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The influence of exogenously applied "anti-stress" agents in the upregulation of the drought response in Iraqi wheat varieties

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The influence of exogenously applied “anti-stress” agents in the upregulation of the drought response in Iraqi wheat varieties.

by

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Dedication

I would like to dedicate my thesis to my beloved parents for their great love and support. I also would like to dedicate my thesis to my dear family.

Author's Declaration

At no time during the registration for the degree of Doctor of philosophy has the author been registered for any other University award. I declare that the work submitted in this thesis is the results of my own investigations except where reference is made to published literature and where assistance where acknowledged.

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Date …….....

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Abstract

Bread wheat (*Triticum aestivum* L.) is one of the most important cereal crops grown in the world. It has great importance because it constitutes a major source of carbohydrate for more than one third of the world’s population (Budak *et al*., 2013). In the last three decades, drought conditions are becoming more widespread in wheat production areas including Europe, Australia and Asia, and it is considered a major cause of reduced wheat growth and productivity in most developing countries with semi-arid climates. Drought constitutes the most important threat for wheat production in Iraq and especially for the Kurdistan Regional Governate owing to the limited source of water during at least some part of the growing period. Because wheat is considered a staple food and has economic importance for the Kurdistan Regional Government research is needed to determine the production capacity of Iraqi wheat varieties under drought stress and the potential for the maximization of the drought tolerance response.

The soil moisture holding capacity of the intended growing medium was measured gravimetrically in pots with and without wheat plants and correlated with the soil capacitance measured using a TDR Theta Probe (Delta-T Devices). This was used to determine the available water content of the soil (AWC) and to control and manage the watering regimes during drought studies. The results of a study of the response of different cultivars of Iraqi wheat (*Triticum aestivum* L.) to watering regimes of 70% and 50% showed that drought stress had a significant effect on the biomass and yield traits especially tiller number and stem bundle weight compared to normal conditions. The highest significant difference was observed for cv. Tamooz 2 in comparison to Adana 99, but there was a little difference between cvs. Rizgary and Sham 6.

The effect of the exogenous application of salicylic acid (SA) and molybdenum (Mo) on drought tolerance of cvs. Tamooz 2 and Adana 99, showed that Tamooz 2 had higher values for growth characteristics and higher yield potential when sprayed with a lower concentration of SA (1.44 mM) under well-watered conditions in comparison with Adana 99. The effect of spraying variety Tamooz 2 with SA at different growth stages indicated that biomass production and yield components (the number of spikes/pot, grain dry weight and average 1000 grain dry weight) significantly increased at both stem+flower as well as leaf+stem+flower sprayings for plants subjected to drought. Also, SA treatments at stem extension and flowering had a positive effect on the up-regulation of the drought response gene *CBF*/*DREB* under drought stress conditions.

These findings indicate that agronomic treatments with exogenous applications of salicylic acid and molybdenum could help to reduce the effects of drought in the field.

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List of Abbreviations

ABA	Abscisic Acid
ANOVA	Analysis of variance
ASA	Acetyl Salicylic Acid
AWC	Available water content
***CBF*s**	C-repeat binding factor gene
Chl	Chlorophyll
Cm	Centimetre
CO_2	Carbone dioxide
CRD	Randomized complete block design
cv.	Cultivar
DNA	Deoxy ribonucleic acid
DRE/CRT	Dehydration-responsive element/C-repeat
Exp.	Experiment
F.wt	Fresh weight
FC	Field capacity
g	Gram
gs	Stomatal conductance
h	Hours
H1	First harvest
H2	Second harvest
K	Potassium
Kg	Kilogram
LA	Leaf area
LSD	Least significant difference test
MC	moisture content
mm	millimetre
Mo	Molybdenum
MPa	Megapascals

N	Nitrogen
^{o}C	Celsius
P	Phosphorus
ppm	Part per million
PSII	Photosystem II
RGR	Relative growth rate
RH	Relative humidity
RQ	Relative quantitative
RT-PCR	Reverse transcription polymerase chain reaction
Rubisco	Ribulose 1, 5 bisphosphate
RWC	Relative water content
SA	Salicylic Acid
SCI	Society of chemical industry
SDW	Shoot dry weight
TDR	Time domain reflectometry
TFs	Transcription factors
V	Voltage
VAR.	Variety
Vs.	Opposed to
WLR	Water loss rate
WP	Wilting point
WUE	Water use efficiency

Chapter 1

1. General Introduction and Literature Review

1.1. General Introduction

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops species in the world and is a major source of nutrition for greater than one third of the world's population (Budak *et al*., 2013). Currently it is second only to rice as the staple source of food in many developing countries, supplying about 19% of the calories and 21% of the protein of human diets (FAOSTAT, 2010). Moreover, the domestication of wheat (*Triticum* spp) was established it as the first food grain to be easily cultivated on a large scale. It is believed that many species of the genus *Triticum* originated from the Levant area of the Middle East, and that it has been grown in the Nile valley since 5000 BC. Later, its cultivation spread out to other regions of the world for instance, the Indus and Euphrates valleys from 4000 BC, China from 2500 BC, and Europe around 2000 BC. There are different species of wheat, but the common wheat hexaploid (*Triticum aestivum*), and tetraploid durum wheat (*Triticum turgidum* ssp. *durum*) are currently cultivated worldwide. Wheat cultivation is not only important to provide the staple food in sufficient quantities for humans but it has also been linked with human history as a key factor enabling the emergence of civilizations of Europe, West Asia, and North Africa for 8000 years and supporting the consistency of population growth through to modern times (Monneveux *et al*., 2012).

It is commonly considered that the Iraqi Kurdistan Region is the centre of diversity of wheat with some of the oldest remains of wheat crops found in Tel-Yarmo dating back to 8000 BC (Bakhteyev and Yanushevich, 1980). Wheat is considered as the main winter cereal crop grown in Iraqi Kurdistan and represents

the largest crop among all other field crops and is grown on 78% of the available rainfed area (Al-Najafi, 2010).

World wheat production is facing major concerns because of the rising demand for wheat consumption as a staple source in the diet of many countries over the last decade, especially in the developing regions of the world. It has been estimated that the productivity of crops must increase annually by about 2% in order to meet global demands. Unfortunately, the availability of arable land is limited for growing crop plants and the negative impacts of environmental conditions on yields caused by a scarcity of water is leading to a crisis in global wheat production. As a consequence, the primary aim of most wheat breeding programmes must be to enhance the yields of wheat on the land that is already being used and which in turn can meet the needs for the future (Lucas *et al*., 2011).

In both natural and agricultural conditions, plants are frequently exposed to abiotic stresses particularly water stress (drought stress). It has been reported that only 10% of the world's arable lands are free from wide spread water deficit conditions (Ashraf and Wu, 1994). Drought is one of the main environmental stresses that adversely affects photosynthesis and plant growth and results in reducing crop productivity in many areas of the world especially semi-arid regions which constitutes more than 50% of world arable land area (Boyer, 1982). Not only inadequate amounts of rainfall give rise to water deficits but also limited supplies of irrigation waters exacerbate dehydration stress (Wilhite, 1994).

The most prominent environment stress factor facing crop plants in Iraq is the exposure of plants to frequent drought for at least some part of the growing period, which can lead to a decrease in the plant growth and/or a delay or acceleration in development. For these reasons, improving drought tolerance of wheat and the stability of grain production has long been a major goal of most wheat breeders around the world where wheat is grown with limited rainfall, including in Iraq.

The aim of this study was to investigate the physiological and agronomical responses of selected Iraqi wheat varieties to drought stress and to study the up-regulation of the drought response regulon in response to the spraying of plants with the "anti-stress" compound salicylic acid (SA) and trace element molybdenum (Mo). This was carried out under normal and stress conditions in order to assess drought tolerance capacity of Iraqi wheat cultivars as well as assessing whether these exogenous applications could improve drought tolerance, or could ameliorate the adverse effects of the constraint of water deficit. Continuous research efforts are required to find preferred cultivars that possess as many desirable yield and quality traits as possible and that are able to tolerate water deficit induced stress.

1.2. Literature Review

This literature review will focus on a number of physiological and biochemical responses observed in plants in order to demonstrate and support the up-regulation of the drought response regulon. Evidence will be presented of altered plant metabolism at the physiological and molecular level following the effects of drought stress on plants. Furthermore, evidence will be presented that demonstrates the effect of exogenous applications of salicylic acid (SA) and other known elicitors that induce an adaptation response to environmental stress such as drought.

1.2.1. Stress on plants

The term stress has general meanings rather than a precise definition and is often used subjectively. In the case of biology, it has been used for representing the stressor agent, that is the unusual factor, or a normal factor modified in a way to be in excess or deficit, that is capable to cause bodily injury or disease. First of all, it was defined by Hans Selye in 1936 as “all factors which can act as pressures that cause specific action for living organisms”; this is especially important for plants because they are rooted in their habitat and cannot escape from most environmental stimuli. The exceptions are deciduous and annual life strategies where plants avoid or minimize the stresses of cold and/or drought by entering a quiescent or dormant state during the period of maximum potential stress. When plants cannot escape a stress they need to have special mechanisms of responses to stress such as protection and adaptation (Lichtenthaler, 1998) to

cope with increased levels of a stressing agent. Studying the responses of living organisms to environmental stress factors has occupied the attention of ecologists since the 1960s. One of the pioneer researchers to investigate the responses of plants to the stress concept was Levitt (1972) who defined stress precisely from the physics concept as a power which is applied on the area of the matter while, the biological meaning of stress is any external impact that has the capability of inducing a potential injurious change in living organisms and it affects an organisms without necessary a force. With respect to strain, in physics this is recognized as the reaction of the material to an applied force such as, change in length. In biology, it is any physical or chemical response of the metabolism and/or morphology, which is made as a consequence of a stress and has not necessarily a change in dimension. And so, the living organisms may show a physical strain for instance a cessation of cytoplasmic streaming or display a chemical strain such as a shift in metabolism. Other researchers such as Lichtenthaler (1996) and Gaspar *et al*. (2002) have also shown that stress is any unfavourable condition or substance for a living organism that affects or blocks growth and development of the organism. Stress can be defined as any change of environmental condition, which is pushing an organism away from its optimal situation. In addition, strain has been defined by Larcher (1987) as physiological changes that occur when species are exposed to unexpected unfavourable conditions that do not necessarily result in a threat to life but will induce an alarm response for instance wilting. Kranner *et al*. (2010) pointed to related fact that the stress response is a major determining factor of an organism's life. An organism's response to stress is similar to the reaction in non-living material even supposing there varying in the nature of the stresses. Hence, organisms are able to deal

with stress to a certain extent, which determines the restrictions of its natural ecosystems. According to this, whenever a plant is subjected to a specific stress such as low soil water potential, the plant will undergo a specific strain that would provoke a response of the plant to prevent or repair the damage. This depends on the mechanisms that enable plants to survive under stress and the plant's response will vary with increasing duration and severity of the stress. If the stress is adequately severe, it will further influence that plant, chiefly altering the physiological state. In this way, the organism is unable to maintain normal function which can lead to damage or death.

1.2.1.1. Physiological effects of stress on plants

Most plants undergo strain during the different stages of their life. Such strain can decrease plant growth and reproduction capacity, so it is necessary to understand some of the physiological mechanisms of stress effect on plants (Osmond *et al*., 1987). Research by Lichtenthaler (1998), Chaves *et al*. (2003) and Pinheiro and Chaves (2011) pointed at the first stage of physiological impact of stress on plants, and concentrated on stress that can change the construction and function of plants. Stress directly decreases physiological and metabolic activities such as the decline of photosynthesis and closure of stomata. As a consequence of stomatal closure, transpiration decreases, and diffusion of CO_2 into the chloroplast tissue is reduced thus reducing photosynthetic rate. These effects ultimately lead to the reduction in growth rate and productivity of the plant. The effects of such restrictions are different according to the strength and/or duration of the stress, whether or not the stress is being applied singly or in combination with other stresses, as well as the species of plant exposed to the stress, its

development stage and its metabolic state. One of the first researches on the stomatal behaviour was conducted by Liang *et al*. (2002) on wheat plants (*Triticum aestivum)*. The osmotic regulation was assessed under the effect of drying and watering conditions. It is proved that leaf stomatal conductance and transpiration rate of the treated plants changed significantly with lessening the soil water content and declined to 58 μmol $m^{-2}s^{-1}$ at the end of the drying period compared to the control pots which varied between 150 μmol $m^{-2}s^{-1}$ and 170 μmol $m^{-2}s^{-1}$. Studies by Shah and Paulsen (2003) and Wang and Frei (2011) concluded that stress had a considerable influence on the physiological traits of many crop plants resulting in reduced yield and crop quality. These results are supported by Dolferus *et al*. (2011) who have shown that stress conditions can cause significant change in grain yield during the early period of reproductive growth especially during pollen development. For this reason, cereals including wheat, rice, and barley and many other plants are acutely sensitive to abiotic stress during the critical period of flowering. It is usually believed that the process of grain filling is regulated environmentally. Based on this fact an investigation into the impact of stress on flowering in crop plants by Yang and Zhang (2006) indicated that soil drying enhance remobilization of carbon from vegetative tissues to grains reducing the net effect of decreased photosynthesis. Also, water stress substantially shortens the grain-filling duration, and increases grain filling rate and interestingly rice plants (*Oryza sativa*) showed a lower grain filling rate than wheat (*Triticum aestivum*).

1.2.1.2. Consequences of stress on plants - acute and chronic

Relatively few studies have examined the consequences of acute mild stress on plants and most have described plants that have been exposed to severe stress often characterized by a common symptom of stress, the occurrence of tissue dehydration. Tissue dehydration caused by drought is a consequence of the difference between root water uptake and leaf transpiration, and plants respond to the stress by decreasing their physiological activities, such as the closing of stomata to conserve water status, but this also results in the decrease of CO_2 absorption leading to the reduced performance of the photosynthetic apparatus as CO_2 becomes limiting. Supporting the work done by Luvaha *et al*. (2008) who reported that extreme water deficit stress caused a greater decrease in CO_2 assimilation rate in the mango plants (*Mangifera indica* L.) than the control, and this limit of CO_2 diffusion through the stomata resulted in a decline in the photosynthesis rate. Severe tissue dehydration can cause wilting and damage of the plant tissues that may rapidly result in the death of those plants which have no resistance to such acute stress. However, chronic stress does not mean that damage necessarily will happen in plants but depends on the intensity and duration of the plants exposure to the stress. Plants will frequently adapt themselves to resist the stress and damage symptoms may be absent or limited (Lichtenthaler, 1998) but such response has a metabolic cost. Lichtenthaler (1996) also explored the difference which reduces growth rates between chronic stress which can be partly repaired by the tolerance of low tissue water potential, and acute stress which often caused substantial damage finally leading to plant death. These responses in plants have been distinguished by using the chlorophyll fluorescence method in order to detect constraints on leaf

photosynthesis, during the application of stress on plants. The findings of Aroca *et al*. (2012) discussed how controlling the root water uptake is more important for plants to resist stress damage, than leaf transpiration, and how some specific conditions of stress could be regulating the response of root cells via increasing concentration of some plant hormones such as abscisic acid (ABA), and an inducer signaling molecule salicylic acid (SA).

1.2.2. Drought stress

Drought is known as an abiotic environmental stress affecting plants due to insufficient amounts of rainfall or irrigation. In relation to agriculture, drought usually refers to a period with a serious decline of soil moisture which occurs in a region when it receives a below average rainfall over a season or a year and this is especially frequent in semi-arid zones. Drought has been recognized as one of the main threats to crop production in the twenty-first century in many developing countries, where the water deficit has a limiting effect on plant growth and crop productivity and is associated with the increase in demand for water where agriculture is considered as the biggest consumer of water accounting for approximately 70 to 95% of water-use in many regions. The occurrence of drought usually depends on several interacting weather conditions, such as high temperatures, high winds, low relative humidity and duration of rain during a crop growing season and the consequences of drought are determined by biological traits and stage of growth of the plant and the physical properties of soil (Wilhite, 1994). A similar description for drought was given by Mishra and Singh (2010) who state that drought is usually due to reduced amounts of water in the soil caused by its evaporation and/or transpiration to the atmosphere. Furthermore,

Ding *et al*. (2011) have suggested that drought in many areas can be caused by the rapid growth of a population of plants and their subsequent transpiration of the water from the soil to the atmosphere leading to increasing competition for water. Moreover, Roelofs *et al*. (2008) point out that drought is one of the major factors negatively affecting soil as a suitable growth medium for plants. For example, Xu et al. (2009b) recently testified how pre-drought of plants following re-watering can markedly affect grass relative growth rate (RGR), and photosynthetic potential. As a response to stress the plants may try to cope with the great loss of the plant's production as showed in the recovery of droughted perennial grass plant *Leymus chinensis*, in comparison to control plants, the extent of plant responses was related to the intensity of soil drought.

1.2.2.1. Definition of drought from a soil point of view

Even though there is some controversy on defining the field capacity (FC), it has been generally defined as the highest amount of available water which can be held by soil after the drainage of excess water (Fazackerley and Lawrence, 2012). In relation to wilting point (WP) this can be defined as the lowest water content of the soil where the plant can no longer extract water. These two parameters of soil water content are used to calculate the available water capacity (AWC) of soil as well as to determine the amount of water that should be used in irrigation of crops (Dahiya *et al*., 1988). One of many studies that has examined water stress from the soil point of view was provided by Jamieson and Ewert (1999) in which wheat crops were exposed to various amount of water deficit so as to investigate the limiting role of roots in water uptake in a deep soil, by comparing plant water uptake with control plants. The results showed that there was a significant

increase in the water uptake and a reduction of soil water for plants treated with moderate to severe drought. It has been documented by Prasad et al. (2008b) that decrease of soil water potentials is not often a reliable parameter for measuring the extent of drought stress. Dryness of the soil under water deficit conditions leads to a decrease in the hydraulic conductivity of the soil, which reduces the rate of water uptake by the plant which stimulates root development to compensate for the water loss from the plant during transpiration (Prasad et al., 2008b). Thus the plant/soil interaction is a dynamic one depending on soil water availability. For example, Jones (2007) emphasised how important it is to choose the most reliable method for specific objectives of an experiment. This aim of drought experiments must be related to the relationship between the effects of different water potentials and the mechanism of plant response to water deficit including “drought-tolerant” plants. The determination of the soil water status is essential for the programme of water irrigation management of crop plants to know how much water the crop needs. Accordingly, the available methods for measuring soil moisture content has been reviewed in this study. These include a direct method that is performed by gravimetric measurement and requires to know volume of soil which is weighed and then dried and reweighed on a balance. In addition, indirect techniques can be used for monitoring the soil moisture content according to their relation to studies of drought tolerance by instruments which include electromagnetic sensors representing time domain reflectometry (TDR). The findings of such studies emphasise the use of the soil water potential indicator rather than plant tissue hydration because it is the measure that most closely indicates the potential ability of plants to extract water. This ability depends on hydraulic conductivity of the soil/plant pathway. In view of

that, these are illustrated that the lack of physiological processes such as photosynthesis showed that lowering water potential by around 1.5 MPa has no significant effect on the photosynthesis rate of a leaf.

1.2.2.2. Definition of drought from a plant point of view

The study of Krasensky and Jonak (2012) emphasized drought stress from the plant point of view. Water stress often changes metabolic processes which ultimately can lead to a reduction in the plant growth and/or a delay or acceleration in development. Hsiao (1973) and Flexas *et al*. (2006) summarized that water stress commonly limits photosynthesis processes relating to decreased CO_2 fixation in higher plants. An experiment on maize (*Zea mays* L.*)* carried out by Li-Ping *et al*. (2006) investigated the water uptake limits of roots by adopting an experiment in which plants from the early stage of seedling up to maturity were subjected to varied water stress. The main focus of this work was to discover the effects of water scarcity on leaf water status and membrane permeability at different growth stages. The results showed that severe drought stress was more influential on maize in mature stages. Severe water stress decreased the leaf relative water content (RWC) but also increased leaf relative conductivity in comparision with well irrigated plants. In contrast, moderate stress had no significant effect on the RWC or the relative conductivity of the leaves. Similarly, earlier work by Teare *et al*. (1982) has proved the relationship between total water potential, stomatal conductivity and the stage of physiological growth by germinating wheat (*Triticum aestivum* L.) in pots in a growth chamber where different vegetetative growth stages were exposed to water deficit. The findings showed that leaves of water-stressed and non-stressed plants had higher total

water potential so that closing the stomata, at the filling of the spike stage recorded a lower total water potential in order to open the stomata. Shah and Paulsen (2003) investigated the effects of drought and high temperature stresses on crops separately so as to compare between their impacts on wheat. Plants were grown normally in well irrigated conditions at 25 ± 2 ^{o}C and were then subjected to droughts with water potentials of -0 to -2.4 MPa at 15, 25, and 35 ^{o}C in a controlled environment. Results indicated that drought led to decreased photosynthesis and stomatal conductance but it actually increased plant water use efficiency (WUE) i.e. growth per unit of water transpired. Siddique *et al*. (2000) performed a different experimentation for measuring water deficit effects on wheat (*Triticum aestivum* L.) cultivars including Kanchan, Sonalika, Kalyansona, and C306 that were grown in pots and then exposed to different levels of water stress at either vegetative or later developed stages. The effect on the stressed plants had a more considerable impact on plant water levels than unstressed plants at both vegetative and reproductive growth stages. As showed in their results, the drought stress reduced leaf water potential from -0.63 MPa in control plants to -2.00 MPa in stressed plants. Sonalika and C306 cultivars had a low leaf water potential at the harvest stage, and the relative water content values diminished significantly from 88% to 45%. Cultivars of Kanchan, Sonalika and Kalyansona had high relative water contents at the development stage. However, C306 had the lowest RWC values. This study suggests that wheat plants are able to recover if they are re-watered after exposure to water stress at the vegetative stage.

1.2.2.3. Drought stress reducing crop yield potential

Several studies have reported that drought reduces crop yield potential including Benveniste-Levkovitz *et al*. (1993) who conducted a comparison between wild wheat (*Triticum kotschyi)* and cultivated wheat (*Triticum aestivum* cv*.* Lakhish) and the influence of water stress. The outcome revealed that a decrease in yield at water potentials of -0.9 MPa, -2.3 MPa was not associated with damage of photosystem II (PS II) whereas the yield of the both species was decreased at less than -2.3 MPa caused by loss of the ability of PSII. Stomatal closure was shown to decrease to a similar extent in both species with increasing water stress. This concurs with the research by Jaleel *et al*. (2009) who clarified that drought is one of the major abiotic stresses which can make changes in metabolic functions of higher plants either by loss or decrease of photosynthetic functionality and accordingly negatively affects crop yield.

The tolerance of any crop species to this adverse effect differs according to severity, time of exposure to stress and also genotype. As an example, in barley plants (*Hordeum vulgare* L.), drought stress reduced the grain yield significantly in four genotypes tested under post anthesis drought condition. The yield reduction for genotypes Arta and Tadmor were by 25%, while Morocco 9-75 and W12291 were by 54 and 51% as compared to the control plants (Li *et al*., 2006). As shown in the table below (Table 1.1) that the productivity of crop plants differed noticeably at different growth stages under water deficiency stress. Maize plants had the largest yield trait at the vegetative growth stage, but the smallest yield was at grain filling.

Table 1.1: A summary table of studies carried out on the yield of some crop plants affected by soil water deficits during growth stages. Modified from Farooq et al. (2009b)

Crop plants	Growth stage	Yield reduction (%)	References
Maize	Reproductive	63-87%	(Kamara et al., 2003)
Maize	Grain filling	79-81%	(Monneveux et al., 2006)
Maize	Vegetative	25-60%	(Atteya, 2003)
Maize	Reproductive	32-92%	(Atteya, 2003)
Rice	Reproductive (mild stress)	53-92%	(Lafitte et al., 2007)
Rice	Reproductive (severe stress)	48-94%	(Lafitte et al., 2007)
Rice	Grain filling (mild stress)	30-55%	(Basnayake et al., 2006)
Rice	Grain filling (severe stress)	60%	(Basnayake et al., 2006)
Rice	Reproductive	24-84%	(Venuprasad et al., 2007)
Durum wheat	Reproductive (severe stress)	48-60%	(Giunta et al., 1993)
Barley	Grain filling	49-57%	(Samarah, 2005)

1.2.3. How plants perceive drought stress

Mahajan and Tuteja (2005) and Lata *et al*. (2015) revealed that drought is firstly perceived by the plant cell membrane through receptors which bind and interact with the extracellular molecules known as elicitors and then leads to the switching on of genes which are responsible for increasing the tolerance to the stress. In this context generally drought has an initial negative effect on plants by disordering the ionic balance of the cell and this leads to changes in calcium concentrations which has an important role as a signaling molecule for the upregulation of many abiotic stress responses. Bray (1993) concured that the perception of plants for information about stress starts was a signal transduction pathway. Accordingly, this pathway leads to response processes of cellular protection from dehydration such as guard cell closure, or regulation of gene expression (Figure 1.1).

The process of stress signal transduction in a cell or tissue may not take place directly. For example, it is recognised that the stomatal conductance and the regulation of transpiration process are linked with soil water deficits sensed in the root by the hydraulic signals and chemical signalling compounds such abscisic acid (ABA) so as to transmit the information from root cells through the xylem to the shoot system (Davies *et al*., 2002). The plant regulator ABA plays a crucial role in abiotic stress response by amplifying the first signal and also starting the creation of the second messengers such as reactive oxygen species (ROS) and inositol phosphates that can follow the same general pathway or initiate a phosphorylation cascade that finally targets stress-responsive genes (Xiong and Zhu, 2001, Raghavendra *et al*., 2010). The ABA signalling pathway leads to the

activation of the regulon *CBF*/*DREB* (Cold-binding factor/dehydration-responsive element binding). The expression of these stress-responsive genes could be directly associated with cellular protection or transcription factors (TFs) that regulate the expression of downstream stress-regulated genes. Also, these genes help the plant's adaptation to adverse environmental conditions like cold and drought stress (Lata and Prasad, 2011, Lata *et al*., 2015). Finally, the secondary signalling pathway plays a crucial role in the metabolic response by synthesis a different stress molecules (Bhargava and Sawant, 2013).

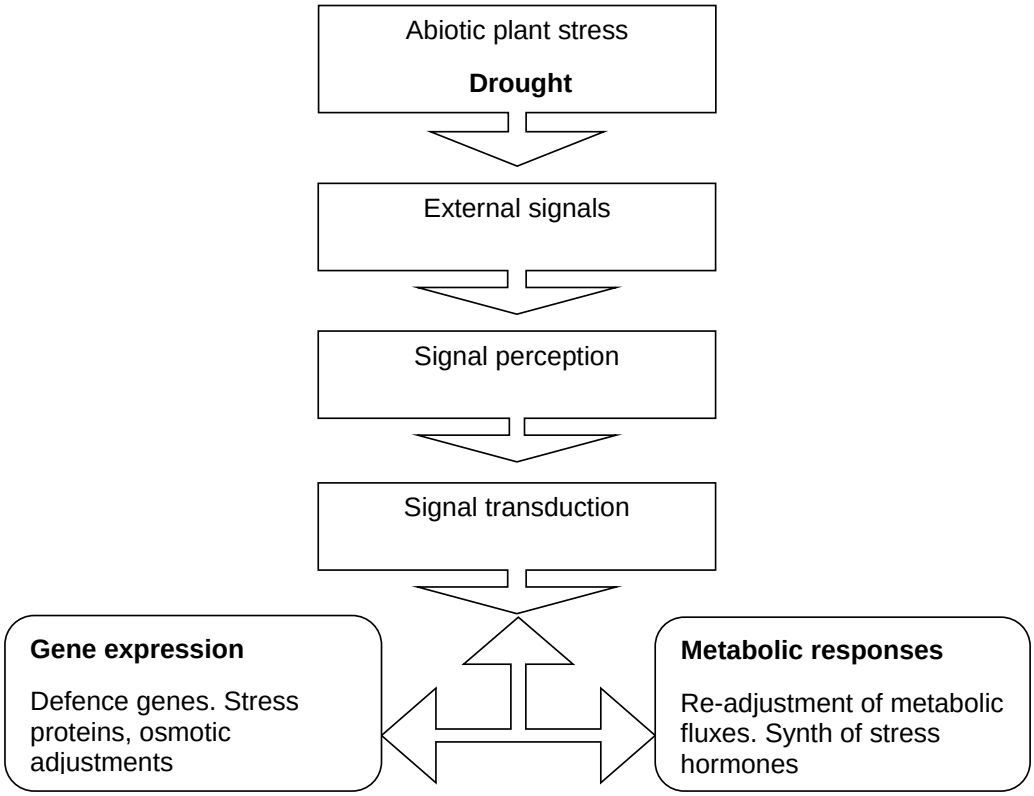


Figure 1.1: Diagram of the stress signal perception and transduction leading to induction of plant responses which are metabolic responses and gene expression (Modified from Lichtenthaler (1998)

1.2.4. Plant responses to drought stress

The plant response to water deficiency is a complex process, and it includes damage and/or adaptation of the plant. The complication of this response is most diverse in herbaceous plants. These kind of plants differ in their capacity of the avoidance of water status changes, and one of the strategies of their resistance to drought is accompanied by the change in the root to shoot ratio by developing deep roots in the soil to maximize the hydraulic conductivity capacity of the plant to extract water (Chaves *et al*., 2002, Chaves *et al*., 2003). For example, In most cereal crops and in rice plants, the reduction of producing new tillers and leaves, as well as growth of a deep root with a limited number of adventitious root represent a major adaptive mechanism for moderating water use and maintenance the water status of leaf tissue under water scarcity stress (O'Toole and Cruz, 1980). Plants usually show different adaptations in semi-arid drought conditions at the morphological and physiological level which enable plants to survive under these conditions, above all the decrease of transpiration rate by closing stomata, and also is associated with decrease of the leaf area, in order to maintain high tissue water potential, thus avoiding the deleterious effects of later severe stress (Hsiao, 1973, Chapin *et al*., 1993). For example, it has been reported that the three nerica rainfed rice varieties of (*Oryza sativa* L.) had variations in rooting pattern and their response to water deficit including decreases in the plant biomass, transpiration rate and stomatal conductance as the water content was reduced, whereby the stressed plants had lower transpiration and stomatal conductance rates than the well watered plants in all the three varieties (Sikuku *et al*., 2010). This elucidation agrees to those gained by Liang *et al*. (2002) who proved that water consumption was steadily reduced

in wheat (*Triticum aestivum*) during water deficiency stress because of the decreased stomatal conductance and transpiration rate, while the ratio of root dry weight to shoot dry weight was increased. However, the study of Rezaeieh and Eivazi (2011) aimed to assess the morphological response of five maize (*Zea mays* L.) cultivars under drought conditions and their relation to the total dry matter and the major character for selecting maize genotypes drought reaction was determined as the root dry weight. Another strategy of drought response of many plants is the rolling of the leaves, which is modulated by water limitation during plant development in order to limit evaporation of the water to air. Also, as reported in earlier studies, rice plants respond to drought with certain physiological changes like reducing leaf elongation to reduce light absorbance through rolled leaves (O'Toole and Cruz, 1980).

1.2.4.1. Plant responses to drought at the physiological level.

Plants express physiological characteristics for responses to low water potential, and these responses are associated with some abilities to tolerate the stress, for example plants with small leaves have a greater efficiency in minimizing water loss (Chaves *et al*., 2002). As shown by Thameur *et al.* (2012) in the work on two strains,Tlalit and Switir, cv. of Ardhaoui barley which were grown in southern Tunisia where control plants were well watered, while other plants were irrigated near to 50% of FC. Their findings showed that the Tlalit strain was more tolerant compared to Switir due to having low leaf area and an ability to keep high leaf RWC under drought conditions. Rampino *et al*. (2006) studied *Triticum* and *Aegilops* seedlings to identify the genotypes which have resistance to water stress. The category of the genotypes in their response to water stress at the

physiological level were determined by RWC, and water loss rate (WLR) which is a function of stomatal density and aperture. The outcomes of the plant response to 24 hour of exposure to dryness stress reported that RWCs of control plants for all genotypes was 98%. While, the genotypes which showed RWCs above 25% were considered as resistant genotypes, and the seedling which exhibited RWCs below 25% were considered as sensitive genotypes.

A different perspective for identifying the correlation between physiological traits and drought tolerance was demonstrated by Li *et al*. (2006), who concluded that measuring the chlorophyll content could be a reliable indicator of drought tolerance in barley (*Hordeum vulgare* L.) cultivars Tadmor, Arta, Morocco 9-75 and WI2291 under “well-watered” and drought stress conditions. The outcomes of this study revealed that all genotypes exhibited a reduction in the chlorophyll content with different degrees under drought stress. The decrease values of chlorophyll content in the two drought tolerant genotypes Tadmor and Arta were 10.7 and 1.6%, but the chlorophyll content which corresponds to drought sensitive genotypes Morocco 9-75 and WI2291 were 31.3 and 30.1% respectively. These effects of drought stress show that the components of the photosynthetic apparatus in drought sensitive genotypes could be considerably damaged in comparison to drought tolerant genotypes which were relatively less affected. This review is in disagreement to view of Efeoglu et al. (2009) who showed that the changes in chlorophyll pigment composition and biomass production measurements would be related to the physiological response of the plants to soil water loss. In this investigation three maize cultivars of (*Zea mays* L.) were subjected to a cessation of irrigation. The findings indicated that after 12

days exposure of the plants to water stress, the Chlorophyll (chl) a, chl b and total chl contents, as well as dry biomass were significantly reduced for all stressed cultivars, but these returned to the control values following rewatering the plants for 6 days. It is well documented that most higher plants share common measure of responses to low water potential through a decrease in many metabolic process in plants, such as rates of photosynthesis and growth parameters. As a consequence their decline in physiological functions, the plants diverge from their standard physiology (Chapin *et al*., 1993). For that reason, the research into physiological responses of wheat plants under soil moisture deficient conditions is becoming increasingly essential, and is probably the best approach to examine drought-tolerance of different Iraqi wheat varieties. Hopefully such studies will contribute to the aim to develop sustainable crop production under water stressful situations.

1.2.4.2. Plant responses to drought at the molecular level.

Plants respond to drought stress at a molecular level by various patterns of gene expression. Several of the genes induced under water shortage have been identified, but the mechanism of activation of these genes is still not fully described and therefore further study is required in order to elucidate activation mechanisms. The results of these proposed studies may eventually be transferred and applied to crop protection strategies. Clearly it is essential that science describes the function of abiotic stress genes but it is recognised that the specific function of these genes is complex (Chaves *et al*., 2003). Research by Agarwal *et al*. (2006) has focused on the role of dehydration responsive element binding transcription factors *DREB/CBF* in plants to regulate the expression of

genes associated with drought stress tolerance. One of these factors are the *DREB*s transcription factors which relate to the ERF family consisting of two subclasses, *DREB*1/*CBF* and *DREB*2 that are induced by cold and dehydration, respectively. Moreover, the expression of the stress-response genes has been studied by the researchers Yang *et al*. (2010) and Lata and Prasad (2011) who emphasized the role of *DREB* transcription factors (TFs) namely, *DREB*1 and *DREB*2 which are involved in regulating abiotic stress responses in plants to improve the tolerance of crop plants to low temperature and dehydration as well as their utility in crop improvement programmes through breeding and marker-assisted selection. The *DREB* proteins specifically bind to the DRE sequence and they are induced by particular signaling molecules such as ABA which can control the expression of different stress-responsive genes in plants and produce a variety of proteins, especially transcriptional factors in order to avoid negative effects of water deficit conditions on plant growth. Numerous *DREB* genes have been isolated from a number of plants exposed to various stresses drought, cold and salinty (Table 1.2). Lately, an expressional analyses of 10 genes from *Triticum turgidum* and *Triticum durum* leaves were monitored by the real-time quantitative PCR for their expression under drought conditions and their role in the damage-repairing and regulating the abiotic stress responses to improve the adaptation. The results revealed that drought induced the transcription factors *DREB*s which were involved in the signal transduction and up-regulation of some genes to high levels. Moreover, the responsive element binding factor genes were not significantly affected by the drought stress (Melloul *et al*., 2014). In the same way, Pandey *et al*. (2014) showed that dehydration responsive element binding (*DREB*) proteins play a significant role in regulating the water stress

response. The efforts were mainly made on the amplification of the *DREB*2 gene using specific primer from a diverse set of Indian wheat genotypes. Transcripts level of the genes in leaves were recorded (17%), but it shown to be (8%) in stem and seed. A review by Wang *et al*. (2006) focuses on the role of dehydration responsive element (DRE) binding protein in the regulation of many stress-inducible genes in plants and enhancing the tolerance to drought. Low temperature and high salt also affect the *DREB* transcription factor with dehydration-responsive element/C-repeat (DRE/CRT) cis-acting element in the promoter region of many stress responsive genes for the signal transduction and action of genes. Supportively, among DRE-binding proteins, the *DREB*1A and *DREB*2A were primarily isolated as cDNAs encoding DRE/CRT-binding protein from *Arabidopsis thaliana*, and they are thought to be the major transcription factors which act as regulators of stress-responsive gene expression under abiotic stresses. The expression of *Arabidopsis* transcription factor *DREB*1A was induced by cold stress, but the transcript *DREB*2A was induced by drought and salinity stresses and not by ABA treatment (Liu *et al*., 1998b). Likewise, in the analysis of Sakuma et al. (2006a) *Arabidopsis thaliana* was used to isolate cDNA homologous to *DREB*1A and *DREB*2A using Microarray and RNA gel blot technique. These transcription factors specifically interact with cis-acting dehydration-responsive element/ C-repeat (DRE/CRT) which are involved in gene expression regulatory networks and tolerance to cold and drought stresses respectively. The results revealed the over expression of active *DREB*2A resulting in regulation of major drought stress tolerance genes. However, *DREB*2A expression does not activate downstream genes of *DREB*1A which also recognizes DRE/CRT and functions in cold stress-responsive gene expression.

However, some gaps still remain in the understanding of these mechanisms and of the molecular mechanisms of activation *DREB*2-type genes to water deficit conditions have not been elucidated fully. This generates a need for conducting genomic research to study the gene expression of *DREB*2A and its role in enhancing drought tolerance in Iraqi wheat cultivar that can be tested by molecular-based approaches.

Table 1.2: Summary table to illustrate the response of DREB genes and their expression to various stresses (drought, cold and saline). Adapted from Lata and Prasad (2011)

***DREB* TFs**	**Plant species**	**Accession**	**Stress response**	**References**
Hv*DREB*1	*Hordeum vulgare*	DQ012941	Drought, Salt, Cold	(Xu *et al*., 2009a)
Ta*DREB*1	*Triticum aestivum*	AAL01124	Cold, Dehydration	(Shen *et al*., 2003)
*DREB*1A	*Arabidopsis thaliana*	AB007787	Cold	(Liu *et al*., 1998a)
Os*DREB*1A	*Oryza sativa*	AF300970	Cold, Salt	(Dubouzet *et al*., 2003)
Os*DREB*1B	*Oryza sativa*	AF300972	Cold	(Dubouzet *et al*., 2003)
Os*DREB*1C	*Oryza sativa*	AP001168	Drought, Salt, Cold	(Dubouzet *et al*., 2003)
Os*DREB*1D	*Oryza sativa*	AB023482	None	(Dubouzet *et al*., 2003)
Os*DREB*1F	*Oryza sativa*		Drought, Salt, Cold	(Wang *et al*., 2008)
Pe*DREB*2	*Populus euphratica*	EF137176	Drought, Salt, Cold	(Chen *et al*., 2009)
W*DREB*2	*Triticum aestivum*	BAD97369	Drought, Salt, Cold	(Egawa *et al*., 2006)
Sb*DREB*2	*Sorghum bicolor*	ACA79910	Drought	(Bihani *et al*., 2011)
Gm*DREB*2	*Glycine max*	ABB36645	Drought, Salt	(Chen *et al*., 2007)
*DREB*2A	*Arabidopsis thaliana*	AB007790	Drought, Salt	(Liu *et al*., 1998a)
Dv*DREB*2A	*Dendrathema*	EF633987	Drought,heat,cold.	(Liu *et al*., 2008)
Sb*DREB*2A	*Salicornia brachiata*	GU592205	Drought,Salt,Heat	(Gupta *et al*., 2010)
Zm*DREB*2A	*Zea mays*	AB218832	Drought, Salt, Cold	(Qin *et al*., 2007)
Pg*DREB*2A	*Pennisetum glaucum*	AAV90624	Drought, Salt, Cold	(Agarwal *et al*., 2007)
Os*DREB*2B	*Oryza sativa*		Heat, Cold	(Matsukura *et al*., 2010)
Os*DREB*2C	*Oryza sativa*	AK108143	None	(Matsukura *et al*., 2010)
Os*DREB*2E	*Oryza sativa*		None	(Matsukura *et al*., 2010)

The two levels of plant responses to drought stress are summarized in (Figure 1.2).

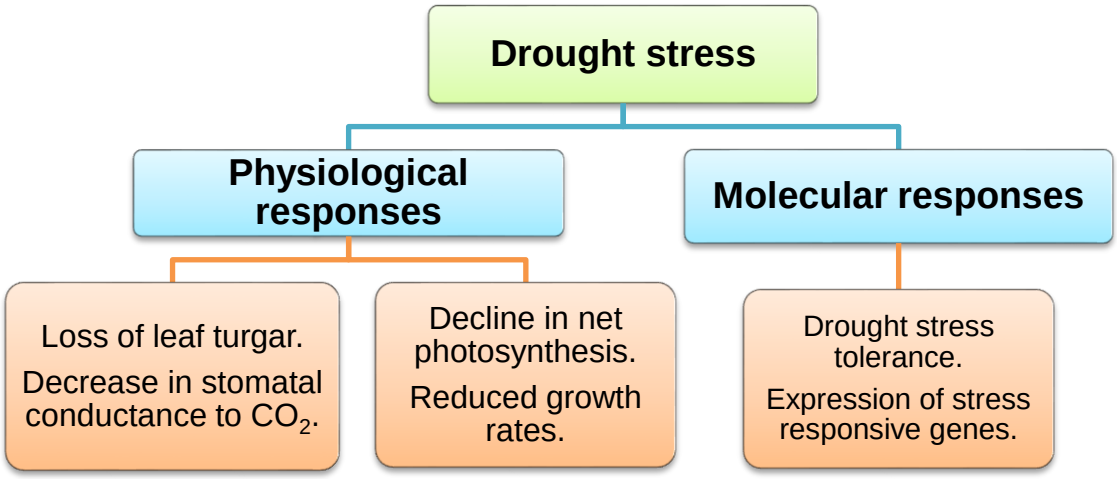


Figure 1.2: The physiological and molecular responses to drought stress in higher plants Adapted from (Reddy et al., 2004)

1.2.5. The limitations of plant generated protection measures from drought stress

Plant protection from drought stress is controlled by a group of genes which include several transcription factors as described above. These genes however, are not actually involved in protecting cells but upregulate other genes which produce the regulatory proteins. The transcription factors also regulate stress signal transduction as well as modulating gene expression. Thereby, they activate the expression of many target genes that are responsible for the control of the stress response (Lata and Prasad, 2011).The manipulation of genes which are involved in cell protection could theoretically increase the survival of plants under severe water stress. Moreover, The maintenance of metabolic homeostasis of leaf water status under water deficit is achieved by reducing transpiration through controlling the stomatal aperture and leaf growth which has

a positive effect on plant water status. However, this has a negative effect on photosynthesis and might be regarded as a constraint of plant protection which contradicts the standard measurement of drought tolerance (Tardieu, 2005).

1.2.6. Plant protection measures

Plants commonly resist drought conditions either by the avoidance of tissue dehydration and maintain tissue water potential at a high level, or by the tolerance of low tissue water potential to maintain metabolic activity. Avoidance is a mechanism associated with a variety of responses against drought which involves minimizing water loss by the closure of stomata so as to maximise water retention and by rolling the leaves which leads to reduced leaf area and therefore to reduce transpiration (Chaves *et al*., 2003). Kauser *et al*. (2006) also assessed the variation in a morpho-physiological mechanism of plant protection on two canola (*Brassica napus* L.) in hydroponic culture. The results indicated that water deficit conditions caused a substantial decrease in leaf area, but the cultivar Dunkeld was less affected than Cyclone. On the other hand, Bhargava and Sawant (2013) in their research discovered the differences in gene expression to tolerate dehydration in barley genotypes which were upregulated in drought tolerant genotypes, while those related with metabolic processes including photosynthesis were downregulated. These workers concluded that the genes are generally involved in protecting plants from drought stress through stress perception, signal transduction, transcriptional regulatory networks in cellular responses to dehydration.

1.2.6.1. Exogenous application of materials

Plant hormones regulate various processes in plants, which enable plants to protect themselves against various abiotic stresses. It has been reported that these regulators are synthesized in roots and translocated to leaves, where they cause stomatal closure and inhibit plant growth, consequently enabling the plant to adapt to stress conditions (Bhargava and Sawant, 2013). This is supported by Farooq et al. (2009b) who showed that exogenous application of substances such as plant growth regulators at very low concentrations can help maintain physiological activity during long periods of drought. In addition, plant growth regulators have been recognized as an important substance for the delay of dehydration damage in water-limited environments by a continued maintenance of cell turgor and other physiological process and giving tolerance against drought injuries by maintaining high tissue water potential. Singh and Usha (2003) indicated the role of salicylic acid (SA) as an important signal molecule regulating the drought response of plants and studied the growth and metabolism of wheat seedlings in response to exogenously applied of SA in the range of concentrations 1-3 mM under water stress. The results revealed that moisture content and dry mass significantly increased in water stressed plants that were treated with SA as compared to the water stress control without SA. Furthermore, the protection was greater particularly at the 3 mM SA treatment.

In the same way, molybdenum (Mo) has been signified as a major growth promoter that can act as a “shotgun” means to improve abiotic stress tolerance in different crops. Mo is essential micronutrient element for the nutrition of cereal crops and helps maintain plant growth, due to the importance of Mo in the

production of two major enzymes in plants nitrogenase and nitrate reductase, that play a role in the process of biological nitrogen fixation from air or soil which is needed for synthesis of compounds such as amino acids, proteins and chlorophyll in plants (Taiz and Zeiger, 2004). The effect of molybdenum spray with three treatments pure water, 0.5% and 1% concentrations on the functional characteristics and yield of wheat under water stress condition has been studied by Ghafarian *et al*. (2013), they found that severe stress treatment induced reduction in grain yield and many of yield components than normal irrigation treatment for example, the values of grain yield had significantly decreased by light and severe stress about 14.6% and 26.7% respectively at flowering stage treatment. Whereas, leaf area index decreased under water stress treatments for both grain filling and flowering stages than normal treatment. Morover, exogenously applied 1% concentration of Mo caused significant increase in the productivity including total seed weight, number of spike and number of grain per spike with 13.9%, 6.5% and 17.4% respectively, and thus reduced water stress damages to protect photosynthetic machinery. Also, the results revealed that number of productive tillers was alleviated by spraying 0.5 Mo treatment as compared with pure water, the values was 413.3 and 423.3 respectively, and concerning spray with 1% Mo concentration led to drop the value to 397.6.

1.2.6.2. Genetic modification of gene function

The approach of genetic modification has become popular as a more rapid and capable mechanism to improve stress tolerance rather than the ordinary reproduction strategy (plant breeding). Such efficiency can provide the necessary tools for the successful application of stress tolerant traits and to search for an explanation of the complicated molecular mechanism of plant stress responses. Since drought stress tolerance is a complex trait it needs a prolonged research time, much research which has studied genetic modification for improved drought tolerance were not agronomic plants but were model plants such as tobacco and *Arabidopsis*. These species have a much greater facility to transform and *Arabidopsis* has a short life cycle in comparison with many crop plants making it an ideal model plant for such research and many of the genetic modifications that improve drought stress tolerance have a growth reduction consdequence. Only a few crop plants have been tested for genetic modification of drought tolerance in the field (Moreno *et al*., 2005) and none have made significant agronomic advances yet.

1.2.6.3. Plant breeding

The plant breeding process is the selection of two plants with desirable traits which are crossed to comnbine their genes and then selected so as to obtain new genetic arrangements in new individuals. The aim of this improvement is to test individual plants for the expression of the favourable characteristic as well as plant regeneration which is now been applied to a significant number of crop species. Thus, the process has opened opportunity for crop improvement genetically through a wide collection of “drought-related” genes and this can be

supported by using recombinant DNA technology which include familiar tags linked to target genes; these are known as molecular markers, which are based on polymorphisms that occur naturally in the DNA sequence (Xoconostle-Cazares *et al*., 2010).

To sum up, it is clear that drought has physiological effects on crop plants and decreases the growth and productivity, so investigating physiological and moleculare responses of wheat plants to water stress are very important for solving the gaps in the current knowledge of this research area.

Chapter 2

2. Soil drying characteristics

2.1. Introduction

The drying characteristics of soils and thereby the Available Water Capacity (AWC) can be assessed using Time-Domain Reflectometry (TDR) measurements using a commercially available measuring device such as the Theta Probe (Delta-T Devices Ltd) calibrated against a gravimetric assessment of soil drying. Volumetric calculation is a more convenient method for expressing soil water content of irrigation systems using TDR, and it relates to the volume of water in the sample to the total volume of soil (Charlesworth, 2005). As has been explained previously, an electronic device TDR probe is widely applied for measuring the time between transmission of voltage impulse along parallel rods down to the soil and receiving the reflected signals by sensors. To calculate the electrical conductivity for all type of soils depends on the average of volumetric water content and this needs to be calibrated with gravimetric measurements (Topp *et al*., 1980, Topp and Davis, 1985). The gravimetric method is assessed as weight and determined by the ratio of watered weight to the dry weight for a particular soil sample. The oven-drying technique is commonly used for drying soil to constant weight so as to determine the total water weight in a soil sample and this can be calculated by difference between the weights of the saturated and oven dry samples. This measurement is a basic method used as the standard for the calibration of all other soil moisture determinations. Also, the accuracy of the application of the TDR method for measuring the soil moisture requires verification using the traditional gravimetric method (Black *et al*., 1965, Reynold, 1970, Olszewska and Nowicka, 2015).

Petropoulos *et al*. (2013) and Ozbek and Kaman (2014) validated the conventional approaches for the determination and monitoring of soil water content and stated that it may not be appropriate to use one method as an effective measurement of the variations in soil moisture. Based on the overview of these studies, characteristics of approaches used for measuring soil moisture content presented as below in Table 2.1.

Table 2.1: Comparison between 2 methods used for measuring soil moisture content.

Methods	Advantages	Disadvantages
TDR probe	Gives the most accurate readings.	
	Without any damage to the soil.	
	Provides faster data collection.	
	Measuring of samples can repeated.	Affected by soil types and particles.
	A new technique and widely used.	
	Not required much labour force.	
	Quick time response less than 1 minute.	
Gravimetric measure	A basis method for calibration with TDR.	Less precise and accurate.
	Easily applied with low cost.	Destructive to soil samples.
	Not affected by soil types and particles.	Slower collection of data.
		Repeat sampling not possible.
		The oldest technqiue.
		Required much labour force.
		Time-consuming is 24-48 hours.
		Affected by sampling equipments.

2.2. Objectives:

Experiment 1- To determine the soil moisture holding capacity and AWC of commercially available topsoil (chosen as the soil substrate for all experiments) in the presence and absence of wheat plants and the calibration with Theta Probe readings.

Experiment 2- To determine the amount of water that should be used for irrigation in relation to the management of the watering regimes during drought studies in pots.

2.3. Materials and methods

The soil used for all subsequent experiments was a bulk “topsoil” product obtained from LBS Horticulture Ltd, England and had a consistent loam texture and was free of stones above 5 mm diameter. Soil was delivered in bags in a moist ready to use condition in accordance with standard horticultural practice. Sufficient bags for an experiment were opened and thoroughly mixed together to ensure homogenisation prior to use. The pH of soil was measured as 5.65 (Basic pH Meter, Denver Instrument company, 300408.1Rev.F, U.S.A) following a 2:1 (wt:vol) protocol according to Rowell (1994).

Two experiments, using two different groups of pots were carried out in the room 201 in Davy building of Plymouth University, UK. In the first group, three pots of topsoil without plants were assesed for characteristics of soil drying over 86 days using a TDR Theta Probe and compared to the soil moisture content assessed

by gravimetric measurements. The second group characterised the soil drying of three pots of topsoil with wheat plants growing in them.

2.3.1. The measurement of soil drying characteristics.

Three 15 litre plastic pots (diameter 28.5 cm, height 26 cm) were prepared and each pot had access ports for the Theta probe drilled in two places (mid and low) 12 cm, 6 cm as measured from the bottom of the pot respectively. Each pot was filled with the same amount of topsoil by the volume of pot and then standard compaction applied by pressing another pot forcibly on the soil surface of filled pots and further soil added to a final weight of approximately 14 kg.

Gravimetric moisture content (% MC) assessment of the soil contained in pots and the soil capicitance using the TDR Theta Probe followed the procedure based on Jones (2007):

1- The loam filled pots were saturated to maximum water content by adding water until drainage occurred and standing in saucers of water overnight to ensure full water saturation through capillary action. Then the saucers were removed and drainage of the excess water allowed to drip out. When drainage ceased the pots were assumed to be at 100% field capacity and at full saturated weight, and were weighed.

2- After the setup of pots to full saturated weight they were never watered again. The fresh weight of the pots was now approximately 17 kg indicating that they had taken up approximately 3 kg (or 3 litres) of water. Pots were then allowed to dry progressively in the growth room and weighed and Theta Probe measured periodically. The average temperature (^{o}C) of the growth room was recorded during the experimental period by data-logger (Tiny Tags) (Figure 2.1)

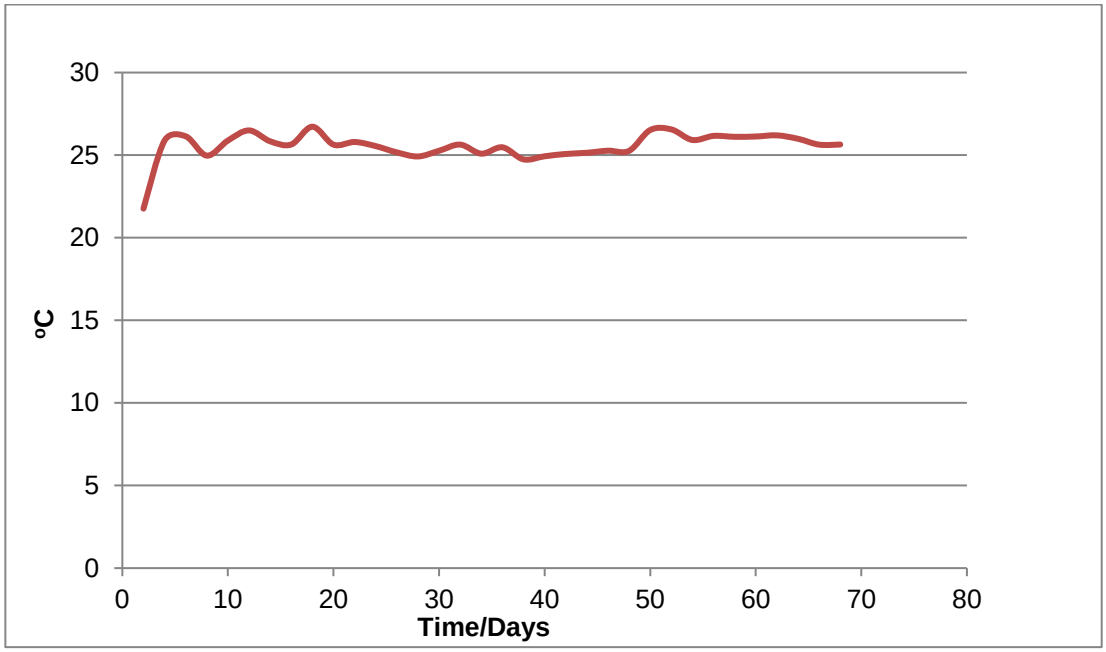


Figure 2.1: Average temperature in the growth room used for the soil drying experiments.

3- A portable TDR Theta probe (type ML2x, Delta-T Devices Ltd Cambridge, UK) was used to measure the drying characteristics of the soil in the pots. The rods of Theta probe were pressed into the soil surface of pots and measurements recorded and subsequently into the two lateral access ports (Plate 2.1). Then the pots were weighed on a digital top-pan balance (Toledo Model 47141, UK). These measurements began in early March 2014 and were repeated every 2 days for a period of 52 days until the readings stabilized.

Plate 2.1: To illustrate the three different sites that were used for the Theta Probe measurements for pots without plants during 52 days.

4- Even when the readings of Theta probe become constant there was still a limited amount of water in the soil. To obtain a true dry weight, pots were emptied into metal trays and the soil spread out and left to air dry for 2 days and reweighed then, left for another 2 days and weighed again. After that, the soil was put in the oven at 105 ^{o}C for 2 days and weighed again this was repeated every 2 days until they reached constant weight. They were finally fully dried after a period of 62 days.

2.3.2. Characterizing the drying capacity of the soil in the presence of wheat plants.

In the second experiment, twenty-five seeds of wheat (*Triticum aestivum* L.) cv. Rizgary were sown in each of three pots. One week after seedling emergence; plants were thinned to a equivalent field density of 250 plants m^{-2} (16 seedlings per pot). The plants were grown during the whole experiment with 12h photoperiod at a photosynthetic photon flux of 77.3 μmol m^{-2} s^{-1} measured by PAR Quantum Sensor (Skye).

After the wheat seedlings reached 3 weeks age and established 3 leaves, the saturation of the pots with water was established (Plate 2.2). Following this the four previously described steps of Theta probe measurement and pot weighing were applied to the seeded pots until permanent wilting point was achieved. Theta probe measurements were taken and repeated every 2 days for a period of 60 days until the readings stabilized. The pots were emptied into metal trays and the soil with wheat plants spread out and left to air dry for 2 days and weighed, left for another 2 days and weighed again. Finally, the soil was put in the oven at 105 ^{o}C for 2 days and weighed again this was repeated for another 2 days until the drying of topsoil reached the constant weight. Soil and plants were fully dried after a period of 68 days.

Plate 2.2: Showing the three different sites top, mid and low were used to measure theta θ^{o} for pots with wheat plants during 60 days.

2.3.3. Soil moisture content.

The moisture content of soil (% MC) was measured as described by Topp *et al*. (1984). The measurement was made by the use of the portable battery-powered TDR Theta probe consisted of a slightly tapered steel rods; the rods were inserted vertically into the soil surface through 15 cm depth for each well watered and droughted pots. TDR measurement determines an electromagnetic pulse passed into the soil measured at 20 Volts (V). The value of soil water content was calculated from a calibration equation below used to convert the TDR readings to water content (% MC) based on (Topp *et al*., 1980).

$$\mathbf{Y = 63.074x^2 + 54.159x + 8.8204}$$

Where; x is Theta θ^o readings which was taken on the soil surface of well watered and droughted pots after period of 23th day of the imposition of the watering regimes.

Statistical analysis

Characteristics of topsoil drying between with and without wheat plants were compared, so as to determine the differences. Curves were fitted to data using MicroSoft Excel 2010 so as to maximise the coefficient of determination (R^2) values by applying sequential polynomial curve fittings by regression.

2.4. Results

2.4.1. The measurement of soil drying characteristics.

The results showed that the Theta probe readings could be related to the soil moisture content as measured gravimetrically in a curvilinear fashion resulting in a calibration curve for the Theta probe for the selected soil type. Differences however were recorded in the relationship between moisture content and Theta probe readings at the top, mid and lower positions in the pots. Theta probe readings were higher lower down in the soil profile (Figure 2.2) presumably as the pots dried from the surface.

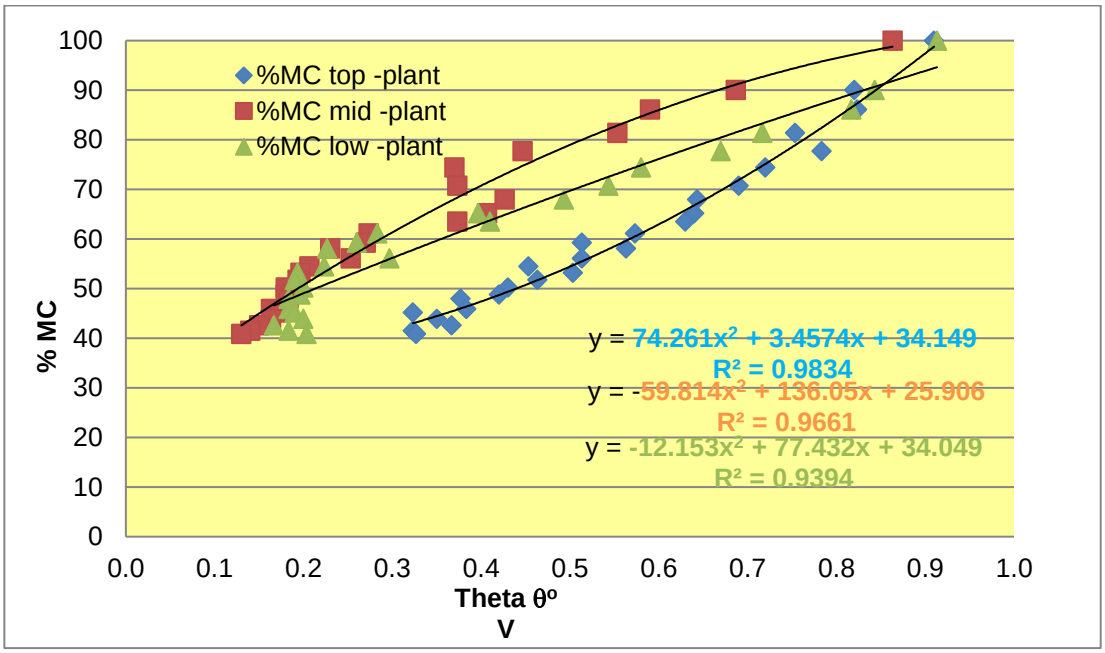


Figure 2.2: Calibration curves of Theta probe vs moisture content measured the top, mid and low profile of soils in pots without wheat n = 3 pots.

2.4.2. Characterizing the drying capacity of the soil in the presence of wheat plants.

In Figure 2.3 the mid and low horizons of pots showed the lowest Theta probe readings at 38% moisture content. While, the minimum amount was in the top of pots at 34 % MC. The results of the correlation coefficient (R^2) of regression analysis showed good correlation between the measurements made with the use of the gravimetric method and the TDR method.

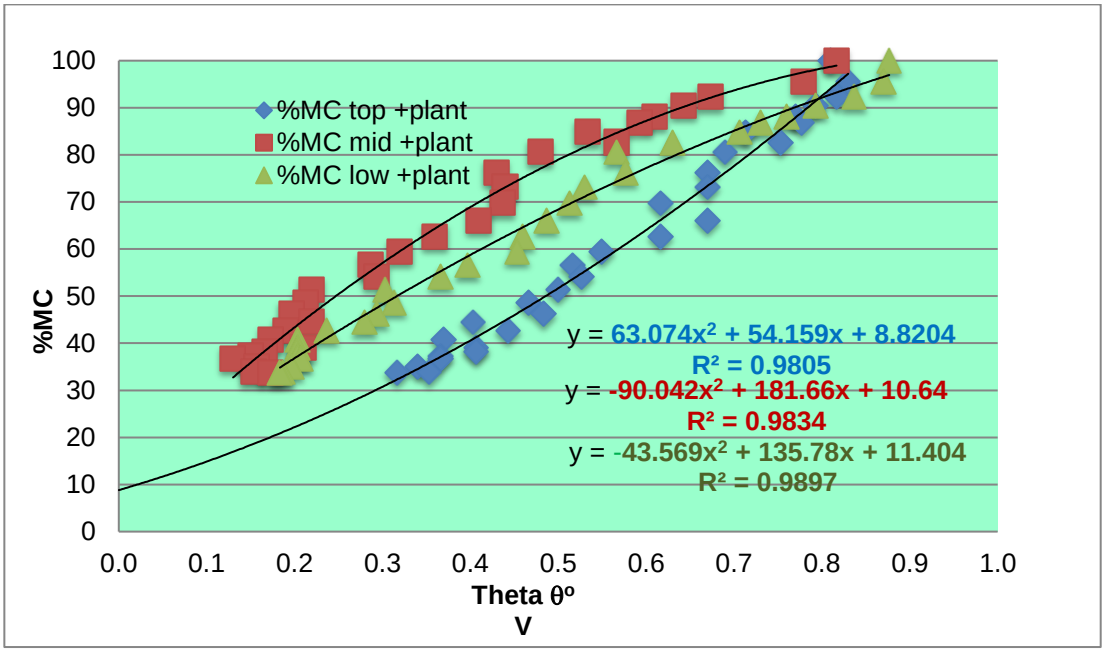


Figure 2.3: Calibration curves of moisture content measured the top, mid and low of pots with wheat vs. theta probe n = 3 pots.

2.5. Discussion

The result of this experimentation showed that the TDR measured values were as reliable as those obtained from gravimetric measurments, and resulted in differences between moisture content and Theta probe readings at the top, mid and low sites of the pots; the relationship was in a nonlinear curve. The analysis of the volumetric water content of soil using TDR instrument in comparsion with values obtained by a gravimetric determination on a sample of soil is in accordance with the method was prescribed by Topp *et al*. (1984) where the water content of the surface soil and the depths of 50 to 300 mm was repeatedly measured in long-term setting up. The finding showed that there is a consistent relationship between TDR measured water content θT and gravimetric water content θvg.

Olszewska and Nowicka (2015) compared the results of moisture measurements conducted with the TDR method and the gravimetric method on two soil profiles, A and B of the medium alluvial in the valley of the Oder river region. The analysis of the results indicated a good correlation between the soil moisture values obtained with the use of those two methods and a higher value of volumetric moisture are observed in profile B located in the valley below the barrage than profile A in the adjacent location to the barrage. However, the data presented in the paper by Reeves and Smith (1992) showed a comparison between the values of volumetric moisture content measured by TDR and the gravimetric measurement for two soil depths of 20 mm and 36 mm under field condition and they concluded that first place had a higher value of moisture contents than second place.

It has been interpreted that variant measurment of soil water content in the characterization of soil drying depends on the soil type and electrical conductivity of the soil water (Topp *et al*., 1984). Reeves and Smith (1992) used TDR method in their studies to measure soil water content. Their conclusion indicated that fewer errors were obtained at sites with sandy loam soils compared to coarse limestone soils. This was referred to the accuracy of TDR probe measurements is affected by the air gaps around probe when inserting the rods. The variability in soil moisture content could be influenced by several factors on the surface, including physical properties of soil such as the soil density, compaction and sensor placement. Also, that reflects the effects of the weather conditions such air temperature and relative humidity which influence the rate of evaporation from the soil (Dorigo *et al*., 2011).

2.6. Conclusion

Although, there were small differences between different soil horizons in the absence and presence of wheat in the relationship of the moisture content to theta probe readings, the experimentation showed that surface Theta probe readings can be used to predict the moisture content of the entire pot. As a consequence the portable Theta probe instrument could be used to obtain reliable water content measurments for soil characterization when applied to the pots with wheat plants in subseqent experimentation. This method allows for the determination of the available water content (AWC) of the soil rapidly with high accuracy; the AWC of soil can be defined as the highest amount of available water which can be held by soil after the drainage of excess water subtracted by the lowest water content of the soil where the plant can no longer extract water. Thus, it can be used with an irrigation plan by knowing the soil moisture holding capacity of the irrigated area. The maximum amount of water that could be added to the chosen topsoil for saturation of the chosen pot size was 3 litres per pot.

This experimentation led to the confident establishment of a pot watering regime for the controlled maintenance of a drought stressing regime that could maintain sufficient level of persistent drought without killing plants outright.

Chapter 3

3. The physiological response of Iraqi wheat varieties to a drought stress regime

3.1. Introduction

Plants show various physiological responses to drought stress that can help prevent plants from damage so as to survive and to maintain their growth and development under these conditions (Chaves, 1991, Chaves *et al*., 2003). The main negative effects of water deficit stress on plants are characterized by affecting various physiological attributes of plants including a reduction in stomatal conductance, photosynthetic rate and plant development (Patel and Golakiya, 1988, Janda *et al*., 2007). The most important physiological responses of plants to low soil water potential are related to stomatal closure and leaf growth inhibition. These traits reflect the tolerance of drought stressed plants in order to cope with excess water loss via reduction of the transpiration process through stomata, which in turn affects the movement of CO_2 into the plant and so protecting the metabolic process functions by the maintenance of water uptake from root system to leaf tissues. These adaptive processes have been used as selection criteria in developing the drought tolerance in crop plants (Flexas *et al*., 2006, Chaves *et al*., 2009, Loka *et al*., 2011).

Crop plants are often considered to have the capacity to respond to drought by modifying leaf cell growth. This characteristic was evaluated most frequently in studies Blum (1996) and Lopes et al. (2011) on sorghum plants because it has better adaptation to water-limiting environments than most other crops, even though leaf area and photosynthesis were affected by the closure of stomata under stressed conditions. As previously pointed out by Hsiao (1973) leaf cell

growth under water deficiency stress is considered to be more associated with the yield of dry matter weight than stomatal responses and CO_2 assimilation.

Biomass production has been considered one of the crucial measurements of the yield under stress and non-stress conditions in studies on wheat plants *(Triticum aestivum* L.) in accordance with the variation among wheat genotypes in terms of their ability to maintain high yield potential (Al-Temimi *et al*., 2013). Soil water content (WC) has also been used as an indicator of physiological measurements due to its main role in cereals crops to sustain many functions of the plant, particularly photosynthesis, plant nutrient transport and enzymatic reactions and transpiration (Hsiao, 1973). For example, moisture capacity of soil was associated with response in legume plants for survival under drought stress (Wery *et al*., 1993).

Total chlorophyll pigments are also considered as an important metabolic character of drought stress resistance in higher plants. Chlorophyll a and b play a key role in leaf physiology mainly in photosynthesis process for harvesting light by which the plants convert water and carbon dioxide to produce carbohydrates. The changes in the pigment composition in plant plastids cause reduction in plant yield (Farooq et al., 2009b). These responses can differ depending on the place, time, duration and severity of the stress in relation to crop growth, species and genotype. As a result of reduced turgor pressure of cells, a stressed plant that positively deals with lack of water and can cope with inadequate water availability is considered to be ‘drought-tolerant’ and is usually more resistant to water loss but has reduced growth parameters (Berger *et al*., 2010). This investigation

aimed to determine how different cultivars of Iraqi wheat seedlings respond to a persistent drought stress regime.

3.2. Objectives

This study aimed to achieve the following objectives:

1. To quantify the physiological responses of four Iraqi wheat varieties of *Triticum aestivum* L. to two irrigation treatments so as to determine the variability in their tolerance toward water stress at the middle and end stages of the crop cycle.
2. To determine whether the varieties are drought resistant or drought sensitive.

3.3. Materials and Methods

Iraqi wheat cultivars demonstrate a range of susceptibility to moisture deficit. The varieties that are given in Table 3.1 were proved as drought tolerant or susceptible (Al-Temimi *et al*., 2013). Four cultivars of bread wheat (*Triticum aestivum*) with alledged different tolerance to drought stress were chosen for the experimental study. According to The Erbil Agricultural Research Centre in Iraq the varieties Rizgary and Tamooz 2 are tolerant to drought stress and, Adana 99 and Sham 6 varieties are sensitive. All seeds batches were tested for germination and vigour and all of the varieties met the International Seed Trade Association (ISTA) minimum standards (greater than 85% germination, in fact all of the varieties exceeded 95% germination).

Table 3.1: Iraqi wheat varieties used and their sources.

Species/Cultivars		Drought Tolerance	Source
Triticum aestivum L.	Rizgary	Tolerant	Erbil Agricultural Research Centre, Erbil, Iraq
	Adana 99	Senstive	Erbil Agricultural Research Centre, Erbil, Iraq
	Sham 6	Senstive	Erbil Agricultural Research Centre, Erbil, Iraq
	Tamooz 2	Tolerant	Erbil Agricultural Research Centre, Erbil, Iraq

Four varieties of Iraqi wheat seeds (*Triticum aestivum* L.) Rizgary, Adana 99, Sham 6 and Tamooz 2 were investigated. The pots were arranged in a randomized complete block design (CRD) with four varieties, two watering treatments (W1 well watered, and W2 drought stressed) with 2 harvests in four replicate blocks where a block was a half bench in the glasshouse (Figure 3.1). Each block included 2 pots per treatment. The allocation of the treatments to the pot positions within a block was randomised using random number tables (Fisher and Yates, 1938).

Rep 1

V3 W1.1	V4 W1.2	V1 W2.1	V2 W1.1	V3 W2.1	V1 W2.2
V4 W2.1	V4 W1.1	V2 W1.2	V2 W2.1	V3 W1.2	V2 W2.2
		V1 W1.1	V4 W2.2	V1 W1.2	V3 W2.2

Rep 2

V1 W1.1	V3 W1.1	V1 W2.1	V4 W1.1	V2 W1.1	V4 W2.1
V2 W2.2	V3 W2.1	V4 W2.2	V1 W1.2	V2 W2.1	V4 W1.2
		V1 W2.2	V2 W1.2	V3 W2.2	V3 W1.2

V3 W2.2	V2 W2.1	V3 W2.1	V1 W1.2	V4 W1.1	V1 W2.2
V1 W1.1	V2 W1.1	V4 W2.2	V3 W1.2	V3 W1.1	V2 W1.2
		V4 W2.1	V4 W1.2	V2 W2.2	V1 W2.1

Rep 3

V2 W1.1	V1 W1.1	V4 W1.2	V1 W2.2	V4 W2.2	V3 W1.2
V3 W2.1	V2 W2.2	V1 W2.1	V2 W2.1	V3 W2.2	V3 W1.1
		V1 W1.2	V4 W1.1	V2 W1.2	V4 W2.1

Rep 4

4R × 4V × 2W × 2H = 64 pots. There were 16 plants/pot

Figure 3.1: Plan showing the layout in the greenhouse of the first drought experiment after randomisation. (R = Replicate; V = Variety; Wx.y = watering regime (1&2) and harvest (1&2).

Four hundred seeds of each wheat variety were pre-germinated in sandwich boxes lined with blue tissue paper and watered with a small amount of distilled water at 20 ^{o}C 16 h light 8 h dark for 72 hour in growth cabinet of (Sanyo Amzga 31311, England) to ensure that only live seeds were planted in the pots. After germination, wheat seeds were transplanted (20 June 2014) into 15 litre plastic pots (n = 64) measuring 28.5 cm (diameter) x 26 cm (height), filled with same amount of loam topsoil and were placed in a greenhouse at Skarden Garden, Skarden Place, Plymouth University UK.

At the beginning of the growth phase all the seedlings were well watered until they were 3 weeks old. In early summer (July 2014) when all the seedlings were established and had 4 leaves (GS14), two watering regimes were applied, well watered and droughted. The well watered pots (controls) were systematically allowed to drop to 70% AWC (Available Water Capacity) and then pots were brought up to fully watered again. In contrast the droughted pots were allowed to drop to 50% AWC and then brought up to 70% AWC again. In order to facilitate ease of irrigation the control pots were labelled with a purple coloured label and the droughted pots were labelled with a yellow coloured label to make them easily noticeable at the time of watering. These regimes were maintained for the entire duration of the experiementation until full seed set when watering was terminated (terminal drought imposed) and plants were allowed to fully dry.

Under well-watered conditions the regime was achieved by adding 2 litres of water to the pots in order to maintain them between 70% to 100% MC whilst the droughted pots were maintained by adding 1 litre of water. The moisture content of a number of representative pots of both treatments of each variety were monitored using the Theta probe (MC %) daily at the soil surface of the pots and readings used to determine the day of watering (Figures 3.2 and 3.3). The temperature in the glasshouse was monitored by data loggers (Tiny Tags) throughout the experimental period (Figure 3.4).

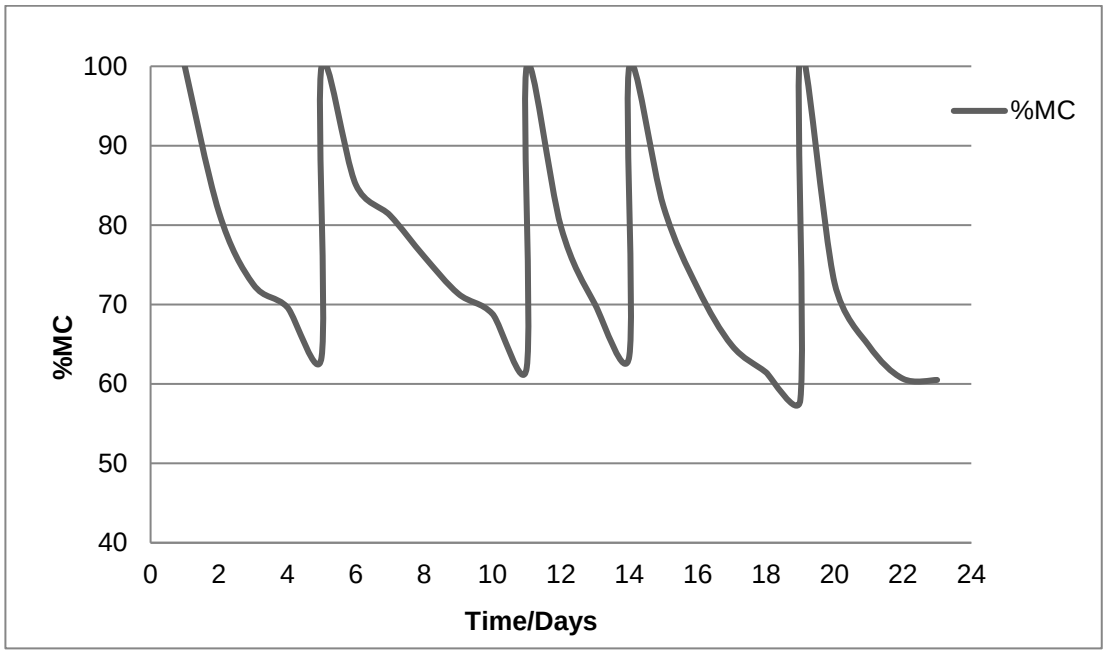


Figure 3.2: The %MC of pots containing wheat plants under the well-watered regime (T1 = control).

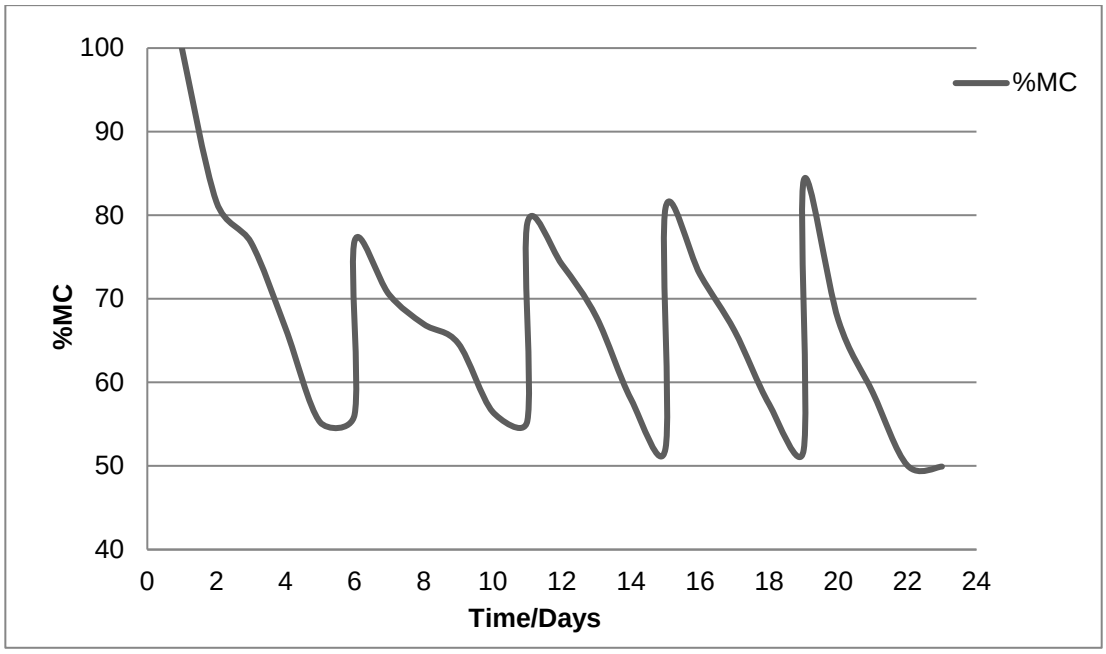


Figure 3.3: The %MC of pots containing wheat plants exposed to the droughted watering regime (T2 = droughted).

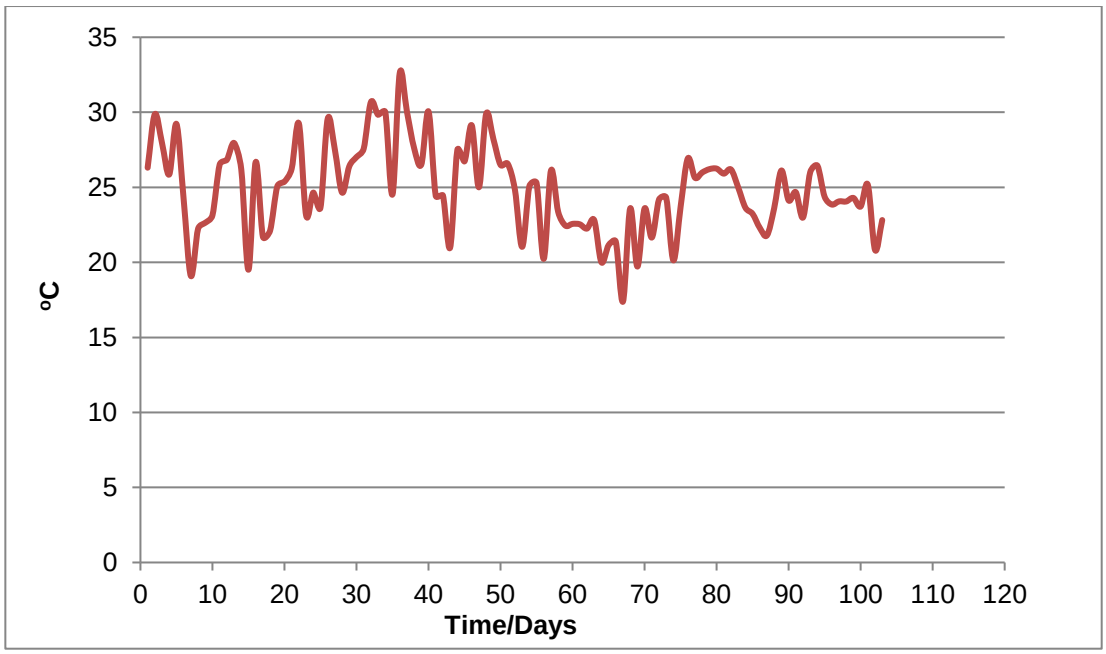


Figure 3.4: Average daily temperature in Skarden Garden glasshouse.

The plants were sprayed for aphids 5 weeks after sowing using Bayer Bug killer (Provado) and later they were sprayed for mildew using the fungicide, Bayer garden Fungus fighter (Systhane). 23 days after imposition of the irrigation regimes all of the pots were left without watering for 2 days to dry out, then half of the pots were harvested (harvest - 1) and measurements taken on 10 flag leaves per pot. The remaining 32 pots continued to be subjected to same irrigation treatments up to maturity. Theta probe measurements continued to the end of the experiment (Figures 3.5 and 3.6).

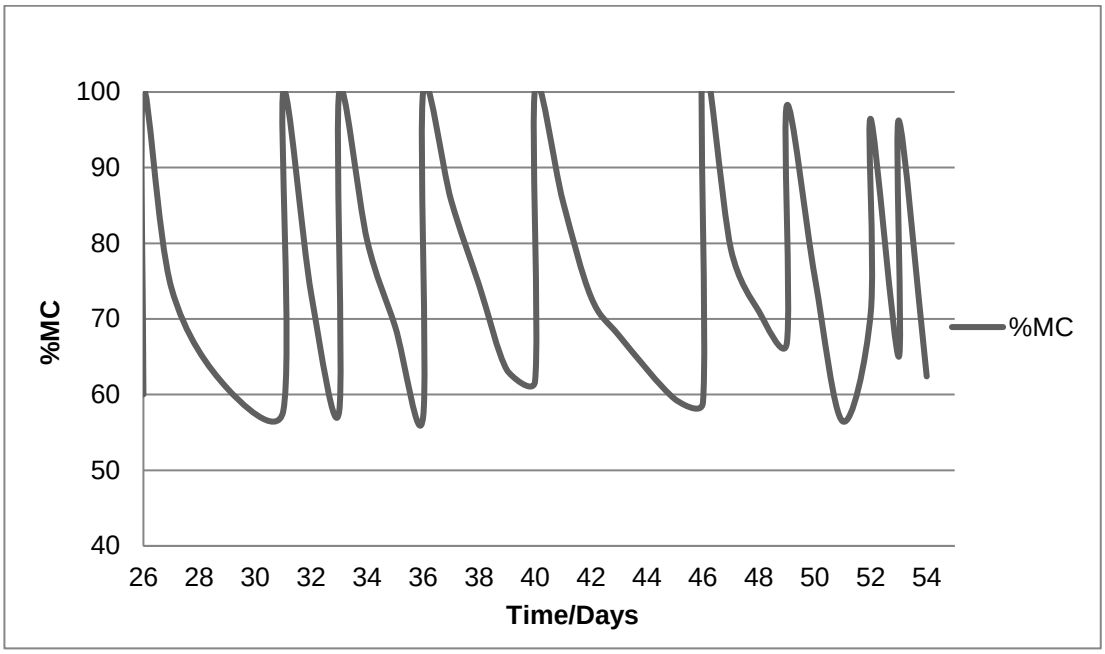


Figure 3.5: The %MC obtained from pots with wheat plants under normal irrigation regime.

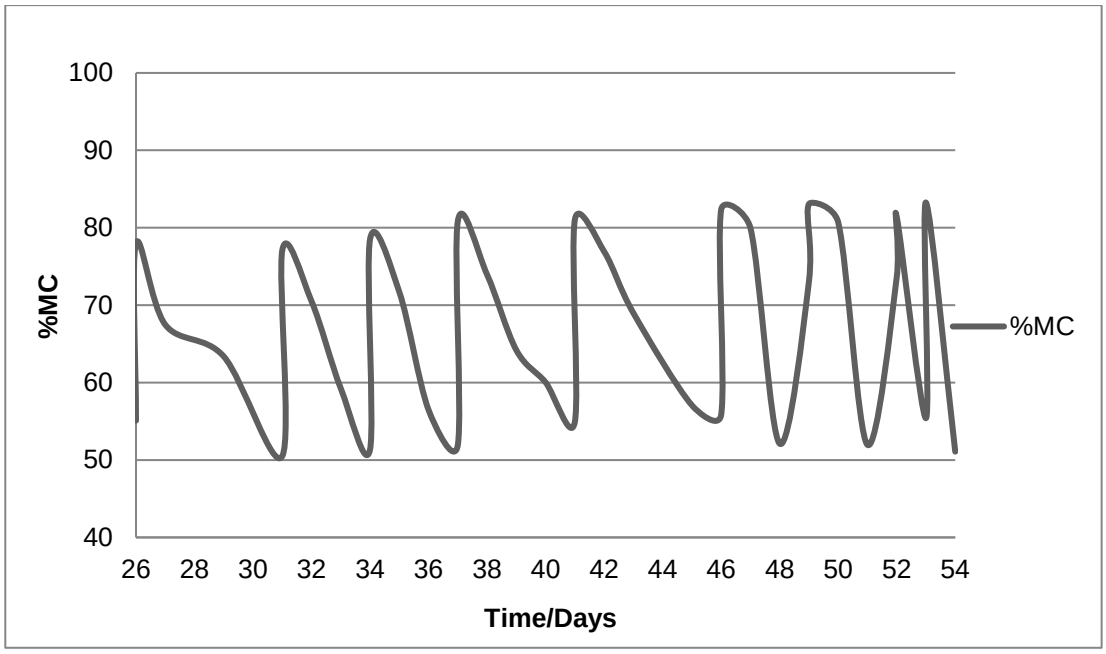


Figure 3.6: The %MC of pots with wheat plants exposed to the droughted watering regime.

3.3.1. Physiological parameters measured at harvest 1.

The response to drought stress was investigated at the ear emergence stage, and before taking the measurements, 10 flag leaves were randomly chosen from both non drought and drought plants of each of the four varieties in each replicate block so as to measure the following parameters.

3.3.1.1. Non-destructive measurements.

The non-destructive measurements comprised leaf stomatal conductance. Measurements were taken on the young fully expanded flag leaves of both non drought and drought stressed plants after the 23rd day of the imposition of the watering regimes and before rewatering.

- Leaf stomatal conductance

The stomatal conductance (gs) was measured on the adaxial surface of young fully expanded flag leaves of the main stem (not tillers) for both control and water stressed plants which were randomly selected in 10 seedlings per pot. Measuring took place at mid-day during a sunny day using a portable Delta T Porometer AP4 (Delta-T devices, Cambridge, UK) (Plate 3.1) so as to record stomatal aperture of leaves in terms of their conductivity to water vapour (gs - cm s^{-1}) at a light intensity of 210 μmol m^{-2} s^{-1} similar to the procedure of Luvaha *et al*. (2008).

Plate 3.1: Showing AP4 Porometer (Delta-T Devices).

3.3.1.2. Destructive measurements (harvest 1).

The measurements of first harvest were determined at half way of the experiment at the ear emergence stage, and the plants were pulled up and roots cut off just above ground. All stems and leaves were separated and put in plastic bags and placed in a polystyrene cool box for later evaluation of the following measurements:

- Leaf Area.

All green leaves on 16 plants per pot for all 4 varieties of each replicate were prepared for measuring the Leaf area (LA) using a Delta-T Leaf area meter and a Hitachi Kp-D40 (Digital color, Japan) for taking the images and calculating the area of leaves. The unit of measurement of the LA was cm^2 pot^{-1}

- Shoot dry weight per pot.

Shoot dry weight (SDW) for each treatment in each replicate for each of the four varieties was obtained by totaling the dry weight of leaves and stems. The leaves and stems were then placed in separate aluminum foil dishes, and put into a drying oven (Gallenkamp Economy Incubator size two, model IH-150, England) at 80 ^oC for 2 days to dry until constant weight (Singh and Usha, 2003).

- Chlorophyll content measurement.

A small sub-sample of fresh leaves was taken from the uppermost recently emerged leaves of control and droughted pots for each variety. The method of measuring the chlorophyll content was performed according to the standard method modified from Coombs *et al*. (2014) as follows:

- 10 fresh leaves (sub-samples) from the 4 varieties of stressed and non-stressed plants were taken, and cut to discs to known weight of leaf material (1 g of leaf).

- Leaf samples were extracted by grinding the leaf tissues in a mortar and a pestle in the presence of 80% ethanol (VWR International Ltd, UK 20821) plus some sand (sand sable GPR™, BDH laboratories supplies, 330945E England) by using 10 mL of 80% ethanol until a homogeneous mixture was obtained.

- The homogenate extract was transferred to a 15 mL centrifuge tube and centrifuged (Hettich Zentrifugen Roto fix 32A, Germany) at 2000 rpm for 2 minutes.

- The supernatant was used and made up to 10 mL with 80% ethanol. The total chlorophyll content of each sample was determined by measuring the absorbance values (ABS) with a Spectrophotometer (Jenway 7315 spectrophotometer, UK) at 647 and 664 nm. The contents of chlorophyll a, chlorophyll b and total chlorophyll were calculated in accordance with the following equations as outlined by Coombs *et al*. (2014).

Chlorophyll a (mg L^{-1}) = 13.19 (A_{664}) – 2.57 (A_{647}) *0.892

Chlorophyll b (mg L^{-1}) = 22.10 (A_{647}) – 5.26 (A_{664}) *0.906

Total chlorophyll a+b (mg g^{-1} fresh wt.) = 7.93 (A_{664}) + 19.53 (A_{647})

Where; A is absorbance at the given wavelength. The chlorophyll content in each sample expressed as mg g^{-1} FW was calculated by multiplying the extraction volume divide by the fresh weight of the sample.

3.3.1.3. Destructive measurements (Second harvest).

The measurements at second harvest (H2 - maturity) were made approximately 6 weeks after the first harvest (H1). 3 days before the irrigation was terminated to let the plants dry naturally. Once the plants were air dry, the roots were cut just above the ground and all plants in a single pot were bundled together as one unit and collected in plastic storage bags, bags were left unsealed to maintain air dryness. Then, they were taken to the laboratory and assessed as follows:

- Stem number (tiller number).

The number of ear bearing stems per pot was counted for 16 plants of each treatment T1 and T2 of four varieties.

- Stem weight (straw weight).

The stem bundle weight per pot was recorded (Precisa balances, Swiss quality, model 400M NO 13909, Switzerland).

Note: grain yield assessment was not possible due to a severe attack by mice over a weekend.

Statistical analysis

To determine the significance variation for all the parameters measured for the study, data was analysed by analysis of variance (Balanced ANOVA) using Minitab statistical programme (Version 17). The Least Significant Difference (LSD) test was applied at the probability level of 5% ($p < 0.05$) to compare the signficant difference between means of treatments for different varieties. In Figures error bars are one standard error of the mean (SE).

3.4. Results

3.4.1. Non-destructive measurements.

Leaf stomatal conductance

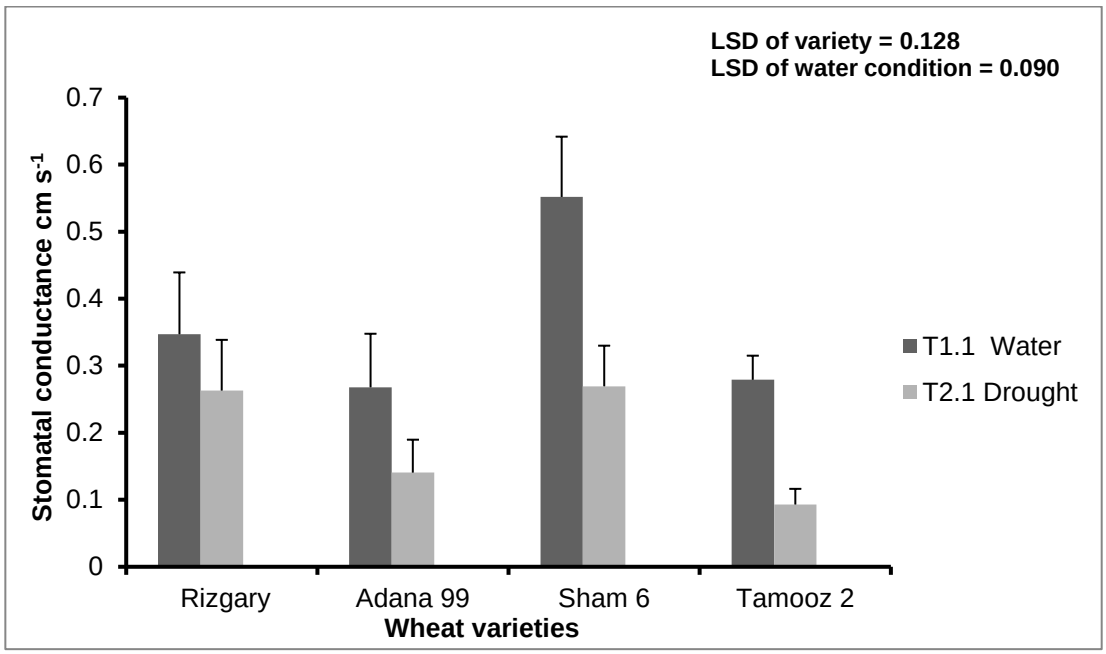


Figure 3.7: The effect of two moisture regimes on stomatal conductance at the flag leaf stage of four Iraqi wheat varieties (*p* = 0.005 for variety. *p* = 0.001 for water conditions. *p* = 0.421 for variety * water condition, error bars = SE).

Variety had a significant effect on the leaf stomatal conductance (*p* = 0.005). The conductance of leaf stomata in cvs. Tamooz 2 and Rizgary was less compared cvs. Adana 99 and Sham 6. Water deficiency had a noticeable effect on stomatal conductance for all stressed plants as compared with their well-watered counterparts (*p* = 0.001) (Figure 3.7). A non significant interaction was observed between variety and watering conditions on leaf stomatal conductance (*p* = 0.421) (Table 3.2 and Appendix 3).

Table 3.2: Summary table of ANOVA results for the effect of two watering regimes on the stomatal conductance of four Iraqi wheat varieties.

Source	F value	*P* value	LSD
Variety	5.71	0.005	0.128
Water condition	15.36	0.001	0.090
Variety * Water condition	0.98	0.421	
Rep	2.74	0.069	

Soil moisture content of the pots.

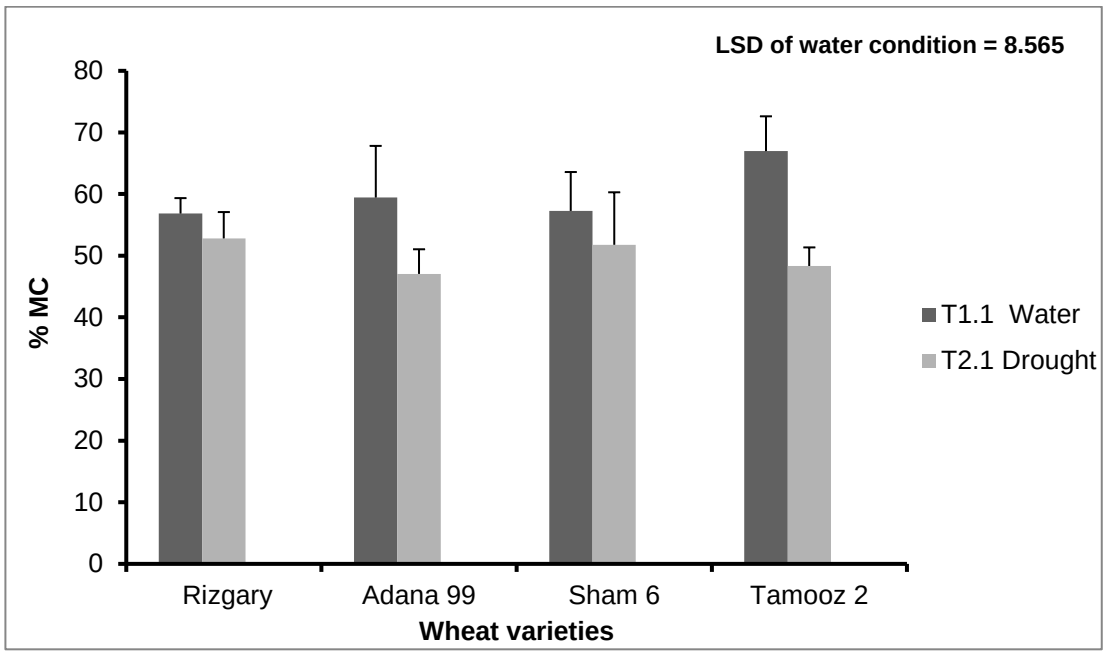


Figure 3.8: Soil moisture content (%MC) in pots of four varieties of wheat (Triticum aestivum L) at different watering regimes (T1.1, T2.1) (*p* = 0.892 for variety. *p* = 0.023 for water conditions. *p* = 0.580 for variety * water condition, error bars = SE).

At the end of the experiment, no significant difference was observed in the soil moisture content among four varieties of wheat plants (p = 0.892). A significant effect was observed in the soil moisture content for all water-stressed plants as compared to their control plants (p = 0.023) (Figure 3.8). There was no significant interaction between variety and water conditions in the soil moisture content (p = 0.580) (Table 3.3 and Appendix 4).

Table 3.3: Summary table of ANOVA results for the effect of two watering regimes on the soil moisture content of four Iraqi wheat varieties.

Source	F value	*P* value	LSD
Variety	0.20	0.892	
Water condition	6.06	0.023	8.565
Variety * Water condition	0.67	0.580	
Rep	0.78	0.518	

3.4.2. Destructive measurements (First harvest).

Leaf Area.

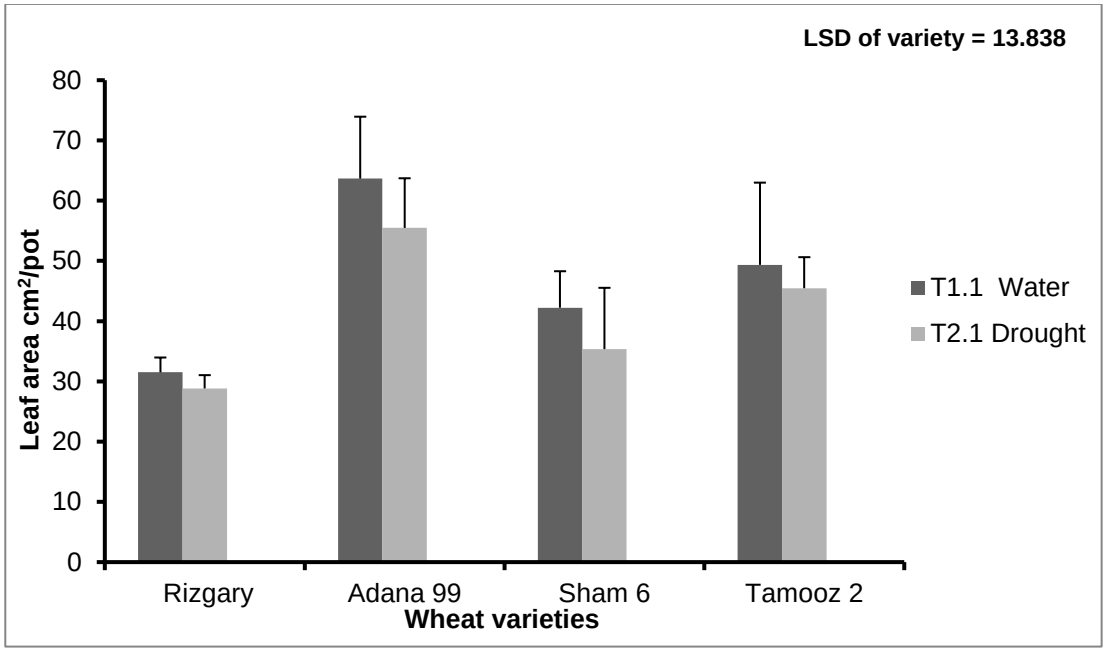


Figure 3.9: The effect of two moisture regimes on total leaf area of 4 wheat varieties (*p* = 0.002 for variety. *p* = 0.264 for water conditions. *p* = 0.973 for variety * water condition, error bars = SE).

A significant effect was found for variety on the leaf area/pot (*p* = 0.002). The leaf area/pot of cvs. Rizgary and Tamooz 2 was less affected compared to cvs. Sham 6 and Adana 99. Watering conditions had no significant effect on the leaf area/pot (*p* = 0.264) (Figure 3.9). There was no significant interaction between variety and water conditions in the leaf area/pot (*p* = 0.973) (Table 3.4 and Appendix 5).

Table 3.4: Summary table of ANOVA results for the effect of two watering regimes on the leaf area of four Iraqi wheat varieties.

Source	F value	*P* value	LSD
Variety	7.11	0.002	13.838
Water condition	1.31	0.264	
Variety * Water condition	0.07	0.973	
Rep	5.12	0.008	

Shoot dry weight per pot.

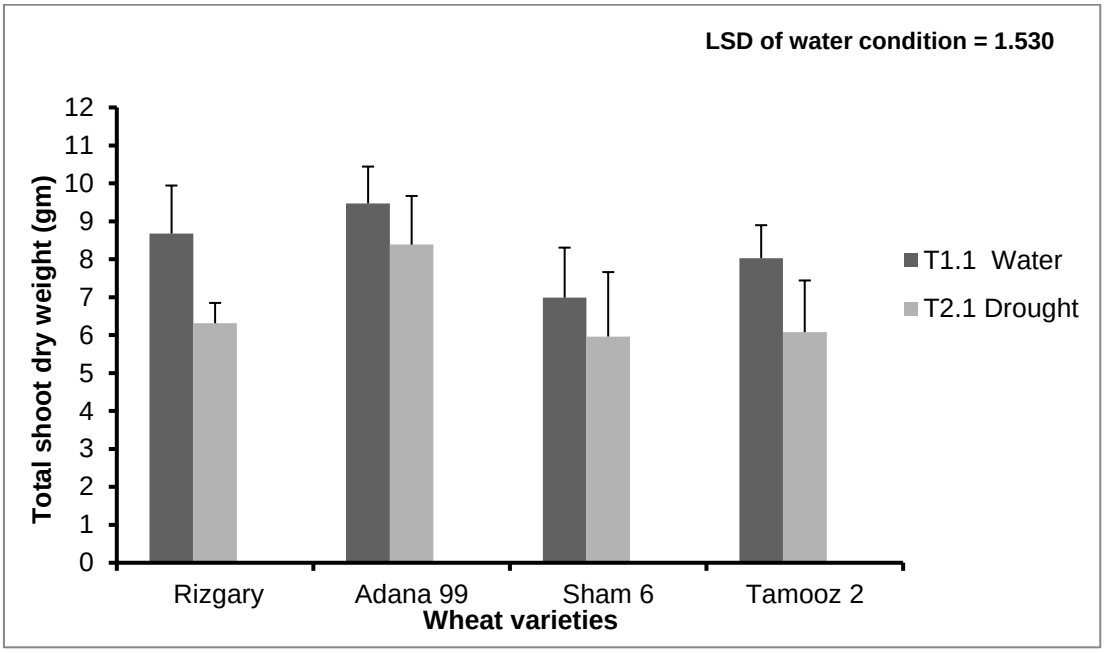


Figure 3.10: Total shoot dry weight of 4 wheat varieties under two watering regimes (*p* = 0.141 for variety. *p* = 0.041 for water conditions. *p* = 0.895 for variety * water condition, error bars = SE).

No significant effect was observed for variety on the total shoot dry weight of wheat plants (p = 0.141). A significant difference was observed on the total shoot dry weight for drought stress effect compared to well watered condition (p = 0.041) (Figure 3.10). There was no significant interaction between variety and watering conditions in the total shoot dry weight (p = 0.895) (Table 3.5 and Appendix 6).

Table 3.5: Summary table of ANOVA results for the effect of two watering regimes on the total shoot dry weight/pot of four Iraqi wheat varieties.

Source	F value	*P* value	LSD
Variety	2.02	0.141	
Water condition	4.76	0.041	1.530
Variety * Water condition	0.20	0.895	
Rep	3.79	0.026	

Chlorophyll content measurement.

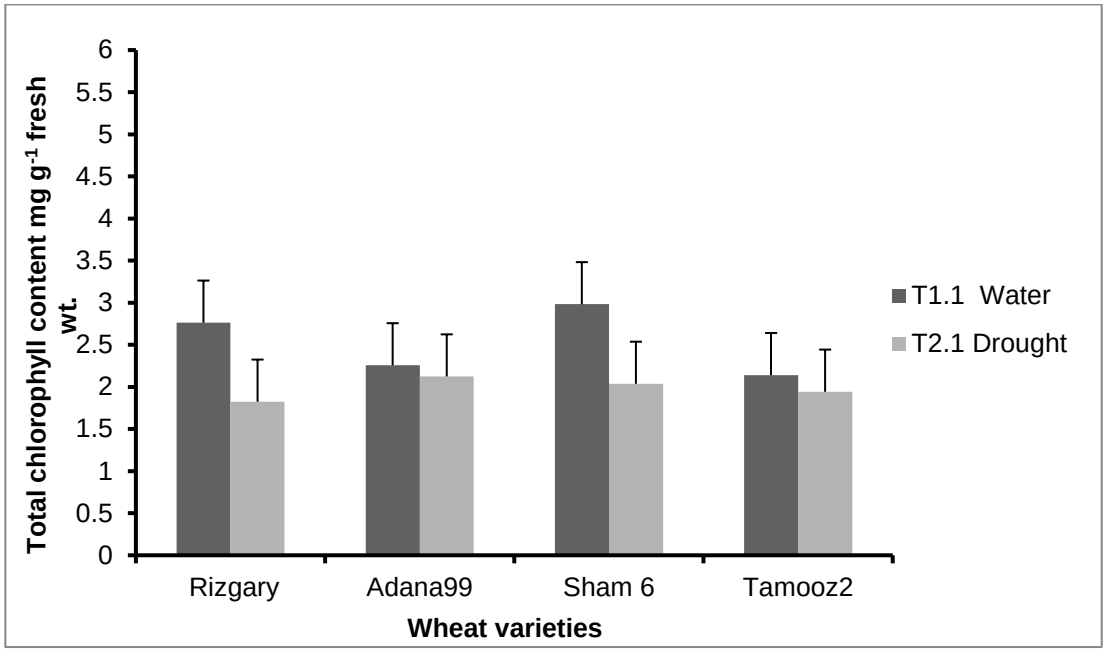


Figure 3.11: Total chlorophyll content of four wheat varieties under two watering regimes for one replicate (*p* = 0.584 for variety. *p* = 0.091 for water condition, error bars = SE).

No significant effect was observed on total chlorophyll content for the four wheat varieties ($p = 0.584$) and there was no significant difference between normal and drought stress conditions on the total chlorophyll contents ($p = 0.091$) (Figure 3.11) (Table 3.6 and Appendix 7).

Table 3.6: Summary table of ANOVA results for the effect of two watering regimes on the total chlorophyll content of four Iraqi wheat varieties for one replicate.

Source	F value	*P* value	LSD
Variety	0.77	0.584	
Water condition	6.07	0.091	
Variety * Water condition			
Rep			

3.4.3. Destructive measurements (H2).

Stem number.

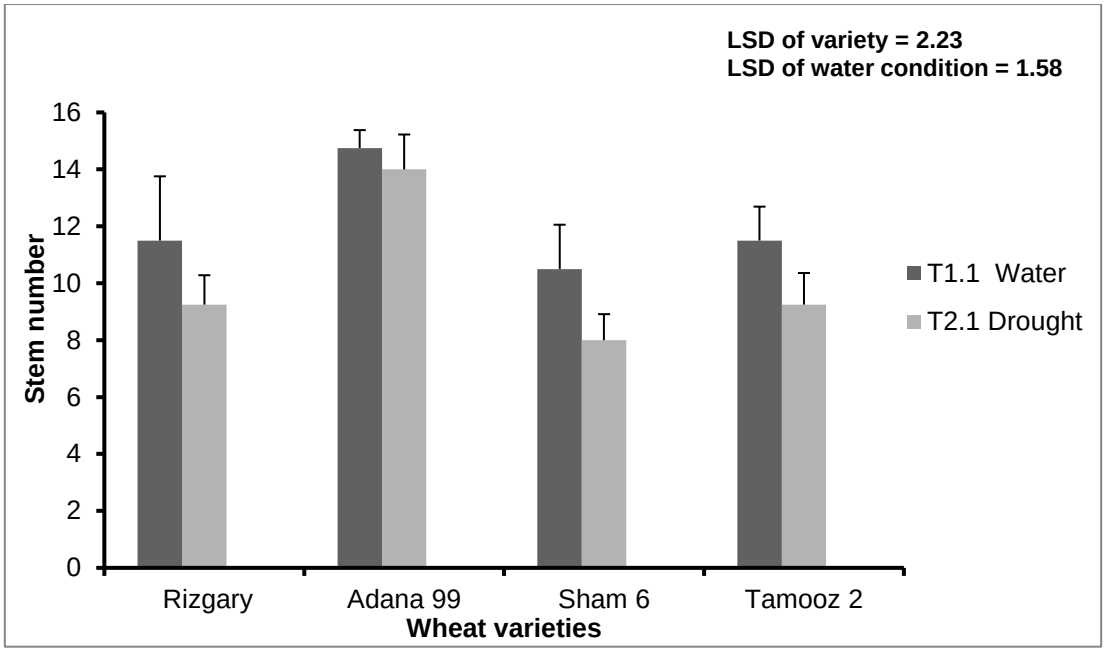


Figure 3.12: Stem number of four varieties under two moisture regimes at the end of experiment (*p* = 0.001 for variety. *p* = 0.018 for water conditions. *p* = 0.840 for variety * water condition, error bars = SE).

Variety had a significant effect on the number of stems at the second harvest (p = 0.001). A greater difference in stem number was observed for Sham 6 as compared to Adana 99. However, both cvs. Tamooz 2 and Rizgary had the same difference in the stem number. There was a significant difference between well watered and drought conditions on the stem number (p = 0.018) (Figure 3.12). A non significant interaction was observed between variety and water conditions on the stem number (p = 0.840) (Table 3.7 and Appendix 8).

Table 3.7: Summary table of ANOVA results for the effect of two watering regimes on the stem number of four Iraqi wheat varieties.

Source	F value	*P* value	LSD
Variety	8.81	0.001	2.23
Water condition	6.52	0.018	1.58
Variety * Water condition	0.28	0.840	
Rep	5.11	0.008	

Stem weight

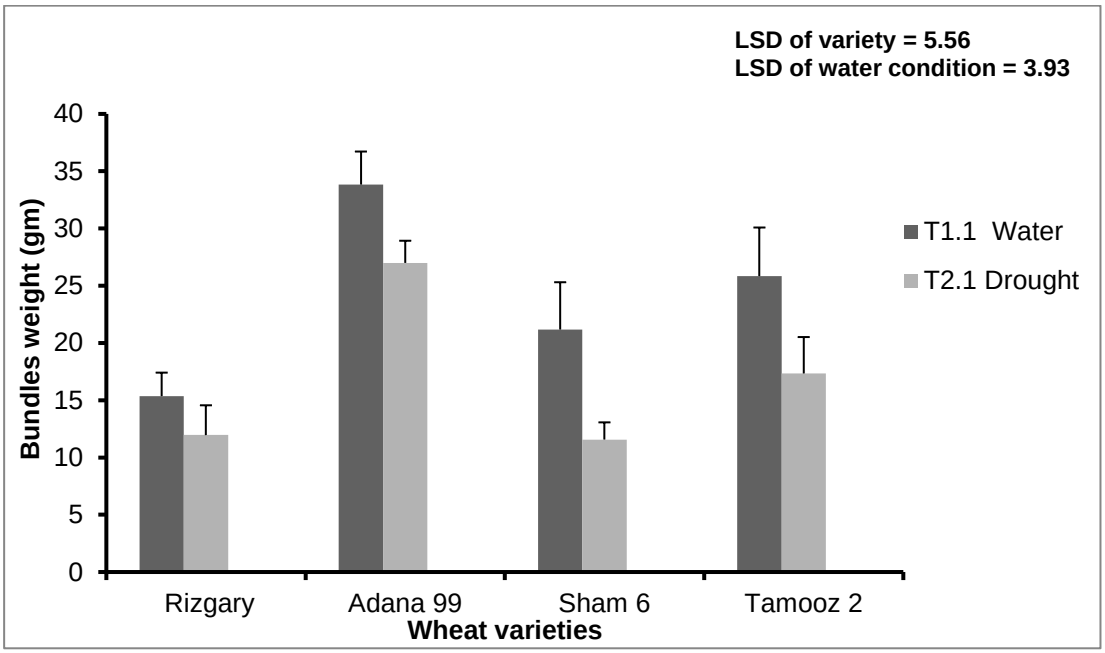


Figure 3.13: Stem weight of four Iraqi wheat varieties under two irrigation regimes ($p \leq 0.001$ for variety. $p = 0.001$ for water conditions. $p = 0.678$ for variety * water condition, error bars = SE).

Variety had a significant effect on the stem weight ($p \leq 0.001$). The biggest significant difference in bundle weight was observed for Adana 99 as compared to Rizgary. A significant effect was observed on the stem weight for drought stress compared to well watering condition ($p = 0.001$) (Figure 3.13). There was no significant interaction between variety and water conditions in the stem weight ($p = 0.678$) (Table 3.8 and Appendix 9).

Table 3.8: Summary table of ANOVA results for the effect of two watering regimes on the stem weight of four Iraqi wheat varieties.

Source	F value	*P* value	LSD
Variety	15.24	< 0.001	5.56
Water condition	14.07	0.001	3.93
Variety * Water condition	0.51	0.678	
Rep	2.85	0.062	

3.5. Discussion

3.5.1. Non-destructive measurements.

Leaf stomatal conductance

The result showed that leaf stomatal conductance was significantly different in wheat varieties being significantly lower in cvs. Tamooz 2 and Rizgary compared to cvs. Adana 99 and Sham 6 (Figure 3.7). These results are also consistent with (Liang et al., 2002) in that drought stress caused a substantial decrease of leaf stomatal conductance in wheat plants when compared to control plants. Dulai *et al*. (2006) also reported that low water content stress led to a significant reduction in stomatal conductance in *Aegilops bicornis* and wheat cultivar Mv9kr1, while *Aegilops tauschii* and *Aegilops speltoides* were not affected significantly. A different outcome has been observed in Mango rootstock seedlings indicating that stomatal conductance only reduced with extreme water deficit (Luvaha *et al*., 2008). Kauser *et al*. (2006) reported that in spite of a negative effect of drought stress on the leaf stomatal conductance, canola cultivars did not differ significantly under both stress and non-stress conditions.

Increased resistance of plants to low water potential might be because of decreased water transport in the leaves further causing a reduction in the stomatal pores acting as an adaptive mechanism. In addition the intensity of water deficiency in the leaves causing quard cells to decrease the turgor and hence limit photosynthetic ability reflects the important role of stomatal response (Tezara *et al*., 2002). It has been emphasized that stomata behavior is in relation to leaf water potential at any stage of physiological development of plants. For example, plants growing under water stress conditions usually minimize the water

vapour loss from their leaves by closure of the stomata of both adaxial and abaxial leaf surfaces (Teare *et al*., 1982).

Soil moisture content.

Soil moisture content results indicated a significant effect for water stress compared to well watering (Figure 3.8). A contradictory effect of drought on soil water potential was observed in Mango rootstock seedligs under water stress. Results of this experiment demonstrated that moisture content of soil reduced as water stress intensified (Luvaha *et al*., 2008). Moreover, the investigation of the change of water supply of soil on drought tolerance of wheat (*Triticum aestivum)* conducted by Liang *et al*. (2002) evidenced that the alternation of "drying-rewatering" caused a decrease in soil water potential of wheat plants. The measures declined significantly to -1.3 MPa after the exposure of wheat to the drying, then increased to -0.5 MPa after the irrigation of treated pots. The variance in water status of the soil could be by reason of not only atmospheric conditions such surface evaporation, transpiration and drainage but also species and the stage of growth. It has been observed that the leaf water potential required for stomatal closure of wheat decreases as plants mature (Teare *et al*., 1982).

3.5.2. Destructive measurements (H1).

Leaf Area.

The result showed that wheat cvs. Sham 6 and Adana 99 had lower leaf area compared to cvs. Tamooz 2 and Rizgary (Figure 3.9). These results are in agreement with the analyses of wheat seedlings responses to moderate soil water deficit (Saidi *et al*., 2010) which showed that leaf area of the plants was significantly larger under adequate water supply (FC) than the -0.02 MPa and -0.04 MPa drought treatments whereas the -0.06 MPa treatment recorded the smallest values. On the other hand, Al-Temimi *et al*. (2013) found that severe soil drought stress caused a significant reduction in the growth characteristics of bread wheat cultivars grown under field conditions, particularly in leaf area where cultivar Tamooz 2 was the most drought tolerant since higher leaf area was obtained compared with the cultivar Sham 6. A study on canola plants (*Brassica napus* L.) in hydroponic culture proved that imposition of water deficit conditions significantly decreased leaf area in both canola cultivars as compared to non-stress conditions, and the tolerant cultivar (Dunked) had a higher value of this growth trait than that the sensitive cultivar (Cyclone) under water stress (Kauser *et al*., 2006). The substantial reduction of leaf area in water-stressed plants has been interpreted as the changes in cell growth and development and is also associated with acclimation during the early drought period, and thus plants develop the adjustments of cell division rate and expansion in response to water availability. These processes are most sensitive to water deficit due to loss of turgor pressure associated with accumulation of ABA that may limit photosynthesis which in turn leads to a reduced rate of leaf growth (Soares‐Cordeiro *et al*., 2009, Tezara *et al*., 2002).

Shoot dry weight per pot.

The results showed a significantly different value of shoot dry weight under drought conditions in comparison with well-watered conditions. The value of shoot dry weight reduction was approximately 2g and reduced to 1g (Figure 3.10). The same was observed in a study conducted by Saidi *et al*. (2010) on response of wheat seedlings to moderate soil water deficit. Their results indicated that shoot growth was promoted by supplying adequate water in wheat, which is known as a relatively drought-resistant crop plant. While, the dry matter growth decreased under water potentials of the soil -0.02 and -0.04 MPa. Similar responses of shoot dry matter were obtained by (Luvaha et al., 2008) on *Mangifera indica* rootstock seedlings grown under water deficit conditions. Observations by (Kauser et al., 2006) also displayed that the drought watering regime reduced shoot dry weight significantly in two canola cultivars as compared to their non-stressed plants under hydroponic culture. The reduction in shoot dry weight may be due to the response of shoot growth to water stress in terms of reduction in leaf and stem formation because of leaf and stem growth rates are reliant on cell expansion resulting from cell turgor effects (Rezaeieh and Eivazi, 2011). Reduction in shoot growth rate has also been attributed to the transmission of abscisic acid (ABA) from roots to leaves through xylem in order to respond water deficiency (Sharp, 1990, Kauser *et al*., 2006).

Chlorophyll content measurement.

The result showed that the total chlorophyll content of four wheat varieties was not significantly different (Figure 3.11) which is a dissimilar finding to a study on the effect of water deficit on the physiological behavior of some Tunisian barley varieties (Raoudha *et al*., 2007). It was shown that a moderate water stress affected photosynthesis and decreased chlorophyll contents for all ecotypes compared to their controls, and two ecotypes showed more tolerance to stress and recorded lower reduction rates of 5.16 and 5.2% than others by having desirable high photosynthetic traits and maintaining higher water potential. This is in agreement with the result of Li *et al*. (2006) who reported that chlorophyll content measurements were significantly different between drought tolerance and senstive genotypes of barley under water deficit conditions.

In contrast differential responses of the test cultivars of bread wheat to water deficit stress has been reported by Al-Temimi *et al*. (2013) who showed that drought tolerant variety Tamooz 2 showed an increase in total chlorophyll content compared to non-tolerant variety Sham 6 which recorded lower values of chlorophyll content. Also in Mango plants (*Mangifera indica*), water stress imposed during the root stock seedlings period showed slightly increased chlorophyll a and total chlorophyll content with the increase of water deficit, whereas the chlophyll b remained constant (Luvaha *et al*., 2008). This variation in chlorophyll content of genotypes implies that the photosynthetic apparatus in tolerant plants is resistant to water deficit, and may be due to continued synthesis of photosynthetic pigments chlorophylls a and b. Conversely, sensitive genotypes of plants are susceptible to soil drying and due to the ability of stress to reduce

the tissue concentrations of chlorophylls more free radicals are generated which induce lipid membrane peroxidation of the thylakoids and thus damage photosynthesis (Osmond *et al*., 1997).

3.5.3. Destructive measurements (H2).

Stem number (tiller number).

Tiller number for varieties Sham 6 and Adana 99 was significantly lesser as compared to the varieties Tamooz 2 and Rizgary (Figure 3.12)**.** This finding is in accordance with Ajalli and Salehi (2012) who assessed drought tolerance indices in barley (*Hordeum vulgare* L.) and reported that moisture deficit stress reduced growth yield by reducing the number of tillers at both flowering and grain filling stage by up to 7%. This was similar to a study in wheat (Al-Temimi *et al*., 2013) which revealed that Tamooz 2 was the most tolerant cultivar as it had the highest number of tillers at high water deficit stress under field conditions compared to the drought-sensitive cultivar Sham 6. This indicates that drought retards the growth of cereals having a negative effect on yield components causing a decrease in the number of fertile tillers per plant.

Stem weight.

There were observed significant influences among all varieties tested in bundle weight. The highest amount of stem weight reduction was observed for cv. Sham 6 as compared to Rizgary (Figure 3.13). This partially agrees with results of Efeoglu *et al*. (2009) in a study of the physiological responses of three maize cultivars to drought stress and recovery. The results indicated a significant reduction in dry biomass among genotypes at the exposure to drought and recovery conditions as compared to their controls and the most resistant cultivar still had the highest dry biomass after 12 and 18 days of growth under control conditions. On the other hand, results of a study by (Liang *et al*., 2002) showed a significant effect of alteration from drought to re-irrigation conditions on the decrease of stem dry mass in wheat plants (*T. aestivum*) under drought compared with that of control plants. The reduction in stem dry weight could be related to reduced rate of leaf production causing reduced number of leaves. Reduction in leaf growth may also be associated with lower rates of cell division and cell extension in the leaves, as well as leading to less photosynthesis. Thus, drought retarded overall plant growth as the resources required for growth processes become limited in supply (Mwai, 2001).

3.6. Conclusion

3.6.1. Non-destructive measurements.

From non-destructive measurements it can be concluded that the degree of drought tolerance of Iraqi wheat varieties can be based on how the physiological functions of the plants can be sustained under low soil water potential. Considerable variation was found among the varieties and also between normal and water stress conditions in leaf stomatal conductance measurement. Results indicated that leaf stomatal conductance for cvs. Tamooz 2 and Rizgary was more affected compared to cvs. Adana 99 and Sham 6.

3.6.2. Destructive measurements (H1).

In view of the results obtained from destructive measurements at the first harvest, it is evident that drought stress significantly decreased the total shoot dry weight/ pot for all stressed plants compared to their controls. Variety had a signifcant effect on leaf area/pot measurement. Both cvs. Rizgary and Tamooz 2 showed better leaf area values compared to cvs. Adana 99 and Sham 6.

3.6.3. Destructive measurements (H2).

In relation to the destructive measurements at the second harvest, it is concluded that variety had a significant influence on the biomass yield traits in terms of the number of tillers and stem bundle weight. Both wheat cultivars Tamooz 2 and Adana 99 showed higher stem dry weights than Rizgary and Sham 6, indicating that they are more suitable for selection as a model system for the study of the drought stress response.

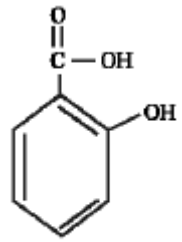
Chapter 4

4. The effect of exogenous applications of salicylic acid and molybdenum in modifying the drought stress responses of wheat

4.1. Introduction

Salicylic Acid (SA) or 2-hydroxy benzoic acid is usually defined as a substance that possesses an aromatic ring with a hydroxyl group and free SA is a crystalline powder (Table 4.1). The first commercial production of synthetic SA was introduced in Germany in 1878 as aspirin. Acetyl Salicylic Acid (ASA) is a close analogue of salicylic acid with similar physiological impacts because it is converted to SA spontaneously by hydrolysis. The name salicylic acid was derived from the Latin word “Salix” for the willow tree, and given to the active ingredient of Salicylate (Salicin) the glucoside of Salicylic alcohol which was isolated from willow bark by Rafacle Piria in 1938 (Raskin *et al*., 1990).

Table 4.1: showing the structure of salicylic acid with some physical and chemical properties (modified from Popova *et al*. (1997))

Physical properties	Molar mass	138.12g/mol
	pH aqueous SA	2.4
	Melting point	157-159 ˚C in H_2O
	Boiling point	211˚C
Chemical properties	Molecular formula	$C_6H_4(OH)COOH$
	Chemical structure	 Salicylic acid (SA)

The first evidence that SA might be involved in plant defence system was provided by White (1979), who found that injection of Aspirin or SA into tobacco leaves enhanced resistance to subsequent infection by Tobacco Mosaic Virus (TMV). Salicylic acid is a naturally occurring derivative of the group of phenolic acid compounds which is distributed in many species of monocotyledonous and dicotyledonous plants including rice, barley, crabgrass and soybean. The amount of SA in leaves and reproductive organs of angiosperms plants has been found to be approximately 1μg g^{-1} fresh weight. The highest concentration of SA was detected in pathogen infected necrotizing inflorescences. In addition, it is known that SA has a regulatory role in a range of physiological processes such as photosynthesis, transpiration, nutrient uptake, chlorophyll synthesis and plant development (Raskin, 1992). SA was later recognized as an important signalling molecule which potentially influences plant tolerance to water stress because of its influence on the regulation of metabolic and physiological activities during the entire lifespan of the plant affecting the growth parameters and bio-productivity of plants (Popova *et al*., 1997).

These responses are directly activated by changing the water status of plant tissues while others are triggered by signalling molecule transduction that regulate growth and development and lead to a cascade of processes responsible for the physiological adaptation of the plant to stress conditions (Reddy *et al*., 2004). Besides this function of growth promotion, it has been reported in the literature during the last decade that exogenous application of SA to plants can play a role in enhancing wheat plant tolerance to some abiotic stresses, such as salinity (Shakirova *et al*., 2003) and drought stress (Singh and Usha, 2003, Waseem et al., 2006, Amin et al., 2008). Several studies support the

stimulatory effect of SA on a number of morphological and physiological processes of plants, including growth, photosynthesis and other metabolic processes. This demonstrates a function as a protective agent in plants modulating the plant response to various biotic and abiotic stresses such in drought, cold, heat and osmotic stress (Hayat *et al*., 2010). Exogenous treatment of drought stressed plants with different levels (0.5 and 1.0 mM) of SA not only caused a decline in the adverse effect of drought in yellow maize (*Zea mays* L.) plants, but also stimulated physiological traits, productivity and plant resistance to drought stress (Elgamaal and Maswada, 2013). Moreover, Farooq et al. (2009a) demonstrated that treatment with SA (100 mg L^{-1}) to leaves of rice (*Oryza sativa* L.) had a positive effect on photosynthesis and plant growth compared to applying other treatments of (50 and 150 mg L^{-1}) and exhibited better resistance to drought stress than soaking the seeds in the same SA solutions.

The first indication of the physiological role of molybdenum (Mo) as an essential micronutrient for plants was reviewed by Bortels (1930), who indicated that supplementation of culture medium with traces of Mo highly increased the fixation of nitrogen and growth by microorganisms. In the biological system, molybdenum as molybdate is the dependent form for more than 50 enzymes in all organisms because of its availability to the cells by combine into the active sites of enzymes to form the molybdenum cofactor. However, five of these molybdo-enzymes have been found in higher plants and are involved in the metabolism processes of nitrogen, purine, sulphur and phytohormone and can act in transducers reduction systems such as hydroxylation of carbon centres under more moderate conditions (Mendel, 2013). These Mo-enzymes are known as nitrate reductase (NR), xanthine dehydrogenase (XDH), sulphite oxidase (SO) and aldehyde

oxidase (AO) respectively and each has a crucial role in diverse metabolic pathways. For these reasons, a shortage of Mo element in the substrate leads to loss of these essential metabolic functions and ultimately to death of the plants (Walker-Simmons *et al*., 1989, Koshiba *et al*., 1996). In view of some reports, molybdenum has been defined as a major growth promoter and it was hypothesized that exogenous application of Mo can also induce abiotic stress tolerance in food crops (Taiz and Zeiger, 2004). In support of this, Mo is currently one of the most studied topics in abiotic stress response research such as drought, cold and salt stress (Ghafarian *et al*., 2013, Sun *et al*., 2009, Al-Issawi *et al*., 2013, Zhang *et al*., 2012).

Although a number of reports have indicated the protective role of SA under environmental stress conditions to date, the research conducted on the effect of exogenous applications of SA on physiological characteristics and yield of wheat under drought stress condition is limited. Therefore, this experiment was conducted to study the influence of spraying plants with “anti-stress” compounds such as SA and Mo on drought tolerance capacity in two Iraqi wheat varieties compared to untreated plants.

4.2. The objective of this study

The present research aimed to:

1. Study how two cultivars of Iraqi wheat respond to drought conditions as well as the influence of exogenous applications of the elicitors salicylic acid and molybdenum on drought stress improvement.
2. To assess whether exogenously applied SA and Mo could adjust the adverse effects of water stress on plant growth and yield, and at which concentration they are most effective.

4.3. Materials and methods

Two Iraqi wheat (*Triticum aestivum* L.) varieties, Tamooz 2 and Adana 99, were chosen as a model system following on from the comparative studies on water stress-induced changes in the first drought experiment (Ch. 3). The 60 pots used in the first drought experiment were used again simulating a 2nd wheat crop and were reweighed using a standing floor balance (Toledo Model 47141, UK), and contained about 13 kg of air dried soil. The pots were placed in pot saucers and then they brought up to full saturation point by adding 4 litres of water to reach around 17 kg fresh weight of soil. Four hundreds seeds of the two varieties were placed on wet absorbent laboratory blue tissue paper in sandwich boxes, and the seeds were allowed to germinate at 25^{o}C in a growth chamber (Sanyo Gallenkamp PLC LE 3202, England) for approximately 72 hour. After 3 days, the germinated seeds were planted (mid November 2014) in the plastic pots (n = 60), and the plants were grown under greenhouse conditions in Skarden Garden

Plymouth University UK with a 12 h photoperiod maintained with sodium vapour supplementary lighting. The experimental arrangement was a completely randomised block design (CRD) with two varieties, two watering regimes (W1 well watered, and W2 drought stressed) and five treatments (Con, SA1, SA2, Mo1, Mo2) in three replicate blocks. The allocation of the treatments to the pot positions within a block was randomised using random number tables (Figure 4.1).

Rep 1

V1 SA1 W1	V2 Mo2 W2	V1 Mo1 W2	V2 Con W1	V1 Con W1	V2 Con W2	V2 SA1 W1
V2 SA2 W1	V2 SA2 W2	V1 SA1 W2	V1 Mo1 W1	V2 SA1 W2	V1 SA2 W2	V1 Mo2 W2
	V2 Mo1 W2	V2 Mo1 W1	V2 Mo2 W1	V1 Con W2	V1 Mo2 W1	V1 SA2 W1

Rep 2

V1 Mo1 W2	V2 Mo1 W1	V2 Mo2 W1	V1 SA2 W1	V1 Mo2 W1
V1 SA1 W1	V2 SA2 W1	V2 Con W2	V1 Mo1 W1	V1 Con W2
V1 Con W1	V1 SA2 W2	V2 Con W1	V2 SA2 W2	V1 SA1 W2
V2 Mo1 W2	V2 SA1 W2	V1 Mo2 W2	V2 SA1 W1	V2 Mo2 W2

Rep 3

V1 Mo2 W2	V2 SA1 W2	V1 SA1 W1	V2 Mo1 W1	V2 SA2 W2	V2 Mo2 W2	V2 SA2 W1
V1 SA2 W2	V2 Con W1	V1 SA2 W1	V1 Mo1 W2	V1 Con W1	V1 Mo2 W1	V2 Con W2
	V1 SA1 W2	V2 SA1 W1	V1 Con W2	V2 Mo1 W2	V1 Mo1 W1	V2 Mo2 W1

3R × 2V × 2W × 5T = 60 pots. There were 16 plants/pot

Figure 4.1: Plan of glasshouse benches showing the layout of pots following randomisation. (R = Replicate; V = variety; W = Watering; Con, SA1, SA2, Mo1, Mo2 = Treatments.

Growth stage codes were given according to Zadoks et al. (1974). At the three leaf stage (GS13), plants were fertilized with 7.5 g pot^{-1} Gromore NPK having the nutrient content of 7:7:7%. One month after sowing, the plants were sprayed with Anti Mildew (Plymouth Garden Centre, 771820 Germany) to control powdery mildew, and spraying was repeated two further times with an interval period of one month. At the four leaf stage (GS14), SA (1.44 and 2.88 mM) and Mo (0.15 and 0.30 mM) were sprayed onto designated pots. Control plants were sprayed with distilled water. Non-stressed plants were maintained in a well-watered condition at 70-100% of field capacity and drought stressed plants were maintained at 50-70% FC as in the first drought experiment:

- Treatment 1 Control (Water spray)
- Treatment 2 SA 1 (SA 1.44 mM)
- Treatment 3 SA 2 (SA 2.88 mM)
- Treatment 4 Mo 1 (Mo 0.15 mM)
- Treatment 5 Mo 2 (Mo 0.30 mM)

One month after first application of fertilizer and at stem extension stage (GS30) plants were fertilized again with a second application of Gromore (NPK 7:7:7%). Thirty eight days after the commencement of the watering regimes, and near to ear emergence (GS51), leaf stomatal conductance and chlorophyll a fluorescence were measured for 10 flag leaves of all treatments for both varieties.

4.3.1. Preparation of salicylic acid solutions

Salicylic acid (Sigma Ltd, S7401.USA) was used to prepare two different concentrations of SA solutions. A stock solution with 1000 ppm of SA ($C_7H_6O_3$) was prepared by dissolving 1 g in distilled water and made up to 1 litre. The pH of the solution measured (Basic pH Meter, Denver Instrument Company, 300408.1Rev.F, U.S.A) and brought up to pH 7 by adding drops of 1M of NaOH. Spray solutions with concentrations of 1.44 (SA1) and 2.88 (SA2) mM SA were prepared from the stock solution by dilution with distilled water as described by Amin et al. (2008). Then 2 drops of Tween' 80 [polyoxyethylene (20) sorbitan mono-oleat] (Atlas Chemical Industries Inc., 56023) were added to each spray solution as a surfactant agent. The plants were sprayed with SA1 and SA2 five weeks from sowing. The SA solutions were sprayed from the top of the plants using a small hand-held sprayer until foliage run-off delivering 200 mL pot^{-1}. To prevent solution drifts to adjacent pots, each pot was shrouded by a cover during spraying (a pot with the base removed inverted over the foliage).

4.3.2. Preparation of molybdenum solutions

Ammonium molybdate tetrahydrate [$(NH_4)6Mo_7O_{24}.4H_2O$] (Fisher scientific, A/5720/53. UK) was used to prepare a stock solution of 1000 ppm by dissolving 1.84 g in distilled water which was then made up to 1 litre. Spray solutions were prepared with 0.15 (Mo1) and 0.30 mM (Mo2) Mo by dilution with distilled water according to Rihan *et al*. (2014). Then, 2 drops of the surfactant agent Tween' 80 were added to each of the spray solutions. The designated plants were sprayed with solutions of Mo1 and Mo2 (200 ml/pot) at the same time of spraying with the two SA solutions (ear emergence). The control plants were sprayed with distilled water (200 ml/pot).

The experimental setup was five treatments (Control, SA1, SA2, Mo1, and Mo2) with three replicates for two varieties arranged in completely randomized design.

4.3.3. Measurements of harvest characteristics of plants:

Once the wheat plants had begun to senesce and were showing signs of yellow, the irrigation was terminated to let the pots dry and the plants to ripen naturally. Two weeks from ending the watering, the plants were ripe for harvest and all the plants were pulled and the roots cut off and discarded. The harvested plants were collected in paper storage bags and transferred to the laboratory for measurement of the following parameters:

Spikes

Each pot was dealt with as one unit (i.e. the plants in a pot were pooled). Spikes were cut from the stems of plants, counted and kept in a separate paper storage bag, one per pot. Then, spike samples were dried in the oven at 80^{o}C for two days until constant weight. The spikes were then weighed (Sartorius balance, model I8400P NO 35039, UK.).

Stems

The numbers of stems were counted (stems/pot) (NB. Not all stems bore a spike). Stems and leaves from plants for each pot were placed in aluminium foil dishes, and dried in a drying oven (Gallenkamp Economy Incubator size two, model IH-150, England) at 80^{o}C for 2 days to dry until constant weight (Singh and Usha, 2003). Then, the dry weight was recorded (Precisa balances, Swiss quality, model 400M NO 13909, Switzerland).

Grain

Total grain dry weight was determined by threshing the spikes so as to separate the grains from the chaff, and then the number of grains for each spike was counted. The grain from all spikes were then pooled and the weight recorded (Sartorius balance, model I8400P NO 35039 UK). From these measurements, the average 1000-grain dry weight per pot, the grain yield per pot and the average grains per spike were obtained (Forno, 1972).

Statistical analysis

The data were checked for normality using Minitab Basic statistics to confirm that the data were normally distributed and met the homogeneity requirements for Analysis of Variance (ANOVA) (Appendix 10 and 11). The data were analysed by Balanced ANOVA using statistical program Minitab v.17. Rather than a combined ANOVA with complex interaction terms, separate ANOVAs were carried out relative to two of the main effects at a time (See Appendices 12-58). Fishers Least Significant Difference (LSD) at the level $p < 0.05$ was applied to discern the significance of differences between means where:

LSD = t x SQRT ((2 x MSE)/N).

Where; t is the t-statistic obtained from error DF from the ANOVA and used in the t-distribution table with a required level of significance (two-tailed) at 5% level). MSE is the value under the mean squares error term in the ANOVA table.

LSD is regularly used as discriminator statistic for agricultural and horticultural experiments (Fisher 1935, Snedecor and Cochran 1967). In Figures, vertical error bars are one standard error of the mean (SE).

4.4. Results

Table 4.2: Summary table of significant and non-significant effects on leaf stomatal conductance measurement (See Appendixes: 12, 13, 15, and 16).

Parameter/ Leaf stomatal conductance	*P* value	LSD
Water conditions * variety		
water conditions	< 0.001	0.039
variety	< 0.001	0.039
water condition * variety	< 0.001	0.055
Chemical treatments of SA and Mo		
treatments	< 0.001	0.066
Chemical treatments * variety		
variety	< 0.001	0.047
treatments	0.001	0.075
treatments * variety	0.270	
Chemical treatments * water conditions		
water conditions	< 0.001	0.043
treatments	< 0.001	0.068
treatments * water condition	0.044	0.097

The effect of water conditions (well watered and drought) on leaf stomatal conductance of varieties Tamooz 2 and Adana 99:

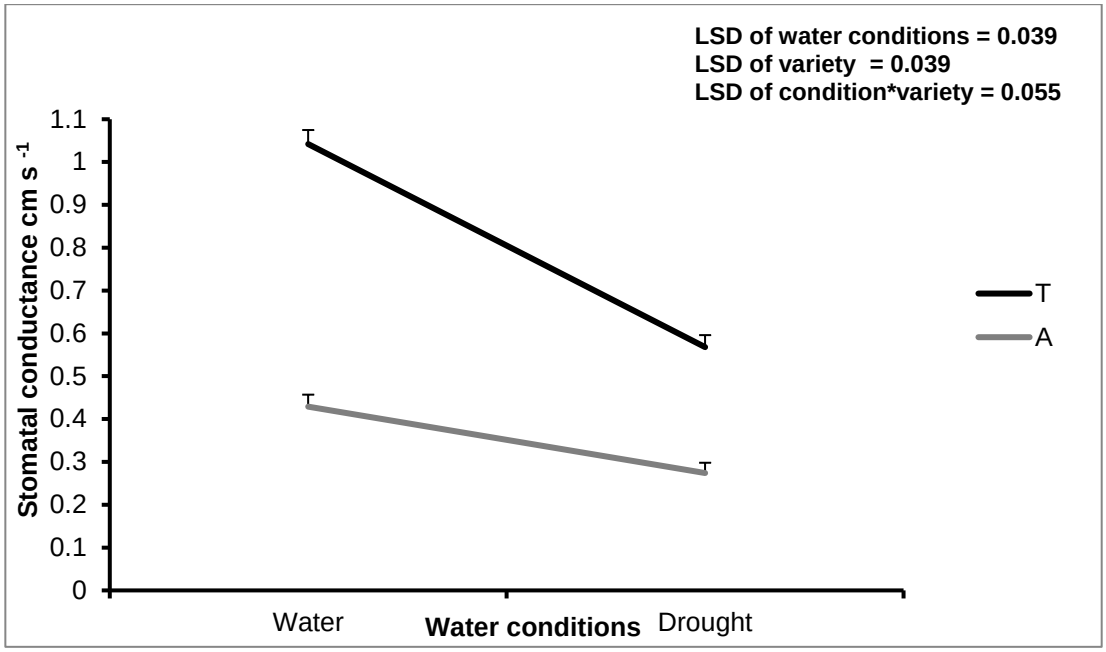


Figure 4.2: Interaction graph to show the effect of water conditions on leaf stomatal conductance of wheat varieties T = Tamooz 2 and A = Adana 99 ($p \leq 0.001$ for water conditions. $p \leq 0.001$ for variety. $p \leq 0.001$ for water conditions * variety, error bars = SE).

A significant effect ($p \leq 0.001$) for the leaf stomatal conductance was observed between drought stress and well-watered conditions. The effect of variety on stomatal conductance was also significantly different ($p \leq 0.001$). The interaction showed a significant difference between watering conditions and variety ($p \leq 0.001$). The most significant decrease in leaf stomatal conductance was noticed for variety Tamooz 2 (0.32) as compared with Adana 99 (0.16) under drought stress (Figure 4.2) (Table 4.2 and Appendix 12).

The effect of chemical treatments (SA and Mo) on leaf stomatal conductance of wheat plants altogether:

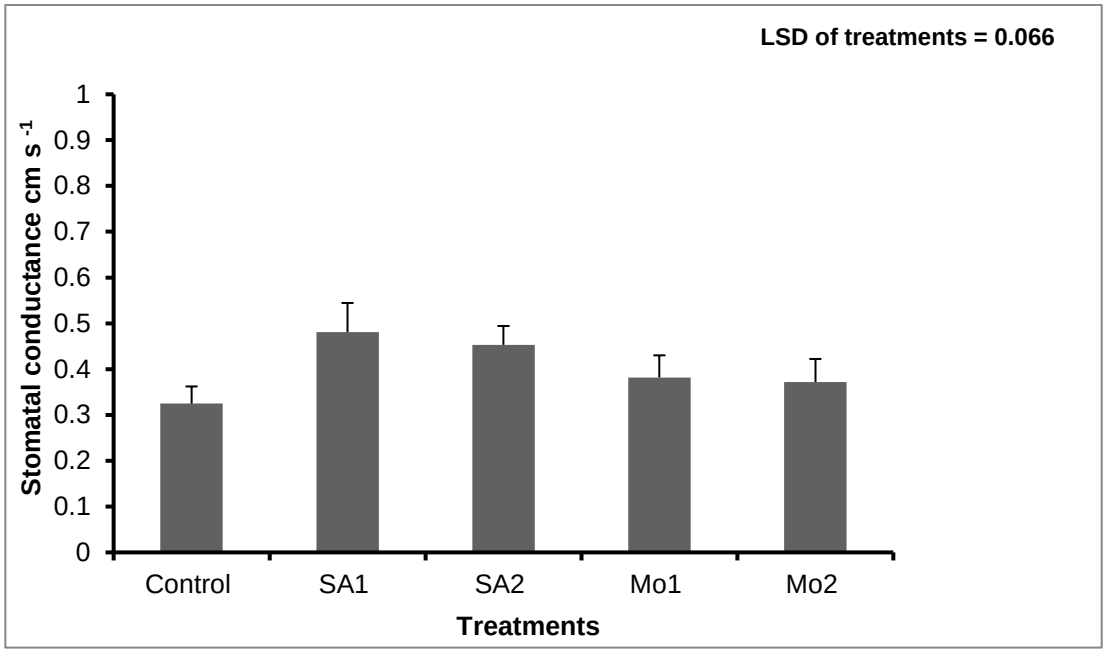


Figure 4.3: Overall effect of spraying plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on leaf stomatal conductance. ($p \leq 0.001$ for chemical treatments, error bars = SE).

The chemical treatments of SA1 and SA2 significantly improved the leaf stomatal conductance compared to the control plants ($p \leq 0.001$). Spraying the pots with the treatments of Mo1 and Mo2 was not significantly different from the control (Figure 4.3) (Table 4.2 and Appendix 13).

The effect of chemical treatments (SA and Mo) on leaf stomatal conductance of varieties Tamooz 2 and Adana 99.

Variety Tamooz 2 was significantly different in leaf stomatal conductance as compared to Adana 99 ($p \leq 0.001$). There was a significant effect for the chemical treatments on leaf stomatal conductance (p = 0.001) (Table 4.2). A non-significant interaction was observed between variety and chemical treatments (p = 0.270) see (Appendixes 14 and 15).

The effect of chemical treatments (SA and Mo) on leaf stomatal conductance of wheat plants under well watered and drought conditions:

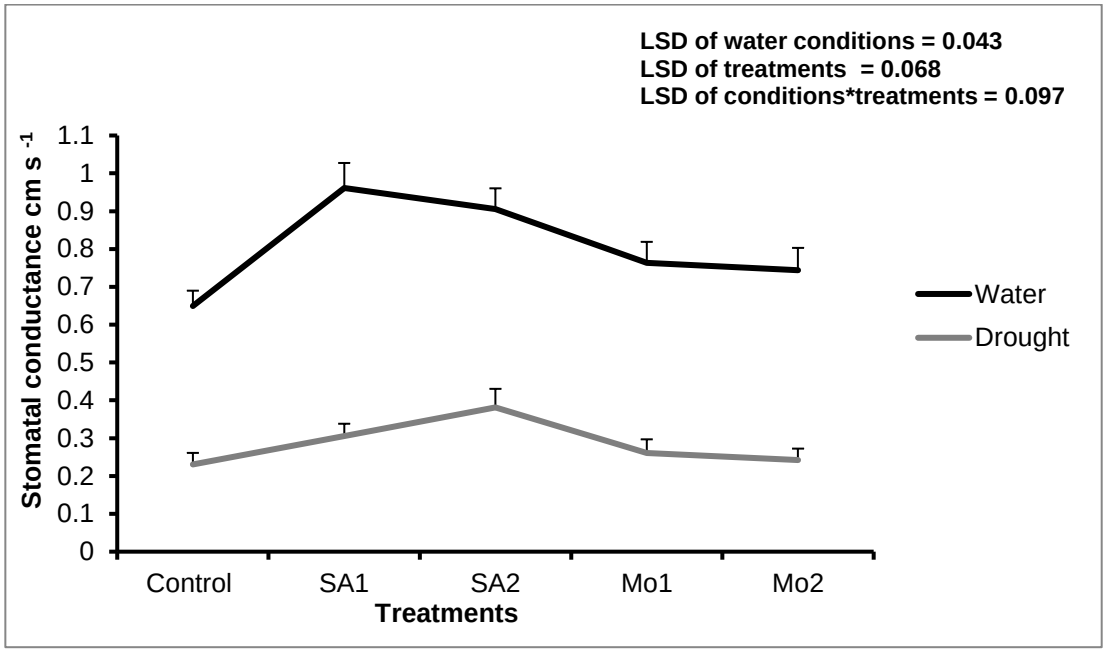


Figure 4.4: Interaction graph to show the effect of chemical treatments SA and Mo on leaf stomatal conductance of wheat varieties Tamooz 2 and Adana 99 treated with water conditions well-watered and drought ($p \leq 0.001$ for water condition. $p \leq 0.001$ for treatments. p = 0.044 for water conditions * treatments, error bars = SE).

There was significant interaction between the water conditions and chemical spray treatments (p = 0.044). The leaf stomatal conductance was significantly decreased by drought stress for all treatments. Under drought stress SA2 significantly improved stomatal conductance compared to the control but not to the levels for SA1 under well-watered conditions (Figure 4.4) (Table 4.2 and Appendix 16).

Table 4.3: Summary table of significant and non-significant effects on number of stems per pot measurement (See Appendixes: 18, 19, 21 and 23).

Parameter/ number of stems/pot	*P* value	LSD
Water conditions * variety		
water conditions	< 0.001	1.025
variety	< 0.001	1.025
water condition * variety	0.398	
Chemical treatments of SA and Mo		
treatments	0.001	1.439
Chemical treatments * variety		
variety	< 0.001	1.033
treatments	0.006	1.634
treatments * variety	0.701	
Chemical treatments * water conditions		
water conditions	< 0.001	0.955
treatments	0.003	1.510
treatments * water condition	0.413	

The effect of water conditions (well watered and drought) on number of stems per pot of varieties Tamooz 2 and Adana 99:

There was no significant interaction between water conditions and variety in the number of stems/pot (p = 0.398). Water conditions had a significant effect on stem number/pot ($p \leq 0.001$) (Table 4.3). The number of stems/pot for cv. Tamooz 2 was significantly different in comparison to cv. Adana 99 ($p \leq 0.001$) see (Appendixes 17 and 18).

The effect of chemical treatments (SA and Mo) on number of stems per pot of wheat plants altogether:

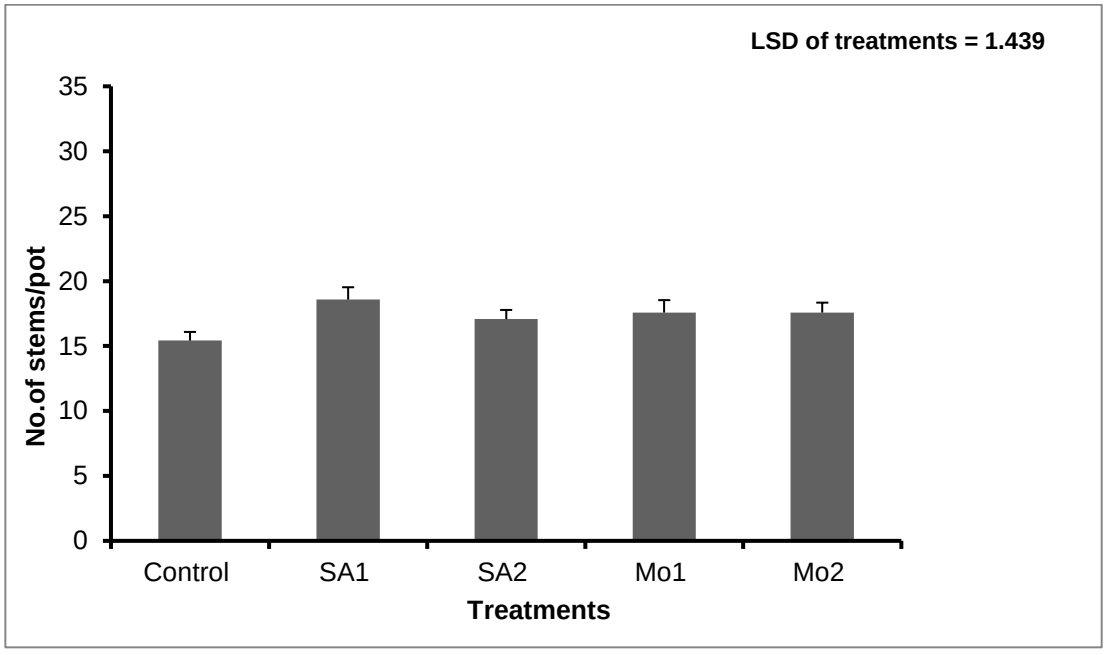


Figure 4.5: Overall effect of spraying plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on number of stems. (p = 0.001 for chemical treatments, error bars = SE).

Spraying the plants with chemical treatments had no significant effect on the number of stems per pot, and only the SA1 treatment was statistically significant compared to the unsprayed the control plants (p = 0.001) (Figure 4.5) (Table 4.3 and Appendix 19).

The effect of chemical treatments (SA and Mo) on number of stems per pot of varieties Tamooz 2 and Adana 99.

A significant difference in the number of stems/pot value observed for cv. Tamooz 2 compared to Adana 99 ($p \leq 0.001$). Chemical treatments had a significant effect on number of stems (p = 0.006) (Table 4.3). There was no significant interaction between variety and chemical treatments in stem number/pot (p = 0.701) see (Appendixes 20 and 21).

The effect of chemical treatments (SA and Mo) on number of stems per pot of wheat plants under well watered and drought conditions:

There was a significant effect on value of the number of stems/pot for well-watered as compared to drought stress conditions ($p \leq 0.001$). Chemical treatments had a significant effect on the stem number/pot (p = 0.003) (Table 4.3). There was no significant interaction between water conditions and chemical treatments in number of stems/pot (p = 0.413) see (Appendixes 22 and 23).

Table 4.4: Summary table of significant and non-significant effects on shoot dry weight per pot measurement (See Appendixes: 24, 25, 27 and 29).

Parameter/ shoot dry weight /pot	*P* value	LSD
Water conditions * variety		
water conditions	< 0.001	1.158
variety	< 0.001	1.158
water condition * variety	0.020	1.638
Chemical treatments of SA and Mo		
treatments	0.001	1.802
Chemical treatments * variety		
variety	< 0.001	1.239
treatments	0.003	1.959
treatments * variety	0.962	
Chemical treatments * water conditions		
water conditions	< 0.001	1.163
treatments	0.001	1.839
treatments * water condition	0.534	

The effect of water conditions (well watered and drought) on shoot dry weight per pot of varieties Tamooz 2 and Adana 99:

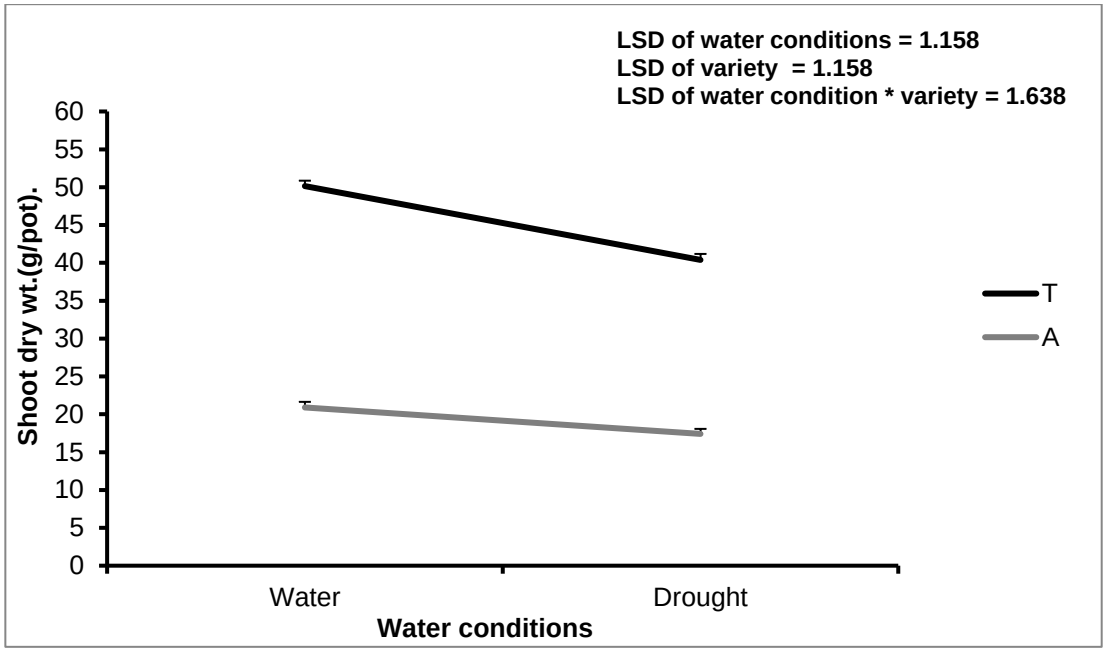


Figure 4.6: Interaction graph to show the effect of water conditions on shoot dry weight of wheat varieties T = Tamooz 2 and A = Adana 99 ($p \leq 0.001$ for water conditions. $p \leq 0.001$ for variety. $P = 0.020$ for water conditions * variety, error bars = SE).

Drought stress significantly ($p \leq 0.001$) reduced the shoot dry weight per pot. The effect of variety on shoot dry weight/pot was also significantly different ($p \leq 0.001$). The most significant difference in shoot dry weight observed for variety Tamooz 2 compared to Adana 99 under well-watered condition. The interaction showed a significant interaction between watering conditions and variety ($p = 0.020$) with Tamooz being affected more by drought stress (Figure 4.6) (Table 4.4 and Appendix 24).

The effect of chemical treatments (SA and Mo) on shoot dry weight per pot of wheat plants altogether:

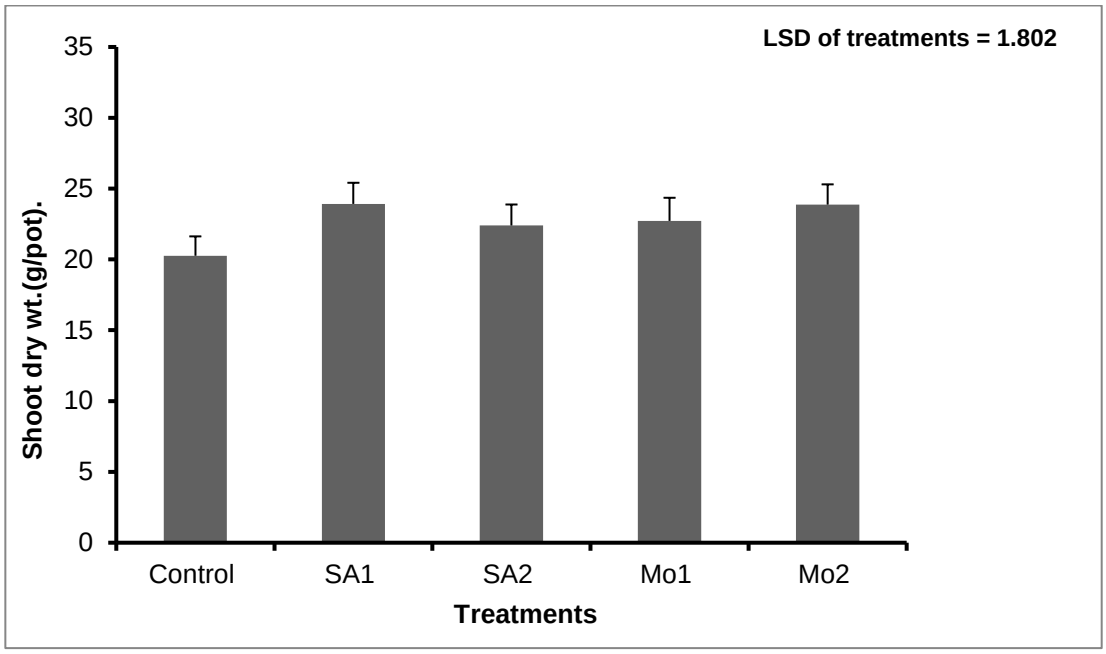


Figure 4.7: Overall effect of spraying plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on shoot dry weight. (*p* = 0.001 for chemical treatments, error bars = SE).

All chemical sprays significantly increased the shoot dry weight in comparison with non-treated control plants (*p* = 0.001). There was no significant effect observed between all the chemical treatments (Figure 4.7) (Table 4.4 and Appendix 25).

The effect of chemical treatments (SA and Mo) on shoot dry weight per pot of varieties Tamooz 2 and Adana 99.

Cultivar Tamooz 2 was significantly different in shoot dry weight/pot compared to cv. Adana 99 ($p \leq 0.001$). There was a significant effect for chemical treatments on shoot dry weight ($p = 0.003$) (Table 4.4). There was no significant interaction between variety and chemical treatments in shoot dry weight /pot ($p = 0.962$) see (Appendixes 26 and 27).

The effect of chemical treatments (SA and Mo) on shoot dry weight per pot of wheat plants under well watered and drought conditions:

A significant effect was observed in the shoot dry weight/pot for well-watered as compared to drought stress conditions ($p \leq 0.001$). Chemical treatments had a significant effect on the shoot dry weight/pot ($p = 0.001$) (Table 4.4). A non-significant Interaction was also observed between water conditions and chemical treatments on shoot dry weight/pot ($p = 0.534$) see (Appendixes 28 and 29).

Table 4.5: Summary table of significant and non-significant effects on number of spikes per pot measurement (See Appendixes: 31, 32, 34 and 36).

Parameter/ number of spikes per /pot	*P* value	LSD
Water conditions * variety		
water conditions	< 0.001	0.921
variety	< 0.001	0.921
water condition * variety	0.562	
Chemical treatments of SA and Mo		
treatments	0.001	1.528
Chemical treatments * variety		
variety	< 0.001	0.982
treatments	0.001	1.553
treatments * variety	0.959	
Chemical treatments * water conditions		
water conditions	< 0.001	0.961
treatments	0.001	1.520
treatments * water condition	0.697	

The effect of water conditions (well watered and drought) on number of spikes per pot of varieties Tamooz 2 and Adana 99:

The drought stress condition had a significant effect on the number of spikes/pot compared to well-watered condition ($p \leq 0.001$). A significant difference was noticed for variety on spikes number/pot ($p \leq 0.001$) (Table 4.5). Spike number/pot for cv. Tamooz 2 was significantly different from cv. Adana 99. There was no significant interaction between water conditions and variety in the number of spikes/pot ($p = 0.562$) see (Appendixes 30 and 31).

The effect of chemical treatments (SA and Mo) on number of spikes per pot of wheat plants altogether:

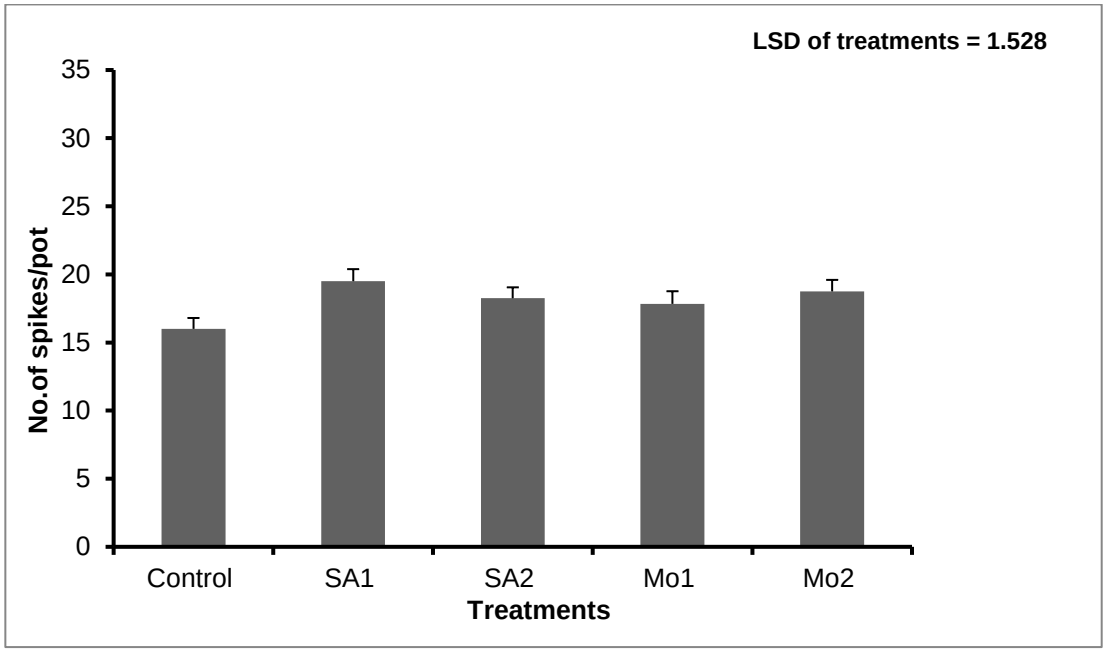


Figure 4.8: Overall effect of spraying plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on number of spikes. ($p = 0.001$ for chemical treatments, error bars = SE).

Spraying the pots with chemical treatments (SA and Mo) improved the spike number/pot compared with non-sprayed control plants. The chemical treatments effect on the spikes number/pot of wheat was non-significant when comparing to each other (p = 0.001) (Figure 4.8) (Table 4.5 and Appendix 32).

The effect of chemical treatments (SA and Mo) on number of spikes per pot of varieties Tamooz 2 and Adana 99.

A significant effect was observed on the number of spikes/pot for cv. Tamooz 2 when compared to cv. Adana 99 ($p \leq 0.001$). Chemical treatments also had a significant effect on spike number (p = 0.001) (Table 4.5). A non-significant interaction was observed between variety and chemical treatments on number of spikes/pot (p = 0.959) see (Appendixes 33 and 34).

The effect of chemical treatments (SA and Mo) on number of spikes per pot of wheat plants under well watered and drought conditions:

A significant effect was observed for well-watered compared to drought stress conditions on the number of spikes/pot ($p \leq 0.001$). Chemical treatments had a significant effect on spike number/pot (p = 0.001) (Table 4.5). A non-significant Interaction was observed between water conditions and chemical treatments on number of spikes/pot (p = 0.697) see (Appendixes 35 and 36).

Table 4.6: Summary table of significant and non-significant effects on spikes dry weight per pot measurement (See Appendixes: 38, 39, 41 and 43).

Parameter/ spikes dry weight /pot	*P* value	LSD
Water conditions * variety		
water conditions	< 0.001	1.976
variety	< 0.001	1.976
water condition * variety	0.622	
Chemical treatments of SA and Mo		
treatments	< 0.001	2.686
Chemical treatments * variety		
variety	< 0.001	2.011
treatments	0.002	3.180
treatments * variety	0.845	
Chemical treatments * water conditions		
water conditions	< 0.001	1.745
treatments	< 0.001	2.759
treatments * water condition	0.970	

The effect of water conditions (well watered and drought) on spikes dry weight of varieties Tamooz 2 and Adana 99:

A significant effect was observed for the watering conditions on spike dry weight/pot ($p \leq 0.001$). Cultivar also had a significant effect on the spike dry weight /pot ($p \leq 0.001$) (Table 4.6). A non-significant interaction was observed between water conditions and variety on the spikes dry weight/pot ($p = 0.622$) see (Appendixes 37 and 38).

The effect of chemical treatments (SA and Mo) on spikes dry weight per pot of wheat plants altogether:

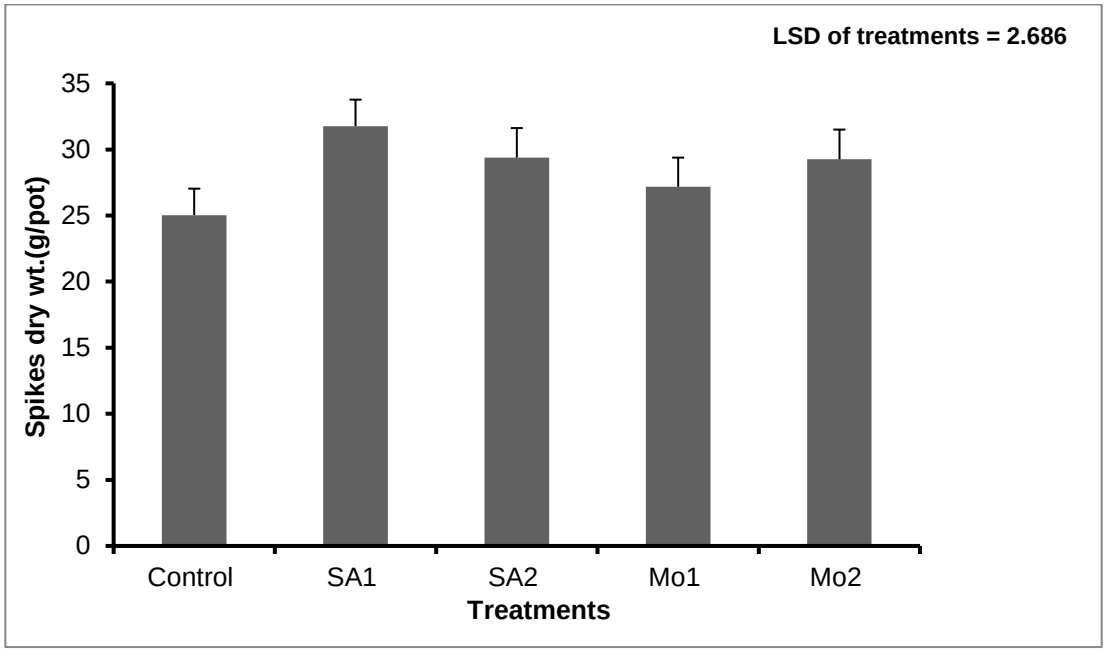


Figure 4.9: Overall effect of spraying plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on spikes dry weight. ($p \leq 0.001$ for chemical treatments, error bars = SE).

Spraying the wheat plants with the chemical treatments SA1, SA2 and Mo2 were significantly different except the Mo1 treatment had a non-significant effect on the spikes dry weight/pot as compared to the non-sprayed control plants ($p \leq 0.001$) (Figure 4.9) (Table 4.6 and Appendix 39).

The effect of chemical treatments (SA and Mo) on spikes dry weight per pot of varieties Tamooz 2 and Adana 99.

Variety had a significant effect on the spikes dry weight/pot ($p \leq 0.001$). A significant effect was observed for the chemical treatments on spike dry weight ($p = 0.002$) (Table 4.6). There was no significant interaction between variety and chemical treatments in spike dry weight/pot ($p = 0.845$) see (Appendixes 40 and 41).

The effect of chemical treatments (SA and Mo) on spikes dry weight per pot of wheat plants under well watered and drought conditions:

Well-watering had a significant improved effect on spike dry weight/pot as compared to drought stress conditions ($p \leq 0.001$). The chemical treatments effect on spikes dry weight/pot was significantly different ($p \leq 0.001$) (Table 4.6). A non-significant Interaction was observed between water conditions and chemical treatments on spikes dry weight/pot ($p = 0.970$) see (Appendixes 42 and 43).

Table 4.7: Summary table of significant and non-significant effects on grain dry weight per pot measurement (See Appendixes: 45, 46, 48 and 50).

Parameter/ grain dry weight /pot	*P* value	LSD
Water conditions * variety		
water conditions	< 0.001	1.401
variety	< 0.001	1.401
water condition * variety	0.775	
Chemical treatments of SA and Mo		
treatments	0.002	2.337
Chemical treatments * variety		
variety	< 0.001	1.429
treatments	0.001	2.259
treatments * variety	0.447	
Chemical treatments * water conditions		
water conditions	< 0.001	1.516
treatments	0.002	2.397
treatments * water condition	0.999	

The effect of water conditions (well watered and drought) on grain dry weight of varieties Tamooz 2 and Adana 99:

Drought stress had a significant negative effect on grain dry weight compared to the well-watered treatment ($p \leq 0.001$). Variety also had a significant effect on grain dry weight ($p \leq 0.001$). Percentage grain yield reduction for variety Tamooz 2 was 12.43% and for Adana 99 was 19.29% (Table 4.7). There was no significant interaction between watering conditions and variety ($p = 0.775$) see (Appendixes 44 and 45).

The effect of chemical treatments (SA and Mo) on grain dry weight of wheat plants altogether:

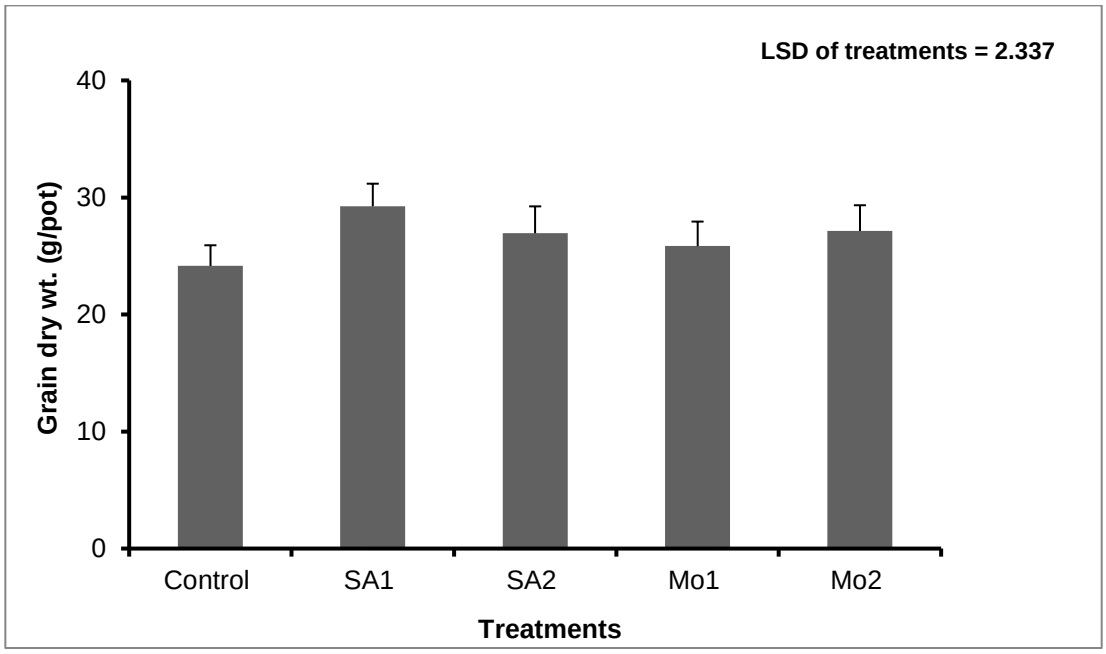


Figure 4.10: Overall effect of spraying plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on grain dry weight. (p = 0.002 for chemical treatments, error bars = SE).

Overall the chemical treatments SA1, SA2 and Mo2 had significant positive effects on grain dry weight, but spraying with the Mo1 treatment was not significant as compared to the control plants ($p = 0.002$) (Figure 4.10) (Table 4.7 and Appendix 46).

The effect of chemical treatments (SA and Mo) on grain dry weight of varieties Tamooz 2 and Adana 99.

A significant effect was observed between cv. Tamooz 2 and cv. Adana 99 in the grain dry weight/pot ($p \leq 0.001$). The chemical treatments also had a significant effect on the grain dry weight ($p = 0.001$) (Table 4.7). A non-significant interaction was observed between variety and chemical treatments on grain dry weight/pot ($p = 0.447$) see (Appendixes 47 and 48).

The effect of chemical treatments (SA and Mo) on grain dry weight of wheat plants under well watered and drought conditions:

There was a significant effect on the grain dry weight/pot value for the well-watered as compared to drought stress conditions ($p \leq 0.001$). The chemical treatments had a significant effect on the grain dry weight/pot ($p = 0.002$) (Table 4.7). There was no significant interaction between water conditions and chemical treatments in grain dry weight/pot ($p = 0.999$) see (Appendixes 49 and 50).

Table 4.8: Summary table of significant and non-significant effects on average 1000 grain dry weight per pot measurement (See Appendixes: 52, 54, 56 and 58).

Parameter/ average 1000 grain dry wt./pot	*P* value	LSD
Water conditions * variety		
water conditions	0.092	
variety	0.260	
water condition * variety	0.900	
Chemical treatments of SA and Mo		
treatments	0.815	
Chemical treatments * variety		
variety	0.267	
treatments	0.842	
treatments * variety	0.989	
Chemical treatments * water conditions		
water conditions	0.092	
treatments	0.834	
treatments * water condition	0.936	

The effect of water conditions (well watered and drought) on average 1000 grain dry weight of varieties Tamooz 2 and Adana 99:

Water conditions had no significant effect on the average 1000 grain dry weight (p = 0.092). There was no significant effect for the variety on the average 1000 grain dry weight/pot (p = 0.260) (Table 4.8). A non-significant interaction was observed between water conditions and variety (p = 0.900) see (Appendixes 51 and 52).

The effect of chemical treatments (SA and Mo) on average 1000 grain dry weight of wheat plants altogether:

Also, the chemical treatments spray of (SA and Mo) had no significant effect on average 1000 grain dry weight values of wheat plants altogether as compared to control plants and also when compared to each other (p = 0.815) (Table 4.8) see (Appendixes 53 and 54).

The effect of chemical treatments (SA and Mo) on average 1000 grain dry weight of varieties Tamooz 2 and Adana 99.

Variety had no significant effect on the average 1000 grain dry weight (p = 0.267). Also, chemical treatments had no significant effect on the average 1000 grain dry weight/pot (p = 0.842) (Table 4.8). A non-significant interaction was observed between variety and chemical treatments (p = 0.989) see (Appendixes 55 and 56).

The effect of chemical treatments (SA and Mo) on average 1000 grain dry weight of wheat plants under well watered and drought conditions:

Watering conditions had no significant effect on average 1000 grain dry weight (p = 0.092). Also, there was no significant effect for chemical treatments on the average 1000 grain dry weight/pot (p = 0.834) (Table 4.8). The interaction between water conditions and chemical treatments was not significantly different (p = 0.936) see (Appendixes 57 and 58).

4.5. Discussion

There was a significant decrease effect in leaf stomatal conductance for Iraqi wheat (*Triticum aestivum* L.) variety Tamooz 2 compared with Adana 99 under drought stress conditions ($p \leq 0.001$). Spraying the plants with the treatment SA1 (1.44) mM significantly increased the leaf stomatal conductance of well-irrigated plants in comparison to the drought stress plants sprayed with SA2 (2.88) mM (Figures 4.2 and 4.4).

The results in this study are in support of those reported by Waseem *et al*. (2006) where it was observed that exogenously applying 5 or 10 mg L^{-1} SA to the rooting medium had a significant effect on increasing leaf stomatal conductance and CO_2 assimilation rate in wheat cultivars under non-stress conditions. Even though the cultivar S-24 showed a slight improvement in the conductance of stomata and cultivar MH-97 in the assimilation rate of CO_2 under drought stress conditions with 5 mg L^{-1} SA, application of salicylic acid did not mitigate the adverse effects which were caused by water deficit stress. Further, it seems these researchers supplied plants with SA through rooting medium which is different from the present experiment where a foliar application was applied. In previous studies, similar responses were reported from applying of SA and its analogues acetylsalicylic acid (ASA) on corn and soybean plants, where stomatal conductance and photosynthetic rates were effectively enhanced in all plants under greenhouse conditions (Khan *et al*., 2003). Further, studies carried out on soybean plants (*Glycine max* L. Merrill) (Kumar *et al*., 2000) and mustard plants (*Brassica juncea*) (Fariduddin *et al*., 2003), with foliar application of SA enhanced the net photosynthetic rate, internal CO_2 concentration, stomatal conductance

and transpiration rate. The results of a study Eraslan *et al*. (2007) revealed that even though there were significant effects of SA on the increase of stomatal resistance in carrot plants (*Daucus carota* L. cv. Nantes) subjected to salinity stress compared to the control plants, the treatment (SA 0.5 mM) was not effective in alleviating salinity stress negative effects of NaCl and Na_2SO_4 combined with Boron toxicity.

The results of other papers reveal that externally applied SA to plants can induce plant tolerance to several abiotic stresses including salt stress (Shakirova *et al*., 2003), drought stress in wheat (Singh and Usha, 2003), cold stress in maize (Janda *et al*., 1999) and Ultraviolet-B (UV-B) radiation stress (Ervin *et al*., 2004) in *Kentucky bluegrass*. Regarding Mo effects on tolerance characteristics of stress, a study by Sun *et al*. (2005) has shown that Mo application on winter wheat plants under low temperature stress led to significant increases in the photosynthetic rate and decreased stomatal conductance and water loss by transpiration. The inducible role of SA in plant tolerance against stresses may have been due to its contributing to various physiological processes like a reduction in transpiration rate by stomatal closure and an enhancement of photosynthesis through the increase of intercellular CO_2 concentration and nutrient uptake processes for example in spring wheat plants under salinity stress (Arfan *et al*., 2007). The efficiency of SA in these processes is determined by the influences of environmental factors including water stress in addition to some physical factors to which the plants may have been exposed for example light or/and temperature stress (Patel and Golakiya, 1988, Janda *et al*., 2007). The effectiveness of SA in ameliorating the adverse effect of stress is not the same for all plants across all stress situations but is dependent on the internal factors

such as those related to the type of plant species and physiological state of the plant.

The stem number/pot for cv. Tamooz 2 was decreased significantly in comparison to cv. Adana 99 whilst the treatment SA1 had a significant effect on increasing the number of stems/pot as compared to the non-treated control plants (Appendix 17 and Figure 4.5). However, Al-Temimi *et al*. (2013) in their study on wheat varieties noticed a significant reduction in stems number due to the water stress influence. The results recorded here showed that the number of stems was more for drought tolerant cultivar Tamooz 2 than the non-tolerant cultivar Adana 99.

Drought stress decreased the dry mass of shoots for variety Tamooz 2 compared to Adana 99 under well-watered condition indicating the significant interaction between variety and water conditions (p = 0.020). All the spray chemical treatments gave a significant increase in shoot dry weight when compared to control plants (p = 0.001) (Figures 4.6 and 4.7).

The results of Khan *et al*. (2003) revealed that applied SA and related compound ASA to corn and soybean plants caused significant increase of the plant dry mass. Waseem *et al*. (2006) also revealed that wheat cultivar MH-97 responded positively to supplying the rooting medium with SA 5 mg L-1 by increasing the shoot dry mass more than S-24 cultivar under drought stress conditions, while the same significant increase of shoot dry weight was observed for both wheat cultivars under control conditions. A significant stimulation in shoot size was observed when wheat seedlings were treated with lower concentration of salicylic acid, thereby enhancing the shoot dry mass of plants treated with SA as compared to control plants (Shakirova, 2007). However, this experiment is

different in the way of SA application because SA use was by the pre-treatment of seeds with SA 0.05 mM along with seedling spray.

An enhanced growth of the shoot system resulted when plants of *Tagetes erecta* were treated with lower concentrations of salicylic acid (Sandoval Yepiz, 2004). Moreover, in a pot experiment carried out by Hussein *et al*. (2007), treatment of wheat plants with the rate of 200 ppm SA improved growth characteristic of shoot dry weight when the plants sprayed with salicylic acid and irrigated with tap water. A decrease in vegetative growth parameters was detected by increasing salt concentrations in the irrigation water. Farooq *et al*. (2009a) applied SA at concentration 100 mg L^{-1} through seed soaking and foliar spraying to rice plants (*Oryza sativa* L.) and revealed that it did alleviate the negative effect of water deficit (50% FC) not affect the rate of photosynthesis and seedling dry weight as compared with plants under well-watered conditions. Yu *et al*. (1999) evaluated the influence of Mo application at the seedling stage on increasing shoot dry matter for most winter wheat cultivars, but to meet Mo requirement for whole growing period for plants, treating seed with Mo was conducted with foliar spraying of Mo in order to provide optimum growth for the plants during their life cycle. Our results are contrary with those reported by Singh and Usha (2003) who presented that treatments of SA 1 and 2 mM did not affect wheat dry biomass of plants under a water sufficient regime. Inversely, dry weight of plants treated with SA were significantly higher than plants without SA application under drought stress and dry biomass for plants treated with application SA 3 mM under water stress was improved as compared to drought control without SA. Moreover, Idrees *et al*. (2010) pointed out the positive effect of salicylic acid on the growth of shoots in varieties Neema and Krishna of lemongrass plants (*Cymbopogon*

flexuosus Steud. Wats) in ameliorating effects of water stress conditions of 75% and 50% FC. Furthermore foliar spray treatments of the plants with a SA concentration 10^{-5} M showed significantly higher values for shoot dry weight than unsprayed SA plants. The results in this observation showed higher reduction of shoot dry weight for var. Krishna than var. Neema as compared to control plants and so Neema variety was identified as more tolerant than Krishna on the basis of the adaptation of variety response to the stress condition and also on the oil yield.

In a hydroponic study it was shown that application of Mo treatment resulted in significant increase in the average of shoot dry weight at 12 days of the exposure cultivars winter wheat plants *Triticum aestivum* to drought stress. The finding exhibited the role of Mo in the adjustment of the osmotic system to protect wheat against stress (Wu *et al*., 2014). It is observed that the effect of SA may be attributed to its inducible effect on enzyme activity of photosynthetic processes namely Rubisco and Phosphoenolpyruvate (PEP) carboxylase. Accordingly, SA-treated plants may be responsible for the improved dry matter in wheat seedling under drought stress by maintaining the stability of membranes (Rajasekaran and Blake, 1999, Singh and Usha, 2003). Otherwise, the decrease in dry mass of this may be attributed to a reduced protection by the adverse effect of drought stress on water stressed plants treated with SA treatments.

According to the current results, a significant effect of variety on yield characteristics was observed in cv. Tamooz 2 compared to cv. Adana 99. The number of spikes/pot was significantly increased with application of the chemical treatments of (SA and Mo) when compared with non-sprayed control plants (Appendix 30 and Figure 4.8). Cultivar Tamooz 2 was significantly different

compared to cv. Adana 99 in the spike dry weight/pot. The applied treatments of SA1, SA2, and Mo2 significantly increased the spike dry weight/pot as comparing to the control plants (Appendix 37 and Figure 4.9).

In support of the above results, studies of (Klessig and Malamy, 1994, Hayat et al., 2007, Hayat et al., 2010) revealed that salicylic acid application enhanced the activities of various physiological and biochemical characteristics such as photosynthetic reactions, flowering and plant development and it may also result in inducing fruit yield and productivity in plants. These studies are in agreement with evidence obtained on treating African violet plants (*Saintpaulia ionantha* Wendl.) with low concentration of SA 0.1 mM. The treatment caused enhanced numbers of flowers per plant and early flowering when grown under greenhouse conditions in comparison with the control plants (Martin-Mex *et al*., 2005). Fariduddin *et al*. (2003) also reported an increase in the number of pods and the seed yield of mustard plant species (*Brassica juncea* Czern and Coss cv. Varuna). The results demonstrated that spraying plants with the lowest SA concentration 10^{-5} M was better than the control plants and also those plants which sprayed with higher concentrations of SA 10^{-4} or 10^{-3} M. The results of the existing investigations are contradictory to some reviews in which it has been demonstrated that exogenous application of SA promotes crop production and counteracts the yield components inhibition caused by abiotic stresses, including drought stress, in wheat plants (Abdelkader *et al*., 2012) and maize plants (Zamaninejad *et al*., 2013). The determined effects of drought stress and genotype variation in drought tolerance could be related with pre-treatment of the plants with SA resulting in the accumulation of ABA which might have contributed to providing pre-adaptation of seedlings under abiotic stress. And then by

exogenous applications of SA affecting flowering as a result of the interaction with other plant regulators e.g. gibberellins, affect cell division rate in root apical meristem of plants by raising levels of IAA and cytokinines in stressed wheat plants and thereby increased growth and productivity of plants (Popova *et al*., 1997, Sakhabutdinova *et al*., 2003, Lata *et al*., 2015). All this evidence indicates the importance of SA in activating other pathways of plant defence responses to survival under stress and provides protection to the stressed plants.

In contrast in a hydroponic study carried out by Wu *et al*. (2014), it was shown that drought stress decreased spikes number and grain yield of winter wheat (*Triticum aestivum*) whether Mo was applied or not. In contrast, in their results about Mo application on stressed plants drought tolerance was significantly enhanced in both wheat cultivars. Ghafarian *et al*. (2013) have also provided evidence of Mo effects on improving the performance of wheat plants when sprayed with concentration of Mo 1% by increasing the yield measurements like the number of spikes of water stressed plants as compared to non-stress plants thereby providing tolerance against the damaging effects of drought stress.

Total grain dry weight of variety plants Tamooz 2 was significantly higher from Adana 99 confirming that it is a higher yielding variety. Furthermore, the spray treatments with SA1, SA2 and Mo2 improved the dry grain weight as compared to non-sprayed the control plants (Appendix 44 and Figure 4.10). Regarding average 1000 grain dry weight there were no significant interaction effects (water conditions and variety, chemical treatments, variety and chemical treatments and also water conditions and chemical treatments) for both plants Tamooz 2 and Adana 99 (Appendixes 51, 53, 55 and 57) indicating that this yield component was stable for these varieties of wheat.

Salicylic acid has the regulatory role in the physiological process of fertilisation process which results in increase the grain dry weight significantly during the flowering. Evidence from a study by Grown (2012) showed that productivity of sunflower plants exposed to drought stress was significantly lower than the non-treated plants. Differing with the existing results of grains yield in current study, a significant improvement was observed in the seed weight/head for water stressed plants sprayed with 75 and 100 mg L^{-1} of SA as compared with fully irrigated plants. Similarly, research by Azimi *et al*. (2013) showed that the damaging effects of moisture stress were alleviated by exogenous application of SA in wheat seedlings and resulted in raising the values of grain dry weight/plant. It was further reported by Zamaninejad et al. (2013) that the application of maize plants (*Zea mays* L.) with salicylic acid (1 mM) modified the negative effects of water stress on grain yield by increasing grain number/spike significantly. The Mo effect in the present results is dissimilar to findings by other researchers. Their findings revealed that the decrease of grain yields under drought was lower in Mo-treated plants (applied by pre-treatment seed/foliar spray) than untreated plants (Yu *et al*., 1999). Likewise, other researchers have shown that Mo treatment enhances stress tolerance (Al-Issawi *et al*., 2013) where the effect of applying Mo through seed soaking/foliar spray was correlated to the tolerance of both spring and winter wheat genotypes plants under normal and low temperature. Additionally, a comprehensive study of Rihan *et al*. (2014) verified that cold tolerance in cauliflower plants (*Brassica oleracea* var. *botrytis*) improved by utilising Mo in artificial seeds. According to the current results, a decline in grain characteristics might be the main reason for grains yield/pot loss for drought-stressed wheat plants which could be explained by the extent of loss in photosynthetic rate and

the carbohydrate influx from leaves and stems into grains, and thereby leading to increased pollen abortion during an early stage of spikelet development and certainly crop plants are more sensitive to drought during self-fertilisation (Kim *et al*., 2000, Cakir, 2004).

It appears that treatment of wheat plants with SA and Mo using only one mode of application with one spray is not sufficient for crop plants to complete their whole life cycle under limited water supply. It can be interpreted that the influence of SA on improvement the plants' tolerance to drought depend on numerous factors, its concentration, the way of application, duration of treatment, species and also physiological state of the plant.

4.6. Conclusion

As shown in the above discussion, drought treatment resulted in significant reduction in growth and yield characteristics in wheat varieties as compared to well-watered controls. Although applying the treatments SA 1.44, SA 2.88 and Mo 0.30 mM led to significant improvements in growth and yield characteristics as compared to control plants, applying SA 1.44 mM was not able to over-ride the negative effects of drought stress although partial alleviation was observed. It was determined that cv. Tamooz 2 had higher values for most growth traits when compared to cv. Adana 99. Furthermore, Tamooz 2 appeared to be a variety with higher yield potential than Adana 99 in terms of grain dry weight (g/pot). As a consequence, Tamooz 2 was chosen as preferable to be taken into the subsequent experimentation where increasing the number of sprays with SA 1.44 mM over the whole lifespan of wheat plants will be compared to spraying at specific growth stages (seedling, stem elongation and flowering).

Chapter 5

5. The effect of exogenous applications of salicylic acid to Iraqi wheat cv. Tamooz 2 at different growth stages on the up-regulation of the drought response regulon

5.1. Introduction

Recently a lot of research has been focused on the influence of drought stress on plant growth and crop productivity, and revealed that crop plants vary in their response to water deficit to different extents depending on the severity of the stress and also the stage of the plant development at which stress occurs (Wilson, 1968, Claassen and Shaw, 1970, Gupta *et al*., 2001). The sensitivity of crops to damage from low soil moisture supply at different stages of growth has been previously reported for maize (*Zea mays* L.) (Denmead and Shaw, 1960, Li-Ping *et al*., 2006) and showed that plants were apparently least affected by moisture stress imposed during the vegetative growth season, and although the plants in this duration appeared to recover from damage, the imposed stress could still result in reductions in grain yield at a later period.

Plants ability to cope with abiotic stresses such as drought, salinity and cold is not only associated with physiological mechanisms but is directed by molecular controls at the cellular level leading to biochemical changes which enhance the ability to survive under these stresses (Dubouzet *et al*., 2003). The molecular mechanisms of stress tolerance in plants involve stress perception and signal transduction followed by the expression of specific molecules (gene products) that play a key role in protecting plant cells from dehydration and damage. The expression of some transcription factors (TFs) that are induced by drought stress varies according to the response system of upregulation to produce different gene products (Reddy *et al*., 2004). It seems evident from the literature that many researchers have focused on the family member of TFs which are the dehydration

responsive element binding gene (*DREBs*) or C-repeat binding factor gene (*CBFs*). The *DREB/CBF* is the most important group of the several families of the transcription factors and it has been known to be induced by environmental constraints such as cold, salt and drought (Liu et al., 1998a, Nakashima et al., 2000, Pellegrineschi et al., 2004, Sakuma et al., 2006b) and to have been determined as important in the regulation of drought response in plants. *DREB*s and *CBF*s have been shown to have considerable homology and more frequently are being considered to be the same gene family.

To elucidate the molecular mechanisms of the gene expression in response to drought stress, many studies have been conducted primarily on the model plant species *Arabidopsis thaliana* (Liu et al., 1998a, Iuchi et al., 2000). Researches has been conducted to identify *DREB1/CBF* expression in transgenic *Arabidopsis thaliana* which was resulted in over-expression of a number of cold responsive/drought responsive (*CRT/DRE)* genes eliciting a higher level of tolerance to salinity and/or drought stress through the abscisic acid (ABA) independent pathway (Kasuga *et al*., 1999, Kitashiba *et al*., 2004, Xianjun *et al*., 2011, Yang *et al*., 2011). This TFs group upregulate the expression of many downstream genes for signal transduction leading to the expression of many stress-responsive gene products that are controlling the protection of cells from stress by the production of important metabolic proteins and other cellular compounds (Agarwal *et al*., 2010).

Chapter 5. The effect of exogenous applications of salicylic acid to Iraqi wheat cv. Tamooz 2 at different growth stages on the up-regulation of the drought response regulon

Analysis of the expression of dehydration-inducible genes has shown the expression of *DREB*/*CBF* genes induced in a variety of plants for example in rice (*Oryza sativa*) where the overexpression of *OsDREB* improved the level of tolerance to drought (Chen *et al*., 2008). Because many DNA markers are linked to drought tolerant traits in cereals, wheat and barley plants have been used in creation of transgenic lines to monitor the expression of *DREB*/*CBF* factors from grains in response to severe drought conditions (Morran *et al*., 2011). Some of these reports have recognised the functional role of SA as a signalling molecule in regulating physiological mechanisms mainly in plants that are exposed to abiotic stress. Accordingly, investigations on wheat plants have shown that treating plants with SA could result in reduction of the negative effects of water deficit on plants under stress (Janda *et al*., 1999, Aydin and Nalbantoglu, 2011). However, there are no reports in the literature linking the application of SA and the gene regulation of the drought response regulon.

The aim of this study specifically focused on the effect of spraying plants with salicylic acid (SA) at various growth stages of wheat and monitoring the effect on drought tolerance and investigating the up-regulation of *CBF/DREB*.

5.2. Objectives

The main objectives of this investigation were to:

1. Assess the effect of SA application at 3 different growth stages on the response of wheat plants (*Triticum aestivum*) to drought stress.
2. Evaluate whether the application of SA treatment has the capacity to induce the expression of *CBF/DREB* gene in wheat plants at different developmental stages of growth.

5.3. Materials and Methods

One variety of wheat (*Triticum aestivum* L.) which was shown to exhibit drought stress tolerance was used in the third glasshouse grown experiment on drought. Before starting the experiment, seeds of variety Tamooz 2 were incubated at 25 ^{o}C in a growth chamber (Sanyo Gallenkamp PLC LE 3202, England) for 3 days. In early November 2015, germinated seeds were transplanted into plastic pots (28.5 cm width, 26 cm height) with 16 seedlings per pot (total n = 64). The experimental arrangement was a completely randomised block design (CRD) with one variety, two watering regimes (W1 well watered, and W2 drought stressed) and eight treatments in four replicate blocks. The allocation of the treatments to the pot positions within a block was randomised using random number tables (Figure 5.1).

Rep 2

V1 L W2	V1 Con W1	V1 F W1	V1 LS W2	V1 LSF W1	V1 S W2
V1 SF W1	V1 LF W1	V1 LS W1	V1 LF W2	V1 SF W2	V1 S W1
		V1 Con W2	V1 F W2	V1 L W1	V1 LSF W2

Rep 1

V1 SF W2	V1 LF W2	V1 SF W1	V1 Con W2	V1 F W2	V1 LS W2
V1 Con W1	V1 L W2	V1 LSF W1	V1 S W2	V1 S W1	V1 L W1
		V1 LSF W2	V1 F W1	V1 LF W1	V1 LS W1

V1 Con W1	V1 S W1	V1 LS W1	V1 F W2	V1 L W2	V1 LSF W2
V1 LF W1	V1 SF W1	V1 LSF W1	V1 Con W2	V1 LF W2	V1 F W1
		V1 LS W2	V1 L W1	V1 SF W2	V1 S W2

Rep 4

V1 S W1	V1 F W1	V1 LS W1	V1 L W2	V1 SF W1	V1 LS W2
V1 LSF W2	V1 F W2	V1 L W1	V1 LF W2	V1 S W2	V1 LF W1
		V1 Con W1	V1 LSF W1	V1 Con W2	V1 SF W2

Rep 3

4R × 1V × 2W × 8T = 64 pots. There were 16 plants/pot

Figure 5.1: Plan showing the layout of pots in the greenhouse after randomisation. (R = Replicate; V = Variety; W = watering; Con, L, S, F, LS, LF, SF, LSF = Treatments.

The plants were grown in the greenhouse (Skarden garden, University of Plymouth) with an average Temperature of 17^{o}C and with supplementary lighting (sodium vapour lamps) to maintain a photoperiod of 12 h.

One month later after three leaves had been formed (GS13) the pots were irrigated to full field capacity (FC). The moisture content of pots was then regularly monitored with a Theta Probe (Delta-T Devices Ltd) and two watering regimes established when the seedling plants had four leaves (GS14). In harmony with the previous experiments, irrigation of pots was carried according to Theta probe readings with the intention that the well-watered treatment was brought back to 100% FC after it had dropped to 70% FC whilst droughted pots were brought back to 70% FC after they had dropped to 50% FC.

The wheat plants were foliar sprayed singly with 1.44 mM SA at three times during the experimental period. The spray was made for seedlings at two months after sowing once the plants had five leaves (GS15). The second application of SA was made during stem extension stage (GS32) one month after the first spray, while, the third spray was applied after a further 4 weeks later at flowering once the ears started to emerge from the sheath (GS51). The stomatal conductance of fully expanded leaf was measured using an automatic Porometer 2 and 10 days after spraying with SA at stem extension and flowering stages.

At stem extension, plants were fertilised with 7.5 g pot^{-1} Gromore fertiliser having the nutrient content of 7:7:7 %. Two months after sowing, all the plants were sprayed with fungicide against powdery mildew (Fungus Clear Ultra, Westland Horticulture limited, Germany), and also sprayed with insecticide against aphids (Bug Clear Ultra, Westland Horticulture limited, Germany). Both sprays were repeated twice more at one month intervals.

The experimental design was a completely randomised block (RCBD) with sixteen treatments (2 watering x 8 SA spray treatments (none, leaf, stem extension, flowering, leaf+stem, leaf+flower, stem+flower, leaf+stem+flower)) in each of four replicate blocks. Destructive leaf samples were collected randomly at 2 and 10 days after each time of SA spraying for molecular analysis of *DREB/CBF* gene from plants of one replicate (one block). At each interval, three leaves were detached from each treatment and placed immediately into plastic bags and placed on ice and then transferred to the -80 ^{o}C freezer. Plants in all four blocks were allowed to grow until the harvest stage. The samples were immediately stored at -80 ^{o}C in the freezer for the future molecular analysis. Plants were harvested in June 2016 in order to evaluate components of yield.

The trace of soil moisture content under both watering regimes was plotted and showed a cyclical pattern of soil drying (Figure 5.2). Greenhouse temperature was recorded using a Tiny Tag data logger throughout the 205 day experimental (Figure 5.3).

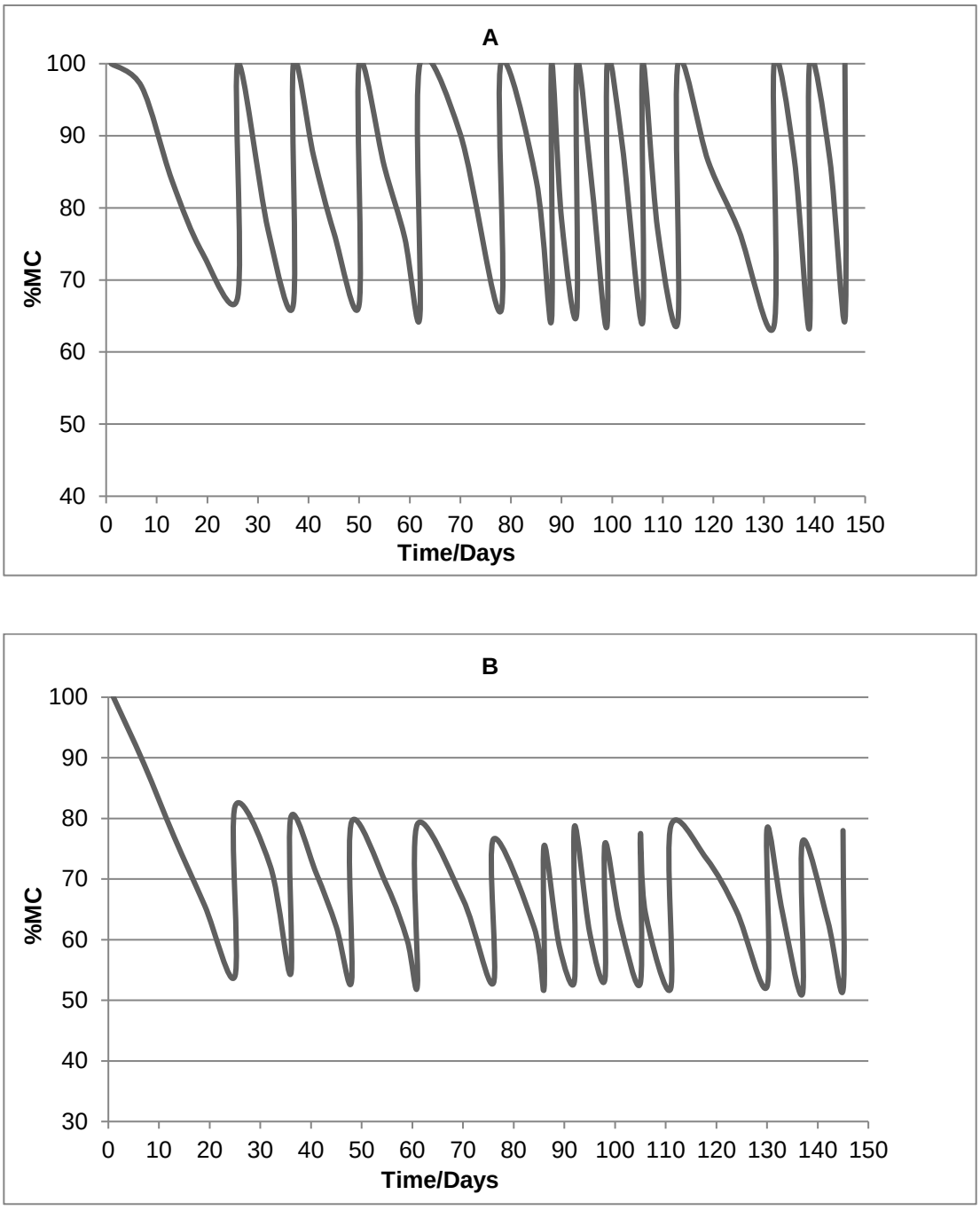


Figure 5.2: The Moisture content of pots over the duration of the experiment as monitored by Theta Probe- A well-watered, B droughted.

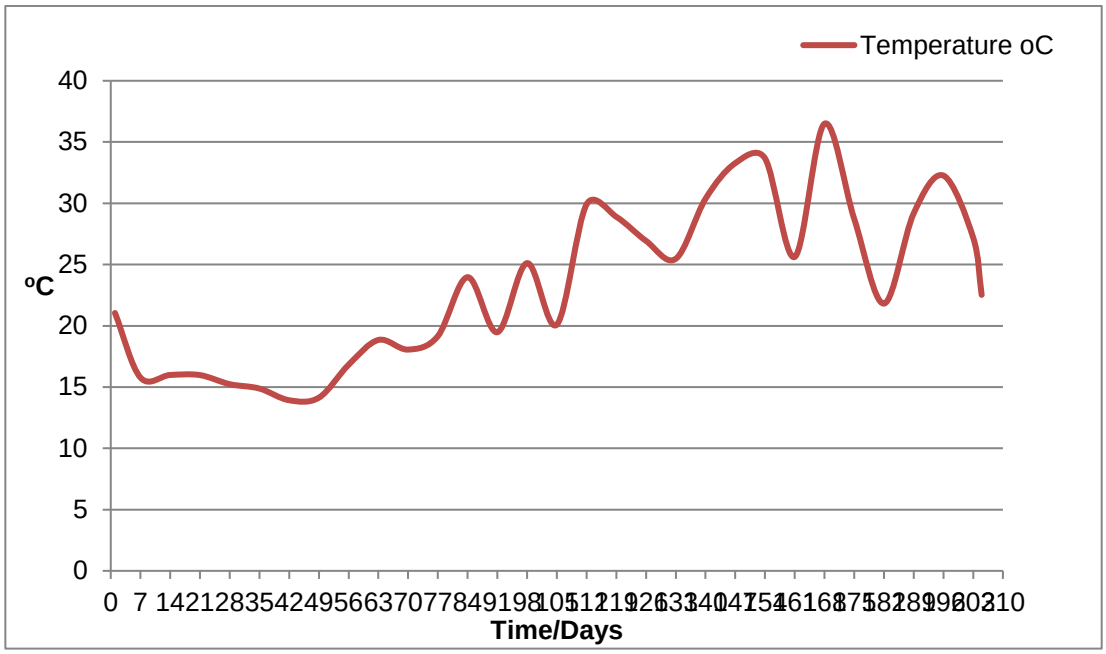
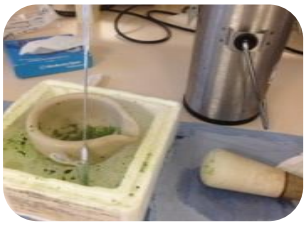


Figure 5.3: weekly average of temperature in Skarden Garden greenhouse during 205 days of the experiment.

5.3.1. Molecular analysis for gene expression of *DREB/CBF*.

The process of gene expression involves the following three major steps (Figure 5.4) modified from Huggett *et al*. (2005)



1- mRNA extraction **(with similar sample size)**

2- cDNA synthesis by reverse transcription **(with similar RNA concentration)**

3- Amplification of cDNA by PCR machine **(with simultaneous measurement of internal reference)**

Figure 5.4: Processes required to generate quantitative PCR result. Embolden text indicates key points for a good normalisation strategy.

5.3.1.1. Extraction of mRNA from plant tissues by RNA analysis.

Total mRNA was isolated from frozen plant samples according to the manufacturer's instructions provided in the Spectrum™ Plant Total RNA kit (Sigma cat, STRN 50 prep) described by Farrell (1998) and Sambrook *et al*. (1989) as follows:

Preparation of plant tissue samples.

- **Grinding plant tissue**

Frozen samples were ground thoroughly under liquid nitrogen to a fine powder using a mortar and pestle. To ensure complete removal of any trace of RNAase contamination, all surfaces and equipment used in the extraction process were

sprayed with RNAase ZAP (Fisher Scientific UK, Cat# 10708345). For best preparation for RNA extraction, the mortar was placed on dry ice and the plant materials were kept frozen at all times.

- **Tissue sample weight**

Liquid nitrogen was allowed to evaporate from the ground powder of the plant tissues and immediately the ground tissue with liquid nitrogen was decanted into an appropriately sized tube of 2 mL RNAase-free microcentrifuge tube (Eppendorf) and 100 mg of frozen tissue powder weighed on a precision balance (Sartorius, Model MSA 2265, UK). The weighed samples were kept on ice before lysis solution was added or stored at -80 ^{o}C freezer until they were used in the RNA isolation step.

- **Preparation Wash Solution 2**

As a prior preparation step for RNA extraction process, 60 mL of absolute Ethanol Electran® (VWR International Ltd, Cat# 437433TDP) was added to the bottle of Wash Solution 2 which was supplied as a concentrate, mixed briefly and then stored as diluted Wash Solution 2.

- **Prepare Lysis Solution/2-ME Mixture**

2-mercaptoethanol (2-ME) was added to Lysis solution in a clean conical tube before use. Accordingly, Master mix was prepared from 1000 µL of the lysis

solution supplemented with 10 µL of 2-mercaptoethanol and mixed briefly to be used for each two sample preparations.

- **Assemble Columns and Collection Tubes**

Filtration columns (indicated by a blue retainer ring) were inserted into a 2 mL collection tubes (provided in the kit) and closed with the lid for the use in step filter lysate. Likewise, binding columns (indicated by a red retainer ring) were also inserted into a 2 mL collection tube and closed the lid for the use in step bind RNA.

- **Lyse tissue sample**

500 µL of the Lysis solution/2-ME Mixture was pipetted directly onto each tissue powder sample of 100 mg in a microcentrifuge tube and vortexed immediately and vigorously for at least 1 minute at the maximum speed (13,000x G). Then the samples were incubated in a heating block (Accublock TM digital dry bath, MBF 131 Labnet international, Inc.) for 3-5 minutes at 56 ^{o}C.

- **Pellet Cellular Debris**

The samples were centrifuged at room temperature and maximum speed (13000-15000 rpm) in a standard microcentrifuge for 3 minutes in order to pellet cellular debris.

- **Filter Lysate**

The lysate supernatants were transferred carefully to a filtration column (blue column, supplied in the Plant Total RNA Kit) seated in a 2 mL free RNase collection tubes by positioning the pipette tip at the bottom of the tube without disturbing the cell debris pellet. Then caps were closed and centrifuged at maximum speed for 1 minute in order to remove residual debris.

- **Bind RNA to Column**

The blue column of each sample was discarded and 500 µL of binding solution was pipetted into the clarified lysate and mixed well immediately by pipetting for at least 5 times or the vortex was briefly used.

- **Bind the RNA**

For each sample, 700 µL of the binding mixture was pipetted directly to the binding column (red column, supplied in the Kit) seated in the 2 mL collection tube and centrifuged at maximum speed for 1 minute so as to bind the RNA. The flow through liquid was poured and the collection tube was turned upside down on clean absorbent tissue in order to drain the residual liquid, and the columns were returned to the collection tubes.

- **Four steps in a washing process were applied:**

1. First Column Wash

500 µL of the wash solution 1 was pipetted into each sample column. Then tubes were closed well and centrifuged at maximum speed for 1 minute. The flow through liquid was poured and the column was returned to the collection tube.

2. Second Column Wash

500 µL of the diluted Wash Solution 2 was pipetted into the sample column and they were centrifuged at the maximum speed for 1 min. The flow through liquid was poured and the column was returned into the collection tube.

3. Third Column Wash

Another 500 µL of the diluted Washing Solution 2 was pipetted into the column and then centrifuged at the maximum speed for 1 minute. The flow through liquid was poured and the column was returned to the collection tube.

4. Dry the Column

The columns were then centrifuged without any addition at the maximum speed for 1 minute to dry. Then columns tubes were removed carefully from the centrifuge in order to avoid splashing the residue flow through the liquid on the dried columns and they were transferred into a new, clean 2 ml collection tubes.

- **Elution the Column**

Columns were transferred into a new clean 2 mL collection tubes. A 50 µL of elution solution was pipetted directly onto the centre of the binding column. Then tubes were closed with their caps and left to set for 1 minute. Finally, they were centrifuged at the maximum speed for 1 minute to elute. The red columns were removed from the tubes and the obtained extracted RNA was ready for immediate use in the next process or storage them at -20 ^{o}C (short term) or -80 ^{o}C (long-term) for the later use.

5.3.1.2. Standard Operating Procedure for *CBF/DREB* gene quantification (SOP-*CBF*/*DREB*).

Based on the use of the SOP-qPCR protocol by Jain *et al*. (2006), two steps of PCR were carried out for the synthesis and amplification of cDNA of *CBF/DREB*. Both of these procedures were obtained from manufacturers instruction kit (Sigma:cat #M 1302) and described as below.

1. mRNA reverse transcription for cDNA synthesis.

After the total RNA was obtained from the tissues by applying the standard operation procedure for RNA extraction, the extracted RNA were kept either chilled on ice for immediate use or placed into a freezer at -20^{o}C for later use. Then, the reverse transcription process was applied to produce cDNA from the extracted mRNA for each of the samples. To synthesise a complementary cDNA

strand, kit M-MLV Reverse Transcriptase (Sigma: M 1302) was used for preparation of the first strand cDNA as described in the following procedure.

1- Since a specific volume of extracted RNA sample was obtained, 2µL of each sample was used and analysed with the NanoDrop 2000 technique. The RNA samples were quantified so as to measure concentration and purity of the RNA. In addition, the absorbance was measured using the A260/A280 ratio procedure (Warburg and Christian, 1941). Because the nucleic acids have a higher absorbance at 260 nm than at 280 nm, the A260/A280 ratio was expected to be ≥ 2 for the pure samples.

The volume of each sample was then calculated by applying the following equation (the RNA concentration is supposed to be less than 1000 in order to obtain the volume).

RNA volume=1000/RNA concentration.

The volume of each RNA sample was added to nuclease-free 0.2 mL Sigma PCR microtubes and made-up to 8 µL volume by adding molecular grade water (Fisher Scientific UK, cat 10505854) to each sample.

2- A master mix consisting of the reagents 1µL of deoxynucleotide dNTPs (Sigma: D7295) and 1µL of random nanomerase primer (Sigma: R7647) was prepared for each sample. The total master mixture was prepared together depending on the sample numbers and mixed gently, and 2µL from the mixture was added to each sample.

3- Samples were incubated in PCR max machine (Applied Biosystem, Veriti) at 70^{o}C for 10 minutes and they transferred quickly to ice for 5 minutes.

4- Samples were kept cold on ice for 5 min and another master mix was immediately prepared by adding the following components to the nuclease-free 0.5 mL microcentrifuge tube.

1µL of M-MLV reverse transcriptase (sigma: M1302)

2µL of M-MLV reverse transcriptase buffer

7µL of molecular grade water

The total mixture was prepared together by multiplying each of these solutions by the samples number.

Once the master mixture was ready for all samples, 10µL from the mixture was added to each reaction sample and incubated at the following thermal cycle for the PCR reaction: 21^{o}C for 10 min, 37^{o}C for 50 min, 94^{o}C for 2 min and holding at 4^{o}C. The generated cDNA was then stored at 4^{o}C for use in quantitative PCR amplification technique.

2. Standard Operating Procedure (SOPqPCR) for cDNA amplification using quantitative Polymerase Chain Reaction.

The genome sequence of forward and reverse primers for the *CBF/DREB* gene as given below were obtained from Eurofins MWG and used as target nucleic acid for the PCR detection system according to Al-Issawi (2013) and according to designed DNA sequences for wheat plants from Blast software, and its suitability for *CBF14* gene detection confirmed using the quantitative PCR protocol. *CBF* 14 was used as the indicator for Transcription Factors *CBF/DREB* because they are identical and have the same sequences, but they appear in different forms.

CBF14-Int-(F 5´-CCGTTCAGCACCGCCAAGGC-3´) and *CBF14*-Int-(R 5´-CCATGCCGCCAAACCAGTGC-3´).

Actin1 was selected as an endogenous control gene in order to normalise the gene expression obtained of *CBF14* in plants (Jain *et al*., 2006). The primers of *Actin 1* as described by Al-Issawi *et al*. (2013) and Rihan (2014) were designed to specifically bind the extremities of the DNA fragment to be amplified and were obtained from (Eurofins MWG/operon. Germany) and used

Actin1 gene: Forward primer (F 5-CCCAAAGGCCAACAGAGAGAAG-3) and reverse primer (R 5-CACCAGAGTCCAGCACAATACC-3).

The expression of the *CBF14* gene in wheat leaves was investigated by use of the quantitative PCR Analysis as presented in kit manufacturer's instructions (Sigma). To amplify the first strand cDNA obtained at the end of reverse

transcription process, Kit (SYBR® Green JumpStart Taq Ready Mix™: cat #S4438-100RXN) used for preparing master mix consisted of all the components for qPCR except the sample cDNA as shown below (per sample):

12.5 µL Syber green master mix.

0.5 µL forward primer

0.5 µL reverse primer

0.25 µL internal reference dye

9.5 µL sterile molecular grade water

A sufficient volume of Syber green master mix and forward/ reverse primers for *CBF* was prepared in an appropriate free nuclease microcentrifuge tube while another Syber green master mix and forward/revers primers prepared for *Actin* in a separate tube. Subsequently, the solution in each tube was mixed gently by inversion to avoid creating bubbles and immediately placed on ice prior to being dispensed to a 96 well plate (Fisher Scientific, Applied Biosystem: MicroAmp cat#VY4346906) as described below:

Each well of the first set of columns was filled with 23.25 µL of Master mix 1 of *CBF* then next set of columns filled by the same volume with Master mix 2 of *Actin*. While, 2 µL cDNA template for each sample was transferred into the wells across rows which labelled to recognise the location of each sample on the plate. Similarly, molecular grade water was added to one of the rows to act as cross-check blanks.

The reaction volume for each sample was 25.25 µL which was obtained by adding 2 µL of template cDNA to 23.25 µL of the master mix. Once the plate was ready, the optical adhesive cover (Fisher Scientific, Applied Biosystems: MicroAmp cat# VY4360954) was placed on the PCR plate and then the PCR plate spinner, (VWR: MBF 154) used briefly to spin down the contents to the bottom of the plate. Then the plate was run in the PCR machine (AB Applied Biosystems: StepOnePlus Real-Time PCR System) under the following thermal cycle:

1- Starting step at 94^{o}C for 10 minutes.

2- Cycling step which consisted of 40 cycles of (95^{o}C for 15 sec followed by 60^{o}C for 1minute.

3- Melting step, this consisted of 95^{o}C for 15 sec followed by 60^{o}C for 1 min followed by 95^{o}C for 15 sec.

Finally, the results obtained were analysed to get the relative quantitation of the expression of the target gene (*Cbf14*) against the endogenous standard gene (18s rRNA).

Statistical analysis

All data were analysed using the statistical software Minitab (version 17) using balanced analysis of variance (ANOVA). Significant differences between means were assessed by the least significant difference test (LSD) at the probability of 5%. In Figures error bars are one standard error of the mean (SE). Because of the complexity of the Figures, significant difference indication by use of letters was not deemed appropriate.

5.4. Results

The effect of different sprays with SA on final biomass of wheat plants under both watered and drought conditions at end of the experment.

Shoot dry weight

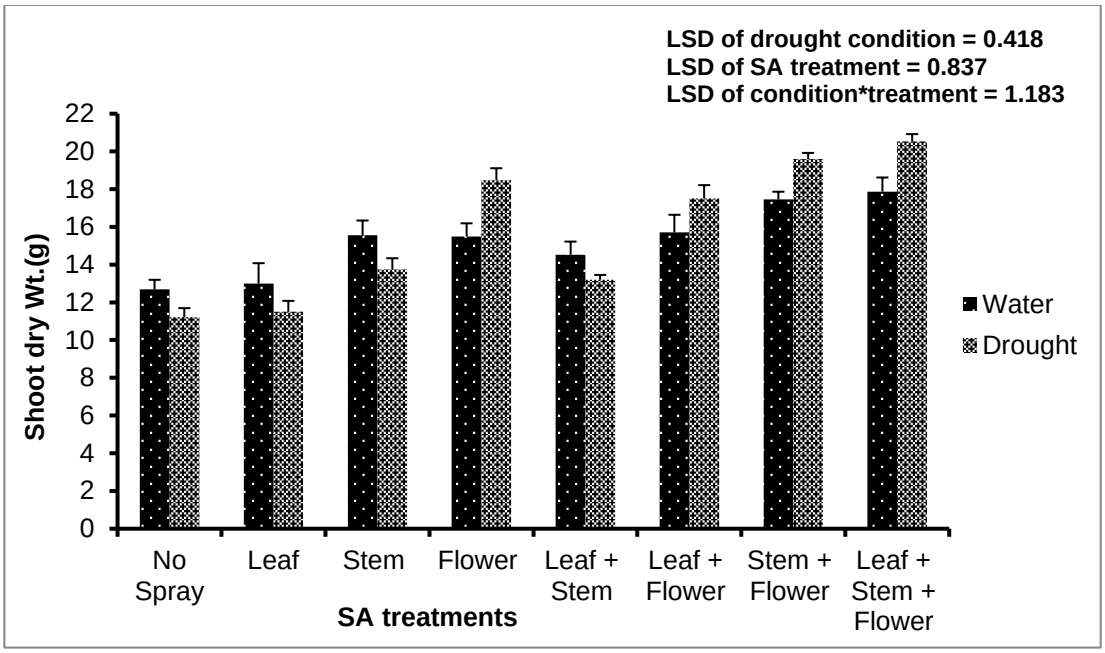


Figure 5.5: The effect of SA sprays on the final shoot dry weight of wheat plants under watered and drought conditions (*P* = 0.043 for drought condition. *P* ≤ 0.001 for SA treatment. *P* ≤ 0.001 for drought condition * treatment, error bars = SE).

The effect of drought on shoot dry weight was significantly different compared to well-watered conditions (p = 0.043). A significant effect ($p \leq 0.001$) on the shoot dry weight was observed for SA treatment. The interaction of drought condition* SA treatment effect on shoot dry weight ($p \leq 0.001$) showed a highly significant difference for droughted plants at SA treatments (stem flower and leaf stem flower) compared to control plants (Figure 5.5) (Table 5.1 and Appendix 59).

Table 5.1: Analysis of Variance results for the effect of different spray with SA on Shoot dry weight.

Source	F value	*P* value	LSD
Drought condition	4.36	0.043	0.418
Treatment	87.63	<0.001	0.837
Drought condition*treatment	13.16	<0.001	1.183
Rep	24.20	<0.001	

The effect of different spray with SA on the yield components of wheat plants under both watered and drought conditions.

Number of spikes per pot.

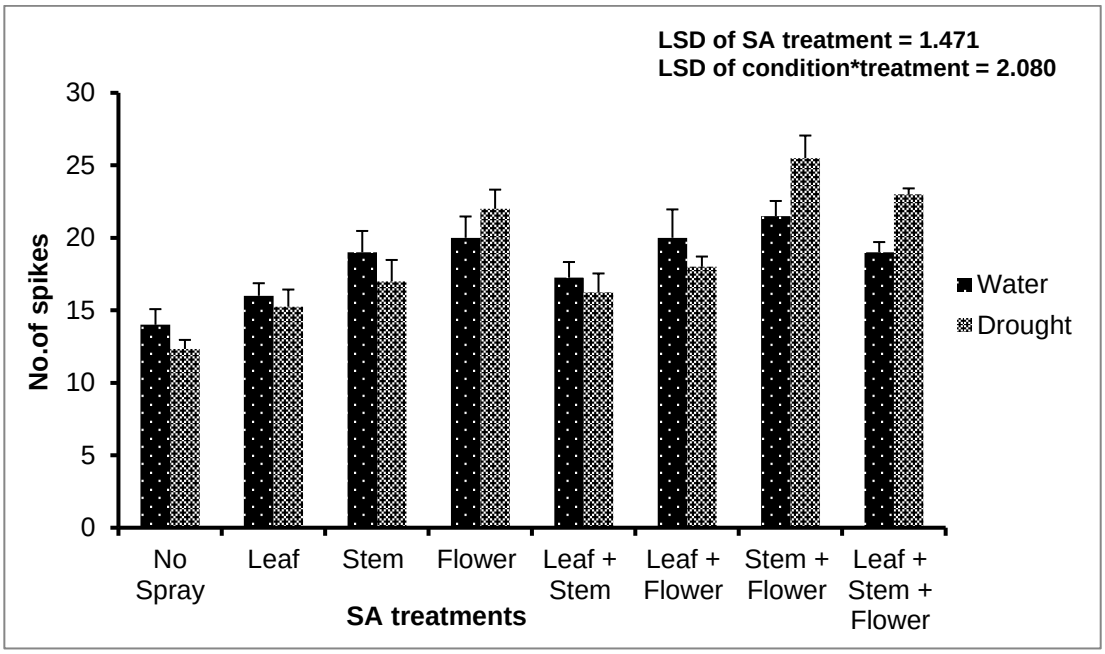


Figure 5.6: The effect of SA spray on the number of spikes at the harvesting stage of wheat plants under watered and drought conditions (*P* = 0.515 for drought condition. *P* ≤ 0.001 for SA treatment. *P* ≤ 0.001 for drought condition * treatment, error bars = SE).

Analysis of variance showed that drought had no a significant effect on number of spikes compared to well-watered conditions (p = 0.515). All SA treatments (except for leaf and leaf+ stem) were improved the spikes number/pot significantly ($p \leq 0.001$). The interaction effect between drought condition and SA treatment was also significant ($p \leq 0.001$). The highest significant difference was for SA treatments stem+flower and leaf+stem+flower for droughted plants

compared to control plants (comparing the difference between means of both treatments to the LSD value) (Figure 5.6) (Table 5.2 and Appendix 60).

Table 5.2: Analysis of Variance results for the effect of different spray with SA on number of spikes/pot.

Source	F value	*P* value	LSD
Drought condition	0.43	0.515	
Treatment	44.22	<0.001	1.471
Drought condition*treatment	6.47	<0.001	2.080
Rep	28.65	<0.001	

Spikes dry weight.

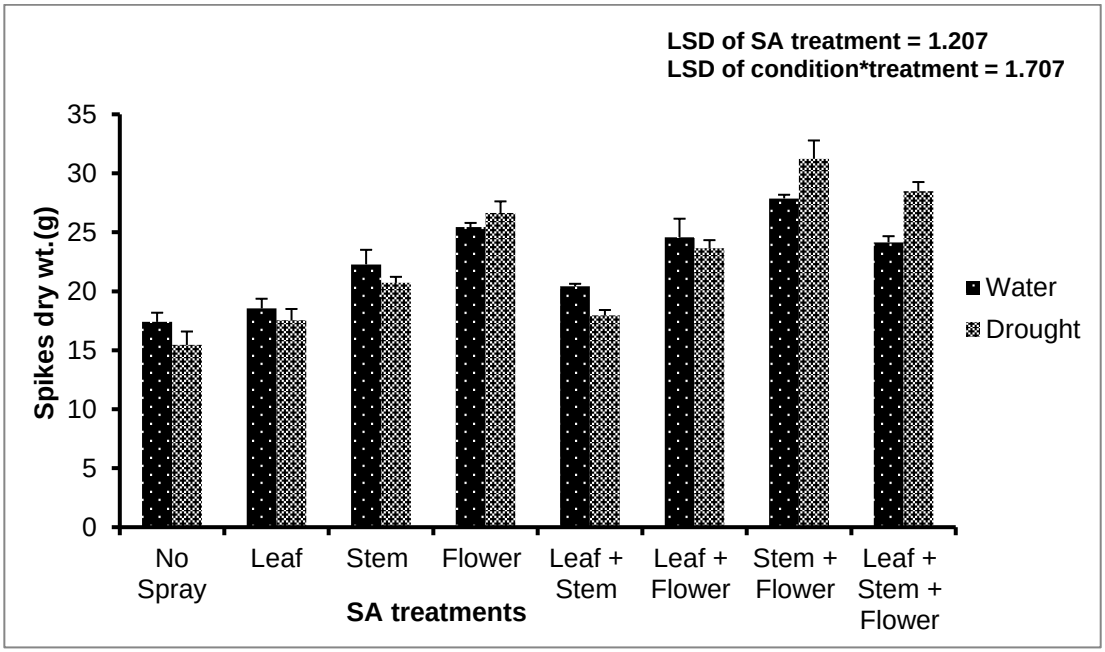


Figure 5.7: The effect of SA on spike dry weight of wheat plants under watered and drought condition (*P* = 0.682 for drought condition. *P* ≤ 0.001 for SA treatment. *P* ≤ 0.001 for drought condition * treatment, error bars = SE).

Drought stress had a non-significant effect on spike dry weight compared with well-watered conditions (p = 0.682). SA treatments significantly ($p \leq 0.001$) increased the spike dry weight at flower, stem+flower and leaf+stem+flower. The interaction of drought condition*treatment had a significant effect on the dry weight of spikes ($p \leq 0.001$). The highest value of significant difference observed for drought stressed plants at SA spray leaf+stem+flower when compared with control plants (Figure 5.7) (Table 5.3 and Appendix 61).

Table 5.3: ANOVA results for the effect of different spray with SA on spikes dry weight.

Source	F value	*P* value	LSD
Drought condition	0.17	0.682	
Treatment	117.58	<0.001	1.207
Drought condition*treatment	9.07	<0.001	1.707
Rep	21.09	<0.001	

Grain dry weight.

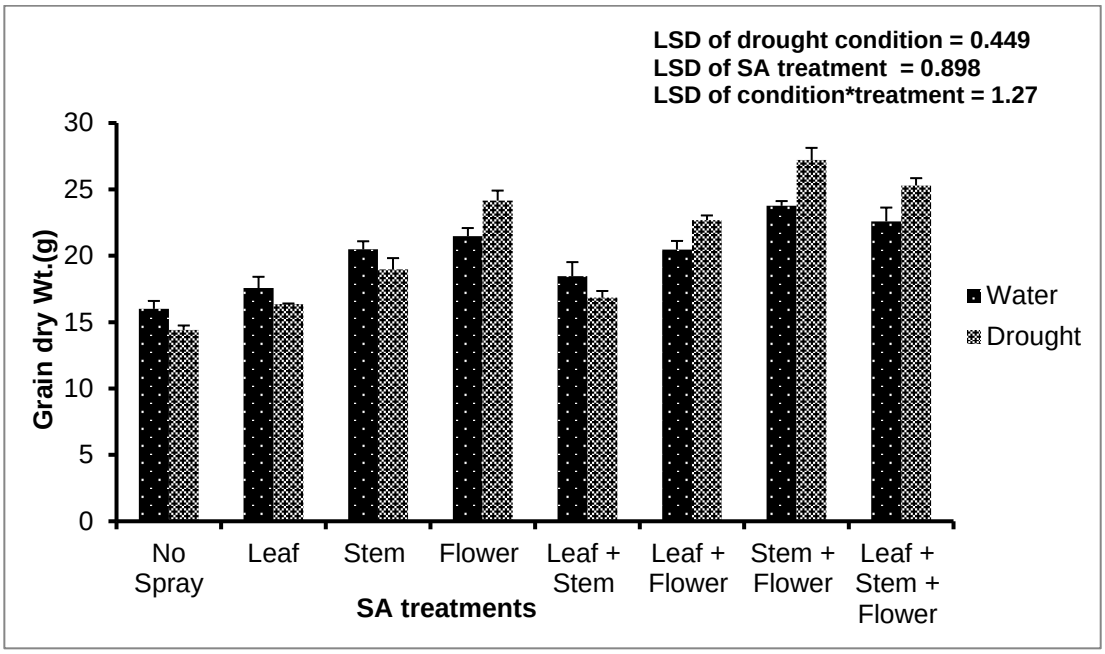


Figure 5.8: The effect of salicylic Acid (SA) spray on grain dry weight of wheat plants under watered and drought conditions (*P* = 0.006 for drought condition. *P* ≤ 0.001 for SA treatment. *P* ≤ 0.001 for drought condition * treatment, error bars = SE).

Grain dry weight for plants under drought stress condition was significantly different from the plants under well-watered condition (p = 0.006). SA treatment had a significant effect on grain dry weight ($p \leq 0.001$). The interaction effect of drought condition and treatment was also significant ($p \leq 0.001$). SA spray on drought plants at leaf+stem+flower, flower and stem+flower showed significant increases in grain dry weight when compared to their non-drought stressed plants. The maximum significant effect observed was the treatment of stem+flower among the drought treatments compared to non-sprayed control plants (Figure 5.8) (Table 5.4 and Appendix 62).

Table 5.4: ANOVA results for the effect of different spray with SA on grain dry weight.

Source	F value	*P* value	LSD
Drought condition	8.44	0.006	0.449
Treatment	132.93	<0.001	0.898
Drought condition*treatment	13.26	<0.001	1.27
Rep	22.98	<0.001	

Average 1000 grain dry weight.

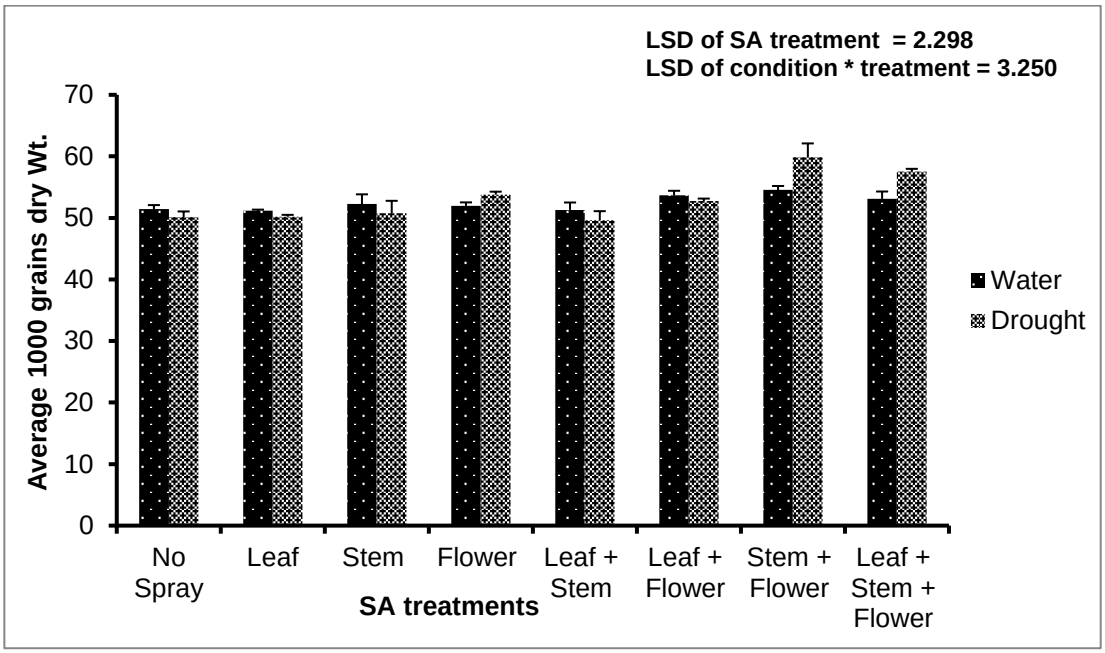


Figure 5.9: The effect of SA spray on average 1000 grain dry weight of wheat plants under well-watered and drought conditions at harvesting stage (*P* = 0.262 for drought condition. *P* ≤ 0.001 for SA treatment. *P* = 0.010 for drought condition * treatment, error bars = SE).

Data in Figure 5.9 showed that the average 1000 grains dry weight was not significantly affected by the drought stress in comparison to the well-watered condition ($p = 0.262$) as much as other yield components. There were significant differences caused by the SA treatments ($p \leq 0.001$). The interaction of drought condition*treatment had a significant effect on the dry weight of average 1000 grains ($p = 0.010$). Both stem+flower and leaf+stem+flower treatments showed a significant increase in average 1000 grain dry weight for plants under drought stress when treated with SA, but other SA treatments were not significantly different compared to non-sprayed control plants (Table 5.5 and Appendix 63)

Table 5.5: ANOVA results for the effect of different spray with SA on average 1000 grains dry weight.

Source	F value	*P* value	LSD
Drought condition	1.29	0.262	
Treatment	9.08	<0.001	2.298
Drought condition*treatment	3.09	0.010	3.250
Rep	0.34	0.798	

The effect of spraying SA on wheat plants on *CBF* gene expression under both well-watered and drought conditions.

A. Early spraying (GS15) of SA

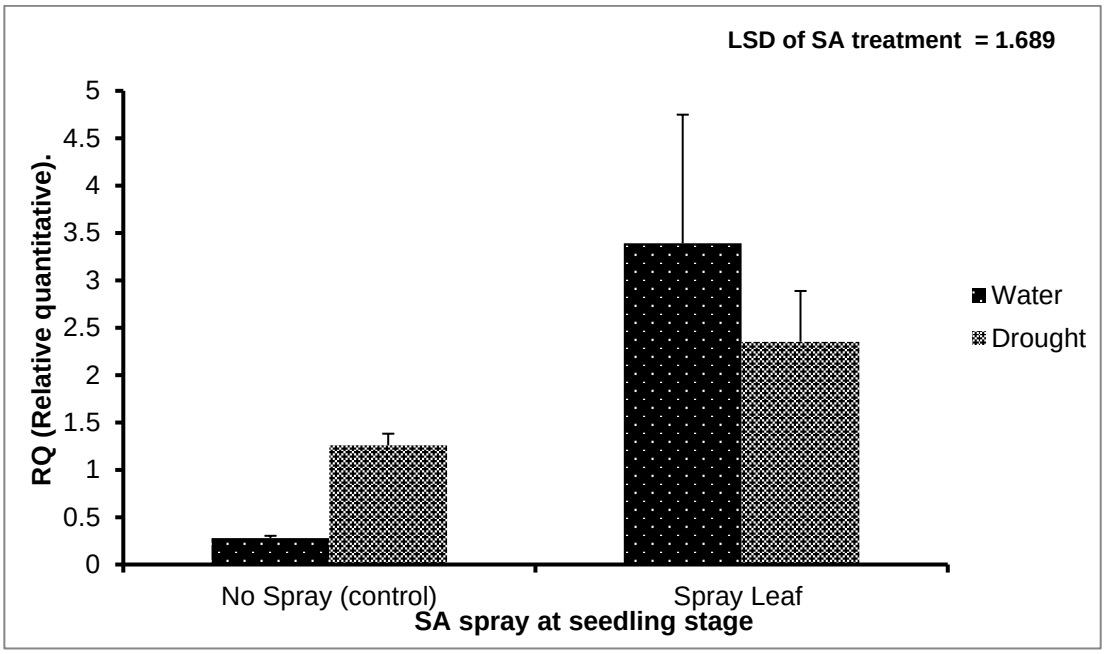


Figure 5.10: The effect of SA on the up-regulation of the *CBF* gene 2 days after spraying of wheat plants (VAR.Tamooz 2) at GS15 under both watered and drought conditions (*P* = 0.968 for water condition. *P* = 0.021 for SA treatment. *P* = 0.206 for water condition * treatment, error bars = SE).

CBF expression under the well-watered conditions was not significantly different compared with drought (p = 0.968). There was a significant effect of SA increasing the gene expression of *CBF* for the wheat plant seedlings 2 days after spraying at GS15 (Spray Leaf) compared to the unsprayed control plants (p = 0.021). There was no significant interaction between watering conditions and SA treatment (p = 0.206) (Figure 5.10) (Table 5.6 and Appendix 64).

Table 5.6: Analysis of Variance results for the effect of SA on the up-regulation of the *CBF* gene 2 days after spraying the leaves of wheat plants (VAR.Tamooz 2) at GS15 under both watered and drought conditions.

Source	F value	*P* value	LSD
Water condition	0.00	0.968	
Treatment	8.23	0.021	1.689
Water condition*treatment	1.90	0.206	

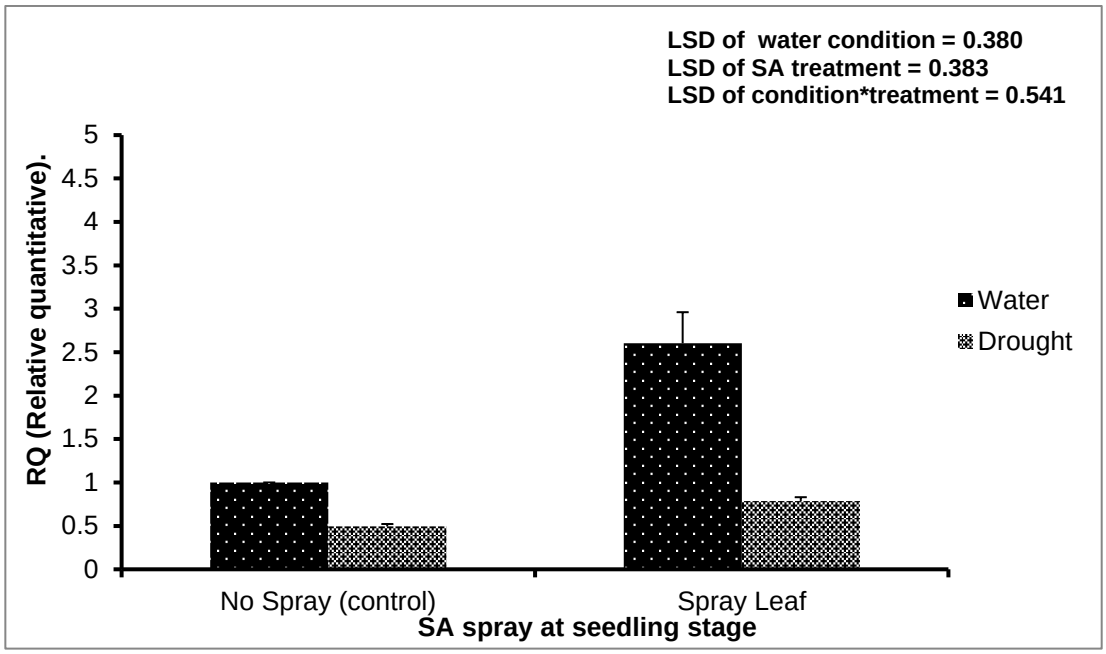


Figure 5.11: The effect of SA on the up-regulation of the *CBF* gene 10 days after spraying the leaves of wheat plants (variety Tamooz 2) at GS15 under well-watered and drought conditions ($p \leq 0.001$ for water condition. $p \leq 0.001$ for SA treatment. $p = 0.002$ for condition * treatment, error bars = SE).

The effect of water condition on *CBF* expression was significantly different compared to the drought condition ($p \leq 0.001$). SA treatment had a significant effect on the expression of *CBF* gene compared to unsprayed control plants ($p \leq 0.001$). There was a significant interaction between the water conditions and SA treatment ($p = 0.002$). Ten days after spraying with SA the expression of *CBF* gene decreased especially for drought stressed plants in comparison to the plants under well-watered conditions (Figure 5.11) (Table 5.7 and Appendix 65).

Table 5.7: Analysis of Variance results for the effect of SA on the up-regulation of the *CBF* gene 10 days after spraying the leaves of wheat plants (variety Tamooz 2) at GS15 under well-watered and drought conditions.

Source	F value	*P* value	LSD
Water condition	41.35	<0.001	0.380
Treatment	27.56	<0.001	0.383
Water condition*treatment	13.15	0.002	0.541

B. Mid-spraying (GS32) of SA

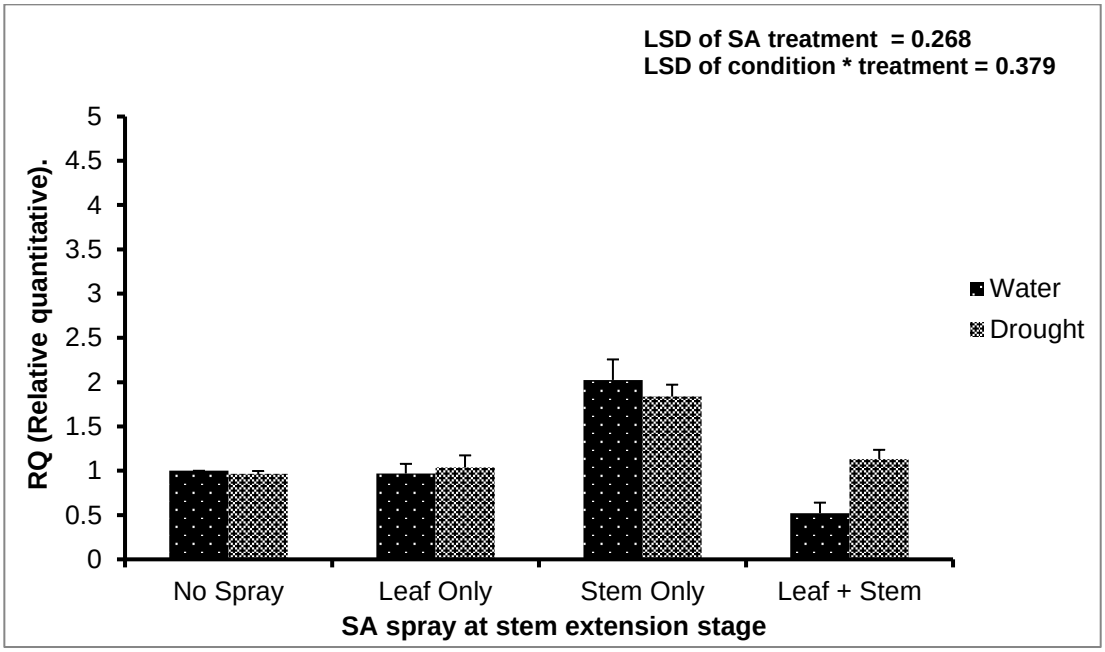


Figure 5.12: The response of *CBF* gene to SA spray of wheat plants variety Tamooz 2 at GS32 two days after spraying under water and drought conditions during stem extension stage (*P* = 0.218 for drought condition. *p* ≤ 0.001 for SA treatment. *p* = 0.031 for condition * treatment, error bars = SE).

Drought condition had a non-significant effect on the expression of *CBF* compared to well-watered (p = 0.218). There was a positive significant effect on the up-regulation of *CBF* gene 2 days after spraying with SA at GS32 (Stem only) compared to the control plants ($p \leq 0.001$). The interaction of drought condition*treatment had a significant effect on the upregulation of *CBF* gene (p = 0.031). When however, the drought stressed plants sprayed earlier at GS15 were resprayed at GS32 (Leaf + Stem) there was little or no further upregulation of *CBF* gene 2 days after spraying (Figure 5.12) (Table 5.8 and Appendix 66).

Table 5.8: Analysis of Variance results for the effect of SA on the up-regulation of the *CBF* gene 2 days after treating with SA under water and drought conditions during stem extension stage.

Source	F value	*P* value	LSD
Drought condition	1.65	0.218	
Treatment	31.78	<0.001	0.268
Drought condition*treatment	3.79	0.031	0.379

CBF gene expression of the wheat plants leaves 10 days after spraying with SA during stem extension stage under both watered and drought conditions.

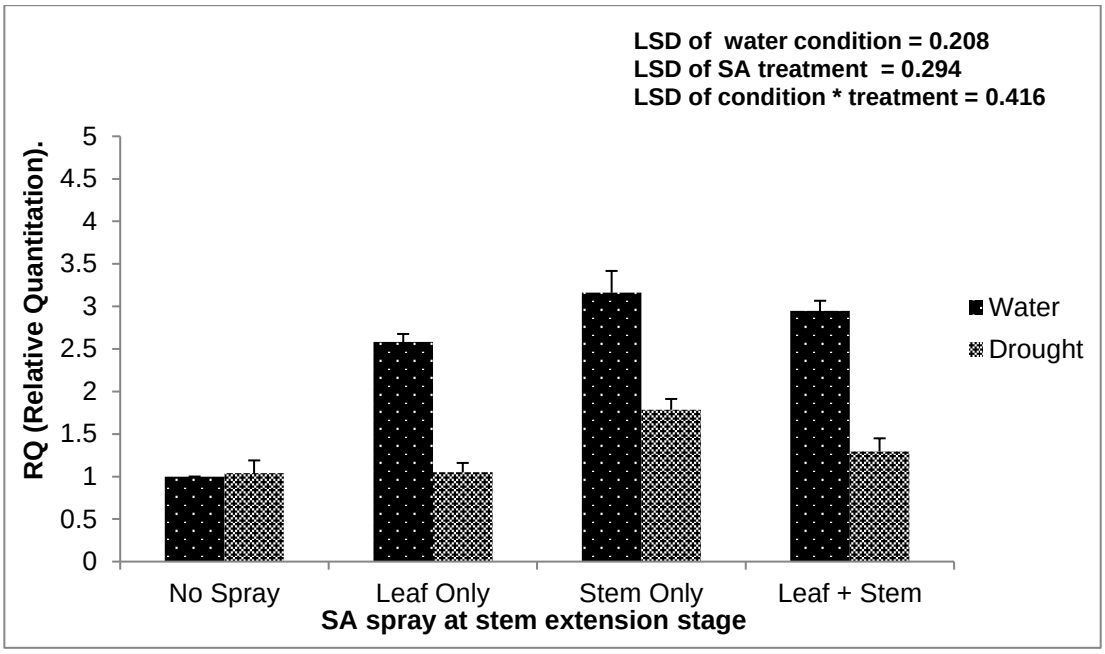


Figure 5.13: The response of *CBF* gene to spraying the wheat plants with SA at GS32 under watered and drought conditions 10 days after spraying ($p \leq 0.001$ for water condition. $p \leq 0.001$ for SA treatment. $p \leq 0.001$ for condition * treatment, error bars = SE).

There was a significant effect on the expression of *CBF* gene observed for the well-watered conditions compared to the drought stress ($p \leq 0.001$). SA treatment had a significant effect on *CBF* expression compared to unsprayed control plants ($p \leq 0.001$). There was a significant interaction between the water conditions and SA treatment ($p \leq 0.001$). The most significant difference in the response of *CBF* gene to SA 10 days after spraying was observed in the well-watered plants sprayed with SA at GS32 (Stem only) compared to drought-stressed plants (Figure 5.13) (Table 5.9 and Appendix 67).

Table 5.9: Analysis of Variance results for the effect of SA on the up-regulation of the *CBF* gene 10 days after treating with SA under water and drought conditions during stem extension stage.

Source	F value	*P* value	LSD
Water condition	125.40	<0.001	0.208
Treatment	37.82	<0.001	0.294
Water condition*treatment	15.19	<0.001	0.416

C. Late spraying at GS 51

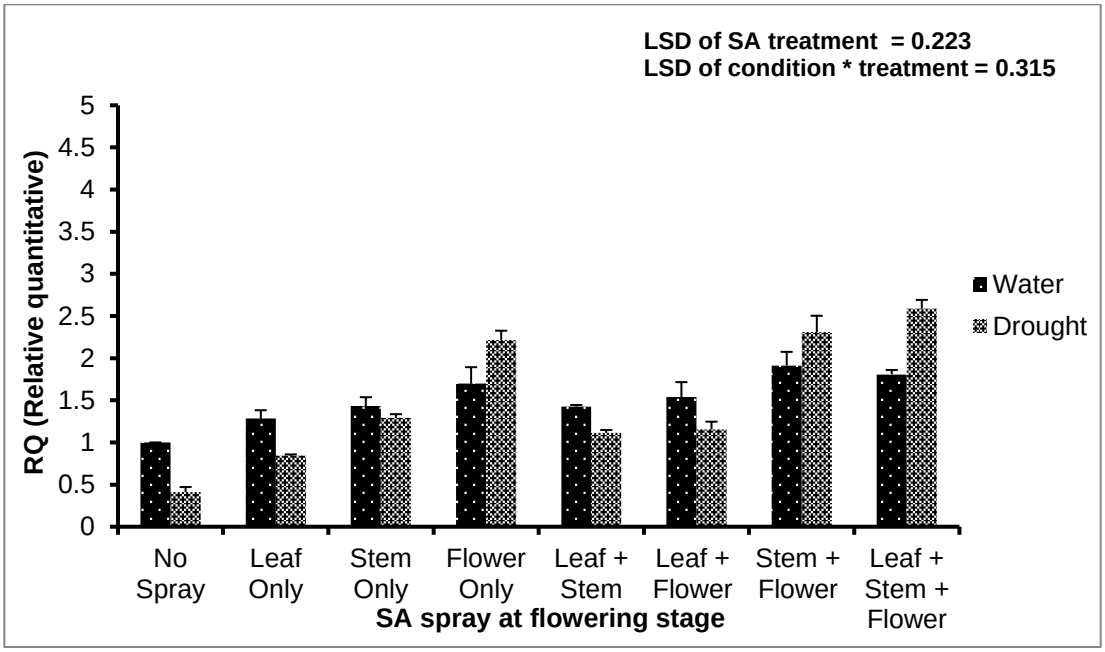


Figure 5.14: The effect of SA on the up-regulation of the *CBF* gene 2 days after of spraying at GS51 wheat plants variety Tamooz 2 under well-watered and drought conditions (p = 0.704 for water condition. $p \leq 0.001$ for SA treatment. $p \leq 0.001$ for condition * treatment, error bars = SE).

The watering conditions had no overall significant effect on the *CBF* expression level ($p = 0.704$). The expression level of *CBF* gene was significantly affected by SA treatments ($p \leq 0.001$) and in addition showed an interaction between water condition and SA treatments ($p \leq 0.001$). The Level of RQ increased significantly 2 days after spraying with SA during the flowering stage for stem+flower, flower and leaf+stem+flower of sprayed plants respectively as compared to their control plants under drought conditions. The highest level of response was for leaf+stem+flower spray. The treatment of stem+flower was higher than all others SA treatments under well-watering (Figure 5.14) (Table 5.10 and Appendix 68).

Table 5.10: Analysis of Variance results for the effect of SA on the up-regulation of the *CBF* gene 2 days after of spraying the wheat plants variety Tamooz 2 under well-watered and drought conditions.

Source	F value	*P* value	LSD
Water condition	0.15	0.704	
Treatment	46.24	<0.001	0.223
Water condition*treatment	10.70	<0.001	0.315

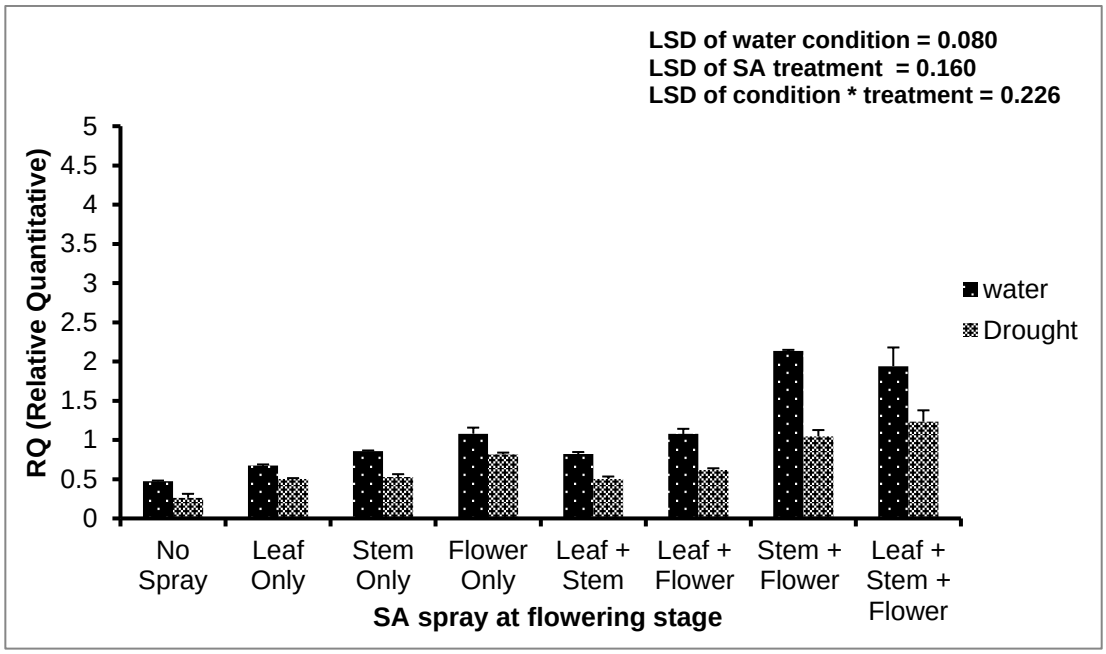


Figure 5.15: The effect of SA on the up-regulation of the *CBF* gene 10 days after of spraying wheat plants variety Tamooz 2 at GS51 under well-watered and drought conditions ($p \leq 0.001$ for water condition. $p \leq 0.001$ for SA treatment. $p \leq 0.001$ for condition * treatment, error bars = SE).

The expression level of *CBF* gene was significantly affected by the well-watering condition compared to drought ($p \leq 0.001$). SA treatments had a significant effect on the *CBF* expression level ($p \leq 0.001$). There was a significant interaction between water conditions and SA treatment ($p \leq 0.001$). The level of *CBF* gene for drought stress plants decreased significantly 10 days after spraying compared to the well-watered plants under the same SA spray stem+flower, flower and leaf+stem+flower (Figure 5.15) (Table 5.11 and Appendix 69).

Table 5.11: Analysis of variance results for the effect of SA on the up-regulation of the *CBF* gene 10 days after of spraying the wheat plants variety Tamooz 2 under well-watered and drought conditions.

Source	F value	*P* value	LSD
Water condition	124.55	<0.001	0.080
Treatment	64.89	<0.001	0.160
Water condition*treatment	7.66	<0.001	0.226

5.5. Discussion:

5.5.1. The effect of different sprays with SA on final biomass of wheat plants under both well-watered and droughted conditions.

There were highly significant differences in shoot dry weight across drought conditions and SA treatments effects with treatments (stem flower and leaf stem flower) for droughted plants in comparison with the control plants (Figure 5.5).

Water deficit is the major stress among other environmental factors and it limits plant growth mostly at the late stage. Growth characteristics of the shoot are considered as the most important parameters determining the yield in wheat plants (Shao *et al*., 2008). Moreover, the applied chemical agents such SA appeared to improve the growth parameters depending on the plant species, developmental stage, the way of application and the concentration of SA (Gutierrez-Coronado et al., 1998, Horvath et al., 2007). Back to earlier evidence, the exogenous application of SA appears to promote growth criteria of the shoot and also appears to counteract the adverse effect of abiotic stresses in a number of crop plants.

In wheat (*Triticum aestivum* L.) for example, SA-treated plants under water stress exhibited a higher dry mass (Singh and Usha, 2003). Shakirova *et al*. (2003) observed a significant effect of SA apply at earlier growth stage on wheat plants increase in plant growth under both salinity and water deficit stress. Also, Khodary (2004) and Gunes *et al*. (2007) elucidated the promotive effect of SA spray on the dry yield of maize plants (*Zea mays* L.) grown under salinity stress. Recently,

application of SA has been found to induce oxidative damage tolerance in barley plants (*Hordeum vulgare* L.) under drought stress (Habibi, 2012).

Although those studies reported improvements from SA applied at early growth stages Pancheva *et al*. (1996) previously observed the effect of exogenously applied seedling with SA concentration range 100 mM on a decrease in the growth and photosynthetic rate of barley plants (*Hordeum vulgare* L.) and Nemeth et al. (2002) indicated that the pretreatment of young maize plants (*Zea mays* L., hybrid Norma) with 0.5 mM SA decreased their net photosynthetic rate and drought tolerance. The decline in dry material weight of plants during seedling stage could be due to adverse effects of drought on photosynthesis rate for stem as a result of the reduction of cell production by meristem thus causing lower dry material accumulation in plants (Bartels and Sunkar, 2005).

Supporting the current results, Aldesuquy *et al*. (2012) have proved that foliar application of SA (grain pretreated at 0.05 M) and glycine betaine (GB - 10 mM) combined with SA on droughted wheat cultivars (*Triticum aestivum* L.) induced an increase in the dry mass of shoot at heading and anthesis when compared to control plants under stress conditions. As well, it has been observed by (Abdelkader *et al*., 2012) the treatments of SA (presoaking grains in 1 mM SA and leaves spray at pre-anthesis stage) significantly increased the plant biomass and shoot dry weight value for wheat plants *Triticum aestivum* var. Gimaza 9 and improved resistance of plants to water deficit as compared to non-stressed plants. In contrast to our results, those reports showed spraying with SA only once which was at one stage of reproductive growth either before or at anthesis.

The observed increase in shoot dry weight of droughted wheat plants might be due to the essential role of SA in the regulation of different physiological processes including stomata behavior in addition to its function as an anti-transparent which reflected on improving the leaf turgidity by regulating the stomata conductivity which is more dependent factor than photosynthetic ability itself at the time of exposing plants to the stress (Davenport *et al*., 1972).

5.5.2. The effect of different sprays with SA on yield components of wheat plants under both watered and drought conditions at the harvesting stage.

The results (Figure 5.6) show that treatment of drought stressed plants with SA at stem flower and leaf stem flower led to significant increase in a number of spikes/pot as compared to well-watered plants and others treatments.

Slatyer (1969), Boyer and Westgate (2004) and Gholamin *et al*. (2010) reports came to the same conclusion that developmental growth period (around anthesis and early grain filling) is frequently sensitive to drought stress. Wheat productivity in arid and semiarid regions climate is influenced dramatically by drought stress during maturation resulting in yield reductions of about 10 percent because of limited rainfall in spring. While, smallest yield decreases occur under moderate stress during the vegetative stage and have no effect on crop plants. A number of reports have been reviewed a crucial role of salicylic acid in regulating physiological processes such as plant growth, development and some other metabolomic processes as photosynthesis, vegetative growth, flowering and grain formation. In addition, it’s effective in increase the plant's tolerance to

adverse effects of various abiotic stress conditions (Popova *et al*., 1997, Khodary, 2004, Hayat *et al*., 2010). The current findings support the evidence that application of SA induces flowering as shown in other plants. In a previous study carried out by Cleland and Ajami (1974), treatment of cocklebur plants with SA was stimulated the flowering and so its concentration increased in the phloem when they were induced to flower by manipulating day duration. Martin-Mex *et al*. (2005) also noted an increase in the number of flowers per plant caused by the spray SA concentrations 1.0 to 0.1 mM on the shoots of African violet plants at early flowering in comparison with that of the controls plant.

In recent years, Elgamaal and Maswada (2013) have studied the ability of exogenously applied SA for stimulating the responses of yellow maize hybrids plants under two watering intervals. They found a decrease in a number of ears under water stress but an increase in productivity for treated plants with SA 0.5 and 1.0 mM resulting in more tolerance to stress. Consistent with that observed by Abdelkader *et al*. (2012) who analyzed the survival of wheat (*Triticum aestivum* var. Gimaza 9) under water deficit using different treatments. Their results showed a significant increase in morphology and yield components. Besides, the highest values of spikes number/m^2 recorded by presoaking grains in 1 mM and SA spray before anthesis comparing to non-stressed plants. Conversely, those reports indicated that low concentrations of SA are required to induce flowering. It could be explained by the reason that effect of water stress and SA plant spray are differing in terms of mode of SA application and its dose. About our results, the more effective treatments applied was SA 1.44 mM and more dependent on the times of spray at different growth stages of plants.

Spraying plants with SA (Figure 5.7) led to significant increase effects on spike dry weight of drought stress plants at each SA spray flower, stem+flower and leaf+stem+flower when compared with non-drought. Leaf+stem+flower spray gave the maximum significant difference. Also, the interaction between drought condition and SA treatments was significant ($p < 0.05$).

Results obtained in Abdelkader *et al*. (2012) support the assertion of the protective role of salicylic acid in the survival of the wheat plants (*Triticum aestivum*) under drought stress. Spikes dry weight/plant was increased by applying SA with Thiourea on droughted stress before anthesis stage. The present study contrasts with that report in terms of applying SA only at one growth stage. This may be attributable to drought effect on the dry weight of spikes and number of fertile heading during the stage of stem elongation and is widely considered as the main factor to loss the wheat yield at anthesis (Gonzalez *et al*., 2003). In order to reach the flowers to the fertile stage, maintain the flow of carbon assimilates through both pollination/ovule development is needed for enhanced the growth of spikes and so grains at anthesis (Slatyer, 1969, Cakir, 2004). Changes in the distribution of carbon assimilate among different plant organs and also the balance between photosynthesis and respiration processes are related to the nature of the drought stress (Flexas *et al*., 2006). Wheat might response to mild soil drying conditions by efficiently remobilize assimilates from stems to grains and SA may facilitate this. This could be due to the effective role of SA in the process of nutrient uptake and also an activation of the transfer of the photosynthesis products from the source to sink to meet the needs of the developmental stage for maturity.

Results of grain dry weight per pot in (Figure 5.8) showed the significant differences for both drought condition and SA treatments effects at all treatments of SA under both well watering and drought conditions. However, stem+flower treatment gave the highest mean value of grain dry weight and there was also a significant interaction between drought condition and SA treatment. In a similar way, the investigation of Nawaz *et al*. (2012) confirmed that grain weight per spike varied in an amount by means of application of water deficit stress at three and six weeks after seedling growth of wheat (*Triticum aestivum* L). In their findings found water deficit applied at later stages of growth more affected on grain yield of wheat plants as compared to its application at early growth stages. Similarly with the review about influences of drought and/or heat stresses on wheat plants, the growth and grain yield of crop plants were more sensitive to both stresses during late reproductive growth stage than early developmental stage (Prasad et al., 2008a). In another study Grown (2012) revealed that growth and yield of two sunflower cultivars were improved by SA spraying on plants grown in sandy soil. As a result, treatments of 75 and 100 mg/l for Sakha 53 and Giza 102 significantly enhanced seeds weight/ head of treated plants as compared with the untreated plants. Similar productivity promoting response resulted in significant high values for grains dry weight/plant and alleviated the moderate drought stress effect. Also, study Azimi *et al*. (2013) on the regulatory effective role of salicylic acid in the physiological process of fertilization which resulting in elevation the grain dry weight significantly near the flowering stage for plants under moisture stress. A rise of grain yield could be interpreted to the final yield of wheat plants more affected by drought stress during stage of early grains development (Saini and

Westgate, 1999). In that way, spraying plants before spike emergence and at the flowering stage can result in an improved weight of grains per spike as well as the spikes number.

The comparison between means showed that both sprays stem flower and leaf stem flower significantly increased average 1000 grains dry weight and it recorded higher level among other SA treatment under drought stress as compared with non-drought (Figure 5.9).

These results similar to that observed by Hussain *et al*. (2008) who demonstrated the positive effects of exogenous application of SA 0.724 mM combined with GB 100 mM in improving the yield of hybrid sunflower (*Helianthus annuus* L.) under different irrigation regimes. They concluded that GB and SA application significantly increased achene yield in terms of average 1000-achene weight under water stress and the effect was more substantial when applied at the flowering stage than at the vegetative stage. Moreover, Azimi *et al*. (2013) found that water stress had a negative significant effect on growth and yield characteristics of unsprayed wheat plants, but treating with SA at anthesis and early grain filling improved the response of Zarrin wheat cultivar under drought stress as compared to control plants and the maximum tillers number/m^2 obtained at concentration of 1.5 mM. SA improved the yield of wheat seed. The present results point to non-significant effect on YW349286D of spraying the plants with SA on X at earlier growth stages, but comparing to SA treatments at late growth stages, the grain yield was increased significantly as a result of remobilization of the carbon from stems to grains which in turn improved number of fertile spikes

per plant and number of grains per main spike. Unlike, Zamaninejad *et al*. (2013) who found an interaction effect of drought stress and spraying maize plants (*Zea mays* L.) with SA 1 mM at the leaf stage increased 1000 kernel weight (gr) in flowering and grain filling. Our observation is in contrast with this finding because their results exhibited a higher grain yield related to SA applied under early drought stress than late drought stress.

5.5.3. Effect of spraying wheat plants with SA on *CBF14* expression at different stages of growth under normal and drought conditions.

The importance of TFs group especially *DREB/CBF* genes is known to regulate the expression of several downstream stress responsive genes such as rd29, late embryogenesis abundant protein (LEA) genes. *DREB* is expressed under different abiotic stresses for example, in the model plant, *Arabidopsis thaliana* the *AtDREB1/CBF* gene is induced by low temperature stress 4 ^{o}C within time 10 min. In general, *DREB* genes and mostly subgroups (*DREB1*) proteins possess conserved sequences, and it contains a highly conserved AP2/ERF DNA-binding domain (Stockinger et al., 1997, Liu et al., 1998a). It has also been revealed that genes including to the *DREB1* subgroup were induced in common in many herbaceous crop plants, such as rice, wheat, barley and maize by cold, drought and salt stresses (Lata and Prasad, 2011).

Monitoring the upregulation of *CBF*/*DREB* is notoriously difficult and it is only possible to take “snap-shots” of its activity at standard times. It appears from the results presented here that it varies temporally i.e. over time after, receiving a stimulus. Furthermore, that stimulus could be the immediate application of SA, a

residual level of SA from a previous application or drought. Furthermore, since drought is a dynamic stress, the actual amount of drought needed to elicit a CBF/DREB upregulation is extremely difficult to determine.

A pattern of the *CBF* gene up-regulation has been reported by Al-Issawi (2013) where the expression of *CBF14* increased on the first day of exposure of vegetative wheat (European and Iraqi genotypes) to low temperature at 4^{o}C but then it started to decrease during the next few days but remained above control levels. In this work, a connection between molybdenum application and molecular mechanism response to drought was established leading to stimulation of drought resistance and improved survival under the stress. Rihan (2014) also confirmed the effect of cold stress on the upregulation of *CBF/DREB1* leading to improved cold tolerance in cauliflower using plant tissue culture techniques. Additionally, Chu *et al*. (2014) have analyzed the up-regulation patterns of two *DREB1* genes in black poplar (*Populus nigra*) by quantitative RT-PCR under normal growth and abiotic conditions with ABA treatment. The expression of *PnDREB68* and *PnDREB69* was rapidly induced by cold stress in leaf and stem tissues exceeding two-fold that of control levels at 2 h after application and a slow response to cold stress was observed in roots relative to the control at 8 h. When salt stress NaCl was applied, the *PnDREB68* and *PnDREB69* showed elevated expression levels in stem tissues at 8 and 48 h after the stress was applied.

Given the vagaries of expression patterns of *CBF*/*DREB* and the complexity of the experiment carried out in this chapter, it is not surprising that a simple picture did not emerge for *CBF*/*DREB* expression. Nevertheless, it is clear that both

drought and more importantly SA could upregulate the *CBF*/*DREB* expression and this could explain the positive biomass/yield effects found.

The *DREB* transcription factors are known to be directly involved in the protection of cells from damage caused by dehydration and include signal transaction cascades and activation/regulation of other transcriptional factors (Agarwal *et al*., 2006). The current research supports the evidence that SA provides the protection to the plants by its act as regulatory molecules in the expression of *CBF/DREB* regulon and it has the role in inducing the mechanism of plant resistance to drought stress (Lata *et al*., 2015). The results Figure 5.10 and 5.12 showed that SA sprays of leaf only and stem only not having the effect increasing *CBF*/*DREB* expression to drought 2 days after spraying. While Figure 5.14 showed some indications of the influence of SA sprays during stem extension plus flowering stages at interval 2 days after treating the plants in up-regulating the *CBF* gene under drought stress as compared with their control plants in wheat plants variety Tamooz 2. Oppositely, the expression level of *CBF* gene decreased 10 days after spraying the plants as presented in Figure 5.15.

Conformity with Morran *et al*. (2011) have shown the expression levels of the *DREB* TFs of control and transgenic wheat and barley plants at the time of 3-4 and 7-10 days after their exposure to moisture content stress. The analysis of stress-inducible genes in transgenic *Triticum aestivum* cv. Chinese Spring plants by the inducible promoter (Rab17 gene from maize) indicated over up-regulation of both downstream genes *TaDREB2* and *TaDREB3* which resulted in plants more tolerant to severe drought and cold stresses. In spite of transcript

expression levels of both genes being strongly up-regulated in leaf tissues by drought and slightly by cold in wheat and barley as compared to grains, the improved survival of the transgenic barley plants was observed through the over expressed *TaDREB2* more than *TaDREB3* as compared with non-transgenic controls. On the contrary, *DREB1A* transcription factors of *Arabidopsis thaliana* that regulate the expression of drought tolerance genes have showed the induction at 10 days of exposure the transgenic bread wheat plants to drought. The *DREBs* was transferred into the wheat plants and it manipulated by the promoter rd29A so as to involve in plant stress signaling and develop stress tolerance (Pellegrineschi *et al*., 2004). The reason of this might be related to having a different category of transcription factors which act as regulators of drought-responsive gene expression in direct or indirect ways (Bray, 2004). These studies are in opposition to the existing experiment due to their performance by transgenic technique under drought stress in addition to using analysis of Northern blot hybridization with q-PCR analysis.

5.6. Conclusion

Based on the results of different sprays with SA on yield components of wheat plants under both watering regimes the watered and drought conditions, SA treatments of stem+flower as well as leaf+stem+flower significantly improved wheat plants exposed to drought stress condition. Furthermore spraying wheat plants with SA had positive effects on the up-regulation of drought response gene *CBF*/*DREB*, and whilst there was variance in the gene expression at different growth stages there was some consistency between treatments. It can be concluded that spraying the plants with SA during growing period of stem extension plus flowering stage is important to develop the crops yield and also to improve drought tolerance in stressed plants through the upregulation of the *CBF/DREB* gene and its downstream regulon.

Chapter 6

6. General Discussion

6. General Discussion

Shortage of water resources is one of the main limiting factors affecting wheat production in many Mediterranean and semi-arid regions and especially challenging in countries with a growing population (Passioura, 2002, Singh and Chaudhary, 2006). It is essential to be making efforts in agronomy and crop improvement to select the most suitable varieties of crop plants for improved agronomic traits such as yield and yield components and sustaining their productivity under dry farming conditions (Xoconostle-Cazares *et al*., 2010). In response, the present study was conducted to study the performance of varieties of Iraqi-bread wheat under two watering regimes, and to investigate whether the application of exogenous elicitors can influence their yield response.

Among major morphological traits in drought environment regions, seed vigour is often considered as a reliable indicator to screen for drought tolerant varieties and helps potentially with the selection of varieties for improving the yield capacity in wheat. This was taken as an indication of wheat improvement by Bin Abdul Hamid (2012) who assumed that seed germination and vigour are basics for increasing the emergence rate and fall stand establishment of winter wheat plants and to produce healthy seedlings, but have less influence on the drought tolerance at a later stage of growth. Therefore, the rate and degree of the seedling establishment are particularly important factors to produce an acceptable crop yield and determine the time of maturity. According to the seed germination capacity, all of the Iraqi wheat varieties met the International Seed Trade Association (ISTA) minimum standards. Also, Bin Abdul Hamid (2012) deduced

that early seedling vigour of winter wheat was associated with genotypes that were adaptated to irrigated system conditions and they found that highest seed vigour was observed for cultivar Goodstreak compared to Harry and Wesley cultivars. They assumed that the evaluation of seed vigour provided the possibility of improving seedling drought resistance ability under low moisture conditions but did not go on to test performance to maturity. Whilst this approach was reasonable, it needed to extend to the full growing season in order to determine whether seedling vigour was correlated with yield under drought conditions.

Moisture holding capacity is an important physical characteristics of the soil related to yield potential especially in low rainfall regions. Measuring moisture holding capacity however is not always straightforward especially in the field and requires probes to estimate the degree of moisture available. There is a reliable relationship between change in the soil drying measurement over time with the water content measured by TDR (Theta Probe) in various soil conditions (Topp *et al*., 1984) but the actual relationship depends on the soil type. Furthermore, where moisture loss is pricipally via surface evaporation, there are expected to be variations in TDR relationships down the soil profile, and this was the case in the current experimentation. The value of water status of the surface layer of soil is commonly affected by climatological conditions in the Mediterranean environments where soil surface temperatures can be high under intense irradiation conditions. For example under drought stress conditions, up to 40% of available water may be lost by evaporation directly from the soil surface (Loss and Siddique, 1994). Measurement of the availability of soil water was required to carry out the drought stress experiments presented in this thesis since stress

had to be managed over an entire growing cycle. The results showed that the Theta Probe values measured for different sites of the pots in the absence and presence of wheat were slightly different from their moisture content measurements so that different curvilinear relationships existed at different profiles in the soil. Nevertheless it was concluded that Theta Probe measurements can be used to obtain reliable soil moisture content estimates by taking measurements at the surface of pots in the presence of wheat plants and was a satisfactory way to monitor drought stress levels in pot experiments subject to variable watering regimes (70% and 50% FC indicating well watered and moderate drought conditions respectively). This agrees with work done by Topp et al. (1980); Topp and Davis (1985) who determined the average volumetric water content in order to calibrate with gravimetric measurement. It is proposed however, that the variation in the characteristic of the water content of the top layer of soil can be related to the distribution (density) of plant species and their subsequent productivity.

Most higher plants possess various protecting mechanisms to respond and adapt their growth to changes in water availability (water stress) at the whole plant level. These responses are dependent on the severity and duration of the stress (Bray, 1997), but also on the developmental stage and physiological parameters of the plant (Hirt and Shinozaki, 2004). Stomatal closure has been considered as an initial drought response feature which can trigger various other mechanisms of physiological responses in plants so as to reduce the loss of water through transpiration and hence maintain water use efficiency in the leaf tissue and improve physiological processes of the crop during the early stages of soil drying (Munir *et al*., 2007). Praba *et al*. (2009) demonstrated that the drought tolerant

varieties in both rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.) maintained lower stomatal conductance and transpiration rates than drought susceptible varieties during severe water-limited conditions at both vegetative and reproductive growth stages.

One of the main characteristics concerning the study of the physiological responses of plants to low water potentials is ultimately biomass production. The importance of drought tolerance in crop plants is not whether they survive under stress, but whether they show good yields under stress conditions (Pereira, 1993). Several studies have focused on the consequences of drought on non-stomatal factors including stem dry matter as well as the correlation to leaf photosynthesis and plant water relations. Bin Abdul Hamid (2012) evaluated drought tolerance in winter wheat cultivars during the early phase of growth under controlled conditions in the greenhouse and reported that shoot dry biomass showed that cv. Wesley was a drought-sensitive cultivar compared to cvs. Harry and Goodstreak because it had lower adaptation to well-irrigated wheat production which was related to a decline in the water use efficiency and leaf photosynthetic rate. Similarly the reduction in final dry matter was reported for oat plants (*Avena sativa* L.) and reflected the severe drought effect at the vegetative stage (Sandhu and Horton, 1977).

In the current work, to determine the tolerance of Iraqi wheat cultivars to a moderate drought stress regime, different cultivars were assessed for various physiological traits. The results indicated that exposure of wheat plants to water deficit stress significantly decreased the leaf stomatal conductance, soil water content and the biomass and its associated yield components. Wheat cultivars Rizgary and Tamooz 2 were more tolerant to water stress than Adana 99 and

Sham 6. A decrease was observed in the photosynthetic rate in response to stomatal closure under water deficit stress and it is suggested that this can be controlled by increased levels of abscisic acid (ABA) in leaves which is linked to the regulation of stomatal movement restricting CO_2 diffusion to the chloroplast and therefore affecting the main enzymes ribulose bisphosphatase carboxylase/oxygenase that correlate to the carbon dioxide fixation for the leaf photosynthesis process (Lawlor, 1995, Medrano *et al*., 2002, Lawlor and Tezara, 2009). Whist ABA was not measured in the current experiments, there is no reason to doubt that this mechanism of stomatal regulation was evident. Flexas *et al*. (2006) also commented that photosynthetic responses under water deficit stress conditions impair the performance of the anabolic process mostly as a result of low carbon dioxide supply. Such inhibitory effects of drought stress on the mechanism of CO_2 assimilation has been reported in a number of crop plants such as maize (*Zea mays* L.) (Boyer and McPherson, 1975), rice (*Oryza sativa* L.) (Cutler *et al*., 1980), wheat (*Triticum aestivum* L) (Johnson and Moss, 1976) and barley (*Hordeum vulgare* L.) (Matsuda and Riazi, 1981).

These physiological traits of plant response to drought are directly activated by changing the water status of plant tissues or may be elicited by chemical signalling molecules that are needed to direct transduction cascades of pathways for improvement of the adaptation of the plant to stress conditions (Reddy *et al*., 2004). For instance, the ABA signaling molecules and second messengers Ca^{+2} ions are the transducers of stress signal information from membrane receptors to activate metabolism and initiate a set of signalling network pathways which regulate physiological responses to environmental stress factors (Xiong *et al*., 2002). Allen *et al*. (2001) have reported that the extent of Ca^{+2} signalling in guard

cells plays a role in the dynamics of stomatal behaviour in terms of stomatal movements (opening and closure). Salicylic acid has been reported to influence the regulation of various physiological and biochemical processes involved in plant growth and development and to induce the mechanism of protection and resistance to abiotic stress (Raskin, 1992, Popova *et al*., 1997, Lopez-Delgado *et al*., 1998). In a study investgating the influence of spraying SA on the regulation of different physiological processes in wheat plants, it was found that the application of a low concentration of SA (100 mg L^{-1}) led to enhanced growth-related characteristics and yield when compared to untreated plant growth. This was interpreted as an increased photosynthetic activity leading to increasing number of tiller and leaves (Amin et al., 2008). In other words, photosyntate "source" was maintained leading to the survival and proliferation of growth "sinks" (i.e. leaves and tillers).

In order to ensure productive wheat plants under challenging environmental conditions, the exogenous application of the chemical elicitor molecule SA has been proposed by several researchers to develop drought stress tolerant in crop plants. Tuna *et al*. (2007) revealed that application of a low dose of SA derivatives had an effective role in decreasing the adverse effect of salinity stressed maize (*Zea mays* l.) plants. The ameliorating role of spray with SA on drought stress was also observed by Singh and Usha (2003) in wheat plants, by Farooq et al. (2009a) in rice plants and by Elgamaal and Maswada (2013) in maize plants.

From the early experiments conducted here, the evaluation of the most significant physiological traits helped to choose both wheat varieties Tamooz 2 and Adana

99 to study the effect of spraying plants with salicylic acid and molybdenum on their responses to water deficit stress. Results of this study confirmed that despite the fact that drought conditions had negative significant effects on the leaf stomatal conductance and shoot dry weight, these measurements were not reflected in their interaction to improve yield and they are not selective parameters of growth to be depended on in the selection drought tolerance of wheat varieties. The exogenous spraying of plants with SA 1.44 mM however, did lead to a decrease in the adverse effect of the drought condition on seedling growth of variety Tamooz 2 rather than Adana 99.

The explanation of the effective role of treatment with salicylic acid may be due to its participation in the regulation of physiological processes in plants such as stomatal closure to improve the rate of carbon assimilation under osmotic stress and also in the protection of functional enzymes and lipids of the plants photosynthetic apparatus by inducing nutrient uptake to accumulate carbohydrates compounds in leaves (Raskin, 1992, Khan *et al*., 2003, Hayat *et al*., 2010)

At the molecular level, plants primarily adapt to a particular stress to survive by the expression of specific stress-induced genes which in turn direct specific biochemical and physiological processes of adaptation. One of the first products of stress-induced molecular responses is the unpregulation of transcription factor (TFs) genes. One of the ubiquitously expressed TF is the dehydration-responsive element binding protein (*DREB*) known to be involved in enhancing the tolerance of many plants to stress (Wang *et al*., 2006). In many plants and in wheat particularly dehydration stress response is regulated by the *CBF* (DRE-binding) dehydration-responsive element regulon which was firstly found by the analysis

of dehydration stress responsive genes in *Arabidopsis thaliana*. It has been concluded that the transcription factor *DREB*2A is a major subgroup of *DREB* proteins which specifically interact with DRE/C-repeat cis-acting present in the promoter region of target genes to activate the expression and upregulation of the dehydration responsive gene *CBF* to improve the drought tolerance in plants. The *DREB*s are known as the members of a family of transcription factors of plants and they classified by having the AP2 DNA binding domain to identify the drought responsive element in target promoters (Liu et al., 1998a, Sakuma et al., 2006a). Interestingly, a number of researchers have focused on the family of TFs which is the *DREB* genes or C-repeat binding factor gene (*CBF*s) and their role in inducing the tolerance of plants to water deficit conditions by controlling the characters related to the responses such as osmoprotection and metabolism processes (Bray, 2004, Shinozaki and Yamaguchi-Shinozaki, 2007, Chen *et al*., 2008). *DREB* and *CBF* genes have very high homology and it is commonly considered that they are the same gene family. The physiological traits of protecting plants against stress however may vary according to the growth stage of plants. It was verified that water deficit stress is the critical factor among various environmental conditions affect on growth stages of the wheat crop but it more affected when the stress occurs at late stages of growth including tillering, booting, anthesis, grain formation and grain filling and it substantially reduces grain yield due to the critical impact of dry matter and stored nitrogen during the pre-anthesis transition stage in Mediterranean environments (Mahpara *et al*., 2014). Also, Mohamed (2013) concluded that net assimilation rate of safflower plants (*Carthamus tinctorius* L.) was significantly decreased by the water stress effect

with the close values at growth stages of stem elongation and beginning of flowering as compared with control plants.

Accordingly, the physiological and yield characteristics of wheat plant tolerance in the existing study were combined with investigating the expression level of the *CBF*14 (a commonly expressed *DREB* family TF) gene at different growth stages under drought stress on cv. Tamooz 2. Tamooz 2 was chosen because it showed an indication of the improvement in growth traits in terms of its biomass production as shown by the results of the second drought stress experiment (Chapter 4). It was observed that spraying Tamooz 2 with SA1 effectively diminished the negative effects of drought stress imposed at the growing period of stem extension to flowering and resulted in increasing the crop yield. Also, it had a significant impact on the improvement of drought tolerance in stressed plants through the upregulation of the *CBF* gene and its downstream regulon. There was some indication of the influence of spraying with SA during stem extension and flowering stages on the expression the *CBF* gene under drought stress at 2 days after the treatment in comparison with their control plants, but the level of *CBF* gene expression decreased 10 days after spraying the plants. The pattern of expression *CBF* gene in this study is related to the evidence presented by Al-Issawi (2013) who has recently investigated the possibility of the up-regulation of *CBF*14 gene in both Iraqi and European wheat plants exposed to cold stress where the *CBF* gene was upregulated after 24h exposure to low temperature (4 ^{o}C) in the early growth stage of spike emergence of Iraqi wheat and then the *CBF* upregulation declined drammatically up to 14 days in the late stage of spike emergence. However, there was no expression in non-acclimated plants of comparative non-adapted european variety of wheat.

The activity of *CBF*/*DREB* class of genes can also be regulated by ABA-dependent cascade signalling pathways (Shinozaki and Yamaguchi-Shinozaki, 1997, Xiong *et al*., 2002, Seki *et al*., 2003) indicating a dual role for ABA, that is, regulation of gene expression of the drought response regulon influencing many gene products by acting as transcriptional regulator molecule to induce the transduction signalling pathway against the effect of abiotic stress and as the regulator of stomatal closure leading to a physiological response to stress to reduce water loss under drought. It is suggested that SA can also act as a regulatory molecule in the expression of *CBF*/*DREB* regulon and has a role in inducing the mechanism of plant protection against drought stress (Lata *et al*., 2015) and this is supported by the results presented in this thesis.

The genetic modification of *CBF*/*DREB* has been attempted in a variety of model species (eg Arabidopsis) and crop plants (eg Barley and Wheat) as an approach to genetically improve drought stress (Zhu *et al* 2004, Moran *et al* 2011). Most modifications report some improvement in stress resistance (cold, salt or drought) but with a growth depression consequence which in itself depresses yield. Other genetic modification approaches are targeting key physiological process limitations such as in C4 and C3 photosynthesis (Rengan *et al* 2016). These approaches offer long-term genetic solutions but are extremely complex due to the numerous genes involved and the interactions between their gene products. It is clear from the work presented in this chapter that exogenous applications of some compounds which could be termed “elicitors” (salicylic acid and molybdenum) have gene regulation functions. It is clear that this needs further investigation to fully explain the function of this interaction.

Chapter 7

7. General Conclusions, Limitations of the study and Recommendation for future work

7.1. General Conclusions

The following conclusions can be drawn from this work on the response of Iraqi bread wheat plants to drought stress conditions.

Watering regime management is needed for the environment of Iraq as many semi-Arid countries of the Mediterranean regions in which water is a limiting factor for plant growth and productivity.

Drought stress treatment caused a considerable reduction in the physiological characteristics of Iraqi wheat varieties such as the leaf stomatal conductance, chlorophyll a fluorescence and soil moisture content compared with the plants under normal watering conditions. As a consequence, drought had a significant influence on biomass yield traits in terms of the number of tillers and stem bundle weight. The best shoot dry weights under water stress condition were observed for both resistant wheat cvs. Rizgary and Tamooz 2 compared to susceptible cvs. Adana 99 and Sham 6.

It was shown that spraying with SA led to reduction of the effect of drought stress in cv. Tamooz 2 compared to Adana 99 by minimising the stomatal conductance and transpiration rate. Cv. Tamooz showed a significant interaction with watering conditions and was characterised to be a tolerant variety based on the grain yield trait. Accordingly, it was determined that variety Tamooz 2 had higher values for most growth traits under well-watered and drought conditions and when sprayed with the lower concentration of salicylic acid (1.44 mM).

Spraying wheat variety Tamooz 2 with a low concentration of SA during stem elongation and flowering significantly enhanced the yield components of spike number and grains dry weight. There was also an indication of a significant induction of the expression levels of drought-responsive gene *CBF*14 (a member of the *CBF*/*DREB* transcription factor family) by the drought stress conditions especially after 2 days of the spray and it is deduced that this assisted the drought tolerance because the yield traits of wheat are more affected by drought at these two critical stages of the growth cycle.

In any agricultural system there are two approaches, agronomic and genetic, to influencing the effects of stress. Agronomic approaches include water management and intervention with sprays whilst genetic approaches include standard selective breeding and genetic modification. Agronomic approaches tend to precede genetic approaches because of the long lead time for genetic improvements (up to 15 years). However, genetic approaches provide a more sustainable long term solution. The work presented in this thesis indicates that an agronomic approach using exogenous applications of salicylic acid and/or molybdenum could be used in the field and offers growers a short-term improvement in wheat yield when stressed with drought. These results need to be verified in the field but could be a very useful treatment in wheat farming in Iraq.

7.2. Limitations of the study

The key difficulties encountered in this study was the identification of accurate drought tolerance traits of Iraqi wheat varieties due to the fact that wheat was grown in environmental conditions very different from its original climate. The varieties were very susceptible to powdery mildew and aphids necessitating constant crop protection spraying.

In the experiment of spraying plants with two different concentrations of SA and Mo, the big challenges was to grow 2 wheat varieties in pots with soil weighing around 14 kg and which were adjacent to each other because of the limited glasshouse space. It was necessary to take great care when spraying plants with treatments to avoid cross contamination. Great efforts and a long time was spent on taking yield component measurement following the harvest.

It was a bit more complicated combining a study of the physiological traits with a molecular level of response for yield improvement in the third drought experiment in the greenhouse because the regulation of specific gene products *CBF*14 in wheat variety Tamooz 2 depends upon the growth stage in which the crop was sprayed with SA. Additionally, owing to the fact that wheat *Triticum aestivum* has some identical sequence of its genome due to the fact that it is hexaploid (2n= 6x) with AA BB & DD chromosomes. As a consequence, transcription factors such as *CBF*/ *DREB*s are expected to be upregulated very strongly under drought stress conditions.

7.3. Recommendation for future work

The results of this investigation proved that drought stress had more affect in the reproductive development stages of growth than the seedling and was associated with the determination of grain yield as well. The application of growth elicitor salicylic acid enhanced plant growth and development and it had a defensive role in contradiction of drought stress. However, the findings demonstrated some indication on the effect of spray with SA on upregulation the expression level of target gene *CBF* after 2 days of spray during stem elongation and flowering stages.

It is recently reported that not only are transcription factors upregulated but also a class of dehydrin protein named COR15a. Researchers Al-Issawi (2013) and Rihan (2014) proved that accumulation of stress protein COR15a contributes in the activation of plant tolerance to stress (cold) conditions of wheat and cauliflower plants respectively.

Because it is supposed that COR15a protein plays the same vital role in dehydration stress tolerance as in cold by promoting the ability of the plants to survive stress cellular tolerance of dehydration through protection the functions of cytoplasm and maintaining the balance of cell water to regulate the hydraulic conductivity of membranes by stomatal regulation (Close, 1997). The upregulation of COR15a is very slow and probably take about 10 days or 2 weeks following spraying the plants to improve plant response to drought and also acclimation. Therefore, one of the interesting suggestions for future research will be to focus efforts on the actual gap in knowledge concerning the analysis of protein COR15a in bread wheat by the exposure to drought at the critical stage

of growth stem extension and flowering under treatment of SA to obtain more valuable information about the molecular mechanism of response of the plants to such stress.

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Appendixes

Appendix 1:

Publications, professional membership and conferences attended

Publications

Effect of foliar application of Salicylic acid on growth, yield components and chemical constituents of Wheat (*Triticum aestivum* L. var. Cham 6). 5th Scientific Conference of Agriculture College/ Tikrit University/ Iraq, 2010.

Effect of Salicylic Acid on Some Biomass and Biochemical Changes of Drought-Stressed Wheat (*Triticum aestivum* L. Var. Cham 6) Seedlings. Research Journal of Duhok University/ Kurdistan region, Iraq, 2011.

The effect of exogenous applications of salicylic acid and molybdenum on the tolerance of drought in wheat. Journal of Agricultural Research and Technology, August 2017.

Professional membership

Kurdish Postgraduate Society, University of Plymouth 2013.

Member of Society of Chemical Industry (SCI- BioResources Young Research) 2014.

Conferences attended

The investigation of drought tolerance of Iraqi wheat varieties. (Poster presentation) - AgriScience into Practice" 4th C.A.R.S PG Symposium - in BBSRC North Wyke Experimental Station, Rothamsted Research, Okehampton, 6th June 2014.

The analysis of physiological response of four Iraqi wheat varieties towards drought stress regime. (Oral presentation) - Sowing the seeds of Land-based Researcher Development" 6th CARS Postgraduate Symposium, Eden Project, Boldeva, Cornwall, UK, 19th November 2014.

The effect of exogenous applications of salicylic acid at different growth stages to Iraqi wheat cv. Tamooz 2 on the up-regulation of the drought response regulon. (Poster presentation) - 3rd Global Summit on Plant Science Conference Series, Rome, Italy, 7-9 August 2017.

Appendix 2:

Postgraduate skills development training and workshops were attended at University of Plymouth:

Molecular Methods in Biological Sciences Workshop .....1st Jul 2013

Bio 5124 Module (Biology) ..... 24th Oct 2013

Induction day for new research students..... 23rd Oct 2013

Plagiarism ..... 11th Dec 2013

Project management ..... 6th Jan 2014

Word 2010 structuring your thesis .....14th Jan 2014

Excel 2010, introduction to essential features .....15th Jan 2014

Research-owning and using..... 16th Jan 2014

Overview to searching and accessing information resources..... 29th Jan 2014

Presenting at conferences ..... 29th Jan 2014

Power point creates and enhances your presentation .....5th Feb 2014

Spss ..... 5th Feb 2014

Excel sorting, filtering and pivot tables ..... 17th Feb 2014

MS project 2010 .....19thFeb 2014

Effective reading ..... 24th Feb 2014

Conditional formatting and charts ..... 25th Feb 2014

Career planning for first year PhD students ..... 26th Feb 2014

Iraqi day conference, University of Plymouth ..... 1st Mar 2014

Appendixes

The interview workshop ..... 4th Mar 2014

Scientific writing skills for environmental scientists workshop .....7th Mar 2014

Word master documents and navigation pane ..... 10th Mar 2014

Making progress: Avoiding defeatism and self-sabotage..... 10th Mar 2014

L and M questioning and listening ..... 11th Mar 2014

Entrepreneuship-maximising research .....12th Mar 2014

The transfer process 14thMar 2014

Research methods. Science, Technology, Engineering and Medicine..... 19th Mar 2014

Careers: Strengths and skills in professional development .....26th Mar 2014

Excel as a database .....2nd Apr 2014

Critical thinking Workshop.....30th Apr 2014

Introduction to EndNote ..... 2nd Jun 2014

Public engagement ..... 3rd Jun 2014

Submit on Pearl..... 10th Jun 2014

Writing up and completing the thesis ..... 11th Jun 2014

The postgraduate society annual conference, University of Plymouth.....17th Jun 2014

Introduction to Statistics for Life Scientists Workshop ..... 5th August 2014

Ecology and Marine Biology Seminar Series..... 2013-2014

Preparing for the Viva..... 17th November 2015

Appendix 3: ANOVA table results for the effect of two watering regimes on the stomatal conductance of four Iraqi wheat varieties.

Analysis of Variance for Stomatal conductance

Source	DF	SS	MS	F	P
Variety	3	0.25762	0.08587	5.71	0.005
Condition	1	0.23113	0.23113	15.36	0.001
Variety*Condition	3	0.04429	0.01476	0.98	0.421
Rep	3	0.12350	0.04117	2.74	0.069
Error	21	0.31606	0.01505		
Total	31	0.97261			

Appendix 4: ANOVA table results for the effect of two watering regimes on the soil moisture content of four Iraqi wheat varieties.

Analysis of Variance for Moisture Content

Source	DF	SS	MS	F	P
Variety	3	83.1	27.7	0.20	0.892
Condition	1	822.5	822.5	6.06	0.023
Variety*Condition	3	272.7	90.9	0.67	0.580
Rep	3	317.5	105.8	0.78	0.518
Error	21	2849.8	135.7		
Total	31	4345.7			

Appendix 5: ANOVA table results for the effect of two watering regimes on the leaf area of four Iraqi wheat varieties.

Analysis of Variance for Leaves Area

Source	DF	SS	MS	F	P
Variety	3	3779.1	1259.7	7.11	0.002
Condition	1	232.9	232.9	1.31	0.264
Variety*Condition	3	39.4	13.1	0.07	0.973
Rep	3	2722.3	907.4	5.12	0.008
Error	21	3719.6	177.1		
Total	31	10493.3			

Appendix 6: ANOVA table results for the effect of two watering regimes on the total shoot dry weight/pot of four Iraqi wheat varieties.

Analysis of Variance for Total shoot drywt.

Source	DF	SS	MS	F	P
Variety	3	26.289	8.763	2.02	0.141
Condition	1	20.632	20.632	4.76	0.041
Variety*Condition	3	2.597	0.866	0.20	0.895
Rep	3	49.303	16.434	3.79	0.026
Error	21	90.961	4.331		
Total	31	189.783			

Appendix 7: ANOVA table results for the effect of two watering regimes on the total chlorophyll content of four Iraqi wheat varieties.

Analysis of Variance for Total chlorophyll

Source	DF	SS	MS	F	P
Variety	3	0.2317	0.0772	0.77	0.584
condition	1	0.6124	0.6124	6.07	0.091
Error	3	0.3027	0.1009		
Total	7	1.1468			

Appendix 8: ANOVA table results for the effect of two watering regimes on the stem number of four Iraqi wheat varieties.

Analysis of Variance for Stems No.

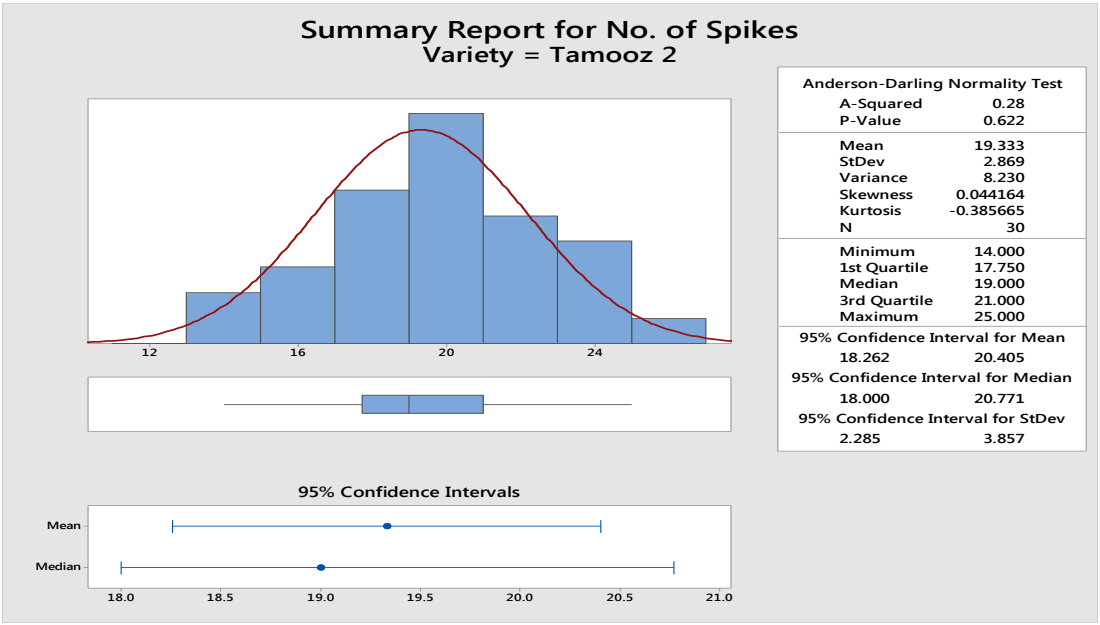
Source	DF	SS	MS	F	P
Variety	3	121.594	40.531	8.81	0.001
condition	1	30.031	30.031	6.52	0.018
Variety*condition	3	3.844	1.281	0.28	0.840
Rep	3	70.594	23.531	5.11	0.008
Error	21	96.656	4.603		
Total	31	322.719			

Appendix 9: ANOVA table results for the effect of two watering regimes on the stem weight of four Iraqi wheat varieties.

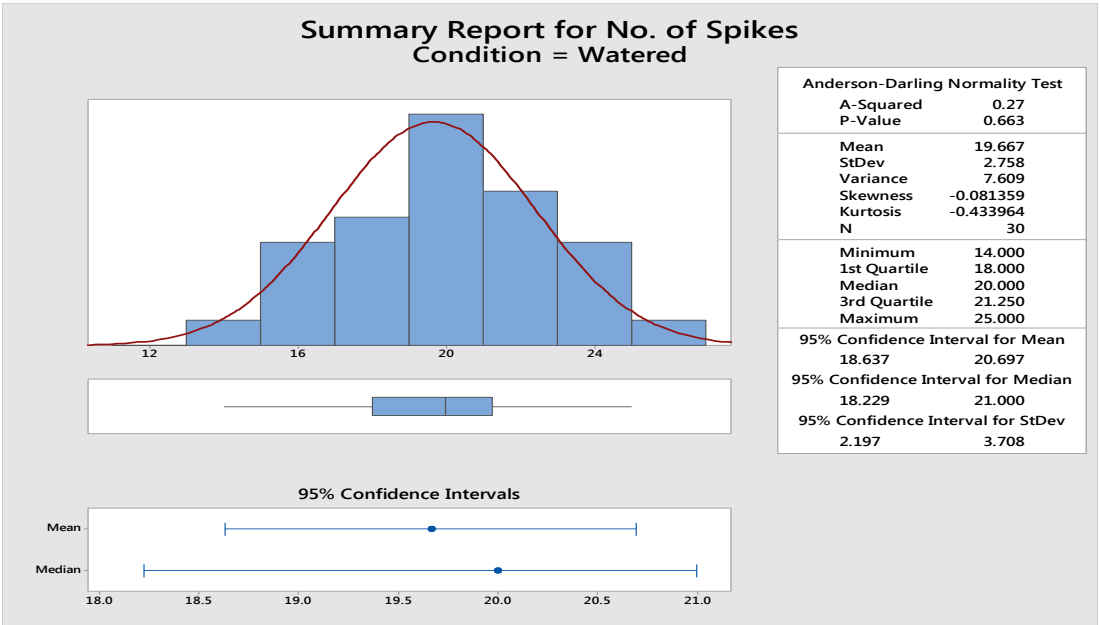
Analysis of Variance for Bundles wt.

Source	DF	SS	MS	F	P
Variety	3	1305.89	435.30	15.24	0.000
condition	1	402.08	402.08	14.07	0.001
Variety*condition	3	43.93	14.64	0.51	0.678
Rep	3	244.49	81.50	2.85	0.062
Error	21	599.93	28.57		
Total	31	2596.33			

Appendix 10: Summary result of the normality test for number of spikes/pot of variety Tamooz 2.



Appendix 11: Summary result of the normality test for number of spikes/pot under well-watered condition.



Appendix 12: ANOVA table results for water condition * variety interaction effect on leaf stomatal conductance measurement.

Analysis of Variance for Stomatal Conductance.

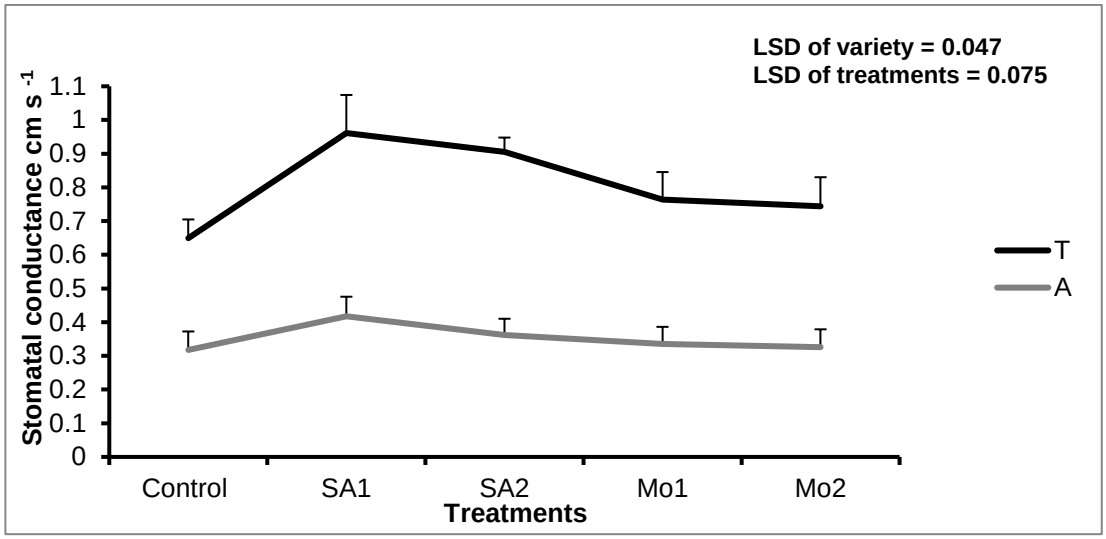
Source	DF	SS	MS	F	P
Variety	1	0.155622	0.155622	27.58	0.000
Condition	1	0.839480	0.839480	148.77	0.000
Variety*Condition	1	0.100950	0.100950	17.89	0.000
Rep	14	0.440520	0.031466	5.58	0.000
Error	42	0.236994	0.005643		
Total	59	1.773566			

Appendix 13: ANOVA table results for chemical treatments of SA and Mo effect on leaf stomatal conductance measurement.

Analysis of Variance for Stomatal Conductance.

Source	DF	SS	MS	F	P
Treatments	4	0.192495	0.048124	7.57	0.000
Reps	11	1.301240	0.118295	18.60	0.000
Error	44	0.279831	0.006360		
Total	59	1.773566			

Appendix 14: Interaction graph to show the effect of spraying the plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on leaf stomatal conductance of varieties Tamooz 2 and Adana 99 ($p \leq 0.001$ for variety. $p = 0.001$ for treatments. $p = 0.270$ for variety * treatments, error bars = SE).



Appendix 15: ANOVA table results for chemical treatments * variety interaction effect on leaf stomatal conductance measurement.

Analysis of Variance for Stomatal Conduct

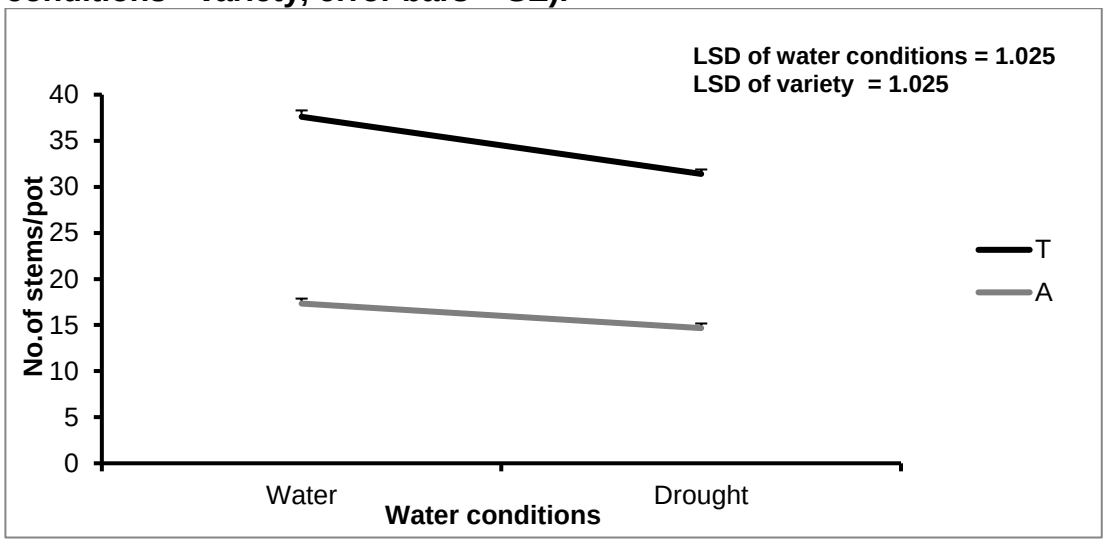
Source	DF	SS	MS	F	P
Variety	1	0.155622	0.155622	18.69	0.000
Treatments	4	0.192495	0.048124	5.78	0.001
Variety*Treatments	4	0.044656	0.011164	1.34	0.270
Reps	5	1.006092	0.201218	24.17	0.000
Error	45	0.374701	0.008327		
Total	59	1.773566			

Appendix 16: ANOVA table results for chemical treatments * water conditions interaction effect on leaf stomatal conductance measurement.

Analysis of Variance for Stomatal Conduct

Source	DF	SS	MS	F	P
Condition	1	0.83948	0.83948	121.27	0.000
Treatments	4	0.19250	0.04812	6.95	0.000
Condition*Treatments	4	0.07414	0.01853	2.68	0.044
Rep	5	0.35594	0.07119	10.28	0.000
Error	45	0.31151	0.00692		
Total	59	1.77357			

Appendix 17: Interaction graph to show the effect of water conditions on number of stems of wheat varieties T = Tamooz 2 and A = Adana 99 ($p \leq 0.001$ for water conditions. $p \leq 0.001$ for variety. $p = 0.398$ for water conditions * variety, error bars = SE).



Appendix 18: ANOVA table results for water condition * variety interaction effect on number of stems/pot measurement.

Analysis of Variance for No. of Stems

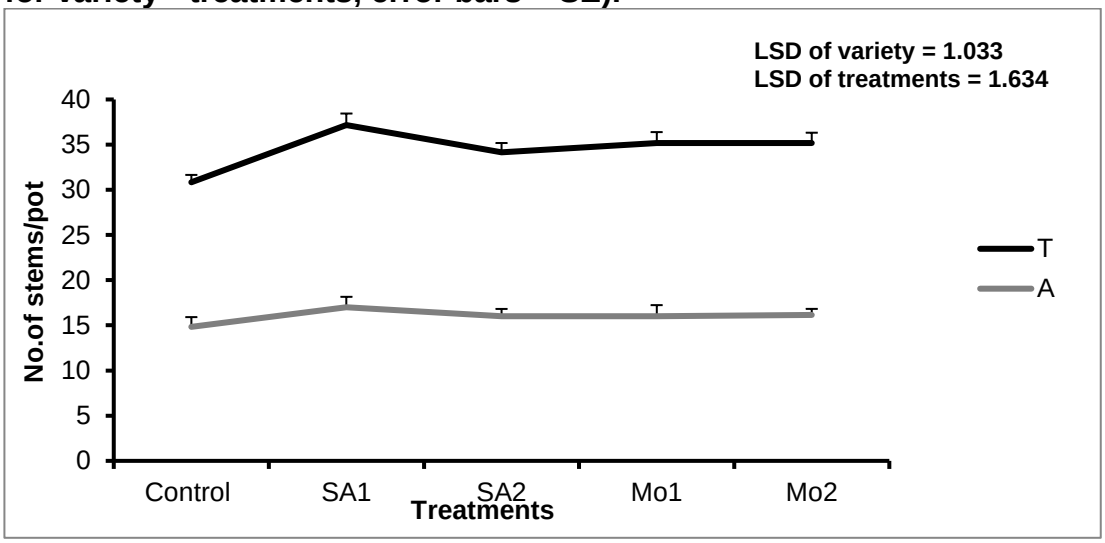
Source	DF	SS	MS	F	P
Variety	1	93.750	93.750	24.23	0.000
Condition	1	144.150	144.150	37.25	0.000
Variety*Condition	1	2.817	2.817	0.73	0.398
Rep	14	102.000	7.286	1.88	0.057
Error	42	162.533	3.870		
Total	59	505.250			

Appendix 19: ANOVA table results for chemical treatments of SA and Mo effect on number of stems/pot measurement.

Analysis of Variance for No. of Stems

Source	DF	SS	MS	F	P
Treatments	4	64.667	16.167	5.29	0.001
Reps	11	306.050	27.823	9.10	0.000
Error	44	134.533	3.058		
Total	59	505.250			

Appendix 20: Interaction graph to show the effect of spraying the plants with two Concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on number of stems of varieties Tamooz 2 and Adana 99 ($p \leq 0.001$ for variety. $p = 0.006$ for treatments. $p = 0.701$ for variety * treatments, error bars = SE).

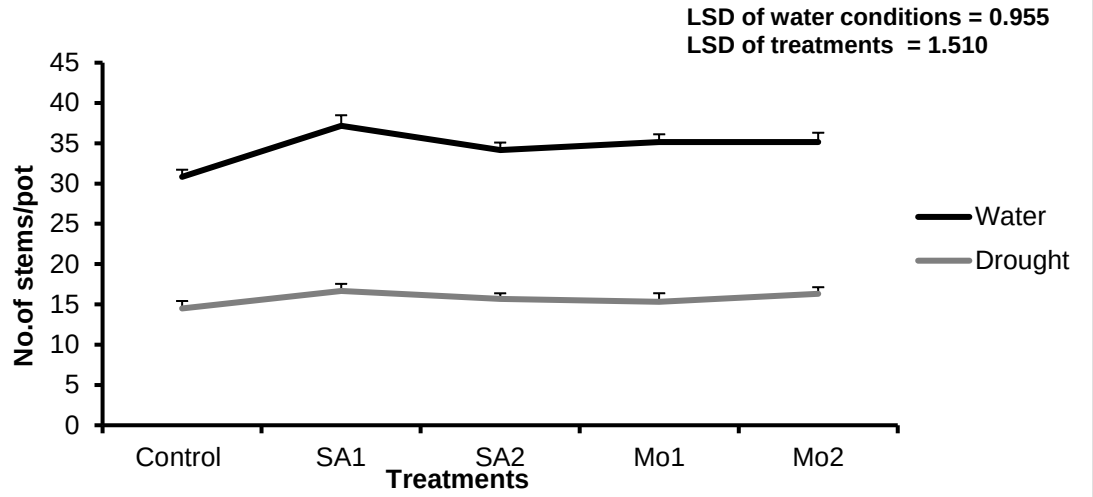


Appendix 21: ANOVA table results for chemical treatments * variety interaction effect on number of stems/pot measurement.

Analysis of Variance for No. of Stems

Source	DF	SS	MS	F	P
Variety	1	93.750	93.750	23.75	0.000
Treatments	4	64.667	16.167	4.10	0.006
Variety*Treatments	4	8.667	2.167	0.55	0.701
Reps	5	160.550	32.110	8.14	0.000
Error	45	177.617	3.947		
Total	59	505.250			

Appendix 22: Interaction graph to show the effect of chemical treatments SA and Mo on number of stems of wheat varieties Tamooz 2 and Adana 99 treated with water conditions well-watered and drought ($p \leq 0.001$ for water condition. $p = 0.003$ for treatments. $p = 0.413$ for water conditions * treatments, error bars = SE).



Appendix 23: ANOVA table results for chemical treatments * water conditions interaction effect on number of stems/pot measurement.

Analysis of Variance for No. of Stems

Source	DF	SS	MS	F	P
Condition	1	144.150	144.150	42.77	0.000
Treatments	4	64.667	16.167	4.80	0.003
Condition*Treatments	4	13.600	3.400	1.01	0.413
Rep	5	131.150	26.230	7.78	0.000
Error	45	151.683	3.371		
Total	59	505.250			

Appendix 24: ANOVA table results for water condition * variety interaction effect on shoot dry weight/pot measurement.

Analysis of Variance for Shoot Dry wt.

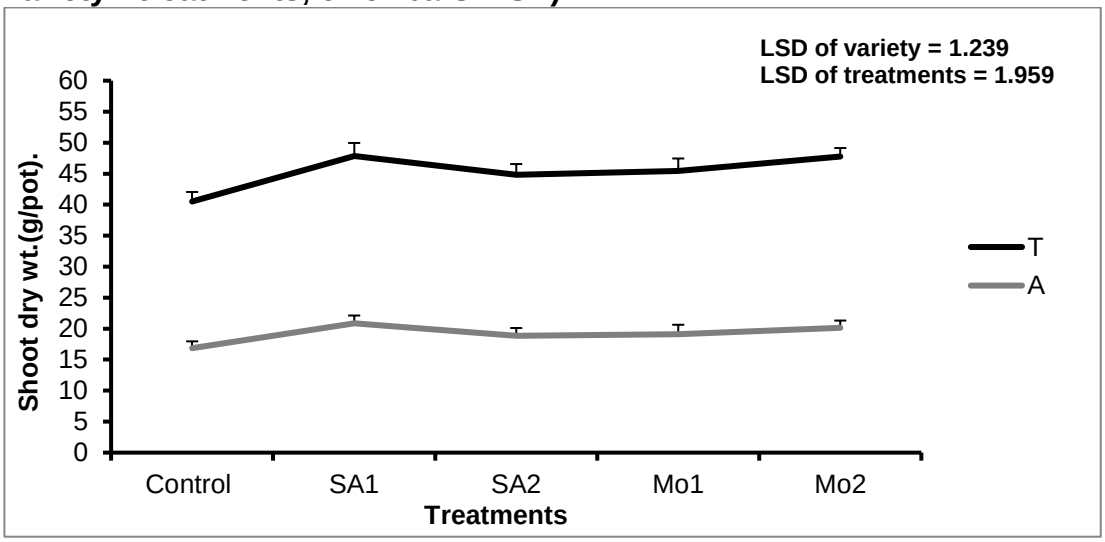
Source	DF	SS	MS	F	P
Variety	1	726.485	726.485	147.05	0.000
Condition	1	356.728	356.728	72.21	0.000
Variety*Condition	1	28.732	28.732	5.82	0.020
Rep	14	237.710	16.979	3.44	0.001
Error	42	207.496	4.940		
Total	59	1557.151			

Appendix 25: ANOVA table results for chemical treatments of SA and Mo effect on shoot dry weight/pot measurement.

Analysis of Variance for Shoot Dry wt.

Source	DF	SS	MS	F	P
Treatments	4	106.903	26.726	5.57	0.001
Reps	11	1239.261	112.660	23.49	0.000
Error	44	210.987	4.795		
Total	59	1557.151			

Appendix 26: Interaction graph to show the effect of spraying the plants with two Concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on shoot dry weight of varieties Tamooz 2 and Adana 99 ($p \leq 0.001$ for variety. $p = 0.003$ for treatments. $p = 0.962$ for variety * treatments, error bars = SE).

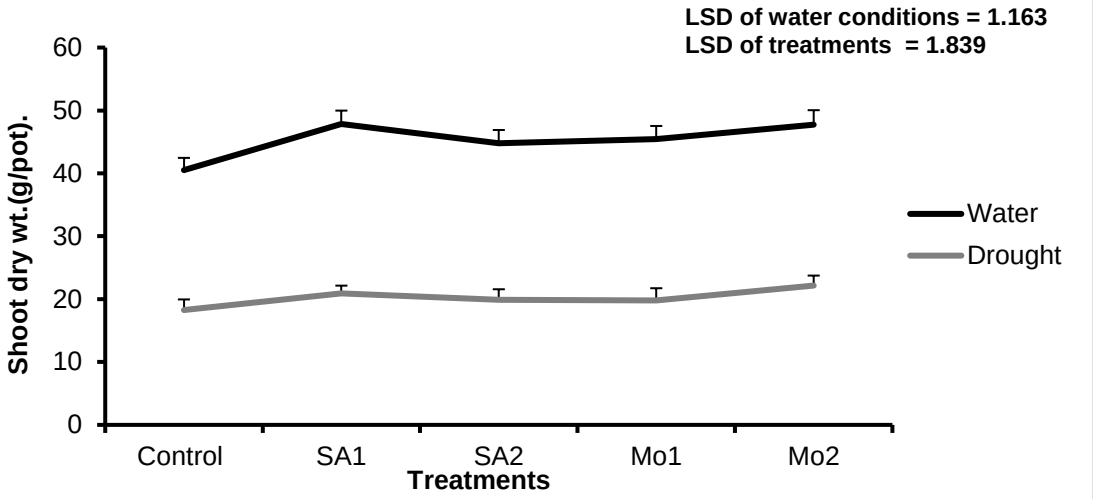


Appendix 27: ANOVA table results for chemical treatments * variety interaction effect on shoot dry weight/pot measurement.

Analysis of Variance for Shoot Dry wt.

Source	DF	SS	MS	F	P
Variety	1	726.485	726.485	127.94	0.000
Treatments	4	106.903	26.726	4.71	0.003
Variety*Treatments	4	3.406	0.851	0.15	0.962
Reps	5	464.838	92.968	16.37	0.000
Error	45	255.520	5.678		
Total	59	1557.151			

Appendix 28: Interaction graph to show the effect of chemical treatments SA and Mo on shoot dry weight of wheat varieties Tamooz 2 and Adana 99 treated with water conditions well-watered and drought ($p \leq 0.001$ for water condition. $p = 0.001$ for treatments. $p = 0.534$ for water conditions * treatments, error bars = SE).

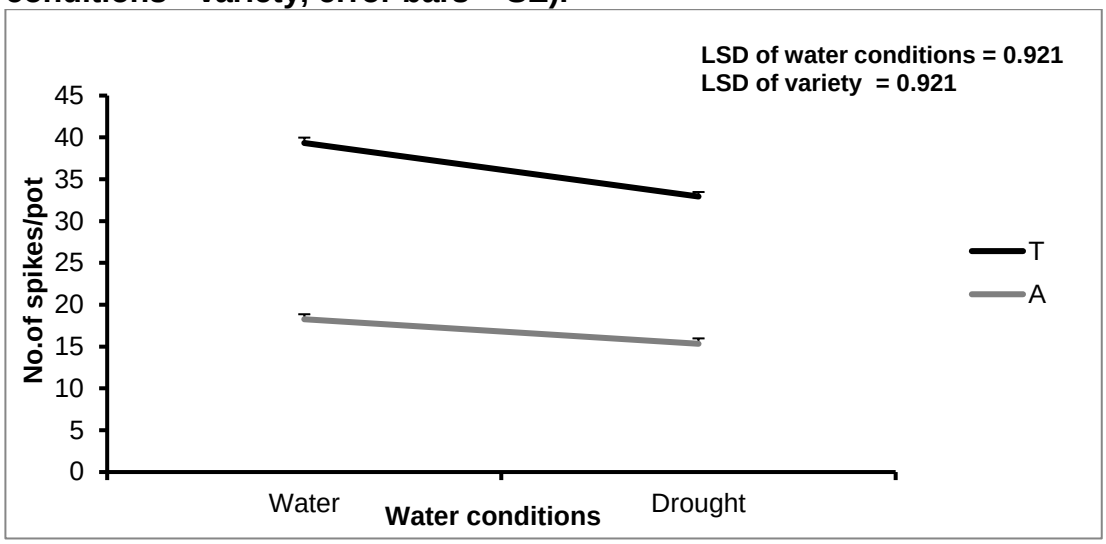


Appendix 29: ANOVA table results for chemical treatments * water conditions interaction effect on shoot dry weight/pot measurement.

Analysis of Variance for Shoot Dry wt.

Source	DF	SS	MS	F	P
Condition	1	356.728	356.728	71.34	0.000
Treatments	4	106.903	26.726	5.34	0.001
Condition*Treatments	4	15.931	3.983	0.80	0.534
Rep	5	852.572	170.514	34.10	0.000
Error	45	225.017	5.000		
Total	59	1557.151			

Appendix 30: Interaction graph to show the effect of water conditions on number of spikes of wheat varieties T = Tamooz 2 and A = Adana 99 ($p \leq 0.001$ for water conditions. $p \leq 0.001$ for variety. $p = 0.562$ for water conditions * variety, error bars = SE).



Appendix 31: ANOVA table results for water condition * variety interaction effect on number spikes/pot measurement.

Analysis of Variance for No. of Spikes

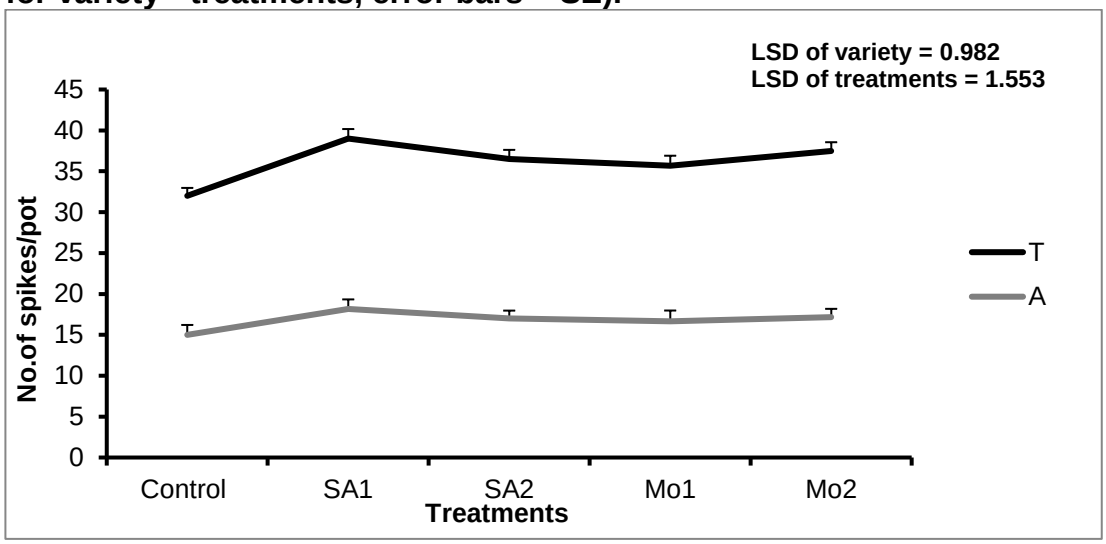
Source	DF	SS	MS	F	P
Variety	1	96.267	96.267	30.85	0.000
Condition	1	153.600	153.600	49.22	0.000
Variety*Condition	1	1.067	1.067	0.34	0.562
Rep	14	179.733	12.838	4.11	0.000
Error	42	131.067	3.121		
Total	59	561.733			

Appendix 32: ANOVA table results for chemical treatments of SA and Mo effect on number spikes/pot measurement.

Analysis of Variance for No. of Spikes

Source	DF	SS	MS	F	P
Treatments	4	82.567	20.642	5.98	0.001
Reps	11	327.333	29.758	8.62	0.000
Error	44	151.833	3.451		
Total	59	561.733			

Appendix 33: Interaction graph to show the effect of spraying the plants with two Concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on number of spikes of varieties Tamooz 2 and Adana 99 ($p \leq 0.001$ for variety. $p = 0.001$ for treatments. $p = 0.959$ for variety * treatments, error bars = SE).

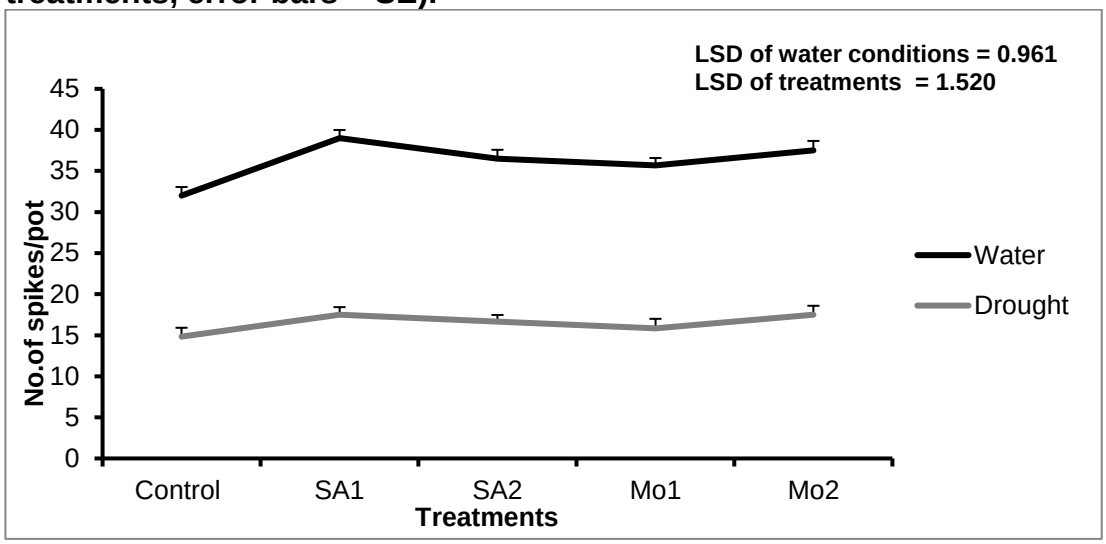


Appendix 34: ANOVA table results for chemical treatments * variety interaction effect on number spikes/pot measurement.

Analysis of Variance for No. of Spikes

Source	DF	SS	MS	F	P
Variety	1	96.267	96.267	26.99	0.000
Treatments	4	82.567	20.642	5.79	0.001
Variety*Treatments	4	2.233	0.558	0.16	0.959
Reps	5	220.133	44.027	12.34	0.000
Error	45	160.533	3.567		
Total	59	561.733			

Appendix 35: Interaction graph to show the effect of chemical treatments SA and Mo on number of spikes of wheat varieties Tamooz 2 and Adana 99 treated with water conditions well-watered and drought ($p \leq 0.001$ for water condition. $p = 0.001$ for treatments. $p = 0.697$ for water conditions * treatments, error bars = SE).

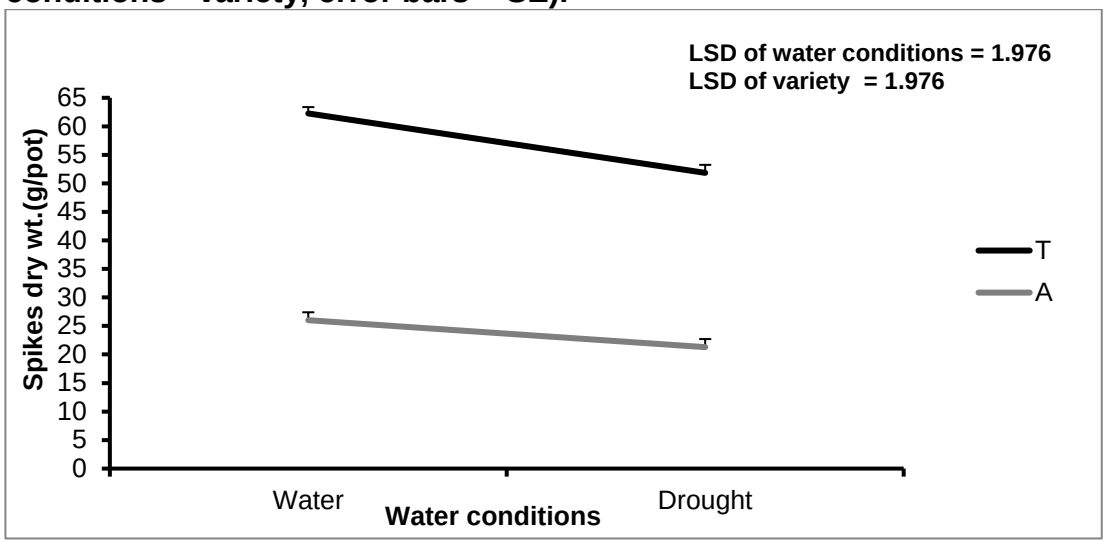


Appendix 36: ANOVA table results for chemical treatments * water conditions interaction effect on number spikes/pot measurement.

Analysis of Variance for No. of Spikes

Source	DF	SS	MS	F	P
Condition	1	153.600	153.600	44.98	0.000
Treatments	4	82.567	20.642	6.04	0.001
Condition*Treatments	4	7.567	1.892	0.55	0.697
Rep	5	164.333	32.867	9.62	0.000
Error	45	153.667	3.415		
Total	59	561.733			

Appendix 37: Interaction graph to show the effect of water conditions on spikes dry weight wheat varieties T = Tamooz 2 and A = Adana 99 ($p \leq 0.001$ for water conditions. $p \leq 0.001$ for variety. $p = 0.622$ for water conditions * variety, error bars = SE).



Appendix 38: ANOVA table results for water condition * variety interaction effect on spikes dry weight/pot measurement.

Analysis of Variance for Spikes dry wt.

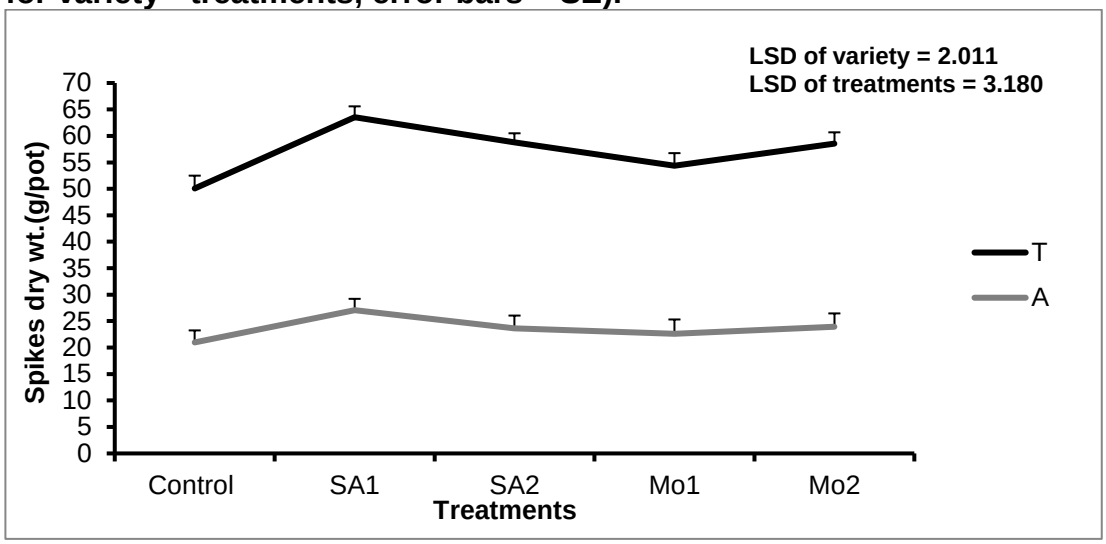
Source	DF	SS	MS	F	P
Variety	1	1425.45	1425.45	99.12	0.000
Condition	1	406.69	406.69	28.28	0.000
Variety*Condition	1	3.54	3.54	0.25	0.622
Rep	14	894.62	63.90	4.44	0.000
Error	42	604.01	14.38		
Total	59	3334.30			

Appendix 39: ANOVA table results for chemical treatments of SA and Mo effect on spikes dry weight/pot measurement.

Analysis of Variance for Spikes dry wt.

Source	DF	SS	MS	F	P
Treatments	4	309.25	77.31	7.25	0.000
Reps	11	2555.94	232.36	21.79	0.000
Error	44	469.11	10.66		
Total	59	3334.30			

Appendix 40: Interaction graph to show the effect of spraying the plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on spikes dry weight of varieties Tamooz 2 and Adana 99 ($p \leq 0.001$ for variety. $p = 0.002$ for treatments. $p = 0.845$ for variety * treatments, error bars = SE).

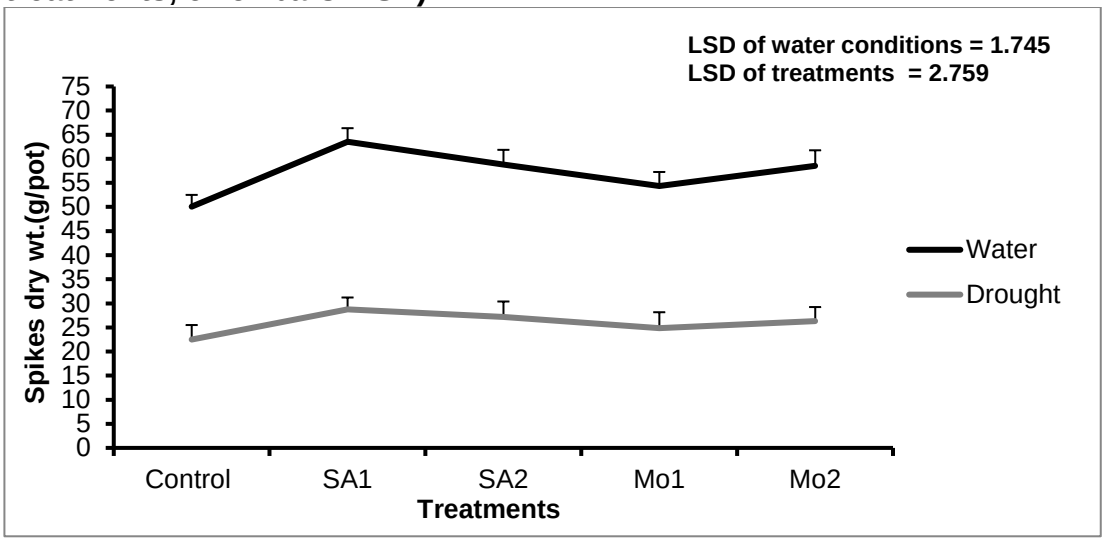


Appendix 41: ANOVA table results for chemical treatments * variety interaction effect on spikes dry weight/pot measurement.

Analysis of Variance for Spikes dry wt.

Source	DF	SS	MS	F	P
Variety	1	1425.45	1425.45	95.28	0.000
Treatments	4	309.25	77.31	5.17	0.002
Variety*Treatments	4	20.76	5.19	0.35	0.845
Reps	5	905.58	181.12	12.11	0.000
Error	45	673.26	14.96		
Total	59	3334.30			

Appendix 42: Interaction graph to show the effect of chemical treatments SA and Mo on spikes dry weight of wheat varieties Tamooz 2 and Adana 99 treated with water conditions well-watered and drought ($p \leq 0.001$ for water condition. $p \leq 0.001$ for treatments. $p = 0.970$ for water conditions * treatments, error bars = SE).

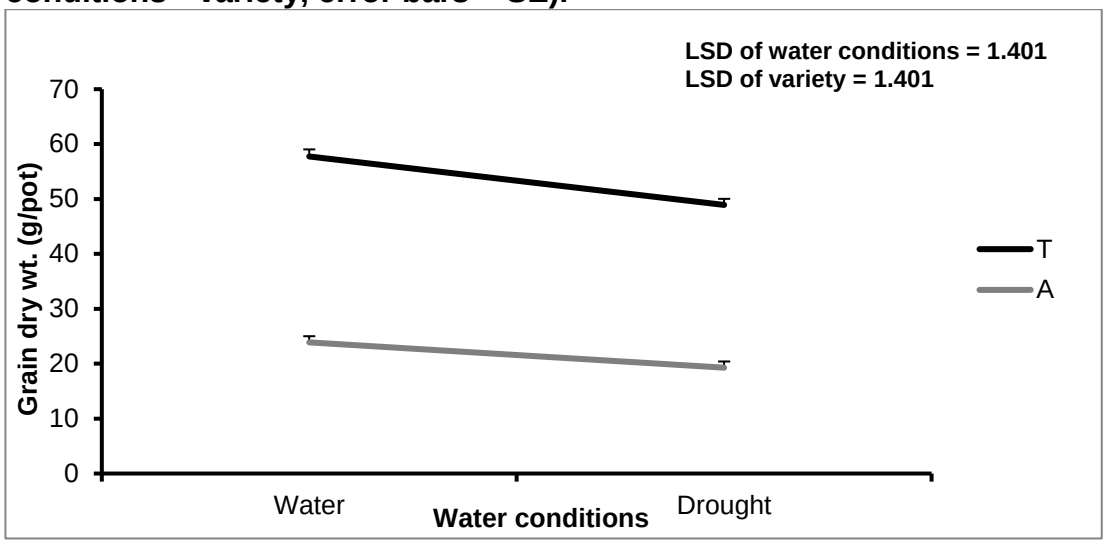


Appendix 43: ANOVA table results for chemical treatments * water conditions interaction effect on spikes dry weight/pot measurement.

Analysis of Variance for Spikes dry wt.

Source	DF	SS	MS	F	P
Condition	1	406.69	406.69	36.11	0.000
Treatments	4	309.25	77.31	6.86	0.000
Condition*Treatments	4	5.92	1.48	0.13	0.970
Rep	5	2105.62	421.12	37.39	0.000
Error	45	506.82	11.26		
Total	59	3334.30			

Appendix 44: Interaction graph to show the effect of water conditions on grain dry weight of wheat varieties T = Tamooz 2 and A = Adana 99 ($p \leq 0.001$ for water conditions. $p \leq 0.001$ for variety. $p = 0.775$ for water conditions * variety, error bars = SE).



Appendix 45: ANOVA table results for water condition * variety interaction effect on grain dry weight/pot measurement.

Analysis of Variance for grain dry wt.

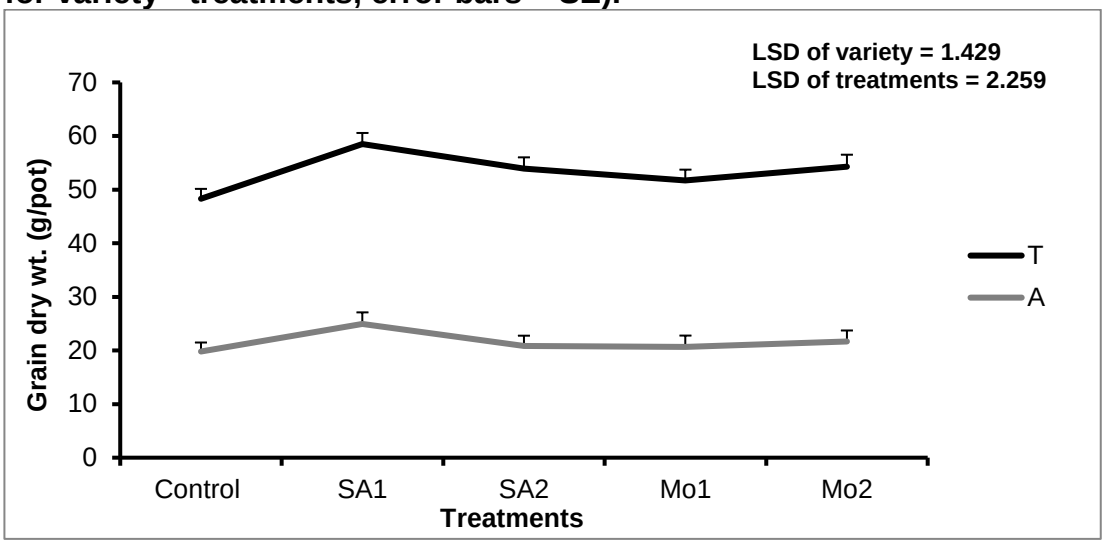
Source	DF	SS	MS	F	P
Variety	1	1548.99	1548.99	214.15	0.000
Condition	1	291.81	291.81	40.34	0.000
Variety*Condition	1	0.60	0.60	0.08	0.775
Rep	14	837.38	59.81	8.27	0.000
Error	42	303.80	7.23		
Total	59	2982.59			

Appendix 46: ANOVA table results for chemical treatments of SA and Mo effect on grain dry weight/pot measurement.

Analysis of Variance for grain dry wt.

Source	DF	SS	MS	F	P
Treatments	4	167.77	41.94	5.20	0.002
Reps	11	2459.67	223.61	27.70	0.000
Error	44	355.14	8.07		
Total	59	2982.59			

Appendix 47: Interaction graph to show the effect of spraying the plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on grains dry weight of varieties Tamooz 2 and Adana 99 ($p \leq 0.001$ for variety. $p = 0.001$ for treatments. $p = 0.447$ for variety * treatments, error bars = SE).

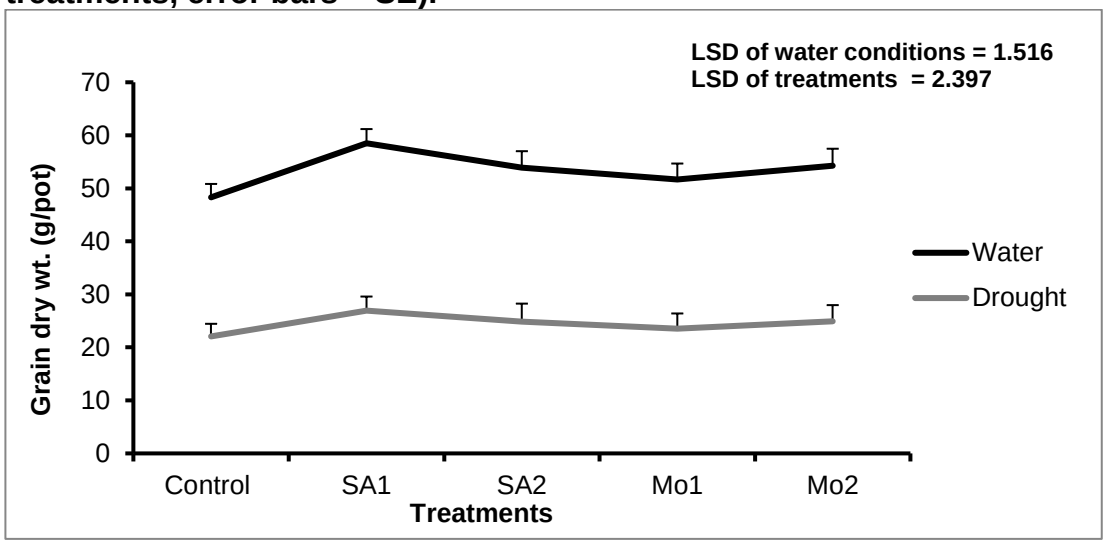


Appendix 48: ANOVA table results for chemical treatments * variety interaction effect on grain dry weight/pot measurement.

Analysis of Variance for grain dry wt.

Source	DF	SS	MS	F	P
Variety	1	1548.99	1548.99	205.09	0.000
Treatments	4	167.77	41.94	5.55	0.001
Variety*Treatments	4	28.53	7.13	0.94	0.447
Reps	5	897.42	179.48	23.76	0.000
Error	45	339.87	7.55		
Total	59	2982.59			

Appendix 49: Interaction graph to show the effect of chemical treatments SA and Mo on grains dry weight of wheat varieties Tamooz 2 and Adana 99 treated with water conditions well-watered and drought ($p \leq 0.001$ for water condition. $p = 0.002$ for treatments. $p = 0.999$ for water conditions * treatments, error bars = SE).

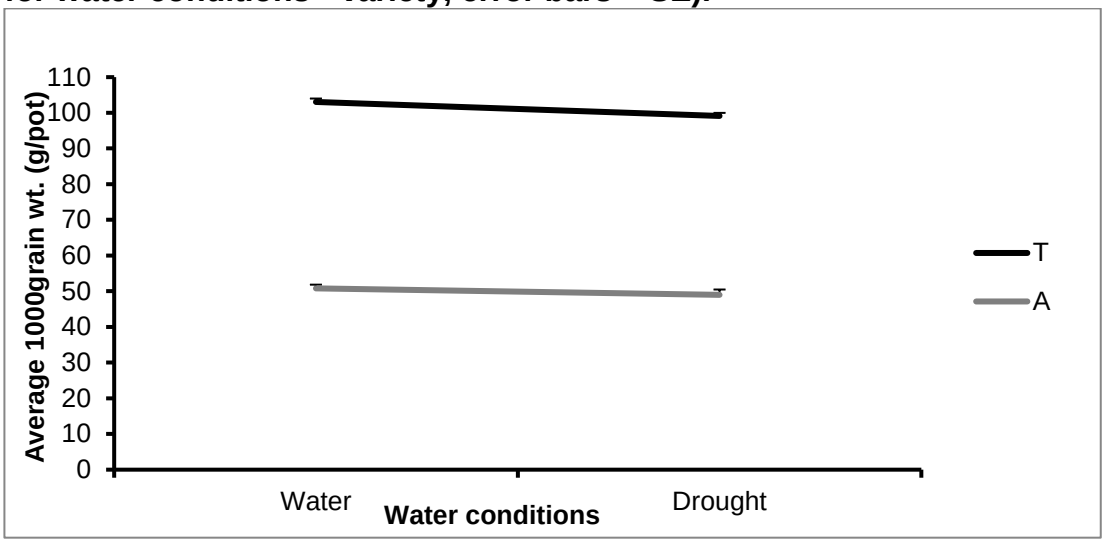


Appendix 50: ANOVA table results for chemical treatments * water conditions interaction effect on grain dry weight/pot measurement.

Analysis of Variance for grain dry wt.

Source	DF	SS	MS	F	P
Condition	1	291.81	291.81	34.32	0.000
Treatments	4	167.77	41.94	4.93	0.002
Condition*Treatments	4	0.62	0.15	0.02	0.999
Rep	5	2139.76	427.95	50.33	0.000
Error	45	382.62	8.50		
Total	59	2982.59			

Appendix 51: Interaction graph to show the effect of water conditions on average 1000 grain dry weight of wheat varieties T = Tamooz 2 and A = Adana 99 (*p* = 0.092 for water conditions. *p* = 0.260 for variety. *p* = 0.900 for water conditions * variety, error bars = SE).

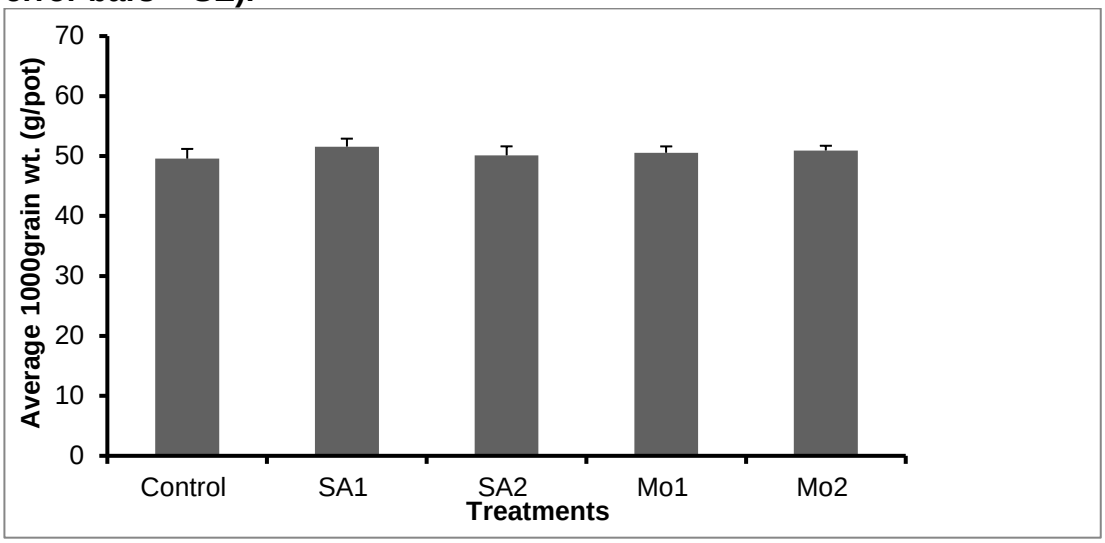


Appendix 52: ANOVA table results for water condition * variety interaction effect on average 1000 grain dry weight/pot measurement.

Analysis of Variance for Average 1000 grain dry Wt.

Source	DF	SS	MS	F	P
Variety	1	25.17	25.17	1.30	0.260
Condition	1	57.64	57.64	2.98	0.092
Variety*Condition	1	0.31	0.31	0.02	0.900
Rep	14	236.00	16.86	0.87	0.593
Error	42	812.03	19.33		
Total	59	1131.15			

Appendix 53: Overall effect of spraying plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on average 1000 grain dry weight. (*P* = 0.815 for chemical treatments, error bars = SE).

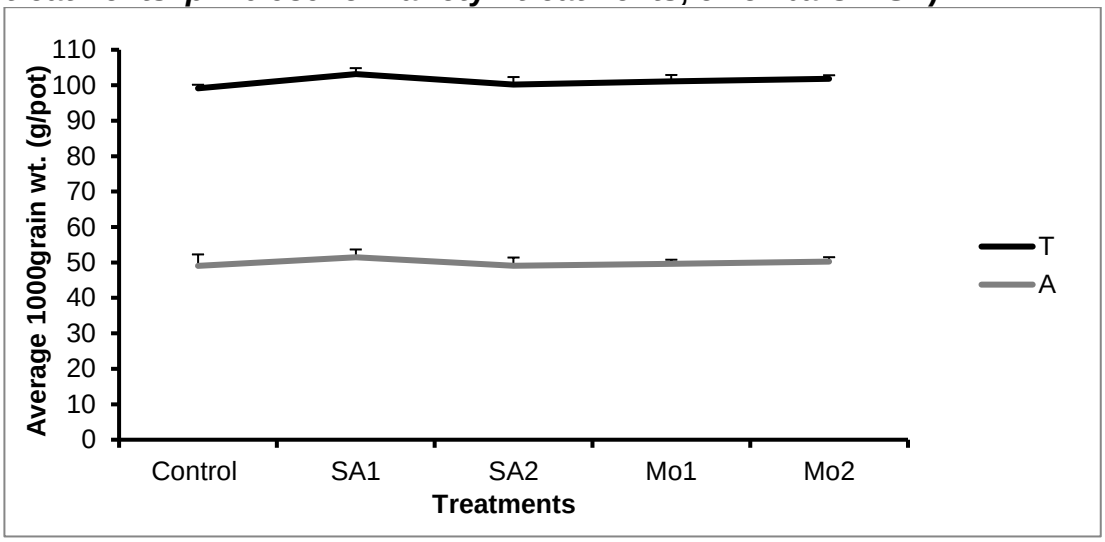


Appendix 54: ANOVA table results for chemical treatments of SA and Mo effect on average 1000 grain dry weight/pot measurement.

Analysis of Variance for Average 1000 grain dry Wt.

Source	DF	SS	MS	F	P
Treatments	4	28.08	7.02	0.39	0.815
Reps	11	310.18	28.20	1.56	0.143
Error	44	792.89	18.02		
Total	59	1131.15			

Appendix 55: Interaction graph to show the effect of spraying the plants with two concentrations of SA (SA1 = 1.44 and SA2 = 2.88 mM) and Mo (Mo1 = 0.15 and Mo2 = 0.30 mM) on average 1000 grains dry weight of varieties Tamooz 2 and Adana 99 (*p* = 0.267 for variety. *p* = 0.842 for treatments. *p* = 0.989 for variety * treatments, error bars = SE).

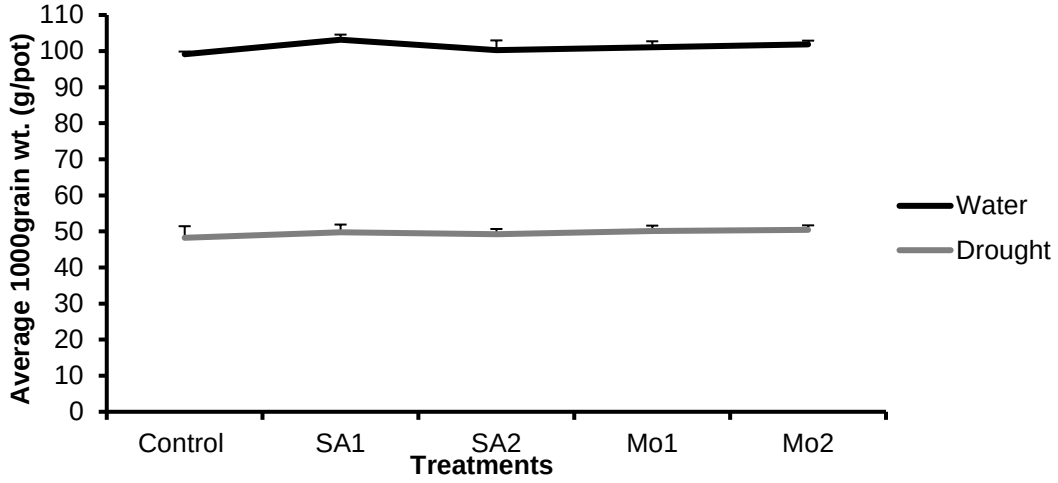


Appendix 56: ANOVA table results for chemical treatments * variety interaction effect on average 1000 grain dry weight/pot measurement.

Analysis of Variance for Average 1000 grain dry Wt.

Source	DF	SS	MS	F	P
Variety	1	25.17	25.17	1.26	0.267
Treatments	4	28.08	7.02	0.35	0.842
Variety*Treatments	4	6.16	1.54	0.08	0.989
Reps	5	173.15	34.63	1.73	0.146
Error	45	898.58	19.97		
Total	59	1131.15			

Appendix 57: Interaction graph to show the effect of chemical treatments SA and Mo on average 1000 grains dry weight of wheat varieties Tamooz 2 and Adana 99 treated with water conditions well-watered and drought (*p* = 0.092 for water condition. *p* = 0.834 for treatments). *p* = 0.936 for water conditions * treatments, error bars = SE).



Appendix 58: ANOVA table results for chemical treatments * water conditions interaction effect on average 1000 grain dry weight/pot measurement.

Analysis of Variance for Average 1000Grain Wt.

Source	DF	SS	MS	F	P
Condition	1	57.64	57.64	2.97	0.092
Treatments	4	28.08	7.02	0.36	0.834
Condition*Treatments	4	15.63	3.91	0.20	0.936
Rep	5	157.38	31.48	1.62	0.173
Error	45	872.42	19.39		
Total	59	1131.15			

Appendix 59: ANOVA table results for the effect of different spray with SA on shoot dry weight.

Analysis of Variance for Shoot dry wt.

Source	DF	SS	MS	F	P
Condition	1	3.007	3.007	4.36	0.043
Treatment	7	423.301	60.472	87.63	0.000
Condition*Treatment	7	63.574	9.082	13.16	0.000
Rep	3	50.109	16.703	24.20	0.000
Error	45	31.054	0.690		
Total	63	571.046			

Appendix 60: ANOVA table results for the effect of different spray with SA on number of spikes/pot.

Analysis of Variance for No. spikes/pot

Source	DF	SS	MS	F	P
Condition	1	0.919	0.919	0.43	0.515
Treatment	7	660.392	94.342	44.22	0.000
Condition*Treatment	7	96.587	13.798	6.47	0.000
Rep	3	183.406	61.135	28.65	0.000
Error	45	96.010	2.134		
Total	63	1037.315			

Appendix 61: ANOVA table results for the effect of different spray with SA on spikes dry weight.

Analysis of Variance for Spike dry wt.

Source	DF	SS	MS	F	P
Condition	1	0.244	0.244	0.17	0.682
Treatment	7	1182.400	168.914	117.58	0.000
Condition*Treatment	7	91.257	13.037	9.07	0.000
Rep	3	90.896	30.299	21.09	0.000
Error	45	64.647	1.437		
Total	63	1429.443			

Appendix 62: ANOVA table results for the effect of different spray with SA on grain dry weight.

Analysis of Variance for grain dry wt.

Source	DF	SS	MS	F	P
Condition	1	6.708	6.708	8.44	0.006
Treatment	7	739.694	105.671	132.93	0.000
Condition*Treatment	7	73.770	10.539	13.26	0.000
Rep	3	54.809	18.270	22.98	0.000
Error	45	35.771	0.795		
Total	63	910.752			

Appendix 63: ANOVA table results for the effect of different spray with SA on average 1000 grains dry weight.

Analysis of Variance for Average 1000 grains dry wt.

Source	DF	SS	MS	F	P
Condition	1	6.713	6.713	1.29	0.262
Treatment	7	331.085	47.298	9.08	0.000
Condition*Treatment	7	112.631	16.090	3.09	0.010
Rep	3	5.285	1.762	0.34	0.798
Error	45	234.290	5.206		
Total	63	690.003			

Appendix 64: ANOVA table results for the effect of SA on the up-regulation of the *CBF* gene 2 days after spraying the leaves of wheat plants (VAR.Tamooz 2) under well-watered and drought conditions during seedling stage.

Analysis of Variance for 2day seedling stage RQ

Source	DF	SS	MS	F	P
Condition	1	0.003	0.003	0.00	0.968
Treatment	1	13.253	13.253	8.23	0.021
Condition*Treatment	1	3.053	3.053	1.90	0.206
Error	8	12.876	1.610		
Total	11	29.185			

Appendix 65: ANOVA table results for the effect of SA on the up-regulation of the *CBF* gene 10 days after spraying the leaves of wheat plants (VAR.Tamooz 2) under well-watered and drought conditions during seedling stage.

Analysis of Variance for 10day seedling stage RQ

Source	DF	SS	MS	F	P
Condition	1	6.7451	6.7451	41.35	0.000
Treatment	1	4.4961	4.4961	27.56	0.000
Condition*Treatment	1	2.1446	2.1446	13.15	0.002
Error	16	2.6101	0.1631		
Total	19	15.9960			

Appendix 66: ANOVA table results for the effect of SA on the up-regulation of the CBF gene 2 days after spraying the leaves of wheat plants (VAR.Tamooz 2) under well-watered and drought conditions during stem extension stage.

Analysis of Variance for 2day stem extension stage RQ

Source	DF	SS	MS	F	P
Condition	1	0.07892	0.07892	1.65	0.218
Treatment	3	4.57085	1.52362	31.78	0.000
Condition*Treatment	3	0.54565	0.18188	3.79	0.031
Error	16	0.76707	0.04794		
Total	23	5.96249			

Appendix 67: ANOVA table results for the effect of SA on the up-regulation of the *CBF* gene 10 days after spraying the leaves of wheat plants (VAR.Tamooz 2) under well-watered and drought conditions during stem extension stage.

Analysis of Variance for 10day stem extension stage RQ

Source	DF	SS	MS	F	P
Condition	1	10.2107	10.2107	125.40	0.000
Treatment	3	9.2373	3.0791	37.82	0.000
Condition*Treatment	3	3.7114	1.2371	15.19	0.000
Error	24	1.9542	0.0814		
Total	31	25.1135			

Appendix 68: ANOVA table results for the effect of SA on the up-regulation of the CBF gene 2 days after spraying the wheat plants (VAR.Tamooz 2) under well-watered and drought conditions during flowering stage.

Analysis of Variance for 2day Flowering stage RQ

Source	DF	SS	MS	F	P
Condition	1	0.0072	0.0072	0.15	0.704
Treatment	7	15.8939	2.2706	46.24	0.000
Condition*Treatment	7	3.6783	0.5255	10.70	0.000
Error	48	2.3568	0.0491		
Total	63	21.9362			

Appendix 69: ANOVA table results for the effect of SA on the up-regulation of the CBF gene 10 days after spraying the wheat plants (VAR.Tamooz 2) under well-watered and drought conditions during flowering stage.

Analysis of Variance for 10day Flowering stage RQ

Source	DF	SS	MS	F	P
Condition	1	3.1455	3.1455	124.55	0.000
Treatment	7	11.4712	1.6387	64.89	0.000
Condition*Treatment	7	1.3537	0.1934	7.66	0.000
Error	48	1.2122	0.0253		
Total	63	17.1827			