinant plasmids.

3.5.3.6.5 Plasmid isolation

The plasmid DNA from clone pTZ57R/T was isolated by alkaline lysis mini-preparation method (Ahn *et al*., 2000). It is fast, reliable and relatively clean way to obtain plasmid DNA from cells. It allows the plasmid DNA to be separated from the bacterial chromosome. Typically, *E. coli* cells that contain the plasmid were lysed with alkali. The cell debris was precipitated using SDS and potassium acetate. This was spun down, and the pellet was removed. Plasmid DNA was precipitated from the supernatant by using isopropanol. A mini prep usually yields 5.0-10.0 µg.

Solutions

Solution I: Resuspension buffer

50.0 mM Tris-CL, pH 8.0

10.0 mM EDTA, pH 8.0

20.0 µg Rnase A

Rnase A stock solution (10.0 mg/ mL) was added freshly to resuspension buffer at 2 µg/ 100.0 mL.

Solution II: Lysis buffer

0.2 N NaOH

1 % SDS

Sterile DDW

(Freshly prepared)

Solution III: Neutralization buffer

3 M sodium acetate, pH 5.5

Media: Luria Broth

TE Buffer : 10.0 mM Tris-CL (pH 8.0), 1.0 mM EDTA (pH 8.0).

Protocol

1. Isolated colonies of clone pTZ57R/T were grown overnight in 2.0 mL of Luria broth medium containing ampicillin (100.0 µg mL^{-1}) at 37 ^{0}C with constant shaking at 180 rpm.
2. The overnight grown culture was transferred into a 2.0 mL microcentrifuge tube and centrifuged at 12000 g for 1 min.
3. The supernatant was discarded and the pellet was suspended in 100.0 µL of resuspension buffer (Solution I). The pellet was vortexed vigorously till it is completely suspended.
4. 100.0 µL lysis buffer (Solution II) was added and mixed gently by inversion.
5. 120.0 µL of neutralization buffer (Solution III) was added to neutralize the lysate, mixed gently and incubated in ice for 3 min.
6. These tubes were centrifuged at 12000 g for 1 min at room temperature and the clear supernatant was carefully transferred to a fresh tube avoiding the white precipitate.
7. To precipitate the plasmid DNA, 200.0 µL of isopropanol was added and incubated at room temperature for 10 min.

8. The plasmid was precipitated by centrifugation at 12000 g for 1 min and the pellet was washed with 70 % ethanol.

9. The plasmid pellet was air dried and dissolved in 50.0 µL TE buffer.

10. An aliquot of the DNA preparation after adding 1/ 6^{th} volume of 6X loading dye was checked by electrophoresis on a 1 % agarose gel containing 0.5 µg/ mL ethidium bromide. The gel electrophoresis was carried out in 1X TBE buffer at 5 V/ cm (Sambrook and Russell, 1989).

3.5.3.6.6 Confirmation of cloning by colony PCR analysis

A single bacterial colony was picked with an autoclaved toothpick or pipette tip and swirled into 25.0 µL of autoclaved distilled water in an autoclaved microfuge tube (the entire colony is not removed as the number of cells required is small). The area that was picked was marked on the bottom of the plate. The mix was heated in a boiling water bath (90-100 ^{0}C) for 2 min. The sample was spun for 2 min at high speed in centrifuge. The supernatant of about 20.0 µL was transferred into a new microfuge tube and 5.0 µL of the supernatant was taken as template in a 50.0 µL PCR standard PCR reaction (**Fig. 3.3.**).

Reagents	Quantity for 50.0 µL of reaction mixture
Sterile distilled water	30.0 µL
10X PCR buffer	5.0 µL
50.0 mM $MgCL_2$	3.0 µL
10.0 mM dNTP	2.0 µL
10.0 µM Forward primers	1.0 µL
10.0 µM Reverse primers	1.0 µL
Template	5.0 µL
Taq polymerase	3.0 µL
Total volume	50.0 µL

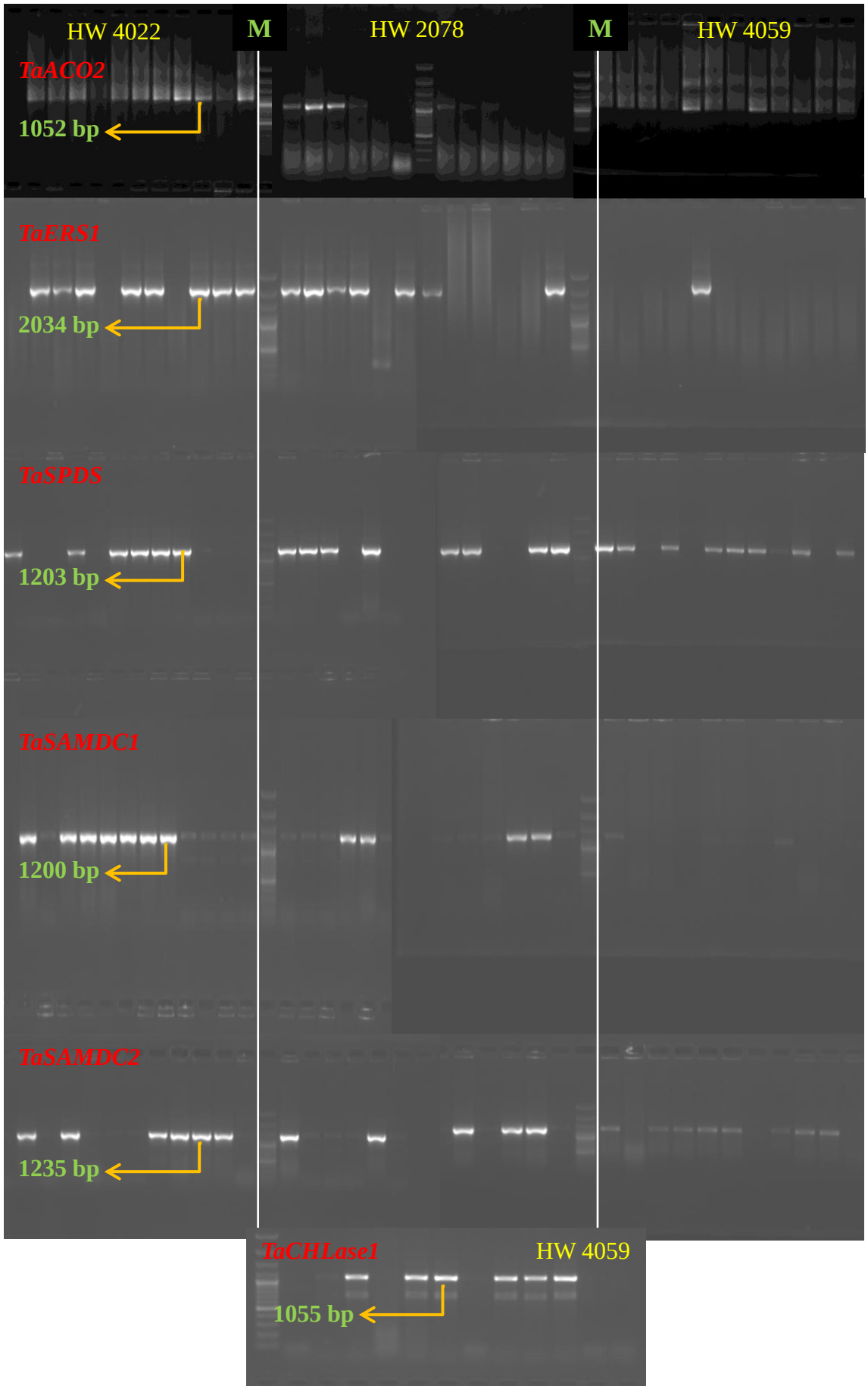


Fig. 3.3 Colony PCR of selected genes (*TaACO2*, *TaERS1*, *TaSPDS*, *TaSAMDC1*, *TaSAMDC2* and *TaCHLase1*) transformed into XL1-Blue strain of *E.coli*

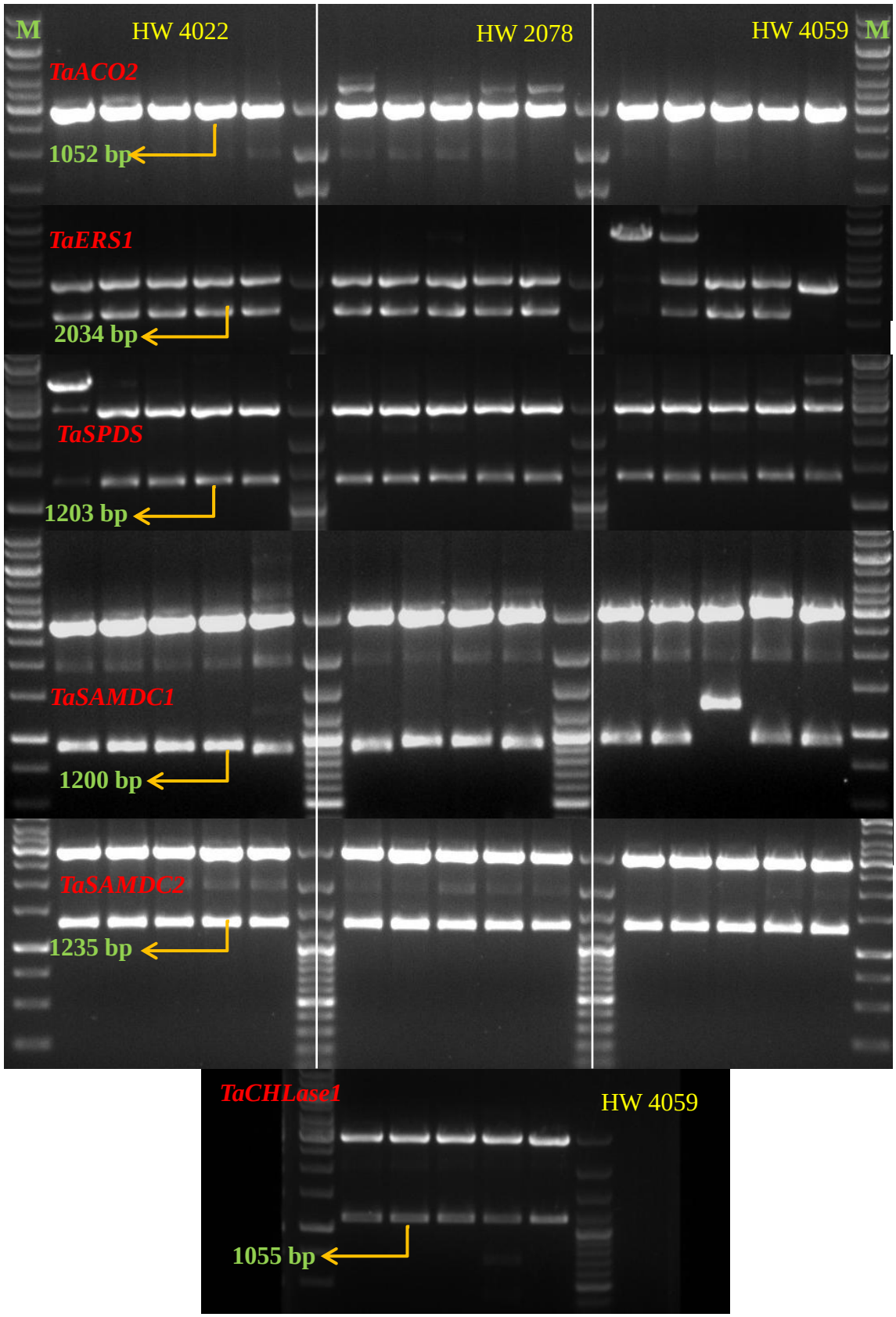


Fig. 3.4 Restriction digestion of selected genes (*TaACO2*, *TaERS1*, *TaSAMDC1*, *TaSAMDC2*, *TaSPDS* and *TaCHLase1*) using *BamH*I and *Eco*RI of isolated plasmids containing the gene insert from transformed XL1-Blue strains of *E.coli*

The reaction mixture used was as follows:

Reagents	Quantity for 25 µL of reaction mixture
Recombinant plasmid DNA (1 µg)	as per calculation
Tango buffer (2X)	5.0 µL
BamHI (10U/ µL)	1.0 µL
EcoRI (10U/ µL)	1.0 µL
Sterile double distilled water	as per calculation
Total volume	25 µL

After restriction, the DNA samples were electrophoresed on 1.0 % agarose gel. Fragment sizes were assessed in comparison with 1 kb DNA ladder as molecular weight marker.

3.5.3.6.8 DNA sequencing

E. coli cells containing desired recombinant plasmid was given to DNA sequencing Lab, Xcelris Genomics, Ahmedabad-380054, for sequencing the cloned insert DNA. Cloned insert DNA in the pTZ57R/T vector was sequenced by dideoxy chain termination method (Sanger *et al*., 1977) using universal M13 primers.

3.5.3.6.9 *In-silico* analysis tools employed in the present study

Tool	Link	References
National Centre for Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/	-
Basic Local Alignment	http://blast.ncbi.nlm.nih.gov/Blast.cgi	Altschul *et al*. (1997)

Search Tool (BLAST)		
Open reading Frame (ORF) Finder	http://www.ncbi.nlm.nih.gov/projects/gorf/	-
Integrated DNA Technologies OligoAnalyzer Tool	https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/	-
Oligo Calculator	http://mbcf.dfci.harvard.edu/docs/oligocalc.html	-
ExPASy Translate	http://www.expasy.org/tools/ http://web.expasy.org/translate/	Gasteiger *et al*. (2003)
Multiple Sequence Alignment ClustalW2	http://www.ebi.ac.uk/Tools/msa/clustalw2/	Larkin *et al*. (2007)
ExPASy ProtParam	http://web.expasy.org/protparam/	Gasteiger *et al*. (2003)
Conserved Domain Architecture Retreival Tool (CDART)	http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps	Geer *et al*. (2002)
ExPASy ScanProsite	http://prosite.expasy.org/scanprosite/	De-Castro *et al*. (2006)
TMHMM Server v. 2.0	http://www.cbs.dtu.dk/services/TMHMM-2.0/	Krogh *et al*. (2001)
Simple Modular Architecture Research Tool (SMART)	http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1	Letunic *et al*. (2002)
TargetP 1.1 Server	http://www.cbs.dtu.dk/services/TargetP/	Emanuelsson *et al*. (2000)

WoLF PSORT	http://www.genscript.com/psort/wolf_psort.html	Horton *et al*. (2007)
MEGA 6.06 software	-	Tamura *et al*. (2013)
BioEdit 7.1 software	-	Hall (2004)

3.6 Statistical analyses

The results are expressed as means with standard error (S.E.). The significance difference (at $P < 0.05$) between control and stressed samples were determined by Duncan's multiple range tests at a significance level of 0.05 for all biochemical parameters and was evaluated by analysis of variance (ANOVA). ANOVA and critical difference value were calculated by using SPSS 10.0 (SPSS Inc., Chicago, IL, USA), OPSTAT (hau.ernet.in/opstat.html) and Microsoft Excel.

4.1. RESEARCH PAPER I

Association of stay-green traits with drought tolerance efficiency under water deficit stress in wheat

4.1.1 Abstract

Stay-green (SG) is a beneficial trait with delayed senescence rate and act as a key indicator of plant adaptation to drought stress. The adverse effect of drought on photosynthesis and yield was documented in numerous studies over the last few decades, but little information exists on how SG wheat genotypes respond to drought stress. An experiment was conducted to determine the association between SG traits and drought tolerance in thirty-five wheat genotypes under water deficit stress condition imposed by with-holding irrigation for 10 days at reproductive stage. Genotypes were assessed for SG traits like SCMR (SPAD chlorophyll meter reading), photosynthesis (Pn), leaf senescence rate (LSR), relative water content (RWC; %) and yield components from both control and water stressed plants. Marked decline in RWC, drought tolerance efficiency (DTE; %), SCMR and Pn, whereas increase in LSR was observed in all genotypes under water deficit stress as compared to normal irrigated condition. However, the decline was relatively less in SG genotypes compared to the non stay-green (NSG) ones. Genotype HW 4022 registered lesser reduction in all the above mentioned traits in response to water deficit stress indicating that the drought tolerance mechanism exist in HW 4022 owing to its better management under limited water supply particularly at reproductive stage in comparison to other genotypes. The data suggested that SG traits are pivotal for regulating LSR and yield formation during water deficit stress and could be used as a best physiological marker in wheat for drought tolerance.

Key words: Stay-green, Photosynthesis, Chlorophyll content, Drought tolerance efficiency, Leaf senescence rate, Wheat.

4.1.2 Introduction

Global climate change is presently considered as the most devastating threat to the natural environment, which is now gaining significant attention from farmers, scientists, and policy makers because of its major impact on crop production (Hasanuzzaman *et al*., 2013). The irregularity of climatic conditions and the complex nature of the environment, made the situation more serious day by day. One of the most devastating abiotic stresses affecting agriculture is drought stress which has marked effects on the plant growth and development. The consequence increases further with a growing water shortage for cultivation. The world population is projected to grow to 8.2 billion by 2030 and 9.2 billion by 2050 and for feeding the growing population with available water, crop yields need to be improved by 40 % on present cultivable land (Nakashima *et al*., 2014). Climate change and global warming further aggravate the challenge and therefore issue of food security need to be addressed through innovations in crop production (Gepstein and Glick, 2013). Among the crop plants, wheat (*Triticum aestivum* L.) is a staple food for more than 35 % of the world population with 30 % of the world's cereal area, and over 220 million ha, often under abiotic stress which consequently lowers its yield (Cossani and Reynolds, 2012). Drought stress is one of the most devastating environmental stresses that limit wheat performance in terms of yield and productivity in many parts of the world (Shahryari and Mollasadeghi, 2011; Nawaz *et al*., 2013). Thus, Improvement of wheat productivity for drought tolerance is a major objective in plant science research. The best option for improving wheat production, yield and yield stability under soil moisture deficit conditions is to develop drought tolerant genotype for the drought prone areas (Li *et al.*, 2011).

A physiological approach would be the most attractive way to develop rapidly wheat genotypes suitable for water limited conditions. In mid 80s, relative water content (RWC; %) was introduced as a best criterion directly reflecting plant water status which, later on was used as RWC instead of plant water potential (Arjenaki *et al.*, 2012). It reflects its relation with cell volume accurately and indicated the balance between amount of the water absorbed and consumed through transpiration in plants (Schonfeld *et al.*, 1988). It has been reported that higher RWC is indication of drought resistance rather than that of drought escape and is a result for higher osmotic regulation of tissue with lower cell wall elasticity (Keyvan, 2010). Thus, RWC may

be considered to be the most reliable physiological parameter for quantifying plant water status during drought stress in wheat.

Stay-green (SG) traits like greenness index reflecting chlorophyll content and photosynthesis are the beneficial traits observed in many crop plants under water limited conditions (Jordan *et al*., 2012; Nawaz *et al*., 2013). SG wheat genotypes retained their green leaves for longer time at reproductive stage resulting in higher photosynthesis rate with improved yield performance, particularly under limited water supply (Christopher *et al*., 2013). Thus, SG traits can act as a promising tool, while screening the genotypes for drought tolerance. SG traits have been used successfully in phenotyping sorghum for yield stability and are a promising selection tool in wheat (Christopher *et al*., 2008). Greenness index measured using chlorophyll meter SPAD-502 (Minolta Camera Co; Ltd, Japan) gives an indication of relative leaf chlorophyll content. Higher the SPAD value reflects higher leaf chlorophyll content (Lopes and Reynolds, 2012). Leaf senescence rate (LSR), one aspect of SG traits, was evaluated through visual scoring under field condition in cereals crops (Kumari *et al*., 2013). Visual scoring for measurement of LSR is a widely practiced non-destructive technique, while assessing large number of wheat germplasms for drought tolerance and a linear relationship was found between visual scoring stay-green values and SPAD- 502 values which would be proved to be useful for crop improvement (Adu *et al*., 2011). Thus, visual scoring for LSR and SPAD value for greenness index might be consider as a reliable indication of leaf senescence rate and need to be used in screening wheat genotypes for drought tolerance.

Selection and breeding for improved grain yield in wheat is the ultimate way to produce stress tolerant genotypes (Golabadi *et al*., 2011). In wheat, drought stress directly affects the grain development that naturally results in lower grain weight and yield (Ahmed *et al*., 2013). Drought tolerance efficiency (DTE; %) is considered to be the most effective selection criteria to assess drought tolerance (Puri *et al*., 2013). Most of the tolerant genotypes showed highest DTE and minimum reduction in seed yield under drought stress, which clearly indicated that improvement in drought tolerance, is possible through simple selection (Bahar and Yildirim, 2010).

Association between SG traits and yield stability is reported in many crops but published studies on a possible association between them are limited (Kumari *et al*., 2013). In addition, screening wheat genotypes for drought tolerance has been carried out by many scientists, but little information is available about the screening on the

basis of their stay-green character at reproductive stage. So, the current study was aimed to screen wheat genotypes for drought tolerance on the basis of their SG traits like delayed leaf senescence rate and drought tolerance efficiency under water deficit condition. In this paper the results revealed that stay-green genotypes exhibits distinctly delayed leaf senescence rate with maximum photosynthesis and drought tolerance efficiency in comparison to non stay-green genotypes under water deficit stress.

4.1.3 Materials and methods

4.1.3.1 Plant material and treatments

A pot culture experiment was conducted during *rabi* season on thirty five wheat genotypes listed in **Table 3.1** showing diversity for SG traits with recommended package of practices for wheat crop. Plants were subjected to water deficit stress by with-holding irrigation for 10 days at 50 % anthesis, while routine schedule of irrigation was practiced for control plants. The response of plants in terms of growth and physiological traits were studied in upper most fully expanded flag leaf at 50 % anthesis stage.

4.1.3.2 Observations recorded

4.1.3.2.1 Relative water content (RWC; %)

To evaluate the plant water status RWC was measured by Barrs and Weatherley (1962) method. Leaf RWC was estimated by recording the fresh weight (g) of leaf samples, thereafter immediately transferring in petridishes containing distilled water for 4 h to record turgid weight (g), followed by drying in hot air oven at 70 ^{0}C till constant dry weight (g) has reached.

RWC (%) = [(Fresh wt. – Dry wt.) / (Turgid wt. – Dry wt.)] x 100

4.1.3.2.2 Photosynthesis rate

Leaves were categorized into green and yellow/ dead leaves and the rate of photosynthesis (Pn) was measured using portable Infrared Gas Analyser (IRGA), LI-6400XT Model (Li-COR Ltd., Lincoln, Nebraska, USA) by operating the IRGA in the closed mode between 10.00-11.00 a.m. when relative humidity, temperature, photosynthetic photon flux density and CO_2 conc. ranged from 50-60 %, 30 to 35 ^{0}C, 1200 µmol $m^{-2}s^{-1}$ and 350 to 360 µmol mol^{-1}, respectively. Fifteen flag leaves were selected at random for Pn measurement and expressed in µmol CO_2 m^{-2} s^{-1}.

4.1.3.2.3 SCMR (SPAD chlorophyll meter reading)

Soil and plant analyzer development (SPAD) values were measured in the middle part of flag leaves using portable Minolta SPAD-502 chlorophyll meter (Minolta camera Co. Ltd., Osaka, Japan) at the end of water deficit stress period for 10 days at 50 % anthesis stage from both control and treated plants. The average readings of 10 leaves per pot was recorded and used for analysis.

4.1.3.2.4 Assessment of leaf senescence rate

Phenotyping for leaf senescence was done visually and LSR was scored using a scale of 0 to 10, dividing the percentage of estimate area that is dead by time duration in days as per Lu *et al*. (2011). A rating of 10 indicated essentially no leaf death, 5-6 indicated approximately 50 % mature leaf area dead, while 0 indicate 100 % leaf senescence.

10 = no leaf dead area; 9 = 10 % dead area; 8 = 20 % dead area; 7 = 30 % dead area; 6 = 40 % dead area; 5 =50 % dead area; 4 = 60 % dead area; 3 = 70 % dead area; 2 = 80 % dead area; 1 = 90 % dead area and 0 = 100 % dead area.

After 10 days of water deficit stress at 50 % anthesis, LSR was scored 6 times after every 2 day interval.

$$\text{LSR} = \frac{\text{Change in the degree of senescence}}{\text{Total time duration (days)}}$$

4.1.3.2.5 Yield and associated parameters

The plants were harvested separately from control and water stressed pots. Measurements on 1000 seed weight, grain yield plant^{-1} were recorded in control and water deficit stressed plants as economic yield. The whole plant dry weight was measured as biological yield. Three replications were taken for each parameter. DTE % was calculated using the formula given by Fischer and Wood (1981) and harvest index (HI; %) was calculated as the ratio of the economic yield to biological yield and was expressed in percentage (Gardner *et al.,* 1985).

DTE (%) = (Yield under water deficit stress/ Yield under irrigated condition) x100

HI (%) = (Economic yield/ Biological yield) x100

4.1.3.3 Statistical analysis

The results are expressed as means with standard error (S.E.). The significance difference (at $P < 0.05$) between control and stressed samples were determined by Duncan's multiple range tests at a significance level of 0.05 for all biochemical parameters and was evaluated by analysis of variance (ANOVA). ANOVA and critical difference value were calculated by using SPSS 10.0 (SPSS Inc., Chicago, IL, USA), OPSTAT (hau.ernet.in/opstat.html) and Microsoft Excel.

4.1.4 Results

4.1.4.1 Effect of water deficit stress on relative water content (RWC; %)

Results on the effect of water deficit stress on relative water content of wheat genotypes are reported in **Table 4.1.1** and **Fig. 4.1.1.** Significant difference was reported for RWC under both water regimes. RWC value for normal irrigated plants ranged between 75-85 % whereas, for water deficit stress treated plants the value decreased to 65-75 %. The genotypes HW 2060, DBW 44, CBW 23, HW 4207, HD 2888 and HW 4022 reported with lowest percent decline in RWC under water deficit stress condition and hence categorized as drought tolerant genotypes. In contrast, the genotypes HD 2789, HW 4059, HW 2040, PBW 590, HW 4024 and DBW 77 showed marked percent decline in RWC value under water deficit stress and categorized as drought susceptible genotypes.

4.1.4.2 Effect of water deficit stress on SCMR and Pn of flag leaves

Both the SCMR value and Pn decreased as the water deficit stress progressed in all the studied wheat genotypes. The genotypes HW 4022, CBW 23, PBW 555 and HW 4010 always exhibited higher SCMR values under water deficit stress whereas, HW 4059, HW 4207, HW 541 and HW 4055 showed greater decline (**Table 4.1.2** and **Fig. 4.1.2**). Similarly, the leaf Pn rate decreases significantly in all genotypes under water deficit stress. Maximum reduction in Pn was in HUW 541, PBW 142, HW 4059 and HW 2083, while HW 2036, HW 4022, HD 2888 and CBW 14 showed minimum reduction under water deficit stress at reproductive stage (**Table 4.1.3** and **Fig. 4.1.3**). Plants with SG traits showed lesser reduction in greenness index (GI) and Pn in comparison to plants with NSG traits under water deficit stress condition. Significant

Relative water content (RWC; %)					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	CBW 14	82.0	71.5	**76.7**	12.9
	CBW 23	80.2	73.0	**76.6**	9.0
	CBW 39	82.9	73.7	**78.3**	11.2
	DBW 39	83.6	71.9	**77.7**	14.1
	DBW 44	80.4	73.3	**76.8**	8.8
	DBW 77	82.3	68.8	**75.6**	16.3
	HD 2789	82.2	66.1	**74.2**	19.6
	HD 2888	81.0	72.9	**76.9**	10.0
	HD 2923	83.6	71.5	**77.6**	14.5
	HI 1544	83.4	71.9	**77.7**	13.7
	HUW 541	82.7	73.2	**78.0**	11.6
	HW 2013	81.9	73.0	**77.4**	10.8
	HW 2021	83.8	74.3	**79.1**	11.3
	HW 2022	87.0	73.1	**80.0**	16.0
	HW 2034	85.3	71.6	**78.5**	16.1
	HW 2036	80.8	72.7	**76.8**	10.1
	HW 2040	83.8	68.4	**76.1**	18.4
	HW 2059	83.4	70.7	**77.0**	15.3
	HW 2060	80.8	74.1	**77.4**	8.3
	HW 2078	84.3	71.7	**78.0**	14.9
	HW 2083	83.0	72.7	**77.8**	12.5
	HW 4010	80.6	70.7	**75.7**	12.3
	HW 4022	80.2	72.0	**76.1**	10.3
	HW 4024	86.1	71.4	**78.7**	17.1
	HW 4026	84.3	74.5	**79.4**	11.7
	HW 4029	83.6	73.6	**78.6**	11.9
	HW 4055	83.0	72.1	**77.6**	13.1
	HW 4059	85.3	69.2	**77.2**	18.9
	HW 4207	81.8	74.2	**78.0**	9.2
	HW 5209	83.3	70.4	**76.8**	15.4
	LOK 64	84.1	70.5	**77.3**	16.2
	PBW 142	80.8	72.2	**76.5**	10.6
	PBW 550	81.4	71.5	**76.4**	12.2
	PBW 555	82.8	69.2	**76.0**	16.5
	PBW 590	82.0	68.0	**75.0**	17.1
	Mean	**82.8**	**71.7**	**77.2**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**N/A**		**2.722**	
	Treatment (T)	**1.839**		**0.651**	
	Interaction G x T	**N/A**		**3.85**	

Table 4.1.1 Effect of water deficit stress on relative water content (RWC; %) in wheat genotypes at reproductive stage

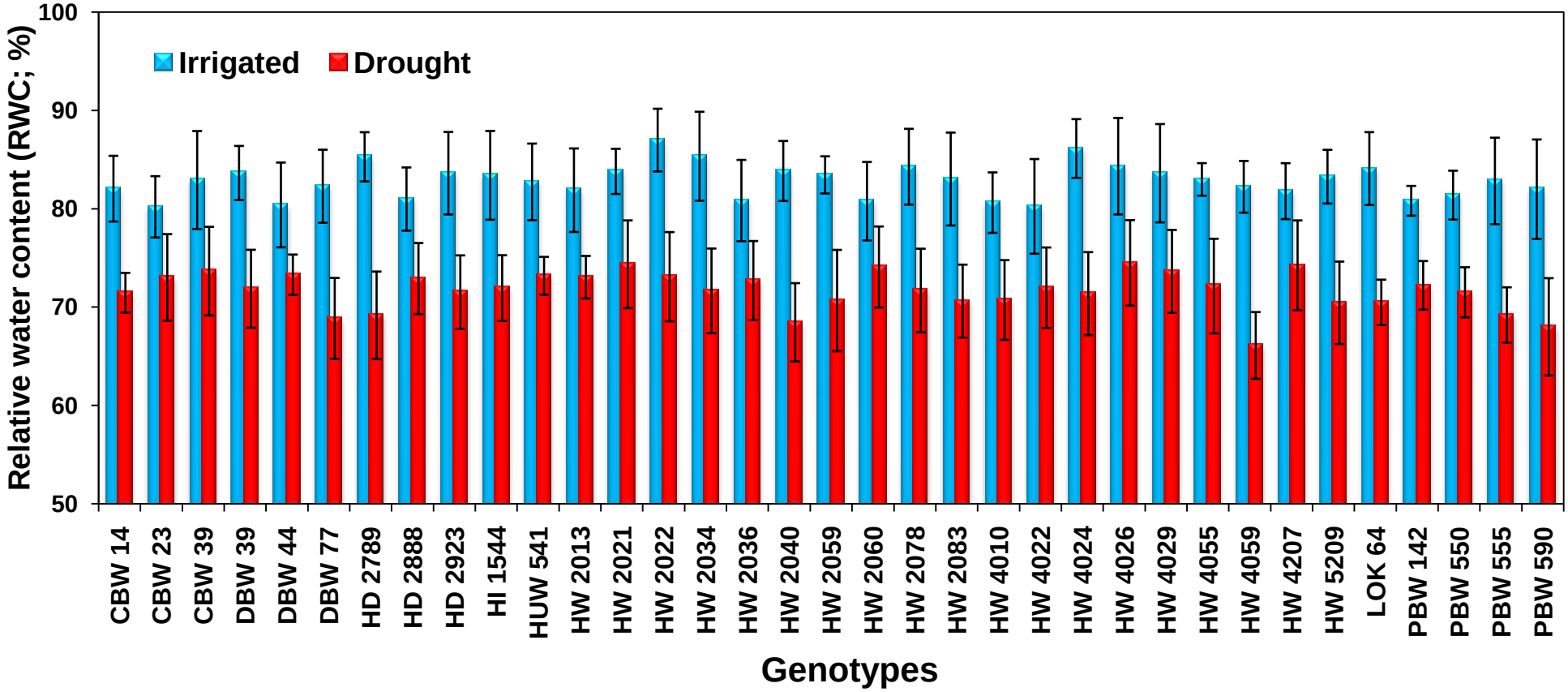


Fig. 4.1.1 Effect of water deficit stress on relative water content (RWC; %) in wheat genotypes at reproductive stage

SCMR (SPAD chlorophyll meter reading)					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	CBW 14	50.4	45.2	**47.8**	10.3
	CBW 23	46.1	42.4	**44.3**	8.0
	CBW 39	46.4	40.7	**43.5**	12.4
	DBW 39	53.9	47.2	**50.5**	12.4
	DBW 44	50.2	45.9	**48.0**	8.5
	DBW 77	51.8	46.1	**49.0**	11.1
	HD 2789	49.9	45.2	**47.6**	9.4
	HD 2888	51.7	44.8	**48.2**	13.4
	HD 2923	54.2	48.7	**51.5**	10.2
	HI 1544	49.1	43.8	**46.5**	10.7
	HUW 541	51.9	44.5	**48.2**	14.2
	HW 2013	50.6	43.5	**47.1**	14.1
	HW 2021	52.3	48.0	**50.2**	8.3
	HW 2022	48.2	43.5	**45.9**	9.8
	HW 2034	53.3	46.4	**49.9**	13.1
	HW 2036	54.0	47.5	**50.8**	12.0
	HW 2040	47.3	42.6	**45.0**	9.9
	HW 2059	42.4	36.5	**39.4**	13.9
	HW 2060	50.3	43.7	**47.0**	13.2
	HW 2078	52.4	46.2	**49.3**	11.7
	HW 2083	50.6	45.7	**48.1**	9.7
	HW 4010	50.4	46.3	**48.3**	8.1
	HW 4022	49.7	46.1	**47.9**	7.2
	HW 4024	56.6	49.1	**52.8**	13.2
	HW 4026	43.5	38.4	**40.9**	11.8
	HW 4029	53.4	48.6	**51.0**	9.1
	HW 4055	42.0	36.0	**39.0**	14.2
	HW 4059	51.3	43.6	**47.4**	15.1
	HW 4207	52.4	44.6	**48.5**	14.8
	HW 5209	49.3	45.2	**47.3**	8.3
	LOK 64	50.5	45.8	**48.2**	9.3
	PBW 142	47.6	41.7	**44.7**	12.3
	PBW 550	54.6	47.9	**51.3**	12.2
	PBW 555	49.4	45.5	**47.5**	8.0
	PBW 590	47.4	41.4	**44.4**	12.5
	Mean	**50.1**	**44.5**	**47.3**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**1.91**		**0.682**	
	Treatment (T)	**0.457**		**0.163**	
	Interaction G x T	**N/A**		**0.965**	

Table 4.1.2 Effect of water deficit stress on SCMR (SPAD chlorophyll meter reading) in wheat genotypes at reproductive stage

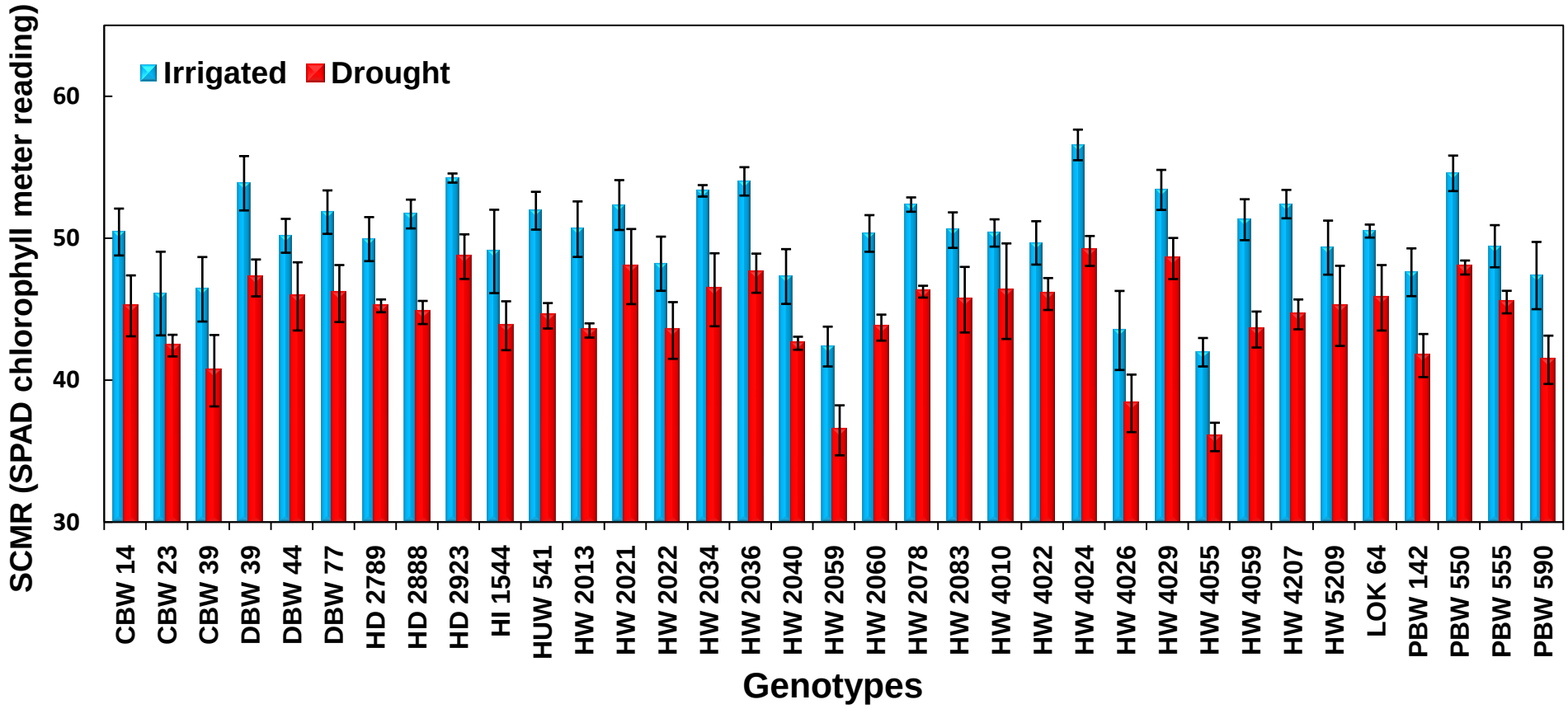


Fig. 4.1.2 Effect of water deficit stress on SCMR (SPAD chlorophyll meter reading) in wheat genotypes at reproductive stage

Photosynthesis rate (µmol CO_2 m^{-2} s^{-2})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	**CBW 14**	25.3	22.4	**23.8**	11.3
	CBW 23	28.6	16.8	**22.7**	41.4
	CBW 39	28.7	15.3	**22.0**	46.6
	DBW 39	27.6	15.4	**21.5**	44.2
	DBW 44	26.0	21.7	**23.8**	16.4
	DBW 77	27.7	16.8	**22.3**	39.2
	HD 2789	28.2	19.0	**23.6**	32.7
	HD 2888	26.8	15.6	**21.2**	41.6
	HD 2923	24.3	16.4	**20.3**	32.5
	HI 1544	23.7	14.3	**19.0**	39.9
	HUW 541	24.9	14.3	**19.6**	42.6
	HW 2013	27.8	14.5	**21.1**	47.9
	HW 2021	27.4	19.6	**23.5**	28.5
	HW 2022	29.1	12.3	**20.7**	57.7
	HW 2034	28.4	14.4	**21.4**	49.4
	HW 2036	27.8	21.4	**24.6**	23.1
	HW 2040	29.9	16.4	**23.1**	45.2
	HW 2059	27.3	20.4	**23.8**	25.2
	HW 2060	29.2	17.2	**23.2**	41.0
	HW 2078	26.7	19.4	**23.1**	27.5
	HW 2083	28.4	12.6	**20.5**	55.4
	HW 4010	28.0	15.0	**21.5**	46.5
	HW 4022	26.4	22.2	**24.3**	16.1
	HW 4024	28.1	21.0	**24.5**	25.4
	HW 4026	24.3	14.7	**19.5**	39.6
	HW 4029	24.8	13.7	**19.2**	44.6
	HW 4055	28.8	17.9	**23.4**	37.8
	HW 4059	26.8	12.3	**19.6**	54.0
	HW 4207	30.2	20.9	**25.6**	30.8
	HW 5209	26.7	12.6	**19.7**	52.6
	LOK 64	25.6	22.8	**24.2**	11.1
	PBW 142	27.0	12.2	**19.6**	54.7
	PBW 550	27.6	15.2	**21.4**	45.1
	PBW 555	24.8	17.2	**21.0**	30.7
	PBW 590	25.9	21.3	**23.6**	17.6
	Mean	**27.1**	**17.0**	**22.1**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**0.706**		**0.252**	
	Treatment (T)	**0.169**		**0.06**	
	Interaction G x T	**0.998**		**0.357**	

Table 4.1.3 Effect of water deficit stress on photosynthesis rate (µmol m^{-2} s^{-2}) in wheat genotypes at reproductive stage

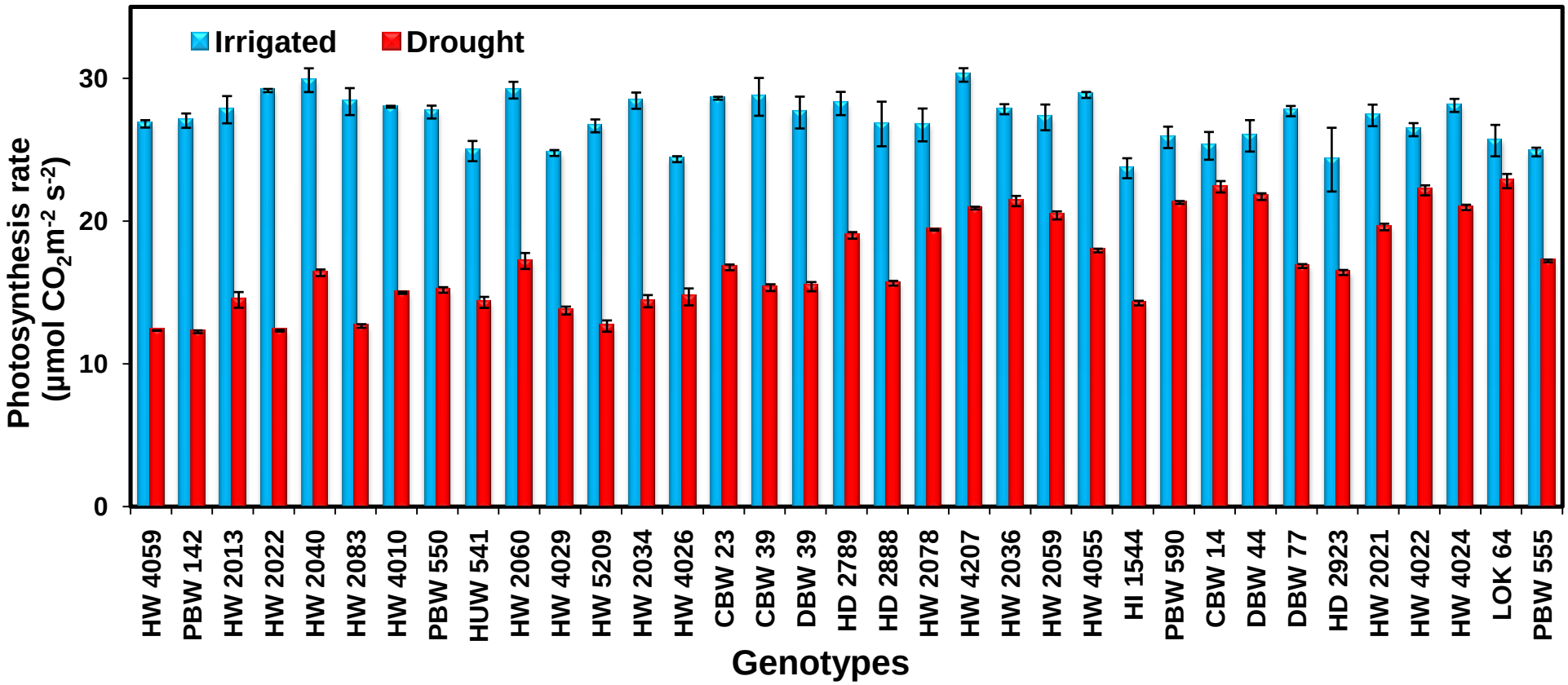


Fig. 4.1.3 Effect of water deficit stress on photosynthesis rate (µmol m^{-2} s^{-1}) in wheat genotypes at reproductive stage

genotypic differences were observed for GI and Pn in SG traits under both water regimes.

4.1.4.3 Visual symptoms of senescence

A significant variation was observed for LSR among all genotypes (**Table 4.1.4.1** and **Fig. 4.1.4**) measured at 50 % anthesis stage after 10 days of water deficit stress treatment by visual scoring (0-10 scale). Genotypes showing $0 \leq LSR \leq 0.35$ were categorized as SG, $0.35 \leq LSR \leq 0.5$ as moderate SG and $LSR \geq 0.5$ as NSG (**Table 4.1.4.2**). LSR was low in PBW 555 followed by LOK 64, HW 4024 and HW 4022 whereas, HW 4059 shows fastest LSR under water deficit stress condition. SG genotypes displayed lesser LSR as compared to NSG genotypes and they continued to maintain their superiority under water deficit stress conditions.

4.1.4.4 Yield and related parameters

There were significant decrease in yield attributes *viz.*, biomass per plant (**Table 4.1.5** and **Fig. 4.1.5**), seed weight per plant (**Table 4.1.6** and **Fig. 4.1.6**), 1000 seed weight (**Table 4.1.7** and **Fig. 4.1.7**), HI % (**Table 4.1.8** and **Fig. 4.1.8**) and DTE % (**Table 4.1.9** and **Fig. 4.1.9**) under water deficit stress in all the genotypes. The genotypes with SG traits were observed with higher yield attributes under both water regimes. Highest value for yield attributes were recorded in HW 4022 followed by HW 2036, HW 4055 and CBW 23; whereas it is lowest in HW 4059, PBW 142, HUW 541 and HW 2022 under water deficit stress condition. Significant differences were recorded among the genotypes on the basis of DTE %, genotype (HW 4022) showed highest DTE % value as compared to all other genotypes while, HW 4059 showed lowest DTE % values.

4.1.5 Discussion

Drought is a serious environmental threat altering plant growth, development, metabolism and ultimately crop yield (Ajithkumar and Panneerselvam, 2014). Plants under drought stress develop several tolerance mechanisms, which enable them to survive and reproduce under water scarcity conditions (Budak *et al*., 2013). The primary response under drought stress is inhibition of growth and acceleration of senescence process (Gepstein and Glick, 2013). By understanding the physiological mechanisms that facilitate crop plants to adapt to drought stress along with optimum

yield and production may possibly help in screening genotypes of economically important crop plants for drought prone areas (Geravandi *et al*., 2011).

Development of improved wheat genotypes with drought tolerance is critical for sustainable wheat production (Li *et al*., 2011). Relative water content (RWC) of leaves is known as best screening measure for drought tolerance (Elham *et al*., 2012). In the present study, the first criteria used for screening of wheat genotypes for drought tolerance is evaluating the RWC under both water regimes. The genotypes that maintained their water relation with higher value for RWC under water deficit stress were categorized as drought tolerant genotypes, while the others with lower value for RWC as drought susceptible genotypes. Comparable findings reported in wheat in previous reports where, drought tolerant genotypes maintained significantly higher RWC as compared to susceptible ones (Bogale *et al*., 2011; Elham *et al*., 2012). Thus RWC of leaves are considered as the most sensitive physiological parameter and indicator of water limited conditions.

In addition, Kumari *et al*. (2013) viewed that there is significant variability for stay-green (SG) traits in wheat and these traits can be used as an effective selection criterion for tolerance to heat and drought stress. The results on SG traits in the present study showed that photosynthesis (Pn) and SCMR values decreased significantly in all the genotypes under water deficit stress conditions. The genotypes *viz*. HW 4022, CBW 23, PBW 555 and HW 4010 maintained higher amount of Pn and SCMR values whereas, HW 4059, HW 4207, HW 541 and HW 4055 showed greater decline for stay-green traits under water limited condition. The findings thus indicate that drought induced senescence leads to a reduction in Pn and SCMR values which were found to be more in non stay-green genotypes (NSG) as compared to SG ones and this might be one of the reasons for their sensitivity to drought stress. In addition, drought induced leaf senescence rate (LSR) was slow in PBW 555 followed by LOK 64, HW 4024 and HW 4022 whereas, HW 4059 reported with fastest LSR. The genotype HW 4022 not only have higher SCMR and Pn values but also sustained it longer throughout grain filling period than the other genotypes. From the data, it was inferred that a better stability in the level of SCMR values under water deficit stress might have kept genotype HW 4022 in privileged situation. The current findings supported by Elshafei *et al*. (2013) where they reported that tolerant wheat genotype maintained higher chlorophyll content than the susceptible genotype with higher LSR under drought stress. Comparable findings in wheat (Tian *et al*., 2013;

Leaf senescence rate (LSR)		
Genotypes	**CBW 14**	0.31
	CBW 23	0.45
	CBW 39	0.45
	DBW 39	0.45
	DBW 44	0.31
	DBW 77	0.31
	HD 2789	0.45
	HD 2888	0.45
	HD 2923	0.31
	HI 1544	0.35
	HUW 541	0.53
	HW 2013	0.54
	HW 2021	0.31
	HW 2022	0.54
	HW 2034	0.47
	HW 2036	0.43
	HW 2040	0.54
	HW 2059	0.43
	HW 2060	0.53
	HW 2078	0.45
	HW 2083	0.54
	HW 4010	0.54
	HW 4022	0.31
	HW 4024	0.31
	HW 4026	0.45
	HW 4029	0.53
	HW 4055	0.41
	HW 4059	0.64
	HW 4207	0.45
	HW 5209	0.53
	LOK 64	0.31
	PBW 142	0.64
	PBW 550	0.54
	PBW 555	0.25
	PBW 590	0.35
	Mean	**0.44**

Table 4.1.4.1 Effect of water deficit stress on leaf senescence rate (LSR) in wheat genotypes at reproductive stage

$0 \leq LSR \leq 0.35$	$0.35 \leq LSR \leq 0.5$	$LSR \geq 0.5$
PBW 555	CBW 23	HUW 541
LOK 64	CBW 39	HW 2031
CBW 14	DBW 39	HW 2022
DBW 44	HD 2789	HW 2040
DBW 77	HD 2888	HW 2060
HD 2923	HW 2034	HW 2083
HI 1544	HW 2036	HW 4010
HW 2021	HW 2059	HW 4029
HW 4024	HW 4207	PBW 550
PBW 590	HW 4026	HW 5209
HW 4022	HW 4055	PBW 142
-	HW 2078	HW 4059

Table 4.1.4.2 Grouping of wheat genotypes on the basis of LSR under water deficit stress condition

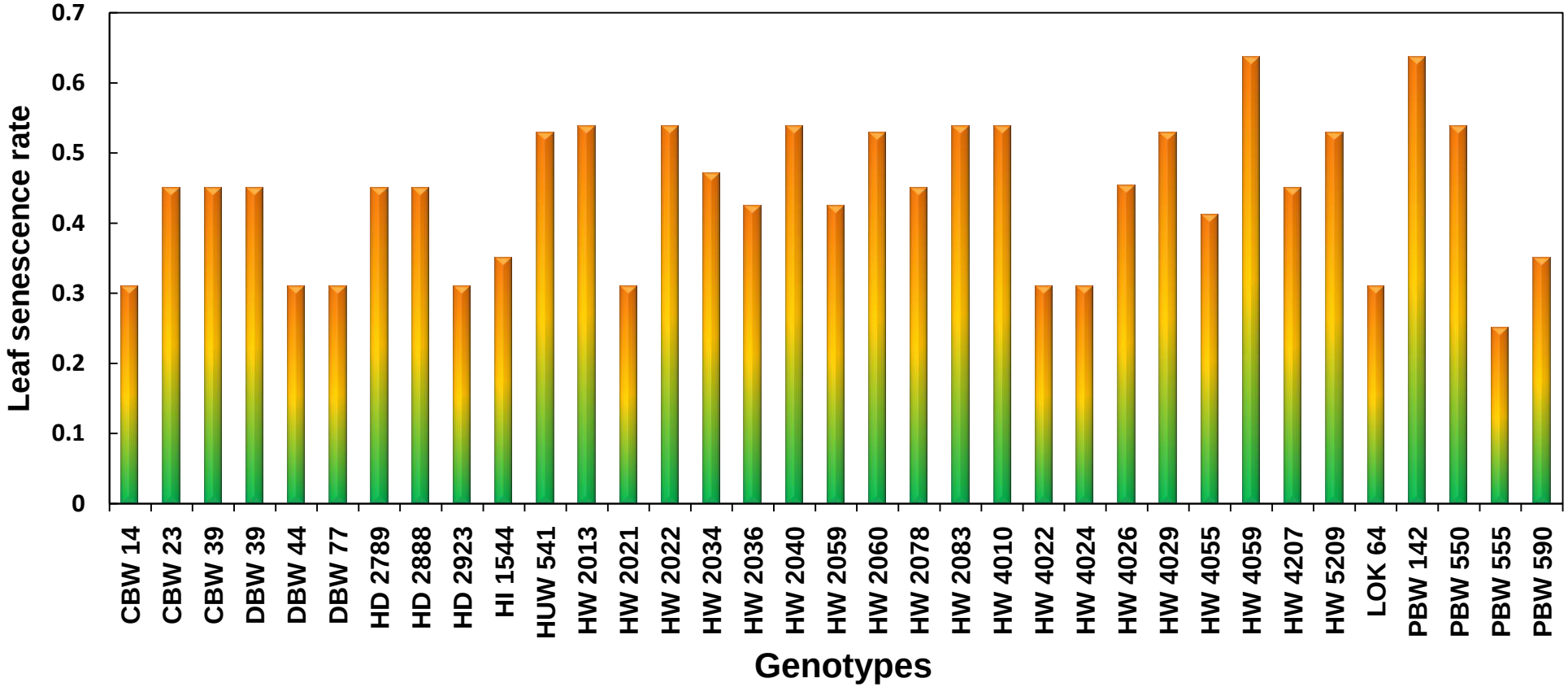


Fig. 4.1.4 Effect of water deficit stress on leaf senescence rate (LSR) in wheat genotypes at reproductive stage

Biomass per plant (g plant^{-1})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	CBW 14	37.1	21.5	**29.3**	42.1
	CBW 23	27.9	16.6	**22.3**	40.5
	CBW 39	24.4	12.6	**18.5**	48.4
	DBW 39	44.6	25.3	**34.9**	43.3
	DBW 44	37.7	22.4	**30.1**	40.4
	DBW 77	43.4	17.7	**30.6**	59.1
	HD 2789	41.1	12.5	**26.8**	69.6
	HD 2888	34.2	21.9	**28.0**	35.8
	HD 2923	37.3	20.5	**28.9**	45.0
	HI 1544	27.7	17.3	**22.5**	37.5
	HUW 541	37.5	9.5	**23.5**	74.7
	HW 2013	43.8	22.9	**33.3**	47.8
	HW 2021	41.0	23.8	**32.4**	42.1
	HW 2022	28.5	8.9	**18.7**	68.7
	HW 2034	47.1	30.1	**38.6**	36.2
	HW 2036	37.6	25.6	**31.6**	32.0
	HW 2040	38.1	13.7	**25.9**	64.2
	HW 2059	34.8	12.8	**23.8**	63.2
	HW 2060	35.9	19.5	**27.7**	45.5
	HW 2078	38.0	22.2	**30.1**	41.6
	HW 2083	38.3	12.5	**25.4**	67.4
	HW 4010	35.5	21.6	**28.5**	39.1
	HW 4022	39.4	32.0	**35.7**	18.7
	HW 4024	40.5	25.1	**32.8**	37.9
	HW 4026	32.9	21.7	**27.3**	34.0
	HW 4029	37.8	19.2	**28.5**	49.2
	HW 4055	28.3	17.7	**23.0**	37.3
	HW 4059	36.4	14.8	**25.6**	59.3
	HW 4207	41.0	11.7	**26.3**	71.5
	HW 5209	40.9	19.0	**30.0**	53.6
	LOK 64	31.2	12.5	**21.8**	60.0
	PBW 142	33.2	10.3	**21.8**	68.9
	PBW 550	24.0	14.7	**19.4**	39.0
	PBW 555	41.0	20.3	**30.6**	50.6
	PBW 590	24.9	15.7	**20.3**	37.0
	Mean	**36.1**	**18.5**	**27.3**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**1.642**		**0.587**	
	Treatment (T)	**0.392**		**0.14**	
	Interaction G x T	**2.322**		**0.829**	

Table 4.1.5 Effect of water deficit stress on biomass per plant (g plant^{-1}) in wheat genotypes at reproductive stage

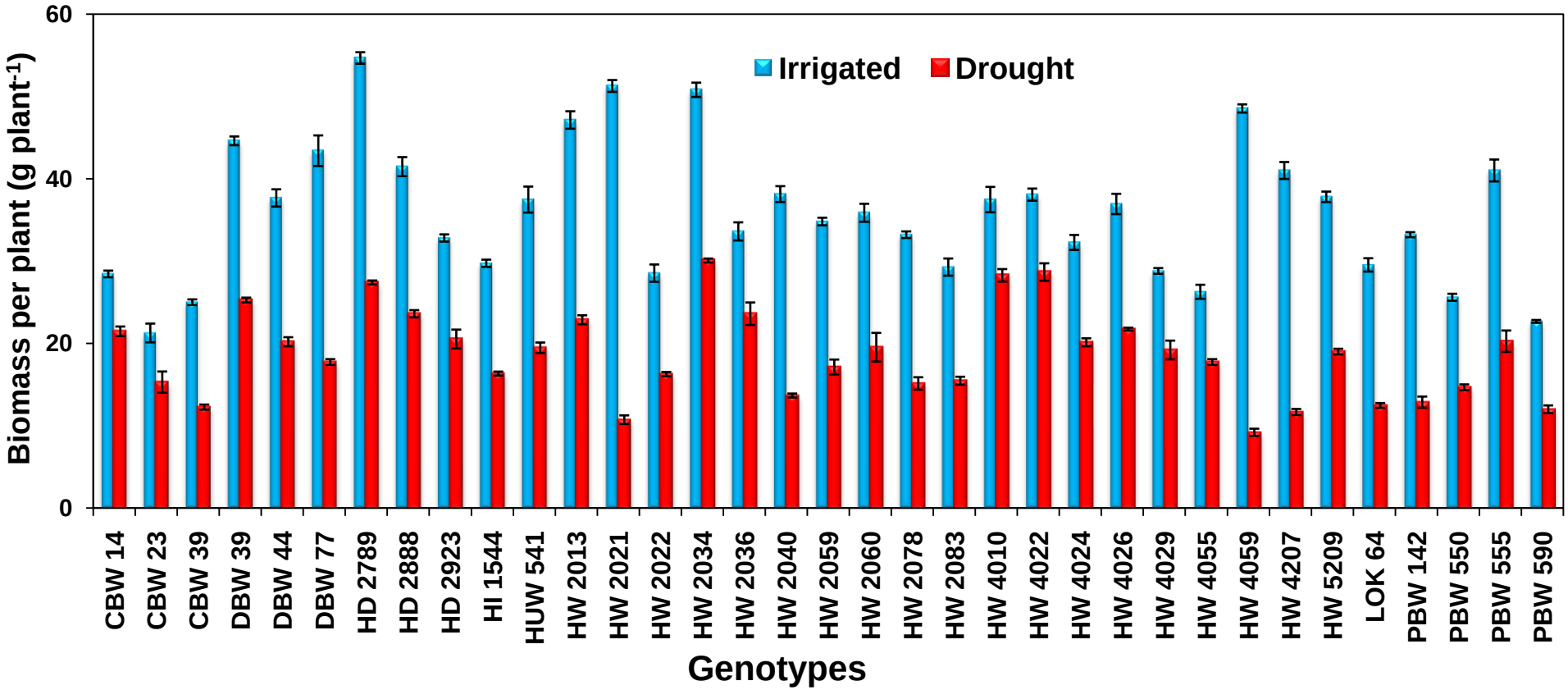


Fig. 4.1.5 Effect of water deficit stress on biomass per plant (g plant^{-1}) in wheat genotypes at reproductive stage

Seed weight per plant (g plant^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**CBW 14**	17.0	7.5	**12.2**	56.1
	CBW 23	11.9	5.7	**8.8**	51.7
	CBW 39	9.9	3.3	**6.6**	66.4
	DBW 39	17.9	6.9	**12.4**	61.2
	DBW 44	16.5	7.3	**11.9**	55.6
	DBW 77	18.0	4.7	**11.3**	74.1
	HD 2789	18.1	3.2	**10.6**	82.3
	HD 2888	16.5	6.7	**11.6**	59.5
	HD 2923	16.7	4.5	**10.6**	72.8
	HI 1544	11.7	4.4	**8.1**	62.5
	HUW 541	15.5	1.7	**8.6**	88.8
	HW 2013	18.6	4.9	**11.8**	73.5
	HW 2021	17.2	7.7	**12.5**	55.1
	HW 2022	12.4	1.7	**7.1**	86.0
	HW 2034	19.7	6.4	**13.1**	67.6
	HW 2036	13.9	7.6	**10.8**	45.5
	HW 2040	16.3	3.7	**10.0**	77.5
	HW 2059	14.4	2.8	**8.6**	80.6
	HW 2060	15.3	6.4	**10.9**	58.3
	HW 2078	16.9	6.7	**11.8**	60.5
	HW 2083	16.3	2.7	**9.5**	83.6
	HW 4010	13.1	6.5	**9.8**	50.2
	HW 4022	18.1	10.8	**14.4**	40.5
	HW 4024	17.7	8.3	**13.0**	53.3
	HW 4026	13.7	5.3	**9.5**	61.0
	HW 4029	15.4	4.4	**9.9**	71.5
	HW 4055	12.0	6.1	**9.1**	48.9
	HW 4059	14.0	2.5	**8.3**	82.1
	HW 4207	16.7	3.6	**10.1**	78.4
	HW 5209	18.3	5.1	**11.7**	72.3
	LOK 64	13.3	3.5	**8.4**	74.0
	PBW 142	14.1	1.9	**8.0**	86.3
	PBW 550	9.9	3.5	**6.7**	64.9
	PBW 555	16.7	5.2	**11.0**	68.9
	PBW 590	10.3	4.3	**7.3**	58.4
	Mean	**15.3**	**5.1**	**10.2**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**0.857**		**0.306**	
	Treatment (T)	**0.205**		**0.073**	
	Interaction G x T	**1.212**		**0.433**	

Table. 4.1.6 Effect of water deficit stress on seed weight per plant (g plant^{-1}) in wheat genotypes at reproductive stage

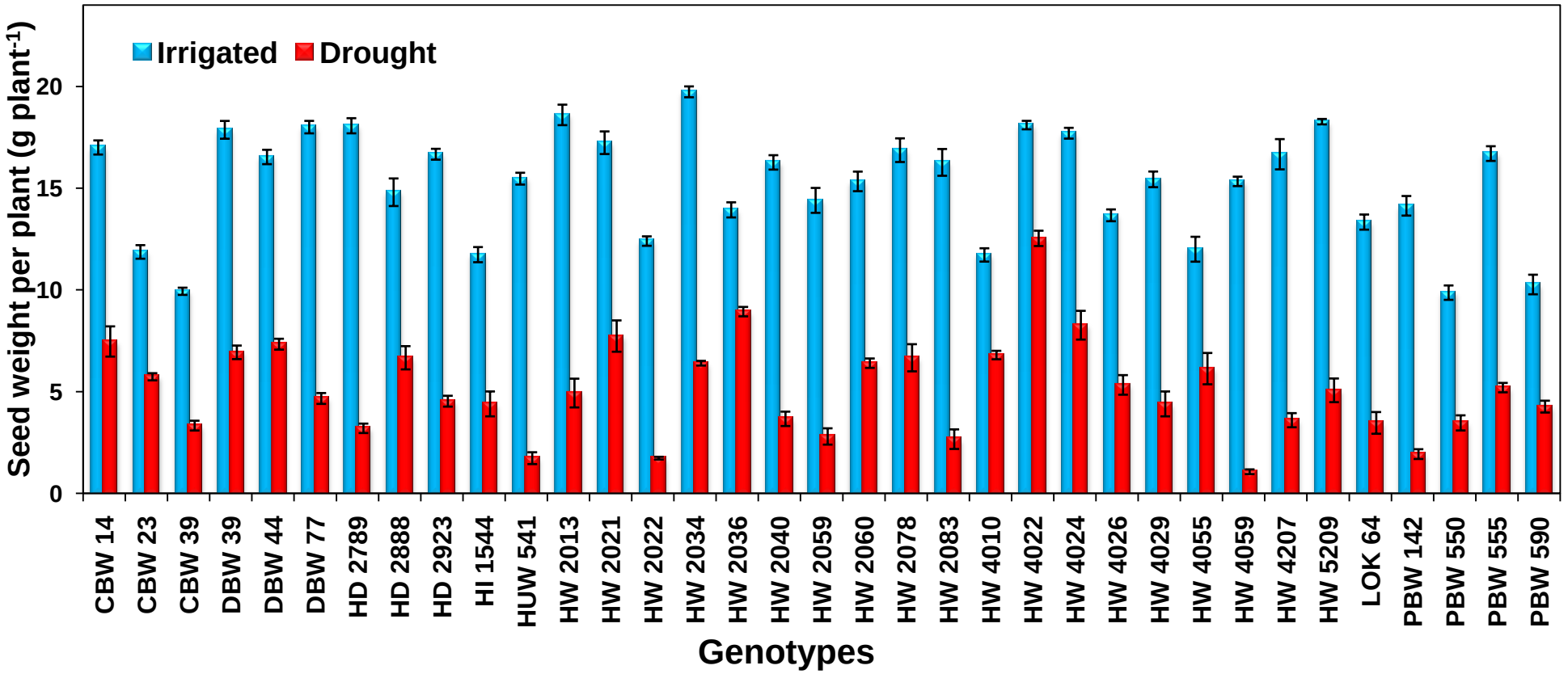


Fig. 4.1.6 Effect of water deficit stress on seed weight per plant (g plant^{-1}) in wheat genotypes at reproductive stage

		Test weight (1000 seed weight; g)			
Water status		Irrigated	Drought	Mean	% Change
Genotypes	CBW 14	47.4	34.5	**41.0**	27.1
	CBW 23	44.1	34.5	**39.3**	21.9
	CBW 39	47.4	30.1	**38.7**	36.6
	DBW 39	50.0	34.5	**42.3**	30.9
	DBW 44	46.2	35.1	**40.6**	24.1
	DBW 77	50.3	31.3	**40.8**	37.8
	HD 2789	50.4	29.2	**39.8**	42.1
	HD 2888	46.1	32.8	**39.4**	28.8
	HD 2923	46.5	29.5	**38.0**	36.4
	HI 1544	43.2	28.3	**35.8**	34.4
	HUW 541	43.2	24.3	**33.7**	43.8
	HW 2013	48.0	30.4	**39.2**	36.7
	HW 2021	48.2	36.5	**42.4**	24.2
	HW 2022	44.0	24.9	**34.4**	43.5
	HW 2034	50.7	33.2	**42.0**	34.4
	HW 2036	48.2	38.3	**43.3**	20.5
	HW 2040	45.3	26.3	**35.8**	42.1
	HW 2059	40.2	23.5	**31.9**	41.5
	HW 2060	42.6	30.1	**36.3**	29.4
	HW 2078	47.1	33.0	**40.1**	30.0
	HW 2083	45.3	25.6	**35.5**	43.5
	HW 4010	47.1	36.6	**41.8**	22.2
	HW 4022	50.5	40.0	**45.2**	20.7
	HW 4024	49.4	38.0	**43.7**	23.0
	HW 4026	46.1	30.0	**38.1**	35.0
	HW 4029	46.1	29.3	**37.7**	36.4
	HW 4055	45.1	35.0	**40.1**	22.4
	HW 4059	43.0	24.3	**33.7**	43.4
	HW 4207	46.5	27.5	**37.0**	40.7
	HW 5209	51.1	32.0	**41.5**	37.4
	LOK 64	42.1	26.5	**34.3**	36.9
	PBW 142	39.4	22.0	**30.7**	44.1
	PBW 550	47.3	34.5	**40.9**	26.9
	PBW 555	46.5	29.1	**37.8**	37.4
	PBW 590	48.1	34.3	**41.2**	28.6
	Mean	**46.4**	**31.0**	**38.7**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**2.302**		**0.823**	
	Treatment (T)	**0.55**		**0.197**	
	Interaction G x T	**3.256**		**1.163**	

Table 4.1.7 Effect of water deficit stress on test weight (1000 seed weight; g) in wheat genotypes at reproductive stage

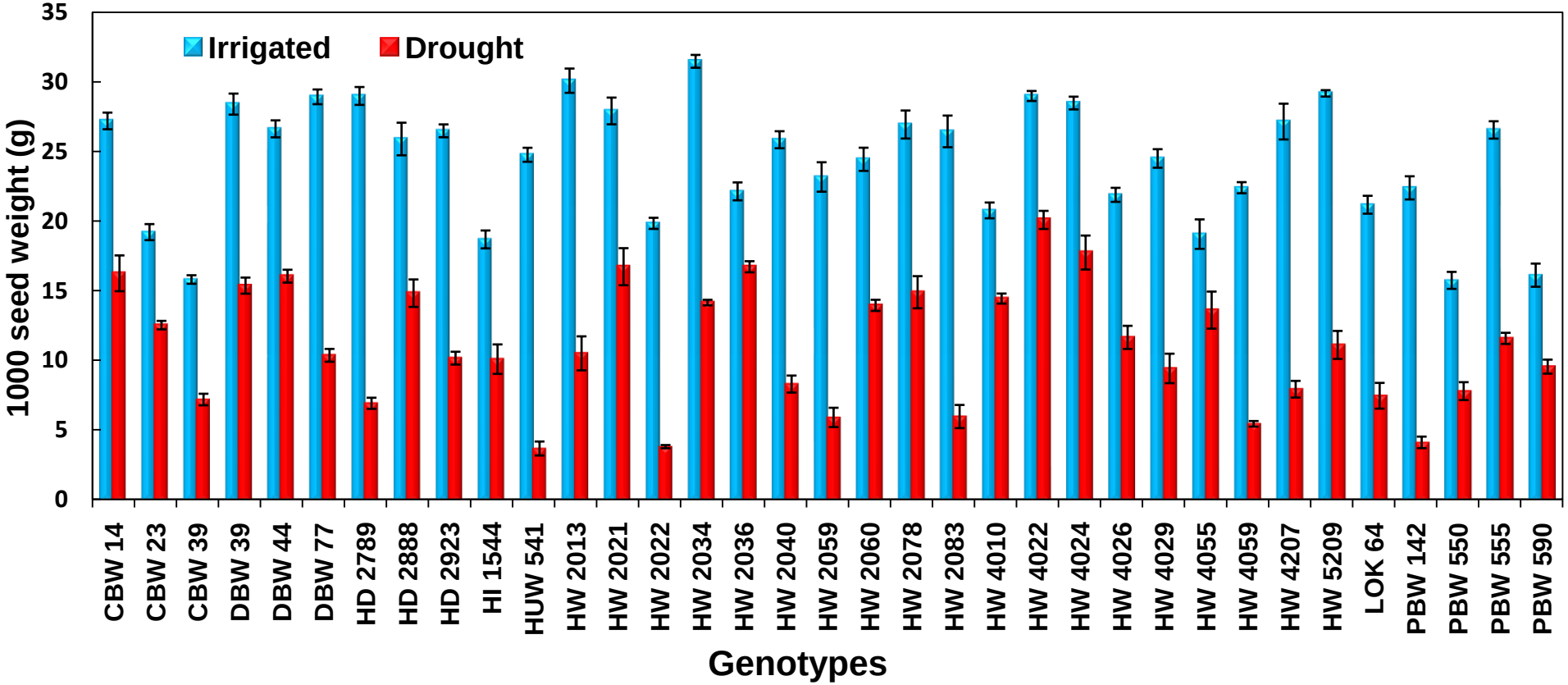


Fig. 4.1.7 Effect of water deficit stress on test weight (1000 seed weight; g) in wheat genotypes at reproductive stage

Harvest Index (HI; %)					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	CBW 14	45.8	34.6	**40.2**	24.4
	CBW 23	42.6	35.0	**38.8**	17.8
	CBW 39	40.7	26.6	**33.6**	34.7
	DBW 39	40.1	27.5	**33.8**	31.5
	DBW 44	43.9	32.7	**38.3**	25.5
	DBW 77	41.7	26.4	**34.0**	36.7
	HD 2789	44.0	25.7	**34.9**	41.6
	HD 2888	48.2	30.4	**39.3**	36.9
	HD 2923	44.7	22.2	**33.4**	50.4
	HI 1544	42.4	25.3	**33.9**	40.2
	HUW 541	41.4	18.6	**30.0**	54.9
	HW 2013	42.6	21.5	**32.0**	49.6
	HW 2021	42.0	32.5	**37.2**	22.7
	HW 2022	43.6	19.4	**31.5**	55.5
	HW 2034	41.9	21.3	**31.6**	49.2
	HW 2036	37.1	29.9	**33.5**	19.6
	HW 2040	42.7	26.9	**34.8**	37.0
	HW 2059	41.4	22.3	**31.8**	46.0
	HW 2060	42.9	33.2	**38.0**	22.7
	HW 2078	44.4	30.3	**37.3**	31.8
	HW 2083	42.6	21.6	**32.1**	49.3
	HW 4010	37.0	30.2	**33.6**	18.4
	HW 4022	46.0	33.6	**39.8**	26.9
	HW 4024	43.8	33.0	**38.4**	24.6
	HW 4026	41.7	24.6	**33.1**	41.1
	HW 4029	40.8	23.1	**32.0**	43.3
	HW 4055	42.6	34.8	**38.7**	18.3
	HW 4059	38.4	16.9	**27.7**	56.1
	HW 4207	40.7	30.8	**35.7**	24.3
	HW 5209	44.6	26.7	**35.7**	40.1
	LOK 64	42.7	27.7	**35.2**	35.3
	PBW 142	42.6	18.6	**30.6**	56.3
	PBW 550	41.0	23.6	**32.3**	42.6
	PBW 555	40.8	25.9	**33.4**	36.5
	PBW 590	41.3	27.2	**34.3**	34.1
	Mean	**42.3**	**26.9**	**34.6**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**4.473**		**1.598**	
	Treatment (T)	**1.069**		**0.382**	
	Interaction G x T	**6.326**		**2.26**	

Table 4.1.8 Effect of water deficit stress on harvest index (HI; %) in wheat genotypes at reproductive stage

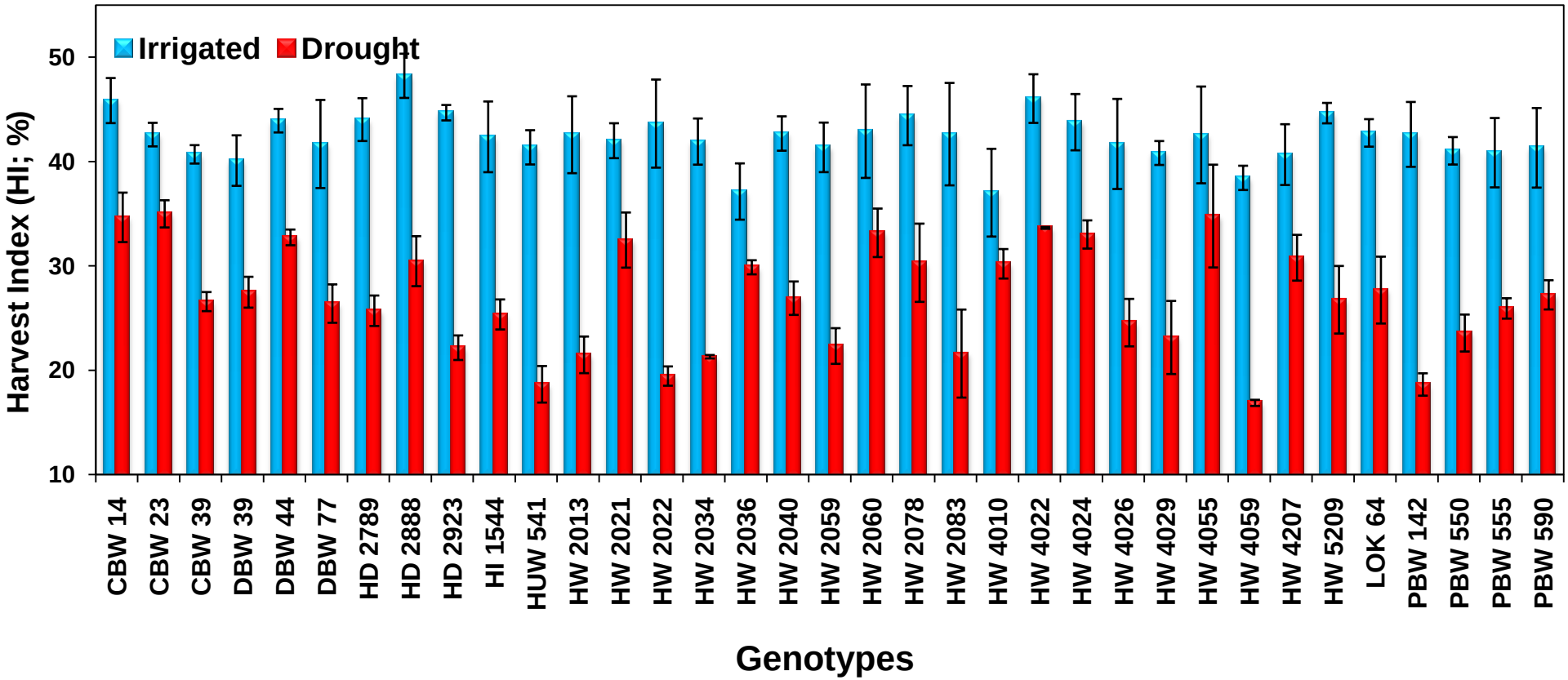


Fig. 4.1.8 Effect of water deficit stress on harvest index (HI; %) in wheat genotypes at reproductive stage

Drought Tolerance Efficiency (DTE; %)			
Genotypes	**CBW 14**	43.8	
	CBW 23	48.5	
	CBW 39	33.5	
	DBW 39	38.9	
	DBW 44	44.4	
	DBW 77	25.9	
	HD 2789	17.7	
	HD 2888	40.7	
	HD 2923	27.3	
	HI 1544	37.6	
	HUW 541	11.3	
	HW 2013	26.7	
	HW 2021	45.2	
	HW 2022	14.0	
	HW 2034	32.4	
	HW 2036	54.6	
	HW 2040	22.7	
	HW 2059	19.6	
	HW 2060	41.8	
	HW 2078	39.9	
	HW 2083	16.4	
	HW 4010	49.9	
	HW 4022	59.5	
	HW 4024	46.8	
	HW 4026	38.9	
	HW 4029	28.6	
	HW 4055	52.1	
	HW 4059	17.9	
	HW 4207	21.7	
	HW 5209	27.8	
	LOK 64	25.8	
	PBW 142	13.6	
	PBW 550	35.1	
	PBW 555	31.2	
	PBW 590	42.0	
	Mean	**33.5**	
Statistics		**CD at 5 %**	**SEM**
	Genotype (G)	**9.688**	**3.428**

Table 4.1.9 Effect of water deficit stress on drought tolerance efficiency (DTE; %) in wheat genotypes at reproductive stage

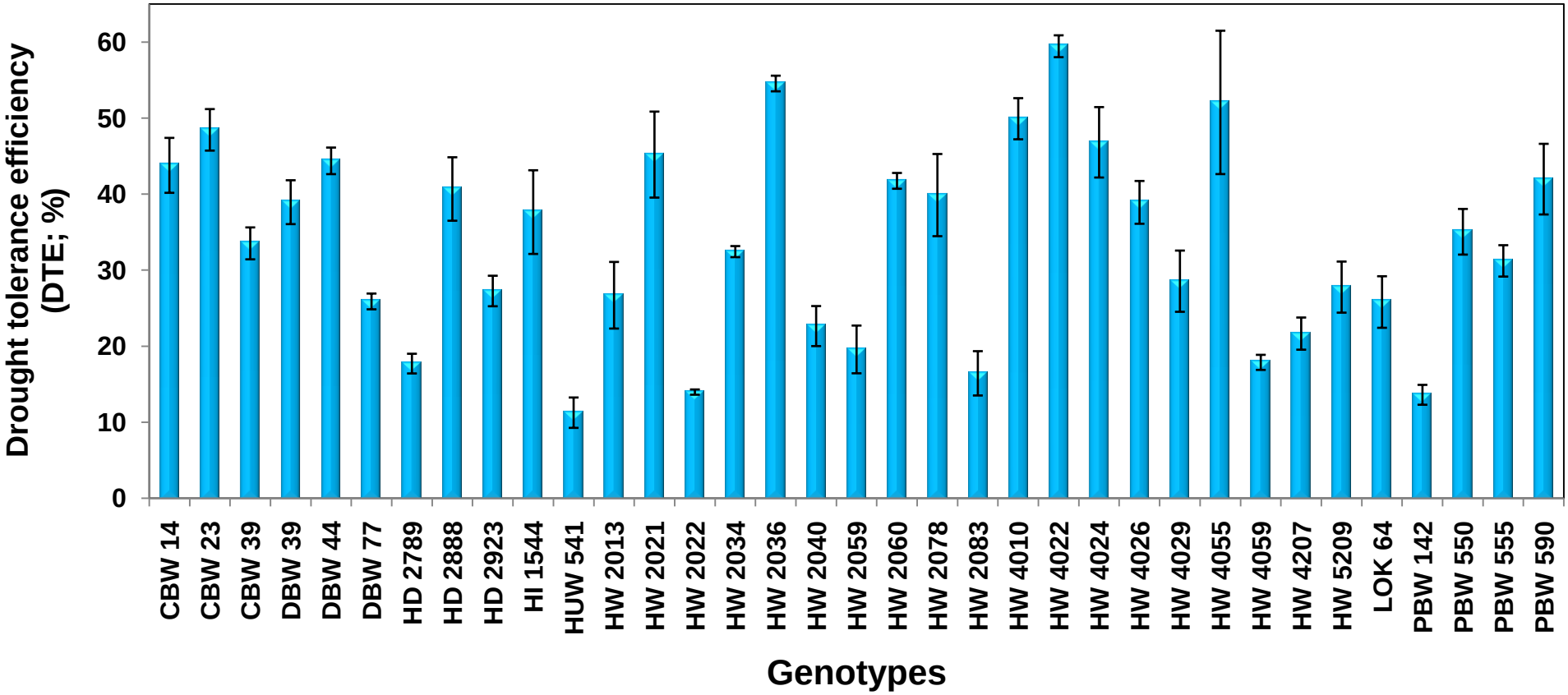


Fig. 4.1.9 Effect of water deficit stress on drought tolerance efficiency (DTE; %) in wheat genotypes at reproductive stage

De-Simone *et al*., 2014) where stay-green mutant with delayed leaf senescence able to maintained their higher photosynthesis characteristic under water limited conditions.

In the global agriculture, wheat production and yield represent a crucial factor during drought stress. Grain yield is frequently used in wheat crop as the main selection criteria for drought tolerance in which genotypes are evaluated for either high yield potential or stable performance under water deficit stress (Mohammadi *et al*., 2013; Yasir *et al*., 2013). A strong association of the SG traits with grain and biomass yield under drought stress and using delayed senescence as a rapid screening tool for drought tolerance especially for large number of samples in breeding programs will help in targeting and developing drought tolerant genotypes (Muchero *et al*., 2013). To detect and improve such traits in field trials will help us in better understanding of the consequences of the drought stress on yield (Kipp *et al*., 2014). In the present study, a general reduction in yield on account of water deficit stress was observed in all genotypes. Significant variations were observed among genotypes for yield, test weight, HI % and DTE %. Tolerant SG genotypes (HW 4022, HW 2036, HW 4010, HW 4055, CBW 23 and HW 4024) were superior in all the yield and related parameters with high DTE % while, drought susceptible NSG genotypes (HW 4059, HUW 541, PBW 142, HW 2022, HW 2083 and HD 2789) showed reduction in all yield attributes studied. The current findings are in parallelism with Mohammadi *et al*. (2011) in wheat and Puri *et al*. (2013) in rice where minimum yield reduction was realized in the genotypes which had the highest DTE % values in water deficit stressed environments. Similar reports seen in chickpea, where drought tolerant genotype had the highest DTE % and minimum reduction in seed yield under drought stress (Parameshwarappa and Salimath, 2010). In addition, drought tolerant pigeon pea genotype was characterized by high DTE %, lowest DSI % with improved grain yield performance (Deshmukh and Mate, 2013). Comparable findings in wheat (Derkx *et al*., 2012) that slow senescing SG genotypes with increased photosynthetic capacity during reproductive stage is a best way to maintained and improve wheat yield under drought stress. Further supportive results in wheat (Nawaz *et al*., 2013) and sorghum (Jordan *et al*., 2012; Tolk *et al*., 2013; Borrell *et al*., 2014) where, genotypes with SG character maintained higher grain weight and grain number per spike and grain yield under drought conditions. As reported by Messmer *et al*. (2011) in maize genotypes under drought stress at reproductive stage, delayed LSR can be positively correlated with higher grain yield. Thus it can suggested that SG character

with longer grain filling rate and duration under drought stress may be used for screening wheat genotypes for drought tolerance at reproductive stages. As SG genotypes exhibited better agronomic performance and produced greater grain yield per plant and could be recommended as a promising parent for wheat breeding programs.

4.1.6 Conclusion

In conclusion, the genotypes screened in this study showed significant variation for the SG traits and LSR was significantly higher in NSG genotypes than in those displaying the SG trait. The result further revealed that the genotype HW 4022 sustained functional SG traits with lower LSR as well as exhibited higher yield stability under water deficit stress owing to the existence of drought tolerance mechanism that can be utilized for increasing yield potential through the improved dry matter production under water limited conditions. In brief, the study suggested that there is a strong association between DTE % and LSR which can be consider as an essential trait for maintaining high yield stability in wheat under water limited condition.

Relative water content (RWC; %)					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	84.35	73.25	**78.80**	**13.17**
	HW 2078	85.40	70.03	**77.71**	**18.00**
	HW 4059	83.95	66.70	**75.33**	**20.55**
	Mean	**84.57**	**69.99**	77.28	
Statistics		CD at 5 %		SEM	
	Genotype (G)	N/A		1.30	
	Treatment (T)	3.308		1.06	
	Interaction G x T =	N/A		1.84	

Table 4.2.1 Effect of water deficit stress on relative water content (RWC; %) in contrasting set of three wheat genotypes at reproductive stage

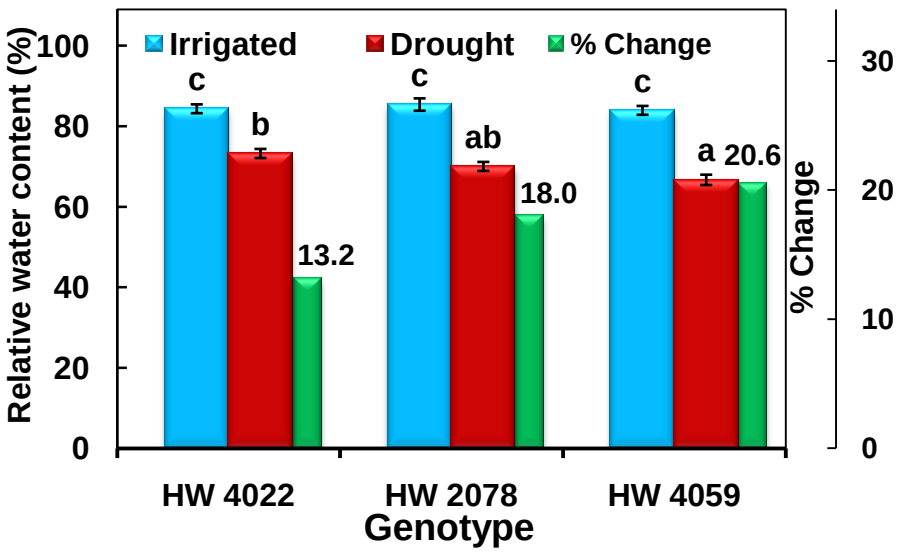


Fig. 4.2.1 Effect of water deficit stress on relative water content (RWC; %) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05).

Membrane Stability Index (MSI; %)					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	86.95	76.73	**81.84**	**11.75**
	HW 2078	87.71	73.08	**80.39**	**16.69**
	HW 4059	88.46	67.24	**77.85**	**23.98**
	Mean	**87.71**	**72.35**	**80.03**	
Statistics		CD at 5 %		SEM	
	Genotype (G)	**2.689**		**0.86**	
	Treatment (T)	**2.195**		**0.71**	
	Interaction G x T	**3.802**		**1.22**	

Table 4.2.2 Effect of water deficit stress on membrane stability index (MSI; %) in contrasting set of three wheat genotypes at reproductive stage

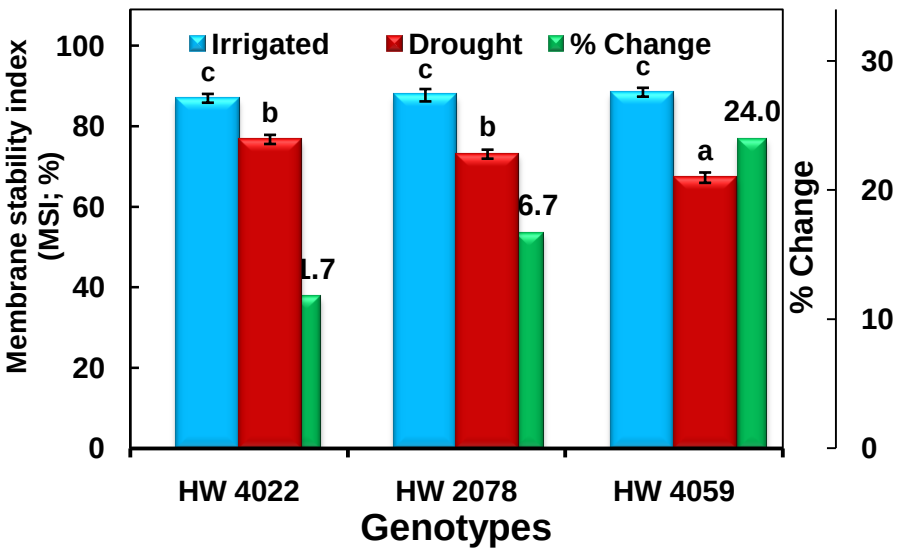


Fig. 4.2.2 Effect of water deficit stress on membrane stability index (MSI; %) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*:** MSI: Membrane stability index

H_2O_2 content (µmol H_2O_2 gDW^{-1})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	0.97	1.15	**1.06**	**18.19**
	HW 2078	1.08	1.33	**1.20**	**23.08**
	HW 4059	1.24	1.58	**1.41**	**27.28**
	Mean	**1.10**	**1.35**	**1.22**	
Statistics		CD at 5 %		SEM	
	Genotype (G)	**0.135**		**0.038**	
	Treatment (T)	**0.11**		**0.031**	
	Interaction G x T	**N/A**		**0.054**	

Table 4.2.3 Effect of water deficit stress on H_2O_2 content (µmol H_2O_2 gDW^{-1}) in contrasting set of three wheat genotypes at reproductive stage

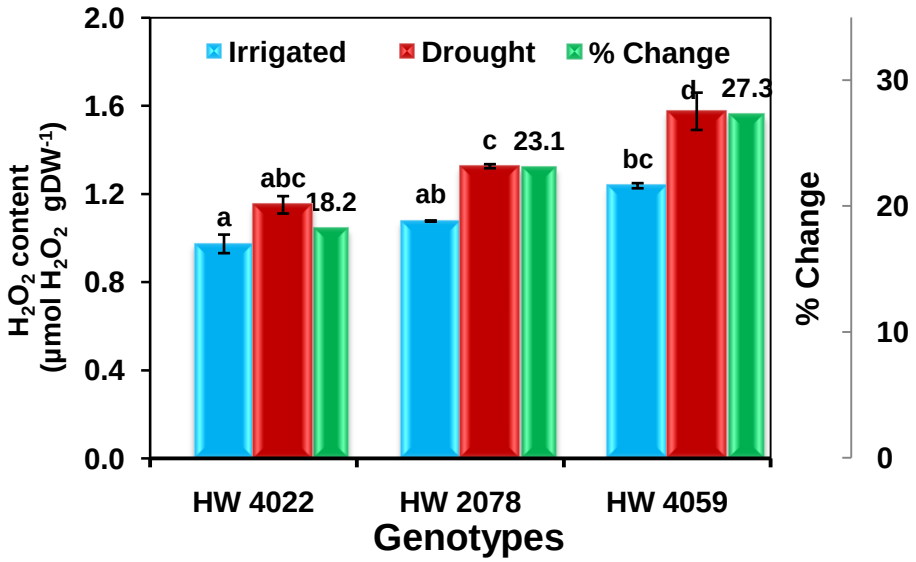


Fig. 4.2.3 Effect of water deficit stress on H_2O_2 content (µmol H_2O_2 gDW^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$)

***Abbreviation*:** DW: Dry weight, H_2O_2: Hydrogen peroxide

and **Fig. 4.2.3**). During water deficit stress susceptible genotype (HW 4059) exhibited enhancement of about 27 % in H_2O_2 content with severe membrane injury as compared to tolerant genotypes HW 4022 and HW 2078 where increase in H_2O_2 content was low ranging from 18 to 23 %.

4.2.4.4 Lipoxygenase (LOX) activity (ΔOD234 mg^{-1}protein min^{-1}) under water deficit stress

LOX activity is highly sensitive to water deficit stress condition and increased in all the genotypes under such circumstances. Under irrigated condition mean LOX activity was non-significant between the genotypes and reverse trend was observed under water deficit stress condition (**Table 4.2.4** and **Fig. 4.2.4**). Tolerant wheat genotype HW 4022 showed comparatively lower LOX activity than the other two genotypes (HW 2078 and HW 4059) under water deficit stress.

4.2.4.5 Total protease activity under water deficit stress

Significant genotypic differences were not observed for protease activities under both water regimes (**Table 4.2.5** and **Fig. 4.2.5**). Beside this, the tolerant genotypes HW 4022 and HW 2078 showed slight increase in protease activity ranging from 2.8 to 3 % as compared to their control while, HW 4059 showed comparatively higher increase of 4.7 % under water deficit stress condition.

4.2.4.6 Lipid peroxidation under water deficit stress

It was observed that effect of water deficit stress on lipid peroxidation in terms of Thiobarbituric Acid Reactive Substances (TBARS) content was higher in all three wheat genotypes (**Table 4.2.6** and **Fig. 4.2.6**). The TBARS content increased significantly between the contrasting genotypes under water deficit stress condition. Moisture stress induced increases in TBARS content was lesser in HW 4022 ranging from 30 to 32 %. This increase was significantly higher in susceptible genotype HW 4059 of about 42 % under water deficit stress.

4.2.4.7 Proline content under water deficit stress

Imposition of water deficit stress led to accumulation of free proline in all the studied genotypes (**Table 4.2.7** and **Fig. 4.2.7**). There was significantly greater enhancement in proline content in case of HW 4022 and HW 2078 under water deficit

stress whereas, HW 4059 showed lesser increment. Genotypes HW 4022 and HW 2078 showed approximately 16 and 13 fold increase respectively, in proline content under water deficit stress in comparison to HW 4059 which showed 6 fold increment in comparison to control condition.

4.2.4.8 Antioxidant enzyme activities under water deficit stress

In this study, activities of SOD, CAT, APOX, POX and GR, increased significantly in all three genotypes after 10 days of drought stress as compared to normal irrigated condition. Significant difference was found for the SOD, CAT, APOX, POX, GR activity among the genotypes under water deficit stress condition while, the genotypes varies non-significantly in case of enzyme activities under irrigated condition (**Fig. 4.2.14**). Maximum SOD activity was obtained in HW 4022 followed by HW 2078 after water stress treatment (**Table 4.2.8** and **Fig. 4.2.9**). There was tremendous increase in CAT activity in HW 4022 and HW 2078 under water deficit stress as compared to control while; in case of HW 4059 slight increment was observed under water deficit stress condition (**Table 4.2.9** and **Fig. 4.2.10**). Maximum activity of GR was recorded in HW 4022 followed by HW 2078 and HW 4059 under water deficit stress condition (**Table 4.2.10** and **Fig. 4.2.11**). Under water deficit stress POX activity in HW 4059 was significantly lower than in HW 4022 and HW 2078 (**Table 4.2.11** and **Fig. 4.2.12**). HW 4059 recorded lesser addition, while HW 4022 and HW 2078 showed significantly higher increase in APOX activity under moisture deficit stress (**Table 4.2.12** and **Fig. 4.2.13**). Thus HW 4022 showed the higher antioxidant enzymes activity followed by HW 2078 as compared to susceptible genotype (HW 4059) under water deficit stress condition. Our results thus revealed that HW 4022, which are more drought tolerant than the other two genotypes (HW 2078 and HW 4059) and maintained its drought tolerance efficiency through increased antioxidant enzymatic activity for better scavenging system against ROS as presented in **Fig. 4.2.8** and **Fig. 4.2.14** under drought stress condition.

4.2.5 Discussion

Drought stress severely affects wheat growth and development resulting in tremendous yield losses. One of the unavoidable consequences of drought stress is enhanced reactive oxygen species (ROS) production. ROS levels increases under drought stress with higher lipid peroxidation and protein oxidation resulting in

LOX activity (µmole linoleic acid oxidised mg protein^{-1} min^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**HW 4022**	1.7	2.5	**2.1**	**49.38**
	HW 2078	1.8	2.7	**2.3**	**50.77**
	HW 4059	1.7	2.9	**2.3**	**73.39**
	Mean	**1.7**	**2.7**	2.2	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**0.129**		**0.045**	
	Treatment (T)	**0.105**		**0.036**	
	Interaction G x T	**0.182**		**0.063**	

Table 4.2.4 Effect of water deficit stress on lipoxygenase (LOX) activity (µmol linoleic acid oxidised mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes grown at reproductive stage

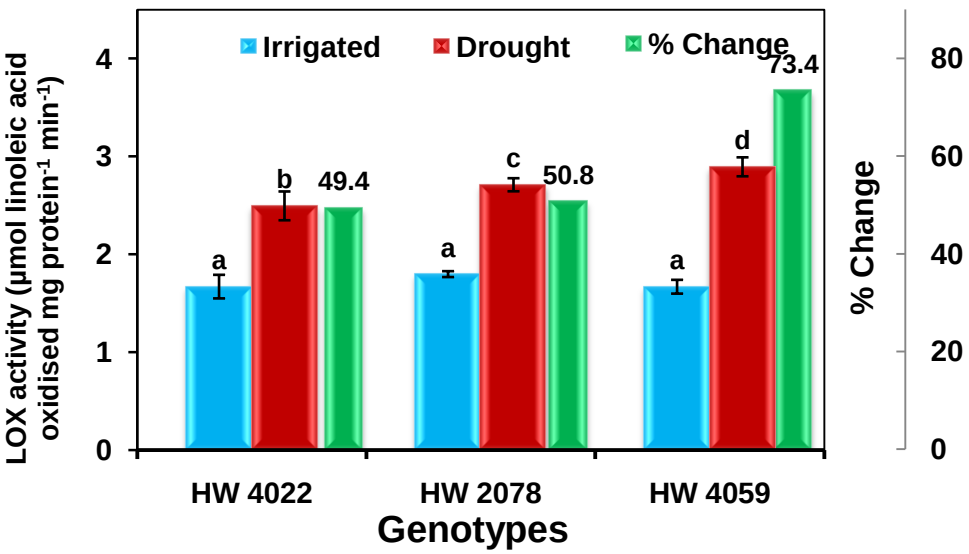


Fig. 4.2.4 Effect of water deficit stress on lipoxygenase (LOX) activity (µmole linoleic acid oxidised mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*:** LOX: Lipoxygenase

Protease activity (µmol mg protein^{-1} min^{-1})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	95.4	98.2	**96.8**	**2.91**
	HW 2078	95.8	98.9	**97.4**	**3.24**
	HW 4059	95.4	100.1	**97.8**	**4.94**
	Mean	**95.5**	**99.1**	**97.3**	
Statistics		CD at 5 %		SEM	
	Genotype (G)	N/A		0.459	
	Treatment (T)	1.321		0.374	
	Interaction G x T	N/A		0.648	

Table 4.2.5 Effect of water deficit stress on protease activity (µmol mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage

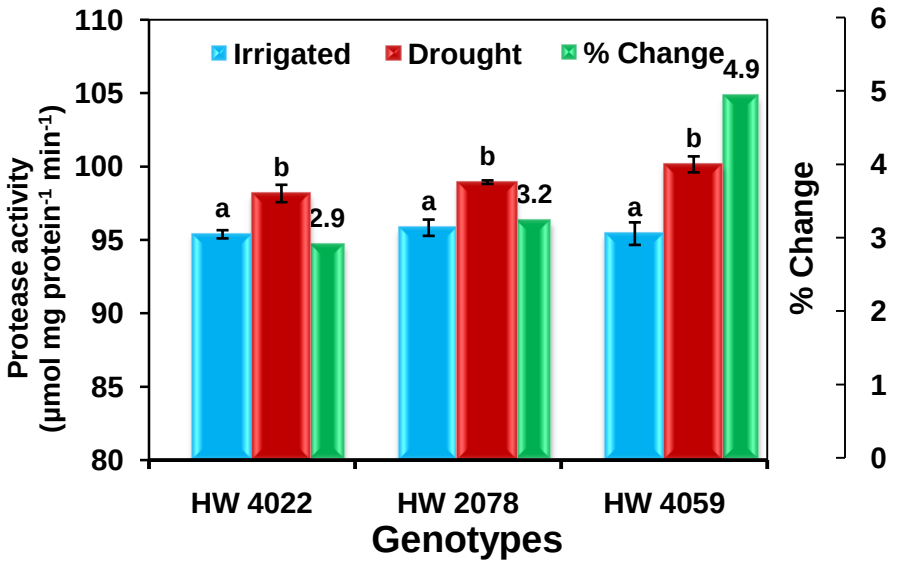


Fig. 4.2.5 Effect of water deficit stress on protease activity (µmol mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

Lipid peroxidation (nmol TBARS gDW^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**HW 4022**	954.13	1242.83	**1098.48**	**30.26**
	HW 2078	1011.53	1349.70	**1180.61**	**33.43**
	HW 4059	940.11	1430.49	**1185.30**	**52.16**
	Mean	**968.59**	**1341.01**	**1154.80**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**N/A**		**32.30**	
	Treatment (T)	**82.158**		**26.37**	
	Interaction G x T	**N/A**		**45.68**	

Table 4.2.6 Effect of water deficit stress on lipid peroxidation (nmol TBARS gDW^{-1}) in contrasting set of three wheat genotypes at reproductive stage

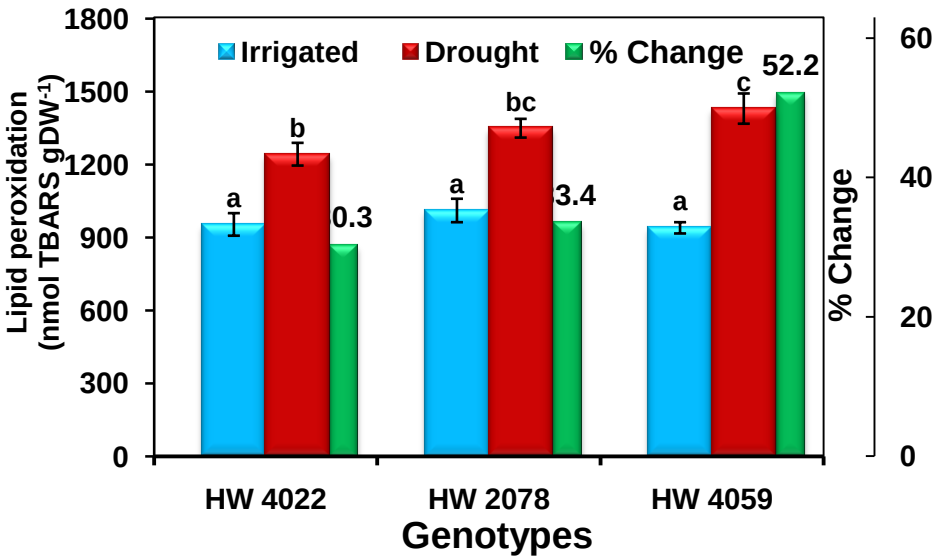


Fig. 4.2.6 Effect of water deficit stress on lipid peroxidation (nmol TBARS gDW^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$)

***Abbreviation*:** TBARS: Thiobarbituric acid reactive substances

Proline content (µg proline DW^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**HW 4022**	213.1	3412.8	**1813.0**	**1501.54**
	HW 2078	215.6	2851.2	**1533.4**	**1222.24**
	HW 4059	260.1	1705.7	**982.9**	**555.68**
	Mean	**229.6**	**2656.6**	**1443.1**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**120.587**		**34.183**	
	Treatment (T)	**98.459**		**27.91**	
	Interaction G x T	**170.536**		**48.342**	

Table 4.2.7 Effect of water deficit stress on proline content (µg proline DW^{-1}) in contrasting set of three wheat genotypes at reproductive stage

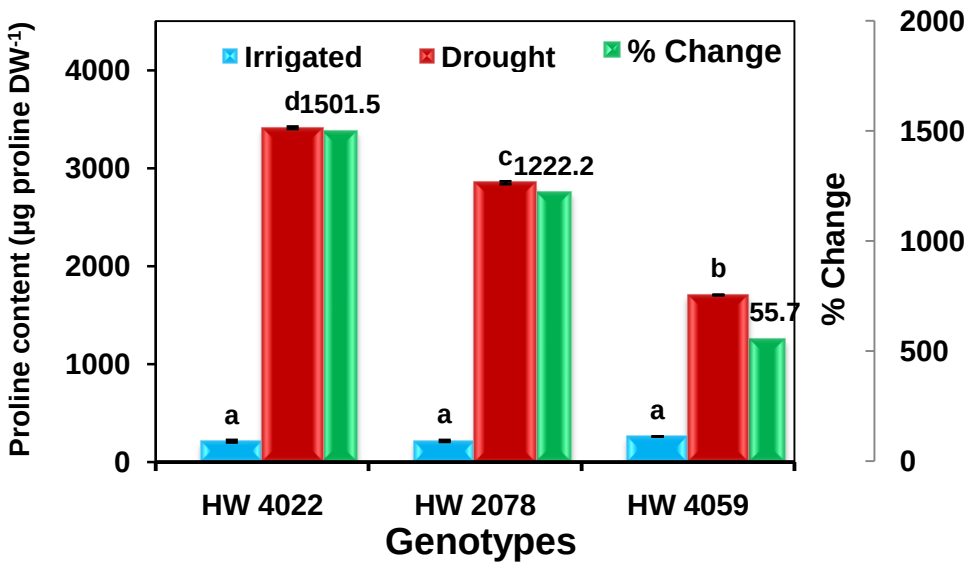


Fig. 4.2.7 Effect of water deficit stress on proline content (µg proline DW^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$)

***Abbreviation*:** DW: Dry weight

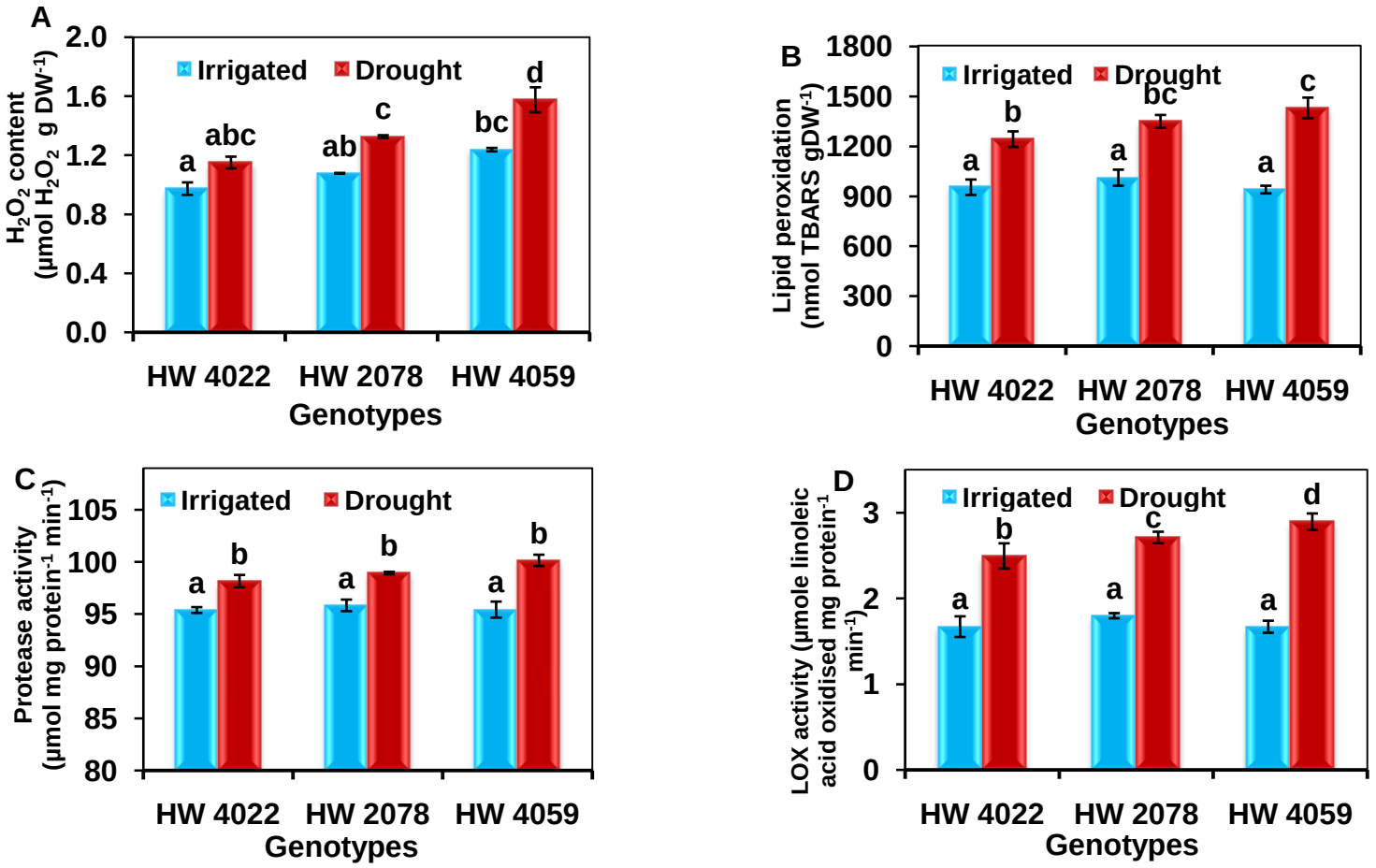


Fig. 4.2.8 Effect of water deficit stress on oxidative indicators (H_2O_2 content, lipid peroxidation, protease activity and LOX activity) in wheat genotypes at reproductive stage. Error bar indicates ±SE of mean (n=3)

Different panels showing various parameters; A) Hydrogen peroxide content B) Lipid peroxidation C) Protease activity D) Lipoxygenase activity

Superoxide dismutase (SOD) activity (unit mg protein^{-1} min^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**HW 4022**	8.15	12.39	**10.27**	**52.04**
	HW 2078	7.33	10.39	**8.86**	**41.82**
	HW 4059	6.53	8.14	**7.33**	**24.60**
	Mean	**7.34**	**10.31**	**8.82**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**1.161**		**0.329**	
	Treatment (T)	**0.948**		**0.269**	
	Interaction G x T	**N/A**		**0.465**	

Table 4.2.8 Effect of water deficit stress on superoxide dismutase (SOD) activity (unit mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage

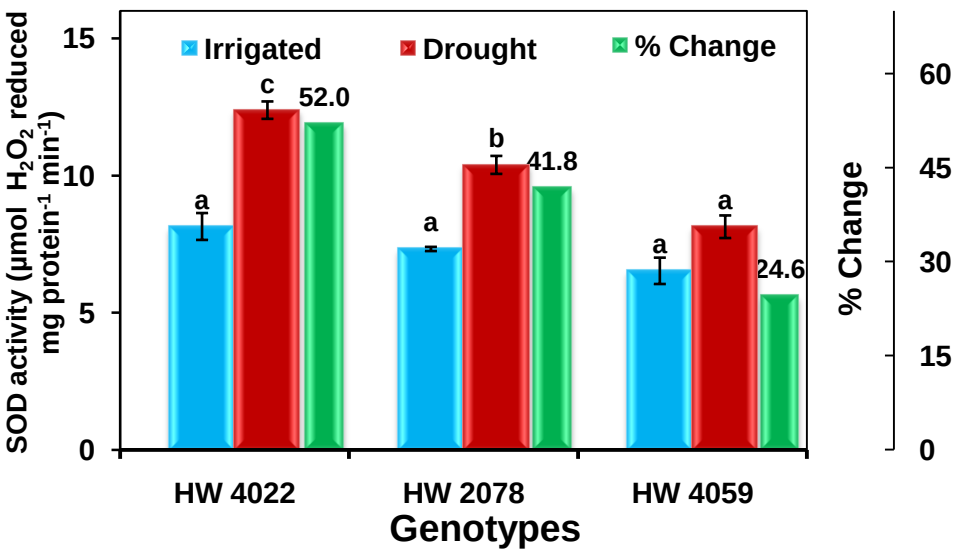


Fig. 4.2.9 Effect of water deficit stress on superoxide dismutase (SOD) activity (unit mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*:** H_2O_2: Hydrogen peroxide, SOD: Superoxide dismutase

Catalase (CAT) activity (µmol H_2O_2 reduced mg protein^{-1} min^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**HW 4022**	0.85	5.63	**3.24**	**561.27**
	HW 2078	0.97	4.76	**2.86**	**390.35**
	HW 4059	0.83	1.75	**1.29**	**112.27**
	Mean	**0.88**	**4.05**	**2.47**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**0.512**		**0.145**	
	Treatment (T)	**0.418**		**0.118**	
	Interaction G x T	**0.724**		**0.205**	

Table 4.2.9 Effect of water deficit stress on catalase (CAT) activity (µmol H_2O_2 reduced mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage

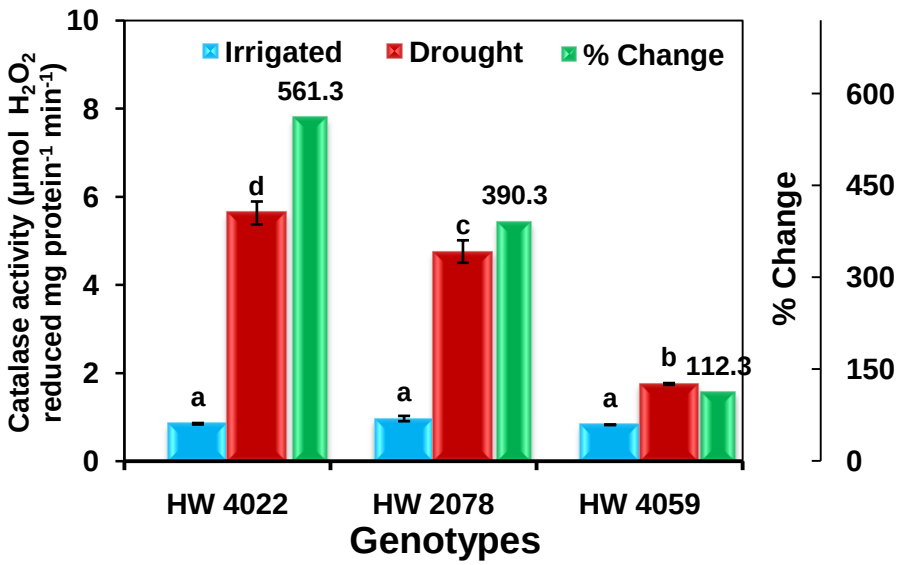


Fig. 4.2.10 Effect of water deficit stress on catalase activity (µmol H_2O_2 reduced mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*:** H_2O_2: Hydrogen peroxide, CAT: Catalase

Glutathione reductase (GR) activity (µmol glutathione reduced mg protein^{-1} min^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**HW 4022**	660.0	783.2	**721.6**	**18.67**
	HW 2078	674.2	748.3	**711.3**	**10.99**
	HW 4059	546.8	617.4	**582.1**	**12.91**
	Mean	**627.0**	**716.3**	**671.7**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**60.686**		**17.202**	
	Treatment (T)	**49.55**		**14.046**	
	Interaction G x T	**N/A**		**24.328**	

Table 4.2.10 Effect of water deficit stress on glutathione reductase (GR) activity (µmol glutathione reduced mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage

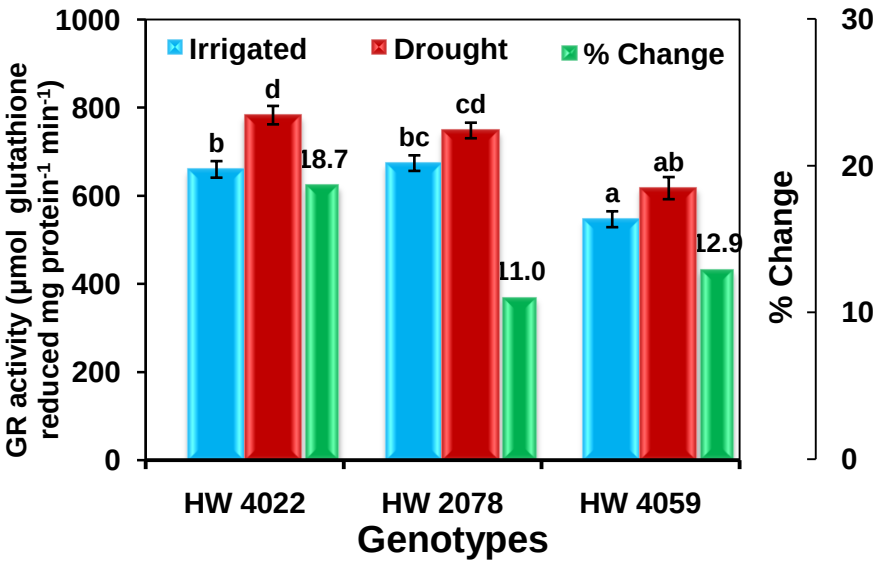


Fig. 4.2.11 Effect of water deficit stress on glutathione reductase (GR) activity (µmol glutathione reduced mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*:** GR: Glutathione reductase

Peroxidase activity (µmol TG formed mg protein^{-1} min^{-1})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	132.5	290.6	**211.5**	**119.43**
	HW 2078	148.0	232.1	**190.1**	**56.87**
	HW 4059	121.3	166.1	**143.7**	**36.93**
	Mean	**133.9**	**229.6**	**181.8**	
Statistics		CD at 5 %		SEM	
	Genotype (G)	32.426		9.192	
	Treatment (T)	26.476		7.505	
	Interaction G x T	45.857		12.999	

Table 4.2.11 Effect of water deficit stress on peroxidase (POX) activity (µmol TG formed mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage

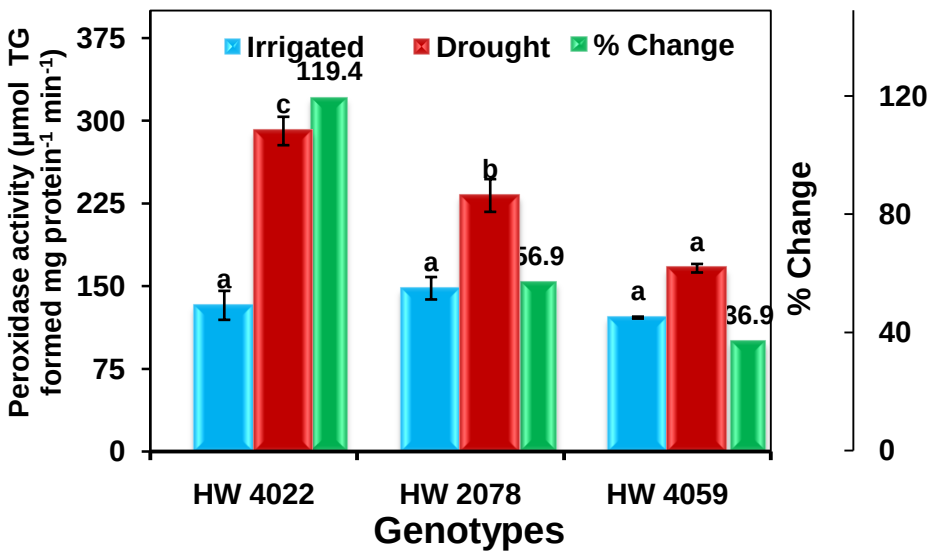


Fig. 4.2.12 Effect of water deficit stress on peroxidase (POX) activity (µmol TG formed mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*:** TG: Tetraguaiacol, POX: Peroxidase

Ascorbate peroxidase (APOX) activity (μmol H_2O_2 reduced mg protein^{-1} min^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**HW 4022**	9.94	21.74	**15.84**	**118.65**
	HW 2078	8.53	17.28	**12.91**	**102.52**
	HW 4059	8.51	13.65	**11.08**	**60.43**
	Mean	**8.99**	**17.56**	**13.28**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**2.532**		**0.718**	
	Treatment (T)	**2.067**		**0.586**	
	Interaction G x T	**3.581**		**1.015**	

Table 4.2.12 Effect of water deficit stress on ascorbate peroxidase (APOX) activity (μmol H_2O_2 reduced mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage

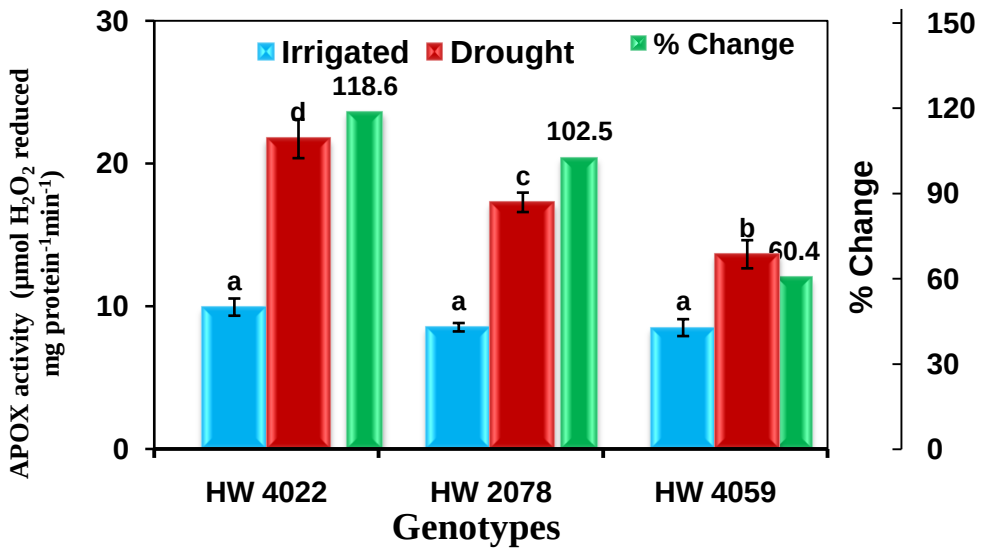


Fig. 4.2.13 Effect of water deficit stress on ascorbate peroxidase (APOX) activity (μmol H_2O_2 reduced mg protein^{-1} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*:** H_2O_2: Hydrogen peroxide, APOX: Ascorbate peroxidase

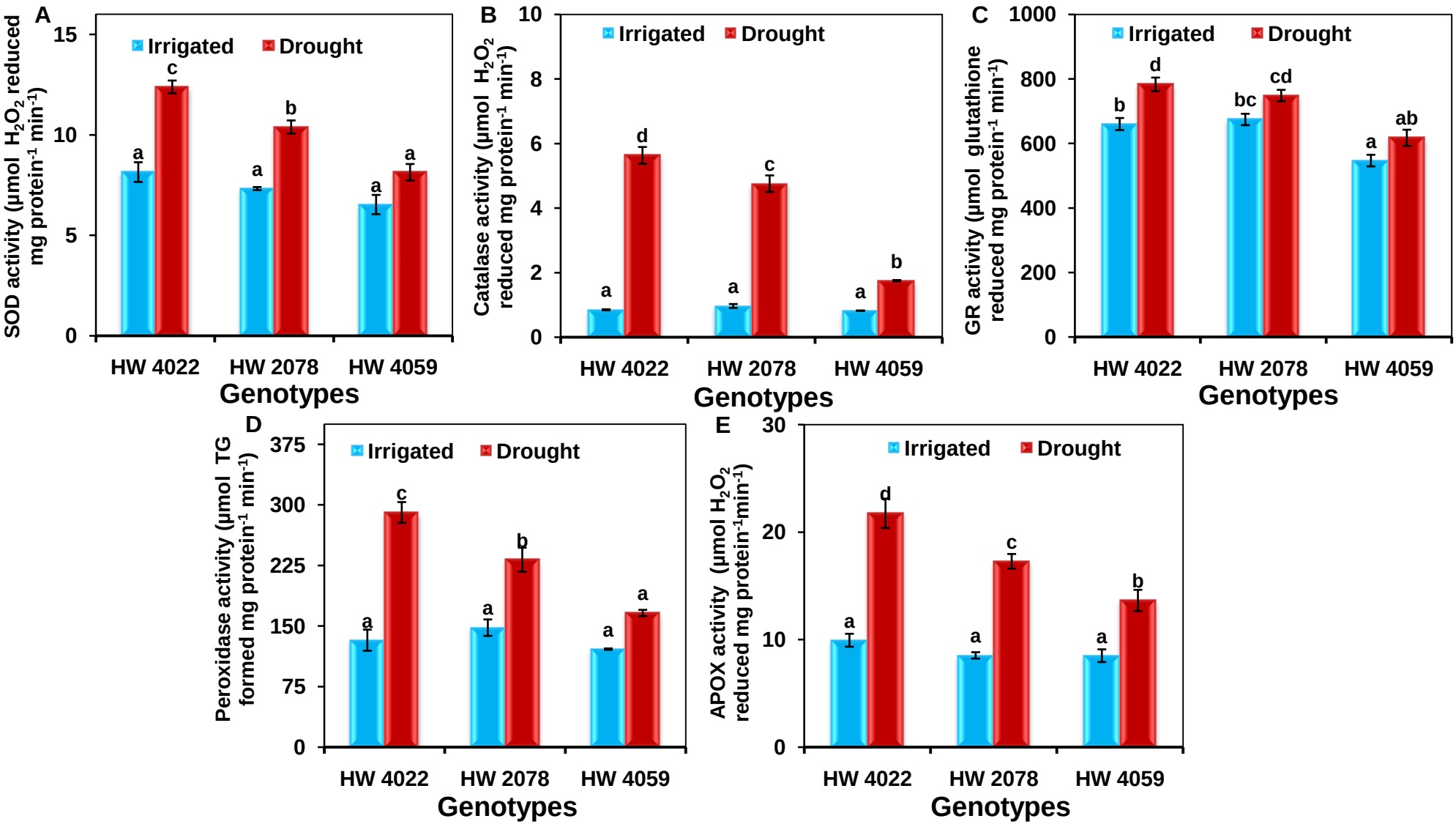


Fig. 4.2.14 Effect of water deficit stress on antioxidant enzyme activity (SOD, CAT, GR, POX and APOX) in wheat genotypes at reproductive stage. Error bar indicates ±SE of mean (n=3). Different panels showing activities of various enzymes; A) Superoxide dismutase B) Catalase C) Glutathione reductase D) Peroxidase E) Ascorbate peroxidase

cellular membrane injury and ultimately cell death (Sharma *et al*., 2011). In the present study, the response of drought tolerant (HW 4022), relatively drought tolerant (HW 2078) and drought susceptible (HW 4059) wheat genotypes to water deficit stress induced oxidative stress and antioxidant management, at reproductive stage has been evaluated. Similar studies were conducted in different crop species including major cereal crops like rice (Walter *et al*., 2013), maize (Zhang *et al*., 2014), barley (Marok *et al*., 2013), sorghum (Dykes *et al*., 2013) sugarcane (Cia *et al*., 2012) etc. Lascano *et al*. (2001) observed no clear correlation between water stress tolerance and antioxidant system behaviour between drought tolerant and susceptible wheat genotypes under field conditions. The present study showed clear difference in the participation of antioxidant defense system in the drought tolerance of wheat genotypes when subjected to water deficit stress for 10 days at reproductive stage. Our findings showed that under water deficit stress, the drought tolerant wheat genotype (HW 4022) maintained better membrane stability index (MSI; %) and higher relative water content (RWC; %) as compared to susceptible genotype (HW 4059). Maintenance of cellular membrane stability is vital for the development of drought tolerance in crop plants. Comparable findings in wheat by Farooq *et al*. (2013) reported that drought stress causes membrane injury due to drought induced oxidative stress. Further report by Chopra and Selote (2007) in wheat that during mild and severe water stress, the drought resistant wheat genotype maintained better leaf water relations in terms of relative water content and MSI as compared to susceptible genotype. Similar results reported by Chakraborty and Pradhan (2012) that lipid peroxidation increased significantly due to drought stress, which was higher in the case of drought sensitive genotypes. Our findings were further supported by Marok *et al*. (2013) in barley during water shortage drought susceptible genotype exhibited elevated levels of lipid peroxidation than drought tolerant genotype. Similar findings was reported by Hameed *et al*. (2011, 2012) in drought sensitive wheat genotype, TBARS content increased by 28 % over the control, while in drought tolerant genotype it remained unchanged under drought stress condition. Comparable findings by Singh *et al*. (2012) in wheat reported that biological membranes unstability in terms of lipid peroxidation was greater in drought sensitive genotypes than drought tolerant genotypes. Lower MSI in susceptible genotype might be due to increased H_2O_2 accumulation and lipid peroxidation as compared to tolerant genotypes (HW 4022 and HW 2078). Better membrane stability in tolerant genotype (HW 4022)

might be due to the higher activity of ROS detoxifying enzymes such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APOX), peroxidase (POX) and glutathione reductase (GR) followed by relatively drought tolerant genotype (HW 2078) under water deficit stress as compared to susceptible genotype (HW 4059). In support to our results findings by Sanchez-Rosales *et al*. (2010) reported that drought tolerant tomato genotype registered lower H_2O_2 content and associated with higher antioxidant activity. In parallel to our findings report by Sekmen *et al*. (2014) in cotton provide evidence that under combined drought and heat stress the sensitive genotypes were associated with decreased CAT and POX activities resulting in higher H_2O_2 accumulation and oxidative stress induced lipid peroxidation whereas, the tolerant genotypes were able to maintained higher SOD, CAT, POX and APOX activities alongwith enhanced proline content. Similar findings in sugarcane under water deficit stress reported by Boaretto *et al*. (2014) in agreement with our observations that improved performance of genotypes under drought stress was associated with a more efficient antioxidant system.

Protein breakdown by proteases enzyme is basic sign of the plant response to abiotic stresses such as drought. Present findings pointed out that under water deficit stress protease activity enhanced in susceptible genotype (HW 4059) in comparison to drought tolerant genotype (HW 4022). Possibly the enhancement in lipid peroxidation due to drought induced oxidative stress and increased proteolysis by enhanced protease activity may be responsible for sensitivity of drought susceptible genotype (HW 4059) under water deficit stress. Earlier reports by Simova-Stoilova *et al*. (2010) supported our result that in wheat both at transcription and activity of cysteine protease was up-regulated under drought stress. Similar findings reported in common bean under drought stress by Budic *et al*. (2013). Comparative findings by Pyngrope *et al*. (2013) in rice drought tolerant genotypes performed better under water deficit stress by maintaining higher level of antioxidative enzyme activities and lower proteolysis activity under water deficit stress condition.

Proline can act as an important osmoprotectant or signaling molecule capable of altering the expression of antioxidant-related genes involved in the plant response to environmental constraints (De-Carvalho *et al*., 2013). Proline accumulation is one of the common characteristics in many crop plants under drought stress conditions. Very high accumulation of cellular proline (above 100 % of the total amino acid pool

under stress as compared to just 5 % under the normal condition) has been reported earlier in many higher plants species due to increased synthesis and decreased degradation under the stress conditions such as salt, drought and heavy metal (Jain *et al*., 2013). In this study we observed burst in the proline accumulation under water deficit stress condition in all genotypes with highest proline content in drought tolerant wheat genotype (HW 4022). In support to our result Bowne *et al*. (2012) reported that drought stress induced the proline content in wheat genotypes. The study where further supported by Bayoumi *et al*. (2008) in wheat, superior genotypes with higher relative water content accumulated more free proline and had lower drought susceptibility index indicating that accumulated proline acts as a compatible solute regulating and reducing water loss from the cell during drought stress.

In the current study, lipoxygenase (LOX) activity was observed under water deficit stress condition in wheat genotypes. Susceptible genotype (HW 4059) showed higher degree of LOX activity compared to tolerant genotypes (HW 4022 and HW 2078). Our findings supported by De-Simone *et al*. (2014) reported that wheat genotypes with delayed leaf senescence rate maintain better redox state due to higher antioxidant activity particularly of SOD, CAT and POX with lower LOX activity. In rice, drought sensitive genotypes were associated with higher induction of endogenous H_2O_2 levels with increment in lipid peroxidation and LOX activity while, the corresponding parameters were much lesser in drought tolerant genotypes which confirms its better ROS scavenging mechanism and hence prominent drought tolerance traits as reported by Basu *et al*. (2010).

4.2.6 Conclusion

In conclusion, results from our study suggest that oxidative stress induced membrane injury due to higher H_2O_2 accumulation resulting in enhanced lipid peroxidation; protease and lipoxygenase activity is due to poor antioxidative defense capacity in susceptible genotype (HW 4059) with lower level of osmoprotectant accumulation like proline. Whereas, the drought tolerant genotypes (HW 4022 and HW 2078) maintained its membrane stability under water deficit stress condition with enhanced antioxidative enzyme capacity. In brief, the present work indicated that the tolerance of a specific wheat genotype to water deficit stress depends upon its responses of antioxidant systems, and the decline of antioxidative systems induced by

drought is associated with its sensitivity, as evident from various oxidative stress induced damages. Our findings thus suggest that coordinated activity of antioxidative enzymes play important role in scavenging ROS and mitigating water deficit stress in wheat genotypes and could serve as an indicator to screen out drought tolerant wheat genotypes.

4.3. RESEARCH PAPER III

Cloning and expression analysis of chlorophyllase gene in wheat (*Triticum aestivum* L.)

4.3.1 Abstract

The present study was conducted to determine the effect of drought stress on chlorophyll degradation in contrasting wheat genotypes differing in their drought tolerance when subjected to water deficit stress for 10 days at reproductive stage. A marked decline in chlorophyll content and photosynthetic traits was found in susceptible genotype (HW 4059) resulting in yield losses as compared to tolerant ones (HW 4022 and HW 2078). In order to understand the roles played by chlorophyllase (CHLase) and pheophorbide a oxygenase (PaO), the key enzymes in chlorophyll degradation pathway, their transcript levels were examined under drought stress. The expression analysis showed that drought/ osmotic stress induced expression of *TaCHLase1* and *TaPaO* was higher in susceptible wheat genotype as compared to tolerant ones. We also studied the effect of ethylene inducer and inhibitors on chlorophyll catabolism genes (*TaPaO* and *TaCHLase1*), ethylene inducer upregulated, while ethylene inhibitors downregulated the expression of *TaCHLase1* and *TaPaO*. In this study a novel gene of chlorophyllase, annotated as *TaCHLase1* from wheat was cloned. The complete cDNA sequence of *TaCHLase1* consisted of 1054 bp nucleotides containing an open reading frame of 960 bp encoding 319 amino acids. The encoded protein contained conserved residue such as lipase motif GXSXGG at position 143-148 and putative active site Ser145. Sequence alignment showed that *TaCHLase1* share higher degree of identity with other monocots and dicots species. These result suggested that *TaCHLase1* may be involved in drought induced leaf senescence in susceptible non stay-green genotype in response to water deficit stress.

Key words**:** *TaPaO*, *TaCHLase1*, Cloning, Gene expression, Drought, Wheat.

4.3.2 Introduction

Global climatic models predicted changing patterns of precipitation with frequent episodes of drought, which has a considerable impact on the ecosystem and agriculture (Farooq *et al*., 2014). Drought is an important limiting factor for crop production and it is becoming an increasingly severe problem in many parts of the world (Shaddad *et al*., 2011). Among cereals, wheat (*Triticum aestivum* L.) is one of the most widely cultivated species, where drought is the major environmental stress threatening its productivity worldwide. It affects wheat performance most critically during flowering and grain filling stages which results in tremendous yield losses (Pradhan *et al*., 2012). In order to minimize the effects of drought stress in crop production, drought tolerant wheat genotypes need to be developed with the maximum water use efficiency out of available water stored in the soil. An improved knowledge of the mechanisms by which water deficit affect leaf gas exchange parameters like photosynthesis, transpiration and stomatal conductance; plant growth and yield traits are necessary to develop potentially drought-resistant wheat genotypes in the field conditions (Yasir *et al*., 2013). Leaf senescence in plants is an internally programmed process, during which the photosynthetic apparatus is gradually disorganized along with chlorophyll degradation and a progressive decline in photosynthetic capability, which involves coordinated regulation of transcriptional networks, hormonal actions, and reactive oxygen species ultimately leading to cell death (Guo, 2013). The most sensitive physiological process to drought stress is photosynthetic activity and thus maintenance of the photosynthetic apparatus is an important strategy for enhancing photosynthetic activity under drought stress (Tian *et al*., 2013). If drought occurs during reproductive stage, rate of leaf senescence accelerated resulting in yield reduction of wheat (Nawaz *et al*., 2013). More than 90 % of crop biomass is derived from photosynthesis and enhancing photosynthesis at the level of the single leaf would increase yield of cereal crops like rice and wheat (Makino, 2011).

Senescence is a physiological process illustrated by an increase of catabolism and a decrease of anabolism leading to the end of cellular viability. However, leaf senescence, defined as a highly organized, energy-requiring, genetically programmed process; is the final developmental phase between maturity and death of a plant or a

plant part and a decline in photosynthetic activity (Xiao *et al*., 2014). Green leaves are essential for plant photosynthesis, while chlorophyll breakdown is one of the earliest symptoms of leaf senescence which results in subsequent loss of leaf greenness However, because of its light-absorbing properties; it is a dangerous molecule and needed to be eliminated to avoid accumulation of phototoxic pigments (Hortensteiner and Krautler, 2011) during leaf senescence under stress. Chlorophyll degradation pathway have been resolved by several researchers (Hortensteiner, 2013; Christ *et al*., 2013; Hortensteiner and Krautler, 2011; Buchert *et al*., 2011); involving a multistep pathway starting with the dephytilation reaction of chlorophyll molecules into specific chlorophyllide, catalyzed by the enzyme chlorophyllase which is a chloroplast membrane protein, followed by removal of the central Mg^{2+} ion by Mg-dechelatase. Pheophorbide a oxygenase (PaO) is another important enzyme in this pathway that catalyses the oxygenation of pheophorbide a, where the porphyrin macrocycle of pheophorbide is oxygenolytically cleaved by the joint action of PaO and red chlorophyll catabolite (RCC) reductase. The product, a primary fluorescent catabolite is further converted to the final non-fluorescent chlorophyll catabolites (NCCs) and transported to vacuole (Shemer *et al*., 2008; Sakuraba *et al*., 2012), as shown in **Fig. 2.2** It is well known that the initiation of leaf senescence is under hormonal control but the mechanisms triggered by hormones are not fully understood. During senescence process levels of many plant hormones are altered and genes involved in biosynthesis and signaling of these were also differentially expressed (Penfold and Buchanan-Wollaston, 2014). Phytohormones like ethylene, jasmonic acid, abscisic acid and salicylic acid promote leaf senescence, whereas cytokinin, auxin, gibberellic acid, nitric oxide, and polyamines suppress the process (Jibran *et al*., 2013). Recently, a network analysis revealed that leaf senescence can be regulated due to altered phytohormone signaling (Li *et al*., 2012). Previous reports have also shown that phytohormones like ethylene accelerate the process of leaf senescence and mutants in ethylene biosynthesis, perception or signaling showed altered rates of senescence (Zhang and Zhou, 2013).

Hence, delaying leaf senescence has become a desirable trait in wheat genotypes under drought stress. The aim of the current research is identifying and characterizing the genes involved in chlorophyll degradation pathway and understanding the mechanisms regulating the process of drought tolerance in wheat.

To test this hypothesis, we performed semi-quantitative expression analysis of genes encoding chlorophyll degradation enzymes *viz.* chlorophyllase (*TaCHLase1*) and pheophorbidae a oxygenase (*TaPaO*) in presence of ethylene inducers, biosynthesis and signaling inhibitors. In addition, as little is known about CHLase in wheat, we cloned *TaCHLase1* gene from wheat. Results from cloning and expression analysis experiments provide evidences that drought tolerant stay green wheat genotype maintained the lower expression of chlorophyll degrading enzyme (*TaCHLase1* and *TaPaO*) and this information can be a promising initiative for deciphering the function of chlorophyllase gene in wheat under drought stress in future.

4.3.3 Materials and methods

4.3.3.1 Plant materials and treatments

From our preliminary experiment conducted in pot culture condition, we selected three wheat genotypes based on their drought tolerance efficiency *viz*., HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). These genotypes were grown with recommended package of practices and subjected to water deficit stress at 50 % anthesis stage by with-holding irrigation for 10 days. Scheduled routine of irrigation was practiced for control plants throughout the crop growth period. For biochemical and molecular analysis flag leaf sampling was done from both control (RWC; 80-85 %) and stressed (RWC; 65-75 %) plants. For *in vitro* analysis, seeds of the above mentioned genotypes were surface sterilized with 2.5 % sodium hypochlorite for 15 min and then washed 4-5 times with distilled water. These seeds were then germinated in petridishes with wetted germination paper at 25 ^{0}C under dark. After 48 h of incubation, uniformly germinated seeds were selected and raised in 50.0 mL test tubes containing half-strength Hoagland solution. The hydroponically raised seedlings were grown for 10 days in growth chamber at 25 ^{0}C day/ 20 ^{0}C night, light intensity 1200 $\mu molm^{-2}s^{-1}$ and 90 % relative humidity. Osmotic stress was imposed to 10 days old seedlings using 20 % polyethylene glycol (PEG-6000) solution giving osmotic potential of -4.91 MPa (Michael and Kaufmann, 1972), where double distilled water served as controls. They were treated with ethylene inducer (ethrel; 10 ppm), ethylene biosynthesis inhibitor (aminoethoxy vinylglycine; AVG, 2 ppm) and ethylene signaling inhibitor (1-methyl

cyclopropene; 1-MCP, 10 ppm). The treatment combinations given to 10 days old seedlings are described in **Table 3.2.**

4.3.3.2 Observations recorded

4.3.3.2.1 Photosynthesis characteristics

4.3.3.2.1.1 Estimation of chlorophyll and carotenoid contents

Chlorophyll and carotenoid contents were estimated by extracting 0.5 g fresh leaf sample in 10.0 mL dimethylsulfoxide (DMSO) (Hiscox and Israelstam, 1979). Samples were heated in an incubator at 65 ^{0}C for 4 h and then after cooling to room temperature; the absorbance of the extracts was recorded at 470, 645 and 663 nm. Chlorophyll content was calculated as per Arnon (1949), while carotenoid content was determined by following the formula given by Lichtenthaler and Welburn (1983). The values thus obtained were in µg/ mL of extract (solvent). Values in mg/ g fresh weight were obtained by multiplying the above values with V/ W x 1000, where V is volume of extract and W is dry weight of sample.

Total chlorophyll content (mg gDW^{-1}) = $[20.2 \times A_{645} + 8.02 \times A_{663}] \times (V/W) \times 1000$

Total carotenoid content (mg gDW^{-1}) = $[A_{470} + (0.114 \times A_{663}) - (0.638\text{-}A_{645})] \times (V/W) \times 1000$

4.3.3.2.1.2 Photosynthesis, transpiration and stomatal conductance

Leaves were categorized into green and yellow/ dead leaves, and the rate of photosynthesis, transpiration and stomatal conductance was measured using portable Infrared Gas Analyzer (IRGA), LI-6400XT Model (Li-COR Ltd., Lincoln, Nebraska, USA) by operating the IRGA in the closed mode between 10.00-11.00 a.m. when relative humidity, temperature, photosynthetic photon flux density and CO_2 conc. ranged from 50-60 %, 30 to 35 ^{0}C, 1200 µmol $m^{-2}s^{-1}$ and 350 to 360 µmol mol^{-1}, respectively. Fifteen flag leaves per treatment were selected at random for measurement.

4.3.3.2.2 Yield and associated parameters

The plants were harvested separately from control and water stressed pots. Measurements on test weight (1000 seed weight), grain yield $plant^{-1}$ were recorded in

control and water deficit stressed plants as economic yield. The whole plant dry weight was measured as biological yield. Three replications were taken for each parameter. DTE % was calculated using the formula given by Fischer and Wood (1981) and harvest index (HI; %) was calculated using the formula by Gardner *et al*. (1985) as the ratio of the economic yield to biological yield and was expressed in percentage.

DTE (%) = (Yield under water deficit stress/ Yield under irrigated condition) x100
HI (%) = (Economic yield/ Biological yield) x100

4.3.3.2.3 Molecular analysis of chlorophyll degradation genes

4.3.3.2.3.1 RNA isolation and cDNA synthesis

In order to determine the semi quantitative gene expression analysis of chlorophyll degrading genes (*TaCHLase1* and *TaPAO*); the total RNA was extracted from the flag leaf after 10 days of water deficit stress in pot culture and leaves from 10 days old seedlings after above mentioned treatment combinations. Isolation of total RNA was carried out by TRIzol® reagent (Invitrogen, USA) and RNase-free DNase I (Promega, USA) was applied to remove contaminating genomic DNA at 37 ^{0}C for 1 h. Quality and integrity of total RNA were then determined by running appropriate amount in a formamide denaturing gel, and quantity of total RNA was determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized according to the instructions of the cDNA Synthesis Superscript® III First- Strand Synthesis System (Invitrogen, USA). Resulting cDNA was stored at -20 ^{0}C and employed as template for two-step RT-PCR reactions following recommended conditions provided in user's manual.

4.3.3.2.3.2 Primer designing

The primers employed in the present study were designed using the bioinformatics tools described in detail as follows. The reported nucleotide sequence of pheophorbide a oxygenase (PaO*)* gene from National Centre for Biotechnology Information (NCBI) database *viz*. GenBank accession no. JN689384 was selected for designing gene specific primers for semi-quantitative gene expression analysis (RT-PCR) in wheat. Search for nucleotide sequence in NCBI database

(http://www.ncbi.nlm.nih.gov/) for chlorophyllase (*CHLase1*) gene in wheat failed to retrieve any results. Hence we selected the protein sequence of *CHLase1* from *Arabidopsis* (GenBank accession no. NP_564094) and performed TBLASTn (Altschul *et al*., 1997) analysis with nucleotide sequence database of NCBI in wheat. We obtained a cDNA sequence from wheat (GenBank accession No. BT009214) having 95 % coverage and 48 % identity with *Arabidopsis CHLase1*. The complete open reading frame (ORF) coverage was confirmed by ORF finder tool of NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/). The deduced amino acid sequence using ExPASy translate tool (Gasteiger *et al*., 2003; http://web.expasy.org/translate/) of the putative *CHLase1* gene showed significant homology with rice, *Arabidopsis* and *Brachypodium* protein sequences by BLASTp analysis (Altschul *et al*., 1990; http://blast.ncbi.nlm.nih.gov/Blast.cgi). This putative sequence of *TaCHLase1* was used for designing gene specific primers for semi-quantitative expression analysis (RT-PCR) and cloning in wheat. The gene specific primers were designed manually and the quality parameters were confirmed using Integrated DNA Technologies (IDT) Oligoanalyzer tool (https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). The primers used for semi-quantitative RT-PCR analysis and cloning were listed in **Table 3.3** Every RT-PCR measurement was performed at least thrice. Expression of *TaActin* was used as an internal standard for normalization.

4.3.3.2.3.3 Cloning and sequencing of the *TaCHLase1* cDNA

PCR amplification with gene specific primers and proof-reading enzyme Platinum Hi-fidelity Taq DNA polymerase (*Invitrogen*^{TM}, USA) gave approximately 1kb fragment that was cloned in TA cloning vector pTZ57R/T (Thermo Scientific, USA). The construct was transformed into *E. coli* strain XL1-Blue and confirmed by blue-white screening in a media containing IPTG (Isopropyl β-D-1-thiogalactopyranoside), X-gal and Kanamycin. Positive clones were subcultured in medium having same composition and further confirmed by colony PCR, restriction digestion and sequencing (**Fig. 2.2** and **Fig. 2.3**).

4.3.3.2.3.4 *In- silico* analysis of *TaCHLase1* gene sequence

The BLAST software online (http://www.ncbi.nlm.gov/blast) was used to analyze the cDNA and protein sequences. Prediction of putative

subcellular localization was carried out using the WoLF PSORT (Horton *et al*., 2007; http://wolfpsort.org), SMART program (Letunic *et al*., 2002; http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) and TargetP 1.1 (Emanuelsson *et al*., 2000; http://www.cbs.dtu.dk/services/TargetP/). Its isoelectric point (pI) and molecular weight (MW) were analyzed by ExPASy ProtParam tool (Gasteiger *et al*., 2003; http://www. expasy.org/protparam). The multiple sequence alignment of *TaCHLase1* and its homologs from other species was carried by ClustalW2 (Larkin *et al*., 2007) tool of BioEdit software and represented using BoxShade server of ExPASy. The phylogenetic tree was constructed with the neighbor joining clustering method (Saitou and Nei, 1987) using MEGA 6.06 software (Tamura *et al*., 2013) with 500 bootstrap replicates for ClustalW2 alignment and the evolutionary distances were estimated using Poisson correction model.

4.3.3.3 Statistical analysis

The results are expressed as means with standard error (S.E.). The significance difference (at $P < 0.05$) between control and stressed samples were determined by Duncan's multiple range tests at a significance level of 0.05 for all biochemical parameters and was evaluated by analysis of variance (ANOVA). ANOVA and critical difference value were calculated by using SPSS 10.0 (SPSS Inc., Chicago, IL, USA), OPSTAT (hau.ernet.in/opstat.html) and Microsoft Excel.

4.3.4 Results

4.3.4.1 Water deficit stress and gas exchange characteristics

Changes in gas exchange parameters (photosynthesis rate, transpiration rate and stomatal conductance) were observed during water deficit stress (RWC; 65-75 %) imposed by withholding irrigation for 10 days at reproductive stage and are compared with normal irrigated condition (RWC; 80-85 %) in three wheat genotypes differing in their drought tolerance efficiency *viz*. HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). In the current study we observed that water deficit stress induced reduction in the gas exchange parameters differ significantly amongst the genotypes as well as between the treatments. Reduction in photosynthesis rate (**Table 4.3.1** and **Fig. 4.3.1**), stomatal conductance (**Table 4.3.2** and **Fig. 4.3.2**) and transpiration rate (**Table 4.3.3** and **Fig.**

Photosynthesis rate (µmol CO_2 m^{-2} min^{-1})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	23.14	13.90	**18.52**	**39.93**
	HW 2078	21.95	12.06	**17.00**	**45.04**
	HW 4059	21.18	10.47	**15.83**	**50.57**
	Mean	**22.09**	**12.14**	**17.12**	
Statistics		CD at 5%		SEM	
	Genotype (G)	**0.63**		**0.217**	
	Treatment (T)	**0.514**		**0.177**	
	Interaction G x T =	**N/A**		**0.307**	

Table 4.3.1 Effect of water deficit stress on photosynthesis rate (µmol CO_2 m^{-2} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage

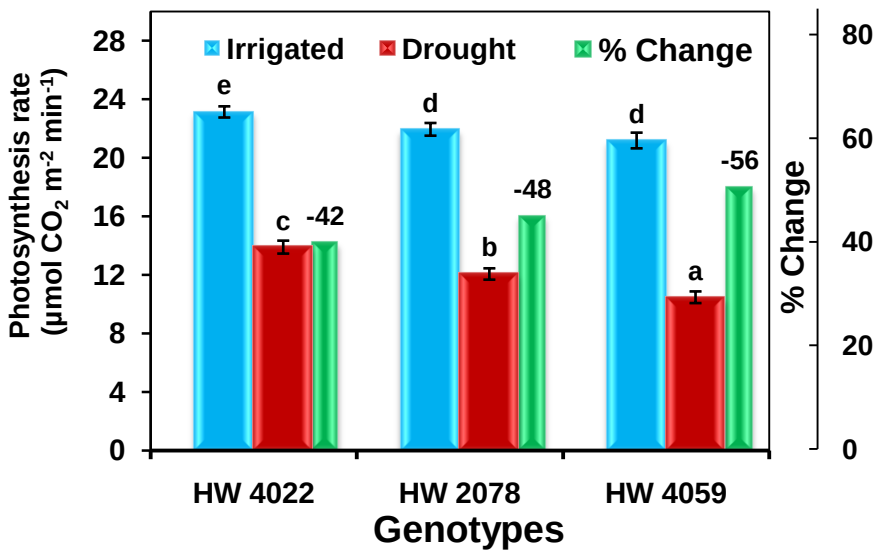


Fig. 4.3.1 Effect of water deficit stress on photosynthesis rate (µmol CO_2 m^{-2} min^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

Stomatal conductance (cm s^{-1})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	0.37	0.20	**0.28**	**46.20**
	HW 2078	0.35	0.16	**0.26**	**52.34**
	HW 4059	0.32	0.13	**0.22**	**57.88**
	Mean	**0.34**	**0.16**	**0.25**	
Statistics		CD at 5%		SEM	
	Genotype (G)	**0.003**		**0.001**	
	Treatment (T)	**0.003**		**0.001**	
	Interaction G x T =	**0.005**		**0.002**	

Table 4.3.2 Effect of water deficit stress on stomatal conductance (cm s^{-1}) in contrasting set of three wheat genotypes at reproductive stage

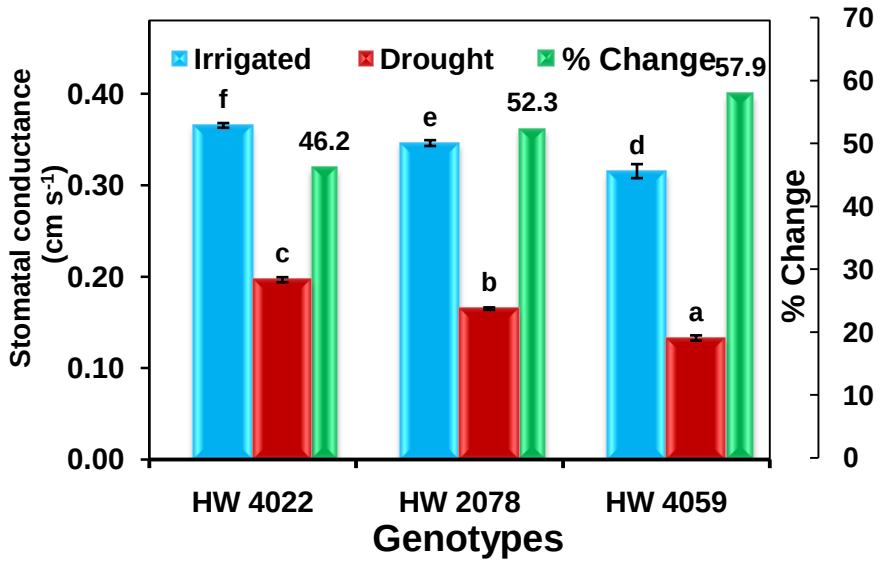


Fig. 4.3.2 Effect of water deficit stress on stomatal conductance (cm s^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

Transpiration rate (mmol m^{-2} s^{-1})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	3.9	2.8	**3.3**	**29.41**
	HW 2078	2.3	1.6	**2.0**	**33.61**
	HW 4059	3.3	2.1	**2.7**	**35.41**
	Mean	**3.2**	**2.1**	**2.7**	
Statistics		CD at 5%		SEM	
	Genotype (G)	0.129		0.044	
	Treatment (T)	0.105		0.036	
	Interaction G x T =	0.182		0.063	

Table 4.3.3 Effect of water deficit stress on transpiration rate (mmol m^{-2} s^{-1}) in contrasting set of three wheat genotypes at reproductive stage

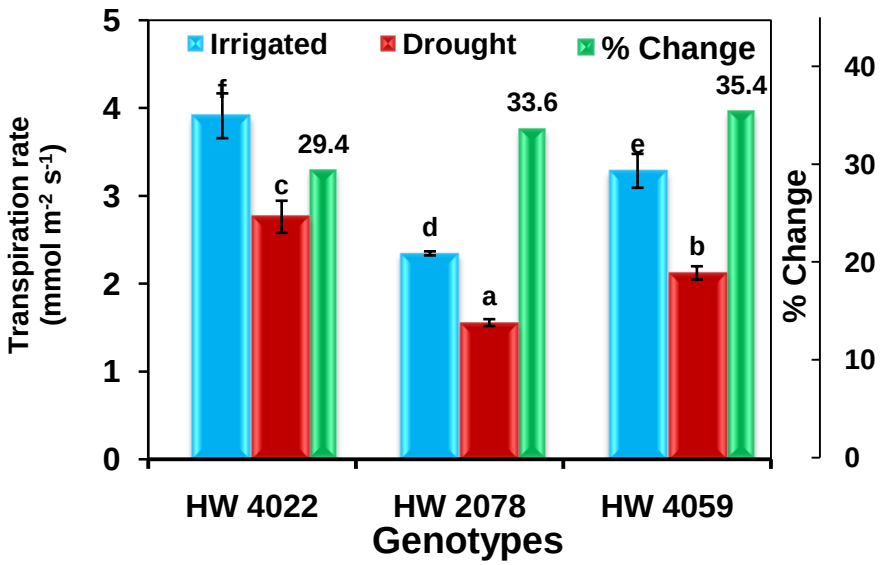


Fig. 4.3.3 Effect of water deficit stress on transpiration rate (mmol m^{-2} s^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

Total chlorophyll content (mg gDW^{-1})					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	11.7	9.2	**10.48**	**21.27**
	HW 2078	11.2	8.2	**9.69**	**26.88**
	HW 4059	11.8	7.2	**9.50**	**38.53**
	Mean	**11.6**	**8.2**	**9.89**	
Statistics		CD at 5 %		SEM	
	Genotype (G)	**0.781**		**0.25**	
	Treatment (T)	**0.638**		**0.21**	
	Interaction G x T =	**1.105**		**0.36**	

Table 4.3.4 Effect of water deficit stress on total chlorophyll content (mg gDW^{-1}) in contrasting set of three wheat genotypes at reproductive stage

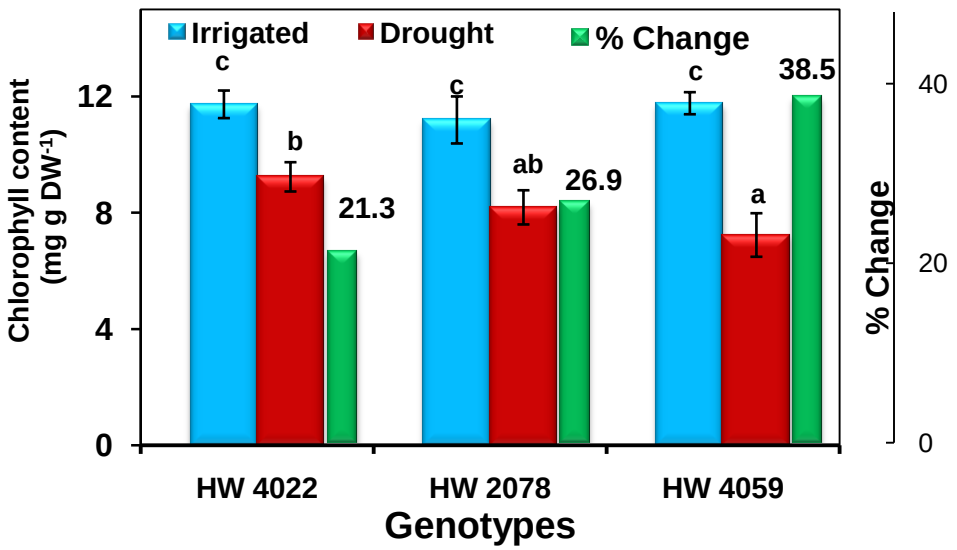


Fig. 4.3.4 Effect of water deficit stress on total chlorophyll content (mg g DW^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$)

***Abbreviation*:** DW: Dry weight

Total carotenoid content (mg gDW^{-1})					
Water status		**Irrigated**	**Drought**	**Mean**	**% Change**
Genotypes	**HW 4022**	0.8	0.7	**0.76**	**15.83**
	HW 2078	0.8	0.6	**0.70**	**25.63**
	HW 4059	0.8	0.5	**0.67**	**42.23**
	Mean	**0.8**	**0.6**	**0.71**	
Statistics		**CD at 5 %**		**SEM**	
	Genotype (G)	**0.039**		**0.01**	
	Treatment (T)	**0.032**		**0.01**	
	Interaction G x T =	**0.055**		**0.02**	

Table 4.3.5 Effect of water deficit stress on total carotenoid content (mg gDW^{-1}) in contrasting set of three wheat at reproductive stage

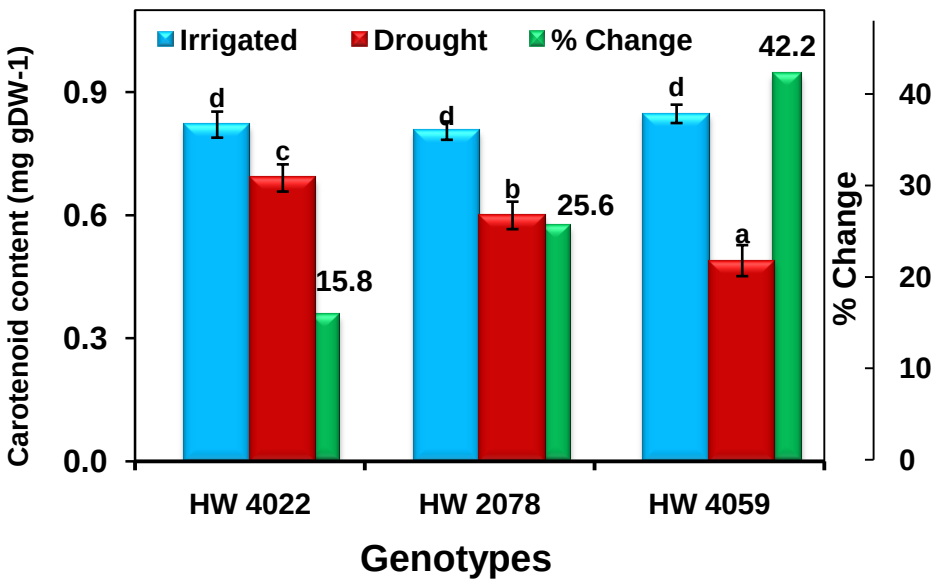


Fig. 4.3.5 Effect of water deficit stress on total carotenoid content (mg gDW^{-1}) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different ($P < 0.05$)

***Abbreviation*:** DW: Dry weight

4.3.3) was more in susceptible non stay-green genotype (HW 4059) as compared stay-green tolerant genotypes (HW 4022 and HW 2078). In addition, significant genotypic differences were observed for both chlorophyll (**Table 4.3.4** and **Fig. 4.3.4**) and carotenoid content (**Table 4.3.5** and **Fig. 4.3.5**) under water deficit stress in all the three genotypes studied. Susceptible genotype (HW 4059), recorded the maximum reduction for both the pigments while, it was comparatively higher in tolerant genotypes under water deficit stress.

4.3.4.2 Water deficit stress and yield attributes

In this study, water deficit stress showed significant decline in 1000 seed weight (**Table 4.3.6** and **Fig. 4.3.6**) and harvest index (**Table 4.3.7** and **Fig. 4.3.7**) in all the three genotypes (HW 4022, HW 2078 and HW 4059). Decline in terms of 1000 seed weight was more in susceptible genotype (HW 4059) as compared to tolerant genotype (HW 4022 and HW 2078) under water deficit stress. Similarly, HW 4022 maintained highest drought tolerance efficiency (DTE; %) followed by HW 2078 while, HW 4059 found to have lowest DTE % value (**Table 4.3.8** and **Fig. 4.3.8**). Results also showed that increase in yield traits in tolerant genotypes is closely associated with higher photosynthesis rate and stomatal conductance under drought stress.

4.3.4.3 Expression profiles of *TaCHLase1* and *TaPaO* under osmotic/ drought stress.

Expression profiling of genes associated with chlorophyll degrading pathway was studied in leaf of 10 days old seedlings and flag leaf of wheat genotypes by semi-quantitative expression analysis (Reverse transcriptase polymerase chain reaction, RT-PCR). Transcript expression profiling of *TaCHLase1* and *TaPaO* genes were studied using gene specific primers resulted in 360 and 300 bp amplicon size, respectively and further confirmed by sequencing (**Table 3.3**) in all the three wheat genotypes in presence of various chemical regulators (ethylene inducers/ inhibitors) (**Fig. 4.3.9** and **Fig. 4.3.11**). Significant difference was observed in gene expression level of *TaPaO* and *TaCHLase1* under control (RWC; 80-90 %) and osmotic stress (RWC; 70-80 %) in 10 days old leaf sample. Amongst the three genotypes studied, susceptible genotype (HW 4059) showed higher expression level of both chlorophyll degrading genes *TaPaO* and *TaCHLase1* followed by relatively drought tolerant

genotype (HW 2078), while lowest expression was observed in case of tolerant genotype (HW 4022) under both water regimes. Ethrel application (ethylene inducer) slightly induced the expression level of *TaPaO* under osmotic stress in HW 4059 and HW 2078 but the genotype HW 4022 maintained the lower expression level, while in case of *TaCHLase*1 gene, HW 2078 showed higher transcript level under both control and osmotic stress. In presence of AVG (ethylene biosynthesis inhibitor, aminoethoxy vinylgylcine) and 1-MCP (ethylene signaling inhibitor, 1-methylcyclopropene) the transcript of *TaPaO* and *TaCHLase1* decreased significantly under both osmotic regimes while, in case of HW 2078 the expression of *TaPaO* was comparatively high under control and osmotic stress condition. In pot culture condition, after imposing drought stress for 10 days at 50 % reproductive stage the expression level of *TaPaO* drastically reduced in HW 4022 and HW 2078 but in HW 4059 the expression level is high under both water regimes (**Fig. 4.3.12**). In case of *TaCHLase1* expression level was maintained to be high in HW 4059 and HW 2078, while tolerant genotype HW 4022 showed comparatively reduced expression level of *TaCHLase1* under irrigated as well as water deficit stress condition (**Fig. 4.3.10**).

4.3.4.4 Cloning and *in-silico* characterization of *TaCHLase1* gene from wheat

A full-length cDNA encoding a polypeptide with sequence similarity to *Arabidopsis* chlorophyllase *(AtCHLase1)* was amplified by PCR of cDNA using gene specific primers and cloned from drought susceptible non stay-green wheat genotype HW 4059. The BioEdit 7.1 software was used for aligning forward and reverse sequencing results and the complete nucleotide sequence was reconstructed having a nucleotide sequence of 1054 base pairs and was annotated as *TaCHLase1* based on the similarity with *AtCHLase1* of *Arabidopsis*. The sequences were translated using ExPASy Translate Tool and deduced amino acid sequences were obtained. These results were confirmed by BLASTp analysis with *Arabidopsis* (http://www.arabidopsis.org/Blast/) sequence as chlorophyllase. The cDNA contained an open-reading frame for a 319 amino acid polypeptide with a calculated molecular mass of 33.84 kDa and isoelectric point of 5.71. The multiple protein sequence alignment (ClustalW2) with *Arabidopsis* and *Brachypodium* showed considerable homology between the sequences by 45 % and 81 % with *Arabidopsis* and *Brachypodium* CHLase1 genes, respectively. Multiple sequence alignment also

1000 seed weight/ test weight (g)					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	48.0	37.0	**42.5**	**22.9**
	HW 2078	46.0	33.0	**39.5**	**28.3**
	HW 4059	43.0	25.0	**34.0**	**41.9**
	Mean	**45.7**	**31.7**	**38.7**	
Statistics		CD at 5 %		SEM	
	Genotype (G)	**3.004**		**0.964**	
	Treatment (T)	**2.453**		**0.787**	
	Interaction G x T =	**4.249**		**1.364**	

Table 4.3.6 Effect of water deficit stress on 1000 seed weight or test weight (g) in contrasting set of three wheat genotypes at reproductive stage

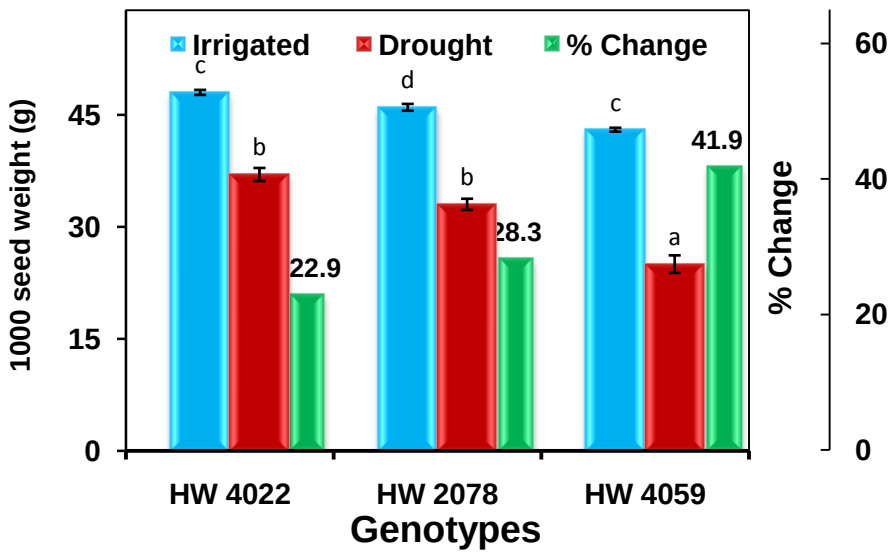


Fig. 4.3.6 Effect of water deficit stress on 1000 seed weight/ test weight (g) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

Harvest Index (HI; %)					
Water status		Irrigated	Drought	Mean	% Change
Genotypes	HW 4022	45.78	36.85	**41.3**	**19.5**
	HW 2078	44.03	34.41	**39.2**	**21.9**
	HW 4059	43.56	28.41	**36.0**	**34.8**
	Mean	**44.5**	**33.2**	**38.8**	
Statistics		CD at 5 %		SEM	
	Genotype (G)	1.522		0.489	
	Treatment (T)	1.243		0.399	
	Interaction G x T =	2.152		0.691	

Table 4.3.7 Effect of water deficit stress on harvest index (HI; %) in contrasting set of three wheat genotypes at reproductive stage

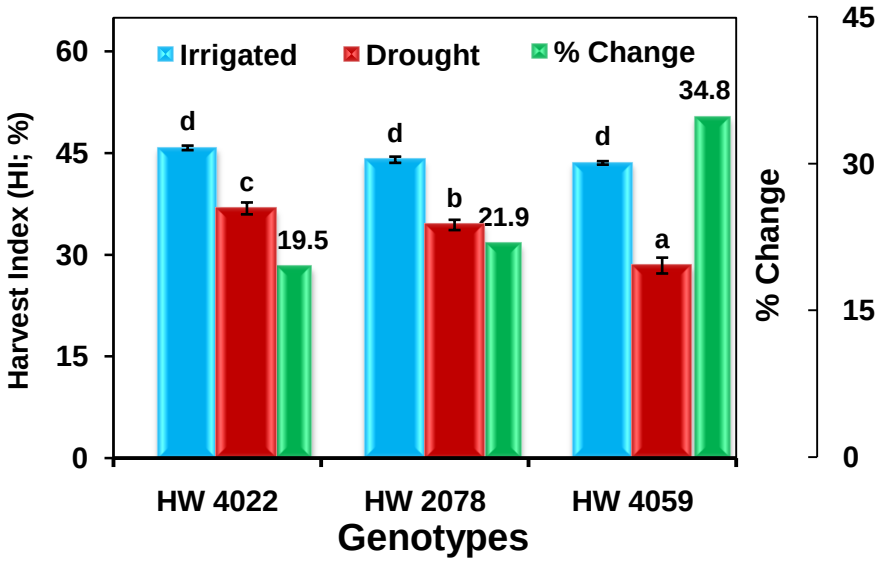


Fig. 4.3.7 Effect of water deficit stress on harvest index (HI; %) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)
***Abbreviation*:** HI: Harvest index

Drought Tolerance Efficiency (DTE; %)			
Genotypes	**HW 4022**	41.31	
	HW 2078	39.22	
	HW 4059	35.99	
	Mean	**38.84**	
Statistics		**CD at 5 %**	**SEM**
	Genotype (G)	**10.754**	**3.05**

Table 4.3.8 Effect of water deficit stress on drought tolerance efficiency (DTE; %) in contrasting set of three wheat genotypes at reproductive stage

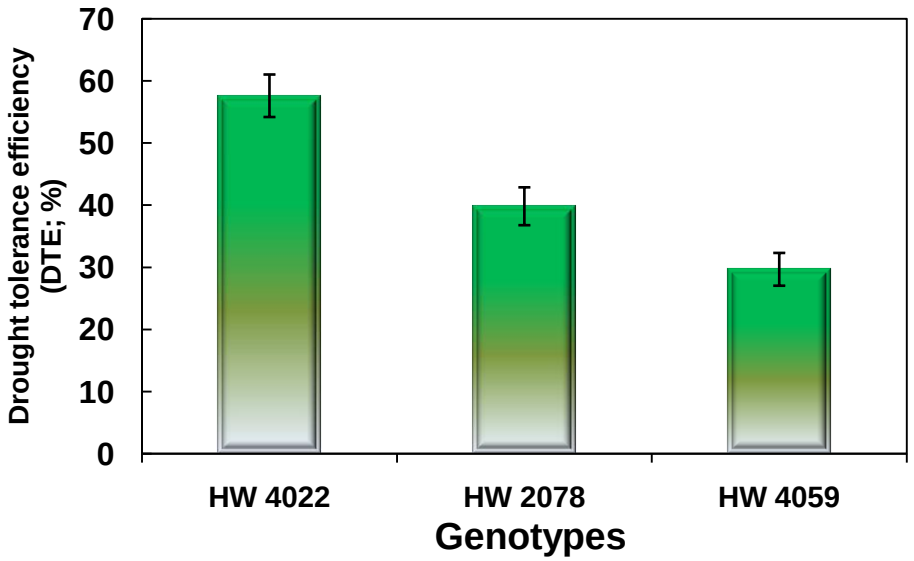
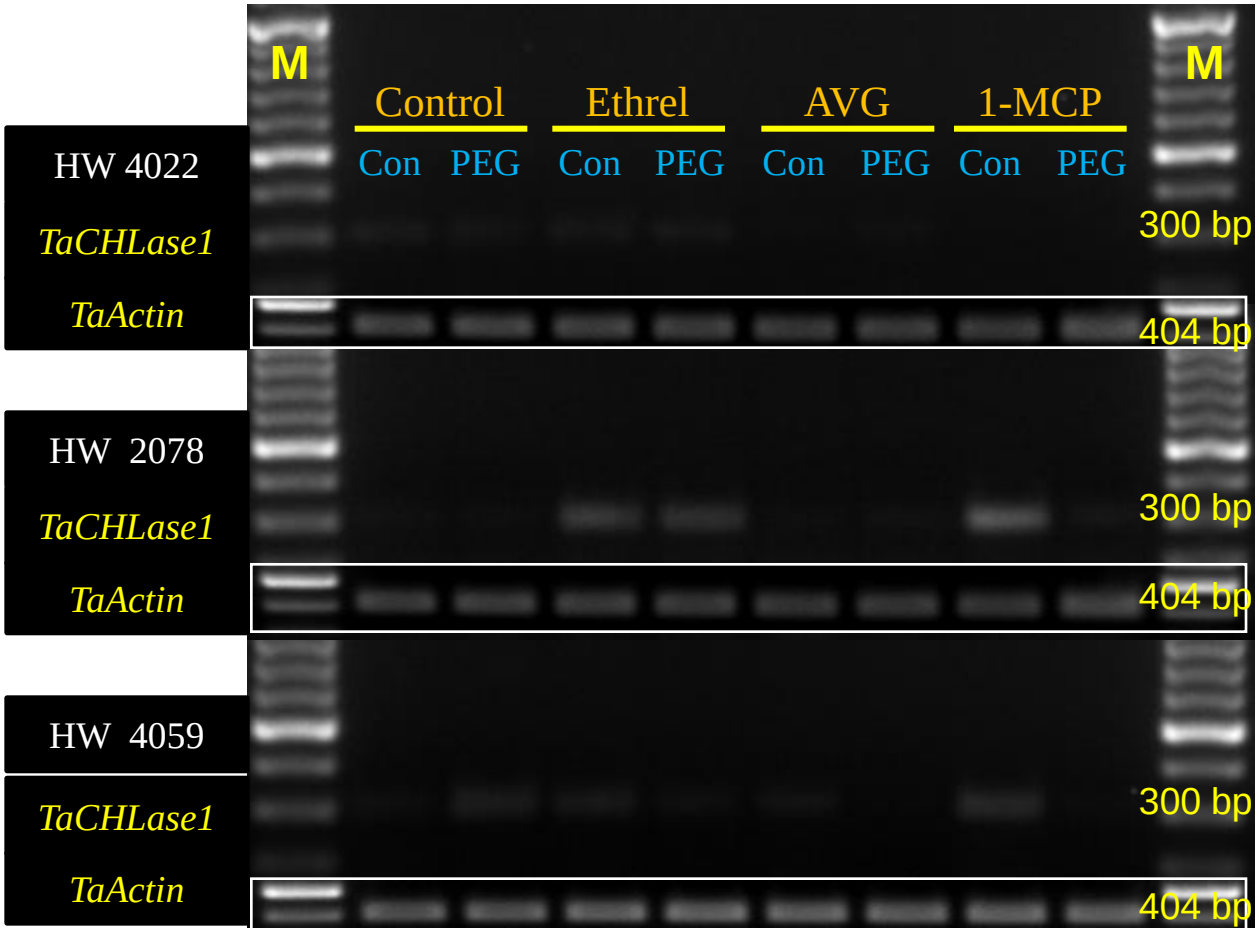


Fig. 4.3.8 Effect of water deficit stress on drought tolerance efficiency (%) in contrasting set of three wheat genotypes at reproductive stage. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)
***Abbreviation*:** DTE: Drought tolerance efficiency

Chlorophyllase



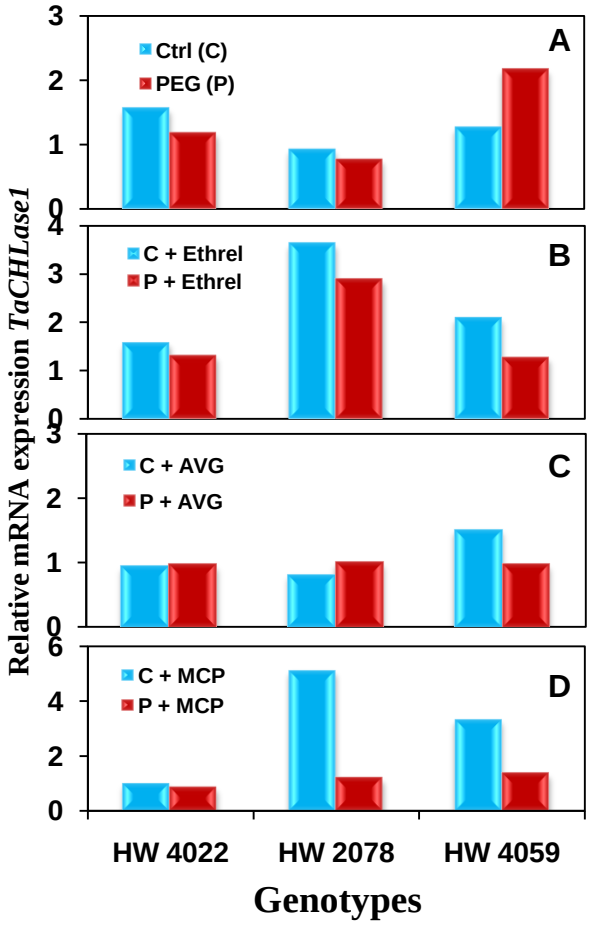
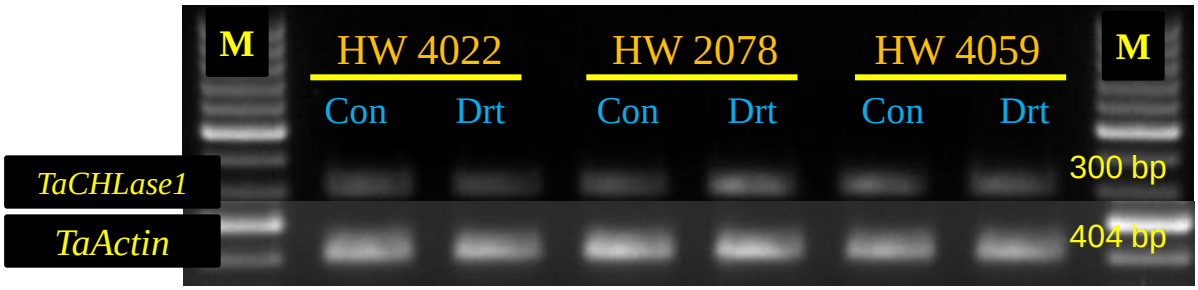


Fig. 4.3.9 Semi-quantitative expression analysis of *TaCHLase1* gene encoding enzyme involved in chlorophyll degrading pathway in 10 days old wheat seedlings. Different panels showing relative mRNA expression under various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions.
B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .
C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.
D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Abbrevation: CHLase: Chlorophyllase; PEG: Polyethylene glycol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

CHLase



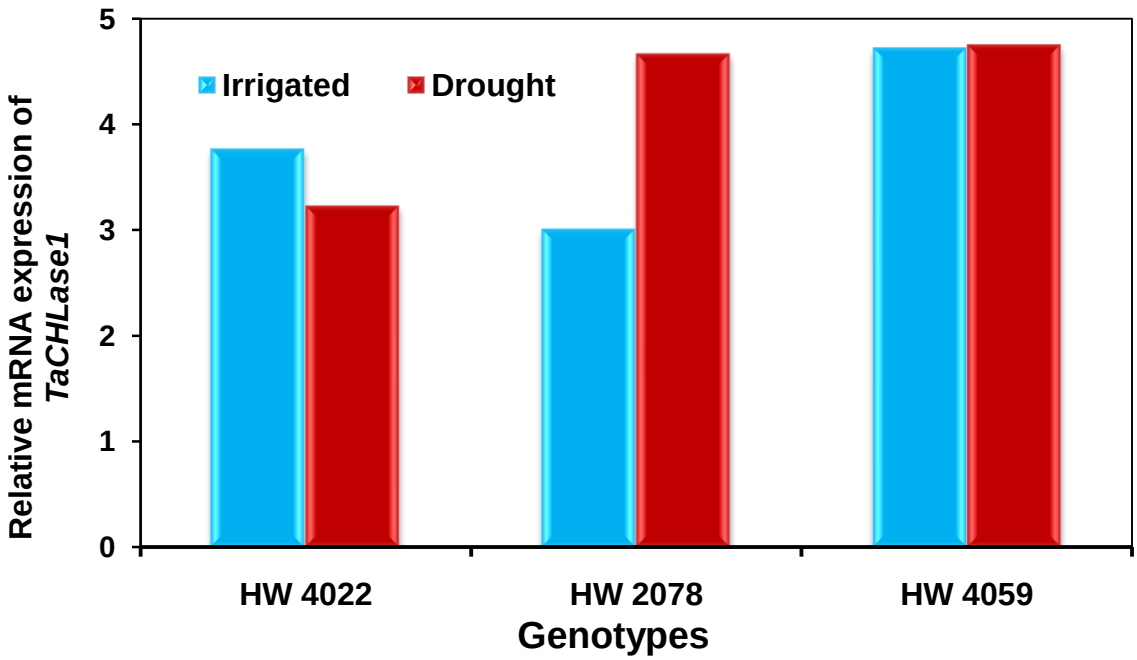


Fig. 4.3.10 Semi-quantitative expression analysis of *TaCHLase1* gene encoding enzyme involved in chlorophyll degradation pathway in flag leaf of wheat genotypes under control (normal irrigation, RWC; 80-85 %) and water deficit stress condition (withholding irrigation for 10 days, RWC; 65-75 %)

Lane M: 100 bp ladder

Abbrevation: CHLase: Chlorophyllase; Con: Control; Drt: Drought

PaO

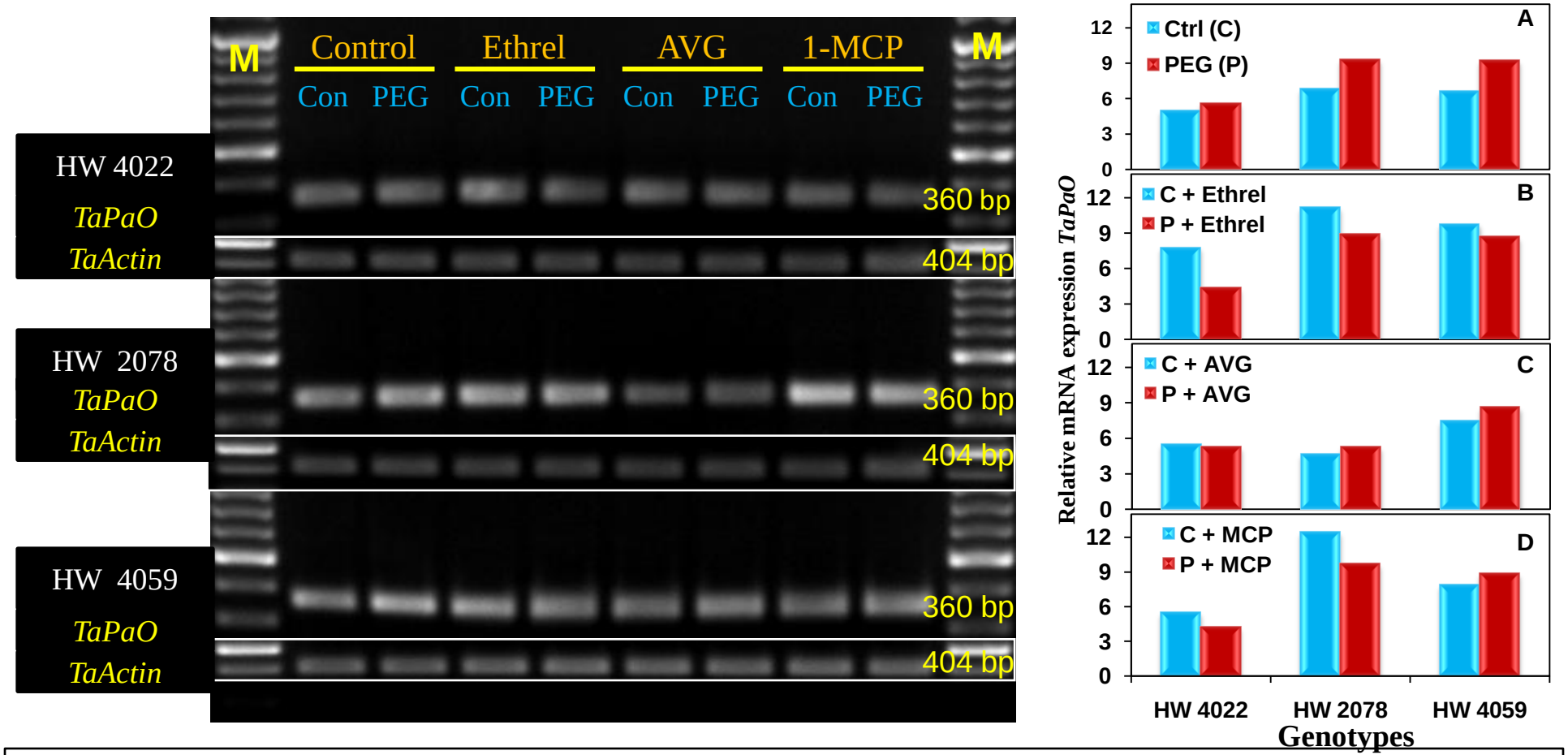


Fig. 4.3.11 Semi-quantitative expression analysis of *TaPaO* gene encoding enzyme involved in chlorophyll degrading pathway in 10 days old wheat seedlings. Different panels showing relative mRNA expression under various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions.
B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .
C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.
D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Abbrevation: PaO: Pheophorbide a oxygenase; PEG: Polyethylene glycol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

PaO

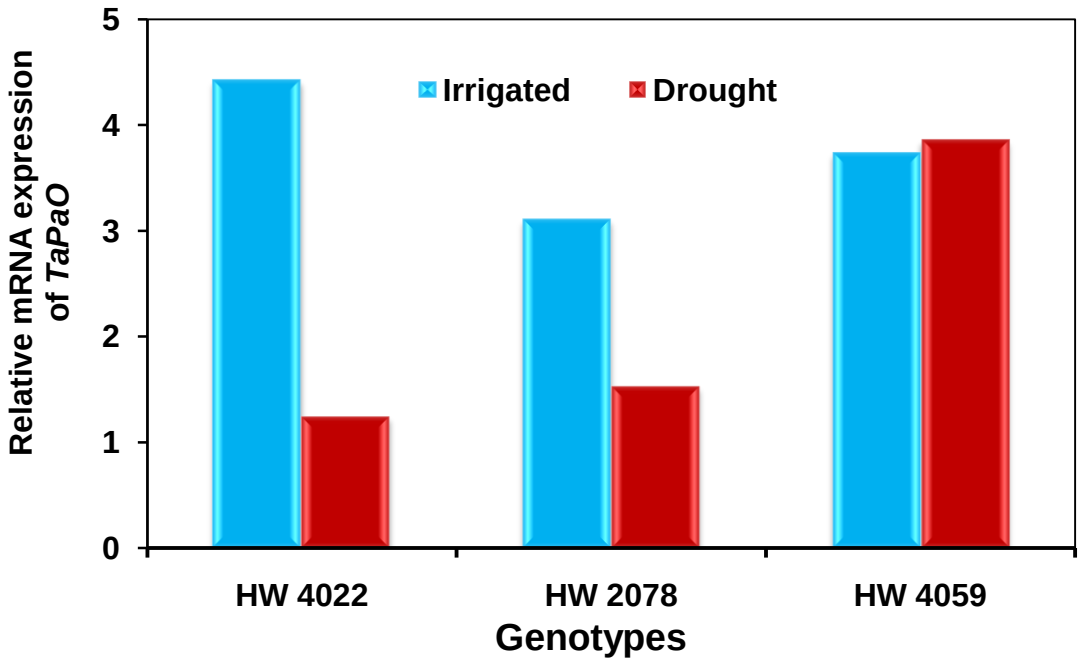


Fig. 4.3.12 Semi-quantitative expression analysis of *TaPaO* gene encoding enzyme involved in chlorophyll degradation pathway in flag leaf of wheat genotypes under control (normal irrigation, RWC; 80-85 %) and water deficit stress condition (withholding irrigation for 10 days, RWC; 65-75 %)

Lane M: 100 bp ladder

Abbrevation: PaO: Pheophorbide a oxygenase; Con: Control; Drt: Drought

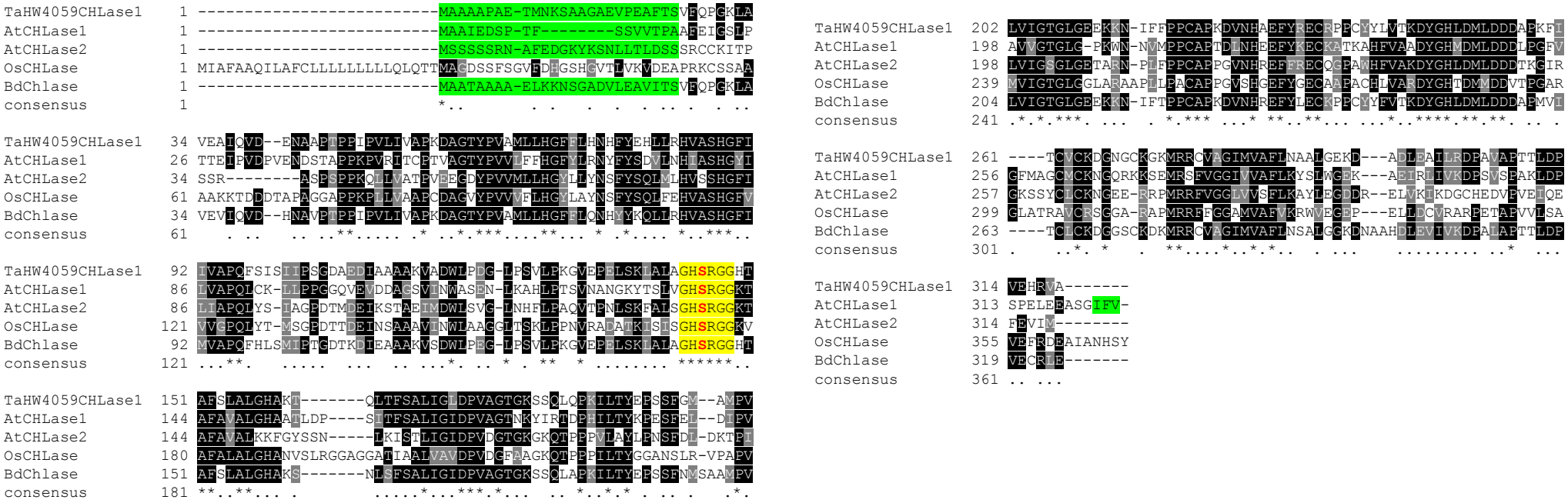


Fig. 4.3.13 Alignment of *TaCHLase1* and other homologous CHLase proteins using ClustalW2 and represented by BoxShade. The sequences used in this alignment are as follows: *Triticum aestivum* (GeneBank accession no. AHJ14565), *Arabidopsis thaliana* (GeneBank accession no. NP_564094), *Oryza sativa* (GeneBank accession no. NP_001064620), *Brachypodium distachyon* (GeneBank accession no. XP_003576870). Dark shaded regions show amino acid residues conserved in all five sequences and grey shaded regions represent similar residues. The consensus sequences are represented by symbols in each line. Various colour code indicates; yellow shade: conserved lipase motif, red letter colour: catalytically active serine, green shade: post-translationally removed motifs at the N and C-terminus.

CHLase

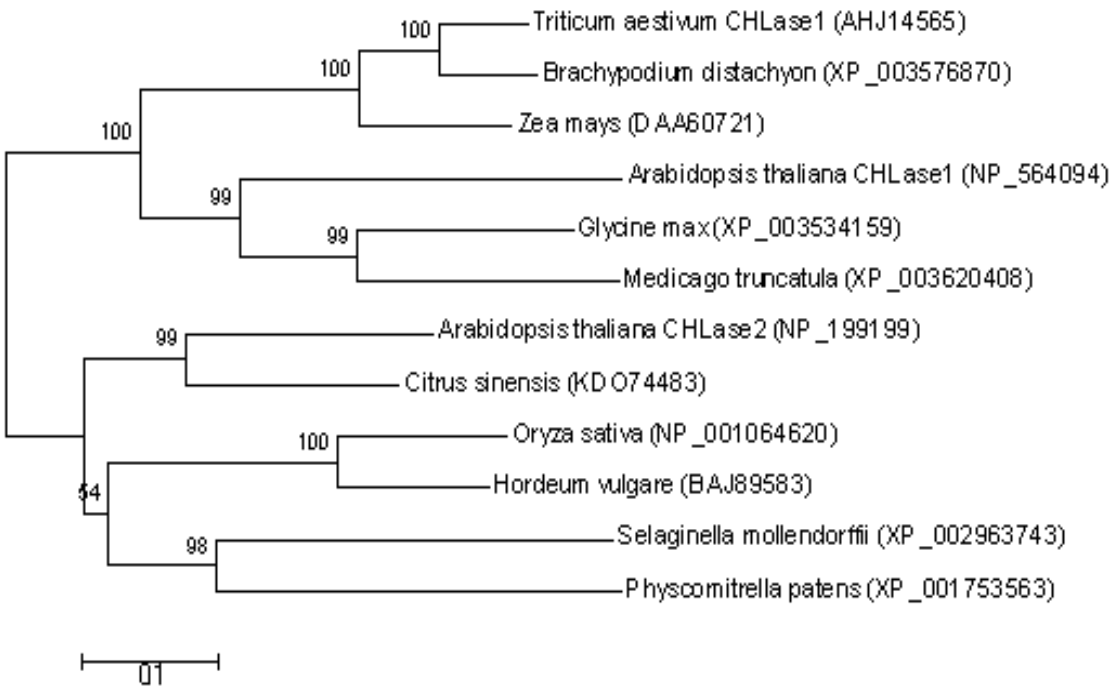


Fig. 4.3.14 Phylogenetic tree constructed by the neighbor-joining method of MEGA 6.06 software with 500 bootstrap replicates of the ClustalW2 alignment. Evolutionary distances were estimated using the Poisson correction model. Phylogenetic tree of CHLase proteins from different plant species along with their accession numbers are given in parentheses

showed conserved residues such as lipase motif (GXSXGG) *i.e.,* GHSRGG of wheat at amino acid position 143-148 and the putative active serine (Ser 145) (**Fig. 4.3.13**).

The phylogeny tree has been constructed (MEGA 6.06 software) using Neighbor joining method for *TaCHLase1* using the amino acid sequence from the selected plant species (http://www.phytozome.net/), which gave light into the evolution of the *TaCHLase1* (**Fig. 4.3.14**). The evolution of *chlorophyllase* can be dated back from the fern (*Selaginella mollendorfii*) and bryophyte (*Physcomitrella patens*) to higher plants like dicots and monocots. The result showed that the available CHLase proteins from various plant species were classified into two groups. The first group mainly included *PpCHLase* (*Physcomitrella. patens*), *SmCHLase* (*Selaginella mollendorfii*), *OsCHLase* (*Oryza sativa*), *HvCHLase* (*Hordeum vulgare*), *CsCHLase* (*Citrus sinensis*) and *AtCHLase2 (Arabidopsis thaliana),* while the second group included *MtCHLase (Medicago truncatula), GmCHLase (Glycine max), ZmCHLase (Zea mays), BdCHLase* (*Brachypodium distachyon*), *AtCHLase1* (*Arabidopsis thaliana*) and *TaCHLase1* (*Triticum aestivum*). Interestingly, the phylogeny tree showed higher identity between *TaCHLase1* and *AtCHLase1* (45 %) than with *OsCHLase* (40 %).

4.3.5 Discussion

Photosynthesis in the chloroplast is one of the most drought sensitive physiological processes where water deficit stress damages the thylakoid membrane, disturbs its functions, and ultimately decreases photosynthesis and crop yield (Tian *et al*., 2013). Photosynthetic pigments like chlorophyll and carotenoids are integrally related to the physiological function of leaves and changes in pigment contents provide further insight into the modification processes taking place in the photosynthetic apparatus. Chlorophyll content can directly determine photosynthetic potential and indirectly the nutrient status of leaf. Changes in gas exchange parameters like photosynthesis rate, transpiration and stomatal conductance are most closely related to changes in chlorophyll and carotenoid contents in flag leaf of wheat under drought stress as reported by Guendouz *et al*. (2014). In the present study also we found that water deficit stress causes reduction in the photosynthesis, transpiration and stomatal conductance in the three wheat genotypes differing in their drought tolerance efficiency *viz*. drought tolerant- HW 4022, relatively drought tolerant- HW 2078 and drought susceptible- HW 4059. Reduction was reported to be higher in

susceptible genotype (HW 4059), while tolerant genotype (HW 4022) maintained the higher values for the above mentioned parameters. The results were consistent with Chen *et al*. (2012) in wheat genotypes, where they reported water stress reduced the photosynthesis traits like transpiration, stomatal conductance and chlorophyll content as well as yield related traits as compared to well watered condition. Prasad *et al*. (2011) also supported these data in wheat of combined drought and heat stress treatment, caused the reduction in chlorophyll content, photosynthesis rate as well as yield traits like number of grains and harvest index. In parallel to current findings, earlier reports also showed that limiting transpiration rate and decreasing stomatal conductance improved grain yield in many crop species like sorghum (Gholipoor *et al*., 2010), chickpea (Zaman-Allah *et al*., 2011), pearl millet (Kholova *et al*., 2010) and maize (Yang *et al*., 2012) under water deficit stress. Further statistical analysis revealed highly significant and positive correlation between 1000 seed weight, harvest index and drought tolerance efficiency when compared with photosynthesis rate in the genotypes studied. Results of the present study hence proved that higher positive correlation exist between photosynthesis traits and yield in wheat under drought stress. In support of this, there are other few reports like in maize (Messmer *et al*., 2011) and rice (Fu *et al*., 2011) also reported that under drought stress at flowering stage, delayed leaf senescence rate by maintaining the photosynthesis characteristics can be positively correlated with higher grain yield.

As stated above, the chlorophyll directly determines the photosynthetic potential of crop plants. So, we also studied the chlorophyll breakdown, which is degraded through a series of reactions catalyzed by chlorophyllase (CHLase), magnesium de-chelatase, and pheophorbide a oxygenase (PaO) (Hortensteiner, 2013). The result in the current study showed positive correlation between chlorophyll and expression of genes encoding chlorophyll degrading enzymes *viz*. *TaCHLase1* and *TaPaO* under water deficit/ osmotic stress condition in presence of ethylene releasing compound (ethrel) and ethylene inhibitors (1-MCP and AVG), respectively. Drought tolerant stay-green genotype (HW 4022) showed reduced expression level for both *TaCHLase1* and *TaPaO* genes as compared to drought susceptible non stay-green genotype (HW 4059) confirming that it has less chlorophyllase and pheophorbide a oxygenase activity due to which it maintained its leaf greenness and high photosynthesis. However, the susceptible genotype (HW 4059) showed increase in expression of *TaPaO* gene which confirms its non stay-green characters with rapid

leaf senescence rate under drought/ osmotic stress condition. In support to our findings in cereals Tang *et al*. (2011) reported that by over-expressing *OsPaO* in rice triggers the senescence and cell death in rice seedlings. As there are fewer reports in cereals, *vis-à-vis* senescence in general and chlorophyll degradation in particular, so we correlate with other vegetables and fruits. Comparable findings reported in Broccoli by Buchert *et al*. (2011) where ethylene treatment induced the rate of senescence by up-regulating the expression of chlorophyllase and pheophytinase (PaO) gene resulting in increased chlorophyll degradation. A recent report by Lira *et al*. (2014) in tomato during ripening where the treatment of ethylene perception inhibitor (1-MCP) resulted in higher level of chlorophyll content which can be positively correlated with lower expression of chlorophyll degrading enzymes. Peng *et al*. (2013) also reported in citrus where they proved that ethylene treatment strongly induced the expression pattern of CHLase1 gene which is negatively correlated with ratio of chlorophyll a/ b and total chlorophyll content. In pear Chen *et al*. (2012) reported that ethylene inhibitors suppressed the expression of chlorophyll degrading enzymes (Chlorophyllase, PaO and Red chlorophyll catabolite reductase) while reverse is the case in presence of ethylene. Zhang *et al*. (2011) in cabbage reported similar results where they showed, the expression of chlorophyll degrading genes was closely associated with chlorophyll degradation during leaf senescence.

The molecular mechanism governing the function of *TaPaO* gene in wheat is already known (Ma *et al*., 2012), but little information is available on the molecular characteristics of *TaCHLase1* in wheat. Chlorophyllase was originally purified from *Citrus sinensis* and *Chenopodium album* which led to the isolation of two genes from *Arabidopsis* designated as *AtCLH1* and *AtCLH2* (Jacob-Wilk *et al*., 1999). It was isolated and partially purified from tissues of a wide range of higher plants and algae, and its catalytic activity under *in vitro* conditions was intensively studied by many researchers (Lee *et al*., 2010; Gupta *et al*., 2011; Gupta *et al*., 2012). Here, we identified the novel and putative chlorophyllase gene from hexaploid wheat (*Triticum aestivum* L.) and annotated as *TaCHLase1* owing to its homology with *AtCLH1*. Cloning of *TaCHLase1* from wheat is a significant breakthrough in studying the stay-green traits phenotype in crop plants. Multiple sequence alignment showed that CHLase is widely distributed in plant kingdom. Consensus with the previous reports (Tsuchiya *et al*., 1999, 2003; Arkus *et al*., 2005; Okazawa *et al*., 2006), the current study on comparative analysis of protein sequences revealed significant homology

between wheat and other higher plants. The sequence analysis provided by various researchers (Tsuchiya *et al*., 2003; Arkus *et al*., 2005; Okazawa *et al*., 2006; Lee *et al*., 2010; Gupta *et al*., 2011; Gupta *et al*., 2012) showed the presence of a lipase motif containing a catalytic site and disruption of these catalytic residues by site-directed mutagenesis disrupts its function (Tsuchiya *et al*., 1999, 2003). The lipase motif obtained from deduced amino acid sequence (GHSRGG) from position 143 to 148 with the catalytically active serine (Ser145) provides light into its role in removing phytol tail from chlorophyll molecule, similar to the findings by Arkus and Jez (2006). The N-terminal processing site of CHLase is reported in two species: *Citrus sinensis* (Jacob-Wilk *et al*., 1999) and *Chenopodium album* (Tsuchiya *et al*., 1999), where it was Thr21 in citrus and before Ser30 in chenopodium. The wheat sequence reported in present study also showed N terminal processing site (Ser26), which upon removal can lead to enzyme activation. The phylogeny tree revealed the evolutionary lineage of wheat chlorophyllase. A search for transit peptide and chloroplast localization using bioinformatics tools (SMART, WolfPSort and ChloroP) did not provide any conserved motifs. But previous studies showed that mature citrus CHLase versions were localized by GFP fused CHLase1 to the chloroplast membrane fraction when expressed in tobacco protoplasts. Further immunodetection of CHLase by Harpaz-Saad (2007) in tobacco protoplasts showed that CHLase precursor (35 kDa) as well as mature protein (33 kDa) is chloroplast membrane localized.

4.3.6 Conclusion

Overall, we concluded that reduction in chlorophyll contents and photosynthesis characteristics in response to water deficit stress in susceptible non stay-green genotype results in wheat yield losses. *TaCHLase1* is involved in drought induced leaf senescence and responses to drought stresses. These findings enhanced and contribute to our knowledge to get insight information regarding the molecular mechanism of chlorophyll degrading enzymes chlorophyllase and pheophorbide a oxygenase during drought stress. In future the sequence information of *TaCHLase1* can be carried further for the functional validation in wheat by reverse genetic approaches for down regulation of *TaCHLase1* in wheat for delayed leaf senescence having stay-green traits for drought tolerance.

4.4. RESEARCH PAPER IV

Interactive role of ethylene and polyamines for drought tolerance in wheat (*Triticum aestivum* L.)

4.4.1 Abstract

This study tested the hypothesis that the cross-talk between ethylene and polyamines may be involved in mediating the effects of osmotic stress in wheat. From our previous study, three wheat genotypes namely, HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) were selected on the basis of their drought tolerance efficiency. Genotypes were raised for 10 days in growth chamber under controlled environmental conditions. Two treatments, well watered and osmotic stress (20 % PEG-6000) were imposed on 10 days old seedlings in combination with chemical regulators like ethylene inducer (ethrel; 10 ppm), ethylene biosynthesis inhibitor (Aminoethoxy vinylglycine; AVG, 2 ppm) and ethylene signaling inhibitor (1-methyl cyclopropene; 1-MCP, 10 ppm). The leaf relative water content (RWC; %) decreases significantly in all the three genotypes under osmotic stress condition in presence of chemical regulators and the reduction was more in susceptible genotype (HW 4059). Osmotic stress increases the polyamines contents (spermidine and spermine), expression levels of polyamine biosynthesis genes (S-adenosyl-l-methionine decarboxylase and spermidine synthase), and decreased the ethylene evolution rate as well as expression of ethylene biosynthesis genes (ACC synthase and ACC oxidase) and ethylene signaling gene (Ethylene response sensor 1) in tolerant genotype (HW 4022). These results were reversed in case of susceptible genotype (HW 4059) under water deficit stress. The outcomes from this study suggested that interaction between ethylene and polyamines biosynthesis pathway responds to water deficit stress and facilitates drought tolerance in wheat crop with increased spermidine and spermine content under osmotic stress.

Key words: Osmotic stress, Ethylene, Polyamines, Wheat.

4.4.2 Introduction

Plants regulate their growth and development in response to ever-changing environmental conditions like drought, temperature fluctuations, nutrient imbalance, pathogens, etc. and these responses are mediated by plant growth regulators (Peleg and Blumwald, 2011). They regulate every aspect of plant growth and development and their responses to biotic and abiotic stresses. These compounds are derived endogenously and can act either at the site of synthesis or transport in the plant. In recent years significant research was carried out which contributed to the understanding of processes associated with plant hormones on their biosynthesis, metabolism, as well as their role in signaling. They don't act in isolation but interacts synergistically or antagonistically by cross-talk mechanisms, so that they can modulate each other's biosynthesis and responses (Liu *et al*., 2013).

Polyamines are organic polycations, which are low molecular weight nitrogen-containing compounds that are found in a wide range of organisms from bacteria to plants and animals (Tiburcio *et al*., 2014). In higher plants, the major polyamines are putrescine, spermidine, and spermine (Pegg and Michael, 2010). Putrescine can be directly synthesized from ornithine by enzyme ornithine decarboxylase (ODC) or indirectly from arginine *via*. arginine decarboxylase (ADC) (Gill and Tuteja, 2010). The synthesis of the triamine spermidine and the tetraamine spermine is catalyzed by enzymes spermidine synthase (SPDS) and spermine synthase (SPMS) respectively *via.* incorporation of aminopropyl moiety to diamine putrescine and spermidine, respectively. The conversion of S-adenosyl-methionine (SAM) to decarboxylated S-adenoylmethionine, a donor of aminopropyl moiety to putrescine and spermidine is catalyzed by the enzyme S-adenosylmethionine decarboxylase (SAMDC). The synthesis of spermidine and spermine is mainly regulated by the level of SAMDC (Tiburcio *et al*., 2014). Interestingly, SAM is a common biosynthetic precursor for ethylene and polyamines (Bitrian *et al*., 2012). Ethylene biosynthesis pathway proceeds from methionine, through SAM and 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene and that the two main enzymes involved are ACC synthase (ACS) and ACC oxidase (ACO). Firstly, ACS catalyses the cyclisation of SAM into ACC, and subsequently ACO catalyses the oxidative conversion of ACC to ethylene (Meng *et al*., 2014). ACS catalyzes the rate limiting step in ethylene biosynthesis pathway (Habben *et al*., 2014).

In plants, polyamines are involved in a wide range of biological processes, including growth, development, senescence, programmed cell death as well as biotic and abiotic stress responses (Moschou and Roubelakis-Angelakis, 2014, Hussain *et al*., 2011 and Gupta *et al*., 2013). The success of polyamines in nature as compared to other compatible solutes can be explained by their peculiar functions especially, putrescine, spermidine and spermine are involved in a wide range of physiological processes, including organogenesis, embryogenesis, floral initiation and development, leaf senescence and ripening (Tiburcio *et al*., 2014). Ethylene is another important gaseous phytohormone which regulates many aspects of plant growth and development such as senescence, fruit ripening, and responses to various environmental stresses like drought, wounding and pathogen attack (Kar *et al*., 2011 and Zhang *et al*., 2013). Previous report by Wan *et al*. (2011) in rice and Beltrano *et al*. (1994) in wheat showed that ethylene negatively regulate the drought responses in crops. In addition, ethylene production is affected through regulation of ethylene biosynthesis genes which further up-regulate the expression of stress related genes and plant adaptation to stress condition (Zhang and Huang, 2010).

Polyamines and ethylene are endogenous plant growth regulators mediating many physiological processes such as morphogenesis, embryogenesis, senescence, and responses to environmental stresses (Torrigiani *et al*., 2012). Both these hormones share the common biosynthetic precursor SAM, and increasing polyamine biosynthesis has a notable effect on ethylene biosynthesis rates. In comparison to ethylene, increased polyamine contents improved grain filling rate in rice under drought stress condition as reported recently by Chen *et al*. (2013). However, little is known about whether and how the two plant growth regulators (polyamines and ethylene) respond during drought stress in wheat crop.

There might be a possibility that both these plant growth regulators *viz.* ethylene and polyamines interact with each other under drought stress condition. However, little is known about the relationship between them in regulation of drought tolerance mechanism in wheat crop. We proposed a hypothesis that higher polyamines and lower ethylene have the beneficial impact on wheat crop under water deficit conditions. To test this hypothesis we performed an experiment whereby, three wheat genotypes selected on the basis of difference in their drought tolerance efficiency in presence of ethylene inducers and inhibitors. Salient findings from our experiments

led to the conclusion that there is a metabolic interaction/ cross-talk between ethylene and polyamines for drought tolerance in wheat crop and it may be facilitates by increased spermidine and spermine content in comparison to ethylene evolution rate during osmotic stress condition.

4.4.3 Materials and methods

4.4.3.1 Plant material and treatments

From our preliminary experiment, we selected three wheat genotypes based on their drought tolerance efficiency *viz*., HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). Seeds were surface sterilized with 2.5 % sodium hypochlorite for 15 min and then washed 4-5 times with distilled water. These seeds were then germinated in petridishes with wetted germination paper at 25 ^{0}C under dark. After 48 h of incubation, uniformly germinated seeds were selected and raised in 50.0 mL test tubes containing half-strength Hoagland solution. The hydroponically raised seedlings were grown for 10 days in growth chamber at 25 ^{0}C day/ 20 ^{0}C night, light intensity of 1200 $Em^{-2}s^{-1}$ and 90% relative humidity. Osmotic stress was imposed to 10 days old seedlings using 20 % polyethylene glycol (PEG-6000) solution giving osmotic potential of -4.91 MPa (Michel and Kaufmann, 1973), where double distilled water (0.0 MPa) served as controls. They were treated with ethylene inducer (ethrel; 10 ppm), ethylene biosynthesis inhibitor (aminoethoxy vinylglycine; AVG, 2 ppm) and ethylene signaling inhibitor (1-methyl cyclopropene; 1-MCP, 10 ppm). The treatment combinations given to 10 days old seedlings were described in **Table 3.2.**

4.4.3.2 Observations recorded

4.4.3.2.1 Relative water content (RWC; %)

To evaluate the plant water status RWC was measured by Barrs and Weatherley (1962) method. Leaf RWC was estimated by recording the fresh weight (g) of leaf samples, thereafter immediately transferring in petridishes containing distilled water for 4 h to record turgid weight (g), followed by drying in hot air oven at 70 ^{0}C till constant dry weight (g) has reached.

RWC (%) = [(Fresh wt. – Dry wt.) / (Turgid wt. – Dry wt.)] x 100

4.4.3.2.2 Ethylene evolution rate (nmol g^{-1}DW h^{-1})

For ethylene estimation, wheat seedlings were grown hydroponically in growth chamber for 10 days and were transferred to 50.0 mL test tubes containing above mentioned treatment combinations. Tubes' mouth was covered with rubber serum stopper and whole test tube set was covered with aluminium foil and finally with black cloth according to Beltrano *et al*. (1994) with little modifications. These test tubes were kept in dark for 24 h for ethylene evolution. After 24 h dark treatment, one mL gas sample was taken out from the respective tubes with the help of specialized syringe for ethylene assay using a gas chromatography (Model HP 5890, Hewlett Packard, USA) which was calibrated using standard ethylene gas (Laser Gases, New Delhi). The gas chromatography was equipped with Porapak-N (80-100 mesh) column and a flame ionization detector (FID). Nitrogen was used as the carrier gas at a flow rate of 30.0 mL min^{-1}, while hydrogen and air were fuel gases which had flow rate of 25.0 and 250.0 mL min^{-1}, respectively. The temperature of injector, column and detector were maintained at 110, 60 and 275 ^{0}C, respectively. The ethylene evolution rate was performed in triplicate and expressed as nmol g^{-1} DW h^{-1}.

4.4.3.2.3 Polyamine contents (µmol g^{-1}DW h^{-1})

Polyamines (putrescine, spermidine and spermine) were quantified by High Performance Liquid Chromatography (HPLC) using the method described by Flores and Galston (1982). Leaf sample were extracted in 5 % (v/ v) cold perchloric acid ($HClO_4$) at a ratio of about 100.0 mg tissues per mL $HClO_4$. After extraction for 1 h in ice bath, samples were pelleted at 48000 g for 20 min, and then supernatant phase containing the polyamine fraction was collected and stored at -20 ^{0}C in plastic vials (avoided glass vials since polyamines binds to glass). Standards and plant extracts were benzoylated according to Redmond and Tseng (1979). One mL of 2N NaOH was transferred to 250.0 µL of $HClO_4$ extract and PAs standards (putrescine, spermidine and spermine). Then 10.0 µL of benzoyl chloride was added and vortexed for 10 sec followed by incubation at room temperature for 20 min. Saturated NaCl, 2.0 mL was added with shaking and 2.0 mL diethyl ether was added. Centrifugation was done at 1500 g for 5 min, 1.0 mL of the upper ether phase was collected and allowed it to evaporate to dryness under a stream of warm air, and then redissolved in 100.0 µL methanol. These derivatized standards and samples were used for estimation

of polyamine contents through HPLC (Model Prostar 210, Varian, Palo Alto, CA, USA) equipped with quaternary pump, UV detector and connected with rheodyne injection system using Lichrospher stainless steel 5 µm particle size reverse-phase (C_{18}) column (250 mm x 4 mm), acetonitrile: 0.1 % O-phosphoric acid (52 : 48) as mobile phase at a flow rate of 1.0 mL min^{-1} and wavelength 254 nm. Standard curve was prepared using concentration ranging between 0.625-5 ppm. Under these conditions, the retention time of putrescine, spermidine and spermine was 3.41, 4.18 and 5.11 min, respectively.

4.4.3.2.4 Gene expression analysis

In order to determine the semi quantitative gene expression analysis of ethylene biosynthesis genes (*TaACS* and *TaACO),* ethylene response sensor (*TaERS1)* and polyamine biosynthesis genes (*TaSAMDC, TaSPDS* and *TaADC)*; total RNA was extracted from the leaves of 10 days old wheat seedlings after above mentioned treatment combinations. Isolation of total RNA was carried out by TRIzol® reagent (*Invitrogen*™, USA) and RNase-free DNase I (Promega, USA) was applied to remove contaminating genomic DNA at 37 ^{0}C for 1 h. Quality and integrity of total RNA were then determined by running appropriate amount in a formamide denaturing gel, and quantity of total RNA was determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized according to the instructions of the cDNA Synthesis Superscript® III First- Strand Synthesis System (*Invitrogen*™, USA). Resulting cDNA was stored at -20 ^{0}C and employed as template for two-step RT-PCR reactions following recommended conditions provide in user's manual. Sequences of gene specific forward and reverse primers employed are described below (**Table 3.3**). Every RT-PCR measurement was performed at least thrice. Expression of *TaActin* was used as an internal standard for normalization.

4.4.3.3 Statistical analysis

The results are expressed as means with standard error (S.E.). The significance difference (at $P < 0.05$) between control and stressed samples were determined by Duncan's multiple range tests at a significance level of 0.05 for all biochemical parameters and was evaluated by analysis of variance (ANOVA). ANOVA and

critical difference value were calculated by using SPSS 10.0 (SPSS Inc., Chicago, IL, USA), OPSTAT (hau.ernet.in/opstat.html) and Microsoft Excel.

4.4.4 Results

4.4.4.1 Effect of water deficit stress on relative water content (RWC; %) in wheat leaves

Osmotic stress induced by 20 % PEG-6000 (-4.91MPa) caused a decrease in RWC in all the three wheat genotypes *viz.* HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) (**Table 4.4.1** and **Fig. 4.4.1.A**). In susceptible genotype (HW 4059) RWC diminished more markedly (20 % reduction over the control) than that of tolerant genotype HW 4022 (13 % reduction in RWC). In HW 2078 the value of RWC was intermediate between drought susceptible and tolerant genotype (18 % reduction in RWC). There were no significant differences observed for genotypes under different treatment combinations (**Table 4.4.1** and **Fig. 4.4.1.B-D**).

4.4.4.2 Estimation of ethylene evolution rate (nmol g^{-1}DW h^{-1}) in wheat seedlings

To verify the role of polyamines and ethylene in the regulation of drought tolerance in wheat, chemicals involved in promoting or inhibiting ethylene level was applied to 10 days old wheat seedlings. Ethylene evolution rate was estimated by gas chromatography in genotypes HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) (**Table 4.4.2** and **Fig. 4.4.2.A-D**). Significant differences were observed amongst the genotypes for ethylene level in presence of chemical regulators like ethylene inducer (Ethrel, 10 ppm), ethylene biosynthesis inhibitor aminoethoxy vinylglycine (AVG, 2 ppm) and ethylene action inhibitor 1-methyl cyclopropene (1-MCP, 10 ppm) under control and osmotic stress condition (20 % PEG-6000 with osmotic potential of -4.91 MPa). Results showed that ethylene level increases consistently from tolerant to susceptible genotypes in both control and osmotic stress (**Table 4.4.2** and **Fig. 4.4.2.A**). Treatment with exogenous ethrel as a source of ethylene significantly promoted ethylene production and HW 4059 displayed the highest level amongst the genotypes under osmotic stress (**Table 4.4.2** and **Fig. 4.4.2.B**), while treatment with AVG drastically reduced ethylene production. The highest reduction was displayed in HW 4022 under osmotic stress

condition (**Table 4.4.2** and **Fig. 4.4.2.C**). In presence of ethylene action inhibitor 1-MCP, the level of ethylene amongst the genotypes followed the similar level as well as trend in their corresponding control and osmotic stress condition (**Table 4.4.2** and **Fig. 4.4.2.D**).

4.4.4.3 Estimation of polyamines level (µmol g^{-1}DW h^{-1}) in wheat seedlings

Polyamines level was estimated by high performance liquid chromatography (HPLC) in 10 days old seedlings of wheat genotypes HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) (**Table 4.4.6** and **Fig. 4.4.6**). Putrescine (**Table 4.4.3** and **Fig. 4.4.3**), spermidine (**Table 4.4.4** and **Fig. 4.4.4**) and spermine levels (**Table 4.4.5** and **Fig. 4.4.5**) in leaves of HW 4022 and HW 2078, increased after 20 % PEG-6000 treatment, contributed mainly by spermidine and spermine content whereas, in HW 4059 its level reduced markedly and became less than the control. Treatment with exogenous ethrel (ethylene inducer, 10 ppm) has no significant influence on content of putrescine, spermidine and spermine among the genotypes under both osmotic stress regimes, but HW 2078 showed remarkable increase in putrescine and spermine level under osmotic stress as compared to other two genotypes. Application of ethylene biosynthesis inhibitor (AVG, 2 ppm) significantly accelerated the content of these amines in HW 4022 and HW 2078 under osmotic stress condition while, in HW 2078 putrescine level hastened to significantly higher level as compared to spermidine and spermine. Genotype HW 4059 produced a reduced level of putrescine, spermidine and spermine under all treatment combinations. When the treatment of exogenous 1-MCP was given, there was a decrease in putrescine level in HW 4022 and HW 4059 under osmotic stress, while HW 2078 showed increase in putrescine, spermidine and spermine level.

4.4.4.4 Expression profiling of ethylene biosynthesis and signaling genes.

4.4.4.4.1 1-aminocyclopropane-1-carboxylic acid synthase 1 *(TaACS1)*

Expression profiling of genes associated with ethylene biosynthesis pathway was studied in 10 days old seedlings in wheat by semi-quantitative expression analysis (Reverse transcriptase polymerase chain reaction, RT-PCR). *TaACS1* (1-aminocyclopropane-1-carboxylic acid synthase 1) gene expression was studied using gene specific primers resulted in 342 bp amplicon size and further confirmed by sequencing (**Table 3.3**) in all the three genotypes (drought tolerant- HW 4022,

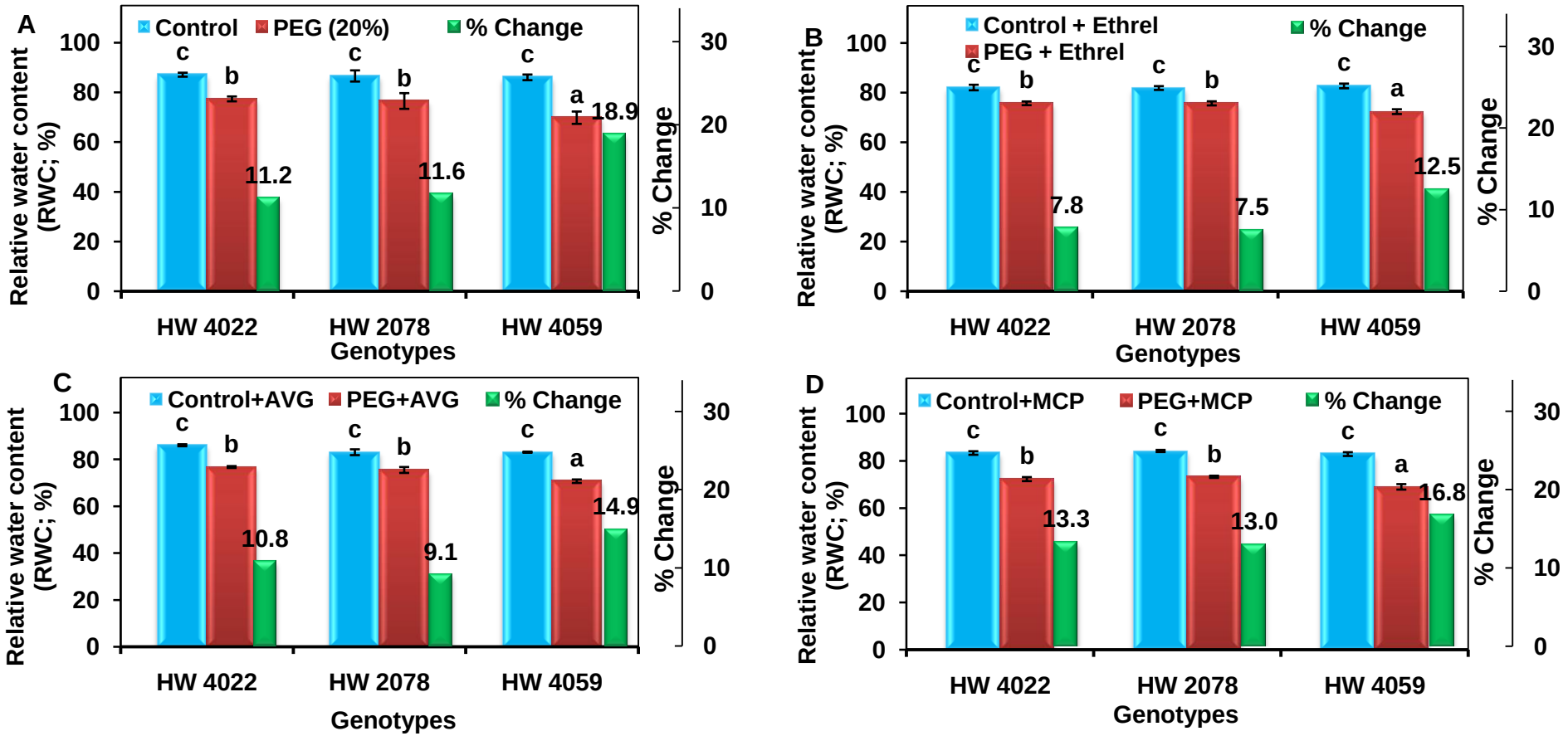


Fig. 4.4.1 Effect of osmotic stress on relative water content (RWC; %) in 10 days old wheat seedlings

Different panels showing various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91MPa) conditions.

B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .

C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.

D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Each value represents the mean (±SE) with three replicates each.

Abbrevation*:* PEG: Polyethylene gylcol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

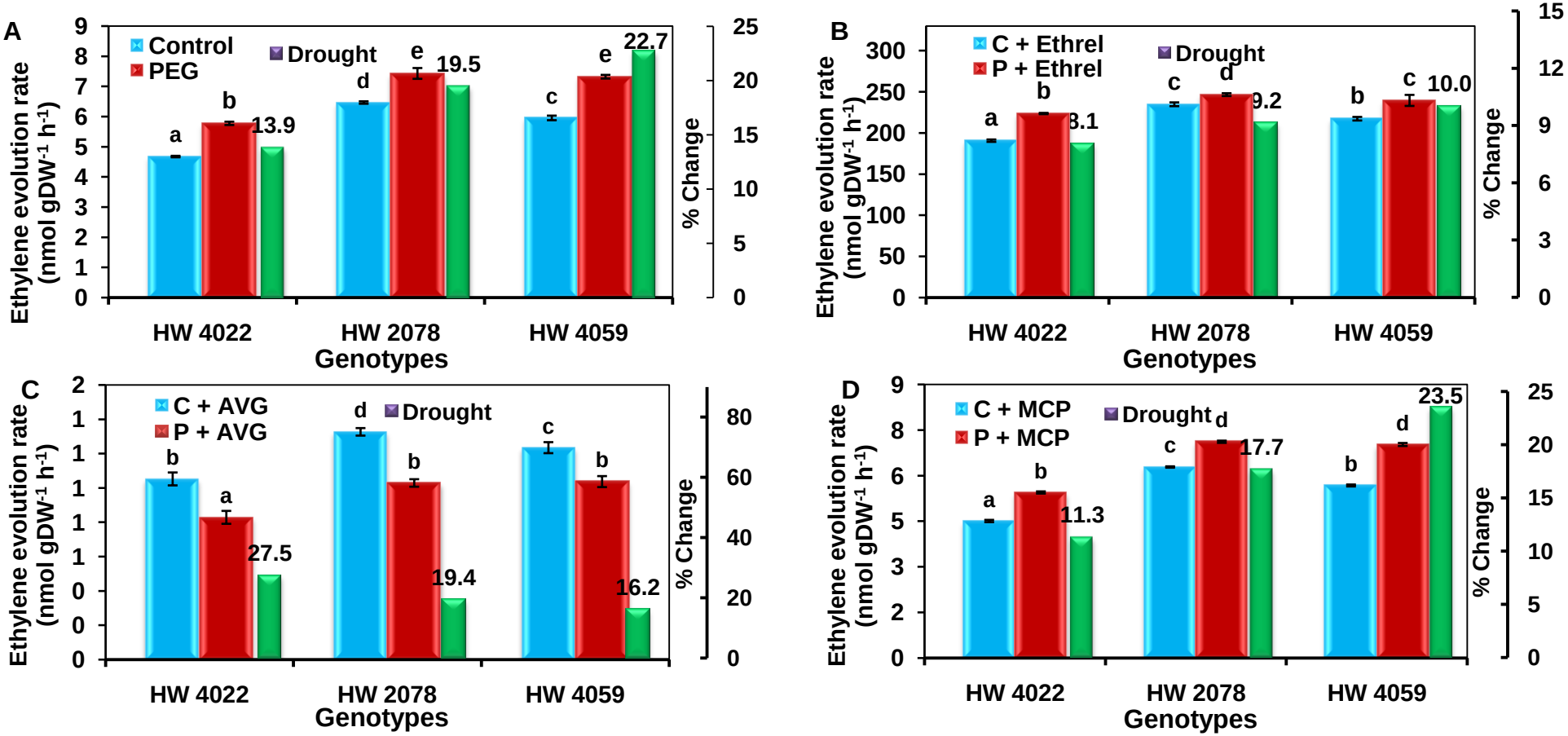


Fig. 4.4.2 Effect of osmotic stress on ethylene evolution rate (nmol gDW^{-1} h^{-1}) in 10 days old wheat seedlings

Different panels showing various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions.

B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .

C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.

D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Each value represents the mean (±SE) with three replicates each.

Abbrevation*:* PEG: Polyethylene gylcol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1 methyl cyclopropene

Putrescine content (µmol g DW^{-1})					
Genotypes		HW 4022	HW 2078	HW 4059	Mean
Treatment combinations	Control	4.26	4.01	1.62	**3.30**
	PEG	4.02	3.83	2.39	**3.41**
	Control+Ethrel	5.50	8.46	2.70	**5.55**
	PEG+Ethrel	5.56	9.12	2.93	**5.87**
	Control+AVG	7.33	5.89	1.94	**5.05**
	PEG+AVG	8.04	9.76	3.87	**7.22**
	Control+1-MCP	6.42	5.90	6.33	**6.22**
	PEG+ 1-MCP	3.84	8.05	2.40	**4.76**
	Mean	**5.62**	**6.88**	**3.02**	**5.17**
Statistics		CD at 5 %			SEM
	Genotype (G)	**0.216**			**0.076**
	Treatment (T)	**0.353**			**0.124**
	Interaction G x T	**0.612**			**0.215**

Table 4.4.3 Effect of osmotic stress on putrescine content (µmol g DW^{-1}) in 10 days old wheat seedlings

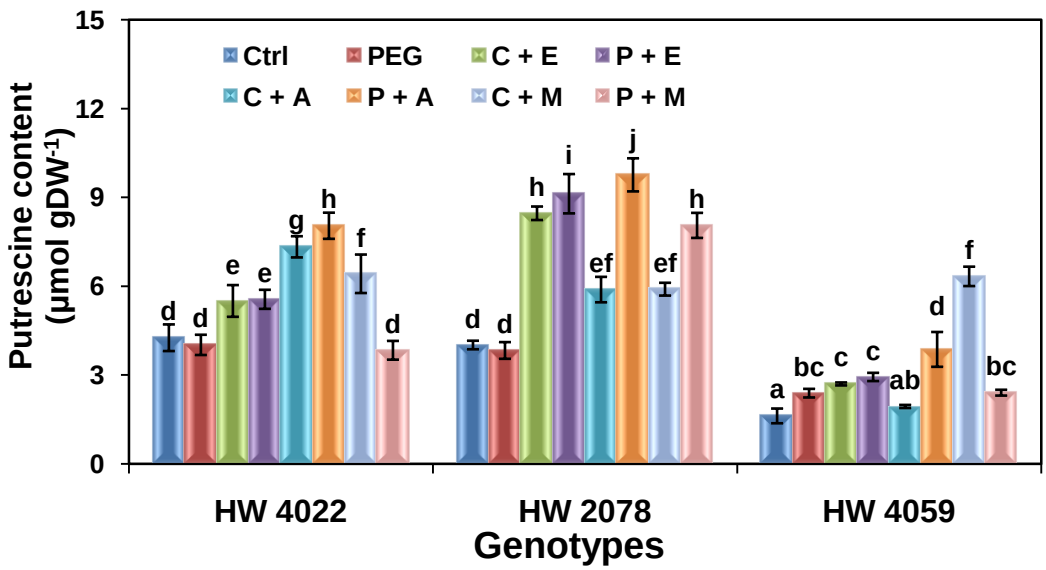


Fig. 4.4.3 Effect of osmotic stress on putrescine content (µmol g DW^{-1}) in 10 days old wheat seedlings. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*: C:** Control, P: PEG-6000, **E:** Ethrel, **A:** 1-Amino-ethoxy vinylglycine, **M:** 1-Methyl cyclopropane

Spermidine content (µmol g DW^{-1})					
Genotypes		HW 4022	HW 2078	HW 4059	Mean
Treatment combinations	Control	1.97	1.79	1.37	**1.71**
	PEG	2.92	2.42	1.05	**2.13**
	Control+Ethrel	2.58	5.51	1.49	**3.19**
	PEG+Ethrel	2.55	5.73	1.42	**3.23**
	Control+AVG	4.20	3.47	1.38	**3.02**
	PEG+AVG	5.37	5.02	1.56	**3.99**
	Control+1-MCP	2.93	3.20	2.12	**2.75**
	PEG+ 1-MCP	2.75	4.28	0.92	**2.65**
	Mean	**3.16**	**3.93**	**1.41**	**2.83**
Statistics		CD at 5 %			SEM
	Genotype (G)	0.115			0.04
	Treatment (T)	0.188			0.066
	Interaction G x T	0.326			0.114

Table 4.4.4 Effect of osmotic stress on spermidine content (µmol g DW^{-1}) in 10 days old wheat seedlings

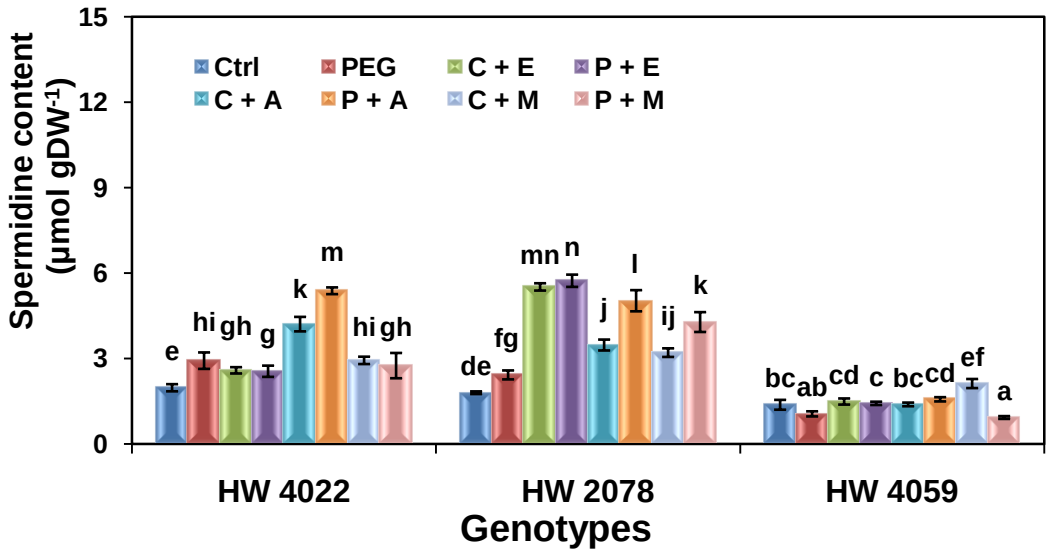


Fig. 4.4.4 Effect of osmotic stress on spermidine content (µmol g DW^{-1}) in 10 days old wheat seedlings. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*: C:** Control, P: PEG-6000, **E:** Ethrel, **A:** 1-Amino-ethoxy vinylglycine, **M:** 1-Methyl cyclopropane

Spermine content (µmol g DW^{-1})					
Genotypes		HW 4022	HW 2078	HW 4059	Mean
Treatment combinations	Control	1.20	1.93	2.19	**1.77**
	PEG	2.24	3.04	1.02	**2.10**
	Control+Ethrel	2.60	5.99	2.05	**3.55**
	PEG+Ethrel	2.37	6.80	1.63	**3.60**
	Control+AVG	5.15	4.76	1.93	**3.94**
	PEG+AVG	5.01	5.00	1.52	**3.84**
	Control+1-MCP	1.96	4.34	1.92	**2.74**
	PEG+ 1-MCP	2.10	5.03	0.91	**2.68**
	Mean	**2.83**	**4.61**	**1.64**	**3.03**
Statistics		CD at 5 %			SEM
	Genotype (G)	0.146			0.051
	Treatment (T)	0.238			0.084
	Interaction G x T	0.413			0.145

Table 4.4.5 Effect of osmotic stress on spermine content (µmol g DW^{-1}) in 10 days old wheat seedlings

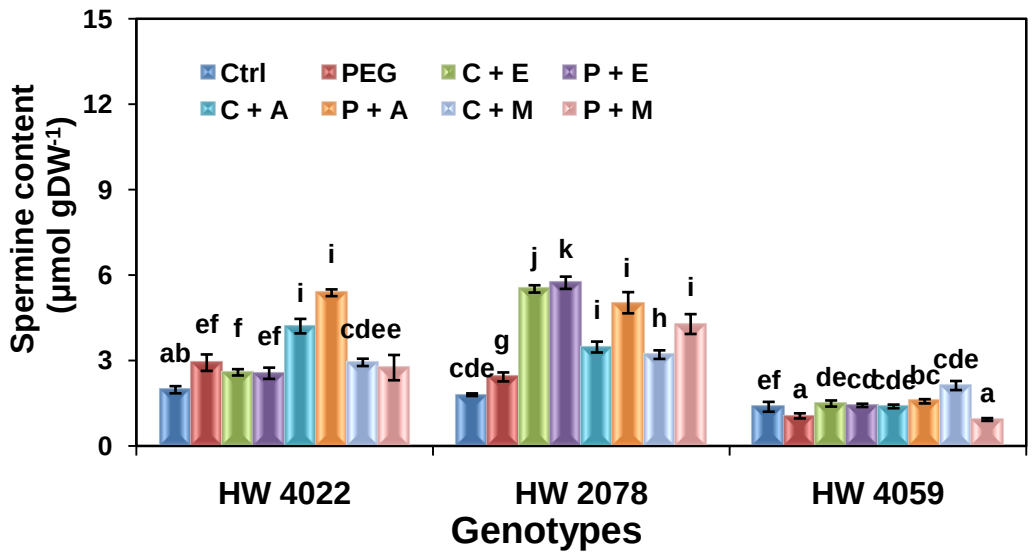


Fig. 4.4.5 Effect of osmotic stress on spermine content (µmol g DW^{-1}) in 10 days old wheat seedlings. Vertical bars indicate ± standard error of mean (n=3). The same letters above the columns indicate that the values are not statistically different (P < 0.05)

***Abbreviation*: C:** Control, P: PEG-6000, **E:** Ethrel, **A:** 1-Amino-ethoxy vinylglycine, **M:** 1-Methyl cyclopropane

Total polyamine content (µmol gDW^{-1})					
Genotypes		HW 4022	HW 2078	HW 4059	Mean
Treatment combinations	Control	7.43	7.74	5.18	**6.78**
	PEG	9.18	9.29	4.45	**7.64**
	Control+Ethrel	10.69	19.96	6.23	**12.29**
	PEG+Ethrel	10.47	21.65	5.98	**12.70**
	Control+AVG	16.68	14.11	5.25	**12.01**
	PEG+AVG	18.42	19.78	6.95	**15.05**
	Control+1-MCP	11.31	13.44	10.37	**11.71**
	PEG+ 1-MCP	8.69	17.36	4.23	**10.09**
	Mean	**11.61**	**15.42**	**6.08**	**11.04**
Statistics		CD at 5 %			SEM
	Genotype (G)	0.333			0.117
	Treatment (T)	0.544			0.191
	Interaction G x T	0.943			0.331

Table 4.4.6 Effect of osmotic stress on total polyamine content (µmol g DW^{-1}) in 10 days old wheat seedlings

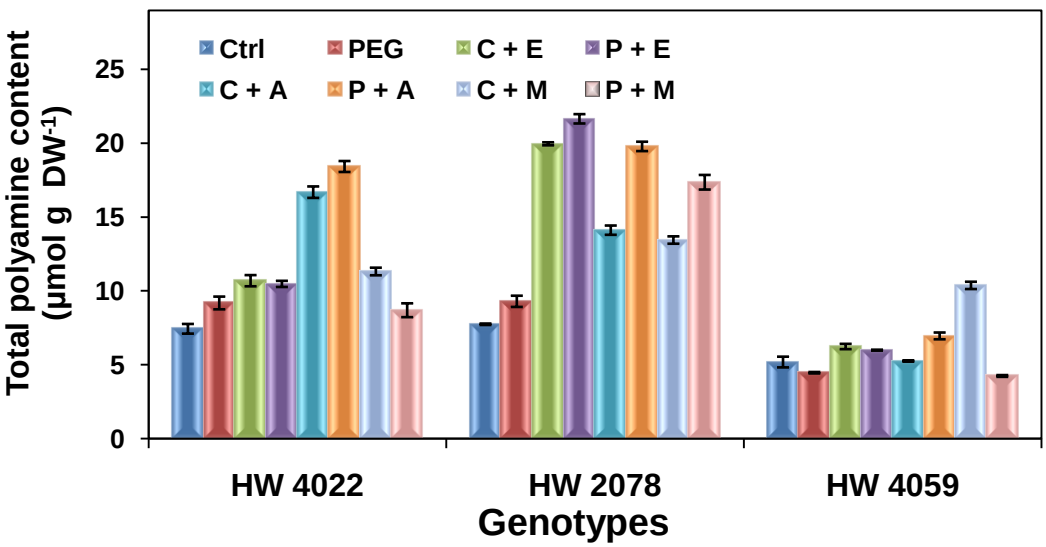


Fig. 4.4.6 Effect of osmotic stress on total polyamine content (µmol g DW^{-1}) in 10 days old wheat seedlings. Vertical bars indicate ± standard error of mean (n=3)

***Abbreviation*: C:** Control, P: PEG-6000, **E:** Ethrel, **A:** 1-Amino-ethoxy vinylglycine, **M:** 1-Methyl cyclopropane

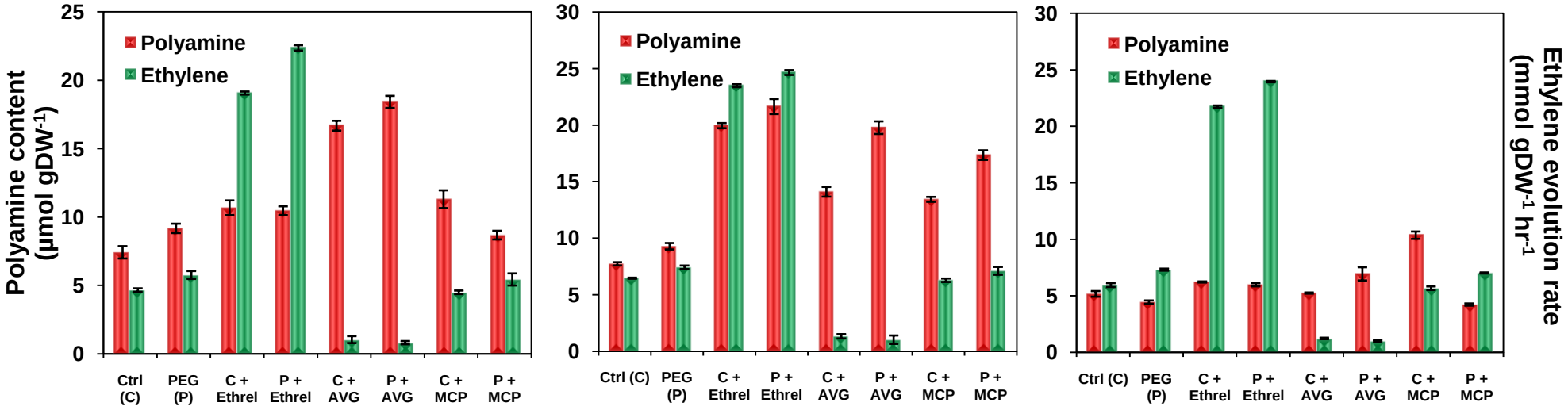


Fig. 4.4.7 Effect of osmotic stress on ethylene evolution rate and polyamine contents in 10 days old wheat seedlings
***Abbreviation*: C:** Control, P: PEG 6000, **E:** Ethrel, **A:** 1-Aminoethoxy vinylglycine, **M:** 1-Methyl cyclopropane

Relative water content (RWC; %)					
Genotypes		HW 4022	HW 2078	HW 4059	Mean
Treatment combinations	Control	87.16	86.66	86.09	**86.64**
	PEG	77.43	76.58	69.85	**74.62**
	Control+Ethrel	82.03	81.82	82.67	**82.17**
	PEG+Ethrel	75.67	75.65	72.31	**74.54**
	Control+AVG	86.02	83.04	83.07	**84.04**
	PEG+AVG	76.73	75.47	70.67	**74.29**
	Control+1-MCP	83.36	84.23	82.90	**83.49**
	PEG+ 1-MCP	72.29	73.27	69.01	**71.52**
	Mean	**80.09**	**79.59**	**77.07**	**78.92**
Statistics		CD at 5 %		SEM	
	Genotype (G)	1.247		0.437	
	Treatment (T)	2.036		0.714	
	Interaction G x T	3.527		1.236	

Table 4.4.1 Effect of osmotic stress on relative water content (RWC; %) in 10 days old wheat seedlings

Ethylene evolution rate (nmol g DW^{-1} h^{-i})					
Genotypes		HW 4022	HW 2078	HW 4059	Mean
Treatment combinations	Control	4.67	6.46	5.95	**5.70**
	PEG	5.77	7.43	7.32	**6.84**
	Control+Ethrel	208.51	229.78	262.10	**233.46**
	PEG+Ethrel	217.21	221.97	255.30	**231.49**
	Control+AVG	1.16	1.16	1.21	**1.18**
	PEG+AVG	0.84	0.95	1.06	**0.95**
	Control+1-MCP	5.62	6.24	6.31	**6.06**
	PEG+ 1-MCP	4.77	7.65	5.57	**6.00**
	Mean	**56.07**	**60.20**	**68.10**	**61.46**
Statistics		CD at 5 %		SEM	
	Genotype (G)	0.984		0.345	
	Treatment (T)	1.607		0.563	
	Interaction G x T	2.783		0.976	

Table 4.4.2 Effect of osmotic stress on ethylene evolution rate (nmol g DW^{-1} h^{-i}) in 10 days old wheat seedlings

relatively drought tolerant- HW 2078 and drought susceptible- HW 4059) by using various chemical regulators (ethylene inducer/ inhibitors) (**Fig. 4.4.8**). Significant differences were observed in gene expression level in control (RWC; 80-90%) and osmotic stress (RWC; 70-80 %). Amongst the three genotypes studied, susceptible genotype (HW 4059) showed higher *TaACS1* expression under osmotic stress condition while, lower expression was observed in case of tolerant genotype (HW 4022) under both osmotic regimes. Ethrel application (Ethylene inducer) showed a proportionate increase in the level of *TaACS1* expression in all the genotypes studied under control, but in HW 4059 its expression got decreased under osmotic stress. While, in presence of AVG (Ethylene biosynthesis inhibitor) the expression level of *TaACS1* gene reduces steadily in all the drought related genotypes studied. But in presence of 1-MCP (Ethylene action inhibitor) the expression of *TaACS1* gene was not following the consistent reduction in gene expression level.

4.4.4.4.2 1-aminocyclopropane-1-carboxylic acid oxidase 2 *(TaACO2)*

Semi-quantitative expression analysis of *TaACO2* (1-aminocyclopropane-1-carboxylic acid oxidase 2) gene expression was studied using gene specific primers resulted in 356 bp amplicon size and further confirmed by sequencing (**Table 3.3**) in all the genotypes (**Fig. 4.4.9**). Between the three genotypes, HW 2078 and HW 4022 showed less expression of *TaACO2* in presence of chemical regulators under both water regimes. Genotype HW 4059 showed higher expression of *TaACO2* in presence of ethrel and 1-MCP while, less expression was seen in presence of AVG under osmotic stress condition.

4.4.4.4.3 Ethylene response sensor 1 (*TaERS1*)

In case of Ethylene response sensor 1 (*TaERS1*) gene, semi-quantitative expression analysis was performed with gene specific primers led to an amplicon of size 401 bp and further confirmed by sequencing (Table 3.1.) in all the three genotypes (**Fig. 4.4.10**). Significant difference in gene expression was observed among the genotypes under control and osmotic stress condition. HW 4022 showed induced expression of *TaERS1* under osmotic stress compared to control condition while reverse trend was seen in HW 2078 and HW 4059. Application of ethylene inducer (ethrel) showed same pattern of expression in all the three genotypes but HW 4022 showed comparatively more expression under both osmotic potential regimes.

Treatment of AVG in HW 4022 slightly reduced the transcript level of *TaERS1* under osmotic stress while reverse pattern was seen in case of 1-MCP application. While, relatively drought tolerant genotype (HW 2078) showed the highest expression amongst the genotypes under osmotic stress condition in presence of both ethylene inhibitors (AVG and 1-MCP). Application of AVG and 1-MCP in HW 4059 resulted in reduced expression of *TaERS1* gene under osmotic stress as compared to control.

4.4.4.5 Expression profiling of polyamines biosynthesis genes.

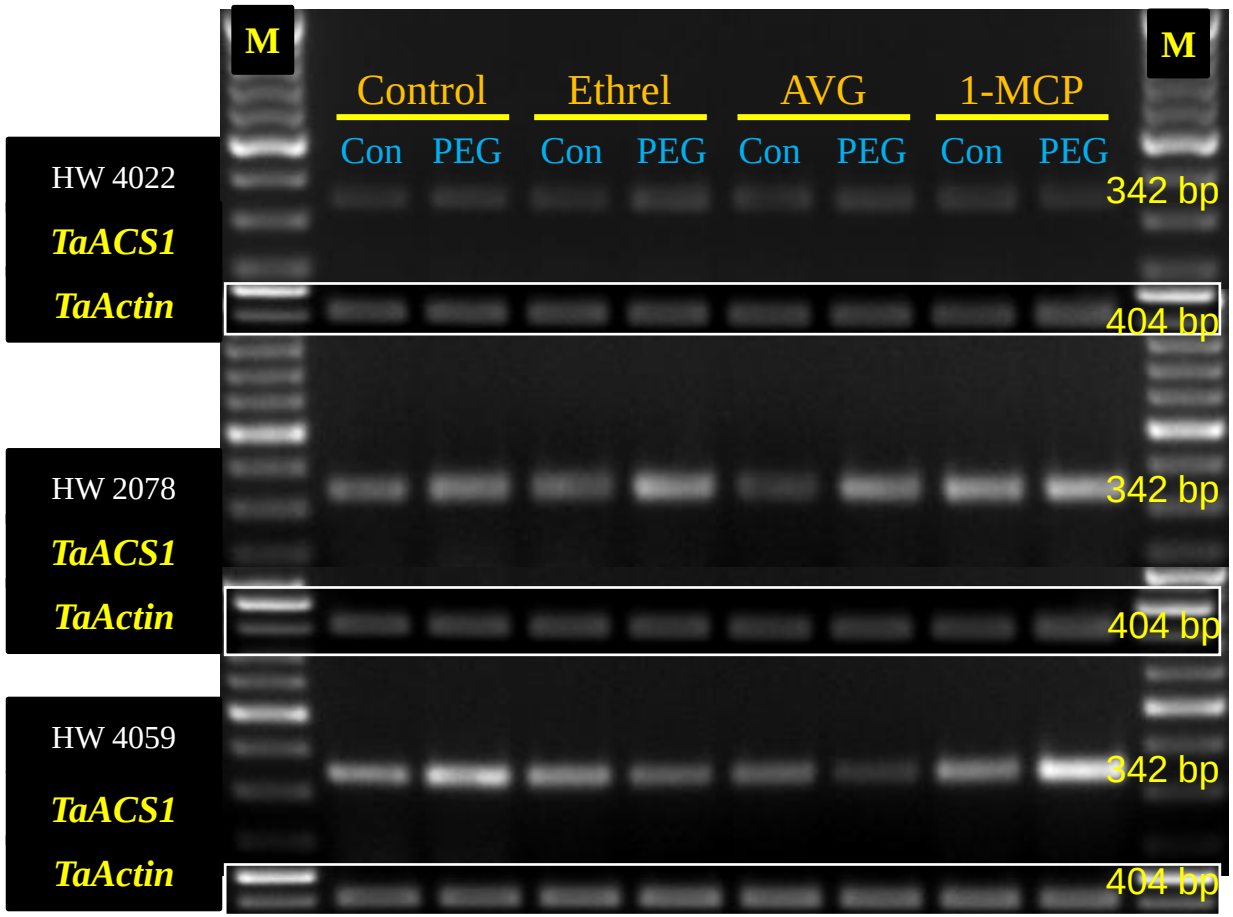
4.4.4.5.1 Arginine decarboxylase 2 (*TaADC2*)

Semi-quantitative expression profiling of putrescine biosynthesis gene arginine decarboxylase *(TaADC2)* using gene specific primers resulted in 315 bp amplicon size, confirmed by sequencing in three genotypes (**Table 3.3**). Significant difference in gene expression of tolerant and susceptible genotypes was observed. Application of PEG-6000 (20 %) showed no significant difference with control in all the genotypes (**Fig. 4.4.1.1**). PEG treatment in combination with ethrel and AVG resulted in increased transcript level of HW 2078 and HW 4059, but not in HW 4022. The 1-MCP treatment in combination with PEG resulted in decrease of *TaADC2* transcripts as compared to its control in HW 4022 and HW 4059 and the reverse trend was observed in intermediate genotype (HW 2078) during osmotic stress.

4.4.4.5.2 S-adenosylmethionine decarboxylase (*TaSAMDC*)

Expression profiling of genes associated with polyamine biosynthesis pathway was studied in wheat by semi-quantitative expression analysis of S-adenosylmethionine decarboxylase (*TaSAMDC*) using gene specific primers resulted in 376 bp amplicon size and further confirmed by sequencing in all genotypes (**Table 3.3**). In presence of chemical regulators significant genotypic difference was observed for gene expression pattern. Between the three genotypes studied, susceptible genotype (HW 4059) maintained the lower expression level of *TaSAMDC* gene under all treatment combinations. While, genotypes HW 4022 and HW 2078 showed comparatively higher expression of *TaSAMDC* under osmotic stress condition (**Fig. 4.4.12**). Application of ethylene inducer (ethrel) resulted in relatively lower expression of *TaSAMDC* in tolerant genotype HW 4022; while in presence of AVG and 1 MCP it maintained its transcript level under osmotic stress condition as compared to control.

ACS



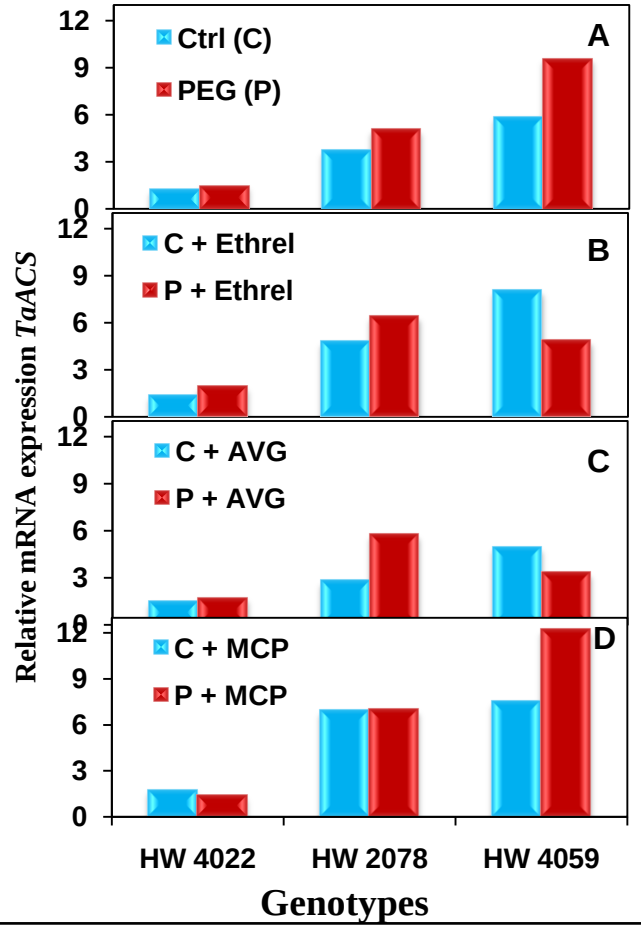
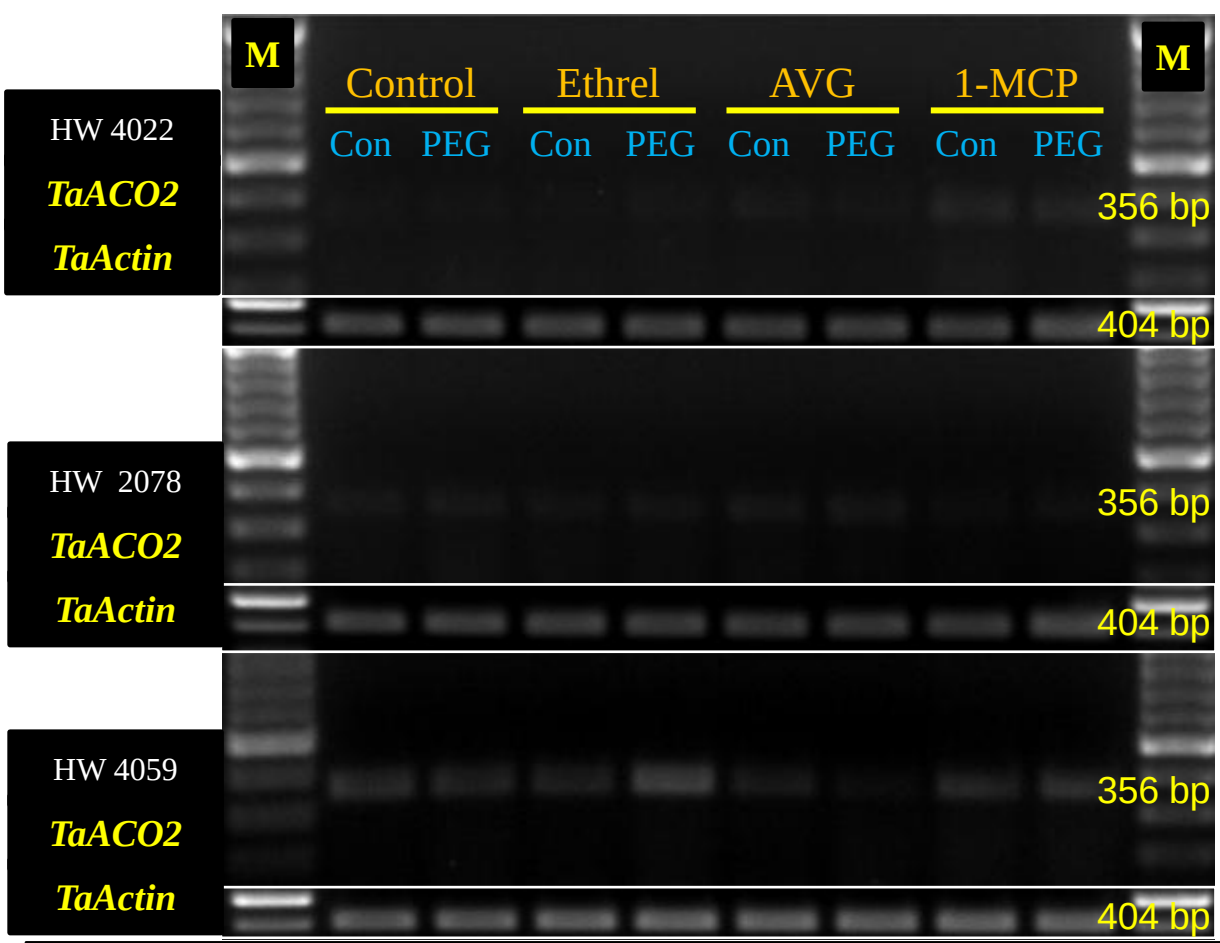


Fig. 4.4.8 Semi-quantitative expression analysis of *TaACS1* gene encoding enzyme involved in ethylene biosynthesis pathway in 10 days old wheat seedlings. Different panels showing relative mRNA expression under various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions
B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress
C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress
D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress

Abbrevation: ACS: 1-aminocylopropane-1-carboxylic acid synthase; PEG: Polyethylene glycol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

ACO



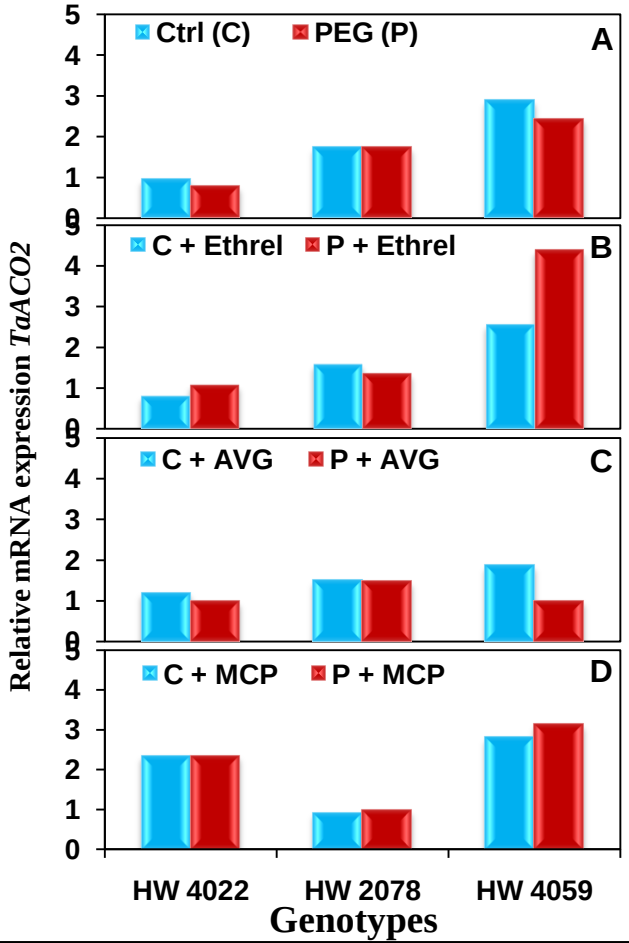


Fig. 4.4.9 Semi-quantitative expression analysis of *TaACO2* gene encoding enzyme involved in ethylene biosynthesis pathway in 10 days old wheat seedlings. Different panels showing relative mRNA expression under various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions.
B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .
C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.
D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Abbrevation: ACO: 1-aminocylopropane-1-carboxylic acid oxidase; PEG: Polyethylene glycol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

ERS

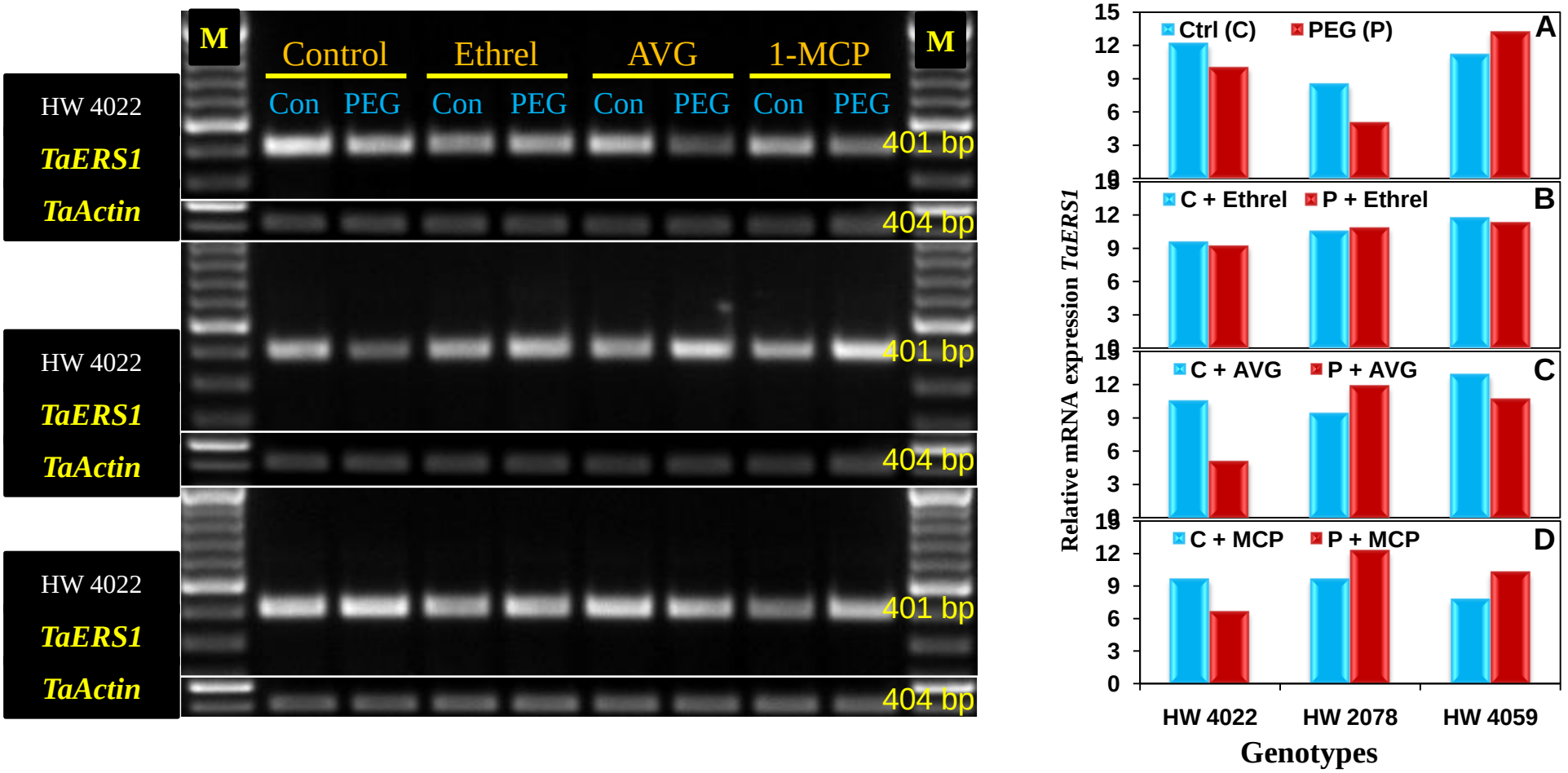


Fig. 4.4.10 Semi-quantitative expression analysis of *TaERS1* gene encoding enzyme involved in ethylene biosynthesis pathway in 10 days old wheat seedlings. Different panels showing relative mRNA expression under various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions.
B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .
C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.
D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Abbrevation: ERS: Ethylene response sensor; PEG: Polyethylene glycol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

ADC

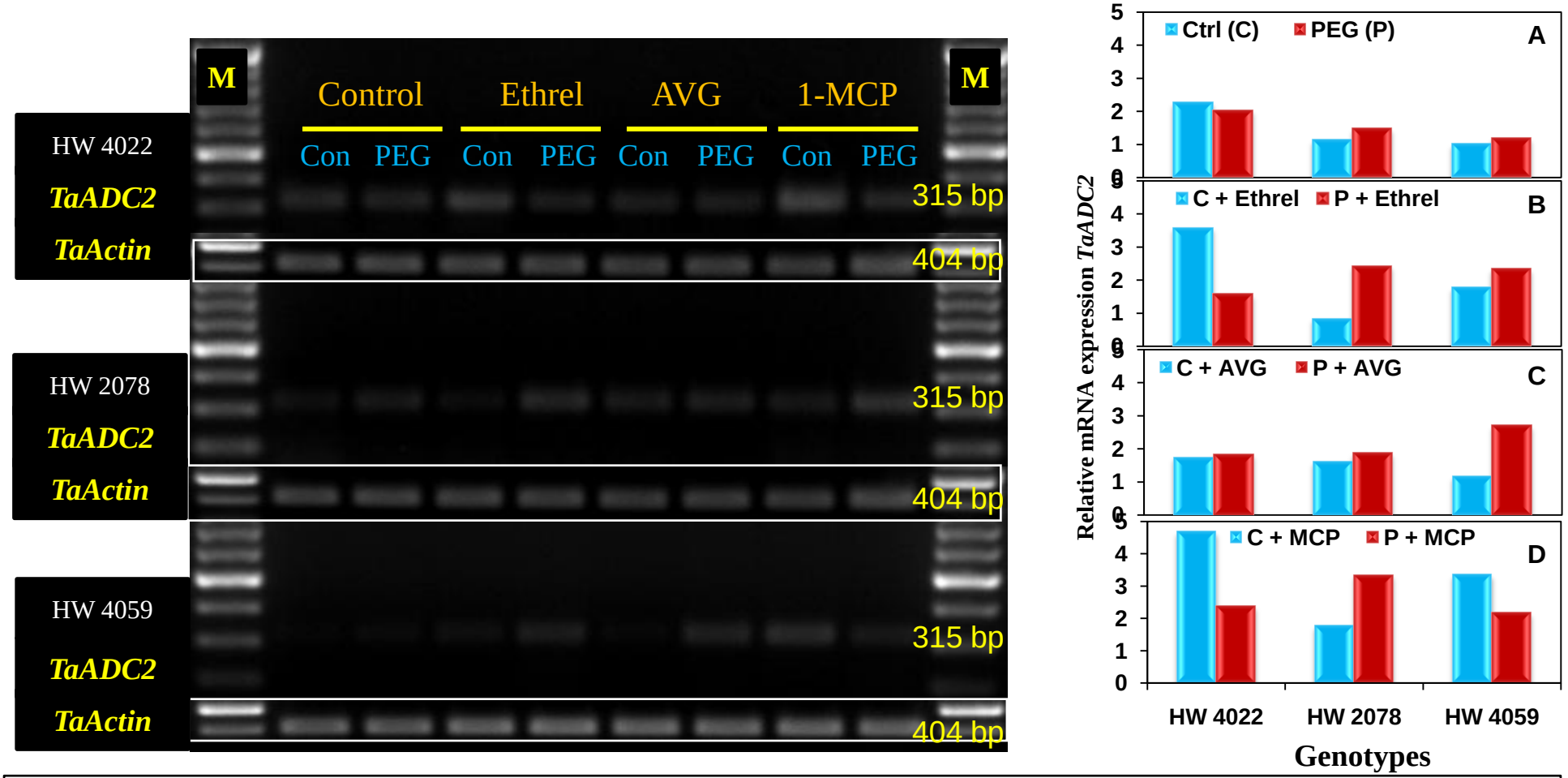


Fig. 4.4.11 Semi-quantitative expression analysis of *TaADC2* gene encoding enzyme involved in polyamine biosynthesis pathway in 10 days old wheat seedlings. Different panels showing relative mRNA expression under various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions.
B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .
C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.
D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Abbrevation: ADC: Arginine decarboxylase; PEG: Polyethylene glycol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

SAMDC

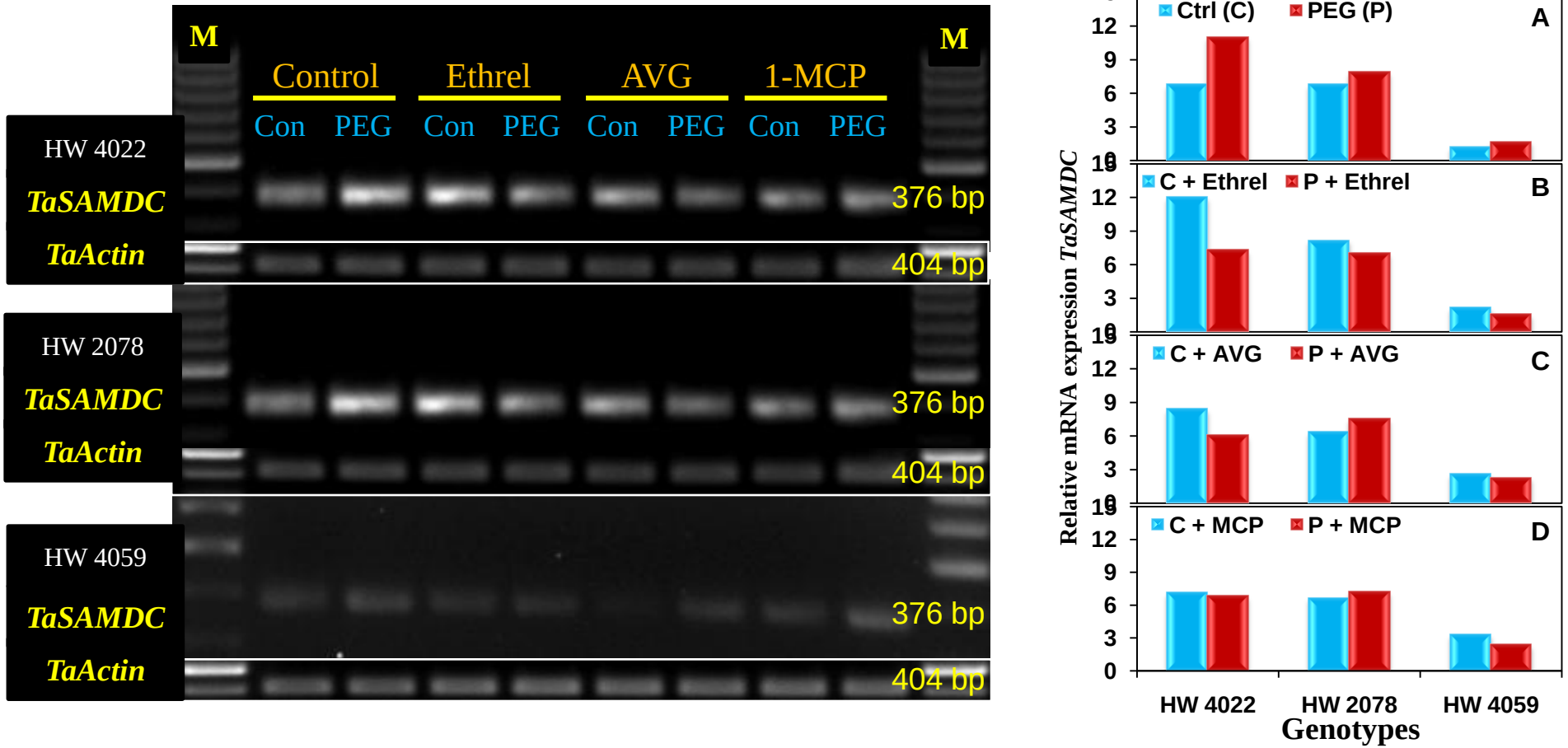


Fig. 4.4.12 Semi-quantitative expression analysis of *TaSAMDC* gene encoding enzyme involved in polyamine biosynthesis pathway in 10 days old wheat seedlings. Different panels showing relative mRNA expression under various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions.

B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .

C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.

D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Abbrevation: SAMDC: S-adenosyl methionine decarboxylase; PEG: Polyethylene glycol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

SPDS

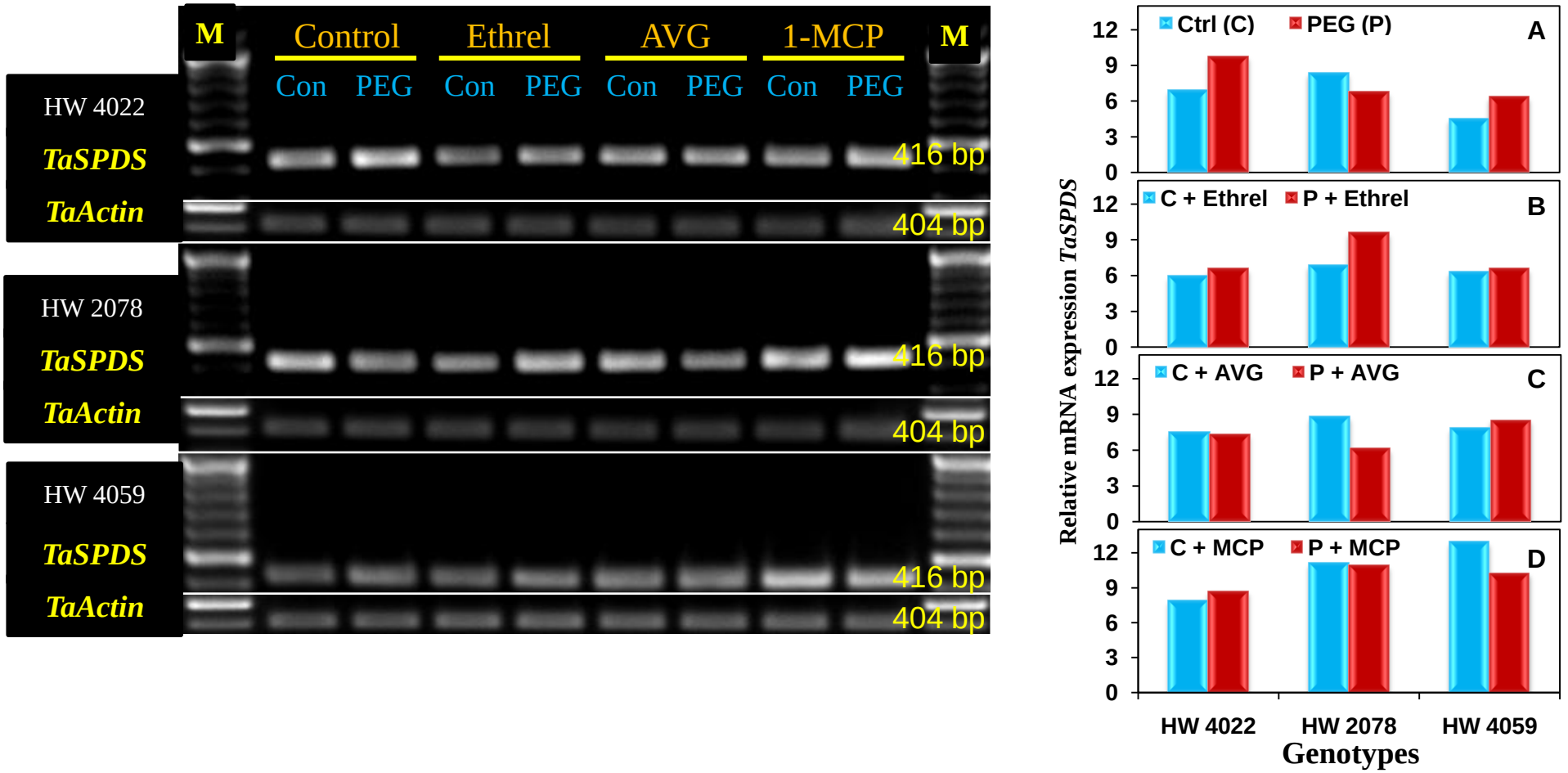


Fig. 4.4.13 Semi-quantitative expression analysis of *TaSPDS* gene encoding enzyme involved in polyamine biosynthesis pathway in 10 days old wheat seedlings. Different panels showing relative mRNA expression under various treatment combinations;

A) Control and osmotic stress (20 % PEG-6000, 4.91 MPa) conditions.
B) Ethylene inducer (Ethrel, 10 ppm) under control and osmotic stress .
C) Ethylene biosynthesis inhibitor (AVG, 2 ppm) under control and osmotic stress.
D) Ethylene action inhibitor (1-MCP, 10 ppm) under control and osmotic stress.

Abbrevation: SPDS: Spermidine synthase; PEG: Polyethylene glycol; AVG: Aminoethoxy vinylglycine; 1-MCP: 1-methyl cyclopropene

4.4.4.5.3 Spermidine synthase (*TaSPDS*)

Semi-quantitative expression analysis of spermidine biosynthesis gene spermidine synthase (*TaSPDS*) using gene specific primers resulted in 416 bp amplicon size, confirmed by sequencing in all genotypes studied (**Table 3.3**). As observed in case of *TaSAMDC* similar trend was also followed in expression profile of *TaSPDS* in tolerant genotype HW 4022 under osmotic stress condition (**Fig. 4.4.13**). In contrast to HW 4022, susceptible genotype HW 4059 showed less expression of *TaSPDS* transcript. Even in presence of ethylene inducer/ inhibitors HW 4022 maintained its optimum transcript level under both osmotic regimes.

4.4.5. Discussion

To clarify the possible role played by ethylene and polyamines for drought tolerance mechanism in wheat crop, three wheat genotypes differing in their response to osmotic stress conditions at 10 days old seedling stage were studied. Genotypes *viz*. HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) were selected from thirty-five wheat genotypes on the basis of their response to water deficit stress in pot culture conditions as reported in our previous study. Although several studies have been published on the response of wheat crop to drought stress condition (Grzesiak *et al*., 2013, Yang *et al*., 2014); the potential regulation of ethylene and polyamines biosynthesis pathway for mediating drought tolerance in wheat crop is not yet clear.

Plant tolerance to water deficit stress is often associated with higher relative water content (RWC; %) of leaves. RWC is a good indicator of plant water status than thermodynamic state variables (water potential, turgor potential and solute potential) (Sinclair and Ludlow, 1985). From the earlier studies by Filek *et al*. (2012) it was known that PEG treatment stimulated osmotic stress condition. Previous work done by Liu *et al*. (2004) reported that osmotic stress induced by PEG decreases the leaf relative water content and increases leaf relative electrolytic leakage in drought sensitive wheat genotype, and these changes were reverse in case of drought tolerant genotype. In the present study, RWC was determined to give an indication of plant water status where it decreased with osmotic stress in all the genotypes studied. Osmotic stress by polyethylene glycol (20 % PEG-6000) showed leaf relative water content of 80-90 % under control and 75-80 % under osmotic stress conditions for

three genotypes. Therefore, from our findings, it was confirmed that HW 4022 is osmotic stress tolerant, HW 2078 relatively osmotic stress tolerant and HW 4059 is osmotic stress sensitive. Similar findings by Marok *et al*. (2013), in two barley genotypes differing in their drought sensitivity, where sensitive genotype showed lower RWC with accelerated wilting as compared to tolerant genotype. The present findings were also in agreement with results of Khanna-Chopra and Selote (2007) that in two wheat genotypes the drought tolerant (C 306) recovered better after severe water stress than susceptible genotype (Moti) by maintaining its leaf water status. Supporting observations in wheat genotypes were studied by Aprile *et al*. (2013) where, susceptible genotype showed lower RWC in comparison to tolerant one reflecting its susceptibility to drought stress. From our findings it was clear that genotypic variations in terms of RWC may be due to differences in osmotic adjustment in order to maintain tissue turgor and normal physiological functions to cope up with water deficit stress conditions.

The tolerant genotype (HW 4022) in our study was observed with higher levels of polyamines in general and particularly, spermidine and spermine under osmotic stress condition. Similar findings were reported by Grzesiak *et al*. (2013) in wheat genotypes differing in drought tolerance when exposed to polyethylene glycol (-1.5 MPa) for two days; tolerant genotypes showed marked increase in polyamines content especially, spermidine and spermine in leaves under osmotic stress condition in contrast to susceptible one. Our results are consistent with previous findings of Do *et al*. (2013) in rice genotypes exposure to drought stress led to a marked decrease in putrescine and corresponding increase of spermidine and spermine level. This is in agreement with our observations that increased spermidine and spermine levels in a drought-tolerant wheat genotype while, a drought-sensitive genotype accumulated higher level of putrescine. To further support our result in wheat, the work by Yang *et al*. (2001) in rice also observed that higher levels of spermidine and spermine promote grain filling and increase the grain weight of rice under drought stress whereas, putrescine had an opposite effect. These reports were also in agreement with recent findings by Chen *et al*. (2013) that under water deficit stress there was increase in spermidine and spermine content during grain-filling stage in rice which accelerates grain filling rates with increased grain yield.

It was known previously that polyamines and ethylene shares a common biosynthetic precursor SAM, and increased in spermidine and spermine biosynthesis are likely to affect the ethylene evolution rate (Liang and Lur, 2002; Feng *et al*., 2011). The present study indicated that the drought susceptible genotype (HW 4059) was more sensitive to osmotic stress with enhanced ethylene evolution rate than that of drought tolerant genotype (HW 4022) and hence seemed to be unfavorable for drought condition, while reverse findings observed in tolerant genotype. These variations in ethylene evolution rate of the studied genotypes are consistent with polyamines level. The result thus indicates that a reduction in ethylene biosynthesis would increase the spermidine and spermine levels confirming our hypothesis that ethylene exhibited an antagonistic relationship with polyamines under osmotic stress condition (**Fig. 4.4.7**). Similar findings reported by Chen *et al*. (2013) in rice genotypes subjected to severe water deficit stress, during which the content of spermidine and spermine increased with decreased ethylene evolution rate. Comparable results were also reported in maize (Feng *et al*., 2011), tomato (Nambeesan *et al*., 2012) and sorghum (Yin *et al*., 2014). Recent report by Yang *et al*. (2014) provide an evidence on the interactive role of ethylene and polyamines during grain filling period in wheat, that increased grain yield under drought stress is positively correlated with the increased ratio of spermidine/ ethylene or spermine/ ethylene.

Our findings suggested that exogenous application of ethylene inducer (ethrel) intensified apparently the PEG-induced injury to drought-sensitive genotype (HW 4059) by increase in ethylene evolution rate together with inhibition of spermidine and spermine content. This reduction may be closely associated with increased transcription of *TaACS1* and *TaACO2* with inhibition of *TaSAMDC* and *TaSPDS* transcript level. Whereas the results was reversed after treatment of ethylene inhibitors (AVG and 1-MCP) confirming that it would be better to have higher content of particular polyamines (spermidine and spermine) in comparison to ethylene under osmotic stress condition. Whereas, tolerant genotype (HW 4022) showed increased transcript for polyamine biosynthesis gene with greater promotion of the PEG induced spermidine and spermine level and down regulation of ethylene biosynthesis genes under above discussed treatments. Previous report by Wang *et al*. (2012) in rice supports our findings that in presence of ethylene inhibitor, ethylene evolution rate decreased significantly during grain filling stage with enhancement in

spermidine and spermine content while, the application of ethylene releasing compound showed an opposite effect. Our study further supported by Locke *et al*. (2000) that blocking the ethylene biosynthesis by exogenous application of polyamines or ethylene biosynthesis inhibitors enhanced putrescine and spermidine content in germinating barley grains. Our findings were also in agreement with Chen *et al*. (2013) in rice where they suggested that polyamine play positive while, ethylene has negative role in cereal crops under water deficit stress condition. The molecular mechanism by which polyamines facilitates drought tolerance is not well understood. In our study we found the down-regulation of ethylene biosynthesis gene *TaACS1* and *TaACO2* in tolerant genotype as compared to susceptible one. Our results are in accordance with Habben *et al*. (2014) that down-regulation of ethylene biosynthesis gene (ACS) improves the performance of maize under drought stress condition. Previous reports in wheat as per Ma and Wang (2003) the expression pattern of *ERS1* was induced in leaf and stem in presence of senescence inducer like wounding, ABA and Jasmonate. We also studied ethylene signaling receptor molecule Ethylene response sensor 1 (*TaERS1*) expression, probably first time, in wheat crop during osmotic stress treatment whereby, tolerant genotype (HW 4022) were observed with higher expression of *TaERS1* under osmotic stress in presence of ethylene action inhibitor 1-MCP in comparison to susceptible genotypes (HW 4059) which contributes to its osmotic stress tolerance. In support to our findings Zhao and Schaller (2004) shown that ethylene reduced the expression level of its receptor *ERS1* in *Arabidopsis* when exposed to salinity or osmotic stress condition and act as a negative regulators of ethylene responses. Recent report by Do *et al*. (2013) in rice provide an evidence that expression of arginine decarboxylase (ADC), spermidine synthase (SPDS) and spermine synthase (SPMS) genes showed a correlation with the drought tolerance in rice genotypes, among which ADC exhibited a higher expression, while SPDS/ SPMS showed lower expression in sensitive genotypes. In our study the response of ADC expression in sensitive genotype (HW 4059) of wheat are on par with the above findings in rice. Similar findings reported in pea seedlings by Gil-Amado *et al*. (2012) and in olive by Wu *et al*. (2011) in that in presence of ethylene, transcript of ADC and SAMDC decreased ultimately resulting in reduced polyamine levels.

4.4.6. Conclusion

In conclusion, results from our study indicate that in contrast to ethylene, polyamines (spermidine and spermine) were associated with osmotic stress tolerance in tolerant wheat genotype. We also found that reduced expression of ethylene biosynthesis genes (ACS and ACO) in presence of ethylene inhibitors (AVG and 1-MCP) was responsible for enhanced polyamine content with increased spermidine/ ethylene and spermine/ ethylene ratio under osmotic stress condition. Further our findings confirmed a cross-talk between polyamines and ethylene at their biosynthesis level which regulates osmotic/ drought stress tolerance. Polyamines, especially spermidine and spermine play an important role in regulating drought tolerance mechanism in wheat crop. Further investigation by reverse genetics approaches is needed to understand the novel role of polyamines for drought tolerance in wheat.

4.5. RESEARCH PAPER V

Cloning and expression analysis of ethylene biosynthesis gene ACC oxidase in wheat (*Triticum aestivum* L.)

4.5.1 Abstract

The phytohormone ethylene regulates many aspects of plant growth and responses to environmental stresses. However, the exact role played by ethylene in drought stress remains unclear. Here, we proposed that ethylene regulates plant responses to drought stress in wheat. To test this hypothesis, an experiment was conducted with three wheat genotypes differing in their drought tolerance efficiency at reproductive stage. Results showed that the drought susceptible genotype (HW 4059) displayed the higher expression of ethylene biosynthesis genes ACC synthase and ACC oxidase (ACC, 1-aminocyclopropane-1-carboxylic acid) under water deficit stress in comparison with tolerant ones (HW 4022, HW 2078). Furthermore, cloning and *in-silico* characterization of ACO showed conserved domains essential for ACC, Fe^{2+} and ascorbate binding and enzyme activity. The multiple sequence alignment had shown the conserved domains specific for this gene family in other plant species. The result suggests that *TaACS1* and *TaACO2* genes are involved in drought tolerance mechanism in wheat. To our knowledge this is the first report on the molecular analysis of ACO gene in wheat and its regulation under drought stress.

***Key words*:** Ethylene, Drought, Cloning, *TaACS1*, *TaACO2*, *Triticum aestivum* L.

4.5.2 Introduction

Global climatic change is predicted to vary rainfall patterns, temperature and increases in frequency of extreme climatic events (Harrison *et al*., 2014). Today, a key challenge for plant growth is water shortage throughout the world, limiting crop yields and this further gain an importance in future as agricultural activities expand to less fertile areas to satisfy food demand of growing population (Flexas *et al*., 2013). Wheat is a staple crop that plays a major role in global food security and one of the most highly cultivated cereal crop in the world. Like other cultivated crops, wheat production is significantly affected by drought stress (Farooq *et al*., 2014). Water deficit stress affects wheat growth at all the phenological stages among which reproductive stage is highly sensitive owing to its impact on grain yield (Pradhan *et al*., 2012). Multiple wheat varieties adapted to different climatic conditions and suitable for different regions have been developed through conventional breeding approaches; however, the molecular basis of such adaptations remains unknown in most cases (Alvarez *et al*., 2014). Plants cannot completely escape from the adverse affects of drought, so they develop various strategies at their physiological and molecular level through alteration in cellular metabolism, changes in gene expression, enzyme activities and synthesis and signaling of phytohormones in a highly coordinated manner (Kohli *et al*., 2013) to cope with the stress. In order to understand better on the mechanism underlying drought tolerance in wheat crop, we have to investigate the role played by phytohormone in response to drought stress.

The phytohormone ethylene is a simple gaseous hormone that plays a key role in regulating plant growth, development and environmental stress responses (Abeles *et al*., 1992, Schaller, 2012, Shakeel *et al*., 2012). Ethylene is biologically active in very small amount and its biosynthesis from the amino acid methionine is a relatively simple metabolic pathway that has been extensively studied and well documented in plants (Meng *et al*., 2014). The methionine is converted to SAM (S-adenosyl-L-methionine) by the enzyme SAM synthetase. The SAM is then converted to ACC (1-aminocyclopropane-1-carboxylic acid) and 5′-deoxy-5′-methylthioadenosine (MTA) by the enzyme ACC synthase (ACS). The conversion of SAM to ACC is the rate limiting step in the ethylene biosynthetic pathway and ACC as a key metabolic intermediate. The ACC oxidase (ACO) catalyzes the final step of conversion of ACC to ethylene along-with CO_2 and hydrogen cyanide

(HCN) gas as by-products and it is an oxygen dependent step and, under anaerobic conditions, its formation is completely suppressed (**Fig. 2.3**). In this reaction, Fe^{+2} and ascorbate are required as a cofactor and a co-substrate, respectively. The other reaction product, MTA must be recycled back into the methionine pathway to provide continuous supply of methionine as substrate for ethylene production. The poisonous gas HCN formed is detoxified by β-cyanoalanine synthase (Yan and Hoffman, 1984; Kende, 1993; Iqbal *et al*., 2013).

Various environmental stresses regulate the expression of ACS and ACO the key enzymes in ethylene biosynthesis pathway. It was reported that under drought stress there is an increment in ethylene production rate and this may be due to stress induced increase in ACS and ACO enzyme activities which is detrimental for plant growth and development. Therefore, by modulating ethylene biosynthesis, we can reduce the stress induced injuries in plants. In plants, ACS and ACO are encoded by multi-gene families in response to external and internal stimuli (Iqbal *et al*., 2013). A substantial amount of information exists on molecular characteristic of ACS in various plant species *viz*. rice (Zarembinski and Theologis, 1993), maize (Gallie and Young, 2004). However, there is no report on cloning and characterization of ACC oxidase in wheat. To aid to our understanding, study was conducted to investigate the role played by ethylene in response to drought stress in wheat crop, we examined the transcript level of ethylene biosynthesis genes (*TaACS1* and *TaACO2*) in wheat genotypes differing in their drought tolerant characteristics. Expression analysis studies showed that there is a marked decrease in transcript of *TaACS1* and *TaACO2* gene in tolerant genotypes (HW 4022 and HW 2078) under water deficit stress, suggesting a negative correlation between ethylene biosynthesis gene and drought tolerance in wheat under water deficit stress. To understand the molecular characteristics of ACC oxidase, a full-length coding sequence of *TaACO2* was cloned for the first time from wheat. The deduced amino acid sequence from this clone showed that the polypeptide contains the conserved Fe^{+2} 2-oxoglutarate dioxygenase domain that are the characteristic of ACO gene family.

4.5.3 Materials and methods

4.5.3.1 Plant materials and treatments

From our preliminary experiment conducted in pot culture condition, we selected three wheat genotypes based on their drought tolerance efficiency *viz*., HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). These genotypes were grown in pot culture with recommended package of practices and subjected to water deficit stress at 50 % anthesis stage by withholding irrigation for 10 days. Scheduled routine of irrigation was practiced for control plants throughout the crop growth period (**Table 3.2**). For molecular analysis flag leaf sampling was done from both control and stressed plants.

4.5.3.2 Molecular analysis of chlorophyll degrading genes

4.5.3.2.1 RNA isolation and cDNA synthesis

In order to determine the semi-quantitative gene expression analysis of ethylene biosynthesis genes *TaACS1* and *TaACO2*; total RNA was extracted from the flag leaf sample of control and water stress plants of three wheat genotypes namely, HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). Isolation of total RNA was carried out by TRIzol® reagent (*Invitrogen*™, USA) and RNase-free DNase I (Promega, USA) was applied to remove contaminating genomic DNA at 37 ^{0}C for 1 h. Quality and integrity of total RNA were then determined by running appropriate amount in a formamide denaturing gel, and quantity of total RNA was determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized according to the instructions of the cDNA Synthesis Superscript® III First- Strand Synthesis System (*Invitrogen*™, USA). Resulting cDNA was stored at -20 ^{0}C and employed as template for two-step RT-PCR reactions following recommended conditions provide in user's manual.

4.5.3.2.2 Primer designing

The primers employed in the present study were designed using the bioinformatics tools described in detail as follows. Search for nucleotide sequence in

NCBI database (http://www.ncbi.nlm.nih.gov/) for ACO gene in wheat failed to retrieve any results. Hence we selected the protein sequence of ACO2 from rice (GenBank accession no. AAC05507) and performed TBLASTn analysis with nucleotide sequence database of NCBI in wheat. We obtained a cDNA sequence (GenBank accession no. AK332340) having 98 % coverage and 82 % identity with rice ACO2 sequence as putative *TaACO2*. The complete open reading frame (ORF) coverage was confirmed by ORF finder tool of NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/) and the deduced amino acid sequence using ExPASy translate (http://web.expasy.org/translate/) of the putative ACO2 gene showed significant homology with rice and *Arabidopsis* protein sequences by BLASTp analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). This putative sequence of ACO2 was used for designing gene specific primers.

Search for nucleotide sequence in NCBI database for ACS gene in wheat retrieved a partial sequence of ACS1 (GenBank accession no.U35779). To obtain a complete sequence towards 3′end, BLASTn analysis with expressed sequence tag (EST) database of NCBI was performed which retrieved a nucleotide sequence (GenBank accession no. CK163652) from wheat. The two sequences were aligned by BioEdit software and the resulting contig was used for designing gene specific primers for ACS1 in wheat. The gene specific primers were designed manually and quality parameters were confirmed using Integrated DNA Technologies (IDT) Oligoanalyzer tool (https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). The primers used for semi-quantitative gene expression analysis (RT-PCR) of ethylene biosynthesis genes (*TaACS1* and *TaACO2*) and cloning of *TaACO2* are listed in **Table 3.3** Every RT-PCR measurements were performed at least thrice. Expression of *TaActin* was used as an internal standard for normalization.

4.5.3.2.3 Cloning and sequencing of the *TaACO2* cDNA

PCR amplification with gene specific primers (Table 1.) and proofreading enzyme Platinum Hi-fidelity Taq DNA polymerase (*Invitrogen*^{TM}, USA) gave approximately 1.2 kb fragment that was cloned in TA cloning vector pTZ57R/T (Thermo Scientific, USA). The construct was transformed into *E. coli* strain XL1-Blue and confirmed by blue-white screening in a media containing IPTG, X-gal and Kanamycin. Positive clones were sub-cultured in medium having same composition

and further confirmed by colony PCR, restriction digestion and sequencing (**Fig. 2.2** and **Fig. 2.3**).

4.5.3.2.4 Sequence analysis of *TaACO2* gene

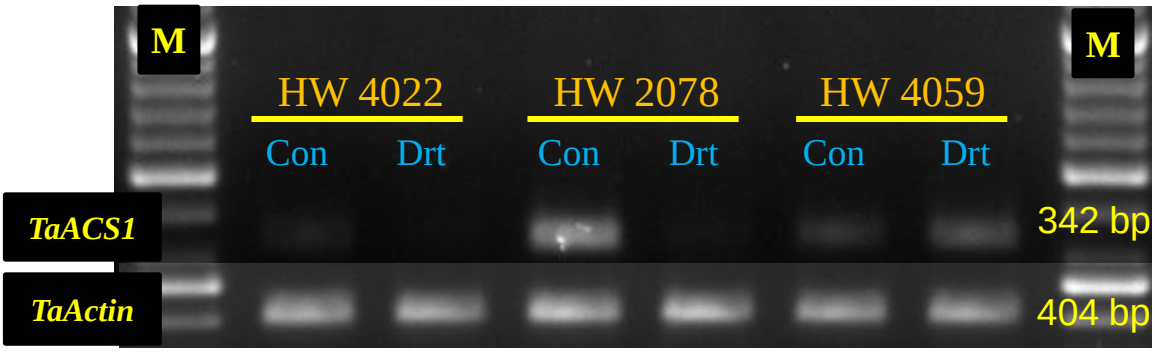
The BioEdit 7.1 software (http://bioedit.software.informer.com/7.1/) was used for aligning forward and reverse sequencing results and the complete nucleotide sequence was reconstructed. The reconstructed sequences were translated using ExPASy Translate Tool (http://web.expasy.org/translate/) and deduced amino acid sequences were obtained for *TaACO2*. These results were confirmed by BLASTp software online (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Isoelectric point (pI) and molecular weight (MW) were analyzed by ExPASy ProtParam tool (http://www. expasy.org/protparam). The multiple protein sequence alignment of *TaACO2* and its homologs from model species *viz*. rice and *Arabidopsis* were carried out by ClustalW2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and represented using BoxShade server (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic tree was constructed with homologous protein sequences from other species by Neighbor joining method using the MEGA 6.06 software.

4.5.4 Results

4.5.4.1 Expression profile of ethylene biosynthesis genes under water deficit stress

Expression profiling of genes associated with ethylene biosynthesis pathway *TaACS1* (1-aminocyclopropane-1-carboxylic acid synthase 1) and *TaACO2* (1-aminocyclopropane-1-carboxylic acid oxidase 2) was studied in flag leaf sample of routinely irrigated control (Relative water content; 80-85 %) and water deficit stress treated wheat plant (Relative water content; 65-75 %) by withholding irrigation for 10 days at anthesis stage. Semi-quantitative expression analysis (Reverse transcriptase polymerase chain reaction, RT-PCR) of genes using gene specific primers of *TaACS1* and *TaACO2* resulted in transcript of 342 and 386 bp amplicon size respectively (**Fig. 4.5.1** and **Fig. 4.5.2**) and further confirmed by sequencing in all the three genotypes (**Table 3.3**). Significant differences were observed in *TaACS1* gene expression level under both control and water deficit stress treated plants. Amongst the three genotypes studied, higher expression was observed in case of susceptible genotype (HW 4059) while the tolerant genotype (HW 4022) showed reduced expression level

ACS



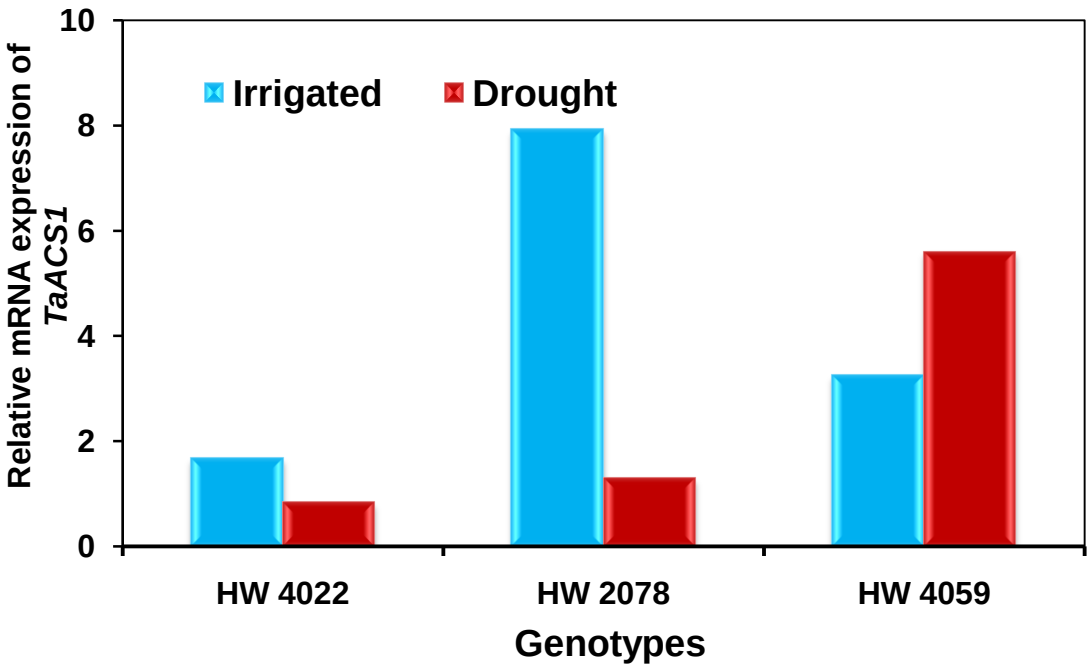
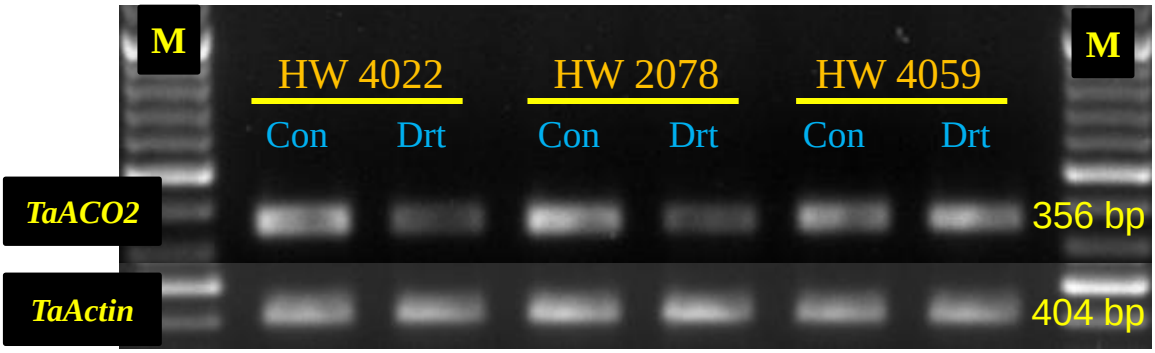


Fig. 4.5.1 Semi-quantitative expression analysis of *TaACS1* gene encoding enzyme involved in ethylene biosynthesis pathway in flag leaf of wheat genotypes under control (normal irrigation, RWC; 80-85 %) and water deficit stress condition (withholding irrigation for 10 days, RWC; 65-75 %)

Lane M: 100 bp ladder

Abbrevation: ACS: 1-aminocylopropane-1-carboxylic acid synthase; Con: Control; Drt: Drought

ACO



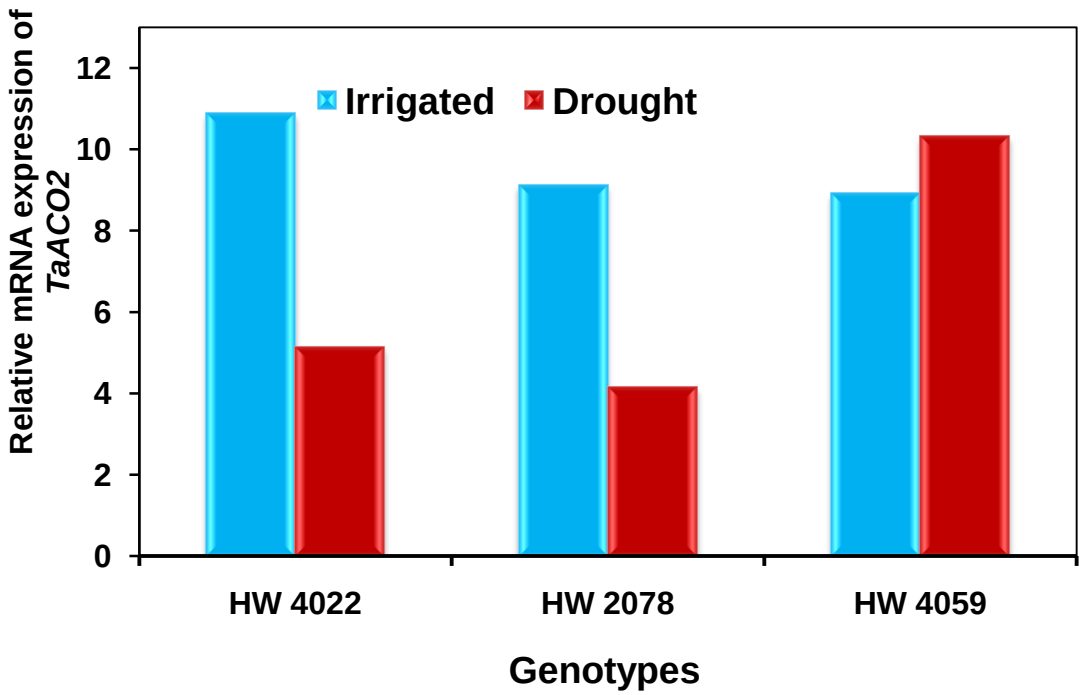


Fig. 4.5.2 Semi-quantitative expression analysis of *TaACO2* gene encoding enzyme involved in chlorophyll degradation pathway in flag leaf of wheat genotypes under control (normal irrigation, RWC; 80-85 %) and water deficit stress condition (withholding irrigation for 10 days, RWC; 65-75 %)

Lane M: 100 bp ladder

Abbrevation: ACO: 1-aminocylopropane-1-carboxylic acid oxidase; Con: Control; Drt: Drought

for *TaACS1* gene under both water regimes. It was observed that under normal irrigation condition relatively drought tolerant genotype (HW 2078) exhibited higher *TaACS1* expression level while under water deficit stress the expression level of *TaACS1* decreased drastically as observed in tolerant genotype. Expression analysis of another gene *TaACO2* (1-aminocyclopropane-1-carboxylic acid oxidase) encoding ethylene biosynthesis enzyme was studied in three wheat genotypes (HW 4022, HW 2078 and HW 4059) by semi-quantitative expression analysis. Compared to normal irrigated condition relative expression of *TaACO2* varies in three wheat genotypes under water deficit stress. In comparatively drought tolerant genotypes HW 4022 and HW 2078 expression level of *TaACO2* reduces under water deficit stress condition as compared to their control while susceptible genotype HW 4059 showed elevated *TaACO2* expression under both water regimes.

4.5.4.2 Cloning and *In-silico* analysis of *TaACO2* gene in wheat

A full-length cDNA encoding a polypeptide with sequence similarity to *Arabidopsis* 1-aminocyclopropane-1-carboxylate oxidase 2 (*AtACO2*) was amplified by PCR of cDNA using gene specific primers and cloned. The BioEdit 7.1 software was used for aligning forward and reverse sequencing results and the complete nucleotide sequence was reconstructed having a nucleotide sequence of 1052 bp and annotated as *ACC oxidase 2* (*TaACO2*) based on the similarity with ACO2 of *Arabidopsis* (**Fig. 4.5.3.A**). The sequences were translated using ExPASy Translate Tool (http://web.expasy.org/translate/) and deduced amino acid sequences were obtained for *TaACO2*. These results were confirmed by BLASTp analysis with rice (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml) and *Arabidopsis* (http://www.arabidopsis.org/Blast/). The cDNA clone covered an open-reading frame (ORF Finder, NCBI) for a 331 amino acid polypeptide with a calculated molecular mass of 36.6 kDa and isoelectric point of 5.27 predicted using Expasy ProtParam tool (http://www. expasy.org/protparam). The search for conserved domains by bioinformatics tools like Expasy ScanProsite Tool (http://prosite.expasy.org/scanprosite/), conserved domain database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and SMART (http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) showed that amino acid positions from 164 to 263 contained the conserved Fe^{+2} 2-oxoglutarate dioxygenase domain. The prediction of sub-cellular localization by WoLF PSORT

tool (http://www.genscript.com/psort/wolf_psort.html) revealed its cytoplasmic location.

The ClustalW2 multiple sequence alignment of nucleotide showed that even though there were single nucleotide polymorphisms (SNPs) at the nucleotide level, the protein sequence did not exhibited any difference between the genotypes studied *viz*. HW 4022, HW 2078 and HW 4059. Multiple protein sequence alignment (ClustalW2), represented using BoxShade server (http://www.ch.embnet.org/software/BOX_form.html) with the deduced amino acid sequence of wheat showed 83.5 % and 64.7 % similarity with rice and *Arabidopsis* respectively. The phylogeny tree constructed (MEGA 6.06 Tool) for *TaACO2* using the amino acid sequence from the selected plant species (http://www.phytozome.net/) gave light into the evolution of the ACO protein (**Fig 4.6.4**).

4.5.5 Discussion

The phytohormone ethylene plays several roles in the plant development and responses to various environmental cues (Merchante *et al*., 2013). Various types of abiotic stresses accelerate ethylene production in different plant tissues (Abeles *et al*., 1992). Despite the progress made in the ethylene research, the molecular mechanisms underlying the regulation of ACC synthase and ACO oxidase, the key enzymes in ethylene biosynthesis pathway under drought stress is not fully understood. Young *et al*. (2004) in maize reported that ACS mutants with delayed leaf senescence rate performed better under drought stress condition. Comparable findings in rice by Chen *et al*. (2013) and maize by Habben *et al*. (2014) suggested that severe drought stress induced the ethylene level as well as transcript of ethylene biosynthesis genes (ACC synthase and ACC oxidase) which has a negative effect on the grain filling rate and yield. Recent findings by Yang *et al*. (2014) in wheat reported that reduced ethylene evolution rate is a desirable physiological trait for drought tolerance. However, the molecular mechanism governing this trait is not very clear. In the current study, we established that ethylene production was closely associated with drought tolerance mechanism in wheat crop. The results of present study revealed that in general, drought tolerant wheat genotype (HW 4022) reported with lower expression of ethylene biosynthesis genes (*TaACS1* and *TaACO2*) whereas, the susceptible genotype (HW 4059) showed marked increase in

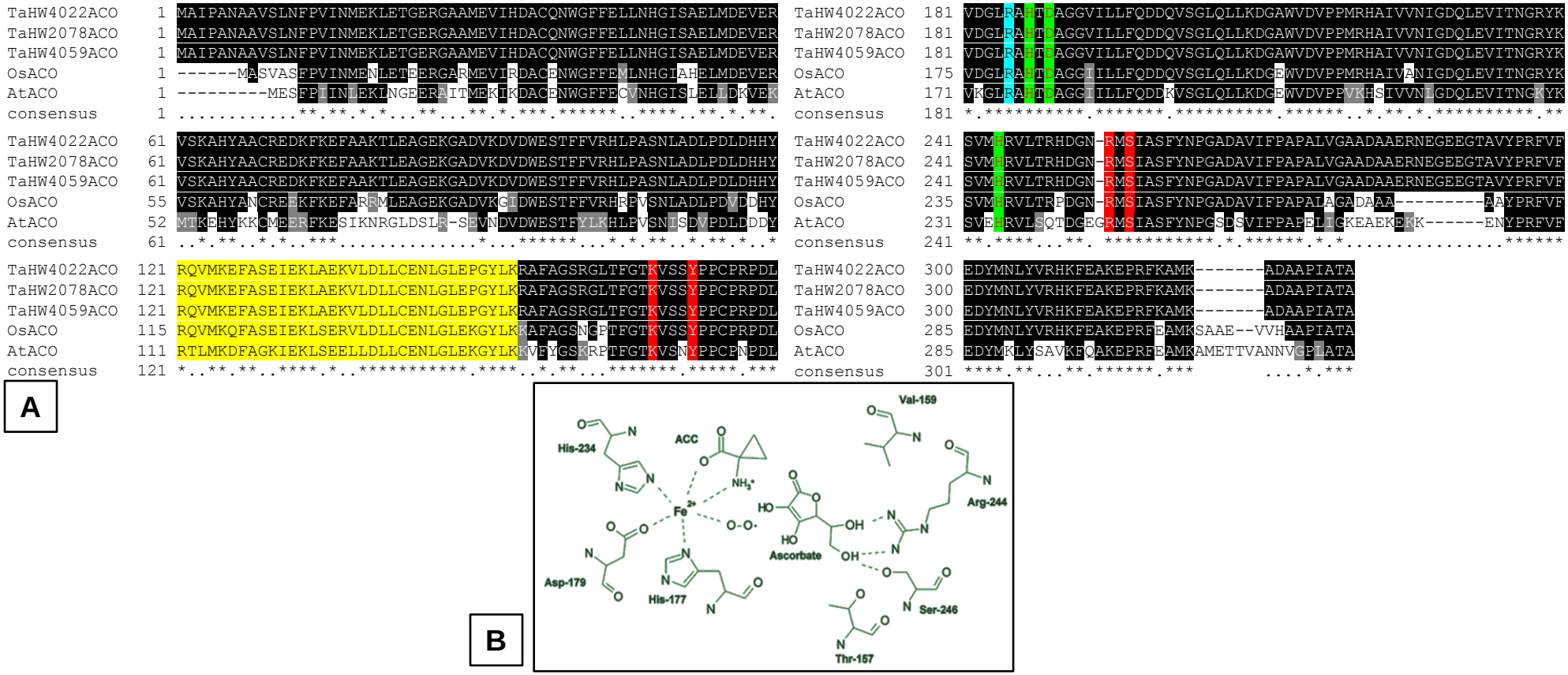


Fig. 4.5.3 Multiple sequence alignment of *TaACO2* and other homologous ACO proteins using ClustalW2. A) Alignment of *TaACO2* and other homologous ACO proteins represented by BoxShade. The sequences used in this alignment are as follows: *Triticum aestivum* (GeneBank accession no. AHJ14562, AHJ14563, AHJ14564), *Oryza sativa* (GeneBank accession no. NP_001064620), *Arabidopsis thaliana* (GeneBank accession no. NP_171994). Dark shaded region show amino acid residues conserved in all five sequences and grey shaded regions represent similar residues. The consensus sequences are represented by symbols in each line. Various colour code indicates; yellow: leucine zipper domain, red: the residues involved in ACC binding including the RXS motif, green: the facial catalytic triad for Fe^{2+} binding, blue: bicarbonate binding residue.

B) Schematic diagram of the residues involved in ascorbate and ACCbinding at the active site of ACO enzyme

ACO

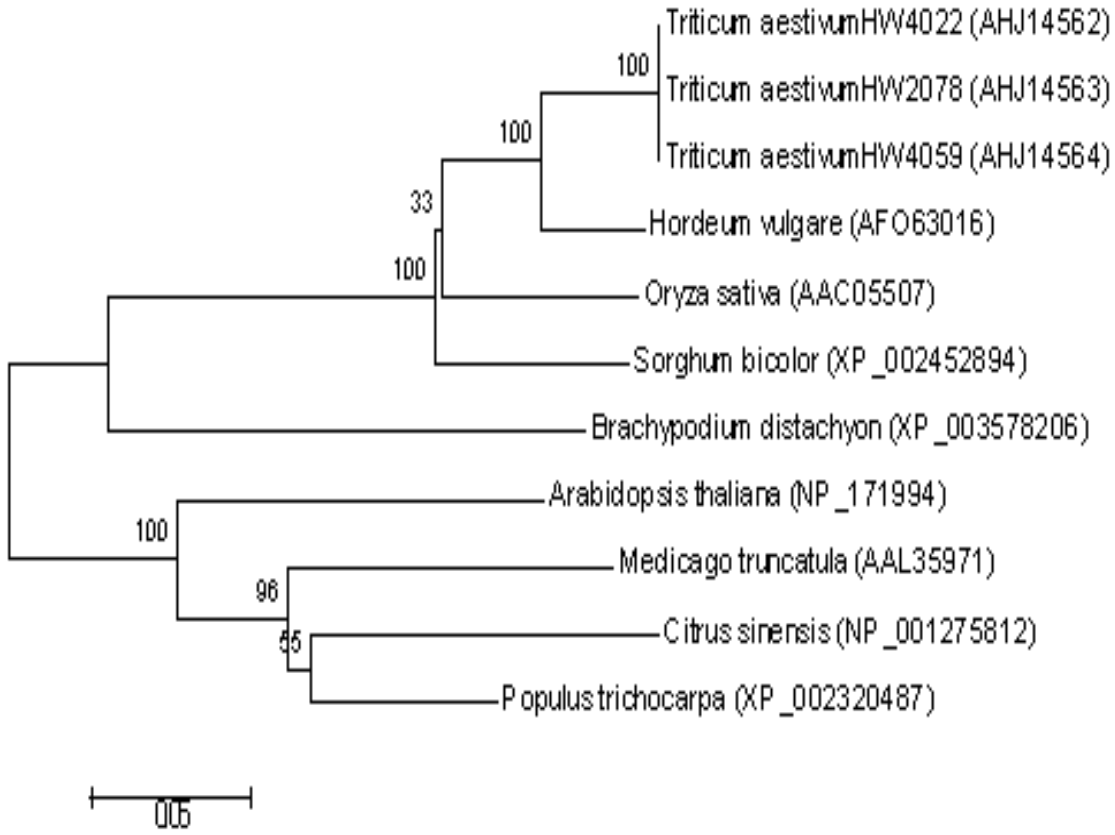


Fig. 4.5.4 Phylogenetic tree of ACO constructed by the neighbor-joining method of MEGA 6.06 software with 500 bootstrap replicates of the ClustalW2 alignment. Evolutionary distances were estimated using the Poisson correction model. Phylogenetic tree of *TaACO2* proteins from different plant species along with their accession numbers are given in parentheses

expression of *TaACS1* and *TaACO2* under water deficit stress. These findings can be supported by our previous reports where, drought stress enhanced ethylene production in susceptible genotype (HW 4059) increasing its sensitivity to water deficit stress. Finally the study highlights that in susceptible genotype (HW 4059) increase in ethylene biosynthesis genes (*TaACS1* and *TaACO2*) and higher ethylene evolution is responsible for its susceptibility to drought stress.

The last step in ethylene biosynthesis pathway was catalyzed by enzyme, 1-aminocyclopropane-1-carboxylic acid oxidase (ACO). It converts ACC to ethylene along with CO_2 and HCN as byproducts, and required Fe^{+2} and oxygen as co-factor, ascorbate as co-substrate, and bicarbonate and CO_2 as activators (Moya-Leon and John, 1995; Bidonde *et al*., 1998; Ryle and Hausinger, 2002). ACO is a member of a super family of non-haem iron oxygenase and oxidase and encoded by a multigene family in all plant species studied (Zhang *et al*., 2004; Lin *et al*., 2009; Jafari *et al*., 2013). The *Arabidopsis* genome encodes five ACO genes while, in tomato six ACO genes exist in the databases. Genes for ACO have already been isolated from several plant species *viz*. tomato (Hamilton *et al*., 1990), avacoda (Mc-Garvey *et al*., 1990), melon (Balague *et al*., 1993), petunia (Tang *et al*.,1993), tobacco (Knoester *et al*., 1995), rice (Mekhedov and Kende, 1996), papaya (Lin *et al*., 1997), peach (Ruperti *et al*., 2001), potato (Nie *et al*., 2002), sugarcane (Wang *et al*., 2003), strawberry (Trainotti *et al*., 2005), white clover (Chen *et al*., 2006), plum (Fernandez-otero *et al*., 2006) and apple (Binnie *et al*., 2009) but no reports on isolation of ACO in wheat (*Triticum aestivum* L.) is available in literature. The ACO gene that we cloned from wheat was having 1052 bp length with an ORF of 996 bp coding 331 amino acid polypeptide which was found to be homologous with previously reported ACC oxidase genes of *Arabidopsis* (64.7 %) and rice (83.5 %) and annotated as *TaACO2*. The deduced amino acid sequence of *TaACO2* revealed a conserved domain from position 121 to 154 that contained the potential leucine zipper as indicated by yellow shade (**Fig. 4.5.3.A** and **B)**. This leucine zipper domain has been shown to be essential for interaction with membrane in consistent with previous findings by Malerba and Bianchetti (1997). The biochemical characterization of ACO protein from citrus revealed the enzyme as ferrous ion-dependent one (Dupille and Zacarias, 1996). The conserved domain search by ExPASy Prosite scan tool revealed three Fe^{2+} binding sites. With reference to the

reports by Chen *et al*. (2003) and Zhang *et al*. (2004) the deduced amino acid sequence of *TaACO2* also contained the facial catalytic triad *viz*. His187, Asp189 and His244 for Fe^{2+} binding. The ACC will then bind to this Fe^{2+} through its amino and carboxylic acid side chains (Seo *et al*., 2004) as represented by green colour. Zhang *et al*. (2004) and Seo *et al*. (2004) also reported the existence of RXS motif along with Tyr and Lys residues which are directly involved in binding of ACC through its carboxylate side chain. The deduced amino acid sequence of *TaACO2* also showed the RXS motif at position 254-256, Lys158 and Tyr162 which are involved in ACC binding as shown by red shade. In addition, the bicarbonate binding residue which is essential for enzyme activation is indicated by blue colour in the reported sequences at amino acid Arg185. Kadyrzhanova *et al.* (1999) reported that mutation at this binding site resulted in decreased enzyme activity. The phylogeny tree was constructed using Neighbor joining clustering method by Mega 6.06 software from the deduced the amino acid sequence of wheat ACC oxidase with other ACO proteins from the selected plant species (http://www.phytozome.net/) which gave light into the evolution of the ACC oxidase, where wheat *ACO* evolved from coniferales like *Picea glauca* and further diverged in higher genera of dicots like *Arabidopsis* and monocots like rice, barley and wheat **(Fig. 4.5.4).**

4.5.6 Conclusion

The current study, thus clearly shown that ethylene is inducible under drought stress and the result highlights the role played by ethylene in plants when subjected to water deficit stress. Further, drought stress induced changes in expression of ethylene biosynthesis genes act as a protective mechanism as the decrease in these ethylene biosynthesis genes (*TaACS1* and *TaACO2*) is closely associated with drought tolerance mechanism in tolerant wheat genotype as prove by the findings from present study. Additionally, the complete CDS of *TaACO2* from wheat identified will help to understand the molecular characteristic and function of ACC oxidase gene in wheat under drought stress in future.

4.6. RESEARCH PAPER VI

Ethylene perception by ERS1 gene in wheat (*Triticum aestivum* L.) under drought stress

4.6.1 Abstract

Ethylene is involved in plant responses to biotic and abiotic stresses. Ethylene perception in plant tissue requires specific receptors and a signal transduction pathway to coordinate downstream responses. Ethylene receptors act as a negative regulator of ethylene response under various environmental stresses. The present invention relates to an isolation of ERS1 from wheat (*Triticum aestivum* L.) encoding an ethylene receptor protein that is useful in improving drought tolerance traits. A drought induced ERS1 gene (*TaERS1*) was cloned from wheat, its expression under drought stress and subcellular localization was studied. The full cDNA of *TaERS1* is of 2034 bp in length with an open reading frame of 1908 bp coding for a polypeptide of 634 amino acids. The protein sequence consisted of three transmembrane domains at N-terminus, GAF domain, histidine kinase domain and HATPase domain at C-terminus. A result of semi-quantitative RT-PCR, expression study in contrasting set of wheat genotypes under drought stress revealed that *TaERS1* was upregulated in tolerant genotype (HW 4022) whereas, downregulated in susceptible genotype (HW 4059) under water deficit stress. The present study showed that *TaERS1* were expressing well under drought stress in tolerant wheat genotype. Therefore, indicating a significant role played by ERS protein under drought in providing ethylene insensitivity and provides us an idea that how plants regulates their ethylene responses.

***Key words*:** ERS1, Cloning, Expression analysis, Drought, Wheat.

4.6.2 Introduction

Ethylene, a gaseous plant hormone, plays critical roles in plant growth, development and response to various biotic and abiotic stress environments. (Abeles *et al*., 1992; Bleecker and Kende, 2000; Chen *et al*., 2009; Vandenbussche *et al*., 2012; Wang *et al*., 2013). Ethylene regulated processes are initiated by the elevation of ethylene biosynthesis, which is under tight control by a complex signaling network (Xu and Zhang, 2014). To understand more in detail about how ethylene exerts its responses, one has to examine the ethylene biosynthesis and signaling cascade pathway. Although ethylene biosynthetic pathway is well understood, but the knowledge underlying ethylene signaling cascade is limited. Production of ethylene in response to stress has been known for many decades and the connection between drought stress and ethylene evolution rate was demonstrated by many researchers in different crops (Wang *et al*., 2012, Habben *et al*., 2014, Larrainzar *et al*., 2014). In phytohormones signaling was initiated upon interaction with their corresponding receptors (Shan *et al*., 2012). In dicotyledonous model plant *Arabidopsis*, the key components in ethylene signaling pathway have been identified through analysis of ethylene response mutants based on triple response. These include five ethylene receptors ETR1 (Ethylene receptor/ response), ETR2, EIN4 (Ethylene insensitive), ERS1 (Ethylene response sensor) and ERS2 (Ju and Chang, 2012). These five members of the putative ethylene receptor gene family in *Arabidopsis* have been cloned and appear to belong to two subfamilies. Subfamily 1 consists of ETR1 and ERS1, whereas subfamily 2 contains ETR2, EIN4 and ERS2 (**Fig. 2.4**). Members of same subfamily have more amino acid sequence similarity with each other along-with conserved intron positions that are not shared between two subfamilies (Hua *et al*., 1998; Arora, 2005). These receptors interact with constitutive triple response 1 (CTR1) leading to its activation in the absence of ethylene (Zhong *et al*., 2008, Mayerhofer *et al*., 2012). CTR1, a Raf-like Ser/ Thr protein kinase upon activation by ethylene receptor directly or indirectly inhibits a central membrane protein EIN2 (Ethylene insensitive 2), a positive regulator of ethylene signaling (Bisson and Groth, 2011). The EIN3/ EIN3-Like1 (EIL1) transcription factors function downstream of EIN2 in ethylene signaling (Zhang *et al*., 2011). All the five ethylene receptors are trans-membrane proteins located in endoplasmic reticulum and are responsible for initiating ethylene signaling cascade (Merchante *et al*., 2013). In the absence of

ethylene, the receptors are constitutively expressed and suppress ethylene responses while the binding of ethylene to receptors prevents receptor activity (Binder *et al*., 2012). The ethylene signaling machinery may be highly conserved in higher plants but may be differentially regulated in different plant species.

Current knowledge about the ethylene signaling cascade in plants comes mainly from dicot plant species. Ethylene signaling components were identified and functionally demonstrated in tomato (Zhong *et al*., 2008) and *Arabidopsis* (Ju *et al*., 2012) which indicates conserved signaling machinery in dicots species. Recently the reports on ethylene signaling (Zhang *et al*., 2012) in rice initiate a step for further studies in monocot crops like wheat. Although a basic framework of the initial events in the ethylene-response pathway is emerging from many previous studies, but there are many issues that remain to be resolved *i.e.* the expression patterns of ethylene receptors in crop plants particularly, wheat under drought stress have not been fully investigated. Although ethylene has been implicated in the drought stress response, the exact role played by ethylene receptors in the regulation of drought stress remains unclear. Furthermore, only few reports are available on cloning and characterization of ethylene receptors in wheat. Here, we undertook a molecular approach to investigate the role of ethylene signaling gene ERS1 under drought stress in wheat. The objective of this study was to investigate effect of water deficit stress on expression of ethylene signaling gene *TaERS1* in contrasting set of wheat genotypes differing in their drought tolerance efficiency. Result showed that *TaERS1* act as a negative regulator of ethylene biosynthesis under water deficit stress as its expression increased in drought tolerant wheat genotype (HW 4022) while, drought stress down-regulated its expression in susceptible genotype (HW 4059). This study will help us to understand the role of ethylene receptor (ERS) during water deficit stress in wheat crop. The cloning of *TaERS1* is an important step to further characterize its function in different signaling pathways related to drought tolerance in wheat.

4.6.3 Materials and method

4.6.3.1 Plant materials and treatment

From our preliminary experiment, we selected three wheat genotypes based on their drought tolerance efficiency *viz*., HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) for the current study.

Plants were subjected to water deficit stress by with-holding irrigation for 10 days from 50 % anthesis stage. Scheduled routine of irrigation was practiced for control plants throughout the crop growth period (**Table 3.2**). For biochemical and molecular analysis flag leaf sampling was done from plants under both water regimes.

4.6.3.2 Molecular analysis of ethylene signaling gene

4.6.3.2.1 RNA isolation and cDNA synthesis

For performing semi-quantitative PCR (RT- PCR) isolation of total RNA was carried out by TRIzol® reagent (*Invitrogen*™, Carlsbad, CA, USA) and RNase-free DNase I (Promega, USA) was applied to remove contaminating genomic DNA at 37 ^{0}C for 1 h. Quality and integrity of total RNA were then determined by running appropriate amount in a formamide denaturing gel, and quantity of total RNA was determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized according to the instructions of the cDNA Synthesis Superscript RT™ (*Invitrogen*™, USA). Resulting cDNA was stored at -20 ^{0}C and employed as template for two-step RT-PCR reactions using recommended conditions.

4.6.3.2.2 Primer designing

The primers employed in the present study were designed using the bioinformatics tools described in detail as follows. Search for nucleotide sequence of ERS in NCBI database (http://www.ncbi.nlm.nih.gov/) did not have any results in wheat. Hence we selected the protein sequence of ERS1 from *Arabidopsis* (GenBank accession no. NP_181626) and performed TBLASTn analysis (Altschul *et al*., 1997; http://blast.ncbi.nlm.nih.gov/Blast.cgi) with expressed sequence tag (EST) database of NCBI in wheat. We obtained three cDNA sequences (GenBank accession no. CV779652; BQ743715; HX062039) covering considerable length having nearly 50 % identity with *AtERS1* sequence, but was unable to produce a complete *TaERS1* sequence. To get the complete open reading frame (ORF) the nucleotide sequences of GenBank accession no. CV779652 and HX062039 were further analyzed by BLASTn (Altschul *et al*., 1990) with EST database of wheat in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) which gave two sequences *viz*. CJ955450 and HX173544 covering 5′ and 3′ ORF, respectively. These five sequences were used to construct a contig by aligning nucleotide sequences using BioEdit 7.1 software (Hall

et al., 2004; http://bioedit.software.informer.com/7.1/). The ORF coverage was confirmed by ORF finder tool of NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/) and the deduced amino acid sequence using ExPASy translate (Gasteiger *et al*., 2003; http://web.expasy.org/translate/) of the putative *TaERS1* gene showed significant homology with rice and *Arabidopsis* protein sequences by BLASTp analysis (Altschul *et al*., 1990; http://blast.ncbi.nlm.nih.gov/Blast.cgi). This contig sequence of *TaERS1* was used for designing gene specific primers in wheat (*Triticum aestivum* L.). The gene specific primers were designed manually and quality parameters were confirmed using Integrated DNA Technologies (IDT) Oligoanalyzer tool (https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). The primers used for semi-quantitative gene expression analysis (RT-PCR) of ethylene signaling gene *TaERS1* and its cloning are listed in **Table 3.3** Every RT-PCR measurement was performed at least thrice. Expression of *TaActin* was used as an internal standard for normalization.

4.6.3.2.3 Cloning and sequencing of *TaERS1* gene

PCR amplification with gene specific primers (**Table 3.3**) and proofreading enzyme Platinum Hi-fidelity Taq DNA polymerase (*Invitrogen*^{TM}, USA) gave approximately 2 kb fragment that was cloned in TA cloning vector pTZ57R/T (Thermo Scientific, USA). The construct was transformed into *E. coli* strain XL1-Blue and confirmed by blue-white screening in a media containing IPTG (Isopropyl β-D-1-thiogalactopyranoside), X-gal and Kanamycin (Zhou and Gomez-Sanchez, 2000). Positive clones were sub-cultured in media having same composition and further confirmed by colony PCR, restriction digestion and sequencing (**Fig. 2.2** and **Fig. 2.3**).

4.6.3.2.4 *In silico* analysis of *TaERS1* gene sequence

The BioEdit 7.1 software was used for aligning forward and reverse sequencing results and the complete nucleotide sequence was reconstructed. The reconstructed sequences were translated using ExPASy Translate Tool (http://web.expasy.org/translate/) and deduced amino acid sequences were obtained for *TaERS1*. These results were confirmed by BLASTp software online (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Isoelectric point (pI) and molecular weight (MW) were analyzed by ExPASy ProtParam tool

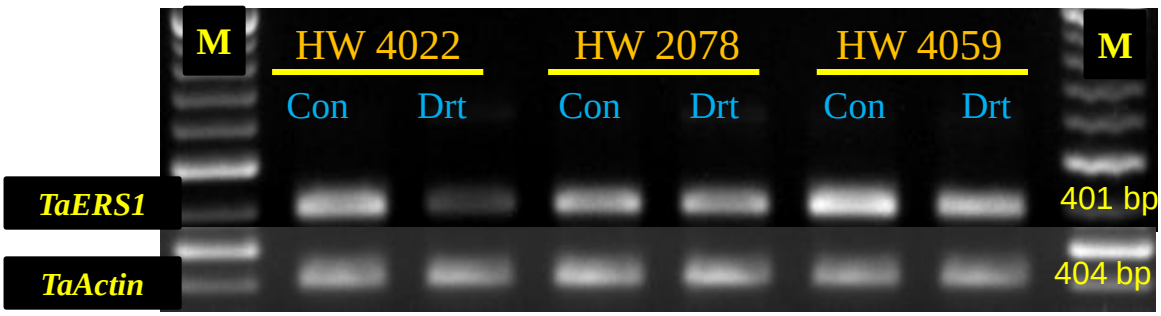
(http://www. expasy.org/protparam). Prediction of putative subcellular localization was carried out using the WoLF PSORT online program (http://wolfpsort.org) and the presence of conserved domains were predicted by SMART program (Letunic *et al*., 2002; http://smart.embl-heidelberg.de/smart/set_mode.cgi?NORMAL=1) and TMHMM Server v.2.0. (Sonnhammer *et al*., 1998; http://www.cbs.dtu.dk/services/TMHMM-2.0). The multiple protein sequence alignment of *TaERS1* and its homologs from model species *viz*. rice and *Arabidopsis* were carried out by ClustalW2 tool (Larkin *et al*., 2007; http://www.ebi.ac.uk/Tools/msa/clustalw2/) of Bioedit 7.1 software (Hall *et al*., 2004) and represented using BoxShade server (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic tree was constructed with homologous protein sequences from other species by Neighbor-joining method (Saitou and Nei, 1987) with 500 bootstrap replicates using the MEGA 6.06 (Tamura *et al*., 2013) software.

4.6.4 Results

4.6.4.1 Expression profiling of ethylene signaling gene (*TaERS1*)

Expression profiling of ethylene signaling gene, ethylene response sensor (*TaERS*) was studied in flag leaf of three wheat genotypes differing in their drought tolerance efficiency *viz*., HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) under normal irrigated (RWC; 80-85 %) and water deficit stress condition (RWC; 65-75 %) by with-holding irrigation for 10 days at reproductive stage. Semi-quantitative expression analysis of *TaERS1* gene was performed using gene specific primers resulted in 401 bp amplicon size (**Fig. 4.6.1**) and further confirmed by sequencing in all the studied wheat genotypes (**Table 3.3**). Significant difference in expression of *TaERS1* gene was observed among the genotypes under both water regimes. Amongst the three genotypes studied, susceptible genotype (HW 4059) showed reduced *TaERS1* expression under water deficit stress as compared to normal irrigated condition. Drought induced expression for *TaERS1* was observed in case of tolerant genotype (HW 4022), while no significant difference was observed in relatively tolerant genotype (HW 2078) under both water status.

ERS1



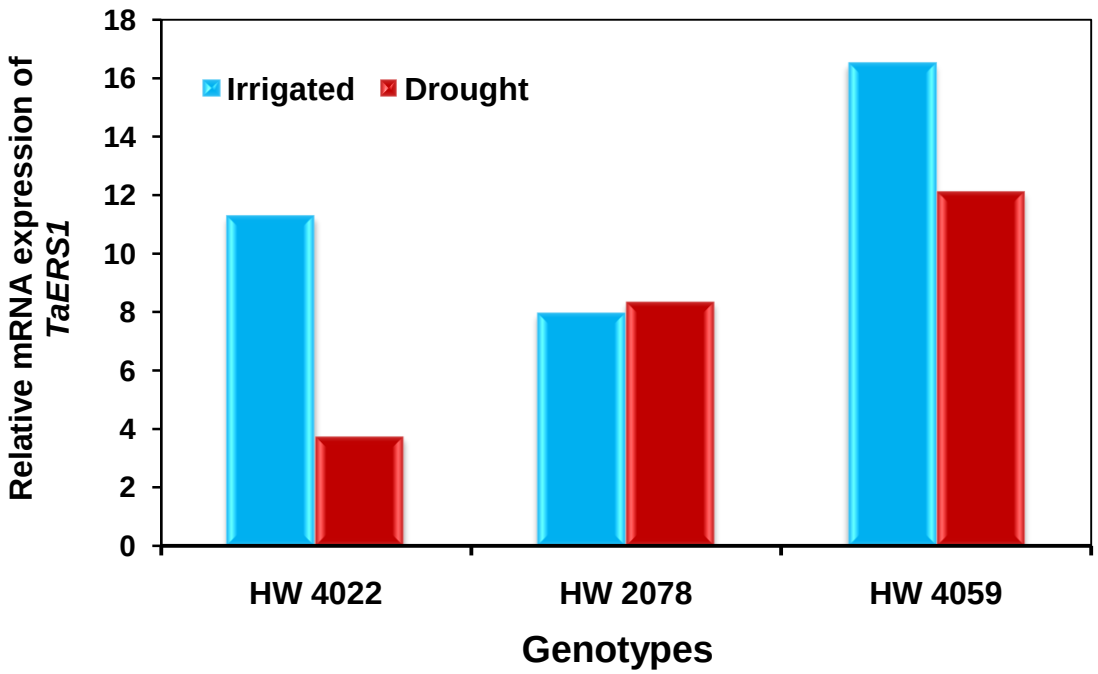


Fig. 4.6.1 Semi-quantitative expression analysis of *TaERS1* gene encoding enzyme involved in ethylene signaling in flag leaf of wheat genotypes under control (normal irrigation, RWC; 80-85 %) and water deficit stress condition (withholding irrigation for 10 days, RWC; 65-75 %).

Lane M: 100 bp ladder

Abbrevation: ERS: Ethylene response sensor; Con: Control; Drt: Drought

4.6.4.2 Cloning and *In-silico* characterization of *TaERS1* gene from wheat

A full-length cDNA of about 2 kb nucleotide sequence length, encoding a polypeptide with similarity to *Arabidopsis* and rice ERS1 was amplified by PCR of cDNA using gene specific primers (**Table 3.3**) and cloned. The BioEdit 7.1 software was used for aligning forward and reverse sequencing results and the complete nucleotide sequence was reconstructed, giving a nucleotide sequence of 2034 bp and was annotated as *TaERS1* based on the similarity with ERS1 of *Arabidopsis* (**Fig. 4.6.2.A**). The cDNA contained an ORF of 1908 bp (ORF Finder, NCBI) encoding 635 amino acid polypeptide with a calculated molecular mass of 70.99 kDa and calculated pI of 6.48 (Expasy-ProtParam Tool). The search for conserved domains by SMART tool retrieved three trans-membrane domains, GAF domain (position 158-317), histidine kinase domain (position 343-408) and HATPase domain (position 455-589). The three trans-membrane domains at N terminus *viz*. positions 21-43, 53-75 and 88-110 was further confirmed by TMHMM Server v.2.0. Multiple sequence alignment by ClustalW2 using the deduced amino acid sequence showed 92 % and 70 % similarity with *Oryza sativa* and *Arabidopsis* sequences, respectively. The drought susceptible genotype (HW 4059) showed only 99.84 % similarity with the other two genotypes (HW 4022 and HW 2078) because of a single amino acid difference from His501 to Asp501 located in HATPase domain. The phylogeny tree constructed (MEGA 6.06 Tool) for *TaERS1* using the amino acid sequence from the selected plant species (http://www.phytozome.net/) gave light into the evolution of the *TaERS1* (**Fig. 4.6.4**).

4.6.5 Discussion

Ethylene is a gaseous phytohormone involved in plant responses to various environmental stresses (Abeles *et al*., 1992; Bleecker and Kende, 2000). Numerous studies on ethylene responses led to the identification of key components in ethylene signal transduction pathway, but little is known regarding the physiological relevance of ethylene receptors in crop plants under drought stress. In ethylene signal transduction pathway its receptors are constitutively active and serve as negative regulators of ethylene responses, which get inactivated in presence of ethylene (Merchante *et al*., 2013). A decrease in receptor levels increases the plant sensitivity to even low levels of ethylene under stress (Tieman *et al*., 2000, Yoo *et al*., 2008). In the current study, we mainly focused on ethylene signaling mediated by *TaERS1* during drought stress in wheat. This prediction have been confirmed experimentally in

the present study where, the ethylene receptor ERS1 showed reduced expression in susceptible wheat genotype (HW 4059) with marked increased in ethylene responses when subjected to water deficit stress. At the same time tolerant genotype (HW 4022) displayed drought induced expression of *TaERS1* proving its drought tolerance ability with reduced ethylene sensitivity and responses. Comparable findings in wheat reported by Hong *et al*. (2012) where, subtractive hybridization between irrigated and drought stressed plants showed increased expression of *TaERS* homologs (*TaERS1*, *2, 3,* and *4*) under drought stress. Similar results were also reported in other crop species like sugarcane where, susceptible genotype showed a reduced expression of ERS gene under water deficit stress (Wang *et al*., 2014). Expression of *Arabidopsis ERS1* gene in coriander by Wang and Kumar (2004) suggested that the transgene can effectively delay the ethylene responses *i.e.* the leaf and flower senescence.

Though the current findings along-with the previous reports gave an idea about the expression of ethylene receptors, ERS in particular for drought tolerance; but the information available are not sufficient enough to get insight knowledge about the molecular mechanism of ERS with respect to its function. To fill this gap, cloning of *TaERS1* was done to evaluate the precise role of this receptor and how its activity is governed under drought stress in wheat. Ethylene response sensor (ERS) genes have been isolated and characterized in various plants (Hall *et al*., 2000; Yau *et al*., 2004; Arora *et al*., 2006, Firon *et al*., 2012, Yan-Yan *et al*., 2013). To give an insight to the molecular mechanism behind ERS1 under drought stress cloning and *in-silico* analysis was performed in wheat genotypes differing in their drought tolerance efficiency. The *in-silico* analysis resulted in deduced amino acid sequence of 2034 bp containing an ORF of 1908 bp encoding for 635 amino acid polypeptide from all the three genotypes. The deduced amino acid sequence showed 71 % identity to ERS1 (GenBank accession no. NP_181626) of *Arabidopsis.* Hence, the isolated ethylene receptor was annotated as *TaERS1*. The deduced amino acid sequence was having three trans-membrane domains and three conserved domains *viz.* GAF domain, histidine kinase domain, HATPase domain and lacked the receiver domain at the C terminus (**Fig. 4.6.3.B**). These domains are typical for members of ETR subfamily I (Mayerhofer *et al*., 2012).

Sub-cellular localization in several species by GFP fusion of ERS1 led to the discovery that N-terminal trans-membrane domains which helps the receptor to

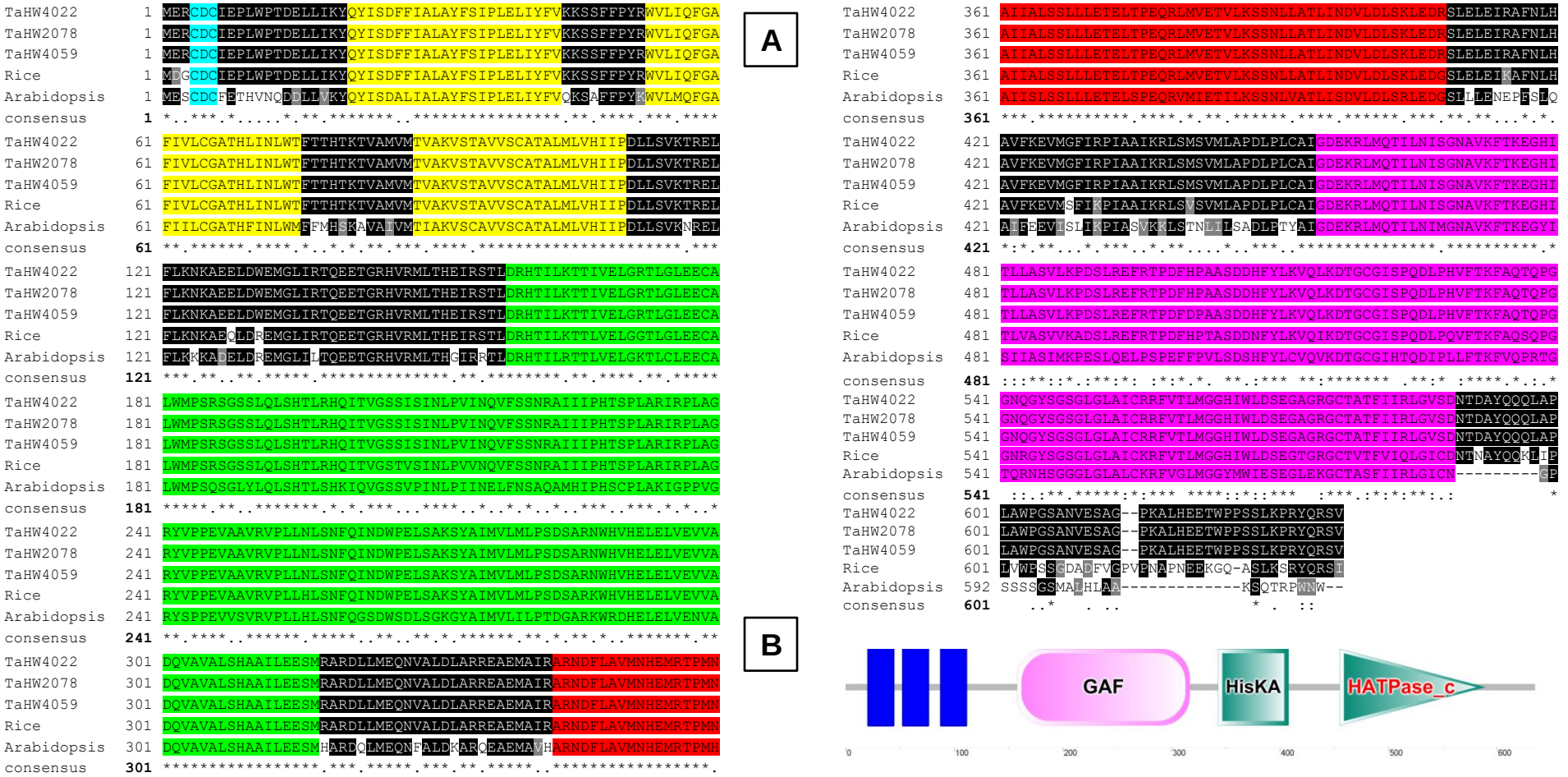


Fig. 4.6.2 Multiple sequence alignment of *TaERS1* and other homologous ERS proteins using ClustalW2. A) Alignment of *TaAERS1* and other homologous ERS proteins represented by BoxShade. The sequences used in this alignment are as follows: *Triticum aestivum* (GeneBank accession no. KJ001485, KJ001486, KJ001487), *Oryza sativa* (GeneBank accession no. AAB72193), *Arabidopsis thaliana* (GeneBank accession no. NP_1814626). Dark shaded region show amino acid residues conserved in all five sequences and grey shaded regions represent similar residues. The consensus sequences are represented by symbols in each line. Various colour code indicates; yellow: transmembrane domains, blue: CXC motif for dimerization, green: GAF domain, red: histidine kinase domain, pink: HATPase domain

B) Schematic diagram of the domains of TaERS1 obtained by CDD analysis

ERS1

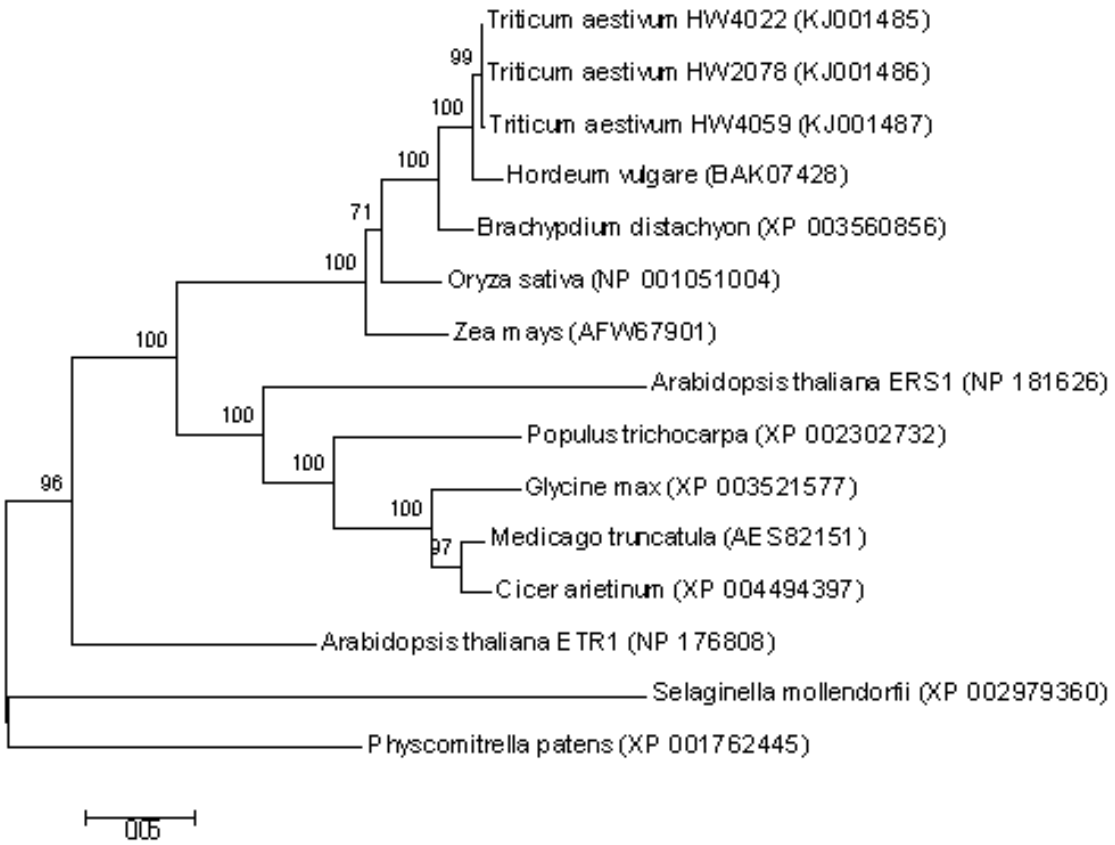


Fig. 4.6.3 Phylogenetic tree of ERS constructed by the neighbor-joining method of MEGA 6.06 software with 500 bootstrap replicates of the ClustalW2 alignment. Evolutionary distances were estimated using the Poisson correction model. Phylogenetic tree of *TaERS1* proteins from different plant species along with their accession numbers are given in parentheses

embed at endoplasmic reticulum membrane that encompass ethylene-binding site, with the C-terminus facing the lumen (Ma *et al*., 2006, Grefen *et al*., 2008). The sequence obtained was also having three transmembrane and ethylene binding domains at the N terminus. Several mutant studies in *Arabidopsis* identified the ethylene binding sites of the receptors. Sequence analysis of *TaERS1* revealed the ethylene binding sites at Cys65 and His69 that coordinate the copper ion to facilitate ethylene binding. The dominant ethylene insensitivity was observed when ETR1 was mutated as cysteine to serine at position 65 and histidine to alanine at position 69, located within the second hydrophobic subdomain and expressed in yeast (Schaller and Bleecker, 1995; Rodriguez *et al*., 1995). Additionally, Ala at 31 and Ile at 62 position, essential for ethylene binding were also found, upon comparing the reports by Hall *et al*. (2000). The binding of 1-MCP to the ethylene binding motifs can constitutively repress ethylene responses (Hall *et al*., 2000). The dimerization domain *viz.* Cys4 and Cys6, originally described by Schaller *et al*. (1995) that form disulfide bridge was also observed in the wheat *ERS1* sequence.

Further, the evolutionary route was analyzed by phylogenetic tree from various plant species for ERS as reported by Yan-Yan *et al*., 2013. From the phylogeny analysis it was observed that ethylene receptor gene has been evolved from primitive plant genera like the moss (*Physcomitrella patens*) and fern (*Selaginella mollendorfii*) to higher plants like *MtERS1(Medicago truncatula), GmERS1 (Glycine max), AtERS1(Arabidopsis thaliana) and CaERS1(Cicer arietinum)* from where it gradually evolved through maize, rice, barley and finally into wheat.

4.6.6 Conclusion

In conclusion, the results from this study may help to understand the regulation of ethylene responses mediated by ethylene receptors under drought stress in wheat. As the wheat ethylene receptors is a multigene family, the significance of other members need to be isolated and characterized in future studies in abiotic stress research.

4.7. RESEARCH PAPER VII

Molecular cloning and expression analysis of polyamine biosynthesis genes in wheat (*Triticum aestivum* L.)

4.7.1 Abstract

Polyamines are low molecular weight phytohormone mediating many physiological processes and plant responses to various environmental stresses. This study was attempted to investigate the connection between polyamines and drought stress tolerance. To analyze the effect of water deficit stress on polyamine biosynthesis genes, *TaSAMDC* (S-adenosylmethionine decarboxylase) and *TaSPDS* (Spermidine synthase), three wheat genotypes differing in their drought tolerance ability was studied at reproductive stage. Gene expression analysis indicated that polyamine biosynthesis genes (*TaSAMDC1*, *TaSAMDC2* and *TaSPDS*) responded much more strongly under drought in tolerant genotype (HW 4022) than in susceptible one (HW 4059) which may be responsible for its drought tolerance characteristics. In this study, we further cloned *TaSAMDC1*, *TaSAMDC2* and *TaSPDS* from hexaploid wheat (*Triticum aestivum* L.). To the best of our knowledge molecular characteristics of *TaSPDS* in wheat under drought stress condition was reported for the first time in literature. *TaSAMDC1*, *TaSAMDC2* and *TaSPDS* were predicted to encode a protein with 388, 392 and 385 amino acids respectively. The multiple sequence alignment of *TaSAMDC1* revealed that in HW 2078 glycine is replaced by glutamic acid at 310^{th} position in HW 4022 and HW 4059, while in case of *TaSPDS* arginine in tolerant genotypes (HW 4022 and HW 2078) at position 59^{th} got replaced by cysteine in susceptible genotype (HW 4059). Prediction of conserved domain indicated the SAM binding sites in *TaSAMDC* while, polyamine biosynthesis domain signature was found in case of *TaSPDS,* from positions 145 to 158. Combining gene expression analysis and cloning, we propose that there is a beneficial drought induced polyamine biosynthesis in tolerant wheat genotypes for the accumulation of spermidine and spermine under drought condition.

Key words: Polyamine, Cloning, Drought, Wheat

4.7.2 Introduction

The impact of global climatic changes on crop production has become the major area of research in the present date and for future. It was assumed that by the year 2025, around 1.8 billion people will face the water shortage and about 65 % of the world's population will live under water-stressed environments (Nezhadahmadi *et al*., 2013). Among the various environmental stresses, drought stress is currently the major threat on world's food supply, limiting crop production particularly of economically important cereal crops like wheat, rice, maize, sorghum, etc. However, mechanisms regulating drought tolerance is complicated since it is a quantitative trait influencing the plant's critical developmental stages (Budak *et al*., 2013). Although plant responses to drought stress have been widely studied in many crop species, the exact mechanisms regulating drought tolerance is still under question. Amongst the cereal crops, wheat is an important staple food for majority of the world population. In different parts of India, wheat productivity is severely affected by drought stress conditions (Chakraborty *et al*., 2013). Improvement for drought tolerance is crucial for stabilizing and increasing wheat production under limited irrigation facilities which can be achieved by identification and introduction of drought-related genes and candidate molecules or loci in wheat genotypes.

Drought stress induces the accumulation of different osmolytes like sugars, proline and polyamines to cope with water deficit stress conditions (Alcazar *et al*., 2011) and surprisingly, drought stress induced changes in polyamines can be highly correlated with drought tolerance mechanism. Polyamines (PAs) are small aliphatic amines ubiquitous in plants, animals and microorganisms which are implicated in a wide range of environmental stresses (Marco *et al*., 2011). In plants, the major polyamines are putrescine, spermidine and spermine. Polyamine pathway in plants has been well documented where, putrescine is formed either by the direct decarboxylation of L-ornithine through ornithine decarboxylase (ODC) or by decarboxylation from L-arginine to agmatine by arginine decarboxylase (ADC) and thereafter to putrescine (Hussain *et al*., 2011). Further conversion of putrescine to spermidine and further to spermine requires the addition of one or two aminopropyl groups from decarboxylated S-adenosylmethionine (SAM) by spermidine synthase

(SPDS) and spermine synthase (SPMS), respectively. The aminopropyl moiety resulting from the decarboxylation of S-adenosylmethionine (SAM) by SAM decarboxylase (SAMDC) (Tiburcio *et al.,* 2014). SAMDC and SPDS are thus the key enzymes in polyamine biosynthesis since they plays major roles in spermidine and spermine synthesis (**Fig. 2.5**).

In order to address the function of polyamines for drought tolerance, it is required to isolate the genes encoding the key enzymes (SAMDC and SPDS) involved in polyamine biosynthesis, particularly spermidine and spermine. SAMDC gene has been isolated and characterized in several species like *E. coli* (Tabor and Tabor, 1987), mammals (Pajunen *et al*., 1988), yeast (Kashiwagi *et al*., 1990) and in plant species like potato (MadArif *et al*., 1994), spinach (Bolle *et al*., 1995), wild barley (Dresselhaus *et al*., 1996), rice (Li *et al*., 1999; Li and Chen, 2000b) and wheat (Li and Chen, 2000a), *Arabidopsis* (Urano *et al*., 2003). Another key enzyme spermidine synthase has three homologs *viz.* SPDS1, SPDS2 and SPDS3 in *Arabidopsis* genome (Hanzawa *et al*., 2002). Among the cereal crops, in rice *OsSPDS* was cloned to get insight knowledge about chilling tolerance (Imai *et al*., 2004). However, little is known about molecular mechanism played by SAMDC and SPDS in wheat to cope with drought stress. The current study hypothesized that manipulation of internal polyamine concentrations might be a more appropriate way of studying the physiological significance of stress-induced changes in polyamine contents in wheat. Genes encoding *TaSAMDC1*, *TaSAMDC2* and *TaSPDS* were cloned and their molecular characteristics were analyzed through bioinformatics survey in the present study. In addition the transcript levels of genes (*TaSAMDC1*, *TaSAMDC2* and *TaSPDS*) encoding enzymes involved in polyamine biosynthesis were also analyzed under drought stress. Eventhough, the putative CDS for SPDS in wheat was reported in NCBI database (GenBank accession no. HQ121400) but its molecular characteristic is not yet reported in literature to the best of our knowledge. In this paper, cDNA cloning and molecular analysis of the S-adenosylmethionine decarboxylase (*TaSAMDC1* and *TaSAMDC2*) and spermidine synthase (*TaSPDS*) genes in wheat were reported and found that drought tolerant wheat genotype maintained the higher expression of polyamine biosynthesis genes under drought stress in field condition.

4.7.3 Materials and methods

4.7.3.1 Plant materials and treatments

From preliminary experiment conducted in pot culture condition, we selected three wheat genotypes based on their drought tolerance efficiency *viz*., HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). These genotypes were grown with recommended package of practices and subjected to water deficit stress at 50 % anthesis stage by with-holding irrigation for 10 days. Scheduled routine of irrigation was practiced for control plants throughout the crop growth period. For molecular analysis flag leaf sampling was done from both control (RWC; 80-85 %) and stressed plants (RWC; 65-75 %).

4.7.3.2 RNA isolation and cDNA synthesis

For semi quantitative gene expression analysis of polyamine biosynthesis genes *TaSAMDC* and *TaSPDS*, total RNA was extracted from the flag leaf of control and water deficit stressed plants of three genotypes differing on the basis of their tolerance to water deficit stress. Isolation of total RNA was carried out by TRIzol® reagent (*Invitrogen*™, USA) and RNase-free DNase I (Promega, USA) was applied to remove contaminating genomic DNA at 37 ^{0}C for 1 h. Quality and integrity of total RNA were then determined by running appropriate amount in a formamide denaturing gel, and quantity of total RNA was determined using a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA). The first-strand cDNA was synthesized according to the instructions of the cDNA Synthesis Superscript® III First- Strand Synthesis System (*Invitrogen*™, USA). Resulting cDNA was stored at -20 ^{0}C and employed as template for two-step RT-PCR reactions following recommended conditions provide in user's manual.

4.7.3.3 Primer designing

The primers employed in the present study were designed using the bioinformatics tools described in detail as follows. For semi-quantitative gene expression analysis (RT-PCR) two reported nucleotide sequence of SAMDC (S-adenosylmethionine decarboxylase) gene in wheat from National Centre for Biotechnology Information (NCBI) database *viz*. GenBank accession no. GU016570 and HQ121401 were selected for designing gene specific primers of SAMDC which

were annotated as *TaSAMDC1* and *TaSAMDC2* respectively. Similarly, the reported nucleotide sequence of SPDS in wheat (GenBank accession no. HQ121400) was used for designing gene specific primers for *TaSPDS*. The complete open reading frame (ORF) coverage was confirmed by ORF finder tool of NCBI (http://www.ncbi.nlm.nih.gov/projects/gorf/) and the deduced amino acid sequence using ExPASy translate (http://web.expasy.org/translate/) of the genes showed significant homology with rice and *Arabidopsis* protein sequences by BLASTp analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The gene specific primers were designed manually and quality parameters were confirmed using Integrated DNA Technologies (IDT) Oligoanalyzer tool (https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/). The primers used for semi-quantitative gene expression analysis (RT-PCR) of polyamine biosynthesis genes *TaSAMDC* and *TaSPDS*; and cloning of *TaSAMDC1, TaSAMDC2* and *TaSPDS* are listed in **Table 3.3** Every RT-PCR measurements was performed at least thrice. Transcript of *TaActin* (GenBank accession no. JQ004803) was used as an internal standard for normalization.

4.7.3.4 Cloning and sequencing of *TaSAMDC1, TaSAMDC2* and *TaSPDS* genes

PCR amplification with gene specific primers and proofreading enzyme Platinum Hi-fidelity Taq DNA polymerase (*Invitrogen*^{TM}, USA) gave approximately 1.2 kb fragment that was cloned in TA cloning vector pTZ57R/T (Thermo Scientific, USA). The construct was transformed into *E. coli* strain XL1-Blue and confirmed by blue-white screening in a media containing IPTG (Isopropyl β-D-1-thiogalactopyranoside), X-gal and Kanamycin. Positive clones were sub-cultured in medium having same composition and further confirmed by colony PCR, restriction digestion and sequencing (**Fig. 2.2** and **Fig. 2.3**).

4.7.3.5 *In-silico* analysis of *TaSAMDC1, TaSAMDC2* and *TaSPDS* genes

The BioEdit software was used for aligning forward and reverse sequencing results and the complete nucleotide sequences were reconstructed. The reconstructed sequences were translated using ExPASy Translate Tool (http://web.expasy.org/translate/) and deduced amino acid sequences were obtained for *TaSAMDC1, TaSAMDC2* and *TaSPDS*. These results were confirmed by BLAST software online (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Isoelectric point (pI) and

molecular weight (MW) were analyzed by ExPASy ProtParam tool (http://www. expasy.org/protparam). The multiple protein sequence alignment of *TaSAMDC1*, *TaSAMDC2* and *TaSPDS* and its homologs from model species *viz*. rice and *Arabidopsis* were carried out by ClustalW2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and represented using BoxShade server (http://www.ch.embnet.org/software/BOX_form.html). The phylogenetic tree was constructed with homologous protein sequences from other species by Neighbor joining method using the MEGA 6.06 software.

4.7.4 Results

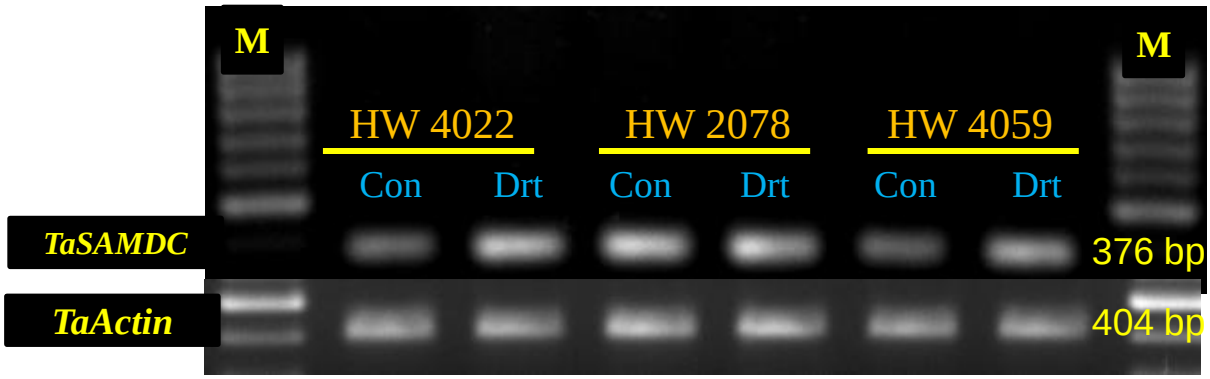
4.7.4.1 Expression analysis of polyamine biosynthesis genes

Expression analysis of genes encoding spermidine biosynthesis *viz*., S-adenosylmethionine (*TaSAMDC*) and spermidine synthase (*TaSPDS*) was studied in flag leaf of wheat genotypes. In this study genotypes employed differ in their drought tolerance efficiency *viz*. HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible) was subjected to water deficit stress by with-holding irrigation for 10 days at reproductive stage. Semi-quantitative expression analysis (Reverse transcriptase polymerase chain reaction, RT-PCR) of *TaSAMDC* and *TaSPDS* was studied using gene specific primers (**Table 3.3**) resulted in 376 and 416 bp amplicon size, respectively. Significant difference was observed in expression pattern of *TaSAMDC* and *TaSPDS* under drought stress (Relative water content; 65-75 %) as compared to irrigated condition (Relative water content; 80-85 %) in all the genotypes studied. Drought stress induced the transcript level of *TaSAMDC*, the tolerant genotype showed higher expression of *TaSAMDC* under water deficit stress while, the lower expression was observed in susceptible genotype HW 4059 (**Fig. 4.7.1**). Similar expression pattern was also observed in *TaSPDS* gene with highest expression level in HW 4022 under water deficit stress (**Fig. 4.7.2**).

4.7.4.2 Cloning and *In-silico* characterization of *TaSAMDC1, TaSAMDC2 and TaSPDS* genes in wheat

The sequencing done on complete coding sequence clones of *SAM decarboxylase* 1 and *2* (*TaSAMDC1* and *TaSAMDC2*) gene yielded a nucleotide sequence of 1200 and 1235 bp containing an open reading frame of 1164 and 1176 bp encoding a polypeptide of 388 and 392 amino acids, respectively. The deduced amino

SAMDC



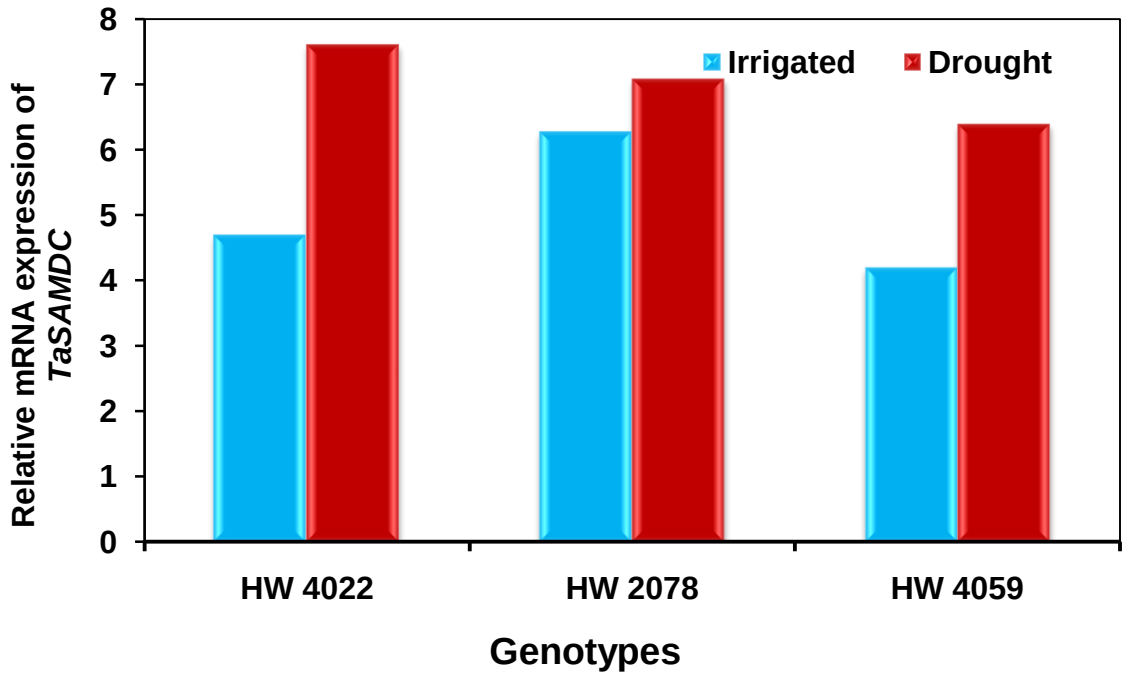
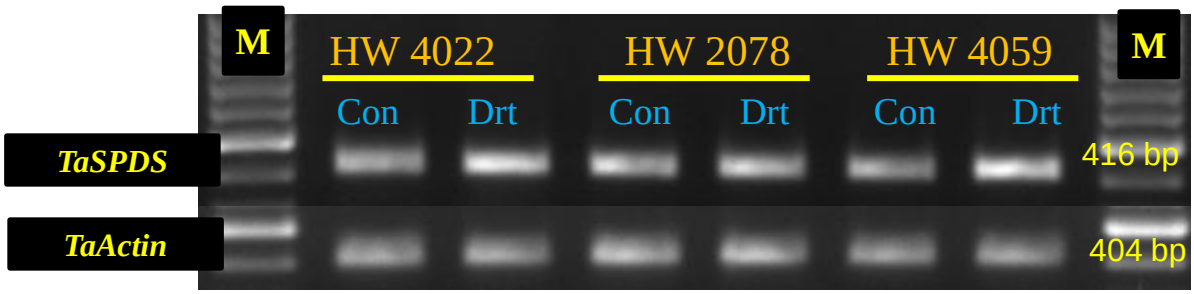


Fig. 4.7.1 Semi-quantitative expression analysis of *TaSAMDC* gene encoding enzyme involved in polyamine biosynthesis in flag leaf of wheat genotypes under control (normal irrigation, RWC; 80-85 %) and water deficit stress condition (withholding irrigation for 10 days, RWC; 65-75 %).

Lane M: 100 bp ladder

Abbrevation: SAMDC: S-adenosyl methionine decarboxylase; Con: Control; Drt: Drought

SPDS



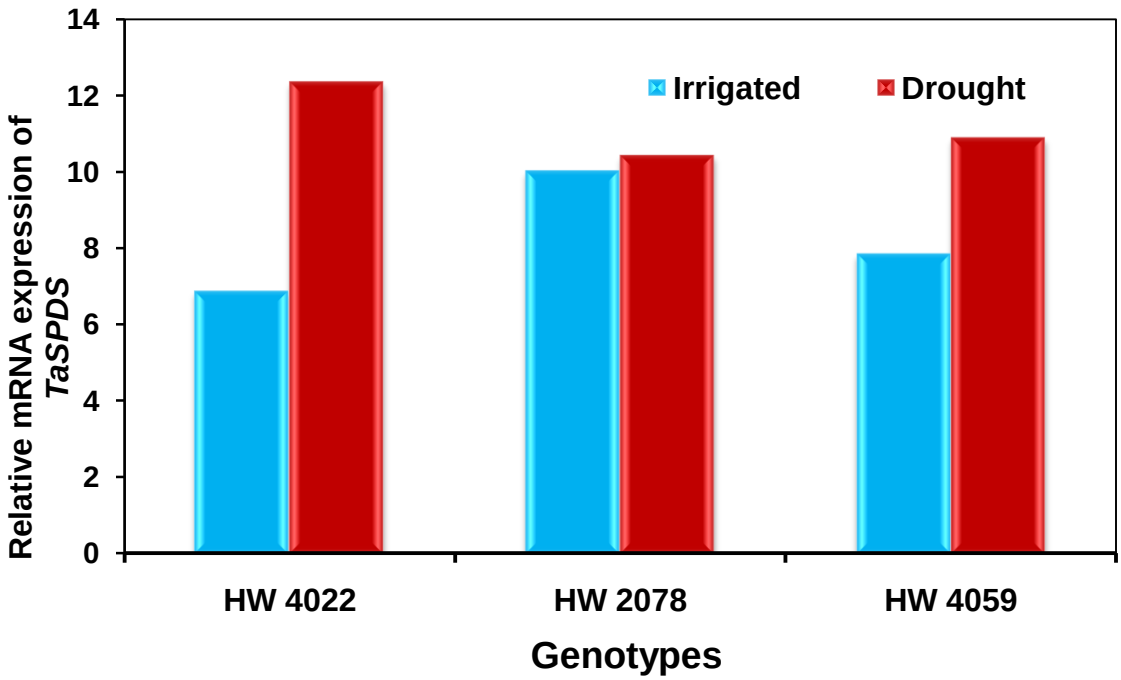


Fig. 4.7.2 Semi-quantitative expression analysis of *TaSPDS* gene encoding enzyme involved in polyamine biosynthesis in flag leaf of wheat genotypes under control (normal irrigation, RWC; 80-85 %) and water deficit stress condition (withholding irrigation for 10 days, RWC; 65-75 %).
Lane M: 100 bp ladder
Abbrevation: SPDS: Spermidine synthase; Con: Control; Drt: Drought

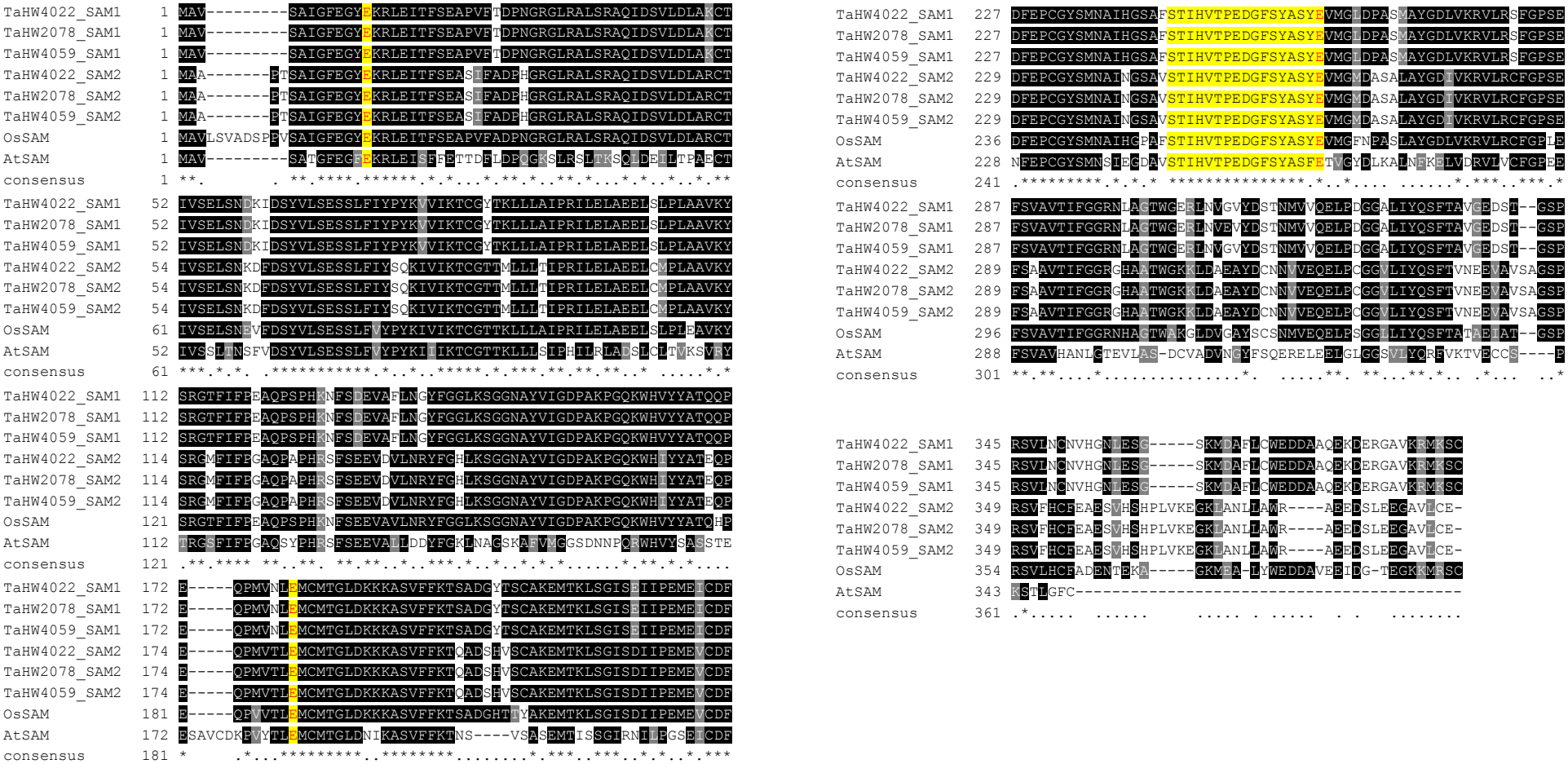


Fig 4.7.3 Multiple sequence alignment of *TaSAMDC1*,*TaSAMDC2* and other homologous SAMDC protein using ClustalW2 and represented by BoxShade. The sequence used in this alignment are as follows: *Triticum aestivum* (GeneBank accession no. AHJ14566, AHJ14567, AHJ14568, AHJ 14569, AHJ14570, AHJ14571), *Oryza sativa* (GeneBank accession no. NP_001047404) and *Arabidopsis thaliana* (GeneBank accession no. NP_189184). Dark shaded region show amino acid residues conserved in all eight sequences and grey shaded regions represent similar residues. The consensus sequences are represented by symbols in each line. Various colour code indicates; yellow: PEST domain, red letters in yellow shade: putrescine activated glutamic acid residues

SAMDC 1 and 2

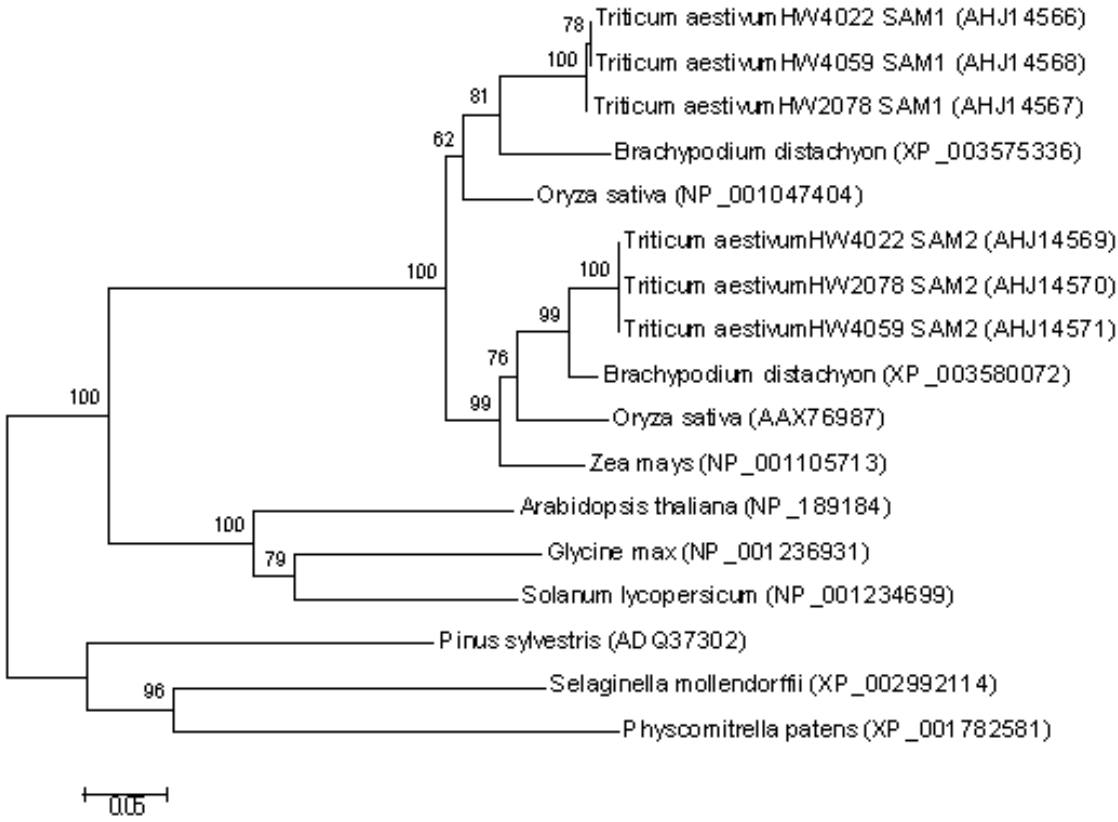


Fig. 4.7.4 Phylogenetic tree of *TaSAMDC1* and *TaSAMDC2* constructed by the neighbor-joining method of MEGA 6.06 software with 500 bootstrap replicates of the ClustalW2 alignment. Evolutionary distances were estimated using the Poisson correction model. Phylogenetic tree of SAMDC proteins from different plant species along with their accession numbers are given in parentheses

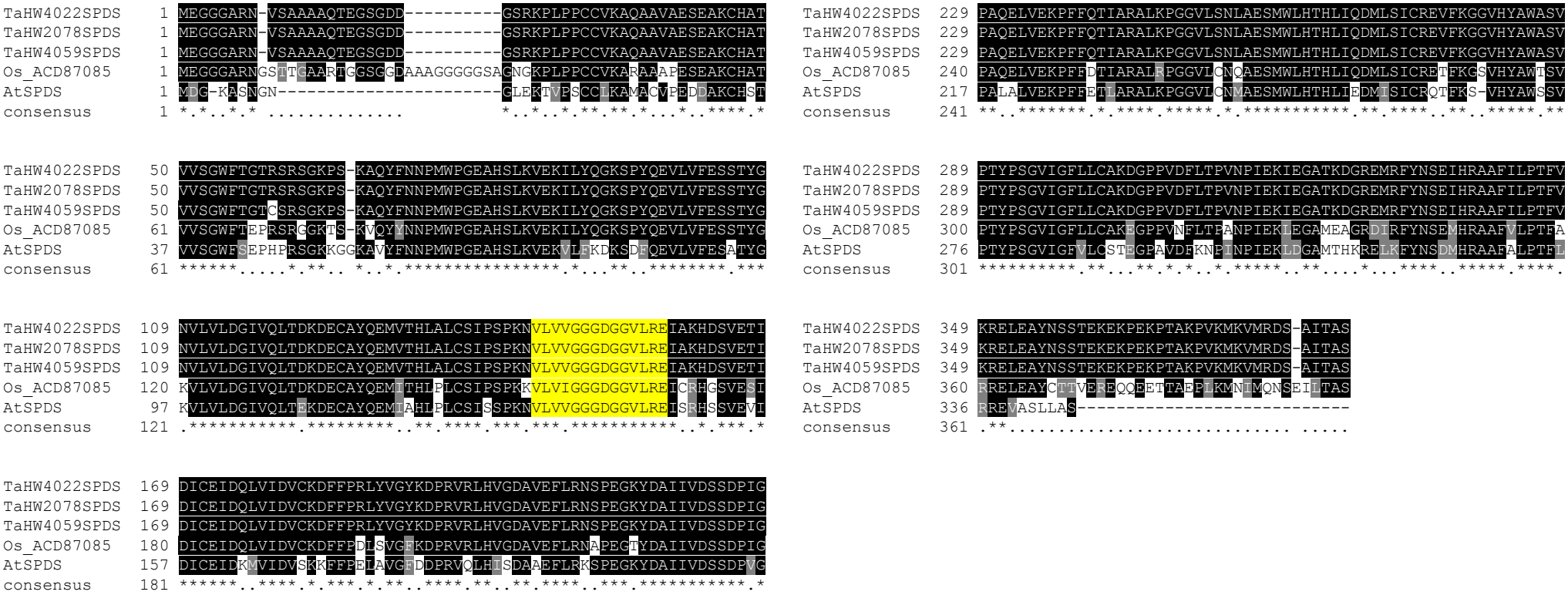


Fig 4.7.5 Multiple sequence alignment of *TaSPDS* and other homologous SPDS proteins using ClustalW2 and represented by BoxShade. The sequence used in this alignment are as follows: *Triticum aestivum* (GeneBank accession no. AHJ14572, AHJ14573, AHJ14574), *Oryza sativa* (GeneBank accession no. CAB61629) and *Arabidopsis thaliana* (GeneBank accession no. AAM64782). Dark shaded region show amino acid residues conserved in all eight sequences and grey shaded regions represent similar residues. The consensus sequences are represented by symbols in each line. The colour code indicates; yellow: spermidine synthase signature motif

SPDS

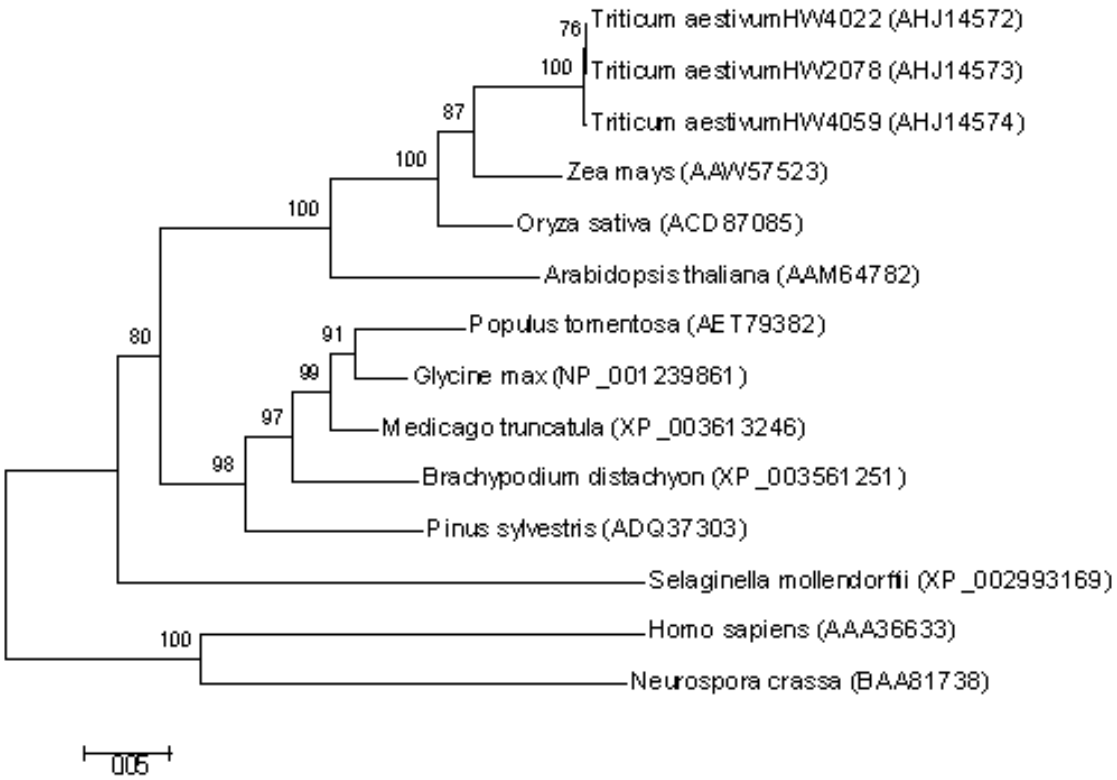


Fig. 4.7.6 Phylogenetic tree of *TaSPDS* constructed by the neighbor-joining method of MEGA 6.06 software with 500 bootstrap replicates of the ClustalW2 alignment. Evolutionary distances were estimated using the Poisson correction model. Phylogenetic tree of SPDS proteins from different plant species along with their accession numbers are given in parentheses

acids by Expasy translate tool produced a protein sequence of 388 amino acids having a molecular mass of 42.37 kDa with pI of 4.93 for *TaSAMDC1* while, it was 392 amino acids having a molecular mass of 42.87 kDa with pI of 4.97 for *TaSAMDC2*. The ClustalW2 multiple sequence alignment of *TaSAMDC1* showed single nucleotide polymorphisms (SNPs) at the nucleotide level which also gave difference in the protein sequence between the genotypes (**Fig. 4.7.3**). The glycine residue of SAMDC1 at 310th position of HW 4022 and HW 4059 is replaced by glutamic acid in HW 2078. The protein sequences of *TaSAMDC1* showed a homology of 54 % and 82 % with *Arabidopsis* and rice SAMDC genes, respectively. Similarly, in case of *TaSAMDC2* the ClustalW2 multiple sequence alignment also showed SNPs at the nucleotide level but no difference in the protein sequence between the genotypes. The protein sequence of *TaSAMDC2* showed a homology of 50 % and 81 % with *Arabidopsis* and rice SAMDC2 genes, respectively. The phylogeny tree constructed using the MEGA 6.06 software gave light into the evolution of SAMDC gene in plant species (**Fig. 4.7.4**).

PCR amplication and cloning of full-length cDNA encoding a spermidine synthase (*TaSPDS*) gene in wheat yielded a nucleotide sequence of 1203 bp. The cDNA contains an open-reading frame (ORF Finder, NCBI) of 1158 bp encoding 385 amino acid polypeptide with a calculated molecular mass (Expasy Compute PI/MW Tool) of 42.04 kDa and pI of 5.91. Conserved domain (Expasy Prosite) from positions 145 to 158 was shown to have polyamine biosynthesis domain signature (**Fig. 4.7.5**). The search for conserved domains in Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) retrieved class I S-adenosylmethionine dependent methyl transferases that flanks three SAM binding sites at positions E 172, G 202 and D 222, in accordance with its molecular function. Due to its close sequence similarity with spermine synthase, the BLASTp provided ambiguous results of *SPDS* and *SPMS* in several species. The susceptible genotype (HW 4059) showed only 99.74 % identity with the other two genotypes (HW 4022 and HW 2078) because of a single amino acid difference from arginine to cysteine at 59th position. The multiple protein sequence alignment (ClustalW2) with *Arabidopsis* and rice showed considerable homology between the sequences of 71 % and 81 %, respectively. The phylogeny tree was constructed for *TaSPDS* in the similar way as done for *TaSAMDC* (MEGA 6. 06 Tool) (**Fig. 4.7.6**).

4.7.5 Discussion

S- adenosylmethionine decarboxylase (SAMDC) is a key enzyme controlling the rate of polyamine production (Spermidine and spermine) in plants resulting in enhanced tolerance to various stresses like drought (Alcazar *et al*., 2011), salinity (Hu *et al*., 2014), cold (Guo *et al*., 2014) and heat (Johnson *et al*., 2014). SAMDC is a small gene family in plants for eg. Four members of SAMDC gene existed in *Arabidopsis* (Ge *et al*., 2006), six SAMDC paralogs exist in rice genome among them *OsSAMDC2* is induced during drought stress (Do *et al*., 2013). The biosynthesis and enzymatic mechanism of SAMDC was first reported by Riley and Snell in 1968 and later cloned and characterized by Tabor and Tabor (1987) in *Escherischia coli*, Kashiwagi *et al*., (1990) in yeast and thereafter in several plant species like potato (MadArif *et al*., 1994), wheat (Li and Chen, 2000a), rice (Li and Chen, 2000b), mustard (Hu *et al*., 2005) etc.

The present study targeted to understand the possible role of polyamine biosynthesis genes in wheat crop in response to drought stress. Semi-quantitative RT-PCR expression analysis was performed in flag leaf for control (RWC; 80-85 %) and after imposing drought stress for 10 days at reproductive stage (RWC; 65-75 %) in three wheat genotypes differing in response to drought tolerance characteristics *viz*. HW 4022 as drought tolerant, HW 2078 as relatively drought tolerant and HW 4059 as drought susceptible genotype. The result showed that water deficit stress induced the synthesis of spermidine in tolerant genotype (HW 4022) by up-regulating the expression of *TaSAMDC* and *TaSPDS* genes, encoding spermidine biosynthesis enzyme S-adenosylmethionine decarboxylase and spermidine synthase, respectively. On the other hand susceptible genotype showed lower transcript of *TaSAMDC* and *TaSPDS* suggesting lower level of spermidine content under water deficit stress condition. The data is consistent with the report in rice by Do *et al*. (2013, 2014) under salinity and drought stress, respectively where they found induced expression of *OsSAMDC* and *OsSPDS* in tolerant genotype whereas it was downregulated in susceptible one. Similar findings in rice reported recently by Chen *et al*. (2014) where transgenic rice plants down regulating *OsSAMDC*, showed reduced level of spermidine and spermine content with increased sensitivity to drought and salinity stress. Additional supporting results from rice by Basu *et al*. (2014) showed increased transcript level of SAMDC under drought, cold, salinity, light and ABA stress,

thereby confirming protective role played by polyamines under various kind of stresses. Comparable findings reported in wheat embryo under drought stress by Du *et al*. (2014) where elevated level of polyamines (putrescine, spermidine and spermine) induced by drought stress enhanced the tolerance of developing wheat embryos.

Further molecular characterization in terms of cloning and *in-silico* analysis of SAMDC homologous i.e. SAMDC1 and SAMDC2 in wheat (*Triticum aestivum* L.) was also performed. The resulting sequences of SAMDC1 and SAMDC2 were of 1200 and 1235 bp in length and containing an open reading frame of 1164 and 1176 bp encoding a polypeptide of 388 and 392 amino acids, respectively. The ClustalW2 multiple sequence alignment of predicted amino acid sequence of *TaSAMDC1* and *TaSAMDC2* with SAMDC proteins from *Arabidopsis* and rice identified several conserved regions. These include the catalytically active putrescine activated glutamic acid residue at positions 12, 179 and 260 in *TaSAMDC1*; and 14, 181 and 262 in *TaSAMDC2*. These residues were already been described by Li and Chen (2000a) in wheat. Another highly conserved region, a putative PEST domain consisting about 16 amino acid (TIHVTPEGFSYASYE) residues from positions 244 to 260 in *TaSAMDC1*and 246 to 262 in *TaSAMDC2* was also identified. The PEST domain was first described by Rogers *et al*. (1986) which are rich in proline, serine, threonine and glutamic acid. PEST domain is responsible for rapid protein turnover and removal of this domain stabilizes the protein (Li and Chen, 2000a). The genes that we cloned coded for SAMDC proenzyme with a cleavage site at position 67th (*TaSAMDC1*) and 69th (*TaSAMDC2*) has already been reported (Kashiwagi *et al*., 1990) and confirmed to be essential for biological function of SAMDC in plants (Schroder and Schroder, 1995; Li and Chen, 2000a).

Another key enzyme spermidine synthase encoding gene *TaSPDS* in wheat (*Triticum aestivum* L.) was cloned and sequenced. The isolated nucleotide sequence was of 1203 bp containing an open reading frame of 1158 bp encoding a polypeptide of 385 amino acids with a molecular mass of 42.05 kDa. Database searches revealed amino acid sequence of *TaSPDS* showed strong similarity with other SPDS proteins in higher plants (Hashimoto *et al*., 1998; Fuell *et al*., 2010). There are few other reports on isolation of SPDS genes has been reported: *Homo sapiens* (Casero *et al*., 1991), *Nicotiana sylvestris* (Hashimoto *et al*., 1998), *Arabidopsis* (Hashimoto *et al*., 1998), *Solanum lycopersicum* (Alabadi and Carbonell, 1999), *Oryza sativa* (Imai *et*

al., 2004), *Caenorhabditis elegans* (Dufe *et al*., 2005), *Cucumis sativus* (Wang *et al*., 2005) and *Zea mays* (Rodriguez-Kessler *et al*., 2006) were characterized till date. To the best of our knowledge, this is the first report on transcriptional regulation and cloning of SPDS gene in wheat under drought stress. The deduced amino acid sequence of (*TaSPDS*) contained the characteristic motifs of SPDS signature (VLVVGGGDGGVLRE) from positions 145-158 (Rodriguez-Kessler *et al*., 2006) which contained catalytically active aspartic acid residue at 152nd position. The other catalytically active positions include tyrosine at 127th position and aspartic acid at 222nd position (Wu *et al*., 2008). The phylogeny tree was constructed to put a light into the evolutionary history of SAMDC and SPDS proteins with animals, fungi, ferns, mosses, pines, monocots and dicots using the ClustalW2 method (Higgins and Sharp, 1988). From our report, the presence of SAMDC can be identified from the fern (*Selaginella mollendorfii*), bryophyte (*Physcomitrella patens*) and pines (*Pinus sylvestris*) to higher plants like dicots (*Arabidopsis thaliana, Glycine max,* etc.) which further diverged into SAMDC1 and SAMDC2 in monocots (*Oryza sativa, Brachypodium distachyon*, *Triticum aestivum*). The presence of *SPDS* can be dated back from the fungi (*Neurospora crassa*), mammals (*Homo. sapiens*) and fern (*Selaginella mollendorfii*) from where it diverged into monocots (*Zea mays*, *Oryza sativa, Brachypodium distachyon* and *Triticum aestivum*) and dicot (*Arabidopsis thaliana, Glycine max,* etc.) species of higher plants. Thus, it was proved that SAMDC diverged from fern into plant species while, evolution of SPDS originated in fungi to land plants.

4.7.6. Conclusion

In summary, we identified and cloned SAMDC homologs (*TaSAMDC1* and *TaSAMDC2*) and *TaSPDS* gene in wheat. To the best of our knowledge this is the first report on cloning of *TaSPDS* in wheat under drought stress. These findings together with previous reports suggests that higher spermidine and spermine level by transcriptional regulation of *TaSAMDC* and *TaSPDS* gene contribute to drought tolerance in wheat. Further work is required to characterize these polyamine biosynthesis genes by reverse genetic approaches to ascertain their biological function under various environmental constraints.

5. DISCUSSION

Drought stress is one of the major threats for wheat production (Gong and Chen, 2012). According to the prediction of current climate change models, the frequency and severity of drought will increase in several regions around the world (Mishra and Singh, 2010). The drought tolerance efficiency of a crop is linked to its ability to access available soil moisture and to use it most productively under water limited conditions (Richards *et al*., 2010). Although drought may affect wheat crop during all growth stages but the reproductive stage is the most sensitive phase for water deficit stress (Pradhan *et al*., 2012). Development of wheat genotypes requiring less water input to produce sufficient biomass along with an accurate site-specific package of production technology is urgently needed to sustain crop productivity in drought-prone areas (Tariq *et al*., 2013). Increasing drought tolerance of wheat is a feasible way to overcome the drought related problems by delaying drought induced leaf senescence, thereby developing stay-green genotypes with normal photosynthesis and higher yield. To prove the hypothesis the present study was conducted with thirty-five wheat genotypes differing in their drought tolerant efficiency under water deficit stress.

In the current study, genotypic variations were revealed for stay-green traits under water deficit stress. All the genotypes showed decline in stay-green traits like greenness index and photosynthesis rate under water deficit stress as compared to normal irrigated conditions. Genotypes *viz*. HW 4022, CBW 23, PBW 555 and HW 4010 that maintained higher stay-green traits under water limited condition with delayed leaf senescence rate are categorized as stay-green genotypes. Those showing faster leaf senescence rate such as HW 4059, HW 4207, HW 541 and HW 4055 were grouped as non stay-green genotypes. Further, genotypes with stay-green traits *viz*. HW 4022, HW 2036, HW 4010, HW 4055, CBW 23 and HW 4024 were superior in yield, harvest index, test weight with higher drought tolerance efficiency (DTE; %) while, non stay-green genotypes (HW 4059, HUW 541, PBW 142, HW 2022, HW 2083 and HD 2789) showed significant reduction in all the yield related traits. For further dissection to know the mechanism of drought tolerance, three wheat genotypes were screened based on their contrasting DTE and stay-green character *viz*. HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible genotype) were selected. In consistence with our findings similar results

reported in chickpea that the drought resistant genotype had highest DTE with minimum reduction in grain yield under moisture deficit stress condition (Parameshwarappa and Salimath, 2010; Deshmukh *et al*., 2013). Puri *et al*. (2013) also reported that rice genotypes with highest DTE could be categorized as the most drought tolerant genotypes that could play important role in extreme climatic situations, where farmers can adopt them as climate resilient genotypes. Further, in sorghum, Borrell *et al*. (2014) reported that stay-green genotypes maintained their photosynthesis activity with higher water use efficiency resulting in increased biomass, grain number and yield under terminal drought situation. Jordan *et al*. (2012) in rice and Saxena *et al*. (2014) in wheat also found a close association of stay-green traits with increased grain yield and this trait can be used as selection tools for physiological breeding programmes to increase the production and productivity of cereal crops under stress.

Relative water content (RWC; %) and membrane stability index (MSI; %) are the good criterion for selection of tolerant genotypes with higher yields under drought condition (Rad *et al*., 2013). In the current study, all the three genotypes showed decline in RWC and MSI under water deficit stress condition. Drought imposed decline in RWC and MSI was less in stay-green genotypes (HW 4022 and HW 2078) as compared to non stay-green one (HW 4059). Similar findings reported by Ashraf and Harris (2013) that under water deficit stress, drought tolerant wheat genotype maintained higher RWC and MSI which can be used as good physiological indices of drought tolerance and improvement of drought tolerance in wheat.

Oxidative stress due to generation of reactive oxygen species (ROS) such as superoxide, hydrogen peroxide (H_2O_2), singlet oxygen, and hydroxyl radical are common events during drought stress (Arora *et al*., 2002; Farooq *et al*., 2013). Drought stress significantly enhanced the membrane deterioration as reflected by increased H_2O_2 level resulting in enhanced lipid peroxidation, protease and lipoxygenase activity (LOX). Results from the current study under water deficit stress showed that drought tolerant genotype (HW 4022) was having lower H_2O_2 level and lipid peroxidation as compared to susceptible genotype (HW 4059). In addition, marked increase in protease and LOX activity was detected in drought susceptible wheat genotype (HW 4059) as compared to tolerant one (HW 4022) under water deficit stress conditions. Parallel findings by Hameed *et al*. (2012, 2013) in wheat

support our results, where drought susceptible genotype under drought stress showed marked increase in H_2O_2 content and lipid peroxidation level along with protease activity resulting in severe membrane deterioration as compared to tolerant genotype. Possibly, the enhanced lipid peroxidation due to drought induced oxidative stress particularly; H_2O_2 accumulation and increased proteolysis by raised protease activities may have compelled the sensitive genotype to perform poorly under water limited condition. Similar reports were observed from tobacco (Chaves *et al*., 2011) and rice (Campo *et al*., 2014). Water deficit stress induced the membrane bound LOX activity in most of the wheat genotypes (Permyakova *et al*., 2012). The higher LOX activity is correlated with higher lipid peroxidation in tomato genotypes subjected to water deficit stress (Sanchez-Rodriguez *et al*., 2012). In conclusion, lower oxidative stress damage in tolerant genotype (HW 4022) is due to reduced level of H_2O_2 accumulation additionally with decreased protease and LOX activities resulting in enhanced membrane stability under water deficit stress conditions.

Plant responds to oxidative stress damage through increased activity of antioxidant enzymes which act as protective scavenging system against ROS species and also osmoregulation by accumulating osmolytes like proline, sugars etc. In the present study, drought tolerant stay-green genotypes (HW 4022 and HW 2078) showed enhancement in their antioxidative defense system with increment in antioxidant enzyme activities like superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), peroxidase (POX) and ascorbate peroxidase (APOX) as compared to drought susceptible non stay-green genotype (HW 4059) under water deficit stress condition. Comparable findings by De-Simone *et al*. (2014) in wheat, where stay-green genotypes showed better redox state generally by higher antioxidant enzymes activities like SOD, CAT and POX along with lower LOX activity as compared to non stay-green ones. The association of osmotic adjustment has also been shown in a number of different cereals crops under drought stress (Gonzalez *et al*., 2008; Hajlaoui *et al*., 2010). The compatible osmolytes such as proline was induced by water deficit stress and have been demonstrated to be involved in sequestration of ROS, resulting in protection against oxidative stress damage in several plant species (Loutfy *et al*., 2012; Jain *et al*., 2013). Under water limited condition, drought tolerant genotypes (HW 4022 and HW 2078) reported with higher proline accumulation as compared to susceptible genotype (HW 4059). The present findings were supported by Bowne *et al*. (2012) under drought stress, tolerant wheat

genotypes reported with higher proline accumulation and better osmotic adjustment in comparison to drought susceptible genotype.

Chlorophyll content is a marker for a stay-green character of plant (Thomas and Howarth, 2000). Stay-green genotypes have been reported to maintain leaf greenness through delayed leaf senescence while, leaf senescence is associated with chlorophyll degradation and a progressive decline in photosynthetic capability (Tian *et al*., 2012, 2013). Drought stress leads to a wide range of physiological responses in addition to the obvious reductions in photosynthesis, stomatal conductance and transpiration rate (Munns, 2011). Photosynthesis is a highly drought sensitive process, while reduced stomatal conductance is the primary cause of decreased photosynthesis rate under water deficit stress (Cornic, 2000; Chen *et al*., 2012). Drought stress causes leaf senescence which results in degradation of chlorophyll and disorganization of the photosynthetic apparatus (Matile *et al*., 1999) resulting in lower crop yields. Results from the present study showed that drought tolerant stay-green genotypes (HW 4022 and HW 2078) recorded with higher chlorophyll and carotenoid content along with photosynthesis characteristics like photosynthesis rate, transpiration rate and stomatal conductance in comparison to non stay-green genotype (HW 4059). To get an insight about chlorophyll degradation pathway, we also analyzed expression of genes encoding chlorophyll degrading enzymes in three contrasting wheat genotypes. Chlorophyll is degraded through a series of reactions catalyzed by enzymes like chlorophyllase (CHLase), magnesium de-chelatase, and pheophorbide a oxygenase (PaO) (Hortensteiner, 2013). In the present study, tolerant genotypes with improved stay-green traits (HW 4022 and HW 2078) showed reduced expression of chlorophyll degradation genes (*TaPaO* and *TaCHLase1*), while susceptible non stay-green genotype (HW 4059) displayed higher expression under both osmotic/ water deficit stress. The complete cDNA sequence of *TaCHLase1* consisted of 1054 bp nucleotides containing an open reading frame of 960 bp encoding 319 amino acids. The encoded protein contained conserved residue such as lipase motif GXSXGG at position 143-148 and putative active site Ser145. Similar findings were reported in support to our study in other plant species mostly in horticulture crops (Zhang *et al*., 2011; Chen *et al*., 2012; Peng *et al*., 2013; Lira *et al*., 2014) and few reports are available for cereal crops. In conclusion, a negative correlation exist between chlorophyll content and expression of chlorophyll degrading enzymes and there is a potential for increasing grain yield by delaying chlorophyll degradation in wheat under drought stress.

Phytohormones are involved in mediating the responses of the plant to abiotic stress. They don't act in isolation but are interlinked synergistically or antagonistically so that they can modulate each other's biosynthesis or responses which play a crucial role in plant adaptation to various abiotic stresses (Wilkinson and Davies, 2010). Controlling the ratio of two hormones' level remains a major challenge, since the hormone levels attained should be moderate in order to maintain a balance between the positive effects of plant hormones on stress tolerance and the negative effects on growth and development (Peleg and Blumwald, 2011). Phytohormones activate the molecular and cellular systems in plants to sense drought and activate signaling networks to enhance drought tolerance in plants (Hitomi *et al*., 2013).

Ethylene is recognized as one of the main factor that trigger the onset of stress-induced leaf senescence and abscission (Abeles *et al*., 1992). Ethylene produced during drought stress can act as a direct yield detriment, notably by reducing grain-filling rates and with enhanced leaf senescence rate in various economically important cereal crops like rice and wheat (Wilkinson and Davies, 2010). The role of polyamines in improving drought resistance has been examined from several perspectives, focusing on improved antioxidant system, improved leaf water status and delayed leaf senescence rate under stress condition (Alcazar *et al*., 2010). Further, changes in plant polyamine metabolism occur in response to a variety of abiotic stresses (Alcazar *et al*., 2011), however, the physiological significance of increased polyamine levels in abiotic stress responses is still unclear (Gill and Tuteja, 2010). Thus, there is a genetic potential for plants to modulate their polyamine levels to cope with stress conditions. Changes in polyamines and ethylene accumulation are a common response of plants to water deficit stress and are considered to be correlated with plant drought tolerance. In the present study sharp increase in ethylene level was observed in drought susceptible genotype (HW 4059) under PEG induced osmotic stress (-4.91 MPa) in 10 days old seedling as well as in presence of ethylene releasing compound (Ethrel; 10 ppm) in comparison to tolerant genotypes (HW 4022 and HW 2078). Ethylene evolution rate decreases in presence of ethylene biosynthesis (AVG; 2 ppm) and signaling (1-MCP; 10 ppm) inhibitors, but the decrease was reported to be more in tolerant genotype (HW 4022). Polyamine levels particularly, spermidine and spermine was found to be higher in tolerant genotypes (HW 4022 and HW 2078) under osmotic stress as well as in presence of ethylene inhibitors, while in susceptible genotype the level was markedly decreased under osmotic stress and various

treatment combinations. Comparable findings reported in economically important crop species like in tomato (Nambeesan *et al*., 2012), cereals like rice (Chen *et al*., 2013), maize (Feng *et al*., 2011) and sorghum (Yin *et al*., 2014), where polyamines act as a positive regulator for drought tolerance with improved yield and ethylene showed negative impact on growth and yield by enhancing drought induced leaf senescence rate under water deficit stress condition. Recent report by Yang *et al*. (2014) provide an evidence on the interactive role of ethylene and polyamines during grain filling period in wheat, that increased grain yield under drought stress is positively correlated with the increased ratio of spermidine/ ethylene or spermine/ ethylene.

To gain further insight into this topic, it is necessary to study the molecular mechanism governing the interconnections of polyamine and ethylene biosynthesis pathway with drought tolerance mechanism in wheat. It was reported previously that both these plant growth regulators share a common biosynthetic precursor S-adenosylmethionine (SAM) (Alcazar *et al*., 2010). Hence, polyamines and ethylene could act in an antagonistic manner competing for the common substrate SAM. To prove this hypothesis we analyzed the expression of the genes encoding key enzymes involved in polyamine and ethylene biosynthesis/ signaling pathway. The result showed that tolerant genotype (HW 4022) displayed higher expression of spermidine and spermine biosynthesis genes (*TaSAMDC* and *TaSPDS*) along-with ethylene signaling gene (*TaERS1*) and lower expression of ethylene biosynthesis genes (*TaACO2* and *TaACS1*) under osmotic/ water deficit stress treatment and even in presence of ethrel as a source of ethylene. Exogenous application of ethylene inhibitors (AVG and 1-MCP), is accompanied by marked increased in polyamine biosynthesis genes and it reduces the expression of ethylene biosynthesis genes in drought tolerant genotype (HW 4022). In contrast to this, susceptible genotype (HW 4059) showed increment in expression of ethylene biosynthesis genes, while decreased expression of polyamine biosynthesis and ethylene signaling genes was observed under the above mentioned treatments. In addition, significant change in gene expression was not recorded in susceptible genotype (HW 4059) even in presence of ethylene inhibitors under osmotic/ water deficit stress. Our findings hereby suggested that exogenous application of ethylene inducer (Ethrel) intensified apparently the water deficit stress induced injury to drought susceptible genotype (HW 4059) by increase in ethylene evolution rate, together with inhibition of

polyamine content. However, tolerant genotype (HW 4022) showed improved drought tolerance mechanism through up-regulation of polyamine biosynthesis and down regulation of ethylene biosynthesis genes under water deficit stress condition and even in presence of various chemical regulators under osmotic stress. Our study had been supported by Locke *et al*. (2000) in barley, Gill-Amado *et al*. (2012) in pea, Chen *et al*. (2013) in rice and Habben *et al*. (2014) in maize, providing evidences to the view that polyamine and ethylene cross-regulation occurs and has important implications for drought tolerance in crops. The result suggested that by down regulation of ethylene biosynthesis genes and increase in expression of polyamine biosynthesis genes improved the performance of wheat crop under water deficit stress condition.

To know more in detail about the genes encoding key enzymes for ethylene biosynthesis (ACO)/ signaling (ERS), we isolated the complete CDS of these genes and studied their molecular characteristics from selected wheat genotypes. Cloning and *in silico* characterization of *TaACO2* using the reported homologues from other species showed conserved domains essential for ACC, Fe^{2+}, ascorbate binding and enzyme activity. The *TaERS1* gene also showed the conserved motifs/ domains typical to ethylene receptors including transmembrane domains which perceive ethylene, GAF domain that activates cyclic GMP, histidine kinase domain and HATPase domains which are essential for ethylene responses. In addition, we were successful in isolating the genes encoding spermidine and spermine biosynthesis, annotated as *TaSAMDC1*, *TaSAMDC2* and *TaSPDS*. SAMDC genes were found to have three glutamic acid residues (E12, E179 and E260 in *TaSAMDC1*; and E14, E181 and E262 in *TaSAMDC2*) essential for putrescine binding and hence the enzyme activity. The gene SPDS, coding for the enzyme spermidine synthase was found to have the spermidine synthase signature motif which contained the catalytically active aspartic acid residue (Asp152). The output from cloning and sequence analysis of these genes helped in understanding the mechanisms governing their catalytic activity.

In summary, the result presented from the current research indicates that delayed leaf senescence rate is a good physiological index for drought tolerance in crop plants. Under water limited conditions, polyamine act as a positive regulator, while ethylene as a negative regulator. In addition, the study further provide an

evidence that there might be a potential metabolic crosstalk between ethylene and polyamines under natural drought situations in plants which may help them to mitigate its impacts. In conclusion, the major point from whole research in relation with wheat crop under drought situation. The evidences proved the hypothesis that genetic manipulation of crop plants with genes encoding enzymes of polyamine biosynthesis pathway may provide better stress tolerance to crop plants under drought stress.

6. SUMMARY and CONCLUSION

The present investigation entitled "**Interactive role of ethylene and polyamines on the drought tolerance in wheat**" was conducted to have physiological and molecular insights about the role played by ethylene, polyamines and their potential metabolic interaction for drought stress tolerance in wheat. The findings in the present study revealed that delayed leaf senescence through enhanced polyamine biosynthesis gene(s) could be used as biochemical or molecular markers for breeding and development of climate resilient wheat crop.

The wheat genotypes were grown in pot culture, Division of Plant Physiology, IARI, New Delhi during two *Rabi* seasons of 2011-12 and 2012-13 with recommended cultural practices. Effect of water deficit stress was studied in thirty five wheat genotypes by with-holding irrigation for 10 days at reproductive stage and routine schedule of irrigation was practiced for control plants throughout the growth phases. Three wheat genotypes differing in their drought tolerance efficiency and stay-green traits (HW 4022, drought tolerant; HW 2078, relatively drought tolerant and HW 4059, drought susceptible) were selected for further study. Biochemical and molecular studies were conducted in laboratory with selected genotypes and osmotic stress was induced by polyethylene glycol (PEG-6000, 20 % with osmotic potential of -4.91 MPa). In addition, treatments were given to 10 days old seedlings with chemical regulators like ethylene inducer (Ethrel), ethylene biosynthesis inhibitor (AVG) and signaling inhibitor (1-MCP). The various physiological and biochemical parameters studied are relative water content (RWC; %), test weight, harvest index (HI; %), drought tolerance efficiency (DTE; %), stay-green traits, photosynthesis characteristics, oxidative stress indicators, antioxidant enzymes' activity, ethylene and polyamine levels. Expression analysis by semi-quantitative RT-PCR of chlorophyll degradation genes (*TaCHLase1* and *TaPAO*), ethylene biosynthesis/ signaling genes (*TaACO2, TaACS1* and *TaERS1*) and polyamine biosynthesis genes (*TaSAMDC* and *TaSPDS*) and cloning and characterization of the genes involved in stay-green traits (CHLase), ethylene biosynthesis/ signaling (ACO and ERS), polyamine biosynthesis (SPDS) and their interaction (SAMDC homologs) were also studied.

The salient features of experimental findings are presented below:

- Genotypic variations were observed among the genotypes under water deficit stress conditions for stay-green traits (greenness index and photosynthesis rate) and leaf senescence rate. Genotypes *viz*. HW 4022, CBW 23, PBW 555 and HW 4010 maintained the stay-green traits under water limited condition with delayed leaf senescence rate (LSR) and categorized as stay-green genotypes. Those showing faster leaf senescence rate such as HW 4059, HW 4207, HW 541 and HW 4055 were grouped as non stay-green genotypes.
- Relative water content decreased under water deficit stress condition in all the genotypes studied but the decline was more in non stay-green genotypes as compared to stay-green ones.
- Genotypes with stay-green traits *viz*. HW 4022, HW 2036, HW 4010, HW 4055, CBW 23 and HW 4024 were superior in yield, harvest index, test weight with higher drought tolerance efficiency (DTE; %) while, non stay-green genotypes (HW 4059, HUW 541, PBW 142, HW 2022, HW 2083 and HD 2789) showed significant reduction in all the yield related traits.
- Genotype HW 4022 with highest DTE and lower LSR were selected as drought tolerant, HW 2078 with intermediate value for both the parameters as relatively drought tolerant and HW 4059 with lowest value for DTE and highest LSR were selected as drought susceptible genotype.
- Drought susceptible genotype (HW 4059) reported with maximum membrane damage due to increased accumulation of H_2O_2 level, lipid peroxidation, higher protease and lipoxygenase enzyme activity which results in membrane damage under water limited condition as compared to tolerant genotypes (HW 4022 and HW 2078).
- Drought tolerant genotype (HW 4022) maintained higher antioxidant enzyme activities of superoxide dismutase, catalase, glutathione reductase, peroxidase and ascorbate peroxidase under water limited condition as compared to susceptible genotype (HW 4059).
- Drought induced increase in accumulation of proline content reported in tolerant genotypes (HW 4022 and HW 2078) which helps in osmoregulation mechanism in comparison to susceptible genotype (HW 4059).

- Drought tolerant genotypes (HW 4022 and HW 2078) with improved stay-green traits showed reduced expression of chlorophyll degradation genes (*TaPaO* and *TaCHLase1*), while susceptible non stay-green genotype (HW 4059) displayed higher expression under both osmotic/ water deficit stress.
- Ethylene level was found to be more in drought susceptible genotype (HW 4059) under osmotic stress in comparison to tolerant genotypes (HW 4022 and HW 2078).
- Ethylene level decreases in presence of ethylene biosynthesis (AVG) and signaling (1-MCP) inhibitors, but the decrease was reported to be more in tolerant genotype (HW 4022).
- Polyamine levels particularly, spermidine and spermine was found to be more in tolerant genotypes (HW 4022 and HW 2078) under osmotic stress as well as in presence of ethylene inhibitors, while in susceptible genotype (HW 4059) ethylene level was markedly decreased under osmotic stress and various treatment combinations.
- Tolerant genotype (HW 4022) displayed higher expression of spermidine and spermine biosynthesis genes (*TaSAMDC* and *TaSPDS*) along-with ethylene signaling gene (*TaERS1*) and lower expression of ethylene biosynthesis genes (*TaACO2* and *TaACS1*) under osmotic/ water deficit stress condition as well as during the presence of ethrel.
- Exogenous application of ethylene inhibitors (AVG and 1-MCP), is accompanied by marked increased in polyamine biosynthesis genes and decrease in expression of ethylene biosynthesis genes in drought tolerant genotype (HW 4022).
- Cloning and sequence analysis of ethylene biosynthesis gene (*TaACO2*) using the reported homologues from other species showed conserved domains for ACC, Fe^{2+}, ascorbate binding which are essential for ACO enzyme activity.
- Cloning and sequence analysis of ethylene signaling gene (*TaERS1*) also showed the conserved domains typical to ethylene receptors including transmembrane domains which perceive ethylene, GAF domain that activates cyclic GMP, histidine kinase domain and HATPase domains which are essential for ethylene responses.
- Cloning and sequence analysis of genes encoding key enzyme regulating ethylene and polyamine biosynthesis by SAMDC (*TaSAMDC1*, *TaSAMDC2*). SAMDC

homologs were found to have three glutamic acid residues (E12, E179 and E260 in *TaSAMDC1*; and E14, E181 and E262 in *TaSAMDC2*) essential for putrescine binding and hence the enzyme activity.

- Cloning and sequence analysis of spermidine biosynthesis gene (*TaSPDS*) was found to have the spermidine synthase signature motif which contained the catalytically active aspartic acid residue (Asp152). In conclusion, selection of wheat genotypes for drought stress tolerance was conducted on the basis of genotypic differences in (i) Stay-green traits (Greenness index, photosynthesis rate and leaf senescence rate) (ii) Yield stability (Harvest index, drought tolerance efficiency) (iii) Oxidative stress indicators (H_2O_2 level, lipid peroxidation and LOX activity) (iv) Antioxidant enzyme activities prove the hypothesis. Further, identification and characterization of polyamine biosynthesis genes need to be done for functional validation by reverse genetics approaches to understand the novel role of polyamines for drought tolerance in wheat.

7. ABSTRACT (ENGLISH)

Interactive role of ethylene and polyamines on the drought tolerance in wheat

Cereals including wheat, rice, maize, sorghum and barley are of primary importance to meet food demands of the increasing population and to ensure food security in the 21st century. The greatest fear of global climatic change is drought which is a serious threat to wheat production and productivity if it occurs during reproductive phases. To address the problem, study was conducted with thirty-five wheat genotypes grown in *Rabi* season of 2011 and 2012 to evaluate the effect of water deficit stress on stay-green traits and yield related parameters. First year sowing was done on 26th November 2011 and water deficit stress was imposed by with-holding irrigation for 10 days at 50 % anthesis stage, while routine irrigation schedule was practiced for control plants along-with recommended cultural practices. The studied genotypes showed significant decline in relative water content (RWC; %), membrane stability index (MSI; %), greenness index, photosynthesis, biomass, harvest index and grain yield under water deficit stress as compared to control condition. Three wheat genotypes were screened out on the basis of leaf senescence rate and drought tolerance efficiency *viz*. HW 4022 (drought tolerant), HW 2078 (relatively drought tolerant) and HW 4059 (drought susceptible). For biochemical and molecular studies, second year sowing was done on 28th November 2012 of three contrasting set of wheat genotypes and treated in similar way as in first year. Water deficit stress induced decrease in RWC, MSI, chlorophyll and carotenoid content, photosynthesis characteristics, yield and related traits was less in drought tolerant genotypes (HW 4022 and HW 2078) as compared to drought susceptible one (HW 4059). Phytohormones such as ethylene and polyamine levels was estimated in 10 days old wheat seedlings grown hydroponically in growth chamber under controlled environmental conditions. Osmotic stresses (20 % PEG-6000) were imposed to seedlings in combination with chemical regulators like ethylene inducer (ethrel; 10 ppm), ethylene biosynthesis inhibitor (aminoethoxy vinylglycine; AVG, 2 ppm) and ethylene signaling inhibitor (1-methyl cyclopropene; 1-MCP, 10 ppm). Osmotic stress increases the polyamines contents (spermidine and spermine) and decreased the ethylene evolution rate in tolerant genotypes (HW 4022 and HW 2078) and the results were opposite in case of susceptible genotype (HW 4059). Expression analysis of

chlorophyll degradation genes (*TaCHLase1* and *TaPAO*), ethylene biosynthesis/ signaling genes (*TaACO2, TaACS1* and *TaERS1*) and polyamine biosynthesis genes (*TaSAMDC* and *TaSPDS*) were also studied in three wheat genotypes during water deficit stress in flag leaf and osmotic stress during seedling stage in presence of various chemical regulators. The results revealed that in drought tolerant genotype expression level of *TaSAMDC, TaSPDS* and *TaERS1* increased while, decreased level of *TaACO2, TaACS1*, *TaCHLase1* and *TaPAO* was observed under water deficit/ osmotic stress condition. Further, also cloned and characterized the sequence of the genes involved in stay-green trait *viz*. chlorophyll degradation (CHLase), ethylene biosynthesis/ signaling (ACO, ERS), polyamine biosynthesis (SPDS) and their interaction (SAMDC homologs). In conclusion, the major point from whole research in relation with wheat crop under drought situation indicates that tolerant genotype maintained its water relation, antioxidant defense mechanism, stay-green and yield related traits, through upregulation of endogenous polyamine levels which act as positive regulators of drought tolerance. The susceptible genotype showed an enhanced leaf senescence rate and oxidative stress damage with increased ethylene levels owing to its sensitivity to drought stress. In conclusion, the major point from whole research in relation with wheat crop under drought situation. The evidences proved the hypothesis that genetic manipulation of crop plants with genes encoding enzymes of polyamine biosynthesis pathway may provide better stress tolerance to crop plants under drought stress.

8. ABSTRACT (HINDI)

गेहूँ में सूखा-सहनशीलता हेतु इथलीन एंव पॉलीअमीन की पारस्परिक क्रिया संबंधी

भूमिका

बढ़ती जनसंख्या की भोजन–आवश्यकताओं को पूरा करने तथा इक्कीसवीं शताब्दी में खाद्य–सुरक्षा को सुनिश्चित करने के लिए खाद्यान्न (अनाज) जिनमें गेहूँ, धान, सोघर्म एंव जौ सम्मिलित हैं, सर्वधिक महत्वपूर्ण हैं। भूमण्डलीय जलवायु–परिवर्तन में सूखा, सबसे अधिक चिंता का विषय है जो गेहूँ के उत्पादन एंव उत्पादिता के लिए एक गंभीर चुनौती है, विशेष रूप से जब यह जनन अवस्थाओं के दौरान होता है। इस समस्या को ध्यान में रखते हुए, सन् 2011 एवं 2012 की रबी ऋतु के दौरान, स्टे–ग्रीन गुणों एवं उपज संबंधी प्राचलों पर जल की कमी के प्रभाव का मूल्याँकन करने के लिए, गेहूँ के पैंतीस जीनप्ररूपों के साथ प्रस्तुत अध्ययन किया गया। प्रथम वर्ष में, 26 नवम्बर 2011 को बुआई की गई तथा 50 % परागोद्भवन (ऐंथेसिस) अवस्था पर 10 दिनों तक सिंचाई न कर, जल–प्रतिबल लगाया गया जबकि कंट्रोल के रूप में लगाए गए पौधों में संस्तुत सस्य क्रियाओं सहित रूटीन सिंचाई शेड्यूल अपनाया गया। कंट्रोल अवस्था की तुलना में, अध्ययन किए गए जीनप्ररूपों ने सूखा–प्रतिबल के अन्तर्गत आपेक्षित जल अंश (आर डब्ल्यू सी), झिल्ली स्थायित्व घातांक (एम एस आई), हरापन घातांक, प्रकाश संश्लेषण, जैवमात्रा, कटाई घातांक एंव दाना–उपज में महत्वपूर्ण रूप से कमी दर्शायी। पर्ण–जीर्णता एंव सूखा सहनशीलता क्षमता के आधार किए गए विविक्तकर निरीक्षण द्वारा गेहूँ के तीन जीनप्ररूप अर्थात एच डब्ल्यू 4022 (सूखा सहनशील), एच डब्ल्यू 2078 (अपेक्षाकृत सूखा सहनशील), एंव एच डब्ल्यू 4059 (सूखा सुग्राही) विभक्त किए गए। जैव–रासायनिक एंव आण्विक अध्ययनों हेतु गेहूँ–जीनप्ररूपों के तीन विपर्यासी सेट की द्वितीय वर्ष में 28 नवम्बर 2013 को बुआई की गई और उन्हें प्रथम वर्ष की तरह ही उपचारित किया गया। आर डब्ल्यू सी, एम एस आई, हरितवर्ण एवं कैरोटेनॉयड्स अंश, प्रकाशसंश्लेषण गुणों, उपज एवं उससे संबंधित गुणों में जल–कमी प्रतिबल से प्रेरित कमी, सूखा सुग्राही जीनप्ररूप (एच डब्ल्यू 4059) की तुलना में सूखा सहनशील जीनप्ररूपों (एच डब्ल्यू 4022 एवं एच डब्ल्यू 2078) में कम पायी गयी। नियंत्रित पर्यावरण–परिस्थितियों के अन्तर्गत वृद्धि–कक्ष में जल संवर्धन (हायड्रोपोनिक्स) विधि द्वारा उगाए गए गेहूँ के दस दिन आयु के पौधों में पादप हार्मोनों यथा, इथायलीन एवं पॉलीएमीन के स्तरों का आकलन किया गया। रासायनिक नियामकों जैसे कि, इथायलीन प्रेरक (इथरेल, 10 पीपीएम), इथायलीन जैवसंश्लेषण संदमक (अमीनोइथॉक्सी विनायलग्लायसीन, एवीजी, 2 पीपीएम) एवं इथायलीन सिगनलिंग संदमक (1–मिथायलसायक्लोप्रोटीन, 1–एम सी पी, 10 पी पी एम) के संयोजन में पौध पर परासरण संबंधी प्रतिबल (20 % पैग 6000) लगाए

गए। परासरण संबंधी प्रतिबल ने सहनशील जीनप्ररूपों (एच डब्ल्यू 4022 एवं एच डब्ल्यू 2078) में पॉलीअमीन अंश (स्पर्मिडीन एवं स्पर्मीन) में बढ़ोतरी की और इथायलीन विकास दर में कमी की जबकि सुग्राही जीनप्ररूप (एच डब्ल्यू 4059) में परिणाम इसके विपरीत थे। गेहूँ के इन तीन जीनप्ररूपों की नवाद्भिद पौध अवस्था में कई रासायनिक नियामकों की उपस्थिति में, फ्लेग लीफ में जल कमी प्रतिबल एवं परासरण संबंधी प्रतिबल के दौरान हरितवर्ण निम्नीकरण जीन्स (टीए सी एचएलएएसई एवं टीए पीएओ), इथायलीन जैवसंश्लेषण सिगनलिंग जीन्स (टीए एसीओ2, टीए एसीएस1 एवं टीए ईआरएस1), एवं पॉलीअमीन जैव–संश्लेषण जीन्स (टीए एसएएमडीसी एवं टीए एसपीडीएस), के अभिव्यक्ति विश्लेषण का भी अध्ययन किया गया। परिणामों ने दर्शाया कि जल की कमी/परासरण संबंधी प्रतिबल अवस्था के अन्तर्गत सूखा सहनशील जीनप्ररूप में टीए एसएएमडीसी, टीए एसपीडीएस एवं टीए ईआरएस1के अभिव्यक्ति स्तर में बढ़ोतरी जबकि टीए एसीओ2, टीए एसीएस1, टीए सीएचएलएएसई 1 एवं टीए पीएओ के स्तरों में कमी देखी गई। इसके अतिरिक्त, स्टे ग्रीन गुण में सम्मिलित जीन्स अर्थात हरितवर्ण निम्नीकरण (सीएचएलएएसई 1), इथायलीन जैव–संश्लेषण/सिगनलिंग (टीए एसीआ, टीए ईआरएस), पॉलीअमीन जैवसंश्लेषण (एसपीडीएस), एवं उनकी पारस्परिक क्रिया (एसएएमडीसी समजातों), में सम्मिलित जीन्स की क्लोनिंग तथा जीन्स के अनुक्रम का अभिलक्षण किया गया। निष्कर्ष के रूप में सूखे की अवस्था के अन्तर्गत गेहूँ की फसल के संदर्भ में किया गया यह शोध कार्य दर्शाता है कि सूखा सहनशील जीन प्ररूपों, अतंर्जात पॉलीअमीन स्तरों के अपरेग्यूलेशन जो सूखा सहनशीता के लिए धनात्मक नियामक के रूप में कार्य करता है, के माध्यम से अपने जल संबंध, प्रतिऑक्सीकारक रक्षा क्रियाविधि, हरे बने रहने एवं उपज संबंधी गुणों को बनाए रखता है। सूखा–सुग्राही जीनप्ररूप ने इथायलीन स्तरों में बढ़ोतरी सहित पर्ण–जीर्णता एवं ऑक्सीकारक प्रतिबल क्षति में भी बढ़ोतरी दर्शायी जो इसके सूखा प्रतिबल के प्रति संवेदनशीलता के कारण थी। इस अध्ययन से मिलने वाले साक्ष्य इस अवधारणा को सिद्ध करते हैं कि पॉलीअमीन जैव–संश्लेषण पाथवे के एंजायमों को क्रोडित करने वाली जीन्स के साथ फसल के पौधों में आनुवंशिक हेर–फेर कर सूखा प्रतिबल के अन्तर्गत फसल के पौधों को उच्चतर प्रतिबल सहनशीलता उपलब्ध करायी जा सकती है।

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