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

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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

1. 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 hectarage (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 supress 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 endogenous abscisic acid (ABA) concentration of (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 m^2 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 than 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 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 half 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.

2. Materials and Methods

2.1 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).

2.2 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.

2.3 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 m^2 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.

2.4 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.

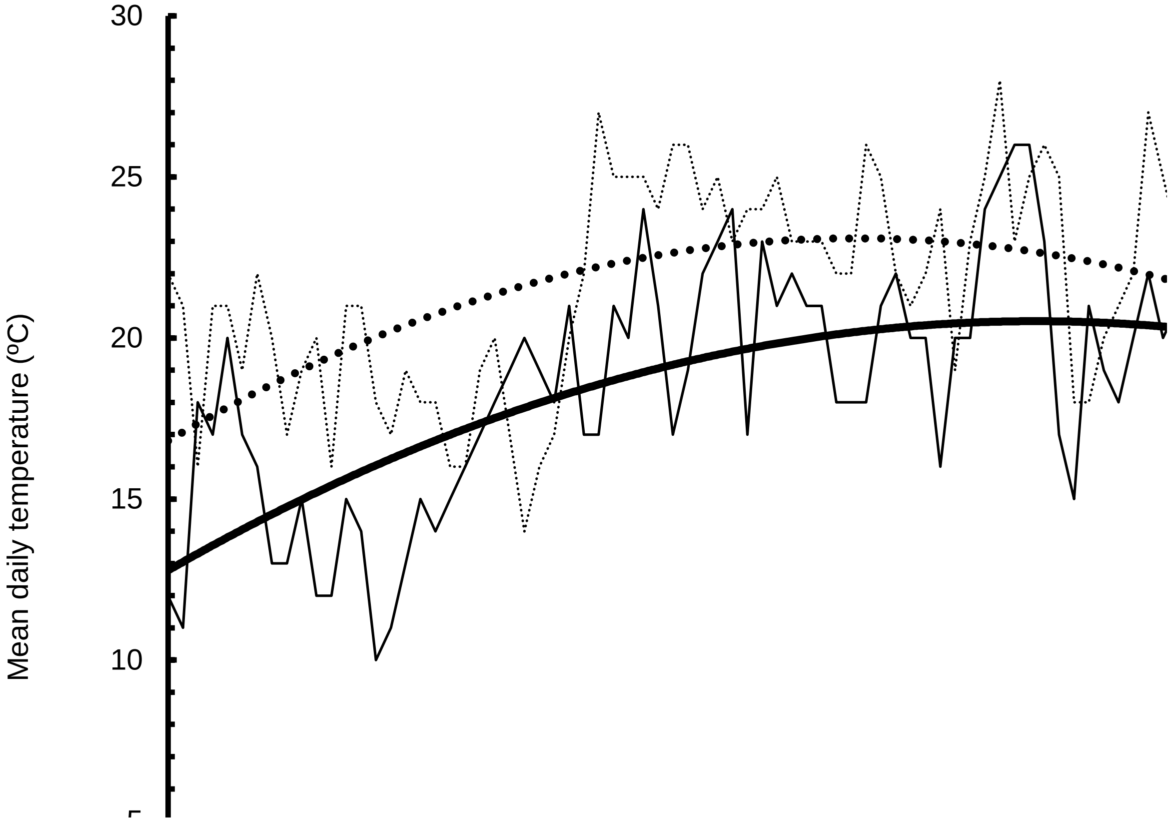
Table 2: Vapor Gard application and ABA sampling schedule.

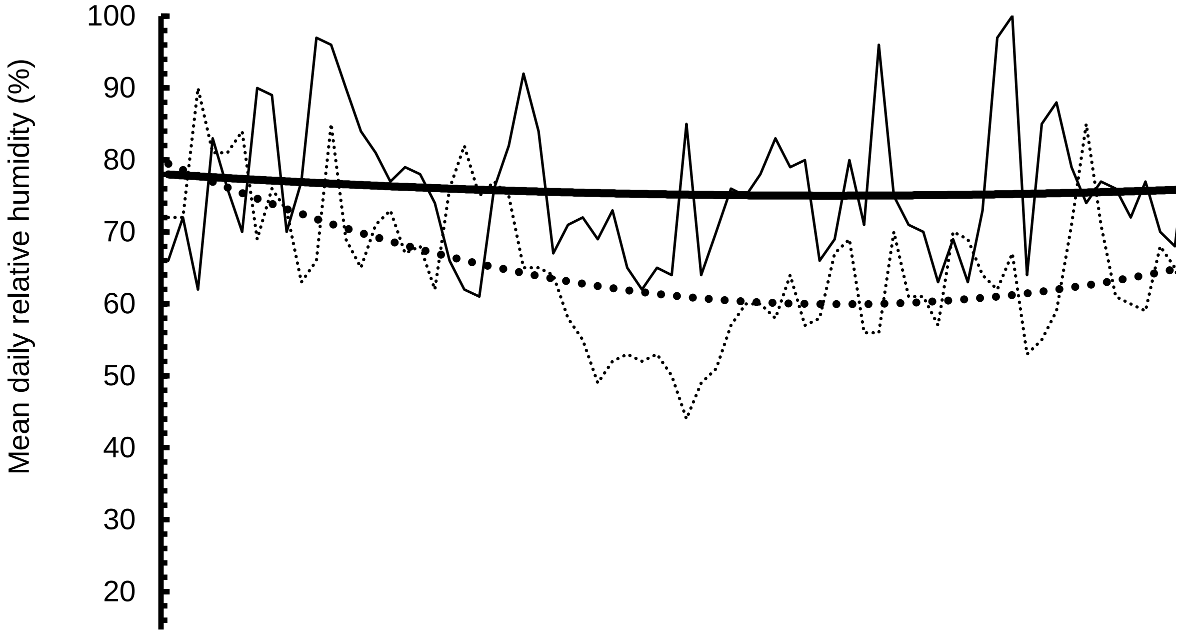
Year	Timing of VG sprays	Spraying at days after planting		Leaf sampling* at days after planting and days after spraying			Spike sampling at days after planting and days after spraying		
	Growth stage	DAP	Date	DAP	Date	DAS	DAP	Date	DAS
2018	GS33	43	6th June	52 69	15th June 2nd July	9 26	N/A		
	GS45	45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

2.5 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 1, a). The seasonal averages were 21 and 19 ºC, for 2018 and 2019, respectively. Between June and August in 2018, there were heat waves in the United Kingdom with the maximum temperature being 33.0ºC (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 ºC. 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 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 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.


Mean daily temperature (ºC)
30
25
20
15
10


Mean daily relative humidity (%)
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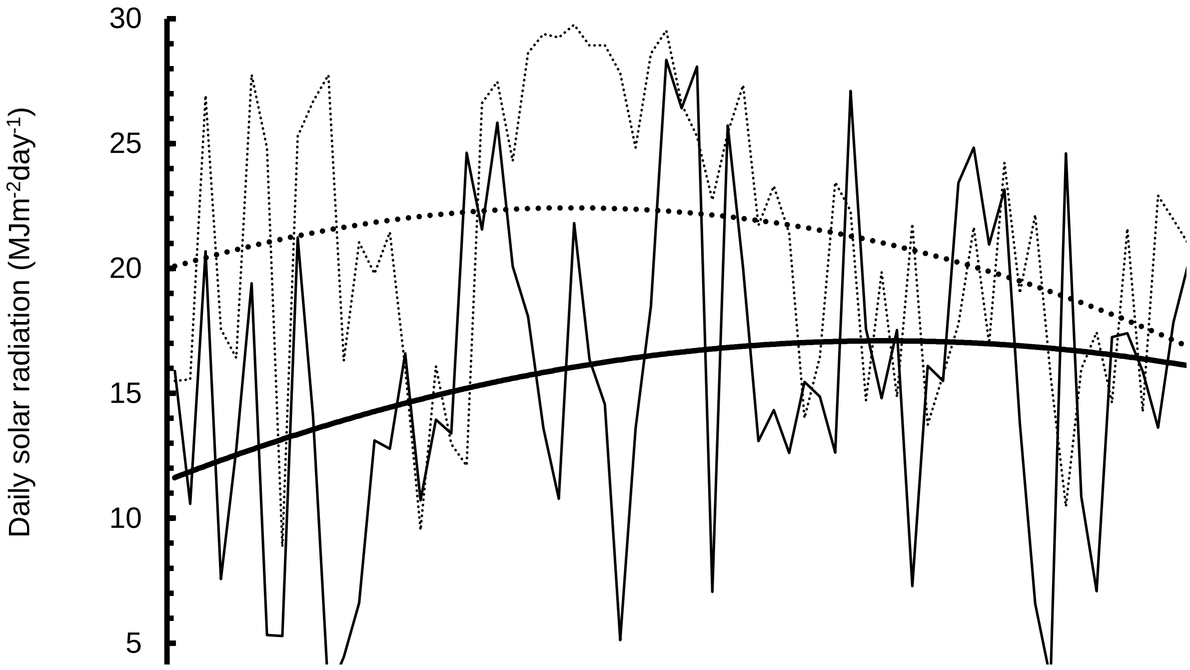


Figure 1: (a) Mean daily air temperature and (b) mean daily relative humidity inside the rain shelters; and daily solar radiation (c) recorded by the meteorological station based at Harper Adams University between 1st June and 31st August. Each of these variables was significantly different ($p < 0.001$) 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-E09159Pl (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

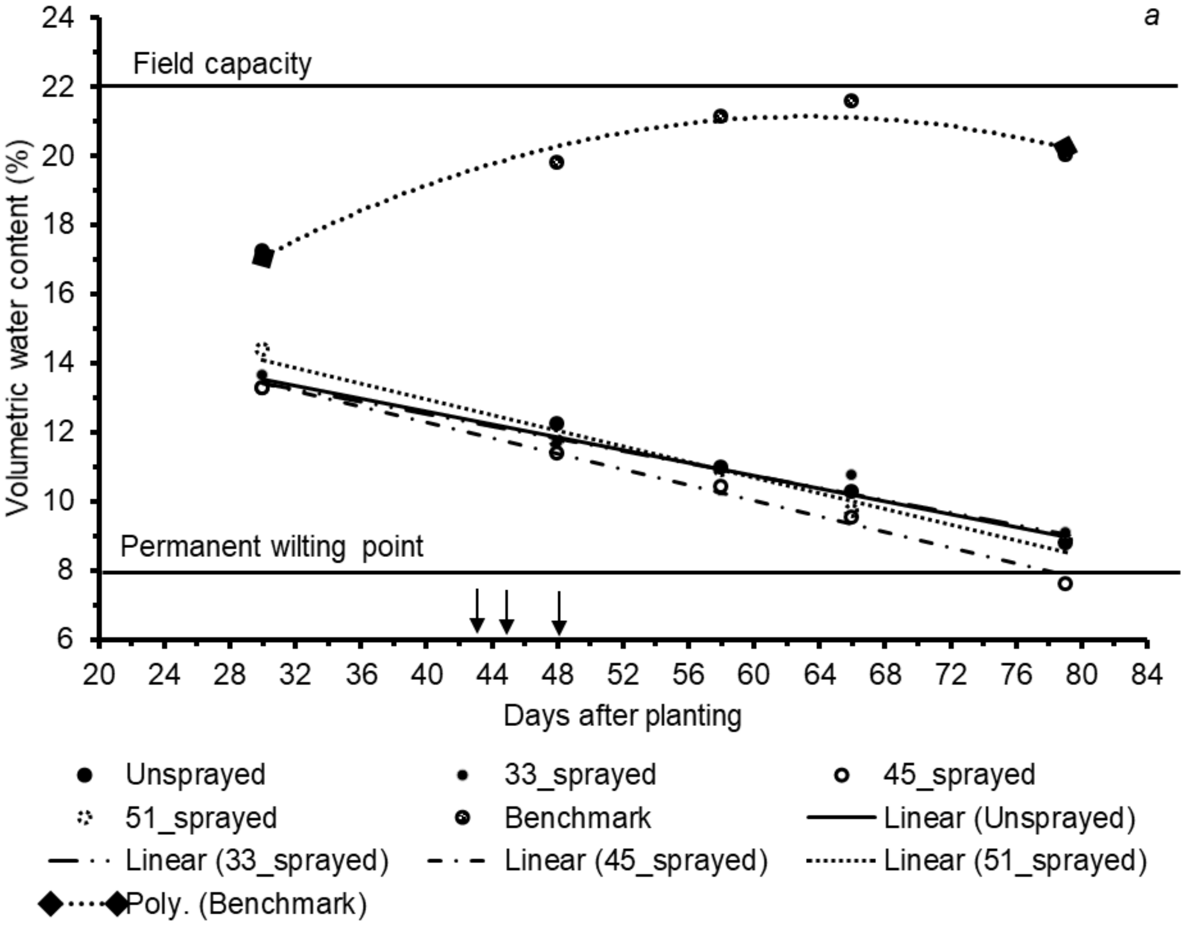
(Farm-Tec, Whitby, North Yorkshire, UK). The number of grains obtained from this weight was also used in the calculation of number of grains per m^2 (Moeller and Rebetzke, 2017). Finally, grain yield per plot was obtained by bulking and weighing all the grain harvested from each plot and converting it 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. Results

3.1 Soil moisture dynamics

The volumetric water content (VWC) in the top 60 cm significantly decreased with increasing DAP (Figure 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 %.

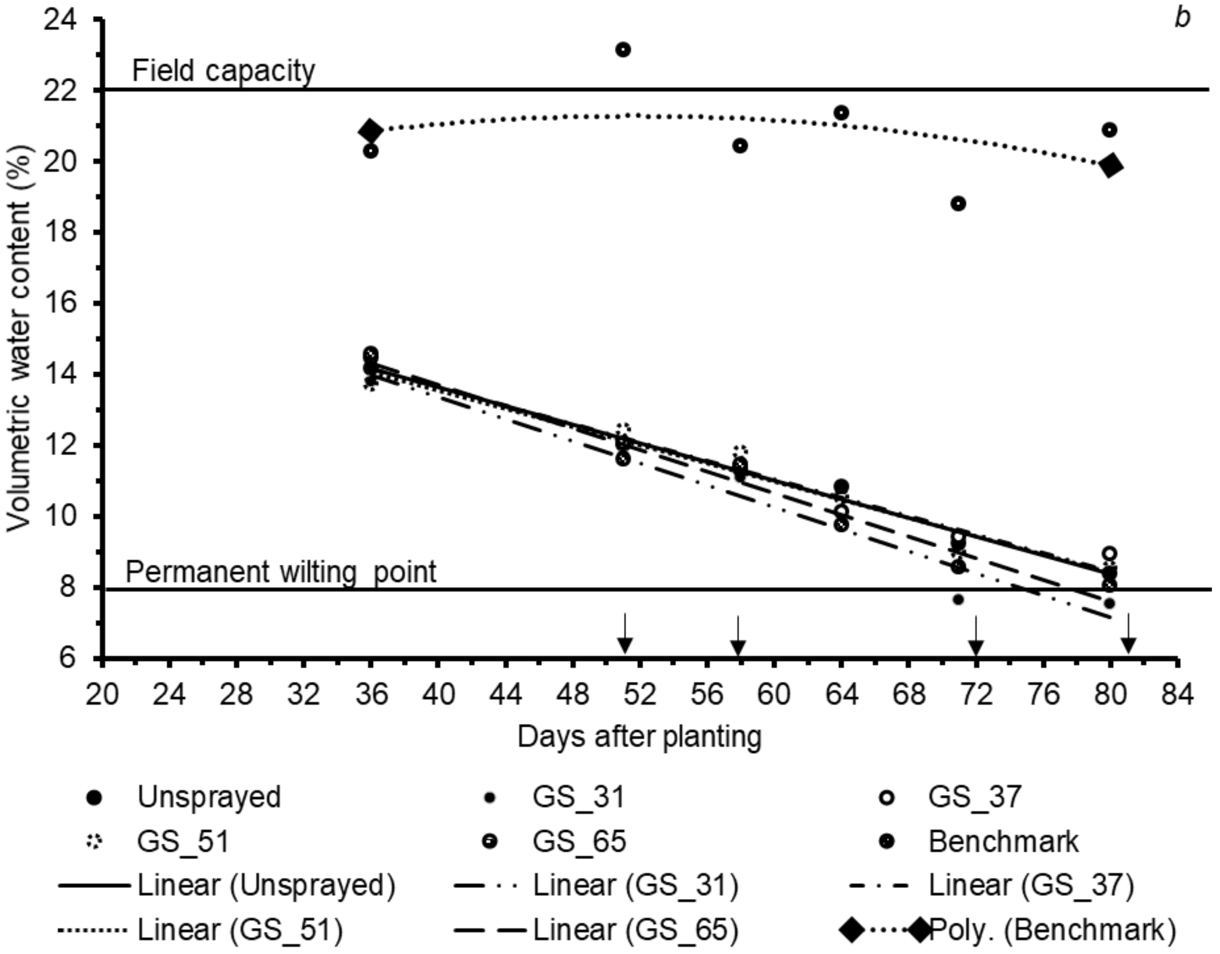


Figure 2: Soil moisture dynamics between 30 and 79 and from 36 to 81 days after planting (DAP) showing significant decrease in (a) 2018 $p < 0.001$) and (b) 2019, ($p < 0.001$). Deviations of slopes from the unsprayed reference were non-significant (GS33: $p = 0.817$, GS45: $p = 0.522$, GS51: $p = 0.161$; GS31: $p = 0.608$, GS37: $p = 0.914$, GS51: $p = 0.822$, GS65: $p = 0.446$) in either year. Each point represents a treatment average of three or four measurements. Arrows indicate DAP on which the antitranspirant was sprayed in the appropriate plots.

3.2 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 4). 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.

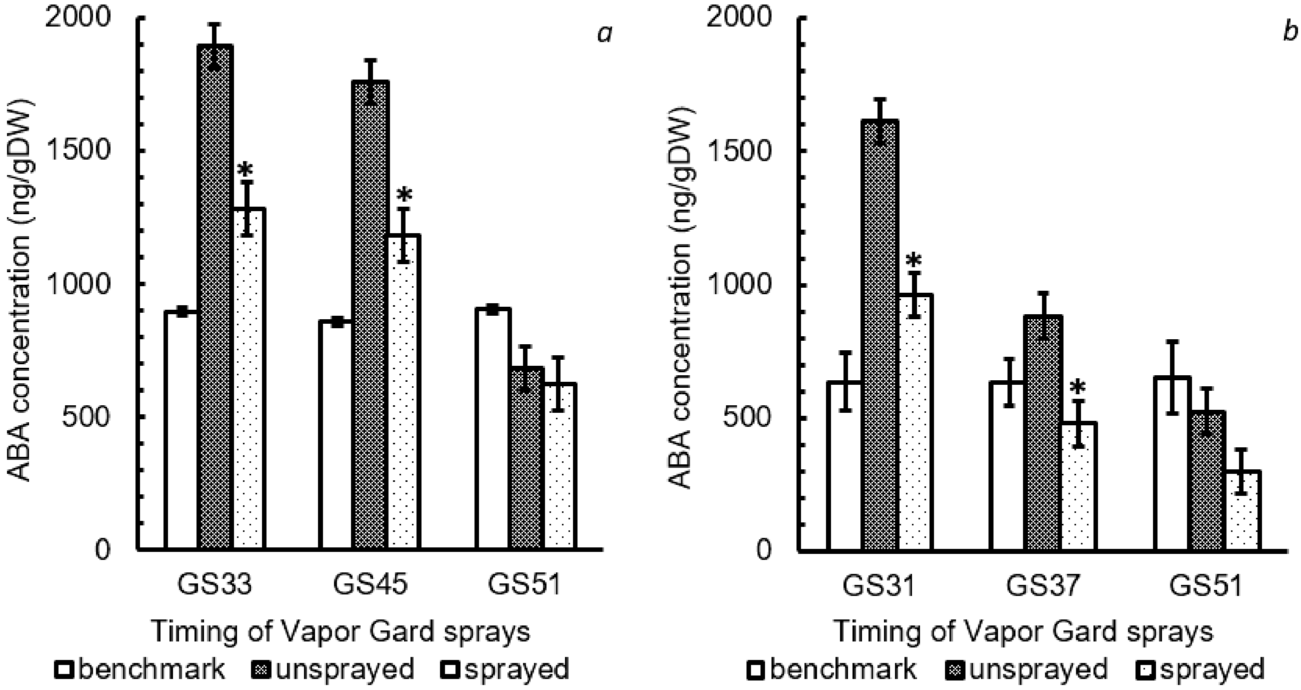


Figure 4: Average ABA concentrations (ng/g DW) in flag leaves sampled at nine days after spraying di-1-*p*-menthene, at three growth stage treatments in droughted plots, showing consistent significant interactions from (a) 2018 ($p = 0.033$) and (b) 2019 ($p = 0.048$). Asterisks represent sprayed treatments 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 5).

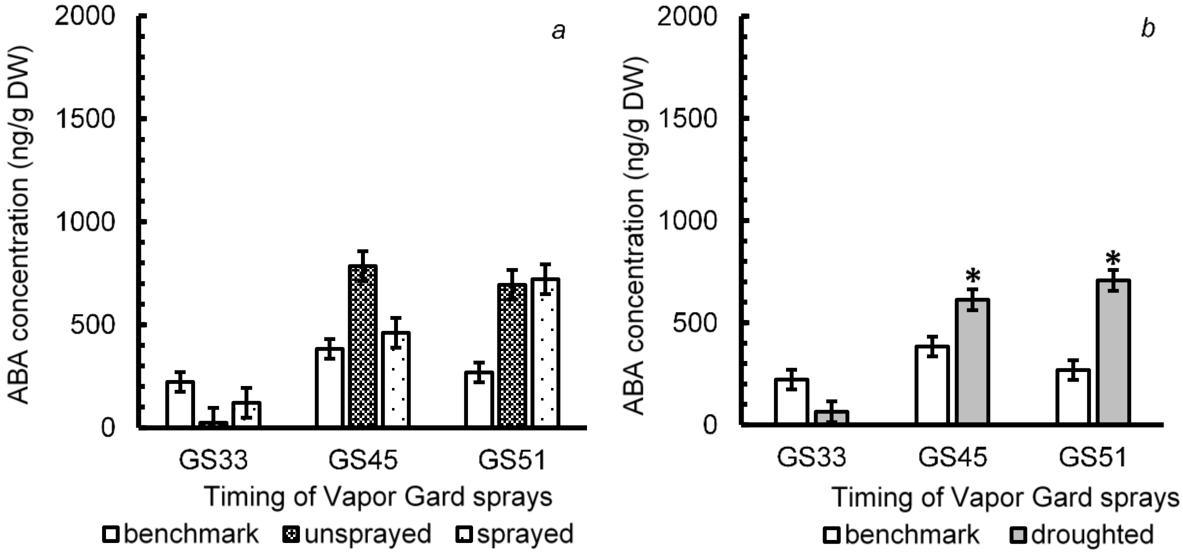


Figure 5: Average ABA concentrations (ng/g DW) in flag leaves sampled at 26 days after spraying di-1-*p*-menthene (2018 only) at three growth stage treatments for (a) sprayed and unsprayed droughted plots compared and (b) sprayed and unsprayed droughted plots combined. Irrigated benchmark plots are shown 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 6). 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.

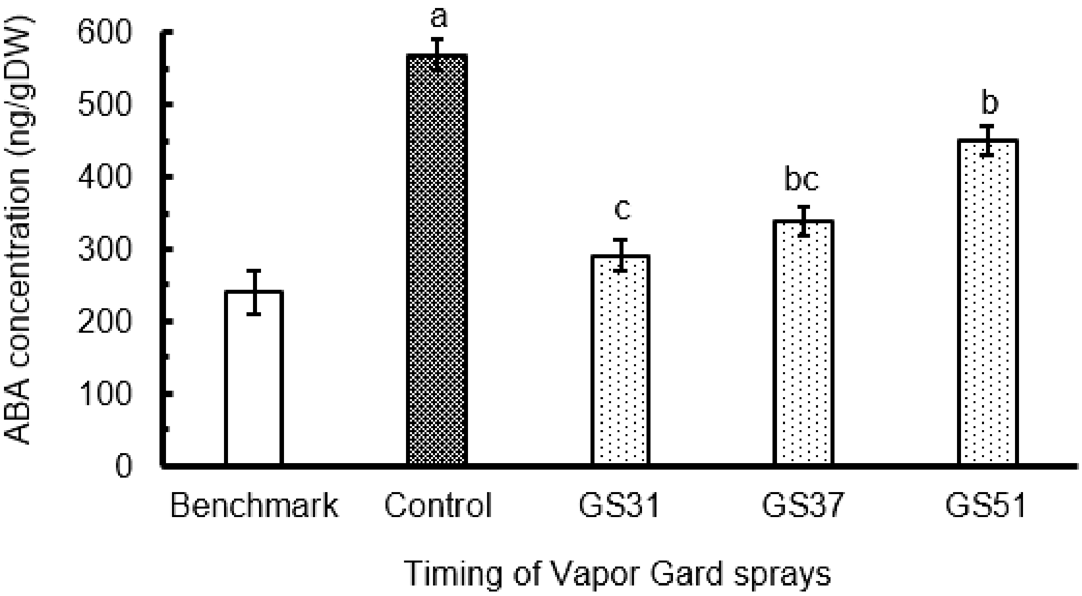


Figure 6: Average ABA concentrations (ng/g DW) in spikes of droughted plants not sprayed (control) and sprayed with di-1-*p*-menthene at three growth stages (GS) in 2019 showing a significant effect ($p < 0.001$, different letters indicate significant differences). All samples were taken at once at GS55, 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.

3.3 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 m^2 by 49 % and 59 % in 2018 and 2019, respectively. Application of di-1-*p*-menthene minimised the loss in grain number per m^2 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 m^2 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 m^2 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: 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. 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 m^2 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 m^2 and fertile spike density which explained 96 % and a maximum of 98 % of variance, respectively (Figure 7). 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.

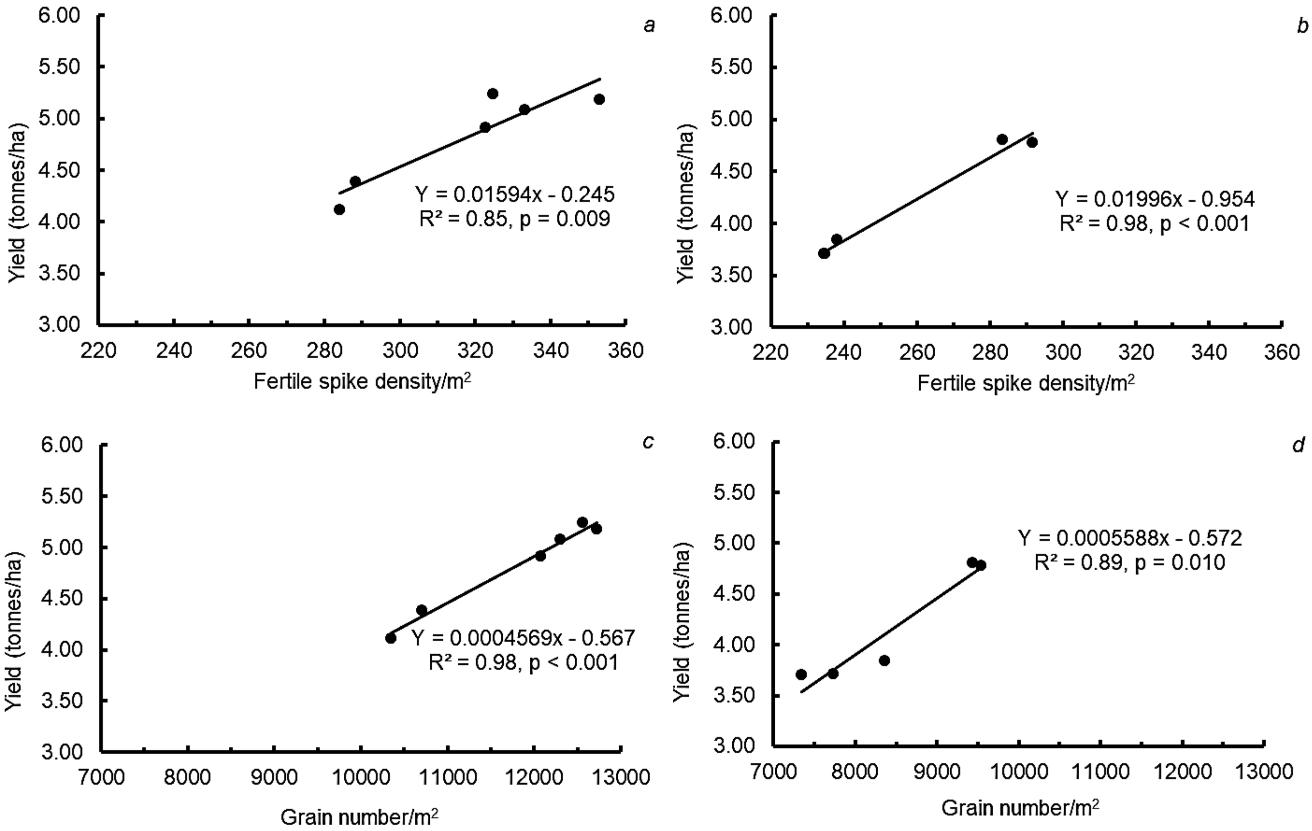


Figure 7: Simple linear regressions of mean values between yield (Y), in tonnes per hectare, and (a, b) number of fertile spikes per m^2 (x) and (c, d) number of grains per m^2 (x) for 2018 and 2019, respectively. Each point represents (a, b) three and (c, d) single measurements per plot in used in the regression analysis to construct the curves.

3.4 Endogenous ABA relationships with yield and yield components

Figure 8 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 m^2 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 8).

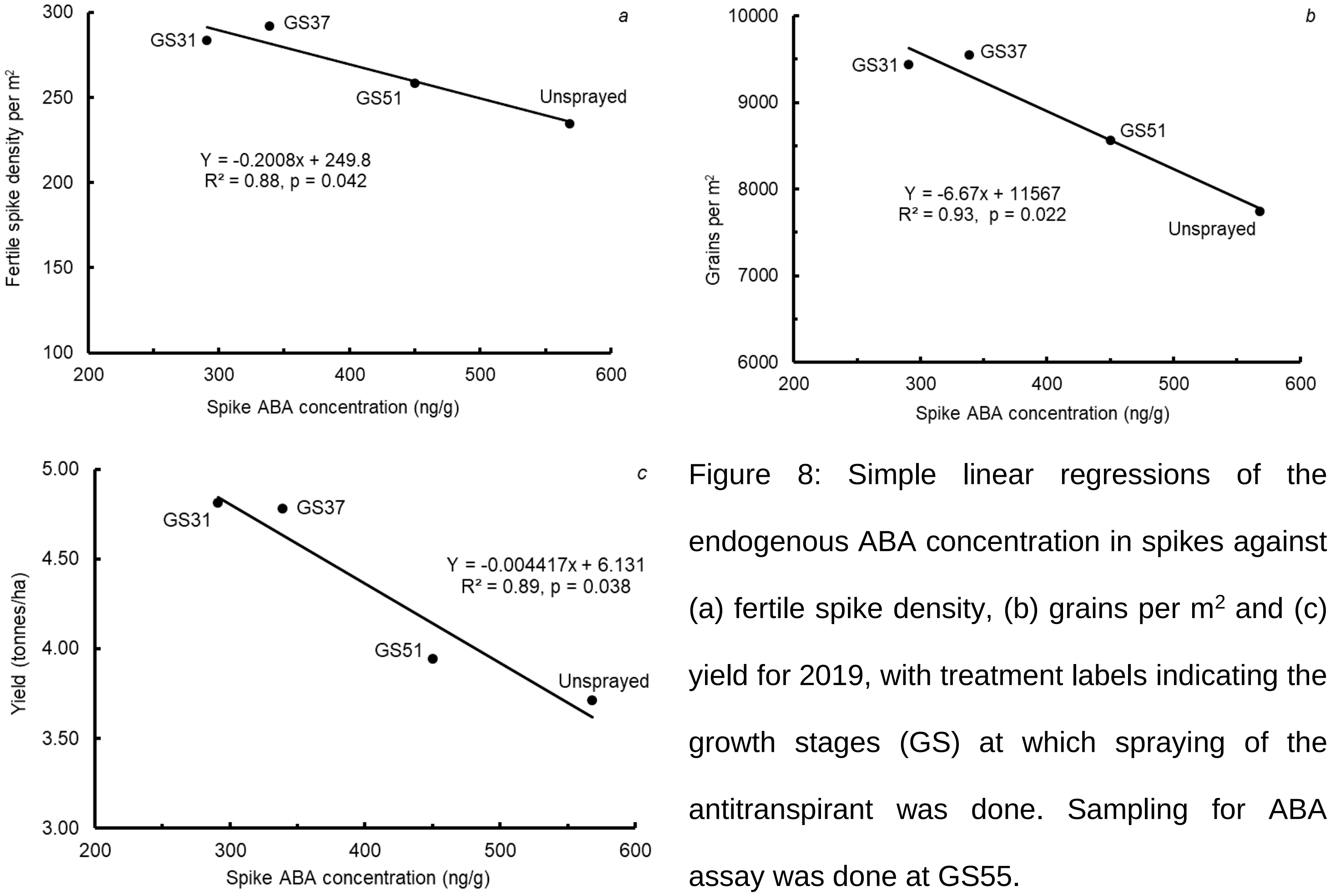


Figure 8: Simple linear regressions of the endogenous ABA concentration in spikes against (a) fertile spike density, (b) grains per m^2 and (c) yield for 2019, with treatment labels indicating the growth stages (GS) at which spraying of the antitranspirant was done. Sampling for ABA assay was done at GS55.

4. 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 m^2.

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 m^2 and so the final yield. Regression analysis showed that endogenous ABA was negatively associated with fertile spike density, and grains per m^2 as well as grain yield (Figure 8). Spike ABA was better associated with these variables (Figure 8) than leaf ABA (results not shown).

The reductions in spike ABA were driven by the antitranspirant, with earlier application being most 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 m^2, 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 4 and 6) also showed improvement in not only fertile spike density but also grain number per m^2 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 m^2 is controlled by fertile floret and spike density per m^2 (Prieto et al., 2018). There is a linear relationship between not only spike fertility but also grain number per m^2 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 m^2 were strongly associated with grain yield per hectare (Figure 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 m^2 (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).

5. 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 m^2 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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7. 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.

8. Conflicts of Interest

The authors declare no conflict of interest.

9. References

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