



**Harper Adams
University**

A Thesis Submitted for the Degree of Doctor of Philosophy at
Harper Adams University

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The Role of Di-1-*p*-menthene and Drought Signalling in Yield Formation in Spring Wheat

**Thesis submitted to the Harper-Adams University as partial fulfilment
for the degree of Doctor of Philosophy**

Agriculture and Environment Department

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April, 2021



**Harper Adams
University**

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General Abstract

Drought is an important abiotic stress affecting wheat production across the globe. The film antitranspirant, di-1-*p*-menthene, has been shown to ameliorate drought and improve wheat grain yield but the mechanisms involved have not been established. As drought induces an accumulation of endogenous ABA, we tested the hypothesis that application of di-1-*p*-menthene improves grain yield of droughted spring wheat by reducing the endogenous concentration of this hormone. To test the hypothesis, we conducted four field experiments and two glasshouse experiments, studying four main objectives: (i) to measure endogenous concentration of ABA in well-watered, droughted and droughted-di-1-*p*-menthene-treated spring wheat plants and relate it to yield and yield components. (ii) To relate the timing of di-1-*p*-menthene application on droughted plants to sensitivity to yield formation; (iii) to compare the effects of exogenous application of ABA to the effects of di-1-*p*-menthene on droughted plants and to well-watered plants on yield and yield components and (iv) to compare the effects of exogenous application of the ABA inhibitor, fluridone, to the effects of di-1-*p*-menthene in droughted plants and to well-watered plants. We found evidence that di-1-*p*-menthene reduces endogenous ABA, photosynthesis and transpiration, and ameliorates terminal drought stress, improving grain yield; and that applications during stem elongation were the most effective. We failed to find evidence that application of exogenous ABA reduced grain yield and yield components. The opposite effect was observed as exogenous ABA ameliorated drought. In contrast, despite fluridone also reducing endogenous ABA and photosynthesis, like di-1-*p*-menthene, it had no effect on transpiration and did not ameliorate drought stress. Overall, the results suggest that di-1-*p*-menthene improvement of droughted

spring wheat is linked to mechanisms that involve curtailing the accumulation of endogenous ABA and suppressing transpirational water loss.

Declaration

I, Wiza Mphande, do hereby declare that the works contained in this thesis being submitted as a partial fulfilment for the award of “Doctor of Philosophy in Crop Physiology” are my own, except where information from other sources has been duly referenced.

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Bless the Lord, all my soul, and all that is within me, bless His holy name.

~Psalm 103: 1~

1. General Introduction

1.1 Background information

Wheat is one of the three most important cereals in the world and accounts for about 44% of global trade (FAO, 2019). It is the most widely grown crop across the globe, including under rain-fed conditions where drought is a common abiotic stress limiting production (Sio-Se Mardeh et al., 2006). In the Mediterranean-type climates, drought is the most important abiotic stress responsible for wide yield gaps in wheat (Maghsoudi et al., 2016; Monneveux et al., 2012). Adaptive technologies are required to mitigate the negative effects of drought on wheat production. One of the adaptive strategies, the application of antitranspirants on crops, is an agronomic technique that has potential to contribute significantly to crop production under water-limited environments. The film antitranspirant, di-1-*p*-menthene (DpM), has been shown to ameliorate drought and improve wheat grain yield (e.g. Abdullah et al., 2015; Kettlewell et al., 2010; Weerasinghe et al., 2016) but the mechanisms involved have not been established.

1.2 Central hypothesis

As drought induces an accumulation of endogenous ABA in plants (Buckley, 2019; Lee and Luan, 2012), and based on past evidence that application of DpM improves grain yield of droughted wheat, it might be that the antitranspirant curtails the accumulation of abscisic acid in plant organs. Therefore, we tested the hypothesis that

Reducing the concentration of endogenous abscisic acid in droughted wheat improves grain yield.

The hypothesis was tested by conducting four field experiments and two glasshouse experiments. All field experiments were conducted in rain shelters to simulate Mediterranean-type drought. In Mediterranean-type conditions, almost all of the rains fall in winter, with little or no summer rain, thus exposing crops to terminal drought, which is water deficit during the reproductive phases of a crop (Rana and Katerji, 2000; Tigras and Tsakiris, 2015). The experiments were conducted under four main objectives stated below and under the respective chapters in which they were addressed.

1.3 Thesis structure

The structure of the thesis is based on published papers, the first two of which are post-prints. For this reason, each experiment-based chapter is made up of the common journal article structure made up of an abstract, an introduction, materials and methods, results, discussion, conclusion and references. Thus, there is no single list of references but a separate one for each chapter. The referencing style used for each chapter was based on author guidelines of the target journal, and so it was not possible to have one general list of references. The manuscripts were modified to take into consideration advice from supervisors, who are duly acknowledged as co-authors. The co-authorship of supervisors is also acknowledged in yet-to-be published papers, including one already submitted to a journal and another targeted for later submission. Chapters 2 and 3 are accepted versions of published articles.

Chapter 2: Runs under the title “**The potential of antitranspirants in drought management of arable crops: A review**”. This chapter was published in the *Agricultural Water Management* journal. The chapter introduces antitranspirants;

provides a historical overview; highlights thematic areas involving antitranspirants and products with antitranspirant effects, by providing a quantitative assessment of each antitranspirant class using published papers to show trends in recent research; and identifies research gaps and challenges.

Chapter 3 is titled “**Yield improvement by antitranspirant application in droughted wheat is associated with reduced endogenous abscisic acid concentration**”. The paper was published in the *Agricultural Water Management* journal. The chapter is based on the first two field experiments (one in 2018 and another in 2019), and addressed the first two objectives:

- (i) To measure endogenous concentration of ABA in droughted and well-watered and DpM - treated spring wheat plants and relate to yield and yield components.
- (ii) To relate the timing of DpM application on droughted plants to sensitivity to yield formation.

Being central to the entire study, objective (i) runs through all the experiment-based chapters

We found evidence that DpM reduces endogenous ABA and ameliorates terminal drought stress improving grain yield; and that applications during stem elongation were the most effective.

Chapter 4 runs under the title “**Metabolic and film antitranspirants both reduce drought damage to wheat yield despite having contrasting effects on leaf ABA**” and was submitted to the *Journal of Agronomy and Crop Science* and at the time of writing it was under review. The chapter is based on the 2018 and 2019 glasshouse experiments, which besides objective (i) addressed the third objective:

- (iii) To compare the effects of exogenous application of ABA to the effects of DpM on droughted plants and well-watered plants on yield and yield components.

We found no evidence that application of exogenous ABA reduced grain yield and yield components, to the contrary, the metabolic antitranspirant ameliorated drought.

Chapter 5: “**Inhibiting ABA in droughted wheat associated with increased yield only if transpiration is also suppressed**”. This manuscript is targeted for submission to the *Agricultural Water Management* journal, whose author guidelines were used in its preparation. This chapter addresses the fourth objective, which was tested in the last two field experiments. The objective was:

- (iv) To compare the effects of exogenous application of fluridone (an ABA biosynthesis inhibitor) to the effects of DpM in droughted plants and on yield and yield components.

Despite fluridone reducing endogenous ABA, it did not ameliorate drought stress, probably because, unlike DpM, it did not reduce transpiration.

Finally, Chapter 6: “**General discussion and general conclusion**”, brings all papers together, the objective being to discuss key findings of the study. Overall, the results suggest that DpM improvement of droughted spring wheat is linked to mechanisms that involve curtailing the accumulation of endogenous ABA if transpirational water loss is also suppressed.

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2 The potential of antitranspirants in drought management of arable crops: A review

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2.1 Abstract

About 80% of global farmland is under rain-fed conditions and most of it prone to drought, which limits crop productivity. Due to climate change, drought will become more frequent and severe threatening world food security. Antitranspirants, materials that reduce transpiration, could potentially result in greater food production by realising more of a crop's potential yield during drought. Despite antitranspirants also reducing photosynthesis, research has shown that they can mitigate drought stress resulting in increased grain yield. Although this paper is not restricted to specific years, part of it is a systematic review of 173 original research articles published between 2009 and 2018. Overall, the analysis suggests that interest in the potential of antitranspirants is growing. One major achievement in antitranspirant research during the past decade was establishing the optimal timing of application of the substances, which is linked to reproductive processes most vulnerable to drought. Despite research

evidence of the efficacy of antitranspirants in ameliorating drought stress, they are not widely used for commercial arable crop production. However, in fruit horticulture, products with antitranspirant effects are being used for various non-antitranspirant purposes such as synchronising fruit ripening, enhancement of nutritional quality, protection against sunburn and controlling diseases and insect pests.

Keywords: Stomatal conductance, terminal drought, drought amelioration

2.2 Introduction

Future crop productivity and world food security will be undermined by severe and more frequent droughts and other abiotic stresses (IPCC, 2018), and reducing transpiration with antitranspirants (ATs) may have a role in ameliorating drought. About 80% of the total cropped area globally is under rain-fed agriculture (Huang et al., 2019, supplementary information), and prone to droughts (<https://youtu.be/Sy0u8LCZK50>, Singh et al., 2017). Under a frequent drought scenario predicted for the future, reducing transpirational water loss will be required to obtain improvements in grain yield. The rapidly increasing world population, projected to reach 9.8 billion by 2050 (United Nations, 2017), will put pressure on food demand. Cereal production must increase by 26% from 2.8 to 3.5 billion tonnes, as from the 2014 baseline (Hunter et al., 2017, supplementary data) in order to avert global food insecurity.

One neglected agronomic technique that has potential to significantly contribute to drought stress amelioration in food crop production is the use of ATs. ATs are

substances that are applied on leaves to reduce transpiration and hence improve plant water potential (del Amor et al., 2010). However, to avoid reducing grain yield biomass due to depression of transpiration, drought is a precondition as past researched demonstrated (e.g. De and Giri, 1978 and del Amor et al., 2010). In addition, a comprehensive study by Kettlewell et al. (2010) showed that AT should target the most drought sensitive stage to avoid counterproductive effects. The potential of ATs to 'waterproof' the most critical crop development stages (Kettlewell, 2014) through reduced transpiration and improve water use efficiency (WUE) during drought is an active area of research.

Detailed reviews of drought avoidance and other plant adaptive strategies to water deficit stress have been dealt with in previous reviews (e.g. Farooq et al., 2009 and Luo, 2010). Also, not discussed here are silicon, and biostimulants like proline and the various compounds extracted from seaweed (such as betaines). These products have drought ameliorative effects which are unrelated to transpiration such as up-regulating flavonoid biosynthesis and antioxidant activities (Ma et al., 2015) and reducing osmotic stress (Ghaffari et al., 2019; Khan et al., 2009).

Thirty-eight years have passed since the last general review on ATs by Solarova et al., (1981). This may in part be due to the negative research conclusion in the late-1970s, which was echoed by Solarova et al., (1981) that ATs were not generally recommended for use in arable crop production. Much of the research on ATs in the past was conducted between 1950 and 1979 (Kettlewell et al., 2010). Researchers at that time found that although ATs improved the water status of plants by reducing the rate of transpiration, the products also reduced the intake of carbon-dioxide (CO₂) and hence the rate of photosynthesis (Kettlewell et al., 2010). In fact, ATs were found to be less permeable to CO₂ than water vapor

(Plaut et al., 2004; Woolley, 1967). Because of this, the conclusion made was that ATs were unsuitable for use in crop production except where survival of the plant was at stake, in which case photosynthesis was of secondary importance (Das and Raghavendra, 1979; Davenport et al., 1972). This explains the decline in AT research after the 1970s and why the technique remains largely neglected. However, it is important to note that photosynthesis is partially reduced and not completely stopped by ATs, and that the reduction in carbon assimilation is outweighed by the benefit if the products are applied to protect the most drought sensitive stage (Kettlewell, 2014). Das and Raghavendra (1979) concluded that AT products were only economically feasible for specific enterprises such as high value fruit production. Although AT products currently have commercial relevance in fruit horticulture (e.g. for protection against sunburn, synchronising ripening and pest protection), there is no evidence that they are being used for drought amelioration. This suggests that they may still be not be cost-effective for low value crop production systems like arable farming.

2.3 Antitranspirant classes

The mode of action of each one of the three classes of ATs and their representative compounds (Table 1) are reviewed below.

Table 2.1: Examples of the main antitranspirant compounds and their modes of action in the surveyed literature between 2009 and 2019.

Antitranspirant classes	Compounds	References
Metabolic	s-ABA	Park <i>et al.</i> 2016
	Chitosan (poly (D-glucosamine))	Li, Zhang, <i>et al.</i> 2017; Rieger <i>et al.</i> 2016
	Fulvic acid	Zhang <i>et al.</i> 2016
Reflective	Aluminosilicate (kaolin)	Cantore <i>et al.</i> 2009
	Calcium carbonate (CaCO ₃)	da Silva <i>et al.</i> 2019
	Calcium oxide (CaO)	da Silva <i>et al.</i> 2019
Film-forming	di-1- <i>p</i> -menthene	AbdAllah <i>et al.</i> 2019
	poly-1- <i>p</i> -menthene	Faralli <i>et al.</i> 2016
	Acrylic polymers	AgroBest Australia, 2017

2.3.1 Research topics between 2009 and 2018

A decadal overview of AT classes used, and research objectives was done using original research articles published between 2009 and 2018. Our sampling of research papers in this decade was for a more quantitative assessment aiming to reveal the trends in research in recent years. Non-drought amelioration uses of ATs were included in the analysis for the purpose of demonstrating the wider range of uses of AT products. Papers were accessed using the search engines *Google Scholar* and the Harper-Adams University's electronic library system, with links to several databases including *BioOne*, *Web of Science* and *ScienceDirect* which were relevant to this study. A total of 173 peer-reviewed original research papers were collated and analysed on the basis of crop type, AT type and

research objective (Table 2). The trend of publications shows that research interest in the potential of ATs is increasing.

2.3.2 Reflective antitranspirants

As their name suggests, reflective ATs function on the basis of reflectance to minimise leaf temperature and consequently the transpiration rate (Glenn, 2012). Once reflective ATs have been applied, the foliar characteristics to absorb, reflect and transmit light are altered, modifying leaf temperature and gas exchange variables (Abou-Khaled et al., 1970). Kaolin (an aluminosilicate, $\text{Al}_4\text{Si}_4\text{O}_{10}(\text{OH})_8$, Cantore et al., 2009) is foremost among the reflective ATs being the most studied as the survey of published literature revealed (e.g. between 2009 and 2018, there were 78 original research papers on kaolin versus 18 on the other reflective ATs). Ordinary kaolin, in its crude form, has impurities of titanium dioxide (TiO_2) and ferric oxide (Fe_2O_3), but as a particle film technology product, it is upgraded to a highly light reflective, fine grained ($< 2 \mu\text{m}$), low-abrasive product of over 99% purity, after a spreader-sticker is added (Brito et al., 2019; Glenn and Puterka, 2005). Applied to the leaf surface, it forms a whitish film (Boari et al., 2015) by which the optical properties of the target leaf are transformed.

With enhanced reflective properties, particle film kaolin is more effective than unprocessed kaolin in minimising the heat load on leaf surfaces, as more infrared radiation and ultraviolet rays are reflected (Brito et al., 2019; Glenn and Puterka, 2005). Despite reduced transpirational cooling, previous studies reported leaf temperature reduction (or canopy temperature depression) effect due to kaolin of $>3^\circ\text{C}$ e.g. by 5.6°C in walnut (Gharaghani et al., 2018), and between 3 and 4°C in Valencia orange - corresponding to a 22-28% decline in transpiration -

(Abou-Khaled et al., 1970), similar to 3.8 °C in snap beans (AbdAllah et al., 2019). Kaolin was effective in reducing transpiration and leaf temperature (by 1.4 °C) in field beans (Tworkoski et al., 2002). However, kaolin can have an opposite effect on leaf temperature if the target plant is not under drought stress. In grape plants sprayed with kaolin, Brillante et al., (2016) observed an increase in leaf temperature of 1.47 °C in well-watered versus a 1.30 °C decrease in water stressed. The increase in leaf temperature under well-watered conditions is attributable to kaolin occluding stomata, reducing the transpirational cooling effect in the process. This means that to reduce the negative effects of high temperature on crop physiology, drought stress is a requisite. This may not be true in all cases as kaolin is widely used to solely protect fruit from sunburn. In mung beans (*Vigna radiata L*), water deficit stress was demonstrated as a prerequisite to kaolin application for improved yield (De and Giri, 1978). Kaolin can also cause an increase in leaf temperature under low photosynthetic photon flux density (Brillante et al., 2016) and also lead to reduced photosynthesis (Brito et al., 2019). In apples, (Gindaba and Wand, 2005) observed up to 1.1 °C increase in leaf temperature. Further, by partially occluding stomata, reflective ATs can also partially contribute to a reduced gas exchange profile of the pores (Boari et al., 2015).

For transpiration to occur, a vapour pressure gradient between the leaf and the surrounding air is necessary (Bloomfield et al., 2019; Medina and Gilbert, 2015). By lowering the leaf heat energy balance, the vapour pressure gradient between the leaf and the air is narrowed, thereby decreasing the transpiration rate (Kostka and Aquatrols Corporation of America, 2018). Additionally, particle films confer protection to the photosynthetic apparatus, particularly photosystem II reaction

centres against excessive irradiance (P.S.O. da Silva et al., 2019; Dinis et al., 2018). This suggests that plants that have been sprayed with particle films can have not only higher but also longer-lasting photosynthesis before initiation of senescence. While reflecting infrared radiation and ultraviolet rays, reflective ATs also reduce the amount of photosynthetically active radiation (PAR) absorbed (Brillante et al., 2016) and hence have a negative effect on photosynthesis. However, depending on the canopy architecture, reflection can result in a positive redistribution of light with an overall enhancement of whole canopy photosynthesis, especially in dense canopies (Brito et al., 2019; Glenn, 2012).

The two calcium-based reflective ATs, CaCO_3 and CaO have a similar mode of action to kaolin. At the time of this review, there was no known study on arable crops involving these ATs, and only a few on non-arable species (e.g. banana (El-Khawaga, 2013) and grapes (P.S.O. da Silva et al., 2019)). This may be explained by the novelty of calcium in particle films (Paulo Silas Oliveira da Silva et al., 2019). In grapes and coffee, CaO was found to have longer lasting physiological effects than CaCO_3 . In 28 days after application the luminosity value of CaCO_3 declined by 31% against 17% for CaO . Besides higher luminosity, CaO maintained reduced leaf temperature and gas exchange variables and higher chlorophyll content (P.S.O. da Silva et al., 2019; Paulo Silas Oliveira da Silva et al., 2019). To obtain a complete and equivalent foliar coverage to that of CaCO_3 , the concentration of CaO needs to be doubled (e.g. 20% w/v CaO versus 10% w/v CaCO_3). The higher concentration of CaO may account for this difference in duration of efficacy (Paulo Silas Oliveira da Silva et al., 2019). Besides calcium compounds, reflective ATs less researched include any other mineral-based products such as magnesium carbonate (MgCO_3 , Al-Desouki et al., 2009) and

magnesium silicate (MgO_3Si , Schrader, 2011). One environmental concern with reflective ATs that needs addressing is the effect on non-target organisms as further reviewed in the last section.

2.3.3 Metabolic or stomata-closing antitranspirants.

Metabolic ATs are a group of substances that have hormone or hormone-like effects, inducing partial stomatal closure by acting on guard cells (AbdAllah et al., 2018). Prominent in this class is exogenous abscisic acid (ABA) in its naturally-occurring bioactive form (*S*)-*cis*-ABA (s-ABA) or a commercially available mixture with the synthetic (*R*)-*cis*-ABA (J. Li et al., 2017). ABA signalling causes an efflux of ions from guard cells, and water by osmosis, leading to flaccidity of the cells with concomitant stomatal closure (Kim et al., 2012; Munemasa et al., 2015). Several studies have reported drought tolerance and yield improvement effects of exogenous ABA e.g. in artichoke (Shinohara and Leskovar, 2014) and wheat (Travaglia et al., 2010; Zhang et al., 2016).

ABA as an AT is perhaps the most prominent product in this group, as chitosan and fulvic acid are not solely ATs but are also used as biostimulants to improve uptake of both major and trace elements - even on crops under well-watered conditions - (Pettit, 2004; Sootahar et al., 2019). Plant biostimulants are substances or micro-organisms that are applied on plants on or in their growth medium to enhance growth by boosting nutrient uptake or tolerance to biotic or abiotic stress (Brown and Saa, 2015). The mechanism of interaction between exogenous ABA as an AT and endogenous ABA is not well understood. However, it is known that benefits from endogenous ABA are short-term and occur only under mild drought stress (Sreenivasulu et al., 2012). This corroborates with the

short-lived (less than seven days) effects of exogenous ABA on stomatal closure in wheat (Travaglia et al., 2010). During reproductive-stage drought stress, endogenous ABA may induce premature senescence with undesirable effects such as reproductive organ abortion (Pang et al., 2017); and loss of grain number if drought coincides with meiosis in grain-bearing crops like cereals and pulses (Dolferus et al., 2011; Ji et al., 2011). Applying ABA as an AT during meiosis may therefore negatively affect crop productivity. In commercial fruit horticulture, ABA is used for fruit quality enhancement, particularly of table and wine grapes (Gonzalez et al., 2018).

Closely connected to ABA signalling is the metabolic pathway regulated by chitosan. In field bean plants (*Phaseolus vulgaris* L.), Iriti et al. (2009) found that a foliar chitosan application led to stomatal closure and a reduction in transpiration. An increase in the endogenous ABA concentration (more than threefold) and that of hydrogen peroxide (H_2O_2) was also observed one day after spraying. The intrinsic WUE was not significantly improved. Chitosan is known to cause stomatal closure by promoting biosynthesis of ABA, but the mechanism by which this is accomplished is not well understood (Hidangmayum et al., 2019). Although Iriti et al. (2009) did not explain the link between ABA and H_2O_2 , in faba beans (*Vicia faba* L.), it is known that ABA signalling downstream induces the production of H_2O_2 , as a secondary messenger, to cause stomatal closure (Arve et al., 2014). Calcium ions, nitric oxide and reactive oxygen species are among the other secondary messengers in the ABA signalling network (Lee and Luan, 2012).

The other biostimulant-antitranspirant in the metabolic AT class is fulvic acid. Fulvic acid is a by-product of organic matter decomposition that dissolves in both alkaline and acidic solutions (Klucakova et al., 2000). It is a variable mixture of

both aliphatic and aromatic organic acids, which are similar to humic acids but more reactive (Pettit, 2004). Along with this diverse chemical constitution, fulvic acid has both AT and biostimulant functions. As an AT, the effects of fulvic acid on gas exchange physiology have been studied in a number of crops and genotypes. In maize growing under drought stress (soil moisture at 35% of field capacity), Anjum et al. (2011) found that fulvic acid applied at the tasselling stage insignificantly increased stomatal conductance by 13% while transpiration rate, net photosynthesis and WUE were significantly improved, implying that fulvic acid acted as a biostimulant instead. Zhang et al. (2016) found that while exogenous ABA significantly reduced both stomatal conductance and transpiration rate in wheat under mild drought stress, fulvic acid was not effective. Zhang et al. (2016) and Anjum et al. (2011) apparently contradict Xudan (1986), who observed that fulvic acid was effective in reducing stomatal conductance in wheat and improving water potential and grain yield. This discrepancy might be attributed not solely to genotypic and interspecific factors but also to the chemical variability of fulvic acid. Working on wheat, Dunstone et al. (1988) confirmed that although fulvic acid reduced stomatal conductance, its effects were highly variable. In a glasshouse experiment, a reduction in stomatal conductance was recorded only in well-watered but not in drought stressed plants. Furthermore, while in the four most responsive genotypes the reduction ranged between 15 and 40%, it was only between 1 and 3% in the least. In addition, for the highly responsive genotypes, the effect was stable in growth cabinets where artificial light was used, but rapidly decayed under natural light, becoming marginally significant to non-significant within four to seven days. The heterogenous composition of the molecular structure of fulvic acids and other biostimulants, particularly the functional groups,

affects their chemical and physico-chemical properties (Bai et al., 2015; Klucakova, 2018) and thus may partly explain the contradictory findings. For, this reason, replicating experiments involving these products is impossible as there is not a single mode of action (Brown and Saa, 2015). A consideration of these findings by different researchers suggests that the mechanism of drought amelioration by fulvic acid may not significantly be mediated by stomatal movement but rather through its biostimulant functionalities. Fulvic acid biostimulant functionalities are its roles or functions for which it has been found to be more effective or better suited as a biostimulant (e.g. enhancing bioactivity of glutamic oxaloacetic transaminase to improve nutrient uptake in wheat, Zhimang et al., 2001 and increasing gas exchange and carbon assimilation, Anjum et al., 2011, which are opposite effects of an AT).

Besides modifying gas exchange variables, both chitosan and fulvic acid confer drought tolerance through biostimulant functions. By inducing antioxidant activities, they enhance scavenging for reactive oxygen species and promote cellular membrane integrity (Bistgani et al., 2017; Z. Li et al., 2017). In addition to improving the water potential of droughted wheat plants, fulvic acid was found to alleviate the loss of chlorophyll and enhance the uptake of phosphorus by roots (Xudan, 1986). Z. Li et al. (2017) found that chitosan not only improved water balance in droughted white clover but also up-regulated antioxidant activity and chlorophyll content.

The short-lived effects of metabolic ATs and that the use of chitosan and fulvic acid is not restricted to drought stress conditions imply that concerns that these ATs may hinder crop growth and productivity in an event of improved soil moisture status (e.g. following rainfall) are unnecessary.

Phenyl mercuric acetate (PMA), a popular subject of metabolic AT research in the past (Sinclair et al., 1975), was found to be toxic (Das and Raghavendra, 1979). It disappeared from AT research but was still used as a fungicide before being banned in some countries (Nandi, 1985). India is apparently the only country where the use of PMA as an AT (e.g. Pandey et al., 2017 and Kumar et al., 2018) remains legal.

Another compound with an ABA-related mechanism is pyrabactin, a synthetic growth inhibitor with a sulphonamide functional group (Cao et al., 2013). Though not among the popularly known ATs, it has a mode of action similar to ABA and is its agonist but not its structural analogue (Fan et al., 2015). In peas (*Pisum sativum* L.), Puli and Raghavendra (2012) found that pyrabactin had similar effects to ABA on stomatal closure. Stomatal apertures decreased by a larger percentage in plants where pyrabactin and ABA were used together, suggesting a synergistic mechanism. Nevertheless, pyrabactin may not play a significant role in drought stress mitigation as its bioactivity in vegetative parts is weaker than in seeds, where it inhibits germination (Cao et al., 2013; Park et al., 2009). There is limited published information on pyrabactin as an AT. None of the studies cited here (and more e.g. Park et al., 2009, Yu et al., 2017 and Han et al., 2019) were conducted in the field or on well established crops to provide conclusive evidence of the potential of pyrabactin as an AT.

2.3.4 Film-forming antitranspirants

The currently available film-forming ATs are mostly water-emulsifiable organic polymers that form films after a spray application has dried (Moftah and Al-

humaid, 2005). The films act as a physical barrier over stomata by which transpirational water loss is reduced.

Di-1-*p*-menthene (pinolene) is the oldest film-forming AT with one of the earliest references being Williamson (1963). Under glasshouse conditions, (Faralli et al., 2016) compared the drought ameliorative effects of di-1-*p*-menthene and poly-1-*p*-menthene on oil seed rape. Both compounds significantly reduced gas exchange, however, di-1-*p*-menthene was not only more effective in suppressing stomatal conductance (by 50% against 11%) but also sustained the effect for a longer period (>14 days versus < nine days). The yield component results were consistent with the stomatal conductance readings, with di-1-*p*-menthene having higher values for number of pods per plant and seed biomass, suggesting that the negative effect on CO₂ fixation was compensated for by increased WUE. The efficacy of di-1-*p*-menthene in suppressing gas exchange has also been demonstrated on horticultural species, especially grapes, and not only for foliar but also bunch transpiration (Fahey and Rogiers, 2018; Vaio et al., 2019). In an AT comparative study on droughted grapes, di-1-*p*-menthene was found to depress photosynthesis but had no effect on intrinsic WUE while kaolin enhanced these variables (Brillante et al., 2016). This suggests that different classes of AT do not induce similar physiological responses.

One group of compounds in this class, paraffinic hydrocarbon waxes - Folicote - (Francini et al., 2011; Fuehring, 1973) was effective in suppressing gas exchange variables and increasing yield (e.g. between 11 and 17% in maize (Fuehring and Finkner, 1983) and five to 17% in sorghum (Fuehring, 1973)). However, paraffinic hydrocarbon waxes do not feature in recent AT research. Other less important compounds, on which limited research has been published include vegetable oils

and acrylic polymers. The former may become important in future as they are not only cheaper (Granger and Trager, 2002) but more readily available than synthetic products.

A film-forming AT end-user advantage over other AT classes is the less frequent application, typically once for 30 days for an acrylic polymer in a glasshouse (Plaut et al., 2004), though for poly-1-*p*-menthene it was less than nine days in the OSR study above. In comparison, weathering agents can significantly reduce the foliar coverage and efficacy of reflective ATs to less than half a month (e.g. CaCO₃ (P.S.O. da Silva et al., 2019). As for metabolic ATs, their effects generally last for a few days (Travaglia et al., 2010), being controlled by the plant biochemistry since they are absorbed unlike the other ATs.

Although the use of ATs is valid only under drought stress conditions, in an event of improved soil moisture status due to rainfall, it would be desirable to remove them from the plant surface, particularly film antitranspirants. The importance of removing film antitranspirants in an event of unexpected rains is one research gap that needs pursuing. However, in the case of cereal stands the impact of film antitranspirants will reduce over time as new leaves emerge and take their place at the top of the canopy. This is particularly relevant in this case as it is these later emerging leaves that act as the source of carbohydrates supplied to the growing grain.

Finally, one other concern during early AT research that is more relevant to film-forming types was the effect on leaf temperature. However, the review by Gale and Hagan (1966) shows that an intersection of high solar radiation and very low wind speed was required to significantly increase leaf temperature due to reduced

transpiration. In fact, a 30% reduction of transpiration attributed to ATs was found to have no significant effect on leaf temperature (Gale and Poljakoff-Mayber, 1965). However due to global warming, the use of reflective ATs may play a more important role in drought stress mitigation.

2.3.5 Carbon-dioxide: the climate change antitranspirant

Anthropogenic activities have elevated levels of atmospheric CO₂ leading to climate change (IPCC, 2018). Elevated CO₂ reduces transpiration rate (e.g. up to 30% in carnation plants), however there is no consensus due to environmental and interspecific differences (Xu et al., 2016). Drought, one of the consequences of climate change, reduces plant nutrient uptake (Nawaz et al., 2012). There is currently limited information on the interaction of antitranspirants, elevated CO₂ and drought. One particular source is del Amor et al. (2010) who tested the effects on pepper. They showed that under elevated CO₂ (2000 ppm) and drought, stomatal conductance was higher with antitranspirant (di-1-*p*-menthene) application four and eight days after spraying (when measurements were taken), although the difference was not significant. The corresponding CO₂ assimilation effect was inconsistent being significantly higher in antitranspirant treated plants four days after spraying and becoming non-significant at eight days after spraying. The response of transpiration rate was consistent with stomatal conductance. Interestingly, benefits of elevated CO₂ were only attainable under well irrigated conditions, with no significant difference between antitranspirant treated and untreated plants. These findings suggest that elevated CO₂ may not be as potent as di-1-*p*-menthene in suppressing gas exchange. Further, this may eliminate concerns of reduced transpiration-driven nutrient uptake by mass flow induced by

elevated CO₂ and other factors that suppress transpiration (Mcgrath and Lobell, 2013). However, further research is needed to make well established conclusions on interactions between drought, ATs and elevated CO₂ and how these affect plant nutrition and growth of various crops. Other impacts of global climate change will reduce the availability of soil moisture in some cases and affect transpirational processes. This may make dynamic drought management become more important.

2.4 The basis for timing of antitranspirant application

Drought has varying effects on crop performance depending on its timing in relation to crop growth stage (GS). In cereals, drought at any time before the end of grain filling will affect grain yield. However, the worst is terminal drought (soil moisture deficit during a crop's reproductive development stages) as it has a grain yield limiting effect by reducing grain number (Saradadevi et al., 2017). Reproductive processes such as meiosis and microgametogenesis that occur during booting are the most susceptible to abiotic stresses such as drought (Barber et al., 2015). There is a strong correlation between grain number per ear and grain yield (Liu et al., 2015). In rice Kato et al. (2008), found that a mild drought stress at meiosis triggered secondary rachis and spikelet abortion per panicle of 70% and 45% respectively while drought at panicle initiation did not cause abortion of these reproductive structures. In oil seed rape (*Brassica napus*) Faralli et al. (2016) showed that improving the water status of drought-stressed plants with film-forming AT during the reproductive stage was positively correlated with not only an increase in the number of pods per plant but also the seed yield. The processes of microsporogenesis (involving meiosis in pollen mother cells) and microgametogenesis (development of microspores into mature pollen grains) are

known to be more sensitive to water stress than their female counterparts (De Storme and Geelen, 2014; Jager et al., 2008). Sensitivity to drought is most critical at the young microspore stage during microgametogenesis (Dolferus et al., 2011; Ji et al., 2010). However, Barber et al. (2015) argued that due to the influence of environmental and genotypic aspects, it was not possible to associate the most sensitive development processes with any one particular GS. Nevertheless, it is considered to be during booting; and in wheat there is evidence that it occurs between GS41 and GS43 (Alghabari et al., 2013; Barber et al., 2015). While it is not until near anthesis that the male gametophyte begins to accumulate starch grains, the process begins earlier in the female gametophyte (Ji et al., 2010; Raghavan, 1988), implying that in early development, pollen grains accumulate less carbohydrate reserves than ovaries. This could partly explain the higher vulnerability of pollen grains to abiotic stress during this period compared to ovaries. It may be for the same reason that most of the abiotic stress research on reproductive performance has focussed on the male gametophyte. A study by Onyemaobi et al. (2017) involving reciprocal crosses between well-watered and water stressed wheat plants provides evidence. Onyemaobi et al. (2017) found that only four out of 13 genotypes showed the female gametophyte to be significantly more sensitive to drought than the male gametophyte. Nearly 70% of cultivars studied showed the male gametophyte to be more susceptible to water deficit stress than the female counterpart. It is the susceptibility of these reproductive processes coinciding with terminal drought that forms the foundation for the timing of AT application.

2.5 Re-evaluating antitranspirants

With reproductive processes identified as the most sensitive to drought, AT application timed to protect these stages is expected to result in the highest possible benefits to grain yield under water stress. One of the important developments in AT research in the recent past has been the identification of the most critical stage at which to apply ATs in order to optimise their usefulness under drought conditions. Overall, early AT researchers overlooked the role of plant GS in modulating the efficacy of ATs leading to the erroneous conclusion in the 1970s that AT use was only recommended if plant survival was at stake due to the negative effects on photosynthesis. In fact, even in the 1970's, the necessity of studying the effects of timing of AT application was mentioned by Davenport et al. (1972).

There are two notable exceptions to early AT researchers overlooking the importance of development stage. Fuehring (1973) researched the effects of rate of application of three ATs (metabolic -atrazine and PMA and film-forming-Folicote) and of irrigation frequency on the transpiration and yield performance of sorghum. In addition, he tested the effects of timing of application of PMA sprayed on three sets of plants at eight days before booting, 20 days after booting and a third set receiving double sprays - once on each of these days- and the unsprayed control plants. He found that the effects on grain yield of the lower rates of atrazine and PMA, and the higher rate of Folicote were not significantly different from the unsprayed control plants. However, the higher rates of atrazine and PMA and lower rate of Folicote increased yield. Concerning timing of PMA application, spraying at eight days before booting resulted in significantly the highest yield

response followed by double sprays. This research showed that not only the type of compound but also the rate of AT applied and timing of AT application modulate agronomic efficacy.

Later, Patil and De (1978) conducted a similar study but on oilseed rape and using Mobileaf (film-forming AT), kaolin (reflective AT) and PMA (metabolic AT). All ATs improved grain yield above the unsprayed and droughted plants. PMA applications were repeated, at the initiation of flowering and during pod development. Since the treatments at these different GSs involved the same plants, there was no basis for a comparative analysis. Nevertheless, it is noteworthy that they focused on the most drought-sensitive stages.

Recent research has been more robust in terms of understanding timing effects of AT application. Results have shown that despite reducing photosynthesis, ATs applied within specific plant GS can improve the performance of crops growing under water stress. Kettlewell et al. (2010) found that di-1-*p*-menthene (film-forming AT) improved grain yield of droughted wheat if the reproductive stages most sensitive to drought stress were protected. They tested the timing response of winter wheat to di-1-*p*-menthene application at five GSs (GS described by Zadoks et al. (1974) as GS37, GS39, GS45, GS55 and GS69). Their results revealed that di-1-*p*-menthene was most beneficial to yield improvement if applied at GS37 and GS39, unlike at GS45. On the other hand, much later applications at GS55 and GS69 were counterproductive in that they reduced crop yield – thus being corroborative of the 1970s research conclusion. Further, the AT was also found to reduce yield if soil water deficit was not high. Soil moisture deficit was earlier found as a precondition for kaolin application to obtain yield improvement in mung beans De and Giri (1978). These findings agree with the analysis presented

by Gale and Hagan (1966). The importance of the shoot/root ratio theory as presented by Gale and Hagan (1966) suggests that antitranspirants would be more effective for plants with high values (i.e. higher shoot-to-root biomass, due to lower actual-to-potential evapotranspiration ratio under well-watered conditions, below 0.9). Their argument implies that antitranspirants should not be used where the shoot/root ratio is lower as evapotranspiration is higher, except in an event of abiotic stress such as reduced mineral uptake from the soil (e.g. due to drought) that leads to the breakdown of the photosynthetic machinery (chlorosis). In such a situation antitranspirants would have a reduced inhibitory effect on photosynthesis as the mesophyll resistance to CO₂ conductance is high. This analysis indicates that shallow-rooted plants, being more prone to drought stress, would need AT application before deep-rooted ones. Taken together, the works by De and Giri (1978) and Kettlewell et al. (2010) demonstrated that not only drought stress was required as a precondition for AT application but the crop growth stage most sensitive to water stress must be protected for ATs to improve crop yield.

As discussed by Barber et al. (2015), the period of pollen development can last several days within a spike and much longer in a crop due to asynchrony between the male and female gametophytes. For this reason, reproductive stages sensitive to drought damage have a wider span in a crop and are not limited to a single GS. Refining limits within which farmers may apply AT to maximise benefits is critical and therefore requires further research. Nevertheless, Kettlewell et al. (2010) established that crop development stages most sensitive to water stress dictate timing of di-1-*p*-menthene application for optimum benefits.

Following the work of Kettlewell et al. (2010) on the role of crop development stage, other researchers have found corroborative evidence. Kettlewell and

Holloway (2010) applied di-1-*p*-menthene at the boots swollen stage, GS45, and found that it improved yield of droughted wheat by as much as 42% compared with droughted-and-unsprayed plants. In a separate study involving two water treatments and two GS, Abdullah et al. (2015) observed 30% more wheat grain yield in droughted plants treated with di-1-*p*-menthene at the booting stage than in the droughted but unsprayed plants, while application at anthesis had no significant effect on yield. Recently, Faralli et al. (2017) reported 22% yield improvement in oil seed rape plants treated with film-forming AT as floral initiation was taking place but with no improvement from applications at initiation of seed development. These findings during the last decade are a refutation of earlier research in terms of ameliorative effects of ATs on droughted crops.

Though in part, the mechanisms by which metabolic and reflective ATs ameliorate drought are better understood than those of film-forming types. However, discrepancies between researchers indicate the need for more research. As Gale and Hagan (1966) indicated, this may be attributed to ambient, edaphic and plant factors such as wind speed, soil water status and turgor of cells, respectively, prevailing at and after AT application - necessitating the need for researchers to provide sufficient details in these areas. In spite of our knowledge that di-1-*p*-menthene applied at the right GS and under drought conditions improves grain yield, the underlying physiological mechanisms by which this compound ameliorates drought beyond blockage of stomata are unknown. A study by Weerasinghe et al. (2016) attributed wheat grain yield improvement under drought conditions using di-1-*p*-menthene to increasing of pollen viability, via increased plant water conservation. Nevertheless, the discovery of this relationship was only correlative and not a conclusive cause-and-effect one. Thus, the underlying

mechanisms by which di-1-*p*-menthene improves reproductive development - be they hormonal or metabolic or otherwise - are yet to be established. In a drought stressed oil seed rape study by Faralli et al. (2016), a four-fold decrease in endogenous ABA concentration following an application of di-1-*p*-menthene was correlated with a 17% yield improvement. This suggests that the drought amelioration effects of di-1-*p*-menthene may be related to increased catabolism and/or reduced biosynthesis of ABA. Increased ABA concentration is known to repress cell wall invertase (CWIN) via down-regulation of genes controlling the synthesis of this enzyme (Ji et al., 2010; Koonjul et al., 2005). The significance of CWIN to reproductive development lies in their hydrolysis of sucrose into glucose and fructose required by anthers and ovaries (Braun et al., 2014; Ruan et al., 2010). Researching the effects of ATs on ABA signalling with respect to CWIN is therefore required. Knowledge of mechanisms, if established, could further feed into developing relevant plant breeding and agronomic management strategies for improving WUE and crop production.

2.6 Challenges in using antitranspirants

This review has shown that the effects of ATs on crop development are modulated by prevailing environmental conditions and intraspecific and interspecific factors. This accounts for apparently contradictory findings by different researchers, hence the need for thorough description of experimental conditions. The type of AT used in research also modulates plant response. Concerning the effect on leaf temperature, Gale and Hagan (1966) downplayed the risk of overheating due to AT use, as stated above. However, global warming is likely to increase the risk of

heat stress. This may see film-forming ATs play a minor role than the reflective types that attenuate solar radiation, thereby reducing the heat load on the leaf.

Due to suppressing both transpiration and photosynthesis, ATs reduce not only carbon assimilation but also the uptake of minerals from the soil. The negative effect can be minimised by selecting appropriate ATs for a given drought scenario. Metabolic ATs would be ideal in situations where a dry spell that coincides with the most drought-sensitive crop growth stage is expected, while film-forming types would suit prolonged drought events as they have longer-lasting effects (30 to 40 days, Plaut et al., 2004). Nevertheless, finding a product that is more permeable to CO₂ than water vapour has been a long standing AT challenge yet to be addressed. Product research is therefore needed to invent suitable ATs. Further, ATs may not be used as a sole drought mitigation technique. However, most if not all the current research focuses on the use of ATs in isolation. An integrated approach is required, for example, De *et al.* (1983) demonstrated reduced irrigation requirement in wheat by using combinations of rice stubble and kaolin. Agarwal (1979) obtained the highest barley yield under a combined stubble mulch-and-kaolin treatment.

The environmental risks associated with the use of the three most popular ATs, ABA, di-1-*p*-menthene and kaolin are likely very low. ABA can be extracted from plants, obtained through microbial fermentation or synthesised (Shi et al., 2017), while di-1-*p*-menthene is extracted from pine resin (Francini et al., 2011). Kaolin, an aluminosilicate, Al₄Si₄O₁₀(OH)₈, is obtained from clay (Cantore *et al.* 2009). However, kaolin is also used to control insect pests, and studies have demonstrated that it is effective (Table 2.2) against some studied species. Unfortunately, almost all papers reviewed in this article focussed on plant

response to ATs, neglecting the potential negative effects on the environment. One exception is Pascual et al., 2010 who showed that while kaolin significantly reduced the incidence of fruit fly (*Bactrocera oleae*) and black scale (*Saissetia oleae*), pests of olives, the AT also caused mortality of their natural enemies (e.g. coccinellids or ladybird beetles: *Scymnus mediterraneus* and *Stethorus punctillum*, among other families of natural enemies). Earlier, Marko et al. (2008) established that severity of infestation with a number of apple pests (e.g. leaf miner moth and woolly apple aphid) was linked to the use of kaolin. The AT was toxic not only to pests but also their natural enemies (e.g. spiders and common black ants). More research is therefore required to establish off-target effects of other ATs, particularly in this class and in other crops, and how the products can be made safe.

Lack of cost-benefit analysis: Clearer, evidence-based messages on the economic feasibility of using ATs in crops production, especially in arable farming, are needed to help prospective adopters make firm decisions. However, the number of research papers with information on the cost-benefit analysis of using ATs in crop production is meagre (e.g. Kettlewell, 2011 and Brahma et al., 2007). This is not surprising since yield improvement has been the primary focus of most researchers. Yield loss due to drought needs to be quantified and valued; and the yield benefit due to AT application demonstrated in monetary terms. Lack of thorough, in-depth analyses on the cost-effectiveness of ATs in most crops may partly be blamed for the erratic response of crop producers in the adoption of ATs as a drought mitigation option. This might also have stifled research in novel products that could be cheaper.

2.7 Conclusion and future prospects

The agronomic benefits of ATs have been highlighted in this review. The physiological mechanisms by which metabolic and reflective ATs ameliorate drought have been described but exactly how they affect reproductive processes is still unknown. Similarly, although timing of AT application was studied using the film-forming product, di-1-*p*-menthene, the mode of action of this class of ATs is yet to be established. The discovery of the role of crop GS in modulating AT efficacy in wheat by Kettlewell et al. (2010) has been a major research breakthrough of the last decade. This might be the reason behind the increasing interest in AT research besides the threat of increasing frequency of droughts.

As the findings of Kettlewell et al. (2010) were based on a film-forming AT, there is need for a comparative study involving all the three classes to determine the most effective types. It would also be interesting to examine interspecific responses to the AT classes. Discrepancies in plant response suggest that species, genotype, environment, chemical nature and rate of AT applied further modulate the efficacy of ATs. In wheat and oil seed rape, film-forming di-1-*p*-menthene has shown consistent positive results in different growth environments. Further research opportunities include understanding possible interaction between ATs and heat stress, testing the efficacy of vegetable oils and integrating ATs with other drought management techniques (e.g. mulching or cover crops). The broader environmental impacts of different types of AT must also be considered. Finally, one of the challenges against achieving world food security ahead of 2050 is not just finding effective drought amelioration techniques but also increasing the knowledge and accessibility to the end users of the technologies. ATs are yet to

enter drought amelioration in arable crop production, despite research providing empirical evidence of agronomic benefits. ATs may still be too expensive for use in low value crop production systems hence the need to find cheaper alternatives. Increasing frequency and intensity of droughts and the associated crop failure may stimulate farmers' adoption of ATs in arable crop production.

Table 2.2: Summary of antitranspirant research based on 173 peer review articles published between 2009 and 2018.

Year	Antitranspirant class	Antitranspirant product	Research area/objective	Crop/plant species	Citation	Some of the reported effects on crop/plant performance
2009	Reflective	Kaolin	Drought and heat stress	Tomato	Cantore et al., 2009	Reduced drought and heat stress and improved marketable yield by 21%
2009	Reflective	Kaolin	Heat stress management	Apple	Glenn, 2009	Reduced heat stress, increased carbon fixation and fruit size
2010	Reflective	Kaolin	Fruit protection from sunburn	Apple	Aly et al., 2010	Increased light reflectance and leaf expansion, reduced sunburn, increased fruit weight and yield
2010	Reflective	Kaolin	Water stress	Apple	Glenn, 2010	Increased photosynthesis, reduced WUE
2010	Reflective	Kaolin	Insect pest and disease control	Wheat	Campolo et al., 2014	Decreased reproduction of beetle, <i>Rhyzopertha dominica</i> , reduced multiplication of yeast moulds
2010	Reflective	Kaolin	Insect pest control	Olives	Pascual et al., 2010	Significant mortality of pests <i>Bactrocera oleae</i> and <i>Saissetia oleae</i> and natural enemies (e.g. <i>Scymnus mediterraneus</i>).
2010	Reflective	Kaolin	Insect pest control	Cotton	Alavo et al., 2010	Reduced oviposition and egg number of bollworms, <i>Helicoverpa armigera</i>
2010	Reflective	Kaolin	Physiological responses	Grapes	Michael Glenn et al., 2010	Reduced leaf temperature, reduced stomatal conductance but increased leaf water potential under well-watered conditions

2010	Reflective	Kaolin	Drought stress	Olives	Roussos et al., 2010	Maintained plant water status, increased canopy biomass, did not improve chlorophyll content and fruit yield
2010	Reflective	Kaolin	Protection from sunburn	Pomegranate	Weerakkody et al., 2010	Reduced sunburn damage, no effect on phenolic compounds and antioxidant activity
2011	Reflective	Kaolin	Insect (Medfly) control	Nectarines and peaches	D'Aquino et al., 2011	Decreased landing and fruit damage
2012	Reflective	Kaolin	Drought stress	Olives	Denaxa et al., 2012	Increased photosynthesis, plant water content, leaf tissue density and reduced leaf temperature
2012	Reflective	Kaolin	Drought stress	Tomato	Lukic et al., 2012	Improved WUE (by 43%) and increased total biomass and economic yield
2012	Reflective	Kaolin plus deficit irrigation	Irrigation management	Grapes	Song et al., 2012	Increased terpene alcohols and anthocyanins, negligible effect on volatile compounds
2012	Reflective	Kaolin, calcium carbonate	Control of cherry fruit fly	Sweet cherry	Yee, 2012	Kaolin more effective than calcium carbonate in decreasing landing and oviposition and also with higher mortality rates
2012	Reflective	Kaolin, magnesium carbonate	Drought stress	Jatropha	Khalil et al., 2012	Increased relative water content, reduced osmotic pressure and carbohydrate content
2012	Reflective	Kaolin	Irrigation management	Strawberry	Santos et al., 2012	Reduced irrigation frequency, saved 20% of water required,

						increased fruit weight
2013	Reflective	Kaolin	Drought and heat stress	Mango	Chamchaiyaporn et al., 2013	Reduced irradiance and leaf temperature and improved stomata conductance and photosynthesis. Increased fruit number and yield by 41 and 44%, respectively
2013	Reflective	Kaolin, magnesium carbonate	Drought stress	Wheat	Desoky et al., 2013	Improved photosynthetic pigments, increased yield
2013	Reflective	Kaolin	Aphid control	Wheat	Nateghi et al., 2013	Reduced damage, improved biomass and grain yields
2013	Reflective	Kaolin	Drought stress	Soyabeans	Javan et al., 2013	Increased yield components, biomass and grain yield
2013	Reflective	Kaolin	Boll weevil (<i>Anthonomus grandis</i>) control	Cotton	Silva and Ramalho, 2013	Reduced oviposition and boll damage
2013	Reflective	Kaolin	Drought stress	Pistachio	Azizi et al., 2013	Increased fresh weight and soluble solids, reduced early nut splitting
2013	Reflective	Kaolin and calcium carbonate	Drought stress	Banana	El-Khawaga, 2013	Reduced irrigation frequency, improved crop productivity
2013	Reflective	Calcium carbonate	Fruit protection from sunburn	grapes	Ahmed et al., 2013	Chlorophyll and carotenoid enhancement, berry setting and yield improvement
2013	Reflective	Kaolin	Drought stress	Grapes	Shellie and King, 2013	Reduced stomatal conductance and temperature, increased anthocyanin and phenolics content and berry fresh weight

2014	Reflective	Kaolin plus other materials	Drought stress	Wheat	Patil et al., 2014	Improved yield components and yield
2014	Reflective	Kaolin	Heat stress	Grapes	Tepkaew et al., 2014	Increased photosynthesis, transpiration, total soluble solids, berry weight, diameter and improved yield by 44%.
2015	Reflective	Kaolin	Fruit quality enhancement	Grapes	Lobos et al., 2015	Effective in reducing leaf temperature through reflectance, did not significantly decrease gas exchange variables, reduced fruit damage, no effect on fruit nutritional quality
2015	Reflective	Kaolin	Irrigation management	Bananas	Gawad, 2015	Decreased transpiration rate and amount of irrigation water, increased yield
2015	Reflective	Kaolin	Drought stress	Gooseberry	Segura-Monroy et al., 2015	Reduced transpiration, leaf temperature, improved stem elongation, total biomass, WUE
2015	Reflective	Kaolin	Irrigation water management	Oil seed rape	Badukale et al., 2015	Reduced irrigation frequency
2015	Reflective	Kaolin	Drought stress, transplant survival	Citrus, beans	Boari et al., 2015	Decreased transplant shock, improved biomass
2015	Reflective	Kaolin	Irrigation water management	Egg plant	El-Said, 2015	Reduced irrigation water by 33%, increased chlorophyll content, fruit weight, nutritional quality, plant biomass and economic yield

2015	Reflective	Kaolin	Drought stress and fruit quality	Olives	Khaleghi et al., 2015	Enhanced chlorophyll and carotenoid contents, increased oleic acid in olive oil
2015	Reflective	Kaolin	Heat and drought stress	Grapes	Correia et al., 2015	Higher active PSII reaction centres, lowered mesophyll limitations, increased net photosynthesis, reduced sunburn, improved yield
2015	Reflective	Kaolin and calcium carbonate	Fruit protection from sunburn	Pomegranate	El-wafa, 2015	Reduced sunburn, increased anthocyanin and total sugar content, fruit weight and yield
2015	Reflective	Potassium silicate	Drought stress	Tomato	El-azm and Youssef, 2015	Increased relative water content, leaf expansion, WUE, plant biomass and yield
2015	Reflective	Kaolin	Pest (whitefly) control	Beans	Nunez-Lopez et al., 2015	Reduced the number of eggs, nymphs and adult insects by 70, 75 and 80%, respectively. Suppressed transpiration by 40%, increased chlorophyll content by 43%
2015	Reflective	Kaolin	Drought stress	Peruvian Ground Cherry	Segura-Monroy et al., 2015	Reduced transpiration, leaf temperature, leaf thickness and trichome density, increased WUE, stem elongation and biomass yield
2016	Reflective	Kaolin	Drought stress and fruit quality	Grapes	Brillante et al., 2016	Improved intrinsic WUE and anthocyanin content
2016	Reflective	Kaolin	Salinity tolerance	Tomato	Boari et al., 2016	Mitigated salinity stress and reduced damage by insects: increased marketable yield and reduced sunburn by 17.7 and

						76.4%, respectively,
2016	Reflective	Kaolin	Drought stress	Tomato	Djurovic et al., 2016	Improved WUE, increased individual fruit weight and total yield by 27%
2016	Reflective	Calcium carbonate	Control of potato psyllid (<i>Bactericera cockerelli</i>)	Irish potatoes	Prager et al., 2013	Reduced oviposition, no effect on mortality
2016	Reflective	Kaolin	Drought stress and fruit quality	Grapes	Conde et al., 2016	Enhanced flavonoid and anthocyanin contents
2016	Reflective	Kaolin	Drought stress	Tomato and pepper	Cosic et al., 2016	Did not affect stomatal conductance and plant water status significantly
2016	Reflective	Kaolin	Drought and heat stress; and fruit quality	Grapes	Dinis et al., 2016	Increased phenol, flavonoid anthocyanin and vitamin C contents by 40, 24, 32 and 12%, respectively, reduced reactive oxygen species and improving fruit quality
2016	Reflective	Kaolin	Heat stress mitigation	Apples	Glenn, 2016	Reduced photosynthetically active radiation interception, increased fruit weight
2016	Reflective	Kaolin	WUE	Apples	Glenn, 2016b	Reduced WUE, increased stomatal conductance and transpiration, improved yield
2017	Reflective	Kaolin	Fruit protection from sunburn	Grapes	Ferrari et al., 2017	Reduced sunburn

2017	Reflective	Kaolin	Control of leafhopper	Grapes	Tacoli et al., 2017	Effective in inducing mortality of nymphs
2017	Reflective	Kaolin	Control of leafhopper	Grapes	Tacoli et al., 2017b	Caused mortality of nymphs through inhibition of feeding; and reduced leaf symptoms and damage
2017	Reflective	Magnesium carbonate, sodium carbonate, potassium nitrate	Drought stress	Soyabeans	Dass and Bhattacharyya, 2017	MgCO ₃ and KNO ₃ improved grain yield and protein content
2017	Reflective	Kaolin, calcium carbonate (with conservation tillage)	Insect (thrips) control	Cotton and groundnuts	Knight et al., 2017	Reduced <i>Frankliniella fusca</i> , decreased incidence of tomato spotted wilt virus in groundnuts. Increased incidence in cotton. No effect on yield in both crops
2017	Reflective	Potassium nitrate, sodium carbonate and magnesium carbonate	Drought stress	Soyabeans	Sanbagavalli et al., 2017	Increased relative water content and biomass and grain yield
2017	Reflective	Kaolin, calcium carbonate	Fruit protection from sunburn	Pomegranate	Abdel-Sattar et al., 2017	Reduced sunburn damage, enhanced total soluble solids
2017	Reflective	Kaolin	Irrigation management	Aubergine	Abd El-Hady and Doklega, 2017	Reduced irrigation frequency and water use, increased yield
2017	Reflective	Kaolin	Light extinction coefficient and radiation use	Pistachio	Vatandoost et al., 2017	Reduced single-leaf light interception but increased canopy absorbance, light

			efficiency			extinction coefficient (K) not significantly affected, improved light use efficiency in some cultivars
2018	Reflective	Kaolin	Drought stress	Olives	Brito et al., 2018	Maintained leaf water status, improved photosynthesis, ameliorated oxidative damage, whole-plant WUE and biomass not improved
2018	Reflective	Kaolin	Irrigation and heat stress management	Pepper	Cosic et al., 2018	Water deficit with kaolin reduced canopy temperature, full irrigation with kaolin increased temperature
2018	Reflective	Kaolin	Heat stress	Walnut	Gharaghani et al., 2018	Decreased heat stress, enhanced photosynthesis, gas exchange and WUE
2018	Reflective	Kaolin	Pest (<i>Diaphorina citri</i>) control	Citrus	Miranda et al., 2018	Reduction in host apparency (40%) and recognition (50%)
2018	Reflective	Kaolin	Heat stress and fruit quality	Grapes	Kok and Bal, 2018	Enhanced contents of phenolic compounds and anthocyanins
2018	Reflective	Kaolin, calcium carbonate	Pest (<i>Diaphorina citri</i>) control	Citrus	Ramírez-Godoy et al., 2018	Decreased photosynthesis (25%), leaf temperature, incidence of imagoes, nymphs and egg laying
2018	Reflective	Kaolin	Fruit protection from sunburn and cracking	Pomegranates	Sharma et al., 2018	Decreased sunburn (47%) and fruit cracking (46%), increased anthocyanins and phenolic contents and antioxidant activity

2018	Reflective	Kaolin	Drought stress	Sunflower	El Mantawy and El Bialy, 2018	Increased chlorophyll and proline contents, reduced stomatal conductance and transpiration, improved relative water content and yield
2018	Reflective	Kaolin	Drought stress	Grapes	Dinis et al., 2018	Enhance stomatal conductance, photosynthesis and intrinsic WUE, reduced ABA concentration, enhance drought stress tolerance.
2018	Reflective	Kaolin	Drought stress	Tomato	AbdAllah et al., 2018	Reduced canopy temperature, irrigation water required, net CO ₂ assimilation rate and marketable yield
2018	Reflective	Calcium carbonate	Drought stress	Tomato	Patane et al., 2018	Reduced transpiration (by 47 - 58%) and leaf temperature, improved vitamin C and total phenols contents by 15 and 12% respectively, increased antioxidant activity and marketable yield
2018	Reflective	Kaolin	Irrigation management	Maize	Ulameer and Ahmed, 2018	Increased leaf number and plant height, improved biomass accumulation
2018	Reflective	Kaolin	Graft survival	Mango	Thorat et al., 2018	Depressed transpiration, enhanced photosynthesis, increased relative water content and survival of grafts
2018	Reflective	Kaolin	Drought stress	Pears	Fayed et al., 2018	Reduced irrigation water applied, elevated indoleacetic acid and gibberellic acid

						activities
2018	Reflective	Kaolin (deficit irrigation; mulch)	Irrigation management	Common Zinnia	El-Deen et al., 2018	Increased plant height, biomass accumulation
2009	Metabolic	ABA	Fruit quality enhancement	Grapes	Lurie et al., 2009	Increased anthocyanin content, reduced fruit firmness, no effect on soluble solids content, titratable acidity, and berry size
2009	Metabolic	Chitosan	Drought stress	Beans	Iriti et al., 2009	Depressed stomatal conductance – reduced transpiration and photosynthesis, mode of action mediated by ABA, elevated endogenous ABA concentration threefold, no effect on intrinsic WUE
2009	Metabolic	ABA	Fruit ripening	Grapes	Wheeler et al., 2009	Promoted fruit ripening and increased berry size
2009	Metabolic	ABA	Vase life extension	Eustoma flowers	Shimizu-Yumoto and Ichimura, 2009	Reduced leaf damage attributed to sucrose solution, slowed loss of fresh weight, extended vase life
2010	Metabolic	ABA	Drought stress	Various woody ornamentals	Hebert et al., 2010	Reduced stomatal conductance, reduced cumulative water loss, increased stem water potential, extended marketability by one to seven days

2010	Metabolic	ABA	Protection from sunburn	Apples	Mupambi et al., 2014	No effect on sunburn, significantly reduced fruit size and weight and total soluble solids
2010	Metabolic	Chitosan	Drought stress	Bean	Ludwig et al., 2010	Reduced stomatal conductance and transpiration
2010	Metabolic	ABA	Vase life extension	Cut roses	Pompodakis et al., 2010	Pre-treatment with ABA did not increase vase life but reduced electrolyte leakage
2010	Metabolic	ABA	Drought stress	Pansy and Viola	Waterland et al., 2010	Induced leaf chlorosis, improved drought tolerance by delaying wilting and maintained marketable quality in the presence of benzyladenine and gibberellic acid
2010	Metabolic	ABA	Drought stress	Seed Geranium, petunia, marigold and others	Waterland et al., 2010a	Reduced water loss, induced chlorosis, delayed wilting by 1.7 to 4.3 days
2010	Metabolic	ABA	Drought stress	Chrysanthe mums	Waterland et al., 2010c	Suppressed reducing stomatal conductance, delayed wilting by 1.2 to 4.0 days, induced recovery from severe drought
2011	Metabolic	Salicylic acid	Vase life extension	Cut roses	Alaey et al., 2011	Enhanced uptake of vase solution increasing relative fresh weight of flowers, improved antioxidant activities of catalase, extending the vase life

2011	Metabolic	ABA	Extension of shelf life	Tomato	Astacio and van Iersel, 2011	Decreased transpiration, stomatal conductance, photosynthesis and irrigation water use. Increased shelf life
2011	Metabolic	ABA	Drought stress	Apples	Tworkoski et al., 2011	Enhanced water potential by 62% and reduced endogenous leaf ABA by 45%.
2011	Metabolic	ABA	Cold tolerance	Citrus	Yang et al., 2011	Reduced electrolyte leakage
2011	Metabolic	Fulvic acid	Drought stress	Maize	Anjum et al., 2011	Maintained chlorophyll content and gas exchange, enhance plant growth and yield
2011	Metabolic	Chitosan	Disease control	Grapes	Iriti et al., 2011	Reduced powdery mildew infestation, upregulated polyphenol content and removal of free radicals
2011	Metabolic	ABA	Fruit quality enhancement	Grapes	Gu et al., 2011	Increased anthocyanin content up to 85%, marginal improvement of total soluble solids contents
2011	Metabolic	ABA	Drought stress	Smoke bush	Li et al., 2011	Elevated endogenous ABA, did not increase relative water content, reduced stomatal conductance, transpiration and photosynthesis, improved drought tolerance
2011	Metabolic	Salicylic acid	Salt stress	Sunflower	Noreen et al., 2011	Promoted plant growth, chlorophyll and calcium contents and improved leaf turgor

2011	Metabolic	ABA	Fruit quality enhancement	Grapes	Sandhu et al., 2011	Upregulated antioxidant activity by 38%, increased anthocyanins and phenolic contents
2011	Metabolic	ABA	Cold tolerance	Grapes	Zhang et al., 2011	Effective in inducing leaf senescence, abscission and shoot dormancy and promoting freeze tolerance
2011	Metabolic	ABA	Vase life extension	Iris flowers	Zhong and Ciafre, 2011	Prevented flag petal expansion, induced ethylene-independent senescence, upregulating proteolytic degradation and electrolyte leakage
2012	Metabolic	ABA	Cold tolerance	Grapes	Zhang and Dami, 2012	Enhanced shoot dormancy and freeze tolerance
2013	Metabolic	ABA	Fruit ripening	Cucumber	Wang et al., 2013	Effective in promoting fruit ripening
2013	Metabolic	ABA	Flowering regulation	Litchi	Cui et al., 2013	Increased flowering per panicle
2013	Metabolic	Fulvic acid	Drought stress	Wheat	Desoky et al., 2013	Improved photosynthetic pigments, increased yield
2013	Metabolic	Chitosan	Drought stress	Soyabeans	Javan et al., 2013	Increased yield components, biomass and grain yield
2013	Metabolic	ABA	Vase life extension	Gladiolus	Kumar et al., 2014	Reduced water uptake, fresh weight, enhanced senescence in cut flowers, decreased vase life by two days
2013	Metabolic	Salicylic acid and acetylsalicylic acid	Drought stress	Fibrous flax.	Heller et al., 2013	Enhanced stem elongation and fibre yield

2014	Metabolic	ABA	Fruit quality enhancement	Tomato	Barickman et al., 2014	Increased both chlorophyll and carotenoid concentrations in leaf and fruit tissue
2014	Metabolic	ABA	Control of blossom end-rot	Tomato	Casey Barickman et al., 2014	Improved uptake of calcium and reduced blossom end-rot
2014	Metabolic	ABA (used with ABA Analog: (+)-8'-acetylene ABA)	Fruit set control and gas exchange	Apples	McArtney et al., 2014	Reduced stomatal conductance limited photosynthesis inducing a decline in fruit set
2014	Metabolic	Chitosan	Drought stress	Pomegranate	El-Khawaga and Mansour, 2014	Decreased sugar and anthocyanin contents, increased fruit set, weight and yield
2014	Metabolic	ABA	Fruit quality enhancement	Citrus	Kawai et al., 2014	Increased fruit size and weight, enhanced potassium content, reduced sugar accumulation
2014	Metabolic	ABA	Fruit quality enhancement	Grapes	Koyama et al., 2014	Did not cluster size or weight, raised anthocyanin content
2014	Metabolic	ABA	Transplant shock	Leafy vegetables	Racsko et al., 2014	Improved quality of seedlings pre-planting, increased marketability of spinach at harvest
2014	Metabolic	ABA	Transplant shock	Artichoke	Shinohara and Leskovar, 2014	Induced stomatal closure, increased plant water potential, reduced electrolyte leakage, enhanced drought tolerance
2014	Metabolic	ABA	Drought stress	Pansies	Weaver and van Iersel, 2014	Decreased leaf chlorophyll content, stomatal conductance and carbon assimilation, induced chlorosis.

2015	Metabolic	ABA	Vase life extension	Lilium Sorbonne	Geng et al., 2015	Increased sucrose uptake from solution, content of simple sugars and fresh weight
2015	Metabolic	ABA	Cold tolerance	Grapes	Dami et al., 2015	Enhanced cold tolerance, did not have an effect on yield and berry quality, no evidence of toxicity
2015	Metabolic	ABA	Fruit quality enhancement	Grapes	Ferrara et al., 2015	Increased anthocyanin content, antioxidant activity and bunch yield
2015	Metabolic	ABA	Fruit quality enhancement	Grapes	Yamamoto et al., 2015	Increased anthocyanins content and improved flavour of juice
2015	Metabolic	Fulvic acid	Drought stress	Tomato	Aggag et al., 2015	Improved relative water content and marketable yield by 24%, irrigation WUE by 34.82%.
2015	Metabolic	Fulvic acid	Drought stress	Oil seed rape	Lotfi et al., 2015	Enhanced maximum quantum efficiency of photosystem II, fluorescence of chlorophyll a, antioxidant activities, ameliorated lipid peroxidation.
2016	Metabolic	ABA	Drought stress	Apple	Al-Absi and Archbold, 2016	Depressed gas exchange variables, water deficit stress not alleviated
2016	Metabolic	ABA	Cold stress	Blueberry	Panicker and Matta, 2016	Reduced electrolyte leakage of floral parts increasing their tolerance to freezing injury
2016	Metabolic	ABA	Cold stress	Grapes	Bowen et al., 2016	Reduced bud damage
2016	Metabolic	Acetylsalicylic acid	Vase life extension	Cut roses	Fanourakis et al., 2016	Reduced transpiration, extended vase life
2016	Metabolic	ABA	Cold stress and fruit quality	Grapes	Li and Dami, 2016	Induced early leaf senescence, abscission, enhanced dormancy, increased cold

						tolerance
2016	Metabolic	ABA	Fruit quality enhancement	Grapes	Katayama-Ikegami et al., 2016	Upregulated flavonoid and anthocyanin biosynthesis genes, increased anthocyanin content
2016	Metabolic	ABA	Fruit quality enhancement	Grapes	Koyama et al., 2014	Increased anthocyanin polyphenolic compounds
2016	Metabolic	ABA	Drought stress	Various bedding plants	Park et al., 2016	Depressed stomatal conductance, improved drought tolerance by delaying wilting by between 1.3 and 3.7 days
2016	Metabolic	ABA	Fruit quality enhancement	Grapes	Singh et al., 2016	Improved berry and bunch sizes, total soluble solids and fruit firmness
2016	Metabolic	Fulvic acid	Water deficit conditions	Wheat	Zhang et al., 2016	Improved WUE, carbon assimilation and yield
2016	Metabolic	ABA	Water deficit conditions	Grapes	Wang et al., 2016	Reduced electrolyte leakage and lipid peroxidation, improved antioxidant activity
2016	Metabolic	ABA	Water deficit	Wheat	Zhang et al., 2016	Improved WUE and yield
2017	Metabolic	ABA	Fruit thinning	Pears	Arrington et al., 2017	Suppression of net photosynthesis by 75-90%, increased fruit size, fruit quality unaffected
2017	Metabolic	Salicylic acid, Benzoic acid	Drought stress	Tomato	Isa et al., 2017	Increased leaf area index, fruit number and marketable yield
2017	Metabolic	ABA	Fruit quality enhancement	Tomato	Barickman et al., 2017	Enhanced the concentrations of fructose, glucose and the contents of chlorophyll and

						carotenoids
2017	Metabolic	ABA	Fruit quality enhancement	Grapes	Gonzalez et al., 2018	Elevated accumulation of ethanol, depressed aroma quality
2017	Metabolic	ABA	Fruit quality enhancement	Grapes	Jia et al., 2018	Increased anthocyanin and aroma volatiles contents, decreased fruit firmness
2017	Metabolic	Salicylic acid	Vase life extension	Cut roses	Kazemi et al., 2017	Sustained membrane stability, attenuated lipid peroxidation, reduced weight loss, enhanced antioxidant activities, extended vase life
2017	Metabolic	Chitosan	Vase life extension	Macaw flower	Banuelos-Hernandez et al., 2017	Increased anthocyanin and flavonoid contents by 48 and 46%, respectively, sustained fresh weight, extended vase life by 10.3 days
2017	Metabolic	ABA	Fruit quality enhancement	Grapes	Neto et al., 2017	Enhanced soluble solids, anthocyanin and total phenolic contents
2017	Metabolic	ABA	Post-harvest preservation	Grapes	F. J. D. Neto et al., 2017	Decreased shatter, decay and loss of fruit weight
2017	Metabolic	ABA	Fruit quality enhancement	Grapes	Olivares et al., 2017	Upregulation of anthocyanin biosynthesis genes, elevated anthocyanins content
2017	Metabolic	ABA	Berry and cluster manipulation	Grapes	Padmalatha et al., 2017	Reduced berry number, improved berry and cluster uniformity

2017	Metabolic	ABA	Extension of marketability	Tomato	Agehara and Leskovar, 2017	Inhibited stem elongation, leaf expansion and shoot biomass accumulation by 22% effectively prolonging market value
2017	Metabolic	Chitosan	Drought stress	White clover	Z. Li et al., 2017	Increased contents of amino acids, sugars, sugar alcohols, antioxidants and other metabolites associated with drought resistance mechanisms
2017	Metabolic	Fulvic acid	Crop productivity	Safflower	Moradi et al., 2017	Improved seed yield by 36% and oil content by 86%
2018	Metabolic	ABA	Stomatal physiology	Cut gerberas	Huang et al., 2018	Increased percentage of closed stomata, reduced stomatal aperture, decreased water loss
2018	Metabolic	ABA	Fruit quality enhancement	Grapes	Kok and Bal, 2018b	Enhanced phenolic compounds and anthocyanin contents, increased grape yield
2018	Metabolic	ABA	Fruit quality enhancement	Grapes	Koyama et al., 2018	Upregulated biosynthesis of anthocyanins and flavonoid genes expression
2018	Metabolic	ABA	Fruit quality enhancement	Blueberry	Oh et al., 2018	Elevated endogenous ABA, increased anthocyanins (malvidin, delphinidin, petunidin glycosides) and fruit softening
2018	Metabolic	ABA	Fruit quality enhancement	Oranges	Rehman et al., 2018	Enhanced colour index, carotenoid content, reduced organic acids, no effect on sugar content

2018	Metabolic	Chitosan	Drought stress	Wheat	Singh et al., 2018	Improved chlorophyll, carotenoid, proline and superoxide dismutase contents
2018	Metabolic	Chitosan, deficit irrigation	Irrigation management	Wheat	Reddy et al., 2018	Increased stem extension and leaf expansion
2018	Metabolic	Fulvic acid	Low rainfall/drought stress	Maize	Yang et al., 2018	Increased kernel number per ear, did not increase yield
2018	Metabolic	Fulvic acid	Drought stress	Tomato	AbdAllah et al., 2018	Reduced canopy temperature, irrigation water required (by 28%), net CO ₂ assimilation rate and marketable yield, increased WUE by 33.45%
2018	Metabolic	Phenyl mercuric acetate	Drought stress	Oil seed rape	Kumar et al., 2018	Increased plant height and total biomass, but not seed oil and protein contents
2009	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Kiwi fruit	Latocha et al., 2009	Enhanced efficiency of Photosystem II and chlorophyll content, non-significant effect on yield
2009	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Sultani fig	Al-Desouki et al., 2009	Reduced sugars and total soluble solids, improved vegetative growth and yield
2009	Film-forming	Di-1- <i>p</i> -menthene	Physiology, nutrient uptake and yield	Pepper	del Amor and Rubio, 2009	Suppressed carbon assimilation, reduced water absorption, no effect on cation uptake and dry weight and yield, reduced incidence of blossom-end rot

2009	Film-forming	Canola oil (other vegetable oils)	Control of powdery and downy mildews	Cucumber	Jee et al., 2009	Reduced powdery and downy mildews by 99 and 96%, respectively
2009	Film-forming	Di-1- <i>p</i> -menthene, poly-1- <i>p</i> menthene	Control of apple scab (<i>Venturia inaequalis</i>)	Apples	Percival and Boyle, 2009	Increased chlorophyll fluorescence, decreased conidia germination and foliar and fruit scab severity, improved yield
2010	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Wheat	Kettlewell et al., 2010	Improved yield at high soil water deficit
2010	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Wheat	Kettlewell and Holloway, 2010	Improved yield
2010	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Bean	Ludwig et al., 2010	Reduced stomatal conductance
2010	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Pepper	del Amor et al., 2010	Reduced gas exchange under ambient CO ₂ concentration, higher water potential and photosynthesis under combined elevated CO ₂ and AT
2010	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Citrus	Mohawesh et al., 2010	Non-significant effect on gas exchange, plant water status, chlorophyll and proline contents
2010	Film-forming	Di-1- <i>p</i> -menthene	Fruit quality control	Grapes	Palliotti et al., 2010	Depressed transpiration and carbon assimilation rates, increased intrinsic WUE and anthocyanin content, reduced yield
2011	Film-forming	Di-1- <i>p</i> -menthene	Ozone stress	Beans	Francini et al., 2011	Reduced membrane damage, maintained stomatal conductance and photosynthesis

2012	Film-forming	Carboxylated hydrophilic polymer (Envy)	Transplant shock	Agarwood	Page and Awarau, 2012	Neither improved growth rates nor reduce stress and mortality of transplants
2013	Film-forming	Castor bean oil	Drought stress	Soyabeans	Javan et al., 2013	Increased yield components, biomass and grain yield
2013	Film-forming	Di-1- <i>p</i> -menthene	Micronutrient and trace element quality	Sweet cherry	Mikiciuk et al., 2013	No effect on quantity of manganese, nickel, cadmium, copper or zinc
2013	Film-forming	Di-1- <i>p</i> -menthene	Control of sugar content	Grapes	Palliotti et al., 2013	Reduced transpiration, photosynthesis, increased intrinsic WUE, reduced anthocyanin content (by 19%) and sugar content
2014	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Wheat and barley	Ouerghi et al., 2014	Improved water potential in both crops, increased photosynthesis in wheat only
2015	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Wheat	Abdullah et al., 2015	Improved plant water status, photosynthesis and yield
2015	Film-forming	Paraffin	Drought stress	Potatoes	Khalel, A. M. S., 2015	Reduced transpiration, improved WUE, total yield and marketable yield
2015	Film-forming	Di-1- <i>p</i> -menthene		Strawberry	Mikiciuk et al., 2015	Reduced transpiration without depressing carbon assimilation, increased relative water content and WUE, no effect on chlorophyll
2016	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Wheat	Weerasinghe et al., 2016	Improved water potential, pollen viability and grain yield
2016	Film-forming	Poly-1- <i>p</i> -menthene and di-1- <i>p</i> -menthene	Drought stress	Oil seed rape	Faralli et al., 2016	Decreased endogenous ABA, improved flower and pod water potential.

2016	Film-forming	Di-1- <i>p</i> -menthene	Fruit quality	Grapes	Brillante et al., 2016	No effect on intrinsic WUE, depressed gas exchange, sugar and anthocyanin content
2016	Film-forming	Di-1- <i>p</i> -menthene	Controlled ripening	Grapes	Gatti et al., 2016	Reduced gas exchange, slowed sugar accumulation
2017	Film-forming	Poly-1- <i>p</i> -menthene and di-1- <i>p</i> -menthene	Drought stress	Oil seed rape	Faralli et al., 2017	Reduced transpiration and stomatal conductance, enhanced water potential and yield
2017	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Oil seed rape	M. Faralli et al., 2017	Reduced CO ₂ assimilation and relative water content, increased intrinsic WUE
2018	Film-forming	Poly-1- <i>p</i> -menthene	Vase life extension	Roses	Di Stasio et al., 2018	Reduced transpiration, increased vase life
2018	Film-forming	Di-1- <i>p</i> -menthene	Drought stress	Grapes	Fahey and Rogiers, 2018	Reduced both leaf and bunch stomatal conductance, photosynthesis and transpiration, elevated leaf and berry temperature

Conflicts of Interest

The authors declare no conflicts of interest.

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3 Yield improvement by antitranspirant application in droughted wheat is associated with reduced endogenous abscisic acid concentration

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3.1 Abstract

Drought stress reduces seed crop yields in part by causing reproductive sterility associated with increased endogenous ABA. Application of the film antitranspirant, di-1-*p*-menthene, during reproductive-stage drought has been shown to ameliorate yield loss in wheat but the physiological processes involved are not understood. Using rain shelters to simulate Mediterranean-type terminal drought, we studied the possibility that application of the antitranspirant reduces both endogenous ABA concentration and the subsequent yield loss. Di-1-*p*-menthene was sprayed on droughted spring wheat plants (cv, Chilham) at 1.0 L/ha in two separate field experiments, at three growth stages between stem elongation and spike emergence in 2018; and at four growth stages between stem elongation and anthesis in 2019, with droughted but unsprayed plants serving as the control. Drought stress increased endogenous ABA but decreased yield. Di-1-*p*-menthene application reduced endogenous ABA concentration by 33 % and 40 %; and improved grain yield by 16 and 15 % averaged across all growth stages in 2018 and 2019, respectively. The consistent effects of di-1-*p*-menthene in both years suggests that the mechanism of drought stress amelioration by the film antitranspirant is related to reduced endogenous ABA concentration during key growth stages.

Keywords: water deficit; drought mitigation; abscisic acid; Vapor Gard

3.2 Introduction

Wheat (*Triticum aestivum* L.) is one of the three most important sources of calories in human diets and accounts for approximately 44 % of cereals traded annually across the globe (FAO, 2013, 2019), despite being widely cultivated in drought-prone areas (Sio-Se Mardeh et al., 2006), which account for 30 % of the global hectareage (Sheoran et al., 2015). In Mediterranean-type climates, wheat is principally grown under rainfed conditions characterised by low productivity due to frequent terminal droughts – prolonged soil moisture deficit inclusive of a crop's reproductive development stages (Tigkas and Tsakiris, 2015). Terminal drought stress is also a common feature of environments where crops are grown under stored soil moisture at the end of the rainy season, such as subtropical areas (Pang et al., 2017). Although the UK as a whole does not experience frequent droughts, almost a third of its wheat is produced in drought-prone regions where water deficit causes a 10 % loss in annual production (Dodd et al., 2011; Foulkes et al., 2007). Drought limits wheat productivity by hampering physiological and reproductive processes. Depending on severity, drought can accelerate senescence and progressively suppress carbon fixation due to low stomatal conductance (Christopher et al., 2016), leading to reduced grain development and reproductive organ abortion (Pang et al., 2017; Turc and Tardieu, 2018).

Drought stress induces an increase in the biosynthesis and concentration of endogenous abscisic acid (ABA) (Lee and Luan, 2012). Loss of cell turgor due to low water potential is correlated with an increase in the ABA concentration (Pierce and Raschke, 1980). That the increase in ABA concentration is related to a decrease in cell turgor is a position that has recently been challenged by Sack et al. (2018), who

argue that it is instead associated with a decline in relative water content or cell volume due to water loss. This argument is corroborative with Zhang and Davies (1990) who found low root water potential as a trigger for increased endogenous ABA. Once produced, ABA is transported through the vascular system acting as a drought signal, e.g. from the roots or leaves to the floral organs. Westgate et al. (1996) found that in wheat, increasing soil moisture deficit induced a much larger increase in endogenous ABA concentration in leaves than in floral organs, and that the turgor pressure in the leaves declined while it remained steady in the floral organs due to their higher water status. This suggests that in studying plant response to changes in soil water status, it is important to consider specific organs as they do not respond uniformly.

Stomatal closure, to reduce transpirational water loss, is modulated by ABA signalling as one of the immediate adaptive responses of plants to decreased soil water status (Buckley, 2019; Lee and Luan, 2012). Reduced transpiration can be simulated agronomically by applying antitranspirants as a drought amelioration technique. Antitranspirants are agronomic products that are applied on leaves to reduce transpiration and hence improve the plant water status (del Amor et al., 2010; Mphande et al., 2020).

Based on the mode of action, antitranspirants are classified into three types. The metabolic or stomata-closing type such as exogenous abscisic acid (ABA) reduce transpiration by physiologically inducing stomatal closure (AbdAllah et al., 2018). The reflective class (e.g. kaolin) enhance the light reflectance properties of leaf surfaces to minimise leaf temperature and consequently the transpiration rate (Glenn, 2012). The third group, called the film-forming antitranspirants (e.g. di-1-*p*-menthene), as

used here, reduce transpiration by physically blocking stomatal pores when a spray application has dried on the leaf surface (Palliotti et al., 2010).

A recent review by Mphande et al. (2020) shows that antitranspirants can improve yield under drought stress conditions in a number of crops. However, yield improvements were not observed in all cases, as at some growth stages the antitranspirants had negligible or no effect (e.g. Kettlewell et al., 2010). The mechanisms of drought amelioration by antitranspirants are not fully understood. Recent studies suggest that the timing of antitranspirant application in relation to reproductive development, especially of pollen development, could be important (Mphande et al., 2020). Pollen viability is an important aspect of reproductive development because it affects the grain number per spike, with drought stressed spikes having less grains than well-watered ones (Ji et al., 2011; Rajala et al. 2009). Ultimately, the number of grains per m² is also affected as grain number per spike is one of its components (Petr et al., 1988). Weerasinghe *et al.* (2016) attributed wheat grain yield improvement under water deficit stress, using di-1-*p*-menthene, to increased pollen viability by increasing plant water conservation. They found that plants not sprayed with the antitranspirant had significantly lower pollen viability. Nevertheless, the relationship was correlative and not a conclusive cause-and-effect one. Thus, the underlying mechanisms by which di-1-*p*-menthene and other antitranspirants improve reproductive development are yet to be established.

A possible explanation could be that the antitranspirant effect of reducing water loss also reduces ABA biosynthesis and thus its downstream effects on photosynthesis, reproductive development or other metabolic processes. It is known that abiotic stresses such as drought and high temperature are among factors that modulate spike development and growth and consequently affect yield (Frank et al., 1987; Gol

et al., 2017; Kondhare et al., 2015). Knowledge of reproductive development processes and their duration is necessary in understanding how they may be affected by abiotic stress. Pre-anthesis development of the apical meristem (Table 1) is divided into three major parts: vegetative, early reproductive and late reproductive phases (Gol et al., 2017). Although it is impossible to precisely assign secondary growth stage to every reproductive development event, due to variations in genotypic and environmental factors (Acevedo et al., 2002; Barber et al., 2015) and the fact that the shoot apex is concealed (Kirby and Appleyard, 1987), there is a general consensus on limits within which most are expected to occur (Table 1). While vegetative and early reproductive phases control the number of spikelet primordia that are set, the late reproductive phase, (which is vulnerable to abiotic stress particularly between beginning of stem elongation and meiosis), limits how many of those primordia will form viable florets; and hence impacts on yield (Gol et al., 2017).

Table 3.1: Summary of pre-anthesis principal wheat development phases and selected secondary growth stages.

Major development phase	Morphological and physiological change	Associated crop growth stage (GS)	Source
Late vegetative phase	Leaf primordia transitions to spikelet primordia	No precise growth stage	Tottman, 1987
Early reproductive phase	Spikelet primordia (at double ridge)	Just before *GS30	Tottman, 1987
Late reproductive phase – spike growth	Stem elongation	GS30 to GS39	Tottman, 1987; Zadoks et al., 1974
	Terminal spikelet – end of spikelet initiation	GS31 to GS32	Barber et al., 2015; Kirby and Appleyard, 1987
	Floret primordia	GS30 to GS37	(Mcmaster, 2009)
	Meiosis - anthers ~1 mm long and light yellow	GS37 to GS41	Kirby and Appleyard 1987; Tottman, 1987; Zadoks et al., 1974
	Booting	GS40 to GS49	Tottman, 1987
	Flag leaf sheath swollen	GS45	Zadoks et al., 1974
	Ear emergence or heading - spike pushed out of flag leaf sheath	GS50 to GS59	Zadoks et al., 1974
	50 % of ear emerged	GS55	Zadoks et al., 1974
	Anthesis	GS60 to GS69	Zadoks et al., 1974
	50 % of anthers shed	GS65	Zadoks et al., 1974

Film forming antitranspirants can protect crops from drought by delaying and reducing the stress experienced by the plants. Therefore, our present hypothesis is

that applying di-1-*p*-menthene on drought-stressed spring wheat plants reduces the endogenous ABA concentration and ameliorates the negative effects of drought on reproductive development and grain yield.

The hypothesis was tested in two separate experiments conducted in consecutive years (2018 and 2019). Di-1-*p*-menthene was applied at three growth stages in each year, all of which fall between stem elongation and anthesis, and its effects on the endogenous ABA concentration and the yield components of spring wheat were determined under drought conditions.

3.3 Materials and Methods

3.3.1 Experimental site description

Two field experiments were conducted in 2018 and 2019 in rain shelters at the Flatt Nook Field, Harper Adams University, UK (52°46'N, 2°25'W). The soil at the site is a loamy sand with good drainage (Beard, 1988). The field capacity (FC) was determined as 22 % (volumetric water content, VWC) and the permanent wilting point 8 % (Weerasinghe et al., 2016).

3.3.2 Experimental design

The experiment was arranged in a randomised complete block design with each of four erected rain shelters serving as a block – i.e. there were four replications in each year. Each block consisted of six progressively drying plots in 2018 and five in 2019 – with plants grown on stored soil moisture throughout the growth cycle to simulate Mediterranean-type climatic conditions. All treatments were grown under drought conditions and included control plots not sprayed with the antitranspirant (designated as *unsprayed*). The 2018 design was a 2x3 factorial with the factors

being the film-forming antitranspirant di-1-*p*-menthene (VG) and growth stage (GS). There was an equal number (three) of unsprayed and sprayed plots at each GS in each block, while in 2019 it was a single factor design. The spray description and growth stages are given under the section on antitranspirant application below. Two additional plots were also included in every block as a benchmark for assessing the extent of drought damage by comparison with droughted plots. The benchmark plots were irrigated using the drip irrigation system, twice for 30 minutes each time, on Mondays, Wednesdays and Fridays. This kept the volumetric water content in the upper 60 cm of the soil profile at an average of 20.02 %, which was 91 % of FC. Benchmark plots were not part of plot randomisation. In order to make irrigation easier and reduce the chance of water moving to the droughted plots, they were located on one side of the rain shelters and thus were not included in statistical analyses.

3.3.3 Planting and agronomic management

Fertiliser application: Based on soil nutrient analysis and the Nutrient Management Guide - RB209 – (AHDB, 2019), nitrogen was applied pre-planting at 100 kg N/ha (as ammonium nitrate) by broadcasting. To incorporate the fertiliser, land was prepared by ploughing using a tractor-drawn plough immediately afterwards. As the soil nutrient analysis showed that the supply of P and Mg were adequate for arable crops, these were not added to the soil. However, as drought limits uptake of nutrients from the soil (Nawaz et al., 2012), nitrogen deficiency symptoms, manifest at GS32, were corrected by applying an inorganic multi-nutrient fertiliser solution (3X Solution, Omex Agriculture Ltd, Norfolk, PE30 2HH, UK) as a foliar spray at a rate of 5.0 L/ha.

Planting: Spring wheat (cv, Chilham) was hand-planted on 24th April in 2018 and on 31st March in 2019, at a rate of 370 seeds per m² by drilling at a depth of approximately 2 cm. Seedling emergence was at five days after planting (DAP) in 2018 but it took 11 days in 2019 due to cooler weather.

Weed control: Weeding was done manually in 2018 but in 2019, the selective herbicide, Zypar (active substances: 6 g/L arylex + 5 g/L florasulam) was applied at GS23 at a rate of 0.75 L/ha.

3.3.4 Antitranspirant application

The film antitranspirant used in the present study was Vapor Gard (96 % di-1-*p*-menthene, Miller Chemical and Fertilizer Corp., Hanover, PA). The aim was to select growth stages within early and late reproductive phases, particularly from those falling between stem elongation and early booting (Table 1), which are said to be most vulnerable to abiotic stress, such as drought (Gol et al., 2017). The antitranspirant was applied at three growth stages described by Tottman (1987) as GS33, GS45 and GS51 in 2018. The growth stages in 2019 were similar but not all identical with the antitranspirant being sprayed at GS31, GS37, GS51 and GS65. The dates on which the antitranspirant was applied are listed below (Table 2), alongside ABA sampling dates. The antitranspirant was sprayed onto plants using a hand-held boom sprayer (Lunch Box Sprayer, Trials Equipment (UK) Ltd, Essex CM7 4EH) at 1.0 L/ha, as used by Faralli et al. (2019) and control plants were not treated with the antitranspirant.

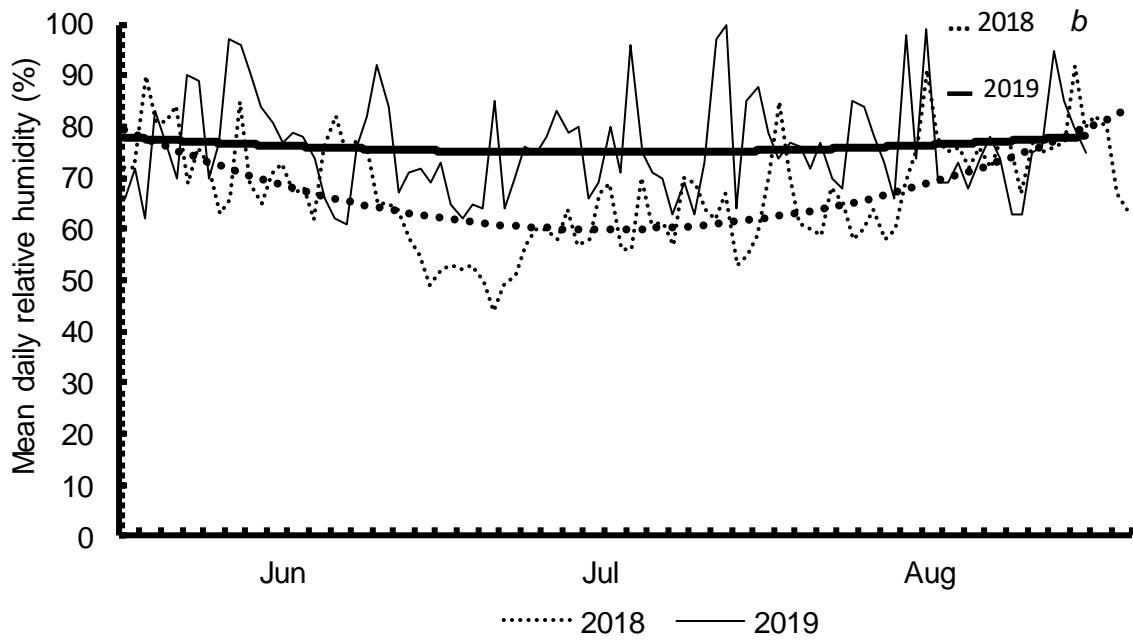
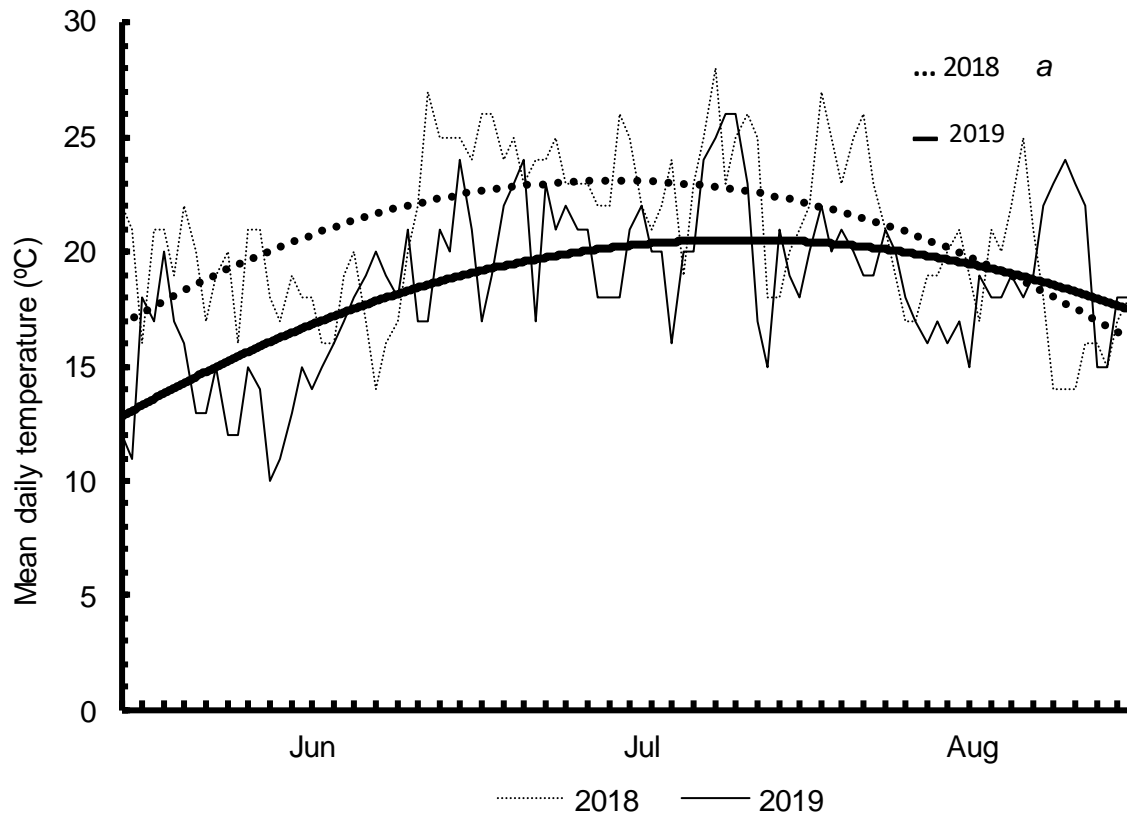
Table 3.2: Vapor Gard application and ABA sampling schedule.

Year	Timing of VG sprays	Spraying at		Leaf sampling* at days			Spike sampling at days		
		days after planting	Date	after planting and days after spraying	after planting and days after spraying	after planting and days after spraying	DAP	Date	DAS
	Growth stage	DAP	Date	DAP	Date	DAS	DAP	Date	DAS
2018	GS33	43	6 th June	52	15 th June	9	N/A		
				69	2 nd July	26			
	GS45	45	8 th June	54	17 th June	9	N/A		
				71	4 th July	26			
	GS51	48	11 th June	57	20 th June	9	N/A		
				74	7 th July	26			
2019	GS31	51	21 st May	60	30 th May	9	75	14 th June	24
	GS37	58	28 th May	67	6 th June	9	75	14 th June	17
	GS51	72	11 th June	81	20 th June	9	75	14 th June	3
	GS65	81	20 th June	N/A		N/A	N/A		

* Droughted-unsprayed control and benchmark plots were sampled on the same dates as droughted-sprayed plots, with the exception of spikes for the benchmark plots whose sampling was 10 days later than the rest due to a lag in development to the required growth stage. DAP, DAS and VG stand for days after planting, days after spraying and Vapor Gard, respectively.

3.3.5 Measurements

Meteorological factors: The temperature and relative humidity readings in the rain shelters were monitored using *Tinytag View 2* (Gemini Data Loggers UK Ltd, Chichester, England) and *Omega OM-24* (Omega Engineering, Inc, USA) data loggers. Solar radiation data were obtained from the meteorological station based at Harper Adams University located within a one-kilometre distance from the research site. The mean daily temperature observed inside the rain shelters during the growing seasons was significantly higher ($p < 0.001$) in 2018 than in 2019 (Figure 3.1, a). The seasonal averages were 21 and 19 °C, for 2018 and 2019, respectively. Between June and August in 2018, there were heat waves in the United Kingdom with the maximum temperature being 33.0°C (McCarthy et al., 2019). Inside the rain shelters, temperatures were even higher, with the highest values on some days ranging from 37.1 to 40.4 °C. June 30th was notably hot and abrupt yellowing of uppermost parts of flag leaves in the benchmark plots indicated that the heat stress was severe. There was no visible evidence of heat-induced necrosis in droughted plants, as they were far ahead in development and already senescing. For relative humidity (RH, %), 2019 had significantly higher ($p < 0.001$) mean daily values (Figure 3.1, b). Seasonal RH averages were 67 and 76 % for 2018 and 2019, respectively. The solar radiation received in 2018 was significantly higher ($p < 0.001$, month x year interaction) than in 2019 during the same period (Figure 3.1, c). Average solar radiation received during the growing seasons were 20.15 and 14.61 MJ m⁻² day⁻¹ in 2018 and 2019, respectively.



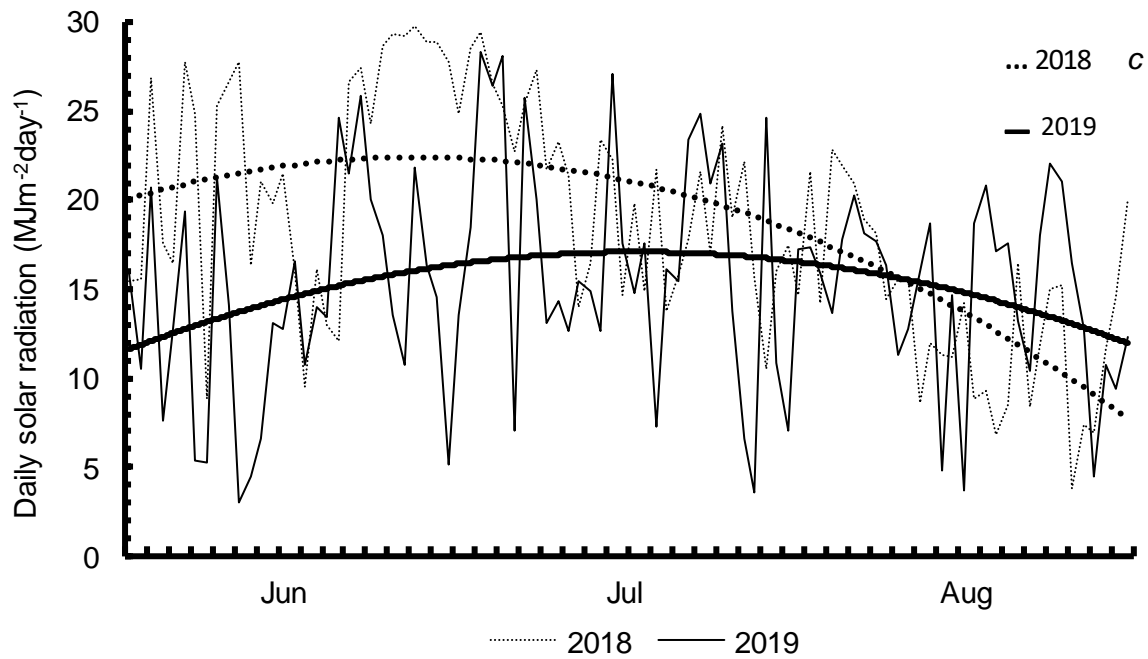


Figure 3.1: (a) Mean daily air temperature and (b) mean daily relative humidity inside the rain shelters; and daily solar radiation (c) recorded by the meteorological station based at Harper Adams University between 1st June and 31st August. Each of these variables was significantly different ($p < 0.001$) between 2018 and 2019. The curves were fitted with quadratic regression.

Soil water content: Soil moisture measurements in the top 60 cm were taken approximately once per week using the using a time domain reflectometry (TDR) probe, (TRIME-TDR, IMKO Micromodultechnik GmbH, Ettlingen, Germany). Readings were recorded in volumetric water content terms (%) at three depths (0 - 20 cm, 20 - 40 cm and 40 - 60 cm).

Determination of endogenous ABA concentrations: For ABA assay, sampling of the uppermost, fully expanded leaves from seven plants per plot was done at nine and 26 days after spraying (DAS) in 2018; and at nine DAS in 2019, while spikes were all sampled at GS55 (2019 only). Each sample was put in a 5.0 mL vial and immediately flash frozen in liquid nitrogen and stored at -80°C . The frozen leaf and

spike samples were freeze dried for two days and further processed and assayed in accordance with the Cusabio ABA ELISA protocol, code CSB-E09159PI (Cusabio Biotechnology Co., Ltd, Wuhan, Hubei Province 430206, China <http://www.cusabio.com>). The ABA standard used in the assay was a mixture of isomeric forms ((+/-) cis-Abscisic acid). Absorbance readings were taken at 450 nm using a BioTek spectrophotometer (BioTek Instruments Ltd, Bedfordshire, SG19, 2NR, UK). The endogenous concentrations of ABA in the sample extracts were calculated after fitting a standard curve.

Yield and yield components: Determination of fertile spike density, i.e. the number of spikes with one or more grains per unit area (m^2), was done at GS89 by counting grain-bearing spikes in three random samples per plot. This is distinguished from the total spike density which may include both grain and non-grain bearing spikes (Fernandez et al., 1997) and can be determined before grains develop in the spikes as well as at maturity (Moeller and Rebetzke, 2017) or at harvest (Fernandez et al., 1997). Sampling was done using a 33 x 33 cm quadrat. At harvest, spikes from each plot were hand-harvested and collected in separate bags, threshed, weighed and oven dried at 105 °C for 40 h. Sub-samples of 50 main stem spikes per plot were kept separately for the determination of the number of grains per spike before being added back to the rest of the grain (for yield determination). All samples were threshed using the electrically powered threshing machine (F. Walter & H. Wintersteiger KG, Austria). Chaff that was not blown off by the thresher was removed by hand to obtain clean grain. TGW was determined by the method described by (Sylvester-Bradley et al., 1985) after weighing 40g of previously oven-dried grain. Counting was done using the FarmTec CountAmatic grain counter (Farm-Tec, Whitby, North Yorkshire, UK). The number of grains obtained from this

weight was also used in the calculation of number of grains per m² (Moeller and Rebetzke, 2017). Finally, grain yield per plot was obtained by bulking and weighing all the grain harvested from each plot and converting it to tonnes per hectare at 15 % moisture content.

Statistical analysis: Analyses of variance (ANOVA) of meteorological factors, endogenous ABA concentration and yield-related parameters were performed using GenStat 19th Edition (Hemel Hempstead, UK) to estimate the significance of main effects. The analysis was based on main factors, namely di-1-*p*-menthene (at two levels, sprayed or unsprayed) and the growth stage at which the antitranspirant was applied; and their interactions in 2018 and di-1-*p*-menthene as the single factor in 2019. *Post hoc* analyses were performed using the Tukey test (at 5 %). Relationships between variables were analysed using simple linear and polynomial regression. Curve fitting and modelling was performed using Excel, with constants in the fitted models derived from GenStat. The irrigated benchmark plots were not part of the randomisation and therefore could not be included in statistical analysis.

3.4 Results

3.4.1 Soil moisture dynamics

The volumetric water content (VWC) in the top 60 cm significantly decreased with increasing DAP (Figure 3.2). In 2018, before and after spraying of di-1-*p*-menthene and taking of leaf samples, the VWC decreased from an average of 13.52 % at 30 DAP to 8.42 % at 79 DAP. A similar trend was observed in 2019, with the VWC decreasing from an average of 14.65 % at 36 DAP to 8.27 % at 81 DAP. The corresponding soil moisture deficit (SMD) increased from 50.88 mm to 81.48 mm in

2018 and from 44.10 mm to 82.38 mm in 2019. Regression analysis of change in VWC with DAP showed that treatment groups sprayed with the antitranspirant did not significantly deviate from the unsprayed control set as a reference. Soil moisture readings in the benchmark plots was maintained at an average of 20.02 %.

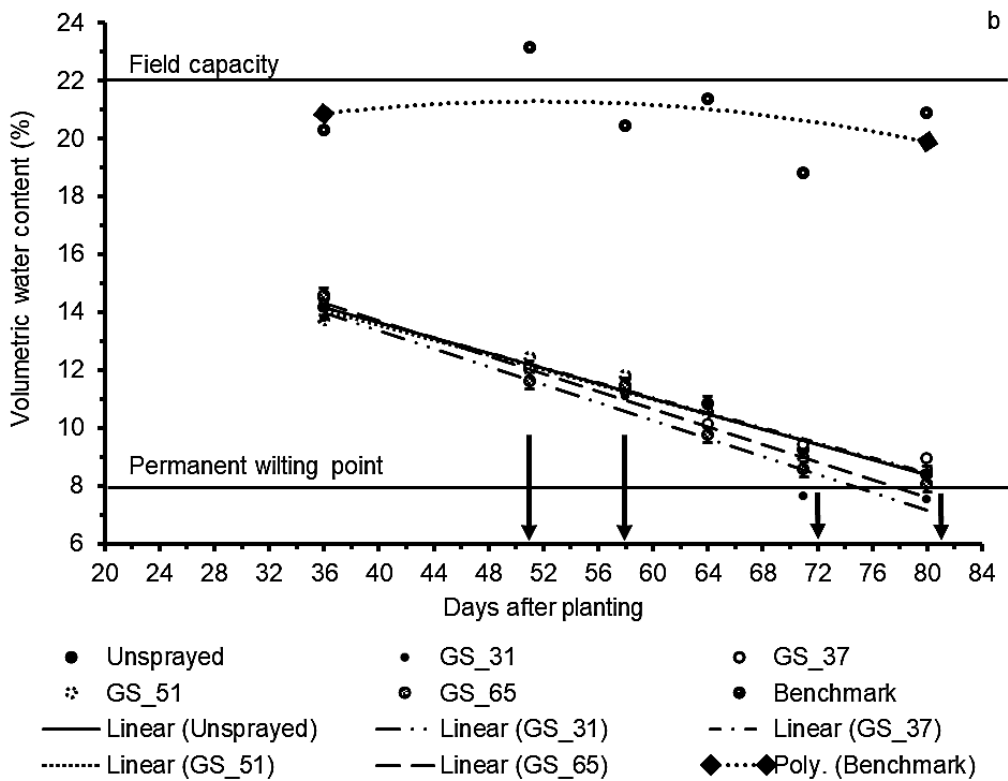
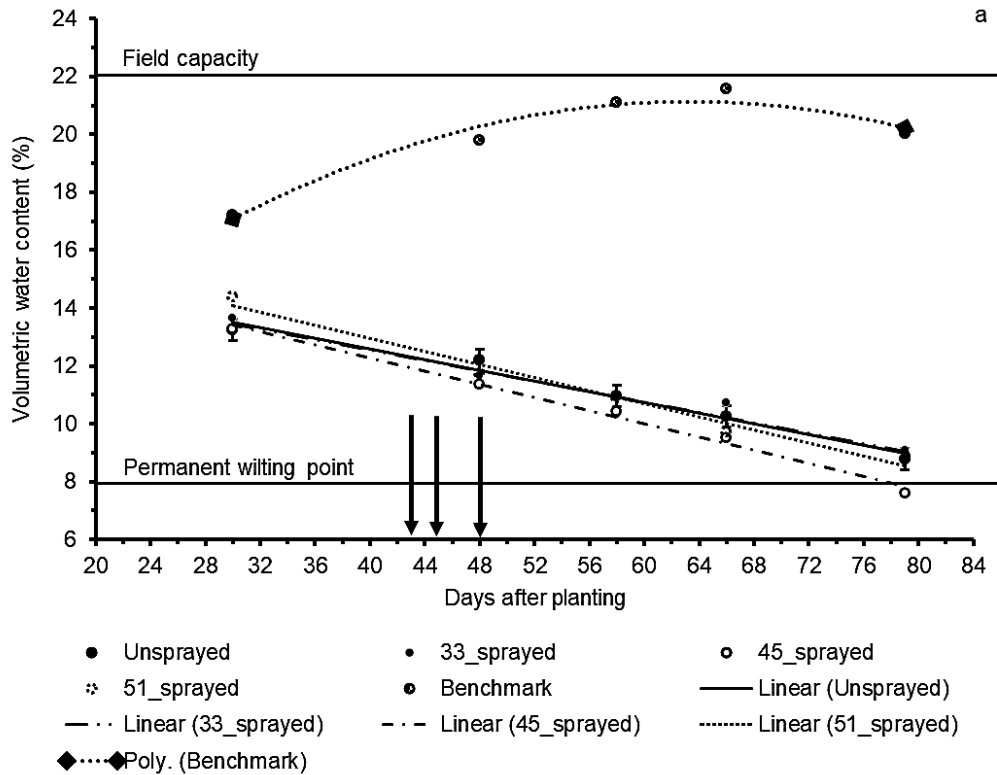


Figure 3.2: Soil moisture dynamics between 30 and 79 and from 36 to 81 days after planting (DAP) showing significant decrease in (a) 2018 $p < 0.001$) and (b, $p = 0.986$, $SER = 0.271$) 2019, ($p < 0.001$). Deviations of slopes from the unsprayed reference were non-significant (GS33: $p = 0.817$, GS45: $p = 0.522$, GS51: $p = 0.161$; GS31: p

=0.608, GS37: $p = 0.914$, GS51: $p = 0.822$, GS65: $p = 0.446$) in either year. Error bars, only on the Water treatment due to space limitation, represent standard error of regression, (a, $p = 0.873$, SER = 0.369) and (b, $p = 0.986$, SER = 0.271). Each point represents a treatment average of three or four measurements. Arrows indicate DAP on which the antitranspirant was sprayed in the appropriate plots.

3.4.2 Effects of di-1-*p*-menthene on ABA concentration

Relative to the irrigated benchmark plots, drought increased the endogenous foliar ABA concentrations (ng/g DW) by a maximum of 113 % and 153 % in 2018 and 2019, respectively, while in the spikes it rose by 137 % (determined in 2019 only) (although benchmark plots could not be included in statistical comparisons). In droughted plants, the endogenous ABA concentrations in leaves were higher in 2018 than in 2019. The analysis of variance showed that spraying di-1-*p*-menthene on droughted plants reduced the foliar ABA concentration at nine DAS, and both main effects (di-1-*p*-menthene and growth stage treatments) were significant (p -value of < 0.001 in both years). The effects of di-1-*p*-menthene moving across the different growth stage treatments modified the levels of ABA accumulation, with GS33 and GS45 treatments (in 2018) and GS31 and GS37 treatments (in 2019) having significantly lower concentrations than their unsprayed counterparts ($p = 0.033$, and $p = 0.048$, respectively, Figure 3.3). Relative to the benchmark, the respective foliar concentrations in the di-1-*p*-menthene sprayed plants at stem elongation treatments were 32 % (GS33) and 40 % (GS31) higher in 2018 and 2019, respectively. This was much lower than in the unsprayed treatments stated above. In both years, the maximum concentrations in unsprayed plants were in samples collected nine days

after the early stem elongation (GS31, GS33) and declined at later development phases.

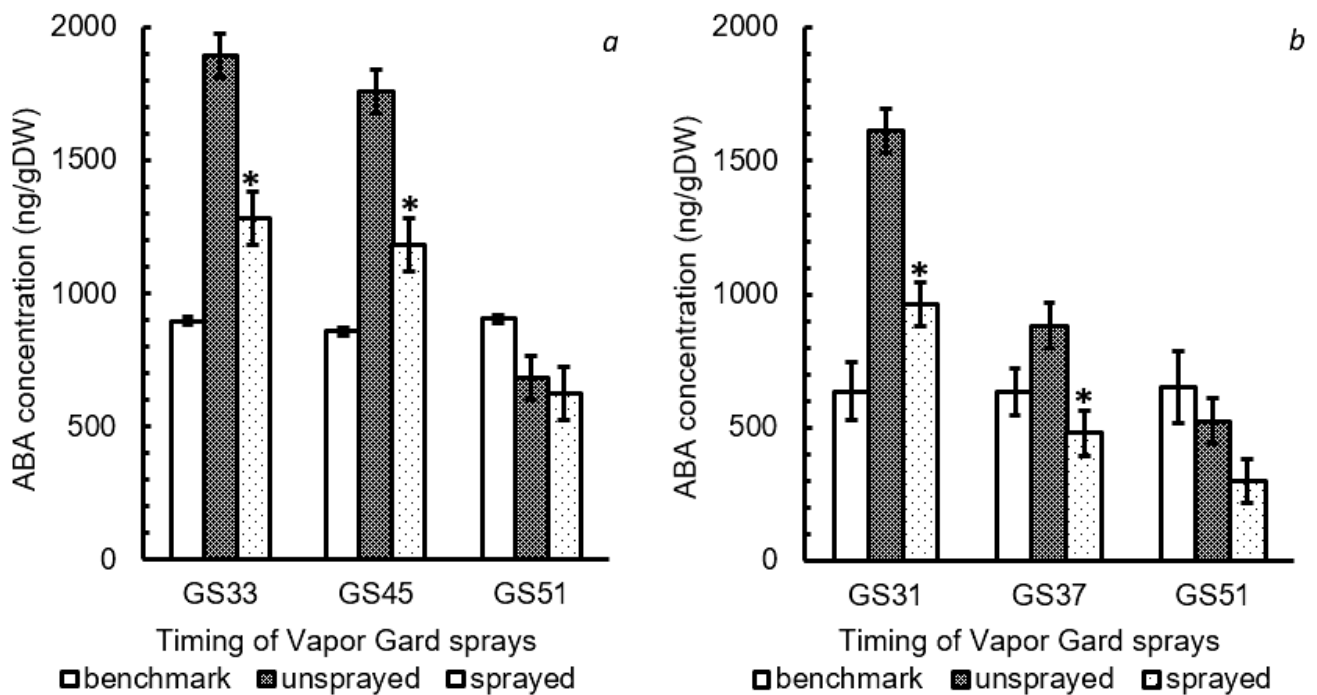


Figure 3.3: Average ABA concentrations (ng/g DW) in flag leaves sampled at nine days after spraying di-1-*p*-menthene, at three growth stage treatments in droughted plots, showing consistent significant interactions from (a) 2018 ($p = 0.033$) and (b) 2019 ($p = 0.048$). Asterisks represent sprayed treatments with significantly lower ABA concentrations than their unsprayed counterparts. The interaction means that moving from GS31 to GS51, the response of endogenous ABA concentration to di-1-*p*-menthene decreased with delayed application, becoming marginal at later applications (at GS51) i.e. as the plants advanced in development. Irrigated benchmark plots are shown for visual comparison only, as benchmark plots were not part of randomisation and are not included in statistical analysis.

Sampling at 26 days after spraying in 2018 showed no significant differences between sprayed and unsprayed plants, but growth stage treatments affected the ABA concentration with GS45 and GS51 having significantly higher concentrations than GS31 ($p < 0.001$, Figure 3.4).

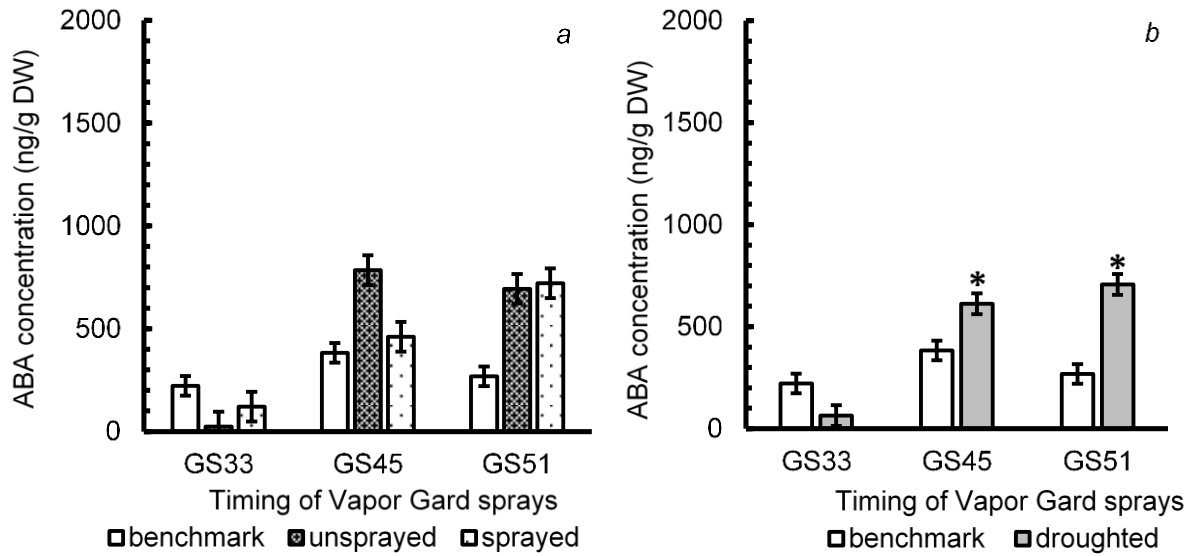


Figure 3.4: Average ABA concentrations (ng/g DW) in flag leaves sampled at 26 days after spraying di-1-*p*-menthene (2018 only) at three growth stage treatments for (a) sprayed and unsprayed droughted plots compared and (b) sprayed and unsprayed droughted plots combined. Irrigated benchmark plots are shown for visual comparison only, as benchmark plots were not part of randomisation and could not be included in statistical analysis. Post hoc testing shows (a) borderline significant interactions ($p = 0.056$) and (b) significant differences between growth stages (*) for the combined data. The interaction effect shows that between growth stages the largest impact of di-1-*p*-menthene on endogenous ABA concentration 26 days after spraying was with application at GS45.

For spike ABA accumulation, spraying di-1-*p*-menthene significantly ($p < 0.001$) reduced the concentration almost by half with application at GS31 (Figure 3.5). The effect diminished with increasing DAP, i.e. plants that were sprayed at an earlier growth stage had lower spike ABA concentration than those in the succeeding one. This was similar to leaf ABA where early application also resulted in the largest reduction in the endogenous ABA concentration.

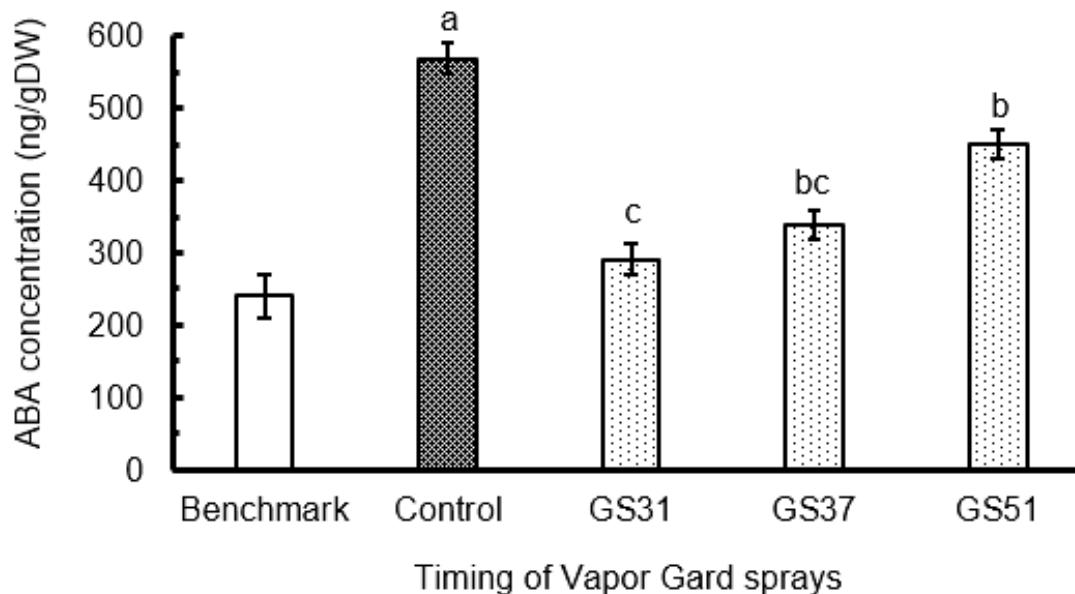


Figure 3.5: Average ABA concentrations (ng/g DW) in spikes of droughted plants not sprayed (control) and sprayed with di-1-*p*-menthene at three growth stages (GS) in 2019 showing a significant effect ($p < 0.001$, different letters indicate significant differences). All samples were taken at once at GS55, when it was 24, 17 and 3 days after spraying at GS31, GS37 and GS51, respectively. Irrigated benchmark plots are shown for visual comparison only, as benchmark plots were not part of randomisation and could not be included in statistical analysis. Benchmark spikes reached GS55 10 days later than the droughted plants.

3.4.3 Yield and yield components

The responses of yield and yield components to drought and the application of di-1-*p*-menthene at three growth stages in each of the two years are summarised in Table 3. Drought negatively affected most of the yield components in both years. Fertile spike density was reduced by 45 % and 53 % in 2018 and 2019, respectively, compared to the benchmark, although non-randomisation of benchmark plots prevented a statistical test of these effects. Spraying di-1-*p*-menthene improved the fertile spike density by 13 % and 12 % across all growth stage treatments in 2018

and 2019, respectively. Drought induced a loss in the TGW in 2018 and number of grains per spike in both years. The decline in the number of grains per spike, was much more pronounced in 2019, with a 33 % reduction compared to 9 % in 2018. Although, di-1-*p*-menthene did not have a significant effect on number of grains per spike or TGW in either year, on average, the antitranspirant improved these traits.

Drought stress reduced the number of grains per m² by 49 % and 59 % in 2018 and 2019, respectively. Application of di-1-*p*-menthene minimised the loss in grain number per m² by an overall average of 13 % in each of the years. The extent to which di-1-*p*-menthene mitigated the reduction in grain number differed with the time of application. In 2019, application at GS37 was the most effective and resulted in a significantly higher number of grains per m² than the unsprayed plots by 25 %. In 2018, although there were no significant differences in the effectiveness of di-1-*p*-menthene applied at different growth stages (Table 3), the highest effect, a 15 % increase above the unsprayed treatment, was obtained with application at GS45. The grain number per m² in 2018 was much higher than for 2019. Similarly, the 2018 grain yield averages per hectare were higher than for 2019, however, relative to the benchmark, drought caused similar losses of 54 and 52 %, respectively.

Table 3.3: Summary of yield and yield components as affected by the application of di-1-*p*-menthene at different growth stages in 2018 and 2019 showing mean values and levels of significance.

Year	Treatments	Fertile spike density per m ²	Grains per spike	Grains per m ²	TGW (at 15 % moisture)	Yield (tonnes per ha) at 15 % moisture
2018	Benchmark	538.4	39.9	21464	44.9	9.66
	GS33 sprayed	333.3a	36.8a	12303a	41.4a	5.08a
	GS33 unsprayed	284b	35.9a	10353b	39.5a	4.11b
	GS45 sprayed	353a	35.6a	12725a	40.6a	5.18a
	GS45 unsprayed	322.9b	37.6a	12080b	41.0a	4.91b
	GS51 sprayed	324.8a	40.8a	12558a	41.4a	5.24a
	GS51 unsprayed	288.3b	35.3a	10707b	41.4a	4.38b
	Significance	*		*		*
2019	Benchmark	500.5	51.6	18684.3	42.0	7.72
	Unsprayed	235.7b	34.8a	7630.4c	49.2a	3.71b
	GS31	283.5a	36.6a	9432.7ab	51.0a	4.81a
	GS37	291.8a	35.3a	9542.0a	50.0a	4.78a
	GS51	243.7b	37.4a	8252.4bc	49.2a	3.94b
	GS65	234.8b	34.2a	7346.3c	49.8a	3.70b
	Significance	**		***		**

Different letters indicate significant differences. *, **, *** represent significance at less than 0.05, 0.01 and 0.001, respectively. In 2018, the significance was attributed to di-1-*p*-menthene – the interaction was not significant. Note that benchmark plots are shown for the purpose of comparison only, as they were not part of randomisation and are not included in statistical analysis.

Timing of antitranspirant application (i.e. growth stage) was not significant in 2018, although di-1-*p*-menthene significantly improved the overall average yield by 16 %

compared to the droughted-unsprayed plots. In contrast, timing of the application of the antitranspirant was significant in 2019, with application at GS31 and GS37, ameliorating drought by an average of 29 % (corresponding to 1.09 tonnes of extra grain per hectare). The overall yield improvement average attributed to the antitranspirant for 2019 was 15 %. Yield was strongly associated with grain number per m² and fertile spike density in a positive way and the association with ABA was negative.

Simple linear regression of yield against yield components was best described by grains per m² and fertile spike density which explained 96 % and a maximum of 98 % of variance, respectively (Figure 3.6). Furthermore, the relationship between yield and timing of di-1-*p*-menthene application was best described by the quadratic expression ($R^2 = 0.63$, $p < 0.001$): $Y = 0.079X - 0.001X^2 + 2.69$, where 'Y' is yield in tonnes per hectare and X is the growth stage, in days after planting, at which the antitranspirant was applied. A simple linear regression did not fit the data ($R^2 = 0.007$, $p = 0.304$) as di-1-*p*-menthene was effective in increasing yield only at early growth stage treatments.

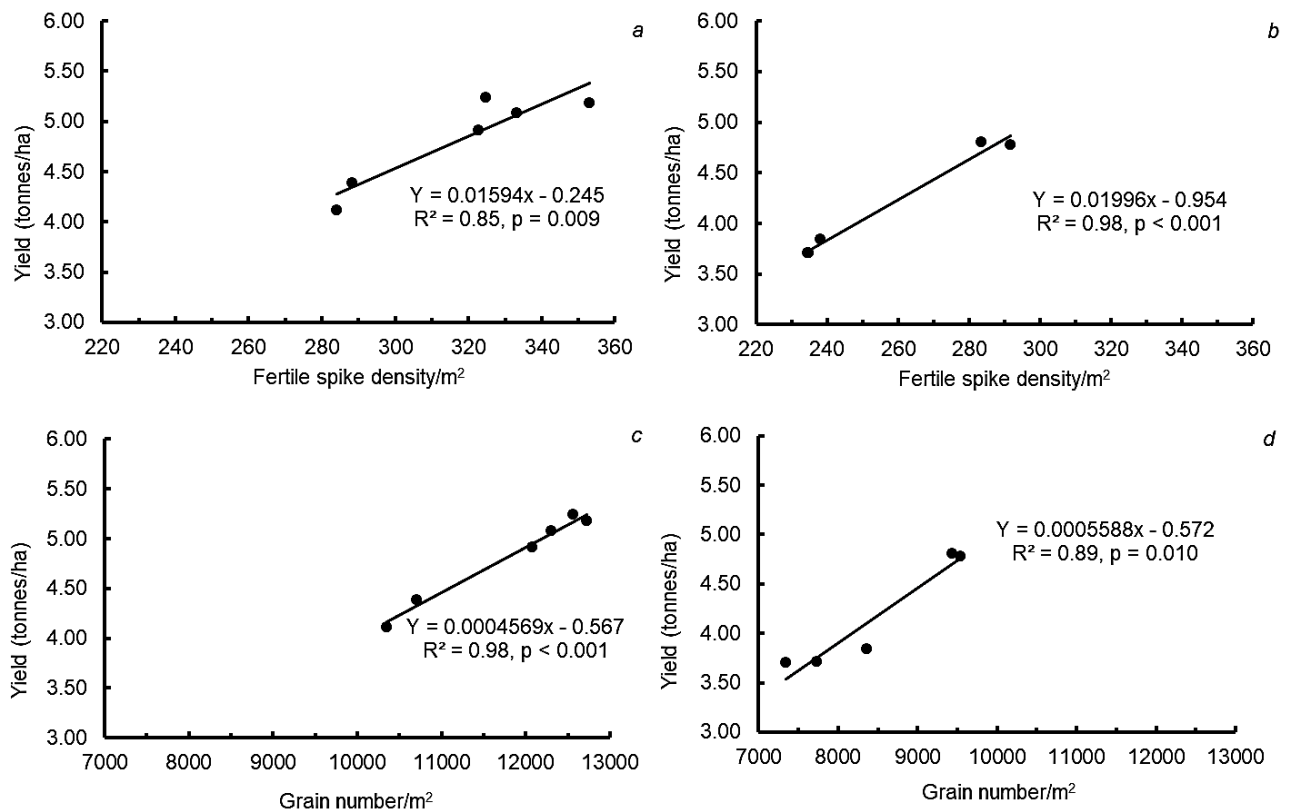
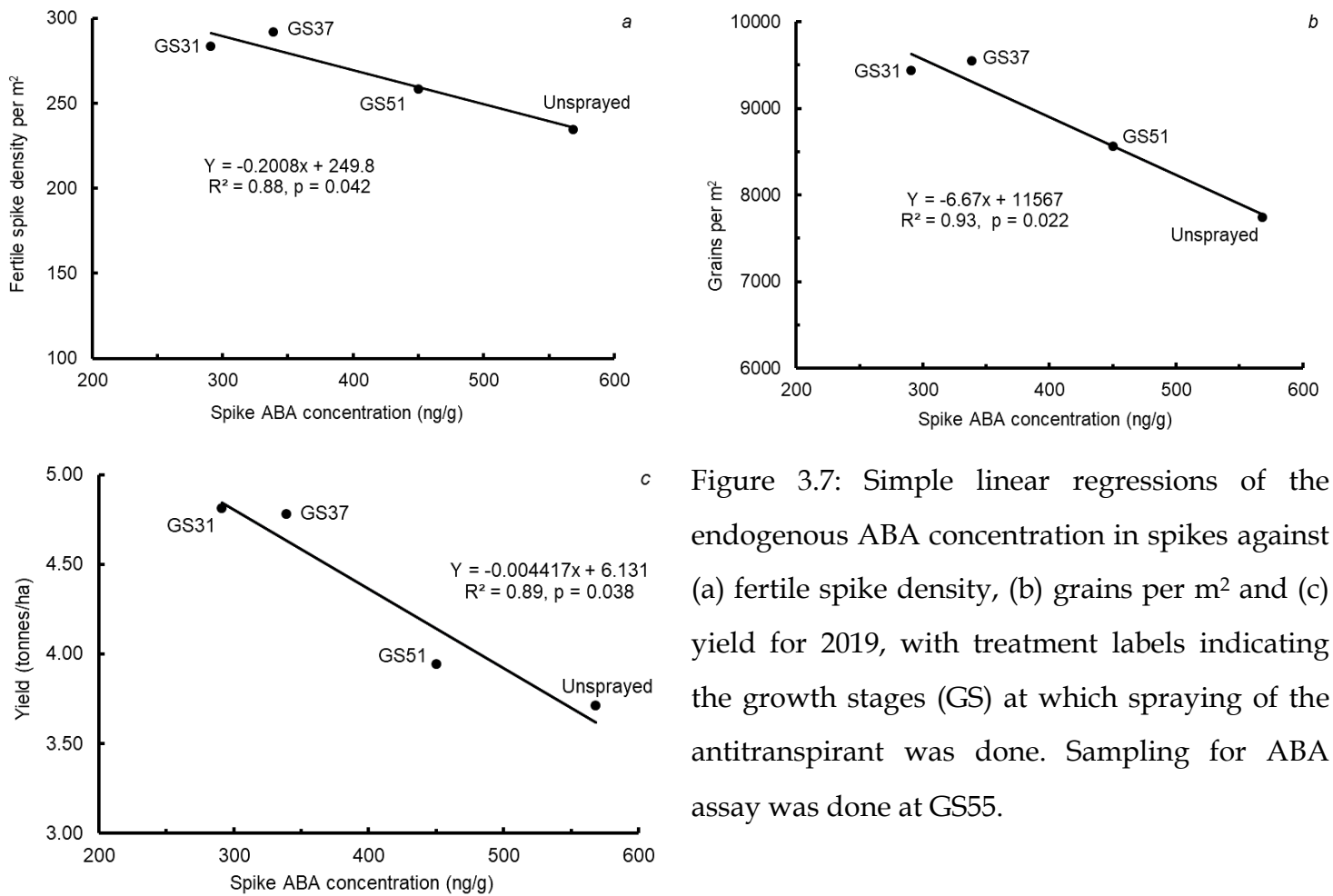


Figure 3.6: Simple linear regressions of mean values between yield (Y), in tonnes per hectare, and (a, b) number of fertile spikes per m² (x) and (c, d) number of grains per m² (x) for 2018 and 2019, respectively. Each point represents (a, b) three and (c, d) single measurements per plot in used in the regression analysis to construct the curves.

3.4.4 Endogenous ABA relationships with yield and yield components

Figure 3.7 shows regression analysis of the significantly altered yield components against endogenous ABA concentration in spikes at GS55. All three yield components, fertile spike density, grains per m² and grain yield, showed a significant negative association with spike ABA. The reductions in spike ABA were driven by the antitranspirants and varied depending on the GS at which it was applied. As the effect of di-1-*p*-menthene on reducing the endogenous ABA concentration increased,

the yield components responded positively with spraying at earlier GS being most effective (Figure 3.7).



c Figure 3.7: Simple linear regressions of the endogenous ABA concentration in spikes against (a) fertile spike density, (b) grains per m² and (c) yield for 2019, with treatment labels indicating the growth stages (GS) at which spraying of the antitranspirant was done. Sampling for ABA assay was done at GS55.

3.5 Discussion

Previous studies have shown that antitranspirants can improve the yield of droughted wheat if applied at the correct growth stage (e.g. Kettlewell et al., 2010). This is supported by our results which also show, for the first time, that this improvement is associated with reduced endogenous ABA concentration during reproductive development. The percentage yield increase in each of the experiments in our study are similar to findings in previous work. Furthermore, we have shown

that to enhance yield, di-1-*p*-menthene significantly improves two yield components - the fertile spike density and the number of grains per m².

Compared to the benchmark and other studies, the SMD and yield data in this study show that the level of water stress was adequate to represent terminal drought. Readings just before the end of grain filling were at 46 % and 38 % of FC in 2018 and 2019, respectively. This was more severe than in a previous study on the same species by Valluru et al. (2016) who maintained soil moisture at 70 % of FC. In a study of barley, mild drought at 50 % of FC during grain filling caused yield loss of 56 % on average (Samarah et al., 2009). The yield losses obtained in this study were similar, being 54 % and 52 % in 2018 and 2019, respectively, and higher than 11 out of the 15 studies reviewed by Farooq et al. (2014).

The water deficit increased foliar and spike ABA concentration by between two and threefold compared to the benchmark, with consistently higher concentrations in leaves at earlier development stages. Work on an ABA-deficient mutant of barley (Az34) demonstrated that ABA homeostasis is necessary for maintaining shoot development (Mulholland et al., 1996; Walker-Simmons et al., 1989), hence the higher accumulation in younger tissues. According to Yoshida et al. (2019), there is a basal ABA concentration that cells maintain even under well-watered conditions, which is necessary for promoting plant growth instead of reducing it. This implies that beyond the basal concentration, ABA begins to negatively affect normal growth and development processes. Our results are comparable to findings by Valluru et al. (2016) in a study involving six spring wheat genotypes contrasting in drought tolerance profiles. Maintaining soil moisture at 70 % of FC, Valluru et al. (2016) found the foliar ABA concentration in three tolerant lines increased by 95 % against 129 % in sensitive ones, with maintenance and loss of shoot dry weight,

respectively. Although this is the first known study on the effects of di-1-*p*-menthene on endogenous ABA concentration in a cereal, the findings are consistent with observations in oil seed rape. In oil seed rape, Faralli et al. (2016) observed that di-1-*p*-menthene reduced endogenous ABA concentration in leaves of droughted oil seed rape plants and improved seed dry weight compared to unsprayed plants.

Elevated ABA during the early reproductive phase seems to impose a sink restriction, by reducing the potential grain number per m² and so the final yield. Regression analysis showed that endogenous ABA was negatively associated with fertile spike density, and grains per m² as well as grain yield (Figure 3.7). Spike ABA was better associated with these variables than leaf ABA (results not shown).

The reductions in spike ABA were driven by the antitranspirant, with earlier application being more effective in controlling spike ABA and limiting the yield loss under drought.

Drought stress is known to induce an increase in endogenous ABA, which inhibits growth as an adaptive strategy (Ng et al., 2014), although a basal concentration of ABA is necessary for leaf expansion, as work on the Az34 (ABA-deficient) barley mutant genotypes has shown (Mulholland et al., 1996; Walker-Simmons et al., 1989). ABA can reduce fertile spike density through a signalling effect, whereby a high endogenous concentration induces sterility. That water stress induction of sterility in wheat spikes might be mediated through ABA signalling was early advanced by Morgan (1980) through both water deprivation and exogenous ABA treatments at late stem elongation (early booting). A strong association has also been found between rising endogenous ABA levels and reproductive organ failure in other species (e.g. soya beans, Liu et al., 2004; chickpeas, Pang et al., 2017; and

maize, Wang et al., 2019), suggesting a role of ABA in regulating yield-impacting physiological processes. The coincidence of accelerated stem elongation and spike growth increases the competition for resources, particularly carbohydrates (Ghiglione et al., 2008; Sreenivasulu and Schnurbusch 2012). Occurrence of drought during these processes, with its concomitant upregulation of ABA (La et al., 2019), enhances the abortion of florets (Ghiglione et al., 2008; Pang et al., 2017). Few studies have characterised ABA effects on early reproductive development, although there are studies on drought and other abiotic stresses. Quarrie and Jones (1977) mimicked the effects of drought by injecting ABA into spring wheat plants during the vegetative phase while concurrently imposing water deficit in another treatment. Compared to the control, both treatments induced significant reductions in the number of spikelets per spike, with drought inducing more severe effects. In our study, applying di-1-*p*-menthene reduced the endogenous ABA concentration and mitigated some of the drought effects on yield. Saini and Aspinall (1982) showed that the effects of exogenous ABA on well-watered plants (inhibition of stem elongation, spike abortion or reduced grain set) correlated with water stress-induced ABA increase. Similarly, Boussora et al. (2019) studied the role of salinity-induced ABA increase on spikelet primordia development in five barley genotypes. They found that an elevated ABA concentration was associated with a decrease in the duration of spikelet development and an increase in spikelet and floret abortion in the developing spikes (number of tillers per plant was also reduced in all genotypes). In the present study, the effects of drought on fertile spike density and grain number per m², indicate that the droughted plants were impacted by a sink limitation, and in agreement with past studies, that this was due in part to an increase in endogenous ABA concentration during spike growth. Treatments in which the endogenous ABA

concentration in both leaves and spikes was reduced due to di-1-*p*-menthene application (Figures 3.3 and 3.5) also showed improvement in not only fertile spike density but also grain number per m² and yield per hectare yield (Table 3) compared to the unsprayed plants. In our study, the lower fertile spike density values for the unsprayed compared to the sprayed plants can be explained by their higher endogenous ABA concentration accumulated due to water stress. Elevated endogenous ABA concentration induces sterility of florets or entire reproductive organs or flowering heads (Pang et al., 2017; Wang et al., 2019) such as spikes in wheat (Morgan, 1980). ABA will therefore reduce the number of grain-bearing spikes per hectare, and in turn the grain number per hectare. Number of grains per m² is controlled by fertile floret and spike density per m² (Prieto et al., 2018). There is a linear relationship between not only spike fertility but also grain number per m² and yield (Bulman and Hunt, 1988; Griffiths et al., 2015). Our findings agree with past studies as both the fertile spike density and grains per m² were strongly associated with grain yield per hectare (Figure 3.7).

The timing of drought stress and of ABA accumulation is important in determining the impact on grain yield. In the present study, drought stress reduced fertile spike density by 45 % (2018) and 53 % (2019) relative to the benchmark. Application of di-1-*p*-menthene at GS37, corresponding to late stem elongation, curtailed the reduction by 13 % (2018) and 12 % (2019). This key growth stage falls within the early portion of the late reproductive phase (Table 1), including growth of the spike and formation of the floret primordia and the anthers. In shoot apical meristem morphogenesis, the early reproductive phase includes the initiation of the spikelet primordia, which signals the end of vegetative development. The late reproductive

stage, between stem elongation and anthesis, includes spike growth and differentiation of floret primordia into florets (Gol et al., 2017; Sreenivasulu and Schnurbusch, 2012). Longstanding evidence indicates that water deficit stress at stem elongation reduces wheat yield by depressing the number of spikes per m² (Day and Intalap, 1970). In fact, water stress anytime between late vegetative phase and late stem elongation reduces grain yield by negatively affecting spikelet primordia initiation, floret formation and spike growth (Gol et al., 2017; Oosterhuis and Cartwright, 1983). This is also true in other cereals, e.g. drought stress at panicle initiation reduced number of panicles and grains per hill in rice (Wopereis et al., 1996). In pearl millet, water deficit during vegetative development and at flowering inhibited or delayed flowering in tillers, reducing number of productive panicles (Winkel et al., 1997). Similar results were observed in sorghum (Craufurd and Peacock, 1993) and barley (Husain and Aspinall, 1970). In our study, these reproductive phases were protected against drought by early application of the antitranspirant. While the protection was marginal when the antitranspirant was applied at GS51 (beginning of inflorescence emergence) and plants sprayed at GS65 (anthesis) were not protected.

If drought occurs during the meiotic-stage (Late reproductive phase - meiosis; Table 1), grain set and grain number decrease with increasing endogenous ABA in the spike (Ji et al., 2011; Westgate et al., 1996). However, in the present study, grain number per spike was not significantly improved with di-1-*p*-menthene application, despite a significant reduction in both foliar and spike ABA. This suggests that di-1-*p*-menthene did not substantively alleviate drought damage during meiosis. Instead the large impact on yield was seen when antitranspirants were applied during stem

elongation (Table 1; Late reproductive phase - stem elongation) and drought amelioration was significantly linked to morphogenesis of spikes (i.e. fertile spike density). The lower fertile spike density and grain number m^2 in the unsprayed plants can be explained by the damaging effects of increased endogenous ABA. Protecting early spike development from drought was therefore crucial to optimising yield improvement, which was associated with di-1-*p*-menthene application only during stem elongation, during which the effect of the antitranspirant in reducing endogenous ABA concentration was also the highest. Application at anthesis was counterproductive, in agreement with Kettlewell et al. (2010).

The slopes of soil moisture depletion of di-1-*p*-menthene-sprayed treatments did not significantly deviate from the unsprayed reference in both years. This does not suggest that sprayed and unsprayed plants used the same amount of soil moisture. The antitranspirant effect on soil moisture conservation was expected to be small and localised, such that the placement of one soil moisture probe per plot could not detect it. Detecting small changes in soil moisture at the plot level required placing several probes near the roots or using appropriate tools such as electrical resistance tomography (Whalley et al., 2017) that can detect spatial changes in soil moisture. We did not measure the leaf water status in the present study, which could have helped assess the effect of the antitranspirant on plant water conservation. The fact that the antitranspirant was sprayed only onto leaves at GS31 and GS37 and yet the spikes had the lowest endogenous ABA but the highest grain yield suggests that di-1-*p*-menthene improved tissue water status. In a past study, improved grain yield in droughted winter wheat was found to be associated with increased plant water conservation following di-1-*p*-menthene application (Weerasinghe et al., 2016). Improving the plant water potential under water deficit conditions reduces elevated

endogenous ABA concentration (Liang and Zhang, 1999) and maintains cell turgor, allowing maintenance of biological activities such photosynthetic activity, assimilate partitioning and growth (Farooq et al., 2009).

Our results agree with Kettlewell et al. (2010) who studied similar growth stages and obtained optimum yield benefit in a winter wheat genotype from applying di-1-*p*-menthene at stem elongation (GS37 and GS39). They showed that applications at GS45 gave a negligible effect while those after ear emergence (GS55 and GS69) were counterproductive. Our 2018 and 2019 yield benefit due to di-1-*p*-menthene averaged across all growth stages was 16 and 15 %. The optimum response was achieved with applications at GS31 and GS37 which gave a yield benefit of 29 %, making our results comparable to Faralli et al. (2019) and Abdullah et al. (2015) who recorded 15 and 30 % yield benefits, respectively.

We have demonstrated yield benefits of di-1-*p*-menthene application under simulated Mediterranean-type drought. Regions with Mediterranean-type climate include the Mediterranean Basin, Western Cape in South Africa, South West and Southern Australia, Central Chile and California-and-Northern Baja California. In these areas, rains principally fall in winter with little or no summer rain (Rana and Katerji, 2000), exposing rainfed crops like winter wheat to terminal drought stress (Tigkas and Tsakiris, 2015). The antitranspirant used in the current study would be a suitable tool for managing water deficit stress under rainfed wheat production in these regions, the semi-arid subtropical climates and elsewhere with predictable drought events.

Based on the January 2020 bread wheat price of approximately £178/t in the UK (<https://ahdb.org.uk/cereals-oilseeds/uk-delivered-prices>), the gross income from the 1.09 tonnes/ha yield benefit would be £194.02/ha. The cost of di-1-*p*-menthene is \$69.9 per US gallon (3.79 litres ~ \$18.44/L = £14.03/L, SeedRanch, 2019). The current national average pesticide spraying cost for the UK is £12.63/ha (National Association of Agricultural Contractors, (NAAC, 2018). This means that the estimated total cost of using di-1-*p*-menthene would be £26.66/ha. At the 2019 optimum application growth stage giving 4.81 tonnes/ha, a yield increase of approximately 3.1 % would be needed to cover this cost at the stated market price. The yield improvement of 1.09 tonnes/ha obtained in 2019 would result in an economic benefit of spraying di-1-*p*-menthene on droughted spring wheat plants of approximately £167.36/ha. Thus di-1-*p*-menthene can make a significant contribution to ameliorating effects of water deficit in the drought-prone regions of the UK, where wheat yield losses range between 1 and 2 tonnes/ha (Dodd et al., 2011; Foulkes et al., 2007).

3.6 Conclusion

In the present study, the mechanism of di-1-*p*-menthene amelioration of drought stress was studied in spring wheat. Drought increased endogenous ABA concentration and had negative effects on yield and yield components. However, application of di-1-*p*-menthene reduced the endogenous ABA concentration consistently in the two years of study. The reduction in endogenous ABA concentration was associated with improved fertile spike density and number of grains per m² in both years. Most importantly, di-1-*p*-menthene significantly increased grain yield of droughted spring wheat plants by 0.70 and 1.09 tonnes/ha in

2018 and 2019, respectively. The mechanism of drought stress amelioration with di-1-*p*-menthene may be related to reduced endogenous ABA, in accordance with our hypothesis. Further work is needed to understand the full consequences of ABA on spike development and yield components.

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Author contribution statement

PK conceived this research. WM, PK, IG, AF and LV designed the research. WM conducted experiments. WM and PK analysed data. WM wrote the manuscript. PK, AF and LV assisted in reading and correcting the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

3.7 References

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4 Metabolic and film antitranspirants both reduce drought damage to wheat yield despite having contrasting effects on leaf ABA

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4.1 Abstract

Film antitranspirants (e.g. di-1-*p*-menthene) and metabolic antitranspirants (e.g. exogenous ABA) can be used to protect grain crops from drought, particularly during reproductive development. Here, we compared effects of di-1-*p*-menthene (1.0 L/ha) and exogenous ABA (100 μ M) on well-watered and droughted spring wheat in two glasshouse experiments. Progressive drought was imposed in the first experiment, controlled drought in the second, both from flag leaf emergence. Antitranspirants were applied at flag leaf emergence, except in the controlled drought experiment additional ABA applications were made at four further stages up to anthesis-complete. Measurements were taken for endogenous ABA, stomatal conductance and photosynthesis during reproductive development, and yield components at maturity. Both progressive and controlled drought elevated leaf ABA, less so in di-1-*p*-menthene-treated plants, whereas in exogenous ABA-treated plants leaf ABA was

elevated further. Overall, both antitranspirants improved yield under reproductive-stage drought, more so for controlled drought. Grain yield was negatively associated with reproductive-stage leaf ABA concentration except that raising leaf ABA concentration with multiple exogenous ABA applications was associated with high yield. In conclusion, both antitranspirants generally reduced effects of reproductive-stage drought on yield despite having contrary effects on leaf ABA.

Keywords: abiotic stress, drought amelioration, drought mitigation, water deficit, water stress.

4.2 Introduction

Abiotic stresses depress crop productivity to levels below their genetic potential (Fita, Rodríguez-Burruezo, Boscaiu, Prohens, & Vicente, 2015). According to the Intergovernmental Panel on Climate Change (IPCC, 2018), drought will increase due to climate change. This poses a threat to world food security, especially in view of the rapidly increasing world population, projected to reach 9.8 billion by 2050 (United Nations, 2017). Wheat, one of the three most important cereals, accounts for about 44% of global trade (FAO, 2019). Adaptive technologies are needed to improve its production to help meet the global food security challenge. One such technique is the use of antitranspirants (ATs) - products sprayed on plant leaves to reduce transpiration (Iriti et al., 2009). Among the ATs are di-1-*p*-menthene (DpM), a film-forming AT, and exogenous ABA (Exo-ABA), which is metabolic. Film-forming ATs, reduce transpiration by covering stomata with a film of low permeability to water vapour, while the metabolic type induce partial stomatal closure by acting on guard cells (Mphande et al. 2020). Both ATs are being studied as possible tools for drought

amelioration. A review by Mphande et al. (2020) provides details on commercial ATs and how they reduce transpiration.

Drought limits crop productivity by suppressing gas exchange and damaging photosynthetic pigments and inactivating photosystem II (Anjum et al., 2011; Batra et al., 2014). More than 90% of water a plant absorbs is lost through transpiration (Morison, Baker, Mullineaux, & Davies, 2008). As soil water availability is limited during drought, reducing transpiration improves the plant water status hence maintaining higher biological function within the cells (Farooq, Wahid, Kobayashi, Fujita, & Basra, 2009). Drought elevates endogenous abscisic acid (endo-ABA) concentration to reduce transpiration via stomatal closure (Lee & Luan, 2012). Although endo-ABA reduces stomatal transpiration, it may also lead to reproductive organ abortion (Pang et al., 2017). ATs are an agronomic approach to reducing transpiration.

ATs also reduce photosynthesis, but benefits outweigh this detrimental effect by improving yield under drought conditions if applied just before the most sensitive crop growth stage (GS). Examples include Kettlewell and Holloway (2010) who found that DpM applied at the boots swollen stage improved yield of droughted winter wheat by as much as 42%; and Faralli et al. (2017) who found that DpM applied at the initiation of flowering improved yield in droughted oil seed rape by 12%. Mphande, et al. (2020) observed that DpM applied at the stem elongation stage in wheat resulted in a 29% yield improvement, which was associated with reduced endo-ABA concentration. Nevertheless, these yield improvements have not been seen in every case and the mechanism of AT drought amelioration has not yet been established. ATs may have positive effects on yield-influencing physiology due to reduced transpiration. Understanding the mechanisms is likely to play a crucial

role in maximising AT potential and developing plant breeding traits for enhancing crop tolerance to drought.

Therefore, glasshouse experiments were conducted to assess the impact of DpM on gas exchange, endo-ABA concentration, yield and yield components in spring wheat under both well-watered and drought conditions, and to compare these effects to similar applications of Exo-ABA. Our hypothesis is that although both antitranspirants are expected to reduce gas exchange, applying DpM maintains yield under drought, in part by reducing leaf ABA; while applying Exo-ABA is less effective at maintaining yield under drought as leaf ABA is elevated.

4.3 Materials and Methods

4.3.1 Experimental site description and design

Glasshouse experiments were conducted at Harper Adams University, UK (52°46'N, 2°25'W) in 2018 and 2019. The light in the glasshouse was set to 16 hrs per day. Photosynthetically active radiation (PAR) was supplied using Osram sodium vapour tubes of the model Vialox NAV-T 400 (with an output of 48, 800 lumens and colour temperature of 2000k, the photosynthetic photon flux was 633 $\mu\text{mol/s}$ per tube (<https://www.waveformlighting.com/horticulture/convert-lumens-to-ppf-online-calculator>)). The experiments were arranged in a randomised complete block design. There were six treatments per block made up of two levels of irrigation, well-watered (WW) and drought and three levels of spray type (unsprayed control, DpM and Exo-ABA); making it a 2x3 factorial design.

4.3.2 Agronomy

Growth medium: In 2018, 5 litre pots were filled with John Innes No. 2 Compost (William Sinclair Horticulture Ltd., UK). Its pot capacity (PC) and permanent wilting point (PWP) expressed in volumetric water content (VWC) terms were determined by Saeed (2008) as 50.33% and 7.00%, respectively. Therefore, its plant available water (PAW) is $50.3 - 7.0 = 43.3\%$ VWC. In 2019, a mixture of air-dry sandy loam soil and moist John Innes No. 2 Compost was used in a ratio of 9:1 by weight, to allow for quicker soil drying. The PC and PWP of the mixture were determined using the pressure plate method as 14.3% and 6.7%, respectively and hence the PAW was 7.6%.

Fertilizer application: In 2018, we applied no additional fertilizer to John Innes no.2 Compost but in 2019 we added Chempak Formula 2 (NPK: 25:15:15; Chempak Products, Hoddesdon, UK) by dissolving 30g of product in 10 litres of water and applying 200ml of the nutrient solution per pot twice, at stem extension and anthesis stages.

Planting: Thirteen seeds of the spring wheat variety, Chilham (KWS UK Ltd), were planted in each pot in 2018. After establishment, thinning was done at GS24 to leave 11 plants per pot. In 2019, plants were thinned to seven and with tiller removal to ensure that all measurements were based on main stems only, unlike in 2018.

Pest control: The common pests in the glasshouse were aphids and powdery mildew. In 2018, aphids were controlled with Gazelle SG (a.s. 20% w/w acetamiprid) at a rate of 250 g/ha while powdery mildew was managed with Eclipse at a concentration of 250 g/litre (24.5% w/w) fenpropimorph and 84 g/litre (8.2% w/w)

epoxiconazole. In 2019, Rose Clear™ Ultra Gun 2, an insecticide-fungicide emulsion mixer of 0.05 g/L cypermethrin and 0.075 g/L myclobutanil, was used for both pests.

Irrigation: Irrigation management was based on the mathematical relationship described by Bellingham (2009): $L[U]SMT = VWC_{PC} - (VWC_{PC} - VWC_{PWP}) \times (MAD)$, where L[U]SMT and MAD stand for lower (or upper) soil moisture target and maximum allowable depletion. With the MAD of 30% for wheat (Hunsaker et al., 2005, higher values may be used e.g. 50%, Bellingham, 2009), the LSMT for the WW pots was 37.0% and 12.0% in 2018 and 2019, respectively. WW pots were irrigated to PC twice a week, on Tuesdays and Fridays, to maintain the moisture content above the LSMT. In the drought treatment, plants were droughted for part of the growth cycle after which normal irrigation was restored. In 2018, drought was progressive (i.e. progressive drought, PD) for 17 days, from GS37 to GS45. In 2019, it was controlled (i.e. controlled drought, CD), with partial irrigation used to sustain plants for a longer period of 50 days from GS37 to GS70. The upper and lower targets for the CD pots was 10.5% and approximately 9.0%, with the MADs of 50% and 70% (Hafez & Farig, 2019), respectively. Approximately 88 ml and 150 ml of water was applied on Tuesdays and Fridays, respectively, to maintain the soil moisture between 9% and 10.5% (VWC).

4.3.3 Spray application

Exogenous (+/-) ABA (A1049; Sigma Aldrich – UK) weighing 52.864 mg and dissolved in methanol (5 ml) and made up to 2.0 L with distilled water to obtain a concentration of 100 µM (5.3 g/ha); and 10 ml DpM (96%, Miller Chemical and Fertilizer Corp., USA) shaken in 2.0 L tap water (1.0 L/ha) were applied using a hand-held boom sprayer (Lunch Box Sprayer, Trials Equipment (UK) Ltd). In each

year, there was one DpM application made when the flag leaf was fully emerged (GS39, Zadoks, Chang, & Konzak, 1974). Under PD, a single Exo-ABA spray was applied at GS39. Under prolonged CD, there were multiple applications of Exo-ABA, once every week for five weeks between GS39 and GS70. The third set of plants not sprayed with any substance served as the unsprayed control.

The dates on which the spray products were applied are listed below (Table 1), alongside ABA sampling dates.

Table 4.1: Spray application and ABA sampling schedule.

Experiment	Spraying				Leaf sampling			Spike sampling	
	GS*	Spray types	DAP*	Date	GS	DAP	Date	DAP	Date
PD (2018)	GS39	DpM*	63	12 th Oct	GS42	68	17 th Oct		N/A
	GS39	Exo-ABA	63	12 th Oct	GS42	68	17 th Oct		N/A
CD (2019)	GS39	DpM	59	16 th Jan	GS62	80	6 th Feb	80	6 th Feb
	GS39	Exo-ABA	59	16 th Jan	GS62	80	6 th Feb	80	6 th Feb
	GS43	Exo-ABA	65	22 nd Jan					
	GS54	Exo-ABA	72	29 th Jan					
	GS62	Exo-ABA	80	6 th Feb					
	GS69	Exo-ABA	87	13 th Feb					

* DAP, DpM, Exo-ABA and GS stand for days after planting, di-1-*p*-menthene, exogenous ABA and growth stage, respectively.

4.3.4 Measurements

Temperature and relative humidity (RH) were monitored and regulated by the TomTech T200 logger (Tomtech (UK) Ltd, Spalding, UK).

Soil moisture: Measurement of soil moisture was done using the HD2 mobile reading device connected to the TRIME-PICO 64 two-rod soil moisture probe (IMKO Micromodultechnik GmbH, Ettlingen, Germany). Readings were recorded as %VWC.

Leaf and spike ABA: Five fully expanded flag leaves were collected from each pot into 5 ml plastic tubes and immediately flash frozen in liquid nitrogen and stored at -80 °C for ABA assay. Sampling was done at five days after spraying (plants at GS42) under PD and at 21 days after spraying (plants at GS62) under CD. Under CD, spikes were also sampled. To perform an ABA assay, the frozen leaf and spike samples were transferred to an Edwards Modulyo 4K freeze drier (Akribis Scientific Ltd, UK) and dried for two days, after which they were ground and assayed according to the Cusabio ABA ELISA protocol, (Cusabio Biotechnology Co., Ltd, Wuhan, China). The ABA standard used in the assay was a mixture of isomeric forms ((+/-) cis-Abscisic acid). Absorbance readings were taken at 450 nm using a BioTek spectrophotometer (BioTek Instruments Ltd, UK). The leaf and spike ABA concentrations in the sample extracts were calculated after a natural logarithmic standard curve was fitted.

Infrared gas analysis: Stomatal conductance (g_s) and net photosynthesis (A) were determined with a portable open-system infrared gas analyser (IRGA) - LCpro-SD (ADC BioScientific Ltd, UK). Light (PAR) was set to 250 and 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in 2018 and 2019, respectively, and CO_2 to 400 $\mu\text{mol mol}^{-1}$. Measurements were taken between 10:00 hrs and 14:00 hrs and recorded when the system was in a steady state (~ five minutes). Measurements were taken at five days after spraying in 2018, and one day after spraying in 2019. Intrinsic water use efficiency (WUE_i) was calculated as a ratio of net photosynthesis, P_N , to stomatal conductance (Sikder et al., 2015).

Yield and yield components: Spikes from each pot were hand-harvested, threshed, cleaned, weighed and oven dried at 105 °C for 40 h. The number of grains per spike was determined by counting the fertile spikes and grains per pot. A spike was

counted as fertile if it was bearing at least one grain. In 2018, 11 main stem and between seven and 28 tiller spikes were harvested and mixed. In 2019 there were only seven main spikes per pot, as tillers were trimmed. Thousand grain weight (TGW) was determined by the method described by (Sylvester-Bradley et al., 1985). Finally, grain yield per spike was obtained by weighing all the grain harvested from each pot and dividing that weight by the number of fertile spikes. Both TGW and grain yield were expressed at 15% moisture content.

Statistical analysis: Analyses of variance (ANOVA) of gas exchange variables, leaf and spike ABA concentration and yield and yield components were performed using GenStat 20th Edition (Hemel Hempstead, UK). The Tukey test (at 5%) was used for *post hoc* analyses. Relationships between ABA and yield components were analysed using simple linear regression. Curves were fitted and modelling done using Microsoft Excel, but with parameters generated from GenStat.

4.4 Results

4.4.1 Temperature and relative humidity

The average, minimum and maximum daily temperature and RH inside the glasshouse for 2018 and 2019 were 19.3 °C, 11.9 °C and 25.1 °C; and 26.4 %, 14.8 % and 56.1%; and 15.9 °C, 14.2 °C and 18.4 °C; and 71.5%, 56.3 %, 78.4%, respectively. Thus, the average temperature for 2018 was significantly ($p < 0.001$) higher than for 2019 while the opposite was true for RH and equally significant ($p < 0.001$) as the values above and Figure 4.1 indicate.

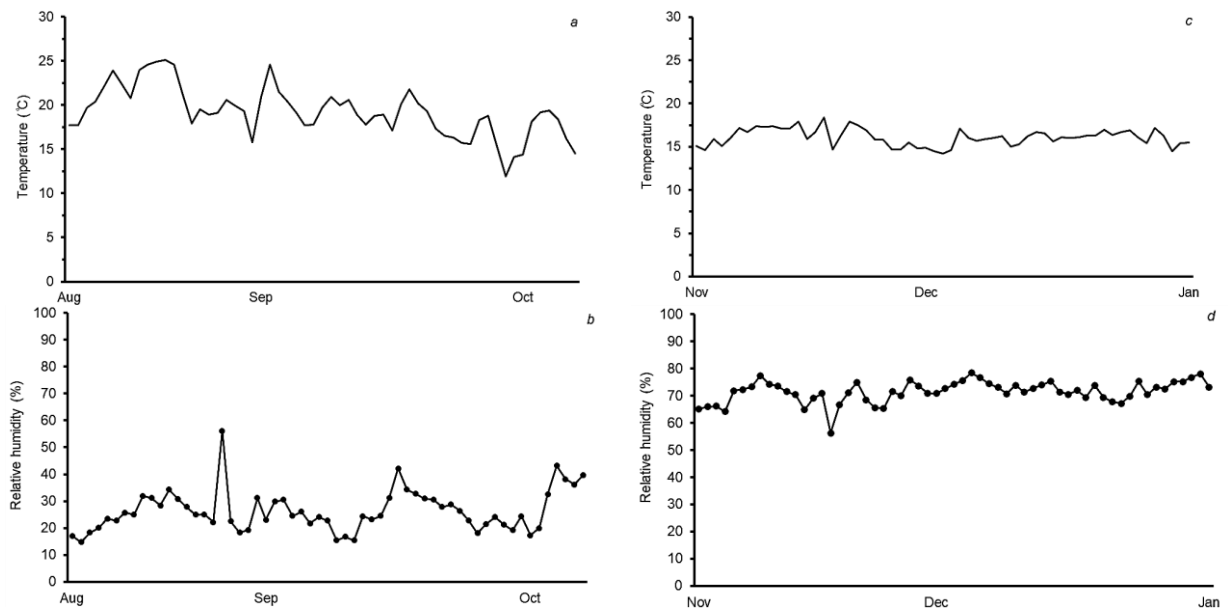
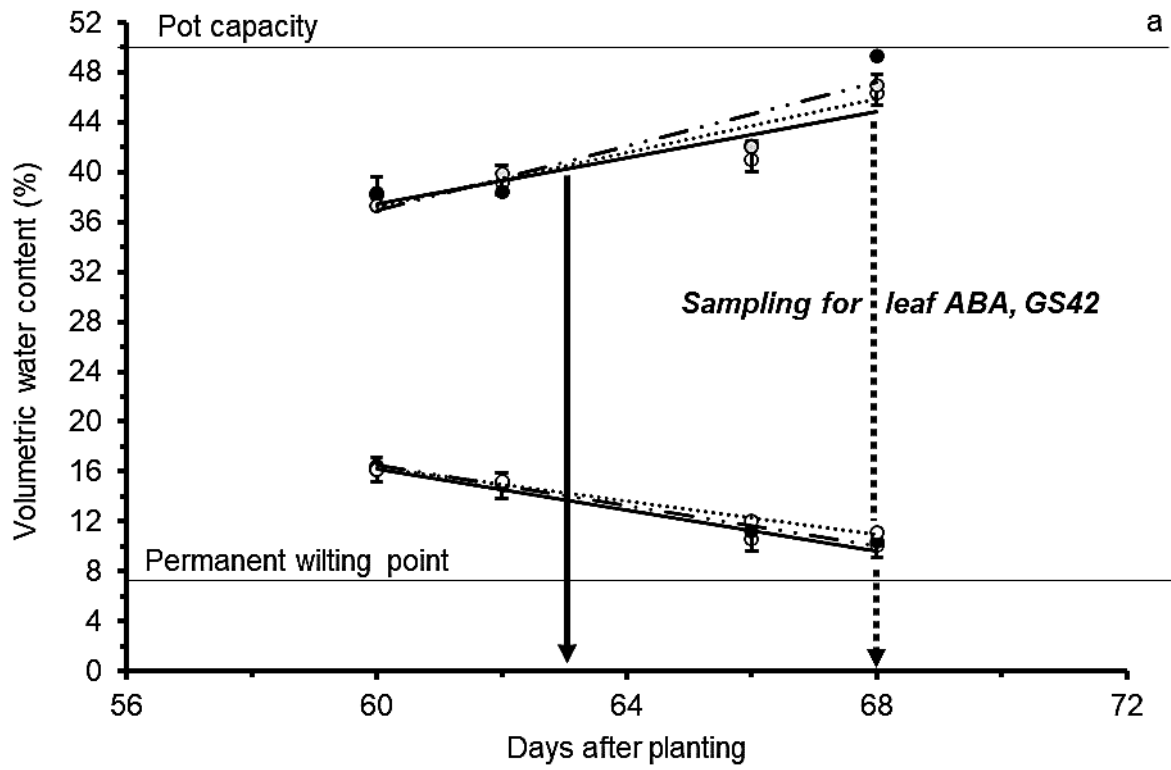


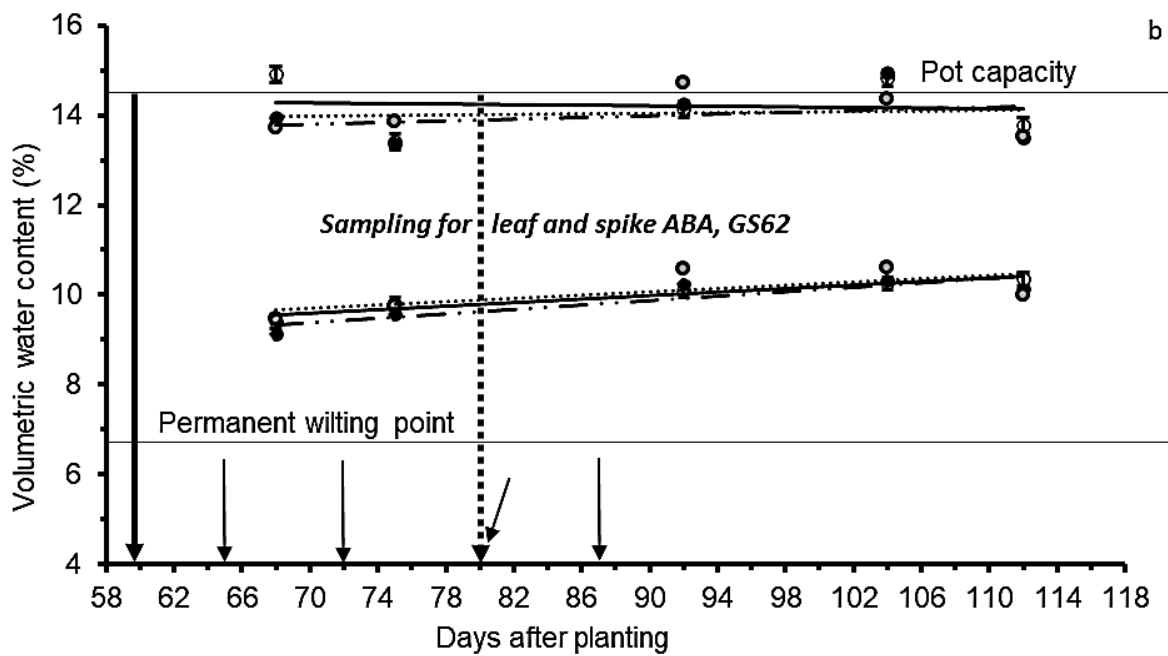
Figure 4.1: Average daily air temperature (a and c) and average daily relative humidity (b and d) inside the glasshouse rain shelters recorded by the TomTech T200 data logger (Tomtech (UK) Ltd, Spalding, UK).

4.4.2 Soil moisture

Under PD (2018), the average fraction of PAW decreased from 22% at 60 DAP to 8% at 68 DAP (Figure 4.2), when watering was resumed. Under CD (2019), the average proportion of remaining PAW fluctuated but measurements taken between 68 and 112 DAP ranged from 35% to 46%. WW plants were kept above their LSMT of 37% and 12% (VWC) in 2018 and 2019, respectively.



○ control ● di-1-p-menthene
 ○ ABA ○ Linear (control)
 — · · Linear (di-1-p-menthene) ····· Linear (ABA)



● di-1-p-menthene ○ aba
 ○ control — · · Linear (di-1-p-menthene)
 ····· Linear (aba) — Linear (control)

Figure 4.2: Soil moisture readings in well-watered (top regression lines) and droughted pots in (a, $p = 0.889$, error bars, only on the Water treatment due to space

limitation, represent standard error of regression, SER = 1.46 and $p = 0.718$, SER = 0.941, respectively) PD between 60 and 68 days after planting (DAP) in (b, $p = 0.664$, SER = 0.182 and $p = 0.747$, SER = 0.146) CD between 68 and 112 DAP. Note that the lower pot capacity for CD was due to a predominantly sandy growth medium instead of John Innes No. 2 Compost for PD. Undotted arrows indicate DAP on which the antitranspirants were sprayed – with smaller lines being for the four exogenous ABA-only sprays.

4.4.3 ABA concentration

The leaf and spike ABA concentrations (ng/g DW) under PD were much higher than under CD, reflective of the respective more severe and milder drought. Due to drought, the ABA concentrations in flag leaves significantly increased above WW plants by 243% and 159% under PD and CD, respectively, while in the spikes it rose by 184% (determined under CD only). The effect of DpM on endo-ABA concentration was consistent in the two experiments, but it varied for Exo-ABA. Application of DpM reduced the endo-ABA concentration in droughted plants to the lowest values at five days after spraying (Figure 4.3). Under drought conditions, Exo-ABA had no significant effect on elevating endo-ABA at five days after spraying (PD). The only sampling for which Exo-ABA significantly elevated leaf ABA in droughted plants was at one day after the fourth spraying under CD (Figure 4.3 b). Under WW conditions, the effect of DpM was negligible. In contrast, Exo-ABA significantly increased the leaf ABA concentration in WW plants in both experiments, regardless of time lapse after spraying. The effect was so large that the concentration rose to similar levels as under droughted conditions at 80 DAP. However, at 68 DAP, it was significantly lower than in the droughted plants but remained significantly higher than in the WW unsprayed control and DpM-treated plants.

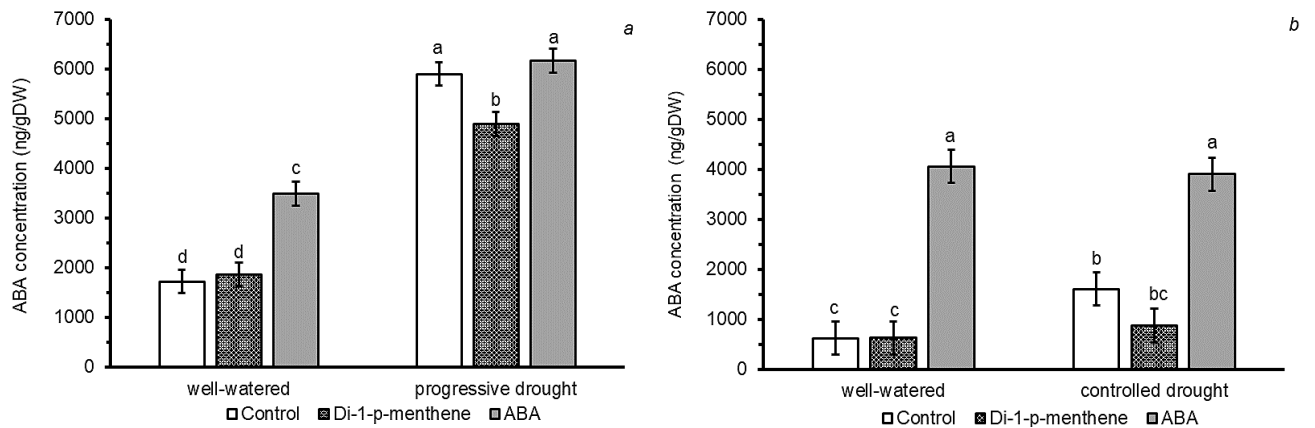


Figure 4.3: The Spray type x Irrigation interaction effects on flag leaf ABA sampled at 68 days after planting (DAP) i.e. five days after spraying (a, $p = 0.008$) under progressive drought; and 80 DAP i.e. 21 days after spraying DpM and one day after a fourth spray of Exo-ABA (c, $p = 0.015$) under controlled drought. Different letters represent significant differences (Tukey test, $p < 0.05$). Error bars represent common standard errors of means from ANOVA table.

Spike ABA response to spray type treatments followed a similar pattern to foliar ABA, with DpM reducing the concentration to the least values and Exo-ABA elevating it (Figure 4.4). However, the effect of DpM was not significant at both levels of irrigation, while Exo-ABA significantly increased the leaf ABA only under WW conditions. Notice that the concentration of ABA in spikes was higher than in leaves of the unsprayed control and DpM-treated counterpart plants.

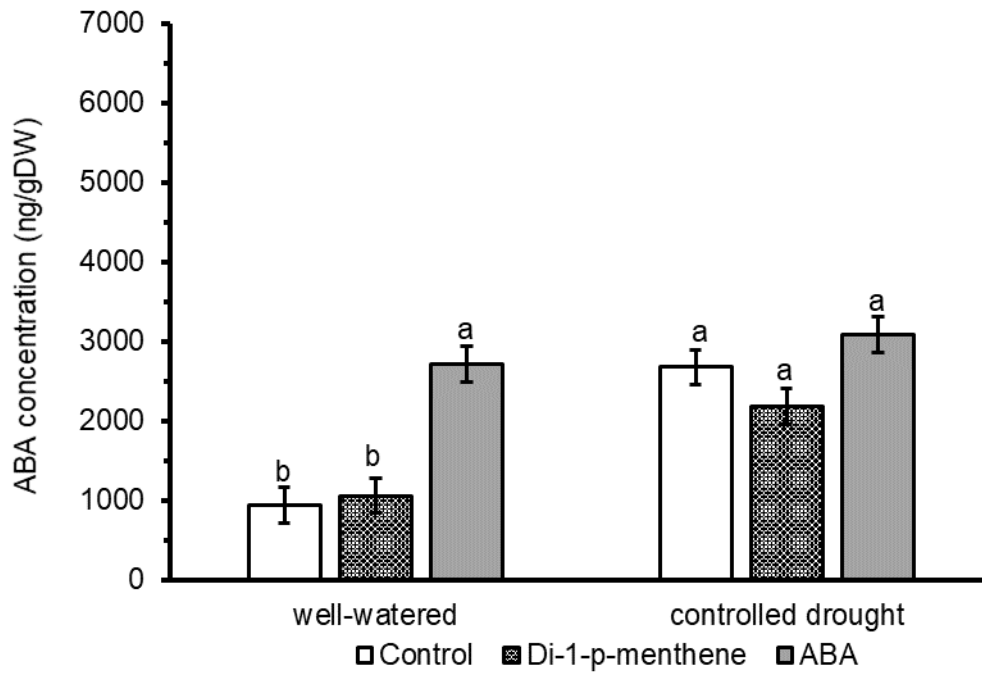


Figure 4.4: The Spray type x Irrigation interaction effects ($p = 0.025$) on spike ABA sampled at 80 days after planting, i.e. 21 days after spraying di-1-*p*-menthene and one day after a fourth exogenous ABA spray under controlled drought. Different letters represent significant difference (Tukey test, $p < 0.05$). Errors bars represent common standard errors of means from ANOVA table.

4.4.4 Gas exchange

Drought suppressed gas exchange both under PD and CD (Figure 4.5). Compared to the WW unsprayed control, stomatal conductance under the PD treatment for unsprayed, DpM and Exo-ABA decreased significantly ($p = 0.025$) by 51%, 92% and 90%, respectively. Under CD (2019), which was mild, the reductions ($p < 0.001$) corresponded to 43%, 61% and 33%. Compared to the unsprayed control, Exo-ABA significantly reduced stomatal conductance under PD but surprisingly induced a nonsignificant increase under mild CD. DpM consistently and significantly reduced

stomatal conductance. Under WW conditions, DpM and Exo-ABA negligibly reduced stomatal conductance in both years.

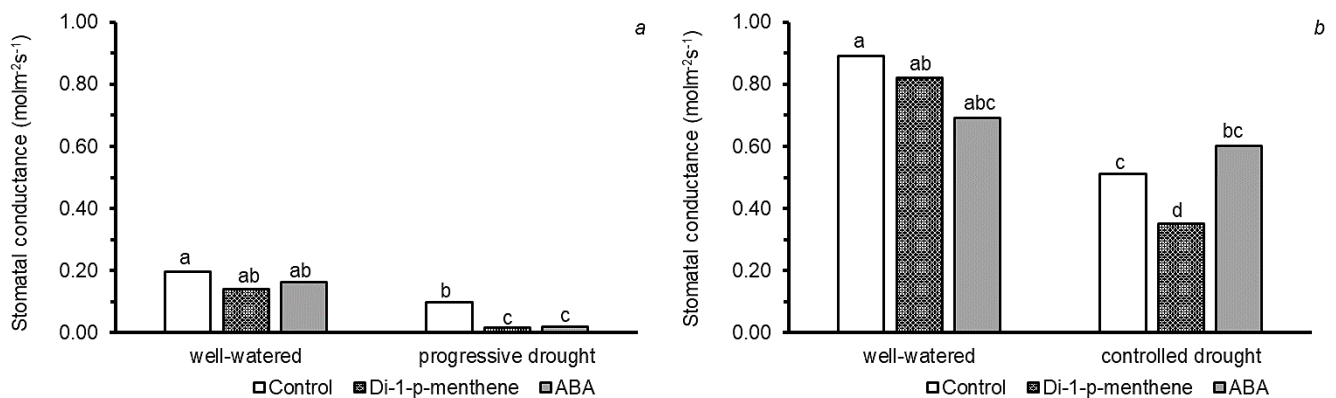


Figure 4.5: Spray type \times Irrigation effects on stomatal conductance measured five days after spraying (DAS, a, $p = 0.025$) and one DAS (b, $p < 0.001$) under progressive drought (PD) and controlled drought (CD), respectively. The photosynthetically active radiation in 2018 was set at $250 \mu\text{mol m}^{-2} \text{s}^{-1}$ and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ in 2019. Different letters represent significant differences (Tukey test, $p < 0.05$). The graphs were constructed from back transformed values. The transformed means and their standard errors (SEM) in the order: well-watered and droughted (control, di-1-p-menthene and exogenous ABA, SEM); were: under PD (square root scale: 0.4434, 0.3759, 0.402 and 0.3108, 0.1244, 0.1382, SEM: 0.02189) and CD (log 10 scale: -0.118, -0.195 and -0.367 and -0.679, -1.043 and -0.514, SEM: 0.0739), respectively .

Photosynthesis values for PD (2018) were much lower than for CD (2019, where a higher light level was used during measurements, Figure 4.6). The effects of DpM and Exo-ABA on photosynthesis for PD were not significant at either water level. Under the mild CD and high level of light during measurements (PAR of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$), DpM significantly suppressed photosynthesis by 44% while Exo-ABA had no significant effect (Figure 4.6, b).

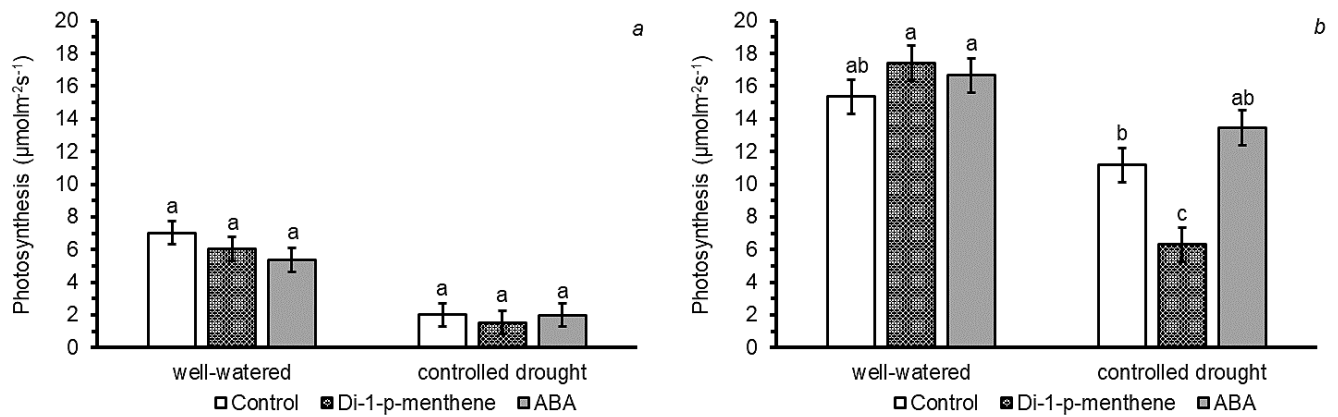


Figure 4.6: Spray type x Irrigation interaction effects on net photosynthesis measured five days after spraying at photosynthetically active radiation (PAR) set at 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under progressive drought (a, $p = 0.488$) and one day after spraying at PAR of 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ under controlled drought (b, $p = 0.004$). Different letters represent significant differences (Tukey test, $P < 0.05$) while error bars are standard errors of means from ANOVA table.

Generally, both drought and ATs increased WUE_i (Figure 4.7). Under the severe PD, the ATs significantly increased the WUE_i to much higher levels than under CD. Under the milder CD, all plants had significantly higher WUE_i compared to unsprayed WW plants, although there were no significant differences for CD plants treated with ATs.

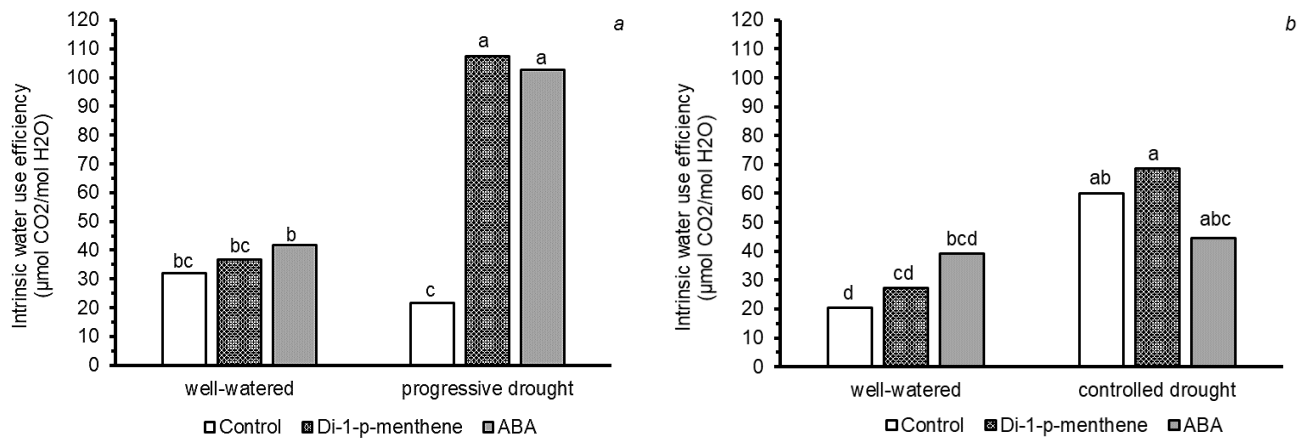


Figure 4.7: Spray type \times Irrigation interaction effects on intrinsic water use efficiency determined on flag leaves (a, $p < 0.001$) under progressive drought (PD) and (b, $p = 0.007$) under controlled drought (CD). Error bars represent standard error of means (SEM). Different letters represent significant difference (Tukey test, $p < 0.05$); graphs were constructed from back transformed values. The transformed means and their SEM in the order: well-watered and droughted (control, di-1-p-menthene and Exo-ABA, SEM); were: under PD (log base 10 scale: 1.50, 1.57, 1.62 and 1.34, 2.03, 2.01, SEM: 0.50) and under CD (square root scale: 4.51, 5.21, 6.25 and 7.75, 8.29, 6.68, SEM: 0.589), respectively.

4.4.5 Yield and yield components

Drought generally reduced most yield components; however, significant differences were seen but not in all cases (Figure 4.8). Under the more severe PD, with plants tillers untrimmed, the number of fertile spike per pot, grains per spike and grain weight, were reduced compared to WW plants, but there were no significant differences (main stem and tiller spikes were mixed). There were no differences in the number of fertile spikes per pot or TGW under both PD and CD. Grain number per spike and grain yield per spike were significantly reduced by CD in unsprayed plants, but application of DpM and Exo-ABA protected these yield components.

Under CD, the average grain number per spike and grain yield per spike were significantly higher than under PD.

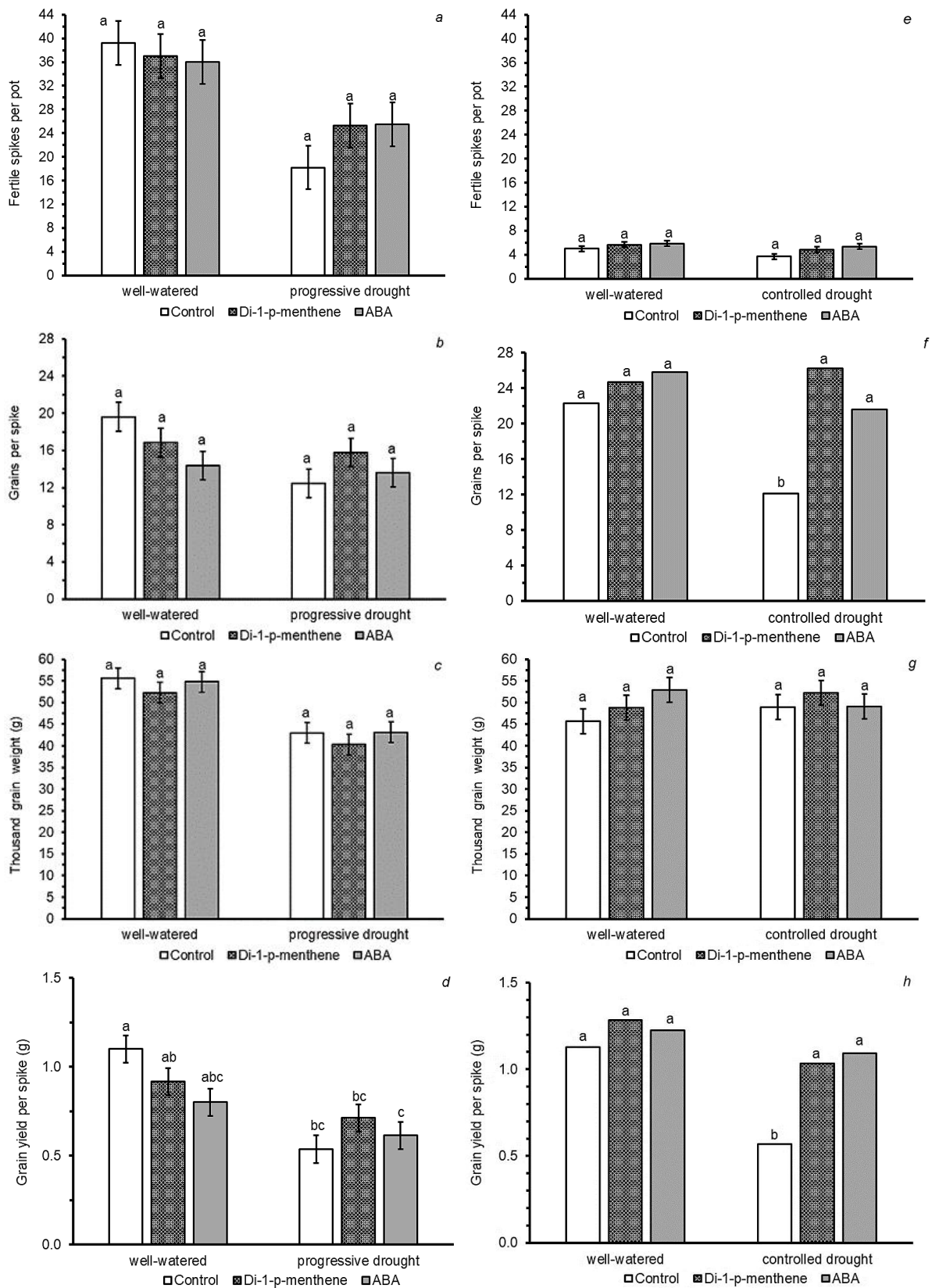


Figure 4.8: Spray type x Irrigation effects on number of grains per spike, thousand grain weight and grain yield per spike for 2018 (a, $p = 0.083$, b, $p = 0.984$ and c, $p = 0.038$) and 2019 (d, $p = 0.043$, e, $p = 0.370$ and f, $p = 0.005$). Error bars represent common standard errors of means (SEM) from ANOVA. Different letters represent significant difference (Tukey test, $p < 0.05$). Figure 4.8 (f and h) were constructed from back transformed values; their means on the square root scale and SEM in the order: well-watered and droughted (control, di-1-*p*-menthene and exogenous ABA, SEM); were (4.72, 4.97, 5.08 and 3.48, 5.12, 4.65, SEM: 0.262) and on the logit scale (-4.474, -4.343, -4.390 and -5.162, -4.561, -4.504, SEM: 0.0750), respectively. Note that for (e) fertile spikes per plot was mitigated, as both plant number and tiller number were controlled

4.4.6 Regression analysis

Yield components were significantly correlated with leaf ABA sampled at the booting stage under PD (Figure 4.9). Leaf and spike ABA sampled at early anthesis under CD showed no significant associations (graphs on spikes not presented). The relationships between leaf ABA and the number of grains per spike, TGW and yield per spike under PD were all significant and linear, their values decreasing with increasing leaf ABA (Figure 4.9 a, b, c and d).

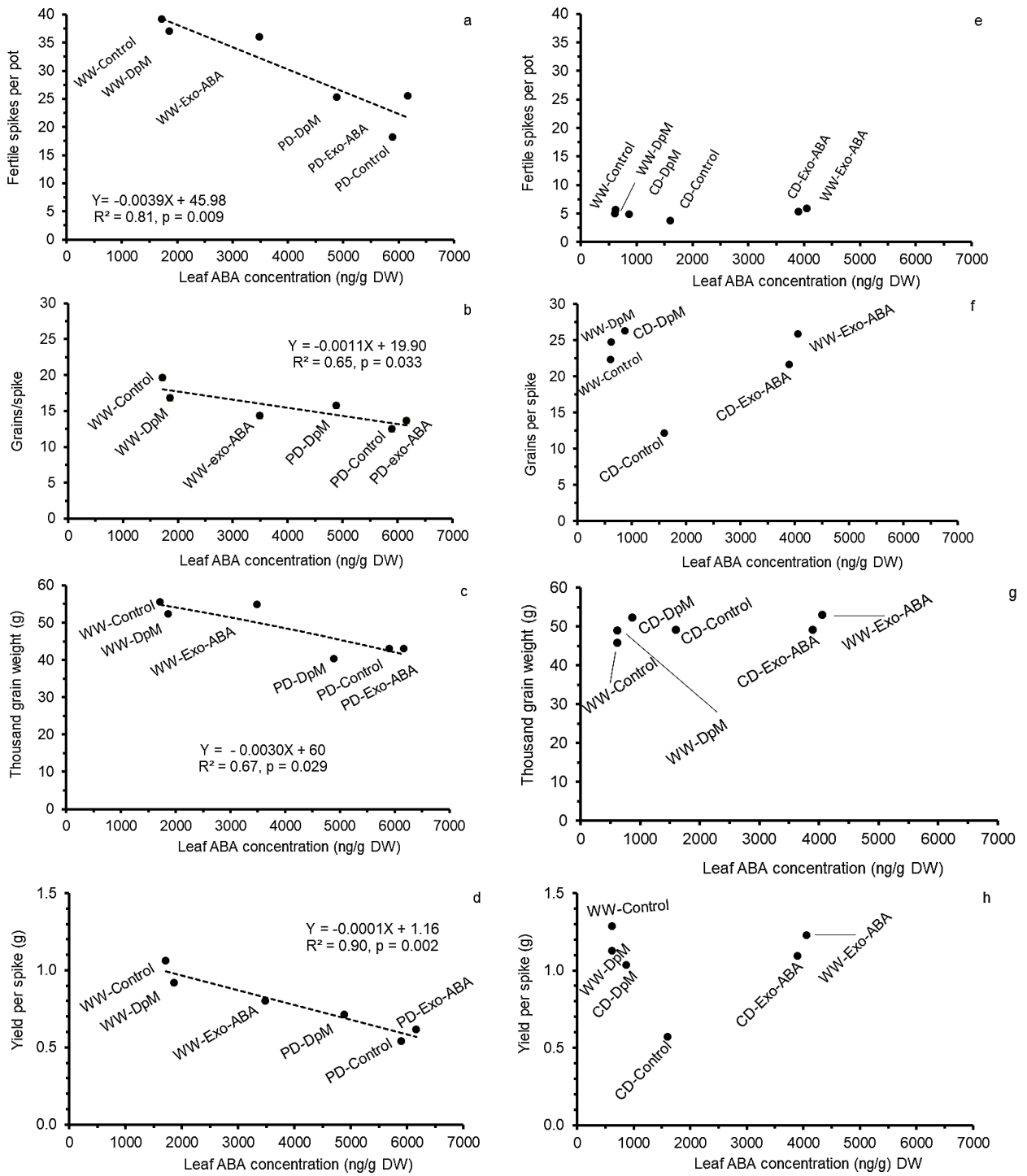


Figure 4.9: Linear regressions of flag leaf ABA concentration against fertile spikes per pot, grain number per spike, thousand grain weight and grain yield per spike under PD (a, b, c and d) and CD (e, f g and h). Under CD, sampling for ABA assay was done at booting (GS42), five days after spraying, and under CD at early anthesis (GS62), 21 days after spraying di-1-*p*-menthene and one day after a fourth exogenous

ABA spray under CD. Note that for (e) fertile spikes per plot was mitigated, as both plant number and tiller number were controlled

4.5 Discussion

Drought is an important yield-limiting abiotic stress because of its impact on key physiological processes such as stomatal conductance, photosynthesis (Acevedo, Silva, & Silva, 2002; Shakeel Ahmad Anjum et al., 2011) and on metabolic processes that determine grain development (Mphande et al., 2021). ATs reduce the negative impact of these processes on yield. In the two glasshouse experiments, drought downregulated stomatal conductance and photosynthesis and upregulated ABA concentration. The higher levels of stomatal conductance under CD reflect not only the greater photosynthetic active radiation used but also the milder stress, as water deficit was more severe under PD than under CD. The application of either DpM or Exo-ABA just before booting (GS39) ameliorated the yield-limiting effects of drought, despite distinct physiological effects of these products.

Drought induces an increase in the endo-ABA accumulation (Pang et al., 2017). Compared to the WW plants, drought upregulated the accumulation of ABA in leaves by 3.4 and 2.6 times under PD and CD, respectively; and in spikes by 2.8 times (determined under CD only). With PAW approaching PWP, droughted plants under PD grew under more severe drought and hotter and drier ambient conditions than under CD, as Figures 4.1 and 4.2 indicate. As expected, application of DpM resulted in less accumulation of leaf ABA during drought, while Exo-ABA resulted in equal or greater leaf ABA during drought (although the differences were not always significant). In wheat, younger leaves accumulate higher levels of ABA (Quarrie & Henson, 1981). This might explain the non-significant effect of DpM on endo-ABA

with sampling at 21 days after spraying (for DpM under CD), being too late. In addition, the weekly irrigation intervals (on two different days) meant that endogenous ABA levels were constantly fluctuating under CD. Under a progressive drought scenario, accumulation of endogenous ABA is known to steadily increase (Itam et al., 2020), but hydration promotes catabolism of ABA (Yang and Zeevaart, 2006). The largest difference in the leaf ABA levels were observed in Exo-ABA - treated plants under CD, which were sampled one day after a fourth application. Although Exo-ABA consistently elevated leaf ABA when it was low, under CD the increase was small and under PD there was no increase (as leaf ABA was elevated due to drought). This suggests that endo-ABA reaches a ceiling under severe drought, beyond which homeostatic mechanisms rapidly catabolise it, probably in not more than two days. Endo-ABA concentration is controlled by a balance between its biosynthesis and catabolism (Nambara & Marion-Poll, 2005). There is evidence that once drought-induced endo-ABA accumulates to a certain level, negative feedback mechanisms mediated by transcription factors, AhAREB1 and AhNAC2, limit its further accumulation (Liu et al., 2016). There are also suggestions that ABA self-regulates (see references in Nambara & Marion-Poll, 2005); and that this is by modulating the negative feedback transcription factors AhAREB1 and AhNAC2 (Liu et al., 2016).

Other than for the effect of Exo-ABA treatment at one day after the fourth spraying (under CD), leaf ABA concentration was expected to be higher than in the spike counterparts, as Mphande et al. (2021) found at ear emergence. It is known that endo-ABA in spikes of wheat and panicles of rice steadily increases after anthesis, reaching maximum at the peak of grain filling, enhancing senescence and remobilisation of fixed carbon from stems to spikes (Mphande et al., 2016;

Tsukaguchi et al., 1999; Yang et al., 2003). Therefore, while the endo-ABA concentration in leaves declines as they age, it increases in spikes as grain filling approaches (Kondhare et al., 2015; Yang, et al., 2004); this might explain why at anthesis spikes accumulated higher levels of the hormone than leaves at the same growth stage. It is known that drought (i.e. high ABA) during grain filling is less damaging to yield than between stem elongation and heading (Khadka et al., 2020). A high concentration of spike ABA pre-anthesis may have damaging effects on some yield components.

By physically blocking stomata, DpM decreases the number of stomatal pores involved in gas exchange. In the current study, DpM significantly reduced stomatal conductance to the lowest values under both PD and CD conditions. Exo-ABA only reduced stomatal conductance under PD, while it resulted in a negligible increase under CD. The non-significant effect of spray type on photosynthesis under PD compared to CD may be attributed to differences in the level of PAR at which measurements were taken. The level of light used ($250 \mu\text{molm}^{-2}\text{s}^{-1}$) under PD was much lower than under ($1000 \mu\text{molm}^{-2}\text{s}^{-1}$) and other studies (e.g. $1200 \mu\text{molm}^{-2}\text{s}^{-1}$, Faralli et al., 2017). Clearly, when the photosynthetic machinery was operating below capacity, no significant differences between treatments were detectable. Our results are consistent with a study on beans by Iriti et al. (2009) who found DpM to be more effective in reducing stomatal conductance than chitosan, another metabolic AT. Droughted plants had higher WUE than well-watered plants, in agreement with previous research (e.g. Mendanha, Rosenqvist, Hyldgaard, Doonan, & Ottosen, 2020). WUE_i can be increased by reducing stomatal conductance or increasing photosynthesis or a combination of both (from the formula: P_N/g_s , (Sikder et al., 2015). This explains why DpM significantly increased the WUE_i under severe

drought (PD) but not under mild CD or WW conditions. Increasing the WUE is one of the drought tolerance traits as water conservation outweighs the penalty on reduced carbon assimilation (Mendanha et al., 2020). Under PD, both DpM and Exo-ABA improved WUE_i but did not significantly increase the fertile spikes per pot, grain number per spike and grain yield per spike most likely due to the masking effect of mixing of main stem and tiller spikes. Iriti et al. (2009) reported DpM to be most effective in increasing WUE_i relative to chitosan and the control under drought.

Saini & Aspinall (1982) showed that high levels of ABA during booting completely inhibited spike fertility in wheat, and Mphande et al., (2021) suggest that some of the positive effects of AT under drought are due to increased spike fertility. The present study has shown negative associations between leaf ABA at the booting stage and yield/yield components (Figures 4.9 a, b, c and d). The number of grains per spike was linearly related to leaf ABA in a negative way. This trait is fixed during spikelet morphogenesis and development but is affected by environmental conditions (Dolferus, Ji, & Richards, 2011; Lin et al., 2020). It is known that pre-anthesis drought at this stage damages florets and entire spikelets, which are the determinants of grain number per spike (Oosterhuis & Cartwright, 1983). In surviving florets, grain number is further repressed by the sporadic effect of drought on pollen meiosis, which has been identified as the most sensitive reproductive process to abiotic stress (Saini & Aspinall, 1982). Reduction in grain number is linked to elevated ABA concentration induced by drought (Morgan, 1980) or other abiotic stresses. Saini & Aspinall (1982) demonstrated that elevating the endo-ABA concentration just before or at the commencement of pollen meiosis significantly reduced grain set and number of grains per spike, but these traits were unaffected if increased concentrations were during pollen development and at the onset of

anthesis. This may explain why elevated leaf ABA concentration at booting in the present study was correlated with number of grains per spike under PD but there was no correlation at anthesis under CD. To the contrary, in a glasshouse study, Weldearegay, Yan, Jiang, & Liu (2012) found that drought at anthesis elevated spikelet ABA levels, reduced grain set and grain yield of spring wheat. The mixing of main stem and tiller spikes under PD masked treatment effects on grain number per spike and other agronomic traits. Unlike main stems, tillers were not at GS39 at the time of spray application, hence drought was not expected to have also affected pollen meiosis in tiller spikes. In addition, even by the time normal watering was resumed (main spikes at GS45), most tillers were still at stem extension. Thus, the treatment effect of stems that were at GS39 was likely overshadowed. The lower number of grains per spike under PD can be attributed to source limitation resulting in competition for growth resources, as there were 11 plants per pot and their tillers against seven tiller-free main spikes under CD. The number of grains per spike reduces with increasing plant density (Li et al., 2016), and water deficit (Lin et al., 2020). Under severe stress, entire flowering heads are aborted, thereby reducing the number of fertile spikes per unit area (Katerji et al., 2009). Thus, under high endo-ABA concentration due to drought, reproductive failure increases. Figure 4.9 (a, b, and c) demonstrates that accumulating high leaf ABA, of over 5000 ng/g DW in this case, had damaging effects being associated with the lowest yield/yield component values.

The significant increase in grain number and yield per spike under CD due to DpM and Exo-ABA application suggests that these compounds had stimulatory or ameliorative effects on these agronomic traits. In droughted spring wheat, Mphande et al. (2021) found an association between yield improvement and reduced endo-

ABA concentration attributed to DpM. DpM is also known to improve plant water balance (Faralli et al., 2017) and this is likely the mechanism linked to reduced endo-ABA. Increasing the plant water status of dehydrated plant cells restores turgor and reduces elevated endo-ABA concentration (Liang and Zhang, 1999) through catabolic pathways of hydroxylation and conjugation (Nambara & Marion-Poll, 2005). Maintenance of cellular turgor is crucial for sustaining biological processes including photosynthesis, assimilate partitioning, growth and reproduction (Farooq et al., 2009).

Application of Exo ABA provides signals to leaves to close the stomata and has been shown to protect wheat from water loss during mild drought, however the amount applied, the interaction with endogenously produced ABA and the timing of the drought can all impact on crop response (Mphande et al., 2020). In wheat, Travaglia et al. (2010) observed that under moderate drought, Exo-ABA sustained chlorophylls *a* and *b*. This agrees with the present study as Exo-ABA increased net photosynthesis with a PAR selection of $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ and not $250 \mu\text{mol m}^{-2} \text{s}^{-1}$, though the effect was not significant. Exo-ABA increasing of stomatal conductance and photosynthesis has also been reported in soyabeans (Liu, Jensen, & Andersen, 2004). Further, Exo-ABA upregulates the expression of glutathione and ascorbate antioxidant activities in droughted wheat (Wei et al., 2015). These mechanisms may explain why Exo-ABA improved grain number and grain yield in our study. Heightened expression of antioxidants is one of the traits conferring tolerance to abiotic stresses like drought in crop genotypes (Vaidyanathan, Sivakumar, Chakrabarty, & Thomas, 2003). However, application of very high levels of Exo-ABA is counterproductive – (e.g. while 20 mg/l (~ 76 μM) during pollen meiosis reduced grain set to less than 15% while 100 mg/l (~378 μM) completely inhibited grain set in

wheat, Saini & Aspinall, 1982). In the current study, 100 μM increased grain set under CD by average of 116%. The inconsistent effects of Exo-ABA concentrations (e.g. 76 μM vs 100 μM) on grain set might be attributed to genotypic differences between the experiments. That the environment is also a factor is clearly demonstrated by the effects of Exo-ABA and DpM on yield and yield components in our study, being less effective under more severe PD than under mild CD. Our results corroborate past findings. A study on oilseed rape by Faralli et al. (2017) reported that the effect of DpM on pod number per plant significantly decreased with increasing drought severity. In wheat, Travaglia et al. (2010) found a significant increase in individual grain weight attributed to Exo-ABA under moderate but not severe drought. Working with three Exo-ABA concentrations (30, 40 and 60 mg/L ~ 113, 151 and 227 μM) on spring wheat, Zhang et al. (2016) observed that grain yield averaged over two years decreased with increasing concentration, supportive of the damaging effects of increased endo-ABA. Other than for the more severe PD, the effect of Exo-ABA on grain yield obtained in this study is consistent with the finding of Zhang et al. (2016). They observed yield improvement in winter wheat grown under both WW and droughted conditions. The effect of Exo-ABA increased with drought from WW to moderate deficit and reached the highest under severe drought. These studies suggest that the mitigative effects of ATs are influenced not only by dose rate but also by genotype, timing and the magnitude of drought. There is need for establishing the interplay between Exo-ABA as an AT for drought mitigation and its potential to cause damage to yield components by elevating ABA concentration. In contrast, Sivakumar, Virendranath, & Srivastava (2001) reported decline in grain weight and grain number per spike, due to Exo-ABA application, of 28% and 22%, respectively, showing that the yield benefits are not in all cases.

4.6 Conclusion

In the present study, the effects of DpM and Exo-ABA on gas exchange and leaf and spike ABA concentration were compared in WW and droughted spring wheat in two glasshouse experiments. We found no evidence of Exo-ABA damaging yield or number of grains per spike. DpM had no significant effect on endo-ABA in WW plants but generally reduced it under drought. As such, the correlation between reproductive-stage leaf ABA concentration and yield, seen here in one experiment and in previous field experiments, might reflect a general association between endo-ABA and drought stress rather than a direct effect of the hormone on grain development, as previously proposed.

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Author contribution statement

PK conceived this research. WM, PK, IG, AF and LV designed the research. WM conducted experiments. WM analysed data. WM wrote the manuscript. PK, AF and LV assisted in reading and correcting the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

4.7 References

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5 Inhibiting ABA in droughted wheat associated with increased yield only if transpiration also suppressed

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5.1 Abstract

In Mediterranean-type climates, terminal drought is one of the important abiotic stresses responsible for grain yield losses in wheat. Drought is known to elevate endogenous abscisic acid concentration and increase sterility of reproductive organs. We studied the possibility of reduced concentration of drought-induced reproductive-stage spike ABA as a mechanism of drought amelioration in two spray application experiments in spring and summer. Mediterranean-type terminal drought was simulated using rain shelters. At flag leaf emergence, we applied di-1-*p*-menthene, a film antitranspirant, at 1 L/ha; and fluridone, a biosynthesis inhibitor of ABA, at 10 μ M, 20 μ M and 50 μ M concentrations, to compare their effects on spike ABA, gas exchange and agronomic traits. Drought induced an increase in spike ABA and downregulated net photosynthesis. Di-1-*p*-menthene and fluridone, at some concentrations, reduced spike ABA but further downregulated net photosynthesis. Averaged across the seasons, di-1-*p*-menthene suppressed transpiration and the

accumulation of spike ABA each by 21% and increased grain yield by 27%. In contrast, although fluridone also reduced spike ABA (16%) and photosynthesis (19%), overall, it did not alter transpirational water and grain yield. Furthermore, when di-1-*p*-menthene was combined with exogenous ABA, much of the water saving and yield benefits remained. The results suggest that yield improvement with application of di-1-*p*-methene under Mediterranean-type drought is mediated through mechanisms that involve the conservation of plant water by suppressing transpiration, and that although this is associated with the reduction of spike ABA concentration, reduced ABA may not be a causal factor.

Key words: terminal drought, crop adaptation, relative water content, yield components

5.2 Introduction

Climate change-driven droughts are forecast to increase causing food shortages across the globe (IPCC, 2019). Wheat, one of the important cereals in human diets, is the most widely grown crop across the world, and climate change-driven drought is likely to further impact on its production in water-limited environments where yield losses greater than 50% of the genetic potential are common (Monneveux et al., 2012). Mediterranean-type climates are among regions with poor rainfall patterns characterised with terminal drought, which is protracted water deficit during a crop's reproductive development stages (Carter et al., 2019; Tigkas and Tsakiris, 2015). Terminal drought does not only reduce grain yield but also undermines grain quality (Gooding et al., 2003). Maintaining and improving crop production through adaptive

technologies is required to avert future global food insecurity. One such technology is the agronomic technique involving the use of antitranspirants - substances that are applied on leaves to reduce transpiration with a view to improving the plant water status and crop productivity (Mphande et al., 2020).

The drought ameliorative potential of antitranspirants has positively been established (e.g. Javan et al., 2013; Kettlewell et al., 2010; Ouerghi et al., 2014), however, the mechanisms are unknown. In a recent study, Mphande et al. (2021) reported an association between di-1-*p*-menthene-induced yield improvement with reduced endogenous ABA in spring wheat. There is evidence implicating ABA in loss of crop yield (e.g. reproductive organ abortion in chickpeas, Pang et al., 2017, abortion of apical and basal spikelets in barley, Boussora et al., 2019, and suppression of seed set in maize, Setter et al., 2001, and wheat, Saini and Aspinall, 1982).

In the present study, to understand whether the mechanism of di-1-*p*-menthene (DpM) amelioration of drought stress is associated with reduced endogenous ABA concentration, we undertook to suppress the biosynthesis of the phytohormone using fluridone. Fluridone (1-methyl-3-phenyl-5-[3-(trifluoromethyl)phenyl]-4(1H)-pyridinone) is an aquatic herbicide whose mode of action involves the inhibition of chlorophyll and carotenoid biosynthesis (Devlin et al., 1978). Chlorophylls and carotenoids play essential roles in photosynthetic physiology, including carotenoids protecting chlorophyll against photooxidation under high light levels but low photosynthesis (Popova, 1995; Sprecher et al., 1998). Carotenoids are also necessary precursors of ABA biosynthesis (Popova, 1995). Fluridone is widely used in research where inhibition of biosynthesis of ABA is desired (Zou et al., 2018). We tested the hypothesis that down-regulation of the biosynthesis of endogenous ABA

with fluridone improves yield of droughted spring wheat, similar to DpM. Physiological traits on which the effects of DpM and fluridone were compared included photosynthesis, transpiration and relative water content (RWC); and yield and yield components.

5.3 Materials and Methods

5.3.1 Experimental site description

Two separate field experiments, one in spring and another in summer, were conducted in 2020 at the Birds' Nest Field, Harper Adams University, Shropshire, UK (52°46'N, 2°25'W). The soil textural classification in the top 60 cm at the site was determined to be a sandy loam (62% sand, 21% silt, 17% clay). The field capacity (FC) and permanent wilting point were determined as 19% (volumetric water content, VWC) and 9%, respectively. This means that the plant available water (PAW) is 10%. To simulate Mediterranean-type climate, we used polythene rain shelters (approximately 26 by 8 metres in size). The land was flooded with irrigation water on multiple days before ploughing, and plants grown under stored soil moisture, except for those in well-watered plots, which were included as a benchmark.

5.3.2 Experimental design and treatments

The experiments were arranged in a randomised complete block design. There were four treatments per block. In the spring experiment, these were the control (sprayed with water), DpM at 1 l/ha, fluridone at a concentration of 10 µM (F10) and a combination of DpM and 100 µM exogenous ABA (DpM+ABA). The experimental unit size was 1.0 m by 1.5 m by four plots per block of six blocks. In summer, two dose rates of fluridone (at 20 µM and 50 µM, F20 and F50, respectively) were used,

with F50 replacing the DpM+ABA mixture. The plot dimensions were 1.2 m by 1.2m and also with four spray types but eight replications. Treatments were randomly assigned to experimental units using GenStat 20th Edition (Hemel Hempstead, UK). A block of well-watered plots was included as a benchmark for assessing the extent of drought on physiological and agronomic variables. Benchmark plots were excluded from randomisation and statistical analyses as they were located at the end of the rain shelters to make irrigation easier and eliminate possibility of water trickling to the droughted plots.

5.3.3 Agronomy

Land preparation: Primary tillage was done using a two-furrow Kverneland tractor-drawn plough (Kverneland Group, UK Ltd). Secondary tillage followed immediately after, using a rotary harrow (Lely UK, Cambourne). This was particularly necessary for the benchmark plots which were under irrigation throughout the growing seasons. Nitrogen was applied at a rate of 100 kg N/ha, just after primary tillage, but before harrowing to incorporate the fertilizer.

Sowing: Planting was done on 23rd March in spring and on 17th June in summer. A fungicide-dressed spring wheat variety, Chilham (supplied by KWS, UK Ltd), used in both spring and summer, was hand-drilled. Hand-drilling was required to ensure comparable sowing rates between plots and experiments. The seed rate was 400 seeds m⁻², and with the germination and establishment percentages being 99% and 90%, respectively, the target plant population was ~356 plants m⁻². The amount of seed sown per plot was determined by weight after counting the number of seeds in weighed subsamples picked from the seedlot.

Weed control: A herbicide mixture of 143g/kg metsulfuron-methyl, 143g/kg tribenuron-methyl and 600g/L mecoprop-P was used to control weeds in the spring experiment. In summer, 500g/kg tribenuron-methyl was mixed with 600g/L mecoprop-P.

5.3.4 Spray application

The spray chemicals were applied on leaves at crop growth stage 37 (GS37) using a hand-held boom sprayer (Lunch Box Sprayer, Trials Equipment (UK) Ltd). In our previous experiment (Mphande et al., 2021), we found that the highest yield response of the spring wheat genotype, being studied, to DpM application under drought was at GS37. Table 1 shows the spray types and their respective concentrations.

Table 5.1: Spray application and endogenous ABA sampling dates

Season	Spraying*				Spike sampling			
	GS	Spray types	DAP	Date	GS	DAP	DAS	Date
Spring	GS37	Water, DpM,						
		DpM+ABA, F10,	57	19 th May	GS68	73	16	4 th June
Summer	GS37	Water, DpM, F20, F50	40	27 th July	GS68	64	24	20 th Aug

*GS - Growth stage; DAP - days after planting; DpM - di-1-*p*-menthene; F – fluridone at concentrations of 10 µM, 20 µM and 50 µM; DpM+ABA - combination of di-1-*p*-menthene and exogenous ABA.

5.3.5 Data collection

Due to logistical challenges associated with the COVID-19 pandemic, collection of some of the data in the spring experiment was delayed.

Meteorological data: Temperature and relative humidity inside the rain shelters were monitored using *Tinytag View 2* (Gemini Data Loggers UK Ltd, Chichester, England) data loggers. The mean daily values were calculated and graphs plotted.

Soil moisture: Soil moisture content in the top 60 cm was determined using a Time Domain Reflectometry (TDR) probe (TRIME-TDR, IMKO Micromodultechnik GmbH, Ettlingen, Germany). TDR access tubes were inserted into the soil just after sowing. Soil moisture levels were monitored at regular intervals from 42 DAP (GS31) in spring and 26 DAP (GS24) in summer (Figure 5.2) and ended at GS87 (physiological maturity), which was 123 DAP and 97 DAP in spring and summer, respectively. Readings were recorded as volumetric water content (%).

Endogenous ABA concentrations: To determine the effects of drought and spray applications on endogenous ABA concentration, three spikes were sampled into plastic tubes at GS68 (Table 1). After collection, each sample tube was immediately flash frozen in liquid nitrogen contained in a Dewar. Samples were stored at -80°C, in accordance with the ABA ELISA protocol. The assay was performed using Cusabio ABA ELISA protocol, code CSB-E09159PI, found at <http://www.cusabio.com>, whose detection range is 0.156µg/ml – 10µg/ml.

Gas exchange: Net photosynthesis and transpiration and leaf temperature were measured simultaneously using an infrared gas analyser (LC *pro-SD*, ADC BioScientific Ltd, England) on dates indicated below (Table 2). The photosynthetic

photon flux density (PPFD) was set to 1,500 $\mu\text{molm}^{-2}\text{s}^{-1}$. Measurements were carried out on a minimum of three fully expanded flag leaves per plot between 10:00 am and 2:00 pm, which is within the time stomata are fully open and conductance is highest (Correia et al., 1997; Grassi et al., 2009). Readings were logged when the system reached a steady state, which on average was after five minutes.

Table 5.2: Gas exchange and relative water content sampling dates

Season	Gas exchange				Relative water content			
	*GS	DAP	DAS	Date	GS	DAP	DAS	Date
Spring	GS75	81	24	12 th Jun	GS77	100	43	1 st July
Summer	GS54	51	11	7 th Aug				
	GS73	70	30	26 th Aug	GS73	69	29	25 th Aug

*GS - Growth stage; DAP - days after planting; DAS – days after spraying

Relative water content: The RWC of a plant is a measure of its water status expressed as a percentage of the fully turgid water content (Boyer *et al.*, 2008). RWC was determined according to the method described by Mullan and Pietragalla (2012) and calculated according to the mathematical expression: $\text{RWC (\%)} = [(FW - DW) / (TW - DW)] \times 100$, where FW, DW and TW represent the fresh weight, oven dry weight and turgid weight of the leaf, respectively. To allow leaves to attain turgidity, which was not achievable using the Mullan and Pietragalla (2012) method, we modified the protocol by making several incisions in each leaf lamina, about 30 mm apart. The incisions were made from one side across the midrib and close to the other margin. The leaves were then incubated in distilled water under light at room temperature for 18 hours before refrigeration at 4 °C for 24 hours. Due to restrictions

associated with the COVID-19 pandemic, sampling in spring was done late unlike in summer (Table 2).

Yield and yield components: We collected data on fertile spike density, grain number per m², fertile tillers per plant (tillering ratio), grain number per spike, thousand grain weight (TGW) and grain yield per hectare. Determination of fertile spike density per m² was done after attainment of physiological maturity (GS87) by counting grain-bearing spikes in three random samples per plot, using a 33 x 33 cm quadrat. Spikes bearing one or more grains were counted as fertile. The number of fertile tillers per plant was determined by systematically sampling 20 plants per plot (following a “W” or “M” pattern across a plot) away from the borders and counting the number of grain-bearing tillers per plant in the sample. Grain number was also determined by systematically sampling as for tillers to obtain ten main spikes and 10 primary tiller spikes. After harvesting, the crops were oven-dried at 65 °C to enhance threshing efficiency. An electrically powered threshing machine (F. Walter & H. Wintersteiger KG, Austria) was used to thresh the bulky samples. Smaller samples for determining number of grains per spike were threshed and counted manually. TGW was obtained by weighing 40g of grain, oven dried at 105 °C and as described Sylvester-Bradley *et al.* (1985). Samples for TGW were collected from the bulky grain of each plot and counted manually. The number of grains in this weight, minus the weight of broken grain, was also used in the calculation of number of grains per m² (Moeller and Rebetzke, 2017). Finally, grain yield per plot was determined by bulking and weighing all grain oven dried at 105 °C for each sample.

Statistical analyses: Analysis of variance (ANOVA) of endogenous ABA concentration, gas exchange, RWC and yield and yield components was performed

using GenStat 20th Edition (Hemel Hempstead, UK) to determine the significance of the treatments on these variables. *Post hoc* analysis was done with the Tukey test (set at 5%). Relationships between ABA and yield components were analysed using simple linear regression. Curves were fitted and modelling done using Microsoft Excel, but with constants in the models generated from GenStat.

5.4 Results

5.4.1 Temperature, relative humidity and vapour pressure deficit

The mean daily temperature inside the rain shelters between the day of planting and attainment of physiological maturity (GS87) for summer was significantly higher ($p < 0.001$) than for spring (Figure 5.1, a). The average mean daily temperature for summer was 19.6 °C, while for spring it was 14.9 °C. For relative humidity, the converse was true, with spring having a significantly higher ($p < 0.001$) mean daily value, 80.2%, than summer, 73.5% (Figure 5.1, b).

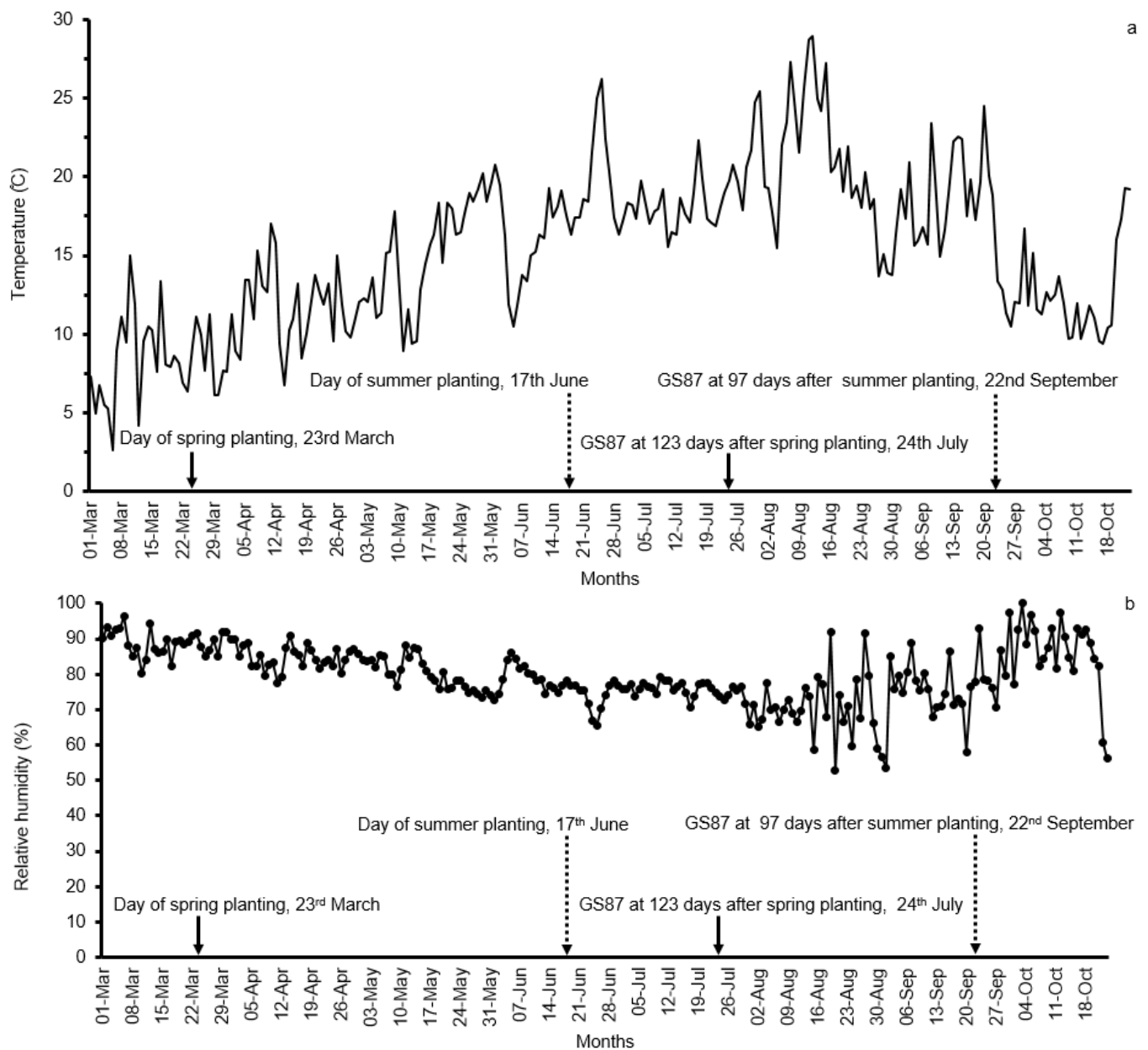


Figure 5.1: Mean daily temperature (a) and mean daily relative humidity (b) inside rain shelters across the spring and summer growing seasons. Bold arrows indicate the starting date and ending date (GS87) of the growing season for the spring-sown experiment while the broken ones are for the summer-planted experiment.

The vapour pressure deficit inside the rain shelters was significantly higher ($p < 0.001$) in summer than in spring. During the growing seasons, the mean daily vapour pressure deficit for spring and summer were 0.322 kPa and 0.531 kPa, respectively.

The vapour pressure deficit graph (not presented) followed a similar trend to the temperature graph.

5.4.2 Soil moisture

Soil moisture readings (%VWC) were taken at five different times between GS31 and GS87 in spring; and GS24 and GS87 in summer. In spring, soil moisture declined from 14.32%, at 42 DAP, to 10.04%, at 123 DAP this was above the PWP (Figure 5.2). The readings in summer were 13.30%, at 26 DAP and 8.92% at 97 DAP, this was slightly below PWP. At GS37, when the chemical treatments were sprayed, in spring, the PAW was depleted by 60% approximately; and in summer by 72%. At physiological maturity, 90% and 110% (below PWP) of PAW was depleted in spring and summer, respectively. Benchmark plots were irrigated to keep them close to field capacity.

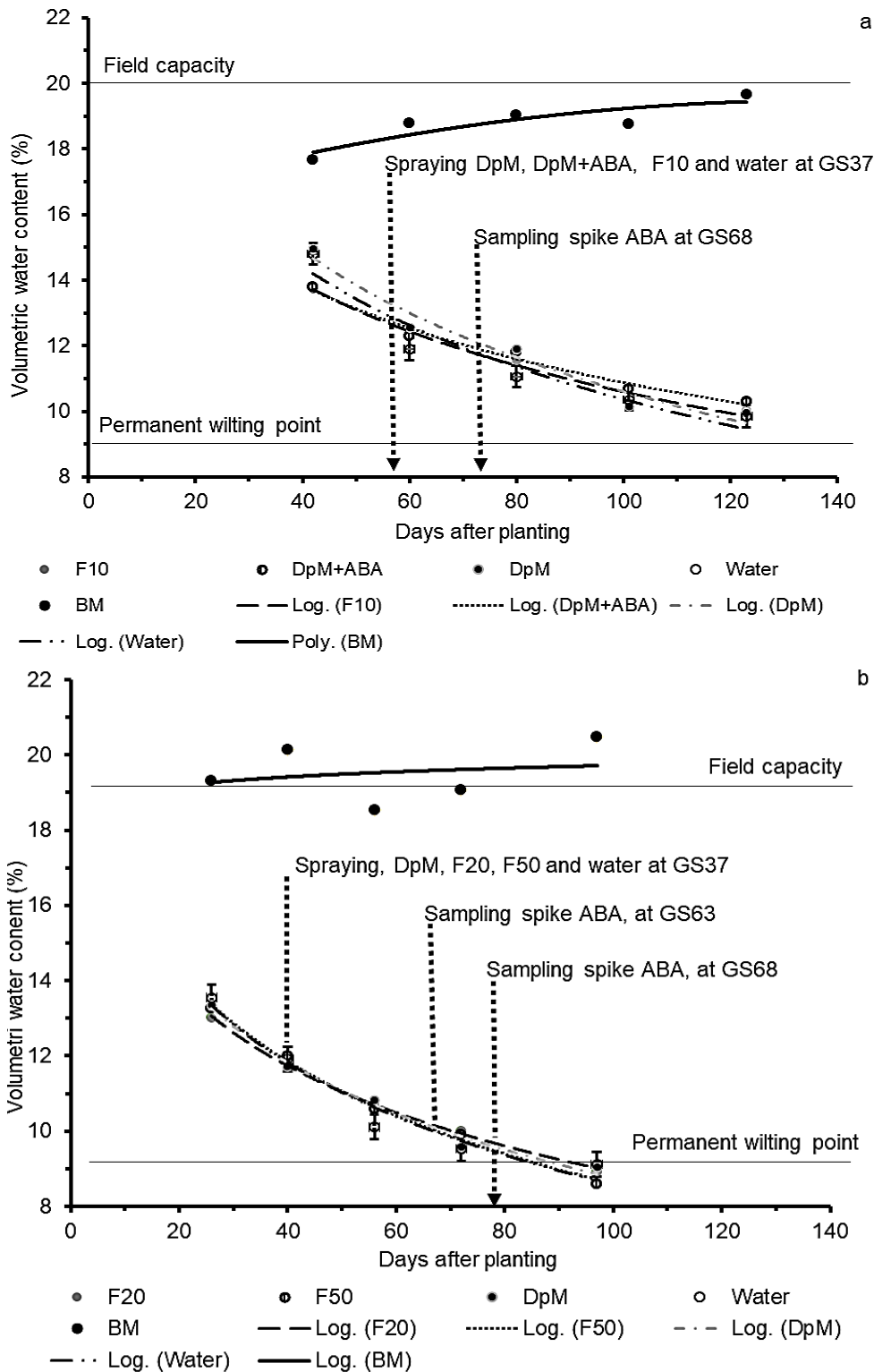


Figure 5.2: Changes in soil moisture content between 42 days after planting (DAP, GS31) and 123 DAP (GS87) in spring (a, $p = 0.971$, error bars, only on the Water treatment due to space limitation, represent standard error of regression, $SER = 0.331$); and 26 DAP (GS24) and 97 DAP (GS87) in summer (b, $p = 0.974$, $SER = 0.329$). BM, DpM, DpM+ABA, F10, F20 and F50 stand for benchmark, di-1-*p*-menthene, a

mixture of di-1-*p*-menthene and abscisic acid, and fluridone concentrations at 10 μM , 20 μM and 50 μM , respectively. Arrows mark days after planting when spray types were applied and spike ABA sampled.

5.4.3 Spike ABA

Drought increased spike ABA concentration by an average of 55% for the two seasons. The increase in summer (57%) was slightly higher than in spring (53%). Comparing the seasonal difference for the water-sprayed treatment, the accumulation was more than twofold higher in summer than in spring (Figure 5.3). In spring, application of F10 and DpM significantly curtailed the accumulation of spike ABA by the same percentage (25%). In summer, DpM and F50 reduced the concentration by 25% and 23%, respectively. Fluridone at the lowest concentration (F10) used in spring was more effective in suppressing the accumulation of spike ABA than at the higher concentrations in summer. The DpM+ABA treatment was not significantly different from the water-sprayed and the DpM-sprayed treatments, but intermediate between them.

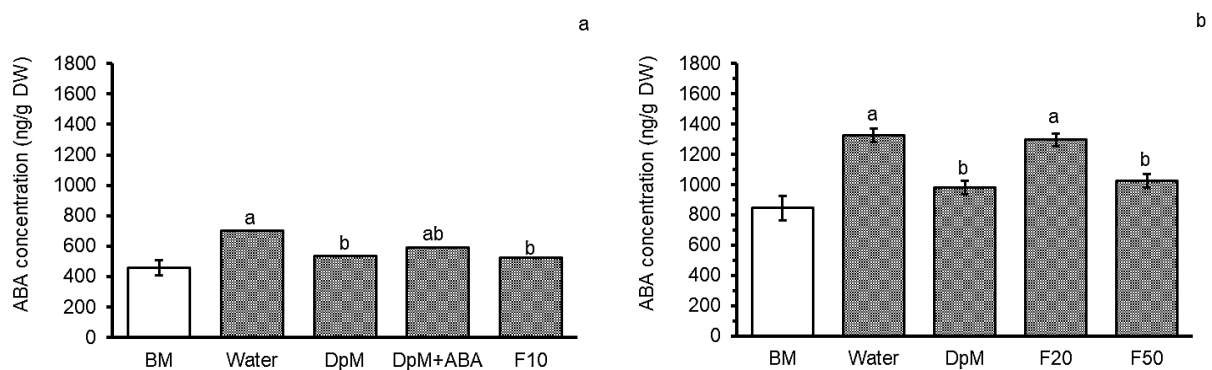
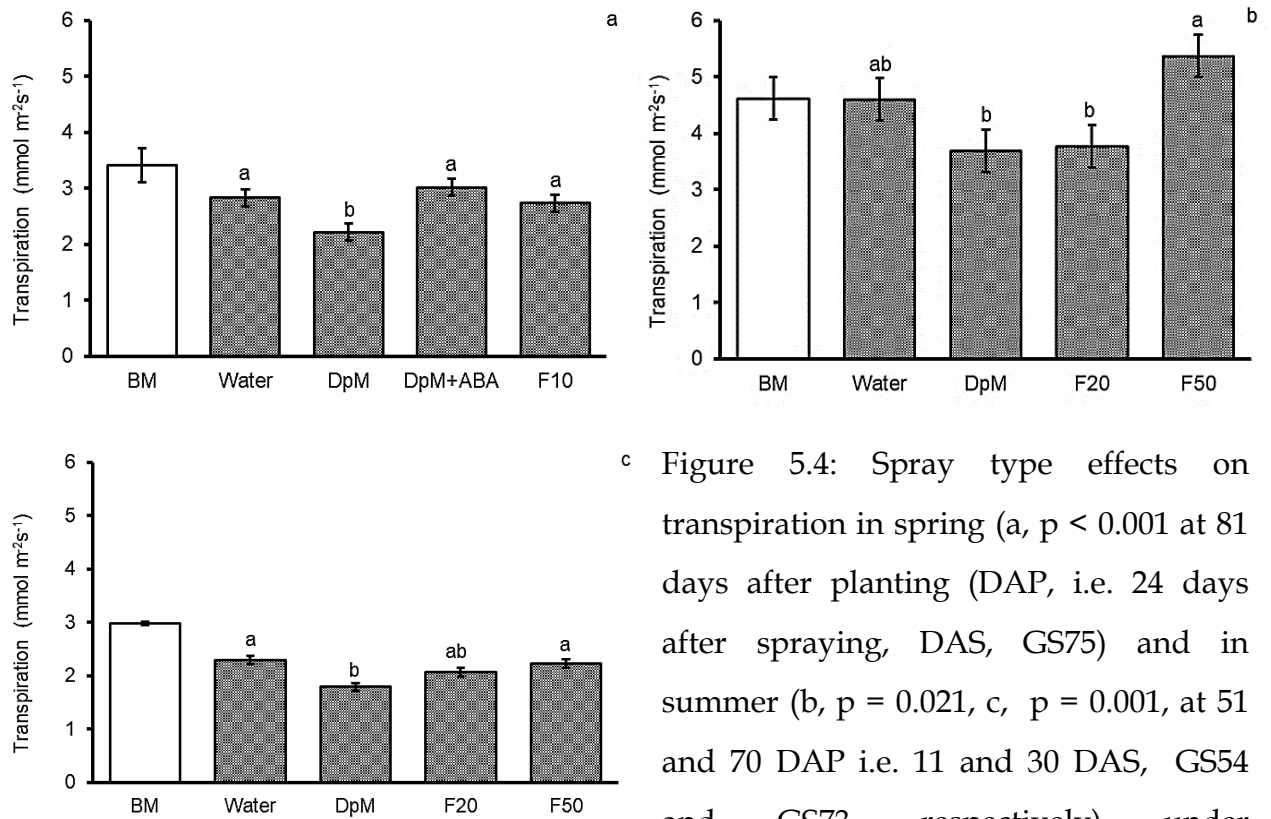


Figure 5.3: Spray type effects on spike ABA concentration in spring (a, $p = 0.017$) at 73 days after planting (DAP, 16 days after spraying, DAS, GS68) and in summer (b, $p < 0.001$) at 64 DAP, 24 DAS, GS68) under progressive drought except for the benchmark (BM). Error bars are common standard errors of means from the

ANOVA table. To facilitate irrigation, the BM plots were not part of randomisation hence data were not included in statistical analysis. Figure 5.3(a) was constructed from back transformed data. The spray type means on the natural log scale in the order water, fluridone at a concentration of 10 μM (F10), di-1-p-menthene (DpM), and DpM+ABA being 6.55, 6.262, 6.285 and 6.386 and SEM (0.0627). F20 and F50 stand for fluridone concentrations at 20 μM and 50 μM , respectively

5.4.4 Gas exchange

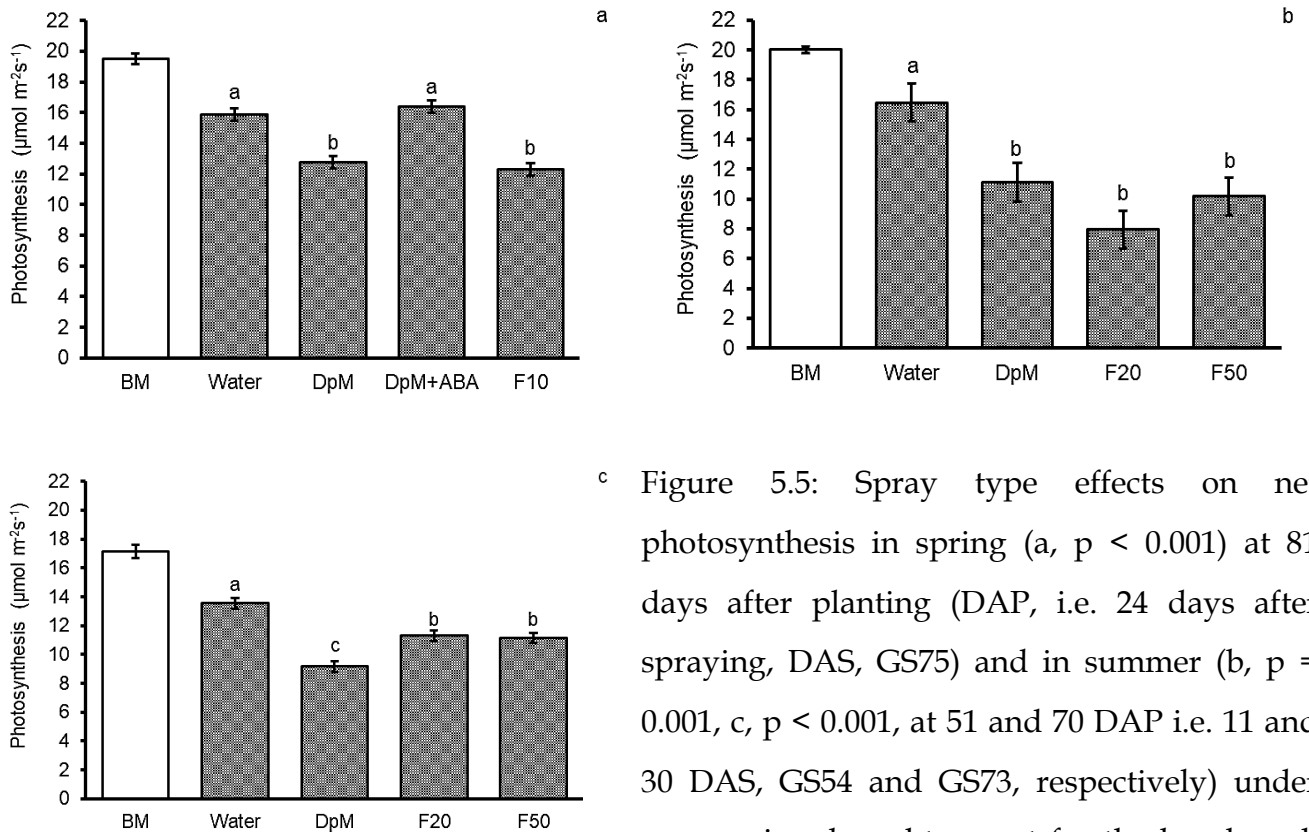
Drought stress reduced transpiration rates at 24 DAS and 30 DAS by 17% and 23%, respectively (Figure 5.4). Measurements in summer were taken twice, at 11 and 30 DAS. The application of fluridone and DpM+ABA had no significant effect on transpiration, although fluridone increased transpiration above that of the water-sprayed plants at 11 DAS (Figure 5.4, b). However, DpM+ABA had significantly higher transpiration compared to DpM. In contrast, DpM suppressed transpiration at 24 DAS and 30 DAS by an average of 21%.



c Figure 5.4: Spray type effects on transpiration in spring (a, $p < 0.001$ at 81 days after planting (DAP, i.e. 24 days after spraying, DAS, GS75) and in summer (b, $p = 0.021$, c, $p = 0.001$, at 51 and 70 DAP i.e. 11 and 30 DAS, GS54 and GS73, respectively) under

progressive drought except for the benchmark (BM). Error bars are common standard errors of means from the ANOVA table. To facilitate irrigation, the BM plots were not part of randomisation hence data were not included in statistical analysis. DpM, DpM+ABA, F10, F20 and F50 stand for di-1-*p*-menthene, a mixture of di-1-*p*-menthene and abscisic acid and fluridone concentrations at 10 μ M, 20 μ M and 50 μ M, respectively.

Drought consistently reduced net photosynthesis, particularly in summer. Application of DpM in spring and DpM, F20 and F50 in summer further reduced net photosynthesis, significantly (Figure 5.5). DpM was the most potent spray type in suppressing photosynthetic activity at 30 DAS.



c Figure 5.5: Spray type effects on net photosynthesis in spring (a, $p < 0.001$) at 81 days after planting (DAP, i.e. 24 days after spraying, DAS, GS75) and in summer (b, $p = 0.001$, c, $p < 0.001$, at 51 and 70 DAP i.e. 11 and 30 DAS, GS54 and GS73, respectively) under progressive drought except for the benchmark

(BM). Error bars are common standard errors of means from the ANOVA table. To facilitate irrigation, the BM plots were not part of randomisation hence data were not included in statistical analysis. DpM, DpM+ABA, F10, F20 and F50 stand for di-1-*p*-menthene, a mixture of di-1-*p*-menthene and abscisic acid and fluridone concentrations at 10 µM, 20 µM and 50 µM, respectively.

5.4.5 Relative water content

Following the protocol for determining RWC described by Mullan and Pietragalla (2012), leaves of the spring wheat genotype, Chilham, failed to attain turgidity after 24 hours of incubation at 4 °C. The saturated leaf weights were almost the same as their fresh weights, therefore giving inflated RWC, which even for droughted plants were as high for the benchmark (Figure 5.6, a). In the summer experiment, a pre-refrigeration treatment involving making several incisions in the lamina, spaced approximately 30 mm apart, followed by an 18 hour-long incubation in distilled water

at room temperature and under light induced turgidity, revealing treatment differences (Figure 5.6, b). Compared with the water-sprayed plants, DpM increased RWC while F50 depressed it.

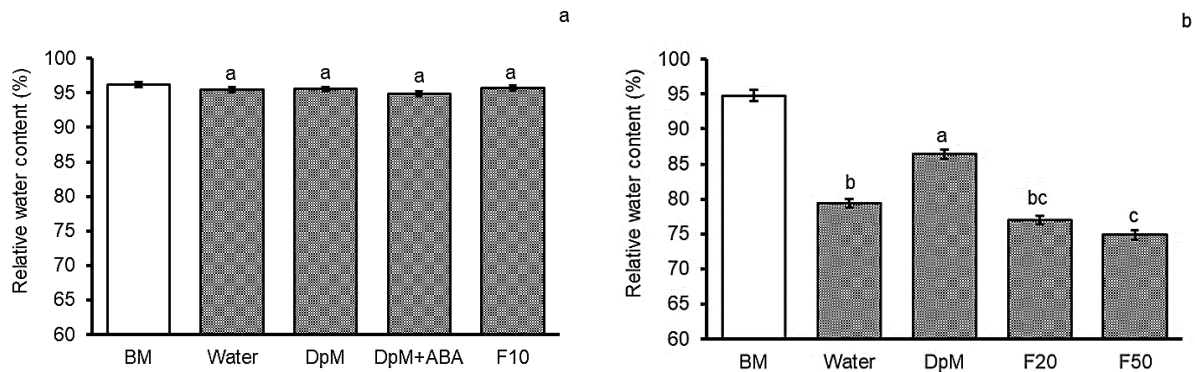


Figure 5.6: Spray type effects on relative water content in spring (a, $p = 0.425$) at 100 days after planting (DAP, i.e. 43 days after spraying, DAS, GS77) and in summer (b, $p < 0.001$, at 69 DAP i.e. 29 DAS, GS73) under progressive drought except for the benchmark (BM). Error bars are common standard errors of means from the ANOVA table. To facilitate irrigation, the BM plots were not part of randomisation hence data were not included in statistical analysis. DpM, DpM+ABA, F10, F20 and F50 stand for di-1-*p*-menthene, a combination of di-1-*p*-menthene and abscisic acid and fluridone concentrations at 10 μM , 20 μM and 50 μM , respectively.

5.4.6 Yield and yield components

All yield component quantities in spring were higher than in summer (Figure 5.7 to 5.9). Drought reduced all yield components except the number of grains per spike in spring and the thousand grain weight in both seasons. Compared to the benchmark, drought reduced the number of fertile tillers per plant (74%), fertile spike density (54%), grains per m^2 (63%) and yield (42%). Plants in spring developed fertile secondary tillers in all plots but the number of grains per spike was not significantly different between treatments (results not shown), however there were much fewer

tertiary tillers. Only a few plots had plants with secondary tillers in summer. In both seasons, application of DpM was effective in improving number of tillers per plant (75%), fertile spike density (37%), number of grains per m² (26%) and grain yield (27%), relative to the water-sprayed plants and all averaged across the two seasons. DpM also improved the number of grains per spike in main stems (20%) but this was only in summer. The only yield component significantly improved with another spray type other than DpM was number of tillers per plant with F20, which was in summer. Overall, the effects of fluridone on grain yield and yield components were negligible.

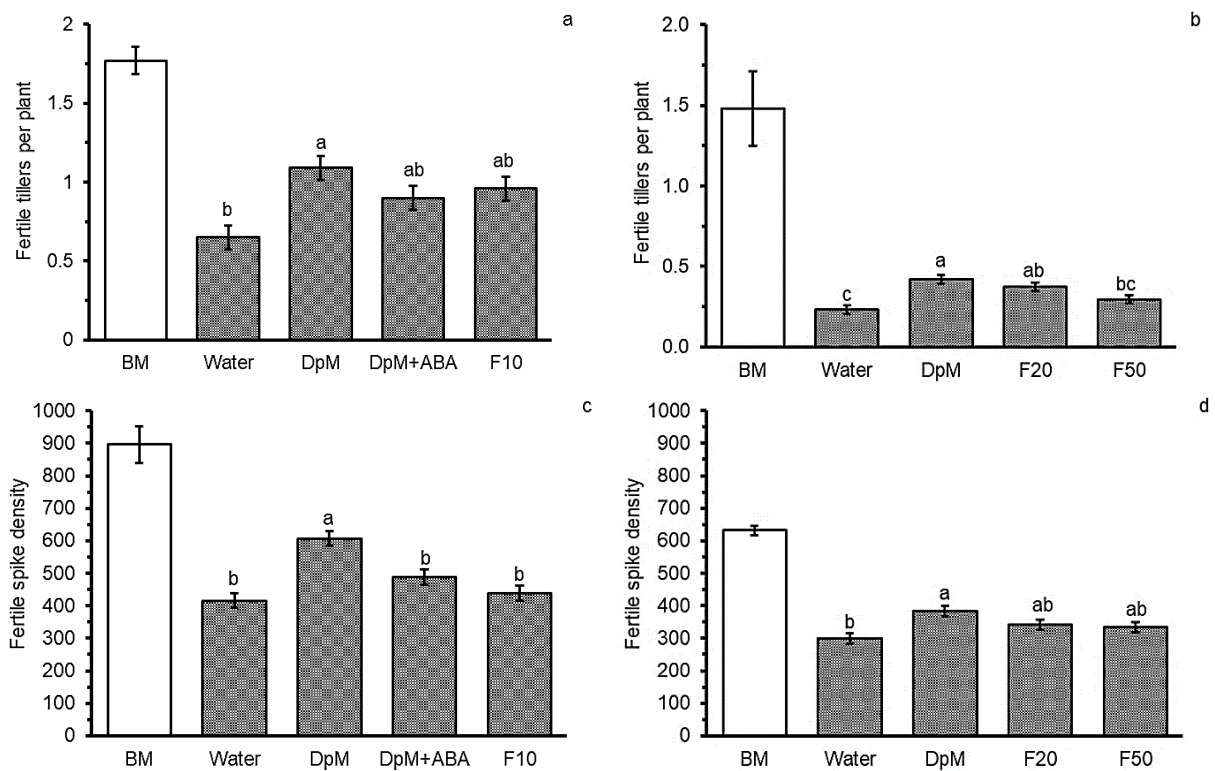


Figure 5.7: Spray type effects on number of fertile tillers per plant and fertile spike density in spring (a, $p = 0.012$, c, $p < 0.001$) and summer (b, $p < 0.001$, d, $p = 0.017$), respectively, under progressive drought except for the benchmark (BM). Error bars are common standard errors of means from the ANOVA table. To facilitate irrigation, the BM plots were not part of randomisation hence data were not included in statistical analysis. DpM, DpM+ABA, F10, F20 and F50 stand for di-1-p-

menthene, a mixture of di-1-*p*-menthene and abscisic acid and fluridone concentrations at 10 μ M, 20 μ M and 50 μ M, respectively.

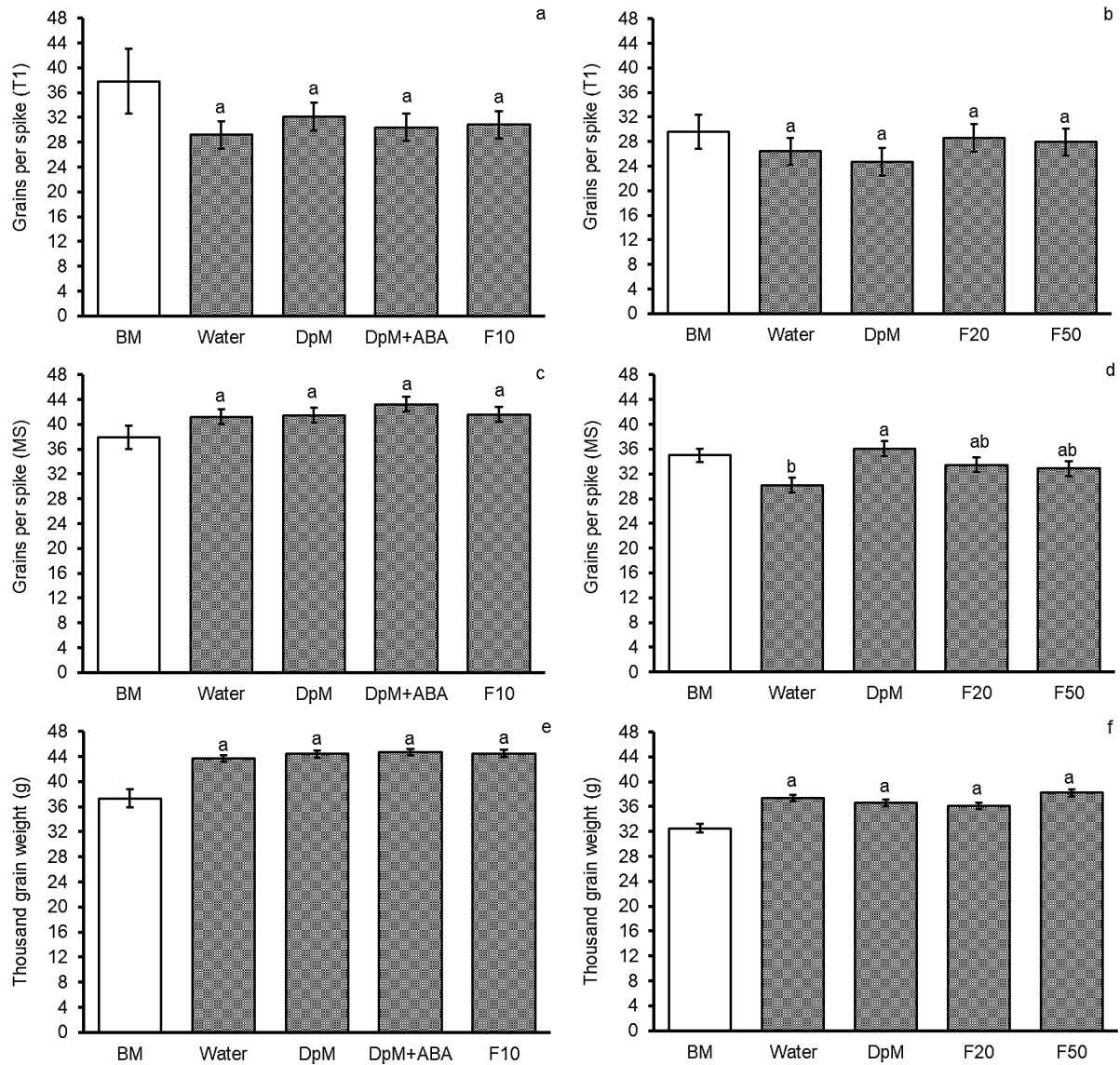


Figure 5.8: Spray type effects on number of grains per spike of primary tillers (T1) and of main stems (MS) and thousand grain weight in spring (a, $p = 0.833$, c, $p = 0.643$, e, $p = 0.604$) and summer (b, $p = 0.630$, d, $p = 0.017$, f, $p = 0.060$), respectively, under progressive drought except for the benchmark (BM). Error bars are common standard errors of means from the ANOVA table. To facilitate irrigation, the BM plots were not part of randomisation hence data were not included in statistical analysis. DpM, DpM+ABA, F10, F20 and F50 stand for di-1-*p*-menthene, a mixture of

di-1-*p*-menthene and abscisic acid and fluridone concentrations at 10 μ M, 20 μ M and 50 μ M, respectively.

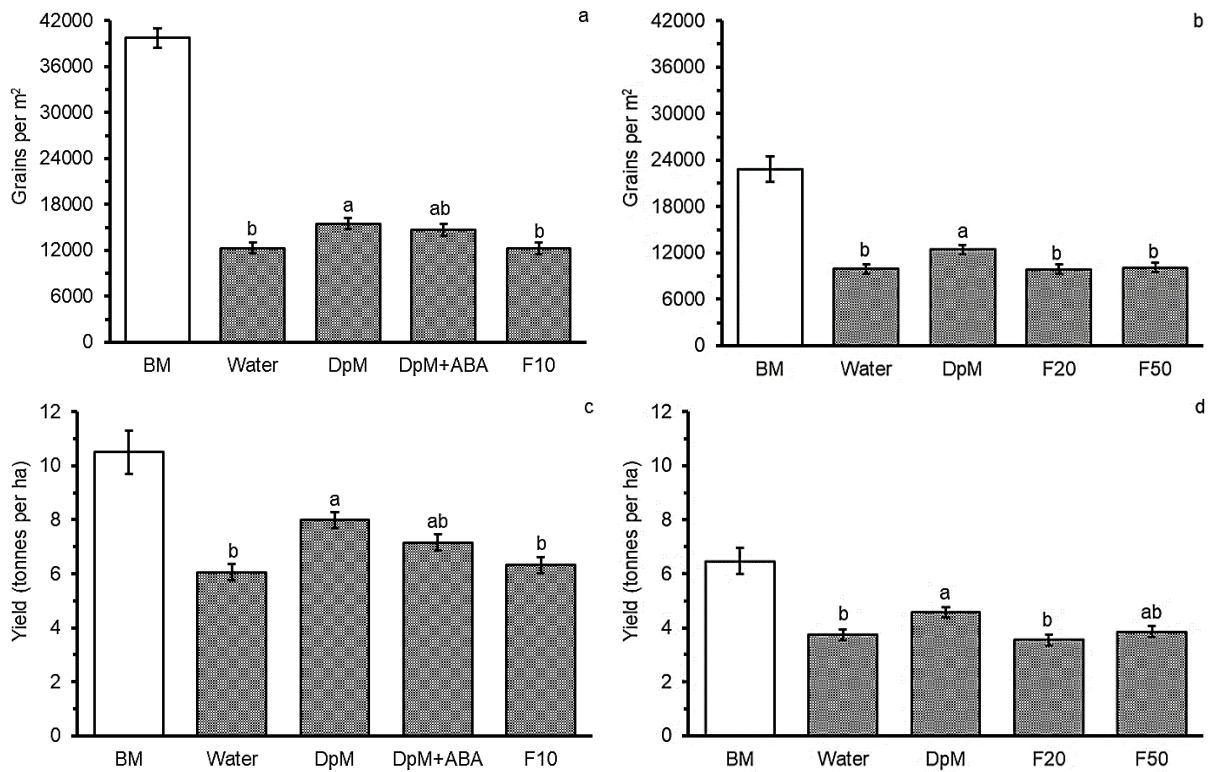


Figure 5.9: Spray type effects on number of grains per m², yield per ha (tonnes) in spring (a, $p = 0.015$, c, $p = 0.003$) and summer (b, $p = 0.012$, d, $p = 0.012$), respectively, under progressive drought except for the benchmark (BM). Error bars are common standard errors of means from the ANOVA table. To facilitate irrigation, the BM plots were not part of randomisation hence data were not included in statistical analysis. DpM, DpM+ABA, F10, F20 and F50 stand for di-1-*p*-menthene, a mixture of di-1-*p*-menthene and abscisic acid and fluridone concentrations at 10 μ M, 20 μ M and 50 μ M, respectively.

5.4.7 Regression analysis

We attempted to run multiple regression analysis of spike ABA and transpiration as explanatory variates against yield and yield components, with season as the grouping factor. However, due to a strong collinearity between spike ABA and transpiration ($r = 0.93$), we performed simple linear regressions. Accumulated analysis of variance showed that the grouping factor was not significant for any response variate. All interactions were also not significant except for number of tillers per plant, with a much weaker interaction effect with spike ABA ($p = 0.045$), against a stronger explanatory variate effect ($p < 0.001$). All the data were therefore best fitted with a common regression line. Figure 5.10 is a summary of analysis of spike ABA with yield and yield components. TGW and number of grains per spike were left out of the analysis, being not significantly and partially significantly affected with spray type treatments, respectively. In spring, although F10 and DpM had the same concentration of ABA there is a very wide yield gap. In summer, there was no yield benefit of reduction in ABA concentration with application of F50 compared to F20. In addition, at about the same level of ABA concentration for DpM and F50, the yield gap is also wide. The gap between DPM and F10 is even wider for grains per m^2 . The associations between transpiration and yield components were also significant (Figure 5.11). The similarity of the associations of spike ABA and transpiration with yield components reflects the collinearity between the explanatory variates.

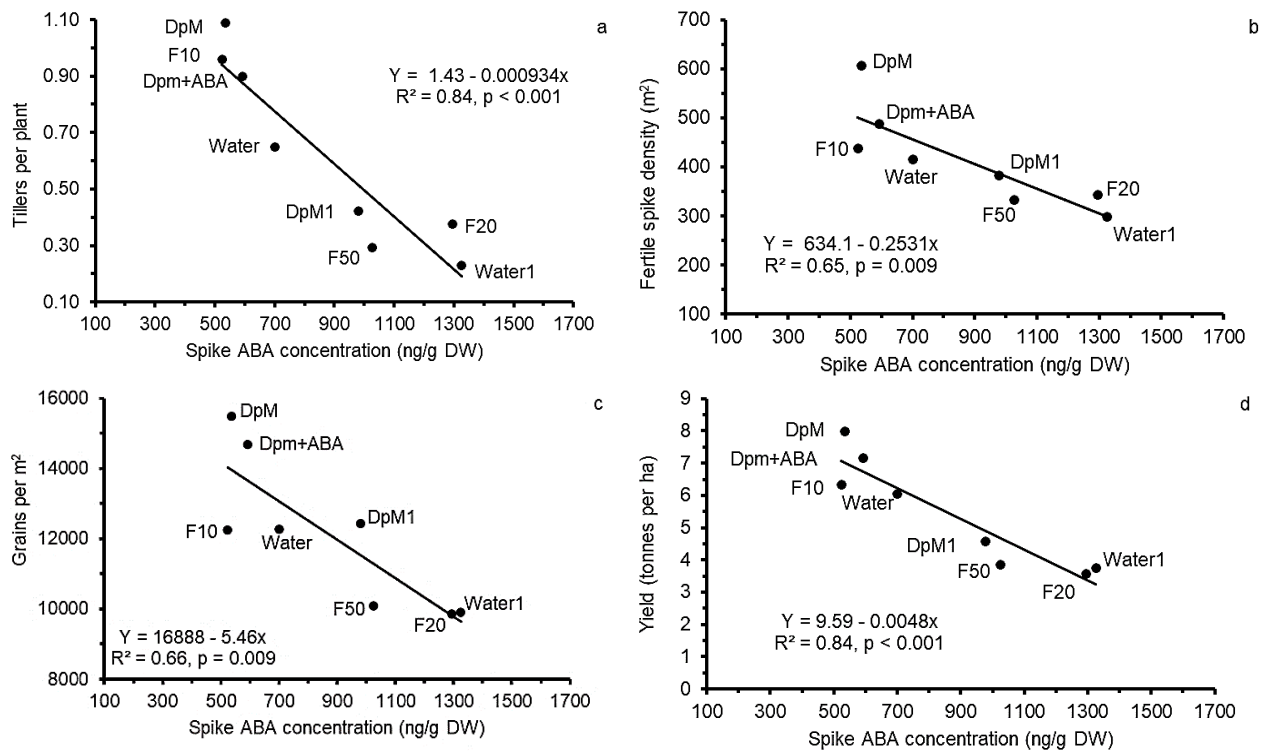


Figure 5.10: Regression analysis with pooled data of spike ABA with tillers per plant (a), fertile spike density (b), grains per m² (c) and grain yield (d). DpM, DpM1, DpM+ABA, F10, F20, F50 and Water1 stand for di-1-*p*-menthene in spring, di-1-*p*-menthene in summer, a mixture of di-1-*p*-menthene and abscisic acid and fluridone concentrations at 10 μM, 20 μM, 50 μM and water treatment in summer, respectively.

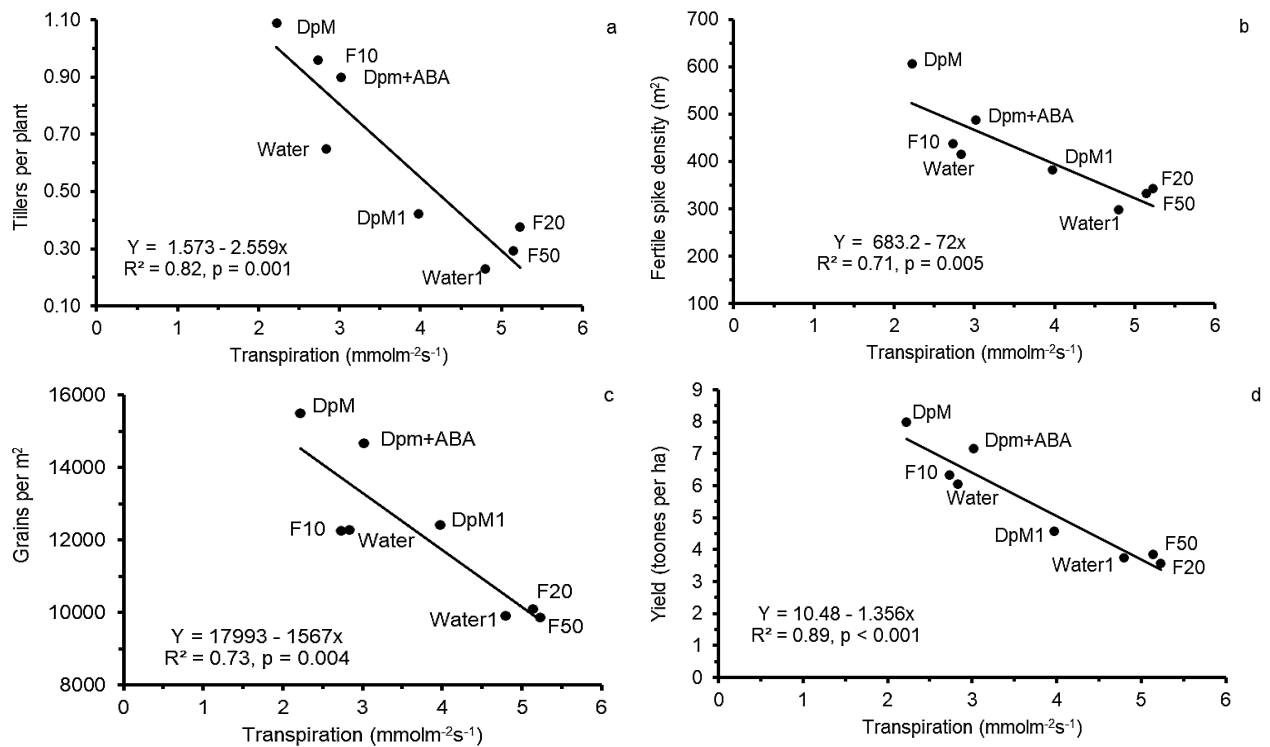


Figure 5.11: Regression analysis with pooled data of transpiration (at 24 days after spraying (DAS) in spring and 11 DAS in summer) with tillers per plant (a), fertile spike density (b), grains per m² (c) and grain yield (d). DpM, DpM1, DpM+ABA, F10, F20, F50 and Water1 stand for di-1-*p*-menthene in spring, di-1-*p*-menthene in summer, a mixture of di-1-*p*-menthene and abscisic acid and fluridone concentrations at 10 μM, 20 μM, 50 μM and water treatment in summer, respectively.

5.5 Discussion

We successfully simulated Mediterranean-type drought in both rain shelter experiments, with no additional irrigation following pre-planting flooding. The level of drought achieved through progressive soil drying was sufficient to induce terminal water deficit stress. On average, for the two experiments, drought increased from 66% depletion of PAW at flag leaf emergence to 80% depletion of PAW at physiological maturity, compared to 45% maximum allowable depletion for wheat (Panda et al., 2003). Drought stress is one of the important environmental factors

responsible for wide yield gaps in wheat production, higher than 50% of genetic potential (Monneveux et al., 2012). Percentage grain yield losses in wheat ranging from 1% to 92% have been reported depending on timing, intensity and duration of drought stress (Farooq et al., 2014; Figueroa-Bustos et al., 2020). In the present study, drought stress reduced grain yield by 42% (average of the two experiments).

Drought stress downregulates photosynthesis, transpiration and RWC (Maghsoudi et al., 2016) but upregulates endogenous ABA (Pang et al., 2017). Under ideal soil moisture conditions, the rate of transpiration is positively correlated with biomass accumulation but not under water deficit, when higher water use efficiency becomes more important (Araus et al., 2002). Reduced stomatal conductance mediated through an increase in drought-induced ABA (Lee and Luan, 2012), contributes to improving the intrinsic water use efficiency (Sikder et al., 2015). In the current study, drought reduced gas exchange overall. That the application of fluridone did not reduce transpiration but negligibly increased it, at the highest concentration (50 μ M, F50), was likely through inhibition of ABA-mediated stomatal closure due to ABA deficiency. Transpiration rates in fluridone-sprayed plants did not significantly rise above the water-sprayed control, probably due to the fact that, unlike other ABA inhibitors such as norflurazon, fluridone does not affect the synthesis of ABA-precursors (carotenoids) already present at the time of its application (Dayan and Zaccaro, 2012). This means that fluridone had no effect on pre-existing levels of endogenous ABA, which would continue to impact on stomatal closure (although to a lesser degree than in water-sprayed plants). Deficiency of ABA might induce wilting due to excessive transpirational water loss, as stomatal closure is impaired (Dodd, 2013; Schippers et al., 2015). Consequently, F50 significantly reduced the RWC. In contrast, by physically blocking stomata and suppressing transpiration, DpM

increased RWC (summer experiment) despite reducing ABA. Improved plant water status under drought conditions is necessary for sustaining biological activities in cells (Farooq et al., 2009). This agrees with regression analysis as transpiration was negatively associated with yield and most of the yield components studied under drought stress conditions. This suggests that the positive linear relationship between transpiration and grain yield (Acevedo et al., 2002; Araus et al., 2002) does not hold under prolonged drought stress but under well-watered conditions, as Siahpoosh and Dehghanian, (2012) observed in wheat. This is because, stomatal closure accounts for not more than 20% of drought-induced inhibition of photosynthesis (Jones, 1998). Under limited soil water conditions, reducing transpiration to conserve water for reproductive processes is one of the desirable drought tolerant traits (Kholova et al., 2010; Sinclair et al., 2017), e.g. a maize genotype with a transpiration-limiting trait under drought stress improved yield by 6.5% (Gaffney et al., 2015). We know that despite different modes of action, antitranspirants suppress both photosynthesis and transpiration, and that for DpM, this is by physically blocking stomata (Mphande et al., 2020). There is abundant evidence in support of DpM improving grain yield of droughted wheat compared to the untreated droughted plants. The reported percentage improvements vary (e.g. Abdullah et al., 2015 (30%), Kettlewell and Holloway, 2010 (42%), Mphande et al., 2021 (29%) and Weerasinghe et al., 2016 (10%). In our study, we found that DpM significantly suppressed both photosynthesis and transpiration nonetheless the grain yield was significantly higher (27%, average for the two experiments) than for the control (water-sprayed) treatment. Application of DpM under drought has been found to reduce transpiration but increase water use efficiency in rapeseed (Patil and De, 1976) and beans (Iriti et al., 2009). Weerasinghe et al., (2016) reported improved

plant water status following DpM application in droughted wheat. As for the DpM+ABA treatment, it might be said that this was a direct test of whether the DpM effect is due to reduced ABA or not. Considering the yield and yield components results indicates that DpM+ABA did reduce the effectiveness of DpM. However, the regressions suggest that overall, ABA concentration might not be the key factor.

Besides inhibiting chlorophyll and carotenoid biosynthesis in higher plants, which are better known fluridone effects, it has other morphophysiological attributes. Fluridone decreases plant water balance through suppression of stomatal closure (Vysotskaya et al., 2018), due to inhibition of ABA biosynthesis (Kondhare et al., 2014; Zou et al., 2018), leading to increased transpiration rates (Hsu and Kao, 2005; Liu et al., 2010). Soil drying induces increased ABA biosynthesis, which causes stomatal closure for water conservation (Saradadevi et al., 2014). Decreased RWC has been reported in stressed maize (Zhang et al., 2012) and rice (Wei et al., 2015). Further, according to Spollen et al. (2000) and Sharp and LeNoble (2002), fluridone inhibits root elongation in maize. Drying of the top layers of the soil profile is a characteristic feature of terminal drought (Saradadevi et al., 2014). Inhibition of root elongation therefore limits accessibility to moisture in deeper soil layers, which is critical during grain filling. Not only root mass but also accumulation of shoot biomass is reduced, and this has been observed both in crops (e.g. canola, Tidemann et al., 2017, and maize, Zhang et al., 2012) and various weed species (Cheshier et al., 2011; Dawuda et al., 2020; Tidemann et al., 2017). In droughted wheat, fluridone downregulated spike ABA and reduced grain filling rates and grain weight (Yang, J. et al., 2006). In our study, induction of negative water balance (F50) may explain why fluridone was ineffective in improving grain yield, as suppression of photosynthetic activity and

curtailing of the accumulation of spike ABA were common to both fluridone and DpM. Thus, it can be said that fluridone lacked the combination of downregulating spike ABA and improving the plant water status of droughted spring wheat. It is this combination that was the likely mechanisms through which DpM conferred ameliorative significance to droughted spring wheat.

Fluridone further suppressed photosynthesis compared to the water-sprayed treatment. Fluridone is a well-known inhibitor of pigment biosynthesis, including chlorophyll and carotenoid and can bleach leaves (Dayan and Zaccaro, 2012; Zou et al., 2018), thus it reduces photosynthesis. Reducing photosynthesis due to drought induces a decline in grain yield (Figuroa-Bustos et al., 2020). Unlike fluridone, downregulation of both photosynthesis and spike ABA accumulation with DpM ameliorated drought effects on yield, increasing it by 27% compared to the water-sprayed control. Here, we have demonstrated that despite DpM reducing photosynthesis, significantly below the water-sprayed treatment, the benefit of applying the antitranspirant outweighed the penalty on photosynthetic activity. This was likely due to the suppression of transpiration, which was significantly lower than in the water-sprayed and in fluridone-sprayed plants except in the summer F20 treatment. The regression analysis (Figure 5.10 d) shows that the general relationship between endogenous ABA and yield is strong, however, it also indicates that for specific treatments, there is a lot of scatter around the regression line. These observations indicate that endogenous ABA was not the sole mechanism controlling yield formation.

Reducing transpiration under terminal drought conditions appears to be more critical than decreasing photosynthesis as stomatal closure affects not more than 20% of

the total drought-induced inhibition of photosynthesis (Jones, 1998). The penalty on photosynthesis is therefore outweighed by reduced transpiration induced by DpM application. The significance of reduced transpiration resulted in increased RWC in DpM-sprayed plants. Improvement of plant water status reduces the negative effects of drought-induced ABA on reproductive performance of wheat (Westgate et al., 1996). Our failure to detect treatment effects on RWC in spring was protocol-related, resulting in droughted plants having values equivalent to the benchmark. Leaves did not absorb sufficient amounts of water, making turgid leaf weights to be almost the same as fresh leaf weights, inflating the RWC values. A similar result was obtained in the summer experiment (results not presented) before modification that reflected treatment differences was made to the commonly used protocol (Mullan and Pietragalla, 2012). The pre-refrigeration modification involved making several incisions in the leaf lamina, spaced approximately 30 mm apart, followed by an 18 hour-long incubation in distilled water at room temperature and under light. The turgid leaf weights were much higher than their respective fresh leaf weights revealing treatment differences.

Application of DpM, which was associated with reduced spike ABA and suppressed transpiration, improved grain yield by 27%, averaged for the experiments. This corroborates Mphande et al. (2021) who found that DpM improvement of grain yield was correlated with reduced endogenous ABA. The levels of spike ABA in the spring experiment for the present study were slightly higher than what Mphande et al. (2021) reported in an experiment that was also established in spring (e.g. for the water-sprayed treatment 700 ng/gDW with sampling at GS68 against just below 569 ng/gDW with sampling at GS55, respectively). However, the concentration in the summer experiment for the same treatment was much higher, being 1325 ng/gDW.

These differences can be attributed to growth stage and seasonal effects. It is known that endogenous ABA increases during grain filling to enhance senescence and remobilisation of water-soluble carbohydrates stored in stems and leaf sheaths (Kondhare et al., 2015; Mphande et al., 2016; Yang et al., 2003). Our findings are consistent with other past reports, which also linked elevated endogenous ABA to reduced crop productivity. Examples include suppression of seed set in maize (Setter et al., 2001) and wheat (Saini and Aspinall, 1982, Westgate et al., 1996) and abortion of reproductive organs in chickpeas (Pang et al., 2017). In barley, elevated ABA in apical and basal spikelets correlated with increased abortion compared with central spikelets, which accumulated lower levels of the hormone (Boussora et al., 2019). An *in vitro* study on wheat spikes and grains by Ahmadi and Baker, (1999) showed that elevated endogenous ABA suppressed the supply of sucrose to grains and also repressed soluble starch synthase and granule-bound starch synthase, enzymes involved in starch synthesis. Thus, repression of assimilate supply to reproductive organs such as developing grain due to high endogenous ABA levels may be the mechanism by which the hormone arrests grain growth and induces organ abortion. Agronomically, DpM improvement of yield was achieved through gains in number of grain-bearing tillers per plant, fertile spike density and grain number per m². Under given environmental conditions, the grain number per spike declines with increasing plant density beyond the optimum (Li et al., 2016). We found limited evidence that DpM improves grain number per spike, as this was only in main stem spikes in the summer experiment. In three of our previous field experiments, which were all established in spring, DpM did not have a significant effect on grain number per spike. This suggests that DpM does not have a large mitigative effect on meiotic-stage drought, which is relevant to grain number per spike (Ji et al., 2011;

Westgate et al., 1996). Grain number per spike therefore might be a less important trait in DpM amelioration of drought. However, there was a significantly higher number of grain-bearing tillers per plant, fertile spike density and grain number per m² attributed to DpM. Severe drought coinciding with reproductive development is known to induce abortion of flowering heads due to elevated ABA (Morgan, 1980; Pang et al., 2017), undermining fertile spike density (Katerji et al., 2009). Reproductive development phase is bounded by end of vegetative development, which is marked by spikelet initiation, and anthesis (Slafer et al., 2015). Some of the key processes in between are spikelet morphogenesis, spike growth and differentiation of floret primordia into florets (Gol et al., 2017; Sreenivasulu and Schnurbusch, 2012). Specific timings of these events can be found in Mphande et al. (2021). It is also known that drought stress during these reproductive phases in wheat shortens the growth cycle; and decreases the number of spikes per m² and grain yield (Day and Intalap, 1970). Application of 60 mg/L (~ 227 µM) of exogenous ABA at stem elongation repressed the number of wheat stems (i.e. spikes) per unit area but it had no effect at later growth stages (Cai et al., 2014). Wheat genotypes that are drought-tolerant prevent spike ABA accumulation under drought conditions through repression of ABA biosynthesis genes and upregulation of catabolic genes (Ji et al., 2011). Our regression analysis supports these reports, as elevated ABA, induced by drought stress, was negatively associated with fertile tillers per plant, fertile spike density and grain number per m², which are the foundation of grain yield. From our present study and the findings of Mphande et al. (2021), it is plausible to suggest that DpM amelioration of drought in spring wheat is more connected to spike morphogenesis and fertility. This effect is mediated directly by improved plant water status and probably indirectly by ABA levels in the spikes affected by plant water

potential. On the other hand, ABA may have a direct role by repressing transport of sucrose to the spikes and suppressing the soluble starch synthase, granule-bound starch synthase and Adenosine diphosphate glucose pyrophosphorylase the starch synthesis enzymes, as Ahmadi and Baker (1999) found for developing grains. Our results demonstrate the potential of DpM as a drought mitigation technique that can contribute significantly to reducing yield losses in spring wheat in Mediterranean-type climates and semi-arid regions that have predictable drought events.

5.6 Conclusion

Although both DpM and fluridone reduced photosynthesis and suppressed the accumulation of spike ABA, only DpM decreased transpiration and improved grain yield. This suggests that amelioration of drought stress with DpM partially requires control of not only the accumulation of endogenous ABA but also transpirational water loss.

Author contribution statement

PK conceived this research. WM, AF, LV, IG and PK designed the research. WM conducted experiments. WM analysed data. WM wrote the manuscript. PK, AF and LV assisted in reading and correcting the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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6 General Discussion and General Conclusion

6.1 General discussion

Effects of drought on physiology and yield and yield components

Endogenous ABA: Drought increased endogenous ABA concentration in all our experiments, in agreement with the long-established knowledge of the effects of water deficit on the hormone (Bray, 1988; Raghavendra et al., 2010). On average, the leaf ABA concentrations in our field experiments between stem elongation and booting growth stages fall within the range observed at the young microspore stage (booting stage, Westgate et al., 1996, Ji et al. (2011)). We consistently observed a decline in leaf ABA concentration from stem elongation to ear emergence, which indicated that accumulation of the hormone declined with increasing leaf age. It has long been known that in wheat, younger leaves accumulate higher levels of ABA (Quarrie and Henson, 1981). While the highest accumulation of leaf ABA in the field experiments was in 2018 (1893 ng/gDW at GS33), values in the glasshouse experiments were much higher, with the peak in the unsprayed control being 5899 ng/g DW, also in 2018. This is likely due to the fact that in pot experiments there is restricted soil volume available for water extraction (Poorter et al., 2012; Van Iersel, 1997), although nutrient uptake may still proceed normally. Consequently, shoot growth and expansion in pot experiments are reduced. For example, the rooting depth for winter wheat and spring wheat can reach up to 2.5m and 1.1m, respectively (Thorup-Kristensen et al., 2009) but pots are shallow (~30 cm in this study). This means that drought in pots developed quicker, triggering a sudden rise in ABA concentration than under field conditions. Our mean spike ABA values in the spring-planted field experiments were 569 ng/g DW and 700 ng/g DW in 2019 and

2020, respectively. These values were comparable to what Ji et al. (2011) reported, which ranged between 331 and 717 ng/g DW. However, in our summer experiment, under a shorter but warmer growing season, the mean value was 1325 ng/g DW. The spike ABA levels in 2019 were much lower than in their leaf counterparts. This observation is in agreement with the findings of other reports (e.g. Westgate et al., 1996 and Ji et al. 2011). As Westgate et al.(1996) found, this is because floral organs retain higher water status than leaves.

Gas exchange: We reported gas exchange variables under the glasshouse experiments and the last two field experiments, Chapters 4 and 5, respectively. Drought suppressed photosynthesis, stomatal conductance and transpiration, as expected (Maghsoudi et al., 2016). Photosynthesis was not responsive in the 2018 glasshouse experiment, likely due to insufficient light stimulation, which was set to a much lower level of $250 \mu\text{mol m}^{-2} \text{s}^{-1}$, against $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ in 2019.

RWC: Drought significantly reduced the RWC of water-sprayed plants. This result was obtained in one out of the five experiments in which RWC was determined (results presented only for the last two experiments). In the four experiments where we failed to detect treatment differences, we used the protocol described by Mullan and Pietragalla (2012). A number of challenges have been recognised in determining RWC, some of which are protocol-related while others are due to species differences (Arndt et al., 2015). In wheat and barley, Boyer et al. (2008) observed excessive hydration, giving inflated turgid weights, which from the mathematical expression for obtaining RWC correspond to lower RWC values. To the contrary, in our studies, spring wheat leaves did not absorb sufficient water to attain turgidity, making the turgid weights similar to fresh weights and hence inflated RWC values. We detected

treatment differences only after modifying the protocol as described in Chapter 5. Finally, drought also affected yield and yield components.

Yield and yield components: Drought reduced yield and almost all yield components. In all field experiments except in 2018, the TGW for droughted plants sprayed or not sprayed with water was higher than for the well-watered counterparts. This was likely due to higher plant densities and number of grains per spike in the well-watered plots causing source limitation and competition for other growth resources. TGW declines with increasing plant density (Li et al., 2016). In addition, elevated ABA in the droughted plants is likely to have contributed to increased remobilisation of stem reserves for grain filling. ABA in spikes and panicles begins to increase from end of anthesis and reaches maximum levels at peak of grain filling (Tsukaguchi et al., 1999; Yang, J. et al., 2006). The effects of drought on fertile tillers per plant, fertile spike density, grain and number per m² was as expected, being decreased, except for grains per spike in the 2020 spring experiment, which was higher than for the well-watered plants.

Effects of DpM, exogenous ABA and fluridone on physiology and yield and yield components

Effects of DpM: DpM application in our 2018 and 2019 field experiments was effective in reducing leaf ABA concentration at GS31, GS33, GS37 and GS45 but not at GS51. As noted above, this may have been due to the leaf aging effect (Quarrie and Henson, 1981), as with and without DpM application, the ABA concentration declined with increasing days after planting. We found DpM to be most effective if applied at GS31 and GS37. That timing of DpM application had no effect

on grain yield in 2018 might have been due to delayed sowing which pushed the growing season further into summer compared to 2019. The longer daylight hours in 2018 decreased the gaps between growth stages, as from GS33 to GS51, it took only five days against 22 days between GS31 and GS51 in 2019 (or 48 days after planting to GS51 in 2018 versus 72 days in 2019). Significant differences in the endogenous ABA concentrations between growth stages in 2018 were not accompanied with corresponding differences in yield and yield components. This was likely due to limited time intervals between the growth stages to allow for sufficient water conservation. Through reduced transpiration induced by physically blocking stomata, DpM improves plant water status (del Amor et al., 2010; Weerasinghe et al., 2016), as was found to be the case in our summer field experiment in 2020. Nevertheless, the fertile spike density, grain number per m² and grain yield were significantly higher in the DpM-treated plants compared to the unsprayed control, consistent with the later three field experiments in 2019 and 2020.

In the glasshouse experiments, DpM significantly reduced leaf ABA with sampling at five days after spraying (booting stage in the 2018 experiment) but not at 21 days after spraying (early anthesis in 2019). This confirmed our field experiment findings where we observed a consistent decline in leaf ABA with increasing leaf age and DpM was not effective in reducing the accumulation of the hormone in leaves at GS51. Therefore, the non-significant effect of DpM on leaf ABA in the 2019 experiment does not suggest that the antitranspirant was ineffective, rather, sampling was too late to detect the effect.

Effects of exogenous ABA: Exogenous ABA was effective in elevating leaf ABA only under well-watered conditions. As suggested in the chapter on glasshouse experiments, there appears to be an endogenous ABA ceiling. In fact, according to Liu et al. (2016), when drought-induced endogenous ABA accumulates to a certain level, negative feedback mechanisms mediated by transcription factors, AhAREB1 and AhNAC2, limit its further accumulation. Endogenous ABA is known to self-regulate (Nambara and Marion-Poll, 2005). Accumulation of ABA in reproductive organs due to drought reduces crop productivity (Ji et al., 2011; Pang et al., 2017). Reproductive failure can also be induced with exogenous ABA application (e.g. ~ 76 μM reduced grainset to less than 15% in wheat, while ~378 μM completely inhibited it, Saini and Aspinall, 1982). However, we found no evidence that exogenous ABA at a concentration of 100 μM had a damaging effect on yield formation in both well-watered and droughted treatments. To the contrary, our 2019 data showed that exogenous ABA had an ameliorative effect on grain number per spike and grain yield of droughted wheat. This might be attributed to the concentration being too weak. In fact, as reviewed in Chapter 2, exogenous ABA is used as a metabolic antitranspirant. As discussed in our paper on the glasshouse experiments, the mechanisms of drought amelioration with exogenous ABA might be linked to its improvement of chlorophylls *a* and *b* (Travaglia et al., 2010) and upregulation of antioxidant (glutathione and ascorbate) activities (Wei et al., 2015). Finally, lack of effect of both DpM and exogenous ABA on yield and yield components in the 2018 experiment can be attributed to masking of the treatments effects as main stem and tiller spikes were mixed at harvesting time.

Effects of fluridone: Fluridone was effective in suppressing the accumulation of endogenous ABA through the indirect mechanism of inhibiting the biosynthesis of

carotenoids, some of which are ABA-precursors (Popova, 1995; Zou et al., 2018). At 10 μM , which was used in spring, fluridone was effective in reducing the accumulation than at the doubled concentration, 20 μM , in summer. This might relate to the higher photolytic potential in summer due to brighter-and-hotter and longer days than in spring. It is known that fluridone is decomposed primarily through photolysis and that the rate of degradation increases with temperature (Cheshier et al., 2011; Wickham et al., 2020). Other effects of fluridone are discussed in later sections below.

Gas exchange: On gas exchange, both DpM and exogenous ABA reduced stomatal conductance, under PD (2018) but only DpM reduced it under mild CD conditions (2019), in our glasshouse experiments. Our finding on the exogenous ABA effect under mild stress agrees with Travaglia et al. (2010) who reported increased stomatal conductance. Only DpM significantly reduced photosynthesis under mild CD. This suggests that the drought effect in 2018 had a far more significant effect in suppressing photosynthesis, and that application of DpM could not significantly suppress it further. Other workers also found exogenous ABA to increase both stomatal conductance and photosynthesis (Liu et al., 2004), which is an unusual response. That exogenous ABA is known to sustain chlorophylls *a* and *b* (Travaglia et al., 2010) may explain why we recorded an increase in net photosynthesis, though negligible at the time of data collection. In the field experiments, both DpM and fluridone suppressed photosynthesis, which, as discussed in Chapter 5 is by physically blocking stomata and inhibiting chlorophyll and carotenoid biosynthesis, respectively. Although fluridone did not significantly increase transpiration in our study, other investigators observed significant increases in rice (Hsu and Kao, 2005) and in a dicotyledonous weed species (Liu et al., 2010). Overall, fluridone had

significantly higher transpiration rates than DpM. This had implications for the plant water balance.

RWC: For RWC, we detected treatments differences following the modification of the protocol, described in Chapter 5. DpM significantly increased RWC above the water-sprayed and F50-sprayed treatments. Our results agree with Weerasinghe et al., (2016) who reported improvement in leaf water potential of droughted winter wheat following DpM application. This was likely due to DpM reducing transpirational water loss, which was significantly higher in both the water-sprayed and F50-sprayed plants.

Overall effects on yield and yield components: Overall, in our field experiments, the yield components that were improved with DpM application were fertile tillers per plant, fertile spike density and grain number per m². Number of grains per spike was improved only twice – once in the only summer field experiment and another in the 2019 glasshouse study. Both DpM and exogenous ABA improved number of grains per spike and grain yield only in the 2019 glasshouse experiment. Thus, our studies suggest that under drought conditions, TGW and grain number per spike were not important yield components through which DpM improved spring grain yield.

Most responsive DpM-growth stages: Our data showed that the ameliorative effect of DpM was most effective at GS31 and GS37, which is during stem elongation. Drought at stem elongation is known to reduce the number of spikes per unit area (Day and Intalap, 1970). Therefore, it may be said that by curtailing the accumulation of endogenous ABA at these growth stages, DpM suppressed the damaging effects of the hormone on spike density. Compared to later growth stages, it might be that applying DpM during stem elongation conserves more plant water,

which induces downregulation of endogenous ABA through catabolism (Yang and Zeevaart, 2006). Irrespective of the relationship between endogenous ABA and yield and yield components, DpM should be applied during stem elongation to maximise yield improvement.

Regression analysis

Endogenous ABA versus yield and yield components: Regression analyses were addressed in Chapters 3 to 5. Out of five experiments in which the relationships between endogenous ABA and yield/ yield components were tested, we found significant negative correlations in three field experiments and one glasshouse experiment. In the four experiments where we found significant associations, it might mean that elevated endogenous ABA, due to drought, has damaging effects on yield formation, since yield/yield components decreased with increasing levels of the hormone. In the one glasshouse experiment where correlations were not significant, multiple spraying of exogenous ABA might have contributed to this result, especially that sampling for endogenous ABA was done one day after application of a fourth spray, which was 21 days after DpM had been applied. In addition, the levels of drought were very different in the 2018 and 2019 glasshouse experiments, being progressive drought and controlled mild drought, respectively. Most likely, the weekly irrigation intervals meant that endogenous ABA levels were constantly fluctuating under mild drought. Under a progressive drought scenario, accumulation of endogenous ABA is known to steadily increase (Itam et al., 2020). This was the droughting protocol under the 2018 glasshouse experiment and the three field experiments conducted under Mediterranean-style drought, where we observed significant associations. Based on the 2020 field experiment results in which there

was no evidence that fluridone improved yield despite reducing the accumulation of endogenous ABA, we performed regression analysis of transpiration against yield/ yield components.

Transpiration versus yield and yield components: Regression analysis involving transpiration was addressed in Chapter 5. Transpiration was negatively correlated with yield and yield components. In prolonged severe drought stress situations, reducing transpiration offers an advantage by conserving soil moisture for grain filling and improved grain yield (Richards et al., 2002; Tardieu, 2012). Where there is little or no rainfall and a crop depends on limited soil moisture, increasing transpiration efficiency is key to enhancing water use efficiency and resilience of crops against drought (Rebetzke et al., 2002; Richards et al., 2002; Siahpoosh and Dehghanian, 2012). Transpiration efficiency, a component of water use efficiency, is the ratio of shoot biomass to amount of water transpired; and can be increased by reducing transpiration (Chenu et al., 2018; Rebetzke et al., 2002). As our results show in Chapters 3 to 5, DpM improved grain yield of droughted spring wheat, in agreement with past research (e.g. in wheat (Abdullah et al., 2015; Kettlewell and Holloway, 2010) and oilseed rape Faralli et al., 2017)). Under water deficit stress, DpM has been found to reduce transpiration, and increase water use efficiency (e.g. in oil seed rape, Patil and De, 1976, beans, Iriti et al., 2009, and wheat, Faralli et al., 2019) and plant water status (e.g. in wheat, (Faralli et al., 2019; Weerasinghe et al., 2016), and wheat and barley, (Ouerghi et al., 2014)). The significantly higher transpiration rate due to fluridone under drought stress compared to DpM may explain why we did not observe increased yield despite fluridone reducing the accumulation of endogenous ABA and considering the transpiration efficiency ratio.

6.2 Hypothesis test result

In five out of six experiments, we found evidence that DpM reduced endogenous ABA in droughted spring wheat. Regression analysis indicated that yield improvement was associated with reduced accumulation of endogenous ABA. However, reducing the accumulation of endogenous ABA was associated with yield improvement only if transpiration was also suppressed. This means that the hypothesis was partially proved to be correct. Reducing endogenous ABA in droughted wheat might therefore not be the sole mechanism explaining yield improvement.

6.3 Research limitations

Physiological, morphological and time constraints: As improved hydration status increases catabolism of ABA (Yang and Zeevaart, 2006), it is impossible to disentangle downregulation of endogenous ABA from reduced transpiration in droughted plants using DpM. Application of DpM and sampling for physiological effects of spray types were based on the growth stages of the main stem spikes but yield and yield components were determined on both main stem and tiller spikes (apart from the 2019 glasshouse experiment). In the last two experiments we undertook to include tillers for physiological measurements. However, we missed out on sampling tillers for all the physiological traits studied due to COVID-19 restrictions in spring and we could not keep abreast with rapid crop development in summer due to long daylength. For challenges associated with the use of antitranspirants in general, refer to Chapter 2.

6.4 Further research

How spray types affect the physiological traits of tillers may reveal useful information. One particular investigation worth considering is determining the endogenous ABA in the vegetative tissues and spikes of tillers, which should be related to tiller fertility. Future research could also consider determining the dynamics of stem water-soluble carbohydrates at a number of growth stages between the spraying of the antitranspirant and end of grain filling. It could be that stem water-soluble carbohydrates play a significant role in DpM improvement of grain yield, considering that the antitranspirant suppresses current photosynthesis. In addition, studying the effects of ABA on sucrose transport and on the activities of starch synthase may yield potential answers to the mechanisms by which DpM improves grain yield in wheat. For other research gaps identified, refer to Chapter 2.

6.5 General Conclusion

We have shown through our studies that DpM reduces endogenous ABA and transpiration of droughted spring wheat. Improvement of grain yield of droughted spring wheat is associated with reduced endogenous ABA if transpiration is also reduced. We found that yield improvement was linked to enhancement of number of fertile tillers per plant, fertile spike density and grain number per m². Regardless of our hypothesis on the relationship between endogenous ABA and yield, we found that the best growth stages to apply DpM was GS31 and GS37 – i.e. during stem elongation!

6.6 References

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