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Memory of past drought stress exposure effects plant responses in subsequent generations in winter wheat (*Triticum aestivum* L.)

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I dedicate this dissertation to my children, Kevin and Ica, with love and appreciation.

Abstract

Current research suggests that plants can memorize the environments experienced by their ancestors to alter their phenotypes. Parental environmental effects have been reported for the first offspring generation, and some studies describe persisting grandparental and great grandparental environmental effects. These inherited environmental effects can include specific developmental adjustments that improve offspring growth under the conditions that induced them. Research on crop stress memory is still in its infancy, despite its potential role in environmental adaptation. The occurrence, persistence, adaptive value, and inheritance of stress memory effects remain unclear, obscuring their evolutionary and ecological significance. Addressing this gap in knowledge would likely improve our ability to breed and manage crops in order to promote stress tolerance. Improving crop response to drought stress is of particular concern, due to the diminishing availability of water in agricultural regions across the globe. Therefore, the overall objective of this research was to investigate the drought stress memory of winter wheat, focusing on the seed, seedling and the reproductive growth stage.

For this purpose, phenotypic plasticity studies were combined with differential gene expression analysis in order to address these outstanding concerns. Winter wheat varieties were grown for two years under drought and controlled moisture conditions to produce seeds with all possible combinations of drought exposure history. Analyses of the seed transcriptome, seedlings and plant biochemical, physiological, and morphological traits, including plant height, above-ground biomass and root architecture alterations illustrated the variability in the expression of memory effects. The performance of plants whose ancestors had been exposed to drought stress in one or more generations was inconsistent, highlighting the strength of each memory effect as well as the complex relationship between cultivars, environments, and their interactions. The results of this research also support the hypothesis that memory imprints include not only the epigenetic marks that alter gene expression but also biochemical and physiological imprints that also modify plant morphology.

While this subject requires further investigation, these results suggest that cultivar specific changes in gene expression due to drought memory may contribute to the regulation of plasticity. The observed cultivar differences underscore the importance of incorporating genetic variation into epigenetic studies. Taken together, the findings in this study indicate that the interactions between different cultivars, environments (offspring environment, parental environment, grandparental environment etc.), and epigenotypes are a meaningful source of phenotypic variation, signifying a promising new direction in plant breeding.

Zusammenfassung

Aktuelle Forschungsergebnisse deuten darauf hin, dass sich Pflanzen die Umwelteinflüsse, denen ihre Vorfahren ausgesetzt waren, einprägen können, um ihren Phänotyp zu verändern. In der Literatur wurden bereits Auswirkungen elterlicher Umwelt-“Erlebnisse“ auf die folgende Generation von Nachkommen beschrieben; einige Studien berichten sogar von Effekten aufgrund Umweltereignisse, die die Großeltern und/oder die Urgroßeltern erfahren haben. Zu diesen generationsübergreifenden (auch als vererbt bezeichneten) Auswirkungen können spezifische Entwicklungsanpassungen gehören, die das Wachstum der Nachkommen insbesondere unter denjenigen Bedingungen verbessern, unter denen diese initiiert wurden. Die Erforschung dieses Stressgedächtnisses von Nutzpflanzen steckt trotz seiner potenziellen Rolle bei der Anpassung an Umwelteinflüsse noch in den Kinderschuhen. Das Auftreten, die Persistenz, der adaptive Wert und die Vererbung des Stressgedächtnisses sind nach wie vor unklar, wodurch ihre evolutionären und ökologischen Bedeutungen noch unverstanden sind. Die Erkenntnis über ein Stressgedächtnis und dessen Bedeutung bei Kulturarten könnte der Pflanzenzüchtung und der Produktionstechnik im Pflanzenbau helfen, die Stresstoleranz in der landwirtschaftlichen Praxis zu verbessern. Insbesondere die Verbesserung der Reaktion von Nutzpflanzen auf Trockenstress ist aufgrund der schwindenden Wasserverfügbarkeit in landwirtschaftlichen Regionen auf der ganzen Welt von besonderer Bedeutung. Daher bestand das übergeordnete Ziel dieser Forschungsarbeit darin, das Trockenstressgedächtnis von Winterweizen zu untersuchen, wobei der Schwerpunkt auf dem Keimlingsstadium und dem reproduktiven Wachstumsstadium lag.

Dazu wurden Untersuchungen der phänotypischen Plastizität mit differenzieller Genexpressionsanalyse kombiniert. Winterweizensorten wurden zwei Jahre lang unter Trocken- und unter Kontrollbedingungen angebaut, um Saatgut mit allen möglichen Kombinationen von Dürreexpositionen zu erzeugen. Transkriptom-Analysen der Samen, Phänotypisierungen von biochemischen, physiologischen und morphologischen Merkmalen verschiedener Wachstumsstadien, Pflanzenhöhe, der oberirdischen Biomasse sowie der Veränderungen der Wurzelarchitektur veranschaulichten die Variabilität der Ausprägung der Gedächtniseffekte. Die Ausprägungen der Gedächtniseffekte von Pflanzen, deren Vorfahren in einer oder zwei Generationen Trockenstress ausgesetzt waren, waren uneinheitlich. Deutlich wurde dieses in der Höhe der entsprechen Gedächtniseffekte sowie in der komplexen Beziehung zwischen Sorten, Umwelt und ihren Wechselwirkungen. Diese Forschungsergebnisse stützen zudem die Hypothese, dass zu den Gedächtnisprägungen nicht

nur epigenetische Prägungen gehören, die die Genexpression verändern, sondern auch biochemische und physiologische Prägungen, die sich auf die Pflanzenmorphologie auswirken. Obwohl dieses Thema weitere Untersuchungen erfordert, scheint es, dass sortenspezifische Veränderungen in der Genexpression aufgrund eines Trockenheitsgedächtnisses zur Regulierung der Plastizität beitragen können. Diese Sortenunterschiede unterstreichen, dass es wichtig ist, die genetische Variation z.B. vor Sorten oder von Akzessionen mit in epigenetische Studien einzubeziehen. Insgesamt deuten die Ergebnisse dieser Studie darauf hin, dass die Wechselwirkungen zwischen verschiedenen Sorten, Umwelteinflüssen auf Vor-Generationen (Umwelt der Nachkommen, Umwelt der Eltern, Umwelt der Großeltern usw.) und Epigenotypen eine bedeutende Quelle phänotypischer Variation sind, was eine vielversprechende neue Richtung in der Pflanzenzüchtung darstellt.

Table of Contents

List of abbreviations

A	CO2 assimilation rate
ABA	Abscisic acid
ANOVA	Analysis of Variance
ARI	Anthocyanin reflectance index
BBCH	Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundessortenamt und CHemische Industrie
BRIWECS	Breeding Innovation in Wheat for Resilient Cropping System
DEG	Differentially Expressed Gene
CIMMYT	International Maize and Wheat Improvement Center
CRI	Carotenoid reflectance index
E	Transpiration rate
ELR	Electrolyte leakage rate
FAO	Food Agriculture Organization
gs	Stomatal conductance
H_2O_2	Hydrogen peroxide
Lea	late embryogenesis abundant
MDA	Malondialdehyde
N	Nitrogen
NDF	Neutral detergent fiber
NIRS	Near infra-red spectrometry
NO	Nitric oxide
PCA	Principal Component Analysis
P5CS	pyrroline-5-carboxylate synthase
Rubisco	Ribulose-1,5-bisphosphat-carboxylase/ oxygenase
ROS	Reactive Oxygen Species
SPAD	Soil Plant Analysis Development
Y(II)	Effective quantum yield of photosystem II
EM-DAT	Emergency Events Database
PSII	Photosystem II

List of Figures

List of Tables

List of Supplementary Material: Chapter 3

Chapter 1 General Introduction

1.1 Water use in crop production

Crop performance and yield result from genotypic expression, which is influenced by constant interactions with the environment. Among the environmental factors, water is one of the most widely limiting for crop production on a global basis. All forms of agriculture absolutely require the supply of freshwater, although the quantity needed differs between different types of agriculture and climatic regions. According to FAO (2020), the global agricultural land area is around five billion hectares (38% of the global land surface). Rainfed agriculture covers 80% of the world's cultivated land and is responsible for about 60% of crop production, while the rest is irrigated agriculture (Dowgert, 2010). This means that the main dominant use of freshwater is agricultural activity, with water usage for irrigation accounting for 70% of the global withdrawals from water resources annually (FAO, 2002; Morison et al., 2008; Alexandratos & Bruinsma, 2012).

Plants require water for development, growth, and subsequent yield. According to McElrone et al. (2013), the importance of water to plants originates from its principal role in photosynthesis and the distribution of molecules. During photosynthesis, opening of the stomata leads to transpiration, thereby bringing a cooling effect to the plant (Chaves et al., 2016). Water serves as a solvent that solubilizes nutrients in the soil and facilitates their absorption and transport through the plant roots. As a medium for movement, it transports nutrients and other molecules within and between cells. It sets up around 80-95% of the entire weight of the growing plant tissue. Protoplasm molecules owe their specific biochemical activities to the water environment in which they exist. In addition, water influences the structure of macromolecules like nucleic acids, proteins, and polysaccharides. Moreover, water in the plant gives it shape and mechanical support (Tyree & Hammel, 1972).

1.2 Drought stress and its effect on crop production

Drought refers to a period in which precipitation is lower than normal, leading to water shortages (Nobre et al., 2016). It is a common and widespread manifestation in nature (Acevedo et al., 2002) and is classified as meteorological, hydrological, and agricultural drought (Shrestha, 2020). During agricultural drought, the rainfall and the soil water content fail to meet the demands of evapotranspiration to sustain optimal growth and production of crops (Liu et al., 2016). A detailed systematic representation is shown in **Figure 1.1**. Therefore, water absorption by the plant and evapotranspiration are the two major processes involved in drought formation. As far as the plant is concerned, root characteristics control water absorption, while stomatal conductance and crop ground cover determine evapotranspiration (Acevedo et al., 2002). However, the severity of the damage to plants caused by drought is usually unpredictable because it is controlled by numerous other factors such as rainfall patterns, soil water-retaining ability, and atmospheric properties such as net radiation and a vapour pressure deficit (Yan et al. 2016). Unlike other abiotic stresses, drought has the longest duration and biggest spatial magnitude globally, which is about 80% of the total cultivated area (Sheffield and Wood, 2012).

While historical droughts are linked to fluctuations in tropic oceanic temperature, current droughts are linked to greenhouse gas emissions as well as recent changes in land use practices (Sheffield & Wood, 2008). According to Trenberth et al. (2014), land degradation, deforestation, and inappropriate water use, and their management, will intensify the strength and severity of the drought. Friedlingstein et al. (2010) report a 150% and 30% increase in methane and carbon dioxide gases, respectively, for over 200 years. Currently, water utilization by agriculture is under increasing scrutiny due to the depletion of groundwater, and climate change will increase the severity and incidence of drought events over the coming years (Dai, 2013).

The impact of drought is estimated to be not only severe in developing nations in Africa and Asia due to the lack of technical means and infrastructures to cope with climate disasters, but also in developed countries (Meza et al., 2020; **Figure 1.2**). The implications of drought events on crop yield are of much concern in agriculture, as they cause about 40% of crop losses each year (Borém et al., 2012). For example, all nations faced severe drought and reduced yields in the years 1987, 2002, and 2007, as reported by Wang et al. (2018). In addition, between 1980 and 2008, drought and high temperatures led to a global yield reduction of up to 1.7% in soybeans, 3.8% in maize, and 5.5% in wheat (Lobell et al., 2011). Furthermore, a dangerous situation has been projected by Li et al. (2009), where the yield of key crops will decrease by more than 50% in 2050 and by around 90% fifty years later.

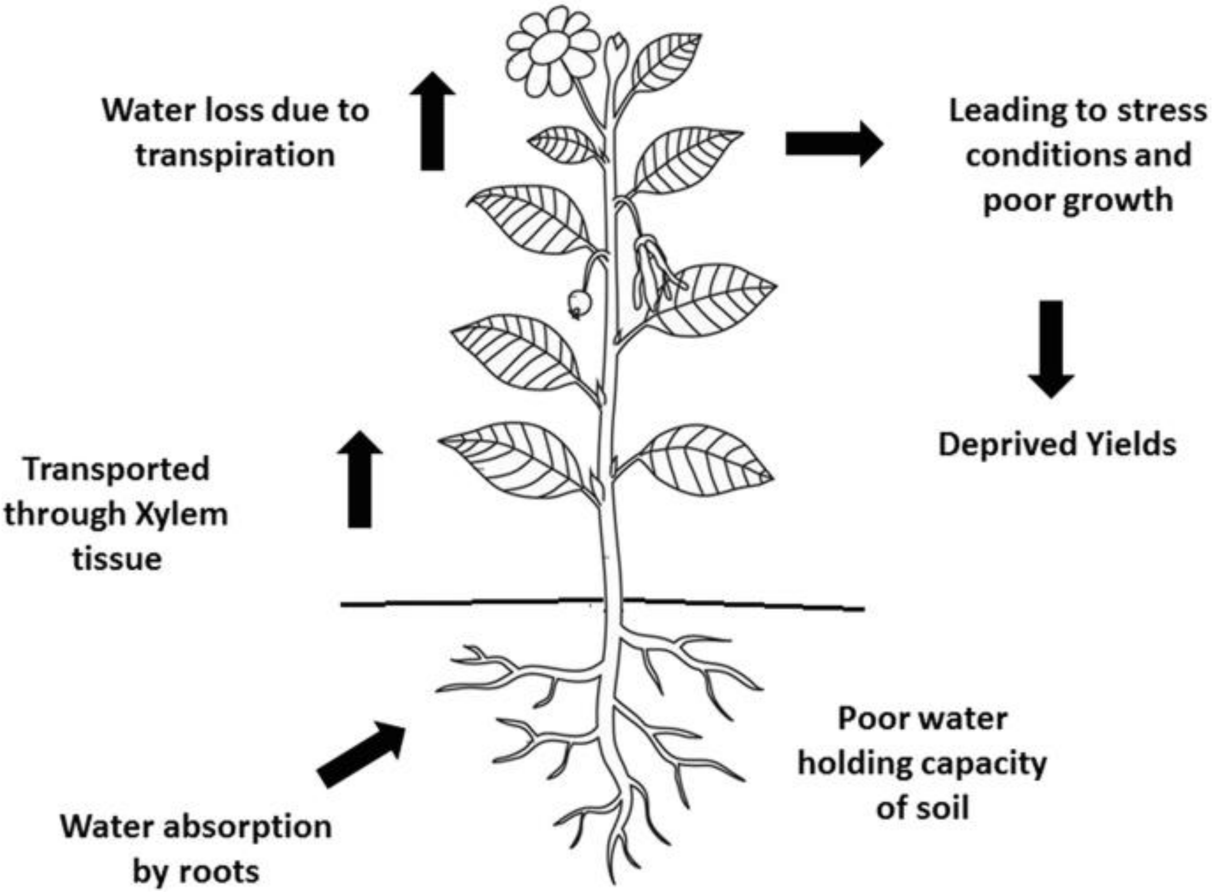

Figure 1.1: Drought stress condition in plants results in deprived yields (Iqbal et al., 2020).

The adverse effect of drought stress conditions on crop production principally depends on the harshness of the stress and the growth stage of exposure (Akram, 2011). A variety of physiological progressions influence yield during this time (Ali et al., 2017). Reduction of anthesis when plants experience drought before affects the filling of cereals because of a reduction in activity of ADP glucose pyrophosphorylase (Farooq et al., 2009). Weakened photosynthesis, reduced assimilate partitioning, and insufficient leaf development all contribute to low yields. The impoverished rate of germination and reduced formation of seedlings are the earliest results of drought conditions on plants (Li et al., 2013). The growth of plants requires the proper division of cells, their differentiation, and subsequent cell enlargement. However, drought stress affects cell elongation and mitosis, thereby resulting in abridged plant growth (Farooq et al., 2009). Reduction in turgor pressure affects growth, which is one of the physiological developments that is sensitive to drought (Nezhadahmadi et al., 2013). As described by Basu et al. (2016), drought stress hinders turgor pressure, a phenomenon that inhibits cell growth, resulting in reduced leaves and leaf area and a subsequent reduction in the fresh/dry weight ratio.

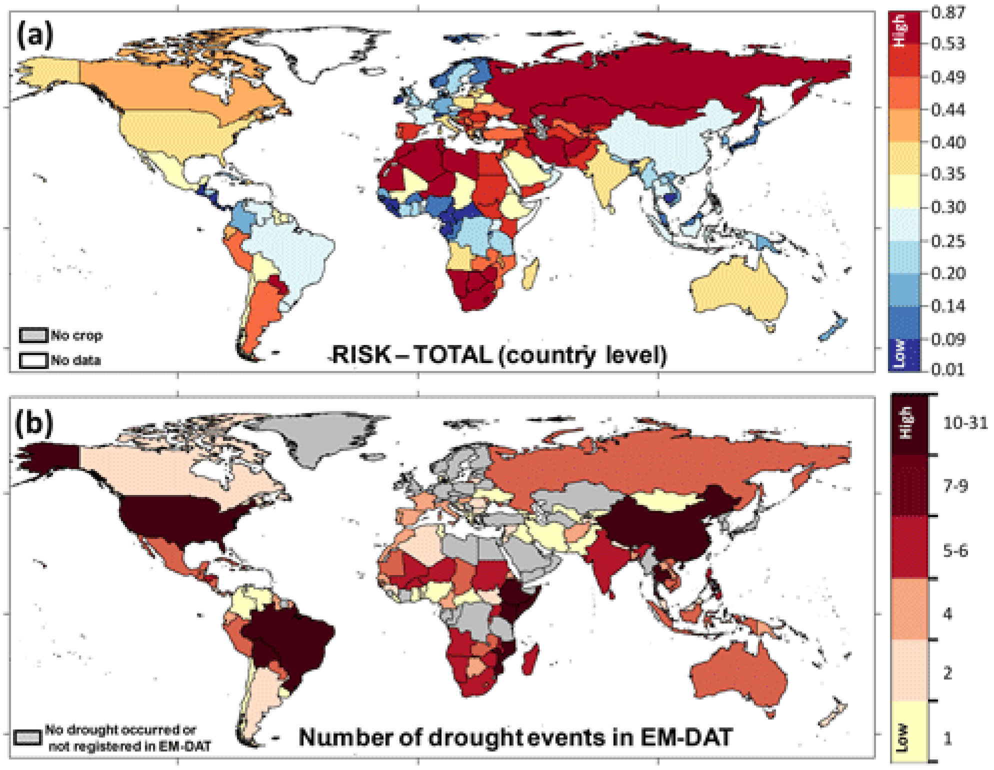

Figure 1.2: The comparison of total drought risks (a) against the number of drought events registered in the Emergency Events Database (EM-DAT) (b) shows clear agreement in most countries. Countries with low drought risk, such as tropical Africa, western and northern Europe, have no or only one registered drought (Meza et al., 2020).

Unlike any other aspect of plant physiology, stomatal conductance is enormously affected by water deficit, leading to a decline in transpiration rate and leaf water potential, thereby intensifying canopy and leaf temperatures (Turner et al., 2001). Photosynthesis is also crucially affected due to the reduced leaf area, insufficient performance of the photosynthetic mechanism, and senescence of the leaf (Wahid et al., 2007). Carbon dioxide availability is curtailed upon closure of the stomata, and damage to photosynthetic pigment due to reduced water accessibility further affects photosynthesis. Reduced carbon dioxide consumption causes oxidative radical damage and assimilation deficiency. **Figure 1.3** illustrates the implication of drought stress on the photosynthesis mechanism.

Sink strength is damaged in drought stress during early grain filling, leading to a reduction of the endosperm cell number and metabolic activity. Moreover, most assimilates like nitrogen are translocated to the roots to refine their water acceptance, a process that is dependent on photosynthesis and sucrose concentration. Under drought conditions, however, damage to the photosynthesis process and a decrease in sucrose content reduce the partitioning from source to sink (Basu et al. 2016; Lawlor and Tezara 2009; Chaves et al. 2002).

If photosynthetic light reactions are continued during drought stress when intercellular carbon dioxide is limited, reduced components of photosynthetic electron transport accumulate, thereby reducing molecular oxygen and causing oxidative damage by the development of reactive oxygen species (ROS) in the chloroplast. These ROS damage different lipids and proteins, thereby posing severe jeopardy to cell functioning (Birben et al., 2012). For this reason, crop breeding and management practices focus on improving cultivars via the reduction of yield losses under water shortages.

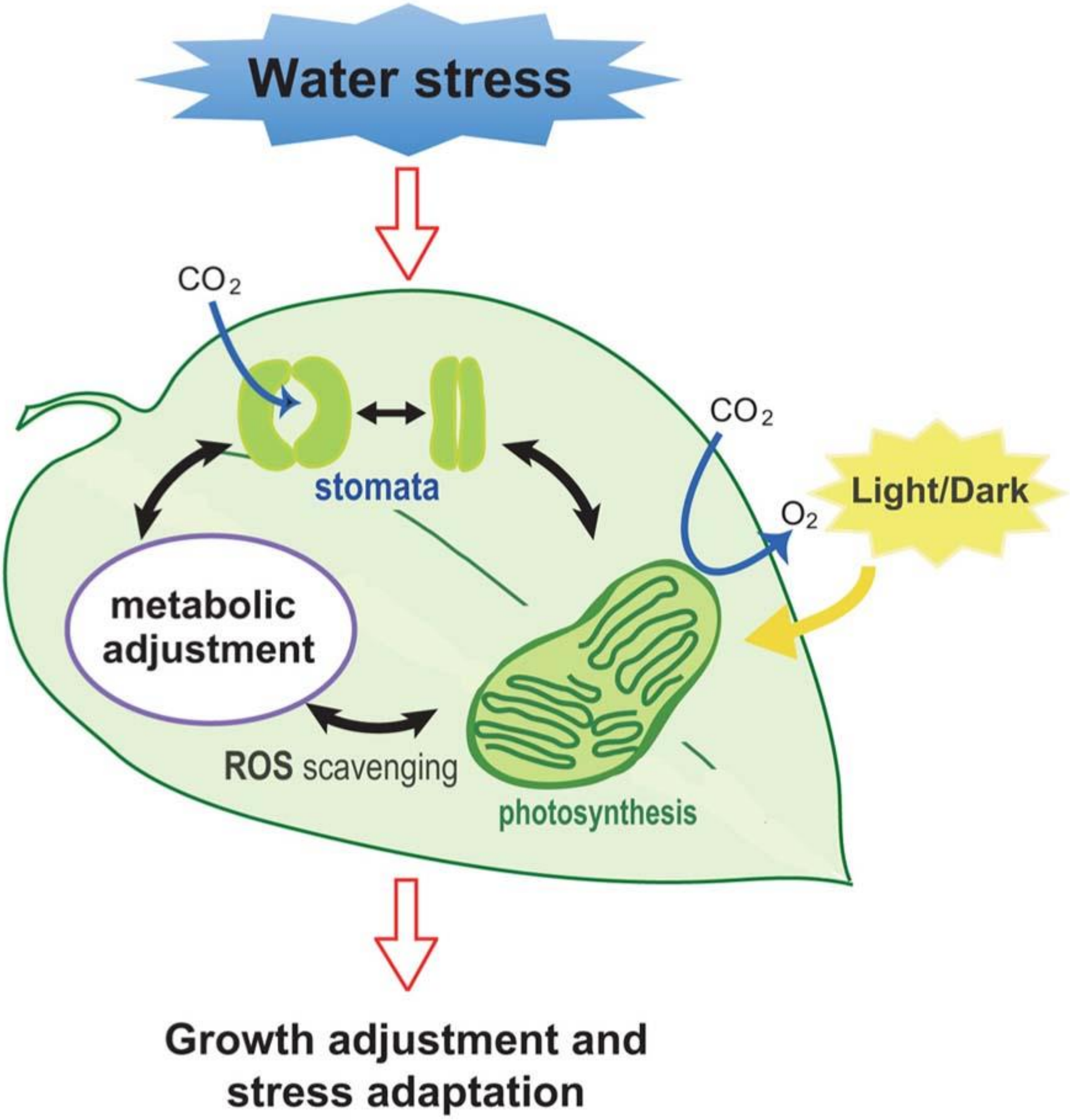

Figure 1.3: Drought stress limits stomatal conductance, which inhibits carbon dioxide consumption and results in the loss of assimilation and the production of ROS, thereby affecting photosynthesis (Osakabe et al., 2014).

1.3 Plant response to drought stress

Under water-stress conditions, plants use a variety of morphological, physiological, and molecular adaptations to maximize water use efficiency (Osakabe et al., 2014). Plants respond to drought by employing avoidance, escape, recovery, and tolerance mechanisms (Zhou et al., 2016). They adapt their lifecycle so that they escape drought and recover from the stress by resuming growth (Mitra, 2001). On the other hand, plants tolerate the drought-induced unwanted biochemical changes through morphological and physiological adjustments. Upon sensing drought, plants increase the production of abscisic acid (ABA), which regulates the closure of stomata, thereby reducing the transpiration rate (Kashtoh & Baek, 2021). The increased ABA production in the guard cell elevates cytosolic calcium concentrations, which in turn initiates a cascade of signaling events, thereby resulting in ion effluxes outside the guard cell and reducing the turgor pressure to cause stomata closure (Kashtoh & Baek, 2021). **Figure 1.4** illustrates stomatal control by ABA during stress.

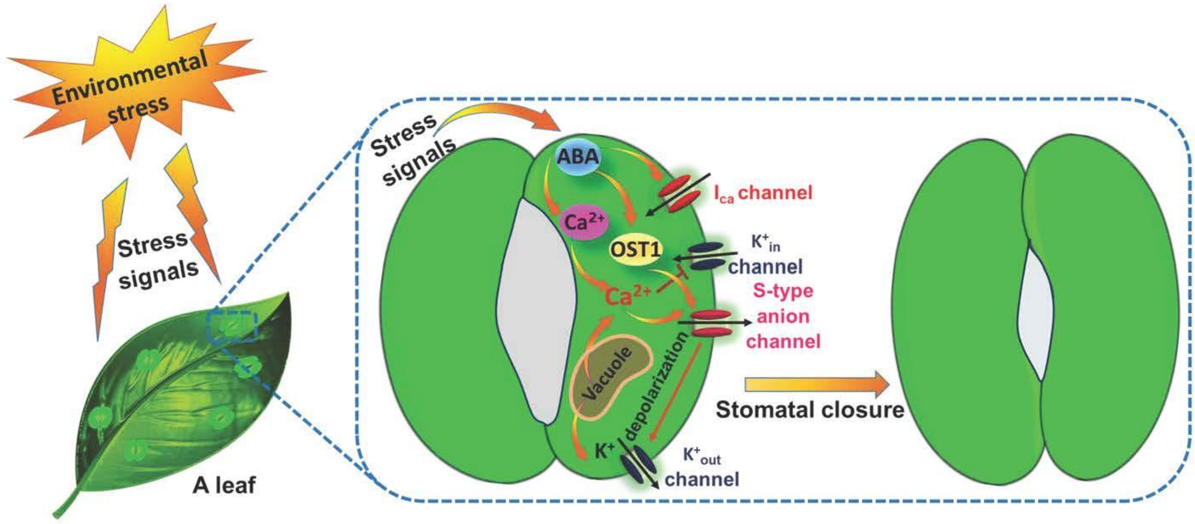

Figure 1.4: ABA is produced during water stress and transported to the guard cells. ABA induces the production of reactive oxygen species like $H_2O_{2,}$ which in turn acts as triggers for production of NO, the inhibition of membrane proton pumps, and Ca^{2+} influx across the plasma and vacuole membranes. Ca^{2+} increases activate slow and rapid type anion channels, resulting in anion efflux from the cells. The anion efflux depolarizes the membrane, causing K+ efflux via K+ out channels across the vacuole and plasma membrane. There is depolarization of the plasma membrane, reduction of the turgor pressure and cell volume, and the stomata close (Kashtoh & Baek, 2021).

Moreover, plants generally depend on the defense of both enzymatic antioxidants like glutathione reductase, superoxide dismutase, catalase, and peroxidases and non-enzymatic antioxidants like carotenoids and glutathione to combat oxidative stress (Farooq et al., 2008; Kasote et al., 2015). As a result, maintaining high levels of antioxidants may be a worthwhile strategy for plants to guard against the destructive impact of reactive oxygen species generated by drought stress conditions (Tripathy & Oelmüller, 2012).

Furthermore, morphological changes like leaf rolling and root adjustment confer tolerance to plants. The study of Denčić et al. (2000) expressed that special attention is paid to wheat due to its morphological traits during drought stress, including leaf (expansion, area, shape, size, senescence, pubescence, waxiness, and cuticle tolerance) and root (density, length, and dry weight). For example, the presence of lateral and small roots is taken as an adaptive strategy due to augmented absorptive surface to increase water uptake (Basu et al., 2016).

Osmotic adjustment, which is a process of solute accumulation in cells upon reduction in water potential, helps in turgor maintenance (Basu et al., 2016). Under drought, osmotic adjustment is implicated in stomatal conductance maintenance, photosynthesis, leaf water volume, and growth. Drought tolerant plants have increased capability for osmoregulation through the accumulation of solutes like inorganic cations, organic acids, free amino acids, and carbohydrates. Solutes such as glycine betaine and proline accumulate to adjust the osmotic balance, detoxify ROS, protect membrane integrity, and stabilize proteins (Chaves & Oliveira, 2004). In wheat, there are numerous genes that are accountable for drought stress tolerance and code for different types of enzymes and proteins such as helicase, late embryogenesis abundant (lea), responsive to abscisic acid (Rab), rubisco, glutathione-S-transferase (GST), carbohydrates, and proline during drought stress (Nezhadahmadi et al., 2013).

Mainly, the developing grains are the primary sinks, while the flag leaf is the source, and drought stress is known to affect the source sink relationship by reducing the strength of the source and thereby reducing yield (Ali et al., 2010; Lawlor & Paul, 2014). Like in many other crops, the increase in genetic yield potential in wheat results from a rise in the harvest index, which is proportionate to the above ground assimilates partitioned to the grains. During the entire plant’s growth, the source organ’s photosynthetic activity has a prominent effect on the consequent sink organ’s demands. This suggests that the source has a feed-forward effect on sink size (Evans & Wardlaw, 2017), implying that adequate sugar supply via photosynthesis, conversion, and transport is recommended during drought stress (Li et al., 2015).

1.4 Stress memory

The effects of stress differ depending on many factors, like the duration and intensity of the stress, the growth phase, genotype, and the imprint(s) that previous stress episodes have left on the plant. These imprints are the stress memory, which can be defined as the modifications that occur biochemically, genetically, and/or structurally due to a stress encounter and which enable the plant to be more resistant (or sensitive in some cases) to future exposure of the same stress factor (and if the future stress is different, the term “cross-stress tolerance” is more appropriate) (Fleta-Soriano & Munné-Bosch, 2016; Bruce et al., 2007; Boyko & Kovalchuk, 2011). According to Boyko & Kovalchuk (2011), stress memory can modify responses to subsequent stress in the same or in the following generation(s). The possibility of stress memories can be traced back to the early nineteenth century. Jablonka & Lamb (1995) show that the inheritance of acquired adaptive traits and their importance in evolution were described by Jean-Baptiste Lamarck in 1809 in the *Philosophie Zoologique* publication. In his work, Lamarck posits that traits acquired by organisms in response to environmental change could be inherited by the next generation. Although the larger scientific community referred to this theory as "soft inheritance" and is inclined more toward Darwinian evolution theory instead, the observations on the inheritance pattern appearing to be Lamarckian cannot be assumed. In 1915, the first non-Mendelian genetics observation was made when a cross of pea plants with a regular and a "rogue" leaf phenotype resulted in a progeny that lacked the anticipated segregation and recovery of the regular phenotype (Bente et al., 2021). The "rogue" phenotype persisted through many generations, an observation that was not consistent with the expected patterns of Mendelian inheritance (Bateson & Pellew, 1915). Recently, according to Koonin & Wolf (2009), the rate of horizontal gene transfer (HGT) and the nature of acquired genes rely on the environment of the receiver organism, and, in some instances, the transferred genes offer a selective gain for growth in that environment, meeting the Lamarckian standards.

The topic of stress memory is controversial because it renews the argument about Lamarckian inheritance mechanisms. One of the fundamental scientific grounds for objection of the idea of stress memory is that only a few known mechanisms are associated with the influences that parental environment has on subsequent generations (Heard & Martienssen, 2014). In addition, if memory mechanisms exist, the resulting memory effects are objected to be of limited importance for long term evolutionary process since they could be transient and erased within a generation (West-Eberhard, 2003).

Stress memory could entail transgenerational epigenetic inheritance that occurs when memory effects are found in generations that are not directly or indirectly exposed to the initial environment or cue that prompted the change (Heard & Martienssen, 2014). In this case, the environmental effects are incorporated into the germ line. Bell & Hellmann (2019) assert that stress memory has substantial evolutionary and ecological consequences even when its effects are only apparent in one generation. For example, stress memory could buffer or prepare offspring for living in a new environment, which may possibly be adequate to allow a population to be established and endure in the new environment.

There is a lot of literature on the evolution of stress memory and associated phenotypic plasticity (Mousseau & Fox, 1998; Schlichting & Pigliucci, 1998; West-Eberhard, 2003; Uller, 2008). Regardless of the evidence that stress memory can be adaptive, dispute exists regarding how its adaptive significance can be assessed, and the necessity to measure the complete set of traits at different multiple points in an organism’s development. For example, Marshall (2008) indicated the potential of stress memory effects to generate offspring traits that are adaptive for overcoming particular stressors, but at the same time decrease general survival. Moreover, stress memory effects could have potentially adaptive advantages at a given life stage, which may cause fitness cost at a later stage (Schlaepfer et al., 2002).

Stress memory can be looked at as a communication process with multiple steps, where environmental signal experienced in one generation influences the phenotype of a later generation (**Figure 1.5**). Parents must first detect the environmental signal, processes the provided information by changing the physiology, hormones and/or gene expression (Moran, 1992; Dall et al., 2015). The parents use this processed information to produce a different signal that is transmitted to the offspring. Different imprints like hormones, microRNAs, and chromatin structure could be transmitted between parents and offspring (Jablonka & Raz, 2009). Offspring must detect a similar environmental signal (in utero or post birth) for the cue transmitted by the parent to influence their response and development. The offspring process the information in the parent’s cue and integrates it with their experiences, genes and other information sources to influence the development of their phenotypes.

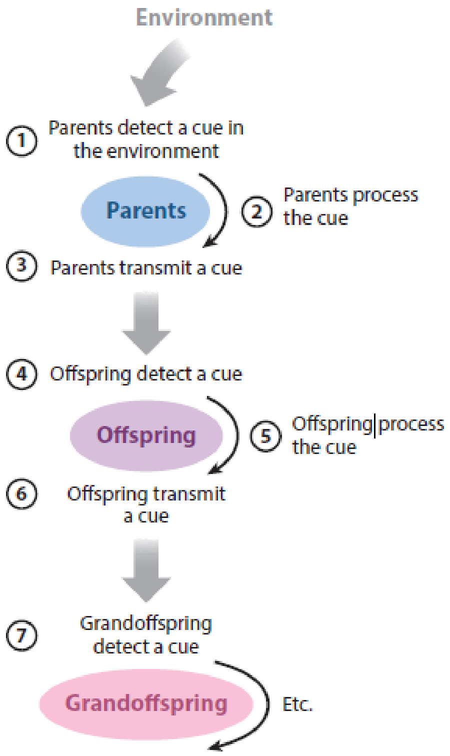

Figure 1.5: A theoretical outline for understanding the steps of stress memory. Offspring use the information from the parent to affect their phenotype and may or may not produce and transmit a cue to the grand offspring etc.

Timing is very important in stress memory transfer. The stage at which an organism experiences environmental stress has important implications for its detection, processing, and transmission to the offspring. Timing is therefore, used in the generation of the different memory types. Somatic stress memory is indicated when stress induces changes in plants that are transient and quickly reset to pre-stressed levels upon removal of the stress. However, these changes could be mitotically heritable and last for several days or the rest period of plant life in the same generation (Liu & He, 2020). Intergenerational stress memory is recognized when there is a direct impact of the stress on gametogenesis, fertilization, and embryonic development, so that the imprints are stored in the seeds to affect the immediate offspring against recurring stress. Moreover, transgenerational memory could arise if the stress imprints from the previous encounter(s) are detectable for at least one non-stressed generation (Liu & He, 2020). Therefore, intergenerational and transgenerational memories are likely to occur when stress is experienced soon before the formation of the offspring and if this cue provides information regarding the likely environment of the offspring (McNamara et al., 2016). In this regard, environmental cues experienced at juvenile stages might be irrelevant for transgenerational transmission, but could generate somatic memory. More importantly, organisms could have time points when they are more likely to be exposed to a given stress, when they are better suited to receive and process it, or when the stress has a particularly strong effect on their physiological state (Zannas & Chrousos, 2017). Timing is also important for the offspring because the manner in which they receive and integrate signals from their parents depends on their developmental stage. In this regard, cues submitted by the parents have the potential to influence the offspring only when the offspring have developed systems capable of detecting them and if they are able to start the required developmental responses to the information provided. Otherwise, the information will be lost. There are different outcomes of stress memory effects, as illustrated in **Figure 1.6**, which include recovery, decline, persistence, accumulation, delay, and reverse patterns (Bell & Hellmann, 2019).

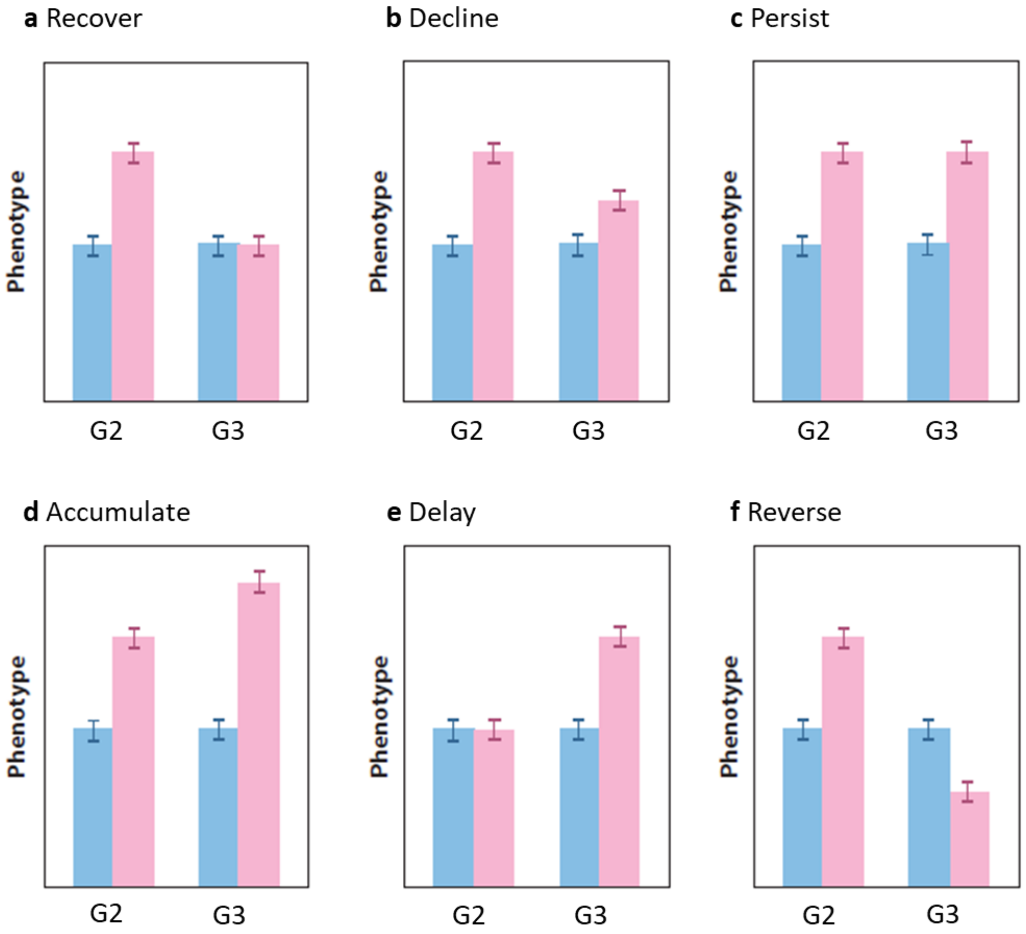

Figure 1.6: Possible outcomes of stress-related memory effects. Here, the assumption is that the first generation (G1) was exposed to environmental stress. (a) The G2 phenotype is influenced by the stress experienced in the G1, but the effects do not persist into the G3 but bounce back. (b) The G2 and G3 phenotypes are influenced by the cues experienced in the G0 generation, but the mean effects decline in the G3 generation (c) The G2 and G3 phenotypes are equally influenced by the cue experienced in the G1 generation to show persistence of phenotype. (d) The G3 phenotype exceeds the mean of the phenotype induced in the G2 generation, meaning that the prompted phenotype accumulates. (e) The phenotype of the G3 generation is influenced by the stress experienced by the G1 generation, but the phenotype of the G2 generation is not. (f) In response to stress in the G1, the G2 and G3 phenotypes change in opposite directions.

Although the depiction in **Figure 1.6** is of scenarios in which the mean phenotype rises in response to G1 cues, the direction is subjective and could be the opposite (Bell & Hellmann, 2019). The question of why and when the experiences of one generation override signals from a previous generation could lead to the assumption that it is always beneficial for the adaptive advantages acquired by a previous generation to be passed on. Still, acquired traits are not always adaptive, especially if they do not fit the current environment (Herman et al. 2014). It is worth noting that the reality of stress-related memory effects is much more complex. For instance, different traits could portray different patterns in a single study. Furthermore, the timing and dosage of stress exposure, as well as genetic variation within a single species, can result in a variety of outcomes (Alexander & Wulff, 1985).

1.5 Wheat as a model of stress memory

Wheat is a global staple food crop and is classified among the three major cereal crops, including rice and maize. It accounts for 20% of the total proteins and calories in the human diet, and the whole grain contains around 60-70% of starch content. Consumption includes not only its primary producing countries but also countries where it is not grown. Worldwide, its production is about 757.6 million tonnes every year, with a yearly consumption of around 734 million tonnes (Schmidt et al., 2020). It is unrivalled in its cultivation range, which includes elevated tropic and non-tropic regions. It is also cultivated in temperate, irrigated, dry, and high-rainfall regions, as well as in warm, humid, dry, and cold environs. Its diversity range and extent to which it is embedded in regions of diverse societies and cultures is also incomparable (Shewry, 2009). **Figure 1.7** illustrates the evolution of wheat production, utilization, and stocks.

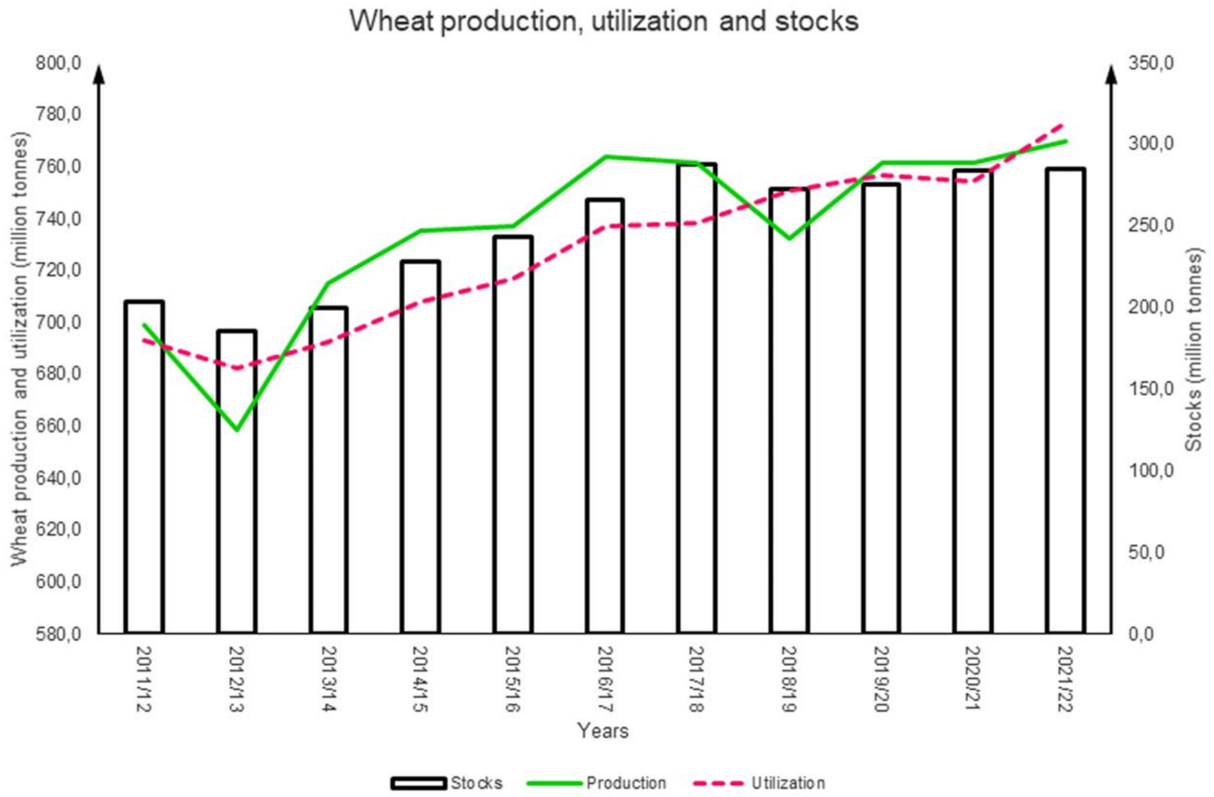

Figure 1.7: Wheat production (green line), utilization (red dashed line) and stocks (bars) from 2011 to 2022 globally (in million tons) (Laugerotte et al., 2022).

Wheat belongs to a diverse family of related grasses that are characterized as members of the *Triticum* genus and was first cultivated around 10,000 years ago during the Neolithic Revolution, thus marking the transition to settled agriculture, which was different from the hunting and gathering of food that was earlier practiced. The earliest cultivated forms were diploid and tetraploid, whose genetic relations show that they originated from Turkey (Dubcovsky & Dvorak, 2007). Hexaploid bread wheat appeared when cultivation spread to the Near East about 1000 years later (Bonjean & Angus, 2001).

Farmers initially selected landraces from wild populations based on high yields and other suitable characteristics. In addition, domestication also included the selection of genetic traits to separate them from their wild relatives. Loss of spike shattering at maturity to prevent loss during harvest and the change from hulled forms to free threshing naked forms are two important traits from domestication syndrome (Nalam et al., 2006; Simons et al., 2006). The current modern tetraploid and hexaploid wheat forms are free-threshing.

Natural populations were domesticated to develop the diploid and tetraploid forms, while cultivation by hybridization of the tetraploid (*Triticum turgidum*, AABB) with the unrelated *Triticum tauschii* (*Aegilops tauschii* or *Ae*. *Squarosa,* DD) resulted in the hexaploid (bread wheat). **Figure 1.8** illustrates the evolution of modern wheat. The tetraploid's A genomes and hexaploid forms relate clearly to the A genomes of wild and cultivated diploid, whereas the hexaploid's D genome is undoubtedly derived from *T. tauschii* (Shewry, 2009). Presently, approximately 95% of the wheat grown globally is hexaploid bread wheat, with almost all of the rest 5% being tetraploid durum wheat.

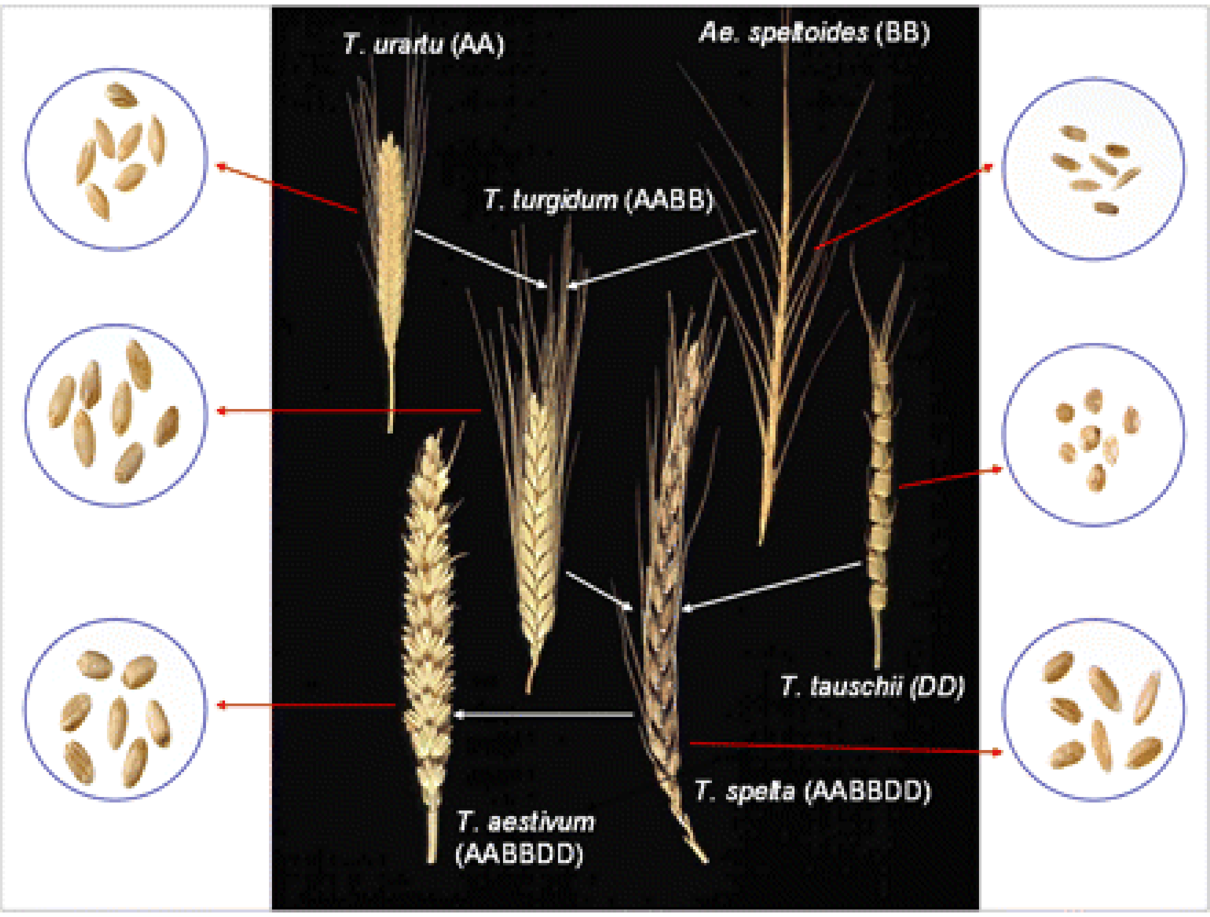

Figure 1.8: The evolution of bread wheat, displaying patterns of spikes and grain (Shewry, 2009). The diploids T. urartu (AA) and Ae. speltoides (BB) hybridized to form tetraploid T. turgidum (AABB), which underwent further hybridization with the unrelated T. tauschii (Aegilops tauschii or Ae. Squarosa, DD) to result in the hexaploid T. spelta (AABBDD), which was domesticated to the free-threshing T. aestivum (AABBDD).

The wheat grain requires 35-45% minimum water content by weight to germinate (Evans et al., 1975). Although the optimal germination temperature is between 12 and 25 °C, wheat can germinate from 4 to 37 °C. Seed size influences growth, development, and eventual yield (Mian & Nafziger, 1994). The response to vernalisation differentiates the two major wheat flowering types, including spring type and winter-type, which show a strong response to vernalisation and therefore need a cold weather period to flower.

Water shortages and swiftly dwindling groundwater tables progressively force growers to limit irrigation numbers and opt for supplementary instead of full irrigation. Therefore, the risk of irrigated wheat getting exposed to drought is on the rise, which is why CIMMYT emphasizes on the development of high yielding drought-tolerant cultivars (Dixon et al., 2009). Acevedo et al. (2002) suggest that together, deficits in water, nutrients, and the effects of pathogens and pests result in reduced global wheat yields of about 2.8 tonne per hector.

Wheat shows sufficient genetic diversity to permit the development of varying types adapted to fit a wide range of environments. Its adaptability and high yield potential have contributed to its success. Indisputably, the complex nature of its genome has enabled this wide adaptation, thereby providing great plasticity to the crop. Cultivated wheat genotypes have high levels of ecophysiological plasticity and can regenerate rapidly following stress (Sadras & Rebetzke, 2013; Le Roux et al., 2020). Although stress response mechanisms in wheat vary between genotypes, interactions between genotypes and resource management in varying environments exist (Zaefyzadeh et al., 2009). Nardino et al. (2022) establish that, alone, genetic variation is inadequate for explaining the differences between genotype responses. An accurate quantification of the phenotypic variance proportion that can be attributed to environment aspects like stress memory and/or epigenetics would clarify wheat’s differences in plasticity between similar genotypes. However, conducting stress memory studies is difficult because it is a big challenge to replicate parental materials, offspring environments, and the occurrence of multiple mechanisms that act simultaneously to produce phenotypes (Herman and Sultan, 2011). On the other hand, it is possible for wheat to overcome these obstacles. According to Naz et al. (2019) and Gaurav et al. (2022), compared to AB genomes, there is an alarming low level of genetic diversity and an abundance of repeated sequences across the D genome due to abrupt changes in chromosomes and a low rate of recombination, thereby creating a bottleneck in the genetic diversity of complex traits. In addition, wheat (*Triticum aestivum* L.) is a self-pollinated species with outcrossing (OC) rates believed to be less than 1% (Lawrie et al., 2006). As such, the offspring are a very close genetic match to their parents, and the observed phenotypic differences under identical environments are unlikely to result from genetic variations between the offspring.

In crops like wheat, increased understanding of drought stress response could potentially contribute to the advancement of cultivars that cope with stressful environments. Historically, this development focused on plant performance under drought in a single season, but there is a major possibility to expand this exploration. Understanding the long-lasting heritable characteristics of stress acclimation is critical to continued development towards adjusting crop production in progressively more volatile environments.

1.6 Aim of the study

Little attention has been paid to the long-term effects of stress on crop performance and production because the stress tolerance breeding strategy involved breeders exposing crops to stress and quantifying their performance in a single generation. The lacking knowledge makes it difficult for breeders and farmers to address the increasing threat of stress on agricultural production. Therefore, a more rigorous study of how stress experienced in the progenitor plants impacts on subsequent generations through stress memory could advance breeding and management programs towards improvement of tolerance to stressors. Water deficit induces drought stress memory, which results to phenotypic differences in various traits in plants, including seed, seedling vigour, root architecture, general plant physiology, and the transcriptome, which require characterization. Using winter wheat as a model system, various questions will be answered in the different chapters of this dissertation. There is a need for extensive phenotyping due to the expectation of an interaction between stress memory and the performance of wheat at multiple growth stages. There is a high likelihood that offspring will grow up in an environment similar to their parents, and various traits will be more accurate in predicting crop development and yield.

1.6.1 Hypothesis

- Offspring grown under the same water regimes but originating from drought-stressed or non-stressed previous generations will respond to drought stress differently.
- The strength and duration of memory increase depending on the number of generations experiencing drought stress and whether they are interrupted by non-stressed generations.
- Tolerant cultivar responds to drought memory effects different from susceptible cultivars
- There is genetic variation in the plasticity of inter- and transgenerational memory effects
- Gene expression changes in some genes (memory genes) are induced and repressed by drought stress memory

1.6.2 Objectives

1. To review the renewed knowledge on stress memory and its regulation in plants experiencing recurrent drought conditions.
    1.1 Classify stress memory in plants and give an overall view using general examples.

   1.2 Focus on drought stress and summarize the epigenetic modifications associated with gene expression control during recurrent drought episodes.
   1.3 Correlate transcriptional and posttranscriptional memory with various drought memory imprints.
2. To examine the intergenerational and transgenerational effects of drought stress on winter wheat (*Triticum aestivum L.*) seed, development, and production.
   2.1 Compare if offspring grown under the same offspring moisture treatment but with a history of drought stress in their grandparental and/or parental generations show different phenotypic responses than offspring without a drought history in their progenitor generations.
   2.2 Determine if the number of exposures (dose effect) and generation (s) during which stress was experienced determine the strength of memory effects.
   2.3 Assess whether grandparental and/or parental drought stress exposures could positively impact drought stress response of offspring.
3. To determine stress memory of physiological and biochemical responses in different wheat cultivars under drought stress.
   3.1 Establish whether physiological and biochemical processes display behaviour consistent with memory.
   3.2 Evaluate whether distinct levels of plant organization, and phases of plant growth show different responses to stress memory.
   3.3 Characterize cultivar-drought-susceptibility-status variation in memory-based responses to drought.
4. To link alterations of seed transcriptome to changes in seedling physiological, biochemical, and morphological responses during repeated drought stress in winter wheat.
   4.1 Establish whether drought stress perceived by prior wheat plant generations induced changes in physiological, biochemical, and morphological responses of seedling leaf and root systems.
   4.2 Identify sets of genes that display coordinated changes in gene expression under different drought histories.
   4.3 Identify putative functions of key genes that may point to physiological, biochemical, and morphological processes that are most strongly involved in drought stress memory.

Different chapters of the dissertation convey the results of the study as follows;

Chapter 2: Stress memory and its regulation in plants experiencing recurrent drought conditions.

Chapter 3: Intergenerational and transgenerational effects of drought on winter wheat (*Triticum aestivum* L.).

Chapter 4: Stress memory of physiological and biochemical responses in different wheat cultivars under drought stress.

Chapter 5: Changes in seedling physiological, biochemical, and morphological responses during repeated drought stress and the associated seed transcriptome of winter wheat.

Chapter 6: General discussion.

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REVIEW

Stress memory and its regulation in plants experiencing recurrent drought conditions

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Abstract

Developing stress-tolerant plants continues to be the goal of breeders due to their realized yields and stability. Plant responses to drought have been studied in many different plant species, but the occurrence of stress memory as well as the potential mechanisms for memory regulation is not yet well described. It has been observed that plants hold on to past events in a way that adjusts their response to new challenges without altering their genetic constitution. This ability could enable training of plants to face future challenges that increase in frequency and intensity. A better understanding of stress memory-associated mechanisms leading to alteration in gene expression and how they link to physiological, biochemical, metabolomic and morphological changes would initiate diverse opportunities to breed stress-tolerant genotypes through molecular breeding or biotechnological approaches. In this perspective, this review discusses different stress memory types and gives an overall view using general examples. Further, focusing on drought stress, we demonstrate coordinated changes in epigenetic and molecular gene expression control mechanisms, the associated transcription memory responses at the genome level and integrated biochemical and physiological responses at cellular level following recurrent drought stress exposures. Indeed, coordinated epigenetic and molecular alterations of expression of specific gene networks link to biochemical and physiological responses that facilitate acclimation and survival of an individual plant during repeated stress.

Introduction

Global warming is one of the most important effects of climate change because it poses the heaviest environmental challenge confronted by mankind at the moment (Rajak 2021). It is not only influencing the air temperature but is also affecting the amount and distribution of precipitation, thereby resulting to future more frequent drought spells (Wang et al. 2014). Drought stress has been reported as one of the most destructive abiotic stress factors globally and generates a huge negative impact on crop production (Vurukonda et al. 2016; Koua et al. 2021). In describing agricultural drought, Trenberth et al. (2014) relate it to deficit in moisture in the topmost of about one meter of soil usually the root zone, thereby impacting crops. A meta-analysis of data collected between 1980 and 2015 showed that drought stress led to 40% yield reductions in maize and 21% yield reductions in wheat (Daryanto et al. 2016). Between the years 2005 and 2015, economic loses induced by drought were estimated to be around 29 billion USD (Trenberth et al. 2014; F.A.O. 2021). Recent droughts have had strong impact on world cereal production and will continue to cause year to year yield fluctuations (F.A.O. 2021), with predictions of having 50% of arable land under drought stress by the year 2050 (Kasim et al. 2013).

Drought stress can occur in every growth stage of a plant and influence the water relations of the plant at all levels including whole plant, organs, cellular and molecular levels (Li et al. 2014; Muscolo et al. 2015). In general, the growth and development of a plant are affected, thereby resulting to production of smaller organs as well as altered production of flowers and grain filling (Farooq, et al. 2009). In addition, stomatal closure is followed by a progressive decline in

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net photosynthetic activity and water-use efficiency, which greatly impair the productivity of plants (Wu et al. 2022).

Different from other organisms, plants are rooted permanently to one location and only respond to environmental cues through adjustment of growth and development patterns. Thus, flexibility is an essential requirement for plants to survive stress, which they maintain through operation of a signal response network (Amtmann & Armengaud 2009; Cutler et al. 2010) that enables them to reprogram their molecular machinery including transcription factors, stress-responsive proteins and secondary messengers (Tani et al. 2019). Plants also respond to drought by adjusting their metabolism/biochemical machinery like ethylene, proline and auxins alterations (Nair et al. 2008; Sharma et al. 2012). In addition, physiological changes involving cell membrane stability and osmotic adjustment (Abid et al. 2018), and morphological changes (phenotypic plasticity) (Basu et al. 2016) occur in plant during exposure to drought.

Recently, researchers have discovered that the ability of plants to adjust response mechanisms in a continuously changing environment shapes their fitness in future and eventually enables them to live in highly diversified habitats (Fleta-Soriano and Munné-Bosch 2016). Upon exposure to stress, plants alter their epigenetic, physiological and metabolomics machineries that modify responses to future similar stress in the same generation (somatic) and/or in the next generation(s) (intergenerational or transgenerational) to adapt and survive in many ways. This popular phenomenon in which an environmental signal prepares a plant for possible future stress exposure is referred to as priming. Xin and Browse (2000) described it as a resource saving strategy of improving plant tolerance to stress. The preservation of a primed state over time forms the basis of stress memory (Haider et al. 2021). Regardless of what plant's future holds, the first stress exposure will leave an imprint in the plant that affects how it responds to later stresses (Liu et al. 2021a). Therefore, stress memory in plants is the capability of a plant upon exposure to stressors to store stress information so that it can respond in a different fashion when challenged by the same stress later (Bruce et al. 2007; Avramova 2015; Bilichak et al. 2015; Crisp et al. 2016; Fleta-Soriano and Munné-Bosch 2016). This capability is an integral part of plant resilience under changing climate.

Available studies exploring the topic of stress memory in plants have so far advanced the understanding of priming by detailing epigenetic, transcriptional, proteomic and physiological alterations resulting to imprints that establish stress memory in plants (Liu et al. 2022a; Sharma et al. 2022; Singh & Prasad 2022). While these studies have described variation between epigenetic marks and their effect on stress response, the integration of altered gene expression due to these modifications with physiological, biochemical and morphological responses of plants during recurrent stress is not well explored. We elucidate the interconnection of these mechanisms during recurrent drought episodes by describing the coordinated stress memory changes (imprints) at different OMICS, cellular and organismal levels that prepare plants to be more responsive to future stress within or across generation(s), which could provide new opportunities for crop improvement to ensure food security (Fleta-Soriano and Munné-Bosch 2016; Godwin & Farrona 2020).

In this review, we (1) classify stress memory in plants and give an overall view using general examples; (2) focus on drought stress and summarize the epigenetic modifications associated with gene expression control during recurrent drought episodes; and (3) correlate transcriptional and posttranscriptional memory with various drought memory imprints.

Classifications of stress memory based on time point of stress and mode of inheritance

Various terms have been devised to describe the different stress memory types, usually based on the stage of the plant when priming is done and the mode of inheritance (Fig. 1).

Somatic stress memory

Stress memory that is limited to one generation in duration is referred to as somatic stress memory (Lämke and Bäurle 2017). While the abiotic stresses occurring at different stages result in a higher risk of injury, plants can experience stress at an early stage during their growth and development, which can induce short-term stress memory to allow the plants to be tolerant if a similar stress strikes in later developmental stages (Li and Liu 2016). Therefore, somatic stress memory lasts for a short period of time, and its memory imprints are inherited mitotically.

Intergenerational versus transgenerational stress memory

Exposing a plant (parental generation) to drought stress during the reproductive phase also exposes its reproductive cells and the resulting seeds to the same drought stress. Therefore, stress memory in the progeny generation could be mediated by cues introduced into the seed or embryo by the parental plant. This type of stress memory is referred to as intergenerational and implies the direct exposure to the stressor of the parental generation and the following generation (progeny) by means of the developing germ cells (Heard & Martienssen 2014; Lämke & Bäurle 2017).

On the other hand, transgenerational transmission is present when effects of the ancestral exposure to an

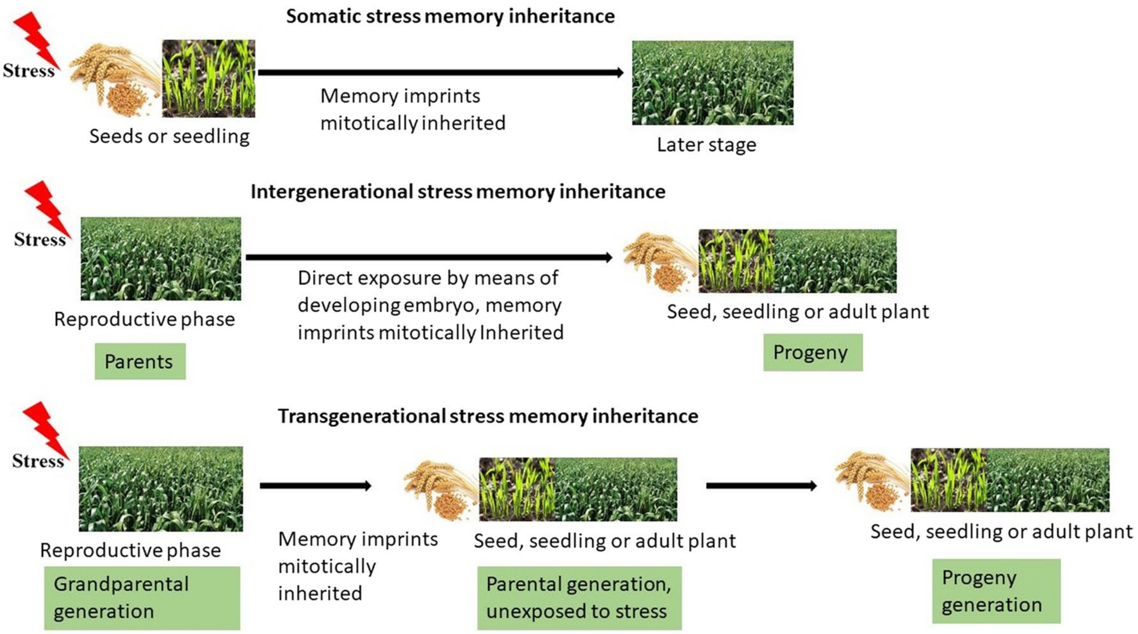

Fig. 1 Somatic, intergenerational and transgenerational stress memory. Memory is dependent on stage of the plant at which priming is done

environment during reproductive stage are present in the generation that is not directly exposed (Klengel et al. 2016). Hence, if grandparental generation was exposed to stress at reproductive stage, true transgenerational inheritance can only be observed in the progeny generation, when the parental generation had been unexposed (recovery period) (Fig. 1).

Stress memory to various stressors in plants

Whether plants can remember is a provocative question that has lately preoccupied scientists. Recent studies addressing priming and stress memory have provided new valuable evidence on responses that are key factors of priming induced stress tolerance (Table 1).

Intensive research has been conducted to study pre-exposure of plants to biotic and abiotic stressors, which trigger stress memory response. These memory imprints enable the plants to be ready to respond to subsequent stressful events (Xin & Browse 2000; Luna et al. 2012; Balmer et al. 2015; Hossain et al. 2015; Wang et al. 2017, 2018; Fan et al. 2018; Leuendorf et al. 2020). For instance, Agrawal (2002) found out that destruction of *Raphanus raphanistrum* L. following attacks from *Pieris rapae* L. during the vegetative phase of growth had influenced the induction of resistance on progeny in a later attack when compared to the controls and additionally reported that herbivory in the maternal generation influenced the growth of the progeny especially on seed mass. Furthermore, other studies in the past had indicated the possibility of memory from attacks by aphids, pathogens and other predators, thereby portraying induced resistance on later attacks (Rogers 1966; Roberts 1983; Lammerink et al. 1984; Shattuck 1993).

In a study on three different plant species that had been grown under two CO_2 concentrations, Lau et al. (2008) discovered that the maternal CO_2 environment during grain filling stage influenced biomass of progeny of all the species. The elevated carbon dioxide (eCO_2) memory increased growth response to a future eCO_2, a finding that had been contradicted by Huxman et al. (2001), who by using *Bromus Rubens* L. found out that the effects of maternal exposure to eCO_2 reduced the performance of the progeny grown under eCO_2 treatment especially by reducing photosynthesis and growth rates.

Whittle et al. (2009) assessed stress memory to find out if *Arabidopsis thaliana* L. plants adaptively responded to environmental conditions experienced by their ancestors. They examined plants that were exposed to mild heat or cold environments in parental and F_1 generation and discovered that previous elevated temperature treatment led to a more than fivefold improvement in fitness in F_3 generation. After checking the persistence of previous stress memory, they reported that improvement due to heat memory in F_3 generation plants remained even when the heat-exposed parental and F_1 plants were grown in a normal temperature regime in F_2 generation. Using *Arabidopsis thaliana* L., the ability of plants to remember salt stress exposure as far as four generations ago was found, and transgenerational as well as somatic effects in almost all analyzed traits were

Table 1 Stress memory development in different crop plants

Stressor	First encounter—when?	Recurrent encounter(s)—when?	Plant species	Memory imprints	Reference(s)
Pseudomonas syringae pv *tomato* DC3000 (*Pst*DC3000)	Seedling stage	Reproductive stage	Arabidopsis (*Arabidopsis thaliana* L.)	Activation of salicylic acid (SA)-inducible defense gene	(Luna et al. 2012)
Herbivores—*Pieris rapae*	Seedling to harvest	Seedling stage	Radish plants (*Raphanus raphanistrum* L.)	Increased seed mass, early plant growth	(Agrawal 2002)
Tobacco mosaic virus (TMV)	Seedling to harvest	Seedling stage	Cultivated tobacco (*Nicotiana tabacum* L.)	Smaller and few lessions	(Roberts 1983)
Brevicoryne brassicae	Pre- to early flowering	Post-flowering and late flowering	Rapeseed (*Brassica napus* L.)	Increased levels of glucosinolate	(Lammerink et al. 1984)
Turnip mosaic virus (TuMV)	8th day of growth	14th day of growth	Mustard (*Brassica campestris* L.)	Increased glucosinolate concentration	(Shattuck 1993)
Carbon dioxide (CO_2) and soil nitrogen (N)	Reproductive stage for 5 seasons	Seedling stage	Sundial lupine, meadow grass and little bluestem (*Lupinus perennis L., Poa pratensis* L. and *Schizachyrium scoparium* L.)	Increased biomass and growth	(Lau et al. 2008)
Carbon dioxide (CO_2)	Reproductive stage	Seedling stage	*Red brome* (*Bromus rubens* L.)	Altered nitrogen dynamics, Reductions in photosynthesis and growth rates	(Huxman et al. 2001)
Heat/ high temperature	Bolting stage till harvest	Bolting stage	*Arabidopsis thaliana* L	Improvement in fitness (increased seed production per individual)	(Whittle et al. 2009)
Salt stress	Reproductive stage	Reproductive stage	*Arabidopsis thaliana* L	Higher expression of AtRad51, higher tolerance to salt, bigger rosette and early flowering plants	(Boyko et al. 2010; Groot et al. 2016)
Physical disturbance			Sensitive plant (*Mimosa pudica L.*)	Leaf-folding habituation, memory of inhibitory modifications and recall	(Gagliano et al. 2014)
Salt (NaCl)	Seeds	seedling	*Rapeseed* (*Brassica napus L.*)	Higher total emergence and dry weight, enhanced proline accumulation	(Farhoudi et al. 2007)
Salt (NaCl)	Seeds	Seedling	Rapeseed(*Brassica napus* L.)	Higher total emergence and dry weight, enhanced proline accumulation	(Farhoudi et al. 2007)
Low Temperature	Seeds	Seedling stage	Okra (*Abelmoschus esculentus* L.)	Increased membrane integrity	(Dkhil et al. 2014)
Heat	Before anthesis	After anthesis	Common wheat (*Triticum aestivum* L.)	Up-regulated the Rubisco activase B encoding gene *RcaB,* higher photosynthesis rate	(Wang et al. 2014)
Salt	Seedling stage	Seedling stage	Maize (*Zea mays* L.)	Increased proline levels	(Tajdoost et al. 2007)

Table 1 (continued)

Stressor	First encounter—when?	Recurrent encounter(s)—when?	Plant species	Memory imprints	Reference(s)
Seed priming with water, $CaCl_2$, moringa leaf extracts and salicylic acid	Seeds	Seedling stage	Maize (*Zea mays* L.)	Reduced the electrical conductivity, increased the leaf relative and chlorophyll contents, increased plant height and yields	(Rehman et al. 2015)
Salt	Seedling stage	Seedling stage	Sorghum (*Sorghum bicolor* L.)	Higher photosynthetic rate, enhanced osmotic resistance and reduction in root Na + uptake	(Yan et al. 2015)

observed (Groot et al. 2016). Similarly, Boyko et al. (2010) had reported that transgenic *Arabidopsis thaliana* L. offspring from salt stress-exposed parents showed increased tolerance to salt and had higher rates of recombination.

In an experiment conducted by Gagliano et al. (2014) using a sensitive *Mimosa pudica* L. plant, whose leaves close rapidly by folding to respond to mechanical disturbance, it was interesting to realize that when the plant was initially dropped to experience mechanical stress, the leaves reacted by closing tight. However, when they dropped the plant repeatedly, its response changed and did not react as expected but the leaves stayed open. This was a clear indication of training and adaptation that suggested learning and memory mechanisms. The authors also noted that the sensitive plants displayed the learned response also when they were placed for a month in a favorable environment without disturbance. In animal studies, memory is considered as long term if one can store information for 24 h and remember (Sánchez-Andrade and Kendrick 2011). Therefore, based on rules routinely used, the mimosa plants had shown that they were capable of learning and remembering what they had learnt.

Drought stress memory as a mechanism of plant adaptation

Plants' responses to drought stress have been widely investigated because drought can occur at any stage of growth, from vegetative to grain filling, thereby negatively influencing yield production. Among other mechanisms of adaptation and tolerance to water scarcity, various studies have demonstrated drought stress memory in several species (Table 2) like in *Brassica napus* L. (Hatzig et al. 2018), *Trifolium repens* L. (Rendina González et al. 2018), wheat (Liu et al. 2020), rice (Zheng et al. 2013), *Polygonum persicaria* L. (Herman et al. 2012), *Arabidopsis thaliana* L. (van Dooren et al. 2020), *Leontodon hispidus* L., *Plantago lanceolata* L. and *Trifolium pratense* L. (Cerda 2020), suggesting that previous drought stress exposure left some stress imprints that were stored to induce improvement in a subsequent stress encounter.

While on the one hand drought stress memory is viewed from an evolutionary perspective as an effective strategy that could prepare a plant for later stress by improving the plant's potential for local acclimation to changing environments, some studies have nevertheless associated it with negative effects like delayed growth and development and reduced yield (Skirycz & Inzé, 2010; Crisp et al. 2016; Wijewardana et al. 2019a, b). Therefore, although mechanisms of drought stress memory could have evolved as adaptive approaches to enhance resistance against drought, the overall performance may be compromised, thereby leading to tradeoffs between yield and stress survival (Godwin & Farrona 2020).

Molecular mechanisms controlling stress memory in plants

Efforts are made to understand the mechanistic basis of stress memory. Liu et al. (2022a) emphasize that investigations on drought stress memory suggests that regulatory mechanisms on the transcriptional level vary in response to a single stress stimulus and repetitive stress stimulations. Several exposures to drought stress enable plants to respond to a new stress by more rapid adaptive changes to gene expression patterns compared with plants not previously exposed to a drought stress (Li and Liu 2016). Growing evidence points to a stress memory that might involve the maintenance of the response to stress by transcriptional, translational or epigenetic (DNA methylation and Histone modifications) means as summarized in Fig. 2 (Sousa et al. 2022). Epigenetic modifications are either mitotically or/and meiotically heritable alterations in gene expression, which are independent of primary DNA sequence changes and potentially affect the outcome of a chromosome or locus without changing the underlying DNA (Bird 2007). According to Godwin and Farrona (2020), DNA methylation and histone modification constitute epigenetic marks within chromosomes that stably change gene expression and other chromosomal properties. Over recent years, it has become increasingly evident that transcriptional regulation cannot be fully understood unless the structural context in which it occurs is considered. Moreover, by frequently influencing the distribution of epigenetic marks, noncoding RNAs can act in a sequence-specific manner to regulate gene expression both at transcriptional and posttranscriptional levels, therefore playing an important role in epigenetic control (Thiebaut et al. 2019). On the other hand, regulation of transcription is a result of the combined effects of chromatin structural properties and the interaction of transcription factors. The transcriptional regulation by transcription factors (TFs) is the major step for the establishment of the gene expression network and has been implicated in the control of stress memory (Crisp et al. 2016). Therefore, we summarize the current findings on gene expression regulation mechanisms associated with drought stress memory by showing their integration with drought memory-responsive genes.

Epigenetic regulation of transcription

Histone modifications and drought memory

DNA is in eukaryotes complexed with eight positively charged histone proteins, consisting of two molecules of each histone (H2A, H2B, H3 and H4), wrapped by 147 negatively charged DNA base pairs to make a nucleosome (Cutter & Hayes 2015). Generally, H2A, H2B, H3 and H4 can undergo covalent modification mostly at lysine and arginine residues by methylation, acetylation, ubiquitination, phosphorylation, biotinylation and ADP-ribosylation (Feng and Jacobsen 2011). Histone marks are a type of chromatin modifications that have been associated with drought-responsive memory genes and the subsequent enhancement of transcriptional response to recurrent drought stress. Kim et al. (2012) found a clear enrichment of H3K4me3 in the coding regions of drought-responsive genes *RD20*, *RD29A* and *AtGOLS2* that increased in response to drought stress and was maintained after gene deactivation by rehydration. In contrast, although H3K9ac increased initially during drought stress, it quickly responded to gene deactivation by rehydration and was drastically reduced on drought-inducible genes. This suggests the possibility of H3K4me3 to function as a stress memory epigenetic mark.

During repeated drought exposures on *Arabidopsis thaliana* L., even though the *RD29B* and *RAB18* genes returned to their initial non-stressed transcript levels when the plants were rewatered, they remained associated with uncommonly high levels of H3K4me3 and Ser5P polymerase II, demonstrating that RNA polymerase II is delayed or hindered in its activity (Ding et al. 2012). This observation supports the findings by Kim et al. (2012) regarding H3K4me3 as a drought stress memory epigenetic marker. The concept of transcriptional memory was clearly illustrated by the observed return of transcript levels to baseline during recovery and a higher induction of transcript levels on a subsequent stress exposure. In *Gossypium hirsutum* L., Tian et al. (2022) revealed that H3K4me3 is necessary for the upregulation of memory genes *GhNCED9*, *GhPYL9-11A*, *GhP5CS1* and *GhSnRK2* during repeated drought, and its level on these genes decreased considerably on the 5th day following recovery. Memory genes with enriched H3K4me3 have also been documented, especially in *P5CS1* in salt stress and *HSP22.0* in heat stress (Feng et al. 2016; Lämke et al. 2016).

DNA methylation and drought memory

DNA methylation is an epigenetic modification where unlike in histone methylation, methylation unvaryingly takes place at the carbon-5 position of cytosine residues (Feng and Jacobsen 2011). Under the action of methylase, the DNA sequence of genes is not altered, but gene function is changed in response to external environmental stimuli. Generally, demethylation events are accompanied by the activation of genes, while methylation in the regulatory or coding regions hampers the expression of target genes (Sousa et al. 2022). This alteration is usually inherited

Table 2 Summary of studies tackling repeated drought stress in various crop species. DAS, days after sowing; FC, field capacity

Plant species	Initial stress	Repeated stress	Memory type	Memory imprints	Reference(s)
Sugarcane (*Saccharum officinarum* L.)	Withdrawal of water	Two more cycles of water withdrawal and recovery, propagules were subjected to water-deficit	Somatic	Faster recovery of CO_2 assimilation and higher instantaneous carboxylation efficiency	(Marcos, Silveira, Marchiori, et al., 2018a)
Oat (*Arrhenatherum elatius* L.)	Early drought stress followed by rewatering	Later drought stress	Somatic	High living biomass, improved photoprotection	(Walter et al. 2011)
Sugarcane (*Saccharum officinarum* L.)	First water-deficit cycle	Second and third water-deficit cycles	Somatic	Increases in intrinsic water-use efficiency, higher root water concentrations	(Marcos et al. 2018b)
Potato (*Solanum tuberosum* L.)	50% FC at tuber initiation stage	Second stress like the first	Somatic	Higher tuber yields, increased antioxidant activity	(Ramírez et al. 2015)
Barley (*Hordeum vulgare* L.)	Withholding of water at full flag leaf stage (BCH 45–47) followed by rewatering	Withholding of water at seedling stage	Transgenerational	Increased thin roots and seed-derived nutrients	(Nosalewicz et al. 2016)
Durum wheat (*Triticum durum* L.)	withholding water and rewatering to field capacity	Withholding of water following the rewatering phase	Somatic	Activated oxygen production and detoxification	(Menconi et al. 1995)
Arabidopsis thaliana L	amino acid b aminobutyric acid (BABA) treatment by soil drench to 5-week-old plants	Drought applied one day after	Somatic	Earlier and higher expression of the salicylic acid-dependent PR-1 and PR-5 and the abscisic acid (ABA)-dependent RAB-18 and RD-29A genes	(Jakab et al. 2005)
Arabidopsis thaliana L	Removing the plants from soil and air-drying for 2 h	Recovery—plants placed 22 h in humid chambers for followed by a similar drought treatment	Somatic	An increase in the rate of transcription and elevated transcript levels of a subset of the stress–response genes	(Ding et al. 2012)
Common wheat (*Triticum aestivum* L.)	21 days after sowing, mild drought then rewatering for 48 h	Severe drought immediately after rewatering	Somatic	Induction of coordinated antioxidant defense, reduced H2O2 accumulation and membrane damage, higher relative water content	(Selote & Khanna-Chopra 2006, 2010)
Common wheat (*Triticum aestivum* L.)	Withdrawal of water 5–7 days at vegetative stage	Water withdrawal at grain filling stage	Somatic	reduced photoinhibition in flag leaves, higher concentration of abscisic acid	(Wang et al. 2015)
Ice plant (*Aptenia cordifolia* L.)	Withholding water for 10 days and rewatering for 4 days	Withholding water for 9 days,	Somatic	Increased abscisic acid	(Fleta-Soriano et al. 2015)
Common wheat (*Triticum aestivum* L.)	Soil relative water content around 35–40% before anthesis	Soil relative water content around 20–25%) 15 d after anthesis	Somatic	Higher photosynthesis rate and ascorbate peroxidase activity, altered protein expression	(Wang et al. 2014)

Table 2 (continued)

Plant species	Initial stress	Repeated stress	Memory type	Memory imprints	Reference(s)
Rice (*Oryza sativa* L.)	Water withdrawal for 6 days followed by 3 days of rewatering	3 days Water withdrawal immediately after rewatering	Somatic	Low MDA, increased peroxidase (POD) and superoxide dismutase (SOD) activities, lncRNA, DNA methylation and endogenous phytohormones	Li et al. 2019; Li et al. 2011)
Orange (*Citrus sinensis L.*)	Withdrawal of water after two years of growth followed by rewatering	Two more subsequent cycles of drought stress	Somatic	epigenetic and hormonal (abscisic acid, auxins and salicylic acid) changes	(Neves et al. 2017)
Arabidopsis thaliana L	Removal of plants from soil and air-drying for 2 h, then rehydration by dripping water to the root for 24 h	Dehydration was repeated	Somatic	Changes in the distribution level of AhATL1expression and AhATL1	(Qin et al. 2021)
Beet (*Beta vulgaris* L.)	Water withdrawn 35–54 DAS, followed by rewatering	86–102 DAS, rewatering, 135–151 DAS, rewatering	Somatic	Alterations in osmotic potential, proline and chlorophyll content	(Leufen et al. 2016)
Soybean (*Glycine max* L.)	Water-deficit at 4-day-old seedlings, recovery	Two more drought phases with recovery in between	Somatic	changing of biochemical parameters (soluble sugar and proline)	(Nguyen et al. 2020)
Common nettle (*Urtica dioica* L.)	Water withdrawal at 49 DAS for 14 days, rewatering	Water withdrawal after flowering	Somatic	Increases in lipid peroxidation	(Oñate et al. 2011)
Rapeseed (*Brassica napus* L.)	Water withdrawal at reproductive stage till harvest	Water withdrawal at seedling stage	Intergenerational	Increased seedling fresh weight and concentrations of several amino acids and nitrogen compound	(Hatzig et al. 2018)
White Clover (*Trifolium repens* L.)	Water withdrawal with rewatering	Water withdrawal with rewatering, 7 more cycles	Intergenerational	Epigenetic change–DNA methylation alterations	(Rendina González et al. 2018)
Durum Wheat (*Triticum durum* L.)	Water-deficit stress was applied from the booting stage		Intergenerational	Differences in microRNA (miRNA) expression	(Liu et al. 2020, 2021b)
Rice (*Oryza sativa* L.)	Water withdrawal from tilling stage to seed filling stage	Water withdrawal from tilling stage to seed filling stage for another five generations and 10 generations	Intergenerational	Changes in DNA methylation patterns, transgenerational epimutations	(Zheng et al. 2013, 2017)
Redshank—*Polygonum persicaria*	Dry soil at seedling for 71 days	Achenes collected and allowed to grow to maturity under dry soil, offspring grown in dry soil	Somatic, intergenerational, transgenerational	longer root systems, increased biomass, greatest provisioning	(Herman et al. 2012)
Arabidopsis thaliana	30% soil moisture content at vegetative stage	30% soil moisture content at vegetative stage	Somatic and intergenerational	Changes in phenotypic, gene expression and DNA methylation	(van Dooren et al. 2020)

Table 2 (continued)

Plant species	Initial stress	Repeated stress	Memory type	Memory imprints	Reference(s)
Soybeans—*Glycine max* L	80, 60, 40 and 20% replacement of evapotranspiration in reproductive stage		Somatic	Reduced germination, seedling vigor and seed quality	(Wijewardana, Raja Reddy, et al., 2019)
Common wheat (*Triticum aestivum* L.)	Tillering or jointing	Post-anthesis	Somatic	Improved leaf water potential, more chlorophyll and ribulose-1, 5-bisphosphate carboxylase/oxygenase contents, enhanced photosynthesis, better photoprotection and efficient enzymatic antioxidant system	(Abid et al. 2016)
Common wheat (*Triticum aestivum* L.)	Polyethylene Glycol (PEG) stress induction at seedling stage	Water withdrawal at jointing stage	Somatic	Physiological and biochemical changes	(Abid et al. 2018)
Rice (*Oriza sativa* L.)	Vegetative stage	Reproductive stage	Somatic	Proteome changes	(Auler et al., 2021b)
Potato (*Solanum tuberosum* L.)	One-month-old plant	One day after priming	Somatic	Photosynthesis, signal transduction, lipid metabolism, sugar metabolism, wax synthesis, cell wall regulation, osmotic adjustment	(Chen et al. 2020)
Soybean (*Glycine max* L.)	Withholding of water 7-day-old plants, rewatering	One day after priming	Somatic	Induction of drought stress memory genes	(Kim et al. 2020)
Rice (*Oriza sativa* L.)	Air-drying of 4-week-old plant for 80 min at 28 °C	22 h after priming Air-drying: 80 min at 28 ◦C and a similar repeat	Somatic	Regulation of alternative splicing events	(Yang et al. 2020)
Common Grapevine (*Vitis vinifera* L.)	Water reduced to 40 field capacity	Water reduced to 40% field capacity for 3 more seasons and then, withdrawn until complete leaf abscission in the 5th season	Somatic	Reduction of xylem hydraulic safety margin	(Tombesi et al. 2018)

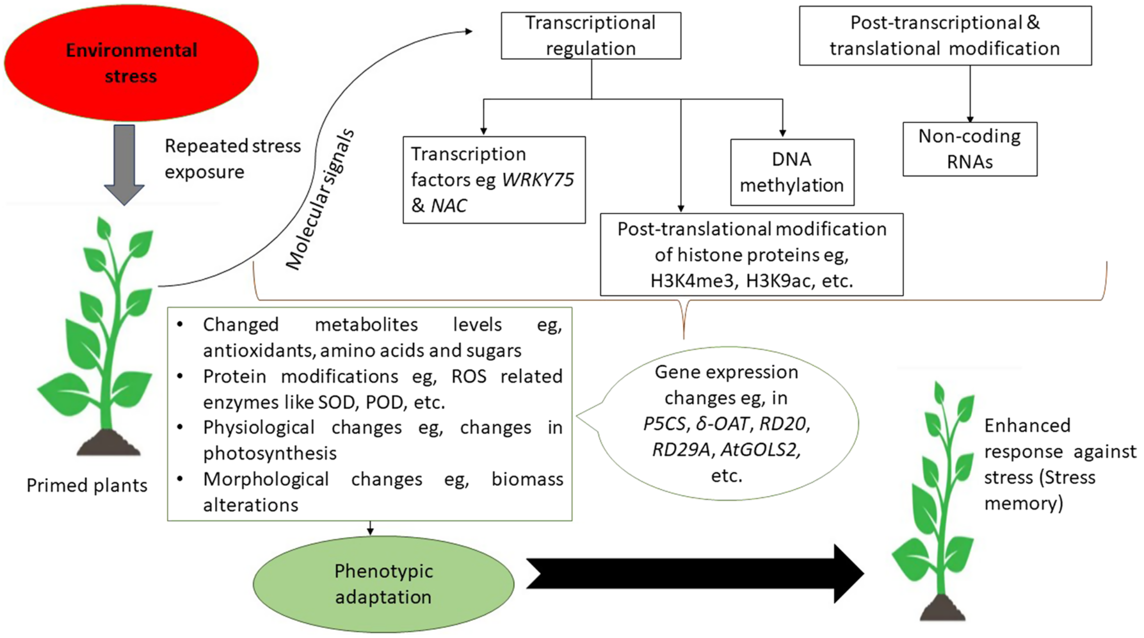

Fig. 2 A graphic presentation of interactions between gene expression control during repeated exposure and stress responses. Inheritance of epigenetic regulators like histone modifications and DNA methylation, and the alteration of regulatory RNAs and transcription factors affect the expression of genes, thereby causing changes in phenotypes of the plant

by future generations to form epigenetic memory, which offers the possibility of breeding new crop varieties that are stress-tolerant.

Selfed progenies of drought-stressed plants showed increased DNA demethylation levels in *P5CS* and *δ-OAT* genes under subsequent drought than under control treatments. This clearly indicated that proline accumulation during repeated drought is facilitated by DNA demethylation, thereby upregulating the expression of these genes. The stability of DNA demethylation of these genes was observed through the increased proline accumulation in both onetime and two-time stressed plants growing under control environment, and subsequent higher levels of gene expression (Zhang et al. 2013).

Examination of the role of DNA methylation variations on rice adaptation to successive drought stress revealed non-random appearances of drought induced epimutations (Zheng et al. 2017), which was consistent to earlier findings that showed the induction of site-specific DNA methylation (Zheng et al. 2013). The authors noted that drought induced DNA methylation alterations were inherited in advanced generations and the genes associated with the discovered transgenerational DNA methylation changes were directly involved in drought-responsive pathways. Based on the Gene Ontology analysis of the non-TE genes related to both transgenerational and recurring DNA methylation alterations, their products are involved in signal transduction, development of flowers and pollination among others. For example, *LOC_Os08g33720* gene encoding a putative lactate/malate dehydrogenase and responding to abiotic stimuli, was found to have 12 hypo-methylated CG-DMPs with recurrence frequencies (Zheng et al. 2017).

The relationship of the expression of memory genes with differentially DNA methylated regions exposed that 5373 drought memory transcripts might be regulated by DNA methylation (Li et al. 2019). Kou et al. (2021) went ahead to examine how DNA methylation is involved in drought stress memory in rice cultivars under recurrent drought stresses and recovery treatments. The study confirmed that the identified differentially methylated regions (DMRs) mediate tolerance by gene expression and transposable elements regulation. Memory (DMRs) were found in promoter region of *LOC_Os05g38150* and in gene body of *LOC_Os01g62900* to directly regulate rice drought memory genes (Kou et al. 2021). Drought in the vegetative stage altered global DNA methylation levels in rice guard cells, and these modifications remained when drought was recurrent in the reproductive stage due to greater genomic stability at this stage (Auler et al. 2021b). Gene expression analysis in this study revealed that protein abundance had a positive correlation with the expression of their coding genes. Neves et al. (2017) revealed alterations in the global DNA methylation patterns that corresponded to an increase in ABA levels in citrus plants that were subjected to three cycles of drought when compared to plants that had experienced drought stress for the first time. However, a different study that investigated

DNA methylome changes in *Arabidopsis thaliana* L. plants and five successive generations subjected to drought stress failed to link the transgenerational memory to epigenetic methylation (Ganguly et al. 2017). Taken together, much evidence indicates a prominent function of chromatin-based mechanisms in transcriptional memory responses linked to drought stress (Godwin & Farrona 2020).

Regulatory RNA and drought memory

Small RNA molecules or microRNAs (miRNAs) are created from intergenic regions, repetitive sequences, transposable elements (TEs) and pseudogenes, accounting for more than 90% of all RNA transcripts (Nguyen et al. 2022). They regulate gene expression in signaling and other developmental pathways. According to Melnyk et al. (2011), systemic movement of drought triggered small RNAs through the symplast and vascular tissues to the meristem leads to DNA methylation by the RNA-directed DNA methylation (RdDM) pathway. Drought stress has been reported to induce expression of miRNAs to suggest their potential use in improving tolerance of plants. Guedes et al. (2018) performed miRNAs expression during the different cycles of drought stress on *Coffea canephora* L. and identified 198 miRNAs (21-nt sequences), from which most targets transcription factors (TFs). Based on differential expression analysis, *miRNA miR408* and *miR398* were highly up-regulated in the different drought stress cycles. Liu et al. (2020) uncovered differences in microRNA (miRNA) expression following repeated drought episodes, whose targets have critical molecular roles in stress adaptations. Liu et al. (2021b) have also reported the association of small RNA and their targets with transgenerational effects of drought stress.

LncRNAs were demonstrated to participate in rice short-term drought memory (Li et al. 2019). They acted as memory factors to activate phytohormone signaling genes that participate in drought memory response. The association analysis of lncRNAs and related mRNAs revealed three memory-related mRNA transcripts (TCONS_00028567, OS02T0626200-01 and OS04T0412225-00) that participate in different pathways. In Switchgrass, the levels of lncRNAs targeting the biosynthesis of ABA and trehalose increased in both first and second drought cycles, but lncRNAs regulating ethylene signaling were suppressed in the second cycle, thereby preventing leaf senescence and supporting plant development (Zhang et al. 2018).

Transcription factors and transcriptional regulation during recurrent drought episodes

Accumulation of transcription factors (TFs) has been shown to be another possible drought memory mechanism in plants (Ding et al. 2012). For example, the transcript and protein levels found for ABF TFs indicated that ABF3 and ABF4 exhibited transcriptional memory behavior although a marginally increased protein levels in response to repeated drought stress (Virlouvet et al. 2014). In a study of epigenetic signatures of stress adaptation using *Zea mays*, Forestan et al. (2020) reveal upregulation of well-characterized transcription factors (TFs) including AP2/EREBP, NAC and WRKY families 7 days following drought recovery.

Gene expression regulation link to physiological, biochemical and morphological responses during repeated drought stress

Gene regulatory networks involved in plants response to drought stress have been studied by examining the genes associated with drought responses, which encode regulatory and functional proteins like transcription factors (Shinozaki and Yamaguchi-Shinozaki 2007; Fujita et al. 2011; Osakabe et al. 2014). Transcriptional reprogramming is a regular aspect of the primed state (Godwin and Farrona 2020). Beyond gene expression control, other aspects have been considered in the study of plant response to reiterated stress including changes in other OMICS approaches like proteomics and metabolomics. A system–biology approach revealed that transcriptional memory correlate with physiological parameters, thereby translating into physiological memory (Virlouvet et al. 2018). In this study, 164 genes classified into four categories related to ABA biosynthesis, stomatal regulation, photosynthesis and pigments pathways were found to encode known drought stress-associated proteins. Taken together, transcripts, proteins and metabolites form interconnected, dynamic networks that mediate drought stress memory in plants (Fig. 3).

On the one hand, some drought stress-responsive genes have been shown to display regulation at transcript level that are significantly different under repeated drought exposures to the responses during their first drought contact (Ding et al. 2012; D'Urso & Brickner 2014). Memory genes according to Ding et al. (2013), are those genes that show altered responses in a subsequent stress different from the non-memory genes that remain unaltered after each round of stress. Comparable to this definition, Forestan et al. (2020) refer transcriptional memory genes, as genes with stable transcriptional changes that persist after drought recovery. Therefore, transcriptional stress memory is said to be evident when there are sustained alterations in activation or repression of genes or from a changed response following a second cue (Lämke and Bäurle 2017). On the other hand, to optimize growth and reproduction in recurrently varying environments, plants have been shown to exhibit a drought stress memory on the physiological level to reduce water loss, reduce cellular oxidative stress by maintaining reactive oxygen species (ROS) homeostasis, reduce membrane damage, reduce inhibition of enzyme activity, increase CO2 assimilation, alter photosynthetic rates and change the general morphology (Fleta-Soriano and Munné-Bosch 2016; Abid et al. 2018). Our focus here is to review transcriptional memory responses in which production of increased levels of transcript and/or enhanced repression has been shown in memory genes upon recurrent drought exposure and their association with physiological, biochemical and morphological responses during repeated drought.

Alterations in photosynthesis and photorespiration

Alterations in photosynthesis and photorespiration mechanisms have been emphasized in different studies tackling drought memory. Generally, damage to the basic organization structure of the plant negatively affects many metabolic processes, carbon assimilation and the photosynthetic apparatus. However, priming has been shown to induce a better maintenance of photosynthetic efficiency during recurrent drought stress. Drought priming of *Triticum aestivum* L. (Abid et al. 2016; 2017) and coffee (Menezes-Silva et al. 2017) led to photosynthetic efficiency and increased Ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) during later stress. Wang et al. (2014) also indicated that drought primed plants before anthesis accumulated more proteins such as Rubisco small subunit, Rubisco activase and ascorbate peroxidase when subjected to another drought stress after anthesis. The propagules from drought-stressed sugarcane plants displayed increased photosynthetic water-use efficiency as well as quicker photosynthesis recovery following rehydration (Marcos et al. 2018a; b). Concurrently, drought memory genes have been by Virlouvet et al. (2018) related to photosynthesis. For example, the identified Calvin-Benson-Basham Cycle, NANDP-Me-type, NAD-ME type, PEPC, PEPCK enzyme type and PEPC kinase memory genes encode proteins that play a role in light harvesting, non-photochemical quenching, energy transfer and general photosynthesis. Memory gene that encode a chloroplast ATP synthase was down-regulated in the second stress to achieve protection of the photosynthetic apparatus.

Oñate et al. (2011) observed that when they subjected *Urtica dioica* L. in a combination of water and nutrient scarcity during juvenile stage, mature leaves revealed improved drought tolerance through modulation of chlorophyll levels during a second stress at reproductive stage. Altered chlorophyll content during subsequent encounter was also observed by Abid et al. (2016; 2017) in *Triticum aestivum* L. Indeed, among the 13 pigment memory genes noted by Virlouvet et al. (2018), two chlorophyll biosynthesis genes were down-regulated while two chlorophyll degradation genes were up-regulated in the second stress encounter. Although situation differ between plants based on the sink–source relationships during stress, a first water stress can improve plant response to a succeeding stress by diminishing the impact of

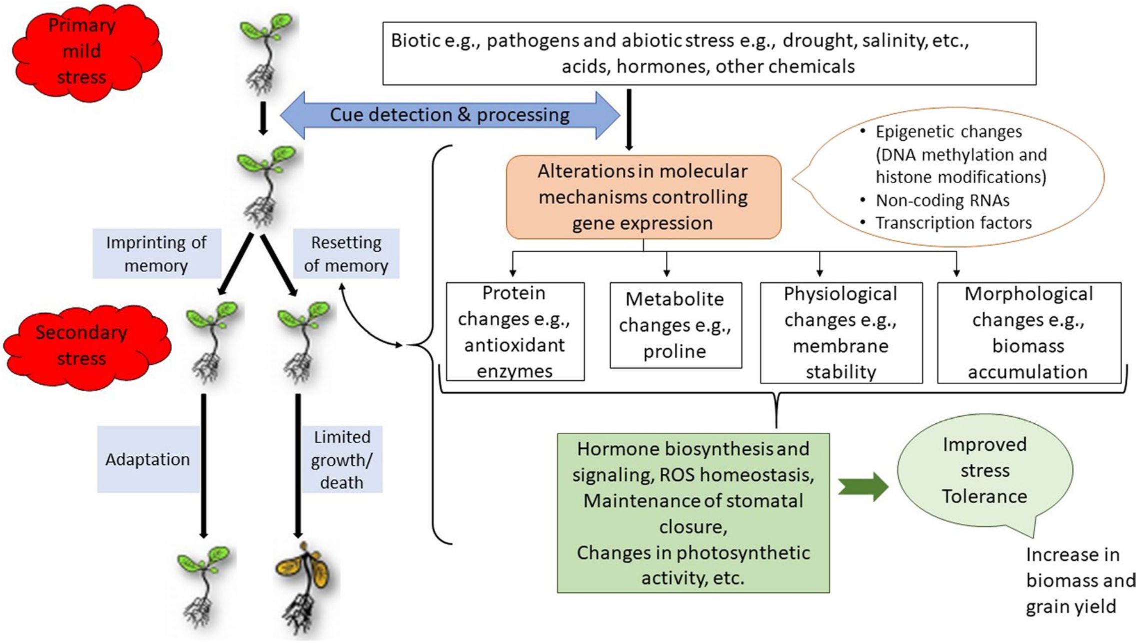

Fig. 3 An overview of stress memory. Molecular and physiological network of drought stress response (Wojtyla et al. 2020). *ROS*, Reactive oxygen species

the second stress on plant photosynthesis and energy mechanisms, thus supporting a better carbon status (Jacques et al. 2021).

Alterations in cell integrity, osmotic and plant water status

Hormones, especially phytohormones, play important roles in the regulation of different processes of plant adaptation to drought environments by modifying cellular functions at molecular levels through diverse cell signaling (Yadav et al. 2021; Iqbal et al. 2022). Abscisic acid (ABA) is a phytohormone that during drought conditions, regulates Ca^{2+} in the guard cells to induce stomatal closure, thereby preventing water loss (Ali et al. 2020). During repeated drought exposures on *Arabidopsis thaliana* L., transcriptional stress memory was displayed by an increased transcription rate and increased levels of transcripts of ABA-inducible *RAB18* (Ding et al. 2012, 2014). The transcripts accumulated progressively in every subsequent drought treatment. In their study, Forestan et al. (2020) noted higher expression levels of genes that speed up ABA biosyntheis steps (*ZEP1*, four *NCEDs* and two *AOs*) indicating stable transcriptional changes that persist after drought recovery and thereby transcriptional memory. The expression levels of ABA and jasmonic acid (JA)-related genes changed significantly in rice during the first drought exposure and the levels were stably maintained following several rounds of treatment (Li et al. 2019).

Higher ABA levels in primed wheat plants under drought stress were associated with improved tolerance to drought that occurred later during grain filling stage and subsequently to higher grain yield compared to the non-primed wheat plants (Wang et al. 2015). Fleta-Soriano et al. (2015) indicated drought memory mediated by modification in ABA by showing that the levels were raised under drought conditions if there was a previous drought exposure on the plant. Moreover, analysis of plant hormone levels in *Aptena cordifolia* L. exposed to reiterated drought revealed that Gibberelin acid went down during the first exposure and remained so in the second one, while ABA was observed to be higher in double-stressed plants compared to single-stressed plants (Fleta-Soriano et al. 2015).

Using an RNA-seq approach to investigate how *Coffea canephora* L. responded to subsequent drought, Guedes et al. (2018) were able to identify differentially expressed genes (DEG) in tolerant and sensitive clones. The findings illustrated that in the tolerant plants acclimatized to multiple drought episodes, memory genes involved in ABA pathways were identified. On the other hand, the sensitive clones were associated with memory genes that triggered an oxidative stress response that probably led to programmed death upon exposure to multiple episodes of drought. The observed transcriptional memory in tolerant and sensitive plant genes suggests the ability of the plants to opt to a mechanism to remember genes that should undergo modulation upon drought stress exposure.

An increase in expression of key ABA biosynthesis modulators including 9-CIS-EPOXYCAROTENOID DIOXYGENASE 3 (*NCED3*) and ALDEHYDE OXIDASE 3 (*AAO3*) has been indicated in previously stressed plants during recovery phase to reduce transpiration in an event of a subsequent stress attack (Virlouvet and Fromm 2015). This locus encodes a vital enzyme in the ABA biosynthesis pathway and performs an important role in signaling in drought stress. As a result of increase in the transcription of many ABA-induced genes in response to repeated drought episodes, plants reduce rates of transpiration by mediating guard cell-specific stomatal memory to keep up the leaf water content (Ding et al. 2012, 2013; Virlouvet and Fromm 2015).

A large proportion of drought memory genes in maize was by Ding et al. (2014) shown to encode for proteins associated in membrane integrity functions including dehydrins, regulators of water and potassium uptake and transport and transmembrane transporters for inorganic phosphate and sucrose. In this regard, plants that had been exposed to repeated periods of both droughts and recovery periods displayed higher retention of leaf water, reduced wilting and increased tolerance to terminal drought stress when compared to plants experiencing the stress for the first time (Jakab et al. 2005; Maseda & Fernández 2006; Ding et al. 2012; Ramírez et al. 2015). The increase in root water was also discovered in multi-generationally stressed sugarcane plants (Marcos et al. 2018b). Seedlings from drought-stressed seeds also displayed reduced membrane damage and increased water retention than the controls (Selote & Khanna-Chopra 2006, 2010; Wang et al. 2018).

Osmotic adjustment for water status maintenance is implicated in water stress plant memory (Jacques et al. 2021). Proline, an amino acid, has been shown to be a critical component of plant drought tolerance due to its role as an osmolyte. Menezes-Silva et al. (2017) reported that plants exposed to multiple drought events adapted to future stress due to the expression of trainable genes related to drought tolerance, which were associated with a deep metabolite reprogramming with concordant adjustments in central metabolic processes. Transcription memory of Δ1-pyrroline-5-carboxylate synthetase 1 (*P5CS1*) and the gene encoding of the proline biosynthetic enzyme were found to be critical in drought stress memory in rice. There was an induction of expression of *LOC_Os01g62900* and *LOC_Os05g38150*, which are *P5CS1* homologous after the initial drought stress and reached a peak during the rewatering, and then stayed constant throughout the succeeding drought stress treatment, corresponding to the level of free proline concentration (Li et al. 2019). Alves et al. (2020) also noted that proline levels in *Dipteryx alata* L. plants rose significantly following recurring drought stress. Its accumulation in the second drought exposure was also reported in peanut plants by Qin et al. (2021). However, Leufen et al. (2016) and Nguyen et al. (2020) revealed lower proline concentrations in sugar beet plants and soybeans, respectively, in the second and third drought stress episodes compared to the first stress.

Memory DMRs also regulated alpha-linolenic acid metabolism, linoleic acid metabolism, biosynthesis of amino acids, glycerophospholipid metabolism, cysteine and methionine metabolism and lysine biosynthesis pathways. Alves et al. (2020) had observed alterations in primary metabolism in *Dipteryx alata* L. plants, especially in osmoprotectants including in sugar, organic acids and amino acid levels. There were significant increases in sucrose, fructose and glucose levels in primed plants. Organic acids like citrate, fumarate, threonic acid and palmitic acid increased their levels in response to successive drought cycles. Amino acids, including glycine, histidine, alanine, GABA and tryptophan increased in plants exposed to three cycles of drought compared to those that experienced just one stress event. Oñate et al. (2011) also observed modulation of malondialdehyde (MDA) in prestressed *Urtica dioica* L. Plants. These findings indicate that past stress exposure determined the response of mature plant as these plants showed acclimation to subsequent stress.

Key proteomic cues and drought stress memory

The general abundance and activity of proteins regulate changes in metabolic pathway activities, thereby influencing metabolite levels. Posttranscriptional regulation through changed protein abundances is an important mechanism of response to stress events, and proteomic analysis under repeated drought revealed an increased abundance of proteins (Alves et al. 2020; Auler et al. 2021a, b; Ding et al. 2013; Schulze et al. 2021). Recently, Schulze et al. (2021) have examined the proteome profiling of recurrent drought events in maize and related it to stress memory responses. The authors found overrepresentation of heat-shock proteins, ribosomal proteins, starch metabolism proteins and proteins involved in photosynthesis photophosphorylation during the first stress encounter. While rewatering recovered these proteins to basal levels, ribosomal proteins remained elevated. The second cycle of drought exposure resulted in abundances in ribosomal, galactolipid synthesis, gluconeogenesis, photophosphorylation and lipid degradation proteins but not heat shock proteins. However, Ding et al. (2013) indicated downregulation of memory genes encoding ribosomal, chloroplast and photosynthetic proteins that are involved in ribosome structure, amino acid biosynthesis and photosynthesis, in addition to memory genes that encode for thylakoid membrane-associated proteins in *Arabidopsis thaliana*. Repeated drought cycles in *D. alata* seedlings led to substantial increase in the activity of superoxide dismutases (*SOD*), pyruvate oxidase (Pox) and glutathione reductase (*GR*), which were not activated by a single drought event (Alves et al. 2020). In rice, Auler et al. (2021a) report decrease in the expression of genes that encode D1and D2 proteins of reaction center of the PSII due to a single drought stress exposure, but double drought stress increased their expression. Rehydration caused the genes to portray an expression level equivalent to that of the control plants. *TRITD1Av1G156270* gene coding for late embryogenesis abundant (LEA) proteins showed variable memory responses in rice and wheat (Sadder et al. 2022). Kim et al. (2020) observed that genes encoding protein phosphatase 2C (PP2C) family proteins and LEA proteins were differentially induced. A number of ABA- and ethylene-responsive genes encoding a putative ABA 8′-hydroxylase, ABA-responsive protein-related and osmotin 34 were highly upregulated under the second drought conditions in soybeans. Comparative proteomics in guard cells between rice plants exposed only once and those with recurrent drought stress exposures at vegetative or/and reproductive stages identified 12 drought-responsive proteins that belonged to the photosynthetic pathway, oxidative stress response and stress signaling such as glucagon-like peptide-1 (GLP-1), glutathione-S-transferase (GST), SOD and those related to protein processing such as small heat-shock proteins in roots (Auler et al. 2021b). Interestingly, the abundance of proteins such as endo-1,3-beta-glucosidase, peroxidase, S-adenosylmethionine (SAMS) and malate dehydrogenase (MDH) significantly increased in roots or leaves depending on the rice genotype. Qin et al. (2021) observed a rapid increase in the expression of Arachis hypogaea abscisic acid transporter like-1 (AhATL1) protein and its levels in the second recovery periods following drought exposure. In turn, the overexpression of AhATL1 raised ABA concentrations and altered the post-response gene type into memory gene type, thereby enhancing the drought tolerance and ability to recover. Generally, these authors concluded that there were changes in protein abundance according to single or repeated drought episodes affecting many pathways in plant.

ROS metabolism cues and drought stress memory

One of the usual consequences of drought stress is the production of ROS in the different cellular compartments, including the peroxisomes, the chloroplasts and the mitochondria. ROS includes singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) (Hasanuzzaman et al. 2020). Its overproduction results in the peroxidation of cellular membrane lipids and degradation of enzyme proteins and nucleic acids (Li and Liu 2016). To alleviate the effect of ROS, plants induce higher antioxidant enzyme activities and higher expression of their related genes, thereby conferring drought stress tolerance and adaptation (Hou et al. 2021). According to Lukić et al. (2023), the anti-oxidative system plays a crucial role in forming a plant stress drought memory through changes in the activity pattern of anti-oxidative enzymes like SOD and peroxidase (POD) as well as non-enzymatic anti-oxidative defense. The authors reported that in *Alopecurus pratensis* L. both enzymes were upregulated in drought treated offspring if the parents were also stressed. Similarly, Lukić et al. (2020, 2023) and Liu et al. (2022b) have pointed out that upregulation of the anti-oxidative system is one of the major mechanisms that mediate transgenerational drought stress memory. In their study, Lukić et al. (2023) found reduced H_2O_2 concentrations in drought-exposed offspring of drought-exposed parents due to increased activity of Catalase (CAT) and POX that converts H_2O_2 to oxygen and water. The upregulation of Superoxide SOD activity and removal of superoxide anion radicals in drought-exposed offspring of drought-exposed parents subsequently resulted to a decrease in oxidative stress levels. Moreover, malondialdehyde levels under transgenerational drought priming could be caused by increased chelation of hazardous ferrous ions that initiate lipid synthesis and the formation of MDA. Menconi et al. (1995) uncovered that two drought periods on wheat obtained by withholding water and rewatering at the end of the first period during seedling stage resulted in improved scavenging of H_2O_2 and control of ROS levels. A second drought stress encounter following recovery period in wheat plant revealed the enhancement of dehydroascorbate reductase, glutathione reductase and ascorbate peroxidase (Menconi et al. 1995). Correspondingly, Li et al. (2015) reported low concentrations of H_2O_2 in wheat leaves if drought priming was done, which could be explained by the high levels of glutathione peroxidase (GPx) in the same plants. A transformed cell structure and the expression of genes mainly encoding proteins related to redox enzymes like APX have been observed in primed plants compared with non-primed plants under drought during grain filling (Wang et al. 2014). The authors postulate that the higher APX activity in primed plants contribute to improve ROS scavenging capacity, to reduce lipid peroxidation in response to a later stress. Wang et al. (2018) found out that the $O_2^{\bullet-}$ release rate and H_2O_2 concentration of wheat flag leaves were significantly increased under drought stress, while they were less affected by drought in the primed plants than in the non-primed plants. Moreover, the authors reported that the activities of antioxidant enzymes like SOD, CAT and APX were increased significantly by drought stress and were much higher in the primed plants than in the non-primed plants. GPX activity was much higher in the primed plants under a second drought encounter. However, only APX gene expression was consistent with its activity levels. Primed rice seedlings displayed increased POX and SOD activity to dissuade the harmful effects caused by oxidative damage in response to subsequent drought stress (Li et al. 2011). According to Yang et al., (2021), when compared with unprimed control, the primed plants showed lower CAT activity, whereas increasing the activity of SOD fivefold. In *Nicotiana tabacum* L., POD activity was linked to reduced H_2O_2 levels in primed plants under drought treatment (Khan et al. 2020). Moreover, transcriptional levels of related genes CAT, APX1 and GR2 were revealed in drought-hardened treatment against drought stress. The expression levels of these genes were considerably increased in drought primed plants in comparison with control, and the expression of these genes was more pronounced in T3 plants than other treatments. In *Glycine max* L., *Zea mays* L. and *Arabidopsis thaliana* L., drought memory genes that encode proteins involved in protective roles including dehydrins and chaperones were discovered (Ding et al. 2012, 2014). Synchronously, KEGG enrichment analysis results showed that the memory DMRs were involved in sesquiterpenoids, triterpenoid and phenylpropanoid biosynthesis and arginine metabolism pathways (Kou et al. 2021). These results suggest that previous drought events modified ROS scavenging systems. Defensive and detoxifying functions are important for plant stress memory since they diminish the impact of drought-induced oxidative stress by sustaining cellular metabolism (Jacques et al. 2021).

Morphological adjustments

Plant morphological characteristics are the most valuable tools in monitoring responses to stressors as they can reveal underlying factors that produce changes in plant conditions. Nosalewicz et al. (2016) have reported the transgenerational effect of severe drought stress on shoots and roots of barley (*Hordeum vulgare* L.). The study revealed that the progeny, whose parental generation was also subjected to drought, showed adaptive morphological alterations such as increased root-to-shoot ratio when compared to the progeny of parental plants that had not been subjected to drought conditions. Backhaus et al. (2014) also reported production of higher amounts of above the ground biomass if there was a pre-exposure to drought when compared to the controls without a previous drought encounter. In agreement with these findings, Marcos et al. (2018b) observed that the plants stored information from the previous stressful events, which led the sugarcane plants that were drought-stressed three times to have increased root dry matter.

Conclusions and future directions for research on drought stress memory and its application in breeding

Changes in the epigenome, transcriptome, proteome and metabolome upon stress encounter confers stress memory, which enable enhanced responses to future stress exposure in plants. Uncovering the potential of this phenomena in crops and how best this discovery can be used in plant breeding programs require an integrated approach. Taken together, the reviewed studies here provide results that point to high variation of species and/ or genotypes specificity to drought stress memory responses. Such studies provide new opportunities for plant breeders and researchers in exploiting different memory capabilities in plants to develop new cultivars in the face of changing climates.

The discovery that plants can memorize past stressful events and pass it to their progeny offers an opportunity to adjust plants' epigenetic architecture and find out how and which genes are expressed to adjust the growth of plant to adapt to the environment. Indeed, exposure to a priming agent could activate a gene or a set of genes. However, instead of reverting to the transcriptionally silent state once the stimulus is removed, an epigenetic modification could perhaps be left, keeping the region in a 'permissive' state. As a result, there is a possibility for quicker and more potent responses to subsequent attacks. This discovery can offer a non-traditional approach to breeding because gene networks that are targeted by this manipulation can be identified without altering the genotype. If a memory gene is identified, it can be regulated to make the plant behave as if it is experiencing the stress, and the mechanisms related to stress tolerance are elicited all the time through the expression of other related genes.

The nature of experiments carried out in the study of stress memory should be assessed for success and applicability. Usually, the recovery period following an initial stress is when stress information is integrated and therefore is crucial for the reinforcement of correct stress memory. In addition, experiments should incorporate different priming stages in the life span of a given crop to evaluate which stage induces most pronounced beneficial impacts. Moreover, it would be necessary to prepare, grow and multiply the seed for an experiment or a selection procedure in exactly the same way, so that the memory does not affect the outcome of the experiment or selection. This would also guarantee that memory effects between experiments are duplicated. Validation is also essential when transforming laboratory or controlled experiment information to the field. As depicted in this review, various imprints including hormones (ABA, Gibberelin acid and JA), enzymes (antioxidants such ascorbate peroxidase) and metabolites like proline are strong causes or consequences of plant memory response. Based on these studies, we propose that investigations on their concentrations during recurrent drought episodes, associated memory genes, as well as related epigenetic marks be carried out. Lastly, there are variable results regarding the usefulness of priming and persistence of the discovered drought stress memory. Therefore, further research is needed to explore the influence of priming on plant population and community structures as it involves plant performances and reproductive success. Researchers should in future also find out how the positive stress memory effects can be increased and prolonged.

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Declarations

Conflict of interest The authors declare that there is no conflict of interests.

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Intergenerational and Transgenerational Effects of Drought Stress on Winter Wheat (*Triticum aestivum* L.)

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Abstract

The environments where the progenitors are grown have the potential to affect the expression of traits in offspring of plants. Currently, there are various hypotheses regarding the evolutionary and ecological importance of stress memory effects. There is uncertainty regarding its occurrence, persistence, predictability, and adaptive value. In this study, fifteen winter wheat cultivars were grown under drought and well-watered (control) treatments for two seasons to produce seeds with all possible combinations of drought exposure histories. A comprehensive analysis to estimate transgenerational (grandparental effects), intergenerational (parental effects), and their combined memory effects on offspring traits under both control and drought moisture treatments was performed. There were significant memory effects in most of the evaluated traits ranging from +787% to -39.0% changes in both seed quality and plant traits. The expression of stress memory was highly dependent on generation and number of exposures, trait and season. Under drought treatment, the combination of grandparental and parental stress memories was additive in all traits, but their strength when considered separately were variable. Stress memory enhanced performance of offspring under similar stressful conditions; increased plant height, above ground biomass, number of grains per plant, grain weight per plant and water potential. This study offers valuable new insights into the occurrence of drought stress memory, the complexities of the effects, possible physiological and metabolic alterations explaining the detected differences, and impacts towards a clearer understanding of their generation and context-dependency.

Key words: *Triticum aestivum* L, drought stress, intergenerational, transgenerational, stress memory, plasticity

Introduction

Under the limiting environmental conditions, global agricultural crop production must increase by approximately 25-70% from current levels to meet a fast-growing food amount demand by 2050. (Hunter et al., 2017). Wheat is one of the key global cereal grains produced in terms of acreage and is consumed as a staple food in household diets (Enghiad et al., 2017), providing around 20% of calories and >25% of proteins (FAO, 2021). However, its production is affected by climatic and environmental changes (Fatima et al., 2020; Harkness et al., 2020; Sarkar et al., 2020) that include abiotic factors like drought, high temperatures, floods, salinity, etc. (Hossain et al., 2021; Khalid et al., 2019).

Compared to other abiotic factors, drought poses one of the biggest risks to food production (World Economic Forum, 2015; Seleiman et al., 2021). It occurs in virtually all climatic regions (Rashid et al., 2022), and the current environmental changes enhance its severity (Wu et al., 2017). In major regions growing wheat in the world, mostly with a Mediterranean climate, average precipitation is lower than mean pan evaporation, particularly during grain filling, leading to terminal drought (Reynolds et al., 2005). It is assumed that by the year 2025, around 1.8 billion people will face an absolute water shortage, and 65% of the world’s population will live in water-stressed environments (Nezhadahmadi et al., 2013).

Oyiga et al. (2019) assert that drought affects plant water relations at the molecular, cellular, organ, and whole plant levels. Reduced soil moisture limits nutrient absorbability and their uptake by the plant. This leads to impaired germination and poor stand establishment. Drought further inhibits dry matter production largely through its repressive effects on leaf expansion and development, and subsequently reduced light capture (Anjum et al., 2011). At the same time, stomatal closure in response to low soil water content decreases the intake of CO2. As a result, photosynthesis is decreased and leaf senescence is accelerated (Farooq et al., 2009). This hampers the redistribution of assimilates from the vegetative tissue to the reserve pools, leading to drought-related reductions in yield and yield components in plants (Lawlor & Cornic, 2002; Flexas et al., 2004; Wasaya et al., 2021; Ahmad et al., 2022).

However, while drought stress has negative effects on the productivity of plants, it leaves imprints that could be passed onto the offspring, affecting both growth and functioning (Nosalewicz et al., 2016; Hatzig et al., 2018; Racette et al., 2019). This phenomenon indicates the potential of plants to remember stressful experiences and thus the concept of stress memory, which is a comparatively unexplored driver of phenotypic plasticity (Herman et al., 2012). A single genotype expresses varying phenotypes based on past exposure (s) to changing environments (Fox et al., 2019). According to Merilä & Hendry (2014), the main model that can explain changes in phenotypes under environmental variation is plasticity, which does not imply changes in DNA sequence.

On one hand, seed provisioning is a crucial means of transmitting parental environmental effects, in which environmental factors like drought may control the amount of resources that a parent plant allocates to the developing seed (Herman & Sultan, 2011). This in turn affects seed mass and shapes various traits in the establishment of a seedling and early growth and development, especially under stressful circumstances (Mukherjee et al., 2019). Stressful parental environments have been shown to reduce the provisioning of seeds (Hussain et al., 2018; Lorts et al., 2020; Wasaya et al., 2021; Zas et al., 2013). For example, Liu et al. (2005) ascribe terminal drought to the most yield losses of any other drought occurring at other times during the plant’s growing season.

On the other hand, environmental effects can be transmitted by mechanisms not directly related to the quantity of maternal resources allocated to seed provisioning. Current research has demonstrated that many environmental conditions may cause epigenetic modifications, including DNA methylation, histone modification, and changes in small non-coding RNAs, which, when passed on, can affect gene activity in the offspring and impact its phenotypes (Boyko & Kovalchuk, 2011; Herman & Sultan, 2011). Epigenetic modifications are either mitotically and/or meiotically heritable alterations in gene expression that are independent of primary DNA sequence changes and potentially affect the outcome of a chromosome or locus without changing the underlying DNA (Bird, 2007).

Effects of the parental environment have been reported for the initial offspring generation, and some studies have shown that environmental effects can persist in the second and subsequent offspring generations (Groot et al., 2016). Intergenerational stress memory refers to offspring memory of the effects of direct exposure of a stressor to the parent. The subsequent generation (offspring) is also exposed by means of the developing germ cells (Heard & Martienssen, 2014). Transgenerational stress memory, on the other hand, is evident when effects of ancestral stressor exposure during the reproductive stage are present in the offspring generation even when its parent is not directly exposed (Klengel et al., 2016).

Only a limited number of studies have looked at the occurrence of stress memory effects in wheat and their implication in drought tolerance. Most have found effects of somatic drought stress memory, which are limited to one generation in duration (Lämke & Bäurle, 2017). For example, osmoprimed wheat seeds resulted in uniform early stand establishment and considerable expansion of traits related to yield even under repeated drought stress at the reproductive stage (Hussain et al., 2018). Further, Abid et al. (2018) showed that osmopriming of wheat seed invoked stress memory against drought induced after germination during the tillering stage. Similar positive outcomes due to somatic effects were reported by Liu et al. (2021) on parental pre-anthesis drought effects on wheat progeny. Wheat cultivars exposed to drought at the vegetative stage showed improved tolerance to post-anthesis stress (Abid et al., 2016; Wang et al., 2015). However, Mendanha et al. (2020) found that somatic drought stress memory had a negative effect on yield reduction in primed plants. Liu et al. (2020) reported parental drought stress effects in wheat; the combination of drought and heat stress during the reproductive stage of the parents negatively affected seed germination and seedling vigour.

Despite these experimental observations, numerous issues remain unsolved. The most important challenge is the inconsistency and lack of reproducibility of memory effects. For instance, while some studies report positive memory effects, others report negative outcomes (Abid et al., 2016; Hussain et al., 2018; Liu et al., 2020; Mendanha et al., 2020). Adaptive stress memories enhance an organism's response when faced with a similar future stress, whereas maladaptive stress memories hinder recovery and affect development and potential yield production (Crisp et al., 2016). Mostly, the expression of memory effects is sensitive to timing, duration, priming agent, and severity of drought stress, thereby complicating comparisons and generalizations between studies. For example, some studies investigated the combination of drought with other stress factors and their resulting memory effects in wheat (Li et al., 2017; Liu et al., 2020; Mendanha et al., 2020). Furthermore, phenotypic responses due to memory effects may also differ between traits and seasons, thus distorting the consistency of results between studies (Liu et al., 2020; 2021).

Another challenge is whether the number of exposures and the generation during which stress was experienced are relevant in determining the strength of memory effects. This raises the unanswered question of whether the strength of grandparental and/or parental effects from separate direct drought exposure on grandparental and/or parental generations can be predicted. Since there is an interruption by a non-stressed parental generation in the formation of transgenerational memory, grandparental effects would be predicted to be weaker than parental ones. On the other hand, combined grandparental and parental drought exposures may be stronger than either grandparental or parental effects acting alone, and the effects may amplify the change in phenotypes in the same direction as either grandparental or parental drought memory. We are not knowledgeable of any related study so far that has tested these hypotheses in wheat.

The major goal of the study presented here was to analyse the occurrence of transgenerational memory, intergenerational memory, and their combined effects on winter wheat. The following questions were addressed: (1) Do offspring grown under the same offspring moisture treatment (OE) but with a history of drought stress in their grandparental and/or parental generations show different phenotypic responses when compared to those without a drought history in their progenitor generations? (2) Does the number of exposures (dose effect) and generation (s) during which stress was experienced by the progenitors determine the strength of memory effects? (3) Could grandparental and/or parental drought stress exposures positively impact the drought stress response of offspring?

Materials and methods

Plant material and experimental set-up

Since wheat produces offspring by self-fertilization, grandparental and/or parental environments are referred to without distinguishing between paternal and maternal parents as they are one individual. Therefore, the wheat cultivars used in this study allow for robust investigation of stress memory effects while entirely holding the genotype constant (Mazer & Gorchov, 1996). In this regard, the term "generation" has been used to refer to reproduction where the transmission of variations is not the result of differences in DNA sequence but the inheritance of epigenetic phenomena between generations and therefore pure lines that are homozygous for every trait (true breeding). In addition, grandparental and parental exposures result in transgenerational and intergenerational memories, respectively, and these terms are used in the text to better clarify the generation when stress was experienced and the associated memory type.

In the grandparents' generation, 200 winter wheat cultivars released between 1948 and 2013 in Europe, Asia, South America, and the USA were used (Voss-Fels et al., 2019a). Each of these cultivars was assigned to drought stress and optimum water supply (control) in 2017/2018 season at Campus Klein-Altendorf, an experimental farm of the University of Bonn in Germany (50.61° N, 6.99° E). The drought stress treatment was performed on the same day for all plants receiving drought under rain out shelter from the BBCH 40 (Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie) (Lancashire et al., 1991), which corresponds to the booting growth stage until harvest (BBCH 99). The control plants were grown under rain-fed conditions. Seeds from both the drought stress (D1) and control treatments (C1) were harvested and stored (**Figure 3.1**). Out of 200 cultivars, 15 cultivars were selected based on the analysis of their drought tolerance and field performance for photosynthesis efficiency, shoot biomass, and yield production under drought stress conditions (Koua et al., 2021). The selected cultivars vary depending on the year of their release and consist of 6 drought-tolerant and 9 drought-sensitive varieties. Details regarding fertilizer application, management practices, as well as soil moisture contents in control and drought moisture treatments were described by Koua et al. (2021).

Seeds produced in the drought and control grandparental moisture treatments (D1 and C1, respectively) from the 15 selected cultivars were used as sowing material in the 2018/2019 season at the Institute of Crop Science and Resource Conservation (INRES) at the University of Bonn, Germany (50.7214° N, 7.0898° E). The seeds were cultivated under semi-controlled conditions in foliar tunnel that was open at both ends and in 12-liter pots that were filled with an equal volume of potting mixture (Terrasoil), which contained 60% natural sand and 40% topsoil. One seed was placed in each of the six 1cm-deep holes per pot and covered with soil. An automated irrigation system was started to water each pot for five minutes in the morning, at noon, and in the evening (3 times x 5 minutes). Seedlings were thinned upon germination to leave four per pot. Treatments were started on the same day for all plants at BBCH 40 by maintaining water supply via an automated drip irrigation system every day (3 times x 5 minutes) in plants under control moisture treatment and once per week (3 times x 4 minutes) in stressed plants until BBCH 99.

Upon harvest, seeds of all replicate plants per cultivar and treatment were pooled, and the resulting seed sets represented all four possible combinations of grandparental and parental moisture environments. The following abbreviations were assigned to denote the drought histories: D1D2 (drought moisture treatment in both grandparental and parental generations), D1C2 (drought moisture treatment in the grandparental generation, control moisture treatment in the parental generation), C1D2 (control moisture treatment in the grandparental generation, drought moisture treatment in the parental generation), and C1C2 (control moisture treatment in both grandparental and parental generations) (**Figure 3.1**). These seed sets were analysed for quality and used to establish the tested offspring generation in the following 2019/2020 and 2020/2021 seasons. Seed quality parameters included ratios of grain nitrogen concentration (nitrogen %), neutral detergent fiber (NDF %), and sedimentation %, which were measured using near-infrared spectroscopy (NIRS) utilizing an AD 7200 diode array feed analyser (Perten Springfield, IL, USA) and following the guidelines of the manufacturer. Near-infrared reflectance spectroscopy is a proven effective method for determining the concentrations of various components in whole grains, allowing for a multi-component analysis to be performed quickly and without the need for sample preparation (Wilcox and Shibles, 2001).

Cultivation and phenotyping of the offspring

To test for the influence of environments where grandparents and/or parents were grown on offspring phenotypes, the D1D2, D1C2, C1D2, and C1C2 seeds (**Figure 3.1**) were grown at the same location as the parent generation for germination and growth with 3 replicates per pot and treatment combination (3 pots per cultivar and treatment and 4 plants per pot). Treatments were also carried out on the same day for all plants as in the parental generation. Investigations were done over two consecutive seasons: 2019/2020 and 2020/2021, on offspring cultivated under control and drought treatment for plant height, days to flowering, above-ground biomass, number of grains per plant, and grain weight per plant. In addition, in the 2020/2021 season, additional traits, including proline content and water potential, were analysed. Soil water content was maintained by automated drip irrigation in the control treatment every day (3 times x 5 minutes) and in the drought treatment once per week (3 times x 4min). The volumetric water content in drought and control moisture treatments was measured from the initiation of drought treatment until harvest using EC-5 soil moisture smart sensors (Onset HOBO U30 USB Remote Monitoring System, USA), and the mean weekly values analysed (**Figure S3.1**). In all the experiments, 3 g of organic nitrogen fertilizer was applied three months after sowing in each pot following the soil test results. The tunnel was 2-3 degrees higher than in the field. Specifically, days to flowering were recorded as days after sowing, when 75% of plants in a pot (3 plants in the pot) had extruded anthers (Zhang *et al.*, (2018). Plant height was measured from soil level to the collar of each wheat ear at BBCH 61 using a ruler in centimetres (Griffiths et al., 2012). Proline concentrations were determined based on the protocol of Bates et al. (1973) and Frimpong et al. (2021), while the protocol by Becker & Knoche (2011) was adapted for the estimation of total water potential in leaves using Scholander Bomb. At harvest, the plant was cut from the soil level and the entire biomass dried in an oven at 105^0 C for 24 hours before the dry weight was collected using an electrical balance (Khan et al., 2017). The Contador optical counter with integrated vibration channel was used to count seeds per plant, which were then weighed using the electrical balance (Milivojević et al., 2022). Each cultivar under each category had three repetitions (pots), and each pot had four plants. It is important to note that data from each of the four plants per pot, except for days to flowering, was averaged, and recoded as one value per pot.

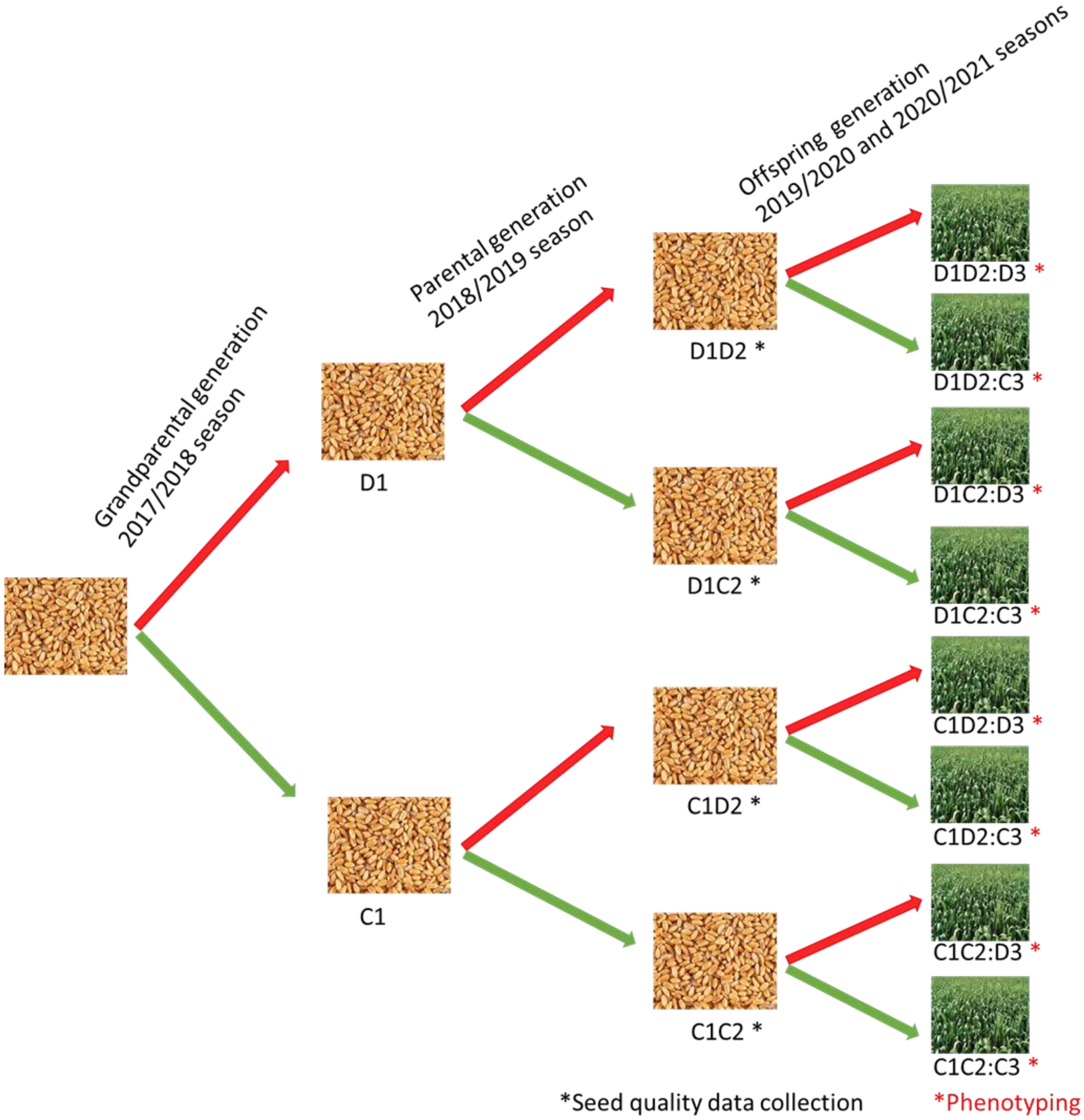

Figure 3.1: Schematic presentation of the experimental design for producing the experimental groups used in the analysis of drought stress memory. Seeds from 15 cultivars were either grown under drought moisture treatment (red arrows) or control moisture treatment (green arrows) for two generations. All of the second-generation seeds (C1C2, C1D2, D1C2 and D1D2) were tested for grain quality, after which they were grown and performance tested under control and drought moisture treatments. D - drought stress, C - control. The numbers (1, 2, and 3) indicate the corresponding generation (grandparental, parental, offspring), respectively.

Statistical analysis

Generalized linear mixed-effect models were fitted to test for drought memory-induced responses in winter wheat. Specifically, we first tested for the presence of overall effects of grandparental moisture treatments (GpE), parental moisture treatments (PE), and offspring moisture treatments (OE) and their interaction on offspring traits across the entire experimental design using generalized linear mixed effect models, where the four parental groups in this study (C1C2, C1D2, D1C2, and D1D2) (**Figure 3.1**) were recorded as a 2*2 factorial design, with GpE, PE, and OE as fixed factors. The cultivars are regarded as a representative random sample of the 200 winter wheat cultivars released between 1948 and 2013 in Europe, Asia, South America, and the USA (Christiansen et al., 2002; Koua et al., 2021; Voss-Fels et al., 2019). The drought response status and repetition were also included as random factors in all models. 95% confidence intervals and p-values were used to analyse variations between experimental groups. Any GpE*OE, PE*OE, GpE*PE, or even GpE*PE*OE interaction would be of interest as it could suggest a conceivable adaptive stress history effect (Groot et al., 2016). Whenever the usual F-test showed differences between the stress histories (C and D) of the offspring, we wanted to know exactly where these differences occurred. As a result, we used the SAS Proc Mixed to perform a contrast analysis. With that analysis, we could test whether the stress applications in the grandparental and/or parental generations, and even more importantly, which generation, had an effect on the performance of the offspring. To specifically test for grandparental, parental, and their combined drought memory effects on offspring phenotypes under offspring moisture treatment (OE) (drought and control environments), the C1C2 group was compared to the C1D2, D1C2, and D1D2 groups, respectively.

The validity of the statistical results is based on the assumption that in each generation, seeds were pooled from each experimental group, and these seed pools are an unbiased set whose characteristics only differ due to the different treatments in their ancestral generations. All analyses were performed in SAS and R.

Results

Grandparental and/or parental generations of drought exposure caused phenotypic plasticity in seeds and plant traits

The effect of ancestral drought exposure on offspring traits was first tested by examining the overall effects of GpE, PE, and OE and their interaction across the entire experimental design, followed by comparisons of offspring whose ancestors were not exposed to drought stress (C1C2) with offspring of sets that had experienced a single generation of drought stress either two generations ago (D1C2; grandparental drought memory effects) or one generation ago (C1D2; parental drought memory effects). For a memory effect the direct effect (GpE, PE or OE) was the most important. Considerable GpE effects were found for all seed quality traits, proline, and water potential (**Tables 3.1 and 3.2**). Strong PE effects were revealed for all of the seed quality traits, plant height, days to flowering, above-ground biomass, and grain weight per plant in one or both of the seasons (**Tables 3.1 and 3.2**). OE impacts were found for all reported traits other than water potential (**Table 3.2**).

Table 3.1: ANOVA results for the effects of grandparental moisture treatment (GpE), and parental moisture treatment (PE) and their interaction on NDF %, nitrogen % and sedimentation %. The bold values show p<0.05.

Source of variation	**NDF %**		**Nitrogen %**		**Sedimentation %**	
	F-Value	ProbF	F-Value	ProbF	F-Value	ProbF
GpE	52.37	**8.8E-11**	4.79	**0.031**	9.59	**0.003**
PE	8.13	**5.3E-03**	772.92	**0.000**	453.37	**0.000**
GpE*PE	0.55	**4.6E-01**	7.61	**0.016**	3.76	0.075

To test whether ancestral drought exposure resulted in a possible growth advantage, we checked for any interaction effect between the factors (GpE, PE, and OE). An interaction effect means that the relationship between OE (drought and control) and a given trait depends on the type of moisture treatment (drought or control) in a previous generation (grandparental and/or parental). It might be that the ranks are still equal, or even that the ranks can change. For seed traits, an interaction effect means that the relationship between PE (drought and control) and a given quality trait depends on the type of moisture treatment in the grandparental generation. GpE*PE interaction effect was recorded for NDF % and Nitrogen %. In the 2019/2020 season, significant PE*OE interactions were discovered for plant height and days to flowering, and for water potential in 2020/2021 season (**Table 3.2**). GpE*OE interactions were found in the two seasons for grain weight per plant, but only for days to flowering in the 2019/2020 season, for plant height, above ground biomass per plant, and number of grains per plant in 2020/2021 season, and for proline (**Table 3.2**). There was a marginally significant GpE*PE*OE interaction ($p = 0.092$) for grain weight per plant in the 2020/2021 season, and these interactions were highly significant ($p = 0.009$) for water potential (**Table 3.2**). Together, these interactions are indications that grandparental and/or parental drought stress exposures could result in specific growth and production advantages in the offspring.

Table 3.2: ANOVA results for the effects of grandparental moisture treatment (GpE), parental moisture treatment (PE), and offspring moisture treatment (OE) and their interaction on plant height, days to flowering, above ground biomass, number of grains per plant, and grain weight per plant in the 2019/2020 and 2020/2021 seasons, and on proline and water potential in the 2020/2021 season. The bold values show p<0.05.

		Plant height (cm)		**Days to flowering**		**Above ground biomass (g)**		**Number of grains/ plant**		**Grain weight/ plant (g)**	
Season	Source of variation	FValue	ProbF	FValue	ProbF	FValue	ProbF	FValue	ProbF	FValue	ProbF
2019/2020	GE	0.20	0.657	1.44	0.231	1.92	0.166	0.38	0.536	0.08	0.782
	PE	6.22	**0.013**	0.22	0.641	7.62	**0.006**	3.30	0.070	4.30	**0.039**
	OE	1226.34	**0.000**	36.47	**0.000**	4469.78	**0.000**	4303.26	**0.000**	4929.37	**0.000**
	GE*PE	0.70	0.403	6.75	**0.010**	0.09	0.766	0.36	0.549	0.16	0.694
	GE*OE	2.60	0.108	8.33	**0.004**	1.60	0.207	2.67	0.103	4.41	**0.037**
	PE*OE	11.16	**0.001**	6.75	**0.010**	0.15	0.695	0.08	0.780	0.01	0.930
	GE*PE*OE	1.44	0.230	0.26	0.610	0.47	0.492	0.23	0.630	0.25	0.617
	GE	2.21	0.138	0.10	0.756	0.93	0.337	0.25	0.618	0.39	0.532
	PE	0.30	0.586	10.43	**0.001**	0.22	0.640	0.29	0.588	0.54	0.462
	OE	208.25	**0.000**	5.74	0.017	241.79	**0.000**	392.08	**0.000**	434.33	**0.000**
	GE*PE	0.63	0.428	2.61	0.107	0.35	0.557	0.39	0.533	0.08	0.780
	GE*OE	4.16	**0.042**	0.09	0.765	11.52	**0.001**	19.75	**0.000**	17.86	**0.000**
	PE*OE	1.24	0.267	0.84	0.361	1.13	0.289	0.95	0.332	1.65	0.201
	GE*PE*OE	0.02	0.886	0.77	0.381	0.38	0.535	1.85	0.175	2.85	0.092

		Proline (μgg^{-1})		**Water potential (MPa)**	
2020/2021	GE	26.71471	**4.91E-07**	8.796559	**0.003254**
	PE	0.211254	0.646196	0.138132	0.710401
	OE	166.8893	**1.94E-29**	0.387386	0.534137
	GE*PE	0.160583	0.688971	17.33272	**4.08E-05**
	GE*OE	21.79571	**5.01E-06**	0.004854	0.944503
	PE*OE	0.002765	0.958104	4.111726	**0.043448**
	GE*PE*OE	0.923909	0.337402	6.911483	**0.008994**

Specifically, grandparental drought (D1C2) effects increased NDF % (**Table 3.3; C1C2 *vs* D1C2, Figure 3.2 b**). In the 2020/2021 season, these effects increased plant height, number of grains per plant, and grain weight per plant only when the offspring was also stressed (**Table 3.4; C1C2 vs D1C2, Figure 3.3; 2020/2021 season, Drought, a, d, and e**). However, drought GpE decreased number of grains and grain weight per plant if the offspring was growing in control OE (**Table 3.4; C1C2 *vs* D1C2, Figure 3.3; 2020/2021 season, Control, d and e**). Further, grandparental effects decreased the expression of proline and water potential under drought OE (**Figure 3.4; Drought a and b**). Parental drought (C1D2) increased sedimentation % and nitrogen % in seeds (**Figure 3.2 a and b**). These effects increased plant height and above-ground biomass per plant only under drought OE in the 2019 /2020 season, but increased days to flowering in the 2020/2021 season only when the offspring was also experiencing drought stress (**Table 3.4; C1C2 *vs* C1D2, Figure 3.3; Drought, 2019/2020 season a and c, 2020/2021 b**). However, parental drought increased water potential of the offspring in control OE (**Figure 3.4; Control b**).

Table 3.3: Results of generalized linear mixed-effects model analysis of a priori contrast tests comparing the control (C1C2) group to the groups that have experienced drought stress in either or both parental and grandparental generations (C1D2, D1C2, and D1D2). Shown are p-values; significant values are indicated in bold (p<0.05).

	Sedimentation %	**NDF %**	**Nitrogen %**
Contrast	p- value	p- value	p- value
C1C2 *vs* C1D2	**0.000**	0.165	**0.000**
C1C2 *vs* D1C2	0.449	**0.000**	0.633
C1C2 *vs* D1D2	**0.000**	**0.004**	**0.000**

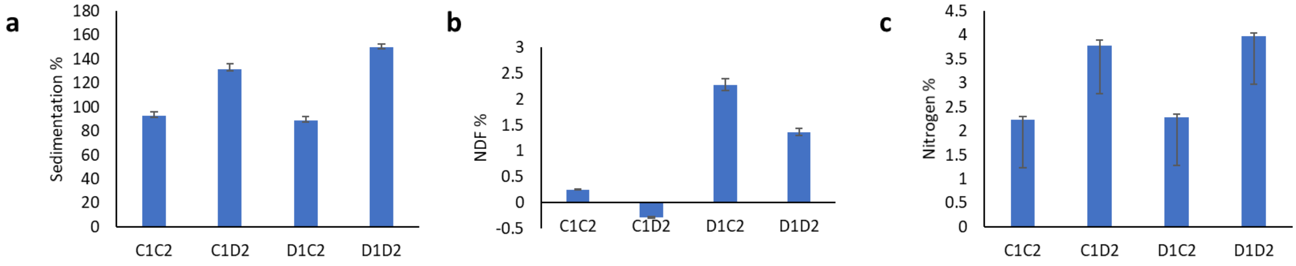

Figure 3.2: Expression of seeds sedimentation %; **a**, NDF %; **b**, and nitrogen %; **c** after exposure of grandparental and/or parental generations to drought stress. Significant differences between groups are shown in **Tables 3.3 and 3.5**.

The combined grandparental and parental drought effects (D1D2) increased sedimentation %, NDF % and nitrogen % (**Figure 3.2 a, b, and c**). They were among the strongest responses in the 2019/2020 season, increasing plant height, above-ground biomass per plant, number of grains per plant, and grain weight per plant if the offspring was also stressed (**Table 3.4; C1C2**

***vs* D1D2, Figure 3.3: see D1D2:D3 bars Drought a, c d and e**). These effects also increased the number of grains per plant and grain weight/plant in the 2020/2021 season (**Figure 3.2**: see D1D2:D3 bars in Drought d and e). further, drought exposure in both grandparental and parental generation reduced proline expression in stressed offspring but increased water potential in the offspring under control treatment (**Table 3.4; C1C2 *vs* D1D2, Figure 3.4; Drought a and Control b**).

The direction of trait changes due to memory effects relative to control allowed the evaluation of whether memory effects elicited drought responsiveness. Generally, under drought OE, offspring whose progenitors had a history of drought had reduced proline content, a higher water potential, higher biomass, were taller, and produced more grains per plant (**Figures 3.3. 3 and 3.4**). Overall, these findings suggest that drought stress memory has the potential to positively influence offspring response in a future encounter with the same type of stress scenario, but that it may be detrimental under control treatment.

Table 3.4: Results of generalized linear mixed-effects model analysis of a priori contrast tests comparing the control (C1C2) group to the groups that have experienced drought stress in either or both parental and grandparental generations (C1D2, D1C2, and D1D2). Each trait was analysed separately per offspring moisture treatment (OE). Shown are p-values; significant values are indicated in bold (p<0.05).

		Plant height (cm)		**Days to flowering**		**Above ground biomass/plant (g)**		**Number of grains/ plant**		**Grain weight/ plant (g)**	
		OE									
		Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Season	Contrast	p- value	p- value	p- value	p- value	p- value	p- value	p- value	p- value	p- value	p- value
2019/2020	**C1C2 *vs* C1D2**	0.743	**0.05**	0.99	0.124	0.446	**0.001**	0.627	0.287	0.483	0.079
	C1C2 *vs* D1C2	0.811	0.542	0.583	0.231	0.202	0.741	0.699	0.218	0.447	0.198
	C1C2 *vs* D1D2	0.58	**0.005**	0.088	0.235	0.86	**0.006**	0.858	**0.000**	0.896	**0.000**
2020/2021	**C1C2 *vs* C1D2**	0.599	0.689	0.292	**0.042**	0.851	0.684	0.933	0.886	0.882	0.622
	C1C2 *vs* D1C2	0.39	**0.061**	0.48	0.49	**0.025**	0.168	**0.011**	**0.004**	**0.024**	**0.000**
	C1C2 *vs* D1D2	0.359	0.106	0.615	0.261	0.209	0.194	0.272	**0.005**	0.471	**0.004**
		Proline ((µgg^{-1}))		**Water potential (MPa)**							
	C1C2 *vs* C1D2	0.352	0.347	**0.032**	0.033						
	C1C2 *vs* D1C2	0.348	**0.000**	**0.018**	**0.000**						
	C1C2 *vs* D1D2	0.246	**0.001**	**0.003**	0.542						

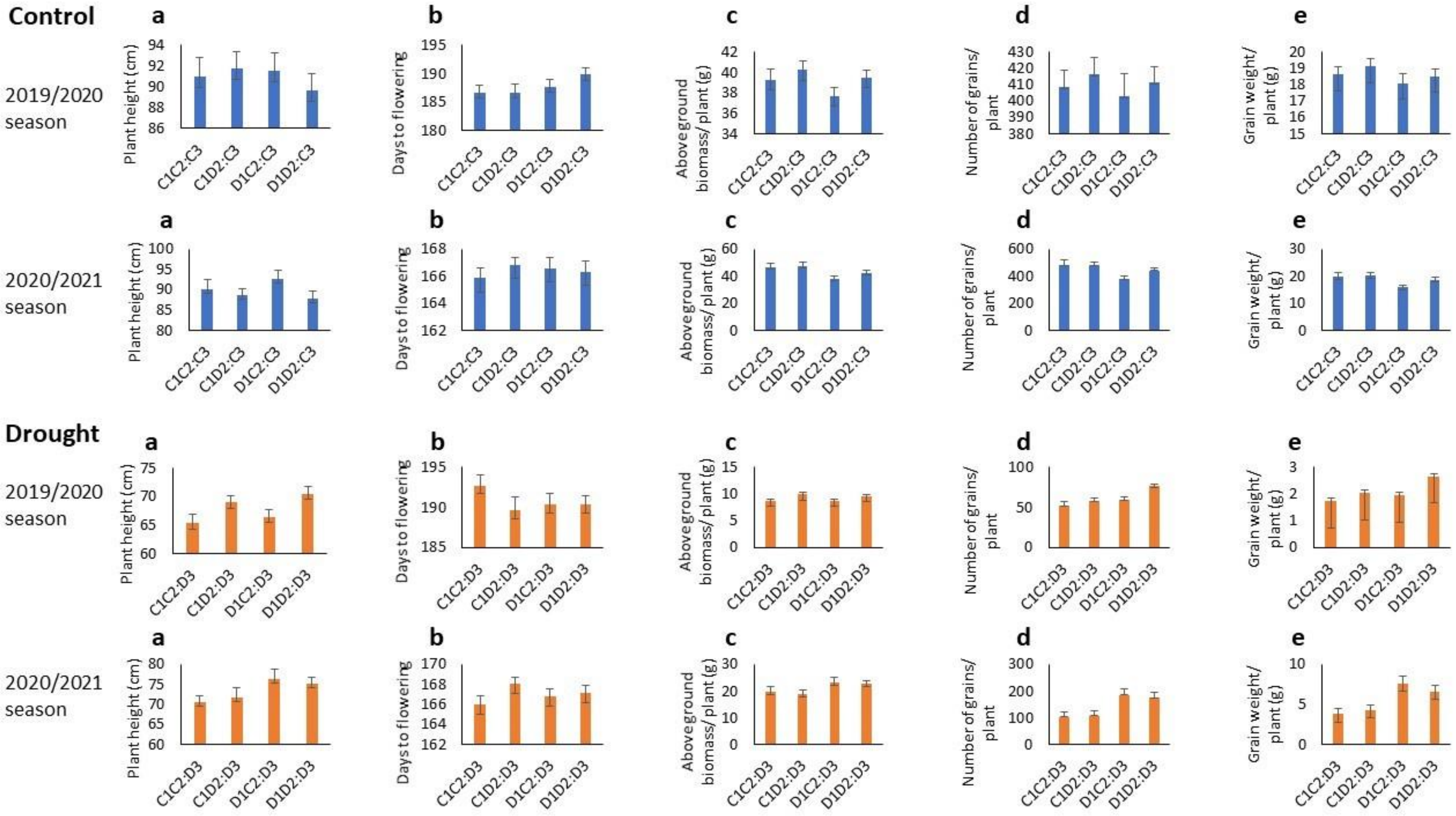

Figure 3.3: Expression of offspring plant height; **a**, days to flowering; **b,** above-ground biomass; **c,** number of grains per plant; **d**, grain weight per plant; **e,** in season 2019/2020 and 2020/2021 in control and drought moisture treatments after exposure of grandparental and/or parental generations to drought stress. Significant differences between groups are shown in **Tables 3.2 and 3.3**.

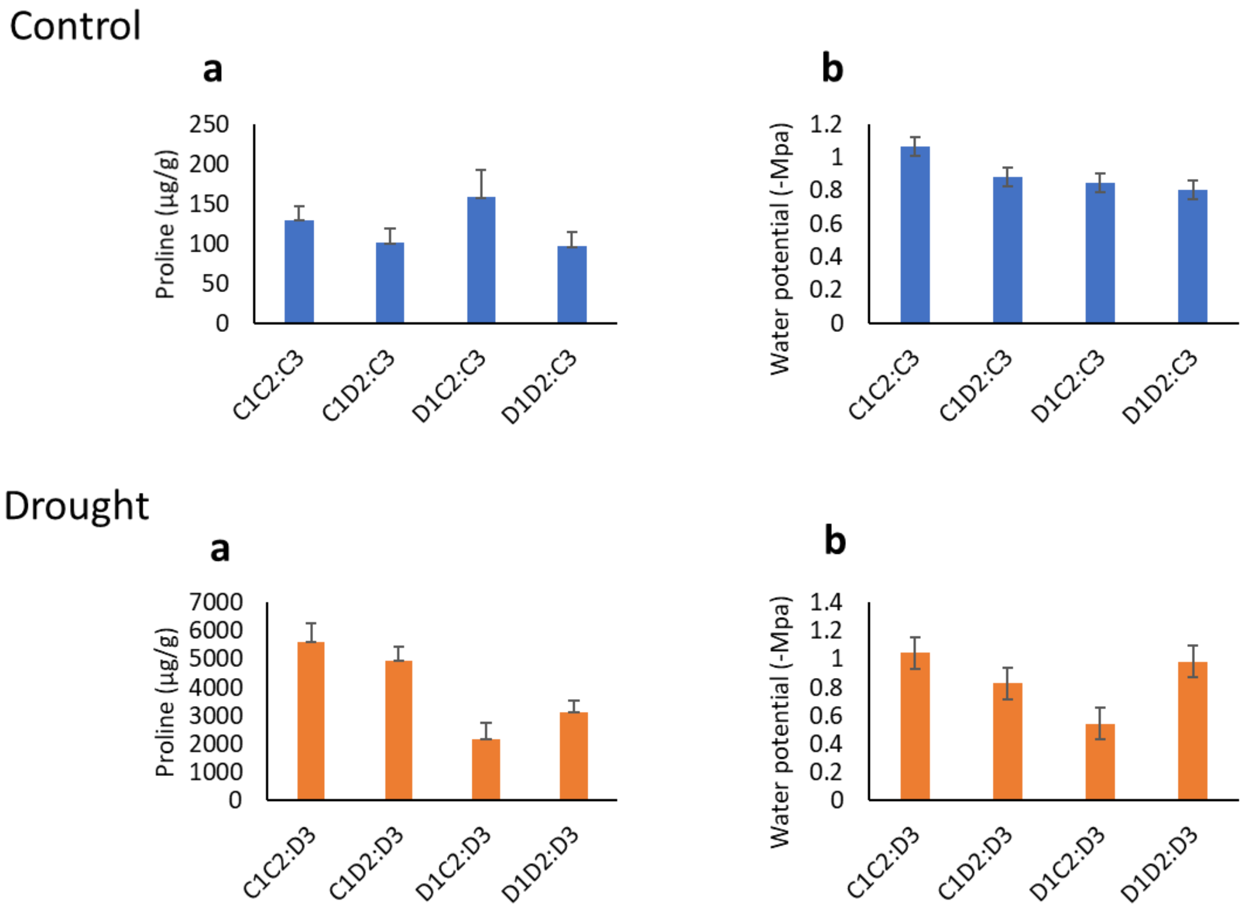

Figure 3.4: Expression of offspring proline; **a**, and water potential; **b,** in control and drought treatments after exposure of grandparental and/or parental generations to drought stress. Significant differences between groups are shown in **Tables 3.2 and 3.3**.

Strength of different memory effects

We used a priori contrasts to investigate if the effects of exposure to drought for two generations are different from those of exposure for a single generation (dose effect) and if drought exposure in the grandparental generation has different effects from exposure in the parental generation on offspring phenotypes. First, we observed that the influence of two consecutive drought exposures was different from that of exposure only in the parental generation for sedimentation % and nitrogen %, and different for sedimentation % and nitrogen % and different from the effect of drought exposure only in the grandparental generation for all the measured seed traits. For example, when compared to seeds without a history of drought (C1C2), the combined impacts of grandparental and parental drought stress exposures (D1D2) resulted in the highest nitrogen (77.7%) and sedimentation (61.8%) increases (**Table 3.5; C1D2 *vs* D1D2 and D1C2 *vs* D1D2 and Table S3.1**). Effects of two successive generations of drought exposure on offspring traits was different from that of exposure only in the parental generation for number of grains per plant and grain weight per plant in both seasons, and for proline when the offspring was also stressed, and different from the effect of drought exposure only in the grandparental generation for plant height, above-ground biomass per plant, number of grains per plant, and grain number per plant only in the drought OE. Moreover, differences in the effects of two consecutive seasons of drought exposure in the ancestral generations were observed for water potential under drought OE when compared to either grandparental or parental effects (**Table 3.6; C1D2 *vs* D1D2 and D1C2 *vs* D1D2**). These dose effects reveal that a single generation of drought exposure had a different influence on offspring phenotypes than two consecutive generations of exposure. Under drought OE, for example, the grandparental drought stress effect was amplified when the parent was also exposed for plant height, number of grains per plant, plant weight per plant, proline, and water potential (**Figure 3.3 and 3.4; Drought**). Most remarkably, in drought OE in the 2020/2021 season, the reduced above ground biomass in C1D2:D3 increased in D1D2:D3 (**Figure 3.3; Drought c**), to imply that the negative effect of parental drought stress exposure on offspring disappeared when grandparents had also been exposed.

Table 3.5: Results of the generalized linear mixed-effects model analysis of a priori contrast tests for seed sedimentation %, NDF % and nitrogen% comparing groups which have experienced a drought stress at different generations during multiplication (C1D2, D1C2 and D1D2). The bold values show $p<0.05$

	Sedimentation %	**NDF %**	**Nitrogen %**
Contrast	p- value	p- value	p- value
C1D2 *vs* D1C2	**0.000**	**0.000**	**0.000**
C1D2 *vs* D1D2	**0.000**	0.269	0.082
D1C2 *vs* D1D2	**0.000**	**0.019**	**0.000**

Secondly, we observed that exposing wheat plants to drought in the grandparental or parental generation generates varying effects on all the measured seed quality traits (**Table 3.5; C1D2 *vs* D1C2, Figure 3.2 a, b, and c**). The effects are also different in the expression of above ground biomass in both seasons in control OE. The differences were also noted in the 2020/2021 in the expression of number of grains per plant and grain weight per plant under both OEs (**Table 3.6; C1D2 *vs* D1C2**). Specifically, under both drought OE, grandparental effects were found to be weaker than parental effects in the 2019/2020 season in the expression of above-ground biomass per plant (p = 0.002). However, in the 2020/2021 season, grandparental effects were stronger than parental effects under drought OE in the expression of number of grains per plant and grain weight per plant but weaker in these traits under control OE (**Table 3.6; C1D2 *vs* D1C2, Figure 3.3**). Grandparental drought effects also differed from parental drought effects in the expression of proline in offspring under both control and drought OEs and water potential only under drought OE. Under control OE, proline was higher due to grandparental effects, while under drought OE, the parental effects increased the values (**Figure 3.4: Control a, and Drought a**). These effects also differed in the expression of water potential under drought OE (**Figure 3.4; Drought b**). These findings suggest that the strength of drought memory impacts varies by season, offspring environment, and generation(s) during which the progenitors experienced drought.

Table 3.6: Results of the generalized linear mixed-effects model analysis of a priori contrast tests comparing groups which have experienced a drought stress at different generations during multiplication (C1D2, D1C2 and D1D2), where each trait was analysed separately per offspring moisture treatment (OE). The bold values show $p<0.05$

		Plant height (cm)		**Days to flowering**		**Above ground biomass/ plant (g)**		**Number of grains/plant**		**Grain weight/ plant (g)**	
		Offspring moisture environment (OE)									
		Control	Drought	Control	Drought	Control	Drought	Control	Drought	Control	Drought
Season	Contrast	p- value	p- value	p- value	p- value	p- value	p- value	p- value	p- value	p- value	p- value
2019/ 2020	**C1D2 *vs* D1C2**	0.929	0.175	0.592	0.731	**0.043**	**0.002**	0.383	0.867	0.145	0.637
	C1D2 *vs* D1D2	0.379	0.383	0.091	0.722	0.559	0.527	0.759	**0.001**	0.406	**0.000**
	D1C2 *vs* D1D2	0.428	**0.027**	0.247	0.991	0.147	**0.015**	0.572	**0.001**	0.529	**0.000**
	C1D2 *vs* D1C2	0.185	0.131	0.809	0.241	**0.017**	0.081	**0.014**	**0.005**	**0.017**	**0.002**
	C1D2 *vs* D1D2	0.699	0.223	0.579	0.359	0.151	0.089	0.313	**0.008**	0.387	**0.018**
	D1C2 *vs* D1D2	0.094	0.686	0.797	0.737	0.258	0.845	0.115	0.717	0.104	0.357
		Proline ($\mu g g^{-1}$)		**Water Potential (MPa)**							
2020/2021	**C1D2 *vs* D1C2**	0.085	**0.001**	0.694	**0.01**						
	C1D2 *vs* D1D2	0.89	**0.013**	0.376	**0.126**						
	D1C2 *vs* D1D2	**0.048**	0.251	0.664	**0.000**						

Discussion

This study showed that environmental conditions experienced by previous generations can influence plant phenotypes. The focus here was only on the differences in the offspring generation plants that were cultivated in the same condition (either control or drought moisture treatment) but were characterized by a different stress history during the previous two generation (s) (grandparental and/ or parental drought exposures). The study's unique full factorial design allowed for comparisons of offspring alterations in responses due to different stress memories. We report that grandparental and/or parental drought memory expression by the offspring depends on offspring moisture treatment, the generation during which drought was in the past experienced, the number of progenitor generations exposed, and the season of testing. In addition, these memory effects could be adaptive for some traits.

Phenotypic plasticity due to grandparental and/ or parental drought stress exposures

The results presented in this study display strong evidence for the occurrence of grandparental, parental, and their combined drought memory effects. The expression of offspring phenotypes is strongly dependent on the past generation(s) that experienced drought stress and the offspring environment. Stress history, regardless of the number of past generations exposed led to alterations in seed quality traits, plant height, days to flowering, above-ground biomass, grain number per plant, grain weight per plant, proline, and water potential. These findings are consistent with previous research that has demonstrated drought stress memory in several species, including *Brassica napus* L. (Hatzig et al., 2018), *Trifolium repens* L. (Rendina González et al., 2018), rice (Zheng et al., 2013), *Leontodon hispidus* L., *Plantago lanceolata* L., and *Trifolium pratense* L. (Cerda, 2020), *Polygonum persicaria* L. (Herman et al., 2012), *Arabidopsis thaliana* L. (van Dooren et al., 2020). This suggests that previous drought stress exposure left some stress imprints in the plant that could be used to induce changes during a subsequent stress encounter. We observed increased biomass, number of grains per plant, plant height, and water potential in the offspring if grandparent and/or parents were stressed. Backhaus et al. (2014) also found that pre-drought exposure resulted in higher above-ground biomass production when compared to controls that had not previously experienced drought. The proline content was highest in offspring that experienced drought for the first time compared to offspring that had a history of drought in their parental generation and remained reduced for the offspring with a history of drought in their grandparental generation, even after interruption by a watered parental generation. This reduction of proline under drought by the memory effect was maintained in offspring whose both parental and grandparental generations had been exposed. This observation is similar to that made by Leufen et al. (2016) and Nguyen et al. (2020) in beet and soybeans, who reported a higher extent of proline content increase when these plants were first drought stressed, with a decrease in the content during the second and third stress cycles. Correspondingly, Li et al. (2019) reported an induction of expression of *LOC_Os05g38150*, which is a *P5CS1* homolog after the initial drought stress, decreasing during re-watering, after which the expression did not vary throughout the subsequent drought treatments. The pattern of water potential also followed that of proline. The C1C2:D3 plants undergoing drought stress for the first time recorded low water potential (more negative values), which increased due to parental or grandparental drought memory, but started to decrease again if the two previous generations had both received drought stress. This could be an indication that C1C2 offspring under drought treatment perceived the stress more strongly than plants with a history of stress, thereby responding quickly to osmotic adjustment through an increase in proline and a subsequent decrease in water potential to avoid further water loss. On the other hand, the reduced expression of proline and water potential by C1D2, D1C2, and D1D2 offspring shows that plants may adapt to recurrent drought by reducing the safety margin at which they operate (Tombesi et al., 2018). According to Campbell (1985), as soil dries, its soil potential decreases, thereby reducing plant water potential and leading to stomatal closure, decreases in transpiration and photosynthesis, and decreased plant production. This is an observation we have made in this study with the plants experiencing drought for the first time (C1C2:D3) having much reduced water potential (more negative values) and, in turn, having reduced grain number and grain weights in the two experimental seasons.

Mostly, the expression of past drought effects differed between the two seasons of testing, which could equate to different offspring environments. Groot et al. (2016) also reported a strong interaction between the offspring environment and the expression of parental salt stress effects. This observation could explain the inconsistency between the findings of different studies performed with different stress inducement conditions and the general environment during the experiment. While testing in uncontrolled environments could allow different stressors to interact to significantly influence plant response, the observed differences between seasons suggest the importance of conducting similar studies in strictly controlled environments to allow for specific quantification of the memory effects of the evaluated stressor.

Strength of different drought stress memory effects

This study permitted the testing of whether grandparental, parental, and their combined effects were all equal on the expression of offspring phenotypes under control and drought OEs. The assumption was that the number of exposures and the generation during which stress was experienced were important in determining the size of memory effects. Since there is an interruption by a non-stressed parental generation in D1C2 offspring, the postulation was that grandparental drought stress effects are weaker than parental drought stress effects. Groot et al. (2017) reported grandparental effects of heat stress that caused strong phenotypic responses in Arabidopsis thaliana offspring, but this contradicted Groot et al. (2016), whose result revealed stronger parental salt stress effects. Our results showed that the strength of parental or grandparental drought stress effects varied based on the trait, season, and offspring moisture treatment. For example, the parental effects were stronger under drought OE, while the grandparental effects were robust under control OE for proline expression.

It was further hypothesized that combined grandparental and parental drought stress effects are additive and would amplify the separate effects of either grandparental or parental drought stress. Under drought moisture treatment, the strength of the combined memory effects was mostly related to the individual direct effects of grandparental or parental exposures. The effects of consecutive drought stress in the grandparental and parental generations had a dose effect on the performance of the offspring under drought most of the time, amplifying the single effects of grandparental and/or parental drought. Most notable was that two successive generations of drought exposure increased number of grains and grain weight per plant in the two testing seasons. Consistent with Herman et al. (2012), our findings reveal that two successive generations of drought stress induced greater growth, generally reduced days to flowering, and increased grain number per plant and grain weight per plant. Most notably, when grandparents were also exposed to drought stress, the small or even negative effect of parental drought stress exposure on offspring increased or disappeared. In this regard, our findings contradict those of Wang et al. (2018), who found no significant differences in the effects of drought stress induced by different generations of priming, implying that any or even one generation of exposure is sufficient to improve the tolerance of offspring to drought. This is the first transgenerational study to our knowledge that has factorially varied both grandparental and parental drought exposures in winter wheat and tested the offspring in a control and drought environment to report on the importance of the number of exposures and generation during which stress was experienced in determining the strength of memory effects.

Drought responsiveness resulting from grandparental and/or parental drought stress exposures

It is common that the environment faced by previous generations (grandparents and parents) is very similar to the environment experienced by the offspring generation (Herman et al., 2012). Therefore, it is critical to comprehend the effects of prior drought stress on future plant performance when confronted with a similar stress. The direction of the trait percentage change due to memory effects relative to control allowed the evaluation of whether memory effects elicited drought responsiveness. Responsive drought memory effects could lead to either a positive or negative percentage change in each trait (Tombesi et al., 2018; Liu et al., 2022). Grandparental drought stress alone increased NDF % in this study, whereas successive drought stress in both generations increased sedimentation %, NDF %, and nitrogen %. Parental drought stress increased sedimentation % and nitrogen % but decreased NDF %. This finding agrees with that by Koua et al. (2021) on the reduction of NDF by drought stress following one generation of exposure. However, whereas Zi et al. (2022) found no significant difference in neutral detergent fiber (NDF) levels between groups in maize due to somatic memory, we found higher NDF levels in wheat seeds due to transgenerational and intergenerational memories.

Water availability affects mineral nutrient concentration, especially in the reproductive phase (Nosalewicz et al., 2016). Increased nitrogen concentrations in seeds from stressed parents, as well as the combination of parental and grandparental drought stresses, are consistent with previous findings by Maleki et al. (2011), who observed increases in mineral concentrations in barley as a result of a water deficit during the seed development stage. The importance of nitrogen accumulation and the increase in sedimentation percentages in seeds have also been illustrated and shown to correlate with protein content (Triboi & Triboi-Blondel, 2002; Nia et al., 2002; Huková & Famra, 2003; Kato, 2012). During drought stress, many genes code for various proteins that include dehydrins and hydrophilic late embryogenesis abundant (LEA) proteins that sustain adequate water potential to help in drought tolerance (Pelah et al., 1997). Together with osmoprotectants, these proteins accumulate in the vegetative tissue and seeds to acclimatize the plant to harsh drought environments (Farooq et al., 2009). As a result, the performance of the plant is improved by these substances, which are also stored in seed and can increase plant yield if exposed to drought in the future (Tabassum et al., 2017; Farooq et al., 2018).

Stress memory is generally considered to cause drought responsiveness when the performance of offspring is enhanced under similar stressful conditions (Boyko et al., 2010; Suter & Widmer, 2013). This could explain the decrease in offspring traits like plant height and number of grains per plant as evidenced under control environments as compared to the increased values under drought if they had a history of drought stress. According to Liu et al. (2022), drought stress memory in plants has the potential to positively influence biomass and grain yield via well-organized regulation of ROS levels, water loss, and photosynthesis. In this study, grandparental and/or parental effects generated offspring responses that were in the same positive and/or negative direction in the two seasons for all the measured traits. It was also observed that the value of each memory type depended on each trait. Stress memory, regardless of type, increased plant height, above-ground biomass, number of grains per plant, and grain weight per plant in the two testing seasons. Therefore, it can be concluded that generally, the tested memory types lead to better drought responsiveness, although they differ in strength of their effects in these traits.

Plants that exhibit minimum loss in biomass in a drought-stress environment are more favourable in a breeding program because they are more tolerant to drought and perform better (Ud-Din et al., 1992). According to Sabaghnia et al. (2011), breeders often determine biomass to select and investigate a way for the development of cultivars suited for drought-stress environments. Additionally, the above-ground biomass increases the likelihood of effective establishment and subsequent productivity and has been widely used as one of the parameters that assess and estimate crop health (Ball et al., 2000; Adamchuk et al., 2010; Korohou et al., 2020; Castro et al., 2013; Groot et al., 2016). In agreement with Tabassum et al. (2018), who reported 14-79% increases in wheat grain yield per plant due to osmopriming, this study discovered a 1.1%-100.9% increase in number of seeds per plant under drought OE, owing to parental and/or grandparental effects.

The offspring's height changed as a result of the combined grandparental and parental drought stress memories. Plant height is among the crop parameters used to estimate biomass and yield (Ehlert et al., 2009; Tilly et al., 2015;Acorsi et al., 2019; Gao et al., 2020). Drought stress alters plant growth, with changes in architecture, which are translated into short height and reduced leaf number and size, thereby leading to less production (Silva et al., 2013). According to Noorka et al. (2007), vigorous plant is imperative in defining plant yield, and plants with better performance during drought stress conditions are considered tolerant. Using the plant height parameter, offspring whose descendants had been previously stressed were taller in this study, indicative of better development than offspring whose descendant had not been previously stressed. These differences in plant development provide a competitive advantage, which is valuable in stressed environments (Esmaeilpour et al., 2015). The increased biomass, plant height, and number of grains per plant observed in offspring whose parents and/or grandparents had been generated under drought conditions obviously reflect enhanced seed quality, which is a known plastic response to drought (Nosalewicz et al., 2016), which is also confirmed in this study.

Offspring belonging to either or both parents and grandparents that had experienced prior drought stress had an increased number of grains per plant in drought environment. This could have resulted from improved tissue water status leading to increased biomass and improved grain formation (Farooq et al., 2008). Low water potential leads to a reduction in photosynthesis due to decreased stomatal opening (Boyer, 1976), which could have led plants experiencing drought for the first time (C1C2:D3) to record reduced seed production. Decreased leaf water potential reduces leaf production by affecting leaf initiation, which occurs in the meristematic tissue, and by affecting cell expansion (Muller et al., 2011). On the contrary, high water potential due to the grandparental drought in D1C2:D3 could have led to increased production of new leaves, as evidenced by the high above-ground biomass. This manufacture of new leaves represents the creation of photosynthetic surface, and these plants recorded the highest number of grains per plant in the 2021 season. According to Hatfield & Dold (2019), production of more grain per unit of water a plant uses describes water use efficiency, and in this circumstance, drought stress memory contributed to this trait.

Plants that exhibit endogenous accumulation of proline are thought to have higher stress tolerance. However, some findings contradict this presumption, such as the analysis of 25 rice cultivars, which revealed no correlation between proline content and tolerance (Lv et al., 2015). Furthermore, Mansour and Ali (2017) provide numerous examples of the negative proline-salt tolerance association. In this study, plants that exhibited the highest proline content under drought offspring environments did not have a history of drought and recorded reduced height, biomass, and grain number per plant. In other reports, Deuschle et al. (2004) and Hellmann et al. (2000) suggest that plants' programmed cell death is activated by proline metabolism's capacity to result in the creation of ROS. These contradictory functions of proline in plant defense are characteristic of redox chemicals, which, depending on the physiological setting, can drive redox reactions in either an oxidative or a reductive direction. For these reasons, Kavi-Kishor and Sreenivasulu (2014) suggest that, in order to adequately assess the role of proline in stress protection, the implication of its accumulation against the activation of its metabolism needs to be reconsidered. Similarly, in the study of drought memory, it is important to comprehend the intricate structure of proline and its metabolism.

The prediction of wheat phenology facilitates the selection of cultivars with specific adaptations to a particular environment. However, the days to flowering of the offspring were highly season dependent, making conclusions about the adaptive value of each stress memory type on this trait under drought treatment difficult. Sometimes, early flowering could be a mechanism used by plants to avoid stresses that would otherwise raise mortality before reproduction starts (Grime, 1977). Similarly, the effects of stress memory enhanced this trait in the first season but not in the second; parental effects delayed flowering time, which could explain the reduced number of grains per plant in that season. This suggests that there is no general response to previous drought stress effects on flowering time in winter wheat.

Interestingly, strong inherited grandparental effects of drought stress that enhanced specific traits that contribute to the success of wheat offspring in drought conditions were observed. Researchers can now explore the epigenome, which is a biological crossing point at which the environment and genetics can interact (Madlung & Comai, 2004; Bossdorf et al., 2008; Richards et al., 2010). Environmental effects could modify the epigenetic landscape of an organism, which can possibly lead to memory effects in offspring in plants. Epigenome-wide association studies could be carried out to enable the identification of genes associated with memory effects on a particular trait through an examination of a genome-wide set of quantifiable epigenetic marks. In particular, the entire plant genome of a large sample size could be studied, looking for small epigenetic marks like DNA methylation, and marks that occur more frequently in plants whose ancestors were stressed than in control offspring can be identified, aiding in the identification of genes that are likely to be involved in stress memory development. Like a population structure, these different ancestral environments might affect the results, and maybe statistical methods could correct for these effects in their analysis.

Conclusion

Drought stress memories in winter wheat are habitual, but complex, sometimes environment and/or trait dependent, and random, and cannot be generalized in winter wheat. Overall, there was manifestation of grandparental (transgenerational memory), parental (intergenerational memory), and their combined drought stress effects in winter wheat to affect seeds and offspring phenotypes. On the one hand, the strength of each stress memory type was difficult to predict from direct drought exposure in either the grandparental or parental generation, as it was difficult to distinguish which effects were stronger than the other. In contrast, under drought treatment, prior exposure of both grandparent and parent generations to drought stress is additive and can amplify either grandparental or parental impacts. The findings indicate that stress memory, regardless of type, has a positive value; stress memory-induced phenotypes that are functionally effective in a drought environment. It is still unclear to what extent these results could be generalized and be representative of the general response of winter wheat to recurrent drought as only 15 cultivars were tested. These findings also show the possibility that epigenetic marks are not completely reset between generations, as indicated by the transgenerational effects (grandparental effects), which points to the necessity of understanding the stability and heritability of epigenetic variation in determining whether the observed variation can be utilized in breeding programs. For a clearer understanding, the use of several genotypes in experiments that are similarly highly controlled in each generation against multiple interacting environmental stressors to allow specific quantification of drought memory effects on plants is proposed.

Author contributions

Carolyn Mukiri Kambona designed and conducted the experiments, data collection, data analyses, and interpreted results and drafted the manuscript. Patrice Ahossi Koua provided plant material and was responsible for correction of the manuscript. Jens Léon analyzed data, interpreted results and was responsible for the critical corrections and revision of the manuscript. Agim Ballvora designed the study, interpreted results and was responsible for the critical corrections and revision of the manuscript. All authors read and approved the final manuscript.

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Data availability statement

All related data can be reached in the manuscript and its supporting materials. Further inquiries can be directed to the corresponding author.

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Competing interests

Authors declare that there is no conflict of interests.

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Stress memory of physiological and biochemical responses in winter wheat under drought stress

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Abstract

Drought is a major stressor for agricultural productivity. Information on how crop plants respond to recurrent external stimuli and form memories of stress responses to ultimately facilitate enhanced tolerance to changes in the environment is essential to developing tolerant cultivars and ensuring yield stability. This study investigated the response to repeated drought exposure of tolerant and sensitive winter wheat cultivars in order to understand general changes at physiological and biochemical levels of organization induced by singular, repeated, or intermittent drought events. For this, winter wheat was subjected for three generations to either drought or well-watered conditions, resulting in three different types of drought stress memory effects, including grandparental, parental, and cumulative effects. Eight traits were measured in the offspring with and without different combinations of stress history in a drought environment. There were memory effects on the efficiency of photosystem II photochemistry, plant water status, oxidative stress reduction, and osmotic adjustment. These effects acted on the traits independently, and generally, offspring from a seed set with previous drought history in any or all of the past generations were able to maintain essential processes for plant survival when compared to those submitted to drought for the first time. Tolerant and sensitive wheat cultivars show differential physiological and biochemical responses to repeated drought events. Sensitive offspring with a history of drought stress exhibited osmotic adjustment through the accumulation of proline and increased non-enzymatic antioxidant activities. The intensity of stress memory-induced modification was not always at the same level in the distinct parameters. Memory effects depend on the generation(s) when the ancestor experienced drought, and cultivars respond differently across the different levels of plant development when exposed to future stress. These findings underlie the biological importance of stress memory and reveal how plants adjust their responses to drought.

Key words; stress memory, intergenerational, transgenerational

Introduction

Plants are constantly confronted with stressful situations that challenge them throughout their lives. Environmental changes are volatile, depending on their duration, frequency of occurrence, and intensity. Therefore, plants have developed the ability to recognize and respond to even the smallest environmental stress cues during their development (Auler et al., 2021). Among the stress factors, drought is a major cause of crop yield reductions worldwide. Following a drought encounter, plants adjust their biochemistry to power a wide array of physiological and molecular changes, supplying cues towards drought adaptive/responsive mechanisms at different levels of plant organization (Bertolli et al., 2014; Sircar & Parekh, 2019).

Although plants frequently encounter fluctuating conditions in their natural habitats, such as alternating stress patterns that may be interrupted by recovery periods, and drought conditions that change between generations, most studies have focused on either single short stress encounters or long-term stress adjustment (Höll et al., 2019). Priming, a process that allows plants to prepare themselves to develop inducible stress responses to a subsequent stress, has been recognized (Hilker & Schmülling, 2019), in which a first stress encounter can prime the organism for an enhanced response to a subsequent stress. When plants are subjected to stress for the first time, they "store" environmental information and initiate a stress state (Mozgova et al., 2019). Memory biochemical processes affecting plant responses to upcoming new potential stressful events support these priming effects (Thellier, M., & Lüttge, 2013; Demongeot et al., 2019; Galviz et al., 2020).

The ability to store information from early stressful experiences and recover that information upon encountering a subsequent stressful situation is referred to as “stress memory.” This memory can ultimately, but not necessarily, improve plant performance. Therefore, memory demands the storage of information, which is accomplished in plants without any kind of nervous system and/or a central brain (Vickers, 2017; Hilker & Schmülling, 2019). Various plant species, including *Arabidopsis thaliana, Zea mays,* and *Oryza sativa,* have been shown to maintain higher relative water content and slow down transpiration in a future stress encounter after experiencing one or more dehydration stress and rehydration cycles (Ding et al., 2012; Ding et al., 2014; Auler et al., 2017; Virlouvet and Fromm, 2015; Virlouvet et al., 2018). These cases reveal that in situations of recurrent stress, plants could improve their resistance to stress by adjusting their physiological state. The processes of plant memory include mechanisms ranging from epigenetic modifications in chromatin and temporary metabolites accumulation to complete alterations in metabolic network pathways (Thellier & Lüttge, 2013; Demongeot et al., 2019). According to Pintó-Marijuan et al. (2017), the mechanisms by which plants remember previous stress in order to better equip themselves to deal with future stress are a progressively hot topic, with the potential to shed light on functioning systems in plant adjustment and adaptation mechanisms.

Despite the general evidence of stress memory in plants, especially wheat, there are still many gaps to be explored. First, although specific genotype adaptation to stress is beneficial, stressful events occur sporadically and rapidly, often overcoming the homeostatic ability of a given genotype and not automatically of another one. Moreover, different scales of plant organization, from physiological and molecular up to whole plant level, can respond in different ways to the same external stimulus (Vítolo et al., 2012). Therefore, studying the contrasting behaviours of tolerant and sensitive cultivars in the same species offers an exceptional system for exploring the variability of the mechanisms involved in drought tolerance (Li et al., 2011). Secondly, the majority of studies on stress memory focus on responses at the transcript level, and only a few emphasize the effects on the agronomic performance of crops, more so on plant physiology and biochemistry. Therefore, it is not clear if stress imprints could be stored in wheat leaves.

In this regard, the central goal of this study was to quantify and recognize the physiological and biochemical impact of drought recurrence in wheat. The focus is on how different histories of drought affect the physiological and biochemical underpinnings of photosynthesis, cell membrane stability, plant water status, oxidative stress reduction, and osmotic adjustment in winter wheat. To achieve this, the study aimed at (1) establishing whether physiological and biochemical processes display behaviour consistent with memory, (2) evaluating whether distinct levels of plant organization, and phases of plant growth show different responses to stress memory, and (3) characterizing cultivar-drought-susceptibility-status variation in memory-based responses to drought.

The memory-based experiments considered different variables at physiological and biochemical scales of plant organization. Physiological responses were verified by evaluation of photosynthetic efficiency through the quantum yield of photosystem II, chlorophyll content, membrane integrity through electrolyte leakage percentage measurements, and plant water status through water potential and osmotic potential. Biochemical responses were verified by performing analyses of oxidative stress parameters like non-enzymatic antioxidants (anthocyanins and carotenoids) and osmotic adjustment analysis through proline determination.

Material and methods

The plant material, experimental set-up, and offspring cultivation were as described in Chapter 3. In summary, drought stress and control treatments had been applied successively in the first, second, and third seasons of winter wheat production on 15 selected cultivars registered in Europe between 1966 and 2013 (Voss-Fels et al., 2019); these included Zappa, Nelson, Jenga, Kalahari, Intro, Inspiration, Sponsor, Urban, Bombus, Konsul, Benni multifloret, Isengrain, Soissons, Sonalika, and Cajeme 71 as outlined in **Table 4.1**. The selection of the 15 cultivars was based on the analysis of their drought tolerance and field performance for photosynthesis efficiency, shoot biomass, and yield production under drought stress conditions (Koua et al., 2021). In this study, some of the obtained seed sets with different levels of previous drought stress experiences were used, including C1D2 (stressed only in the second generation), D1C2 (stressed only in the first generation), D1D2 (stressed for two generations), and D1D2D3 (stressed for three generations), where C and D represent control and drought treatment, respectively, and numbers 1,2, and 3 represent the respective production generations. Chlorophyll content, quantum yield of photosystem II, electrolyte leakage, water potential, osmotic potential, anthocyanins, carotenoids, and proline levels were measured in offspring under drought conditions during the reproductive stage, and chlorophyll content, quantum yield of photosystem II levels were measured also in seedlings under drought.

Table 4.1: Cultivar names, their year of release, and their drought tolerance status

Name of Cultivar	Cultivar ID	Drought tolerance status	Year of release
Zappa	ID1	Tolerant	2009
Nelson	ID2	Tolerant	2011
Jenga	ID3	Tolerant	2007
Kalahari	ID4	Tolerant	2010
Intro	ID5	Tolerant	2011
Inspiration	ID6	Tolerant	2007
Sponsor	ID7	Sensitive	1994
Urban	ID8	Sensitive	1980
Bombus	ID9	Sensitive	2012
Konsul	ID10	Sensitive	1990
Benni multifloret	ID11	Sensitive	1980
Isengrain	ID12	Sensitive	1996
Soissons	ID13	Sensitive	1987
Sonalika	ID14	Sensitive	1967
Cajeme 71	ID15	Sensitive	1971

Photosystem II quantum efficiency

Estimation of the effective quantum yield of photosystem II was done on the second leaf for each cultivar within each category in three repetitions using the MINI-PAM-II photosynthesis yield analyser (Heinz Walz GmbH, Germany).

Chlorophyll content

Leaf chlorophyll content was measured using a Soil Plant Analysis Development (SPAD) meter (SPAD 502 Plus Chlorophyll Meter, Spectrum Technologies, Inc., Aurora, IL, USA) on the flag leaf (fully expanded leaf) (Konica Minolta, 2009). A leaf sample was inserted on the receptor window, the measuring head closed, and a measurement taken. Three readings per sample were taken based on light absorption by chlorophyll between the wavelengths of 650 nm and 940 nm, and the average value was automatically calculated and recorded as the final value before proceeding to the next sample.

Electrolyte leakage as a measure of cell membrane integrity/stability through measurement of Electro Conductivity using conductivity meter

Six leaf discs (9 mm in diameter) were cut and briefly rinsed in Millipore water before being put in a 50-ml Falcon tube containing 30 ml of Millipore water. The Falcon tubes were shaken horizontally for 4hours at room temperature before the discs were removed and the electrical conductivity (L_A) measured by a conductivity meter (WTW GmbH, Weilheim, Germany) (**Figure 4.1**). To destroy the cells and release the maximum amount of electrolytes, the leaf discs were placed overnight at -20 degrees Celsius. The samples were thawed the following day, shaken for 4 hours at room temperature, then the discs were removed, and electrical conductivity was measured (L_B). The electrolyte leakage rate was calculated as a percentage of the maximum leakage using the formula:
Electrolyte leakage rate (ELR) =$L_A/L_B * 100$.

Since conductivity is dependent on temperature, the standard temperature of 25°C was used. In cases where the measurements were done at a different temperature, a correction was applied for standardization. To determine the corrected value, the measured value was multiplied by the corresponding temperature correction factor to get the actual conductivity at 25 degrees centigrade.

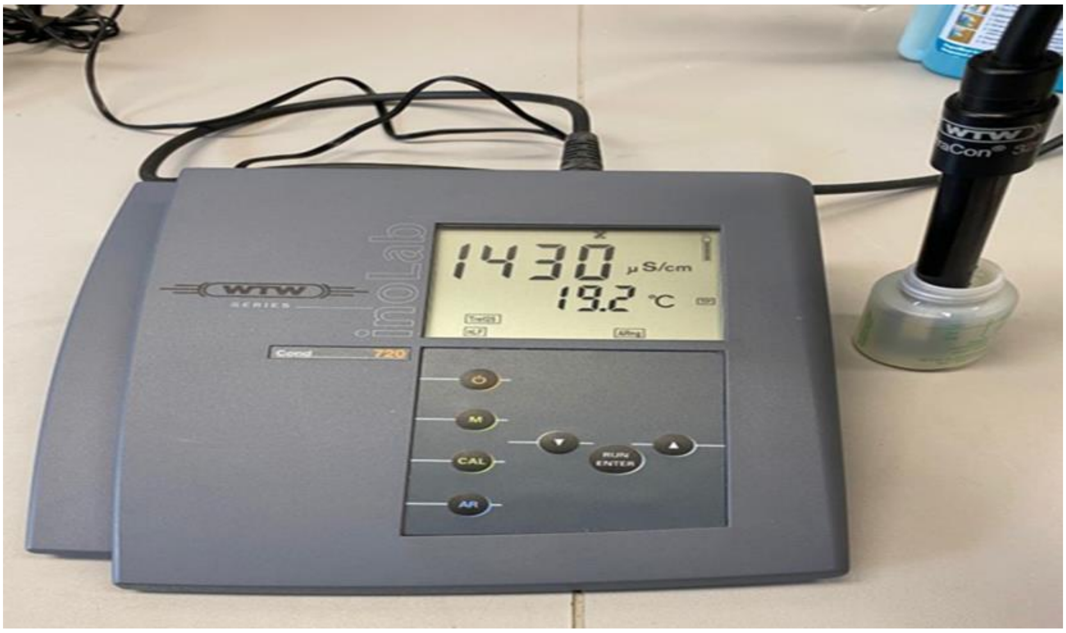

Figure 4.1: WTW inolab PH/Con device used to measure electrical conductivity

Total water potential estimation using the Scholander Bomb (Ψleaf)

The total water potential measurement was made using the leaves and part of the stem. For each measurement, the stem was cut off smoothly and quickly pulled through the hole in the rubber plug so that the cut protrudes about 2 to 3 cm from the top of the plug (**Figure 4.2**). The sealing compound was used to ensure a tight seal. The stopper was then inserted in the cannulation of the bomb cover so that the interface of the stem protrudes from the cover. The seal was carefully checked again, after which the lid was firmly anchored on the lower part of the bomb. By opening the pressure valve, the gas pressure in the chamber is slowly increased, approximately by 20 to 30 kPa/second. At the same time, the protruding interface of the stem was carefully observed with a magnifying glass. As soon as water droplets emerged from the xylem, the compressed air supply was stopped and the value indicated on the pressure gauge was noted down (Becker & Knoche, 2011).

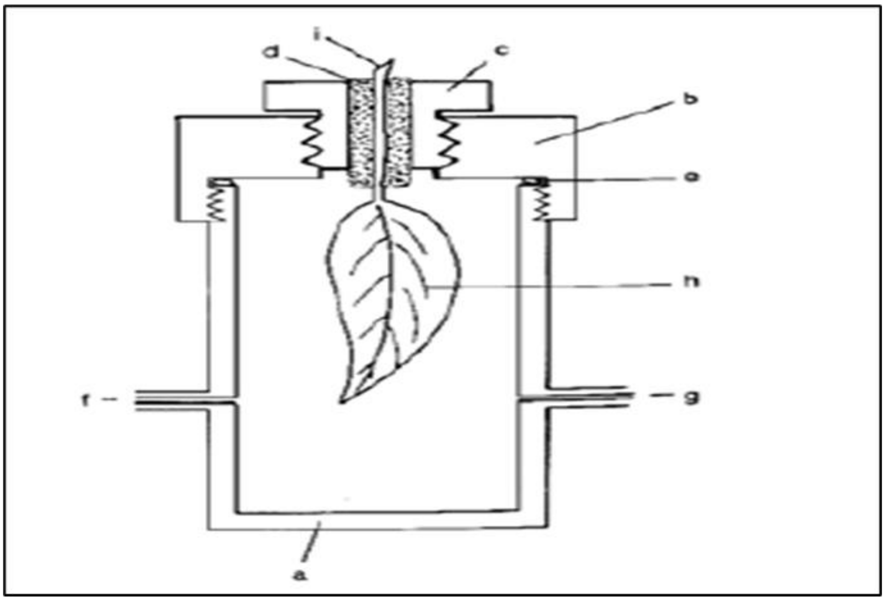

Figure 4.2: Scholander bomb, a) pressure bomb, b) pressure bomb cover, c) replaceable insert, d) sealing compound, e) cover gasket, f) pressure line, g) drainage line, h) leaf, i) cut surface (Maes et al., 2009).

Osmotic potential (ΨS) in leaves

Osmotic potential was measured according to Kautz et al. (2014). Samples were stored at -20°C until the time of analysis. The sap (150 µl) was pipetted into a 1.5-ml tube and centrifuged for 5 minutes at 14,000 rpm. After that, 15 µl of the sample was pipetted into a 0.5-ml tube, and osmolarity was measured using a freezing-point depression osmometer (Osmomat Model 3000 Basic, Genotec GmbH, Berlin, Germany). At the beginning of the measurements, the osmometer was calibrated using preformed Genotec vials (850 mmol kg−1 H2O) and distilled water (0 mmol kg−1 H2O). The measurement was repeated twice, and the average for each sample was recorded.

Anthocyanin Reflectance Index (ARI) and Carotenoid Reflectance Index (CRI)

Spectral measurements of the leaves, including the anthocyanin reflectance index (ARI) and carotenoid reflectance index (CRI), were conducted using the Polypen (PolyPen RP400, Photon Systems Instruments, Drasov, Czech Republic), which was calibrated with the special reflectance standard "Spectralon." Three readings were taken for each cultivar in each stress history category.

Proline

Proline concentrations were determined based on the protocol of (Bates et al., 1973; Frimpong et al., 2021). The second leaf was collected for each sample and immediately submerged in liquid nitrogen. Samples were pulverized using a pestle and mortar on ice. One hundred mg of the pulverized samples were weighed and extracted with 1.5 ml of 3% salicylic acid in chilled 2 mL tubes, vortexed, and centrifuged at 12,000 rpm for 10 min. Five hundred µL of the supernatant was directly transferred into cylindrical glass tubes (fitted with lids) on ice and 500 µL of glacial acetic acid and 2.5% ninhydrin reagent were added. The mixture was then vigorously vortexed and incubated for 1 hour in a water bath at 95˚C. The reaction was quickly terminated on ice. 1.5 mL of toluene was added, and the mixture was kept at room temperature for 30 minutes after mixing. One hundred µL of the upper phase was then pipetted into 96-well plates, and the absorbance at 520 nm was measured using a microplate reader (Tecan Infinite 200 PRO) (**Figure 4.3**). A calibration curve based on eight points of proline standard concentrations (0, 10, 20, 30, 50, 70, 90, and 100 µg/g) yielded a linear regression between proline concentration and the measured absorbance at 520 nm (R2 = 0.9969). This linear model was subsequently used for proline concentration calculations in the samples.

Figure 4.3: Tecan Infinite 200 PRO microplate reader for absorbance measurement

Data analysis

The influence of experimental season, stress history (SH), cultivar, and susceptibility to drought status on offspring traits (chlorophyll, quantum yield of PSII, water potential, osmotic potential, electrolyte leakage, ARI, CRI, and proline) was analysed using the ANOVA package in R. All the possible combinations between the main effects were considered. A significant SH * Susceptibility interaction would indicate susceptibility variation in stress memory.

Apart from the measurements of water potential, osmotic potential, electrolyte leakage, and proline, analyses for other parameters were first conducted using season as a fixed effect before each season was individually analysed. The following general linear mixed model was used:

$$Y_{iklmn}= \mu+ S_i+ C_k +R_m+ C_k (R_m)+ P_l +(CRP)_{kml}+(CRS)_{kmi}+(PS)_{li}+(SR)_{im}+(PR)_{lm}+(CRPS)_{kmli}+(SPR)_{ilm}+ \varepsilon_{iklmn}$$

Where Y_{iklmn} is the response variable, μ is the overall mean, S_i represents the fixed effect of season (i =2019/2020, 2020/2021), C_k is the random effect of cultivar, R_m is the fixed effect of susceptibility to drought (m=Tolerant and sensitive), $C_k(R_m)$ is the random effect of cultivar nested in susceptibility, P_l is the fixed effect of SH, $(CRP)_{kml}$ represents the random effect of the cultivar nested in susceptibility * SH interaction, $(CRS)_{kmi}$ is the random effect of the cultivar nested in susceptibility * season interaction, $(PS)_{li}$ is the fixed effect of the SH * season interaction, $(SR)_{im}$ is the fixed effect of season * susceptibility to drought, $(PR)_{lm}$ is the fixed effect of SH * susceptibility to drought, $(CRPS)_{kmli}$ represents the random effect of the cultivar nested in susceptibility * SH * season interaction, $(SPR)_{ilm}$ represents the fixed effect of season * SH*Susceptibility to drought and ε_{iklmn} represents the random residual effect.

Individual analysis of each season for these parameters as well as the analysis of water potential, osmotic potential, electrolyte leakage and proline were done using the following linear model:

$$Y_{klmn}= \mu+C_k+P_l+R_m+ C_k (R_m) +(CRP)_{kml} + \varepsilon_{klmn}$$

Where Y_{klmn} is the response variable μ is the general mean, C_k is the random effect of cultivar, P_l represents the fixed effect of stress history (SH), R_m is the fixed effect of susceptibility to drought (m=Tolerant and sensitive), $C_k (R_m)$ is the random effect of cultivar nested in susceptibility, $(CRP)_{kml}$ is the random effect of the cultivar nested in susceptibility * SH interaction, and ε_{klmn} represents the random residual effect. Whenever a considerable ANOVA result was found for any trait, Tukey's HSD (honestly significant difference) test was done for pairwise comparisons of the combinations of the experienced stress histories against the control seed set (C1C2) and to separate mean between SH and susceptibility groups. Alpha values of $p<0.1$ '.', $p<0.05$ '*', $p <0.01$ '**', and $p<0.001$ '***' were used for the analysis.

Results

Effect on physiological response

To test for the effects of drought memory on wheat physiological responses, mean trait expressions (quantum yield of photosystem II, chlorophyll content, electrolyte leakage percentage measurements, water potential, and osmotic potential) were compared between offspring from seeds that had a history of drought (D1C2, C1D2, D1D2, and D1D2D3) and the control offspring from seeds that did not have a history of drought (C1C2 or C1C2C3). An analysis of the quantum yield of photosystem II across the two seasons indicated grandparental and parental effects ($P<0.001$) (**Table 4.2**). When season was considered a factor, only parental effects influenced chlorophyll expression ($P<0.05$). We found evidence of cultivar variation for grandparental, parental, and combined effects (grandparental and parental effects) on chlorophyll content (SH: Cultivar). When each year was analysed separately, there was an overall decrease in the quantum yield of photosystem II in 2019/2020 season ($P<0.001$) due to grandparental effects, but chlorophyll content increased in the 2020/2021 season (2.2%, $P<0.05$) (**Table 4.3**). Parental effects increased the quantum yield of photosystem II in both seasons and chlorophyll in the 2019/2020 season. Specifically, in the 2019/2020 season, there was a 3.9% increase in chlorophyll and a 16% increase in the photosynthetic efficiency in offspring from C1D2 under drought treatment (**Figure 4.4**). There was cultivar variation for memory effects on chlorophyll in either of the seasons (SH: Cultivar) (**Table 4.3**). When averaged across all the cultivars in the two separate seasons, two prior successive drought exposures did not influence the offspring differently in these traits from the control offspring under drought stress (**Table 4.3**).

Table 4.2: F and P values from the combined season ANOVA analysis for the effect of drought history on winter wheat cultivars on chlorophyll, ARI, CRI, and quantum yield of photosystem II characteristics

Memory effect type	**Source of variation**	Df	**Chlorophyll** F value	Pr(>F)	**Anthocyanin** F value	Pr(>F)	**Carotenoids** F value	Pr(>F)	**Quantum yield of PSII** F value	Pr(>F)
Grandparental effects	Season	1	282.7	0.000	169.3	0.000	4.6	0.000	206.6	0.000
	SH	1	0.6	0.454	0.0	0.889	0.0	0.856	26.1	0.000
	Susceptibility	1	12.2	0.001	28.9	0.000	31.3	0.000	5.6	0.019
	Cultivar	14	2.6	0.003	5.9	0.000	7.3	0.000	1.5	0.134
	Season: SH	1	10.0	0.002	0.3	0.603	0.4	0.508	17.7	0.000
	Season: Susceptibility	1	1.2	0.269	13.7	0.000	7.1	0.009	0.4	0.531
	SH: Susceptibility	1	1.6	0.205	0.3	0.618	4.3	0.042	0.1	0.806
	Season: Cultivar	9	1.3	0.264	2.0	0.050	2.3	0.022	0.8	0.621
	SH: Cultivar	13	1.9	0.036	1.9	0.037	0.9	0.590	1.3	0.190
	Season: SH: Susceptibility	1	8.2	0.005	0.0	0.845	6.7	0.011	0.1	0.756
	Season: SH: Cultivar	9	3.7	0.000	2.4	0.016	1.0	0.416	1.3	0.218
	Residuals	102								
Parental effects	Season	1	248.8	0.000	138.2	0.000	0.1	0.721	60.9	0.000
	SH	1	5.7	0.019	7.7	0.006	1.4	0.231	13.0	0.000
	Susceptibility	1	5.3	0.023	13.6	0.000	11.7	0.001	2.3	0.133
	Cultivar	13	3.3	0.000	5.1	0.000	7.7	0.000	1.2	0.280
	Season: SH	1	2.2	0.143	1.0	0.324	0.0	0.982	4.0	0.047
	Season: Susceptibility	1	0.2	0.661	11.1	0.001	2.1	0.147	0.1	0.743
	SH: Susceptibility	1	1.0	0.318	0.0	0.942	0.2	0.650	0.1	0.748
	Season: Cultivar	13	1.6	0.088	0.9	0.580	1.1	0.337	0.5	0.919
	SH: Cultivar	13	2.0	0.027	1.1	0.340	1.0	0.430	0.6	0.858
	Season: SH: Susceptibility	1	0.6	0.443	0.2	0.659	1.6	0.210	2.2	0.143
	Season: SH: Cultivar	13	1.7	0.066	0.7	0.784	0.5	0.926	0+N25K3:K39	0.402
	Residuals	120								
Combined effects	Season	1	236.4	0.000	149.4	0.000	0.6	0.440	103.5	0.000
	SH	1	0.3	0.607	1.0	0.315	0.1	0.795	0.6	0.440
	Susceptibility	1	13.5	0.000	3.8	0.052	4.5	0.036	2.0	0.164
	Cultivar	13	2.9	0.001	3.3	0.000	5.2	0.000	0.9	0.582
	Season: SH	1	0.0	0.846	0.9	0.346	0.2	0.671	1.0	0.312
	Season: Susceptibility	1	0.1	0.702	1.0	0.325	0.0	0.934	1.2	0.271
	SH: Susceptibility	1	0.0	0.944	3.1	0.083	0.4	0.508	0.1	0.771
	Season: Cultivar	13	2.0	0.023	1.6	0.085	1.2	0.271	0.8	0.640
	SH: Cultivar	13	2.6	0.003	2.4	0.008	1.4	0.171	0.4	0.976
	Season: SH: Susceptibility	1	0.0	0.981	4.0	0.047	0.0	0.876	0.4	0.547
	Season: SH: Cultivar	13	2.1	0.018	1.3	0.197	0.9	0.566	1.8	0.050
	Residuals	120								

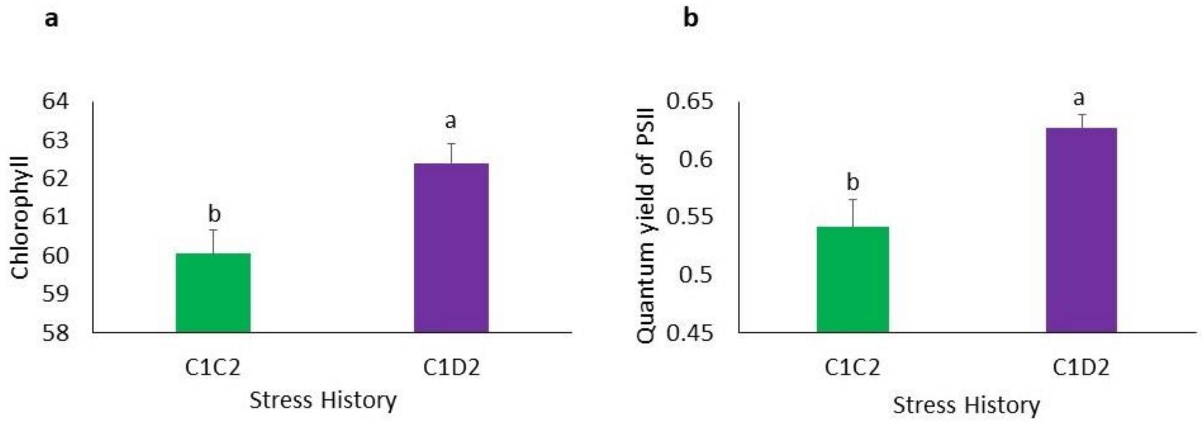

Figure 4.4: Parental effects on **a**; mean chlorophyll content and **b**; quantum yield of PSII of winter wheat offspring. Letters “a” and “b” in each bar indicate the results of the post hoc Tukey test. Error bars represent the standard error of the mean.

Table 4.3: F and P values from the individual season ANOVA analysis for the effect of drought history on 15 winter wheat cultivars on chlorophyll, ARI, CRI, and quantum yield of photosystem II characteristics

				Chlorophyll		**Anthocyanin**		**Carotenoids**		**Quantum yield of PSII**	
Memory effect type	Season	Source of variation	Df	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
Grandparental Effects	**2019/2020**	SH	1	2.89	0.094	0.225	0.637	0.272	0.604	20.74	0.000
		Susceptibility	1	3.62	0.062	3.922	0.052	9.77	0.003	1.928	0.17
		Cultivar	13	2.835	0.003	7.554	0.000	6.267	0.000	0.845	0.612
		SH: Susceptibility	1	0.944	0.335	1	0.321	0.017	0.896	0.026	0.873
		SH: Cultivar	13	1.088	0.387	0.63	0.819	0.983	0.479	0.823	0.635
		Residuals	60								
	2020/2021	SH	1	6.275	0.016	0.011	0.918	0.013	0.910	0.367	0.546
		Susceptibility	1	8.92	0.005	24.64	0.000	20.89	0.000	0.738	0.392
		Cultivar	10	1.393	0.217	2.884	0.010	4.583	0.000	0.957	0.48
		SH: Susceptibility	1	6.492	0.015	0.007	0.934	5.327	0.026	0.335	0.564
		SH: Cultivar	9	3.953	0.001	2.518	0.022	0.993	0.461	1.216	0.293
		Residuals	42								
Parental Effects	**2019/2020**	SH	1	10.481	0.002	4.768	0.033	1.665	0.202	9.316	0.003
		Susceptibility	1	2.439	0.124	0.187	0.667	4.363	0.041	0.856	0.359
		Cultivar	13	1.841	0.057	5.176	0.000	5.935	0.000	0.373	0.973
		SH: Susceptibility	1	2.211	0.142	0.176	0.676	0.671	0.416	0.739	0.393
		SH: Cultivar	13	1.469	0.156	0.82	0.638	0.968	0.493	0.595	0.849
		Residuals	60								
	2020/2021	SH	1	0.32	0.574	4.245	0.044	0.452	0.504	4.007	0.047
		Susceptibility	1	2.915	0.093	14.68	0.000	7.608	0.008	1.36	0.245
		Cultivar	13	2.784	0.004	2.519	0.008	3.962	0.000	1.438	0.148
		SH: Susceptibility	1	0.021	0.885	0.079	0.780	0.947	0.334	1.548	0.215
		SH: Cultivar	13	2.065	0.030	0.914	0.544	0.697	0.759	0.944	0.51
		Residuals	60								
Combined Effects	**2019/2020**	SH	1	0.064	0.801	0.01	0.92	0.05	0.824	1	0.321
		Susceptibility	1	10.21	0.002	1.866	0.177	6.675	0.012	1.8	0.185
		Cultivar	13	2.592	0.006	7.879	0.000	8.21	0.000	0.454	0.941
		SH: Susceptibility	1	0.005	0.941	0.115	0.735	0.446	0.507	0.066	0.797
		SH: Cultivar	13	1.318	0.229	1.731	0.077	1.993	0.037	0.938	0.521
		Residuals	60								
	2020/2019	SH	1	0.211	0.648	1.078	0.304	0.143	0.707	0.003	0.959
		Susceptibility	1	4.515	0.038	2.444	0.123	1.408	0.240	0.335	0.563
		Cultivar	13	2.408	0.011	1.695	0.087	2.273	0.017	1.281	0.231
		SH: Susceptibility	1	0.001	0.976	3.743	0.058	0.176	0.677	0.531	0.468
		SH: Cultivar	13	3.073	0.002	1.849	0.057	0.975	0.486	0.841	0.617
		Residuals	60								

Table 4.4: F and p values from the ANOVA analysis for the effect of drought history on 15 winter wheat cultivars on water potential, osmotic potential, electrolyte leakage and proline content characteristics

			Water potential		**Osmotic potential**		**Electrolyte leakage**		**Proline**	
Memory effect type	Source of variation	Df	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)	F value	Pr(>F)
Grandparental effects	SH	1	24.015	1.47E-05	1.198	0.2799	0.745	0.393	37.969	2.80E-07
	Susceptibility	1	1.821	0.1844	5.755	0.021	1.053	0.311	0.363	0.55
	Cultivar	10	2.234	0.0341	2.091	0.0471	1.117	0.372	12.421	9.78E-09
	SH: Susceptibility	1	3.415	0.0717	1.634	0.2082	1.047	0.312	1.235	0.273
	SH:Cultivar	9	1.287	0.2723	2.585	0.0181	0.73	0.679	19.597	1.35E-11
	Residuals	42								
Parental effects	SH	1	3.422	0.0693	0.383	0.5384	2.689	0.106	0.625	0.432
	Susceptibility	1	0.381	0.5393	3.038	0.0864	0.136	0.714	0.432	0.513
	Cultivar	13	2.119	0.0257	1.336	0.2187	1.235	0.279	18.954	< 2e-16
	SH: Susceptibility	1	2.661	0.1081	0.003	0.9549	0.866	0.356	1.999	0.163
	SH:Cultivar	13	1.062	0.4084	1.088	0.3867	0.693	0.762	17.145	1.43E-15
	Residuals	60								
Combined effects (2 seasons)	SH	1	0.572	0.4523	0.044	0.8342	0.044	0.8342	30.968	6.46E-07
	Susceptibility	1	2.333	0.1319	1.533	0.2206	1.533	0.2206	0.465	0.498
	Cultivar	13	1.402	0.1851	1.688	0.0871	1.688	0.0871	12.549	1.19E-12
	SH:Response.status	1	0.647	0.4243	1.121	0.294	1.121	0.294	1.035	0.313
	SH:Cultivar	13	1.708	0.0825	1.927	0.0446	1.927	0.0446	8.268	3.3E-09
	Residuals	60								
Combined effects (3 seasons)	SH	1	1.489	0.2271	13.539	0.000502	1.732	0.193	24.5	6.35E-06
	Susceptibility	1	0.472	0.4947	2.434	0.123966	2.209	0.142	45.33	7.13E-09
	Cultivar	13	1.571	0.1195	1.14	0.345663	1.224	0.286	13.6	2.25E-13
	SH: Susceptibility	1	2.202	0.143	0.245	0.622381	1.633	0.206	22.66	1.26E-05
	SH:Cultivar	13	1.782	0.0673	1.637	0.100143	1.09	0.385	17.05	1.61E-15
	Residuals	60								

There was a difference between the performance of tolerant and susceptible cultivars in the expression of chlorophyll content in both seasons due to grandparental effects, with an interaction in the 2020/2021 season. The tolerant cultivars had higher chlorophyll content compared to the susceptible cultivars. Grandparental effects on the susceptible cultivars reduced the expression of chlorophyll content ($P<0.05$) when compared to tolerant cultivars from the control category and when compared to tolerant cultivars from the same stress history category ($P<0.05$) (**Figure 4.5a**). Susceptible offspring whose parents had been exposed to drought had increased chlorophyll content than those from C1C2 ($P<0.05$) (**Figure 4.5b**).

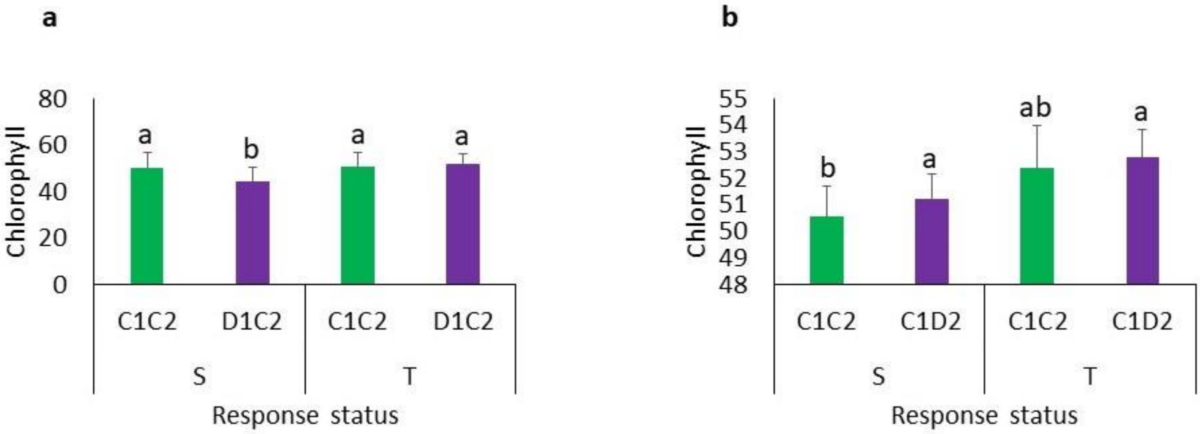

Figure 4.5: Grandparental (a) and parental effects (b) on chlorophyll expression by tolerant and susceptible offspring under drought-stress conditions. Letters “a” and “b” in each bar indicate the results of the post hoc Tukey test. Error bars represent the standard error of the mean.

Both tolerant and sensitive offspring had comparable reduced quantum yield of PSII if their grandparents had been exposed compared to the control offspring (**Figure 4.6a**). This observation was reversed in both seasons if it was the parents that had been previously exposed to drought (**Figure 4.6b**). Sensitive offspring whose parents had been exposed had higher quantum yield of photosystem II compared to tolerant offspring, but sensitive offspring from parents that had not been previously exposed had the lowest values (**Figure 4.6b**).

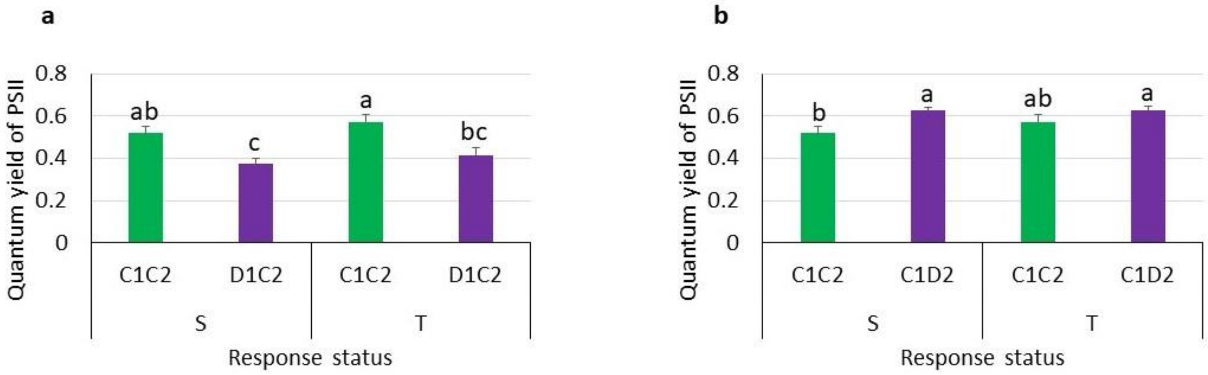

Figure 4.6: Grandparental (**a**) and parental effects (**b**) on quantum yield of PSII expression by tolerant and susceptible offspring under drought stress conditions. Letters “a” and “b” in each bar indicate the results of post hoc Tukey test. Error bars represent standard error of the mean.

Grandparental effects increased the water potential (51.1%) in the offspring compared to the control offspring. When the performance of tolerant and sensitive cultivars was considered, both the sensitive and tolerant offspring had increased water potential if their grandparents had been exposed (**Table 4.4**, **Figure 4.7**). Moreover, parental effects increased water potential, while previous successive droughts for three years increased osmotic potential in the offspring. Generally, grandparental, parental, and their combined effects in successive seasons did not affect electrolyte leakage (**Table 4.4**).

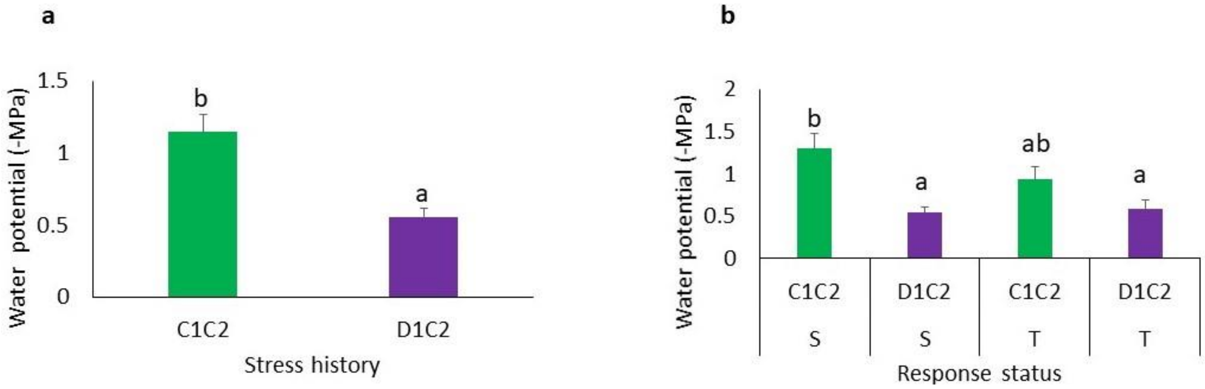

Figure 4.7: Grandparental parental effects on water potential expression (**a**), and its impact on the performance of tolerant and susceptible offspring (**b**) under drought stress conditions. Letters “a” and “b” in each bar indicate the results of post hoc Tukey test. Error bars represent standard error of the mean

Effect of drought stress memory on biochemical responses

Oxidative stress parameters like non-enzymatic antioxidants (anthocyanins and carotenoids) and osmotic adjustment evaluation through proline determination were analysed to assess the effect of previous drought exposure on the performance of offspring under similar stress treatment. There were no memory effects on carotenoids expression and no SH: cultivar interactions when season was considered. Nevertheless, there was cultivar variation for grandparental and combined effects on anthocyanin expression (**Table 4.1**). When seasons were analysed independently, susceptible offspring of D1C2 increased carotenoids compared to those of C1C2 and compared to the tolerant offspring from the same seed category in the 2020/2021 season (**Figure 4.8**). Parental drought stress increased anthocyanins by 72.2 and 24.8% in the two seasons, respectively and susceptible offspring exhibited increased levels compared to their tolerant counterparts ($P<0.05$). The sensitive offspring with a history of drought stress in the parental generation had higher anthocyanin levels than the tolerant offspring with no history of drought stress ($P<0.051$). In addition, tolerant control offspring (C1C2) had much reduced anthocyanins compared to tolerant offspring whose grandparents and parents had previously been stressed (D1D2). Proline content was also altered by grandparental drought exposure and successive drought exposure for two and three years, but not by parental effects (**Table 4.4**). There were differences between cultivars in the expression of proline, and the sensitive offspring generally had more proline if previously exposed to drought than if experiencing stress for the first time. For example, sensitive offspring from D1D2D3 seeds had more proline ($p<0.05$) than sensitive offspring from the control (C1C2C3). Generally, there was evidence of cultivar variation for memory effects on proline in all categories (SH: Cultivar) (**Table 4.4**).

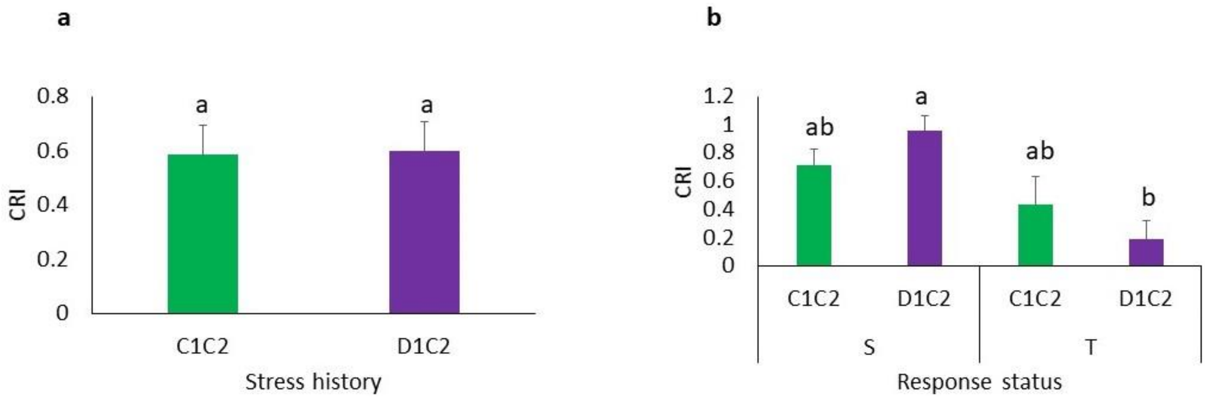

Figure 4.8: Grandparental effects on CRI expression (a), and their impact on the performance of tolerant and susceptible offspring (b) under drought stress conditions. Letters “a” and “b” in each bar indicate the results of the post hoc Tukey test. Error bars represent the standard deviation of the mean.

The expression of the various categories of memory effects varied across the measured traits. Grandparental effects influenced the expression of chlorophyll content, the quantum yield of photosystem II, water potential, and proline content. Parental effects influenced the expression of chlorophyll content, quantum yield of photosystem II, anthocyanins, and water potential. The combined effects (2 consecutive seasons of drought stress) only altered proline content expression, while combined effects (3 consecutive seasons of drought stress) changed the expression of osmotic potential and proline (**Tables 4.2 and 4.3**). Furthermore, cultivar susceptibility to drought influenced their responses across the measured traits as regulated by specific memory effects.

To test if distinct phases of plant growth exhibit different responses to previous stimulus, the quantum yield of photosystem II and chlorophyll content measured during seedling and reproductive stages were compared. Offspring from D1C2 had different quantum yields of photosystem II and chlorophyll content at both seedling and reproductive stages when compared to the controls. However, although offspring of C1D2 had differences in these traits in the reproductive stage, stress memory had no effect on them during the seedling stage. The opposite was observed in D1D2 offspring, where drought memory had no effect on these traits during the reproductive stage but did have an effect during the seedling stage. Similarly, although memory was expressed in chlorophyll content during the reproductive stage in the offspring of D1D2D3, these effects were not expressed in the seedling stage (**Table 4.5**).

Table 4.5: F and P values from seedling ANOVA analysis for the effect of drought history on winter wheat cultivars on chlorophyll and quantum yield of photosystem II characteristics

Memory effect type	Source of variation	Df	Chlorophyll		Quantum yield of PSII	
			F value	Pr(>F)	F value	Pr(>F)
Grandparental effects	SH	1	4.87	0.0495	3.528	0.0871
	Susceptibility	1	1.688	0.2205	1.045	0.3287
	Cultivar	2	1.88	0.1984	0.061	0.9407
	SH: Susceptibility	1	2.581	0.1365	0.728	0.4117
	SH: Cultivar	2	0.052	0.9498	1.458	0.2743
	Residuals	11				
Parental effects	SH	1	0.642	0.436	0.13	0.724
	Susceptibility	1	38.713	2.23E-05	0.024	0.879
	Cultivar	4	2.457	0.094	2.104	0.134
	SH: Susceptibility	1	0.041	0.843	1.073	0.318
	SH: Cultivar	4	1.594	0.231	1.786	0.188
	Residuals	14				
Combined effects (2 seasons)	SH	1	12.82	0.00593	4.476	0.0635
	Susceptibility	1	1.216	0.29867	0.154	0.7037
	Cultivar	2	1.326	0.31285	0.707	0.5188
	SH: Susceptibility	1	2.566	0.14363	0.202	0.6636
	SH: Cultivar	2	0.197	0.82437	0.945	0.4242
	Residuals	9				
Combined effects (3 seasons)	SH	1	1.473	0.2483	0.52	0.484
	Susceptibility	1	2.304	0.1549	0.533	0.479
	Cultivar	2	2.962	0.0901	0.9	0.432
	SH: Susceptibility	1	1.748	0.2108	0.305	0.591
	SH:Cultivar	2	0.427	0.6622	0.899	0.433
	Residuals	12				

Using a principal component analysis (PCA) for all parameters with significant differences between offspring from each category with past drought exposure and the control treatment, clear spatial separations between the offspring were found (**Figure 4.9**). Since all offspring were tested in a drought environment, it is evident that the most important determinant of the separations was the stress history, while cultivar specificities were secondary. The variance of the control offspring receiving drought for the first time appears to be larger when compared to the already drought-primed offspring, as seen in the trait variances.

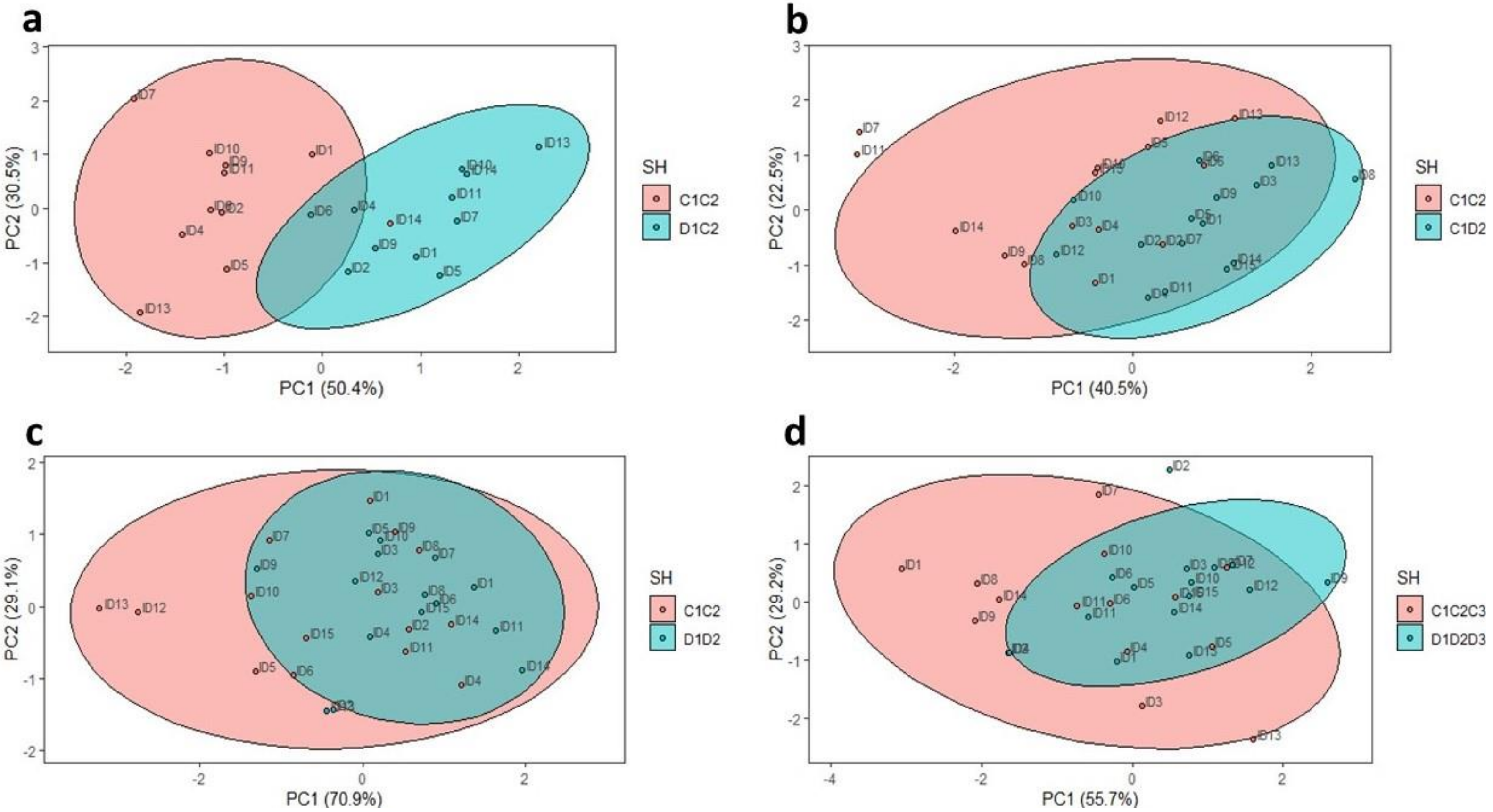

Figure 4.9: Two-dimensional principal component analysis showing the trait variation of winter wheat cultivars under drought conditions. Offspring from control (C1C2) are shown in pink, while those with a history of stress in any one, two, or three generations are shown in blue. Proximity between samples shows similarities in the tested responses. The X and Y axes indicate the first and second principal components, along with the percentage of variation explained by them in brackets.

Discussion

Under climate change conditions, the drought tolerance of wheat in its reproductive stage is very crucial for yield potential and stability (Mu et al., 2021). In this study, the first examination was to check for the presence of drought memory by quantifying the effects on alterations in physiological and biochemical signatures of winter wheat leaves in response to a current drought treatment during reproductive stage. The expectation was that there would be quantifiable changes in physiological and biochemical parameters studied due to the memory of the previous drought stress. In addition, it was expected that there would be cultivar differences in response to the stress memory based on their drought susceptibility status. Offspring expressed traits differently due to memory effects. While grandparental effects influenced the quantum yield of PSII, water potential, and proline, parental effects altered chlorophyll, ARI, the quantum yield of PSII, and water potential. Combined effects (2 successive generations of drought exposure) only changed the expression of proline, while combined effects (3 successive generations of drought exposure) influenced osmotic potential and proline. Distinct stages of wheat growth showed varied responses to previous drought exposure when quantum yield of PSII and chlorophyll content were considered, which makes it difficult to assess stress memory fitness consequences without measuring an entire suite of traits at multiple points in development. This observation agrees with Bell & Hellmann's (2019) assertion that stress memory could have potentially adaptive benefits at one life stage that might generate fitness costs later in life. According to these authors, timing is very important from the perspective of the offspring because the manner in which they receive and integrate stress memory depends on the developmental stage at which they receive the cue.

The most sensitive process to various stressful environments is photosynthesis. When a plant is exposed to stressors such as drought, photosystem II is the most vulnerable component of the photosynthetic process. According to Krause & Weis (1991), photosynthetic efficiency usually decreases before modifications in other physiological processes can be detected. For this reason, measuring the yield of chlorophyll fluorescence provides information regarding changes in the efficiency of photochemistry (Maxwell & Johnson, 2000). In this study, the efficiency of Photosystem II photochemistry was measured as a parameter that indicates the proportion of light absorbed by chlorophyll associated with PSII, which is used in photochemistry. This way, it provides a measure of the rate of linear electron transport, thereby indicating overall photosynthesis. The change in quantum yield of photosystem II in the offspring of C1D2 under drought treatment in the two seasons indicated that the photosynthetic process of wheat was adjusted by the previous drought encounter, while the reduction in offspring from C1C2 (the control) confirms that the lack of any prior stress memory negatively impacts photosynthetic activity. More recently, researchers have determined that under stress conditions, plants with increased rates of photosynthesis also have higher yields in soybean and rice, supporting the possibility of increasing photosynthesis efficiency more in crops (Monteoliva et al., 2021).

Chlorophylls found in the antenna complex of the chloroplasts are the crucial pigments that trap the light to be converted into carbohydrates during photosynthesis. Therefore, chlorophyll content is a photosynthesis-related trait that has been underutilized as a trait to screen genotypes for drought tolerance. However, several studies have found that chlorophyll content is reduced in response to drought and that its maintenance correlates with drought tolerance (Monteoliva et al., 2021; Anjum et al., 2003; Jaleel et al., 2008b). Offspring from seeds with a previous history of drought, regardless of the number of generations of exposure, had significantly higher chlorophyll contents than the offspring that had no previous history of drought. Therefore, drought memory confers high chlorophyll levels to the offspring. Increases in chlorophyll levels guarantee an efficient photosynthesis system, which is a vital process in plant cells. This was observed in the offspring of D1C2 and C1D2 seeds, which in addition to having high levels of chlorophyll also recorded an increased quantum yield of photosystem II. While water stress reduces chlorophyll content in plants due to disintegration of the thylakoid membrane caused by dehydration of cells, drought tolerant genotypes maintain higher levels (Zeng et al., 2016). Smirnoff (1995) argues that under drought conditions, plants record reduced chlorophyll because of the damage to the chloroplast by reactive oxygen species. This observation was also reported by Anjum et al. (2011), who showed that the low chlorophyll levels indicate a distinctive symptom of oxidative stress, which could be the result of photo-oxidation of photosynthetic pigments.

However, plants utilize antioxidants to respond to reactive oxygen species (ROS), which depends on chemical characteristics, the strength of the signal as well the plant’s developmental stage. Healthy vegetation is known to have good absorption in the visible region while exhibiting high reflectance in the near infrared region due to the presence of leaf pigments such as xanthophylls, chlorophylls, and carotenoids, which strongly absorb the visible region with little to no absorption in the near infrared region (Bayoumi & Emam, 2015). We reveal increases in carotenoids and anthocyanins in plants with a stress history. These antioxidants are essential in helping plants resist drought stress because they participate in the scavenging of single oxygen, which means that their relative amounts in a plant determine its tolerance (Jaleel et al., 2008). Lichtenthaler & Wellburn (1983), Lohithaswa et al. (2013), and Zhang et al. (2021) findings report higher carotenoid contents in drought-tolerant plants. According to these studies, photosynthetic pigments decreased with the severity of drought stress, and genotypes that exhibited higher carotenoid contents were classified as tolerant. Although carotenoids function as accessory pigments in the light-harvesting complex to bind to the photosynthetic complexes for more efficient light harvesting and to influence membrane structure and fluidity, they also increase chloroplast photoprotection by quenching chlorophyll fluorescence, scavenging ROS, and dissipating excess energy through NPQ non-photochemical quenching (Pintó-Marijuan, et al., 2017). In addition to acting as an antioxidant, anthocyanin accumulation is also associated with the production of different osmolytes that are known to contribute to the detoxification of ROS under stress (Gould et al., 2002; Sperdouli & Moustakas, 2012; Naing & Kim, 2021).

Proline is one of the most common compatible osmolytes in drought stressed plants (Hayat et al., 2012). In this study, offspring from the categories of seed sets with a history of drought stress recorded proline levels variably when compared to the control offspring. Offspring experiencing drought for their first time increased proline, and the levels went down with subsequent drought treatments (C1D2:D3 and D1D2:D3) and even after re-watering (D1C2:D3), but went up again in the offspring experiencing drought for the fourth time (D1D2D3:D4). According to Mafakheri et al. (2010), drought increased proline severely at the flowering stage compared to the vegetative stage, which is also confirmed in this study, where some offspring recorded very high proline amounts in the reproductive stage. Increases in proline content adjust osmotic potential, thereby resulting in drought stress avoidance. However, offspring of C1D2 that did not record increases in proline recorded significant adjustments in osmotic potential, and water potential was less negative in offspring of D1C2 despite the decreases in proline content to show how drought memory response varied generally. As noted by Do Amaral & Souza (2017) and Pinheiro & Chaves (2011), memory effects on plants are not homogeneous, and observations of independent effects of the different levels of plant organization can lead to misconception.

Being the initial spot of perception for reacting to external stimuli, the plasma membrane can be adversely affected by unfavourable environmental conditions like drought (Couchoud et al., 2019). Specifically, severe drought stress triggers cell membrane disturbance, leading to membrane integrity loss. In this study, drought memory in D1C2 and C1D2 did not affect the plasma membrane, but offspring of D1D2 seeds leaked more electrolytes than those of C1C2 seeds. This damage to the cell membrane could be explained by the low antioxidants, which also relate to the low quantum yield of PSII and chlorophyll content as compared to the offspring of C1D2 and D1C2. Tolerance at the cellular level is among the mechanisms that plants develop to resist drought, which is essential to allow them to maintain cellular homeostasis. Cell membrane damage in the offspring of D1D2 was resolved in the offspring of D1D2D3, which also recorded significantly more negative values of water potential. This illustrates the dynamic changes in the ability of plants to express drought memory and adjust its functioning, by preventing quick, permanent cell damage largely because of membrane degradation resulting from lipid biosynthesis inhibition (Gigon et al., 2004).

We demonstrate cultivar variation in the stress memory of physiological and biochemical traits upon exposure to drought stress. There was evidence of stress memory and its interaction with cultivars as manifested in various characteristics, illustrating that stress memory can affect the adaptive capacity of offspring. This suggests that previous drought exposure is an important influence on wheat response to future stress, and the interactions between stress history (SH) and cultivar show cultivar dependent memory effects on the expression of the traits. Surprisingly, expression of the memory effect depended on the trait of interest, with offspring displaying evidence of stress memory in certain traits and a lack of evidence of stress memory in other traits. This observation demonstrates the variability in response to stress memory in wheat, a finding that was also reported by Racette et al. (2019), who noted cultivar variability in transgenerational stress memory in seedling vigour of peanuts and quoted some genotypes that did not display evidence of transgenerational stress memory. Further, drought susceptibility status of cultivars has almost no general effect on the drought memory response. Unlike Abid et al. (2016) who reported different response of drought tolerant and sensitive cultivars primed at a particular growth stage towards the subsequent drought stress, our findings are variable. While trait values in sensitive cultivars would decrease under drought due to a certain drought memory type, the values would increase in response to a different memory type. In general, the drought-primed offspring showed fewer changes in traits in the current drought environment, whereas the control plants showed greater changes, explaining the observed broader variance (Ullah et al., 2022).

Conclusion

In this study, both physiological and biochemical processes in offspring from seeds with a previous history of drought in any one or all of the previous generations displayed behaviour consistent with drought memory. The memory effects on biochemical responses could or could not match those on physiological responses, and this depended on the type. Cultivars also vary in the expression of memory effects based on the memory type and the trait. Undoubtedly, there is no clear relation between the different memory levels within and between the results of the physiological and biochemical traits considered. Although the relationship between stress memory and adaptation to stress is complex, this study adds to the growing body of literature showing the critical role that stress memory can play in determining the phenotypes of offspring. Eventually, these phenotypes can contribute to plant adaptation to stress, although the strategies that may be utilized by plants to achieve stress memory may vary. Based on this study, examples of such strategies could be photosynthetic, osmotic, membrane integrity, plant water status adjustments, as well as oxidative stress reduction. Our study reveals the result of the beneficial memory effects caused by drought stress pre-exposure in wheat to overcome subsequent stress, which are independent of the cultivar-drought-susceptibility status.

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Changes in seedling physiological, biochemical, and morphological responses during repeated drought stress and the associated seed transcriptome of winter wheat

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Abstract

Plants recall their past stress experiences to alter their responses to subsequent stresses and confront them more promptly and efficiently. Coordinated responses of cells, genes/genomes, and epigenetic modifications, including altered physiological responses, gene repression and induction, and chromatin modification, are considered necessary for the formation of stress memories. However, evidence about these mechanisms in winter wheat is still very limited. We generated seeds with different stress histories from different ancestral generations and studied gene expression by MACE sequencing in the seeds and the resulting response patterns of seedlings to reveal the physiological, biochemical, root morphological, and molecular mechanisms of drought stress memory formation in winter wheat. As histone modification, H3-K14 and H3-K9 acetylation play an important role in (+/-) transcript memory. Heat shock proteins are implicated in the (-/+) memory type. Generally, the probable biological significance of memory genes is indicated in the context of overlapping strategies that are usually used by plants during drought and that include osmotic adjustment, detoxifying functions, growth, and readjustment of cellular homeostasis. We also show both grandparental and combined (grandparental and parental) memory effects in winter wheat. Our result demonstrates that the transcriptional responses after repeated exposures to stress are distinct from the common responses occurring during a single exposure. Modifications of histones and other proteins (heat shock proteins, alpha-amylase, and protease inhibitors) participate in drought memory, possibly acting as memory factors to activate drought related memory transcript pathways like responses to reactive oxygen species and osmotic stress to respond to successive stress.

Keywords, drought memory, memory genes, histone modification, epigenetics

Introduction

Understanding plant responses to drought is vital for upholding productivity in both agricultural and natural ecosystems in the face of changing climates (Porter and Semenov, 2005; Zhu et al., 2010; Lobell and Gourdji, 2012; Trenberth et al., 2014). Plant responses to dehydration involve a variety of physiological, biochemical, and morphological mechanisms, which could have cost-benefit compromises (Kozlowski and Pallardy, 2002; Chaves et al., 2003). Photochemistry uses the proportion of the light absorbed by chlorophyll associated with Photosystem II (PSII) to measure the efficiency of PSII photochemistry and thus overall photosynthesis. During photosynthesis, inorganic carbon from atmospheric carbon dioxide (CO_2) is converted to organic compounds in a process referred to as carbon fixation or assimilation. In drought conditions, plant roots sense the drying soil and produce signals, which, on transmission to shoots, trigger stomatal closure to regulate water loss through transpiration. The plant hormone abscisic acid (ABA) plays a crucial role in the control of guard cell function (Kim et al., 2010; Kollist et al., 2014). However, transpiration enables plants to absorb water and mineral salts from the soil, facilitates the movement of soil water, and cools the plants. Therefore, there is usually a tradeoff between CO_2 gain and water loss, which is a particular problem for plants growing in drought-prone environments. Although water loss is avoided upon the closure of stomatal pores, carbon dioxide is decreased and the concentration of oxygen in the airspace increases (Schulze, 1986; Flexas et al., 2004, 2007). In principle, increases in stomatal conductance (g_s), which regulates gas exchange (CO_2 and water), can permit plants to increase their CO_2 uptake and subsequently enhance photosynthesis. Research has shown that the rate of photosynthesis is directly related to the amount of carbon assimilated by the plant. Moreover, drought influences certain morphological traits of wheat like seed germination, shoot length, root length, and volume. These characteristics are derived from physiological processes like those controlling chlorophyll concentration, photosynthetic rate, and osmotic adjustment (Sharma et al., 2022).

Plants experience repeated drought episodes season after season, sometimes even with an intervening water recovery. Recent studies have shown that pre-exposure to drought events alters responses to subsequent incidents (Fleta-Soriano & Munné-Bosch, 2016; Xin & Browse, 2000; Abid et al., 2018). These observations propose that plants possess “memory” that helps them alter responses to succeeding stress (Bruce et al., 2007; Waters et al., 2008). However, despite such evidence, it is still not clear whether seedling responses display memory of drought experienced by the preceding generations or how changes in gene expression due to recurrent stress could regulate these responses to optimally adjust the phenotype to repeated transgenerational stress cycles and recovery.

In Arabidopsis, soybean, and maize, two types of gene response groups were identified following repeated drought and rehydration cycles (Ding et al., 2012, 2013, 2014; Kim et al., 2020). Some genes produced similar transcript levels in response to each stress and returned to their initial pre-stressed levels during rehydration and were classified as non-memory genes. Other genes, known as memory genes, showed a transcriptional response to subsequent stress that was significantly different from the response to the first stress encounter. It is still not clear whether transcriptional memory could be translated in an integrated physiological and morphological response to repeated drought. This information could be valuable in understanding stress memory and categorising important genes and pathways for drought tolerance improvement in wheat and other agricultural crops.

While many studies have focused on periodic drought with minimal focus on multigenerational drought experiences on crops, it has recently been envisaged that drought memory responses could be looked at as a system of coordinated changes resulting from an initial stress encounter, which triggers altered gene expression that directly or indirectly causes physiological, biochemical, and morphological responses to achieve a new homeostatic state (**Figure 5.1**).

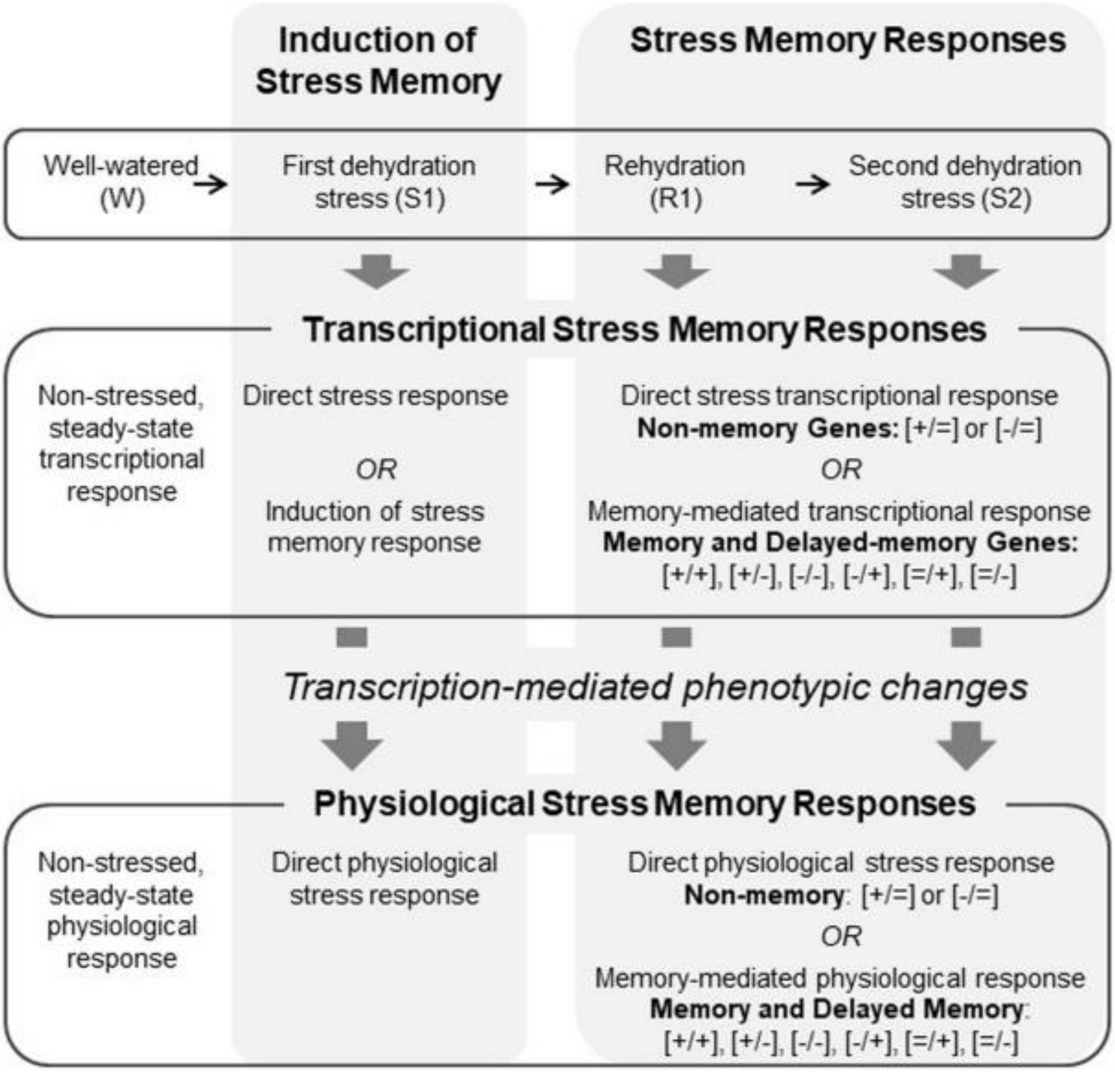

Figure 5.1: A conceptual framework illustrating the interplay between transcriptional and physiological responses in drought stress memory. Where the first sign indicates the gene expression values in the first drought exposure were not statistically different [=], were lower [−], or were higher [+] than values in the non-stressed samples, while the second sign indicates the gene expression values in the subsequent drought stress exposure were similar [=], were lower [−], or were higher [+] compared to those in the first drought exposure (Virlouvet et al., 2018).

We used wheat, one of the most important cereal crops, in this study to identify memory induced by repeated drought stress. Although plants have developed a range of mechanisms to withstand drought stress in nature (Guo et al., 2016), drought stress continues to be one of the major limiting factors in wheat production. Our major goal was to quantify and understand the impacts of drought exposure history on wheat as well as the role of potential drought stress memory genes in the adaptation of wheat to drought recurrence events by focusing on how different drought histories affect the underpinnings of different traits in the offspring. Our specific objectives were to: 1. establish whether drought stress perceived by prior wheat plant generations induced changes in physiological, biochemical, and morphological responses of seedling leaf and root systems, 2. identify sets of genes that display coordinated changes in gene expression under different drought histories, and 3. identify functions of key genes that may point to physiological, biochemical, and morphological processes that are most strongly involved in drought stress memory. Changes in gene transcript levels were studied in the resulting seeds that were used to generate the seedlings whose physiological, biochemical, and morphological parameters were measured. We therefore present the transcriptome changes associated with recurrent drought stress, and how these changes could translate to physiological, biochemical, and morphological measurements.

Material and Methods

In a previous study, a large population of winter wheat cultivars representing broad genetic diversity was screened for their response to drought stress (Koua et al., 2021). After selection of drought-sensitive and -tolerant cultivars, their seeds were repeatedly exposed to control or drought stress conditions over three subsequent generations. This allowed us to collect sets of seeds experiencing different parental stress histories: (i) only one round of previous drought stress; (ii) drought stress over 2 or 3 consecutive generations; and (iii) an intermittent drought stress history. In this study, seeds collected in the second generation were used and included D1D2 (drought stress in the first and the second generations), D1C2 (drought stress only in the first generation), C1D2 (drought stress only in second generation), and C1C2 (no history of drought stress). Two cultivars with contrasting drought tolerance were used: The Intro cultivar released in 2011 was considered drought tolerant, and the Sonalika cultivar released in 1967 was considered drought sensitive. Throughout the text, ID5 and ID14 are used to represent Intro and Sonalika wheat cultivars, respectively.

RNA, MACE sequencing, read processing, mapping to the reference genome, and bioinformatics analysis

To examine the effects of ancestral environment on the seed transcriptomes, total RNA from seeds of each set of seeds category for the two cultivars derived from the same lot of seeds used for the seedling phenotyping was isolated following the protocol by Li & Trick (2005). For each cultivar and ancestral environment, there were two biological replicates that consisted of around four seeds per replicate. Therefore, there were in total two biological replicates of four treatments (C1C2, C1D2, D1C2, and D1D2) for each of the two cultivars, giving a total of 16 Massive Analysis of cDNA Ends (MACE) sequencing samples. A 3′ mRNA sequencing approach using Illumina reads of fragments that are derived from 3′ mRNA ends according to Zawada et al. (2014) was used to sequence biotinylated 3’-end fragments from 16 to 200 bp. Adapters were removed from the reads using the Cutadapt tool (Martin, 2011). FastQC carried out the quality control of the libraries, and Trimmomatic removed the short reads with less than

35 bp (Bolger et al., 2014). These procedures were carried out at GenXPro GmbH (Frankfurt, Germany). The reads were finally mapped to the *Triticum aestivum* genome (version 2.1) found in the International Wheat Genome Sequencing Consortium (IWGSC) using the ENSEMBL genome browser. The HISAT2 splice-aware aligner tool was used to align the reads to the exons of the reference genome. Quantification of reads from BAM files was done using HTSeq count, which is a part of the HTSeq Python package that takes a file with aligned sequencing reads plus a list of genomic features and counts how many reads map to each feature. Deduplicated alignment files and estimation of the amount of read duplication were done by the markdup tool from SAMtools (Li et al., 2009). Normalization of the read count data was done to counts per million and used for the differential expression analysis using the iDEP v1.0 tool that utilizes DESeq2 (Ge et al., 2018).

Drought stress memory genes (DSMGs) were identified by comparing the expression fold changes between C1D2/C1C2, followed by D1D2/C1D2, and D1C2/C1D2. Genes that showed upregulation, downregulation, or no significant changes in expression were denoted by the "+," "-," and "=" symbols, respectively. For example, a gene in the (+ +) category would be upregulated between C1D2/C1C2 and between D1D2/C1D2. Therefore, eight categories of differentially expressed genes (DEGs) could be defined: (+ +), (+ -), (- +), (- -), (+ =), (- =), (= +), and (= -). The (= +) and (= -) categories would describe genes that in C1D2/C1C2 did not have a significant expression change but significantly changed transcription in D1D2/C1D2 or in D1C2/C1D2. Formally, these genes do not belong to the initial C1D2/C1C2 dehydration-stress responding category. The (+ =) and (- =) would describe the non-memory genes.

Seedling growth and phenotyping

Germination

In a separate experimental set-up, seeds of the two cultivars (ID5 and ID14) in every seed category were exposed to 45 °C for 24 h to remove the inherent differential dormancy before planting. In total, there were 2 cultivars x 4 seed set categories (D1D2, D1C2, C1D2, and C1C2) x 2 treatments (drought and control) x 4 repetitions where each pot received 6 seeds each. Germination data was taken from the first day of emergence for a duration of five days. A seed was recorded as having germinated upon the emergence of the seedling (coleoptile) from the soil. The climate conditions in the greenhouse were set at 20/16°C day/night temperatures with a photoperiod of 16 hours and a relative humidity of 70%.

When evaluating the number of normal seedlings at the time of the final count, seedlings were thinned to retain 4 per pot with uniform spacing, and the seedling length was measured per pot and averaged.

Seed Vigor Index (SVI)

Calculations were done for the germination percentage and the average seedling length of the same seed lot. SVI was calculated by multiplying the germination percentage (%) with seedling length (mm). The seed lot showing the higher seed vigour index is more vigorous (Abdul-Baki and Anderson, 1973). SVI=germination%* Average shoot length.

After growing all the seedlings under optimal water supply for 6 weeks after sowing, drought treatment was applied to the pots designated for drought treatment, and the rest of the measurements were collected after the following 3 weeks.

Seedling physiological and biochemical traits

Photochemical quantum yield of photosystem II; calculated based on F and FM′ chlorophyll fluorescence measurements

Three measurements were done on the second leaf for each cultivar within each seed set per treatment with the aid of the MINI-PAM-II photosynthesis yield analyzer (Heinz Walz GmbH, Germany).

Stomatal conductance to water (gsw), assimilation rate (A), and transpiration rate (E)

These gas exchange parameters were estimated by the LI-6800 fluorometer (LI-6800 Portable Photosynthesis System, Li-Cor Biosciences, Lincoln, NE, USA) on the latest fully expanded leaf.

Leaf samples were collected and frozen in liquid nitrogen for later use in the determination of ABA and proline contents.

ABA determination

An accumulation level of abscisic acid was evaluated in pooled leaves using the Plant Hormone Abscisic Acid (ABA) Elisa Kit (CUSABIO, www.cusabio.com, CSB-E09159Pl). The analysis was performed according to manufacturer instructions in three biological replicates at each time-point. The ABA concentration in the leaf samples was measured by an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody for ABA (AFRC MAC 252), according to Asch et al. (2001).

Proline determination

Proline concentrations were determined based on the protocol of (Bates et al., 1973; Frimpong et al., 2021). The second leaf was collected for each sample and immediately submerged in liquid nitrogen. Samples were pulverized using a pestle and mortar on ice. One hundred mg of the pulverized samples were weighed and extracted with 1.5 ml of 3% salicylic acid in chilled 2 mL tubes, vortexed, and centrifuged at 12,000 rpm for 10 min. Five hundred µL of the supernatant was directly transferred into cylindrical glass tubes (fitted with lids) on ice and 500 µL of glacial acetic acid and 2.5% ninhydrin reagent were added. The mixture was then vigorously vortexed and incubated for 1 hour in a water bath at 95 ˚C. The reaction was quickly terminated on ice. 1.5 mL of toluene was added, and the mixture was kept at room temperature for 30 minutes after mixing. One hundred µL of the upper phase was then pipetted into 96 well plates, and the absorbance at 520 nm was measured using a microplate reader (Tecan Infinite 200 PRO). A calibration curve based on eight points of proline standard concentrations (0, 10, 20, 30, 50, 70, 90, and 100 µg/g) yielded a linear regression between proline concentration and the measured absorbance at 520 nm (R2 = 0.9969). This linear model was subsequently used for proline concentration calculations in the samples.

Shoot water content

The seedling shoots were harvested at the end of the 3rd week following drought application, and immediately the fresh weight was determined. Thereafter, they were oven dried at 70°C for 48 hours, and the dry weight was measured. Shoot water content was calculated by subtracting the dry weight from the fresh weight.

Seedling root morphology

In a separate experiment, seeds from D1D2, D1C2, C1D2, and C1C2 of cultivars ID5 and ID14 were selected for germination. Clean, transparent germination trays of 29 * 22.5 cm were prepared with filter papers (Whatman paper) placed on them. To remove surface fungi and bacteria, the selected seeds were immersed in 1% sodium hypochlorite (NaOCl) for 5 minutes, followed by washing under running tap water to remove NaOCl and drying with tissue paper before planting. For each cultivar within a seed set, 15 seeds were sown by placing them on the tray, followed by sprinkling 20 ml of water, and finally covering each germination tray with a transparent plastic lid. The growth chamber was set at 25°C and 40% relative humidity.

One germinated seed per cultivar and seed set category was transplanted 1 cm in the tubes filled with Aquagran filter quartz, 2-3.15 mm (Euroquarz GmbH, Dorsten, Germany), in each of the four hydroponic systems in the greenhouse in three repetitions. Each box was served with the same amount of modified Hoagland nutrient solution as described by Tavakkoli et al. (2010), which was circulated once every hour using EHEIM Universal-pump 1046 (EHEIM GmbH and Co., Deizisau, Germany) and an automatic switch timer. The nutrient solutions were changed every 7 days, accompanied by an adjustment of the pH to 5.5. Thereafter, the solution pH was monitored daily and adjusted to 6.0. The nutrient solution temperature varied from 14.1 to 21.7 °C. The greenhouse used SOD AGRO 400W 230 V lightbulbs (DH Licht GmbH) and was set for 12 hours of lighting from 7am to 7pm, humidity of 40% during the day and 70% at night, and temperatures of 21°C and 25°C, respectively.

All plants were growing under the same normal conditions in the two systems for four weeks. In the beginning of the fifth week, one of the systems was subjected to 20% osmotic concentrations of PEG-6000, corresponding to a final osmotic potential (MPa) of -0.80 MPa and the remaining system remained at 0% PEG to serve as a control.

After one week of treatments, the seedlings were harvested for further analysis of root architecture parameters using a WinRHIZO pro-optical scanner (Regent Instruments LA2400 Scanner) that scanned the roots and analyzed the image with WinRHIZO software (Regent Instrument Software) for seven root parameters including root length, root surface area, root diameter, root volume, number of tips, number of forks, and number of crossings. Separated root samples per cultivar within a seed set were first weighed to get the fresh weight before they were preserved in a 120-ml plastic labelled container containing 50% diluted alcohol to completely submerge the roots for longer storage. Following scanning, the root samples were subjected to 24 hours of drying at 105 °C to obtain their dry weight. Two independent experiments, designated E1 and E2, with three replications each, were done. In each experiment, comparisons were made between the 20% osmotic concentration and the control conditions (0%).

Statistical analysis

Basically, an ANOVA was conducted to compare the effects of stress history and genotype on the values of the different measured parameters. Tukey post hoc tests were used to compare individual means, as indicated in the figure legends. All effects were considered significant at $p < 0.05$. Statistical analyses were performed using the ANOVA package in R (R Core Team, 2020).

Results

Germination and seed vigor

Analysis of variance (ANOVA) revealed considerable differences between the two cultivars in seedling length and seed vigor index but not germination percentage (**Table 5.1**). In addition, stress history led to differences in the expression of germination % and seed vigor index, while there were notable interactions between stress history and cultivar in germination percentage, seedling length, and seed vigor (**Table 5.1**). The maximum seed germination percentage (100%) was recorded in the ID14 cultivar from D1C2 seeds, while the minimum was in the ID5 cultivar from D1D2 seeds (75%). Specifically, ID14 from C1D2 had the lowest percentage at 78% compared to the same cultivar from the other different stress histories. Generally, the average germination percentage of seeds from control seeds without a history of drought (C1C2) was 94%, which was reduced in C1D2 and D1D2 seeds by 8.3% and 11.5 % respectively, but increased in D1C2 seeds by 4.2% (**Figure 5.2b**). The ID14 cultivar from C1C2 had the highest seedling length of 94.7 mm, while seedlings of the same cultivar from C1D2 seeds were 77.2 mm tall. The ID5 cultivar from C1D2 recorded the highest length of 84mm compared to the same cultivar from the other treatments (**Figure 5.2c**). In general, D1D2 seedlings were non-considerably longer (87.8 mm), while those from C1D2 were the shortest (80.6 mm) (**Figure 5.2d**). The calculated seed vigor index (SVI) was lower in ID14 from C1D2 and in ID5 from D1D2 when compared to the same cultivars across other treatments (**Figure 5.2e).** While parental drought (C1D2) reduced the seed vigor index in ID14, the effects increased the seed vigor index in ID5. In general, D1C2 had the highest seed vigor index of 8202 compared to that of C1C2 (8190), while other treatments recorded reduced seed vigor indexes (**Figure 5.2f**).

Table 5.1: ANOVA output for the effects of stress history (SH), cultivar, and their interactions on germination percentage, seedling length, and seed vigor of wheat. “.”, “*”, “**”, and “***” denote significance at the $p < 0.1$, $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels; ns indicates non-significant.

Trait	Variation source	Df	F value	Pr(>F)	
Germination %	SH	3	6.722	0.000592	***
	Cultivar	1	0.314	0.57765	ns
	SH: Cultivar	3	8.58	8.86E-05	***
	Residuals	56			
Seedling length (mm)	SH	3	1.738	0.16959	ns
	Cultivar	1	7.97	0.00657	**
	SH: Cultivar	3	4.034	0.01144	*
	Residuals	56			
Seed vigour index (SVI)	SH	3	3.969	0.0123	*
	Cultivar	1	3.03	0.0872	.
	SH: Cultivar	3	11.463	5.78E-06	***
	Residuals	56			

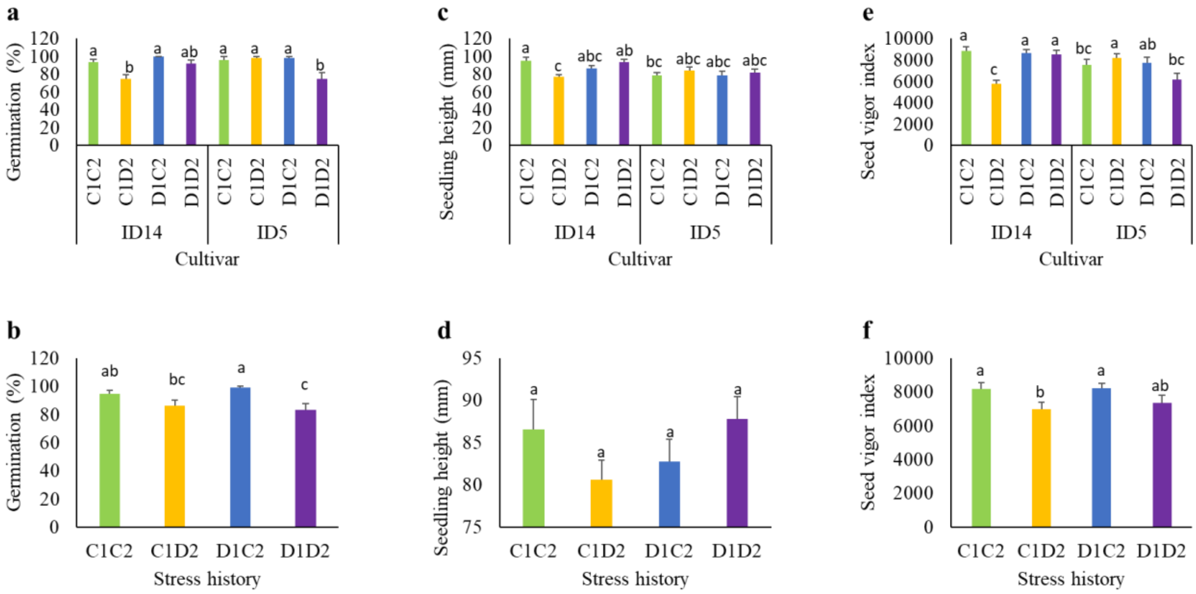

Figure 5.2: Germination percentage (a), seedling length (c), and seed vigor (e) of ID14 and ID5 wheat cultivars from the C1C2, C1D2, D1C2, D1D2 seed set categories. Different letters indicate statistically significant differences in treatment ($p < 0.05$). Error bars represent the standard error of the mean.

Considering the means of the two cultivars, it was found that D1C2 expressed the highest photochemical quantum yield of photosystem II and C1C2 had the lowest under drought treatment (**Table 5.2, Figure 5.3 Drought b**). Under control treatment, the three seed sets with a history of drought in the previous generation(s) had invariably the highest photochemical quantum yield of photosystem II compared to seedlings from C1C2 (**Figure 5.2 Control b**). Specifically, ID5 from D1C2 had the highest value compared to the same cultivar in C1C2, while ID14 showed no statistical difference but C1C2 recorded the least in the same cultivar (**Figure 5.3**).

Table 5.2: Output of a two-way ANOVA for the effects of stress history (SH) and cultivar as well as their interactions on leaf parameters, including quantum yield of PSII, chlorophyll content, shoot water content, stomatal conductance, transpiration rate, assimilation rate, ABA concentration, and proline content of wheat seedlings under drought and control treatments. ".", "*", "**", and "***" denote significance at $p < 0.1$, $p < 0.05$, $p < 0.01$, and $p < 0.001$ levels; ns indicates non-significant.

Treatment	Source of variation	Quantum yield of PSII		Shoot water content		Stomatal conductance		Transpiration rate	Assimilation rate	ABA		Proline
Drought		Df		Df		Df				Df		
	SH	3	**	3	.	3	***	**	***	3	***	ns
	Cultivar	1	ns	1	ns	1	**	**	ns	1	***	ns
	SH: Cultivar	3	ns	3	ns	3	***	***	***	3	***	ns
	Residuals	56		24		178				16		
Control	SH	3	***	3	*	3	***	***	***	3	***	**
	Cultivar	1	ns	1	ns	1	ns	ns	ns	1	.	ns
	SH: Cultivar	3	ns	3	ns	3	**	**	**	3	***	ns
	Residuals	56		24		186				16		

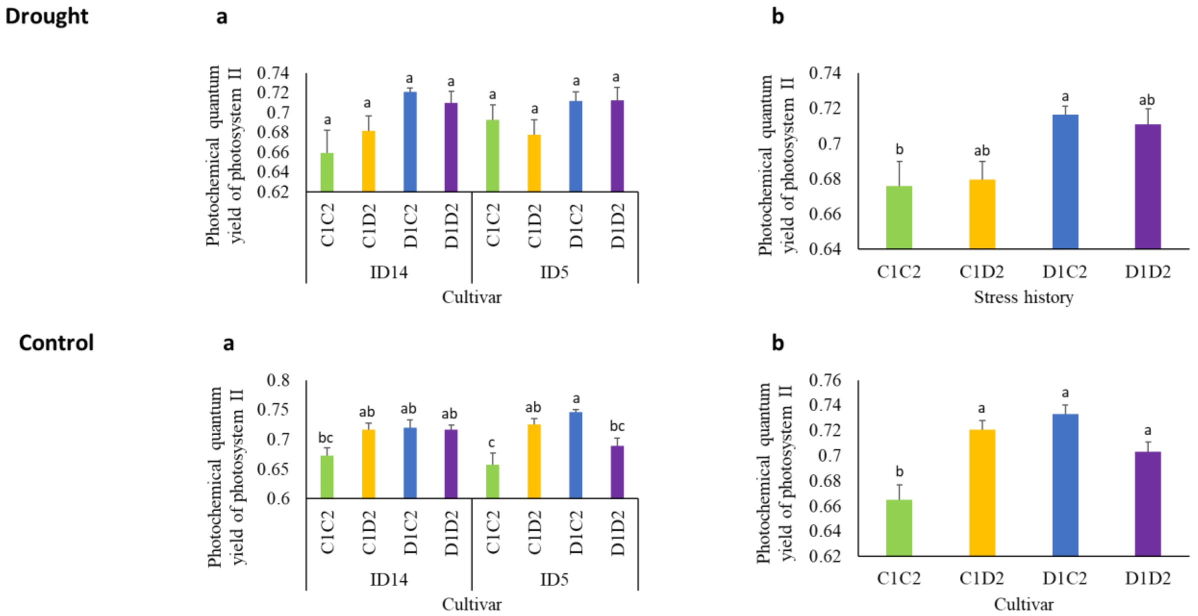

Figure 5.3: Photochemical quantum yield of photosystem II of ID14 and wheat cultivars from the C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

Regardless of drought history, cultivars expressed shoot water content in both drought and control treatments invariably (**Table 5.2, Figure 5.4 Drought a, and Control a**). However, generally, under drought treatment, offspring from D1C2 recorded the least, while under control treatment the opposite was true (**Figure 5.4 Drought b and Control b**).

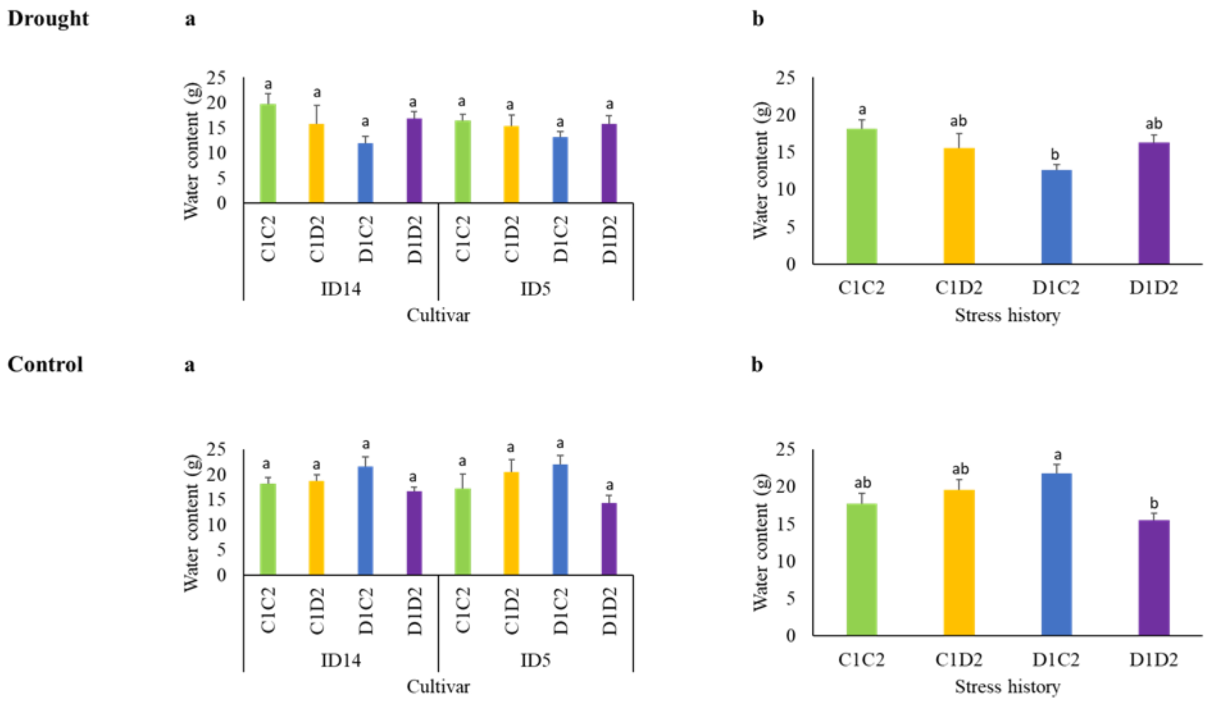

Figure 5.4: Shoot water content of ID14 and ID5 wheat cultivars from C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

Under both drought and control treatments, the cultivars expressed stomatal conductance variably based on the drought history type (**Table 5.2**). ID14 offspring of C1C2 had the lowest values compared to offspring of the same cultivar from other seed sets, with those from D1C2 recording the highest stomatal conductance under drought (**Figure 5.5 Drought a**). Offspring of ID5 from C1D2 recorded the highest stomatal conductance values under drought treatment compared to offspring of the same cultivar from C1C2 seeds. Generally, C1C2 had the lowest mean score value (**Figure 5.5 Drought b**). Under control, offspring from C1C2 similarly expressed stomatal conductance as those from D1C2, while those from C1D2 and D1D2 recorded reduced values (**Figure 5.5 Control b**).

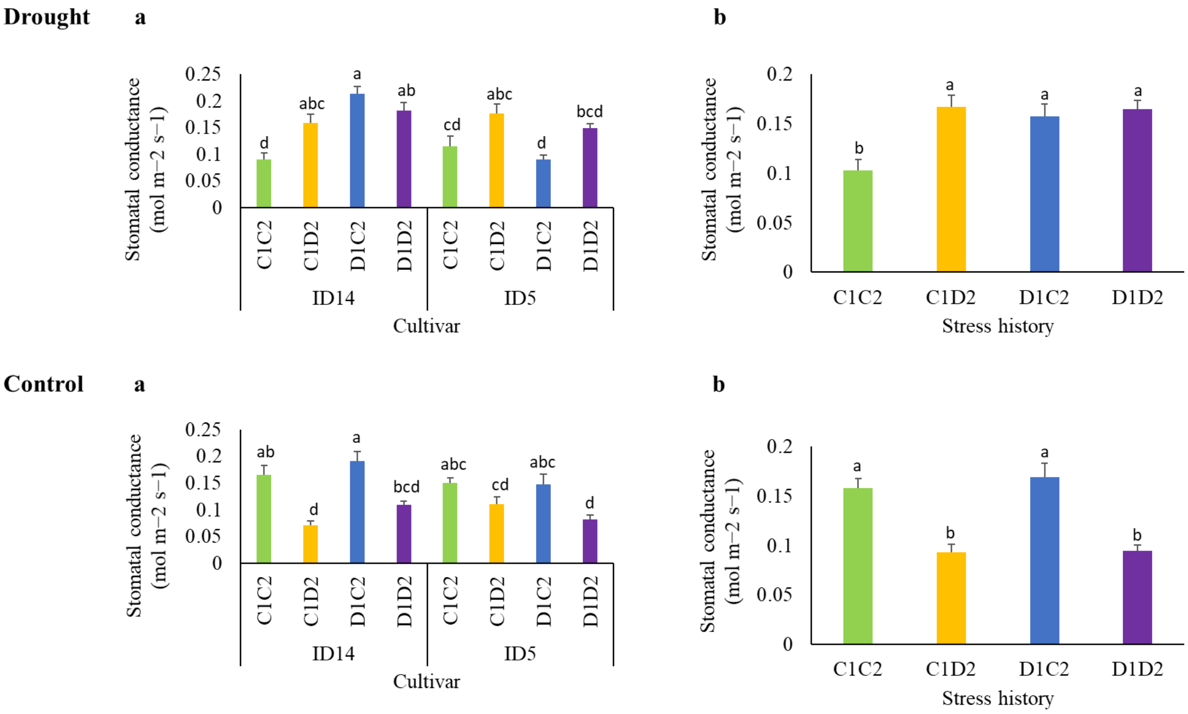

Figure 5.5: Stomatal conductance of ID14 and ID5 wheat cultivars from C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p< 0.05$) (Tukey test). Error bars represent the standard error of the mean.

Transpiration rate under drought treatment also followed the same trend as stomatal conductance, with offspring of ID14 showing increased values if the ancestor experienced drought (**Figure 5.6**). Generally, when the means were compared, offspring from the control seeds expressed a lower transpiration rate than the others under drought treatment. However, under control treatment, offspring from C1C2 had similar high transpiration rates as those of D1C2, while offspring of C1D2 and D1D2 had reduced values, which could be attributed to the reduced values in ID14 and ID5, respectively.

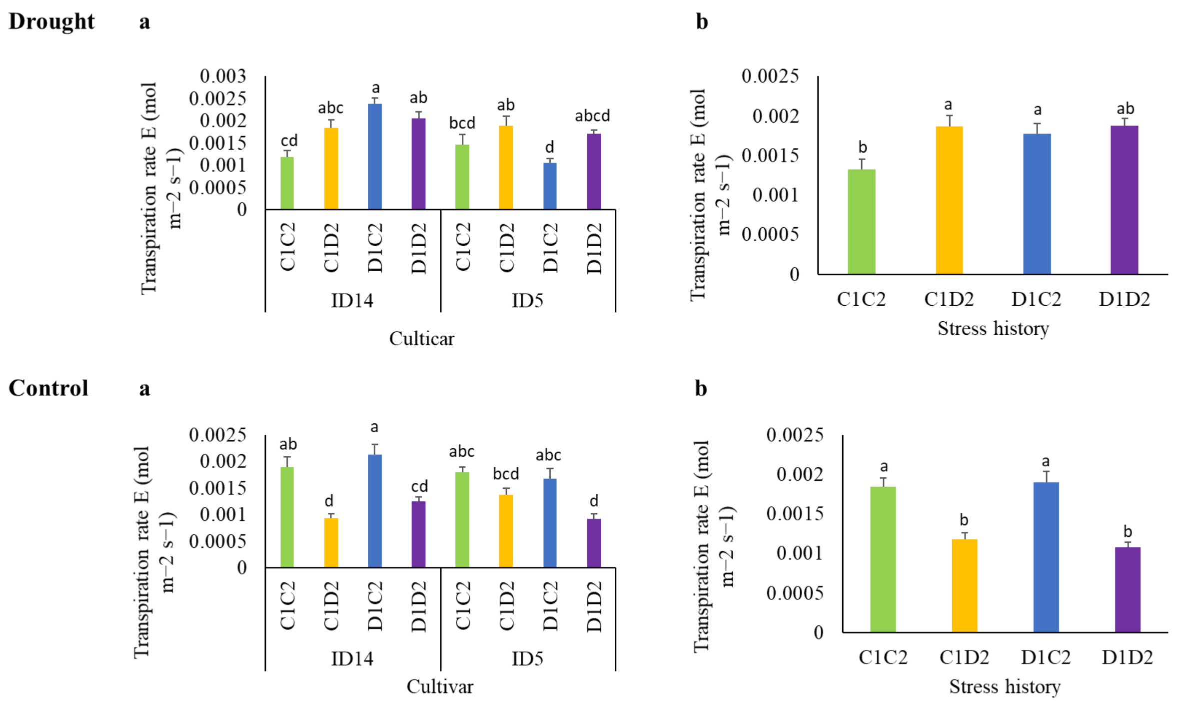

Figure 5.6: Transpiration rates of ID14 and ID5 wheat cultivars from the C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

Assimilation rate as expressed by the two cultivars also followed a similar pattern to that of transpiration rate and stomatal conductance under both drought and control treatments. When the means were compared under drought, offspring from C1C2 had a lower assimilation rate than the offspring from seed sets with a history of drought stress. Under control treatment, seedlings from D1D2 seeds recorded the least rate of assimilation, while those from D1C2 recorded the highest assimilation rates, which could be attributed to the high values recorded from the two cultivars (**Figure 5.7**).

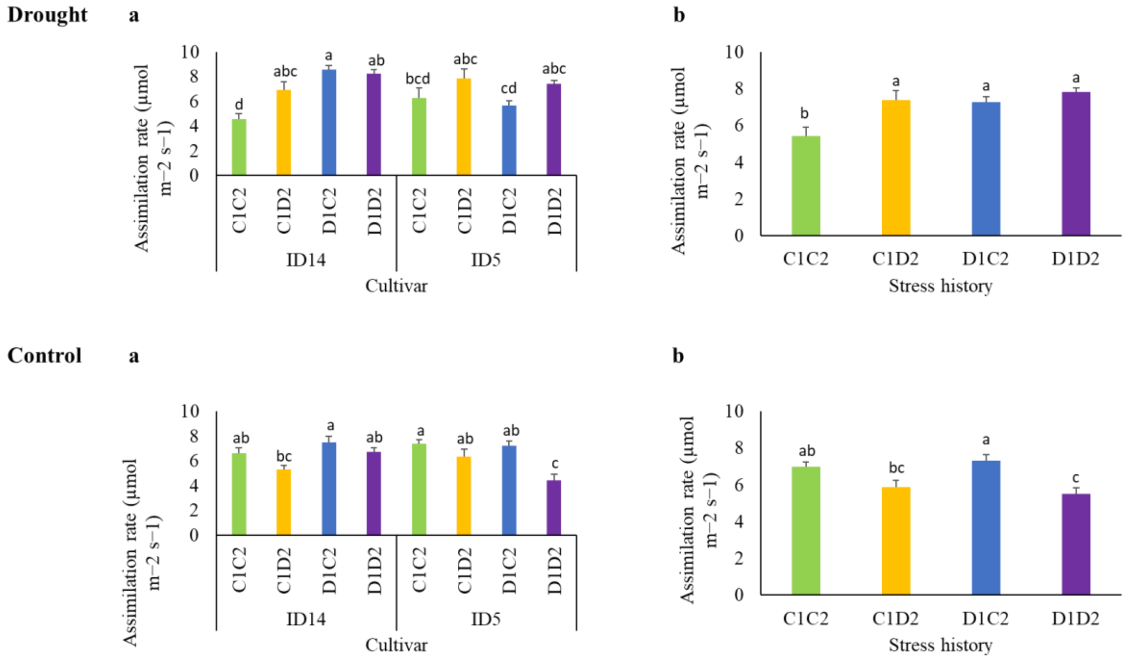

Figure 5.7: Assimilation rates of ID14 and ID5 wheat cultivars from the C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

Under drought treatment, ABA values were highest in ID14 seedlings originating from C1C2 and were considerably lower in the seedlings of the same cultivar originating from seeds with a history of drought. ID5 seedlings from D1C2 had higher ABA levels than the others from the same cultivar. Under control treatment, seedlings of ID5 from C1C2 had reduced ABA concentrations, while ID14 from D1C2 had the smallest value. When the means were compared across the four drought stress histories, seedlings from C1C2 and D1C2 had reduced ABA concentrations (**Figure 5.8**).

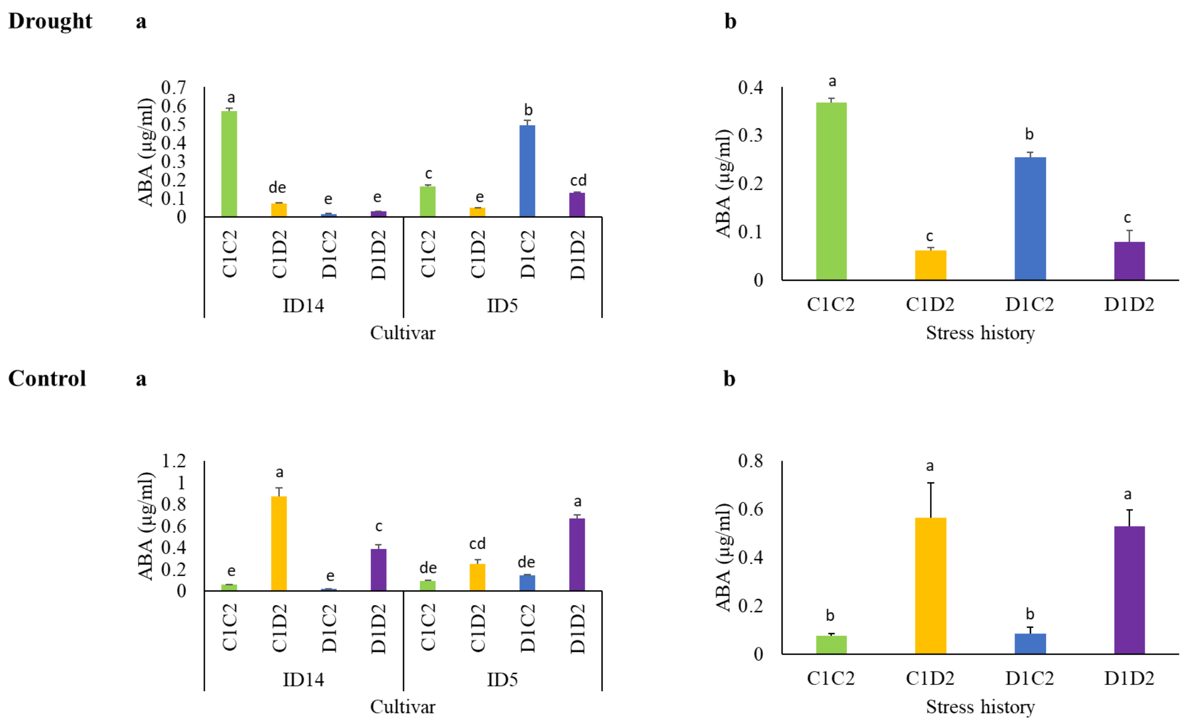

Figure 5.8: ABA content of ID14 and ID5 wheat cultivars from C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

Under drought treatment, the proline values were generally non-significantly lower in offspring from C1C2 seeds. Both cultivars from each of the four categories of previous drought exposure increased proline values in drought compared to the control treatment. C1C2 seedlings generally expressed reduced proline concentrations under control treatments while those from C1D2 and D1D2 expressed increased values when the means were considered (**Figure 5.9**).

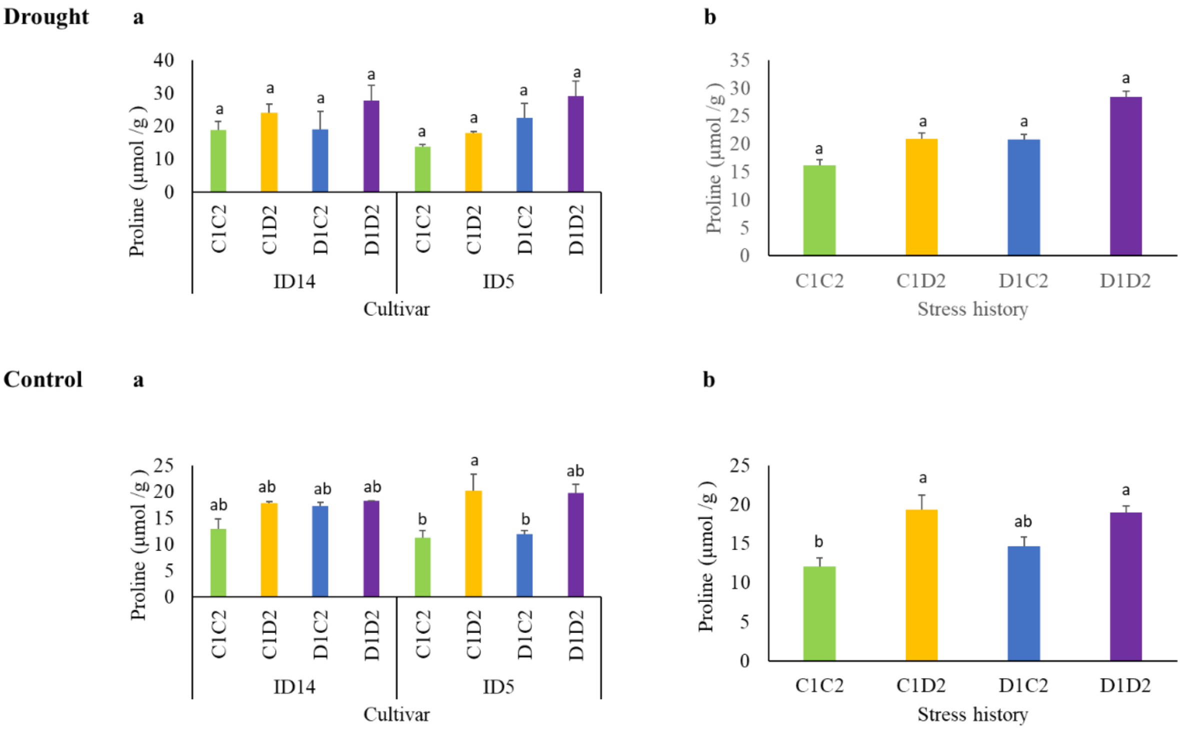

Figure 5.9: Proline content of ID14 and ID5 wheat cultivars from C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

An analysis of variance (ANOVA) showed significant differences among the tested stress history categories for most root traits, including root length, surface area, volume, forks, and crossings, under both drought (20% PEG) and control (0%) treatments. Under control conditions, the cultivar interacted with the stress history in expressing fresh and dry root weights, where seedlings of ID14 from D1D2 were the heaviest (**Table 5.3, Figure 5.10**). Under drought conditions, seedlings from D1D2 and D1C2 had longer roots, while those from C1C2 had the shortest roots. Cultivars also expressed root length differently under the same environment based on the seed set category from which they originated. ID14 from D1D2 had the longest roots, while ID5 seedlings from the same seed category (D1D2) had the shortest roots. While seedlings of D1D2 showed longer roots under the drought environment, those of D1C2 were the longest under the control treatment, and seedlings of C1C2 had the shortest roots in both the two environments (**Figure 5.11**). Root surface area also took on the same pattern of expression as the root length. However, the expression of root diameter by the two cultivars was similar under control but varied greatly under the drought environment based on the drought memory type. ID14 from the C1C2 seed set had the smallest root diameter, while ID5 from the same seed category had the biggest. Root volume was highest in ID14 from D1C2 and D1D2 under both treatments, and the least in seedlings from C1C2. When the means were considered, seedlings from C1C2 had generally reduced root volume compared to the seedlings from the other seed categories with a history of drought (**Figure 5.12**). This trend was like that observed in the expression of tips, forks, and crossings. However, while the two cultivars did not perform differently under drought, the memory type determined their behaviour under controlled conditions for the expression of tips, forks, and crossings. Both ID14 and ID5 from the C1C2 seed category recorded lower values, while ID14 from D1C2 and D1D2 recorded the highest values for these traits.

Table 5.3: Output of a two-way ANOVA for the effects of stress history (SH) and cultivar as well as their interactions on root morphology parameters of wheat seedlings under drought and control treatments. “.”, “*”, “**”, and “***” denote significance at p< 0.1, p< 0.05, p< 0.01, and p< 0.001 levels; ns indicates non-significant.

Treatment	Source of variation	Fresh weight		Dry weight		Root length		Root surface area		Root diameter		Root volume		Root tips		Forks		Crossings	
20% PEG		Df		Df		Df		Df		Df		Df		Df		Df		Df	
	SH	3	ns	3	ns	3	*	3	*	3	ns	3	.	3	ns	3	.	3	*
	Cultivar	1	ns	1	ns	1	ns	1	ns	1	*	1	ns	1	ns	1	.	1	.
	SH:Cultivar	3	ns	3	ns	3	ns	3	ns	3	***	3	ns	3	ns	3	ns	3	ns
	Residuals	16		16		16		16		16		16		16		16		16	
0% PEG	SH	3	ns	3	ns	3	**	3	*	3	ns	3	*	3	.	3	*	3	**
	Cultivar	1	*	1	.	1	.	1	*	1	ns	1	*	1	*	1	*	1	*
	SH:Cultivar	3	*	3	*	3	**	3	**	3	ns	3	*	3	**	3	*	3	*
	Residuals	16		16		16		16		16		16		16		16		16	

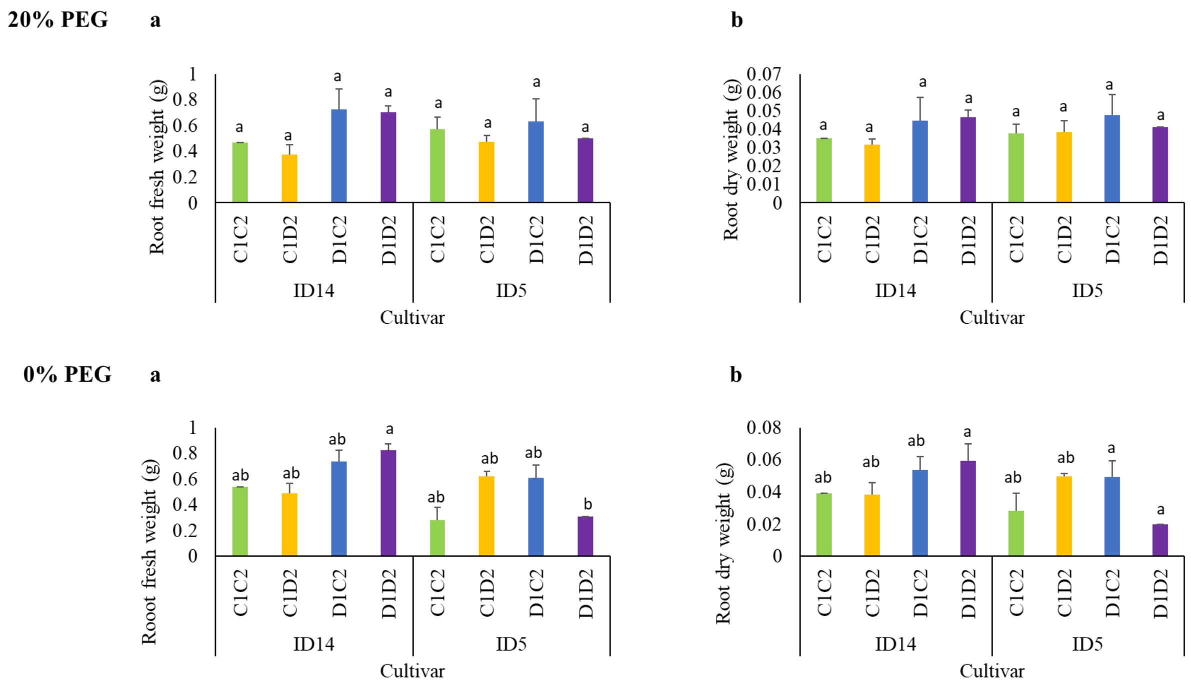

Figure 5.10: Root fresh and dry weight of ID14 and ID5 wheat cultivars from C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

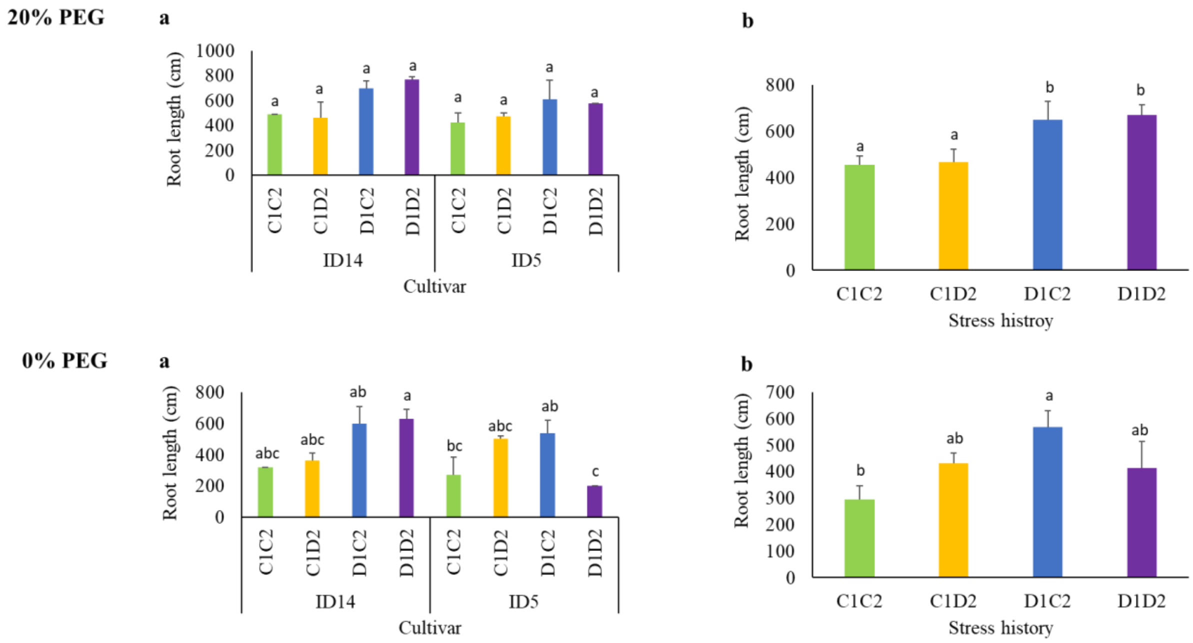

Figure 5.11: Root length of ID14 and ID5 wheat cultivars from C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

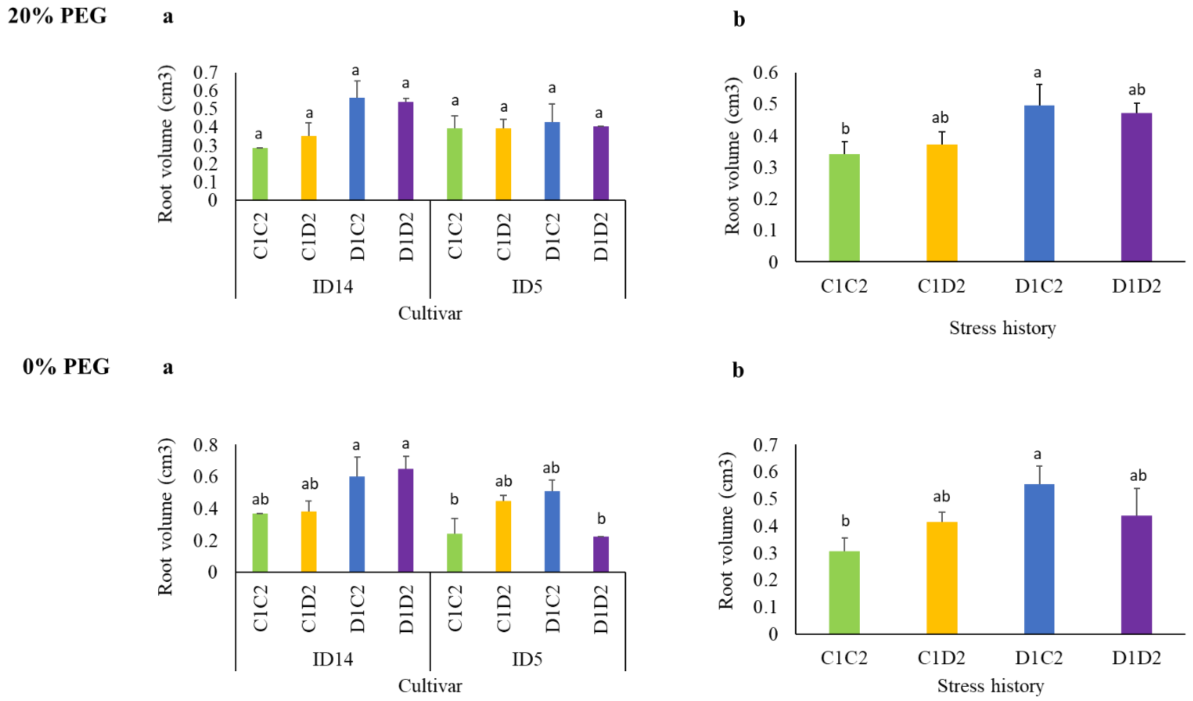

Figure 5.12: Root volume of ID14 and ID5 wheat cultivars from C1C2, C1D2, D1C2, and D1D2 seed set categories. Different letters correspond to statistically significant differences between treatments ($p < 0.05$) (Tukey test). Error bars represent the standard error of the mean.

MACE library read counts

Genome-wide gene expression profiling of the two cultivars was done by MACE to identify differentially expressed genes in the seeds with an ancestral drought history when compared to the control seeds (without a drought history). Therefore, RNA from the seeds was used to generate 16 MACE libraries (2 cultivars (ID5 and ID14) * 4 seed sets (C1C2, C1D2, D1C2, and D1D2) * 2 repetitions). There were in total 61,436,418 reads (MACE tags) in the 16 samples following the removal of duplicates, trimming of polyA-sequences, and elimination of low-quality reads and reads that could not be mapped at all. Of these reads, cultivar ID14 and ID5 recorded 5,923,217 and 8,127,265 from the control (C1C2), 8,011,345 and 10,715,859 from C1D2, 6,549,167 and 7,413,052 from D1C2, and 8,565,749 and 6,130,764 from D1D2 seeds, respectively. Across all the libraries, these 61,436,418 reads accounted for 91730 different genes. Counts per million (CPM) were calculated by normalizing the read counts by the total counts per sample. The data was normalized by the cpm function in edgeR. A gene had to have more than 0.5 counts per million (CPM) in at least one sample. After filtering, 61,852 genes passed the filter and were converted to ENSEMBEL gene IDs. Each sample had an average total read count of around 3 million (**Figure 5.13**).

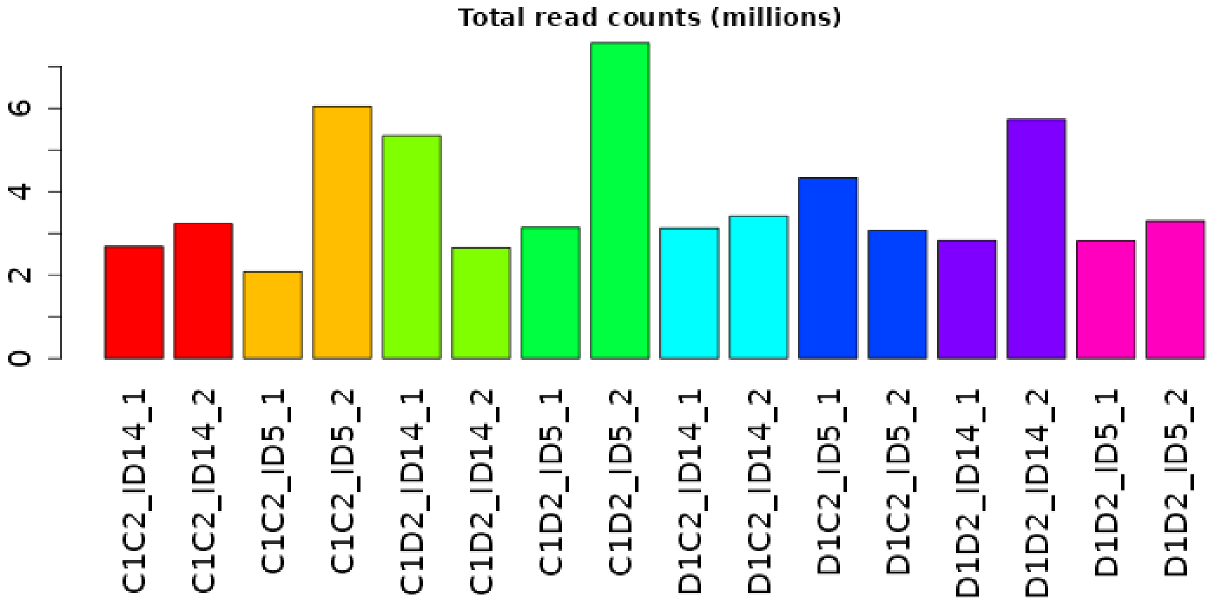

Figure 5.13: Total read counts (millions) per library (seed RNA)

Hierarchical clustering with a heatmap was done using the transformed data. All genes were ranked by standard deviation across all samples, and the top 2000 genes were used in hierarchical clustering using the heatmap.2 function with a cut-off Z score of 3. The data was cantered by subtracting the average expression level for each gene. The distance matrix is 1- *r*, where *r* is Pearson's correlation coefficient. The average linkage was used. The correlation matrix was computed using the *cor* function in R and using the top 75% of genes regarding expression level. The graph was generated using ggplot2 in iDEP. The replicates were grouped together, indicating reliability of the data (**Figure 5.14**).

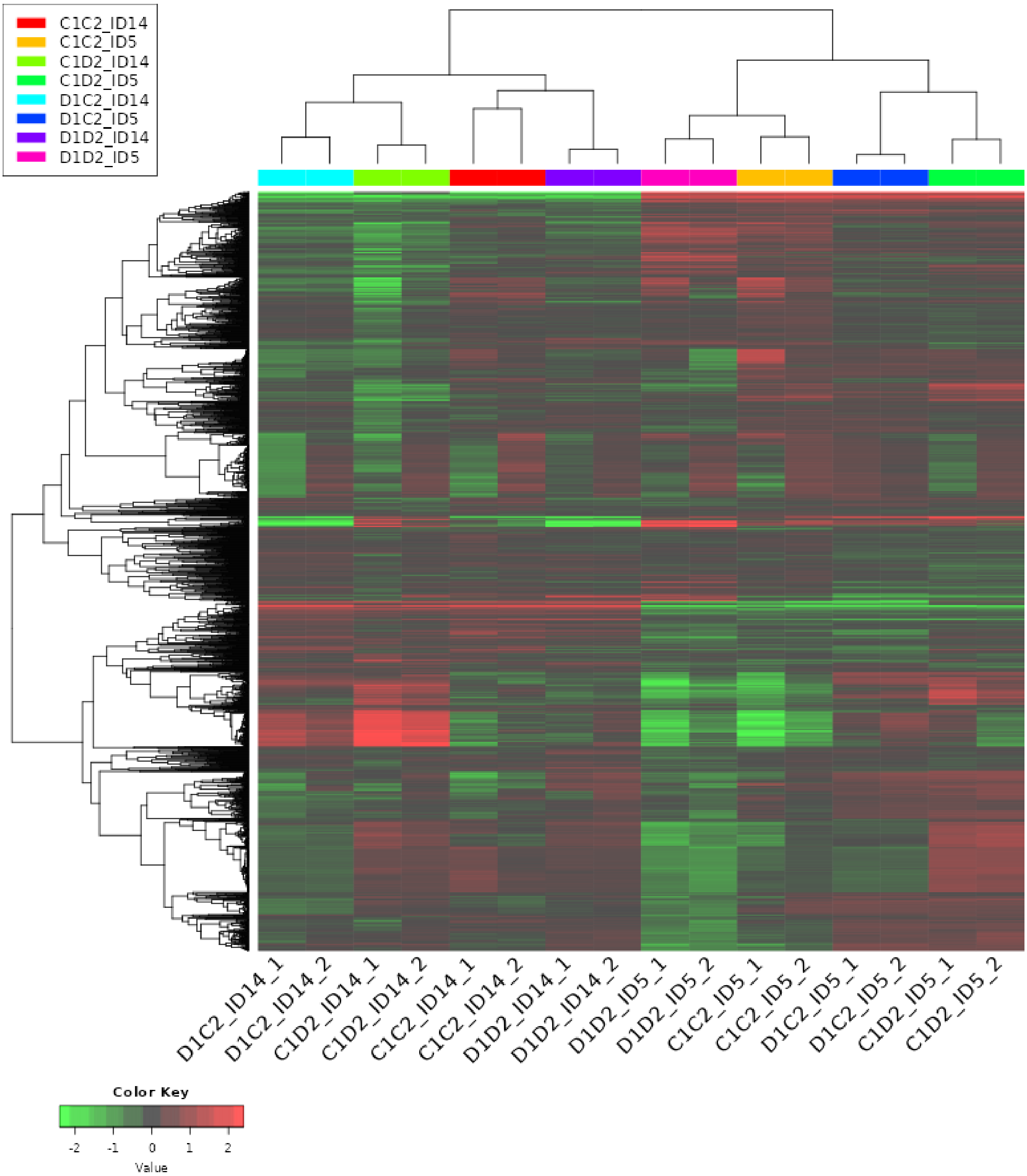

Figure 5.14: Hierarchical clustering using gene expression values for each replicate. The replicates for each sample are grouped together.

Using PCA, we produced a two-dimensional plot of the expression profile. The first principal component captured 26% of the variance, while the second component captured 12% of the variance. The first principal component was correlated with the cultivar, with ID14 on the left and ID5 on the right despite the treatments (**Figure 5.15**). The second principal component differentiates the stress history.

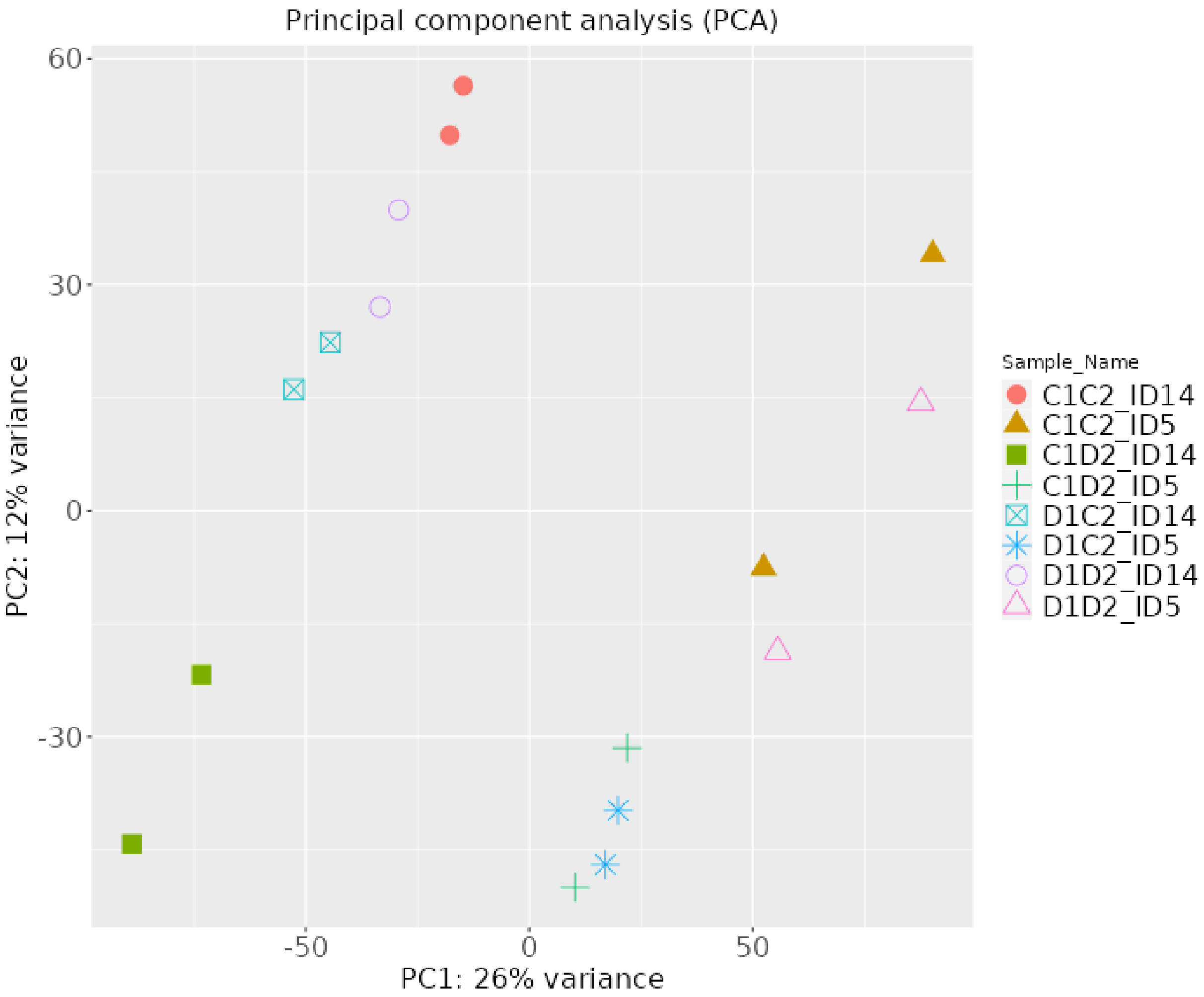

Figure 5.15: Principal component analysis groups the two cultivars separately along PC1, and stress history along PC2. Cultivar ID14 is grouped on the left, while ID5 is on the right, regardless of treatment.

Dehydration stress memory response genes of winter wheat

The iDEP tool ran differential gene expression analysis on all pairs of sample groups using the DESeq2 method. The genes were selected according to FDR< 0.05 and foldchange > 4. We chose the comparisons of interest from the output, and the summarized numbers of differentially expressed genes (DEGs) are shown as a bar graph (**Figure 5.16**).

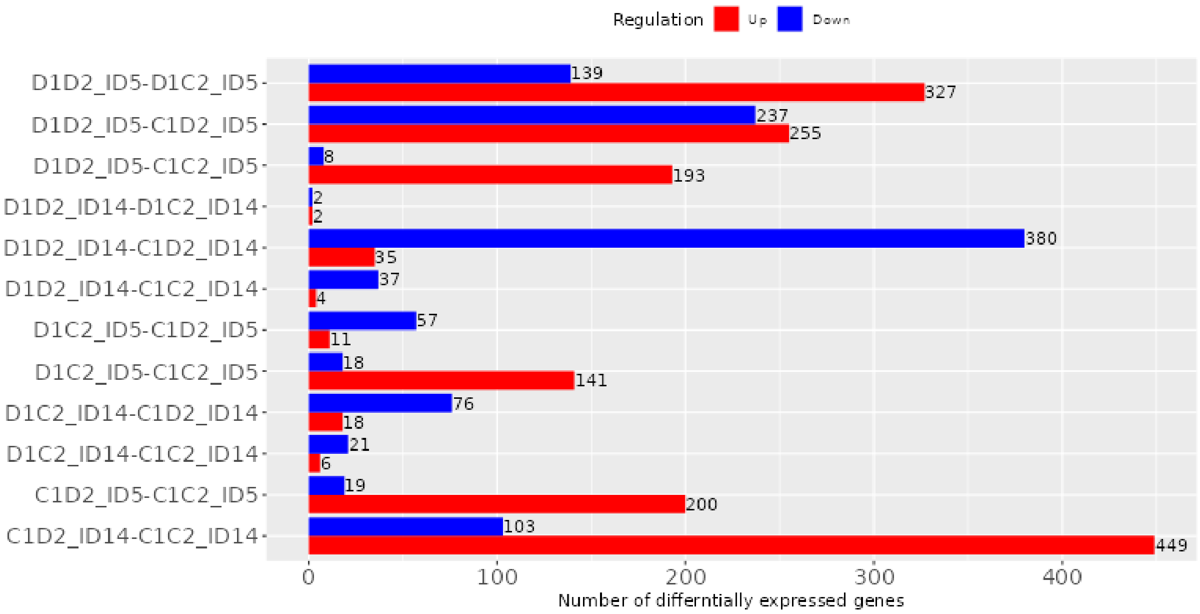

Figure 5.16: Number of differentially expressed genes for each of the considered comparisons. Red indicates upregulated genes, and blue indicates downregulated genes.

Scatter plots were used to examine each of the comparisons. The points above the diagonal represent genes with higher expression values in the sample plotted on the y-axis, while those below the diagonal represent genes with higher expression in the sample plotted on the x-axis (**Figure 5.17**).

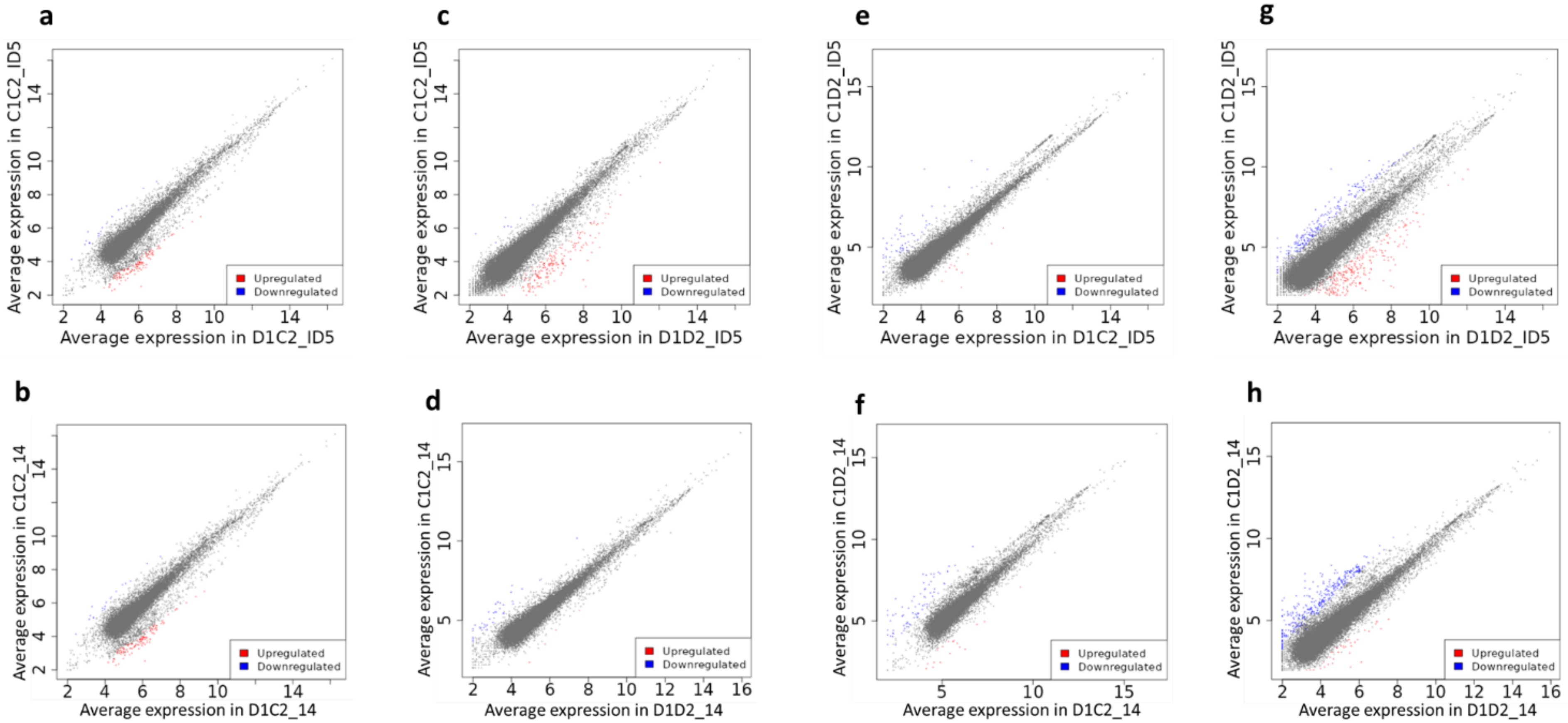

Figure 5.17: Scatter plots indicating upregulated (red) and downregulated (blue) genes for each comparison. **a**; ID5 up and down regulated genes between C1C2 and D1C2, **b**; ID14 up and down regulated genes between D1C2 and D1D2, **c**; ID5 up and down regulated genes between C1C2 and D1D2, **d**; ID14 up and down regulated genes between C1C2 and D1D2, **e**; ID5 up and down regulated genes between C1D2 and D1D2, **f**; ID14 up and down regulated genes between C1D2 and D1C2, **g**; ID5 up and down regulated genes between C1D2 and D1D2 and **h**; ID14 up and down regulated genes between C1D2 and D1D2.

An analysis of the expressed fold changes for DEGS between C1D2 and C1C2, between D1C2 and C1D2, and between D1D2 and C1D2 was done to reveal DMGs categories, non-memory genes, and late response genes. In total, during the first stress period (C1D2), there were 219 and 552 drought responsive genes in ID5 and ID14, respectively (C1D2 versus C1C2). Since our definition of memory genes was those genes with transcript levels in subsequent stresses that are significantly different from their levels during the first stress period, we used this initial set to look at significant responses in D1C2 versus C1D2 and D1D2 versus C1D2. Memory types of winter wheat drought stress response genes were revealed by constructing Venn diagrams using Venn 2.1 that showed overlaps of DEGs of different comparisons.

In ID14, there were 449 and 6 genes up-regulated in C1D2 and D1C2, respectively, of which 4 genes were common genes (genes up-regulated in both grandparental and parental generation, of drought stress exposure) (**Figure 5.18a**). None of the gene, fell into the (+ +) category. There were 103 and 21 genes that were downregulated in the same cultivar in C1D2 and D1C2, respectively, and 4 common genes that were downregulated in both generations (**Figure 5.18a**). Of these genes, none was classified in the (- -) category. However, 33 genes were classified under the (+ -) and 16 under the (- +) category of memory genes (**Figure 5.20 a and b**). In ID5, there were 200 and 141 genes up regulated in C1D2 and D1C2, respectively, of which 32 genes were common genes (**Figure 5.18c**). None of the genes fell into the (+ +) category. There were 19 and 18 genes that were downregulated in the same cultivar in C1D2 and D1C2, respectively, and 4 common genes that were downregulated in both generations (**Figure 5.18d**). Of these genes, none was classified in the (- -) category. However, 30 genes were classified under the (+ -) and 1 under the (- +) category of memory genes (**Figure 5.20c and d**). Two genes were common for ID 14 and ID5 in the (+ -) category. In addition, 2 genes that were differentially upregulated in ID14, were downregulated in ID5 in D1C2 seeds.

In ID14, there were 449 and 4 genes up regulated in C1D2 and D1D2, respectively, of which none were common in both seed sets and none fell in the (+ +) category when DEGs in C1D2 and D1D2 were compared (**Figure 5.19a**). There were 103 and 37 genes that were downregulated in the same cultivar in C1D2 and D1D2, respectively, and 4 common genes that were downregulated in both seed sets (**Figure 5.19b**). Of these genes, none was classified in the (- -) category. However, 247 genes were classified under the (+ -) and 18 under the (- +) category of memory genes (**Figure 5.21a and b**). In ID5, there were 200 and 183 genes up regulated in C1D2 and D1D2, respectively, of which 12 were common in both seed sets and none fell in the (+ +) category when DEGs in C1D2 and D1D2 were compared (**Figure 5.19c**).

There were 19 and 8 genes that were downregulated in the same cultivar in C1D2 and D1D2 respectively, and 3 common genes that were downregulated in both seed sets (**Figure 5.19d**). Of these genes, none was classified in the (- -) category. However, 116 genes were classified under the (+ -) and 7 under the (- +) category of memory genes (**Figure 5.21c and d**). There were 8 genes common to ID14 and ID5 in the (+ -) category. One small heat shock protein gene that was upregulated in ID14 was down regulated in ID5 in both D1C2 and D1D2 seeds. In ID14, there were 32 genes and 11 genes in the (+ -) and (- +) categories, respectively that were common in D1C2 and D1D2 seeds. In ID5, 15 genes in the (+ -) category were identified in both D1C2 and D1D2 seeds.

Finally, when considering grandparental effects as represented by D1C2 seeds, in ID14, 416 genes fell into the (+ =) category and 87 genes into the (- =) category of non-memory genes. 43 and 2 genes fell into the (= -) and (= +) categories of late response gene types, respectively. In ID5, 170 genes fell into the (+ =) category and 18 genes into the (- =) category of non-memory genes. 27 and 10 genes fell into the (= -) and (= +) categories, respectively. When considering combined memory effects as represented by D1D2 seeds, in ID14, 202 genes fell into the (+ =) category and 85 genes into the (- =) category of non-memory genes. 133 and 17 genes fell into the (= -) and (= +) categories of late response gene types, respectively. In ID5, 84 genes fell into the (+ =) category and 12 genes into the (- =) category of non-memory genes. 121 and 248 genes fell into the (= -) and (= +) categories, respectively (**Figures 5.20 and 5.21**).

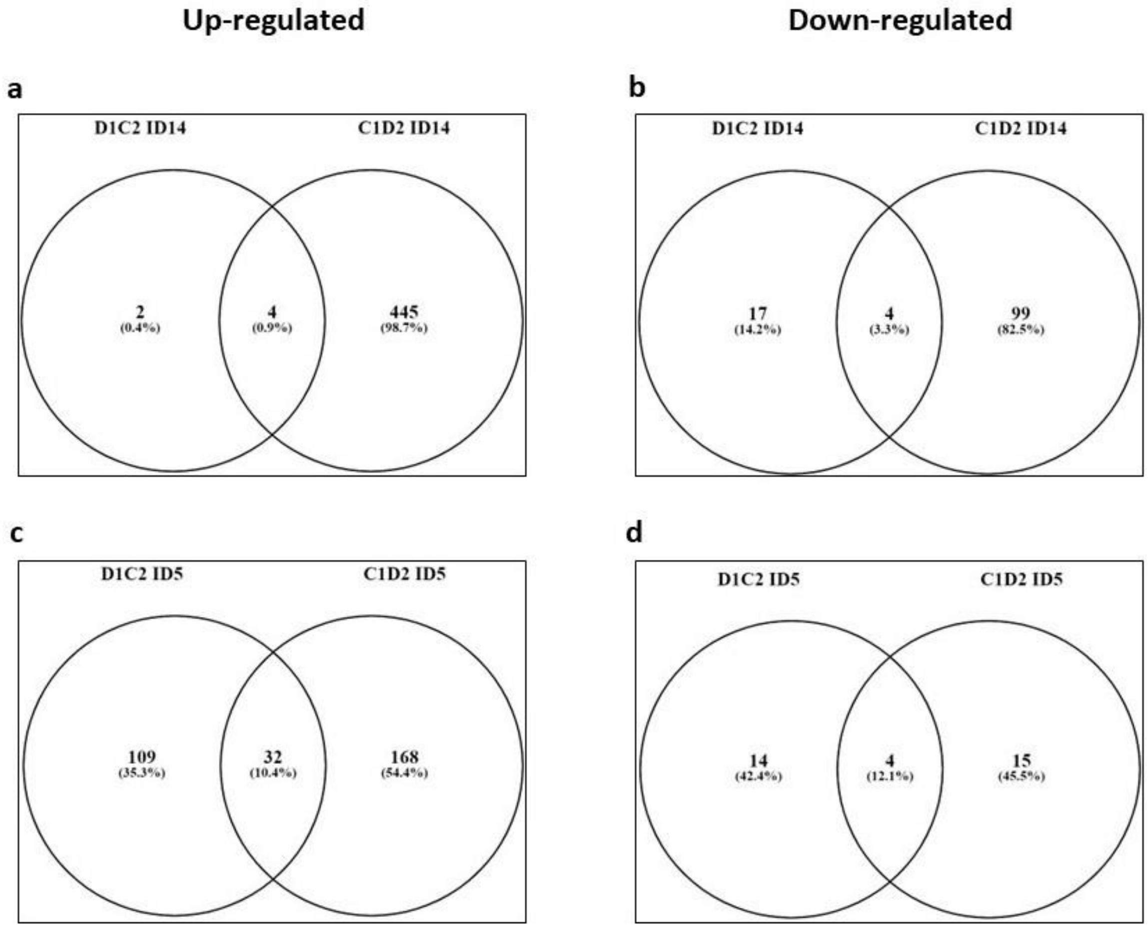

Figure 5.18: Venn diagrams indicating the common genes upregulated (a and c) or downregulated (b and d) in ID14 and ID5 respectively by both D1C2 and C1D2 seeds.

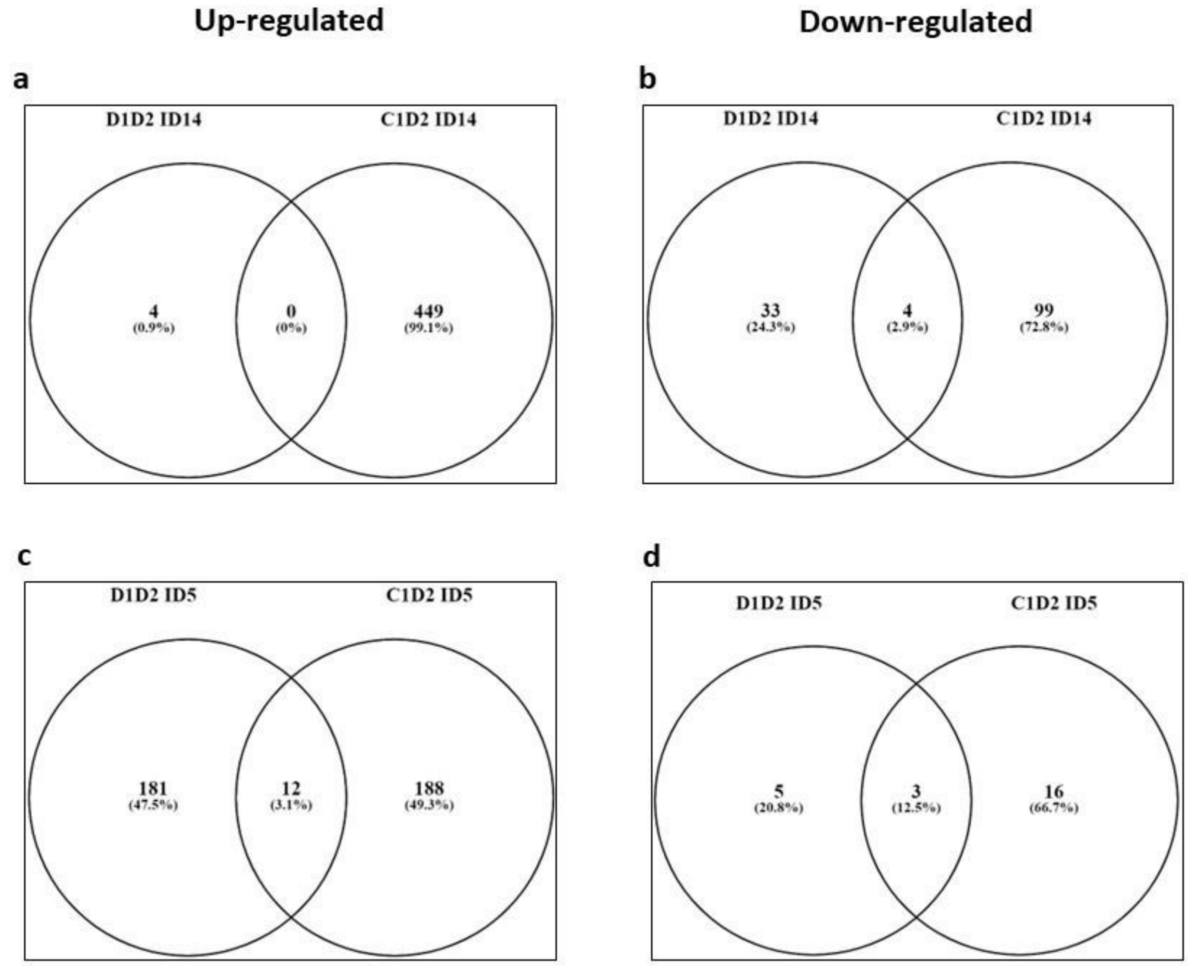

Figure 5.19: Venn diagrams indicating the common genes upregulated (a and c) or downregulated (b and d) in ID14 and ID5 respectively by both D1D2 and C1D2.

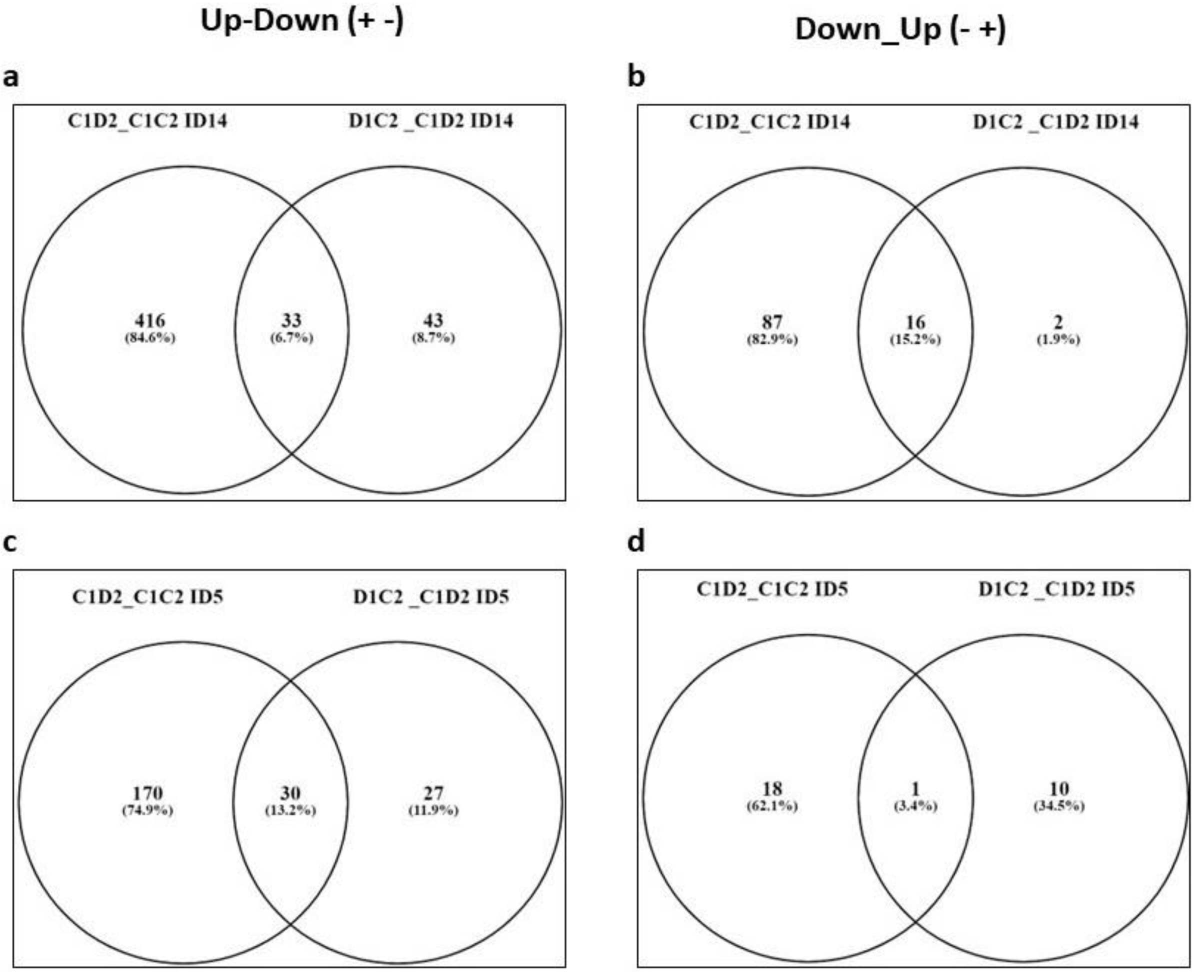

Figure 5.20: Venn diagrams indicating the (+ -)) and (- +) drought memory genes (DMGs) in ID14 (a and b respectively) and ID5 (c and d respectively) by comparing differential expression between C1D2 and D1C2.

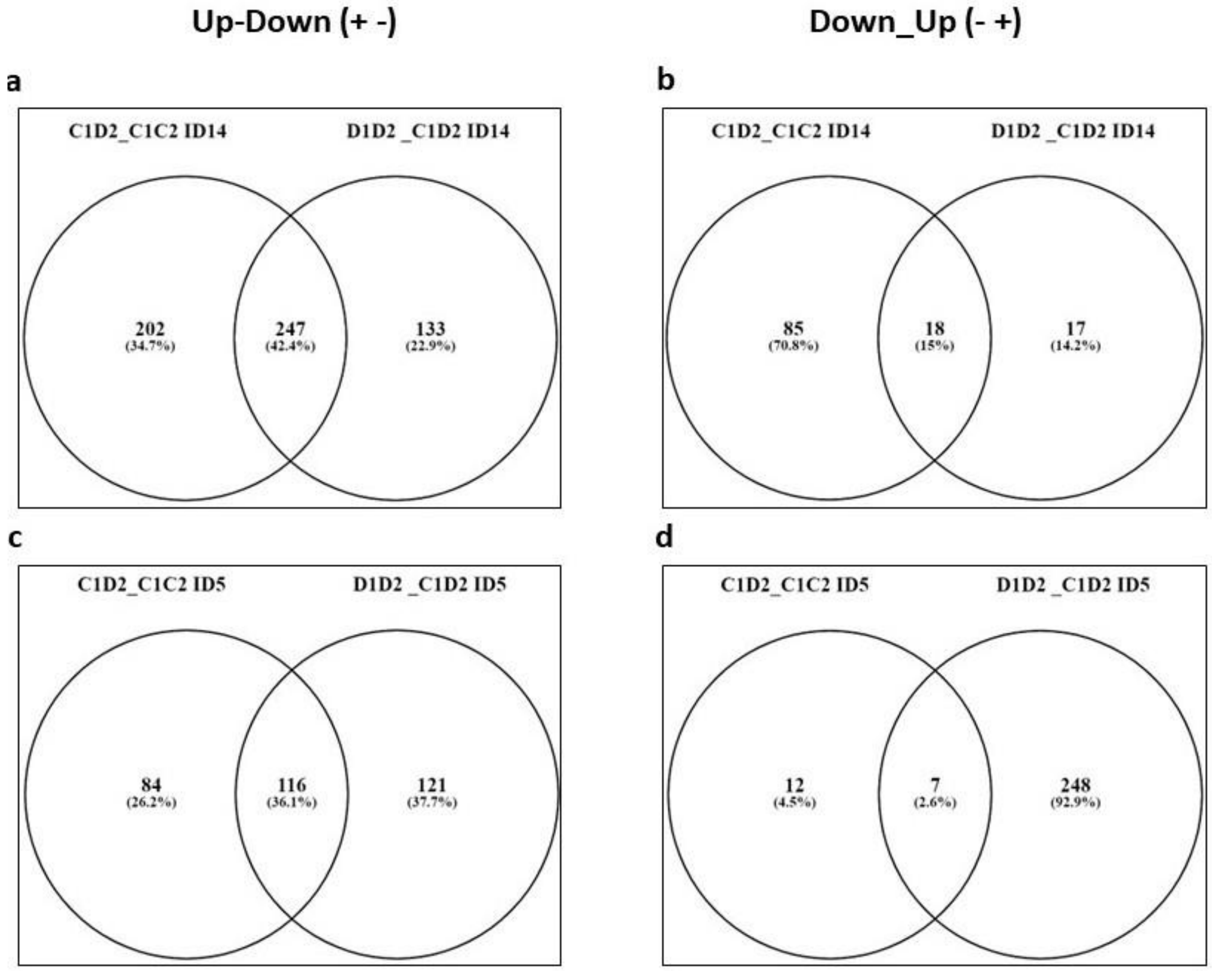

Figure 5.21: Venn diagrams indicating the (+ -) and (- +) drought memory genes (DMGs) in ID14 (a and b respectively) and ID5 (c and d respectively) by comparing differential expression between C1D2 and D1D2.

Functional analysis of the identified DMGs of winter wheat

To evaluate the functions of DMGs and determine whether there is a preferential association of cellular and biological function with any of the two identified memory types, ((+ -) and (- +)) genes, these gene lists were used to conduct enrichment analysis using gene ontology (GO) classification (**Figures 5.22 and 5.23**). Among the memory genes in the (+ -) category in ID14, we found significant enrichment for biological pathways involved in, among others, histone H3-K14 and H3-K9 acetylation, regulation of the wax biosynthetic process (TRAESCSU02G003200), regulation of cellular protein metabolic process, regulation of molecular function, negative regulation of catalytic activity, negative regulation of hydrolase activity , negative regulation of peptidase activity, negative regulation of proteolysis (TRAESCS3B02G038700, TRAESCS4B02G328000, TRAESCS5B02G419900 TRAESCS5D02G004000), biological process involved in interspecies interaction between organisms , response to external stimulus (TRAESCS1A02G398200, TRAESCS1D02G405700, TRAESCS2A02G528200) (**Figures 5.22a**). Among the memory genes in the (- +) category in ID14, significant enrichment for biological pathways were found to be involved in response to reactive oxygen species, response to osmotic stress, protein folding, and response to abiotic stimulus (TRAESCS3B02G049900, TRAESCS3D02G046600) (**Figure 5.22b**). Among the memory genes in (+ -) in ID5, significant enrichment for biological pathways were found involved in nitrogen utilization, regulation of fatty acid biosynthetic process, and anthocyanin-containing compound biosynthetic process (TRAESCS1A02G076100) (**Figure 5.23a**). Significant enrichment for cellular and molecular functions among the (- +) memory genes category in ID5 were found involved in licheninase activity and anchored component of the plasma membrane (TRAESCS1A02G234600) (**Figure 5.23b**).

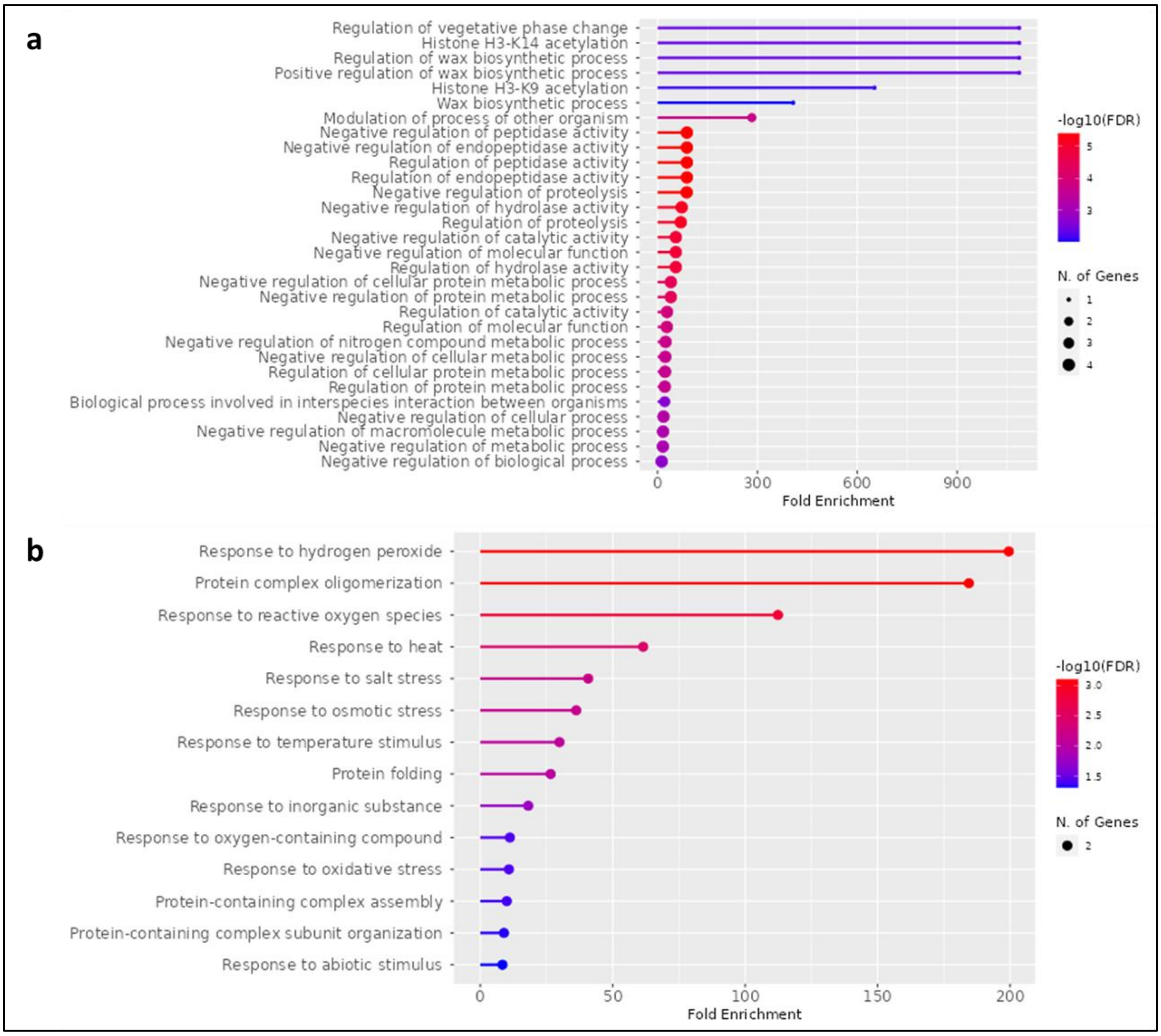

Figure 5.22: Gene Ontology (GO) terms enriched in (+ -) DMGs (a) and in (- +) DMGs (b) in ID14.

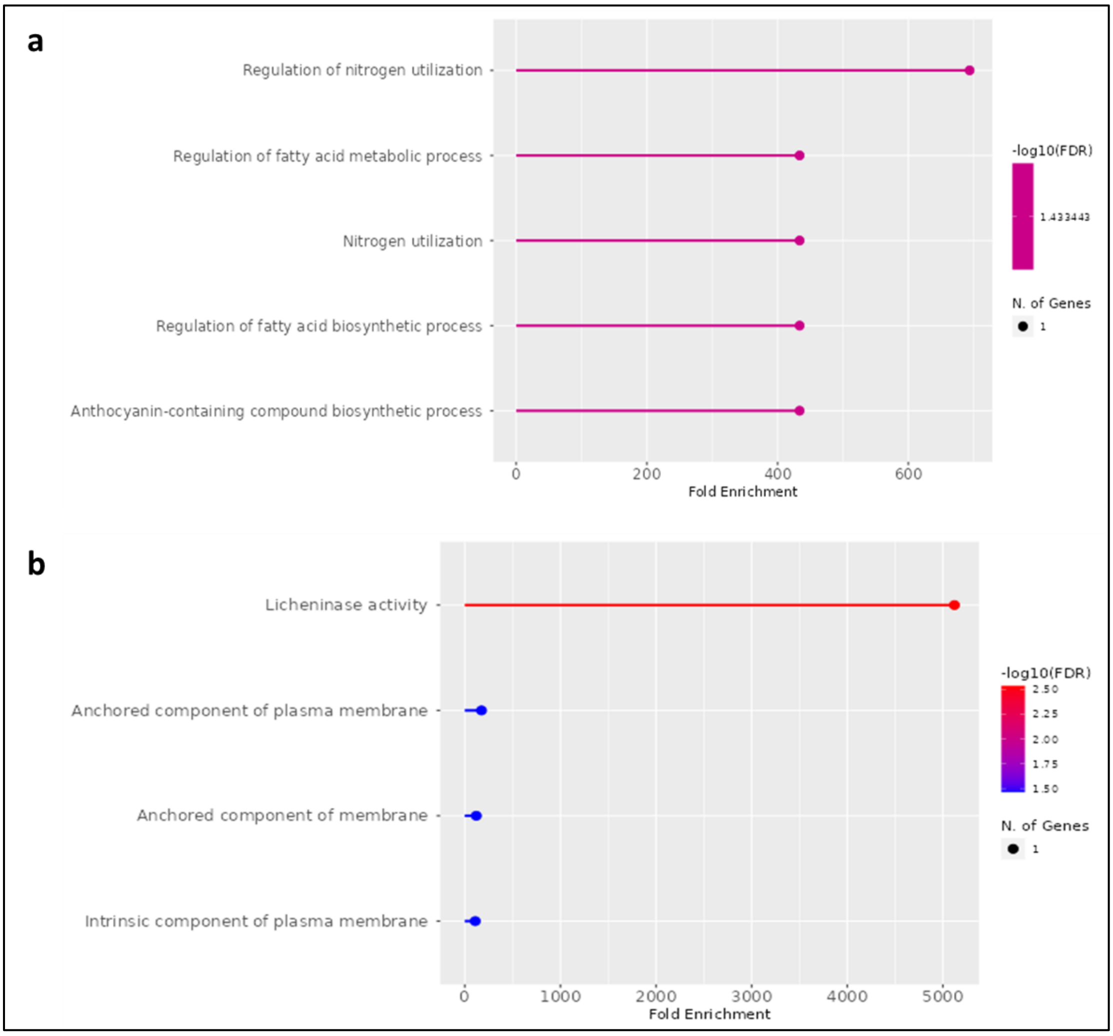

Figure 5.23: Gene Ontology (GO) terms enriched in (+ -) DMGs (a) and in (- +) DMGs (b) in ID5.

Grandparental effects memory genes (D1C2) include response genes in the (= -) category, of which in ID14, 8 genes were implicated in the negative regulation of translation, 12 genes in the negative regulation of cellular protein metabolic processes, and 10 genes in the negative regulation of biological processes. Of the two genes in the (= +) category, one is a delta 12 fatty acid desaturase (FAD2), which has been shown to play a significant role in stress responses in Arabidopsis during plant growth and seed development (Yuan et al., 2012). The (= -) gene category in ID5 are associated in mitotic cell cycle and cell cycle G1/S phase transition. One gene (TRAESCS6A02G153100) in the (= +) category functions in ABA-mediated stomatal closure. Combined effects memory genes (D1D2) in the (= -) category in ID14 are associated with negative regulation of translation, negative regulation of proteolysis and negative regulation of metabolic processes. Three genes in the (= +) are associated with responses to nitrate (TRAESCS6B02G364600), water transport (TRAESCS3D02G540900), and calcium ion homeostasis (TRAESCS3B02G165400). In ID5, the (= -) were associated with response to reactive oxygen species (ROS), and protein folding pathways, while the (= +) were implicated in the negative regulation of proteolysis, negative regulation of molecular and cellular processes.

Discussion

Changes in physiological and morphological parameters in response to repeated drought stress

Exposure of plants to environmental stresses like drought can alter the plants' own response to future stress. Seedlings with a history of drought stress, regardless of which generation they came from, had altered responses compared to those from seeds without a previous history of stress. This is an indication that the memory pattern has been formed, leading to better drought adaptation capability.

There were notable increases in ABA levels in C1C2 seedlings under drought that could have caused stomatal closure, leading to low stomatal conductance, which translates to low carbon dioxide and hence a reduced assimilation rate. Consequently, seedlings of C1C2 had the lowest photochemical quantum yield of photosystem II, hence a reduced photosynthesis when compared with seedlings with a history of drought. According to Onyemaobi et al. (2021), the ability of the drought-tolerant line to retain stomatal conductance correlates with suppression of ABA synthesis, an observation that is evident in seedling with drought history. The cultivars vary greatly in some traits like seedling length, stomatal conductance, ABA, and root dimeter, and the same traits also show the existence of interaction between SH and genotype, indicating that the pattern of memory is different across cultivars. This observation was also revealed by Racette et al. (2019), who noted genetic variability in transgenerational stress memory in the seedling vigour of peanuts. In addition, while drought significantly reduces root length and volume (Figueroa-Bustos et al., 2020), this study reveals a longer and bigger root system if the seedling has a history of drought, which could be linked to the slower decline in stomatal conductance leading to an increased carbon assimilation rate and therefore growth (Chaves, 1991; Zlatev & Lidon, 2012). Therefore, increased carbon assimilation could be a drought stress memory adaptation mechanism.

When considering both the drought and control environments of the offspring, it is revealed that the conditions of the external offspring environment can influence the expression of the previous drought stress exposures. In agreement, Mousseau & Fox (1998) also confirm that the intensity of memory-related expression is a function of the environment quality experienced by the offspring. In addition, the relationship between offspring environment quality and the intensity of memory expression could vary depending on cultivar.

Drought-induced transcriptional memory behavior and its biological relevance

Genes demonstrating transcriptional memory are among the ones responding to the initial drought stress encounter. By changing their expression levels in subsequent stresses, they allow the plant to fine-tune its response to the currently on-going or future stress. Osmotic adjustment is crucial for water uptake and maintenance, membrane protection, and ROS scavenging in plants subjected to water stress (Ashraf et al., 2011). The probable biological relevance of memory genes is reflected in the context of overlapping strategies that are generally used by plants during drought and that include osmotic adjustment, detoxifying functions, growth, and readjustment of cellular homeostasis.

A series of transcriptional activations or repressions takes place for plants to respond and adapt to drought stress. The complex chromatin structure changes caused by epigenetic modifications control these transcriptional activations or repressions (Li et al., 2021). Often, drought stress causes histone acetylation changes in “drought-responsive” genes and other genes to cause genome-wide histone acetylation modifications in plants (Ueda & Seki, 2020). The amount of histone acetylation, which is controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), establishes whether the chromatin is open or closed, thus controlling the entry of DNA-binding proteins for transcriptional activation (Li et al., 2021). One gene (TRAESCSU02G003200) belonging to the histone acetyltransferase family was found in the (+ -) category of DMGs and involved in chromatin remodelling (histone H3-K14 and H3-K9 acetylation) and transcription by RNA polymerase II. Kim et al. (2012) and Lämke (2016) have implicated H3k9ac as an epigenetic memory mark in *Arabidopsis thaliana* during recurrent drought. This mark was reported to have been enriched on many drought-responsive genes, which also had the presence of RNA polymerase II, but during recovery this mark was rapidly removed, and activity of RNA polymerase II fell. On the contrary, we found downregulation of genes controlling acetylation in the D1D2 seeds that were not undergoing recovery from drought stress. The involvement of H3K14ac has been studied by Kim et al. (2008), who reported that H3K14ac modification was not affected in response to drought stress and that its enrichment does not occur on the coding regions of the drought-inducible genes but could function as a structural landmark for the other histone H3 modifications on the coding region of some genes. On the other hand, Lämke & Bäurle (2017) argue that accumulation of H3K14ac is critical for hyperinduction of stress induced genes. In our current study, under recurrent drought conditions, the downregulation of histone modification of H3-K9/K14ac could have occurred on drought stress downregulated genes. Therefore, it would be important to study the drought responsive genes marked with these epigenetic marks (H3K9/K14ac) during recurrent drought.

Winter wheat seeds with a history of drought stress exhibited differential expression of genes related to osmotic and oxidative stress responses. Two genes (TRAESCS3B02G049900 and TRAESCS3D02G046600) belonging to the Heat-Shock Protein 20 Family Member (PTHR11527) were downregulated in C1D2 but upregulated in D1C2 and D1D2 seeds ((- +) memory genes category). Cellular proteins in plants under stressful environments are usually irreversibly damaged (Apel & Hirt, 2004). Hence, maintenance of normal protein conformation and cellular homeostasis is very critical for the survival of plants under stress. Heat shock protein 20 (Hsp20) is a major family of heat shock proteins that mainly function as molecular chaperones. Their concentration increases markedly in cells when organisms are subjected to environmental stress and they have a significant role in the processing of proteins, especially in preventing the irreversible unfolding or wrong protein aggregation by binding to partially folded or denatured substrate proteins (Sun et al. 2002). They are also involved in membrane quality control and, in turn, maintain membrane integrity under stress conditions (Xiang et al., 2018). Consistent to our findings, after examining the proteome profiling of recurrent drought events in maize, Schulze et al. (2021) found an overrepresentation of heat shock proteins in maize with a history of drought. Similarly, Auler et al. (2021) analysed guard cells by comparative proteomics and identified increased levels of small heat shock proteins in the rice roots of plants that had experienced recurrent drought exposure. Therefore, the memory component of these genes allows the plant to adjust its stress tolerance in the context of protection.

In tomato, Endo-1,4-beta-glucanase was implicated as one of the organism’s growth and development-related drought-responsive genes only in drought-tolerant genotypes (Gong et al., 2010). Similarly, in potatoes and common beans, the drought-tolerant varieties upregulated beta-1,3-glucanase (Gupta et al., 2019; Ponce et al., 2022). Consistent with this finding, we found overexpression of (1,3; 1,4) beta glucanase (TRAESCS1A02G234600) in ID5 (a tolerant cultivar), belonging to the Glucan Endo-1,3-Beta-Glucanase family. Lee et al., (2008) correlated the increases of beta-1,3- glucanases in clover leaves during drought stress with increased proline levels. Although implicated in drought tolerance studies, this gene has not previously been reported to be associated with drought memory responses. The discovery of this gene implies that regulation of plant growth and development are crucial in memory transfer to acclimatize to water deficits in wheat.

The overexpression THI1 (thiamine thiazole synthase 1) represses the kinase activity of CPK33 (Ca2+-dependent protein kinase 33), which reduces anion channel activity and increases the impact of ABA on stomatal closure in the guard cells (Li, 2016). We revealed three genes belonging to the Thinin-2.1 family (TRAESCS1A02G398200, TRAESCS1D02G405700, and TRAESCS2A02G5282009) that belonged to the (+ -) category of memory genes in D1C2 seeds. Presumably, these genes help in the restoration of homeostasis as the plant responds to drought stress. Under drought stress, alpha-amylase inhibitor CM 16 subunit and BBI protease inhibitors have been implicated (Zhou et al., 2016; Malefo et al., 2020; Dhanushkodi et al., 2018). We report 4 genes (TRAESCS3B02G038700, TRAESCS4B02G328000, TRAESCS5B02G419900, and TRAESCS5D02G004000) belonging to these families that were categorised in (+ -) DMGs type found in both D1C2 and D1D2 seeds of ID14. These genes are responsible for negative regulation of cellular protein metabolic process and negative regulation of molecular function. One gene belonging to the NITROGEN REGULATORY PROTEIN P-II HOMOLOG family (TRAESCS1A02G076100) was identified in ID5, which is responsible for nitrogen compound metabolic processes, and belonged to the (+ -) memory gene category in both D1C2 and D1D2. According to Huergo et al. (2013), P (II) proteins are pivotal players in nitrogen metabolism and control the activities of many enzymes and transcription factors, however, their role during drought conditions is not well documented. In general, the complementary transcriptional and functional patterns exhibited by (- +) and (+ -) memory genes support the fine coordination arising between metabolic and energy modifications in adaptation to drought.

We uncovered that processes of known importance in plant drought response demonstrated conflicting memory patterns depending on the cultivar. While in one cultivar the drought induced transcriptional changes were correlated with responses to oxidative stress due to repeated stress, the alterations were in the opposite direction for the other cultivar. Memory genes and late response genes were found to have a strong and consistent correlation with specific biological processes, implying that some physiological and biochemical responses involved in drought memory are associated with expression changes in discrete networks of interdependently operational genes. More research is needed to validate the found effects in further experiments and use more cultivars to check for genetic variance in these responses.

Conclusion

Stored seed transcriptomes reveal the existence of two transcriptional memory patterns of response (+ -) and (- +), and two late response patterns (= +) and (= -). By altering these transcript levels and, most likely, the levels of proteins encoded, the cellular responses and crosstalk between overlapping physiological and biochemical pathways are altered. Drought memory gene behaviour adds a new component to the understanding of plants' response to stress and to current models of different signalling systems interactions. The current study suggests that different molecular mechanisms could be involved in both short and long-term memory, as is evident in the growth and development of the offspring under repeated drought stress. Both phenotypic and transcriptomic changes associated with stress memory are not generalized within wheat. Considering that epigenetic marks are also important regulators of gene activity, epigenetics plays a major role in plant adaptation to new environmental conditions and could potentially be employed as a tool to improve crop production and food security. Notably, we found evidence that the H3K9/K14ac epigenetic mark participate in winter wheat drought memory.

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General Discussion

Drought is a prominent abiotic factor causing huge yield losses in wheat because of its effects on plant water and nutrient uptake (Mohammadi, 2018). Therefore, plants have developed several mechanisms, as detailed in Chapter 1, to reduce resource utilization and adjust growth to cope with drought. Various adaptive mechanisms that make plants more tolerant to the hostile effects of drought stress have been developed through evolution and differ from the molecular level up to the plant level. Stress avoidance (e.g., stomatal closure, leaf rolling, root length), escape (e.g., plasticity development, early maturity, remobilization of photosynthates), and tolerance (e.g., osmotic adjustment, desiccation tolerant enzymes, solute accumulation) are the three key persistence strategies that plants employ when exposed to drought stress (Seleiman et al., 2021). Moreover, when plants encounter stress for the first time, they "store" environmental information and activate a stress state, namely the "priming effect" (Mozgova et al., 2019). As explained in Chapter 2, these primed plants develop somatic, intergenerational, and/or transgenerational stress memories of the experience to deal with future stress (Sadhukhan et al., 2022). As a response to future environmental conditions, primed plants display phenotypic plasticity, which enables them to mount fast and protective responses under successive stress. However, it is not clear if such memory effects are common in winter wheat to last over a whole vegetation period, even following harvest, and be transmitted to the next generation(s). Moreover, the underlying mechanisms for such memory transmission have not been fully elucidated. The main aim of this study was to evaluate the behaviour of winter wheat with varying previous drought experiences to future drought stress by examining the growth and behaviour in successive generation(s), including transcriptomic, physiological, biochemical, and morphological responses. Drought stress memory effects are complex and depend not only on plant physiology and other molecular mechanisms but also on the growth stage and generation(s) at which the plant experiences stress. To the best of our knowledge, this is the first stress memory study that has factorially varied both grandparental and parental drought exposures in winter wheat and tested the offspring in a control and drought environment using different cultivars to report on the importance of the number of exposures and generation during which stress was experienced in determining the mechanistic, importance, and strength of memory effects.

6.1 Drought primed wheat at reproductive stage influences the growth and reproduction performance under future stress encounter(s)

In chapter 3, in experiments performed under drought stress conditions, we found that regardless of the generation at which the plants were previously exposed to drought stress, the primed status influenced the growth parameters (plant height and above ground biomass) and increased the production of grains per plant, when compared to the plants that had no previous history of drought encounter. Moreover, the seeds displayed varied phenotypes based on the presence or absence of drought stress exposure in the previous generations. Our results were in line with earlier studies that reported higher seedling vigour in *Brassica napus* L. (Hatzig et al., 2018), *Polygonum persicaria* L. (Herman & Sultan, 2011), and *Arrhenatherum elatius* L. (Walter et al., 2011) compared to non-stressed controls. A link between changes in seed traits, including nitrogen content and plant vigour traits, was found. Similarly, Nosalewicz et al. (2016) quoted seed derived nutrients as the major determinants of the observed transgenerational drought effects in *Hordeum vulgare* L.

6.2 Changes in Physiological and biochemical traits could explain memory formation in wheat under recurrent drought stress

Cell-level responses, including changed physiological and biochemical traits, are considered necessary for the formation of stressed memories (Tian et al., 2022). In Chapter 4, we tested if seedlings and plants with a history of stress portray a different physiological and biochemical response when compared to those without a history of drought when stress comes again. For that, we revealed the physiological and biochemical imprints of stress memory formation in wheat including quantum yield of photosystem II, chlorophyll, carotenoids, anthocyanins, and proline, which could be involved in mediating photosynthesis, cell membrane stability, plant water status, oxidative stress reduction, and osmotic adjustment. These results also showed that the pathways for memory generation are different based on the memory type, cultivar, and plant developmental stage. Our findings agree with those of Tabassum et al. (2018), who reported that crops raised from the seeds collected from terminally drought-stressed plants accumulated more osmolytes and resulted in lower lipid peroxidation than the offspring of well-watered crops. In addition, in other plant species (Auler et al. (2021a and b) and Li et al. (2019) in rice, Liu et al. (2022) in mulberry (Tian et al., 2022) in cotton), showed different physiological and biochemical responses manifestation in primed plants to influence drought memory. Although several studies have performed thorough research on the physiological and biochemical responses to a single stress cycle in wheat (Ahmad et al., 2018; Camaille et al., 2021; Itam et al., 2020; Mu et al., 2021; Thapa et al., 2018), our research provides results to the yet unexplored area of how these responses adjust to confer stress memory to wheat.

6.3 Physiological, biochemical and morphological variation of memory effects on offspring germination and growth in response to drought stress and the seed transcriptome of winter wheat

Alterations in gene expression patterns during recurrent drought could happen, in addition to the memory responses shown by plants at organismal levels (Ding et al., 2012). This change demonstrates the idea of "transcriptional drought stress memory" and suggests the presence of drought stress "memory" genes. Based on previous studies, transcriptional behaviour during repeated stress encounters differs from the behaviour in a primary drought stress. This implies the complexity of stress memory, as it results from the coordination of responses from several signalling pathways (Ding et al., 2012, 2014). Drought-responsive genes are those that respond to an initial stress. When these genes portray a significantly different response in a subsequent stressful situation, they are classified as "memory genes". "Non-memory" genes respond equally to each stress cycle.

Information on whether such memory responses exist in wheat seeds and whether memory is an evolutionarily conserved response to recurrent drought stresses has been missing. In Chapter 5, we determined the transcriptional responses of wheat seeds that have been harvested from plants that experienced repeated exposures to drought stress in comparison with seeds from plants that had not encountered the stress. We further checked the physiological, biochemical, and morphological responses of the resulting seedling and whether these responses reveal memory during a repeated exposure to drought stress. We used the "+," "-," and "=" symbols to denote genes that showed upregulation, downregulation, or no significant changes in expression, respectively, and came up with eight categories of differentially expressed genes (DEGs) that included (+ +), (+ -), (- +), (- -), (+ =), (- =), (= +), and (= -). The first sign (=, -, or +) in each category indicated the gene expression values in the first drought exposure were not statistically different, were lower, or were higher than values in the non-stressed samples, respectively. The second sign (=, -, or +) in each category indicated the gene expression values in the subsequent drought stress exposure were similar, were lower, or were higher compared to those in the first drought exposure (Virlouvet et al., 2018).

We identified memory genes in the (+ -) and (- +) categories as well as late response genes in the (= -) and (= +) categories, which together point to the distinctness of the transcriptional responses during repeated exposures to stress from the usual responses occurring during a single exposure. These genes operate in coordinated strategies during recurrent droughts to ensure osmotic adjustment, detoxification, growth, and readjustment of cellular homeostasis. Similar to our findings, Li et al. (2019) reported that plant memory genes are involved in multiple signalling pathways, which indicates that memory genes are widely present and have an important role in plant growth and development. Taken together, these data suggest that physiological responses to repeated stress are transcriptionally mediated, in addition to the commonly held view that biochemical changes are involved.

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Supplementary Materials

Supplementary Figure

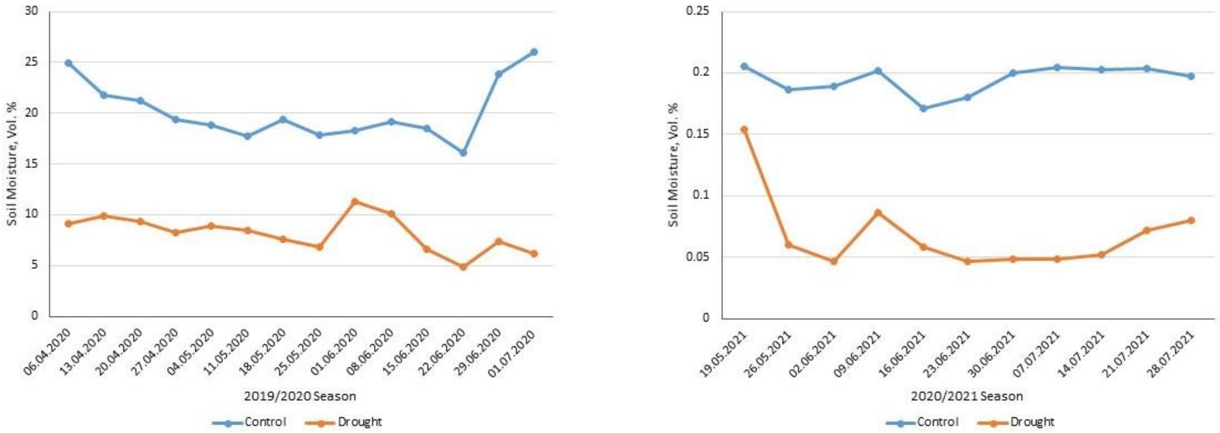

Figure S3.1: Soil moisture content (0 -30 cm depth) in control and drought stress of the experimental plots 2019/2020 and 2020/2021 growing seasons. Mean weekly values were recorded and analysed.

Supplementary Table

Table S 3.1: Percentage (%) change in offspring trait due to grandparental drought stress exposure, parental drought stress exposure and their combined exposure effects relative to control offspring. The percentage changes are recorded under control and drought offspring moisture treatment, except for seed quality traits. All significant percentage changes in traits are bolded (p<0.05).

Memory effects	NDF %	Nitrogen %	Sedimentation %
Grandparental	**787.0**	2.4	-4.1
Parental	-207.8	**68.9**	**42.0**
Combined	**432.5**	**77.9**	**61.8**

Treatment	Season	Memory effects	Plant height (cm)	Days to Flowering	Above Ground Biomass/Plant (g)	Number of Grain/Plant	Grain weight/plant (g)	Water Potential (-MPa)	Proline (µg/g)
Control	2019/2020	Grandparental	0.6	0.5	-4.1	-1.5	-2.9		
	2020/2021	Grandparental	-8.9	0.4	**-31.9**	**-39.0**	**-39.0**	**-20.5**	22.5
	2019/2020	Parental	0.9	0.0	2.5	1.8	2.6		
	2020/2021	Parental	-1.3	0.6	2.4	0.3	2.4	**-17.1**	-22.0
	2019/2020	Combined	-2.3	1.7	0.6	0.7	0.7		
	2020/2021	Combined	-3	0.3	-10.7	-9.8	-7.3	**-24.2**	-25.3
Drought	2019/2020	Grandparental	2.1	-1.3	2.4	11.2	12.4		
	2020/2021	Grandparental	**8.8**	0.3	17.6	**100.9**	**102.0**	**-48.0**	**-61.8**
	2019/2020	Parental	**5.9**	-1.7	**15.4**	9.6	17.0		
	2020/2021	Parental	4.6	**1.4**	-3.2	1.1	10.4	-20.6	-12.4
	2019/2020	Combined	**8.4**	-1.3	**12.7**	**43.0**	**54.0**		
	2020/2021	Combined	6.6	0.7	13.6	**64.2**	**67.9**	-5.9	**-44.6**

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