Physiological Responses of Wheat to Drought and Antitranspirants and Transcriptomic Changes in Anthers

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Declaration

I, Misbah Sehar, declare that I have written this thesis, and all the work contained in this has been completed by me, unless stated otherwise. All the other information from different sources has been fully acknowledged with references.

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ABSTRACT

Climate change is affecting wheat production, prompting scientists to find alternative ways to improve drought tolerance. Antitranspirants applied at the critical growth stages of wheat have shown promising results in conserving water and improving crop yield in various studies, possibly by enhancing pollen viability. However, the molecular mechanisms behind these improvements are not yet well understood. Therefore, in this thesis, two field experiments (in rain shelters), one glasshouse experiment and the transcriptomic study of early meiotic anthers (leptotene-zygotene), were conducted to understand the effects of the film (Vapor Gard (VG), di-1-p-menthene) and metabolic (Abscisic acid (ABA), 20% S-ABA) antitranspirants at different growth stages: VG at stem elongation (GS31), VG or ABA before the start of meiosis (GS39) and ABA at grain filling stage (GS71-73).

Field and the glasshouse experiments revealed no significant effects from antitranspirants, assessed via different physiological parameters (mainly relative water content, pollen viability, yield and most of the yield components) when droughted unsprayed and antitranspirants sprayed plants were compared. The main reason for this might be low soil moisture levels when antitranspirants were applied. However, significant variations at the gene expression level were observed in anthers, and most of the differentially expressed genes (DEGs) were downregulated under drought stress and applying antitranspirants further downregulated these genes (3,959 downregulated genes in both VG and ABA samples), with fewer upregulated genes (830 genes). The number of downregulated genes was higher in VG-treated plant anthers (3,325 genes) compared to ABA-treated anthers (634 genes). DEGs involved in biological processes, especially related to carbohydrate/sugar metabolism, were mainly affected. Overall, the results suggest that plants are more sensitive to antitranspirants at the molecular level as observed in anthers, than at the whole plant level, and less severe drought stress may be needed for the altered gene expression responses to significantly impact crop yield.

CHAPTER 1. General introduction

Wheat is the most widely grown staple food crop globally, and drought is one of the major abiotic factors for its reduced yield, thus threatening global food security and crop production. If the appropriate mitigation steps are not taken, either to improve the crop to cope with these severe climatic conditions or to mitigate these climatic changes, 60% of the area under wheat will face severe drought by the end of this century (Trnka et al., 2019). Therefore, soil, water and crop management (Dodd et al., 2011), along with selection and breeding for drought-tolerant varieties, are becoming the priorities of research in regions where drought is predicted to become severe (Khadka et al., 2020). Scientists are looking for alternative ways to reduce water loss from plants under drought stress conditions, such as the use of antitranspirants to improve crop yield under water-deficit environments (Kettlewell et al., 2010; Abdullah et al., 2015; Mphande et al., 2021b, 2024).

Antitranspirants mainly work by reducing the rate of transpiration from the plant surfaces, and they are categorised into film-forming, stomatal closing, reflecting and growth retarding compounds depending upon their mode of action (Pandey et al., 2017; Sow and Ranjan, 2021). Using antitranspirants to alleviate moisture stress has been investigated in many cereal and horticultural crops. They can not only improve crop yields but are also effective in improving several other physiological, quality and disease resistance characteristics, as summarised in different review papers (Koteswara et al., 2018; Mphande et al., 2020; Guleria and Shweta, 2020). Various studies on wheat with antitranspirant treatments at sensitive growth stages under water stress conditions have demonstrated improved yield and yield components (Kettlewell et al., 2010; Abdullah et al., 2015; Abdallah et al., 2019; Mphande et al., 2021a, 2021b, 2024) which is promising, considering the situation of global warming and food security risks, especially in drier climates.

The application of antitranspirants at specific growth stages sensitive to drought is also crucial, as they may not improve yield when applied at less sensitive stages (Kettlewell et al., 2010). Treatment of film antitranspirants (forms a waxy layer on the leaf surface) before the start of meiosis (Kettlewell et al., 2010; Weerasinghe et al., 2016; Mphande et al., 2021a, 2021b, 2024) has the potential to increase wheat crop yield and might be linked with improved pollen viability (Weerasinghe et al., 2016). It is not possible to assign a specific growth stage number at which the reproductive phase starts, as it depends on genotype and environmental conditions (Barber et al., 2015), but it is generally understood that meiosis in wheat coincides with the boot stage (Acevedo et al., n.d.) therefore the period of GS41-49 is

considered to be appropriate for meiosis duration to be vulnerable to heat and drought stress (Barber et al., 2015). Drought during meiosis is a significant cause of pollen sterility as it is related to changes in carbohydrate metabolism, carbohydrate availability and distribution, changes in hormonal pathways and altered gene expression, which leads to reduced crop yield (Yu et al., 2019). Abscisic acid (ABA) produced in plants under water stress has been suggested as a factor responsible for reduced pollen viability (Huang et al., 2011), while Mphande et al. (2021b) indicated that the yield improvement with film antitranspirant (di-1-p-menthene) under drought stress is associated with reduced endogenous ABA concentration which might be the main cause of improved pollen viability.

Exogenous application of ABA at the flowering stage of wheat has been demonstrated to increase crop yield by accelerating the grain filling rate, starch accumulation rate and 1000-grain weight of two wheat cultivars with different stay-green characteristics (Yang et al., 2014). Another wheat study (exogenous ABA sprayed at the anthesis stage) also observed an improvement in 1000-grain weight and yield under mild and moderate water stress conditions. However, under severe water stress, it decreased grain weight, yield and water use efficiency of plants (Luo et al., 2021). Moreover, two types of antitranspirants (di-1-p-menthene and exogenous ABA) were sprayed at the flag leaf stage of wheat in two glasshouse experiments to understand their effect on reproductive stage drought (under progressive and controlled drought conditions). In the case of controlled drought, ABA was applied at four additional stages up to anthesis (Mphande et al., 2021a). Improved yield was observed in both types of treated plants despite having contrasting effects on the endogenous concentration of leaf ABA.

These studies indicated that film antitranspirants improve grain yield when applied before meiosis, and the use of exogenous ABA also showed promising results in some studies under droughted conditions. So, to investigate it further, exogenous application of ABA at specific growth stages of wheat (before the start of meiosis and grain filling stage), in comparison with film antitranspirant (before the start of meiosis), were explored and whether these applications could mitigate water stress or improve pollen starch accumulation and yield. Also, changes in anthers (during early meiosis) at the transcriptomic (gene expression) level were observed and compared between different samples. This study could provide valuable insights in future research for understanding how drought affects the process of cell division in early meiotic anthers with disruption of processes related to carbohydrate/sugar metabolism that could impact pollen starch accumulation and how different antitranspirants can influence these distinct molecular mechanisms under drought stress.

The central hypothesis of this PhD project is:
The mechanism of increased grain number and yield of drought-stressed wheat from film antitranspirant and exogenous ABA application involves less water stress, leading to reduced inhibition of transcription of enzymes associated with improved pollen starch accumulation.

Main objectives of the central hypothesis:

- To evaluate the improvement in pollen starch accumulation, grain number and yield with the reduction in water loss with film antitranspirant and exogenous ABA application.
- To examine transcriptomic responses of anthers to less water stress due to exogenous spraying of antitranspirants.

The first main objective of the central hypothesis is covered in two chapters of this thesis, which are as follows:

Chapter 3, with the title “Understanding the effect of antitranspirants (film and metabolic) on physiological responses of wheat under drought stress”, is based on two field experiments (in rain shelters) (one in 2022 and the second in 2023) with the main hypothesis of:

Spraying antitranspirants (film or metabolic) at different growth stages before the start of meiosis or at the grain filling stage is responsible for reduced water loss with improvement of pollen viability (pollen starch accumulation), grain number and yield under drought stress.

There was no evidence of improved pollen viability, grain number or yield with the application of both types of antitranspirants at different growth stages.

Due to no effective response from antitranspirants from field experiments, one glasshouse experiment was conducted, with the same main hypothesis as field experiments, which is covered in **Chapter 4** with the title “Responses of wheat to drought stress and antitranspirants at critical growth stages in a glasshouse environment”

There was a slight increase in pollen viability with antitranspirants application at GS39, however, it was not significant, with no evidence of improved yield.

The second main objective of the central hypothesis, which was to understand the transcriptomic responses in wheat anthers with the spraying of antitranspirants, is covered in **Chapter 5** with the title “Transcriptomic responses of wheat anthers at the early meiosis stage (leptotene-zygotene) to drought stress and antitranspirants (film and metabolic)”

This is the first study in wheat following the use of antitranspirants, and there was evidence that most of the biological processes (mainly carbohydrate/sugar-related processes) were transcriptionally downregulated, with the spraying of antitranspirants in comparison to unsprayed plants under water stress.

The last **Chapter 6**, “General discussion and conclusion”, summarises all the findings and conclusions based on the previous three chapters, along with the limitations of the experiments.

Overall, the results suggest no significant improvement in pollen viability, grain number or yield from spraying either film or metabolic antitranspirants, which might also depend on the soil moisture levels and environmental conditions at the time of application. On the other hand, hundreds to thousands of differentially expressed genes were found in different antitranspirant-treated plant anthers, with most of them showing the downregulation of most of the biological processes involved at the early meiotic stages of anthers. Further studies could provide deeper insights by investigating specific genes of interest or by creating knockouts based on this study that could give more answers related to the possibility of improving pollen viability and subsequently influencing crop yield.

CHAPTER 2. Literature review

2.1 Soil and plant water relations

Soil water is important for plants and living organisms to survive. Plants take up their nutrients and other forms of organic minerals from soil with the help of water, which acts as a transport medium. The process of transpiration, photosynthesis, turgor pressure, cell expansion and many other vital functions of plant growth and development depend on water availability in the soil (Blatt et al., 2014). Therefore, an optimum level of soil moisture is crucial for the normal functions of plants.

Forces that hold water within the soil and energy associated with these forces for the movement of water molecules are referred to as water potential. It is usually expressed in bars or kilopascals with negative values, as the movement of the water molecules in the soil or plants is affected by forces such as pressure, gravity or solute concentration, as it cannot move freely due to these forces. Total water potential is the sum of these forces. Soils can store and retain water in varying amounts depending upon their type and capacity, also known as soil retention capacity (Geroy et al., 2011). It is directly related to soil water potential and an indirect measurement of water available to plants, also best represented in the form of soil moisture curves or water retention curves, depending upon the soil texture (Figure 2.1, a). Saturated soil has almost zero water potential, as all pores are filled with water. After excess water drains away, the soil is at field capacity and at this, water is easily available to plants as it has a water potential of around -0.1 to -0.3 bar (-10 to -30 kPa) (Brady and Weil, 2007). Field capacity is the maximum amount of water a soil can hold after drainage, while at permanent wilting point water, soil moisture is low and held with a tension (-15 bar or -1500 kPa) that plants cannot take up from the soil, therefore they either wilt or die if it goes beyond this level. Water which is held between the field capacity and the permanent wilting point is referred to as plant available water, and it becomes limited with time due to the process of evapotranspiration until irrigation is applied or more rain occurs to prevent it from going beyond the permanent wilting point to avoid wilting of plants (Figure 2.1, b). Different soil types (based on their textures) with their field capacity, permanent wilting point and plant available water are given in Table 2.1. Water moves from high (less negative) (wet soil or from roots) to low water potential (more negative) (dry soil or to top leaves in plants as water loss via transpiration), which results in the movement of water from the soil to different parts of plants (Bilskie, 2001; Schoonover and Crim, 2015; Cassel and

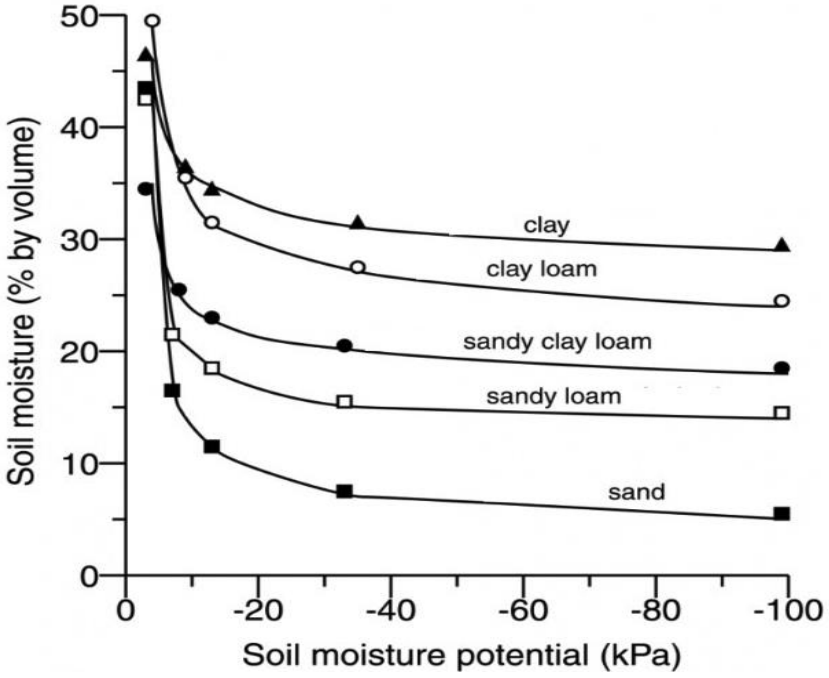
Nielsen, 2018; Agriculture and Food, 2021, Anderson and Flores, n.d.; Brady and Weil, 2007).

There are different ways to determine the moisture content of the soil via the direct method (gravimetric method by weighing a soil sample before and after oven drying to calculate the mass of water/mass of dry soil or via calculating volumetric water content = volume of water/volume of soil if the sample of known volume is taken (Bilskie, 2001)) and the other indirect methods of using different kinds of sensors: dielectric, heat flux and neutron probe (Bittelli, 2011).

Table 2.1: Different soil types with their field capacity (FC), permanent wilting point (PWP) and water available to plants (Hanson et al., 2000).

Soil Type (texture)	FC (%)	PWP (%)	Plant Available Water (%)
Sand	10	4	6
Loamy sand	16	7	9
Sandy loam	21	9	12
Loam	27	12	15
Silt loam	30	15	15
Sandy clay loam	36	16	20
Sandy clay	32	18	14
Clay loam	29	18	11
Silty clay loam	28	15	13
Silty clay	40	20	20
Clay	40	22	18

(a)



(b)

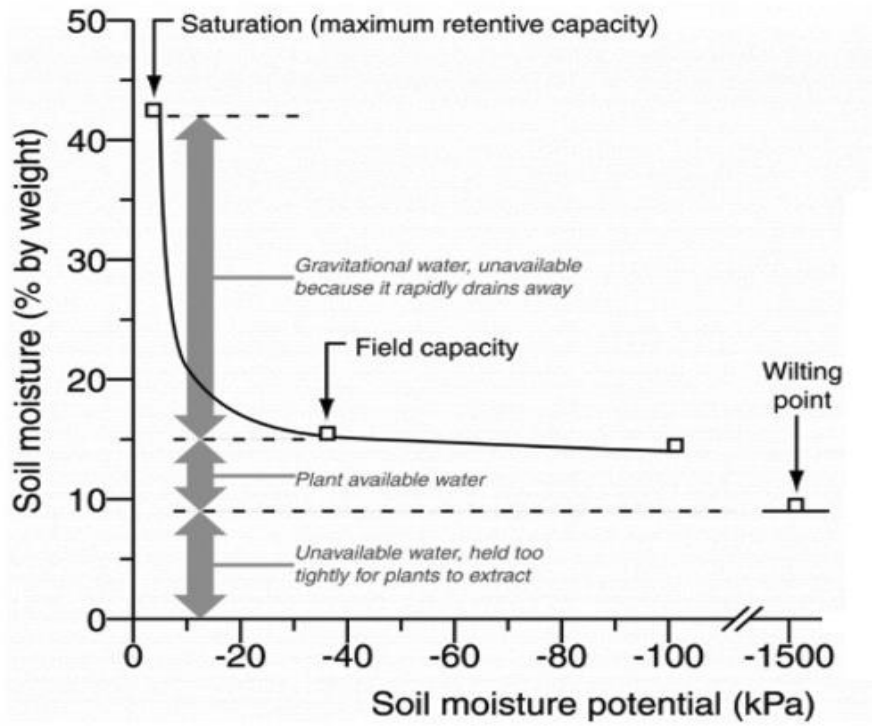


Figure 2.1: Soil moisture curves or water retention curves of different soils with relation to soil water potential (a), taken from Moorberg and Crouse (2021a). Soil moisture curve with the depiction of field capacity, plant available water and permanent wilting point with relation to soil moisture potential values (b), taken from Moorberg and Crouse (2021b).

2.2 Process of transpiration, stomatal conductance and photosynthesis

Transpiration is the natural process in plants in which water evaporates (97 - 99%) from plants through stomata, cuticle (outermost layer of plants) or lenticel (porous tissue on the stem and roots of plants). Water loss through stomatal pores can reach up to 99% in a fully hydrated plant. However, it also depends on many other factors such as air temperature, humidity, soil moisture level and several other morphological features of plants. In hot weather conditions, it helps plants to keep cooler canopies, but if there is less water in the soil, then plants produce abscisic acid (ABA) hormone that closes stomatal pores, thus reducing the water loss from leaves to cope in stressed environments (Kane et al., 2020; Trimble, 2022).

When leaf stomata are open, carbon dioxide can enter the leaf, and oxygen and water vapour can exit. The rate of gaseous exchange from leaf stomata is also referred to as stomatal conductance. The turgor pressure of guard cells around stomatal pores changes according to the environmental signals, thus determining the degree of stomatal opening and regulating the process (Figure 2.2). Many factors, such as light, temperature, humidity, water availability and carbon dioxide concentration, affect the process of stomatal conductance (Kinhal, 2022). Under favourable conditions for photosynthesis, during the day, phototropins detect blue light, causing proton pumps (ATP-dependent) to export protons (H^+) from guard cells, which causes influx of chloride (Cl^-), potassium (K^+) and nitrate (NO_3^-) ions to enter the cells. Starch in guard cells breaks down to sucrose and malate. This high concentration of ions and solutes lowers water potential inside guard cells, causing water to enter via osmosis, which increases turgor pressure within guard cells, causing them to expand, thus opening the stomatal pore (Figure 2.2, a). Stomata response to ABA-induced stomatal closure under water stress involves changes in osmotic/turgor pressure through the transport of different solutes and ions, in and out of the guard cells, causing stomatal pores to close (Figure 2.2, b). When ABA binds to its receptors on guard cells, it causes calcium channels to open, resulting in an increased concentration of calcium (Ca^{2+}) ions inside the cells, which activates calcium-dependent protein kinases (CPKs) that are important for further signalling events for stomatal closure. The presence of ABA in guard cells activates specific ion channels (with the help of CPKs), which facilitate the efflux of potassium (K^+), chloride (Cl^-), malate and nitrate ions from guard cells, resulting in decreased solute concentration inside guard cells. Therefore, water exits the cells via osmosis, decreasing turgor pressure within guard cells, resulting in stomatal closure. ABA also inhibits protein phosphatases (PP2Cs) that negatively regulate CPKs activity, thus helping CPKs to remain

active to maintain high levels of Ca^{2+} ion signalling for effective stomatal closure (Daszkowska-Golec and Szarejko, 2013; Ha et al., 2020; Pan, 2024).

The process of photosynthesis and stomatal conductance are closely linked. During photosynthesis, plants use light energy to convert water and carbon dioxide into glucose and oxygen (Figure 2.3). Stomatal conductance balances plant CO_2 and water loss via transpiration, significantly impacting the rate of photosynthesis and water use of different crop plants during the growing season, which determines the crop yield and productivity. Natural variation exists among different crop cultivars (wheat, rice, soybean, cotton, tomato) for stomatal traits, which can be exploited to develop new crop varieties with better water use efficiency with maximum or improved yield under different environmental conditions (Faralli et al., 2019). A glasshouse study was conducted on rice to assess the relationship between stomatal conductance and photosynthesis under different water stress conditions (severe, mild and no water stress) applied from the flowering stage onwards. The results revealed that severe water stress significantly affected and reduced the linear relationship between photosynthesis and stomatal conductance, thus, it was suggested that crop models which used the linear relationship (between photosynthesis and stomatal conductance) should also consider the water stress environments when estimating carbon dioxide and water fluxes from the crop canopies (Asargew et al., 2024). In another study, relationships were assessed for maximum stomatal conductance, photosynthetic rate, stomatal density and length among 45 woody plants on the Loess Plateau (China). The results revealed that plants with large and few stomata had low stomatal conductance rates but maintained a sufficient photosynthetic rate, which was suggested to be beneficial for plants under increased carbon dioxide and low water availability situations under varying climatic conditions in future (Yin et al., 2020). However, these relationships might vary under different environmental conditions and among different crop species. Thus, these studies show that photosynthesis and stomatal conductance are closely linked processes, and under droughted conditions, the photosynthetic process is affected due to the closure of stomatal pores to lower the transpiration rate. Therefore, breeding and exploiting varieties which perform better under limiting water conditions, with reduced stomatal conductance without affecting much of the CO_2 uptake to maintain a sufficient photosynthetic rate, would be promising for the changing environmental conditions.

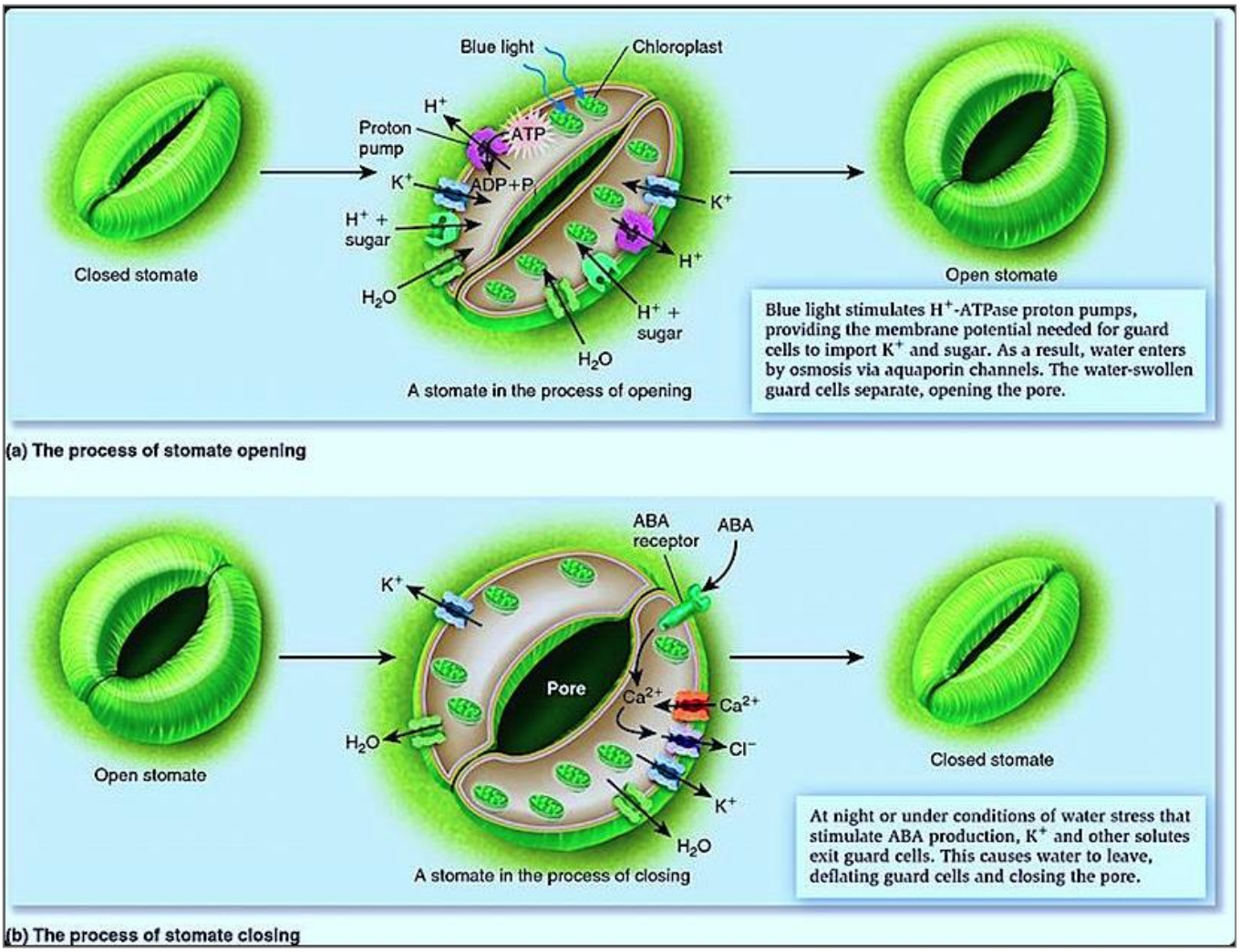


Figure 2.2: The process of stomata opening (a) and closing (b) in response to light or water stress. Figure taken from Cotthem, (2015). Stomata open in response to light (blue and red), with phototropins in guard cells, which are the main blue light receptor proteins for stomatal opening. Blue light activates the plasma membrane H^+ pump, referred to as H^+ -ATPase in guard cells, which exports protons (H^+) resulting in increased membrane potential, stimulating the influx of potassium (K^+), chloride (Cl^-) and nitrate (NO_3^-) ions inside guard cells. Other organic solutes, such as malate^{2-} and sugars, can be derived from starch degradation in guard cells, CO_2 fixation of guard cells or mesophyll photosynthesis and their subsequent import to guard cells. This influx of ions and solutes drives water into guard cells via osmosis, resulting in increased turgor pressure, causing them to expand, thus opening the stomatal pore (Inoue and Kinoshita, 2017; Ha et al., 2020; Flütsch and Santelia, 2021). At night or under water stress conditions that induce ABA production, calcium ions (Ca^{2+}) enter the cell via channels, while K^+, Cl^-, NO_3^-, malate^{2-} ions and other solutes exit the cell, resulting in decreased membrane potential. Water leaves the cell via osmosis, and the turgor pressure decreases, deflating guard cells, thus closing the stomatal pore (Ha et al., 2020).

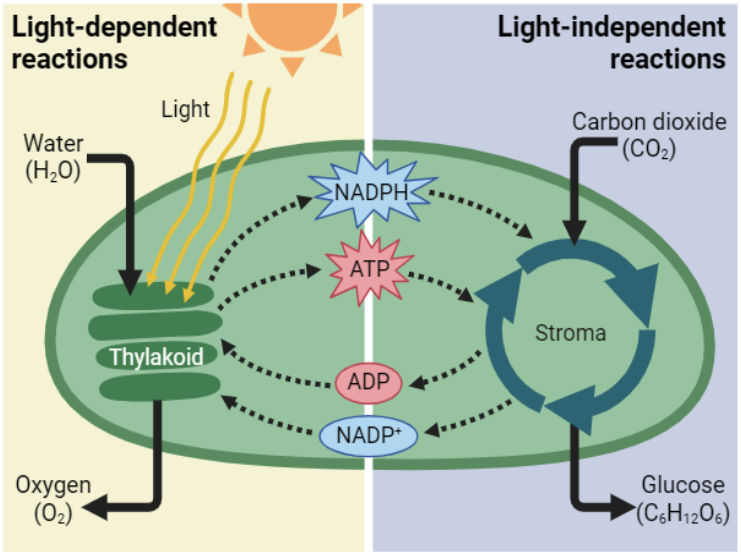


Figure 2.3: The photosynthetic process occurs in two stages (first stage, light-dependent reactions, in which plants use the light energy to make hydrogen carrier NADPH and the energy storage molecule ATP with oxygen release; second stage, light-independent reactions, products from the first stage are used to fix CO_2 which are later converted, into glucose or other carbohydrate products. Figure taken from Khan Academy (2023).

2.3 Drought and its effect on wheat growth stages with approaches for better yield

Drought or water stress is a major problem in agriculture, and the ability to withstand this stress is of immense economic importance (Jaleel and Llorente, 2009). Forty percent, of the world's population depend on agriculture as their main source of income and the drought is putting their lives at risk due to changing climatic conditions, especially in arid and semi-arid areas and in developing countries where it leads to 80% losses in agriculture (Food and Agriculture Organization, n.d.). Drought causes changes in many morphological, physiological, biochemical, and molecular processes in plants. It results in stunted plant growth, affects the process of photosynthesis and transpiration, and the amount of ABA and antioxidants increases to cope with limiting water conditions (Seleiman et al., 2021). Moisture stress at the early developmental stages of plants can lead to wilting or even death, and if it occurs at the reproductive stage, it can cause pollen sterility or ovary abortion, which results in lower or poor fruit/crop production (Silva et al., 2013). The sensitivity of crop plants to drought also depends on soil types in various regions, mainly their texture, structure and composition, as soils with high clay content have greater water-holding capacity and thus

have reduced drought susceptibility in comparison to sandy soils, which have low water-holding capacity (Yu et al., 2023b). Soil depth and compaction are some other factors which can influence plant growth (Hirzel and Matus, 2013; Mondal and Chakraborty, 2023) and thus influence their response to drought.

Wheat is the world's most widely grown food crop, providing a fifth of food calories and protein to the global population (Erenstein et al., 2022). Among abiotic stresses, drought and heat stress significantly impact global wheat production, and the frequency of these stresses will increase in most of the wheat-growing areas due to changing climatic conditions (Trethowan, 2022). Drought stress at critical growth stages of the wheat crop is the main cause of the decline in its production, which is becoming more severe and frequent. Water stress affects seed germination and tillering at the early stages. It affects plant growth and development if it occurs at the jointing and booting stage. The process of anthesis and grain filling is also very sensitive to insufficient moisture supply, as it affects the process of cell division in reproductive parts of the plant and reduces the transport of photoassimilates in grains, which causes a reduction in grain number and quality (Sidorenko and Chebotar, 2020). Drought at the flowering and grain filling period (terminal drought) leads to substantial yield losses in wheat, depending on the severity and duration of stress (Farooq et al., 2014). Different growth stages of wheat, according to the Zadoks (1974) scale, can be seen in Figure 2.4.

In the UK, an average of 10% of wheat yield is lost due to insufficient soil moisture levels, which increase in dry years. Most of the UK’s wheat is grown as a rainfed crop, with 30% of the acreage grown on drought-prone soils (AHDB, 2021). The drought risk in the UK generally increases from June to July in wheat, which usually coincides with flowering to grain-filling stages (critical growth stages) of winter/spring wheat, depending upon the sowing date. However, drought can occur at any growth stage of wheat, depending on the severity and duration of water stress and the soil type. Stem elongation and booting stage are also sensitive to drought, which can significantly impact crop yield (*The wheat growth guide*, 2008; Eser et al., 2024). Soil moisture deficit for the severity/intensity of drought is usually represented as a percentage of field capacity in the literature. However, the exact figure of FC to represent and understand the severity/intensity of drought in wheat varies slightly depending on the study, crop variety, soil type, growth stage and environmental conditions. Generally, around 60% of FC refers to mild drought, 40 - 50% refers to moderate drought, and 30% - 40% of FC or below represents severe drought stress. FC of 70% and above shows crop plants with no water stress (Fang et al., 2017; Zhao et al., 2020; Ahmad et al., 2024; Ali et al., 2023; Wang et al., 2024a).

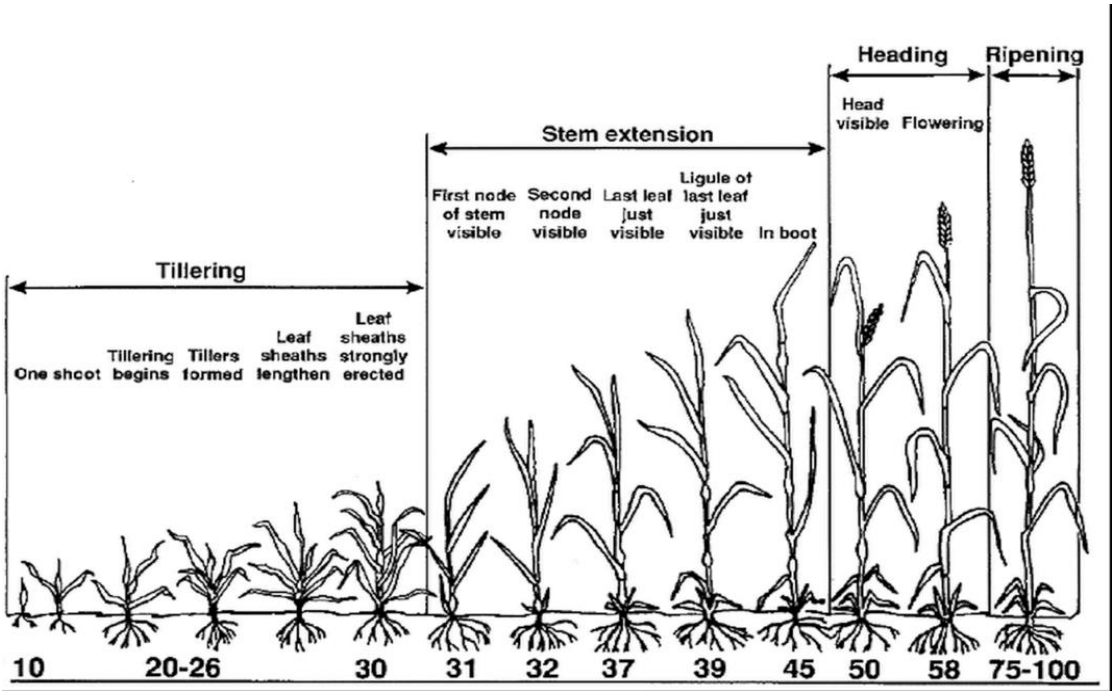


Figure 2.4: Wheat growth stages according to Zadoks (1974) scale, taken from Basden et al. (2016).

Good management practices such as row spacing, different seed enhancement techniques (Farooq et al., 2015) and nitrogen fertiliser application depending upon rainfall of a particular region (Sadras, 2002) to enhance wheat crop yield under terminal drought are among the few practices that can be considered. Selecting and exploiting wheat genotypes based on their root system and ABA responses in regulating water use under terminal drought are some of the other options that can be deployed in screening cultivars (Saradadevi et al., 2017; Figueroa-Bustos et al., 2020). Additionally, droughted and well-watered wheat genotypes revealed the link between leaf and spike enzymes involved in sugar metabolism and antioxidants, with important yield traits which can be used as biomarkers to select drought-tolerant genotypes (Shokat et al., 2020). Selecting and screening drought-tolerant wheat genotypes based on drought adaptability genes, canopy and root traits (such as assessing canopy green area using RGB-based vegetation index for canopy senescence dynamics at anthesis and a high number of crown roots per shoot) can also be a useful option to consider (Nehe et al., 2021). Selecting wheat inbred lines for stay-green traits, such as lines with higher photosynthetic rate and duration of leaf area at the anthesis stage along with retaining a higher level of chlorophyll content, indicated by upregulation of chlorophyll biosynthesis genes at the anthesis stage under drought and heat stress can help attain yield

stability (Kumar et al., 2021). Breeding and selecting drought and heat tolerant wheat varieties, based on different approaches such as direct selection of varieties under different stresses, targeting specific physiological traits, modifying expression of genes involved in stress tolerance and utilising various sources of variation (elite germplasm, landraces or wild relatives, gene editing, genomic selection and genetic engineering) has already been explored and is the focus of many research activities across the globe. However, more work is required to fully utilise the knowledge to enhance wheat yield production under changing climatic conditions (Langridge and Reynolds, 2021).

Drought and heat stress negatively affect crop growth and yields due to changes in morphological responses, physiological disruptions and biochemical changes in plants (Fahad et al., 2017). Drought and heat stress frequently co-occur in wheat-growing regions at the sensitive growth stages, causing significant yield losses by reduction in grain number and weight. The combination of both stresses is detrimental to plants, resulting in shortened grain-filling duration, reduction in stomatal conductance, affecting photosynthetic processes and disrupting plant water relations (Tricker et al., 2018). At the booting and anthesis stage, the combination of both stresses severely affects wheat grain development by disrupting meiosis, causing pollen sterility and reducing stigma receptivity, thus reducing pollination efficiency, abortion of ovules and seeds, reduction in days to anthesis and maturity, resulting in early seed maturity (Zahra et al., 2021). Some of the proposed plant traits to measure in genetic mapping populations that can be beneficial for combined heat and drought tolerance include the following: a root system able to grow fast upon water availability, fine-regulated transpiration via small, dense stomata with the ability to respond to micro-environment, ability to retain water in essential organs, optimal hydraulic conductance in different tissues, efficient heat shock proteins to protect membrane and enzymes under high temperatures, efficient ROS scavenging system with efficient process of carbohydrates synthesis and remobilisation etc. Therefore, more research on the combined drought and heat stress is required via the development of high-throughput phenotyping methods for stress tolerance and the integration of physiological and genetic approaches to understand the complex interaction between heat and drought stress in wheat to improve its productivity under challenging environmental conditions (Tricker et al., 2018).

Overall, the yield potential of the crops can also be enhanced by focusing on improving processes of nutrient partitioning and remobilisation, photosynthetic processes, leaf longevity, seed filling and plant organ growth and development as summarised in the review by Burgess et al. (2022) (Figure, 2.5), with a focus on using single or multiple aspects of these processes under stress environments can also be beneficial for breeding varieties for

tolerance with the possibilities of achieving the high yield potential in crops grown under different environmental conditions.

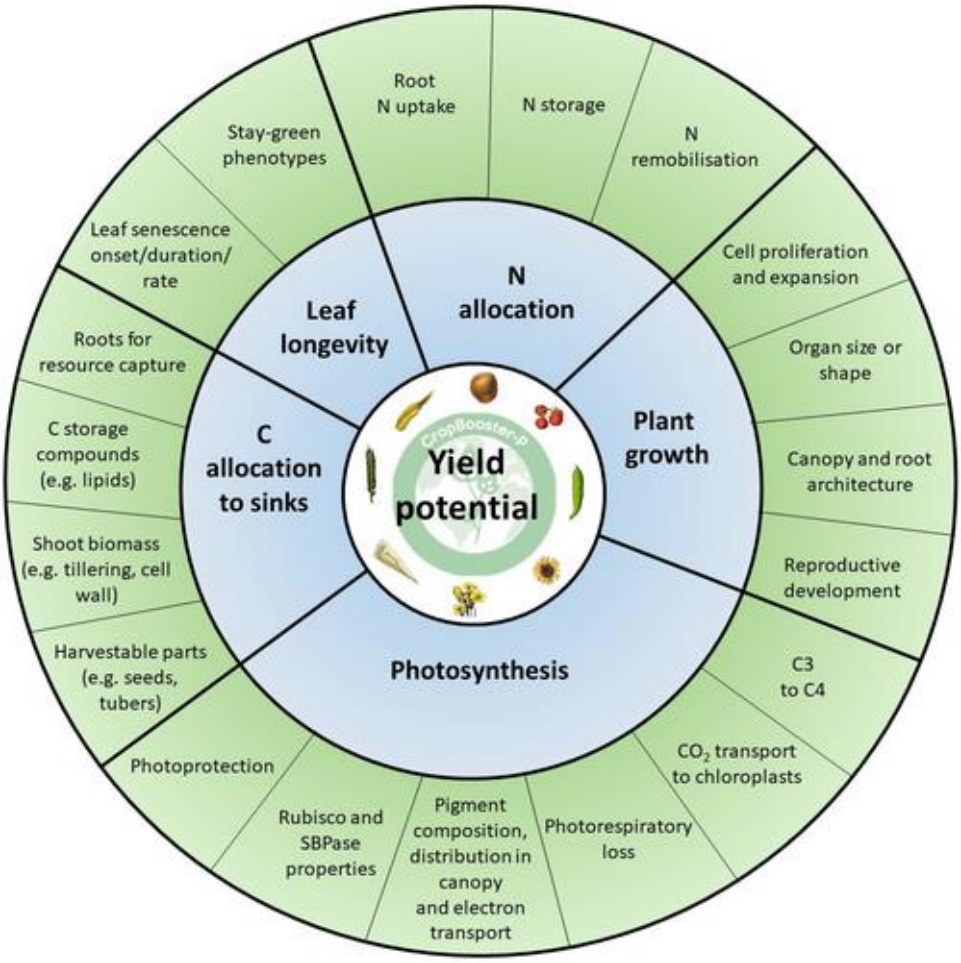


Figure 2.5: Different options for improving crop yield potential via optimisation of a single process (green) or multiple aspects of the main process (blue) to enhance crop productivity. Further exploration and research on these parameters and utilising them in breeding programs is crucial for the food security and futureproofing of current crops (figure taken from Burgess et al., 2022).

2.4 Drought and irrigation management strategies

Crop plants experience water stress conditions if available soil moisture drops below 50%, so it would be ideal to irrigate plants when they have used 40 - 50% of plant available water to avoid stress (Mathesius et al., 2021). Maximum allowable depletion (MAD), also known as management allowable depletion, is the portion of plant available water that can be used by

plants without experiencing stress (Figure 2.6). With increased depletion of available water, plants will face more stress until they reach a permanent wilting point, leading to wilting or death of plants if water is not applied (Datta et al., 2018).

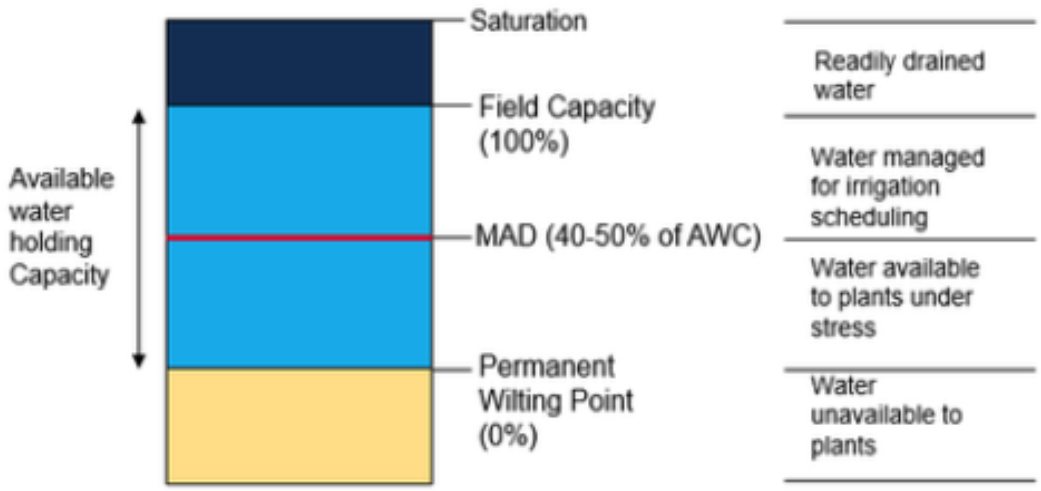


Figure 2.6: Soil water components depict the amount of water available to plants and the limit of maximum allowable depletion (MAD) for irrigation scheduling below which plants would be under stress due to low availability of water (figure taken from Sharma, 2019).

Wheat plants extract most of the water from the 0 - 45 cm of soil profile, therefore, it is recommended to manage irrigation accordingly for wheat grown in sandy loam soil (subtropical regions) under water-scarce conditions (Panda et al., 2003). Thus, to obtain high grain yield and water use efficiency for wheat grown under limited water conditions, irrigation can be scheduled at 45% depletion of available soil moisture for the non-critical growth stages in subtropical, subhumid regions (Panda et al., 2003). Some irrigation management strategies can be adopted to minimise the effect of drought on wheat crop yield, including supplemental irrigation, deficit irrigation, partial root zone drying, drip irrigation (Si et al., 2023; Liu et al., 2022; Iqbal et al., 2020), variable rate irrigation and precision irrigation (Usman, 2024).

Supplementary irrigation is an effective strategy for applying limited amounts of water to rainfed crops to improve their yields and productivity and to provide sufficient moisture for normal plant growth when there is insufficient rainfall (Nangia et al., 2018). One study revealed that supplemental irrigation in winter wheat increased grain yield, nitrogen use efficiency and water productivity when applied at critical growth stages such as jointing and

anthesis. Also, the cultivars used in the study revealed that the nitrogen nutrition index at anthesis could be an important indicator to manage irrigation for improved crop yield, nitrogen utilisation and water use efficiency (Liu et al., 2022).

Deficit irrigation is applying less amount of water than the crop needs, with the main irrigation focus on drought-sensitive growth stages and water restriction limited to less sensitive growth stages. The term deficit irrigation is mainly referred to fully irrigated crops (Geerts and Raes, 2009). The meta-analysis by Yu et al. (2020) for understanding the effect of deficit irrigation on wheat's water use efficiency and crop yield showed that this irrigation method improved WUE by 6.6% but decreased crop yield by around 16%. However, these results varied depending on the timing and amount of water, irrigation type and several other environmental factors. Thus, to minimise the trade-off between both parameters (WUE and yield), this type of irrigation would be more suitable in areas where total rainfall is < 200 mm and the soil is sandy or loamy. Also, there is more chance of getting high WUE and yield with border and furrow irrigation than with drip or sprinkler irrigation (Yu et al., 2020).

Another irrigation strategy which can be beneficial to crops in arid and semiarid conditions is partial root zone drying, which involves irrigating one part of the root zone while the other part remains dry and alternating the two parts periodically (Iqbal et al., 2020). Mehrabi and Sepaskhah (2019) conducted a winter wheat study to understand the effects of partial root-zone drying (via a variable alternate furrow irrigation system to compare with ordinary furrow irrigation), planting methods (on-ridge and in-furrow planting) and three different nitrogen treatments on physiological and agronomic parameters of the crop. It was concluded that in areas with limited water conditions, the combination of alternate furrow irrigation, in-furrow planting with nitrogen application of 150 kg/ha could lead to improved water use efficiency and yield stability of the winter wheat crop.

Drip irrigation is another efficient way of watering plants under water scarcity conditions to improve water use efficiency and crop yield however, it is rarely used for wheat (Wang et al., 2024b). In drip irrigation, water is applied via tubes with small emitters placed near plants, which allow water to drip slowly into plant roots. One study on winter wheat compared the effects of traditional flood irrigation, surface drip irrigation and sprinkler irrigation on crop yield. It was observed that both drip and sprinkler irrigation significantly increased water and nitrogen use efficiency, along with the crop yield. This was due to a delay in the senescence process, which promoted dry matter accumulation after anthesis, resulting in increased grain weight. Also, higher root length density below 80 cm of the soil profile was observed with

micro-irrigation (drip and sprinkler), which promoted the absorption of water and nitrogen from the deeper layers of soils (Li et al., 2018).

The latest technologies that can be adopted to improve crop performance and water use efficiency in drought-prone areas include variable rate irrigation (VRI) and precision irrigation. VRI irrigation management strategy involves data from sensors and mapping tools to apply water variably across the field based on the requirement of plants in a particular area, thus allowing more efficient use of water. The other (precision irrigation) strategy involves using soil moisture sensors, along with weather data, to manage the irrigation thus helping to improve crop performance based on real-time conditions (Usman, 2024).

2.5 Some parameters for determining drought stress

2.5.1 Canopy temperature

Canopy temperature (CT) is the temperature of plant leaves or vegetative cover and is measured and assessed by various methods or techniques such as infrared thermometers/sensors, infrared thermal imaging, thermal resistance temperature measurement and thermocouple measurement. Each of these methods has its characteristics. Infrared temperature measurements are widely used nowadays and are more common because of their fast response and suitability for covering large areas. It gives information about the water status of plants, evapotranspiration rate of different genotypes/cultivars and irrigation requirements and can also be used to forecast yield (Yu et al., 2016; Farm Progress, 2012; American Meteorological Society, 2012). CT shows the relationship between plants, soil and atmosphere and has been recognised as an important indicator of the water status of plants and can be used to identify drought-tolerant cultivars (Bhandari, 2016).

CT was used as a selection index for drought tolerance under changing soil moisture conditions in various wheat genotypes to assess yield stability (genotypes were sown in a rain shelter either with full irrigation or under moisture stress that increased during the crop growth period). Results revealed a positive correlation between CT and drought susceptibility index, indicating reduced yield in drought susceptible genotypes with high CT during midday as compared to drought-tolerant ones, which had cooler canopies (Blum et al., 1989). In a wheat and maize study of diverse genotypes under different water conditions, CT was assessed to determine its relationship with yield by taking images using a thermal

imaging camera. The study revealed the genotypic variations in maize and wheat cultivars, indicating that cooler canopies under water stress can produce high yields (Bhandari, 2016). Similarly, Bhandari et al. (2021) observed the negative correlation between CT and grain yield measured in different wheat genotypes under drier conditions. The CT images taken at anthesis and during the grain-filling stage revealed that CT can be used to identify different genotypes for drought tolerance.

Infrared thermal imaging can also be used to differentiate Arabidopsis mutants for stomatal conductance as investigated by Merlot et al. (2002), where mutants were identified based on their altered stomatal responses under drought when compared with wild-type plants. The use of film-type antitranspirant (methene/pinolene-based) on citrus fruit trees increased leaf or fruit temperature when compared with the two reflectant types (calcium-based) in separate treatments; however, in one combined treatment, reflectant sprayed after antitranspirant treatment counteracted the temperature effect of antitranspirant by lowering leaf/fruit temperature and less sunburned fruits. Thus, it was concluded that spraying film antitranspirants only would not be beneficial for fruit trees, however, spraying reflectants or a combination of both can be helpful to mitigate heat/radiation stress and sunburn on citrus trees (Rodriguez et al., 2019). Thermal imaging was used to record the leaf temperature of rapeseed sprayed with different concentrations of film antitranspirant (Vapor Gard) at the flowering stage under droughted conditions in the field (in rain shelters). It was observed that with the increasing concentration of film antitranspirant, the leaf temperature of plants also increased in comparison to well-watered plants (Xiang et al., 2023), which shows the basic mechanism of how film antitranspirants work by blocking stomata thus reducing transpiration from plants which might be the cause of increased leaf temperature, and in improving their water status to get enhanced yield as observed by Xiang et al. (2023). These studies suggest that getting a positive response or benefits from applying a similar type of antitranspirant (such as film forming) also depends on the type of crop and the environment it is in, otherwise, it won't be beneficial as in Rodriguez et al. (2019) study reflectants were better as compared to film antitranspirant in lowering leaf/fruit temperature to get improved yield. Thus, these studies indicate that CT can be the quickest and simplest method to determine the water status of plants. Also, many other indices can be derived from CT, as mentioned by Nanda et al. (2018), to reduce the variability from air temperature, vapour pressure deficit, solar radiation or wind speed.

2.5.2 Relative water content (RWC)

RWC is one of the known selection criteria used to determine the water status of plants under moisture-stress conditions. It is the amount of water a plant/leaf has at the time of selection and how much it can hold when it is fully turgid, which is usually calculated as: RWC (%) = [(fresh weight of sample – dry weight) / (turgid weight – dry weight)] x 100. For getting turgid weight, leaf samples are hydrated to attain full turgidity and then dried in the oven to get dry weight. A fully transpiring leaf has RWC of 98%, while it ranges between 30% - 40% in severely moisture-stressed leaves (*Leaf Relative Water Content (RWC)* (n.d.); Barr and Weatherley, 1962). Under water deficit conditions, the RWC of the plant decreases, and wheat cultivars with the lowest decrease in RWC under drought stress treatments at different growth stages showed improved yield, which determines their tolerance under drought (Keyvan, 2010) and similar kind of results were observed in a potato study where low yield loss in a cultivar was linked with high RWC values under drought stress (Soltys-Kalina et al., 2016).

RWC has been used in various studies as one of the selection/screening tools for drought tolerance in many cereal crops such as barley and wheat (Matin et al., 1989; Larbi and Mekliche, 2004; Lugojan and Ciulca, 2011; Arjenaki et al., 2012). Determining RWC at the anthesis stage in wheat is more effective (Dhanda and Sethi, 1998). The use of antitranspirants to improve the RWC of plants under water stress conditions has been investigated in various studies on different cereal and horticultural crops (Mphande et al., 2020). The application of different types of antitranspirants in tomato cultivars indicated high RWC values in plants with better stomatal control (Srinivasa Rao, 1986). Studies with the application of antitranspirants in barley and wheat cultivars have been shown to improve the RWC of plants under water stress conditions, thus concluding that antitranspirants can be effective to use in arid or semi-arid areas where drought causes a major reduction in yields (Hellal et al., 2020; El-hady et al., 2018). Film antitranspirant (di-1-p-menthene) application with increasing concentrations (1% to 3%) on rapeseed under terminal drought increased yield, which was concluded to be mediated through improvement in the leaf water status of plants (Xiang et al., 2023). Also, increased leaf RWC with film antitranspirant (di-1-p-menthene) spraying (at GS37) was observed in wheat by Mphande et al. (2024). Thus, it is one of the reliable methods to determine the water status of plants under different water stress environments.

2.5.3 Endogenous abscisic acid content of plants

One of the most common plant hormones produced under abiotic stress conditions is abscisic acid (ABA), also called the stress hormone, which leads to stomatal closure and the process of transpiration is either reduced or stopped to conserve the water status of the plant. ABA also regulates other processes of plant growth and development, such as it is involved in seed/bud dormancy, germination, senescence, storage/transport of starch in seeds, abscission, plant defence against various pathogens and many others (Mongrand et al., 2003; Zeevaart, 2003; Sah et al., 2016; Nambara, 2017; Alazem and Lin, 2017).

ABA is transported via xylem sap from roots to shoots during initial stress conditions. When the soil starts to dry, ABA synthesis increases in roots, while in the leaf, ABA is produced when leaf turgor reaches zero (Hartung et al., 2002). It also depends on the genotype's ability to produce ABA or their stomatal sensitivity to ABA in stress conditions, which can either fully or partially induce stomatal closure (Saradadevi et al., 2017). This ABA production in crop plants during stress conditions affects their yield (Sah et al., 2016) due to the reduced transpiration rate and photosynthesis upon stomatal closure.

Exogenous application of ABA (1 mM) at different phenological stages (at the beginning of shoot enlargement and repeated at anthesis) in wheat results in improved crop yield under moderate water stress conditions (Travaglia et al., 2010). In another study, exogenous ABA application (20 mg/l) increased drought tolerance in wheat plants by stomatal closure and accelerated the process of starch accumulation in grains under mild and moderate water stress due to the acceleration of assimilates transport and the rapid senescence phase. This resulted in improved yield due to enhanced drought tolerance with increased levels of antioxidant enzymes and proline content in flag leaves, but a reduced ratio of endogenous salicylic acid/abscisic acid (SA/ABA) hormones. In contrast to this, under severe water stress, exogenous ABA treatment reduced drought tolerance with low levels of antioxidant enzymes and proline content of flag leaves in the rapid loss phase, but an increased ratio of SA/ABA. This made photoassimilates redundant as it shortened their transport time from stems to grains, leading to a reduction in grain yield (Luo et al., 2021). To describe it further, Luo et al. (2019) explained that the wheat flag leaf senescence process can be divided into two phases, the persistence phase and the rapid loss phase, in a study to identify the hormones and genes that regulate the flag leaf senescence under water stress condition at the grain filling stage. It showed that a shorter average senescence rate, shorter persistence phase and a short duration of flag leaf being photosynthetically active were slightly linked with low grain weight. Thus, Luo et al. (2021) found that, although exogenous ABA

application reduced the initial senescence rate, rapid senescence phase and improved persistence phase of flag leaves in all four treatments (well-watered, mild, moderate and severe water deficit), however, there were inconsistent effects in the relocation of photoassimilates and grain weight between different treatments as explained earlier.

Film forming (di-1-p-menthene, 1 l/ha) and metabolic (exogenous ABA, 100 µM) antitranspirants were assessed for their role in endogenous leaf ABA content and wheat yield under moisture stress. Both types of antitranspirants improved crop yield despite their contrasting effects on endogenous leaf ABA, as film antitranspirant reduced leaf ABA while exogenous ABA elevated this level (Mphande et al., 2021a). Another study by Mphande et al. (2024) on wheat to understand the response of film antitranspirant (di-1-p-menthene, 1 l/ha) and fluridone (an ABA inhibitor, with three different concentrations 10, 20 and 50 µM) application (at GS37) on spike ABA concentration, showed that film antitranspirant reduced spike ABA and transpiration by 21% and increased grain yield by 27%. However, fluridone (10 and 50 µM) reduced spike ABA by 16% but did not change transpiration or grain yield. Thus, it indicates that although fluridone reduced spike ABA, however, the leaf stomatal pores were not closed enough to reduce the transpiration process, which is why transpiration was not affected; therefore, spraying exogenous ABA as an antitranspirant might solve this problem by closing stomatal pores and reducing transpiration to improve crop yield. A significant reduction in stomatal conductance was observed in wheat leaves with exogenous ABA application (at GS39) under a progressive drought glasshouse experiment by Mphande et al. (2021a). Thus, it suggests that using different antitranspirants (film or metabolic) under drought stress can be beneficial for crop plants in improving grain yield; however, it also depends on the intensity of water stress, plant growth stage, type of cultivar/genotype or environmental conditions. Moreover, it also shows that different studies observed variations in the endogenous ABA content in response to spraying different types of antitranspirants, which might improve or reduce crop yield depending on several factors, as mentioned earlier, especially soil moisture conditions and plant growth stages.

2.5.4 Pollen viability

Mature plant pollen contains different types of carbohydrates such as polysaccharides (e.g. starch), disaccharides (e.g. sucrose) and monosaccharides (e.g. glucose and fructose) (Pacini, 1996). Starch-deficient pollen or impaired starch accumulation induces male sterility (Lee et al., 2022) and can be evaluated as one of the indicators for assessing pollen viability in crop plants. Pollen viability is vital for the survival of the next generation of plants/species, and it refers to the functionality of pollen, its ability to live, mature, germinate and transfer its

male gametes in the embryo sac to complete the fertilisation process before losing its viability. Common techniques to assess pollen viability are staining of pollen (using Lugol's potassium iodine solution for detecting starch accumulation or assessing viable pollen via fluorescein diacetate staining), germination of pollen, seed set and pollen germination on excised stigma. To assess pollen viability, it is better to use a different combination of techniques to get a reasonable indication (Dafni and Firmage, 2000; Heidmann et al., 2016; Impe et al., 2020). Pollen viability is generally assessed manually after staining and counting pollen under the microscope; therefore, overall statistical power is low. Thus, analysing pollen images to check their viability using a fluorescence microscope with different imaging software/systems can provide a foundation for automated qualitative and quantitative evaluation of pollen (Ascari et al., 2020).

Pollen viability is greatly affected by abiotic stress conditions, which result in poor crop yields across the globe. Drought is one of the major abiotic factors, and if it occurs during the reproductive stage of a crop cycle it can affect the process of cell division in anthers, causing meiocytes degeneration, microspores disorientation, changes in hormonal balance, alteration in genes involved in meiosis and sugar transport (Yu et al., 2019). A strong link was observed between spikelet fertility and the number of germinated pollens on stigma in rice genotypes under different stress treatments of high temperature, drought and a combination of both at the time of flowering. High temperature and a combination of heat and drought showed a negative cumulative effect on the fertility of rice spikelets, which can be considered when screening genotypes for stress tolerance (Rang et al., 2011).

The use of film antitranspirant has been shown to increase wheat crop yield when applied before meiosis and is considered to be associated with improved pollen viability (Kettlewell, 2014; Weerasinghe et al., 2016), the same kind of improvement was observed in corn pollen, by using antitranspirant agents (glycerol 4%, green miracle 0.3% with zinc) by Abbas and Abdul-Razak (2019). Therefore, evaluating different types of antitranspirants for increasing pollen fertility in other cereal crops and genotypes under moisture-stress environments is crucial.

2.6 Effect of drought at the reproductive stage

The reproductive stage in plants is most vulnerable to drought stress, which leads to losses in crop yields across the globe, especially in rainfed areas. In wheat, both male and female parts are sensitive to moisture stress during meiosis (Fotovat et al., 2017; Onyemaobi et al.,

2017); however, female parts are observed to be more resilient in comparison to anthers (Ji et al., 2010).

Meiotic and mitotic division in anthers is highly vulnerable to drought and, is the main cause of pollen sterility in crops, and this may be due to the changes at the morphological, physiological, and molecular levels and the interactions between these processes (Jin et al., 2013; Yu et al., 2019). In tomatoes, drought can lead to abnormalities in anther development, such as anther indehiscence, pollen abortion or inadequate pollen starch accumulation (Lamin-Samu et al., 2021). The impairment of starch synthesis and enzymes involved in sugar metabolism is believed to be the cause of pollen sterility in rice by stress (Sheoran and Saini, 1996).

Water stress affects the activity of cell wall invertases (enzymes involved in sugar metabolism – converting sucrose to hexoses (glucose and fructose)) around meiosis before hindering pollen development that leads to pollen sterility, as two invertase genes (*IVR1* and *IVR5*) in wheat showed downregulation in a study conducted by Koonjul et al. (2005). Based on this study, Weerasinghe (2013) applied film antitranspirant on wheat at GS33, intending to improve pollen starch accumulation by reducing water loss from plants during meiosis. *IVR5* gene indicated downregulation, however results were not significant when antitranspirants and unsprayed droughted samples were compared. Furthermore, to understand the effect of chemically induced male sterility on carbohydrate metabolism in wheat anthers, a study was conducted by Zhu et al. (2015) using similar invertase genes (*IVR1* and *IVR5*). Invertase genes were significantly downregulated at the young microspore stage in anthers with a lower expression of the sucrose transporter gene (*TaSUT1*) in sterile lines, indicating that this defect in sugar metabolism and transport is the main cause of sterility (Zhu et al., 2015), with the effects seemed similar when carried out under water stress environment.

Changes in the expression of various genes related to meiosis, tapetum development, sugar transport, reactive oxygen species and genes involved in ABA and gibberellic acid pathway were also observed in anthers of crops under water stress (Yu et al., 2019; Lamin-Samu et al., 2021). Draeger and Moore (2017) defined the meiosis stage from interphase to late leptotene to be affected by heat stress in wheat, which can be similar under drought to study changes in pollen mother cells. This heat stress during the earlier stage of meiotic division prevented its progress and resulted in lower crop yield. This all shows that the tolerance of wheat to changing climatic conditions during the reproductive stage is important for yield stability (Senapati et al., 2019).

Many enzymes and genes are involved in carbohydrate metabolism and accumulation in the reproduction organs of plants (Datta et al., 2002; Hedhly et al., 2016), and any abnormality in these processes can lead to male sterility if it occurs during the process of anther and pollen development (Liu et al., 2021). The process of starch biosynthesis in maize pollen was observed in cytoplasmic male sterile (starch deficient) and male fertile (starch positive) genotypes at the early and late stages of pollen mitosis. Changes in the expression pattern of many genes (related to carbohydrate metabolism) were observed in male fertile genotypes, but less in starch deficient genotypes. Significant reduction in hexose sugars was observed in male sterile lines at all stages (early, late mitosis), but also during meiosis as compared to male fertile lines, thus, this sugar reduction and its low flux during carbohydrate synthesis lead to temporal changes in expression patterns of genes resulting in either reduced pollen viability or sterility (Datta et al., 2002), similar kind of results were observed in rice male sterile and fertile lines by Kong et al. (2007). Also, by using bioinformatics and RNA-Seq data analysis of maize anthers, many putative genes involved in genic male sterility of sugar metabolism have been predicted with suggested orthologues in other plants, most of which showed high expression patterns during middle or late developmental stages in anthers *(*Liu et al., 2021).

Abnormal starch accumulation in rice anthers under drought stress was observed due to genes affected more frequently by stress, such as genes involved in tapetum/microspore development, sugar synthesis and cell wall formation. This also indicated a strong interaction between starch metabolism, signalling pathway and reproductive development of plants under water stress (Jin et al., 2013). In a study of water stress in wheat anthers during the meiosis stage, decreased activity of invertase, lower sucrose synthase activity and increased amount of soluble sugars were observed compared to control plants. The study indicated that pollen sterility in stressed plants is because of the defect in the metabolic process of conversion of sucrose to hexose, not due to inhibition of starch enzymes or starch deprivation (Dorion et al., 1996). To understand this mechanism at the molecular level, four genes (*Inv1, Inv3, Raftin and AGP*) involved in carbohydrate metabolism of anther/pollen were observed in two contrasting wheat cultivars (drought-sensitive and tolerant) under water stress at the meiosis stage. Expression of these genes was significantly increased in a tolerant cultivar under stress compared to well-watered plants, while there were no significant changes in expression in the drought-sensitive cultivar. Thus, introducing these genes in drought-sensitive genotypes can decrease the problem of male sterility with an improved level of starch accumulation in pollen (Zare et al., 2016). These studies show the sensitivity of anthers to drought stress, therefore, in one of the experiments in this thesis, the effects of antitranspirants were investigated during the early phase of meiotic division in

wheat anthers, to understand that by using these products, the process of cell division might improve leading to better starch accumulation in pollen (resulting in improved pollen viability), which could enhance crop yield under water stress conditions.

2.7 Molecular/transcriptomic studies

Under stressed environmental conditions, plants produce different types of proteins to cope with abiotic stress, such as high temperatures, drought, flooding, salinity, or exposure to UV light (Sachs and Ho, 1986). These stress conditions are ideal for understanding the mechanisms involved in the regulation of genes (Matters and Scandalios, 1986), therefore, gene expression studies play a crucial role in understanding the type of genes involved, which can be manipulated to produce resistant/tolerant genotypes to cope in these stressful environments, however, it is not always easy as it depends on the type and number of genes involved for a particular trait.

Drought is a quantitative trait; therefore, it is considered complex when it comes to molecular studies with several genes involved, and finding the right targeted genes is a challenge to study their direct effects on plants. Many drought-related genes and quantitative trait loci (QTLs) can be identified in wild wheat relatives that can be transferred to modern wheat cultivars for their improved performance under stressed environments, as modern cultivars have lost their diversity due to domestication and selection processes (Budak et al., 2013). Several single nucleotide polymorphisms (SNPs) markers and genes related to drought resistance have been identified in *Aegilops tauschii* (the progenitor of wheat's D-genome) that can be used in marker-assisted selection for identifying drought-resistant cultivars (Qin et al., 2016). Recently, several QTLs or markers related to yield and drought have been identified in spring wheat, which can increase yield and are involved in drought tolerance (Bennani et al., 2022; Ahmed et al., 2022).

A study on winter wheat varieties revealed that dehydrin genes (stress proteins) were highly expressed in wheat seedlings, and transcription factors DREB1 and WRKY2 (involved in stress responses) were upregulated and downregulated, respectively, indicating different mechanisms involved in stress responses (Vuković et al., 2022). Several transcriptional factors (TFs) play a significant role in the tolerance of wheat to drought, along with several QTLs, genes and markers associated with genes that have been identified and reviewed in various studies (Budak et al., 2013; Gahlaut et al., 2016; Kulkarni et al., 2017; Urbanavičiūtė et al., 2021). Genes of different transcription factor families (dehydration response element

binding (DREB), heat shock factors (HSF), ethylene response factors (ERF) and zinc finger protein (ZFP), WRKY and MYB were identified to be both positive and negative regulators of drought responses in wheat, rice, maize and Arabidopsis (Kulkarni et al., 2017). Moreover, five gene families gained more attention: DREB (AP2/EREBP), bZIP, MYC/MYB, NAC and WRKY, as these play a significant role in drought tolerance response in plants, with some of the reported TFs in wheat as reviewed by Gahlaut et al. (2016). Furthermore, a list of wheat genes controlling QTLs or alleles involved in drought tolerance in different wheat genotypes related to some of these transcription factors is also given by Bhanbhro et al. (2024). All of these gene families are involved in several processes, such as signalling and transcriptional regulation, osmotic adjustment, stomatal closure, ROS scavenging, osmolyte accumulation, and protection of membrane and protein structure under stressful environmental conditions, with overexpression of most of these genes have shown to enhance drought tolerance in wheat and other plants (Gahlaut et al., 2016; Kulkarni et al., 2017; Bhanbhro et al., 2024). Calcium-dependent protein kinases (CPKs) have been demonstrated to be involved in plant growth, development and response to environmental stresses (Dekomah et al., 2022). These modulate abiotic stress tolerance via activating several genes, enzymes, transcription factors and ion channels (Atif et al., 2019). One gene TaCPK34 is suggested to play a positive regulatory role in response to drought via directly or indirectly regulating the expression of ABA-dependent genes (Li et al., 2020). BES1/BZR1 family transcription factor regulates plant stress resilience, growth and development via signal transduction pathways through the regulation of hormone synthesis and growth-related genes in response to external stress (Li et al., 2025).

Furthermore, several genes are responsible for drought tolerance in wheat, producing different proteins and enzymes under stress, such as proline, rubisco, helicase, abscisic acid responsive, late embryogenesis abundant (lea), glutathione-S-transferase (GST) and carbohydrates (Nezhadahmadi et al., 2013). Also, the production of many types of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase and peroxidase (GPX) in response to water stress has been shown as an adaptive mechanism in wheat and barley (Sallam et al., 2019). One study showed that an increase in the expression pattern of genes encoding catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase and peroxidase (GPX) was observed in the drought-tolerant wheat genotypes (Dudziak et al., 2019). Additionally, various types of genes involved in drought tolerance of crop plants fall into two main categories. First, signal transduction factors (ABA receptors, protein kinases and phosphatases, transcription factors and cofactors) and second, functional factors (genes related to metabolism, osmotic adjustment, protein turnover, protein modification, ROS-

related, transporters and wax-related genes) as reviewed by Wang et al. (2021). These genes could be exploited further to improve drought tolerance in crop plants.

A study on wheat seedlings for understanding drought stress tolerance responses using genome-wide association studies (GWAS) identified 85 candidate genes encoding drought stress. These genes belong to the protein kinase domain, MYB/SANT-like domain, WSD1-like domain, cytochrome P450, leucine-rich repeat domain, WD40 repeat domain, ubiquitin-like domain, BURP domain, BTB/POZ domain, etc. (Gudi et al., 2024). Another genome-wide study of drought tolerance in wheat identified 53 SNPs significantly associated with drought susceptibility and tolerance for the targeted traits (plant height, root length and root and shoot dry weight in wheat seedlings), with their diverse function in stress responses, plant growth and development that could be further explored in crop improvement strategies (Nouraei et al., 2024). Furthermore, a wheat study under different conditions of terminal drought by Zhang et al. (2024) identified 5320 drought-related significant SNPs via GWAS and identified around 17000 image-based traits (i-traits) related to drought via high-throughput phenotyping. Also, an advanced prediction model for wheat yield and drought resistance was developed using 24 i-traits via machine learning that could provide a rapid breeding approach for developing drought-resistant wheat varieties. Thus, these GWAS studies can provide a deeper understanding of the complex drought tolerance mechanisms in wheat plants, with several key identified genes in response to drought stress, which could be valuable for future breeding programs aimed at developing drought-tolerant varieties. Additionally, a GWAS study on a wheat diversity panel with *Aegilops tauschii* introgressions was conducted to evaluate them under heat and combined heat-drought stresses under field conditions. Several genes, alleles and QTLs were identified for different traits (including grain yield and related traits, loci for leaf traits such as canopy temperature, vegetative index and carbon isotope composition) that could be beneficial to use in the breeding programs to improve wheat adaptation under heat or combined heat-drought conditions (Itam et al., 2021).

In the last decade, more focus has been on transcriptomic studies (RNA molecules) to understand how genes are expressed in various organisms (plants, animals, cells or tissues) under different environmental conditions. Two wheat cultivars (TAM111 and TAM112) with different drought stress tolerance mechanisms were investigated for transcriptomic and physiological responses in greenhouse conditions. TAM112 gave a high yield, more biomass, elevated ABA in leaf samples and showed more changes at the transcriptomic level from flag leaf samples as compared to the other cultivar. It was concluded that ABA played the main role in regulating these differential changes at both physiological and

molecular levels, which led to the higher yield of TAM112 (Reddy et al., 2014). Also, transcription factors related to the WRKY family (involved in biotic and abiotic responses) were investigated for drought response in wheat, and ten TaWRKY genes were detected using a transcriptome-wide identification from publicly available RNA-Seq data. Relative expression of these genes was measured in the root and leaf tissues of a drought-tolerant and a susceptible cultivar. Five genes showed differential expression in both tissues for the first time under drought stress in wheat, and all quantified transcripts were upregulated in the root tissue of the drought-tolerant cultivar that would be useful in further studies and understanding of drought tolerance responses (Okay et al., 2014). Moreover, a reproductive stage drought stress study was conducted on rice to examine the transcriptomic changes and floral development. Using florets of different sizes, microarray analysis revealed more than a thousand drought-responsive genes in one or two particular sizes of florets, suggesting changes in gene expression response according to the developmental stage of rice florets. Genes involved in starch synthesis, cell wall formation/expansion and tapetum/microspore development were more frequently affected by drought with changes in GA signalling and ABA catabolism pathways (Jin et al., 2013).

Another transcriptomic study of drought stress in wheat during the reproductive stages of spikes (pistil and stamen differentiation stage, anther differentiation stage, tetrad stage, early flowering stage and grain formation stage) was conducted in field conditions (rain shelters). Several differentially expressed genes (DEGs) were identified that were involved in important processes related to stomata, photosynthesis and floral development, with proteins of drought-responsive DEGs that seemed to be present in different cell organelles, thus playing a crucial role in drought tolerance of wheat (Ma et al., 2017). Furthermore, twelve hub genes (*TraesCS7D01G417600 (PP2C)*, *TraesCS5B01G565300 (ERF)*, *TraesCS4A01G068200 (HSP)*, *TraesCS2D01G033200 (HSP90)*, *TraesCS6B01G425300 (RBD)*, *TraesCS7A01G499200 (P450)*, *TraesCS4A01G118400 (MYB)*, *TraesCS2B01G415500 (STK)*, *TraesCS1A01G129300 (MYB)*, *TraesCS2D01G326900 (ALDH)*, *TraesCS3D01G227400 (WRKY)*, and *TraesCS3B01G144800 (GT)*), and three main modules were identified in wheat, using a gene co-expression network analysis to understand the drought resistance mechanism at critical growth stages, which can be used to improve the crop yield by using these candidate genes in transgenics or molecular marker-assisted selection (Lv et al., 2020). Additionally, to understand the transcriptomic response of drought and cold stress separately and by combining both in a wheat tolerant and sensitive cultivar, a study was conducted, and different genes were identified, with similar expression patterns under both stress environments and compared with available transcriptomics results in other varieties. Gene expression was different in the

homoeologous gene group, with some genes showing significant expression patterns while others were expressed less. Also, several SNPs were identified in the genomes of different varieties. Thus, transcriptomic studies can provide a valuable foundation for genome-wide screening, and varieties can be selected with enhanced stress tolerance responses (Konstantinov et al., 2021). A comparative transcriptomic analysis of two wheat varieties under drought stress revealed mass differential expression of genes, including flavonoid biosynthesis, plant hormones, phenolamides and antioxidant pathways, with altered expression of about 700 genes. Co-expression analysis revealed that bHLH and bZIP transcription factors might be involved in regulating various pathway genes, whereas the application of melatonin increased drought tolerance. Thus, this transcriptomic study provides more understanding of the complex tolerance mechanism against drought stress, exploring insights into secondary metabolites and phytohormones (Niu et al., 2023).

As explained earlier, male reproductive parts of plants are more sensitive to water stress than female parts, especially if the process of cell division (meiosis and mitosis) is affected in anther/pollen, which is the main cause of male/pollen sterility. A comparative transcriptomic study of wheat and rice anthers showed that 129 RNA transcripts were involved during the process of meiotic anther development (Crismani et al., 2011). A transcriptomic data of maize meiocytes (from anthers) at the stage of early meiosis (leptotene-zygotene of prophase I) was generated to identify the genes involved and to understand the molecular mechanisms at this stage of gamete formation. It was suggested that it could be beneficial to understand the regulatory processes involved during this stage and to link these with various functions/mechanisms in other related plant species (Dukowic-Schulze et al., 2014). While in barley anthers and meiocytes, most significant transcriptional changes were observed during the pre-meiosis to the leptotene-zygotene stage, and these were enriched in long non-coding RNAs showing significant down-regulation at early meiosis, thus indicating a complex dynamic transcriptional network and processes during prophase I (Barakate et al., 2020). Thus, these studies indicate that cereal crop anthers at the early meiotic stages are transcriptionally more active, which implies that any stress condition could alter these dynamic complex regulatory processes involved in anther development or meiotic division that can cause male sterility.

2.8 Use of antitranspirants

Stomatal closure is associated with reduced plant transpiration to conserve water during drought stress. Following this approach, the use of antitranspirants has been exploited to

reduce water loss from plants under moisture stress conditions in different crops. Antitranspirants are chemical compounds that conserve moisture in many ways, such as by forming an artificial film on the leaf surface (also called film forming type), causing stomatal closure by acting metabolically (also referred to as stomatal closing/ metabolic type), reflectance type (substances that create a thin particulate coating on the leaf surface that reflect solar radiation and can also cause partial or full stomatal blocking if particles settle over stomatal pores thus reducing the transpiration process) and even growth retardants which decrease shoot growth and increase root length so that they can extract water from the deeper layers of soil (Hanson, 2016; Kumari, 2017; Thakur, 2018; Mphande et al., 2020).

Antitranspirants have the potential to improve yield in a variety of arable crops, ultimately resulting in increased crop production (Mphande et al., 2020); however, these can only be effective if applied at specific growth stages of plants that are sensitive to drought (Kettlewell et al., 2010). Moisture stress at these critical growth stages of a crop is also the main cause of the decline in its yield. Similarly, choosing the appropriate antitranspirant at the right time is the key question that needs to be considered for each crop. Although many previous studies indicated the improvement in wheat crop yield with the use of antitranspirants under limiting water conditions (Kettlewell et al., 2010; Abdullah et al., 2015; Weerasinghe et al., 2016; Abdallah et al., 2019; Mphande et al., 2021a, 2021b, 2024), however, it is also revealed that they reduce photosynthesis, respiration and the uptake of nutrients from soil. Also, antitranspirants are helpful in the improvement of several other physiological, quality and disease-resistance characteristics, as summarised in different review papers (Koteswara et al., 2018; Mphande et al., 2020; Guleria and Shweta, 2020). Furthermore, drought is considered a precondition for antitranspirant application; otherwise, it reduces yield (Kettlewell et al., 2010) and applying antitranspirant also depends on the level of moisture stress, as under severe moisture, it might not show any effect. Faralli et al. (2017a) observed that the application of film antitranspirant (at the flowering stage) on oilseed rape did not improve pod formation but seed production only under severe moisture stress (10% of volumetric water content), whereas, under water stress (20% of volumetric water content) film antitranspirant improved both pod formation and seed production in comparison to unsprayed plants. A review of antitranspirants and their potential use in various arable crops grown under limiting water environments by Mphande et al. (2020) covers more detail on the different types of antitranspirants.

Film-forming antitranspirants reduce evaporation from plants by covering stomata, which in turn reduces photosynthesis and gas exchange and can increase the leaf temperature of plants, thus plants have to deal with photosynthesis-transpiration compromise (Davenport et

al., 1974; Chalker-Scott, n.d.). However, this compromise in photosynthesis can be compensated with an improved yield of many crops grown in arid and semi-arid regions. A study of film antitranspirant (di-1-p-menthene) in raspberry on foliar and fruit transpiration indicated that it could be more effective in reducing water loss when applied to the lower surface of the leaf, while in fruit/berries at the immature stage when natural transpiration is high (Moroni et al., 2020). Improvement in the yield of oilseed rape (Faralli et al., 2016) and wheat was observed when applied during the reproductive period under moisture stress conditions (Abdullah et al., 2015) or if sprayed before the start of meiosis (Kettlewell et al., 2010; Weerasinghe et al., 2016; Mphande et al., 2021a, 2021b), which is associated with improved pollen starch accumulation (Kettlewell, 2014; Weerasinghe et al., 2016).

The function of abscisic acid (ABA) in plants under stress is important as it induces stomatal closure by changing their osmotic potential, which causes them to shrink and close (Tombesi et al., 2015). Exogenous ABA application as an antitranspirant can increase crop production in arid climates (Travaglia et al., 2010). ABA application at the grain-filling stage in wheat in PEG-induced drought showed that the expression of the *psbA* gene involved in photosynthesis was less depressed when compared with plants before ABA was applied, therefore showing that the drought tolerance mechanism is linked with *psbA* expression through ABA regulation (Wang et al., 2011). A study conducted by Yang et al. (2014) showed that exogenous ABA can improve wheat crop yield by accelerating the grain filling rate, which results in increased starch accumulation in grains, while Luo et al. (2021) indicated that under severe water stress, ABA application decrease grain yield and water use efficiency of wheat plants but improves under mild and moderate water stress (Travaglia et al., 2010). Similarly, a decrease in leaf hydrogen peroxide content was observed with increased activity of antioxidant enzymes with exogenous ABA application, thus helping wheat seedlings in reducing damage from free radical accumulation due to drought (Kong et al., 2021), with almost similar results reported in maize by Jiang et al. (2022). Drought-tolerant cultivars of wheat grown in PEG-induced stress (polyethylene glycol (PEG) is used to induce drought stress in plants by reducing water availability thus inducing osmotic stress) in hydroponics, showed better osmotic adjustment by the increase in proline and carbohydrate content as compared to susceptible cultivars when sprayed with ABA or salicylic acid (Marcińska et al., 2013).

Two wheat cultivars were investigated for drought tolerance with ABA application (10 µM ABA) as a soil drench. Moisture stress was applied at the jointing or booting stage by withholding water one day after the ABA treatment until all plant available water was used, whereas non-stressed pots were kept above 90% of field capacity by daily watering.

Application of ABA reduced leaf relative water content, stomatal conductance, decreased lethal leaf water potential and reactive oxygen species (ROS), while the leaf ABA level increased and the activities of antioxidant enzymes. The yield was reduced in both cultivars at moderate water stress with ABA treatment, however, ABA application helped in slowing water use and enhancing antioxidant defence during soil drying (Du et al., 2013). Another study of exogenous ABA (10 μM) on wheat seedlings grown in PEG-induced drought increased the amount of glutathione (GSH) and ascorbate (ASA) in leaf and root tissues. Genes involved in the synthesis of GSH and ASA enzymes were upregulated and showed differential expression in both tissues. This study could provide more information on the molecular mechanism of drought tolerance induced by ABA, which is linked with the synthesis of GSH and ASH enzymes (Wei et al., 2015). No improvement in the grain yield of soybean was observed, with exogenous soil drench ABA applied after 45 days of sowing in pots, however, it improved the rate of photosynthesis in leaves, relative water content and water use efficiency (WUE) in moderate water stress. Also, stomatal conductance was decreased, and the ABA content of the leaf increased with exogenous ABA (He et al., 2019). A detailed review of mechanisms of ABA in plants under drought stress at the physiological and molecular level by Muhammad Aslam et al. (2022) and Ali et al. (2020) can give a clear understanding of its role in the tolerance of plants against stresses.

2.9 Research gap

So far, there has been no transcriptomic study in wheat following the use of antitranspirants under drought stress at different growth stages. This study of investigating the role of antitranspirants (film and metabolic) on crop yield and transcriptomic responses in wheat anthers during early meiosis would provide valuable information for future research on understanding the type of genes involved and finding the differentially expressed genes in different antitranspirant-treated plant anthers and control ones (unsprayed droughted and well-watered).

The main objective of the two field experiments in Chapter 3 was to understand the physiological responses of a spring wheat variety under drought stress after the application of film and metabolic antitranspirants at different growth stages.

The hypotheses, according to the main objective, were as follows:

- The spraying of film (VG) and metabolic (ABA) antitranspirants reduces water loss from plant leaves and improves pollen viability and crop yield under drought stress.

- Application of VG or ABA antitranspirant at the flag leaf stage (GS39) reduces water loss and improves pollen viability and crop yield of droughted plants.
- VG application at stem elongation (GS31) or ABA spray at flag leaf stage (GS39) is responsible for reduced water loss from plants with improved pollen viability and grain yield under drought stress, or ABA spraying at watery to milky grain stage (GS71-73) is responsible for improved crop yield due to faster accumulation of photoassimilates.

The main objective of Chapter 4 was almost similar to the field experiments in Chapter 3, with the only difference that it was conducted under a glasshouse environment. The objective was to understand the physiological responses of plants under drought stress with the application of film and metabolic antitranspirants at different growth stages in a glasshouse setting.

The main hypotheses were as follows:

- Spraying of antitranspirants reduces water loss and improves pollen viability and crop yield under drought stress compared to unsprayed droughted plants.
- Application of VG or ABA at the flag leaf stage (GS39) is responsible for reduced water loss and improvement in pollen viability and crop yield under drought stress, or spraying of ABA at the watery to milky grain stage (GS71-73) improves grain yield by the faster accumulation of photoassimilates.

The main objective of Chapter 5 was to understand the transcriptomic responses in different anther samples, with the hypothesis that less water stress from spraying of antitranspirants (VG or ABA) leads to reduced inhibition of enzymes associated with improved pollen viability.

CHAPTER 3. Understanding the effect of antitranspirants (film and metabolic) on physiological responses of wheat under drought stress

3.1 Introduction

Climate change is a major driving force projected to reduce global wheat production by 1.9% by 2050, with 15% and 16% average yield reduction in Africa and South Asia, respectively (Pequeno et al., 2021). Drought is considered one of the major constraints, responsible for lower crop yield due to extreme changes in climatic conditions, and developing countries will suffer the most if mitigation steps are not taken to address the issues related to water stress, especially in arid and semiarid areas (Zaveri et al., 2023).

One mitigation practice is using antitranspirants to improve crop yield under limited water conditions at critical growth stages. Spraying film antitranspirant at the flag leaf stage in droughted wheat (Kettlewell et al., 2010; Mphande et al., 2021b) and flowering stage of *Brassica napus* has been shown to increase grain yield (Faralli et al., 2017b). Drought during the meiosis stage in wheat induces male sterility, which is linked with changes in carbohydrate metabolism and a decrease in the activity of invertase enzymes in wheat anthers (Dorion et al., 1996). Also, the accumulation of abscisic acid hormone in cereals plays an important role in male fertility under reproductive stage drought stress, as a high accumulation of ABA in wheat spikes and anthers was observed in drought-sensitive varieties in comparison to drought-tolerant ones (Ji et al., 2011). Weerasinghe et al. (2016) indicated that the application of film antitranspirant (di-1-p-menthene) before the meiosis stage in wheat (application at GS33) increases crop yield that might be associated with improved pollen starch accumulation, while Mphande et al. (2021b) concluded that the improved yield with film antitranspirant (di-1-p-menthene) might be associated with reduced endogenous ABA concentration and reduced transpiration (Mphande, 2021) with effective applications during stem elongation.

A study by Yang et al. (2011) indicated that exogenous application of ABA at the initial grain-filling stage in wheat enhances grain weight and improves grain filling process along with increasing the endogenous ABA concentration of grains, while Yang et al. (2014) revealed that the application of exogenous ABA (at the anthesis stage (GS60) for three days) can improve crop yield by accelerating the grain filling rate resulting in increased starch accumulation. Another study indicated that under severe water stress, exogenous ABA application (at the anthesis stage for four days) decreases grain yield and water use

efficiency of wheat plants but improves them under mild and moderate water stress conditions (Luo et al., 2021). Thus, these findings show that the application of exogenous ABA improves yield if applied at the anthesis or initial grain filling stage, regardless of whether it is grown under droughted conditions or not; however, it might depend on the severity of drought stress as indicated by Luo et al. (2021). In another study, Mphande et al. (2021a) suggested an association between endogenous ABA and drought stress rather than a direct effect of the hormone on grain development. Improved yield was observed with the application of two different antitranspirants (film - VG and metabolic - ABA) at critical growth stages (film or metabolic antitranspirant spraying before the start of meiosis or multiple metabolic antitranspirant spraying at later stages till anthesis in one experiment) in two glasshouse experiments conducted under progressive and controlled drought conditions. Both antitranspirants (film and metabolic) reduced the effect of reproductive stage drought stress despite having contrasting effects on leaf ABA, with film antitranspirant decreasing the leaf ABA concentration and exogenous ABA increasing the leaf ABA concentration; however, both antitranspirants improved yield.

Thus, to investigate these mechanisms further, two field experiments were conducted to understand the role of antitranspirants (both film and metabolic antitranspirants – Vapor Gard (VG) and exogenous ABA, respectively) in decreasing water loss, improving pollen viability and wheat yield under droughted conditions at critical growth stages (before the start of meiosis and at the grain filling stage) and understanding their role in alleviating the effects of drought. The main reason for doing field experiments was that they give a real picture of plants' performance in a natural environment compared to a glasshouse setting, as daily environmental variations can be considerable in the field, which can affect plants' source: sink ratio and thus overall morphology and physiology (Poorter et al., 2016).

The main hypotheses of the two field experiments were as follows:

- Spraying antitranspirants reduces water loss and increases pollen viability, grain number and yield of plants under drought stress.
- Film antitranspirant (VG) or exogenous ABA application (ABA) at the flag leaf stage (GS39) before the start of meiosis reduces water loss and increases pollen viability, grain number and yield of droughted wheat.
- Antitranspirant application at different growth stages; VG at stem elongation (GS31), ABA at flag leaf stage (GS39) before the start of meiosis, or ABA at watery to milky grain stage (GS71-73) are responsible for getting improved grain number and yield under water deficit conditions.

3.2 Materials and methods

3.2.1 Experimental site and design

Two experiments of spring wheat variety (Chilham) were sown in a randomised complete block design in four polytunnels (rain shelters) at the Flatt Nook Field of Harper Adams University, Shropshire, UK (Grid Reference SJ 71806 19726), in 2022 and 2023. The soil at the site is loamy sand with a field capacity (FC) of 22% volumetric water content (VWC) and a permanent wilting point of 8% VWC (Mphande et al., 2021b).

In 2022, there were eight experimental plots in each polytunnel: six droughted plots and two well-watered plots, each with sizes of 1 x 1 m (Appendix 1 and 2), while in 2023, there were fourteen experimental plots: twelve droughted and two well-watered, each of the same size as in the previous year (Figure 3.1, Appendix 3 and 4). The well-watered plots were not randomised as they were present at the front corner of each polytunnel to enable watering by drip irrigation (5 tubes of 1 m length in each well-watered plot) and avoid water entering the droughted plots. Therefore, these were not included in the statistical analysis in both years. Water was given to well-watered plots two to three days a week (Monday, Wednesday, and Friday) for one hour to keep the mean soil moisture value up to 16% - 18% VWC (around 70% - 80% of field capacity) in the top 50 - 60 cm of the soil profile in 2022 and 2023.

3.2.2 Planting, agronomic practices and management

Experimental plots were marked in the polytunnels before sowing, and soil moisture access tubes were inserted in four randomly selected droughted plots of each polytunnel and one in the well-watered plot in 2022 (Appendix 1 and 2). In 2023, soil moisture access tubes were inserted in each experimental plot of the four polytunnels after sowing (14 tubes in one polytunnel: 12 in droughted plots and 2 in well-watered plots) (Appendix 4).

Based on the previous crop grown in the polytunnels and the nutrient management guide (RB209) (AHDB, 2019), nitrogen fertiliser was broadcast in the polytunnels at the rate of 130 kg N/ha (as ammonium nitrate) before planting in 2022, and four weeks after planting in 2023. Even after broadcasting nitrogen fertiliser in 2023, leaves were showing symptoms of yellowing as drought stress negatively affects plant nitrogen (N) and phosphorous (P) uptake due to low availability of water (He and Dijkstra, 2014); therefore, a multi-nutrient foliar

fertiliser (5 l/ha) was sprayed (3X Solution with 10% w/v of N, Omex Agriculture Ltd, Norfolk, UK) six weeks after planting.

For the first field experiment, seeds were manually sown in four polytunnels between 16 – 20 June 2022 (it was delayed because the first germination in May 2022 was poor, so plots were resown). Well-watered plots were sown first on 16th June, and then sowing of droughted plots was completed in each polytunnel in the next four days. Seedlings emerged after 9 - 16 days of sowing, with water given to each plot two to three days a week up to the growth stage of GS31 (first node visible) (Zadoks et al., 1974) to help the germination process and also to store enough moisture in the soil. So, when progressive drought started after GS31 until physiological maturity, soil moisture remained above the permanent wilting point (8% VWC).

For the second field experiment, manual sowing of seeds in the four polytunnels was completed between 03 – 09 April 2023 (in a week). Well-watered plots in the four polytunnels were sown on 03 April, and in the next six days, the sowing of droughted plots was completed. Seedlings emerged after 14 days of sowing, with water given to each plot (two to three days a week) up to 21 days from sowing to support germination, and progressive drought was started after that till physiological maturity.

In both years, seeds were sown at a depth of 2 cm with a rate of 400 seeds per m^2. There were six rows in each experimental plot with a row spacing of 14 - 15 cm. The sowing of well-watered plots was always completed on the first day in both years, as manual hand sowing in all the polytunnels took 5 to 7 days each year, respectively. The main reason for sowing well-watered plots earlier was that they were almost ready to harvest at the same time as droughted plots.

The crop was grown in polytunnels in both years, and soil moisture readings were measured up to a depth of 50 cm. However, the roots of spring wheat can go up to a depth of 1 m (Thorup-Kristensen et al., 2009), so soil moisture readings below 50 cm were estimated to be very low, as polythene sheets were not removed from polytunnels in winter after the previous crop, to store enough moisture in deeper layers of soil. Also, the soil below 55 cm depth might be stony sand with single grain structure based on the general information of soil profile description around that area (LandIS, 2025), which could suggest that water retention with this soil texture at lower depths is very low, leading to soil moisture in negligible amount which might not be possible for plants to extract.

Plots in the polytunnels were weeded manually during the cropping season, and to keep out birds, rabbits, and other animals, the front and back of the polytunnels were covered with netting and rabbit fencing. There was an issue of powdery mildew in the well-watered plots in both years, but it was not that severe; therefore, no fungicides were sprayed. For the mice problem, near the end of the crop's physiological maturity in 2023, traps were set inside the polytunnels.

3.2.3 Antitranspirant treatments and application

Two types of antitranspirants ((Vapor Gard (VG) (96% di-1-p-menthene, Miller Chemical and Fertilizer LLC., Hanover, PA) at 1 L/ha with an application rate of 200 l/ha (film antitranspirant) as used by Mphande et al. (2021b) and exogenous ABA (20% S-Abscisic Acid, ProTone SG Plant Growth Regulator Soluble Granule, Valent Bioscience LLC, Libertyville, IL) at 30 g/ha of the chemical product (containing 6 g/ha of S-ABA) with an application rate of 200 l/ha (metabolic antitranspirant)) were sprayed at the flag leaf stage (GS39) before the start of meiosis in droughted experimental plots in 2022. There were two plots of each antitranspirant treatment in one polytunnel, along with two unsprayed plots and two well-watered plots.

In 2023, both types of antitranspirants were sprayed at different growth stages (VG at GS31, ABA at GS39, and ABA at GS71-73) with three plots of each treatment, along with three unsprayed and two well-watered plots in each polytunnel. The growth stage of VG at GS39 in the previous year was changed to GS31 in 2023, as there was no improvement in crop yield with VG spraying at GS39, as observed by Mphande et al. (2021a) when results from 2022 field experiments were analysed. Also, because of the poor germination in 2022, the planned treatment plots of VG at GS31 were excluded. Therefore, in 2023, only VG-GS31 treatment was included (excluding VG-GS39) along with ABA antitranspirant treatments at GS39 and GS71-73, respectively. Furthermore, in another study of winter wheat variety, the application of VG at GS31 or GS33 improved pollen viability, which was suggested to be linked with enhanced crop yield (Weerasinghe, 2013). This was also the reason for selecting the GS31 growth stage instead of the GS39 for VG treatment in 2023.

In both years, antitranspirants were sprayed with a handheld sprayer (5 L Hozelock pressure sprayer) on the plant canopy from an approximate height of 0.5 m. The details of antitranspirant treatments, timing and sample collection dates in both years are given in Table 3.1.

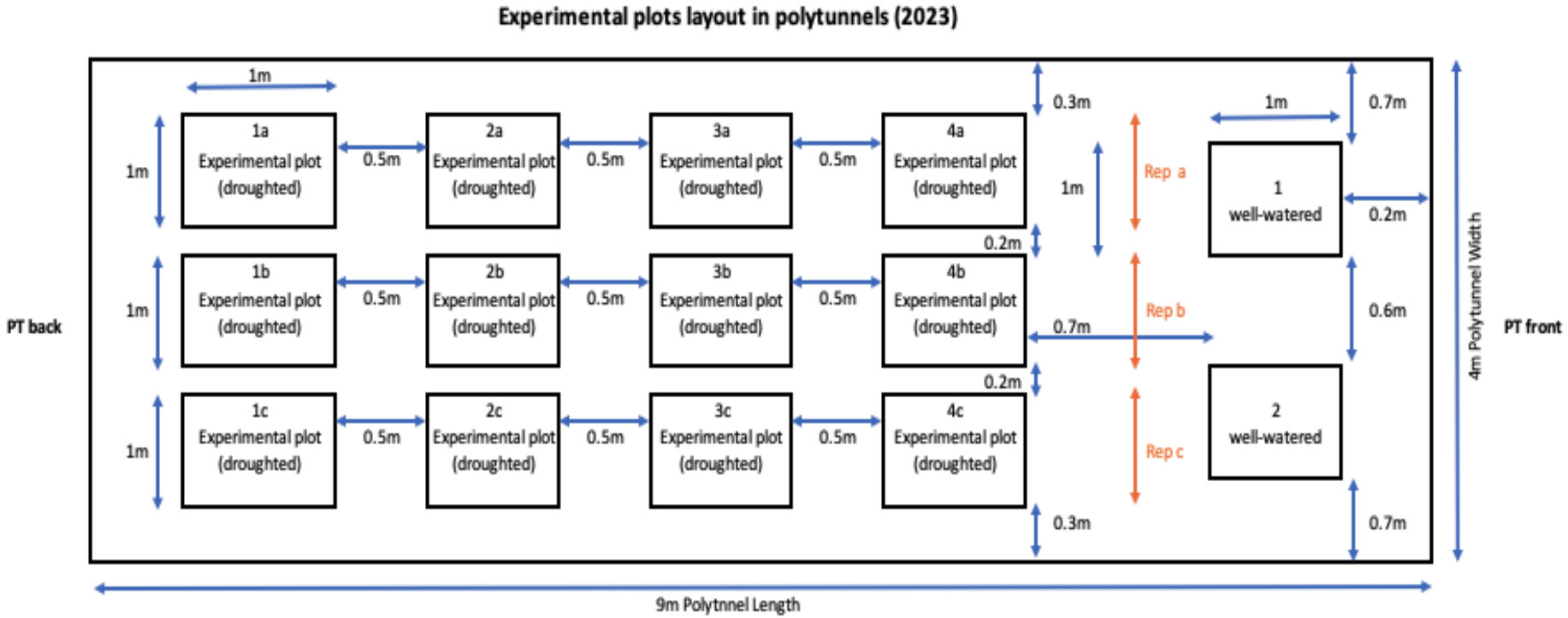


Figure 3.1: Layout of experimental plots in one of the polytunnels (rain shelters) in 2023. Different treatments and their replicates were randomised in each polytunnel (randomisation detail in Appendix 3), except for the well-watered plots that remained at the front corner of all four polytunnels to avoid water entering the droughted plots.

Table 3.1: Timing of antitranspirant (VG and ABA) treatments and sampling dates.

Treatment - Growth Stage (GS)	Spraying Date	Days after Planting (DAP)	Leaf sampling for ELISA ABA analysis and relative water content. DAP - Date Days after Spraying (DAS)	Anthers collection from spikes at leptotene-zygotene stage of meiosis. DAP Dates	Anthers collection for checking pollen viability. DAP Dates	Spike sampling for ELISA ABA analysis from all treatments. DAP Date
2022						
VG-GS39 ABA-GS39	04 Aug 22	47	55 DAP 12 Aug 22 8 DAS	Started 45 DAP 02 Aug 22 to 26 Aug 22	Started 55 DAP 12 Aug 22 to 24 Aug 22	76 DAP 02 Sep 22
2023						
VG-GS31	23 May 23	47	55 DAP 31 May 23 8 DAS	N/A	Started 69 DAP 14 Jun 23 to 19 Jun 23	92 DAP 07 Jul 23
ABA-GS39	05 Jun 23	60	68 DAP 13 Jun 23 8 DAS			
ABA-GS71-73	29 Jun 23	84	N/A		N/A	

3.2.4 Measurements and sampling

3.2.4.1 Temperature, humidity, and solar radiation

Air temperature and humidity data inside the polytunnels was recorded daily with Tinytag Ultra 2 (Gemini Data Loggers Ltd, Chichester, UK) data loggers, and solar radiation data

was taken from the meteorological station based at Harper Adams University (approximately one kilometre away from the location of the research field) in 2022 and 2023.

3.2.4.2 Soil moisture

Soil moisture content was measured (via access tubes inserted randomly in droughted and well-watered plots in 2022 and from tubes inserted in all plots in 2023) once a week up to a depth of 50 cm with a time-domain reflectometer (TDR) (TRIME-PICO IPH/T3, IMKO Micromodultechnik GmbH, Ettlingen, Germany). Readings (in the form of percentage volumetric water content) were taken from four depths (20 cm, 30 cm, 40 cm, and 50 cm) of each access tube. Measurements were started three weeks after sowing in both years and continued until physiological maturity.

3.2.4.3 Canopy temperature

Thermal images were captured to record the canopy temperature (CT) of experimental plots in both years using the FLIR thermal imaging camera (FLIR Systems, Inc., Wilsonville, USA). Images were taken one day before antitranspirant treatments and then starting from one day after the treatment up till six or seven days (skipping one day in between e.g. 1st, 3rd, 5^{th} or 7^{th} day). Later, FLIR Research Studio software was used to get mean canopy temperature data from image pixels for each experimental plot, excluding the soil temperature pixel data (Appendix 5).

3.2.4.4 Relative water content

For determining the relative water content (RWC) of leaves after antitranspirant treatments, six top leaves from each experimental plot were cut randomly, after eight days of each treatment at different growth stages in 2022 and 2023. The top and end sections or damaged parts of leaves were cut off, and only the middle 5 - 6 cm section of each leaf was taken and stored in pre-weighed tubes. Further steps were followed according to the method described by Pask et al. (2012). Leaf samples were collected from unsprayed and well-watered plots, along with antitranspirant-treated plots.

3.2.4.5 Endogenous ABA concentration

To determine the ABA concentration inside the leaf and spike samples of antitranspirant-treated, unsprayed, and well-watered plants, sampling of 8 - 10 top leaves from each experimental plot was done after eight days of different treatments in 2022 and 2023, while spike samples (three spikes from each plot) were collected at GS71-73 in both years.

Samples were collected in 50 ml tubes and were immediately flash-frozen in liquid nitrogen in the field. Later, these were stored at a -80 °C freezer until further processing was completed according to the Cusabio ELISA ABA protocol (Code CSB-E09159PI). For processing, the frozen samples were first freeze-dried for 3 - 5 days and ground in an electric grinder before further steps were carried out according to the protocol. If any sample weight was less than the recommended 0.5 g after freeze-drying and grinding, then the amount of buffer was adjusted accordingly by keeping the ratio 1:9 (sample: buffer). Absorbance readings were recorded at 450 nm using a BioTek microplate spectrophotometer, and the exact ABA concentration of each sample was calculated after fitting the standard curve using the CurveExpert 1.4 software.

3.2.4.6 Assessment of pollen viability

Pollen viability was determined by the presence and absence of pollen starch accumulation via staining of pollen grains in both years. It was assessed according to the method described by Weerasinghe et al. (2016) by randomly collecting 10 - 15 freshly dehisced anthers from the spikes of treated, unsprayed, and well-watered plots in 1.5 ml dark Eppendorf tubes containing Lugol's solution. After collection, tubes were stored in the refrigerator at 4 °C and within 2 - 3 weeks, counting of viable (darkly stained) and non-viable (partially or unstained) pollen was done under a light microscope (10x objective, Zeiss Primostar 3) using a Sedgewick Rafter counting chamber (Appendix 6). Three replicates of ten random grids were counted for each sample, and the mean percentage of viable pollen was calculated.

3.2.4.7 Yield and yield components

To assess the performance of antitranspirant-treated plants and unsprayed plants under droughted conditions in the four polytunnels in 2022 and 2023, yield and yield components were measured. Data from the well-watered plots was also collected but was not included in the statistical analysis, as these were not randomised in the polytunnels.

Spike density per m^2 was calculated according to the method described by Sylvester-Bradley et al. (1985) by measuring 50 cm of two adjacent rows and counting the number of spikes in them using the formula of mean spike count per sample divided by mean row width (cm) and multiplying by 100. In those plots where it was not possible to measure the 50 cm of two adjacent rows due to poor plant density (gap in rows because of poor/no germination or mice damage), only rows with good plant density were chosen to count spikes of that

length to get the total length of 100 cm. Each sample was put in separate bags for threshing manually and weighing to calculate the number of grains per m^2 and grain yield per plot.

The number of grains per spike was calculated by randomly collecting 20 spikes from each plot in separate bags, manually threshing them and counting the number of seeds to calculate the mean number of grains per spike.

Before manually threshing all samples, spikes were put in an oven at 60 °C for 1 - 2 days to improve the threshing efficiency. After manual threshing, samples were oven-dried at 105 °C for 40 hours and weighed to calculate yield per plot.

A subsample of 40g was extracted from the spike density samples to calculate thousand grain weight (TGW), according to the method described by Sylvester-Bradley et al. (1985). Seeds from the samples were counted, and any broken and damaged seeds were removed. Samples were weighed again, and TGW was calculated using the formula: fresh weight of subsample (g) divided by the number of grains in the subsample and multiplied by 1000. The number of grains obtained from TGW was also used to calculate the number of grains per m^2.

3.2.5 Statistical analysis

R Studio was used to apply ANOVA with a randomised complete block design to compare different antitranspirant-treated and unsprayed plots. Before performing ANOVA, Levene's test was performed to check the homogeneity of variances between different samples, the Shapiro-Wilk test to check the normality of data and the Bonferroni outlier test to detect any outliers. Well-watered plots were not included in the statistical analysis.

In 2022, ANOVA contrasts were performed to check for any differences between unsprayed and treated plots and a direct comparison of GS39 treatments of VG and ABA sprayed plots. Covariates were added for green plant row length (for each plot) (Appendix 7) in the yield analysis, as plant rows were not even in density because of poor germination. In 2023, a contrast between unsprayed and treated plots was performed, and soil moisture data from each plot was included as a covariate in the yield components analysis. A Tukey post-hoc test was performed in both years to see which treatments showed the difference.

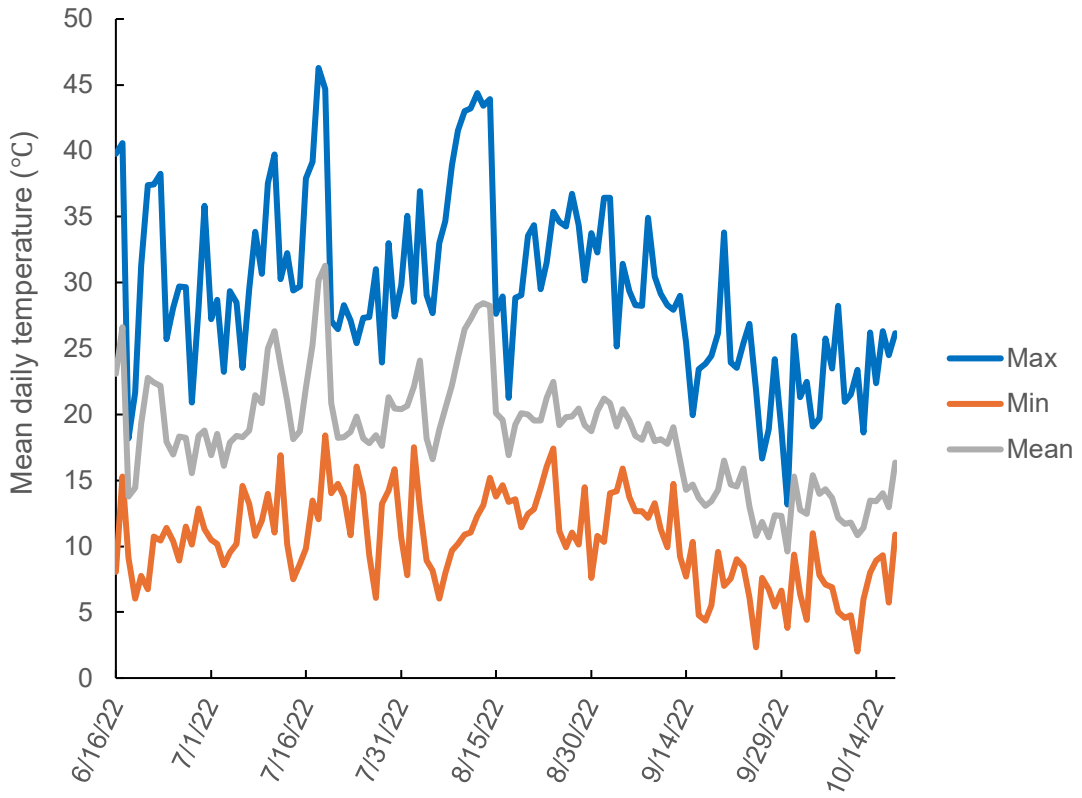
3.3 Results

3.3.1 Temperature, humidity and solar radiation

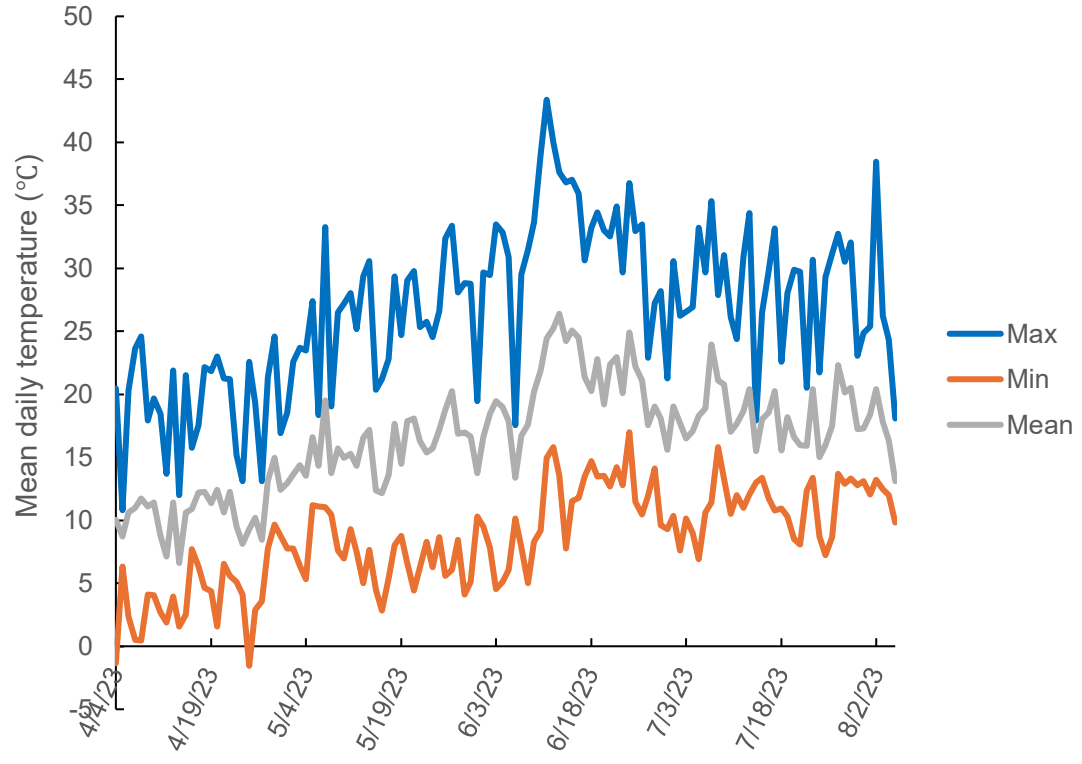
The daily mean temperature and humidity were around 18 °C and 72%, respectively, during the cropping period in 2022, with a mean of 14 MJ/m^2/day of solar radiation (Figure 3.2, a, c, e). There were two heatwaves during the cropping season, where the maximum day temperature was above 40 °C on two days in July and six days in August 2022 inside the polytunnels.

In 2023, mean daily temperature, relative humidity, and solar radiation were 17 °C, 75% and 17 MJ/m^2/day, respectively (Figure 3.2, b, d, f). Data in both years were taken from the Harper Adams meteorological station located one km away from the experimental field site.

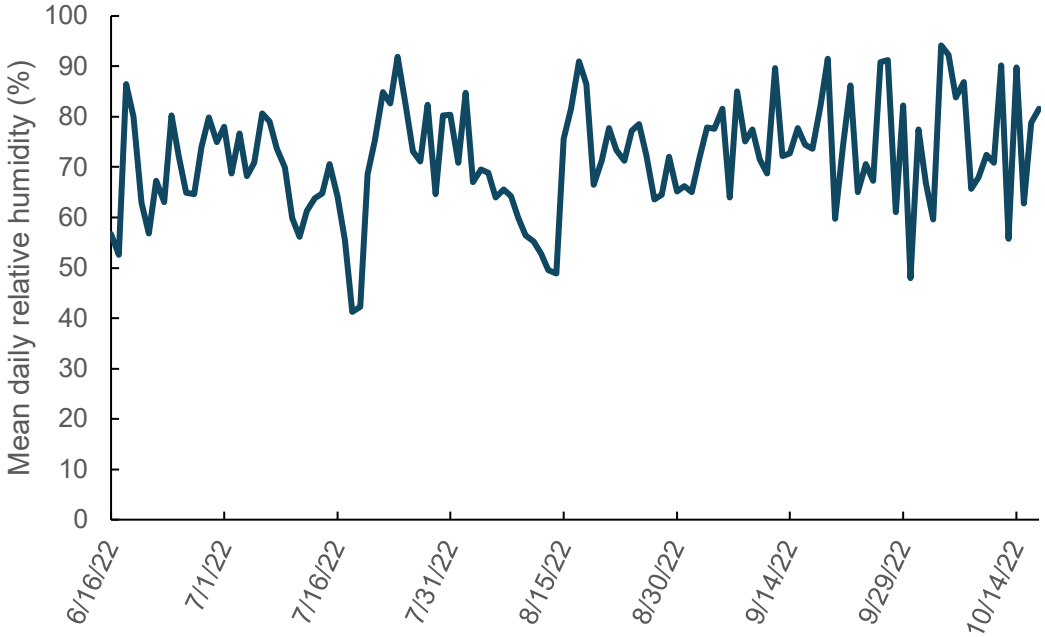
(a)


Mean daily temperature (℃)
50
45
40
35
30
25
20
15
10
5
0
6/16/22
7/1/22
7/16/22
7/31/22
8/15/22
8/30/22
9/14/22
9/29/22
10/14/22
Max
Min
Mean

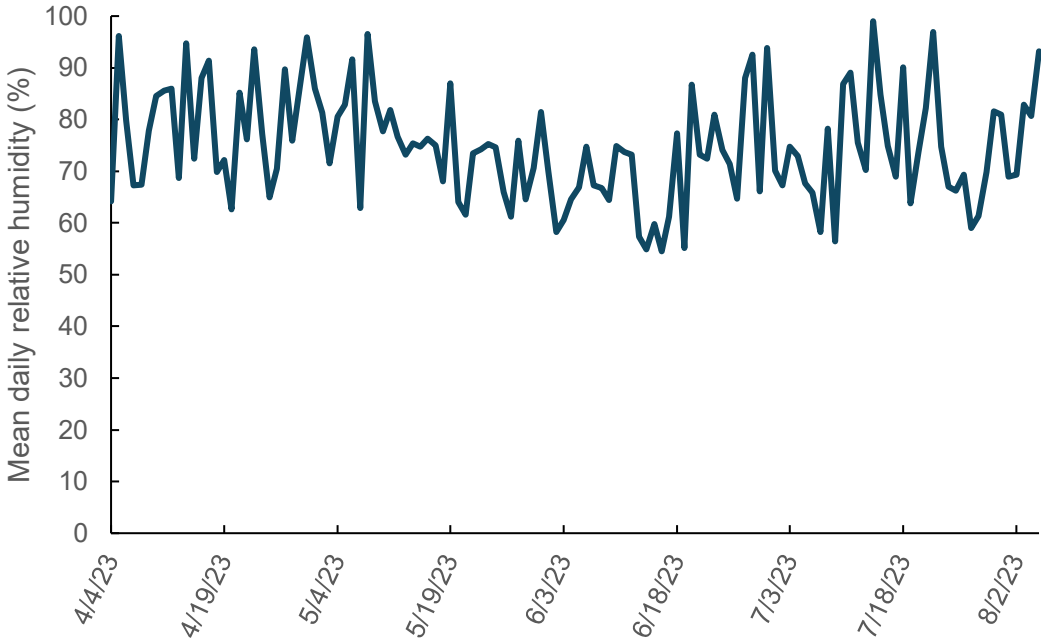
(b)


Mean daily temperature (℃)
50
45
40
35
30
25
20
15
10
5
0
-5
4/4/23
4/19/23
5/4/23
5/19/23
6/3/23
6/18/23
7/3/23
7/18/23
8/2/23
Max
Min
Mean

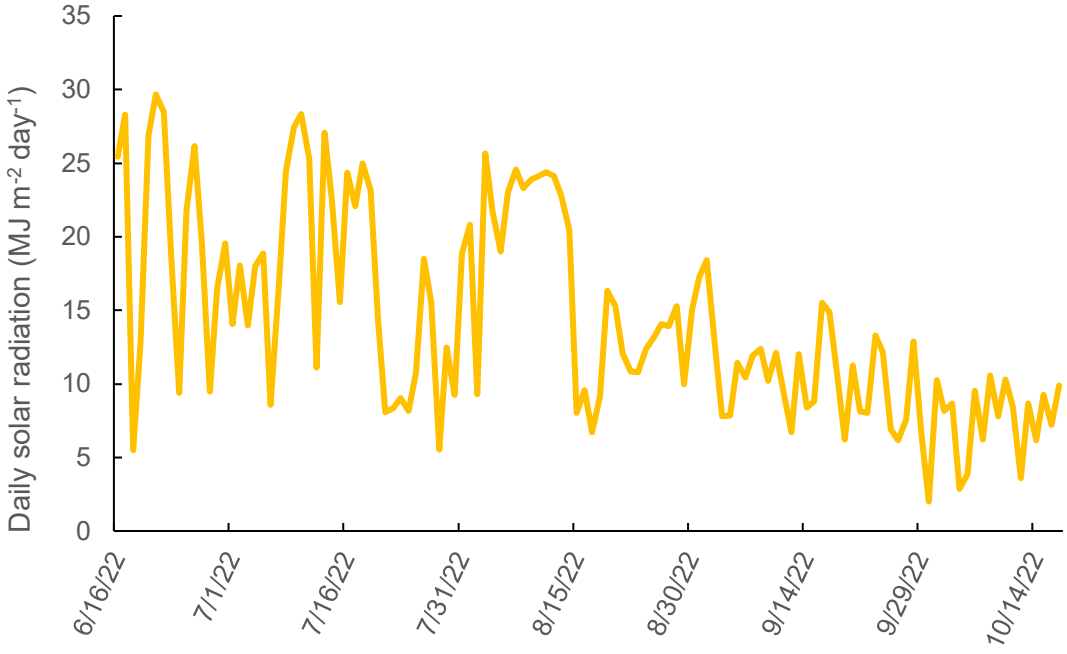
(c)


Mean daily relative humidity (%)
100
90
80
70
60
50
40
30
20
10
0
6/16/22
7/1/22
7/16/22
7/31/22
8/15/22
8/30/22
9/14/22
9/29/22
10/14/22

(d)


Mean daily relative humidity (%)
100
90
80
70
60
50
40
30
20
10
0
4/4/23
4/19/23
5/4/23
5/19/23
6/3/23
6/18/23
7/3/23
7/18/23
8/2/23

(e)



(f)

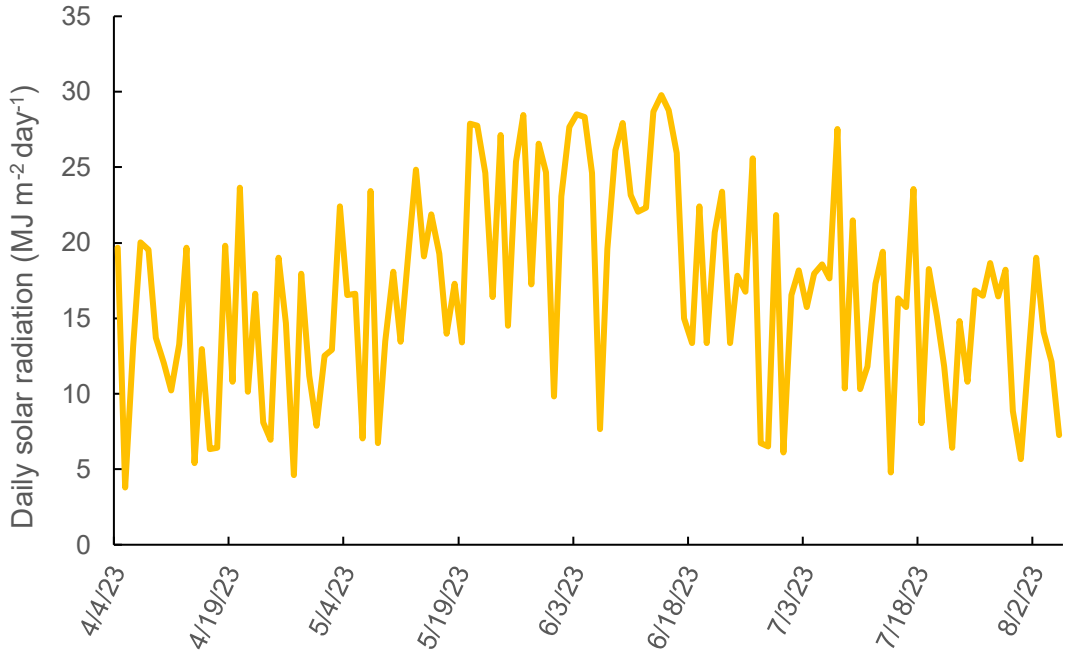


Figure 3.2: Mean daily temperature (a, 2022 and b, 2023) and relative humidity (c, 2022 and d, 2023), recorded inside the polytunnels during the whole cropping period of the field experiments in 2022 and 2023 using Tinytag Ultra 2 data loggers. Daily solar radiation data (e, 2022, f, 2023) was taken from Harper's meteorological station for the cropping period in both years.

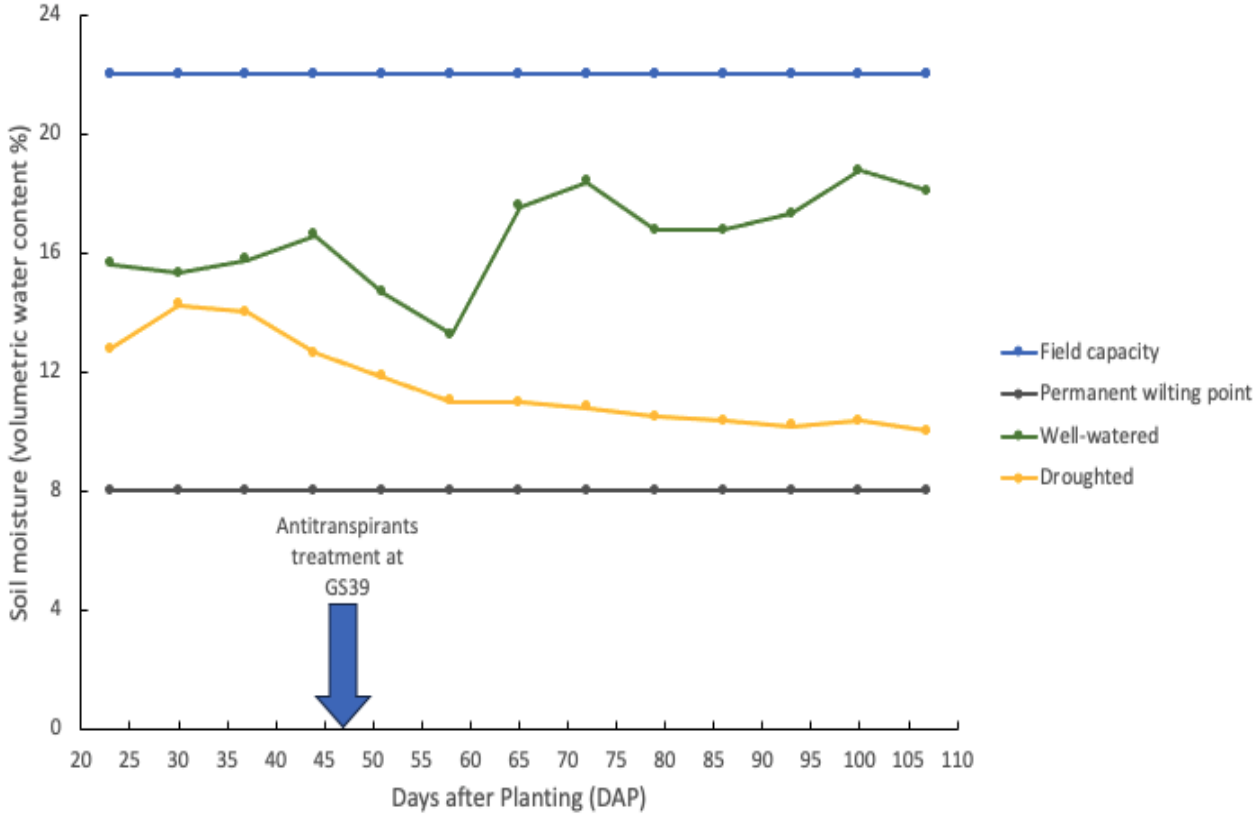
3.3.2 Soil moisture

Soil moisture in the top 50 cm of soil decreased significantly as days after planting increased in the droughted plots of the polytunnels in the two field experiments (2022 and 2023) (Figure 3.3, a and b).

In 2022, the volumetric water content (VWC) of the soil was around 13% (59% of field capacity (FC) with 36% of plant available water and soil-water potential around -80 kPa) when measurements were started after 23 days of planting, and it decreased to around 10% (45% of FC with 14% of plant available water and soil-water potential around -800 kPa) at the end of the grain filling period in the droughted plots. The mean soil moisture value in the well-watered plots was around 17% (77% of FC with 64% of plant available water and soil-water potential around -10 kPa) during most of the cropping season except on a few days where it fell between 13% - 15% due to a heatwave at that time (Figure 3.3, a).

In 2023, soil moisture was approximately 16% (73% of FC with 57% of plant available water, soil-water potential -50 kPa) when moisture readings were started after 26 days of planting, and it reduced to 8% (36% of FC with almost no available plant water in the top 50 cm of soil as soil-water potential reached to -1500 kPa near to the permanent wilting point) during the grain filling period in the droughted plots. In the well-watered plots, mean soil moisture during the whole cropping period remained around 17%, similar to the previous year (Figure 3.3, b). Appendix 8 shows the water retention curve of the soil from the Flatt Nook field.

(a) Year 2022



(b) Year 2023

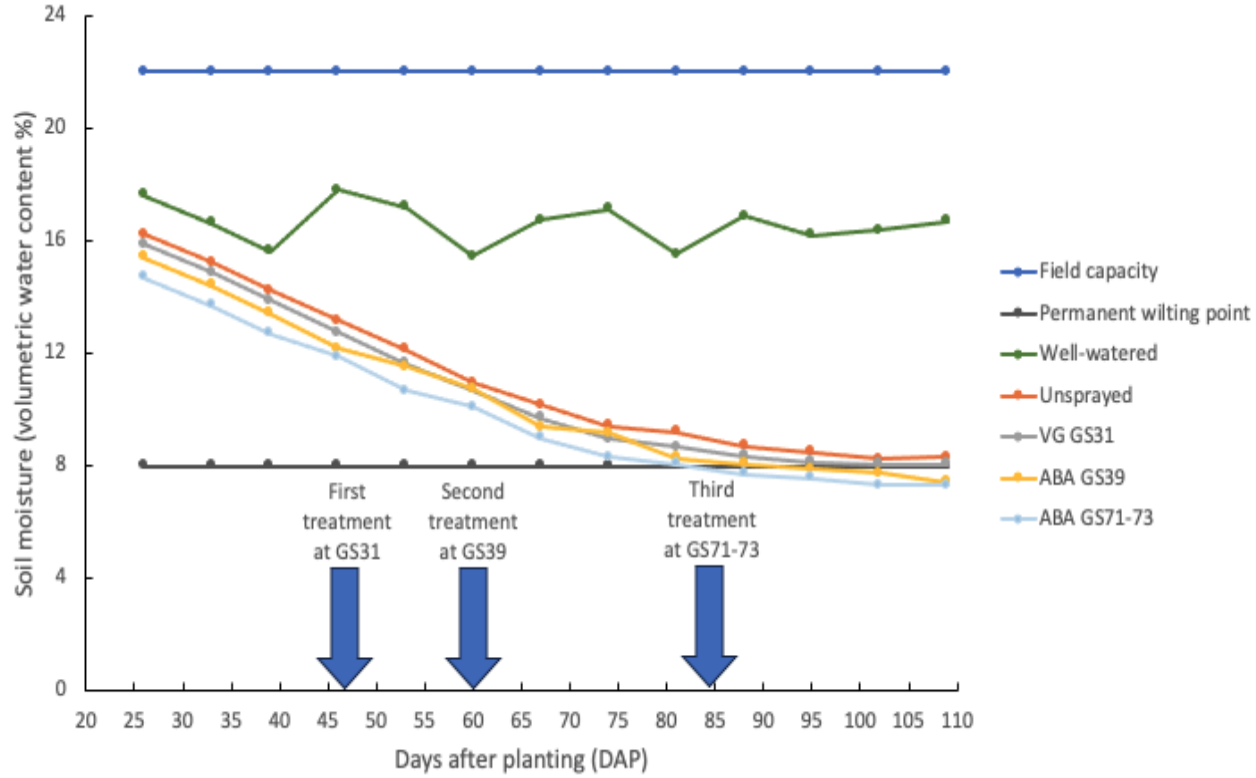


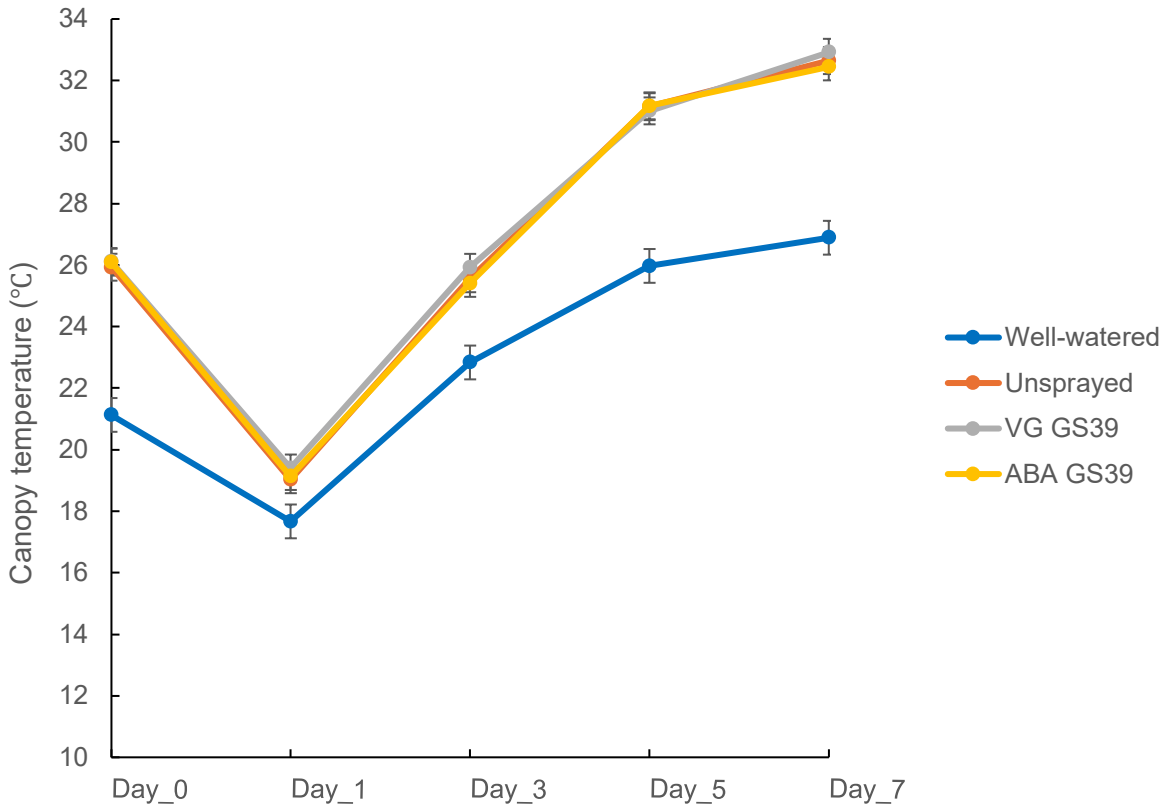
Figure 3.3: Volumetric water content in the top 50 cm of soil of the two field experiments conducted in 2022 (a) and 2023 (b) from the four polytunnels (rain shelters) with values of field capacity (FC) and permanent wilting point (PWP) along with mean soil moisture values (VWC %) of well-watered, unsprayed and antitranspirants treated plants at different growth stages (VG-GS39, ABA-GS39 in 2022 and VG-GS31, ABA-GS39, ABA-GS71-73 in 2023). Blue arrows on each graph represent the days after planting when antitranspirants were sprayed at different growth stages in the two years. Moisture values were taken once a week, and water was given to the well-watered plots for one hour, three days a week, until physiological maturity. In 2022 (a) droughted plots were given water up to 37 days from the date of planting/sowing, and in 2023 (b) water was given up to 21 days from the date of planting, after which progressive drought was started up till physiological maturity in both years.

3.3.3 Canopy temperature

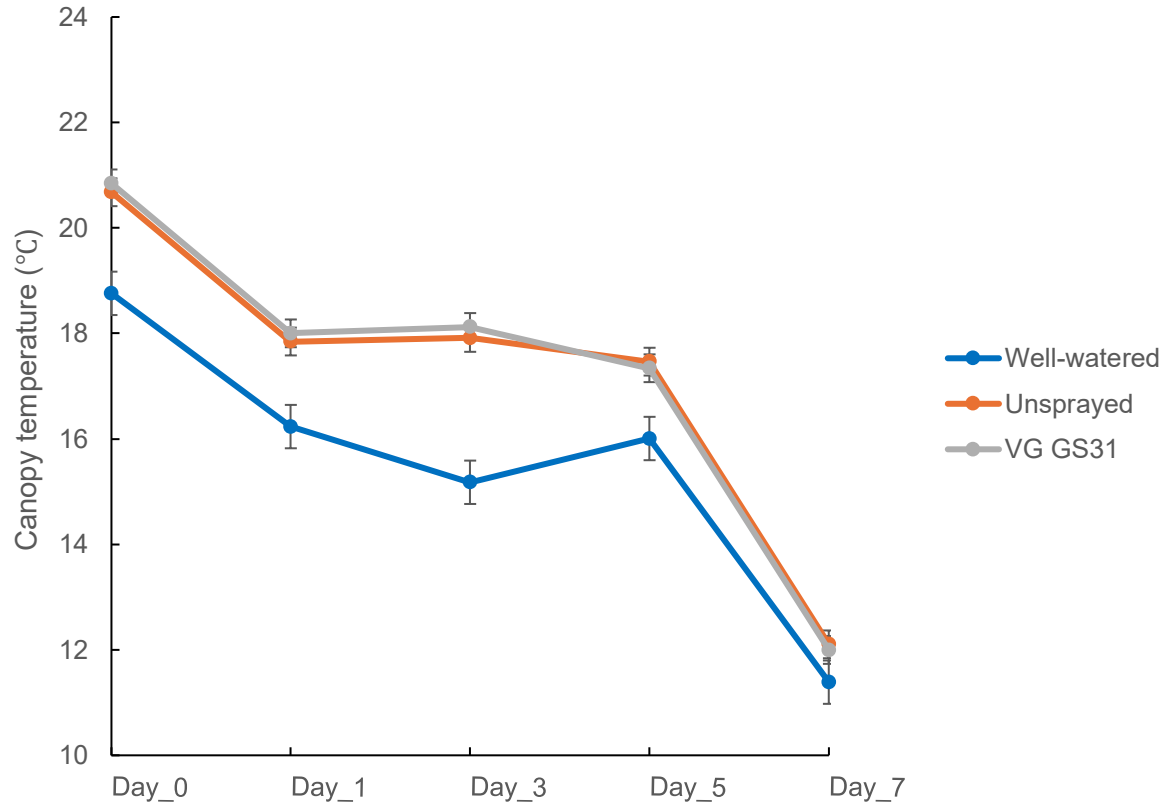
Canopy temperature was lower (by 18% at GS39 in 2022, 11% for GS31 and 15% for GS39 in 2023) in the well-watered plants in comparison to droughted plants in both years of the field experiments (Figure 3.4; a, b and c). However, as the well-watered plots were not randomised in the polytunnels, these were not included in the data analysis. Only data from the droughted unsprayed and antitranspirant-treated plots was used in the repeated measures ANOVA analysis.

There were no significant differences between the canopy temperature of unsprayed and treated plants in both years ($p = 0.797$ in 2022 for GS39 treatments, $p = 0.722$ and $p = 0.326$ in 2023 for data collected after GS31 and GS39 treatments), however, significant differences between days were observed in each year when the data was recorded, which was only due to daily changes in the atmospheric temperature, not because of spraying antitranspirants. Also, the interaction between treatments and days was not significant in both years.

(a)


Canopy temperature (°C)
34
32
30
28
26
24
22
20
18
16
14
12
10
Day_0
Day_1
Day_3
Day_5
Day_7
Well-watered
Unsprayed
VG GS39
ABA GS39

(b)


Canopy temperature (°C)
24
22
20
18
16
14
12
10
Day_0
Day_1
Day_3
Day_5
Day_7
Well-watered
Unsprayed
VG GS31

(c)

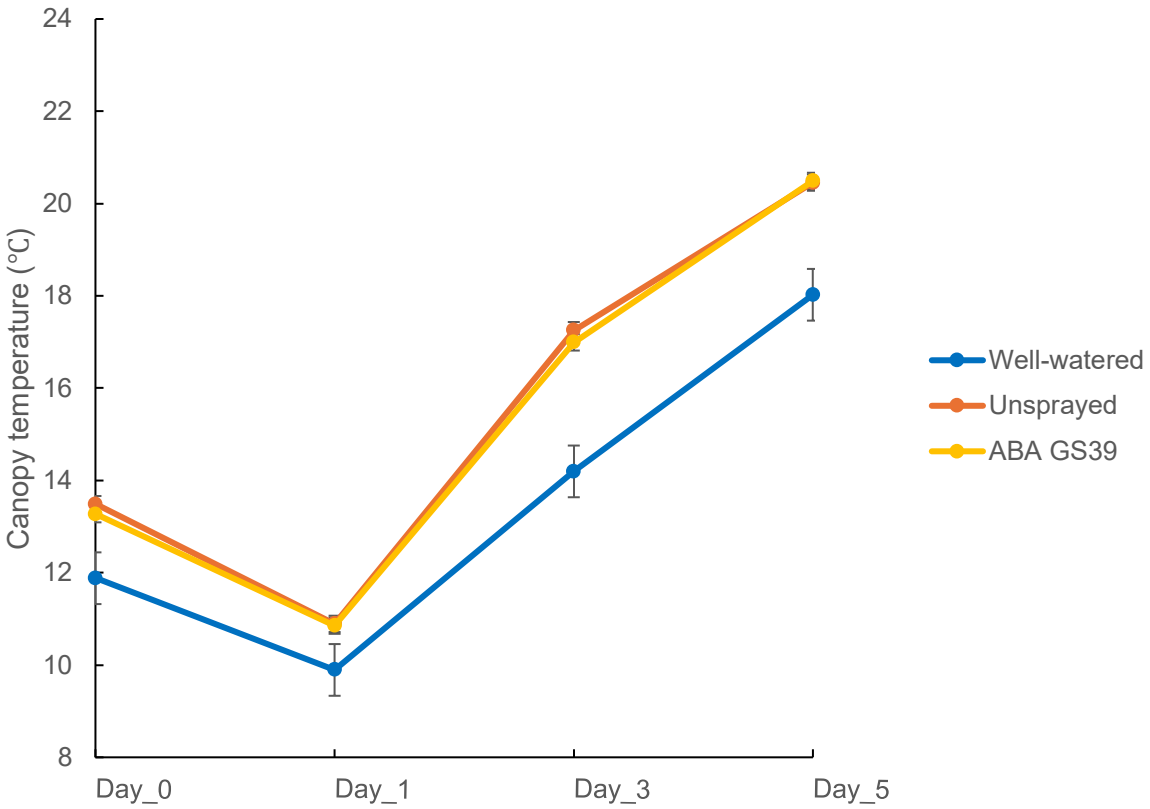


Figure 3.4: Canopy temperature from one day before spraying (Day_0) and one day after spraying (Day_1) up till five or seven days skipping one day in between, in the well-watered (blue line), unsprayed droughted (orange line) and antitranspirants treated plants (VG-GS31, grey line or ABA-GS39, yellow line) in the field experiments of 2022 (a) and 2023 (b and c). Using the FLIR Research Studio software, each data point is the mean of 8 - 12 canopy image values of each treatment, with mean data taken from the pixels of the canopy or leaves of each image, excluding soil pixels with the help of software. Well-watered values are given for comparison purposes only, as these were not included in the statistical analysis. There were no significant differences between different treatments in both years, with $p = 0.797$ for 2022 GS39 treatments (a) and $p = 0.722$ for GS31 and $p = 0.326$ for GS39 treatment in 2023 (b and c respectively), along with no interaction between treatments and days with $p = 0.974$ in 2022 (a) and $p = 0.472$ for GS31 (b), $p = 0.398$ (c) for GS39 in 2023 for the CT of droughted plots excluding the well-watered.

3.3.4 Relative water content

In 2022, the RWC of the droughted plant leaves decreased by 6% compared to well-watered plants, whereas it reduced by 1% and 5% in droughted plants sprayed at GS31 and GS39,

respectively, in 2023. However, as well-watered plants were not included in the statistical analysis; therefore, no significant differences were observed between unsprayed and antitranspirants sprayed plants in both years ($p = 0.464$ in 2022 and $p = 0.701$ at GS31, $p = 0.506$ at GS39 in 2023) (Table 3.2).

In the 2022 experiment, ANOVA contrasts between unsprayed and antitranspirants sprayed (VG-GS39 and ABA-GS39) plants, and a direct contrast between VG-GS39 and ABA-GS39 plants revealed no significant effect between treatments. In 2023, two samplings of leaf RWC were performed, one after the GS31 treatment of VG and one after the GS39 treatment of ABA. In each sampling, leaves were collected from treated, unsprayed and well-watered plants. However, no significant differences were observed between treatments in two RWC samplings in 2023.

3.3.5 Endogenous ABA concentration

Endogenous ABA concentration of leaves collected after GS39 spraying in 2022 ranged between 240 – 258 ng/g in all samples, showing a mean increase of 5% in the droughted leaf samples in comparison to well-watered. In 2023, two leaf samplings were performed, one after GS31 treatment and one after GS39 treatment, the level of mean ABA concentration in the droughted samples was around 430 ng/g and 208 ng/g, respectively, with 0.5% decrease and 59% increase in concentration as compared to well-watered samples at each growth stage.

In both years, spikes were also collected at the watery to milky grain stage (GS71-73) to determine their endogenous ABA concentration, which ranged between 124 – 135 ng/g in 2022 and 224 – 268 ng/g in 2023 in all the samples (including well-watered), with 3% decrease and 14% increase in the droughted samples, respectively, as compared to well-watered (Figure 3.5, a and b).

Endogenous ABA concentration either in leaf or spike samples in 2022 and 2023 did not reveal a significant effect of antitranspirants, when compared with droughted unsprayed samples. Also, no significant differences were observed in the contrast comparisons between unsprayed and sprayed samples and in a direct comparison of VG and ABA treatments at GS39 in 2022 (Table 3.2).

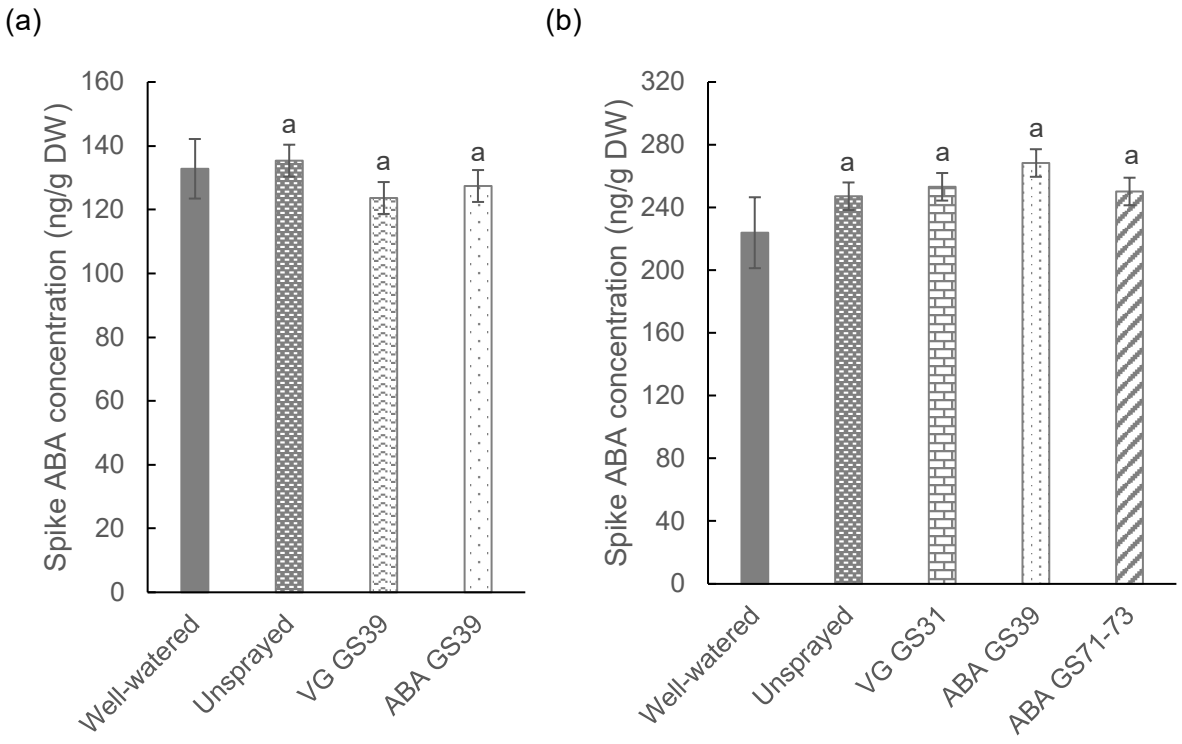


Figure 3.5: ABA concentration in spikes (ng/g DW) from 2022 (a) 2023 (b) field experiments, sampled from well-watered, droughted unsprayed and different antitranspirant-treated plants at GS39 (VG and ABA) in 2022, and at GS31 (VG), GS39 (ABA) and GS71-73 (ABA) in 2023. There was no significant difference between unsprayed and treated spike samples in both years ($p = 0.288$ and $p = 0.345$ in each year, respectively), as represented by similar letters from the Tukey post-hoc test. Well-watered bars are shown for comparison purposes. The error bar indicates the standard error of the mean (SEM) from the ANOVA analysis (SEM = 5.002 and SEM = 8.794 in 2022 and 2023, respectively). Spikes were collected 76 days after planting (DAP) in 2022 and 92 DAP in 2023 from droughted and well-watered plots.

3.3.6 Pollen viability

There was a 12% decrease in pollen viability of stressed plants in 2022 (81%, mean of unsprayed and treated plants) when compared to well-watered (92%) plants, with around 4% reduction in antitranspirant-treated plants (80%, mean of antitranspirants) in comparison to unsprayed (83%). In 2023, it was reduced by 5% in the droughted plants (84%) in comparison to well-watered (88%), with just a 0.4% decline in the viability of antitranspirant-treated when compared to unsprayed plant pollen.

Pollen viability showed no significant difference between different treatments either in 2022 or 2023, p = 0.197 and p = 0.808 in each year, respectively, with the main comparison between antitranspirant-treated and unsprayed plants excluding well-watered (Table 3.2) (Figure 3.6, a and b).

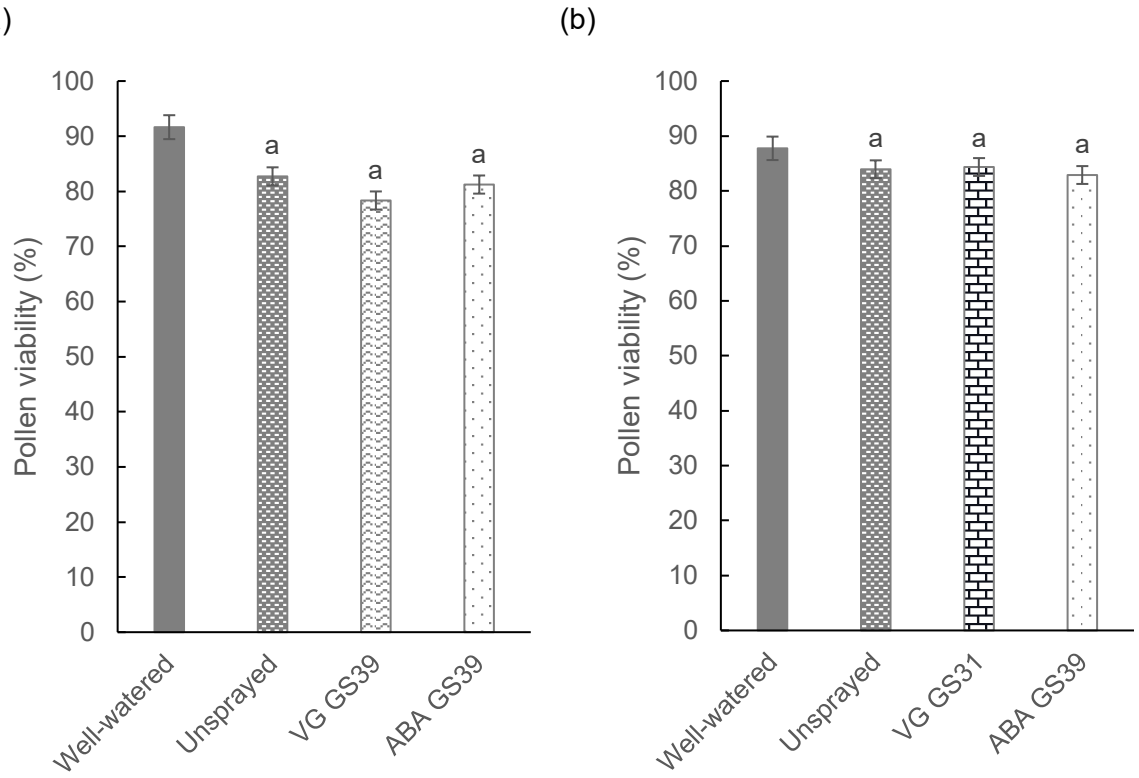


Figure 3.6: Pollen viability (%) of well-watered, unsprayed droughted and different antitranspirant-treated plants at GS39 (VG and ABA) in 2022 (a) and at GS31 (VG) and GS39 (ABA) in 2023 (b). There was no significant difference between unsprayed and sprayed treatments (p = 0.197, SEM = 1.644 and p = 0.808, SEM = 1.623 in 2022 and 2023, respectively). The same letters from the Tukey post-hoc test show no significant effect between treatments. Collection of anthers for assessing pollen viability started 55 days after planting (DAP) in 2022 and 69 DAP in 2023, with all samples completed in two- and one-week period in both years, respectively.

3.3.7 Yield and yield components

The drought stress decreased the yield and yield components of the droughted plants in 2022 and 2023 in comparison to well-watered, except in TGW (Table 3.3 and Figure 3.7); however, as the well-watered plots were not randomised, these were not included in the

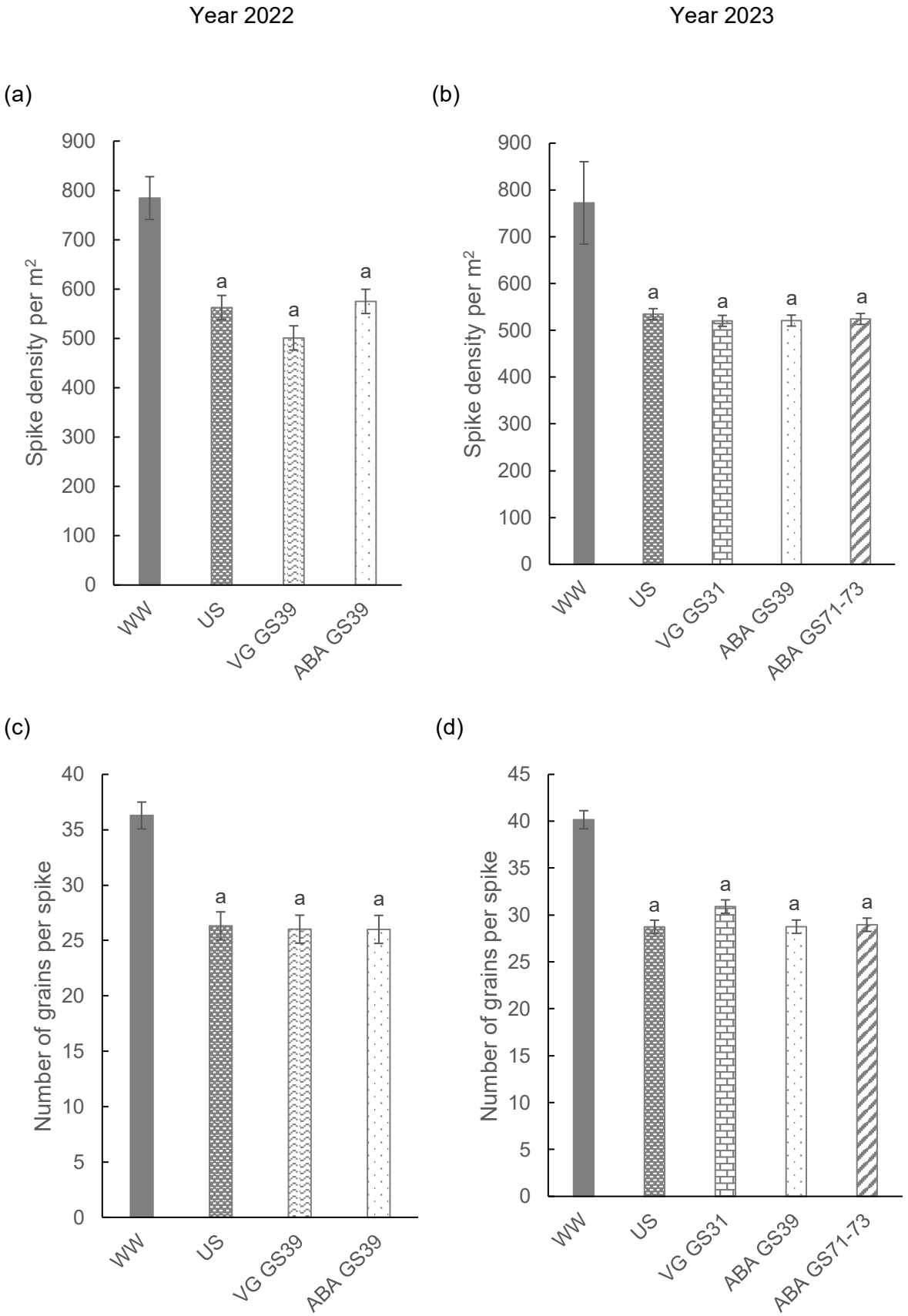
statistical analysis in both years. There was no significant effect of antitranspirants on the plants in different treatments in all the yield and yield components except in ANOVA analysis of TGW in 2023, when sprayed and unsprayed plants were compared ($p < 0.01$). AVOVA contrasts in both years also revealed no significant differences between unsprayed and sprayed plants in all the yield components except for TGW in 2023, where a significant decrease was observed ($p = 0.043$) between unsprayed and treated plants. One of the direct contrasts between VG-GS39 and ABA-GS39 in 2022 also showed no significant differences in all yield components (Table 3.3).

Spike density per m^2 was reduced by 30% and 32% in 2022 and 2023, respectively, in the droughted plots (unsprayed and treated ones), while drought stress resulted in a 28% and 27% reduction in the number of grains per spike in 2022 and 2023 when compared with the well-watered plants.

The number of grains per m^2 decreased by 46% and 53% under drought stress in comparison to well-watered samples in 2022 and 2023, respectively. Spraying antitranspirants further reduced the grain number by 6% and 2%, respectively, in each year when compared to unsprayed samples; however, it was not a significant reduction as mentioned earlier, with most of the results indicating no significant effect from spraying antitranspirants.

Grain yield (tonnes per hectare) also declined by 36% in 2022 and by 52% in 2023 under water stress when compared with the well-watered plots, and the yield of unsprayed plots was a little better (but not significant) as compared to sprayed ones. In the sprayed plots, yield was reduced by 9% and 5% in 2022 and 2023, respectively, in comparison to unsprayed plots.

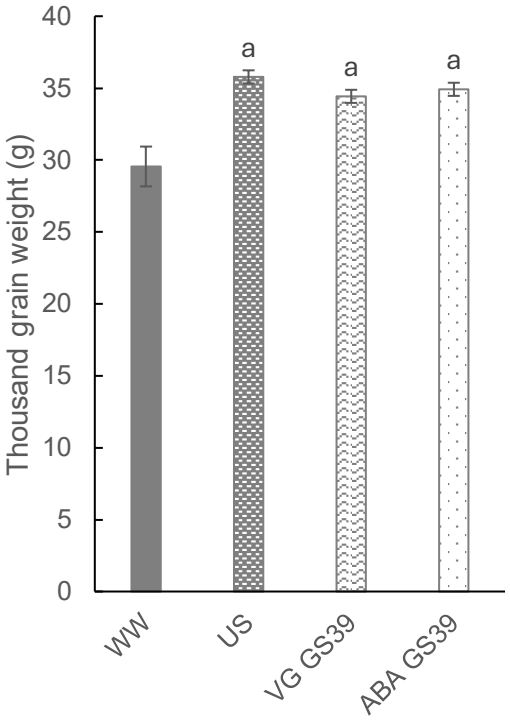
Thus, the overall application of antitranspirants at different growth stages in both years under droughted conditions showed no effect on yield and yield components except in TGW in 2023, which revealed a significant effect of antitranspirants (significant reduction) when compared with unsprayed plants.


Year 2022
Year 2023
(a)
(b)
(c)
(d)
Spike density per m2
Number of grains per spike
WW
US
VG GS39
VG GS31
ABA GS39
ABA GS71-73
a

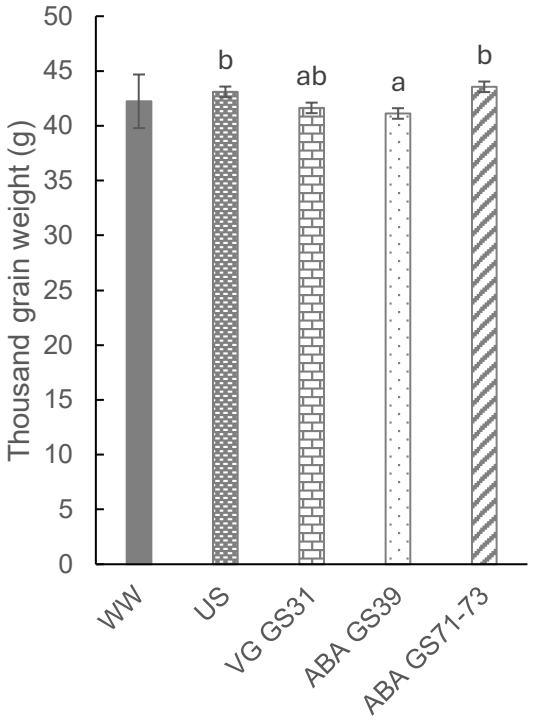
Year 2022

Year 2023

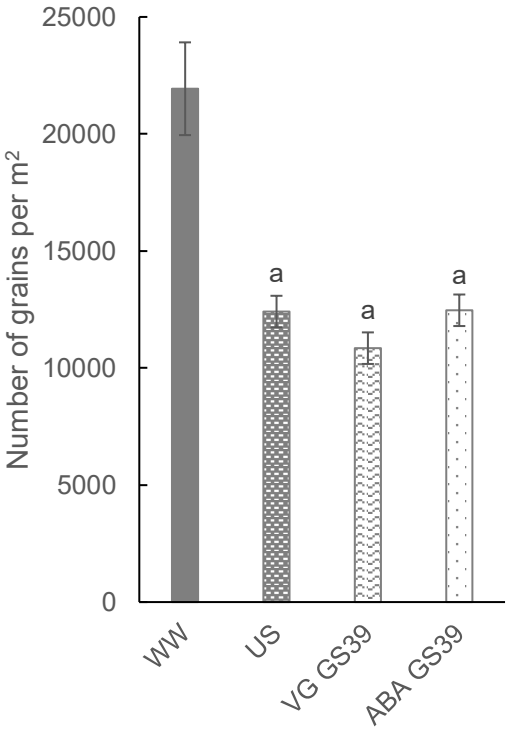
(e)



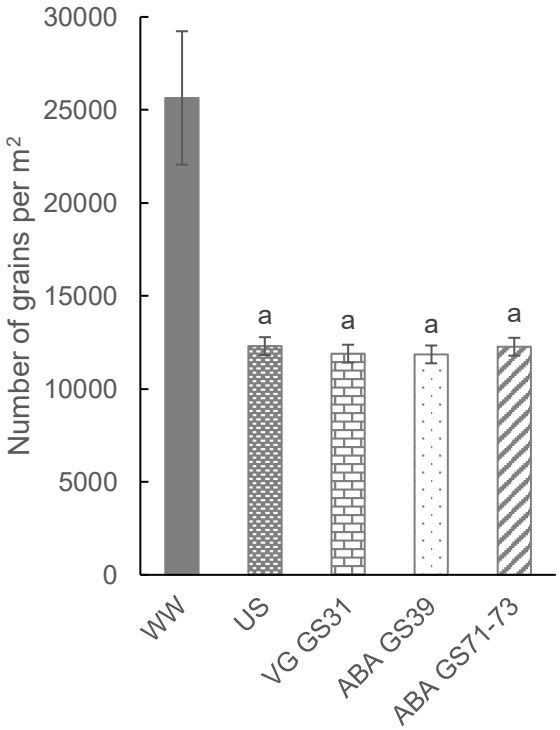
(f)



(g)



(h)



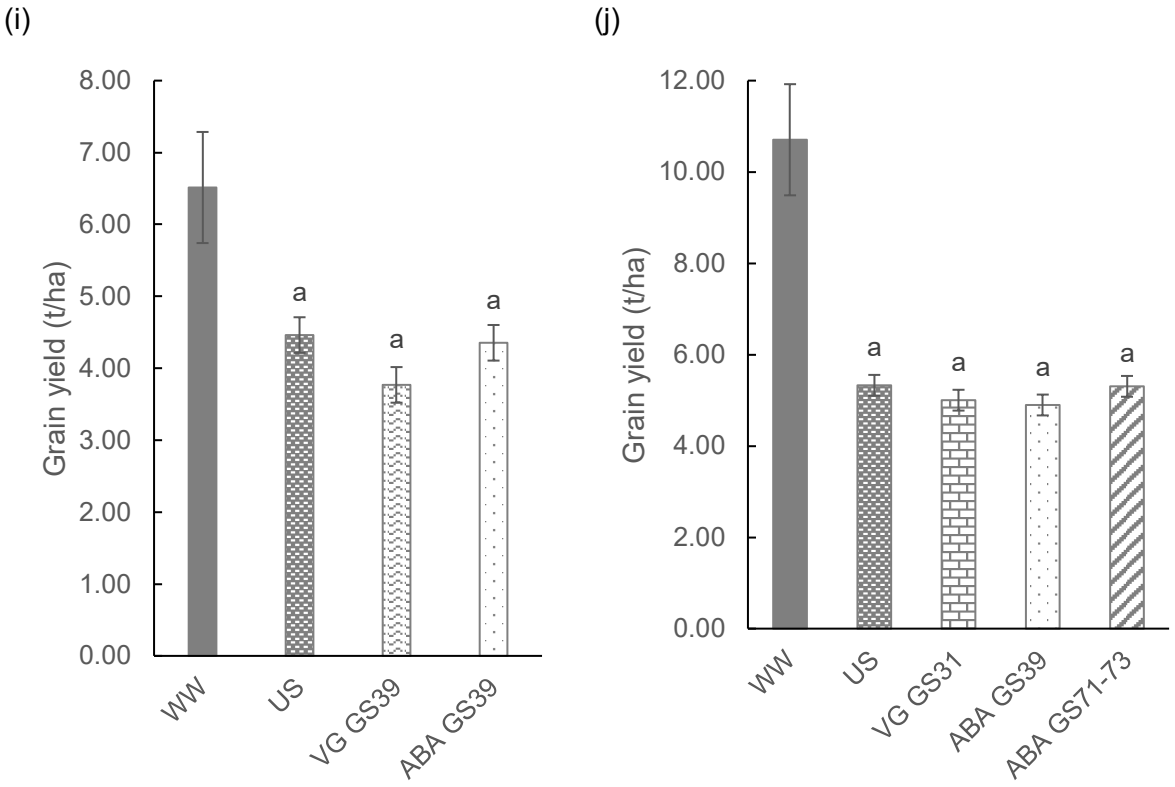


Figure 3.7: Yield components, spike density per m^2 (a, b), number of grains per spike (c, d), thousand grain weight (e, f), number of grains per m^2 (g, h) and grain yield (t/ha) (i, j) from the field experiment 2022 and 2023, respectively. All the yield components data was collected from the well-watered, droughted unsprayed and antitranspirant-treated plants (VG and ABA) at different growth stages. VG and ABA treatment at GS39 in 2022 for all yield components (a, c, e, g and i) and at GS31, GS39 and GS71-73 for all yield components (b, d, f, h and j) in 2023. The same or different letters on the bars represent no or significant differences from the Tukey post-hoc test. SEM and p values of each field component are as follows: (a) p = 0.111, SEM = 24.581, (b) p = 0.804, SEM = 11.702, (c) p = 0.980, SEM = 1.265, (d) p = 0.110, SEM = 0.711, (e) p = 0.143, SEM = 0.455, (f) p = 0.009, SEM = 0.481, (g) p = 0.195, SEM = 674.025, (h) p = 0.864, SEM = 480.009, (i) p = 0.142, SEM = 0.247, (j) p = 0.450, SEM = 0.227. Well-watered (WW) bars are for comparison purposes only, as WW samples were not included in the statistical analysis.

Table 3.2: Mean values of treatments of different parameters (relative water content, pollen viability and endogenous ABA concentration of leaf and spike samples) of the field experiment 2022 and 2023, along with results (p values - degree of freedom (df)) from the ANOVA analysis.

Year	Treatment	Relative water content (%) - leaves collected 8 DAS		Pollen viability (%)	Endogenous ABA concentration (ng/g DW) - leaves collected 8 DAS		Endogenous ABA concentration (ng/g DW) - spikes
2022	Well-watered	89.80		91.63	240.03		132.79
	Unsprayed (US)	85.42 (a)		82.70 (a)	250.91 (a)		135.34 (a)
	VG-GS39	84.85 (a)		78.33 (a)	257.56 (a)		123.63 (a)
	ABA-GS39	83.23 (a)		81.22 (a)	246.36 (a)		127.40 (a)
	ANOVA - p value (df)						
	p value (treatment)	0.464 (2)		0.197 (2)	0.622 (2)		0.288 (2)
	Contrast 1 (US vs AT-treated)	0.386 (1)		0.168 (1)	0.916 (1)		0.169 (1)
	Contrast 2 (VG-GS39 vs ABA-GS39)	0.380 (1)		0.234 (1)	0.341 (1)		0.445 (1)
	SEM (residual df)	1.260 (14)		1.644 (14)	8.048 (14)		5.002 (13)
2023	Well-watered	96.41	95.05	87.76	432.12	130.64	223.90
	Unsprayed	95.27 (a)	90.16 (a)	83.94 (a)	426.46 (a)	212.86 (a)	247.16 (a)
	VG-GS31	95.18 (a)	N/A	84.35 (a)	433.48 (a)	N/A	253.18 (a)
	ABA-GS39	N/A	90.91 (a)	82.89 (a)	N/A	202.19 (a)	268.35 (a)
	ABA-GS71-73	N/A	N/A	N/A	N/A	N/A	250.19 (a)
	ANOVA - p value (df)						
	p value (treatment)	0.701 (1)	0.506 (1)	0.808 (2)	0.706 (1)	0.669 (1)	0.345 (3)
	Contrast (US vs AT-treated)			0.875 (1)			0.556 (1)
	SEM (residual df)	0.155 (11)	0.774 (11)	1.623 (22)	12.853 (11)	17.171 (11)	8.794 (33)

Well-watered values in all the parameters are given for comparison purposes only, as these were not included in the statistical analysis. Similar letters with mean values of each treatment indicate no significant differences between treatments from the Tukey post-hoc test. N/A means that no samples were taken for that treatment. SEM is the standard error of mean, DAS is days after spraying, and AT represents antitranspirants.

Table 3.3: Mean values of yield components (spike density per m^2, number of grains per spike, thousand grain weight, number of grain per m^2 and grain yield) of the field experiments 2022 and 2023, along with results (p values - degree of freedom (df)) from the ANOVA analysis.

Year	Treatment	Spike density per m^2	Number of grains per spike	Thousand grain weight	Number of grains per m^2	Grain yield (t/ha)
2022	Well-watered	784.44	36.29	29.55	21932.86	6.51
	Unsprayed	562.50 (a)	26.32 (a)	35.78 (a)	12412.31 (a)	4.46 (a)
	VG-GS39	500.83 (a)	26.02 (a)	34.43 (a)	10848.62 (a)	3.77 (a)
	ABA-GS39	575.00 (a)	26.00 (a)	34.92 (a)	12467.05 (a)	4.35 (a)
			ANOVA - p value (df)			
	p value (treatment)	0.111 (2)	0.980 (2)	0.143 (2)	0.195 (2)	0.142 (2)
	Contrast 1 (US vs AT-treated)	0.428 (1)	0.845 (1)	0.068 (1)	0.377 (1)	0.209 (1)
	Contrast 2 (VG-GS39 vs ABA-GS39)	0.052 (1)	0.992 (1)	0.461 (1)	0.113 (1)	0.118 (1)
	SEM (residual df)	24.581 (13)	1.265 (13)	0.455 (13)	674.025 (13)	0.247 (13)
2023	Well-watered	772.28	40.16	42.24	25641.49	10.71
	Unsprayed	534.72 (a)	28.73 (a)	43.10 (b)	12300.35 (a)	5.33 (a)
	VG-GS31	520.25 (a)	30.90 (a)	41.64 (ab)	11893.52 (a)	5.00 (a)
	ABA-GS39	520.83 (a)	28.75 (a)	41.13 (a)	11852.43 (a)	4.90 (a)
	ABA-GS71-73	524.31 (a)	28.96 (a)	43.56 (b)	12265.05 (a)	5.31 (a)
			ANOVA - p value (df)			
	p value (treatment)	0.804 (3)	0.110 (3)	0.009 (3) **	0.864 (3)	0.450 (3)
	Contrast (US vs AT-treated)	0.943 (1)	0.545 (1)	0.043 (1) *	0.655 (1)	0.388 (1)
	SEM (residual df)	11.702 (32)	0.711 (32)	0.481 (31)	480.009 (32)	0.227 (32)

Well-watered values in all the parameters are given for comparison purposes only, as these were not included in the statistical analysis. Similar or different letters with mean values of each treatment indicate no significant differences or significant effects between treatments from the Tukey post-hoc test. *, ** represents significance less than 0.05 and 0.01, respectively, from the ANOVA analysis.

3.4 Discussion

The spring wheat variety (Chilham) used in the two field experiments (2022 and 2023) was similar, as investigated by Mphande et al. (2021b) on the same site and soil type. The main objective of the study was to investigate the effect of film (VG) and metabolic (ABA) antitranspirants at different growth stages for any improvement in the yield under water stress conditions, as reported in the previous study. The type of film antitranspirant used was the same, but the growth stages selected for spraying were different, along with variation in the sowing dates (end of April and March by Mphande et al. (2021b), whereas the middle of June and start of April (present study) in two years of each study, respectively). Also, the type of metabolic antitranspirant (ABA) used in this case was different ((+)-ABA or S-ABA, which is a naturally active form of isomer) than that used by Mphande et al. (2021a and 2024) ((±)-ABA, which is a mixture of active and inactive form of isomers).

Soil moisture during the anthesis till grain filling period was around 10% - 11% VWC (45% - 50% of FC with 14% - 21% of available water) in 2022 and 8% - 9% VWC (36% - 41% of FC with 0% to 7% of available water) in 2023 was enough to characterise as moderate to severe drought stress according to the study by Zhao et al. (2020). This is mainly referred to as terminal drought (during the anthesis and grain filling period) as it leads to substantial yield losses in wheat, as summarised in a review by Farooq et al. (2014). Due to terminal drought, yield declined to 36% and 52% in 2022 and 2023, respectively, in comparison to well-watered plants, while Mphande et al. (2021b) indicated the average yield loss of 53% of a similar variety under drought stress with soil moisture of 9% (41% of FC) during the grain filling period. This suggests that soil moisture conditions were almost similar (8.5%, 39% of FC in 2023 at GS71-73) during the grain filling period to understand the effect of antitranspirants, however, there was no evidence of improved yield with antitranspirants (film or metabolic) application at different growth stages in contrast to Mphande et al. (2021b), who found enhanced yield with film antitranspirant on the same variety.

One reason for these contrasting results might be due to the differences in soil moisture conditions when antitranspirants were sprayed. Mphande et al. (2021b) indicated soil moisture values around 12% at GS31-GS37 in 2019 when film antitranspirant was applied, while in the present study, it was around 12% and 10.6% at GS39 in 2022 and 2023, respectively, which seemed adequate in 2022 but lower in 2023. Moreover, in 2022 plants reached the GS39 growth stage (47 days after planting) almost two weeks earlier than Mphande et al. (2021b), as the process of development was faster due to the sowing in

June, while in 2023, plants reached GS39 in 60 days which can be compared to Mphande et al. (2021b) study in which plants reached GS37 in 58 days in 2019 (one of the field experiments). This suggests that differences in antitranspirant responses of this study in comparison to Mphande et al. (2021b) might be related to different soil moisture conditions when antitranspirants were applied as it was lower (10.6%, 48% of FC) before the start of meiosis (GS39) and near to permanent wilting point (8.5%, 39% of FC) at watery to milky grain stage (GS71-73) in 2023 experiment.

There could be another reason that soil moisture values might be overestimated, based on which field capacity was calculated to compare the effect of drought with the previous studies. Soil moisture values were recorded up to a depth of 50 cm in both years. However, spring wheat roots can extend to a depth of around 100 cm (Thorup-Kristensen et al., 2009). There was also an indication from the 2023 experiment that plant roots started to extract water from 50 cm depth after 5 - 6 weeks of sowing (Appendix 12a). This might have led to the overestimation of soil moisture values and, hence, the level of drought stress when antitranspirants were applied. Plants might not be under stress when VG was applied at GS31 (instead of around mild stress with 58% of FC, as data suggested) and under mild drought stress at GS39 when ABA was sprayed, instead of around moderate stress, as data indicated with 48% of FC. However, plants were likely around moderate stress at GS39 (if most of the roots extracted water from the top 50 cm of soil), but if water was extracted from below 50 cm depth before the spraying at GS39, plants might be under severe moisture stress as there might not be sufficient water to extract from deeper layers of soil because the polythene sheet was not removed from the polytunnels after harvesting of the previous crop so, that enough moisture can be stored in soil depth for the next crop (as already explained in section 3.2.2 of this chapter). This might explain the lack of response on grain yield from antitranspirants sprayed at GS31 and GS39 growth stages in the 2023 field experiment. Moreover, it might also depend on selecting the appropriate growth stage at which antitranspirants were applied, such as GS71-73 in the 2023 experiment might seem late to get an effective response from the metabolic antitranspirant used in this study as soil moisture level by then was near to the permanent wilting point (8.5%, 39% of FC). Mphande et al. (2021b) also observed no response of improved yield from film antitranspirant sprayed at GS51 and GS65 with soil moisture around 8% - 9% in one of the experiments, which might also be the result of low soil moisture level at that time. Thus, it indicates that drought stress at critical growth stages leads to high yield losses if the optimum amount of water is not available, especially during the anthesis and grain filling period. Furthermore, even if antitranspirants are sprayed near the reproductive or grain-filling stage to help plants survive

in water deficit conditions, it might also depend on the magnitude of drought stress to get any improved response from antitranspirants.

Plants either fully or partially close their stomata to reduce water loss from the leaf surface under water deficit environments, which helps them to improve their water status and to survive under harsh conditions. Canopy temperature (CT) has been used as a stress indicator in many studies, and it is an indirect way of measuring transpiration (Deery et al., 2019). It can be an alternative to repeated assessment of stomatal conductance among a large number of early-generation wheat breeding lines, as it is a quick method for indirect screening of low or high stomatal conductance (Rebetzke et al., 2013). Furthermore, various studies revealed differing effects of antitranspirants, based on their type, on the leaf/canopy temperature of diverse crop plants. Reflective antitranspirants (such as kaolin) were indicated to reduce leaf temperature in different fruit crops such as mango, gooseberry, and tomatoes under drought stress as summarised by Mphande et al. (2020), however, methene and pinolene-based antitranspirants were reported to increase fruit and leaf temperature in citrus (Rodriguez et al., 2019). The present study revealed a big difference in CT of well-watered and droughted plots. Well-watered plots had lower CT, indicating cooler canopies compared to droughted plots, as also observed by Kurunc et al. (2023) in wheat plants under different drought stress levels and control plants. This suggests that the transpiration rate was higher as indicated by cooler canopies in the well-watered plots, which leads to high yield compared to droughted ones. However, as the well-watered plots were not included in the data analysis, therefore, it is not certain whether the difference in CT values with the droughted plots was significant or not. Furthermore, CT was almost similar in all the droughted plots (with negligible differences that were not significant), thus indicating no effect of antitranspirants on CT of droughted plots, as all the plots had the same CT, either sprayed or not.

Relative water content is an efficient and simple method of measuring the water status of plants under water stress conditions. Lugojan and Ciulca (2011) assessed the leaf water content of winter wheat genotypes, their parents and hybrid crosses for early screening of drought tolerance, and they observed that 24% of the hybrids showed a 10% increase in RWC of leaves as compared to their parents which suggests that genetic makeup of plants improved in hybrids for RWC related traits. The use of antitranspirants (kaolin as reflective and chitosan as metabolic) increased the leaf water potential, relative water content and total chlorophyll with improved yield in maize by reducing the rate of transpiration, as observed by Morsy and Mehanna (2022). A previous study by Mphande, (2021) on a spring wheat variety revealed that film antitranspirant (VG) increased the RWC of plants when compared with

water-sprayed plants, while in this study same variety was used, but both antitranspirants (film – VG and metabolic – ABA) did not show any significant differences in RWC when compared with unsprayed droughted plants. There was a slight reduction of RWC by 2% in 2022 at GS39 with both antitranspirants and a slight increase of 1% in RWC from ABA spraying at GS39 in 2023; however, all these were not significant, indicating no effective response from antitranspirants at any of the growth stages. As there was no improvement in the RWC of plants with spraying antitranspirants in both years, that might also be the reason for getting the consistent response of no effect on yield.

Plants produce abscisic acid under drought stress that helps to regulate physiological, biochemical and molecular changes in plants to cope in stressed environments. The literature revealed that exogenous ABA spraying (S-ABA, 1 mM/1000 µM applied at different growth stages) can improve the transport of photoassimilates from the leaves and stem to the developing grains, thus increasing the sink strength of grains (Travaglia et al., 2010). Exogenous ABA application on winter wheat at the flowering stage (10 mg/l or 38 µM/l for three days) revealed that it increased grain filling rate and starch accumulation content, resulting in increased 1000 grain weight and yield (Yang et al., 2014) and a similar kind of improvement in the grain filling process of maize was indicated by Yu et al. (2024). In the present study, exogenous ABA (S-ABA 6 g/ha) application in two separate treatments, one before the start of meiosis (GS39) and one at the watery to milky grain stage (GS71-73), were assessed for their impact on grain yield under drought stress, however, no significant improvement in the yield was observed, and it was almost same in the unsprayed droughted plots. Furthermore, endogenous ABA content inside leaves and spike samples from different treatments was increased in most of the droughted samples in comparison to well-watered ones; however, spraying ABA did not make much of a difference in the endogenous ABA content, suggesting that a single dose of exogenous ABA application was not effective. The reason for this could be that by the time sampling was done after eight days of spraying, all the droughted samples had almost the same concentration of endogenous ABA as the effect of spraying exogenous ABA might be diluted away by then or spraying ABA under low moisture conditions did not make much of the difference to the endogenous ABA content. However, the concentration of ABA might also play an important role, as a high exogenous concentration of ABA (100 µM) reduced the process of starch synthesis in grains when compared with a low concentration (1 µM) in a wheat study by Ahmadi and Baker (1999). Another study on winter wheat with foliar application of ABA (different concentrations ranged from 0.03 to 0.06 g/l (113 to 227 µM/l) with two, three and four applications) and fulvic acid in water stress conditions revealed that ABA worked better with less frequency and concentration in improving the grain yield performance and transfer of assimilates (Zhang et

al., 2016). Mphande et al. (2021a) used 100 µM (5.3 g/ha (±)-ABA) of ABA with one or multiple applications in two glasshouse experiments, while in the present study, only one application of 113 µM/l (6 g/ha S-ABA) of exogenous ABA was used either at GS39 or GS71-73. The concentrations of applied ABA might vary a little in the previous studies along with the composition (such as S-ABA is the naturally occurring active form of ABA while (±)-cis, trans-ABA contains both active and inactive forms of isomers (Finkelstein, 2013)), application level/rate of the ABA products, along with the applied growth stages that can also influence plants in terms of their response in grain yields of different crops. Furthermore, the association of low endogenous ABA concentration was suggested to be linked with film antitranspirant application, resulting in ameliorating the effects of drought by Mphande et al. (2021b); however, there was no evidence of this association in the present study in both years with film antitranspirant.

Reproductive stage drought is the main cause of pollen sterility that leads to lower crop yields (Yu et al., 2019). Even if pollens are viable, drought can affect their germination or fertilisation process; thus, the whole reproductive process is at risk if plants face stressed conditions. Under normal conditions, pollen accumulates starch to germinate and form the pollen tube, while this process is affected under water stress and sterile pollen either has less or no starch (Jin et al., 2013). A study on the improvement of pollen viability was observed in wheat after the application of film antitranspirant (at GS31 and GS33) that also enhanced the grain number and yield under limited water conditions (Weerasinghe et al., 2016), while in another study on corn, a significant increase in pollen viability was observed with the spraying of antitranspirants agents (glycerol 4%, green miracle 0.3%) with zinc at 6 - 8 leaf stage and a flowering stage (Abbas and Abdul-Razak, 2019). These studies suggest that the use of antitranspirants under stress conditions can be beneficial for the crop to produce fertile pollen, which can increase crop yield. However, in the present study, no improvement in the pollen viability was observed with antitranspirants (film and metabolic) application at GS31 or GS39 in the two field experiments. In 2022, pollen fertility was checked from VG-GS39 and ABA-GS39 sprayed plots, while in 2023, VG-GS31 and ABA-GS39 plots were assessed, but there was no effect of antitranspirants on pollen fertility in comparison to droughted unsprayed plots. There was a slight decrease in the pollen viability (by 4% and 0.4% in 2022 and 2023, respectively) with the spraying of antitranspirant either at GS31 or GS39, however, it was not significant. Thus, these are the contrasting results as observed by Weerasinghe et al. (2016) that film antitranspirant application on wheat at GS31 or GS33 significantly increased pollen viability, however, Weerasinghe et al. (2016) did not observe any significant differences in pollen viability between film antitranspirant sprayed plants at GS41 and unsprayed droughted plants. As the GS39 growth stage in the present

study was almost near to GS41 (as observed in the Weerasinghe et al. (2016) study) that might be one of the reasons that pollen viability was not improved when sprayed at GS39 as it might be late for film or metabolic antitranspirant to alleviate the effect of drought by that time, however, spraying of film antitranspirant (VG) at GS31 in 2023 also did not show any significant differences in results thus contradicting to the previous study. As pollen viability was not affected (only a slight decrease with no significance) by antitranspirants, therefore, yield data indicated a similar pattern of no effect (a slight reduction with no significance, 9% and 5% in 2022 and 2023, respectively) on the yield with antitranspirants application at any of the observed growth stages in both years. Some of the reasons for these contradicting results in comparison to Weerasinghe et al. (2016) might be the level of soil moisture at the time of antitranspirant application, its dosage, the type of varieties used or environmental conditions at that time. Also, only one growth stage (GS31) can be directly compared with the Weerasinghe et al. (2016) study, where VG was applied in the present study in 2023, but not the others in the 2022 field experiment (such as VG at GS39 or ABA at GS39).

Leaves are the primary source of biomass production in plants, and the resulting photoassimilates produced via carbon fixation are distributed throughout the plant. The sites of assimilation and distribution are referred to as sources (leaves), and those organs that receive assimilates are referred to as sinks (spike/grains) (Thomas, 2016). Drought stress during the reproductive stage of plants can significantly impact the source strength by reducing leaf growth and photosynthetic capacity, thus limiting the production of photoassimilates, which are required for further growth and development (Farooq et al., 2009; Basu et al., 2016; Senapati et al., 2019). Pre-anthesis drought in wheat reduces the sink capacity by decreasing the number of spikes, grains per spike and reducing water-soluble carbohydrate reserves for grain filling, leading to reduced grain size and weight (Yang and Liang, 2025). On the other hand, drought stress during the grain-filling stage impairs the photosynthesis in the flag leaf and inflorescence and accelerates leaf senescence, which reduces the photosynthetic capacity and duration of grain filling, resulting in a significant reduction of TGW, grain yield, biomass and harvest index of wheat cultivars (Saeidi and Abdoli, 2015; Luo et al., 2021). Thus, this source-sink relationship is crucial for determining the crop yield, and drought can impact these dynamics (Basu et al., 2016), affecting the grain yield when it occurs at different growth stages of a crop. In the present study, moderate to severe drought stress from the reproductive to the grain-filling stage of the crop reduced the spike density, number of grains per spike, TGW, number of grains per m^2 and the grain yield of the crop in both years. Moreover, applying antitranspirants to ameliorate the effects of drought revealed no significant response in comparison to the unsprayed droughted plants for most of the yield components except TGW. This suggests

that due to limited photoassimilates production in leaves under drought stress, transport and storage of assimilates into sinks (spikes and grains), along with their capacity, were affected, resulting in lower grain yield, and even applying antitranspirants did not mitigate the effect of drought.

According to Mphande et al. (2021b) film antitranspirant (di-1-p-menthene) improved fertile spike density and number of grains per m^2 when sprayed at different growth stages before and during the reproductive stage (GS31, GS33, GS37, GS45), however, in the present study there was no effect of both film and metabolic antitranspirant on the spike density per m^2 and number of grains per m^2. Film antitranspirant was sprayed at GS39 in 2022 and at GS31 in 2023, while ABA was sprayed at GS39 in both years, with one treatment spray at GS71-73. Both spike density and number of grains per m^2 were reduced slightly with antitranspirant treatments in comparison to unsprayed droughted plots (but it was not significant). This indicates that spraying antitranspirants might not significantly affect crop yield, as also consistent with spike density and number of grains per m^2 results, if sprayed under water-stressed environments. Furthermore, the number of grains per spike reduced by an average of 28% in droughted plots when compared to well-watered ones, while applying either VG or ABA at different growth stages (GS31, GS39, GS71-73) did not show any significant improvement compared to unsprayed ones, which is consistent to Mphande et al. (2021b) results, suggesting no significant impact of antitranspirants on spike fertility.

TGW of droughted plants (including both sprayed and unsprayed) in 2022 and 2023 increased by 19% and 0.3%, respectively, in comparison to well-watered plants, which is contradictory to Poudel et al. (2020), as it indicated that drought decreased TGW by 10.6% compared to irrigated conditions. However, it also depends on the type of genotype response to drought stress, which determines how efficiently assimilates are translocated to the sink organs, which might either maintain/increase or cause the least effect on TGW (Ali et al., 2023). These results of increased TGW under drought stress of the present field experiments compared to well-watered plots are also consistent with one of the Mphande et al. (2021b) field experiments of film antitranspirant treatments at different growth stages. Moreover, the use of kaolin 3% and 6% as an antitranspirant was reported to increase 1000 grain weight (by 9% and 13% respectively) in wheat grown under 40% water holding capacity as compared to control plants, which suggests that antitranspirants can be beneficial in improving crop yield via increasing 1000 grain weight of a crop (Abdallah et al., 2019). Another study of chitosan spraying at the flowering stage on pearl millet under drought stress showed increased TGW and yield (Priyaadharshini et al., 2019). These studies indicated the improvement in TGW with different antitranspirant applications, while in

the recent experiments, TGW of the ABA sprayed plants at GS39 in 2022 and at GS71-73 in 2023 was almost similar to the unsprayed ones, indicating no significant effect from the metabolic ABA treatments. This can be compared with Mphande et al. (2021a and 2021b) findings, who also observed consistent, no significant improvement in TGW with film or metabolic antitranspirant application at different growth stages of the same wheat variety. However, the present study revealed a significant reduction of TGW with film and metabolic antitranspirants at GS31 and GS39, respectively, in 2023, which contradicts the previous findings of either improved TGW or no effect on TGW with antitranspirant application. It suggests that antitranspirants spraying at the stem extension (GS31) stage or before the start of meiosis (GS39) might significantly reduce the sink capacity (spike or grain number/size) to store sufficient photoassimilates. Or it might be possible that spraying antitranspirants before the reproductive stage affected the production of photoassimilates in leaves due to stomatal closure, so there were not enough assimilates produced that could be transported to the developing grains, in contrast to unsprayed plants and ABA sprayed ones at GS71-73. On the other hand, this significant reduction in TGW did not affect the crop yield, as it was the same in the sprayed and unsprayed droughted plants (with no significant differences). Thus, it indicates that applying different types of antitranspirants at critical growth stages for the improvement of TGW or yield might not always significantly increase these parameters, however, it might also depend on the type of crop, its physiology, type of antitranspirants, growth stages, soil moisture condition or several other environmental factors at the time of application.

Grain yield is the most important parameter that shows the effect of antitranspirants appropriately, no matter how these influence other physiological parameters. Many studies indicated the improvement in yield with different antitranspirants in diverse crops, such as wheat, maize, rapeseed (Mphande et al., 2021b; Morsy and Mehanna, 2022; Xiang et al., 2023), sunflower and soybean (Javan et al., 2013; Sanbagavalli et al., 2017). However, in this study, both antitranspirants (film – VG and metabolic – ABA) did not improve the yield but reduced it by 9% and 5% in 2022 and 2023, respectively, when compared with unsprayed plots, although it was not significant thus, it indicates that spraying antitranspirant might not always be effective on plants even when sprayed at critical growth stages. Luo et al. (2021) indicated that exogenous ABA application on wheat at the anthesis stage reduced grain yield and water use efficiency of plants under severe drought stress (30% - 40% soil moisture content), while it was improved under mild and moderate drought stress. However, the soil moisture (45% of FC) was not that severe in this study near the end of the grain filling period in the 2022 field experiment, which can explain reduced grain yield (although not significant) from antitranspirant applications. There might be other reasons for these

results; sowing was delayed in 2022; therefore, development of the plants was faster during the critical growth stages of the crop, which could be the reason that antitranspirants did not make much of a difference in the treated plots. Also, both high temperatures (two heatwaves were observed during the growth period) and droughted conditions during the growth period might be the cause of no effect from antitranspirants. Whereas, in 2023, sowing was done at the optimum time in April, and there were more replicates to understand the effects of antitranspirants, yield reduction with antitranspirants was low (by 5%, which was not even significant) in comparison to the previous year. One reason for this might be the low soil moisture (10.6%, 48% of FC) in the soil near the time of meiosis when metabolic ABA was applied at GS39 compared to Mphande et al. (2021b) study in which film antitranspirant sprayed at GS37 with around 12% soil moisture value (55% of FC). Thus, the low soil moisture at GS39 of the present 2023 study was further reduced to 8.5% (39% of FC) during the grain filling period (GS71-73), at which metabolic ABA was also sprayed in one of the treatments, but did not show a significant effect. Also, plants might be under severe moisture stress (instead of around moderate stress (48% of FC) at GS39), resulting in no response from metabolic antitranspirant when sprayed either at GS39 or GS71-73. This suggests that spraying antitranspirants at any growth stage might also depend on the soil moisture conditions when applied, and spraying antitranspirants under these conditions might not always work or show any effect on plants and can reduce the yield of plants instead of improving it (although this reduction with antitranspirants was not significant in comparison to unsprayed plants). Moreover, this also depends upon the physiological and molecular responses of plants under different environmental conditions, although there was not much of a difference in the environmental conditions in the 2023 experiment compared to the Mphande et al. (2021b) experiment of 2019 with the same wheat variety.

Furthermore, all the other measured parameters (relative water content, canopy temperature, endogenous ABA content, pollen viability and other yield components except TGW) revealed the same results of no significant effect (even the variability (coefficient of variation) was within limits for all the parameters ranging between 1% - 17% expect for endogenous leaf ABA concentration which was around 29%) from both antitranspirants at different growth stages, indicating the same pattern as observed in the final product (grain yield). Some other physiological measurements, such as stomatal conductance and photosynthesis, might be more sensitive to antitranspirant application; however, these were not measured directly, only canopy temperature as an indirect measurement of stomatal conductance. Even if these were measured and had shown any differences between different treatments under drought stress, that would have been minimal to affect the crop yield as observed in this study.

3.5 Conclusion

Film antitranspirant – Vapor Gard (first node stage of stem elongation, GS31) and metabolic antitranspirant – ABA (exogenous ABA, also referred to as S-ABA) application at two different growth stages (flag leaf stage before the start of meiosis, GS39 and watery to milky grain stage, GS71-73) of a spring wheat variety, revealed no significant differences in results after assessing different parameters such as relative water content, pollen viability, canopy temperature and endogenous ABA concentration (leaf and spike samples) between droughted unsprayed and antitranspirants sprayed plots of the two field experiments (2022 and 2023, grown inside the polytunnels (rain shelters)). Additionally, no significant effect of antitranspirants was observed on yield and other yield components except on TGW of the 2023 experiment, in which spraying antitranspirants significantly reduced mean TGW by 2%. However, this significant decrease did not affect the crop yield, and it was almost the same as in unsprayed droughted plots (with no significant response). Although there was a slight reduction in yield in both years with antitranspirant treatments (either VG or ABA) in comparison to unsprayed plants, however, it was not significant.

These results do not support any of the hypotheses of improved pollen viability, grain number and yield with antitranspirants spraying at different growth stages and reduction in water loss from plants, as expected based on the previous studies. This suggests that antitranspirants (either film or metabolic) might not significantly increase or decrease different physiological parameters in plants; however, it might also depend on soil moisture conditions for plants to show any significant effect, as under low soil moisture levels or mild stress, they might not show any response. Additionally, it might also depend on several factors such as type of plant species, critical growth stages, antitranspirant type/dosage, and environmental conditions to consider before applying so that they can work efficiently. However, further studies are required to understand their effect in a controlled glasshouse environment and at the molecular level, which are discussed in detail in Chapters 4 and 5, respectively.

CHAPTER 4. Responses of wheat to drought stress and antitranspirants at critical growth stages in a glasshouse environment

4.1 Introduction

Due to extreme climate changes, drought has become a global issue for crop production. It affects crop yields and can be very detrimental to plants if it occurs for an extended period or at critical growth stages. Also, the probability of hot and dry weather extremes during the growing season of crops has increased, with wheat showing the largest increase in such events (up to six-fold), which negatively affects its yield globally (Heino et al., 2023). To cope with these climatic changes, one of the adaptive technologies is the application of antitranspirants. These products reduce plant transpiration and can increase crop performance when applied at sensitive growth stages of a crop under droughted conditions (Mphande et al., 2020; Mphande et al., 2023). However, the effect of these products also depends on several other factors, such as type of antitranspirant, applied dose, plant species and other environmental factors (Kocięcka et al., 2023).

Antitranspirants have proven to improve wheat crop yield by reducing the rate of stomatal conductance either by acting as a physical barrier on plant leaves (film antitranspirants) or by acting metabolically via partial/full stomatal closure (metabolic antitranspirants, such as ABA) (Mphande et al., 2021a). One controlled temperature glasshouse wheat study revealed that spraying film antitranspirant (Vapor Gard) under drought stress before the drought-sensitive boot stage reduced yield by 14%, whereas in unsprayed control plants, it was reduced by 40% in comparison to well-watered plants (Abdullah et al., 2015). Additionally, the application of film antitranspirant during the reproductive-stage drought has been shown to decrease the endogenous ABA levels in wheat plants at key growth stages, resulting in enhanced yield by reducing stress responses and thus helping plants with better water status (Mphande et al., 2021b; Mphande et al., 2024). On the other hand, ABA application as an antitranspirant at critical growth stages (at flag leaf emergence and four further stages up to complete anthesis) has also been reported to improve wheat yield, but its effect on leaf ABA levels differs from that of film antitranspirant (Mphande et al., 2021a). A study by Nayyar and Walia (2004) showed that ABA application (five days after anthesis) on stressed wheat plants increased the endogenous ABA content, accelerated the accumulation of osmolytes with improved water status of flag leaf and grains, resulting in higher grain weight, especially in the drought-sensitive genotype. Thus, it indicates that ABA application at critical growth stages (tillering, flowering or grain filling stage) of wheat increases drought resilience by

improving water status and various physio-biochemical parameters of plants, thus helping plants mitigate the detrimental effects of drought (Zulfiqar et al., 2024).

The flag leaf stage before the start of meiosis and the grain-filling stage are two of the most critical growth stages that can affect crop yield. Therefore, to understand the effects of antitranspirants, two field experiments were conducted in 2022 and 2023 (as discussed earlier in Chapter 3), however, no significant results were observed from the two types of antitranspirants (film antitranspirant - VG and metabolic antitranspirant - ABA) at GS39 and ABA spraying at GS71-73 on yield and other measured parameters. Therefore, a glasshouse experiment was conducted with a randomised complete block design to investigate this further in a semi-controlled environment. The purpose was to reduce some of the environmental variations in the field experiments that may have contributed to the lack of response from antitranspirant application as compared to Mphande et al. (2021b).

Furthermore, although glasshouse and growth chamber experiments can provide a stable environment for light, temperature or humidity settings during the growth period of a crop, without the complex and dynamic interaction of these factors as in the natural field environment, which also significantly impacts morphological and physiological responses of plants (Poorter et al., 2016; Sales et al., 2022). Results from field and glasshouse experiments cannot be compared accurately and are not always consistent (Pooter et al., 2016; Forero, 2019). However, glasshouse experiments can be conducted throughout the year without seasonal constraints, unlike field experiments and allow for a better understanding of plants' developmental processes without the environmental variations, also reducing the risk of pests and diseases as in an open field. Therefore, to get more insights into the physiological responses of plants with antitranspirant (VG or ABA) treatments at different growth stages, a semi-controlled (light and temperature setting) glasshouse experiment was conducted.

The main hypotheses of this experiment were similar to those in the field experiments:

- Spraying film or metabolic antitranspirants under drought stress reduces water loss and improves pollen viability and crop yield compared to unsprayed plants.
- The application of film (VG) and metabolic (ABA) antitranspirants at the flag leaf stage before the start of meiosis (GS39) reduces water loss and increases pollen starch accumulation, grain number and yield of wheat plants.
- Metabolic ABA application at the watery to milky grain stage (GS71-73) increases yield by faster accumulation of photoassimilates during the grain filling stage.

4.2 Materials and methods

4.2.1 Experimental site and design

A glasshouse experiment was conducted at Harper Adams University with a light setting of a 16-hour day length and minimum temperatures of 15 °C during the day and 5 °C at night. There were two days (04 and 05 Feb 2023) when the lights were off in the glasshouse bay, which was 2 - 3 days after the spraying of antitranspirants at GS39. Also, in the next five days (06 – 10 Feb 2023), the mean day temperature was in the range of 6 – 10 °C, which was lower than the mean temperature of 15 °C during the growth period. Moreover, later in the growth period, after five days of spraying at GS71-73, the light mechanism was faulty again, as the automatic light system was not turning off, and the ventilation system in the bay was not working, resulting in the maximum temperature going up to 40 °C inside the bay for three days (22 - 24 Mar 2023) during the grain filling period, which might have also impacted plants and results of this experiment, as some of the plant samples (leaves and spikes) and thermal image data was collected during those days.

The experimental pots were arranged in a randomised complete block design consisting of six blocks, with each block containing five types of treatments: well-watered, unsprayed droughted, and three types of droughted spray treatments (VG-GS39, ABA-GS39, and ABA-GS71-73) (Table 4.1) (Appendix 9).

Table 4.1: Layout of randomised experimental pots of the spring wheat variety Chilham in the glasshouse experiment (2022). WW referred to well-watered pots, US to unsprayed droughted pots, VG39 referred to Vapor Gard sprayed at the droughted GS39 stage, ABA39 to ABA sprayed at GS39, and ABA71-73 referred to ABA sprayed at the GS71-73 stage.

Treatment No.	Block 1	Block 2	Block 3	Block 4	Block 5	Block 6
1	ABA39	US	WW	ABA71-73	VG39	WW
2	US	VG39	ABA71-73	ABA39	WW	US
3	WW	ABA39	VG39	US	ABA71-73	ABA39
4	VG39	ABA71-73	ABA39	WW	US	VG39
5	ABA71-73	WW	US	VG39	ABA39	ABA71-73

4.2.2 Planting, agronomic practices and management

J. Arthur Brower's John Innes No. 2 compost was used to fill 5-litre pots (22.5 cm diameter at the top, 16.5 cm at the base, and 18 cm in height). The values for pot capacity and permanent wilting point were 50.33% and 7% volumetric water content (VWC), respectively, as determined by Saeed (2008). Thus, the water available to the plants (50% - 7%) was around 43% VWC.

Twelve seeds of the spring wheat variety Chilham were sown in each pot on 29th November 2022. Seedlings emerged a week after sowing. At GS22-23, plants were thinned from 12 to 10 plants in each pot. Water was given to all pots twice a week up to the pot capacity for five weeks from the sowing date. At GS31, controlled drought was started in the droughted pots until physiological maturity by keeping the moisture values between 15% - 20% VWC (30% - 40% of the pot capacity and 18% - 30% of plant available water thus keeping them around 70% - 80% of available water depletion), whereas the moisture values of the well-watered pots were maintained between 35% - 40% VWC (70% - 80% of pot capacity and 65% - 76% of plant available water thus keeping them around 30% of available water depletion). These values were kept to create similar soil moisture conditions, as were present in the field experiments (Chapter 3, section 3.3.2), keeping in mind the moisture calculations performed by Mphande et al. (2021a).

Once controlled drought was started at GS31, moisture readings were taken three times a week (Monday, Wednesday, and Friday), and water was given accordingly to maintain the soil moisture values within the specified ranges for both the well-watered (35% - 40% VWC) and droughted pots (15% - 20% VWC).

Nitrogen fertiliser (ammonium nitrate) was added to each pot at a rate equivalent to 50 kg N/ha after seven weeks of sowing, as the leaves were showing symptoms of yellowing due to drought stress.

A fungicide (Cyflamid 0.5 l/ha) was sprayed for the control of powdery mildew after 11 weeks of sowing, and for aphid control, Decis Protect (420 ml/ha) was sprayed after 14 weeks of sowing. Both pesticides were sprayed with a handheld sprayer (5 L Hozelock pressure sprayer).

4.2.3 Antitranspirant treatments and application

Film antitranspirant, Vapor Gard (VG) (96% di-1-p-menthene, 1 l/ha) and a metabolic antitranspirant, exogenous ABA (20% S-Abscisic Acid, 30 g/ha of the chemical product (ProTone) containing 6 g/ha of S-ABA) were sprayed (both with an application rate of 200 l/ha using a custom-built automatic pot sprayer in the glasshouse) at the flag leaf stage (GS39) of the droughted experimental pots. ABA was also sprayed at the watery to milky grain stage (GS71-73) of the droughted pots, resulting in three types of antitranspirant-treated pots, along with unsprayed droughted and well-watered pots. The details of the antitranspirant treatments, including timing and sample collection dates, are mentioned in Table 4.2.

Table 4.2: Timing of antitranspirant (VG and ABA) treatments and sampling dates.

Treatment - Growth Stage (GS)	Spraying Date	Days after Planting (DAP)	Leaf sampling for ELISA ABA analysis and relative water content. DAP - Date Days after Spraying (DAS)	Anthers collection for checking pollen viability. DAP Dates	Spike sampling for ABA analysis from all treatments. DAP Date
VG-GS39 ABA-GS39	02 Feb 23	65	73 DAP 10 Feb 23 8 DAS	Started 83 DAP 20 Feb 23 to 10 Mar 23	115 DAP 24 Mar 23
ABA-GS71-73	16 Mar 23	107	N/A		

4.2.4 Measurements and sampling

4.2.4.1 Temperature and humidity

Temperature and relative humidity inside the glasshouse bay were recorded daily with a Tinytag Ultra 2 data logger (Gemini Data Loggers Ltd, Chichester, UK).

4.2.4.2 Soil moisture

Soil moisture content was measured using a FieldScout TDR 100 soil moisture meter by Spectrum Technologies, Inc., IL, USA, using a medium rod length of 4.7 inches. Measurements were started two weeks after sowing and continued (three days a week) until physiological maturity.

4.2.4.3 Canopy temperature

Thermal images were taken to record the canopy temperature of plants using a FLIR thermal imaging camera (FLIR Systems, Inc., Wilsonville, USA). Images were captured one day before and then one day after the treatments for seven days, skipping one day in between. The FLIR Research Studio software was used to record mean canopy temperature data from plant leaf pixels of each image.

4.2.4.4 Relative water content

The RWC of wheat leaves was determined after the antitranspirant treatments at GS39. Three top leaves from each experimental pot were randomly cut after eight days of VG and ABA treatment. Only the middle 5 - 6 cm section of each leaf was taken and stored in pre-weighed tubes. Leaf samples from the unsprayed and well-watered pots were also collected. Further steps for assessing RWC were followed according to the protocol by Pask et al. (2012).

4.2.4.5 Endogenous ABA content

ABA concentration inside the leaf and spike samples of antitranspirant-treated, unsprayed, and well-watered plants was determined by sampling six top leaves from each pot after eight days of GS39 treatments. Additionally, three spike samples were collected from each pot after the GS71-73 treatment. All samples were collected in 50 ml tubes and were immediately flash-frozen in liquid nitrogen and then stored at a -80 °C freezer until further processed according to the Cusabio ELISA ABA protocol (Code CSB-E09159PI). The frozen samples were freeze-dried for 3 - 5 days, and an electric grinder was used to grind them before following the further steps outlined in the protocol. BioTek microplate spectrophotometer was used for absorbance readings at 450 nm, and the ABA concentration of each sample was determined after fitting the standard curve via CurveExpert 1.4 software.

4.2.4.6 Assessment of pollen viability

Pollen viability was determined via staining of pollen, indicating the presence and absence of pollen starch accumulation as assessed by Weerasinghe et al. (2016). A random collection of ten freshly dehisced anthers was done from the spikes of antitranspirant-treated, unsprayed, and well-watered plants into 1.5 ml dark Eppendorf tubes containing iodine solution (instead of Lugol's solution). The tubes were stored in the refrigerator at 4 °C after collection, and later, counting of viable (darkly stained) and non-viable (partially or unstained) pollen was performed under a light microscope (10x objective, Zeiss Primostar 3) using a Sedgewick Rafter counting chamber. The mean percentage of darkly stained (viable) pollen was determined by counting ten random grids of three replicates from each sample.

4.2.4.7 Yield and yield components

Different yield components were calculated to determine the yield of droughted antitranspirant-treated, unsprayed droughted, and well-watered plants in the glasshouse experiment. Spikes from each pot were harvested and stored in separate bags, and these were later counted to calculate the number of spikes per pot. Spikes were threshed manually, and grains were counted to calculate the mean number of grains per spike. Later, all the samples were oven-dried at 105 °C for 40 hours and weighed to calculate the yield per pot. Additionally, the thousand grain weight (TGW) was calculated according to the method described by Sylvester-Bradley et al. (1985).

4.2.5 Statistical analysis

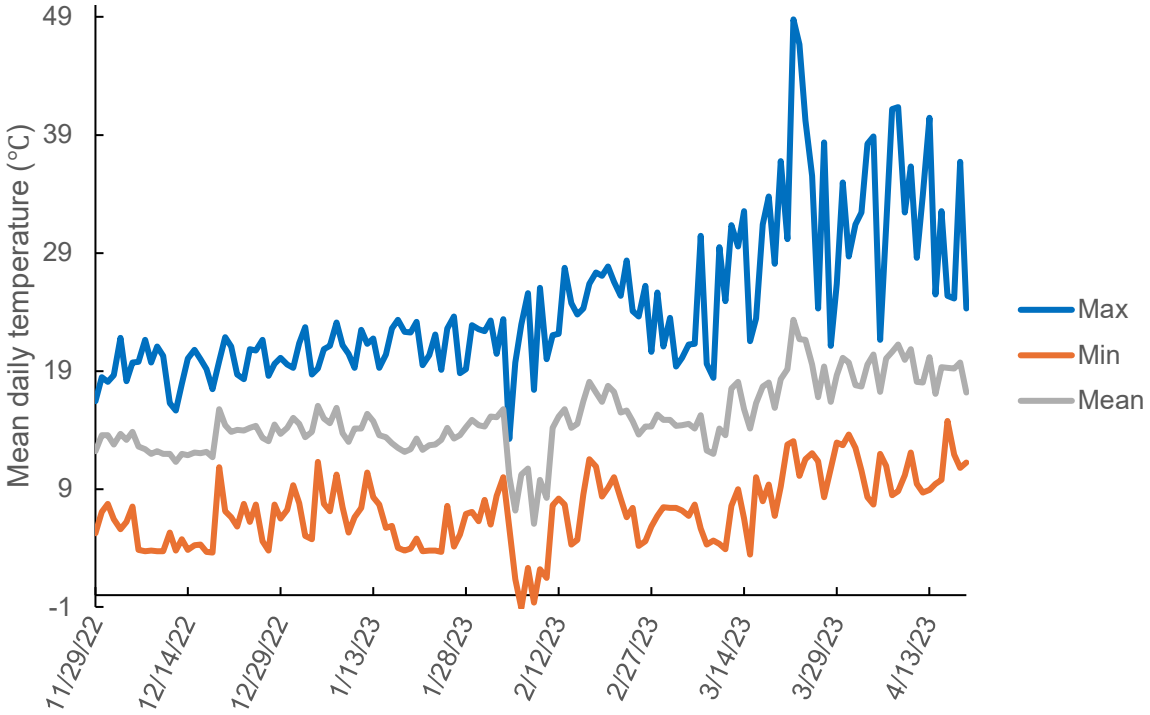
ANOVA with a randomised complete block design was applied using R Studio to compare different antitranspirant-treated, unsprayed, and well-watered plants. Before applying ANOVA, Levene’s test was performed to check the homogeneity of variances between different samples and the Shapiro-Wilk test was used to check the normality of data. ANOVA contrasts were also performed to check for any differences between well-watered and droughted plants, unsprayed and treated plants, and lastly for a direct comparison of GS39 treatments of VG and ABA sprayed plants. Additionally, a Tukey post-hoc test was performed to identify the differences between any significant treatments.

4.3 Results

4.3.1 Temperature and humidity

The mean daily temperature and relative humidity inside the glasshouse bay were 15.02 °C and 62.61%, respectively, with a maximum temperature of 48.78 °C and a minimum temperature of -1.08 °C recorded during the growth period (Figure 4.1, a and b). In March 2023, most of the recorded maximum temperature values during the daytime were above 30 °C or even near 40 °C, as can be seen in Figure 4.1 (a), which might be due to the faulty light mechanism at that time.

(a)



(b)

Figure 4.1: Mean daily temperature (a) and relative humidity (b) inside the glasshouse experiment 2022-23 recorded by a Tinytag Ultra 2 data logger during the growth period.

4.3.2 Soil moisture

The volumetric water content of compost fluctuated during the growth period in the well-watered pots ranging between 17% - 46% VWC with a mean around 32% (58% of plant available water), while in controlled droughted pots it ranged between 3% - 25% VWC with a mean around 9% (5% of plant available water) (Figure 4.2). One reason for this lower range of values than the set range was that measurements were taken before watering, and later water was given to keep the minimum level of 35% for well-watered and 15% for droughted pots.

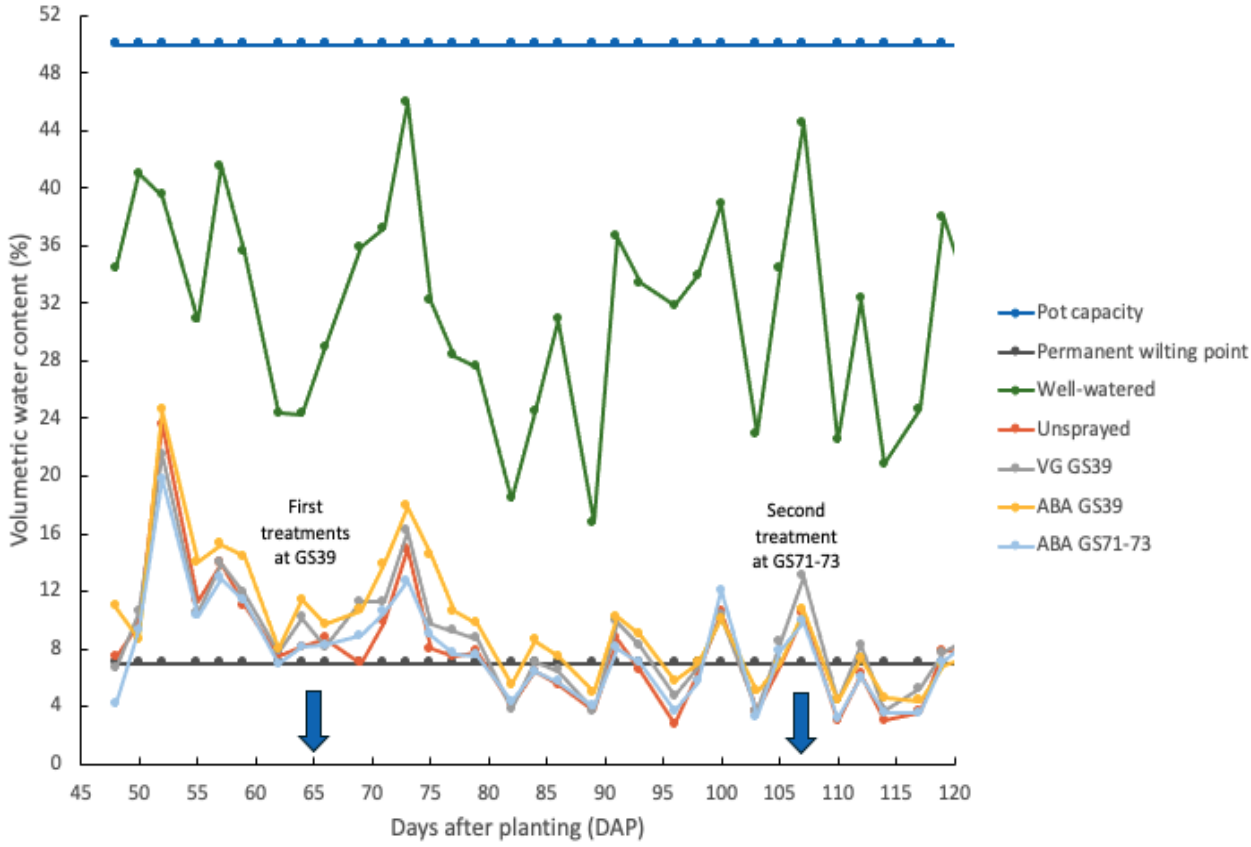
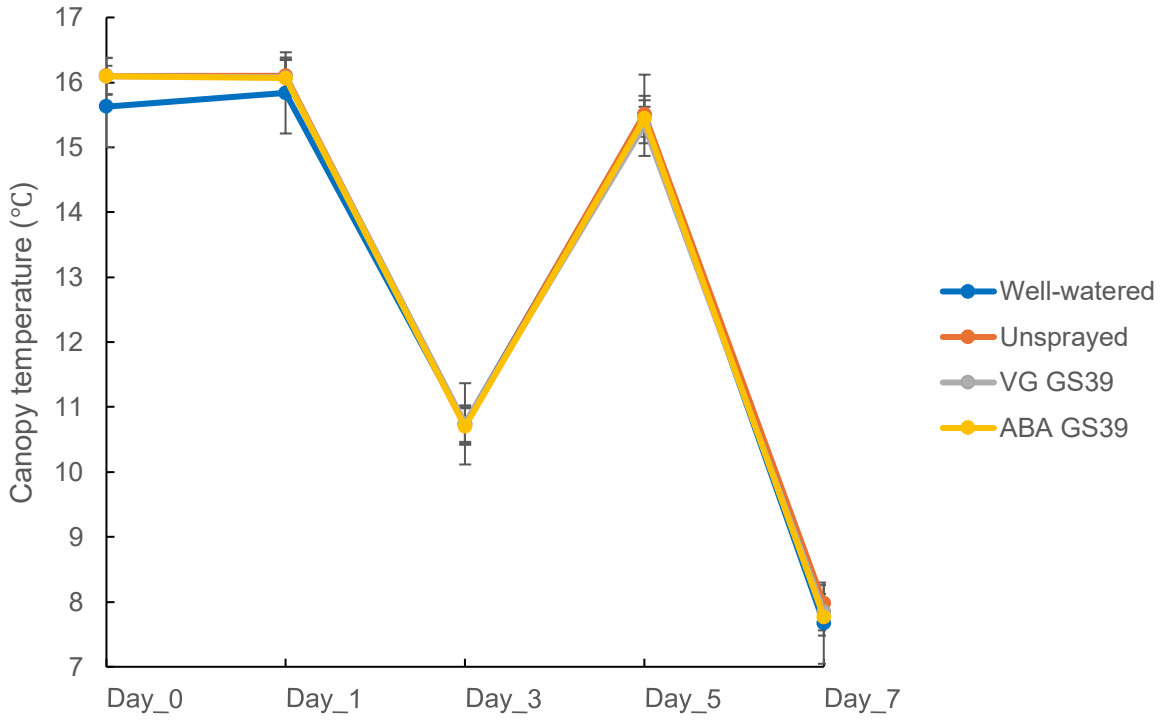


Figure 4.2: Line graph depicting the volumetric water content of John Innes No. 2 compost in 5 L pots with values of pot capacity and permanent wilting point along with mean moisture values of well-watered, unsprayed and antitranspirant-treated plants at different growth stages (VG-GS39, ABA-GS39 and ABA-GS71-73). Blue arrows on the graph represent the days after planting when antitranspirants were sprayed at two different growth stages. Each moisture value was taken before watering three times a week (Monday, Wednesday, and Friday), and then water was given accordingly to keep the volumetric water content of both types of pots in a specified range (well-watered = 35% - 40% VWC, droughted = 15% - 20% VWC).

4.3.3 Canopy temperature

Repeated measures ANOVA was used to analyse canopy temperature data, taken from thermal images after GS39 treatments of antitranspirants (VG and ABA), along with unsprayed and well-watered plants. There were no significant differences between treatments ($p = 0.496$) from ANOVA analysis, and a t-test for pairwise comparison between different treatments also revealed no significant results. However, significant differences were observed between different days ($p < 0.001$), which was expected due to variations in daily temperature. Even though the well-watered plants were included in the analysis, there was very little to no difference in the canopy temperature in comparison to droughted, treated and unsprayed ones after GS39 treatments (Figure 4.3, a). The interaction between treatments and days was also not significant ($p = 0.082$). Air temperature and humidity data inside the glasshouse bay on the days (time between 09:00 am - 12:00 pm), when thermal images were captured, can be seen in the two separate graphs in Figure 4.3 (b) and (c), respectively, for comparison purposes with the mean canopy temperature of plants in Figure 4.3 (a).

(a)



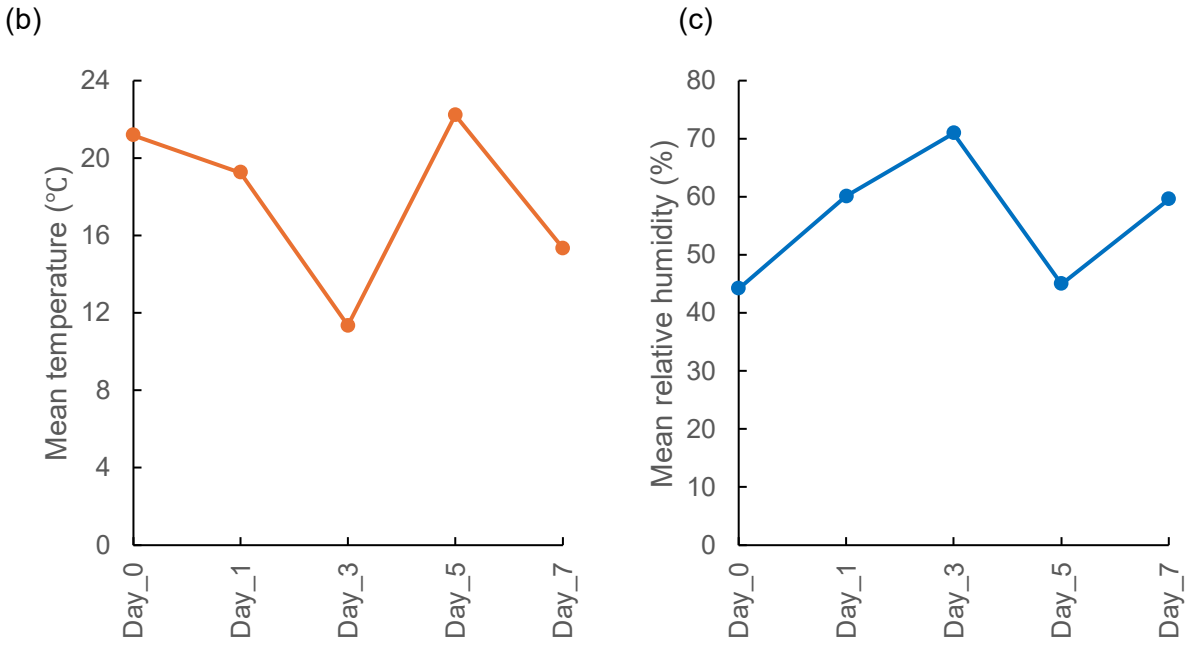


Figure 4.3: Canopy temperature data from the glasshouse experiment (2022) (a) of antitranspirants (VG and ABA) treated plants at GS39 along with unsprayed and well-watered plants one day before the antitranspirant treatments (Day_0) and then one day after spraying (Day_1) up to seven days (Day_7) skipping one day in between. Each treatment is represented with a line of different colours. A single value of each treatment is the mean of six replicates, with data of each point taken from the thermal image pixels of leaves from each plant pot. There was no significant difference between treatments, with p = 0.496, and similar results for the interaction between treatments and days, with p = 0.082 from the repeated measures ANOVA analysis. The mean temperature (b) and the mean relative humidity (c) graphs of different days show the mean values from 9:00 am to 12:00 pm when thermal images were captured.

4.3.4 Relative water content

RWC showed no significant differences between droughted antitranspirants treated, droughted unsprayed and well-watered plants (p = 0.215). Values for RWC were almost similar in all droughted and well-watered plants (Figure 4.4, a). The three different contrasts between well-watered and droughted, unsprayed and antitranspirant-treated, and a direct contrast between VG-GS39 and ABA-GS39 also revealed no significant effect among treatments in the ANOVA analysis (Table 4.3).

4.3.5 Pollen viability

Pollen viability was determined via the presence or absence of pollen starch accumulation. Iodine solution gave colour to starch accumulated in pollen, therefore pollen were checked and counted under the microscope. Mean pollen fertility was approximately 89% in the droughted samples (including unsprayed and antitranspirant-treated plants), while in the well-watered samples it was approximately 91% (Figure 4.4, b). It reduced by 2% in the droughted samples compared to well-watered ones, while spraying antitranspirants improved pollen viability by 2%, when compared with unsprayed, but it was not significant. ANOVA analysis revealed no significant results between different treatments (p = 0.935), and in three contrast comparisons (Table 4.3).

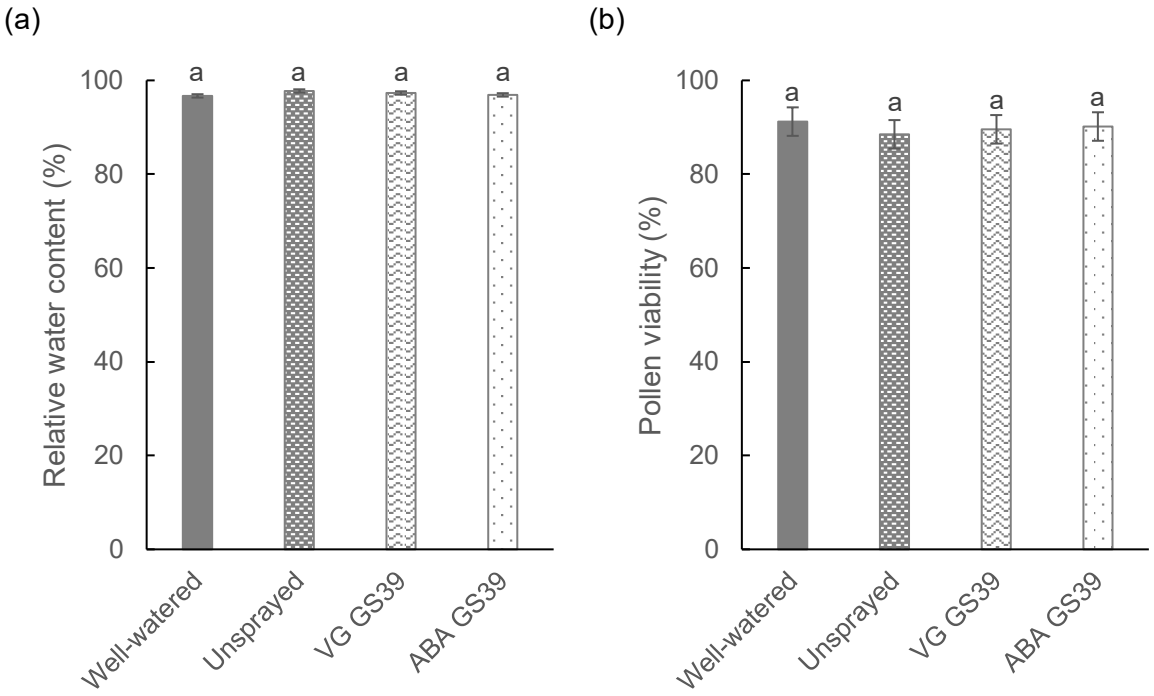


Figure 4.4: Relative water content of leaves (%) (a) and pollen viability (%) (b) of well-watered, unsprayed droughted and antitranspirants treated (VG-GS39 and ABA-GS39) plants sprayed at GS39 showing no significant effect between different treatments (p = 0.215 (a) and p = 0.935 (b)). The same letters on the bars indicate no significant differences from the Tukey post-hoc test. The error bar represents the common standard error of mean from the ANOVA table (SEM = 0.361 (a), SEM = 3.039 (b)). Leaf samples for the relative water content were collected 73 days after planting (DAP) and 8 days after spraying at GS39, whereas anther samples for assessing the pollen viability were collected between 83-100 DAP.

4.3.6 Endogenous ABA concentration

ABA concentration of leaf samples was determined from droughted VG and ABA sprayed (GS39) plants along with droughted unsprayed and well-watered plants. The range of ABA concentration in droughted samples was between 29 - 40 ng/g with a mean of 36 ng/g, while in well-watered ABA concentration was 38 ng/g (Figure 4.5, a). There was high variation in the endogenous ABA content of leaves (coefficient of variation value going up to 53%), and statistical analysis showed no differences between various treatments ($p = 0.789$) and three types of contrasts (well-watered vs droughted, unsprayed vs antitranspirants-treated and lastly a direct contrast between VG-GS39 and ABA-GS39 treated plants) (Table 4.3).

The ABA content of spikes from all treatments was assessed after the last treatment of metabolic antitranspirant (ABA) was applied at the grain-filling stage (GS71-73). ABA concentration was higher in spikes in comparison to leaf samples ranging between 118 - 144 ng/g with a mean of 131 ng/g in all the droughted samples, whereas it was 115 ng/g in the well-watered spikes; thus, ABA concentration increased by 14% in the droughted spikes as compared to well-watered ones. There was an 8% increase in the ABA content of sprayed spike samples compared to unsprayed spikes. However, ANOVA indicated that there was no significant effect of antitranspirants on the ABA content of droughted spike samples, either compared with unsprayed or well-watered samples, as can also be seen in the contrast comparisons in Table 4.3.

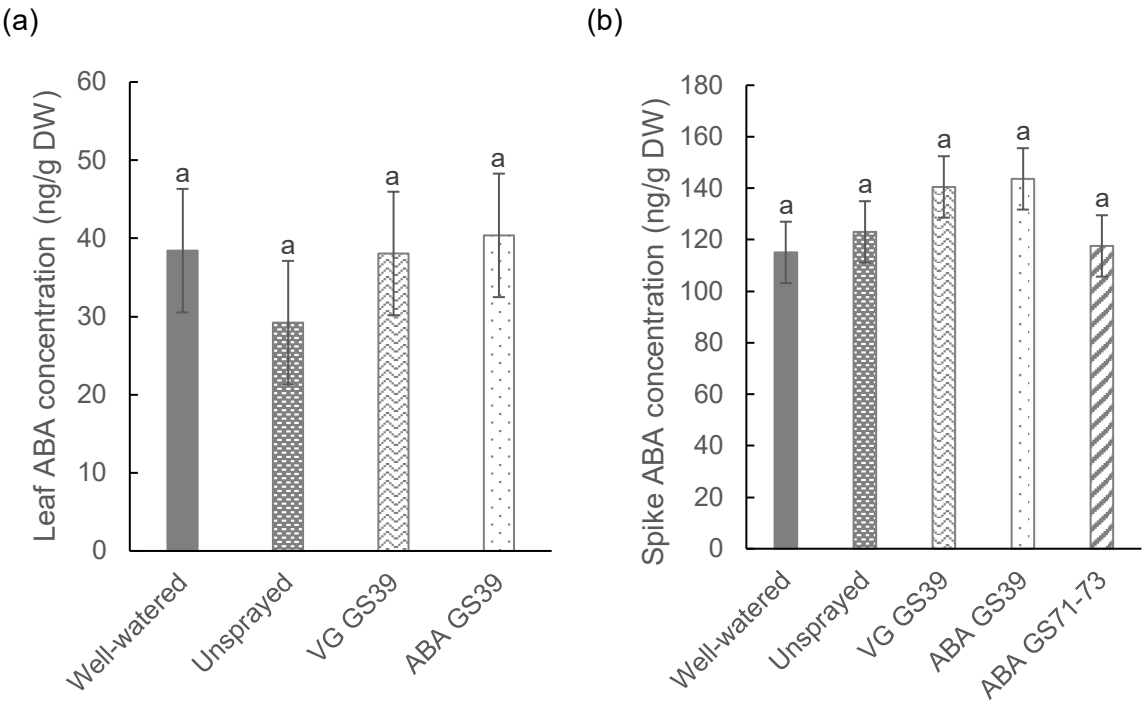


Figure 4.5: ABA concentration (ng/g DW) in top leaves (a) sampled after GS39 spraying from well-watered, droughted unsprayed, and droughted VG and ABA treated plants and in spike samples (b) collected from all treatments (Well-watered, Unsprayed, VG-GS39, ABA-GS39 and ABA-GS71-73) after GS71-73 spraying, which was 115 days after planting. There were no significant effects between different treatments in both leaves ($p = 0.789$ (a)) and spikes samples ($p = 0.329$ (b)). The same letters on the bars indicate no significant differences from the Tukey post-hoc test. The error bar represents the standard error of mean from the ANOVA (SEM = 7.901 (a), SEM = 11.926 (b)).

4.3.7 Yield components

Different yield components (number of spikes per pot, number of grains per spike, TGW and grain yield per pot) were measured to assess the performance of the glasshouse plants treated with two types of antitranspirants (VG and ABA) at two different growth stages (GS39 and GS71-73) under droughted condition along with droughted unsprayed and well-watered plants. Significant effects of different treatments were observed in all the yield components from ANOVA results, mainly because of the inclusion of well-watered plants, which was not possible in the two field experiments, as mentioned in the previous Chapter 3. It can be seen clearly in one of the contrast comparisons between well-watered and droughted plants, showing significant differences in all the yield components (Table 4.4). The Tukey post-hoc

test revealed the treatments that showed significant differences when compared with each other (Figure 4.6 and Table 4.4).

For the number of spikes per pot, there were significant differences between different treatments, with spikes significantly decreasing by 20% in the droughted plants as compared to well-watered ones. There was a mean reduction of 10% in the number of spikes with the spraying of antitranspirants in comparison to unsprayed pots, especially spike number significantly decreased in plants sprayed at GS39 either with VG or ABA, while the number of spikes in the unsprayed and ABA sprayed plants at GS71-73 were the same as can be seen in Figure 4.6, (a).

The number of grains per spike showed significant differences between treatments ($p < 0.05$) in the ANOVA analysis; however, the Tukey post-hoc test indicated no such differences (Figure 4.6, b). These were significantly reduced by 18% in the droughted pots when compared with well-watered plants, according to one of the contrast comparisons ($p < 0.05$) (Table 4.4). Spraying antitranspirants increased the number of grains per spike by 9% in comparison to unsprayed plants; however, it was not significant either in Tukey's test or in one of the contrast comparisons between unsprayed and sprayed plants.

TGW was significantly lower in the well-watered plants ($p < 0.001$) (Figure 4.6, c), while the grain yield per pot was significantly higher in the well-watered plants when compared with the droughted ones ($p < 0.01$) (Figure 4.6, d). There were no significant differences between droughted plant treatments (sprayed or unsprayed) either for TGW or grain yield per pot (Table 4.4).

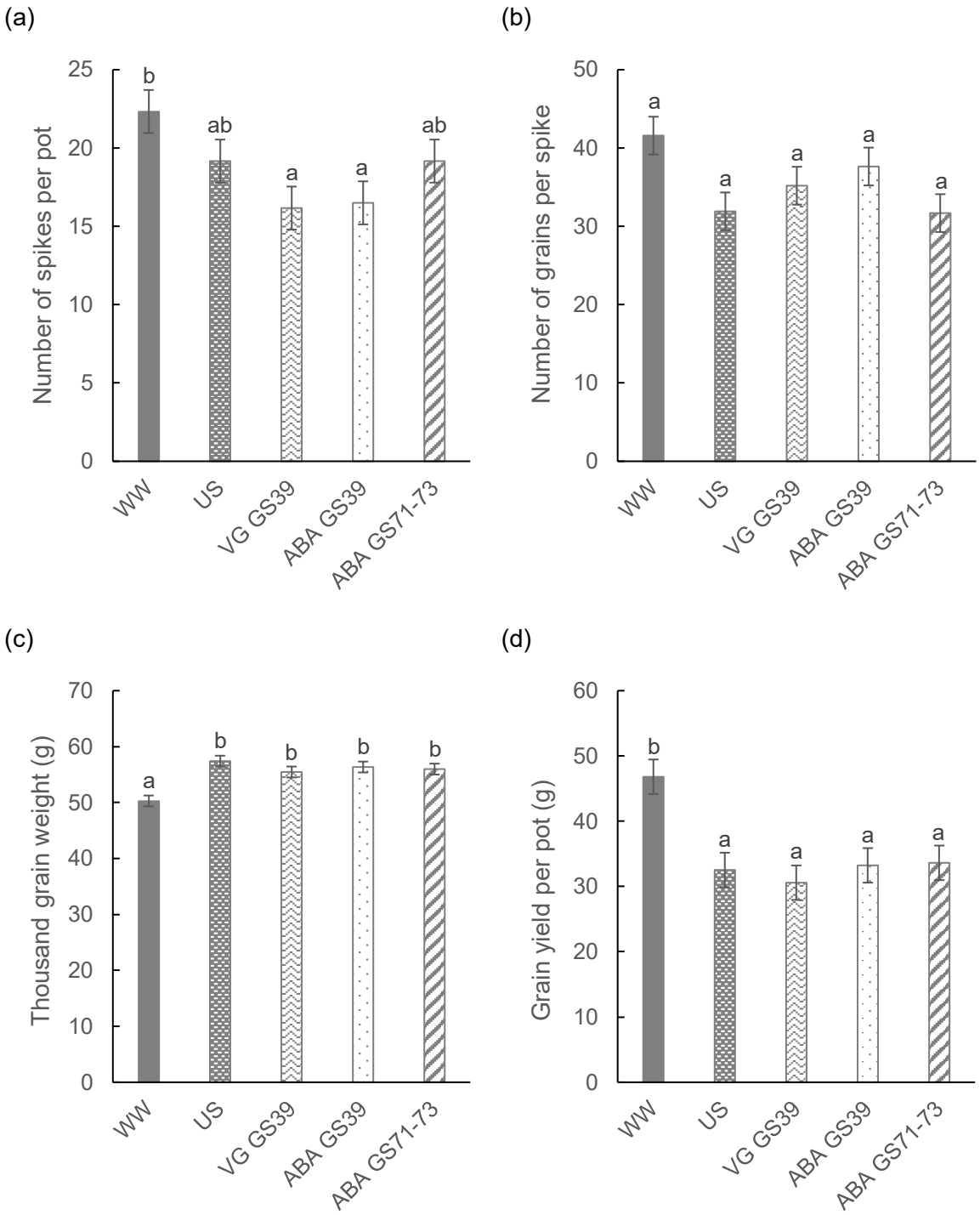


Figure 4.6: Yield components, number of spikes per pot (a), number of grains per spike (b), thousand grain weight (c) and grain yield per pot (d) from the glasshouse experiment (2022-23) calculated from well-watered (WW), droughted unsprayed (US), and droughted VG and ABA treated plants at different growth stages (VG-GS39, ABA-GS39 and ABA-GS71-73). Different letters on bars indicate significant differences from the Tukey post-hoc test. The error bar represents the standard error of mean from the ANOVA (SEM = 1.374 (a), 2.415 (b), 0.974 (c) and 2.645 (d), respectively).

Table 4.3: Mean values of treatments of different parameters (relative water content, pollen viability, and endogenous ABA concentration of leaves and spikes samples) of the glasshouse experiment 2022, along with results (p values - degree of freedom (df)) from ANOVA analysis.

Treatment	Relative water content (%) - leaves collected 73 days after planting (DAP) and 8 days after spraying (DAS)	Pollen viability (%) - anthers collected between 83-100 DAP	Endogenous ABA concentration (ng/g DW) - leaves collected 73 DAP and 8 DAS	Endogenous ABA concentration (ng/g DW) - spikes collected 115 DAP
Well-watered	96.69 (a)	91.21 (a)	38.42 (a)	115.08 (a)
Unsprayed	97.74 (a)	88.50 (a)	29.21 (a)	123.04 (a)
VG-GS39	97.31 (a)	89.57 (a)	38.06 (a)	140.50 (a)
ABA-GS39	96.91 (a)	90.16 (a)	40.38 (a)	143.64 (a)
ABA-GS71-73	N/A	N/A	N/A	117.59 (a)
ANOVA - p value (df)				
p value (treatment)	0.215 (3)	0.935 (3)	0.789 (3)	0.329 (4)
Contrast 1 (WW vs droughted)	0.239 (1)	0.615 (1)	0.779 (1)	0.106 (1)
Contrast 2 (US vs AT-treated)	0.104 (1)	0.719 (1)	0.351 (1)	0.475 (1)
Contrast 3 (VG-GS39 vs ABA-GS39)	0.479 (1)	0.892 (1)	0.839 (1)	0.454 (1)
SEM (residual df)	0.361 (13)	3.039 (15)	7.901 (14)	11.926 (20)

Similar letters with mean values of each treatment indicate no significant differences between treatments from the Tukey post-hoc test. VG is Vapor Gard (96% di-1-p-menthene), while ABA represents (20% S-Abscisic Acid). SEM indicates the standard error of mean values, while N/A means that no samples were collected for that specific treatment.

Table 4.4: Mean values of yield components (number of spikes per pot, number of grains per spike, thousand grain weight, and grain yield per pot) of the glasshouse experiment 2022-23, along with results (p values - degree of freedom (df)) from the ANOVA analysis.

Treatment	Number of spikes per pot	Number of grains per spike	Thousand grain weight (TGW)	Grain yield per pot (g)
Well-watered	22.33 (b)	41.59 (a)	50.29 (a)	46.81 (b)
Unsprayed	19.17 (ab)	31.91 (a)	57.41 (b)	32.53 (a)
VG-GS39	16.17 (a)	35.19 (a)	55.47 (b)	30.58 (a)
ABA-GS39	16.50 (a)	37.64 (a)	56.36 (b)	33.24 (a)
ABA-GS71-73	19.17 (ab)	31.69 (a)	55.99 (b)	33.62 (a)
ANOVA - p value (df)				
p value (treatment)	0.031 (4) *	0.045 (4) *	0.000 (4) ***	0.002 (4) **
Contrast 1 (WW vs droughted)	0.007 (1) **	0.012 (1) *	0.000 (1) ***	0.008 (1) **
Contrast 2 (US vs AT-treated)	0.247 (1)	0.306 (1)	0.214 (1)	0.472 (1)
Contrast 3 (VG-GS39 vs ABA-GS39)	0.865 (1)	0.482 (1)	0.612 (1)	0.170 (1)
SEM (residual df)	1.374 (20)	2.415 (20)	0.974 (18)	2.645 (20)

Different letters with mean values of each treatment indicate significant differences between treatments from the Tukey post-hoc test. *, **, *** represent significance less than 0.05, 0.01, and 0.001, respectively. WW = well-watered, US = unsprayed droughted plants. VG is Vapor Gard (96% di-1-p-menthene), while ABA represents (20% S-Abscisic Acid). Both antitranspirants (AT) were sprayed at the flag leaf stage (GS39) of wheat, and ABA was also sprayed at the watery to milky grain stage (GS71-73) in one of the treatments. SEM indicates the standard error of mean values.

4.4 Discussion

Drought affects wheat crop productivity due to changes in morphology, physiology and biochemical changes (Nyaupane et al., 2024). Drought stress during the reproductive development and grain filling stage can cause a significant reduction in grain number and yield losses (Farooq et al., 2014; Senapati et al., 2019). Antitranspirants can mitigate the effects of drought, mainly di-1-p-menthene or Exo-ABA, when applied at GS39 as investigated by Mphande et al. (2021a) in the glasshouse experiments of spring wheat variety Chilham. To investigate this further, in a glasshouse experiment, the same wheat variety was used and antitranspirants, VG (di-1-p-menthene, same as used in the previous study) and ABA (S-ABA, containing natural and biologically active isomers as compared to the one used in the Mphande et al. (2021a) study who used (±)-ABA containing a mixture of active and inactive isomers**)** were sprayed at GS39 and ABA was also sprayed at GS71-73 (watery to milky grain stage) to determine its effect before the start of reproductive processes and during the grain filling period. Controlled drought was applied from GS31 till physiological maturity, and significant differences were observed in most of the yield components, mainly because the well-watered plants were included in the analysis in contrast to the field experiments (2022 and 2023), where it was not possible (Chapter 3). However, contrast comparisons between unsprayed and sprayed plants in the different yield components revealed no significant effect of antitranspirant treatments. These results of no effect of antitranspirants in most of the yield components and other measured parameters at GS39 or GS71-73 were consistent with field experiments as discussed in the previous Chapter 3.

These contradictory results, as compared to Mphande et al. (2021a) study, might be due to severe drought stress under controlled drought and fluctuations in temperature values during the growth period. In the present study, mean plant available water fluctuated (set range was 18% - 30%) during the growth period and it mostly remained near (9% VWC soil moisture with 5% of plant available water when readings were taken before watering) the permanent wilting point (7%), which was much less in comparison to the Mphande et al. (2021a) study that ranged between 35% to 46% of plant available water (9.4% – 10.2% VWC with a mixture of John Innes compost and air-dry sandy loam soil) under controlled drought of the same wheat variety. This suggests that severe moisture stress might be the reason for no response from antitranspirants. Although this low soil moisture significantly reduced yield by 31% in the droughted plants in comparison to well-watered ones, however, applying antitranspirants did not affect or reduce the yield when compared with the unsprayed plants

in the present experiment. Thus, it also explains no significant changes in the pollen fertility and endogenous ABA concentrations of leaf and spike samples in the treated plants as compared to unsprayed or well-watered plants. On the other hand, Mphande et al. (2021a) observed that controlled drought significantly reduced (by around 17%) grain yield per spike in unsprayed plants when compared to well-watered while spraying antitranspirants (VG or ABA) ameliorated the effect of drought as grain yield per spike was same (with no significant reduction) as in well-watered plants, also with significant differences in endogenous leaf ABA concentration in different treated and control (either well-watered or unsprayed) plants. This shows that the response of the same wheat variety can vary with antitranspirant applications depending on the severity of moisture stress, as observed in the present glasshouse experiment.

Furthermore, although the mean glasshouse temperature during the growth period was around 15 °C in the present study, the same as Mphnade et al. (2021a) (15.9 °C), however, there was a wide variation in minimum and maximum recorded temperatures (-1.08 °C minimum to 48.8 °C maximum) in the recent study in comparison to 14 °C minimum to 18 °C maximum, in the Mphande et al. (2021a) study of controlled drought experiment. Also, in the present experiment, the mean temperature was lower for a week after GS39 treatments ranging between 6 – 10 °C due to defects in the light mechanism in the glasshouse bay (lights were off for two days, 2 - 3 days after GS39 treatments and might not be working properly in the next five days, as the mean temperature was lower for next five days (around 10 °C) with minimum going below 0 °C on two days as can be seen in figure 4.1, a) at that time, which might also be the reason for high variations in endogenous leaf ABA content results (with variability value around 53%) as sampling was completed during those days along with thermal images taken for canopy temperature data. Moreover, during the grain filling period till the physiological maturity of the present experiment, the maximum temperature was recorded above 30 °C or near to 40 °C (duration range from a minimum of half an hour to 2:30 hours maximum on some days), which might also be due to a faulty light mechanism at that time (automatic lights were not turning off even during the day time, even in full sunlight, resulting in temperature going above 40 °C on three days when spike sampling was conducted). Moreover, mean relative humidity values also varied during the growth period in both studies, with a mean of 63% in the recent experiment and 72% in the Mphande et al. (2021a) controlled drought experiment. All these differences, either in soil moisture level, temperature/light or humidity conditions during the growth period of the same wheat variety in the recent study, might have contributed to the contrasting results in comparison to the Mphande et al. (2021a) controlled drought experiment. Low light and low temperature conditions around GS39-41 might have impacted plant growth by reducing

photosynthesis and can also cause sterility, affecting spike fertility, which leads to reduced yield (Yang et al., 2020; Zhang et al., 2022).

Pollen development and fertility decrease under drought stress, leading to a reduction in grain number and yield (Dong et al., 2017). The recent study indicated that under controlled drought, pollen viability decreased in comparison to well-watered plants (but it was not significant), similarly, antitranspirants (VG or ABA) showed no significant effect on pollen fertility, thus contradicting to Weerasinghe et al. (2016) study that showed that using di-1-p-methene (at GS33) significantly increased the pollen viability of wheat plants. Although there was a slight improvement in pollen viability by 2% with spraying antitranspirants at GS39 in comparison to unsprayed plants in the recent glasshouse experiment, however, it was not significant. Furthermore, the type of wheat variety used and dosage of film antitranspirant were different in Weerasinghe et al. (2016) field experiments than in this glasshouse experiment, thus indicating that it might also depend on these factors as well as soil or environmental conditions at the time of treatment. Similarly, improvement in pollen fertility with antitranspirants was not observed in the two field experiments (2022 and 2023), as already discussed in Chapter 3, so it suggests that antitranspirants might not always be associated with a significant increase in pollen viability under droughted conditions. However, it also indicates that applying antitranspirants might not significantly damage or decrease the pollen fertility of crop plants, which might be beneficial for plants in improving yield if moisture stress is not very severe.

The concentration of ABA hormone in plants increases under drought stress (Duvnjak et al., 2023); however, endogenous ABA content of wheat flag leaves was almost similar in well-watered and droughted plants after eight days of spraying with VG and ABA treatments at GS39. Both antitranspirants made no significant changes in the leaf ABA content of plants. Also, as results from leaf ABA concentration were very variable (with variability value (CV) around 53%, as the mean temperature inside the glasshouse was lower (mean temperature around 6 – 10.7 °C with a minimum ranging between -1.1 – 5.7 °C for six days) when leaves were collected after eight days of spraying due to faulty light system at that time) therefore it cannot be related to other studies properly. In spikes, ABA content increased by 14% in the droughted samples in comparison to well-watered plants, with a 9% increase in antitranspirant sprayed plants in comparison to unsprayed ones, however, both were not significant in the ANOVA analysis indicating that spraying either VG (at GS39) or ABA (at GS39 or GS71-73) did not affect the endogenous ABA content of spikes that was also reflected in no significant differences in the grain yield. However, in the previous study by Mphande et al. (2021a) of the same wheat variety, improved yield was observed with di-1-p-

menthene or VG (at GS39) and one or multiple spraying of exogenous ABA (from flag leaf stage till complete anthesis) with significant contrasting effects on leaf ABA under progressive and controlled drought condition respectively. This shows that even on a similar wheat variety, antitranspirant applications might not always improve yield or give significant changes at a biochemical level, as many other factors (as described in earlier paragraphs) might also be responsible for these contradictory results during the growth period of a crop.

This glasshouse experiment revealed that drought significantly reduced the yield and yield components under controlled drought, except for TGW (which significantly increased), in comparison to well-watered plants. Spraying antitranspirants (VG or ABA) at GS39 significantly decreased the number of spikes compared to well-watered and unsprayed plants, while the number was the same in the unsprayed plants and the ones sprayed with ABA at the watery to milky grain stage (GS71-73). There were an equal number of plants in each pot, thus it suggests that spraying antitranspirants might reduce the number of tillers or spikes in wheat if sprayed at GS39, which is consistent with Abdullah et al. (2015) results that revealed a reduction in the number of tillers, emerged spikes and mature spikes with film antitranspirant treatment at GS39. However, Mphande et al. (2021a) did not observe significant differences in the number of fertile spikes for the same variety (Chilham) under progressive and controlled drought, either with untrimmed or trimmed tillers under both droughted conditions, respectively. This shows that responses of the same variety can vary with antitranspirant treatments that might depend on different biological responses of a variety under those conditions. One reason for this could be that the mean glasshouse temperature was around 10 °C with a minimum going to minus for two days as lights were not working properly (off for two days with a mean temperature around 10 °C for the next five days compared to the mean of 15 °C during the growth period) after spraying at GS39, and that might be the reason for temperatures below mean up to seven days after spraying. Severe moisture stress might also be the reason for this significant decrease in the spikes number at GS39, however, it did not affect the grain yield, which is contradictory as observed by Luo et al. (2021) that the exogenous application of ABA at the anthesis stage caused a reduction in the grain weight, yield and water use efficiency of wheat plants under severe moisture deficit condition. Moreover, Faralli et al. (2017a) indicated that under severe water stress (10% VWC) application of film antitranspirant on oilseed rape did not sustain pod formation but only seed production, whereas both were sustained in plants under water stress up to 20% VWC. This suggests that the magnitude of water stress also plays a crucial role in determining the effectiveness of antitranspirants.

The number of grains per spike decreased in the droughted unsprayed and antitranspirants sprayed plants in comparison to well-watered, however, it was not observed in the Tukey test, with all treatments being the same, with no significant difference between well-watered and droughted plants. However, in one of the contrasts between well-watered and droughted plants, ANOVA revealed a significant difference between both treatments. Furthermore, there was no evidence that spraying antitranspirants increased the number of grains per spike, as it was not reflected either in the number of grains per spike or grain yield results. Contrary to this, Mphande et al. (2021a) observed that grain number per spike significantly reduced in unsprayed plants, and the application of antitranspirants (VG and multiple applications of ABA) improved it. A study by Abdallah et al. (2019) also indicated that antitranspirants (kaolin or potassium sulfate) improved the yield of wheat plants by increasing spikelet number per spike, grain number per spike and TGW in two pot experiments conducted at different watering holding capacities (80%, 60%, 40%) to create water stress conditions. Even though these studies indicated improved grain number per spike with different antitranspirant applications (40 - 47 days after cultivation by Abdallah et al. (2019) and at GS39 by Mphande et al. (2021a)) at different soil moisture regimes, the present study showed no improvement in the grain number per spike with antitranspirants (VG or ABA) application. This implies that it also depends on the type of antitranspirants, their timing, crop variety and different watering and environmental conditions at the time of application.

TGW significantly increased by 12% in the controlled drought plants in comparison to well-watered ones in the present experiment, whereas no significant differences were observed by Mphande et al. (2021a) in the previous study under both progressive and controlled drought conditions for the same variety when compared to well-watered plants. As the number of spikes in the droughted pots was less, therefore, the controlled drought condition may have caused efficient translocation of assimilates to the remaining developing spikes compared to well-watered pots, where the number of spikes or grains was more, resulting in less assimilates available to fill in all the grains in spikes. Therefore, this might be the reason that grain size was less, resulting in lower TGW of well-watered plants compared to droughted, sprayed or unsprayed plants. Also, the current study indicated that spraying antitranspirants did not show any significant effect on the TGW of plants in comparison to unsprayed ones, as consistent results of no significant differences in the different antitranspirant treatments were observed for TGW by Mphande et al. (2021a).

Grain yield was significantly affected in droughted plants compared to well-watered ones, but there was no significant difference in the unsprayed and antitranspirant-treated plants,

suggesting that spraying antitranspirants did not affect grain yield of plants in contrast to, as observed in the previous studies on wheat (Abdullah et al., 2015; Abdallah et al., 2019; Mphande et al., 2021a). There could be many reasons for these no or negligible effects from antitranspirants in the present study, as already mentioned in the earlier paragraphs (mainly soil moisture and environmental variations and fluctuations). Furthermore, this indicates the varied response of different antitranspirants on wheat plants in previous studies, with some showing no effect on a particular yield component or an improved response of the same parameter in another, depending on several factors that can alter the responses of plants under specific environmental or soil moisture conditions.

Overall, although the same wheat variety and one similar growth stage (GS39) was used for spraying both types of antitranspirants as investigated in the previous study (Mphande et al., 2021a) (although with a different type of ABA antitranspirant and its multiple applications in a controlled drought experiment); it was observed that antitranspirants did not show any variation in most of the yield components under droughted conditions, as their response was almost related to droughted unsprayed plants except for the number of spikes per pot. Also, these results were consistent with the two field experiments, as discussed in the previous Chapter 3. This shows that even if the same variety is used to understand the effects of antitranspirants, it might not be possible to get consistently improved results in terms of yield, depending on the physiological and molecular state of the plants under the environmental or soil moisture conditions at that time.

4.5 Conclusion

In conclusion, film and metabolic antitranspirant application (at the flag leaf stage before the start of meiosis – GS39 and watery to milky grain stage – GS71-73) on a spring wheat variety (Chilham) under controlled drought condition in a glasshouse experiment indicated no significant changes in most of the measured parameters (relative water content, canopy temperature, pollen viability, endogenous ABA concentration in leaf and spike samples) as compared to unsprayed droughted plants. Similarly, most of the yield components showed the same response except for the number of spikes per pot that showed a significant reduction when antitranspirants were sprayed at the flag leaf stage, although it was not translated into yield, as grain yield was not affected by antitranspirants and was almost the same as in unsprayed plants.

These findings also do not support any of the hypotheses of improvement in the yield or water status of plants with antitranspirants application at any of the growth stages, as expected to be found under controlled environmental conditions. There was a slight improvement in pollen viability (by 2%) in sprayed plants at GS39 compared to unsprayed ones; however, it was not significant, and it was not translated into improved yield. Additionally, most of these results were consistent with the two field experiments (in Chapter 3), which suggest that even under a semi-controlled glasshouse environment, plants might not respond to different antitranspirant treatments. Moreover, it might also depend on the soil moisture, as the percentage of available water was less during the growth period and environmental conditions (as these were fluctuating during the days when antitranspirants were applied), along with the physiological state of plants. However, to understand their effect at the molecular level and to understand what happens to the genes linked to the key physiological responses, further studies are required. For this purpose, a transcriptomic study was conducted on early meiotic wheat anthers collected (from the antitranspirant-treated plants at GS39 along with unsprayed and well-watered plants) from the field experiment (2022) that is discussed, in detail in the next Chapter 5.

CHAPTER 5. Transcriptomic responses of wheat anthers at the early meiosis stage (leptotene-zygotene) to drought stress and antitranspirants (film and metabolic)

5.1 Introduction

In the last decade, transcriptomic research has gained more importance in different arable crops to understand the function of expressed genes and other physiological and molecular studies. Transcriptomics is the study of all the RNA molecules expressed in an organism under different environmental conditions. These varied gene expression patterns under different environments give more detail about the biology of an organism and allow us to infer its functions accurately (Lowe et al., 2017). By understanding the role and kind of genes involved in a particular physiological function of a plant, these can be manipulated and targeted easily to alter the trait of interest. Drought tolerance mechanisms vary in different crops and varieties under diverse climatic conditions, depending on their gene expression patterns at the molecular level, which might later determine their yield. One of the transcriptomic (flag leaf samples) and physiological studies by Reddy et al. (2014) on two wheat varieties with different drought tolerance mechanisms indicated that a variety with higher yield and elevated leaf ABA content showed more changes at the transcriptomic level than the other with lower crop yield. Therefore, it was concluded that the differential role of ABA in plants during the grain-filling stage may regulate the major changes at the molecular and physiological levels that can lead to higher yield and superior adaptation of a variety than the one with less grain yield under water deficit conditions. Another transcriptomic study by Ma et al. (2017) on wheat grown in rain shelters revealed that the early reproductive stages (spike differentiation stages) are more sensitive to drought and can affect crop development, gene expression patterns, and crop yield more than drought stress during the flowering stage. Additionally, some drought-responsive differentially expressed genes (DEGs) were found to link to photosynthesis, stomatal movement, and floral developmental processes.

Furthermore, drought stress during the reproductive stage of anthers is the leading cause of pollen sterility in many crops as it affects the processes related to carbohydrate availability, metabolism and distribution, hormonal signalling and pathways and alters their gene expression patterns to cope with stress environments as summarised by Yu et al. (2019). Reduced pollen fertility from the reproductive stage water stress is the main cause of grain loss, which might be associated with sink strength and carbohydrate supply to anthers to maintain pollen fertility under drought conditions (Ji et al., 2010). In rice, male fertility is

drastically affected by abnormal starch accumulation in anthers, and mainly, genes involved in microspore/tapetum development, formation of the cell wall, and starch synthesis are affected (Jin et al., 2013). Carbohydrate/sugar metabolism, including biosynthesis, transport, degradation and regulation, plays a key role in the male reproduction of plants. Defects or disruption of sugar metabolism during anther and pollen development of crop plants often lead to male sterility, as reviewed by Liu et al. (2021). These sugar pathways lead to starch biosynthesis and breakdown in rice pollen and play an important role in pollen fertility (Lee et al., 2022). Drought-tolerant wheat germplasm can maintain carbohydrate supply and accumulation in reproductive organs under water stress, leading to fertile anthers and ovary (Ji et al., 2010), while low sucrose availability can induce stress-related hormone synthesis (ABA and JA), which impacts spikelet fertility in wheat (Sun et al., 2023). Thus, it shows the crucial role of sugar and starch-related pathways/genes in anther or pollen development of crop plants, which could be affected under stressed environmental conditions. One study by Weerasinghe (2013) investigated the expression of one invertase gene (*IVR5*) in wheat anthers (using the RT-PCR method) for its role in improving pollen viability to understand the molecular mechanism of increased yield with film antitranspirants (applied at third node stage) under drought stress, as Koonjul et al. (2005) observed that the downregulation of *IVR1 and IVR5* invertase genes in wheat anthers under water stress might cause reproductive failure and lead to pollen sterility. However, Weerasinghe (2013) observed no significant difference in the expression level of the *IVR5* gene between film antitranspirant and unsprayed treatments; therefore, it was concluded that there was no evidence that film antitranspirants affect the expression pattern of these genes to improve pollen viability to alleviate drought stress.

Several transcriptomic studies have focused on wheat for drought stress and on reproductive parts of various crop plants to understand different tolerance mechanisms; however, there has been no transcriptomic study in wheat following the use of antitranspirants. Therefore, this study was conducted to understand the effects of antitranspirants at the transcriptomic level in wheat anthers (at the early meiosis stage – leptotene-zygotene) and whether any of these transcriptional changes could be linked to changes at the physiological level and to crop yield.

The main hypothesis was that spraying antitranspirants results in less water stress, which leads to improved crop yield due to reduced inhibition of transcription of enzymes associated with enhanced pollen viability.

5.2 Materials and methods

Samples for transcriptomic study were collected from one of the polytunnels from the field experiment 2022 (Appendix 1). Replicates were collected from only one experimental plot of each treatment to reduce the variability at the transcriptomic level.

5.2.1 Collection of anthers at leptotene-zygotene of meiosis I

Spraying of the film (VG) and metabolic (ABA) antitranspirants for the field experiment 2022 was done at GS39 (flag leaf stage) before the start of meiosis, as already mentioned in Chapter 3 (section 3.2.3). For the collection of anthers at the early meiosis stage (leptotene-zygotene of meiosis I), wheat stems were collected (during the three weeks, 4 - 5 stems daily or every other day depending upon the distance between penultimate leaf and flag leaf sheath) around GS41 from four plots (well-watered, unsprayed, ABA and VG-treated plants) in one of the polytunnels (if the distance between auricles of the penultimate leaf and the flag leaf sheath was between 4.5 - 6 cm then anthers could be found in the spike (3 - 3.5 cm) at the leptotene-zygotene (lepo-zygo) stage of meiosis I) (Appendix 10). Stems were collected during the three weeks. Collected stems were taken to the laboratory where well-watered stems were put in a beaker with water, while droughted ones were placed in a 5% PEG-400 solution (polyethylene glycol was used to simulate drought stress condition as it induces osmotic stress in plants by changing water potential and reducing the availability of water (Steuter et al., 1981; Peršić et al., 2022)), to keep the water potential around -0.2 bars for droughted stems, based on water potential measurements assessed on the Chilham wheat variety stems from the preliminary glasshouse experiment. Both beakers with stems were placed in a refrigerator at 4 °C until anthers were extracted between 1 - 9 days from different samples or replicates of each treatment (Table 5.1).

Anthers were extracted (using No. 5 fine forceps) from spikes under a dissecting microscope (Zeiss Stemi 305). One anther was taken from one of the spikelets in the middle of a spike (either from the first or second floret) and put on a glass slide, one drop of 0.5% acetocarmine solution was added, and then heated for 5 seconds over a flame of a Bunsen burner. A coverslip was placed on the anther and gently tapped with the back of a forceps to squash it. The glass slide was placed under a light microscope (Zeiss Primostar 3) under 40x - 100x objectives to see if the anther was at the lepo-zygo stage or not, and if it was, then the remaining two anthers (from the same floret) were collected from the spikelet and placed in 500 µl RNAlater solution in a 1.5 ml Eppendorf tube. Approximately 50 anthers

were collected in one Eppendorf tube for each sample, checking each time by taking one anther from each spikelet to confirm they were at the right stage of meiosis.

There were four main anther samples (well-watered, unsprayed, from ABA-treated plants, and VG-treated plants) with three replicates (each replicate was different from each other based on the dates at which stems were collected, or anthers were extracted as mentioned in Table 5.1) of each treatment, making a total of 12 samples. Each anther sample contained anthers from multiple tillers. Each sample was stored in a refrigerator at 4 °C overnight after collecting 50 anthers in an Eppendorf tube to allow thorough penetration of RNAlater solution into the anthers. The next day, each sample was stored in a -80 °C freezer until further processing.

5.2.2 RNA extraction from anthers

RNA extraction from anthers was completed according to the protocol described by Blánquez (2022) (using an RNA extraction kit from Zymo Research). However, anthers were used rather than wheat stigmas in the original protocol. Samples were first placed in a refrigerator from -80 °C freezer to defrost, and later RNAlater solution was removed (using a micropipette to suck it out carefully so that only anthers remained in the tube) from the samples and 800 µl of TRIzol reagent (phase separation step) was added following the grinding of anthers in a tissue homogenizer. Further steps (to purify the aqueous phase) were followed according to the protocol. In the end, Nanodrop was used to assess the quantity and quality of extracted RNA. Later, samples were sent for sequencing to the company (GENEWIZ UK Ltd, with an order of 30 million read pairs per sample and 150 bp paired-end reads) and raw data (in the form of FASTQ files) from sample files was processed further, using the following steps to perform transcriptomic analysis.

Table 5.1: Details of wheat anthers extraction from plant stems at the early meiosis stage, along with antitranspirants treatment dates and anthers sampling period.

- Antitranspirant (ABA and VG) treatments were sprayed on 04/08/22 at the flag leaf stage (GS39) (47 days after planting).
- Wheat stems were collected (cut with scissors in the middle of plant stem with 3-4 leaves) from field and taken to lab (sampling 02/08/22 – 25/08/22).
- Droughted stems were put in 5% PEG-400 solution in a beaker until extraction was done. WW plant stems were kept in water (dipped up to 2-3 cm).
- Anthers at the leptotene-zygotene stage of meiosis I were extracted from spikes (sampling period 03/08/22 - 26/08/22).
- 50 anthers per sample were collected in 1.5 ml Eppendorf tubes containing 500 µl of RNAlater and stored at -80 °C freezer until further processing.
- All the samples were collected from the polytunnel one (East) in the Flatt Nook field at Harper Adams University, Shropshire, UK.

Four main samples (WW, US, ABA, VG) with three replicates of each, making a total of 12 samples altogether.
The unique name of each sample with number shows difference in timing of anther extraction from the other sample of that treatment.

Sample No.	Sample Name	Replicate No.	Dates range, wheat stems were collected & placed in water or PEG	Days' range, stems with leaves were stored in water or PEG solution	Anther collection date
1	WW1	1	02 Aug 2022	1 day	03 Aug 2022
2	WW2	2	02-03 Aug 2022	2 days	04 Aug 2022
3	WW4	3	02-07 Aug 2022	6 days	08 Aug 2022
4	US1	1	15 Aug 2022	0 day	15 Aug 2022
5	US3	2	15-16 Aug 2022	1-2 days	17 Aug 2022
6	US4	3	15-17 Aug 2022	1-3 days	18 Aug 2022
7	ABA2	1	15-21 Aug 2022	7 days	22 Aug 2022
8	ABA3	2	16-22 Aug 2022	1-7 days	23 Aug 2022
9	ABA5	3	17-23 Aug 2022	1-7 days	24 Aug 2022
10	VG1	1	16-24 Aug 2022	1-9 days	25 Aug 2022
11	VG2	2	16-24 Aug 2022	1-9 days	25 Aug 2022
12	VG5	3	17-25 Aug 2022	1-9 days	26 Aug 2022

WW = Well-watered plant anthers, US = Unsprayed droughted plant anthers, ABA = Abscisic acid-treated droughted plant anthers, and VG = Vapor Gard treated-droughted plant anthers.

5.3 Transcriptomic data analysis

5.3.1 Pre-processing of raw data files

Reads were trimmed using a trimmomatic tool to remove the adapters and reads of less than 80 base pairs (bp). Trimmed sample files were pseudoaligned using Kallisto (v0.46.1) to RefSeqv1.0 annotation v1.1 (International Wheat Genome Sequencing Consortium (IWGSC), 2018). After mapping, tximport (v1.16.1) was used to combine the count and transcript per million (TPM) data of all samples into one data frame.

5.3.2 Differential expression analysis

DESeq2 (v1.28.1) (Love, Huber and Anders, 2014) was used to compare samples between different conditions. After running DESeq2 on raw count data of all samples and removing low confidence genes, the count data was transformed using the vst function to create the PCA plot (via plotPCA function) to visualise the overall effects between different treatments and their replicates. PCA plot showed that there was high variability within replicates of each treatment (Figure 5.1); therefore, instead of running DESeq2 across samples from all treatments, a set of three contrasts was compared separately to find the differentially expressed genes. Three different contrasts (well-watered vs unsprayed (WW/US), ABA vs unsprayed (ABA/US), and Vapor Gard vs unsprayed (VG/US) were analysed in the DESeq2 analysis for finding differentially expressed genes in each contrast comparison. For each pair of treatment samples in the contrast, the raw count data was used to perform DESeq2 analysis using unsprayed samples as a reference. Using unsprayed as a reference, even with well-watered samples, made it easier to properly understand the gene expression patterns in the other contrast comparisons between VG or ABA anther samples with unsprayed. This process was performed for each contrast separately to find the differentially expressed genes (DEGs) using a p-adjusted value of < 0.05 and log2 fold change value of > 1 and < -1 to find upregulated and downregulated DEGs, respectively. Log2 fold change shows how much gene expression changes between the compared samples, while a positive value means an increase in expression (upregulation), and a negative value means a decrease in expression (downregulation). Before performing the differential analysis, data of each contrast was filtered to include only high-confidence genes expressed at > 0.5 TPM in contrast samples and all the low-confidence genes with low expression were removed (Ramírez-González et al., 2018). In the well-watered 55,754 genes, 56,213 genes in unsprayed, 55,868 genes in ABA and 55,890 genes in VG samples were retained at > 0.5 TPM.

5.3.3 GO enrichment analysis

For the gene ontology (GO) enrichment analysis, first GO terms for RefSeqv1.0 were converted to v1.1 annotation as performed by Borrill et al. (2019) by keeping genes that were > 99% identical to > 90% of the sequence (from v1.0 to v1.1). Then GOseq (v1.40.0) was used to get the GO enrichment terms for each contrast separately using the upregulated and downregulated genes of each contrast obtained from the differential analysis (padj < 0.05 and log2fold change > 1 or < -1).

5.3.4 Filtering invertase genes from expressed genes

Two lists of 126 and 130 invertase genes were obtained from Wang et al. (2022a) and Ye et al. (2022), respectively, and compared with the genes expressed in this study. The gene IDs found in the list from Wang et al. (2022a) were from the RefSeqv2.0 model, so BioMart in EnsemblPlants was used to find the sequences of those genes. Then, the sequences were BLAST (a bioinformatic program to search for sequence similarity) to get a 100% ID match according to the RefSeqv1.1 model, which was used in this case to do the comparison. Also, homoeologs of genes were found, and grouped to see whether all or one of them were expressed in different compared samples.

5.3.5 Starch and sucrose synthesis genes

Lists of starch-related genes obtained from Chen et al. (2023) and Zhao et al. (2024), along with some taken from the wGRN (http://wheat.cau.edu.cn/wGRN/) database, were compared with the expressed genes of this study. Also, the sucrose biosynthesis gene list given by Wang et al. (2022b) and sucrose synthase genes from Hou et al. (2014) and EnsemblPlants database were compared with DEGs of different samples.

5.3.6 Filtering some of the drought tolerance/response DEGs

A list of drought stress tolerance wheat genes and their alleles given in Bhanbhro et al. (2024), compiled from different literature studies, was compared with the DEGs expressed in different contrast comparisons of this study. Some other drought response/tolerance genes from the literature and EnsemblPlants / wGRN database were also compared with the DEGs in different anther samples. Also, the list of transcription factors (TFs) expressed under drought stress in rice florets or spikelets at various developmental stages, given by Jin et al. (2013), was compared with anther samples of this study. For this purpose, rice orthologs of wheat DEGs were found using EnsemblPlants via BioMart. It was not possible to obtain rice

orthologs of all the wheat DEGs, due to evolutionary differences and genome complexity. So, only DEGs with rice orthologs were compared with the Jin et al. (2013) rice list.

5.4 Results

Antitranspirants (ABA and VG) were sprayed on droughted plants at GS39, and anthers were harvested from plants around GS41 (sampling started 10 days after spraying) at the early meiosis stage. Later, RNA was extracted from different anther samples (from well-watered and droughted, including unsprayed, ABA-treated, and VG-treated plants) and sequenced. The sequencing data was aligned with RefSeqv1.1 using Kallisto. On average, samples had 33M reads, and 26M reads were pseudoaligned (80%) using Kallisto (Appendix 11). DESeq2 analysis was performed to find the differentially expressed genes (DEGs) in different anther samples.

Principal component analysis of transformed count data showed the variability between different types of samples and how the replicates of different treatments vary from each other (Figure 5.1). It can be seen in Figure 5.1 that replicates are not closely clustered together, which may be due to plant stems and anthers harvested on different dates (Table 5.1) for each of these replicate samples, which explains the variability between replicates in the PCA plot or it might be because of their innate biological variability. Therefore, for understanding the transcriptional changes between different treatments, three main types of contrasts (WW/US, ABA/US and VG/US) were compared in DESeq2 separately using unsprayed as a reference.

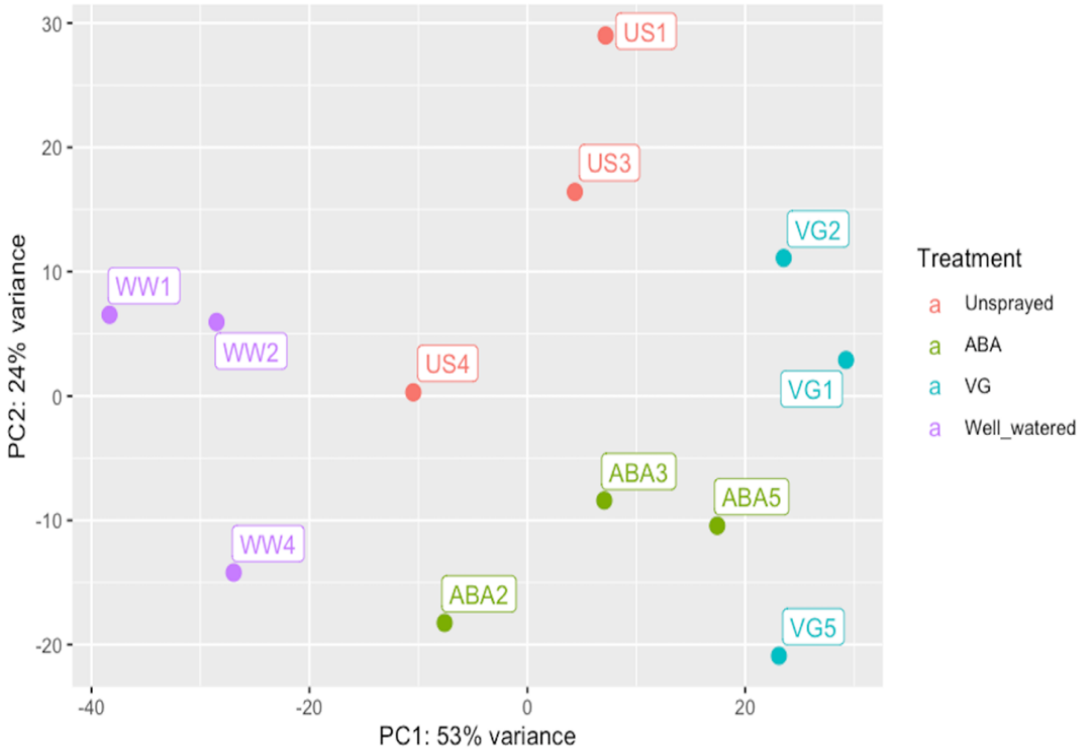


Figure 5.1: Principal component plot of anther samples showing the maximum and second most variation (PC1 and PC2) between different treatments and their replicates. More details of each sample can be seen in Table 5.1.

More than 5,000 (5,641 genes) differentially expressed genes (DEGs) were identified in different contrast comparisons. The total number of downregulated DEGs in two droughted contrasts (ABA/US and VG/US) were very high (3,959 genes) in comparison to upregulated DEGs (830 genes) while in contrary to this, the number of downregulated genes was less (226 genes) in the well-watered contrast (WW/US) in comparison to upregulated DEGs (626 genes) (Figure 5.2). This suggests that under drought stress, most of the genes involved in different developmental processes in anthers at the early meiosis stage were transcriptionally downregulated due to water stress, while in well-watered anthers, most of these processes were upregulated. Moreover, in droughted contrasts, the number of downregulated DEGs was higher in VG/US contrast (3,325 genes) than in ABA/US (634 genes), with upregulated 697 genes and 133 genes in each, respectively (Figure 5.2). This shows that VG (film antitranspirant) treated plant anthers had a high number of differentially expressed genes, while most of these genes were not differentially expressed in ABA-treated plant anthers, suggesting a unique response in two types of antitranspirants.

Furthermore, the up- and downregulated DEGs from all contrasts were used to make an UpSet plot to find the overlapping genes between different contrast comparisons or treatments (Figure 5.3). In two antitranspirant contrasts (ABA/US and VG/US), 85 upregulated genes were common and 479 downregulated genes. There were 20 upregulated overlapping genes between the WW/US and VG/US contrast, 4 with ABA/US, and 6 overlapping genes between the three contrasts. Most of the DEGs that were found in ABA anthers were also found in VG anthers for both up- and downregulated genes, which suggests that VG induced most of the ABA responses but also had additional effects. Moreover, there were unique genes (up- and downregulated DEGs) in both types of antitranspirant-treated plant anthers (ABA – 37 & 92 genes and VG – 578 & 2700 genes, respectively) as shown in Figure 5.3, indicating some differences in molecular responses. Thus, the number of overlapping genes was less in comparison to the unique genes in different contrasts.

In the case of overlapping DEGs that were upregulated in well-watered (WW/US) anthers but downregulated in ABA/US or VG/US anthers showed that 116 common DEGs were upregulated in well-watered anther samples but downregulated in VG samples, thus indicating that the genes of different processes were transcriptionally suppressed by spraying of film antitranspirant. Whereas only 12 overlapping DEGs were downregulated with the spraying of metabolic ABA, and these were also common in VG samples. Additionally, there were 7 overlapping downregulated genes in WW/US anthers that were upregulated with the spraying of film antitranspirant (VG) with one common gene in both ABA and VG anthers (Figure 5.3). Thus, a few genes were upregulated with the spraying of antitranspirants that were otherwise downregulated in well-watered samples.

Moreover, there were also overlaps of DEGs that were downregulated in well-watered (WW/US) anthers and the application of antitranspirants further downregulated those genes, 40 common genes with metabolic ABA treatment and 7 genes with VG treatment, while 11 DEGs were downregulated with both antitranspirants as in well-watered anthers (Figure 5.3).

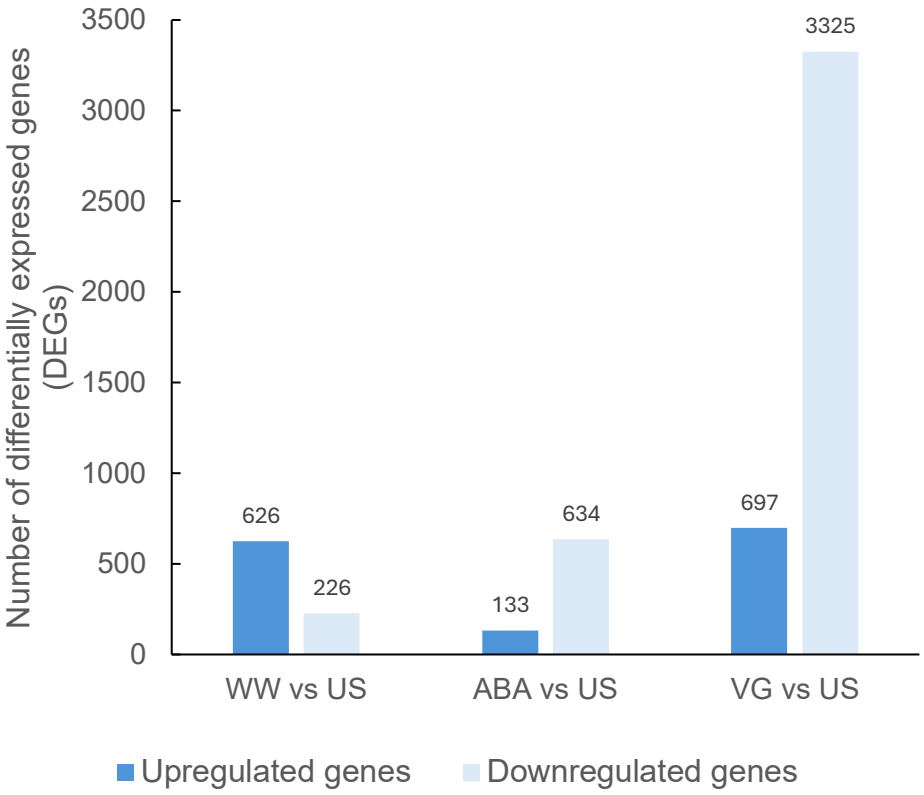


Figure 5.2: Number of differentially expressed upregulated and downregulated genes (padj < 0.05, log2 fold change > 1 (upregulated) and < -1 (downregulated) in three types of contrasts comparisons (well-watered vs unsprayed, ABA vs unsprayed and VG vs unsprayed) after DESeq2 analysis.

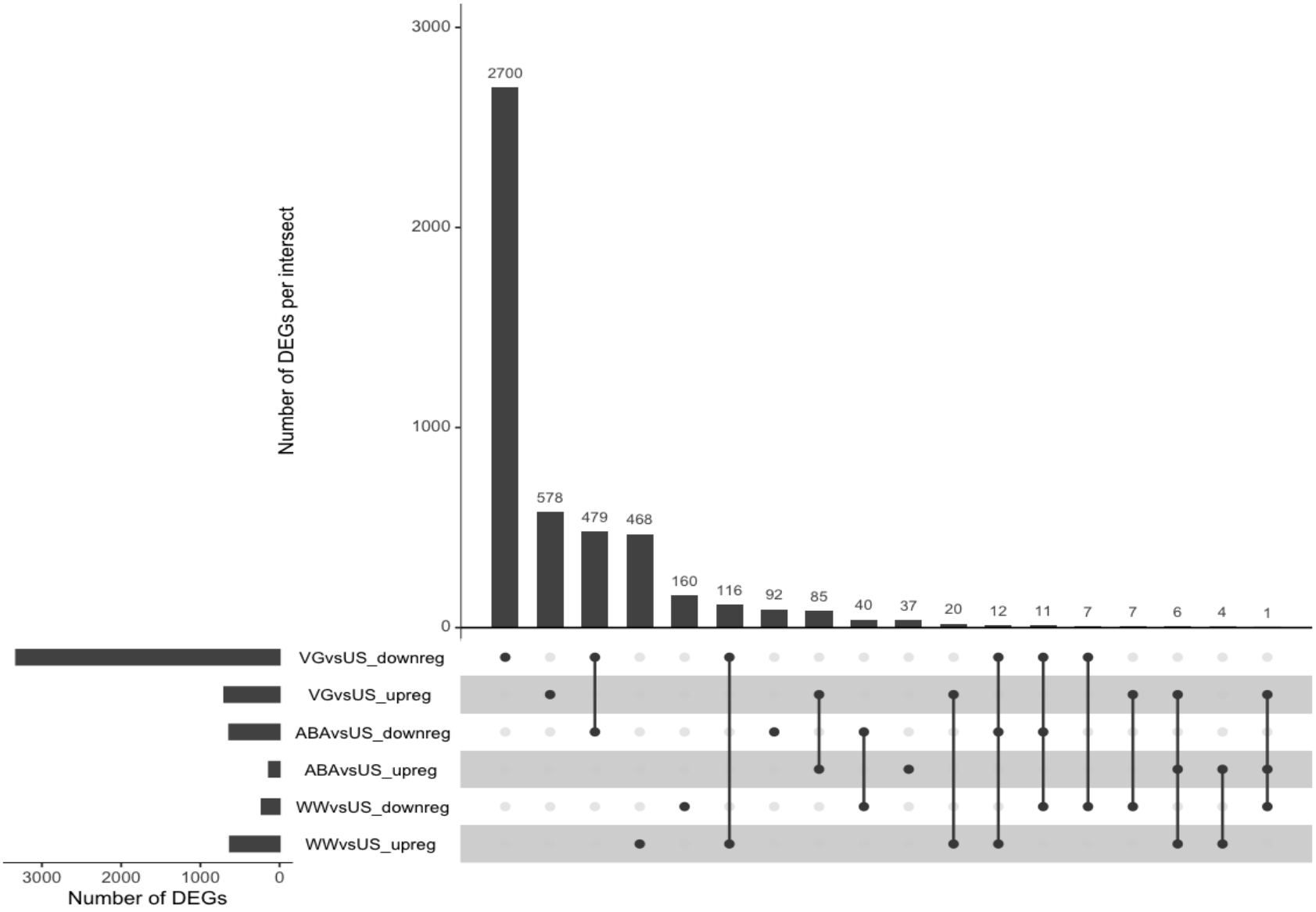

Number of DEGs per intersect
3000
2000
1000
0
2700
578
479
468
160
116
92
85
40
37
20
12
11
7
7
6
4
1
VGvsUS_downreg
VGvsUS_upreg
ABAvsUS_downreg
ABAvsUS_upreg
WWvsUS_downreg
WWvsUS_upreg
3000
2000
1000
0
Number of DEGs

Figure 5.3: Differentially expressed upregulated and downregulated genes (padj < 0.05, log2 fold change > 1 (upregulated) and < -1 (downregulated)) in different contrast comparisons of well-watered vs unsprayed (WW vs US), ABA vs unsprayed (ABA vs US) and VG vs unsprayed (VG vs US) anther samples shown in an UpSet plot. The plot shows the number of up- and downregulated DEGs that overlap across different contrast samples into intersects. The vertical bars represent the number of either up- or downregulated DEGs in each intersect, with the filled circle below showing which contrast sample it belongs to, while the horizontal bars represent the total number of either up- or downregulated DEGs for that sample.

5.4.1 GO enrichment terms

To understand the functions of genes involved in different biological processes, GO enrichment analysis was performed. In the first contrast (WW/US), the GO terms related to cell cycle (heterochromatin/chromosome organisation, nucleosome assembly), carbohydrate/fructose metabolism, and transport were highly enriched amongst upregulated genes. A few main GO terms related to water stress, such as hydrogen peroxide, heat, reactive oxygen species, protein folding/unfolding, and abscisic acid response, were enriched amongst downregulated genes in the well-watered in comparison to unsprayed droughted samples.

There were very few GO terms enriched for upregulated genes in the second contrast (ABA/US), as the number of upregulated genes was lower compared to other contrasts of well-watered and VG samples. Genes enriched for GO terms involved in transcription, respiratory or oxidative burst were the prominent ones. A few key terms enriched for downregulated genes were related to carbohydrate metabolism, oxidation-reduction, flavonoid biosynthesis, photosynthesis, nutrient ion transport, sporopollenin (a biological compound which is the major component of the cell wall of pollen grains or plant spores) and hormones.

In the final contrast comparison of VG and US samples, a few top GO terms associated with upregulated genes were related to water stress, oxidative burst, heat, transcription, hormones, nutrient ion transport and pollen development. In downregulated genes, the few main GO terms enriched for biological processes involved in photosynthesis, cell cycle, carbohydrate metabolism, oxidation-reduction, protein dephosphorylation, hormones, and nutrient ion transport. As the number of downregulated genes in this contrast was the highest (3,325 genes), therefore GO terms enriched for different biological processes were many (281 GO terms), which showed the huge variability of expressed genes involved in diverse biological processes in VG-treated plant anther samples. Figures 5.4, 5.5 and 5.6 show some of the main GO terms categories (related to hormones, anther/pollen and carbohydrates/sugars processes) that were either differentially expressed or showed no differential expression in two antitranspirant-treated plant anthers (ABA/US and VG/US) and well-watered anthers (WW/US). The detailed lists of all the overrepresented GO terms (for upregulated and downregulated genes) enriched for different biological processes in the early meiotic anthers for each contrast comparison are given in Appendix 13.

5.4.1.1 Few common and dissimilar GO terms in ABA and VG contrasts

Common genes and GO terms related to these genes that were expressed in both ABA and VG-treated plant anthers were filtered. Biological processes enriched for upregulated genes in both antitranspirant-treated plant anthers were related to the regulation of transcription, respiratory burst involved in defence response and heat response. For common downregulated genes, some of the main GO terms enriched were associated with carbohydrate metabolism, nutrient ion transport, photosynthesis, oxidation-reduction process, flavonoid biosynthetic process, sporopollenin and pollen exine formation, hormones, secondary metabolites, and oxidative stress response. GO terms enriched for biological processes that were different in both antitranspirant contrasts were mainly related to different stress responses, hormones, nutrient ion transport, cell cycle and sugar metabolism.

5.4.1.2 Hormonal responses and pollen/anther related GO terms

A few GO terms related to hormones were separated to observe gene expression changes in different samples. Some of the main hormonal GO terms enriched for DEGs were related to abscisic acid, gibberellin, jasmonic acid, salicylic acid, ethylene, indoleacetic acid and cytokinin. Most of the hormonal terms enriched for DEGs were observed in droughted anthers in comparison to well-watered anthers. In ABA anther samples, all the hormonal terms were enriched for downregulated genes, while in VG samples, both up- and downregulated enriched terms were observed (Figure 5.4). Abscisic acid signalling pathway and abscisic acid response GO terms were enriched for upregulated genes in the VG samples, whereas none of these terms were enriched in ABA anther samples; instead abscisic acid metabolic process term was enriched for downregulated genes in ABA samples. GO terms related to gibberellin biosynthetic and metabolic processes were enriched for upregulated genes in VG samples, and in ABA samples, one GO term related to gibberellic acid homeostasis was enriched for downregulated genes. GO terms enriched for jasmonic acid and salicylic acid signalling pathway showed transcriptional downregulation in both antitranspirant-treated plant anthers when compared with unsprayed, while no differential transcriptional response was observed in the well-watered anthers. Ethylene response and signalling pathway GO terms were upregulated, and response to cytokinin and indoleacetic acid biosynthetic process was downregulated in VG samples only.

GO terms related to pollen exine formation (pollen wall) and sporopollenin biosynthetic process indicated a clear difference between well-watered and droughted, treated anthers (both ABA and VG) with significant upregulation and downregulation of these processes in

each condition, respectively (Figure 5.5). GO term enriched for pollen development showed contrasting expression in droughted ABA anthers (downregulation) and VG anthers (upregulation) when compared with unsprayed, while in well-watered, no GO term/differential genes were observed (Figure 5.5).

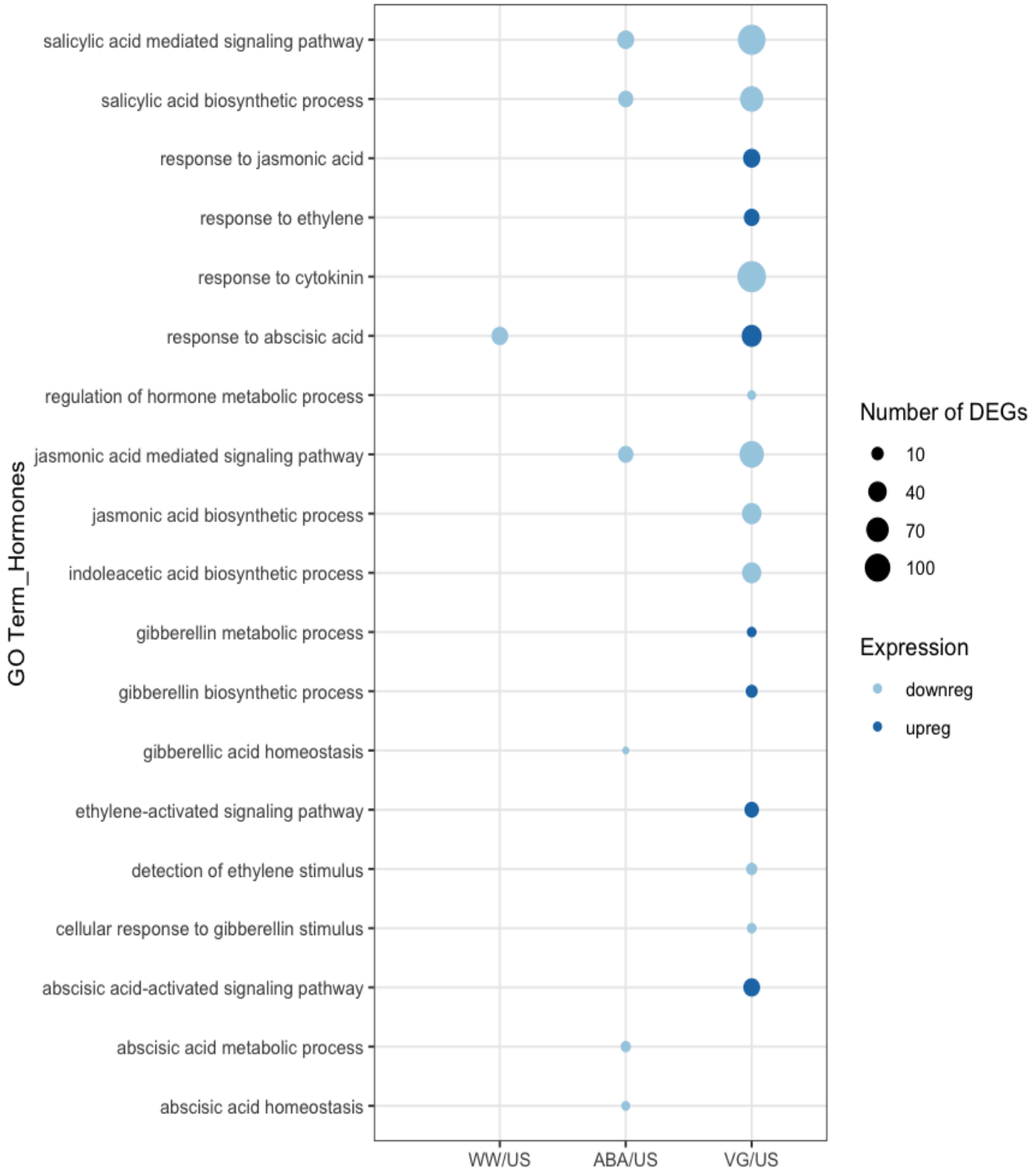


Figure 5.4: GO terms enriched for biological processes related to hormones in three contrasts comparisons of well-watered, ABA and VG (droughted) treated plant anthers in comparison to unsprayed. Dark blue colour represents the GO terms enriched for upregulated genes, and light blue represents the GO terms enriched for downregulated genes. The size of each circle shows the range of the number of differentially expressed genes (DEGs) linked to the GO term overrepresented in different contrasts.

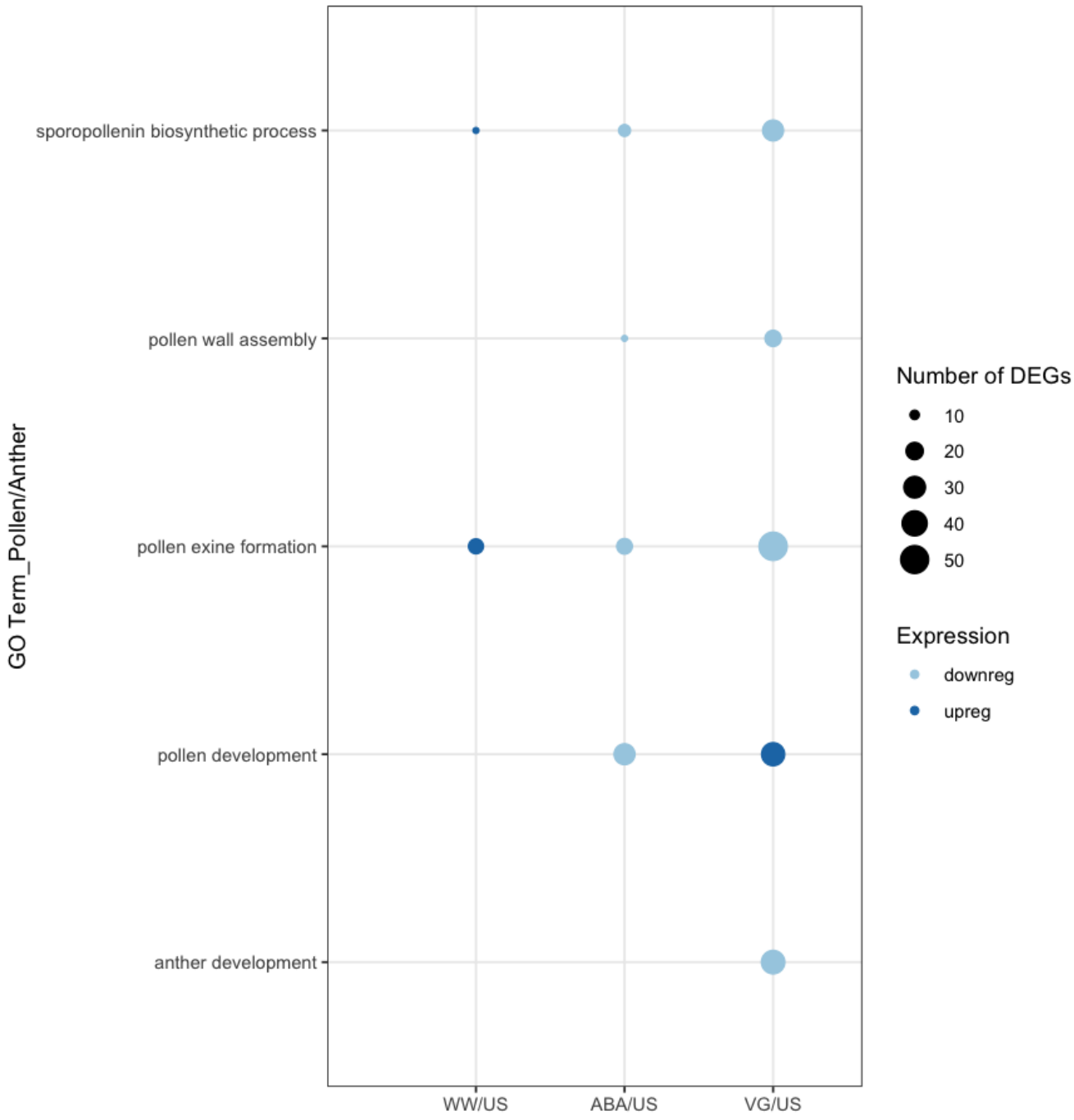


Figure 5.5: GO terms enriched for pollen/anther-related biological processes in three contrasts comparisons of well-watered, ABA and VG (droughted) treated plant anthers in comparison to unsprayed. Dark blue colour represents the GO terms enriched for upregulated genes, and light blue represents the GO terms enriched for downregulated genes. The size of each circle depicts the range of the number of differentially expressed genes (DEGs) linked to the GO term overrepresented in different contrasts.

5.4.1.3 Carbohydrate/sugar metabolism

Literature research showed that genes related to carbohydrate/sugar metabolism played a significant role in terms of pollen fertility (Liu et al., 2021). So, GO terms related to carbohydrate/sugar metabolism and transport were separated (Figure 5.6), which indicated that in the well-watered plant anthers, genes involved in the carbohydrate/sugar metabolism processes were upregulated and in droughted conditions (antitranspirants treated) both in ABA and VG plant anthers, genes were downregulated when compared to unsprayed. It also revealed some genes or related GO terms that were not differentially expressed in some samples while expressed in others, suggesting variable responses in different antitranspirant-treated plant samples and well-watered ones.

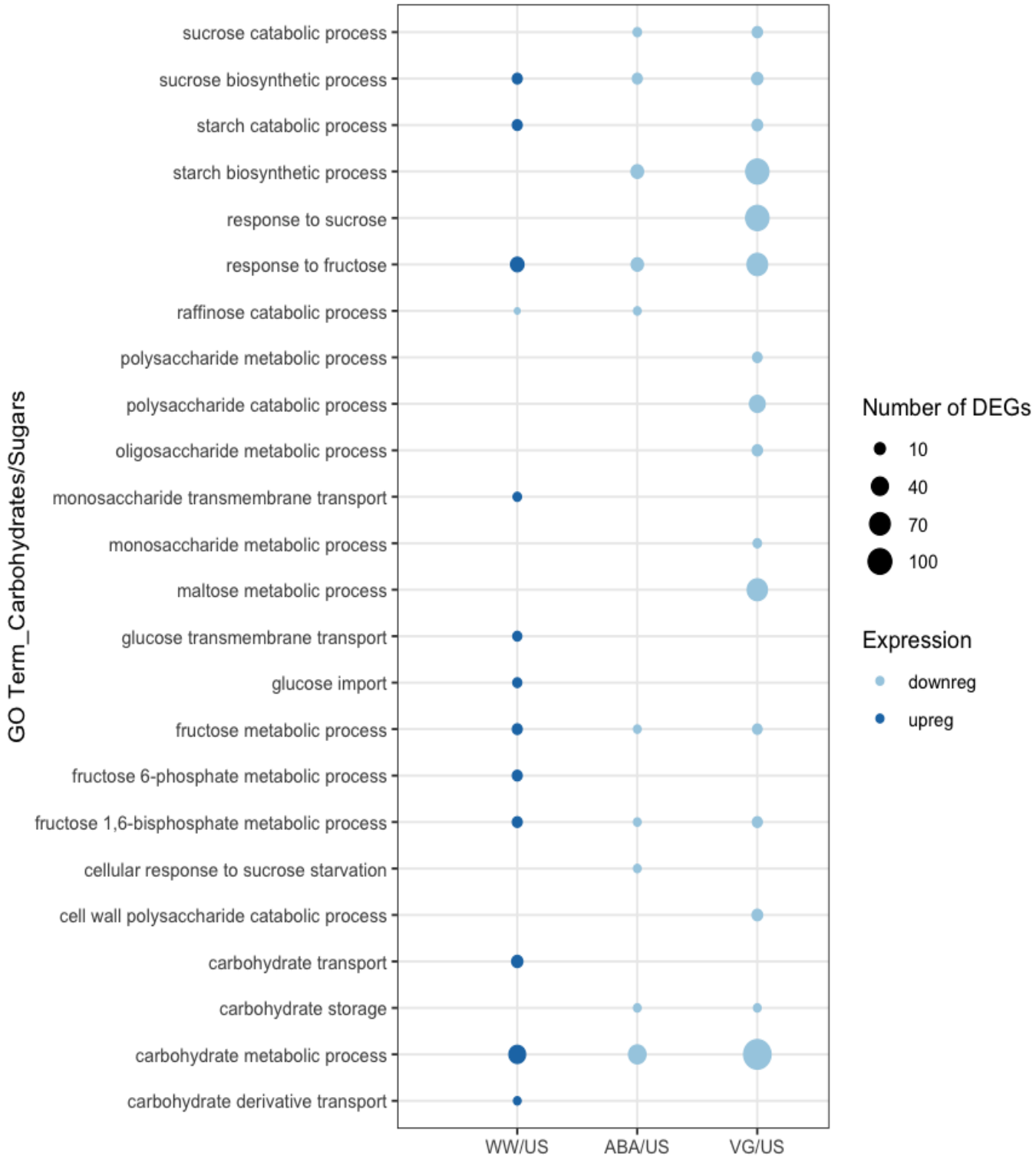


Figure 5.6: GO terms enriched for carbohydrate/sugar metabolism and related processes in the three contrasts comparisons of well-watered, ABA and VG (droughted) treated plant anthers in comparison to unsprayed. Dark blue colour represents the GO terms enriched for upregulated genes, and light blue represents the GO terms enriched for downregulated genes.

5.4.2 Invertase genes

Previous studies showed the importance of invertase genes in carbohydrate metabolism; 17 invertase genes were found with differential expression in various samples (Figure 5.7). One cell wall invertase gene *(TraesCS4B02G356800)* showed a clear difference between samples, with significant upregulation in WW/US contrast and downregulation in two other droughted contrasts (ABA/US and VG/US), while another cell wall invertase gene *(TraesCS1A02G214700)* expressed only in first contrast (WW/US) with significant upregulation in well-watered anthers, while it was not differentially expressed in droughted anthers. All the other expressed invertase genes were downregulated, with only five genes differentially expressed in the ABA/US contrast comparison, 16 genes in VG/US contrast, with five genes common in both.

Homoeolog genes were grouped together to see their expression pattern; out of 13 homoeolog gene groups, only one homoeolog was expressed in 10 groups, while in the other 3, all the homoeolog genes were expressed. Invertase genes in 8 of these homoeolog groups belonged to the cell wall invertase gene family (*TaCWI,* invertase genes expressed in cell wall), four belonged to the vacuolar gene family (*TaVI*, invertase genes expressed in vacuole), while one belonged to the cytoplasmic (*TaCI*, invertase genes expressed in cytoplasm) gene family (Figure 5.7).

Gene Type	Gene ID	WW/US	ABA/US	VG/US
TaCWI	TraesCS2A02G295400			
TaCWI	TraesCS2B02G311900			
TaCWI	TraesCS2D02G293200			
TaCWI	TraesCS3A02G516900			
TaCWI	TraesCS3B02G584500			
TaCWI	TraesCS3D02G524600			
TaCWI	TraesCS4A02G321600			
TaCWI	TraesCS5B02G557100			
TaCWI	TraesCS5D02G552000			
TaCWI	TraesCS5A02G526200			
TaCWI	TraesCS4B02G356800			
TaCWI	TraesCS4D02G350500			
TaCWI	TraesCS2A02G489000			
TaCWI	TraesCS2B02G516800			
TaCWI	TraesCS2D02G489300			
TaCWI	TraesCS2A02G488900			
TaCWI	TraesCS2B02G516700			
TaCWI	TraesCS2D02G489200			
TaCWI	TraesCS3B02G028500			
TaCWI	TraesCS3D02G015900			
TaCWI	TraesCS1A02G214700			
TaVI	TraesCS7A02G009800			
TaVI	TraesCS7D02G009400			
TaVI	TraesCS7A02G009200			
TaVI	TraesCS7D02G008800			
TaVI	TraesCS4A02G484800			
TaVI	TraesCS7D02G010000			
TaVI	TraesCS7A02G010200			
TaCI	TraesCS2A02G304200			
TaCI	TraesCS2B02G320900			
TaCI	TraesCS2D02G302800			

Figure 5.7: Invertase genes differentially expressed in well-watered, ABA and VG (droughted) treated plant anthers in comparison to unsprayed. Dark blue represents upregulated genes, light blue represents downregulated genes, and light yellow represents the expressed homoeologs genes (in different contrast samples) in 13 different homoeolog gene groups separated by a solid black line. *TaCWI* represents cell wall invertase genes, *TaVI* represents vacuolar invertase genes, and *TaCI* represents cytoplasmic invertase genes.

5.4.3 Starch and sucrose synthesis related genes

For understanding the expression pattern of starch-related genes (mainly those involved in starch synthesis) in the early meiotic anthers, which might play an important role in pollen viability (Yu et al., 2023a), wheat starch-related gene lists obtained from Chen et al. (2023) and Zhao et al. (2024) study and some taken from the wGRN database, were compared with the DEGs list of different anther samples. Thirteen DEGs were found, indicating downregulation in VG anthers, with only four expressed in ABA anthers and no DEGs in well-watered samples (Figure 5.8).

Gene Name	Gene ID	WW/US	ABA/US	VG/US
Starch synthase	TraesCS1A02G091500			
Starch branching enzyme	TraesCS7A02G549300			
Starch branching enzyme	TraesCS2B02G327300			
Disproportionating enzyme	TraesCS2A02G123800			
Beta amylase	TraesCS2A02G215100			
Beta amylase	TraesCS2B02G240100			
Beta amylase	TraesCS2D02G220900			
Beta amylase	TraesCS2A02G215300			
Alpha amylase	TraesCS7A02G383900			
Purple acid phosphatase	TraesCS4A02G400600			
Purple acid phosphatase	TraesCS4B02G348600			
Purple acid phosphatase	TraesCS4D02G343000			
Purple acid phosphatase	TraesCS7D02G095900			

Figure 5.8: Starch-related genes expressed in different anther samples. Light blue colour depicts the downregulation of differentially expressed genes (DEGs), while no colour shows that genes were not differentially expressed in that contrast comparison.

For genes involved in sucrose synthesis, a list of 312 sucrose biosynthesis-related genes obtained from Wang et al. (2022b) genome-wide study in wheat, sucrose synthase genes in Hou et al. (2014), with some taken from EnsemblPlants database (genes related to sucrose synthase) were compared with DEGs expressed in different anther samples. After comparison, at least 30 DEGs were found in the three contrasts (Figure 5.9), showing that all the expressed genes in antitranspirant samples (ABA and VG) were downregulated compared to well-watered anthers. Only one sucrose synthase gene was downregulated in well-watered anthers but was not differentially expressed in ABA or VG anthers. Moreover, eight DEGs related to starch (including starch synthase, one starch branching gene

(*TraesCS2B02G327300*), alpha-glucanotransferase, alpha and beta amylases) given in Figure 5.8 also come under the category of genes involved in sucrose synthesis, which are not present in Figure 5.9 as already presented in Figure 5.8. Also, filtered a few DEGs related to sucrose transport, of which three genes showed upregulation in only one of anther sample (well-watered or ABA or VG), with no differential expression of the same gene in the other two samples, while one sucrose transport gene (*TraesCS7A02G090700*) was downregulated in both antitranspirants (ABA and VG) anthers (Figure 5.9).

Gene Description	Gene ID	WW/US	ABA/US	VG/US
Fructose-bisphosphate aldolase	TraesCS4A02G206400			
Fructose-bisphosphate aldolase	TraesCS4B02G109900			
Fructose-bisphosphate aldolase	TraesCS4D02G107400			
Fructose-bisphosphate aldolase	TraesCS5A02G108000			
Fructose-bisphosphate aldolase	TraesCS5B02G115300			
Fructose-bisphosphate aldolase	TraesCS5D02G122700			
Fructose-bisphosphate aldolase	TraesCS7A02G381100			
Fructose-bisphosphate aldolase	TraesCS7B02G283000			
Fructose-1,6-bisphosphatase	TraesCS1A02G273900			
Fructose-1,6-bisphosphatase	TraesCS1B02G283600			
Fructose-1,6-bisphosphatase	TraesCS1D02G274000			
Fructose-1,6-bisphosphatase	TraesCS1D02G274200			
Fructose-1,6-bisphosphatase	TraesCS3A02G377600			
Fructose-1,6-bisphosphatase	TraesCS3B02G410400			
Fructose-1,6-bisphosphatase	TraesCS3D02G370700			
Fructose-1,6-bisphosphatase	TraesCS4A02G093100			
Fructose-1,6-bisphosphatase	TraesCS4D02G212000			
Fructose-1,6-bisphosphatase	TraesCS7D02G471600			
Fructose 6-phosphate	TraesCS5D02G085500			
Fructose 6-phosphate	TraesCS7B02G193600			
Fructose 6-phosphate	TraesCS7D02G228500			
Sedoheptulose-1,7-bisphosphatase	TraesCS3A02G367000			
Sedoheptulose-1,7-bisphosphatase	TraesCS3B02G398300			
Sedoheptulose-1,7-bisphosphatase	TraesCS3D02G359900			
Triose phosphate	TraesCS3A02G161300			
Triose phosphate	TraesCS3B02G192400			
Triose phosphate	TraesCS3D02G168600			
ATP-dependent 6-phosphofructokinase	TraesCS7A02G106800			
Sucrose phosphate synthase	TraesCS3B02G461800			
Sucrose synthase	TraesCS4A02G140000			
Sucrose transport protein	TraesCS2A02G505000			
Sucrose transport protein	TraesCS2B02G533300			
Sucrose transport protein	TraesCS7A02G090700			
Sucrose transport protein	TraesCS4D02G286500			

Figure 5.9: Sucrose biosynthesis-related and transport genes expressed in different anther samples. Dark blue represents the upregulation of the gene, whereas light blue represents the downregulation of the gene in three contrast comparisons.

5.4.4 Drought response/tolerance related genes

Identified wheat genes and their alleles involved in drought stress tolerance regulation, compiled from various literature studies mentioned in Bhanbhro et al. (2024), were compared with the DEGs in different anther samples. As the gene list was compiled from a wide variety of experimental conditions, plants or tissues, it was not possible to find most of the genes in anther samples. Therefore, out of 114 genes provided by Bhanbhro et al. (2024), only twelve DEGs were found, most of which were expressed in VG anthers compared to other samples (Figure 5.10). One MYB and trihelix transcription factor gene was upregulated in VG samples along with zinc finger protein and ABA receptor genes, but these were not differentially expressed either in well-watered or ABA anthers. Ferritin and expansin genes were downregulated in VG or ABA samples.

Gene Description	Gene ID	WW/US	ABA/US	VG/US
MYB transcription factor	TraesCS2A02G206400			
Zinc finger protein	TraesCS5A02G477400			
Zinc finger protein	TraesCS5B02G490600			
Zinc finger protein	TraesCS5D02G491000			
Ferritin	TraesCS5B02G151000			
Ferritin	TraesCS5D02G157600			
Trihelix transcription factor	TraesCS2A02G389200			
Abscisic acid receptor	TraesCS2A02G089400			
Abscisic stress-ripening protein	TraesCS4B02G112000			
Abscisic stress-ripening protein	TraesCS4D02G109500			
Expansin - EXPB7	TraesCS1B02G225700			
TabZIP2 transcription factor	TraesCS6D02G332500			

Figure 5.10: Some drought tolerance response genes expressed in different anther samples. Dark blue represents the upregulation of the gene, whereas light blue represents the downregulation of the gene in three contrast comparisons.

Different types of transcription factors (TFs) were expressed in anther samples of this study after comparing with the rice TFs list of Jin et al. (2013) drought stress study of rice florets/spikelets at different developmental stages (Figure 5.11). Some heat stress (shock) factors (HSF) were upregulated in both ABA and VG anthers, along with one ERF gene. Whereas some bHLH and C2H2-zinc finger protein (ZFP) factors were upregulated, and a few MYB-related TFs were downregulated in well-watered anthers but not differentially expressed in other anther samples. The bZIP transcription factors were downregulated in

both ABA and VG anthers, whereas GRAS transcription factors were downregulated in VG anthers only. Some other drought-related genes, either found from the literature or EnsemblPlants / wGRN database, are given in Table 5.2, with their differential expression, either in well-watered, ABA or VG anther samples.

TF	Wheat Gene ID	Rice Ortholog	WW/US	ABA/US	VG/US
C2H2-ZFP	TraesCS3B02G435800	Os11g0442900			
C2H2-ZFP	TraesCS3D02G397400	Os11g0442900			
bHLH	TraesCS7A02G185300	Os06g0226500			
bHLH	TraesCS7B02G090500	Os06g0226500			
bHLH	TraesCS7D02G187000	Os06g0226500			
bHLH	TraesCS6A02G264200	Os02g0691500			
bHLH	TraesCS7B02G211600	Os11g0523700			
bHLH	TraesCS7D02G308300	Os11g0523700			
WRKY	TraesCS7A02G508800	Os06g0649000			
HSF	TraesCS2A02G401600	Os04g0568700			
HSF	TraesCS2B02G419600	Os04g0568700			
HSF	TraesCS2D02G399000	Os04g0568700			
HSF	TraesCS4D02G276500	Os03g0161900			
HSF	TraesCS7A02G270100	Os08g0546800			
HSF	TraesCS7B02G168300	Os08g0546800			
HSF	TraesCS7D02G270600	Os08g0546800			
ERF	TraesCS7A02G057800	Os06g0127100			
ERF	TraesCS7D02G052500	Os06g0127100			
ERF	TraesCS7D02G052600	Os06g0127100			
ERF	TraesCS2D02G015200	Os06g0127100			
MYB	TraesCS6A02G173800	Os02g0187700			
MYB	TraesCS6B02G201700	Os02g0187700			
MYB	TraesCS6D02G162900	Os02g0187700			
MYB	TraesCS7A02G130700	Os03g0578900			
MYB	TraesCS7D02G130100	Os03g0578900			
bZIP	TraesCS7A02G488600	Os06g0662200			
bZIP	TraesCS7B02G391800	Os06g0662200			
bZIP	TraesCS7D02G475100	Os06g0662200			
GRAS	TraesCS2A02G194600	Os07g0589200			
GRAS	TraesCS2B02G212300	Os07g0589200			
GRAS	TraesCS2D02G193300	Os07g0589200			

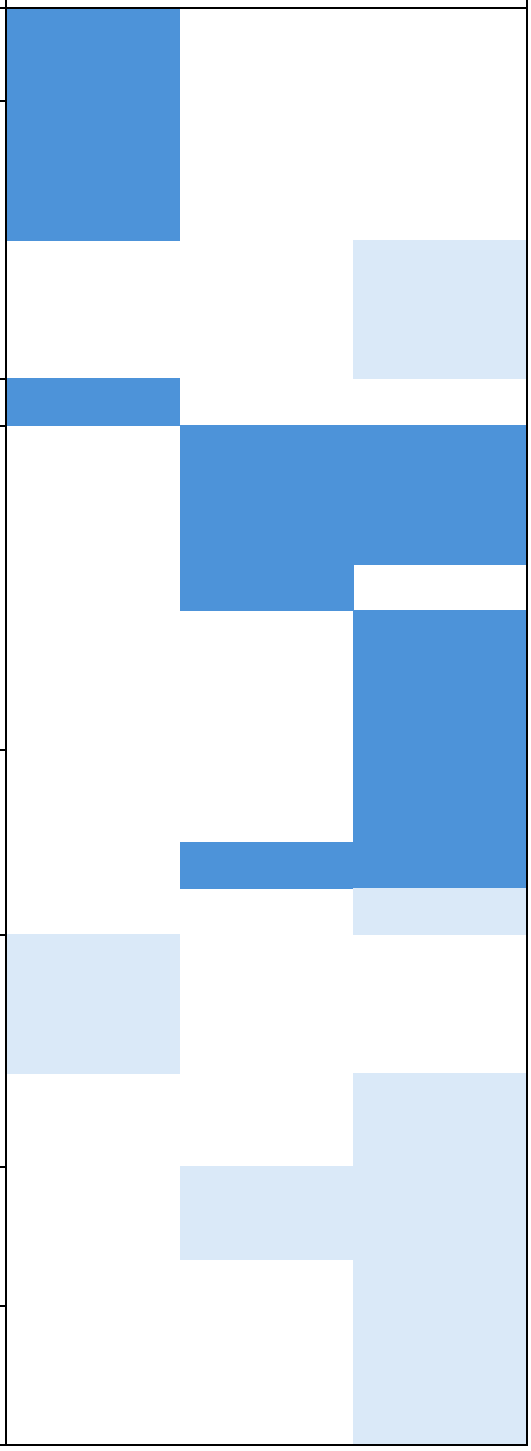
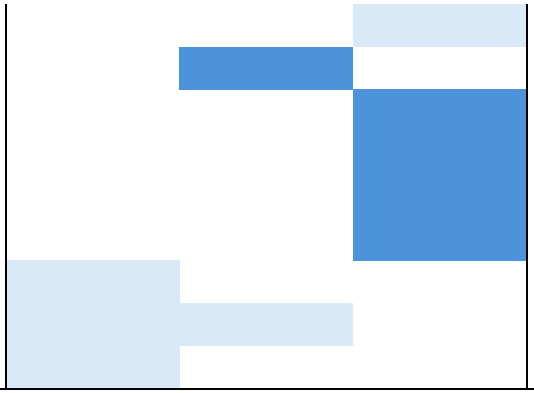


Figure 5.11: Transcription factors (TFs) that were differentially expressed in three types of anther samples. Each type of transcription factor (TF) is separated with a solid line, along with the expressed wheat gene ID and its rice ortholog. Dark blue represents the upregulation of the gene, whereas light blue represents the downregulation of the gene in different contrast comparisons. C2H2 zinc finger protein (C2H2-ZEP), basic Helix-Loop-Helix protein (bHLH) transcription factor, WRKY transcription factor, Heat shock factor (HSF), Ethylene responsive factor (ERF), MYB transcription factor, Basic leucine zipper (bZIP) transcription factor, GRAS transcription factor derived from three members: gibberellic-acid insensitive (GAI), Repressor of GAI (RGA) and Scarecrow (SCR) (Hirsch and Oldroyd, 2009).

Table 5.2: List of a few genes related to drought response/tolerance taken from literature or EnsemblPlants / wGRN database, with their expression pattern in different anther samples. Dark blue shows the upregulated genes, and light blue represents the downregulated genes.

Gene Description	Gene ID	WW/US	ABA/US	VG/US	Reference
BES1/BZR1 protein	TraesCS3A02G123500				Wang et al., 2023a
BES1/BZR1 protein	TraesCS3B02G142600				Wang et al., 2023a
BES1/BZR1 protein	TraesCS3D02G125100				Wang et al., 2023a
Serine/threonine-protein kinase	TraesCS4A02G010000				wGRN, Jin et al., 2013
Serine/threonine-protein kinase	TraesCS4B02G294300				wGRN, Jin et al., 2013
Phytochrome interacting factor	TraesCS5D02G428400				EnsemblPlants, wGRN
NAC transcription factor	TraesCS5A02G049100				wGRN, Zhang et al., 2024
NAC transcription factor	TraesCS5B02G054200				wGRN, Zhang et al., 2024
NAC transcription factor	TraesCS5D02G059700				wGRN, Zhang et al., 2024
Hydrophobic polypeptide	TraesCS4D02G197700				Zhang et al., 2024; Jin et al., 2013
Hydrophobic polypeptide	TraesCS4B02G197300				Zhang et al., 2024; Jin et al., 2013
Phosphoinositide phospholipase	TraesCS1D02G071800				Zhang et al., 2024; Deng et al., 2018
Brassinosteroid-responsive protein	TraesCS6D02G240300				wGRN, Zhang et al., 2024
Annexin	TraesCS6B02G330100				EnsemblPlants, wGRN
myo-inositol oxygenase	TraesCS7A02G357800				wGRN, Zhang et al., 2024
myo-inositol oxygenase	TraesCS7D02G364900				wGRN, Zhang et al., 2024
Calcium-dependent protein kinase	TraesCS4A02G283400				wGRN, Zhang et al., 2024
hypothetical protein/small peptide	TraesCS5B02G157600				Jin et al., 2013; Zhang et al., 2024
hypothetical protein/small peptide	TraesCS5D02G165000				Jin et al., 2013; Zhang et al., 2024
WRKY transcription factor	TraesCS1A02G300900				Ye et al., 2021
WRKY transcription factor	TraesCS5A02G225600				Ye et al., 2021; Zhang et al., 2024
Polygalacturonase	TraesCS3B02G258600				Nouraei et al., 2024
Lipid phosphate phosphatase	TraesCS5B02G546500				Gudi et al., 2024

Serine carboxypeptidase	TraesCS6B02G261700		Gudi et al., 2024; Zhang et al., 2024
Abscisic acid receptor	TraesCS1D02G195300		Mao et al., 2021
Protein phosphatase 2C (PP2C)	TraesCS5A02G183600		Mao et al., 2021; Zhang et al., 2024
Protein phosphatase 2C (PP2C)	TraesCS5B02G182000		Mao et al., 2021; Ma et al., 2017
Protein phosphatase 2C (PP2C)	TraesCS5D02G188600		Mao et al., 2021; Ma et al., 2017
Homeobox-leucine zipper protein	TraesCS2A02G389400		Mao et al., 2021; Zhang et al., 2024
Dehydrin	TraesCS6A02G350700		Mao et al., 2021; Zhang et al., 2024
Dehydrin	TraesCS5A02G369800		Zhang et al., 2024
Dehydrin	TraesCS5B02G372100		Zhang et al., 2024

5.5 Discussion

In this study, transcriptomic changes in antitranspirant-treated plant anthers at the early meiosis stage and well-watered plant anthers were compared with unsprayed droughted ones. A total of 852 genes were differentially expressed in well-watered anthers and 4,789 genes in droughted, treated-plant anthers (ABA and VG) (including all the up- and downregulated DEGs of each contrast, Figure 5.2). The strongest transcriptional changes were detected in VG/US contrast (4,022 genes, including up- and downregulated DEGs), whilst lower numbers of DEGs were found for ABA/US (767 genes) and WW/US (852 genes) contrast comparisons. The number of overlapping genes between different contrasts was less, which shows how gene expression varies in different anther samples, with most expressed genes being different in each contrast. The variations in the number of DEGs and their expression patterns showed contrasting results between film antitranspirant (VG) and metabolic antitranspirant (ABA) treated-plant anthers for some biological processes obtained via GO enrichment analysis. The key changes in expression patterns of genes involved in hormonal responses, pollen/anther-related genes, and carbohydrate/sugar-related processes were observed, and the genes involved in these processes were affected by drought stress as indicated in pollen/anther studies of different crop plants (Yu et al., 2019). At the physiological level, antitranspirants did not affect yield and yield components (Chapter 3, section 3.3.7 and Table 3.3 of the 2022 field experiment from which anthers were extracted for this transcriptomic study), in contrast to the significant transcriptional changes that were observed at the molecular level.

5.5.1 Antitranspirants application induces major transcriptomic changes

Many differentially expressed genes have been identified or investigated in different crop anthers to understand their molecular mechanism under drought conditions, such as in wheat (Koonjul et al., 2005; Ji et al., 2010), rice (Jin et al., 2013) and tomato (Lamin-Samu et al., 2021). After performing pairwise DESeq2 analysis on three types of contrast comparisons (WW/US, ABA/US and VG/US), hundreds to thousands of differentially expressed genes were found in different anther samples in the present study. In general, most of the genes in droughted anthers showed significant downregulation patterns in comparison to well-watered ones that were mostly upregulated. This altered and significant expression pattern of genes in different crop anthers was also summarised by Yu et al. (2019), according to which drought is responsible for changes in crop pollen development that altered the expression of genes involved in sugar transport, hormonal response,

reactive oxygen species and meiotic process related genes. This altered expression might help plants/anthers in repairing or avoiding any drought damage and protect their development in stressful environments. Based on this review, genes and GO terms linked with some biological processes were observed in this study, and consistent results were found that drought stress affected the mechanism related to carbohydrate/sugar metabolism, hormones, and pollen/anther related genes in droughted treated-plant anthers in comparison to well-watered anthers as most of the genes related to these processes were downregulated under droughted condition following the use of antitranspirants.

The number of downregulated genes was highest in VG-treated plant anthers (3,325 genes) in comparison to ABA anthers (634 genes), which might suggest that plants with VG antitranspirant treatment were more sensitive or prone to the highest changes at the gene expression level with most of the biological processes showing downregulation under water stress. Most of the genes that showed differential expression in VG anthers did not express in ABA anthers or showed no differential expression of genes involved in various biological processes; this indicates the unique drought tolerance mechanisms involved in two different types of antitranspirant-treated plant anthers and their varied responses at the gene expression level. This also suggests that VG-treated plants were more active in the downregulation of biological mechanisms involved under a stressful environment in comparison to ABA-treated ones, and this might be one of the ways of coping with the stress condition. Also, the mechanisms of both antitranspirants used to reduce stomatal conductance from plants were different. VG works by forming a physical barrier on the plant leaf surface to reduce transpiration, while ABA acts metabolically by either partially or fully closing stomatal pores to decrease transpiration from plants. This might also be the reason for their varied gene expression responses under a stressed environment.

One other reason for this could be that plant stems from which VG anthers were harvested, dipped in the PEG-400 solution longer (between 1 - 9 days maximum) than ABA ones (between 1 - 7 days maximum), and this could be one of the reasons for this highest number of genes expressed and downregulated in VG anthers. Another reason for this could be that a longer duration of dipping in PEG-400 might have caused toxicity due to ion accumulation in the leaves, as Kaufmann and Eckard (1971) indicated increased cations (K^+, Na^+, Ca^{2+} and Mg^{2+}) accumulation in the root xylem of pepper plants with PEG-400. However, as it is a lower molecular weight PEG, therefore, it is considered to have less effect on plant leaves in comparison to higher molecular weight PEGs (Lawlor, 1970), but as plant stems were dipped for a longer period, that might have some kind of toxic effect that was not related to either drought or antitranspirants. Also, if VG antitranspirant had a toxic effect on plants, that might

have caused this high number of genes to be differentially expressed; however, this has not been examined/reported in any studies related to this. However, one study by Abduallah et al. (2015) indicated that spraying film antitranspirant at GS39 on wheat reduced the number of tillers, emerged spikes and matured spikes, which implies that VG spraying before the start of the reproductive stage could affect developing spikes/anthers under drought stress, thus significantly impacting the gene expression responses at the molecular level. This might be the reason for a high number of differentially downregulated genes in VG anthers compared to ABA anthers.

In well-watered anthers, most of the genes involved in different biological processes showed transcriptional upregulation when compared with unsprayed droughted ones, suggesting the importance and sensitivity of genes involved at the molecular level that could later affect the physiology of plants (Reddy et al., 2014). This also indicates that under normal conditions, most of the processes related to the growth and development of plants are highly active at the transcriptional level and drought stress can significantly affect and alter these transcriptional responses to cope in stressed environments.

In GO enrichment analysis of differentially expressed genes, the two common GO terms (for upregulated genes) similar in both ABA and VG anthers were respiratory burst (also called oxidative burst) involved in defence response and regulation of transcription/DNA templated. It indicates that some of the coping mechanisms involved in tolerance were similar in both types of antitranspirant-treated plants, and genes related to these processes were highly upregulated in comparison to unsprayed plants to survive in water stress condition. Studies revealed that plants produce reactive oxygen species (ROS) under water stress, which act as a signalling pathway to other processes and defence mechanisms and help them to cope under stressful environments, but overproduction of ROS can even lead to cell damage or death (Cruz de Carvalho MH, 2008; Sachdev et al., 2021). Therefore, the upregulation of ROS-related genes/enriched GO term in droughted anthers (both ABA/US and VG/US, GO: 0002679) confirmed the water stress response in comparison to well-watered ones (no enriched GO term), while the GO term enriched for upregulation of transcription process (due to the involvement of transcription factors in drought tolerance response) might be linked to the altered gene expression of various processes and mechanisms under stress environments (Hu et al., 2022) to survive these harsh conditions.

5.5.2 Variation in hormonal responses and anther/pollen related processes

Hormonal balance in reproductive parts of plants is very important, with abscisic acid, gibberellic acid, jasmonic acid, auxin and cytokinin being the main ones responsible for reproductive development in plants and involved in drought tolerance mechanisms (Yu et al., 2019). For understanding the hormonal responses in early meiotic anthers, GO terms related to different hormones were separated. Some of the GO terms enriched for upregulated genes in VG anthers for abscisic acid, gibberellins, jasmonic acid and ethylene responses, while in ABA anthers, either different but related GO terms were enriched for downregulated genes for some hormones or no differential expression was observed. Thus, it indicates altered tolerance mechanism responses related to hormones with the involvement of different genes in two types of antitranspirant-treated plant anthers.

Abscisic acid, also called stress hormone, significantly increases in plants under drought stress (Muhammad Aslam et al., 2022) and is responsible for inducing ABA biosynthesis genes in wheat anthers (Ji et al., 2011). Spraying exogenous ABA has been shown to increase the level of endogenous ABA, while VG spraying is suggested to be linked with low endogenous ABA concentration in the flag leaves of the same wheat variety (Mphande et al., 2021a) used for the present transcriptomic study. Furthermore, the present study indicated contrasting responses of ABA-related GO terms in VG and ABA anthers, with upregulation and downregulation of different genes involved in each case, respectively. This might be due to the different mechanisms of how VG and ABA work and how they act on the endogenous ABA concentration, contrary to as investigated by Mphande et al. (2021a). However, despite these contrasting responses of DEGs from two antitranspirants in anther samples, endogenous ABA concentration of the top leaves selected from the field experiment 2022 (Chapter 3, section 3.3.5) after GS39 treatments from which anthers were collected later for this transcriptomic study, no significant differences (slight increase by 0.4% in sprayed plants compared to unsprayed, however, it was not significant) in the endogenous ABA content were observed between antitranspirant sprayed plants when compared with unsprayed droughted (Chapter 3. Table 3.2). This shows how the same process can vary in terms of their responses at the molecular and physiological levels, indicating more sensitivity at the molecular level than at the latter.

Gibberellic acid (GA), another key plant hormone, plays an important role in rice anther development as it regulates the processes involved in exine formation and programmed cell death of tapetal cells (Aya et al., 2009). Another study on rice anthers indicated that GA-responsive genes were downregulated under drought stress (Jin et al., 2013), whereas, in

VG anthers, genes involved in the GA biosynthesis and metabolic processes were upregulated, with no differential expression of these genes or enriched GO terms observed in ABA anthers. According to Kim et al. (2021), genes related to jasmonic acid (JA) response are upregulated under abiotic stress conditions in many crop plants, however, in this study, a few genes were upregulated, and a few were downregulated in VG anthers, while either no differential expression or downregulation was observed in ABA anthers. Thus, it indicates the clear difference in some hormonal responses in both types of droughted treated-plant anthers, whereas, in well-watered anthers, most of the GO terms for these plant hormones were not enriched, showing no differential expression of genes, indicating the normal response of anthers in the well-watered condition in contrast to the droughted ones. Cytokinin (CK) hormone plays a key role in cell division processes of male and female reproductive parts of plants and is essential for anther or ovary development, which can be impacted under drought stress due to repressing of CK signalling mechanism to adapt under stress conditions (Kinoshita-Tsujimura and Kakimoto, 2011; Li et al., 2016). Present findings revealed that cytokinin-related genes were downregulated (as depicted by the response to cytokinin GO term, Figure 5.4) with spraying of VG antitranspirant compared to unsprayed plant anthers, but no differential expression was observed in other anther samples. Thus, it suggests that spraying film antitranspirant further repressed the CK-related genes under drought stress in wheat anthers of crop plants, which might impact the anther or pollen-related processes.

GO enriched biological terms related to anther/pollen in different samples indicated that pollen wall formation and sporopollenin processes were enriched for downregulated genes in VG and ABA anthers in contrast to well-watered ones. These results are consistent with a study by Jin et al. (2013), which revealed that genes related to cell wall development, microspore development and starch synthesis are mostly affected by drought in rice anthers/florets, as observed in this case. However, pollen development GO term showed contrasting results in ABA and VG anthers with transcriptional downregulation and upregulation in each case, respectively. This suggests that although genes related to pollen wall formation and its composition products were affected by water stress, some genes in VG anthers were transcriptionally higher in their expression, showing a unique response in comparison to ABA anthers. Ortolan et al. (2023) summarised the importance of transcription factors (basic/helix-loop-helix (bHLH) family) involved in the anther development and other anther-specific processes in rice and Arabidopsis. Thus, a further investigation of pollen/anther related genes is required to understand the varied gene expression responses in wheat plant anthers following the use of different antitranspirants to understand their effect and mechanism on the anther/pollen development under water deficit environments.

5.5.3 Antitranspirants affect carbohydrate/sugar metabolism & related processes and role of invertase genes

Changes in genes and mechanisms involved in carbohydrate/sugar metabolism in reproductive parts of plants are significantly affected under stressful environments and are the leading cause of poor pollen fertility and lower crop yield in different crop plants, e.g. rice (Sheoran and Saini, 1996; Jin et al., 2013), wheat (Ji et al., 2010; Zhu et al., 2015) and tomato (Lamin-Samu et al., 2021). In this study, GO terms enriched for processes involved in carbohydrate/sugar metabolism and other related processes were separated to understand the transcriptional response of DEGs in different anther samples. Most of the observed GO terms showed transcriptional downregulation of these processes in droughted, treated-plant anthers (VG and ABA) when compared with unsprayed, while in well-watered anthers, upregulation was observed. It suggests that anthers are sensitive to antitranspirants application and drought stress, and as a result, normal metabolic/transport processes related to carbohydrate/sugar can be significantly affected, which can lead to pollen sterility or poor pollen starch accumulation under water-stressed environments. However, under well-watered conditions, these processes are highly active in anthers, which are responsible for proper starch accumulation in pollen, thus indicating fertile pollen leading to good crop yield. This also suggests that antitranspirant spraying (either VG or ABA) did not rescue plants from drought or ameliorate the effect of drought because gene expression observed in well-watered plants was not restored or further downregulated the genes involved in various processes with antitranspirants spraying. It was also reflected in the pollen viability and grain yield results (Chapter 3, Table 3.2 and Table 3.3), with antitranspirants showing no significant effect on plants in comparison to unsprayed plants. There was a slight reduction in the pollen viability (by 4%) and yield (by 9%) in sprayed plants compared to unsprayed ones; however, it was not significant. It can be concluded that transcriptional downregulation of carbohydrate/sugar-related processes in sprayed plant anthers might be the cause of this slight reduction in pollen viability or yield in comparison to unsprayed plants, and these altered responses at the transcriptomic level can lead to negligible effects at the physiological level. However, more studies are required to further confirm these findings. Furthermore, in well-watered plants, pollen viability and grain yield were good in comparison to droughted plants (Chapter 3, Table 3.2 and 3.3), but as they were not included in the statistical analysis, therefore, it is not certain they were significantly different from unsprayed or treated plants.

Different studies revealed the importance of invertase genes in plants as these are linked to carbohydrate/sugar metabolism and play a significant role in their development (Roitsch and

González, 2004; Wang et al., 2022a). These are involved in the conversion of sucrose into glucose and fructose, hormonal control mechanisms and responses under stressful environments that affect seed or fruit sets, as summarised by Ruan et al. (2010). Drought stress leads to changes in carbohydrate metabolism and a decline in invertase activity in wheat anthers that affect the process of pollen development (Dorion et al., 1996; Koonjul et al., 2005). In Koonjul et al. (2005) study, three types of invertases were investigated: two cell invertases (*IVR1 and IVR3*) and one vacuolar invertase (*IVR5*) in wheat anthers under water deficit near the time of meiosis. Downregulation of two invertase genes (*IVR1 and IVR5*) was observed with no effect on the *IVR3* gene during meiosis but downregulated later during the stress period. Whereas in the present study, 17 different types of invertase genes were expressed in different anther samples with most of them showing significant downregulation patterns in droughted antitranspirant-treated plant anthers in comparison to unsprayed ones and were mostly cell wall invertases (*TaCWIs*) including one same *IVR1* invertase as investigated by Koonjul et al. (2005) labelled here as *TraesCS3D02G015900* in Figure 5.6 showed differential expression in VG anthers only. Moreover, six vacuolar invertases (*TaVIs*) showed transcriptional downregulation in VG anthers, while no differential expression of these vacuolar invertases was observed in ABA and well-watered anthers. Also, the same vacuolar invertase (*IVR5*) from Koonjul et al. (2005) study referred to here as *TraesCS7D02G010000* in Figure 5.6 also showed differential expression in VG anther only. Additionally, one common cell wall invertase gene *(TraesCS4B02G356800)* showed a contrasting effect in well-watered and droughted anthers with significant upregulation and downregulation under well-watered and stressed conditions, respectively (Figure 5.6). It could be interesting to investigate this gene in the future to understand its function in anther/pollen development or starch accumulation, as one study revealed the importance of cell wall invertases in pollen development (Ye et al., 2022).

These recent findings are contradictory to Weerasinghe (2013) study, which investigated one vacuolar invertase (*IVR5*) gene to understand the effect of film antitranspirants on improved wheat pollen viability and crop yield. However, Weerasinghe (2013) gene expression results were not significant (between sprayed and unsprayed plants), indicating no association of the downregulated invertase genes mechanism linked with film antitranspirant in alleviating drought effects by improving pollen viability. However, the present study revealed several differentially expressed invertase genes, including *IVR5* (used by Weerasinghe, 2013), between treated and unsprayed anther samples; however, no significant effects of antitranspirants (film and metabolic) were observed either on pollen viability or crop yield at the physiological level. Overall, this indicates that drought stress and antitranspirants significantly reduced the expression of invertase genes in wheat anthers, and the number

and type of these expressed genes could vary according to the antitranspirants used. Also, this can be linked with affected carbohydrate/sugar metabolism processes as observed in droughted anthers in comparison to well-watered ones. Also, homoeologs of expressed invertase genes were investigated to see whether one or all homoeologs were expressed or not in different anther samples. It revealed that in most of the expressed genes, only one homoeolog was differentially expressed, which can be helpful in future research to create knockouts to understand the effect of any of these genes in anther/pollen development or fertility under stressed environments.

5.5.4 Antitranspirants downregulate the genes involved in starch and sucrose synthesis

Starch and sugar metabolic pathways play a vital role in the pollen fertility of crop plants, with photoassimilates transport from leaves must pass through the apoplastic space from anthers to pollen, where it is stored as starch (Lee et al., 2022). Accumulation of sugars and transient starch in the developing anthers serves as a vital source of energy, which is essential for the normal process of cell division and pollen maturation, determining their viability and germination. Any disturbance in the process of sugar utilisation and starch deposition in stressed anthers can cause pollen abortion (Datta et al., 2002; Guan et al., 2023). Furthermore, reproductive stage drought stress significantly reduces the expression of starch and sucrose synthesis genes in plant anthers, ultimately affecting pollen development and fertility due to the lack of necessary energy reserves (Jin et al., 2013; Yu et al., 2019). Datta et al. (2002) indicated the significant reduction in the levels of hexose sugars in developing pollen of male-sterile maize genotype in comparison to male-fertile one, along with their reduced flux in starch biosynthesis, suggested to lead to temporal changes in gene expression and ultimately pollen sterility. Thus, to understand the gene expression pattern of starch and sucrose synthesis genes in response to drought and antitranspirants of this study, lists of genes from the literature were compared with the DEGs list of different anther samples.

Wheat starch is composed of linear and branched glucose polymers, named as amylose and amylopectin, respectively (Yu et al., 2015), comprising 25~30% amylose and 70~75% amylopectin of wheat grain starch (Kim and Kim, 2021). Different types of enzymes, such as ADP-glucose pyrophosphorylase, starch synthase, starch branching and debranching enzymes, are key for starch synthesis in crop plants and are associated with the conversion of sucrose to starch (Lü et al., 2008; Pfister and Zeeman, 2016). The formation of amylopectin is primarily catalysed by multiple starch synthase and starch branching

enzymes, whereas granule-bound starch synthase is involved in amylose formation (Kim and Kim, 2021). A significant decrease in the activity of these enzymes under drought or heat stress, or a combination of both affects the starch synthesis process in wheat grains due to the substantial decline in starch accumulation rate and duration time for grain filling, leading to decreased starch content and grain yield (Lu et al., 2019). In this study, some starch-related genes showed differential downregulation in antitranspirant-treated plant anthers, with no differentially expressed genes in well-watered anthers. This showed that applying antitranspirants under drought stress further downregulated the starch-related genes compared to unsprayed droughted anthers. Moreover, the significant downregulation of DEGs related to starch biosynthetic process as observed in antitranspirant-treated plant anthers, was also evident from the GO term (GO:0019252 for ABA/US and VG/US downregulated contrasts, Appendix 13) and confirmed from the differential downregulation of starch synthase and branching enzymes in VG anthers as shown in Figure 5.8. However, there was no significant reduction in pollen viability or grain yield from the 2022 field experiment when droughted, unsprayed and antitranspirant-treated samples were compared. This might suggest complex interactions of different processes and genes involved in plant anthers that might not significantly affect pollen viability or yield even with the downregulation of starch synthesis genes at the early meiotic stage, or it could be due to the limitations of this study that might have caused variation in DEGs results due to longer storage/extraction of VG anthers from plant stems in comparison to other anther samples. Additionally, purple acid phosphatases (PAPs) hydrolyse organic phosphorus compounds into inorganic phosphate (Pi), thus mediating phosphate acquisition, utilisation and redistribution in plants. As starch synthesis requires Pi, which is used to produce ADP-glucose (a substrate for starch synthesis), therefore, by influencing Pi availability, PAPs can directly or indirectly influence starch synthesis in cereal grains. These are also involved in carbon metabolism, biotic and abiotic responses, signalling, flowering, senescence, root and seed development (Kuang et al., 2009; Sun et al., 2012; Liu et al., 2017; Xu and Yi, 2021; Bhadouria and Giri, 2021). Four purple acid phosphatase genes were downregulated in VG anthers, while only two were expressed in ABA anthers, thus showing the suppressed response of these genes with antitranspirants (VG or ABA) spraying.

Furthermore, beta and alpha amylases, along with disproportionating enzyme (4-alpha-glucanotransferase), are involved in starch degradation, thus helping plants in growth and development and responding to drought and other stresses. This breakdown of starch increased the soluble sugars content under drought to contribute to osmotic adjustments, thus helping plants to improve their tolerance under stress conditions (Kaplan and Guy, 2004; Zhu et al., 2021; Kim and Kim, 2021; Aljabi and Pawelzik, 2022; Yang et al., 2023;

Zhang et al., 2023b). Four beta amylase genes and one alpha amylase gene of this experiment showed downregulation in VG anthers, with only two beta amylase genes showing differential expression in ABA anthers (Figure 5.8), indicating the same downregulating pattern of starch degradation genes like starch synthesis genes with antitranspirants treatments under drought stress compared to unsprayed ones.

In plants, photoassimilates convert to sucrose which moves from leaves to different parts of plants to support growth and development and are used to synthesise storage substances in sink organs (Wang et al., 2022b). Different genes/proteins are involved in sucrose synthesis and transport in crop plants. There are two pathways of carbon flux from photoassimilates to sucrose formation. Under favourable light, triosephosphates (TPs) are exported to the cytoplasm by triose-phosphate translocators from chloroplast and converted to sucrose (TP-sucrose branch) via a series of catalytic reactions by aldolases, fructose-1,6-biphosphatases, glucose-6-phosphate isomerases, phosphoglucose mutases, UDP-glucose pyrophosphorylases, sucrose-phosphate synthases and sucrose-6-phosphate phosphatases. The Calvin cycle or TP-sucrose branch intermediates (fructose 6-phosphate, glucose 6-phosphate, glucose 1-phosphate) convert to ADP-glucose to form starch in chloroplast, which is then transiently degraded (to maltose and glucose) by a series of enzymes such as beta amylases and phosphoglucan phosphatases, which then export to cytoplasm for continued sucrose synthesis for sustaining cell metabolism and growth. TP-sucrose branch is the main branch of sucrose biosynthesis in wheat leaves (Wang et al., 2022b). Some of the DEGs of this study involved in sucrose biosynthesis (fructose-biphosphate aldolase, fructose-1,6-biphosphatase, fructose 6-phosphate, triose phosphate, sucrose phosphate synthase, sucrose synthase, etc.) indicated a similar downregulation pattern in antitranspirants anthers (same as genes involved in starch biosynthesis) when compared with unsprayed anthers (Figure 5.9), whereas a few sucrose transport genes were upregulated either in well-watered, ABA or VG anthers.

This showed that all the genes involved in starch or sucrose biosynthesis were affected under drought stress, and the application of antitranspirants further suppressed those genes. Consistent results of the downregulation of genes involved in starch or sucrose metabolism were observed due to drought stress from several other studies of crop plants (Jin et al., 2013; Kim and Kim, 2021; Wang et al., 2022c; Li et al., 2022). However, Wang et al. (2022c) also observed that vacuoles invertase and beta amylase genes were upregulated throughout drought stress during jointing-booting stage in rice leaves. While in this study, suppression of invertase or beta amylase genes was observed in VG-sprayed plant anthers compared to

unsprayed ones under drought stress, which shows an opposite impact of antitranspirant spraying in early meiotic anthers.

5.5.5 Antitranspirants induce variations in the expression pattern of genes involved in drought response/tolerance

Drought stress is responsible for inducing many morphological, physiological and biochemical changes in crop plants, along with altered gene expression, to mitigate the negative effects of drought (Seleiman et al., 2021). ABA is the primary signal in plants to respond to various environmental stresses and triggers many physiological processes such as stomatal conductance, modulation of the root system, modification in gene expression responses and metabolic changes (Aslam et al., 2022). Under drought stress, ABA regulates the expression of many target genes through ABA-responsive element (ABRE)-binding proteins / ABF (ABRE binding factor) transcription factors (Pandey et al., 2022). ABA receptors (PYR/PYL/RCARs) perceive increased ABA concentration under drought stress, which leads to the inhibition of protein phosphatase 2C (PP2C). PP2C acts as a negative regulator of the ABA signalling pathway via dephosphorylating and inactivating serine/threonine protein kinases (SnRK2s). The released SnRK2s regulate ABA-responsive gene expression under drought stress by phosphorylating AREB/ABFs regulon genes (Singh and Laxmi, 2015).

Some other drought response genes, including bZIP, MYB/MYC, DREB, WRKY, NAC, ERF transcription factors, protein kinases, protein phosphatases (He et al., 2016; Pandey et al., 2022; Bhanbhro et al., 2024) and several proteins and enzymes are responsible for tolerance, including rubisco, helicase, proline, late embryogenesis abundant and carbohydrates (Nezhadahmadi et al., 2013). Literature suggested that ferritin genes might play an important role in plant stress responses (Zhang et al., 2023a), and expansins are involved in wheat leaf growth and water stress response, with higher expansin gene expression and activity suggested to be involved in drought tolerance (Zhou et al., 2015). However, two ferritin and one expansin gene found in VG anthers were suppressed under drought stress with film antitranspirant application. Thus, it shows a contradictory response to antitranspirants application under drought stress compared to the Zang et al. (2017) and Li et al. (2011) findings of overexpression of ferritin and expansin genes shown to be linked to drought tolerance under stress conditions. Furthermore, ABA stress-ripening (ASR) proteins are expressed in response to different abiotic stresses (drought, heat and salinity) and induce stress tolerance in many plant species (Yacoubi et al., 2021). A study by Hu et al. (2013) indicated that the overexpression of TaASR1 gene in tobacco increased drought

tolerance, contrary to which VG spraying downregulated the expression of one ASR gene (*TraesCS4D02G109500*) under drought stress, whereas no differential expression of this gene was observed in other samples. Moreover, one ASR gene was upregulated in well-watered anthers compared to unsprayed ones, with no differential expression in antitranspirant anther samples (Figure 5.10).

Several transcription factors (TFs) which revealed differential expression in different anther samples in response to drought stress and antitranspirant treatments were related to MYB, ZFP (zinc finger protein), trihelix, bZIP, bHLH, WRKY, HSF (heat shock factor), ERF, GRAS, BES1/BZR1 and NAC gene families. This suggests that drought stress and antitranspirants application induced several TFs in the early meiotic anthers to withstand the stressed environment. Similar differential expression of some of these TFs was observed in various literature studies (Jin et al., 2013; Wei and Chen, 2018; Wei et al., 2020; Ye et al.,2021; Sun et al., 2022; Wang et al., 2023a; Tao et al., 2024; Zhang et al., 2024). A few genes from NAC, WRKY, ERF and HSF transcription factor families were upregulated in both antitranspirant-treated plant anthers (VG and ABA). Whereas some transcription factors were upregulated in VG anthers only (related to MYB, ZFP, ERF, HSF, trihelix and phytochrome interacting factor), with no differential expression in other anther samples. Moreover, some TFs (related to bHLH, bZIP, GRAS, BES1/BZR1and MYB family) showed the downregulation pattern in VG anthers, with differential downregulation of some bZIP, WRKY and BES1/BZR1 related TFs in ABA anthers. According to Jin et al. (2013), most of the same TFs expressed under drought stress (with the same wheat DEGs orthologs of this study) in different developmental stages of rice florets were downregulated, thus showing a consistent response. A few showed contradictory results, such as upregulation of HSF and ERF transcription factors in droughted-antitranspirant samples. Moreover, bHLH (*Os06g0226500*) and C2H2-ZFP (*Os11g0442900*) transcription factors that were upregulated under drought stress in Jin et al. (2013) study, with the same expressed wheat orthologs of this experiment, also showed contradictory results with upregulation in well-watered anthers only. This indicates the mixed response of spraying antitranspirants on a few TFs in comparison to Jin et al. (2013). Furthermore, some of the contrasting results of this study, such as the upregulation of heat shock factor (HSF) in response to antitranspirants application under drought stress, might be due to late sowing of the crop or sampling limitations (described in the limitation section of Chapter 6 and earlier in the discussion of this chapter). Moreover, in this study, anthers were extracted from the field experiment, while Jin et al. (2013) findings using the whole rice florets or spikelets of different developmental stages were from the controlled greenhouse study, which might also be the reason for some of the variations in gene expression responses of both studies, along

with different crop types. Thus, it might not be an accurate comparison of DEGs of both studies but could provide a general idea of the types of genes expressed in this anther study.

Some other drought response/tolerance genes found from the literature, such as serine/threonine-protein kinase, annexin, myo-inositol oxygenase, calcium-dependent protein kinase, lipid phosphate phosphatase, serine carboxypeptidase, polygalacturonase and dehydrin, indicated the downregulation pattern in antitranspirants-treated plant anthers (Table 5.2). A few genes that were upregulated with antitranspirant treatments (mainly in VG anthers) were related to ABA receptor, phosphoinositide phospholipase brassinosteriod-responsive protein, hypothetical protein and homeobox-leucine zipper protein along with protein phosphatase 2C (PP2C). Similar upregulation of PP2C genes was observed in Ma et al. (2017) plant samples collected at the pistil and stamen differentiation stage under drought stress conditions in polytunnels, which suggests that VG spraying further upregulated these genes in anthers. PP2C genes are considered both positive and negative regulators of drought tolerance in crop plants (He et al., 2019; Zhang et al., 2021; Wang et al., 2023b). Serine/threonine-protein kinase (SnRK) genes showed the downregulation in VG anthers in contrast to the upregulation of the same orthologs of these genes in rice florets under drought stress by Jin et al. (2013). Some of the differentially expressed genes that were expressed in response to mild drought stress in drought-sensitive and drought-tolerant wheat varieties from leaves and spike samples by Zhang et al. (2024) under a glasshouse setting were also observed in the expressed DEGs of this study, as a few are shown in Table 5.2. Moreover, as there were some limitations in this study (as already explained earlier in the discussion), which might have also contributed to some of the variations in drought response/tolerance genes, therefore, more studies are required to validate these findings.

Also, various studies from which gene lists or genes were taken to compare with the present study to find the expression pattern in different anther samples, there were some overlaps between genes from different plant tissues or plant types from a wide variety of experimental designs, which were expected as some of it already discussed earlier, due to the similar type of stress condition. However, this study also revealed genes that indicated altered gene expression patterns due to the spraying of antitranspirants that could explain the response of antitranspirants at molecular level, and it might also depend on how the samples were collected or stored before anthers extraction, which might have also caused the differential expression of some unique genes, therefore, future studies can provide more answers about how spraying antitranspirants can be beneficial for plants under moisture stress conditions as indicated in various previous studies, and which genes regulate these processes for getting improved yield response, which was not observed in this study.

5.6 Conclusion

Hundreds to thousands of differentially expressed genes were found from the transcriptomic analysis of wheat anthers, collected from the polytunnel-grown (rain shelters) droughted antitranspirants (VG and ABA) treated plants and compared with unsprayed (total 4,789 DEGs from ABA/US and VG/US contrast comparisons). Anthers from well-watered plants were also collected. Most of the DEGs were downregulated (3,959 genes, including both VG and ABA) in droughted anthers, with 830 genes indicating an upregulated expression pattern in contrast to well-watered anthers, whereas the number of downregulated genes (226 genes) was lower than upregulated genes (626 genes). There were variations in hormonal responses of both ABA and VG, treated-plant anthers, and most of the pollen/anther-related GO enriched terms showed transcriptional downregulation under drought stress. Also, carbohydrate/sugar metabolism and related processes in anthers were affected by antitranspirants treatments on plants under water stress, with most of the expressed invertase genes in anthers also downregulated. Sucrose and starch synthesis genes also showed downregulation in antitranspirant samples. This indicates that drought stress and antitranspirants suppress many genes involved in different tolerance mechanisms in contrast to well-watered plants. Some drought-related genes were upregulated, whereas some showed downregulation with antitranspirant treatments. However, no significant differences were observed in physiological parameters (pollen viability, yield and yield components) between droughted antitranspirants-treated plants and unsprayed ones.

Overall, it shows that wheat plants are more sensitive (especially male reproductive parts) to drought stress at the transcriptomic level, and their gene expression patterns are altered accordingly to cope in a stressed environment. Applying antitranspirants can significantly change the transcriptomic mechanisms in the early meiotic anthers. However, these transcriptomic changes might not lead to significant differences at the physiological level, such as in terms of crop yield. Furthermore, as the number of genes and expression patterns varies depending on the type of antitranspirant used, as observed in this study, more research (with increasing the number of replicates and pooling the samples from different replicated plots) is required (to overcome the limitations of this study in terms of plant sampling and anther extraction, as already discussed in this chapter and also given in detail in next Chapter 6) to fully understand how drought tolerance mechanisms work in different types of antitranspirant treatments and if applied at different growth stages of crop plants. Target genes can then be manipulated accordingly based on their transcriptional expression patterns under different stress environments and can be beneficial for breeders in crop

improvement or for creating knockouts to understand the function of genes of interest for a specific trait.

CHAPTER 6. General discussion and conclusion

6.1 General discussion

The first main objective of this thesis study was to measure the reduction in water loss and improvement in pollen viability, grain number and yield with film (VG) and metabolic antitranspirant (ABA) application under droughted conditions. For this purpose, two field experiments (Chapter 3) and one glasshouse experiment were conducted (Chapter 4). In these experiments, growth stages GS31 (first node stage), GS39 (flag leaf stage before the start of meiosis) and GS71-73 (watery to milky grain stage) were assessed for their antitranspirants (VG or ABA) application responses. In the first field experiment (2022), both VG and ABA antitranspirants were sprayed at GS39 and in the second field experiment (2023), VG was sprayed at GS31, and ABA was sprayed at GS39 and GS71-73 in two different treatments. Different physiological parameters were measured, such as relative water content of leaves, pollen viability, endogenous ABA content of leaves and spikes, canopy temperature and yield and yield components.

Chapter 3: Results from both field experiments (Chapter 3) revealed no significant effect of antitranspirants (either with VG or ABA) in all of the measured physiological parameters except for TGW in the 2023 field experiment, in which spraying antitranspirants at GS31 and GS39 significantly reduced TGW. However, it was not translated into yield when droughted, sprayed and unsprayed plants were compared. Spraying antitranspirants did not make much of a difference to the water status of plants. Also, there was a slight decrease in pollen viability and yield with the spraying of antitranspirants in both years at different growth stages, although it was not significant. This might show that if there is no significant effect on the water status of plants (as assessed via RWC and indirectly via canopy temperature) by spraying antitranspirant at different growth stages, there might not be any significant effect on the other parameters (pollen viability, yield and yield components).

Antitranspirants (VG or ABA) significantly reduced TGW in the 2023 field experiment of the present study, which is contradictory to Mphande et al. (2021a and 2021b) results, as no significant effect of either VG or ABA was observed on TGW with spraying at key growth stages. This significant decrease in TGW might be due to reduced photoassimilates production due to antitranspirants application, either at GS31 or GS39, resulting in insufficient assimilates available for the grain filling period compared to unsprayed droughted plants. This shows that spraying antitranspirants (either VG or ABA) might hinder the

production of assimilates due to the effect on photosynthetic processes from stomatal closure, and if the drought stress continues in later growth stages, there won't be enough assimilates produced that could be translocated to grains, resulting in decreased TGW. Also, the TGW of ABA (GS71-73) sprayed plants was not affected, and it was almost the same as in unsprayed droughted plants. The main reason for ABA spraying at this grain-filling stage was that it could increase the grain filling rate and could increase TGW, as indicated by Yang et al. (2011). However, it might also depend on the severity of the drought at the time of spraying, as it was near the permanent wilting point at that stage, which might be the cause of no effective response of ABA at that late stage.

In contradiction to the findings of the field experiments with no significant effect from antitranspirants (VG or ABA) applications in most of the yield components, Mphande et al. (2021b) showed significant improvement in fertile spike density, grains per m^2 and yield with a significant reduction in ABA concentration with film antitranspirant spraying at the key growth stages. Weerasinghe (2013) also observed increased pollen viability with film antitranspirant application at GS31 or GS33, along with enhanced grain number and yield, in contrast to the results of this study. Furthermore, Weerasinghe (2013) investigated the spraying of film antitranspirant (VG) at GS39 on two winter varieties in one of the field experiments, but no significant differences in the yield of sprayed and unsprayed droughted plants were observed. This seems consistent with the present study (with no response from ABA at GS39); however, as varieties, sowing dates, level of VG dosage, and environmental conditions were completely different to the present field experiments, therefore, it cannot be compared accurately. However, it might seem that at GS39 antitranspirants might not show much effect in terms of crop yield of it being late as compared to earlier stem elongation stages (GS31, GS33, GS37 according to the previous studies) to get significant results, but Mphande et al. (2021a) observed promising results with ABA or VG spraying at GS39 in the two glasshouse experiments. Thus, one other reason for getting no response from metabolic antitranspirant (ABA) could be that spraying either at GS39 or GS71-73 cannot enhance crop yield if the soil moisture level is low (it was at 48% of FC at GS39 in the top 50 cm of soil when sprayed in 2023) or near to the permanent wilting point (around 39% of FC, with 36% of FC was the wilting point) as observed in the present field experiments or due to the other physiological responses of plants at that stages. Luo et al. (2021) findings also indicated that exogenous spraying of ABA (multiple applications at the anthesis stage) causes a reduction in grain weight, yield and water use efficiency of wheat plants under severe water deficit (30% - 40% of soil water content) in contrast to the mild or moderate water deficit conditions. However, there were no significant crop yield differences in the present field experiments, as mentioned earlier, with only one ABA application either at

GS39 or GS71-73, in contrast to multiple ABA applications at the anthesis stage by Luo et al. (2021). Moreover, as the application levels and type/dosage of exogenous ABA and some growth stages used for spraying in wheat by Luo et al. (2021) or Mphande et al. (2021a) were different from the present field experiments, thus, future studies by changing the growth stages for film/metabolic antitranspirant, its application level under moderate water stress (with soil moisture between 50% - 55% of field capacity as observed by Mphande et al. (2021b) with film antitranspirant application) might give promising results.

Chapter 4: To explore the effect of antitranspirants (VG and ABA) using similar objectives as the field experiments, a controlled drought glasshouse experiment (in 2022, with light and temperature settings) (Chapter 4) was conducted to reduce some of the environmental variations. Spraying of antitranspirants, VG and ABA treatments at GS39 and ABA spraying at GS71-73 indicated some significant responses, but only because well-watered plants were included in the statistical analysis, which was not possible in the field experiments. However, in all the yield components, there were no significant effects from antitranspirants, considering only droughted sprayed and unsprayed plants, except in the case of the number of spikes per pot, which was significantly reduced when antitranspirants were sprayed at GS39. However, it did not affect crop yield as compared to unsprayed plants. Furthermore, other physiological parameters, like in the two field experiments (Chapter 3), revealed no significant differences between different treatments, even with the inclusion of well-watered plants.

Drought induces pollen sterility, with a drought-susceptible wheat genotype exhibiting a two-fold decline in seed setting and pollen viability compared to a drought-tolerant one, as shown by Mehri et al. (2020). Contrary to Mehri et al. (2020) and Weerasinghe (2013), in the present case, there was no significant effect on the pollen viability of droughted unsprayed and treated or well-watered plants, which resulted in no significant impact on the grain yield of sprayed and unsprayed plants. However, despite no significant decline in pollen viability of droughted plants, grain yield was significantly reduced under drought stress compared to well-watered ones, indicating complex subsequent physiological responses of plants. It is possible that although pollen viability was not significantly affected in droughted plants, drought might have caused defects in fertilisation or ovary abortion, which can directly impact the seed-setting process, resulting in a significant reduction of yield in droughted plants compared to well-watered ones. One study revealed that drought significantly reduced the relative water content, stomatal conductance, net photosynthesis, 100-grain weight and grain yield of all wheat genotypes grown in earthen pots with three types of water treatments (control-well watered, mild water stress with 60% water holding capacity and severe water

stress with 40% water holding capacity (Wasaya et al., 2021). In comparison to the present glasshouse experiment, the controlled drought was set in a range of 30 to 40% of FC, however, there was no significant difference in the relative water content of well-watered and droughted plants, which is contradictory to Wasaya et al. (2021) with some consistent results of the significant reduction in yield and yield components except for TGW (which increased) under drought stress compared to well-watered plants. This contrasting result of significantly increased TGW under drought stress compared to well-watered plants might be due to better allocation of assimilates into the smaller number of spikes in droughted pots compared to well-watered ones.

Drought significantly reduced the number of spikes compared to well-watered plants, and the application of film and metabolic antitranspirant at GS39 reduced it further compared to unsprayed ones. This is consistent with Abdullah et al. (2015) results of reduced tillers and number of spikes with film antitranspirant application. This suggests that applying antitranspirants before the start of the reproductive stage could affect the physiological processes of plants, as shown by a reduced number of spikes, therefore a negative impact from antitranspirants application is possible at this stage. However, as it did not affect the crop yield in the present experiment thus, these can be considered negligible. Moreover, film antitranspirant application under drought stress indicated reduced water loss from wheat plants soon after application and maintained more grains per spike and yield compared to control unsprayed plants (Abdullah et al.,2015). Additionally, Mphande et al. (2021a) also noted improved yield (grains per spike and grain yield per spike) with both film and metabolic antitranspirant application under reproductive stage drought with VG spraying at GS39 or multiple ABA spraying (at GS39, GS43, GS54, GS62 and GS69) under controlled drought. However, contrary to both studies, the present experiment did not show any significant improvement in the number of grains per spike and grain yield with the application of either VG or ABA at GS39 or ABA at GS71-73 compared to unsprayed droughted plants. This indicates that applying antitranspirants and getting an effective response is not always possible, which might be due to the interaction and complexity of different plant physiological and molecular responses or due to different type/dosage of the metabolic antitranspirant or other factors of this experiment. There were fluctuations in temperature around GS39 and GS71-73 (due to the faulty light system as mentioned earlier in Chapter 4), along with the low available moisture (below or within a range of 18% to 30% of plant available water, which created severe water deficit condition) during the growth period of the present glasshouse experiment. Thus, temperature fluctuations and low levels of available water may have resulted in no effect on the crop yield from antitranspirants (either VG or ABA) application, as also observed by Faralli et al. (2017a) that severe water stress (10% VWC)

with film antitranspirant application (flowering stage) on oilseed rape did not show much effect on pod formation in comparison to unsprayed plants.

Overall, these findings of physiological parameters from both field experiments and a glasshouse experiment suggest that antitranspirants might not always work even when applied at critical growth stages as it might also depend on the soil moisture and environmental conditions at the time of application to work efficiently along with the physiological state of plants under those conditions, otherwise, there is no benefit of applying antitranspirants. It might also depend on the type of antitranspirant, its rate/dosage and application level at different growth stages. Additionally, more studies are required to determine if multiple applications of ABA or at other critical growth stages can work, keeping in mind the soil moisture conditions to work effectively to get improved yield responses. Applying antitranspirants under moderate stress instead of severe drought stress, along with increasing the number of replicates and running a controlled glasshouse experiment without any extreme fluctuations in temperature, might give effective responses from antitranspirants. Also, using contrasting wheat varieties with different drought tolerance levels will provide a deeper understanding of applying antitranspirant (VG or ABA) at critical growth stages, which would be beneficial in improving or maintaining crop yields under water deficit conditions, and it can be investigated further in field or glasshouse environments.

Chapter 5: To understand the effects of antitranspirants on the early meiotic wheat anthers, a transcriptomic study was conducted (Chapter 5) to find any molecular mechanism that could link improved pollen viability with antitranspirant (film or metabolic) application as suggested by Weerasinghe et al. (2016) with film antitranspirant. This is the first transcriptomic study on wheat anthers to understand the mechanisms behind antitranspirant spraying that might also explain any changes at the physiological level, and this was the second main objective of the thesis. Many studies revealed that drought affects the expression patterns of genes involved in different processes of crop anthers/pollen development to cope in water stress environments and to repair drought-induced injuries, as reviewed by Yu et al. (2019). One study showed the downregulation of carbon metabolism genes in short-term drought on the post-meiotic stamens in barley (Lange et al., 2024), while another study indicated the decrease in anther starch and ATP synthesis in drought-stressed cotton anthers by Hu et al. (2020). Consistent results from this study showed that genes related to the carbon metabolic process and other carbohydrate/sugar related processes were upregulated in well-watered anthers compared to unsprayed droughted plant anthers, thus indicating a significant impact on these processes under water stress. The majority of differentially expressed genes (DEGs) were downregulated in the droughted antitranspirant-

treated plant anthers (VG or ABA) in comparison to well-watered anthers. This indicates that spraying antitranspirants further downregulated the genes involved in various biological processes (mainly genes related to carbohydrate/sugar metabolism, storage, pollen/anther-related processes, and several other biological processes linked to DEGs listed in Appendix 13 GO term table). Moreover, invertase genes, starch and sucrose synthesis genes were also suppressed in antitranspirant-treated plant anthers compared to unsprayed ones, which shows the disturbance of carbohydrate/sugar metabolic processes with antitranspirants (VG or ABA) application. This could be the reason for no improved pollen starch accumulation in antitranspirant-treated plants compared to unsprayed ones; thus, no significant improvement in pollen viability was observed, which is contradictory to Weerasinghe et al. (2016).

There were contrasting DEGs responses in hormonal processes and related genes with VG-treated samples showing the upregulation for some of the hormones (ABA, GA, JA and ethylene), while in ABA-treated samples, these were either downregulated or not differentially expressed when compared with unsprayed samples (Chapter 5, Figure 5.4). Moreover, at the physiological level, the endogenous ABA content was measured from leaves and spike samples from unsprayed, treated and well-watered plants, but the concentration of the endogenous ABA content was almost similar in all the antitranspirants sprayed and unsprayed samples (with no statistical difference, Chapter 3, Table 3.2, field experiment 2022). Thus, it might suggest that these major hormonal changes (mainly upregulation of ABA-related genes in VG anthers) in anthers might not be directly associated with ABA levels in leaf or spike samples, as anthers are more sensitive to drought stress than other reproductive parts of plants. Additionally, present findings are contradictory to the previous studies which revealed that film antitranspirant spraying at critical growth stages (such as at GS31, GS33, GS37, GS39, GS45 or GS51) significantly reduced the endogenous leaf or spike ABA concentrations in wheat under drought stress compared to unsprayed control plants (Mphande et al., 2021a, 2021b, 2024), and similar type of reduction was observed in leaves and reproductive organs of oilseed rape when film antitranspirant was applied at the flowering stage (Faralli et al., 2016). Therefore, future studies by creating knockouts of genes involved in ABA synthesis or using mutant plants with antitranspirant applications will provide more insights into plants' hormonal response at the transcriptomic level.

Most of the pollen/anther related GO terms indicated downregulation in antitranspirants sprayed anther samples, while one pollen development GO term showed the upregulation in VG anthers compared to ABA anthers, which might suggest that VG spraying significantly improved the expression of genes involved in pollen development that could improve pollen

viability, however, there was no significant effect of VG on pollen viability at the physiological level from the field experiment. As there were some limitations to this experiment and extraction of anthers from VG sprayed plants was a bit delayed than other samples (Chapter 5, Table 5.1), that might also be the cause of some significant changes at the molecular level. Therefore, more studies in future without the limitations of the present experiment might give deeper insights into these results. Moreover, the pollen development genes that were linked with pollen development GO term which showed the upregulation with VG spraying (Appendix 14) can be explored further based on literature studies and in other crop species that might explain the role of VG in improving pollen viability or crop yield as suggested by Weerasinghe et al. (2016).

Several transcription factors are expressed under abiotic stress conditions in crop plants and are involved in various stress tolerance mechanisms (Hrmova and Hussain, 2021). The present study showed that some DEGs related to drought response/tolerance were upregulated with antitranspirants application (such as a few DEGs related to ZFP, HSF, ERF, NAC and WRKY transcription factor gene families), while some were suppressed (such as some DEGs related to bHLH, bZIP, MYB and GRAS transcription factor gene families). Several other drought tolerance/response genes were also differentially expressed with antitranspirants application as discussed in Chapter 5. This indicates that spraying antitranspirants induces significant changes in expression patterns of genes related to drought stress/tolerance that might also play a key role in helping plants to survive or maintain/improve yield when applied at the critical growth stages, as shown from the various previous studies (Kettlewell et al., 2010; Abdullah et al., 2015; Weerasinghe et al., 2016; Abdallah et al., 2019; Mphande et al., 2021a, 2021b, 2024). However, in this case, grain yield was not affected by the spraying of antitranspirants despite major changes at the molecular level. More studies are required without the limitations of the present transcriptomic study due to delayed crop sowing, high temperatures during the early growth period and some limitations in sampling and storing method (as described in detail in the limitation section 6.3 of this chapter) that might have also contributed to no effective response from antitranspirants at the physiological level along with expression of some unique genes which might not have been expressed if sown in spring instead of summer. Further transcriptomic studies can be conducted either in glasshouse or field settings under moderate stress conditions with different antitranspirants applications either at stem elongation stages or before the start of meiosis, using leaf/anther samples with improved protocol in terms of sample collection/storage, to get more accurate results which could provide beneficial insights in light of present findings.

6.2 General conclusion

This thesis study explored the responses of film and metabolic antitranspirants at different growth stages (GS31, GS39 and GS71-73) under droughted condition (in rain shelters for the two field experiments and the glasshouse experiment with spraying at GS39 and GS71-73) using different physiological parameters and tried to understand the antitranspirants responses at the transcriptomic level in the early meiotic anthers which has never been reported or investigated before. The main conclusions from the experiments of this thesis are as follows:

- Spraying film (VG) antitranspirant at GS31 and metabolic antitranspirant at GS39 and GS71-73 did not improve water status, pollen viability or yield of crop plants under drought stress. There was a slight reduction in pollen viability or crop yield with antitranspirants, however, it was not significant. Low soil moisture levels at the time of antitranspirant applications at some growth stages (GS39 and GS71-73) or any extreme temperature fluctuations (late sowing date of one of the field experiments and temperature variations in the glasshouse experiment) along with physiological responses of plants under those conditions might have contributed to no significant responses from antitranspirants (VG or ABA) treatments.
- Most of the differentially expressed genes were downregulated (3,959 genes) in the droughted antitranspirant-sprayed plant anther samples (VG/US and ABA/US), whereas the number of upregulated genes was less (830 genes). The number of downregulated DEGs was highest in VG-sprayed plant anthers (VG/US contrast, 3,325 genes) compared to ABA-sprayed ones (ABA/US contrast, 634 genes).
- Processes and genes involved in carbohydrate/sugar metabolism were downregulated under drought stress and spraying either VG or ABA did not restore these processes but caused them to further decrease their transcriptional activity in the early meiotic anthers. The activity of all the differentially expressed invertase genes (mainly involved in carbohydrate metabolism) was also transcriptionally downregulated in treated-plant anthers. Moreover, homoeologs of the expressed invertase genes indicated that for some invertase genes, all homoeologs were expressed, while in some, only one homoeolog was differentially expressed.
- Genes involved in starch and sucrose biosynthesis showed the downregulation pattern in antitranspirant-treated plant anthers.
- Antitranspirants upregulated some drought tolerance/response genes under water deficit condition, while some were downregulated.

- GO term enriched for processes/genes related to anther/pollen also showed downregulation patterns in antitranspirants sprayed plant anthers, whereas one pollen development GO term showed upregulation in VG anther compared to ABA ones.
- There were contrasting gene expression responses of GO terms enriched for DEGs related to hormonal responses in both VG-treated and ABA-treated plant anthers, with up- and downregulation of some expressed hormonal genes in VG anthers, whereas only downregulation or no differential expression of similar genes in ABA anthers.
- The number of common or overlapping DEGs expressed in three contrast comparisons (WW/US, VG/US, ABA/US) for upregulated or downregulated genes was less, suggesting more unique genes were expressed in different comparisons that might be beneficial to investigate further in future studies.
- Significant changes at the transcriptomic level might not lead to significant differences at the physiological level, such as in terms of pollen viability or crop yield, however, more studies are required under moderate stress (with FC values between 50% - 55% at the time of antitranspirant application) conditions, to further validate the transcriptomic responses of the present study.

6.3 Limitations and further studies

There were some limitations to the experiments included in this thesis. Sowing was delayed for the first field experiment (2022), from which samples for transcriptomic analysis were also collected. It was sown in June instead of March/April, as the first sowing during the start of May failed due to poor germination. This delayed sowing during the period of high daily temperatures (mean daily temperature 20 °C with maximum going up to 31 °C and minimum around 11 °C from the day of sowing till the first spraying of antitranspirants (VG and ABA) at GS39) resulted in faster development and growth of plants than in normal spring temperatures, especially during early phases of plant growth stages, which might have contributed to the lack of yield improvement from antitranspirant treatments. Furthermore, these high temperatures (two heatwaves before the droughted plant sampling for anthers) might also be responsible for the expression of some of the DEGs related to heat stress in droughted anther samples, which would not have been expressed if sown in March/April.

For the second field experiment in 2023, although it was sown in April, however, the soil moisture limit in the top 50 - 60 cm of the soil was lower in comparison to Mphande et al.

(2021b) when the crop reached GS39 (first treatment of metabolic antitranspirant was applied) and later at GS71-73 it was near to the permanent wilting point that might be the reason of no effect from metabolic antitranspirant applications either at GS39 or GS71-73. Also, although plant roots were below the depth of 50 cm around GS39 (before the start of the reproductive stage) and could be extracting some soil moisture from the deeper layers, which might have resulted in an overestimation of soil moisture values at the earlier growth stages (at GS31), however, it might not be sufficient to mitigate the effect of drought in later growth stages, and plants could be under severe stress during the reproductive stages. However, more studies are required to confirm that either low soil moisture is the reason for no response from metabolic antitranspirant, or multiple applications are required to get any improvement in the yield or would be effective if sprayed at different growth stages. Also, comparing the response between different varieties with different drought tolerance levels might give new insight into understanding how metabolic antitranspirant responses vary at the physiological and molecular levels.

During the glasshouse experiment, there were extreme fluctuations in daily temperature due to the faulty light mechanism (already covered in detail in Chapter 4, section 4.2.1) during the time of meiosis and the grain filling period, along with the lower set range of plant available water, which might have resulted in no effect from antitranspirants treatment at any of the growth stages. Further investigation without any sudden temperature variations and increasing the level of available water to plants might give more information on the responses of antitranspirants under a glasshouse environment. Also, as mentioned earlier, changing growth stages for metabolic antitranspirant application with multiple applications or changing the dosage might give more insights into the responses of plants under a glasshouse environment.

There were some limitations regarding how samples for transcriptomic analysis were collected and stored before anthers were extracted, as most of it is already described in detail in Chapter 5 (Table 5.1). Wheat plant stems (with 3 - 4 leaves) were cut (with scissors from the middle of a plant stem) from the field plots and taken to the lab to store in a refrigerator in a beaker containing either water for well-watered samples or 5% PEG-400 solution for droughted samples. It was done to keep stems with leaves from wilting before spikes were extracted to collect anthers under a dissecting microscope. One anther sample was prepared each day, containing 50 anthers from multiple stems with anthers at the leptotene-zygotene stage. It is not clear how many days different stems were dipped in either water or PEG solution before anthers were extracted from the spikes; however, a rough idea of the number of days is given in Table 5.1, Chapter 5. Improving the plant

sampling method and storage to reduce the limitations of the present transcriptomic study would give more reliable results for future research. Reducing the time for extraction by decreasing the number of anthers collected for each sample (30 - 35 anthers per sample instead of 50 anthers per sample) might quicken the sampling processes for future studies. If the sample size is small ≤ 20 then it would be possible to complete 2 - 3 anther samples per day (instead of one in this study) from the freshly collected stems without dipping them in a PEG solution for droughted stems. Once anthers are extracted from those stems, it would be better to collect fresh stems again for more samples and repeat the cycle till all anther samples are extracted. It might be possible to collect plant stems with anthers at the premeiotic stage during the 1 - 2 week period if sprayed around GS39; however, it depends on how fast plants are growing. If the development of plants is faster and it is not possible to collect fresh stems daily, then if stored in a PEG solution for droughted samples, anthers should be extracted in one or two days, no longer than that, so there would be less possibility of any contamination or toxic effect from PEG if there are any. Another option is to collect anther samples from the glasshouse experiment, where environmental conditions are more controlled, and the whole plant pots can be taken to the laboratory without needing to store them, either in water or PEG solution. Furthermore, increasing the number of replicates and collection of plant stems from different randomised blocks from the field or plant pots from the glasshouse experiment and pooling them together for anther extraction would give more insights at the transcriptomic level.

Many unique genes or genes related to different processes have been revealed from the transcriptomic analysis of this study from both types of antitranspirant (VG and ABA) applications that can be investigated in future studies, such as for creating knockouts to understand the function of genes related to pollen/anther development or invertase genes that were expressed either in some or all anther samples to explore their role further in pollen fertility that would give more insights into the pollen viability mechanism or to understand how antitranspirants mechanism work in improving yield. As yield was not improved with antitranspirants application in this study, so further investigations by applying antitranspirants at different growth stages with less extreme temperature fluctuations (as observed in this study due to late sowing of the 2022 field experiment from which transcriptomic study samples were collected) and then understanding their response at the molecular level might give more reliable transcriptomic findings. Also, this first transcriptomic study in wheat following the use of antitranspirants has given a basic understanding of gene expression responses related to various processes in anthers that can be explored further in breeding programs for studies on drought tolerance and how spraying different antitranspirants can affect certain genes or processes, which can influence crop yield.

Additionally, based on these findings, different orthologues can be identified in other related crop species to understand the transcriptomic responses in anthers and how their expression pattern varies in comparison to wheat and genes of interest (involved in anther/pollen related processes) can be explored further.

Once candidate genes of interest are found (especially with further investigation of pollen development genes that were upregulated with spraying of VG and might give an understanding of film antitranspirant role in improving pollen viability as suggested by Weerasinghe et al. (2016)), these can be further validated using mutant plants (related to the specific gene of interest with combined evidence from literature). The mutant plants (such as TILLING mutants) can be used to create gene knockouts, which can be identified using KASP genotyping and can then be compared to control plants (well-watered or unsprayed) to identify the function of specific genes and how they will respond to the spraying of antitranspirants at a critical growth stage of a crop. Mutant plants can also be generated using the CRISPR gene editing technique. Once the mechanism of antitranspirants in improving pollen viability or crop yield is confirmed and candidate genes are found or traits linked with those genes, markers can be developed for those genes (using SNPs or targeted sequencing to identify variations within the candidate genes), which can later be integrated into the breeding program for marker-assisted selection or to develop varieties with improved performance with antitranspirant spraying under water deficit environments.

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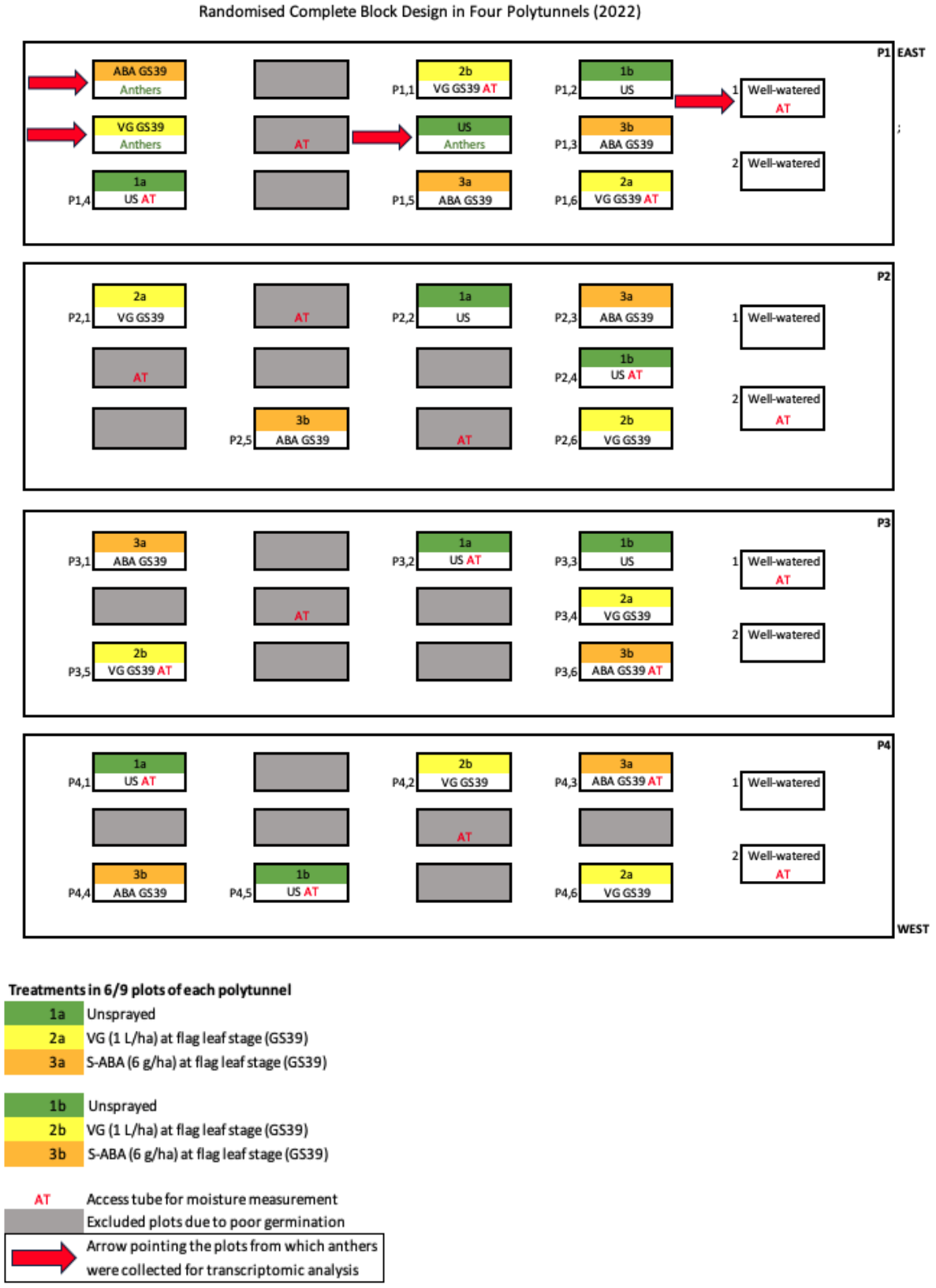
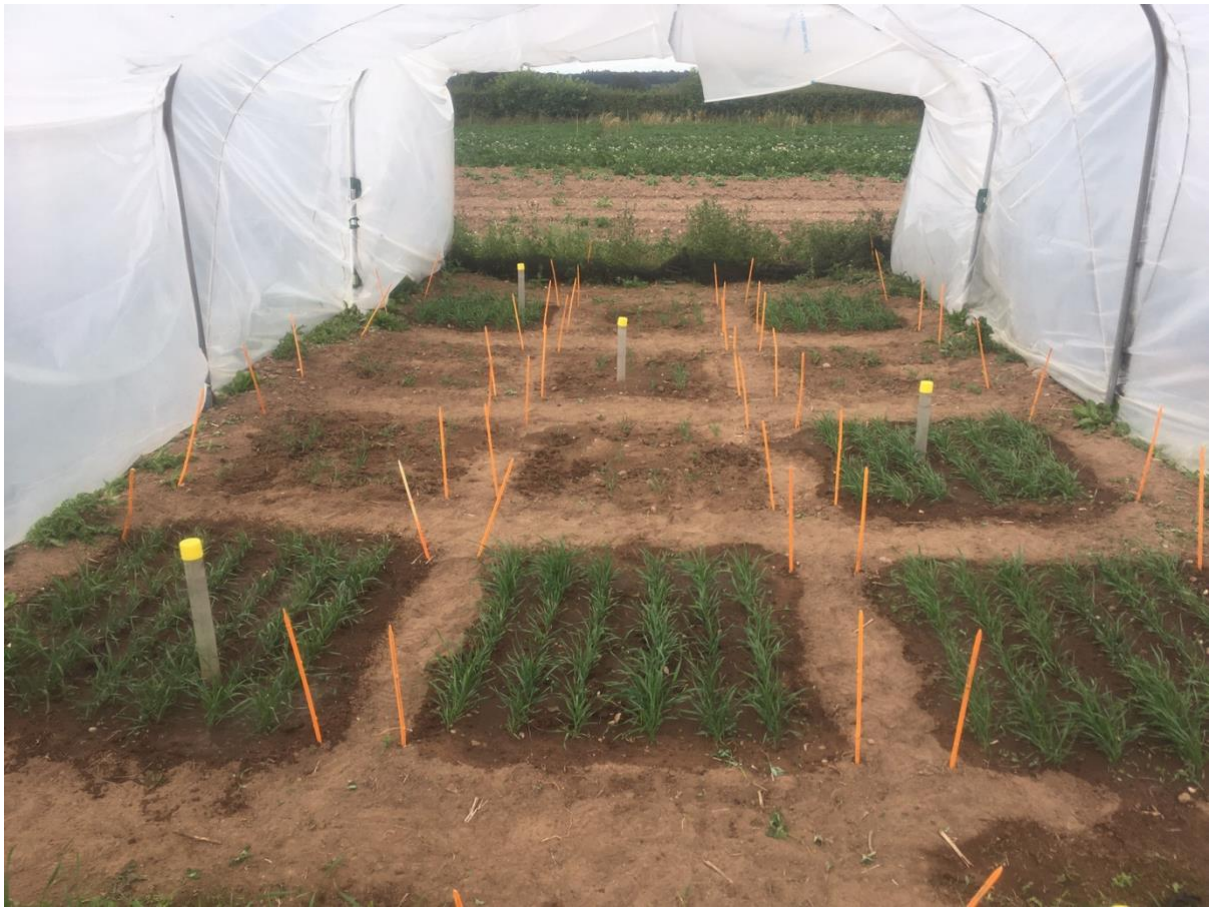
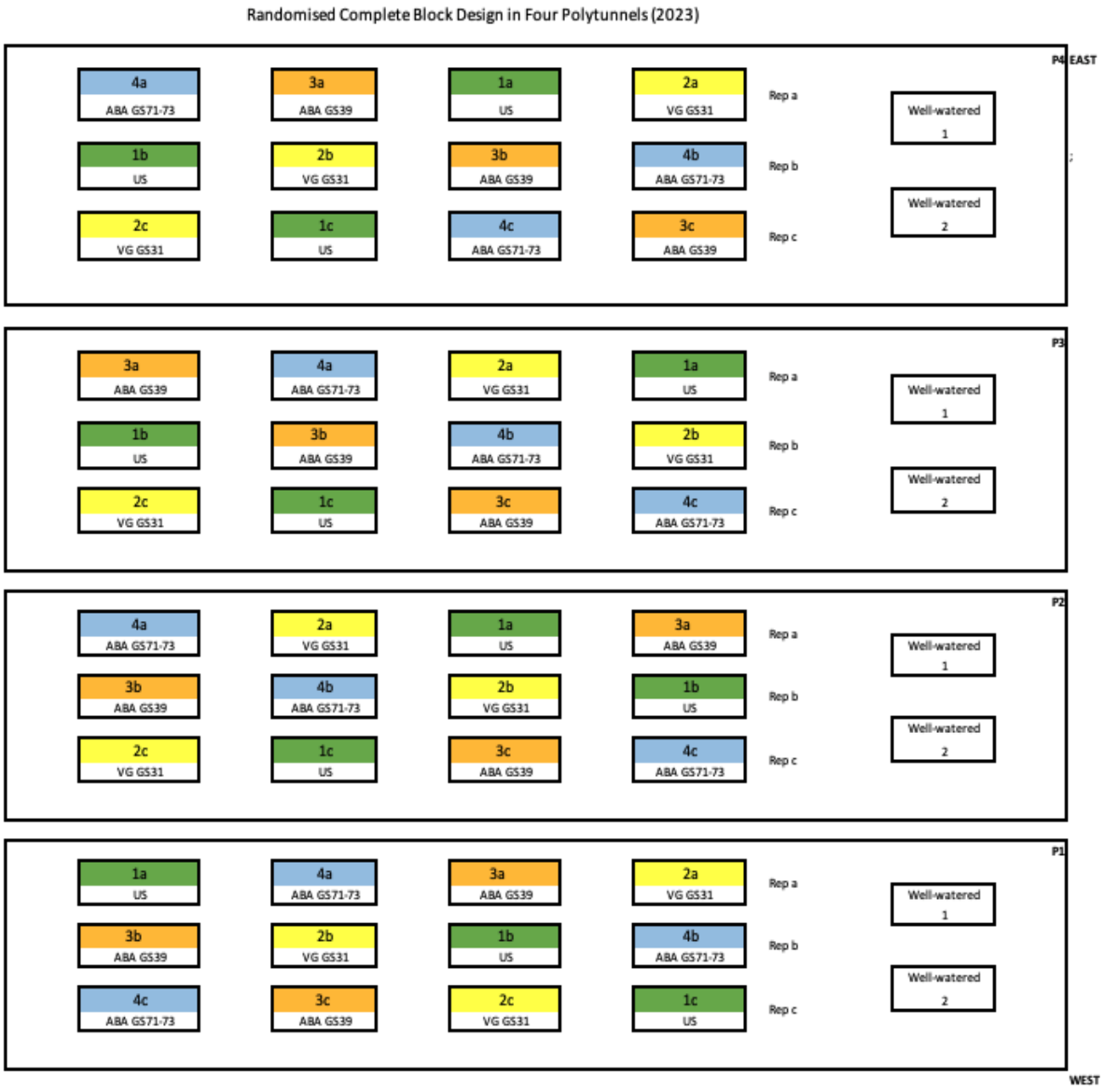
Appendices



Appendix 1: Randomised complete block design of field experiment 2022 inside the four polytunnels, colour-coded with different treatments, excluded plots (grey in colour) and plots from which anthers were collected for transcriptomic analysis (pointed with red arrow).



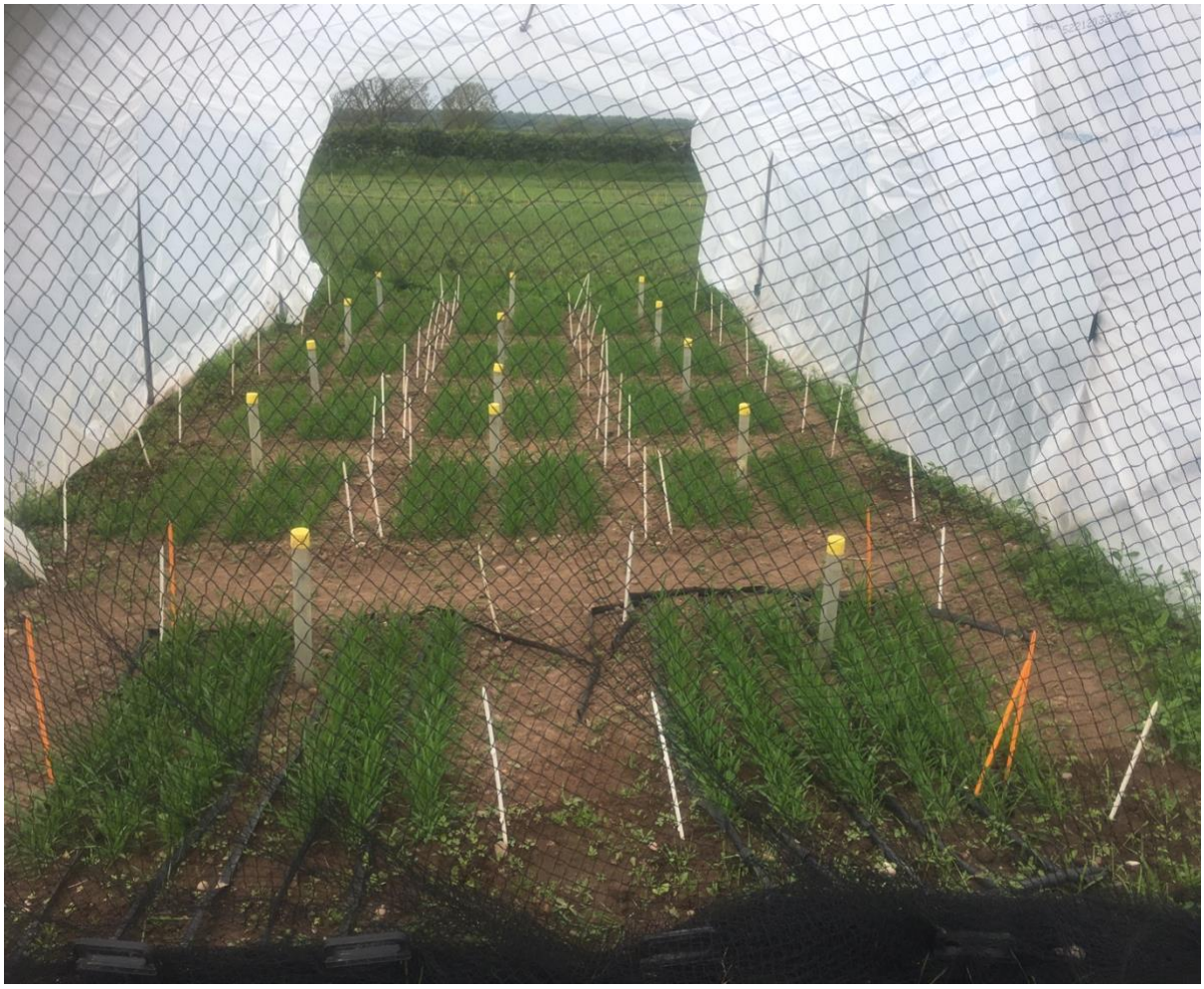
Appendix 2: Picture of plots in one of the polytunnels in 2022 with some of the empty plots that were excluded due to poor germination and can also be seen in Appendix 1 layout shown in grey colour.



Treatments in 12 plots of each polytunnel

1a	Unsprayed
2a	VG (1 L/ha) at 1st node visible GS31
3a	S-ABA (6 g/ha) at flag leaf GS39
4a	S-ABA at (6 g/ha) at watery to early milk grain GS71-73
1b	Unsprayed
2b	VG (1 L/ha) at 1st node visible GS31
3b	S-ABA (6 g/ha) at flag leaf GS39
4b	S-ABA at (6 g/ha) at watery to early milk grain GS71-73
1c	Unsprayed
2c	VG (1 L/ha) at 1st node visible GS31
3c	S-ABA (6 g/ha) at flag leaf GS39
4c	S-ABA at (6 g/ha) at watery to early milk grain GS71-73

Appendix 3: Randomised complete block design of field experiment 2023 inside the four polytunnels colour-coded with different treatments.

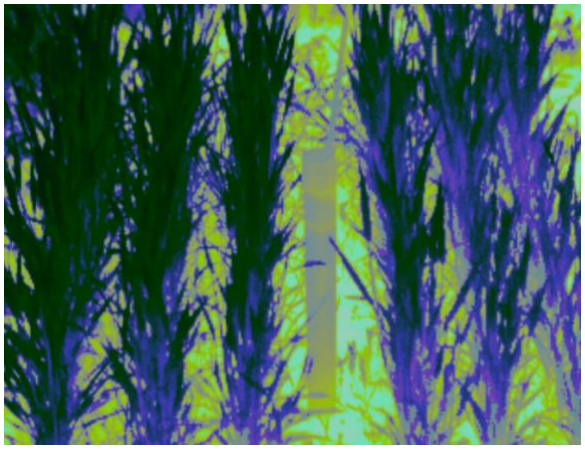


Appendix 4: Picture of plots in one of the polytunnels in 2023 with soil moisture access tubes inserted in each plot to measure the soil moisture readings once a week. Two front plots with drip irrigation pipes were well-watered, while the rest of the plots at the back were the droughted ones. Also, netting covered both the front and back of the polytunnel to prevent birds and rabbits from damaging plants. The picture was taken four weeks after sowing.

(a) (P1_Well-watered)

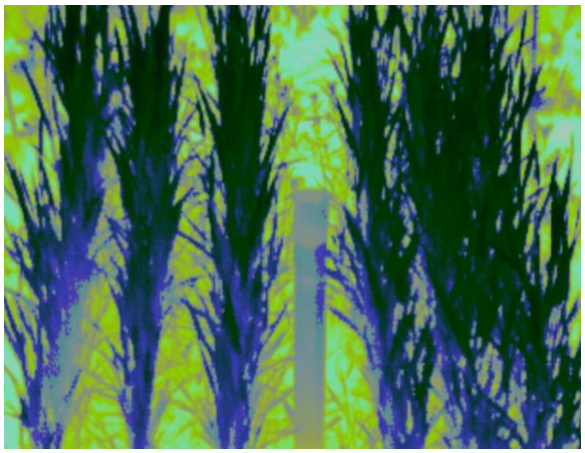
(b)

(c) (P1_ABA_sprayed)



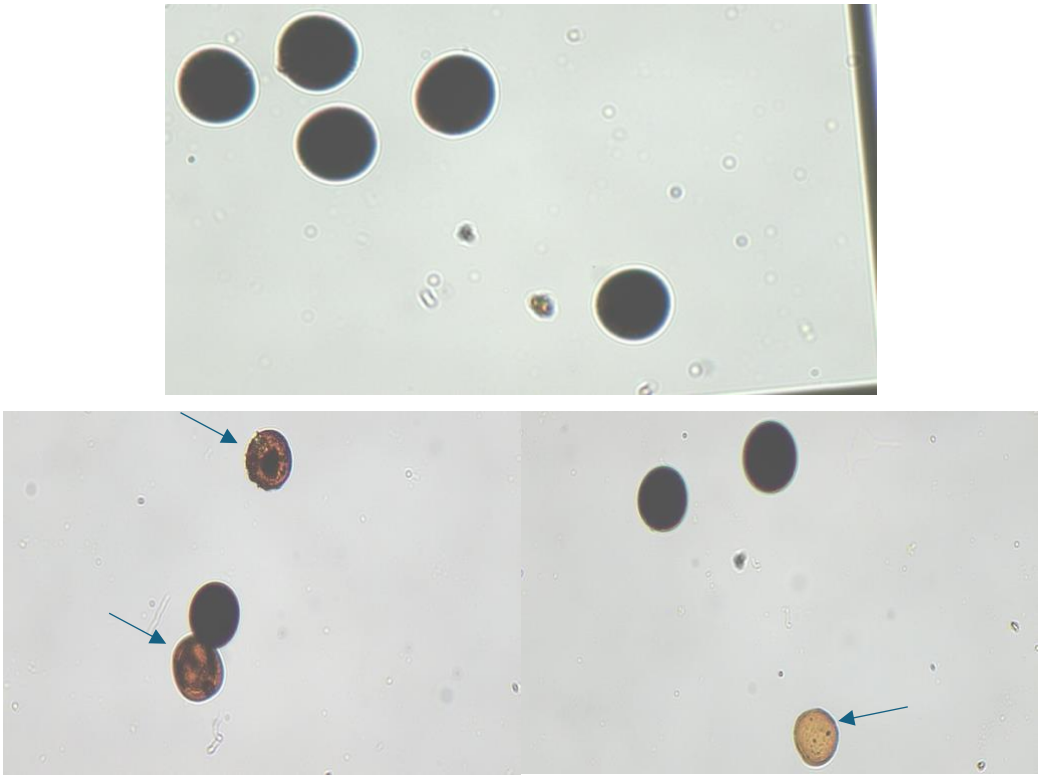
(d)

(e) (P1_Unsprayed)



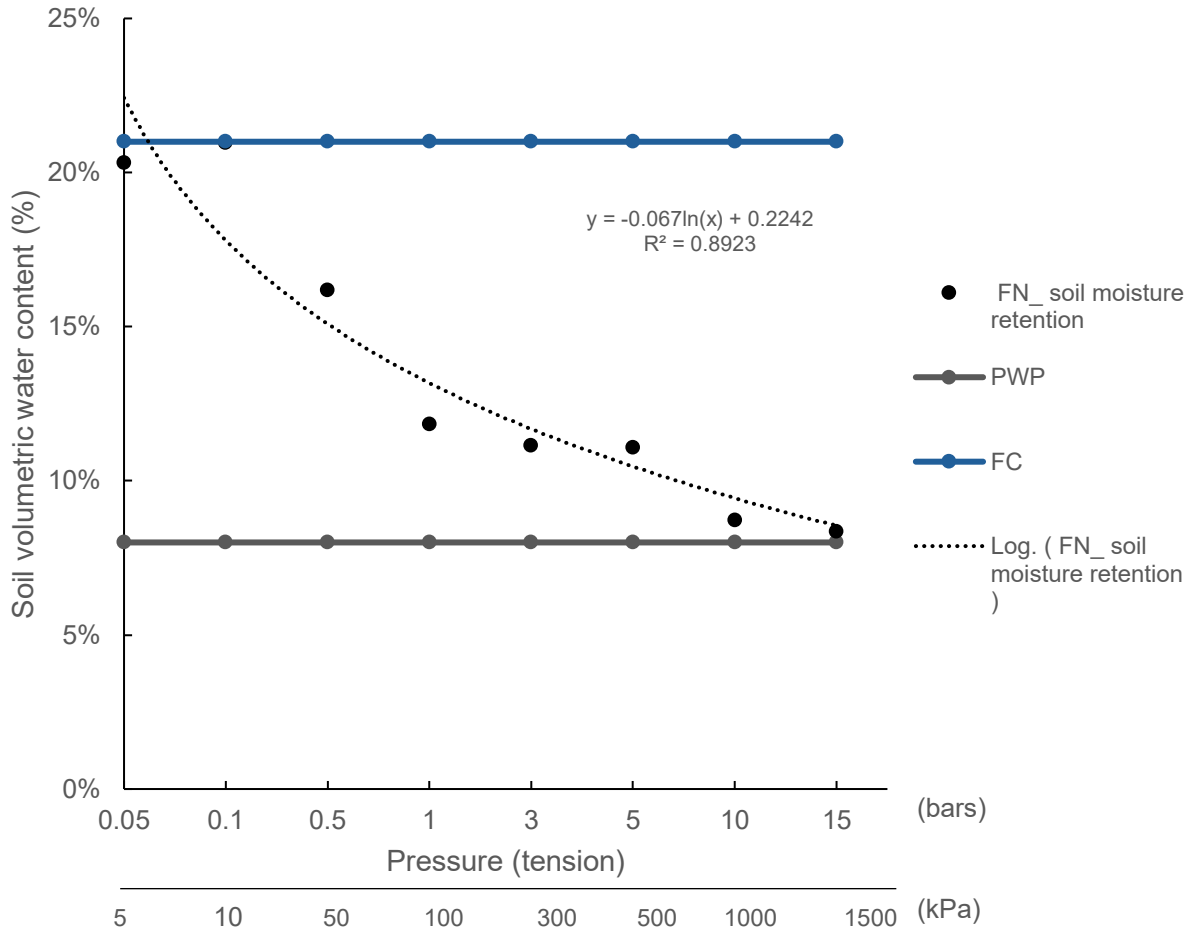
(f)

Appendix 5: Well-watered (a, b) and droughted (c, d, e, f) plots with their original pictures (a, c, e) and thermal images (b, d, f) from the field experiment of 2023 (in one of the rain shelters). These images were captured one day after spraying of the GS39 treatment of ABA antitranspirant. FLIR Research Studio was used to take the mean canopy temperature data from the thermal image pixels of each picture, excluding the soil temperature pixels data.

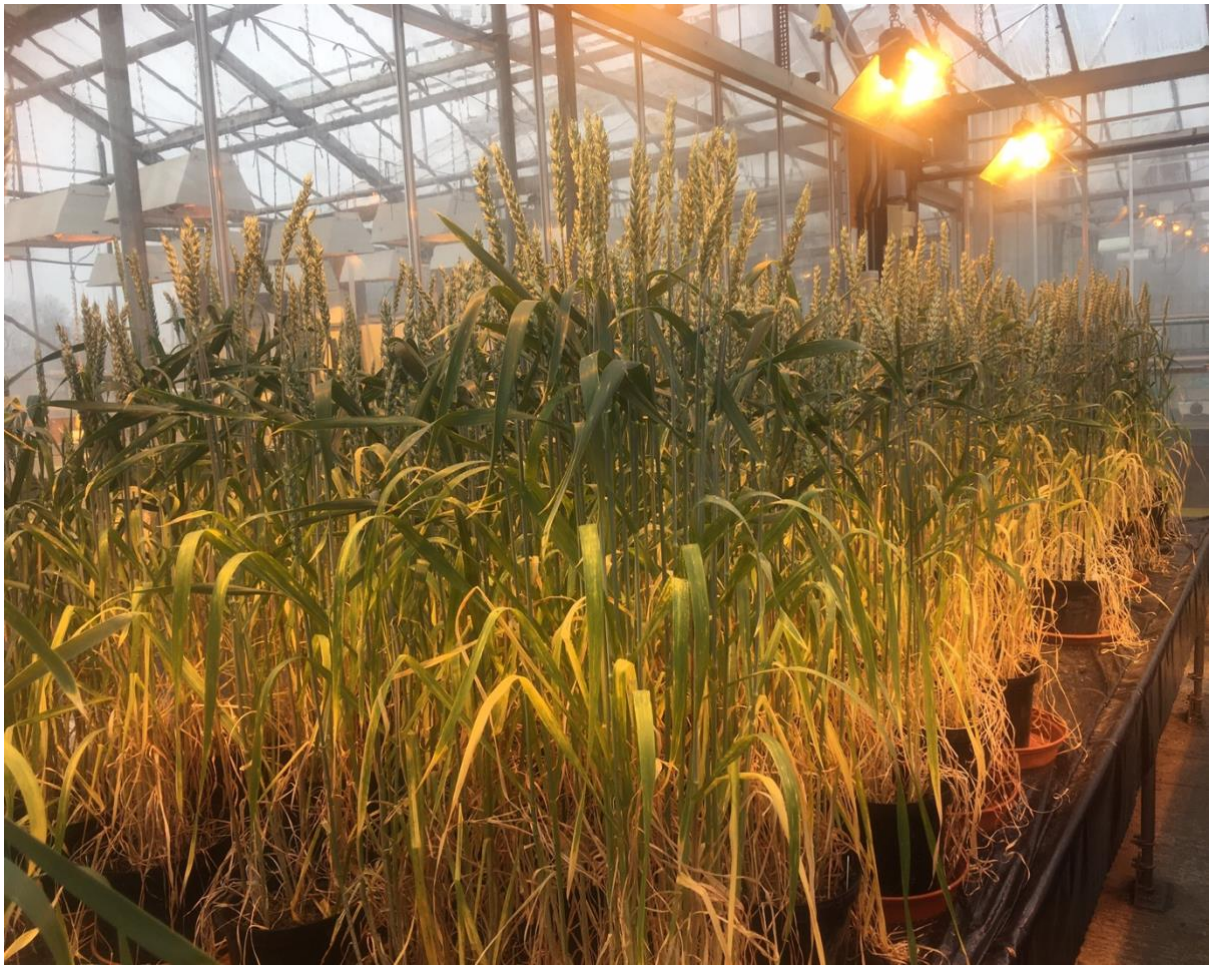


Appendix 6: Pictures of darkly stained pollen (viable pollen) (top picture) and partially or unstained pollen (non-viable) (bottom pictures with arrows) from the field experiment 2023 counted under the microscope (10x objective) using Sedgewick Rafter counting chamber. Three replicates of ten random grids were counted for each sample using the counting chamber, and the mean percentage of viable pollen was calculated.

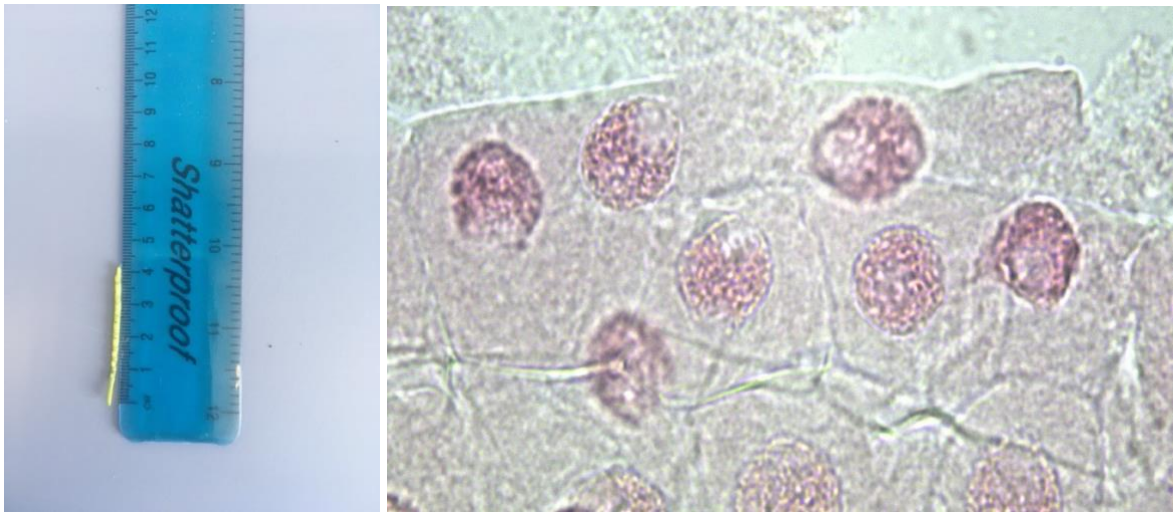
Appendix 7: Mean plant row length (for plant density) was measured separately from each plot from the plot images of each polytunnel in the 2022 field experiment, using the ImageJ software by setting a scale of the known distance of 1 cm using a straight line and then pixels were set accordingly, to set it same for each image. This was done as plant density was not even in each row of the plot due to poor germination because of late sowing. The mean row length of each plot was then used as a covariate in the ANOVA analysis.



Appendix 8: Soil water retention curve of the Flatt Nook field (loamy sand soil) with field capacity (FC) and permanent wilting point (PWP) limits to understand how much energy was required to extract water from the soil (Jie Xiang, personal communication). The normal range of soil-water potential at which water is easily available to plants ranges between -10 kPa to -30 kPa. This is also called the upper limit of plant available water, also referred to as field capacity, whereas -1500 kPa is the lower limit, also referred to as the permanent wilting point, at this water is very tightly held between the soil particles and is not available to plants (Brady and Weil, 2007; Schoonover and Crim, 2015).



Appendix 9: Wheat plant pots in the glasshouse experiment of 2022-23 at GS71-73 stage in a randomised complete block design.



(a) (b)

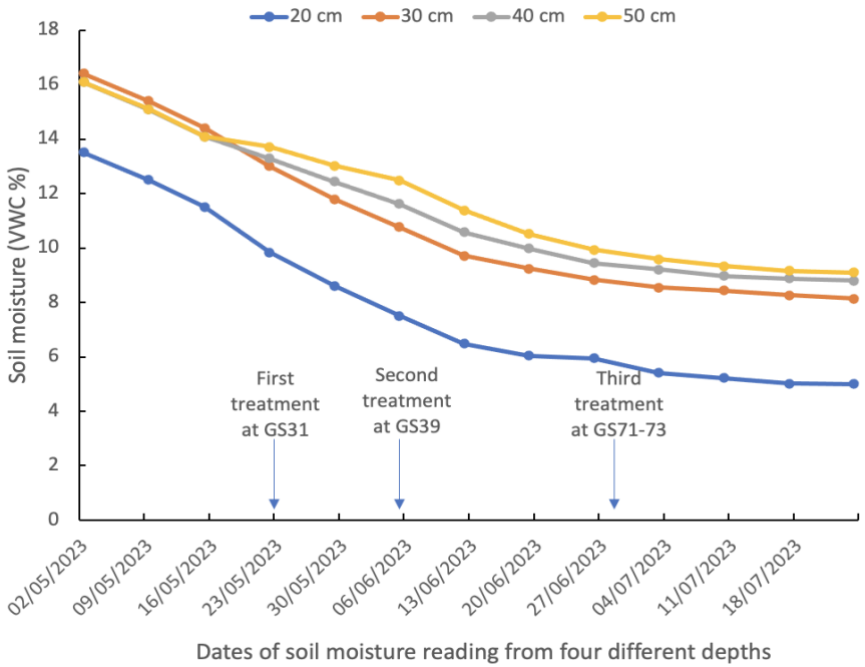
Appendix 10: Immature wheat spike (a) containing anther cells (b) (stained with 0.5% acetocarmine solution) at the leptotene-zygotene stage of meiosis I. Wheat anthers were collected at this meiotic stage (around GS41) for the transcriptomic study from the field experiment (2022) from the well-watered, droughted unsprayed, and VG and ABA treated plants after spraying at GS39.

Appendix 11: Total number of reads and pseudoaligned reads per sample.

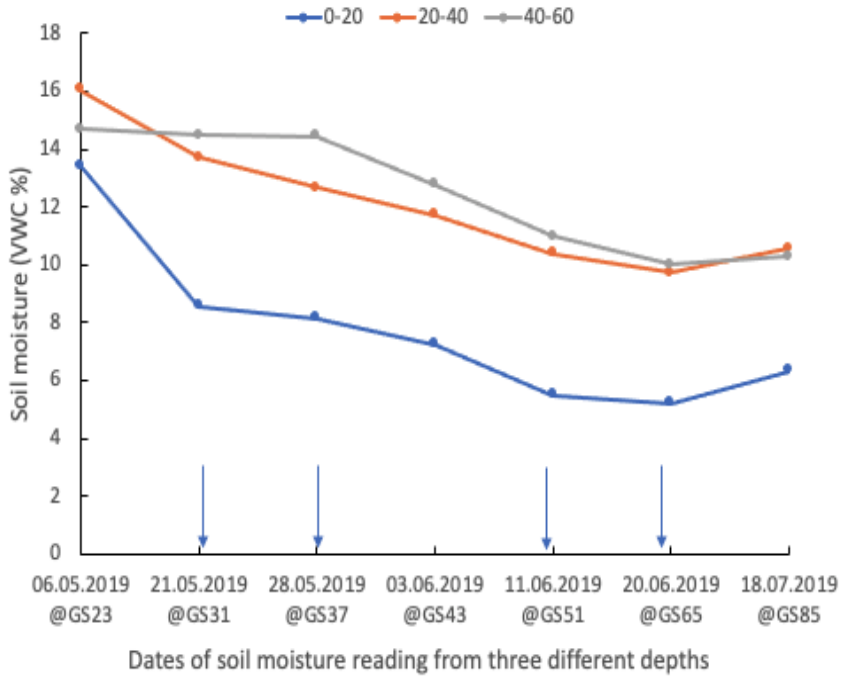
Sample Name	Replicate	Total reads	Pseudoaligned reads	Percentage of reads pseudoaligned (%)
WW1	1	30219463	23928397	79.2
WW2	2	32917685	26172252	79.5
WW4	3	28362672	22394683	79.0
US1	1	27770315	21814153	78.6
US3	2	34928335	28112061	80.5
US4	3	34874095	27990867	80.3
ABA2	1	28745196	23084672	80.3
ABA3	2	35218922	28409624	80.7
ABA5	3	30071716	24208566	80.5
VG1	1	37306431	29874990	80.1
VG2	2	42926786	34734636	80.9
VG5	3	33852418	27213428	80.4

Min	27,770,315	21,814,153	78.6
Max	42,926,786	34,734,636	80.9
Average	33,099,503	26,494,861	80.0

(a)



(b)



Appendix 12: Soil moisture readings measured from different soil depths with the help of a TDR probe via access tubes inserted in the droughted plots of polytunnels. Readings from 4 soil depths (20, 30, 40 and 50 cm) of the 2023 field experiment during the growth period of the crop with antitranspirant treatments (VG-GS31, ABA-GS39 and ABA-GS71-73) at three different growth stages (a). Reading from 3 soil depths (0-20, 20-40 and 40-60 cm) from the Mphande et al. (2021b) 2019 field experiment with film antitranspirant treatment (VG) at four different growth stages of wheat crop indicated via blue arrows (b). The data for Appendix 12(b) was taken via Wiza Mphande's personal communication and is given for comparison purposes only with the 2023 field experiment readings.

Appendix 13: GO term tables from the transcriptomic analysis of different contrast comparisons (WW vs US, ABA vs US and VG vs US) of the upregulated and downregulated DEGs. The first column shows the GO term category, the second column shows the description of the GO term, third depicts the number of differentially expressed genes in that category, the fourth column shows the number of genes generally linked with that GO term category, and the last column shows overrepresented padj < 0.05 values of each category.

Contrast 1_upreg2fold (WW vs US)				
category	GO term	numDEInCat	numInCat	over_rep_padj
GO:0070828	heterochromatin organization	27	82	0.0000
GO:0006334	nucleosome assembly	40	348	0.0000
GO:0006342	chromatin silencing	34	528	0.0000
GO:0008283	cell population proliferation	31	577	0.0000
GO:0051276	chromosome organization	15	131	0.0000
GO:0005975	carbohydrate metabolic process	39	1137	0.0000
GO:2000117	negative regulation of cysteine-type endopeptidase activity	7	14	0.0000
GO:0030388	fructose 1,6-bisphosphate metabolic process	7	16	0.0000
GO:0006000	fructose metabolic process	7	19	0.0000
GO:0001539	cilium or flagellum-dependent cell motility	10	88	0.0000
GO:0006810	transport	47	2203	0.0001
GO:0055085	transmembrane transport	31	1225	0.0002
GO:0006002	fructose 6-phosphate metabolic process	7	59	0.0003
GO:0009750	response to fructose	20	524	0.0003
GO:0005983	starch catabolic process	7	65	0.0003
GO:0005986	sucrose biosynthetic process	7	71	0.0004
GO:0006265	DNA topological change	9	132	0.0010
GO:0043067	regulation of programmed cell death	7	67	0.0011
GO:0030001	metal ion transport	13	269	0.0013
GO:0048830	adventitious root development	8	104	0.0017
GO:0016118	carotenoid catabolic process	3	6	0.0019
GO:0016124	xanthophyll catabolic process	3	6	0.0019

GO:0033494	ferulate metabolic process	3	5	0.0019
GO:0090431	alkyl caffeate ester biosynthetic process	3	5	0.0019
GO:0009239	enterobactin biosynthetic process	9	119	0.0020
GO:0010584	pollen exine formation	16	509	0.0021
GO:0008643	carbohydrate transport	11	218	0.0034
GO:0043086	negative regulation of catalytic activity	10	194	0.0041
GO:0006863	purine nucleobase transport	8	111	0.0041
GO:0015749	monosaccharide transmembrane transport	4	21	0.0042
GO:0006828	manganese ion transport	5	40	0.0044
GO:0080110	sporopollenin biosynthetic process	9	144	0.0047
GO:0055071	manganese ion homeostasis	3	9	0.0050
GO:0048864	stem cell development	3	12	0.0062
GO:0010047	fruit dehiscence	4	23	0.0064
GO:0009715	chalcone biosynthetic process	3	8	0.0081
GO:0030639	polyketide biosynthetic process	3	8	0.0081
GO:0016121	carotene catabolic process	3	10	0.0086
GO:1904659	glucose transmembrane transport	5	52	0.0098
GO:0071836	nectar secretion	5	46	0.0113
GO:0006869	lipid transport	11	272	0.0116
GO:0006741	NADP biosynthetic process	3	11	0.0128
GO:0010586	miRNA metabolic process	3	17	0.0131
GO:0035019	somatic stem cell population maintenance	3	17	0.0131
GO:0080160	selenate transport	3	16	0.0131
GO:0040034	regulation of development, heterochronic	3	19	0.0145
GO:0070574	cadmium ion transmembrane transport	3	15	0.0145
GO:0009830	cell wall modification involved in abscission	4	26	0.0153
GO:0010599	production of lsiRNA involved in RNA interference	3	21	0.0196
GO:0071555	cell wall organization	20	882	0.0196
GO:0010589	leaf proximal/distal pattern formation	3	19	0.0201
GO:0080117	secondary growth	3	18	0.0201
GO:0019674	NAD metabolic process	3	13	0.0207

GO:1902358	sulfate transmembrane transport	3	19	0.0235
GO:0098655	cation transmembrane transport	12	406	0.0244
GO:0046323	glucose import	5	69	0.0244
GO:0071244	cellular response to carbon dioxide	2	3	0.0261
GO:0052325	cell wall pectin biosynthetic process	3	17	0.0279
GO:0015691	cadmium ion transport	6	110	0.0286
GO:0006649	phospholipid transfer to membrane	3	13	0.0317
GO:0009699	phenylpropanoid biosynthetic process	8	170	0.0359
GO:1901264	carbohydrate derivative transport	3	16	0.0392
GO:0045490	pectin catabolic process	5	64	0.0403
GO:0008152	metabolic process	87	6221	0.0410
GO:0035195	gene silencing by miRNA	3	20	0.0418
GO:0010086	embryonic root morphogenesis	3	21	0.0425
GO:0009813	flavonoid biosynthetic process	14	470	0.0468
GO:0009611	response to wounding	41	2387	0.0470
GO:0006825	copper ion transport	5	71	0.0470
Contrast 1_downreg2fold (WW vs US)				
category	GO term	numDEInCat	numInCat	over_rep_padj
GO:0042542	response to hydrogen peroxide	54	1164	0.0000
GO:0009644	response to high light intensity	53	1052	0.0000
GO:0009408	response to heat	56	1346	0.0000
GO:0006457	protein folding	52	1158	0.0000
GO:0010286	heat acclimation	38	523	0.0000
GO:0006950	response to stress	42	801	0.0000
GO:0051259	protein complex oligomerization	16	50	0.0000
GO:0006970	response to osmotic stress	34	1167	0.0000
GO:0034976	response to endoplasmic reticulum stress	27	837	0.0000
GO:0009651	response to salt stress	46	3701	0.0000
GO:0000302	response to reactive oxygen species	13	223	0.0000
GO:0043335	protein unfolding	4	6	0.0000

GO:0009415	response to water	8	59	0.0000
GO:0010187	negative regulation of seed germination	7	67	0.0000
GO:0009961	response to 1-aminocyclopropane-1-carboxylic acid	8	72	0.0000
GO:0009414	response to water deprivation	32	2540	0.0000
GO:0090332	stomatal closure	7	92	0.0000
GO:0009615	response to virus	10	237	0.0000
GO:0009737	response to abscisic acid	29	2477	0.0000
GO:0045471	response to ethanol	4	7	0.0000
GO:0008202	steroid metabolic process	5	37	0.0000
GO:0048316	seed development	11	449	0.0003
GO:0019295	coenzyme M biosynthetic process	3	7	0.0003
GO:0006979	response to oxidative stress	21	1640	0.0005
GO:0010608	posttranscriptional regulation of gene expression	3	9	0.0009
GO:0012502	induction of programmed cell death	2	2	0.0011
GO:0046685	response to arsenic-containing substance	6	129	0.0021
GO:0009793	embryo development ending in seed dormancy	23	2402	0.0026
GO:0009239	enterobactin biosynthetic process	6	119	0.0026
GO:0009790	embryo development	7	235	0.0060
GO:0051782	negative regulation of cell division	3	16	0.0063
GO:0019538	protein metabolic process	4	70	0.0113
GO:0009631	cold acclimation	8	301	0.0136
GO:0071367	cellular response to brassinosteroid stimulus	3	25	0.0152
GO:0046688	response to copper ion	4	51	0.0161
GO:0042906	xanthine transport	2	5	0.0161
GO:0007264	small GTPase mediated signal transduction	8	327	0.0183
GO:0015720	allantoin transport	2	6	0.0214
GO:0015857	uracil transport	2	6	0.0214
GO:0010199	organ boundary specification between lateral organs and the meristem	3	28	0.0249
GO:0006694	steroid biosynthetic process	5	144	0.0273
GO:0034484	raffinose catabolic process	2	9	0.0285

GO:0016485	protein processing	4	98	0.0328
GO:0071705	nitrogen compound transport	2	9	0.0388
GO:0050832	defense response to fungus	20	2321	0.0417

Contrast 2_upreg2fold (ABA vs US)				
category	GO term	numDEInCat	numInCat	over_rep_padj
GO:0006351	transcription, DNA-templated	33	3998	0.0000
GO:0002679	respiratory burst involved in defense response	14	509	0.0000
GO:0006355	regulation of transcription, DNA-templated	36	5908	0.0000
GO:0010200	response to chitin	13	1320	0.0143
GO:0009408	response to heat	13	1343	0.0145
GO:0050832	defense response to fungus	16	2321	0.0186
GO:0010203	response to very low fluence red light stimulus	2	8	0.0268
GO:0009584	detection of visible light	2	9	0.0319
GO:0010201	response to continuous far red light stimulus by the high-irradiance response system	2	11	0.0486
Contrast 2_downreg2fold (ABA vs US)				
category	GO term	numDEInCat	numInCat	over_rep_padj
GO:0050832	defense response to fungus	67	2321	0.0000
GO:0005975	carbohydrate metabolic process	44	1135	0.0000
GO:0055114	oxidation-reduction process	94	3612	0.0000
GO:0009657	plastid organization	22	233	0.0000
GO:0009611	response to wounding	64	2377	0.0000
GO:0009813	flavonoid biosynthetic process	26	468	0.0000
GO:0019684	photosynthesis, light reaction	23	337	0.0000
GO:0042742	defense response to bacterium	60	2433	0.0000
GO:0009595	detection of biotic stimulus	22	379	0.0000
GO:0010103	stomatal complex morphogenesis	23	548	0.0000
GO:0035435	phosphate ion transmembrane transport	8	34	0.0000

GO:0006817	phosphate ion transport	8	41	0.0000
GO:0006098	pentose-phosphate shunt	27	611	0.0000
GO:0080167	response to karrikin	37	1092	0.0000
GO:0016036	cellular response to phosphate starvation	22	484	0.0000
GO:0009809	lignin biosynthetic process	19	333	0.0000
GO:0009682	induced systemic resistance	11	119	0.0000
GO:0009773	photosynthetic electron transport in photosystem I	13	146	0.0000
GO:0009625	response to insect	14	208	0.0000
GO:0007568	aging	14	187	0.0000
GO:0080110	sporopollenin biosynthetic process	12	142	0.0000
GO:0010207	photosystem II assembly	21	447	0.0001
GO:1900366	negative regulation of defense response to insect	5	24	0.0001
GO:0009862	systemic acquired resistance, salicylic acid mediated signaling pathway	30	942	0.0001
GO:0052576	carbohydrate storage	3	3	0.0001
GO:0009697	salicylic acid biosynthetic process	21	526	0.0001
GO:0008152	metabolic process	104	6221	0.0001
GO:0046475	glycerophospholipid catabolic process	4	7	0.0001
GO:0043900	regulation of multi-organism process	14	250	0.0001
GO:0030643	cellular phosphate ion homeostasis	5	20	0.0002
GO:0006979	response to oxidative stress	41	1641	0.0002
GO:0010310	regulation of hydrogen peroxide metabolic process	20	492	0.0002
GO:0006364	rRNA processing	28	857	0.0002
GO:0010466	negative regulation of peptidase activity	8	57	0.0002
GO:0015839	cadaverine transport	3	3	0.0002
GO:0019288	isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway	25	708	0.0002
GO:0019344	cysteine biosynthetic process	22	562	0.0002
GO:0019761	glucosinolate biosynthetic process	22	584	0.0002
GO:0010363	regulation of plant-type hypersensitive response	37	1413	0.0003
GO:0005986	sucrose biosynthetic process	7	70	0.0003

GO:0009759	indole glucosinolate biosynthetic process	8	78	0.0003
GO:0010208	pollen wall assembly	9	117	0.0004
GO:0015706	nitrate transport	19	500	0.0004
GO:0002229	defense response to oomycetes	10	150	0.0004
GO:0080040	positive regulation of cellular response to phosphate starvation	4	9	0.0004
GO:0006952	defense response	55	2785	0.0004
GO:0009409	response to cold	61	3028	0.0004
GO:0080027	response to herbivore	6	45	0.0004
GO:0009699	phenylpropanoid biosynthetic process	11	168	0.0005
GO:0016311	dephosphorylation	16	383	0.0007
GO:0010584	pollen exine formation	17	519	0.0007
GO:0010167	response to nitrate	19	526	0.0010
GO:0046622	positive regulation of organ growth	7	69	0.0010
GO:0051792	medium-chain fatty acid biosynthetic process	7	69	0.0010
GO:0031408	oxylipin biosynthetic process	9	135	0.0011
GO:0051791	medium-chain fatty acid metabolic process	7	72	0.0011
GO:1902603	carnitine transmembrane transport	3	5	0.0011
GO:0032544	plastid translation	6	42	0.0012
GO:0006542	glutamine biosynthetic process	4	15	0.0013
GO:0042430	indole-containing compound metabolic process	7	73	0.0013
GO:0040009	regulation of growth rate	7	74	0.0014
GO:0034484	raffinose catabolic process	3	9	0.0015
GO:0048316	seed development	17	454	0.0017
GO:0042128	nitrate assimilation	7	92	0.0020
GO:0009651	response to salt stress	69	3692	0.0021
GO:0006826	iron ion transport	11	217	0.0022
GO:0010338	leaf formation	7	83	0.0023
GO:0016573	histone acetylation	7	87	0.0024
GO:0044550	secondary metabolite biosynthetic process	8	118	0.0025
GO:0019295	coenzyme M biosynthetic process	3	7	0.0026
GO:0019953	sexual reproduction	10	164	0.0027

GO:0010106	cellular response to iron ion starvation	12	261	0.0028
GO:0097237	cellular response to toxic substance	3	6	0.0028
GO:0009805	coumarin biosynthetic process	12	262	0.0030
GO:0010089	xylem development	17	622	0.0032
GO:0009687	abscisic acid metabolic process	5	41	0.0036
GO:0044036	cell wall macromolecule metabolic process	8	135	0.0039
GO:0009617	response to bacterium	30	1258	0.0042
GO:0009416	response to light stimulus	25	948	0.0047
GO:0019375	galactolipid biosynthetic process	10	230	0.0047
GO:0015979	photosynthesis	21	641	0.0050
GO:0019253	reductive pentose-phosphate cycle	6	58	0.0050
GO:0034050	host programmed cell death induced by symbiont	3	7	0.0052
GO:0010951	negative regulation of endopeptidase activity	6	53	0.0054
GO:0051259	protein complex oligomerization	6	53	0.0055
GO:1902265	abscisic acid homeostasis	3	9	0.0059
GO:0019676	ammonia assimilation cycle	4	26	0.0060
GO:0010120	camalexin biosynthetic process	8	135	0.0063
GO:0071732	cellular response to nitric oxide	6	64	0.0065
GO:0071836	nectar secretion	5	43	0.0067
GO:0048830	adventitious root development	7	107	0.0068
GO:0015824	proline transport	10	188	0.0071
GO:0035265	organ growth	7	101	0.0071
GO:0009063	cellular amino acid catabolic process	3	13	0.0073
GO:0009399	nitrogen fixation	4	26	0.0074
GO:0006811	ion transport	12	377	0.0078
GO:0009642	response to light intensity	5	42	0.0078
GO:0005987	sucrose catabolic process	4	32	0.0082
GO:0010336	gibberellic acid homeostasis	2	3	0.0082
GO:0070981	L-asparagine biosynthetic process	3	13	0.0082
GO:0040007	growth	11	293	0.0106
GO:0030042	actin filament depolymerization	4	29	0.0108

GO:0030397	membrane disassembly	3	21	0.0122
GO:0009698	phenylpropanoid metabolic process	12	328	0.0125
GO:0007623	circadian rhythm	16	559	0.0133
GO:0006452	translational frameshifting	3	10	0.0135
GO:0045901	positive regulation of translational elongation	3	10	0.0135
GO:0045905	positive regulation of translational termination	3	10	0.0135
GO:0010114	response to red light	17	534	0.0147
GO:0009767	photosynthetic electron transport chain	6	72	0.0153
GO:0071722	detoxification of arsenic-containing substance	4	26	0.0154
GO:0009867	jasmonic acid mediated signaling pathway	22	906	0.0167
GO:0034440	lipid oxidation	3	24	0.0167
GO:0009808	lignin metabolic process	10	340	0.0177
GO:0000162	tryptophan biosynthetic process	7	124	0.0189
GO:0019310	inositol catabolic process	2	3	0.0196
GO:0043617	cellular response to sucrose starvation	3	17	0.0198
GO:0019438	aromatic compound biosynthetic process	3	13	0.0198
GO:0043085	positive regulation of catalytic activity	12	340	0.0198
GO:0019464	glycine decarboxylation via glycine cleavage system	3	15	0.0203
GO:0006744	ubiquinone biosynthetic process	8	162	0.0218
GO:0042343	indole glucosinolate metabolic process	3	15	0.0225
GO:0019762	glucosinolate catabolic process	4	34	0.0226
GO:0009641	shade avoidance	7	126	0.0237
GO:0009646	response to absence of light	11	293	0.0237
GO:0006569	tryptophan catabolic process	11	289	0.0240
GO:0009555	pollen development	28	1477	0.0240
GO:0019252	starch biosynthetic process	16	596	0.0242
GO:0010112	regulation of systemic acquired resistance	7	125	0.0244
GO:0016042	lipid catabolic process	11	313	0.0261
GO:0006473	protein acetylation	4	43	0.0295
GO:0010286	heat acclimation	16	526	0.0296
GO:0030388	fructose 1,6-bisphosphate metabolic process	3	16	0.0302

GO:0098712	L-glutamate import across plasma membrane	2	4	0.0323
GO:0031640	killing of cells of other organism	4	32	0.0340
GO:0009750	response to fructose	15	517	0.0342
GO:0009817	defense response to fungus, incompatible interaction	11	349	0.0356
GO:0006000	fructose metabolic process	3	19	0.0356
GO:0010030	positive regulation of seed germination	6	99	0.0356
GO:0052696	flavonoid glucuronidation	8	180	0.0361
GO:0071398	cellular response to fatty acid	3	16	0.0364
GO:1990641	response to iron ion starvation	3	16	0.0364
GO:0006612	protein targeting to membrane	22	962	0.0386
GO:0052544	defense response by callose deposition in cell wall	12	505	0.0411
GO:0042906	xanthine transport	2	5	0.0419
GO:0009627	systemic acquired resistance	25	1218	0.0455
GO:0008284	positive regulation of cell population proliferation	9	275	0.0479
GO:0071281	cellular response to iron ion	6	119	0.0479
GO:0009804	coumarin metabolic process	2	6	0.0479
GO:0006278	RNA-dependent DNA biosynthetic process	3	21	0.0479
GO:0070838	divalent metal ion transport	9	214	0.0480
GO:0046477	glycosylceramide catabolic process	3	21	0.0481

Contrast 3_upreg2fold (VG vs US)				
category	GO term	numDEInCat	numInCat	over_rep_padj
GO:0009414	response to water deprivation	75	2566	0.0000
GO:0071289	cellular response to nickel ion	11	38	0.0000
GO:0010200	response to chitin	48	1332	0.0000
GO:0042538	hyperosmotic salinity response	37	944	0.0000
GO:2000280	regulation of root development	12	83	0.0000
GO:0009408	response to heat	44	1349	0.0000
GO:0045926	negative regulation of growth	11	75	0.0000
GO:0006351	transcription, DNA-templated	87	3998	0.0000

GO:0002679	respiratory burst involved in defense response	25	521	0.0000
GO:0006355	regulation of transcription, DNA-templated	114	5910	0.0000
GO:0009643	photosynthetic acclimation	11	92	0.0000
GO:0009409	response to cold	68	3052	0.0001
GO:0071456	cellular response to hypoxia	9	77	0.0002
GO:0009611	response to wounding	55	2427	0.0006
GO:0009631	cold acclimation	15	299	0.0014
GO:0050832	defense response to fungus	52	2368	0.0021
GO:0080167	response to karrikin	32	1124	0.0021
GO:0009686	gibberellin biosynthetic process	9	126	0.0056
GO:0009753	response to jasmonic acid	32	1246	0.0057
GO:0051403	stress-activated MAPK cascade	6	51	0.0068
GO:0006979	response to oxidative stress	39	1669	0.0087
GO:0051707	response to other organism	17	465	0.0093
GO:0010224	response to UV-B	25	891	0.0093
GO:0009062	fatty acid catabolic process	10	168	0.0095
GO:0000186	activation of MAPKK activity	6	56	0.0096
GO:0009737	response to abscisic acid	51	2512	0.0157
GO:0009416	response to light stimulus	26	962	0.0166
GO:0009873	ethylene-activated signaling pathway	18	549	0.0170
GO:0010286	heat acclimation	18	529	0.0173
GO:0009555	pollen development	34	1498	0.0175
GO:0031098	stress-activated protein kinase signaling cascade	6	68	0.0223
GO:0043462	regulation of ATPase activity	3	9	0.0223
GO:0010466	negative regulation of peptidase activity	6	61	0.0223
GO:0001944	vasculature development	13	363	0.0293
GO:0009685	gibberellin metabolic process	4	25	0.0293
GO:0015691	cadmium ion transport	7	110	0.0322
GO:0006950	response to stress	22	794	0.0346
GO:0042981	regulation of apoptotic process	6	78	0.0362
GO:0009738	abscisic acid-activated signaling pathway	29	1221	0.0362

GO:0009723	response to ethylene	24	945	0.0403
GO:0010686	tetracyclic triterpenoid biosynthetic process	2	3	0.0403
GO:0090069	regulation of ribosome biogenesis	2	3	0.0418
GO:2000232	regulation of rRNA processing	2	3	0.0418
GO:0008631	intrinsic apoptotic signaling pathway in response to oxidative stress	2	3	0.0418
GO:0009605	response to external stimulus	2	3	0.0418
GO:0045893	positive regulation of transcription, DNA-templated	32	1453	0.0418
GO:0010241	ent-kaurene oxidation to kaurenoic acid	2	3	0.0418
GO:0023014	signal transduction by protein phosphorylation	7	126	0.0445
Contrast 3_downreg2fold (VG vs US)				
category	GO term	numDEInCat	numInCat	over_rep_padj
GO:0015979	photosynthesis	260	630	0.0000
GO:0009657	plastid organization	136	233	0.0000
GO:0010114	response to red light	192	542	0.0000
GO:0010218	response to far red light	167	426	0.0000
GO:0010207	photosystem II assembly	174	442	0.0000
GO:0019684	photosynthesis, light reaction	151	332	0.0000
GO:0009637	response to blue light	158	433	0.0000
GO:0009773	photosynthetic electron transport in photosystem I	100	146	0.0000
GO:0006098	pentose-phosphate shunt	185	607	0.0000
GO:0006364	rRNA processing	198	856	0.0000
GO:0009768	photosynthesis, light harvesting in photosystem I	59	65	0.0000
GO:0019288	isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway	170	706	0.0000
GO:0070828	heterochromatin organization	64	81	0.0000
GO:0015995	chlorophyll biosynthetic process	117	382	0.0000
GO:0009765	photosynthesis, light harvesting	59	80	0.0000
GO:0019344	cysteine biosynthetic process	131	560	0.0000
GO:0006334	nucleosome assembly	111	351	0.0000
GO:0018298	protein-chromophore linkage	58	116	0.0000

GO:0009416	response to light stimulus	160	962	0.0000
GO:0055114	oxidation-reduction process	395	3648	0.0000
GO:0035304	regulation of protein dephosphorylation	87	317	0.0000
GO:0008283	cell population proliferation	115	578	0.0000
GO:0009769	photosynthesis, light harvesting in photosystem II	37	47	0.0000
GO:0010027	thylakoid membrane organization	121	616	0.0000
GO:0070838	divalent metal ion transport	72	219	0.0000
GO:0006342	chromatin silencing	97	531	0.0000
GO:0010196	nonphotochemical quenching	34	45	0.0000
GO:0030003	cellular cation homeostasis	73	249	0.0000
GO:0019253	reductive pentose-phosphate cycle	36	58	0.0000
GO:0010155	regulation of proton transport	56	167	0.0000
GO:0005975	carbohydrate metabolic process	145	1143	0.0000
GO:0010103	stomatal complex morphogenesis	85	549	0.0000
GO:0043085	positive regulation of catalytic activity	72	338	0.0000
GO:0009595	detection of biotic stimulus	76	386	0.0000
GO:0019252	starch biosynthetic process	94	596	0.0000
GO:0042742	defense response to bacterium	241	2461	0.0000
GO:0009902	chloroplast relocation	62	287	0.0000
GO:0043900	regulation of multi-organism process	55	250	0.0000
GO:0006636	unsaturated fatty acid biosynthetic process	57	260	0.0000
GO:0016117	carotenoid biosynthetic process	59	296	0.0000
GO:0009735	response to cytokinin	134	1029	0.0000
GO:0009862	systemic acquired resistance, salicylic acid mediated signaling pathway	123	953	0.0000
GO:0080167	response to karrikin	142	1124	0.0000
GO:0015706	nitrate transport	78	502	0.0000
GO:0032544	plastid translation	23	42	0.0000
GO:0034660	ncRNA metabolic process	48	229	0.0000
GO:0009697	salicylic acid biosynthetic process	78	525	0.0000
GO:0009744	response to sucrose	96	689	0.0000

GO:0051276	chromosome organization	35	132	0.0000
GO:0009645	response to low light intensity stimulus	17	24	0.0000
GO:0019761	glucosinolate biosynthetic process	83	589	0.0000
GO:0010363	regulation of plant-type hypersensitive response	153	1418	0.0000
GO:0000023	maltose metabolic process	66	435	0.0000
GO:0009814	defense response, incompatible interaction	70	495	0.0000
GO:0019748	secondary metabolic process	37	148	0.0000
GO:0042744	hydrogen peroxide catabolic process	57	329	0.0000
GO:0010167	response to nitrate	76	531	0.0000
GO:0009767	photosynthetic electron transport chain	27	71	0.0000
GO:0010310	regulation of hydrogen peroxide metabolic process	72	494	0.0000
GO:0009809	lignin biosynthetic process	57	343	0.0000
GO:0015977	carbon fixation	17	33	0.0000
GO:0010304	PSII associated light-harvesting complex II catabolic process	22	67	0.0000
GO:0019760	glucosinolate metabolic process	35	159	0.0000
GO:0006817	phosphate ion transport	17	43	0.0000
GO:0009409	response to cold	260	3052	0.0000
GO:0019953	sexual reproduction	37	170	0.0000
GO:0006546	glycine catabolic process	34	154	0.0000
GO:0009699	phenylpropanoid biosynthetic process	36	179	0.0000
GO:0016556	mRNA modification	40	243	0.0000
GO:0009813	flavonoid biosynthetic process	66	492	0.0000
GO:0000272	polysaccharide catabolic process	32	190	0.0000
GO:0009750	response to fructose	69	521	0.0000
GO:0050832	defense response to fungus	196	2368	0.0000
GO:0009805	coumarin biosynthetic process	43	268	0.0000
GO:0010106	cellular response to iron ion starvation	43	257	0.0000
GO:0044272	sulfur compound biosynthetic process	27	115	0.0000
GO:0009698	phenylpropanoid metabolic process	47	333	0.0000
GO:0009611	response to wounding	203	2427	0.0000
GO:0006733	oxidoreduction coenzyme metabolic process	27	121	0.0000

GO:0006766	vitamin metabolic process	27	121	0.0000
GO:0009106	lipoate metabolic process	27	121	0.0000
GO:0009108	coenzyme biosynthetic process	27	121	0.0000
GO:0006979	response to oxidative stress	153	1669	0.0000
GO:0031408	oxylipin biosynthetic process	27	142	0.0000
GO:0008152	metabolic process	425	6264	0.0000
GO:0000911	cytokinesis by cell plate formation	62	669	0.0000
GO:0009617	response to bacterium	118	1267	0.0000
GO:0071555	cell wall organization	81	873	0.0000
GO:0000165	MAPK cascade	69	617	0.0000
GO:0000226	microtubule cytoskeleton organization	61	725	0.0000
GO:0031348	negative regulation of defense response	128	1526	0.0000
GO:0016572	histone phosphorylation	30	207	0.0000
GO:0030245	cellulose catabolic process	13	44	0.0000
GO:0043086	negative regulation of catalytic activity	33	189	0.0000
GO:0006826	iron ion transport	35	220	0.0000
GO:0000096	sulfur amino acid metabolic process	27	139	0.0000
GO:0016036	cellular response to phosphate starvation	57	495	0.0000
GO:0009072	aromatic amino acid family metabolic process	27	136	0.0000
GO:0044347	cell wall polysaccharide catabolic process	9	14	0.0000
GO:0009117	nucleotide metabolic process	27	139	0.0000
GO:0080110	sporopollenin biosynthetic process	28	152	0.0000
GO:0045490	pectin catabolic process	17	64	0.0000
GO:0030643	cellular phosphate ion homeostasis	9	21	0.0000
GO:0009867	jasmonic acid mediated signaling pathway	88	913	0.0000
GO:0046475	glycerophospholipid catabolic process	6	7	0.0000
GO:0080001	mucilage extrusion from seed coat	11	35	0.0000
GO:0019216	regulation of lipid metabolic process	15	58	0.0000
GO:0009627	systemic acquired resistance	107	1245	0.0000
GO:0010584	pollen exine formation	51	533	0.0000
GO:1900366	negative regulation of defense response to insect	8	24	0.0000

GO:0010224	response to UV-B	80	891	0.0000
GO:0035435	phosphate ion transmembrane transport	11	34	0.0000
GO:0006569	tryptophan catabolic process	40	299	0.0000
GO:0006612	protein targeting to membrane	90	955	0.0000
GO:0009682	induced systemic resistance	21	125	0.0000
GO:0042547	cell wall modification involved in multidimensional cell growth	6	9	0.0000
GO:0009641	shade avoidance	23	134	0.0001
GO:0010226	response to lithium ion	19	89	0.0001
GO:0015994	chlorophyll metabolic process	17	79	0.0001
GO:0009644	response to high light intensity	102	1051	0.0001
GO:0009759	indole glucosinolate biosynthetic process	17	86	0.0001
GO:0045492	xylan biosynthetic process	44	435	0.0001
GO:0009624	response to nematode	57	646	0.0001
GO:0042430	indole-containing compound metabolic process	16	81	0.0001
GO:0009772	photosynthetic electron transport in photosystem II	10	27	0.0001
GO:0009684	indoleacetic acid biosynthetic process	44	363	0.0001
GO:0006275	regulation of DNA replication	32	311	0.0001
GO:0045036	protein targeting to chloroplast	23	145	0.0001
GO:0019438	aromatic compound biosynthetic process	7	13	0.0002
GO:0044550	secondary metabolite biosynthetic process	20	127	0.0002
GO:0040007	growth	35	301	0.0002
GO:0006949	syncytium formation	16	64	0.0002
GO:0009965	leaf morphogenesis	70	849	0.0002
GO:0042793	plastid transcription	29	205	0.0002
GO:0046622	positive regulation of organ growth	15	77	0.0002
GO:0051792	medium-chain fatty acid biosynthetic process	15	77	0.0002
GO:0046477	glycosylceramide catabolic process	8	21	0.0002
GO:0010389	regulation of G2/M transition of mitotic cell cycle	23	186	0.0003
GO:0009828	plant-type cell wall loosening	15	63	0.0003
GO:0090391	granum assembly	7	13	0.0003
GO:0080027	response to herbivore	11	46	0.0003

GO:0051791	medium-chain fatty acid metabolic process	15	80	0.0003
GO:0030104	water homeostasis	7	15	0.0003
GO:0009827	plant-type cell wall modification	38	349	0.0005
GO:0030388	fructose 1,6-bisphosphate metabolic process	7	16	0.0005
GO:0040009	regulation of growth rate	15	82	0.0005
GO:0018316	peptide cross-linking via L-cystine	5	6	0.0006
GO:0080153	negative regulation of reductive pentose-phosphate cycle	5	6	0.0006
GO:0048451	petal formation	19	154	0.0006
GO:0009411	response to UV	46	527	0.0007
GO:0048453	sepal formation	18	144	0.0007
GO:0009625	response to insect	28	218	0.0008
GO:0030397	membrane disassembly	6	21	0.0008
GO:0016042	lipid catabolic process	36	322	0.0008
GO:0009073	aromatic amino acid family biosynthetic process	27	193	0.0008
GO:0010425	DNA methylation on cytosine within a CNG sequence	4	7	0.0009
GO:0010208	pollen wall assembly	18	125	0.0010
GO:0009664	plant-type cell wall organization	57	676	0.0011
GO:0052865	1-deoxy-D-xylulose 5-phosphate biosynthetic process	3	3	0.0011
GO:0055062	phosphate ion homeostasis	12	62	0.0011
GO:0010338	leaf formation	15	90	0.0012
GO:0009831	plant-type cell wall modification involved in multidimensional cell growth	13	54	0.0013
GO:0009800	cinnamic acid biosynthetic process	8	33	0.0013
GO:0052576	carbohydrate storage	3	3	0.0013
GO:0031222	arabinan catabolic process	5	14	0.0014
GO:0071398	cellular response to fatty acid	7	17	0.0014
GO:1990641	response to iron ion starvation	7	17	0.0014
GO:0050896	response to stimulus	29	176	0.0015
GO:1905011	transmembrane phosphate ion transport from cytosol to vacuole	4	8	0.0015
GO:0010133	proline catabolic process to glutamate	4	6	0.0015
GO:0007389	pattern specification process	22	185	0.0017

GO:0009294	DNA mediated transformation	20	155	0.0018
GO:0042549	photosystem II stabilization	7	17	0.0019
GO:0034440	lipid oxidation	6	25	0.0022
GO:0005987	sucrose catabolic process	8	35	0.0023
GO:0019464	glycine decarboxylation via glycine cleavage system	6	15	0.0025
GO:0002229	defense response to oomycetes	20	158	0.0027
GO:0046506	sulfolipid biosynthetic process	5	12	0.0031
GO:0005986	sucrose biosynthetic process	11	71	0.0031
GO:0009808	lignin metabolic process	29	350	0.0033
GO:0022900	electron transport chain	34	276	0.0033
GO:0015839	cadaverine transport	3	3	0.0034
GO:0016139	glycoside catabolic process	8	28	0.0034
GO:0010413	glucuronoxylan metabolic process	33	350	0.0035
GO:0006562	proline catabolic process	3	3	0.0035
GO:0010258	NADH dehydrogenase complex (plastoquinone) assembly	6	12	0.0035
GO:0048830	adventitious root development	16	115	0.0035
GO:0051726	regulation of cell cycle	38	410	0.0035
GO:0005996	monosaccharide metabolic process	4	6	0.0036
GO:0016311	dephosphorylation	39	382	0.0037
GO:0071732	cellular response to nitric oxide	13	67	0.0038
GO:0071454	cellular response to anoxia	5	9	0.0043
GO:0035265	organ growth	16	109	0.0043
GO:0009658	chloroplast organization	49	551	0.0044
GO:0015717	triose phosphate transport	3	3	0.0046
GO:0010075	regulation of meristem growth	51	718	0.0047
GO:0060236	regulation of mitotic spindle organization	5	14	0.0048
GO:0090333	regulation of stomatal closure	9	46	0.0049
GO:0010206	photosystem II repair	8	31	0.0049
GO:0006629	lipid metabolic process	57	645	0.0050
GO:0019375	galactolipid biosynthetic process	25	239	0.0051
GO:0030154	cell differentiation	76	989	0.0056

GO:0071370	cellular response to gibberellin stimulus	4	10	0.0059
GO:0006000	fructose metabolic process	6	19	0.0063
GO:1903428	positive regulation of reactive oxygen species biosynthetic process	3	3	0.0066
GO:0010478	chlororespiration	3	3	0.0069
GO:0070291	N-acylethanolamine metabolic process	4	10	0.0074
GO:0042128	nitrate assimilation	13	94	0.0074
GO:0046283	anthocyanin-containing compound metabolic process	14	79	0.0088
GO:0006559	L-phenylalanine catabolic process	9	52	0.0090
GO:0032973	amino acid export across plasma membrane	4	7	0.0090
GO:0051567	histone H3-K9 methylation	41	554	0.0095
GO:0010430	fatty acid omega-oxidation	4	10	0.0097
GO:0006101	citrate metabolic process	7	27	0.0097
GO:0006102	isocitrate metabolic process	9	42	0.0098
GO:0010245	radial microtubular system formation	3	6	0.0098
GO:0007623	circadian rhythm	48	560	0.0102
GO:0006270	DNA replication initiation	21	220	0.0107
GO:0080184	response to phenylpropanoid	3	3	0.0107
GO:0048653	anther development	35	456	0.0108
GO:0019762	glucosinolate catabolic process	8	34	0.0111
GO:0042221	response to chemical	3	3	0.0113
GO:0009629	response to gravity	6	19	0.0114
GO:0009780	photosynthetic NADP+ reduction	3	3	0.0115
GO:0006032	chitin catabolic process	10	44	0.0117
GO:0045454	cell redox homeostasis	40	357	0.0118
GO:0046202	cyanide biosynthetic process	4	11	0.0138
GO:0080028	nitrile biosynthetic process	5	13	0.0141
GO:0006073	cellular glucan metabolic process	11	53	0.0141
GO:0031640	killing of cells of other organism	9	32	0.0142
GO:0009695	jasmonic acid biosynthetic process	46	481	0.0143
GO:0012502	induction of programmed cell death	2	2	0.0144
GO:1902475	L-alpha-amino acid transmembrane transport	4	8	0.0145

GO:0009834	plant-type secondary cell wall biogenesis	16	145	0.0147
GO:0098869	cellular oxidant detoxification	10	49	0.0148
GO:0010054	trichoblast differentiation	11	59	0.0151
GO:0005976	polysaccharide metabolic process	6	24	0.0155
GO:0043489	RNA stabilization	5	13	0.0159
GO:0009311	oligosaccharide metabolic process	8	42	0.0162
GO:0071482	cellular response to light stimulus	9	48	0.0163
GO:0051258	protein polymerization	13	106	0.0191
GO:0009807	lignan biosynthetic process	7	22	0.0193
GO:0010069	zygote asymmetric cytokinesis in embryo sac	4	16	0.0207
GO:0032350	regulation of hormone metabolic process	3	6	0.0213
GO:0045930	negative regulation of mitotic cell cycle	3	6	0.0213
GO:0042343	indole glucosinolate metabolic process	5	16	0.0214
GO:1902603	carnitine transmembrane transport	3	5	0.0224
GO:0009819	drought recovery	11	79	0.0227
GO:0015824	proline transport	24	189	0.0227
GO:0010120	camalexin biosynthetic process	17	145	0.0241
GO:0009820	alkaloid metabolic process	4	12	0.0257
GO:0042335	cuticle development	34	465	0.0270
GO:0045491	xylan metabolic process	5	16	0.0270
GO:0042372	phylloquinone biosynthetic process	8	39	0.0281
GO:0051171	regulation of nitrogen compound metabolic process	3	6	0.0286
GO:0000302	response to reactive oxygen species	24	228	0.0291
GO:0008652	cellular amino acid biosynthetic process	30	303	0.0292
GO:0046741	transport of virus in host, tissue to tissue	4	8	0.0296
GO:0000162	tryptophan biosynthetic process	16	132	0.0297
GO:0006306	DNA methylation	35	527	0.0298
GO:0007568	aging	24	193	0.0299
GO:1990937	xylan acetylation	4	13	0.0308
GO:0006066	alcohol metabolic process	4	13	0.0312
GO:0055072	iron ion homeostasis	16	141	0.0329

GO:0006857	oligopeptide transport	20	209	0.0342
GO:1901979	regulation of inward rectifier potassium channel activity	8	58	0.0352
GO:0009635	response to herbicide	6	26	0.0366
GO:0080040	positive regulation of cellular response to phosphate starvation	4	9	0.0367
GO:0009727	detection of ethylene stimulus	7	33	0.0369
GO:0052544	defense response by callose deposition in cell wall	35	513	0.0371
GO:0005983	starch catabolic process	9	66	0.0374
GO:0009751	response to salicylic acid	77	1043	0.0393
GO:0030187	melatonin biosynthetic process	5	16	0.0396
GO:0080050	regulation of seed development	2	3	0.0414
GO:0009817	defense response to fungus, incompatible interaction	32	356	0.0419
GO:0042938	dipeptide transport	10	85	0.0427
GO:0006744	ubiquinone biosynthetic process	19	170	0.0449
GO:0006537	glutamate biosynthetic process	3	9	0.0455
GO:0071836	nectar secretion	8	44	0.0482
GO:0009835	fruit ripening	10	69	0.0492
GO:1901657	glycosyl compound metabolic process	6	32	0.0495

Appendix 14: Upregulated DEGs for pollen development GO term (GO:0009555) that come up after GO analysis in VG sprayed plant anther samples when compared to unsprayed. There were 34 DEGs that were linked to this pollen development GO term, given with their rice orthologs. Not all genes have rice orthologs; therefore, “N/A” is written to represent that in the table. Also, some wheat genes have more than one rice ortholog, as obtained after using the BioMart tool on the EnsemblPlants website.

Gene No.	Gene ID	Ortholog No.	Rice Ortholog
1	TraesCS5D02G491600	1.1	N/A
2	TraesCS5A02G477400	2.1	N/A
3	TraesCS2D02G359500	3.1	Os04g0507000
4	TraesCS2B02G437400	4.1	Os04g0616200
5	TraesCS1A02G360300	5.1	Os01g0888500
5	TraesCS1A02G360300	5.2	Os05g0104650
6	TraesCS7D02G004000	6.1	Os08g0223900
7	TraesCS5A02G478300	7.1	N/A
8	TraesCS5D02G321100	8.1	Os09g0526700
9	TraesCS5D02G491500	9.1	N/A
10	TraesCS5D02G491000	10.1	N/A
11	TraesCS5B02G491200	11.1	N/A
12	TraesCS5B02G386300	12.1	Os03g0742400
13	TraesCS3A02G300400	13.1	Os01g0766000
14	TraesCS5B02G490900	14.1	N/A
15	TraesCS3B02G179600	15.1	Os01g0227500
16	TraesCS2D02G415700	16.1	Os04g0616300
16	TraesCS2D02G415700	16.2	Os04g0616400
16	TraesCS2D02G415700	16.3	Os04g0616500
16	TraesCS2D02G415700	16.4	Os04g0616600
16	TraesCS2D02G415700	16.5	Os04g0616700
17	TraesCS1D02G364700	17.1	Os01g0888500
17	TraesCS1D02G364700	17.2	Os05g0104650
18	TraesCS5B02G490600	18.1	N/A
19	TraesCS6B02G420200	19.1	Os02g0811600
19	TraesCS6B02G420200	19.2	Os02g0811400
19	TraesCS6B02G420200	19.3	Os02g0812000
19	TraesCS6B02G420200	19.4	Os02g0811800
19	TraesCS6B02G420200	19.5	Os02g0808800
20	TraesCS2D02G331700	20.1	Os04g0470600
21	TraesCS5D02G026300	21.1	N/A

22	TraesCS4D02G081700	22.1	Os11g0173432
22	TraesCS4D02G081700	22.2	Os07g0251900
23	TraesCS5D02G490900	23.1	N/A
24	TraesCS5B02G064400	24.1	N/A
25	TraesCS6A02G003800	25.1	N/A
26	TraesCS4B02G023300	26.1	Os08g0494100
27	TraesCS7B02G052600	27.1	N/A
28	TraesCS7D02G426000	28.1	Os06g0691800
29	TraesCS1A02G366500	29.1	Os02g0108800
30	TraesCS6B02G319700	30.1	N/A
31	TraesCS6D02G242100	31.1	Os02g0686100
32	TraesCS7D02G523800	32.1	N/A
33	TraesCS4B02G304300	33.1	Os03g0188100
34	TraesCS5B02G491000	34.1	N/A