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The effects of exogenous monoterpenes on drought response in tomato

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Declaration

This thesis has not been submitted in support of an application for another degree at this or any other university. It is the result of my own work and includes nothing that is the outcome of work done in collaboration except where specifically indicated. Many of the ideas in this thesis were the product of discussion with my supervisor Dr Kirsti Ashworth and Prof. Ian Dodd.

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Abstract

Interactions between biogenic monoterpenes and drought stress remain poorly understood and characterised. Even the nature of the response of biogenic monoterpene emissions to water limitation is controversial, possibly depending on the severity, intensity and duration of the drought. Whether monoterpenes regulate plant physiological response to drought stress is currently unknown. In this research, 6-week-old *Ailsa Craig* wild-type (WT) and ABA-deficient (*notabilis*) tomatoes were either well-watered or exposed to deficit irrigation (by watering pots with 25% of daily evapotranspiration) in a factorial combination with selected-monoterpenes applied exogenously as a foliar spray. Both genotypes showed similar physiological and biochemical responses to water deficit. Compared to well-watered controls, drought stress significantly reduced net photosynthesis rate and stomatal conductance, increased hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) concentrations, and also significantly inhibited PSII maximum (*Fv'/Fm'*) and operating (φPSII) efficiency under severe stress. Drought stress significantly increased foliar abscisic acid (ABA) accumulation in WT plants, whereas *notabilis* remained ABA-deficient. Applying exogenous monoterpenes decreased net photosynthesis and stomatal conductance of WT plants under moderate drought conditions. Although foliar H_2O_2 content (a proxy of oxidative stress) was not affected by exogenous monoterpenes, their application significantly decreased the production of MDA (which indicates damage caused by drought-induced oxidative stress). The monoterpene spray also significantly inhibited ABA accumulation under severe stress, possibly by interfering with the methylerythritol (MEP) pathway and thereby reducing production of ABA precursors. Although exogenous monoterpenes increased plant antioxidative capacity by reducing lipid peroxidation, this did not appear to protect photosynthetic activities as the PSII efficiencies or net photosynthesis rate were not affected. That these effects were not observed in *notabilis* suggests that monoterpenes have ABA-dependent impacts on plant photosynthetic biochemistry.

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1 Introduction

1.1 Physiology

Drought is generally defined by eco-physiologistics in terms of soil water deficit and by hydrometrorologists as an extreme climate event relative to the normal local conditions (Dai, 2011). However, there is no universally agreed metric of drought. The extent, severity and frequency of drought is projected to double, and prolonged drought (> 12 months) is expected to become three times more common under the impact of global warming by the end of 21st century (Sheffield and Wood, 2008; Dai, 2011; IPCC, 2013). Numerous studies have investigated the impacts of drought stress on plants and their biochemical and physiological responses (Henckel, 1964; Xu et al., 2010; Joshi et al., 2016). During periods of drought, soil moisture and water potential (ψ_{soil}) decreases, which reduces the movement of water through roots to plant cells (Osakabe et al., 2014). As transpiration (*Tr*) continues cellular water is lost through the stomata, reducing cellular turgor and hence leaf water potential (ψ_{leaf}) (Passioura, 2010), which is a widely accepted as an essential measure of physiological water stress in plants (Boyer and Kramer, 1995). When plant cells are dehydrated for long periods and cellular turgor approaches zero, photosynthesis and turgor pressure are extremely reduced, plant cellular expansion and biomass accumulation will be severely inhibited, respectively (Boyer, 1982). However, plants can reduce water loss through transpiration by closing the stomata. Stomatal aperture can be decreased if the guard cells dehydrate and shrink, and/or via biochemical mechanisms (Bray, 2007; Bodner et al., 2015).

The physiological and biochemical defensive mechanisms of plants are prime factors that determine whether plants are able to acclimate to the drying environment, mitigate damage caused by drought stress, delay hypersensitive responses and programmed cell death to enable plants to survive periods where water is scarce (Henckel, 1964; Zhu, 2002; Bodner et al., 2015).

1.2 Biochemistry

ABA biosynthesis, signalling and regulation

In addition to the physical mechanisms, biochemical activities change under water deficit as important signalling responses (Zhu, 2002). The most crucial of these is the accumulation of phytohormone abscisic acid (ABA) (Boursiac et al., 2013; Finkelstein, 2013). The action of ABA in plants requires synthesis and metabolism, transport and perception. Terpenoids are derived from the C5 (isopentenyl diphosphate, IPP; and dimethylallyl diphosphate, DMAPP; Fig 1.1) precursor(Pulido et al., 2012; Boursiac et al., 2013). It was once thought that all terpenoid were synthesised through the mevalonate (MVA) pathway (Lichtenthaler, 1999). However, more recently, it has been found that the synthesis also occurs through the methylerythritol 4-phosphate (MEP) pathway, which includes isoprene, monoterpenes (MTs) and ABA formed by cleavage of carotenoids (C40) (Schwender et al., 1996; Eisenreich et al., 2004). *De novo* ABA synthesis occurs in the roots with subsequent transport to the shoots and leaves via the xylem, or by direct synthesis in the leaves and guard cells (Nambara and Marion-Poll, 2005). This triggers a battery of physiological responses, of which stomatal closure is the most significant. This reduces plants transpiration and water loss, which acts to maintain plant water status and enhance water use efficiency (WUE) (Christmann et al., 2005; Georgopoulou and Milborrow, 2012; Osakabe et al., 2014). However, stomatal conductance (*Gs*) can also be regulated by hormones other than ABA such as jasmonic acid (JA) (Suhita et al., 2004), and the effects of leaf-pool ABA and xylem transport on stomata and transpiration cannot yet be fully explained (Cochard et al., 2002; Lee et al., 2006; Brodribb and McAdam, 2013).

Gas exchange, photosynthetic activities and reactive oxygen species

Although stomatal closure makes a significant contribution to maintaining plant water status under drought stress, it also limits the uptake of CO_2 and reduces carbon assimilation. It is also usually accompanied by elevated leaf temperature as leaf transpiration is the primary cooling mechanism (Farquhar and Sharkey, 1982; Jones, 1999; Galmes et al., 2007), although this also depends on other factors such as air temperature, relative humidity, vegetation type, and leaf thickness (Chaves

et al., 2012; Poirier-Pocovi and Bailey, 2020). Multiple studies have demonstrated clear relationships between intercellular CO_2 concentration (*Ci*), transpiration (*Tr*), and photosynthesis rate (*A*) with stomatal aperture under water deficit and after re-watering (Mott, 1988; Farquhar et al., 1989; Souza et al., 2004; Gago et al., 2016). Stomatal closure and hence the regulation of gas exchange and carbon assimilation depends on the extent and intensity of the drought and morphological and physiological differences between plant species (Mediavilla and Escudero, 2003; Pirasteh-Anosheh et al., 2016). Gs is primarily responsible for the control of leaf CO2 concentrations under short-term moderate or mild water deficit in many species (Laffray and Louguet, 1990). More sensitive stomatal control and faster adjustments of whole plant water-use-efficiency (WUE) appear to improve the drought tolerance of a species. Under long-term and severe drought stress, however, non-stomatal limitation of photosynthesis becomes increasingly important (Ackerson and Krieg, 1977; Drake et al., 2017). One of the most significant factors is mesophyll resistance, which refers to the physical and biochemical factors that limit CO_2 diffusion across the mesophyll into the chloroplast, reducing transport within the chloroplast and carboxylation (Jones, 1973; Tholen and Zhu, 2011).

As a result of decreases in *A*, and hence *Ci*, competing biochemical and photochemical processes increase or accelerate, fuelling further damage (Ali and Ashraf, 2011; Wang et al., 2018). For instance, the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) enzyme may be diverted from the Calvin cycle for photorespiration or chlororespiration, which oxygenates ribulose-1,5-bisphosphate (RuBP) (Peltier and Cournac, 2002; Rivero et al., 2009); photosystem II (PSII) may be damaged by changes in the electron transport rate (ETR), reducing PSII photochemistry efficiency (Epron et al., 1992; Long et al., 1994; Atkin and Macherel, 2009); and reduced metabolic and enzyme activities may occur especially under serious water stress (Hu et al., 2010).

Another notable effect is the production of reactive oxygen species (ROS). ROS form in several organelles, such as chloroplasts during photooxidation (Asada, 2006), peroxisomes during photorespiration (Noctor et al., 2002), and mitochondria (Rhoads et al., 2006). Under drought conditions, photosynthetic

efficiency and photosynthesis are inhibited, and electrons act directly on molecular oxygen to trigger superoxide radicals that are then converted into hydrogen peroxide, further stimulating the antioxidant protection mechanism (Asada, 2006). Low levels of ROS can participate in ABA signalling and transduction, optimise stomatal response to drought or directly affect guard cells for stomatal regulation via Ca^{2+} based signalling (McAinsh et al., 1996; Desikan et al., 2004; Hu et al., 2016). However, as the drought continues, over-production of ROS results in membrane lipid peroxidation, which produces malondialdehyde (MDA). This may cause permanent oxidative damage to photosystems (Apel and Hirt, 2004), ultimately limiting crop productivity (Miller et al., 2010). Plants synthesise and mobilise a range of antioxidants to scavenge ROS, of which the most important are superoxide dismutase (SOD) and enzymes such as catalase, ascorbate peroxidase (APX) and glutathione reductase (DeJong et al., 2007; Caverzan et al., 2012; Couto et al., 2016). SOD can rapidly decompose superoxide - one of the first ROS be produced - to oxygen and hydrogen peroxide (H_2O_2), which is then scavenged by catalase and APX (C Bowler et al., 1992; Willekens et al., 1997). Glutathione reductase acts either directly or indirectly as a nonenzymatic antioxidant and reducing agent to remove the by-product (dehydroascorbate) of the reduction of H2O2, reducing oxidative stress (Noctor and Foyer, 1998; Hasanuzzaman et al., 2017). The greater a plant's antioxidant capacity, the better it copes with a decline in water availability and leaf water potential during droughts. Recently, volatile terpenoids have also been shown to have antioxidant properties and therefore protect the photosynthetic apparatus of plants from oxidative stresses caused by drought (Lado et al., 2004; Ryan et al., 2014).

1.3 Terpenoids and Drought Stress

Vegetation emits a range of volatiles, of which the terpenoids, isoprene (C_5H_8) and monoterpenes (MTs, $C_{10}H_{16}$), are the most abundant, accounting for 44% and 11% of global emissions respectively). The global BVOCs emission flux from biosphere to atmosphere is estimated to be approximately 1150 Tg C $year^{-1}$ (Guenther et al., 1995). Many terpenoids are highly reactive and play a significant role in tropospheric chemistry and composition. Biogenic volatiles affect composition and air quality at the point of emission, and also over long distances

by forming secondary pollutants, such as ozone and secondary organic aerosols and by regulating the persistence of greenhouse gases (Arneth et al., 2011; Fineschi et al., 2013). Advances have been made over the past two decades regarding scientific understanding of the biosynthesis and emission of plant volatiles. However, research has primarily focused on isoprene (Monson et al., 1992; Llusià and Peñuelas, 1998; Tiiva et al., 2008; Guenther, 2013), which means that knowledge of the sources and regulation of other biogenic compounds remain limited (Wiedinmyer et al., 2004). Terpenoid emission capacity varies widely between plant species (Peñuelas and Staudt, 2010; Mendoza et al., 2019). making the prediction of both the magnitude and composition of emissions highly uncertain (Guenther et al., 1993; Guenther et al., 1995).

The carbon released by vegetation through the emission of volatiles is estimated to be about 0.1-2% of photosynthetic carbon assimilation and may even be higher in species such as poplar and under environmental stress conditions (Goldstein et al., 1998; Possell and Loreto, 2013; Seco et al., 2015). It is therefore reasonable to assume that volatiles play a beneficial role in plant biochemistry and functioning of ecosystems (Sharkey et al., 2007).

1.4 Responses to Environmental Stresses

Both biotic and abiotic stresses alter volatile synthesis and emissions as well as plant physiology and metabolism (Kesselmeier and Staudt, 1999). Environmental stresses usually promote foliar volatile production and emissions. For instance, herbivory and pathogens induce green leaf volatiles (GLVs, e.g. fatty alcohols, acids) terpenoids, and oxygenated terpenoids (Alborn et al., 1997; Shiojiri et al., 2006). These compounds are used for chemical signalling as part of the defence mechanisms adopted by plants, which includes signalling to prime nearby plants (Dicke et al., 2003), repelling herbivores, attracting predators and increasing defence-related protein levels and enzyme activities (e.g., to quench ROS) (Stout et al., 1999; Dicke and Baldwin, 2010)

The responses of biogenic volatile synthesis and emissions to abiotic stresses are more complicated and the mechanisms are highly uncertain. Peñuelas and Staudt (2010) summarised hundreds of research studies exploring the response of >50

plant species to short- and long-term environmental changes. Of these, ~70% focused on isoprene. What is particularly striking is that increases, decreases, and no change in emissions have all been observed. It is now generally accepted that isoprene is produced to protect photosynthesis systems from elevated temperatures (Velikova et al., 2005; Sharkey et al., 2007). There is, however, some evidence to show that elevated atmospheric CO_2 concentrations reduce isoprene emissions (Rosenstiel et al., 2003; Possell et al., 2004), as well as MTs (Loreto et al., 2001a), making estimations of future fluxes highly uncertain. The effect of drought stress on isoprene emissions appears to depend on the intensity of drought with mild drought stress stimulating emissions and severe droughts inhibiting them (Fortunati et al., 2008; Jiang et al., 2018).

This research focuses on the impact of drought stress on MT emissions. In the early stage of drought, stomatal closure decreases intracellular CO2 concentration. Increased mesophyll resistance causes substrate restrictions, limiting the photosynthetic process (Possell and Loreto, 2013). Initially, however, increased leaf temperature as a result of reduced transpiration accelerates the diffusion of some compounds, including MTs, out of tissues and storage pools (Niinemets and Reichstein, 2003; Guenther, et al., 2013). Other studies have reported that mild drought has no impact on terpenoid emissions (Staudt et al., 2002; Pegoraro et al., 2004). As drought continues and intensifies, further reductions in *Gs* and *A* lead to an increased resistance to diffusion and energy supply shortages, respectively, inhibiting the production and emission of volatiles (Sharkey and Loreto, 1993; Marron et al., 2003; Peñuelas and Staudt, 2010). Observations appear to show minimal stomatal regulation of emissions. For example, even when stomata are closed by 90% under drought stress, isoprene emissions from oak were not significantly affected (Tingey et al., 1981), and the isoprene emission rate in aspen leaves was maintained when net photosynthesis and *Gs* were substantially reduced (Fall and Monson, 1992).

Although the impact of drought on MT emissions has not been widely studied, similar behaviour has been observed. For instance, acute and long-term water deficit suppressed MT (α-pinene, β-pinene, myrcene, limonene) emissions (Lavoir et al., 2009). However, in a laboratory study on *Quercus ilex* during which soil

moisture fell from 25% to 5%, MT emissions initially fell but then increased as drought intensified, with a burst in emissions following re-watering (Peñuelas et al., 2009). Reducing the relative humidity around *Q. ilex* decreased Gs substantially with minimal impact on the emissions of the dominant MT α-pinene (Loreto et al., 1996).

On an ecosystem scale, seasonal variations in the effects of drought on MTs have been reported. Leaf level MT emissions from Mediterranean shrubs were less affected by drought in the winter than the summer, while the opposite effect has been observed in woodlands (Llusià et al., 2006). Further evidence of MT emission behaviours for different species and in different conditions is required to elucidate the mechanisms underpinning these emissions and behaviours.

1.5 Emission Mechanisms and Functions

Constitutive volatiles are synthesised and released throughout the whole developmental period of the plant. They may be emitted directly upon synthesis (*de novo*) or stored in specific storage structures such as glandular hairs and then released gradually by diffusion. Additional emissions can occur, through the same route when plants are exposed to environmental stresses (stress-induced emissions) (Possell and Loreto, 2013). Volatiles are biosynthesised via two main biochemical pathways. GLVs are produced through the lipoxygenase (LOX) pathway, which involves lipid peroxidation processes (Feussner and Wasternack, 2002). By contrast, most terpenoids are produced from the same initial precursor (isopentenyl diphosphate; IPP) via the mevalonate (MVA) or methylerythritol 4-phosphate (MEP) pathways (Fig 1.1) in higher plants. The MVA pathway takes place in the cytosol; which is where most sesquiterpenes ($C_{15}H_{24}$, SQTs) are produced. The MEP pathway converts IPP to Dimethylallyl pyrophosphate (DMAPP) in the plastids and lays the foundation for terpenoid synthesis (Rohmer, 1999). There is, however, the potential for intermediate molecules to be exchanged between the two pathways (Vranova et al., 2013). Recent studies have found that IPP can be transported to the mitochondria for SQT biosynthesis (Okada et al., 2000; Vranova et al., 2013). Moreover, stable isotope labelling indicates that the

MEP pathway provides C5 units for cytosolic SQTs (Dudareva et al., 2005), while the MVA pathway has the potential to produce monoterpenes (Cikoš et al., 2019). The interconnections between MVA, MEP and possible pathways in the mitochondria still need clarification (Laule et al., 2003; Rosenkranz and Schnitzler, 2013).

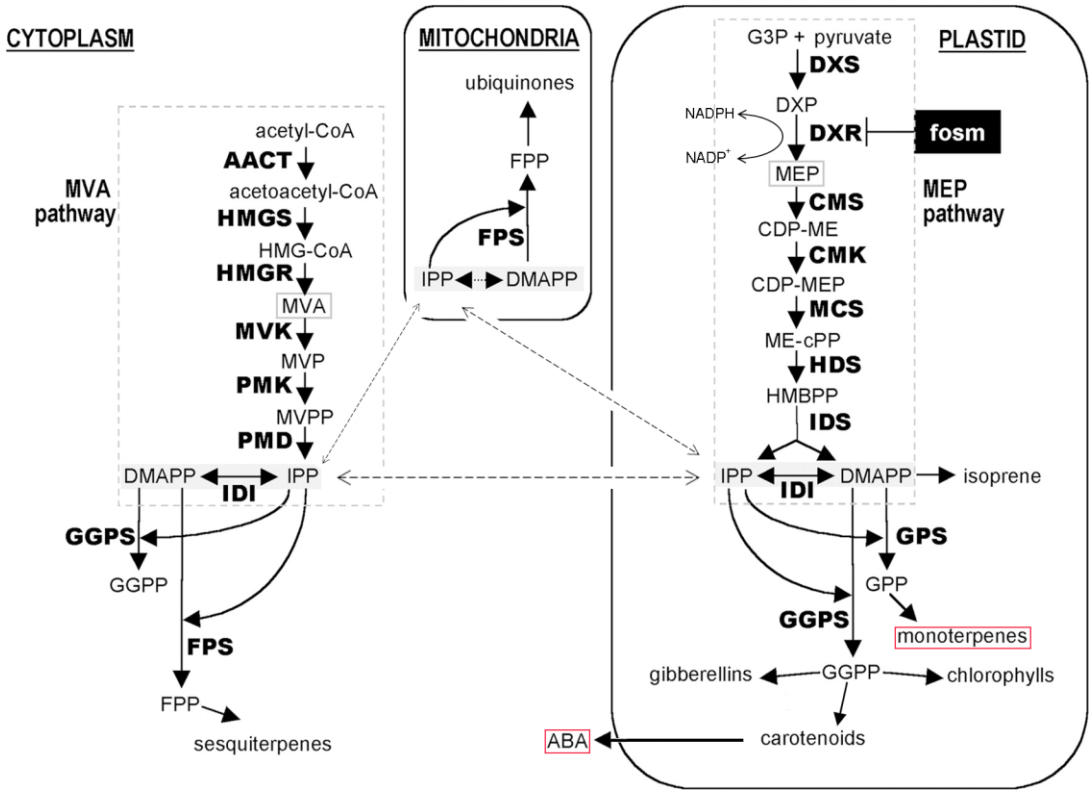

Figure 1. 1 Biosynthetic pathway of terpenoids and precursors (IPP, DMAPP). Left is the mevalonate pathway (MVA) in cytoplasm, right is the methylerythritol phosphate pathway (MEP) in plastid. Substrates are in normal text, HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA; MVA: mevalonate; MVP: mevalonate-5-phosophate; MVPP: mevalonate pyrophosphate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate; FPP: farnesyl diphosphate; G3P: glyceraldehyde 3-phosphate; DXP: 1-deoxy-D-xylulose 5- phosphate; MEP: 2-C-methylerythritol 4-phosphate; CDP-ME: 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; ME-cPP: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; HMBPP: (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; GPP: geranyl diphosphate. Enzymes are in bold text, AACT: acetyl-CoA C-acetyltransferase; HMGS: 3- hydroxy-3-methylglutaryl CoA synthase; HMGR: 3-hydroxy-3-methylglutaryl CoA reductase; MVK: mevalonate kinase; PMK: 5-phosphomevalonate kinase; PMD: phosphomevalonate decarboxylase; IDI: isopentenyl diphosphate isomerase; GPPS: geranyl diphosphate synthase; FPS: farnesyl pyrophosphate synthase; DXS: 1-deoxy-d-xylulose 5-phosphate synthase; DXR: 1-deoxy-d-xylulose 5-phosphate reductoisomerase; CMS: 2C-methyl-D-erythritol-4phosphate cytidylyltransferase; CMK: 4-(cytidine 5′-diphospho)-2-C-methyl-d-erythritol kinase; MCS: 2C -methyl- D -erythritol 2,4-cyclodiphosphate synthase; HDS: 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; IDS: 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; IDI: DMAPP isomerase; GPS: geranyl pyrophosphate synthase. Action of the chemical inhibitor fosmidomycin (fosm) indicated in black box, it blocks DXP isomerase to MEP where NADPH dehydrogenated (Zhao et al., 2013).

The MEP pathway also produces more complex isoprenoids including carotenoids, some of which are known to be ABA precursors. Because ABA strongly moderates stomatal gas exchange, it has been hypothesised that leaf terpenoid (e.g., isoprene) and ABA concentrations are correlated (Barta and Loreto, 2006). Applying chemical inhibitor, fosmidomycin, of the MEP pathway to *Phragmites australis* and *Quercus ilex* decreased concentrations of terpenoids and ABA precursors (carotenoids) and increased *Gs* and *Tr* (Zeidler et al., 1998). Moreover, enhancing ABA concentrations during water stress activates the MEP pathway, and maintains isoprene biosynthesis (Marino et al., 2017). Similarly, terpenoid emissions were directly related to foliar ABA content formed by the MEP pathway which regulated stomata (Barta and Loreto, 2006). Furthermore, plants have several ABA synthesis pathways and storage pools (Li and Walton, 1987). Under drought conditions, ABA accumulates from the roots and is transported to the leaves by xylem sap. However, even in the leaves, drought stress may induce other carotenoids to produce ABA through different pathways (Manzi et al., 2015). The interaction between ABA and terpenoids in various species is yet to be explored, although there are much less data for the relationship with monoterpenes and sesquiterpenes than for isoprene (Ghirardo et al., 2014; Marino et al., 2017).

The MEP pathway occurs in the chloroplasts, and monoterpene biosynthesis ultimately relies on the carbon that harvested by photosynthesis. Most species have developed specialised storage pools for those compounds (Niinemets et al., 2002). For isoprene, though, emission rate is directly associated with *A*, but enzyme and substrate activities also affect isoprene emissions (Niinemets et al., 1999). Although monoterpene emissions have traditionally been thought to be related to temperature alone, increasing evidence suggests they are strongly controlled by photon flux density and photosynthesis rate (Tingey et al., 1980; Winer et al., 1992; Schürmann et al., 1993). Moreover, the control of monoterpene emissions by photosynthesis has been reported in field studies (Kesselmeier et al., 1996; Peñuelas and Llusià, 1999). When *A* is limited due to stress (e.g. drought), the response (reduction) of monoterpene emissions lags behind (Bertin and Staudt, 1996). Suggesting extensive emissions could occur from storage pools or alternative carbon source , such as starch (Tingey et al., 1991; Bouwmeester et al., 1998).

1.6 The Role of Monoterpenes in Plant Responses

Most studies of different abiotic environmental stresses have concentrated on isoprene (Guenther et al., 1995; Peñuelas and Staudt, 2010; Possell and Loreto, 2013). Isoprene can significantly improve plant photochemical efficiency, reduce non-photochemical quenching and enhance physiological heat dissipation (Pollastri et al., 2014), and stabilise membranes (Siwko et al., 2007). Isoprene provides thermal and oxidative protection under a wide range of stresses, including drought and exposure to airborne pollutants such as ozone, by effectively reducing the accumulation of ROS in cells and damage to membranes (Loreto et al., 1998; Velikova et al., 2005; Ryan et al., 2014). Maintained of isoprene emission can enhance plant recovery from drought or heat stress (Sharkey and Loreto, 1993; Velikova and Loreto, 2005; Brilli et al., 2007).

Although not as widely studied as isoprene, it is believed that monoterpenes may have a similar protective function, especially with regard to heat and oxidative stresses (Loreto and Schnitzler, 2010; Possell and Loreto, 2013). Loreto et al., (2002) hypothesised that the reduction of monoterpene emissions results from suppressed enzyme activities. When *Quercus ilex* saplings were exposed to elevated temperature (30 – 55 ℃), emissions of some specific monoterpenes (α-pinene, β-pinene, limonene, myrcene) increased. When the plants were fumigated with those chemicals, photosynthesis rate and operating efficiency of PSII were significantly higher than in control plants, appearing to confirm that monoterpenes provide specific photosynthetic protection. Copolovici et al. (2005) further demonstrated the thermal protection role of monoterpene on photosynthetic electron transport. Moreover, monoterpenes also prevent ROS accumulation and membrane lipid peroxidation. By exposing non-isoprene emitting plants to ozone, monoterpene synthesis was stimulated. When exogenous chemical inhibitor (fosmidomycin) was applied to leaves (see Fig 1.1), monoterpene emissions were significantly reduced, and the ozone rapidly inhibited photosynthesis and increased ROS and MDA contents (Loreto et al., 2004).

Since isoprene and monoterpenes are both synthesised thought the MEP pathway, it is possible that monoterpenes have the same oxidative protection property as isoprene. As heat stress is often accompanied by oxidative stress (Llusià et al.,

2006), so, monoterpenes induced as antioxidant may also help maintain membrane integrity during heat stress (Copolovici et al., 2005). On the other hand, terpenoids can also directly react with ROS to act as antioxidants (Loreto et al., 2001b; Loreto and Velikova, 2001). In the atmosphere, highly reduced terpenoids can be oxygenated by reactive nitrogen and oxygen species. In plants, different terpenoid compounds may have high or low reductive properties (Lado et al., 2004), and the reaction may occur on the surfaces of membrane or in the intercellular space (Loreto and Velikova, 2001; Vickers et al., 2009).

1.7 Research Knowledge Gaps, Aims, Objectives, Hypotheses

As highlighted above, biosynthesis and emissions of biogenic terpenoids are altered under abiotic stresses. However, the role of monoterpenes in particular in plant defences against drought or other abiotic stresses remains to be investigated. Moreover, the interactions between terpenoids and hormones such as ABA, both synthesised via the MEP pathway, has attracted recent attention (Barta and Loreto, 2006; Ryan et al., 2014).

This research aimed to determine whether drought stress affects monoterpene emissions in tomato and if monoterpene synthesis and emissions protect tomatoes against drought stress

This research will be based on following hypotheses:

1. Deficit irrigation (supplying less water than plant evapotranspiration) will change monoterpene emission rates

Previous studies have observed increases, decreases and no change of monoterpene emissions in drought-stressed plants. We do not understand how monoterpene emissions in tomato respond to drought stress, so we hypothesise that water deficit will alter the synthesis of monoterpenes and hence both the magnitude and composition of emissions.

2. Due to competition for carbon between monoterpene and ABA synthesis, we expect higher constitutive emission of monoterpenes in an ABA-deficient tomato mutant, (*notabilis*) with drought stress decreasing emission rate in wild-

type (WT) tomatoes when ABA production increases. However, drought stress is expected to increase emission rate in the ABA-deficient mutant as ABA accumulation is limited

Carbon assimilated via photosynthesis is allocated to the MEP pathway (Fig 1.1) to support terpenoid biosynthesis. Since tomato does not synthesise isoprene (Pazouki et al., 2016), the carbon will be used to produce monoterpenes, ABA and other related hormones. Since drought stress stimulates, ABA production (to regulate stomatal closure and preserve leaf water status), more carbon will be diverted to ABA, and monoterpene production will be reduced consequently.

3. Monoterpene synthesis and emissions maintain photosynthetic system efficiency, protect the photosynthetic apparatus, and sustain photosynthesis rate by limiting ROS damage resulting from drought

Monoterpenes can protect the photosynthetic system and, enhance PSII maximum and operating efficiency under heat stress, and reducing ROS accumulation or subsequent damage under oxidative stresses caused by e.g. high temperature (Loreto et al., 1998; Niinemets et al., 2002). We hypothesise that MT can also protect photosystems via the same mechanism under drought stress.

To assess these hypotheses, wild-type (WT) and ABA-deficient (*not*) tomatoes were cultivated under different water regimes to compare variables between treatments and genotypes. Leaf-level gas exchange, stomatal conductance, leaf water potential and chlorophyll fluorescence were measured, with monoterpene samples collected at the same time as leaf-level measurements. Relationships between these physiological measurements and biochemical responses were determined, by assessing foliar ABA, hydrogen peroxide and malondialdehyde (MDA) concentrations. Moreover, to demonstrate whether monoterpenes affected these physiological and biochemical responses, a factorial experiment that exogenously sprayed selected monoterpenes was performed.

2 Methodology

2.1 Plant Materials and Growth Conditions

Seeds of tomato (*Solanum lycopersicum* L.) genotypes *Ailsa Craig* (wild-type; WT) and its ABA-deficient mutant *notabilis* (*not*). In *notabilis*, the enzymes encoding the rate-limiting step of ABA biosynthesis, a 9-cis-epoxycarotenoid dioxygenase (NCED), is a null mutation, hence, ABA synthesis is blocked at the conversion of the intermediate neoxanthin to Xanthoxin (Parry et al., 1988; Burbidge et al., 1999; Thompson et al., 2000). Seeds were obtained from the Plant and Soil Ecology Lab, Lancaster Environment Centre (Lancaster, UK). On 23rd September, 80 WT and 40 *not* seeds were sown and germinated in John Innes No. 2 potting compost (Westland Horticulture Ltd, Tyrone, UK) in plastic seed trays (5 cm x 5 cm x 5 cm). Three weeks after sowing, uniform-sized seedlings were selected and transferred, one seedling per pot, to 2-litre plastic pots (14 cm top, 10.5 cm base, 18.5 cm depth), which were then filled with John Innes No. 2 to 15 cm depth.

The seedlings were subsequently grown in 1 m^3 semi-controlled environment Teflon-covered growth chambers (Fig 1a.) similar to those described by Stockes et al. (1993). Plants were grown in chambers for another 3 weeks before the start of the experiment. Briefly, a photosynthetic photon flux density (PPFD) of approximately 400 ± 20 μmol photons m^{-2} s^{-1} was provided to each of the four chambers by growth lamps (Powerstar HQI-BT, 600 W/D daylight, OSRAM, Munich, Germany) for 12 h per day (07:00 to 19:00). Air temperature in the chambers was regulated by pumping purified air through the chambers at a flow of 2 m^3 min^{-1} to each chamber. Day : night temperatures were maintained at 22 °C : 16 °C ± 1.0°C, relative humidity (RH) at 40 : 60 ± 10% and CO_2 at 380 ± 10 ppm. Relative humidity, PPFD, and ambient temperature in the growth chambers were recorded at 10-minute intervals by RH2nl-02 Humidity Sensors, QS2 Quantum Sensors and Fenwal UUA32J2 2K Thermistors (Delta-T Devices Ltd, Cambridge, UK) respectively via a DL2e data logger. Sensors were located in the middle left of each chamber (see Fig 2.1b). Growth conditions remained relatively

constant in each chamber throughout the experiment which ran for 3 weeks from 04th Nov – 21st Nov.

Pots were randomly assigned to treatments and were rotated within the chambers every day and between chambers every week to minimise "chamber effect", i.e. any heterogeneity of air temperature, PPFD, relative humidity, and airflow between chambers and therefore treatments. Pots were irrigated once per week with a solution of 15 ml Miracle-Gro® All Purpose Soluble Plant Food (The Scotts Company Ltd, Surrey, UK) dissolved in 4.5 litres of water.

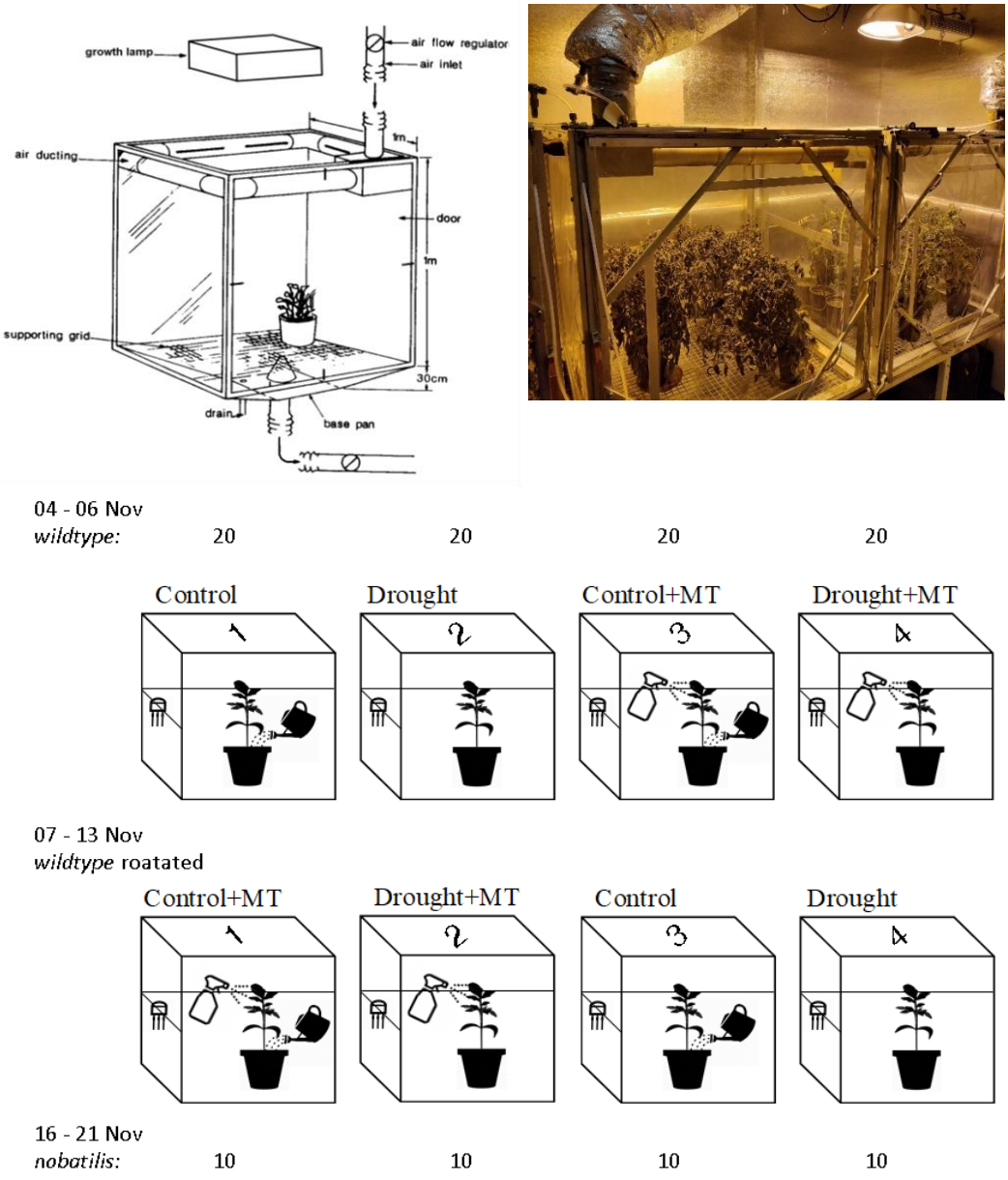

Figure 2. 1 Schematic of (a) growth chambers, (b) experimental design. WT experiments ran from 04th-13th November with 20 plants in each treatment, treatments were switched between chambers at the end of Day 6 and *not* from 16th-21st November with 10 plants in each treatment.

2.2 Treatments

Before transplanting the seedlings, each filled pot was watered to excess (defined as the point water starts dripping from the bottom) and placed on the grid floor of the chambers for 24 hours. Each pot was then weighed after transplanting to determine the container water capacity. Prior to applying drought, all plants were irrigated twice a day (8am and 6 pm) to maintain well-watered conditions (WW); i.e. the replacement of 100% of the daily pot water loss determined by weighing pots. Pots were weighed after measurements and sampling.

Half of the WT (40 plants) and *not* (20) were subjected to a water deficit treatment (WD) in which only 25% of daily pot water loss was added at the end of sampling day (6 pm). The WD treatment was continued until half of the plants were visibly wilted at which point all of the pots were re-watered to container water capacity. The remaining half were kept under WW conditions throughout (control). Half of each control and treatment were used to apply a second treatment factor - exogenous application of monoterpenes by spray (MT). There were thus four treatments applied in parallel in each experiment: WW without spray (control), WD without spray (treatment), WW with spray (Control + MTs) and WD with spray (WD+ MTs).

The first factorial (WD + MT spray) experiment was conducted on WT, with the four control and treatment groups kept in separate chambers to prevent priming or signalling effects on bVOC synthesis and emissions between treatments. Once the WT experiment finished (after 2 weeks) the process was repeated for *not* (a further 1 week).

Monoterpene solutions for the spray treatments were prepared by dissolving 200µl of each of α-pinene, β-pinene, 3-carene, ocimene, limonene, ϒ-terpinene, and terpinolene (Sigma-Aldrich, Dorset, UK) in 8 ml methanol. The solution was transferred to a 1-litre volumetric flask and topped up with distilled water to make a 1.25mM treatment solution. The control spray solution consisted of a solution of 8ml of 0.8% methanol and distilled water. The spray was applied evenly to both sides of the leaves to the point of incipient runoff using a 2-liter hand-held

pressurised sprayer every morning commencing one day before the drought conditions were applied.

2.3 Sampling Protocol

Daily sampling started one day before the drought treatment was first applied (Day 0), at which time all the plants were well-watered, to measure baseline data. Sampling of WT tomatoes began in their 6^{th} growth week, and *not* in their 7^{th} growth week since *not* grew slower. Plants within each treatment were randomly selected for three different sets of measurements: 1) BVOC; 2) morphology; 3) BVOC plus morphology. Three *BVOC* replicates from each treatment were used for BVOC sample collection, leaf gas exchange measurements, and non-destructive measurements which included pot weight, substrate moisture, light-adapted chlorophyll fluorescence. These same three plants were sampled throughout the experiment to counteract any difference in physiology or BVOC emissions between plants. In addition, one *morphology* replicate was destructively harvested for morphological measurements. A final replicate was allocated for both *BVOC* plus *morphology* (destructive) measurements.

One leaflet on the newest fully expanded leaf, which was chosen for gas exchange and BVOC sampling, was subsequently harvested for ROS and ABA assays. Strips of approximately 0.04 g and 0.100 g were cut from a leaflet adjacent or opposite side to the one sampled for gas exchange (on the same leaf), using a razor blade and collected into different 1.5 ml Eppendorf tubes for H_2O_2 and lipid peroxide assays respectively. The rest of the leaflet was collected into a 15 ml Falcon tube for ABA analysis. The Eppendorf and Falcon tubes were frozen in liquid nitrogen immediately and then stored at -80 °C.

The same sampling protocol was implemented on each day of the experiment for all treatments in both experiments.

2.4 Gas Exchange and Chlorophyll Fluorescence

A LI-6400XT portable photosynthesis system (Li-Cor Environmental Ltd, Cambridge, UK) with integral leaf chamber fluorometer (LCF) was used for leaf gas exchange and chlorophyll *a* fluorescence measurement from light-adapted leaf.

A blade on the youngest fully developed leaf (as described in 2.3)was selected and clamped inside the sampling cuvette. The conditions inside the cuvette were set to match those in the growth chamber as closely as possible; conditions were maintained throughout the sampling period for all replicates.

The leaf fan was set to 'Fast' and the flow of air to the cuvette set to a constant 500 μmol s^{-1} to maintain a constant leaf humidity of 60 ± 5%. PAR was supplied to the cuvette by red + blue LED lights in LCF and set to 400 μmol m^{-2} s^{-1} with 10% blue light. A target reference of 400 μmol s^{-1} CO_2 was transmitted to the cuvette by a 6400-01 CO_2 mixer which was calibrated every day before measurements started. The block temperature was maintained at 22 °C. The desiccant scrubber was fully bypassed to maintain the relative humidity. Once the sample cuvette was clamped on to the blade, auto-logging was initiated to record the photosynthesis rate (*A* μmol m^{-2} s^{-1}), transpiration rate (*Tr* mmol m^{-2} s^{-1}), intercellular CO_2 concentration (*Ci* μmol CO_2 mol air^{-1}), stomatal conductance (*Gs* mol m^{-2} s^{-1}), and the maximum fluorescence during a saturating light flash (F_m'), photosynthetic steady-state (F_s'), and minimum fluorescence (F_o') during a momentary darkness, every minute for 30 minutes. The cuvette environment was allowed to stabilise for 10 mins before 20 mins of BVOC sampling was undertaken. by diverting a proportion of the outlet flow (100 ml min^{-1}) to be drawn through a sorbent cartridge (Markes International Ltd, Llantrisant, UK) packed with 0.2g Tenax and 0.1g Carbotrap. An activated carbon filter (Whatman, GE Healthcare UK Ltd, Little Chalfont, UK) was used on the inlet of the LI-6400XT throughout the experiments to ensure the air entering the chamber was free from hydrocarbons. All of the parameters recorded during the 20-min BVOC sampling period were averaged to give one value per plant.

The intrinsic water use efficiency (*i*WUE) is defined as *A*/*Tr* (μmol CO_2 mmol H_2O^{-1}; Ehleringer and Cerling, 1995). PSII operating (φPSII) and maximum efficiency (F_v'/F_m') were estimated as follows:

$$\Phi_{PSII} = \frac{\Delta F}{F_m'} = \frac{F_m' - F_s'}{F_m'} \qquad \text{Equation (1)}$$

$$\frac{F_v'}{F_m'} = \frac{F_m' - F_o'}{F_m'} \qquad \text{Equation (2)}$$

Where F_m', F_s', F_o' refer to light-adapted maximum, steady-state and minimum chlorophyll florescence. φPSII measures the proportion of chlorophyll-absorbed light that is used for photosystem II, it is also often used to calculate the electron transport via PSII, and further represent the overall photosynthesis (Maxwell and Johson, 2000). Whereas PSII maximum efficiency F_v'/F_m' indicates the maximum operating efficiency after light adaptation, a decrease of this parameter reflects an increase in the conversion of light energy to heat (non-photochemical quenching) (Murchie and Lawson, 2013).

2.5 Substrate Water, Plant Water and Morphological Status

Substrate water was measured using a soil moisture sensor (WET-2, Delta-T Devices Ltd, Cambridge, UK) at the end of each sampling period. For morphological replicates, the leaf water potential of the leaf used for the gas exchange measurements was assessed by pressure chamber (Soilmoisture Equipment Corp., CA, USA) after completing the measurement. In brief, the youngest fully developed leaf was cut from the stem using a sharp razor blade, ensuring the cross-profile of the cut was straight and clean. The leaf was then inserted inside the pressure chamber, so that the cut side protruded from the seal gasket. Once the chamber had been closed and sealed, the pressure was gradually increased using a compressed air cylinder until the pressure inside the chamber became equal to that of the xylem, at which point water was exuded on the cut surface and the leaf water potential (MPa) could be read from the chamber gauge.

All shoots were then cut off the morphological replicates to measure shoot and leaf fresh weight (FW), and then dried at 65 °C for 48 hours for dry weight (DW).

2.6 Monoterpene Sampling and Analysis

BVOC samples were collected simultaneously with gas exchange measurements using stainless steel thermal desorption sorbent cartridges (3½-inch [89 mm] long x ¼-inch [6.4 mm] o.d. Markes International Ltd, Llantrisant, UK). Each tube was filled with 0.2g Tenax® Porous Polymer and 0.1g Carbopack™ Adsorbent Matrix (Sigma Aldrich Ltd, UK). The outlet of the LI-6400XT chamber head was connected with a VOC-free three-way PVC T-piece from which one outlet tube

linked with the sorbent cartridge and the other fed back to the IRGA. A total of 2L of air were loaded into the cartridge at a rate of 100 ml min^{-1} for 20 minutes.

Cartridge samples were analysed for volatile content. Samples were first desorbed using an Auto Thermal Desorber (ATD, TurboMatrix150, PerkinElmer, Beaconsfield, UK). The tube was heated to 280℃; the valve temperature set at 230℃; and the trap temperature increased from -30℃ to 300℃ at a rate of 40℃/s. The desorbed samples were injected into an AutoSystem XL Gas Chromatographer/TurboMass Gold Mass Spectrometer (PerkinElmer, Beaconsfield, UK) with a quadrupole mass analyser. The compounds were carried with helium at 11.5 psi and separated in a non-polar Ultra 2 capillary column (5%-phenyl-methylpolysiloxane, Agilent). The column temperature was initially maintained at 35℃ for 2 minutes. The temperature was then ramped up at a rate of 4.0℃ per minute, to reach 160℃ and then further increased at a rate of 45℃ per minute to 300℃. The column was maintained at this temperature for 10 minutes. The inlet line and ion source temperatures were 200℃ and 250℃, respectively and the extractor electron impact (EI) ion source was set to 70 eV. The full mass spectrometer scan range was 50-300 m/z. This follows the standard methodology for high-resolution separation of monoterpenes developed by the biosphere-atmosphere research group at Lancaster University.

Cartridges pre-loaded with a known mass of 10 common monoterpenes were put through the ATD and GC-MS with each batch of samples for calibration. These calibration standards were produced using standard compounds from Sigma-Aldrich Co. (Gillingham, UK): α-pinene (98%), β-pinene (99%), β-myrcene (≥ 90%), limonene (99%), ocimene (≥ 90%), α-phellandrene (99%), 3-carene (99%), γ-terpinene (99%), and terpinolene (≥ 90%). 20 µl of each standard compound was mixed in a 200 ml volumetric flask with methanol. 20 µl of the mixture was transferred into another 200 ml volumetric flask and again made up with methanol to give a final concentration of each chemical of 3.5 ng/µl. 2, 4, and 8 µl of the final dilution were flushed with helium into adsorption tubes, identical to those used for sampling. A total of two of each of those mass standards were loaded into the ATD at intervals and analysed with sample tubes. New standards were made for each run.

Chromatographic peaks and spectrometric data were processed using the TurboMass Software, Version 5.4.2 (PerkinElmer Inc., Shelton, CT, USA) with identification based against the NIST (National Institute of Standards and Technology) 2008 Libraries (Version 2.2.0, PerkinElmer Inc., Shelton, CT, USA). Calibration curves of peak area *vs.* (known) mass were drawn for each individual monoterpene included in the calibration standards. The peak area of each monoterpene identified in the samples were then used to deduce the mass collected by comparison against these calibration curves. Identified compounds that were not included in the standards were quantified using the calibration curve of the standard compound with the nearest retention time. The leaf level BVOC emission rate was calculated by:

$$E_{MT} = \frac{Mass}{Area \times Time} \qquad \text{Equation (3)}$$

Where Mass is the mass of collected compound as determined by the GC-MS quantification method, Area is the sampled leaf area (1 cm^2 = 0.0001 m^2), and Time refers to the 20 mins sampling time (1200s).

2.7 Abscisic Acid Analysis

The concentration of foliar abscisic acid (ABA) was determined by radioimmunoassay using the monoclonal antibody MAC 252 (Quarrie et al., 1988). The fresh leaf tissues that had been placed in 15 ml Falcon tubes were freeze-dried for 48 h. The dried tissues were chopped using dissecting scissors, and ground to a fine powder with a CryoMill. About 20 mg of the ground dry tissue sample were transferred to a pre-weighed 1.5 ml Eppendorf tube which was then re-weighed. Distilled water was added to each tube in a ratio of 1 : 25 (dry weight : water) to extract the ABA. Samples were extracted on a shaker overnight at −4°C and centrifuged at 15,000 rpm for 4 minutes. 50 µl standards or sample supernatants were mixed with 500 µl phosphate buffer saline (PBS) in 1.5 ml centrifuge tips and 100 µl each of 3^H-ABA and MAC252 of each were carefully added. All tubes were centrifuged at 15,000 rpm for 1 min, then refrigerated for 45 mins. 0.5 ml saturated ammonium sulphate was added to each tube to precipitate the complex of ABA-antibody. Tubes were then tipped upside down to fully mix

the solutions and placed in the dark at room temperature for 30 mins. Mixtures were again centrifuged at 15,000 rpm for 4 mins, following which a small white pellet formed at the base of each tube. All supernatants were completely poured out and 1.0 ml 50% ammonium sulphate was placed in each tube which was vigorously shaken to re-suspend the pellets and remove excess unbound radioactivity. Tubes were re-centrifuged for a further 5 mins at 15,000 rpm. All excess liquid was poured into radioactive waste sink and 100 µl deionised water was added before the tubes were vibrated using a cyclone vortex mixer (Whirlimixer, Fisher Scientific Ltd, Loughborough, UK) to re-suspend the pellets. and then injected with 1.5 ml Ecoscint H scintillation solution was then injected, and each tube again mixed on the vortex mixer until all the pellets had dissolved. Tubes were placed inside a clean glass scintillation vial and loaded into a liquid scintillation analyser (Tri-Carb 1600 TR, PerkinElmer Inc. (Packard BioScience), Wellesley, USA). ABA concentrations were calculated from the radioactivity read by the scintillation counter as counts per minute (cpm). Calibration curves were constructed with the highest radioactive counts (B_{max}) taken from the water standards (as these do not contain any unlabelled ABA to compete with the anti-body/3^{H}-ABA binding reaction) and the lowest (B_{min}) from the mixture without antibody binding. Six ABA standards of 62.5, 125, 250, 500, 1000, 2000 pg per vial were run in each batch of samples. All counts were then corrected by subtraction of B_{min}. The linear regression was developed by plotting logit transformed of corrected counts against log of unlabelled ABA in each vial:

$$B = cpm - B_{min} \qquad \text{Equation (4)}$$

$$logit\left(\frac{B}{B_{max}}\right) = \ln\frac{B/B_{max}}{1-B/B_{max}} \qquad \text{Equation (5)}$$

where B is the corrected cpm. ABA concentrations of samples were calculated by interpolating corrected counts to the regression line of the calibration graph.

2.8 Reactive Oxygen Species Assay

Lipid peroxide and H_2O_2 content in leaf tissues were measured as indicators of plant oxidative stresses that resulted from drought. H_2O_2 is a common reactive oxygen species, and an inevitable product of aerobic metabolism (Sharma et al.,

2012). As previously described, drought stress causes the disruption of cellular homeostasis increasing the production of ROS and hence H_2O_2 (Mittler, 2002; Cruz de Carvalho, 2008). As drought conditions continue, increasing lipid peroxidation coincides with the accretion of ROS. When ROS crosses a threshold value, lipid peroxidation causes extensive cell damage and death (Dat et al., 2000; Ayala et al., 2014). Hence, lipid peroxidation is widely used to assess oxidative damage and ROS formation under drought stress. The biomarker malondialdehyde (MDA) is one of the main secondary products of peroxidation (Moller et al., 2007). The thiobarbituric acid-reactive-substances (TBARS) assay (Heath and Packer, 1968), which is based on the staining reaction between MDA and thiobarbituric acid (TBA), was applied to determine the MDA equivalent to the leaf lipid peroxide concentrations.

Hydrogen peroxide

Frozen leaf material (100 mg FW) was homogenised using an ice-cooled mixer mill (Retsch Ltd, Hope, UK) for 5 mins at 30 Hz with 1 ml of 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 15,000 rpm for 30 mins. 0.4 ml of the supernatant was then added to 0.4 ml of 10mM potassium phosphate buffer (pH 7.0) and 0.8 ml of 1M potassium iodide (KI). The coloured reaction product of H_2O_2 with KI develops within 25 mins and is stable for at least 2 h. The absorbance of the supernatant at 360 nm was determined using a spectrophotometer (Jenway 6300, Cole-Parmer Ltd., Stone, UK). A calibration curve was produced using 0.4 ml of 0, 1, 5, 10, 20, 30, 40, 50, 80, 100 μM H_2O_2 in place of the 0.4 ml of the supernatant samples and the H_2O_2 content of each sample calculated using that calibration curve.

Lipid peroxide

Frozen leaf material (40 mg FW) was homogenised in 1 ml of 0.1% (w/v) TCA using an ice-cooled mixer mill (Retsch Ltd, Hope, UK) for 5 mins at 30 Hz. The homogenate was then centrifuged at 4°C at 15,000 rpm for 30 mins. 0.5 ml of the supernatant was mixed with 1.0 ml of 20% TCA containing 0.5% (w/v) thiobarbituate acid (TBA) in a 2ml Eppendorf and the mixture heated for 30 mins at 95°C. The reaction was then immediately stopped in an ice bath and the mixture

centrifuged at 10,000 rpm for a further 5 mins at 4 °C. The absorbance of the supernatant at two wavelengths (532 and 600 nm) was determined using a spectrophotometer. Two wavelengths were used to correct for nonspecific turbidity. To calculate the lipid peroxide content of the samples, an absorption coefficient of 155000 μM^{-1} cm^{-1} was used (Heath and Packer, 1968).

$$MDA\ equivalents, A\ (nmol\ ml^{-1}) = \frac{A_{532} - A_{600}}{155000} \times 10^6 \qquad \text{Equation (6)}$$

$$MDA\ contents\ (nmol\ g^{-1}\ FW) = \frac{A \times 3 \times 1ml}{40mg} \qquad \text{Equation (7)}$$

where A is the MDA concentration in nmol ml^{-1}; 3 is the sample dilution factor (0.5ml supernatant + 1ml TBA/TCA solution); 1ml 0.1% (w/v) TCA and 40mg samples were used for each extraction.

2.9 Statistical Analysis

A General Linear Model (GLM) with ANOVA, post-hoc Tukey, and Bonferroni correction was used. Both 2- and 3-way ANOVA were tested based on treatments (drought, monoterpene, genotypes) that applied in this experiment. A post-hoc Tukey test was used to compare across drought and MT combinations. Paired t-tests compared each independent variable within treatments and genotypes throughout the drought and re-watering cycle.

Curve estimation (non-linear regression analysis) was applied to determine the dependency of monoterpene emissions and gas exchange on soil or leaf water status. All analyses were conducted with IBM SPSS Statistics (version 25, IBM Corp., New York, USA); $p < 0.05$ was considered to indicate a statistically significant difference.

3 Results

3.1 Soil and leaf water status

Individual pots in the drought treatment were watered at 25% of individual daily pot water loss until more than half of the plants in the chamber were visibly wilted. At this point, the drought treatment was ended when plants were re-watered and returned to initial soil water content. Measurements were made on a daily basis from Days 1 to 9 in wild-type and Days 1 to 5 in *not* (Fig. 1a, c), i.e. to include both the drought and recovery periods. Soil moisture declined from 51.8 ± 2.9% to 15.4 ± 3.1% and 52.9 ± 2.2% to 27.4 ± 6.5% in wild-type and *not* drought treatments respectively, before plants were re-watered. In brief, wild-type WD reached wilting point at Day 6 and wild-type WD+MT at Day 7. There followed 2 days of re-watering. Both *notabilis* treatments wilted on Day 3 after a similar rate of soil moisture decline; again, there followed 2 days of re-watering. MT spray appeared to have different impacts on the rate of soil drying in the two genotypes.

For wild-type , the soil moisture of drought treatment without monoterpene spray (wild-type Treat) declined more quickly than that with monoterpene spray (wild-type WD+MT) over the first 4 days whereas soil moisture responses were similar in both *not* drought treatments (*not* WD and *not* WD+MT). After re-watering, soil moisture recovered to the baseline level (WW) by the end of Day 6 (wild-type Treat) and Day 7 (wild-type WD+MT), while recovery to baseline soil moisture for both *not* treatments was complete by the end of Day 3.

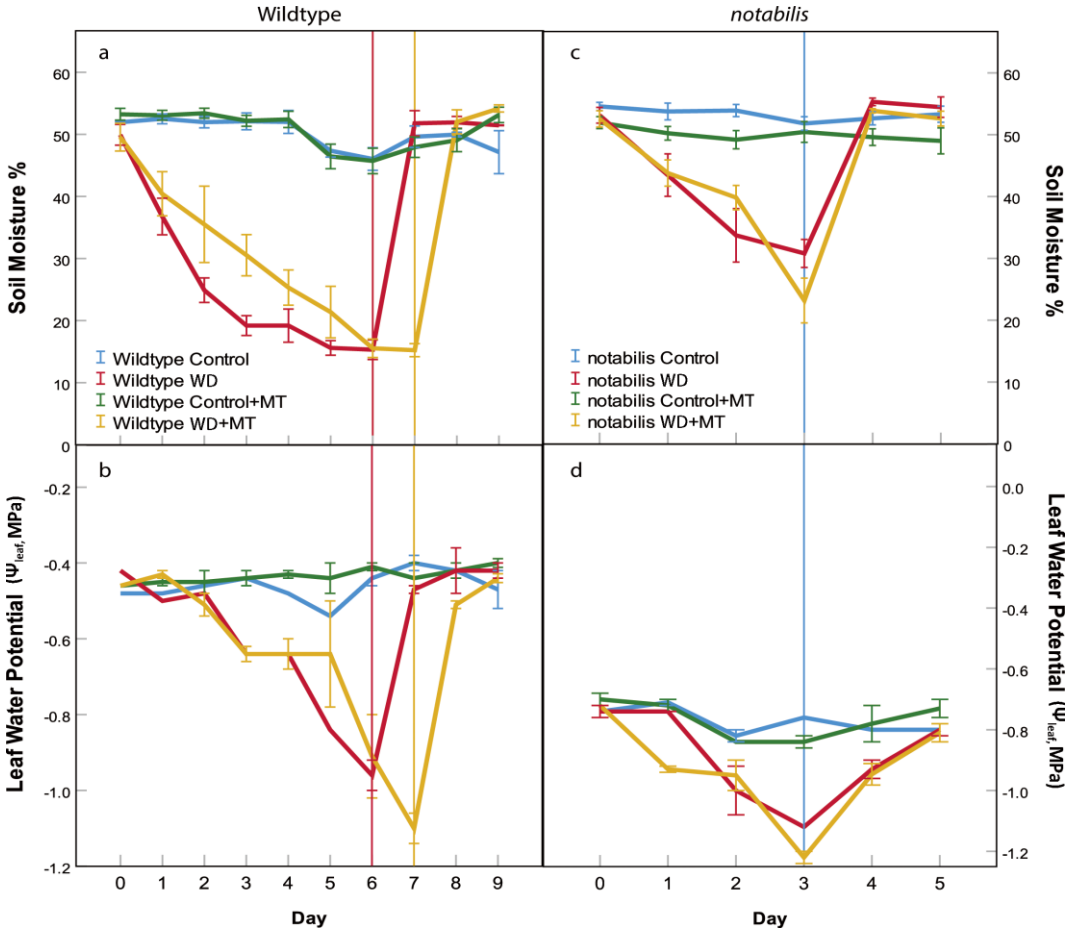

Figure 3. 1 Time series of soil moisture (SM; %, a, c) and leaf water potential (Ψ_{leaf}; b, d) of wild-type (*Ailsa Craig*, a, b) and *notabilis* (c, d) showing means (± standard error, SE). n ≥ 4 plants for soil moisture and n = 2 or 3 for leaf water potential. Treatments: well-watered (Control; blue lines), drought treatment (Treat; red lines), control with MT spray (Control+MT; green lines) and WD with MT spray (WD+MT; yellow lines). The red and yellow vertical lines on wild-type indicate re-watering day for WD and WD+MT, respectively. The blue vertical line on *not* indicates re-watering day for both WD and WD+MT.

Leaf water potential (Ψ_{leaf}; Fig. 3.1b) in wild-type WD fell gradually from -0.46 ± 0.02 to -0.54 ± 0.1 within 3 days before decreasing rapidly to its wilting point of -0.96 ± 0.04 MPa on Day 6. Although Ψ_{leaf} showed the same initial decline in wild-type WD+MT, it then remained constant for two days (Days 3-5) before then falling to its slightly lower wilting point (-1.10 ± 0.04 MPa) on Day 7. The differences however were not significant (P = 0.283). The initial (well-watered) Ψ_{leaf} of -0.72 ± 0.02 MPa was much lower for *not* than wild-type but it fell to a similar value (-1.19 ± 0.04 MPa) of WD at wilting point on Day 3 (Fig. 3.1d). The two genotypes differed in their Ψ_{leaf} response to decreasing soil moisture (Figure 3.2). In wild-type , Ψ_{leaf} remained relatively constant at ~-0.50 MPa for soil

moisture between 53-38 % , declined from -0.50 to -0.68 MPa, i.e. by ~40 %, between 38-23 % and dropped rapidly to wilting point of ~-1.30 MPa below a threshold soil moisture of 23 %. By contrast, in *not*, Ψ_{leaf} fell approximately linearly with soil moisture, and was significantly lower than wild-type for the same soil moisture throughout the experiment ($P < 0.001$, Table 3.1). Based on the Ψ_{leaf} responses of wild-type , we define three distinct stress levels for our subsequent analysis of drought impacts: well-watered (WW, SM $\geq$ 38 %), moderate drought (MD, 23 % $\leq$ SM < 38 %) and severe drought (SD, SM < 23 %).

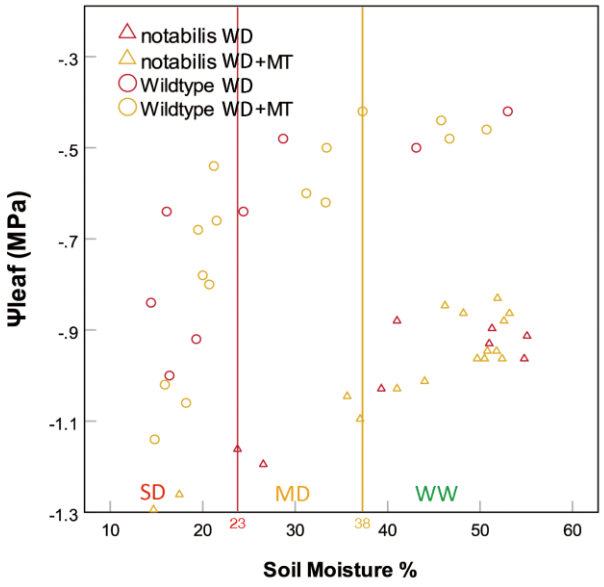

Figure 3.2 Response of leaf water potential (Ψ_{leaf}) to soil moisture (SM %) in wild-type (*Ailsa Craig*, open circles) and ABA-deficient (*notabilis*, open triangles) genotypes in control and drought treatments without (red) and with MT spray (yellow). Each symbol is an individual plant. The yellow line at 38% SM represents a water stress transition from well-watered (WW) to moderate drought (MD) and the red line at 23% the transition from moderate to severe drought (SD).

To sum up, *notabilis* had significantly lower leaf water potential and responded to soil drying more quickly ($P < 0.001$) than wild-type and the application of exogenous monoterpene spray did not affect leaf water status during deficit irrigation in either genotypes.

Table 3.1. The significance of the effects of water deficit treatment (WD: control, WW, MD, SD), genotype (G: *Ailsa*, *not*) and monoterpene treatment (MT) on soil moisture (SM, %), leaf water potential (Ψ_{leaf}, MPa), carbon assimilation rate (*A*, μmol m^{-2} s^{-1}), stomatal conductance (*Gs*, mol m^{-2} s^{-1}), intrinsic water use efficiency (*i*WUE, μmol CO_2 mmol H_2O^{-1}), foliar ABA (ABA, ng g^{-1} DW), hydrogen peroxide (H_2O_2, ng g^{-1} FW), malondialdehyde equivalents (MDA, ng g^{-1} FW) concentrations, PSII maximum (*Fv'/Fm'*) and operating (φPSII) efficiencies.

Factor	SM	Ψ_{leaf}	*A*	*Gs*	iWUE	ABA	H_2O_2	MDA	F_v'/F_m'	φPSII
WD	***	***	***	***	***	***	***	n.s.	*	*
G	**[c]	**	n.s.	***	***	***	***	***	*	n.s.
MTs	n.s.	n.s.	*	*	n.s.	n.s.	n.s.	*	n.s.	n.s.
WD x G	n.s.	n.s.	n.s.	***	n.s.	***	***	n.s.	n.s.	n.s.
WD x MTs	*[c]	n.s.	**	*	***[c]	n.s.	n.s.	n.s.	n.s.	n.s.
G x MTs	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
WD x G x MTs	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.	*	n.s.

*, $P = 0.05$; **, $P = 0.001$; ***, $P < 0.001$; ns, no significant difference; [c] denotes chamber effects.

3.2 Leaf physiological response, gas exchange and growth environment impacts

While the soil moisture responded differently over time in wild-type WD and wild-type WD+MT (Fig 3.1), the relationship between SM and Ψ_{leaf} was the same for both treatments irrespective of MT application (Fig 3.2). Moreover, Table 3.1 indicates that MT spray had no significant impacts ($P > 0.005$) on either SM or Ψ_{leaf}. Taken together, these suggest that the differences seen in the timeseries were not driven by MT treatment and we therefore checked other environmental conditions in the growth chambers.

To reduce chamber effects, plants were rotated within chambers every day and between chambers every week from the day the plants were first transferred to the growth chambers. At the start of the experiment wild-type Control, wild-type Treat, wild-type Control+MT and wild-type WD+MT were growing in chambers 1, 2, 3 and 4 respectively (as shown in Fig 2.1). On Day 3 wild-type Control, wild-type Treat, wild-type Control+MT and wild-type WD+MT were rotated to chambers 3, 4, 1, and 2 respectively. Initially, the vapor pressure deficit (VPD) was slightly higher, by about 0.2-0.45 kPa, i.e. by 10 – 18 %, in wild-type WD (chamber 2) than wild-type WD+MT (chamber 4) from day 0 to 2 when most plants still had well-watered conditions. This was reversed following the rotation of chambers from which time significant differences appeared (Fig 3.3a) in all measured variables.

The change in chambers, and hence VPD, at the end of Day 2 had a marked impact on transpiration (*Tr*). *Tr* and chamber VPD were strongly correlated (Fig 3.3c). Initially, transpiration was higher by nearly 36 % in wild-type Treat, caused by the higher chamber VPD. From Day 3 onwards, however, wild-type WD+MT had substantially higher (by up to 50%) transpiration until re-watering occurred on Days 6 and 7. Soil drying dominated the reduction of *Tr* from Day 3, when soil water moisture was reduced to below 30 % (Fig 3.1a), at which point there was less available water for the plants and stomatal conductance and, hence, transpiration was reduced to minimise leaf water loss.

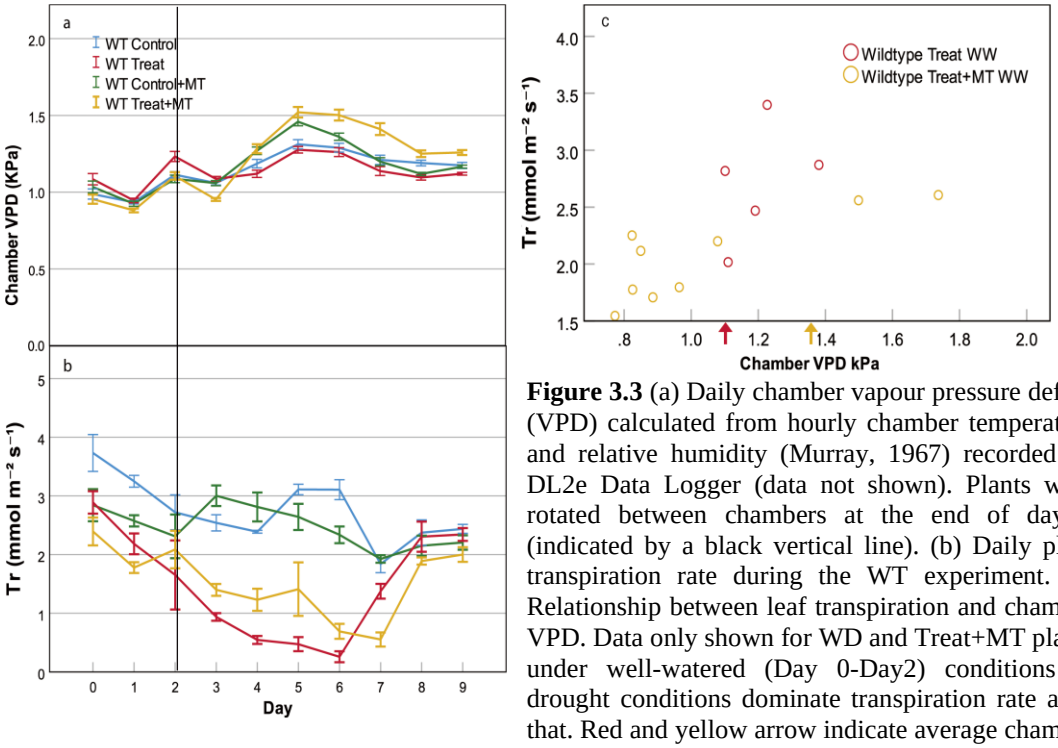

Figure 3.3 (a) Daily chamber vapour pressure deficit (VPD) calculated from hourly chamber temperature and relative humidity (Murray, 1967) recorded by DL2e Data Logger (data not shown). Plants were rotated between chambers at the end of day 2 (indicated by a black vertical line). (b) Daily plant transpiration rate during the WT experiment. (c) Relationship between leaf transpiration and chamber VPD. Data only shown for WD and Treat+MT plants under well-watered (Day 0-Day2) conditions as drought conditions dominate transpiration rate after that. Red and yellow arrow indicate average chamber VPD from Day 5 to 9 in WT WD and WT Treat+MT.

In this experiment, chamber VPD had a negative impact on *Tr* (Fig 3.3b) in wild-type . Between Days 0-3, the higher chamber VPD resulted in higher evapotranspiration and quicker soil water loss in wild-type Treat. However, after wild-type WD+MT was transferred to the higher VPD on Day 3, leaf *Tr* remained relatively constant for 2 days. By contrast, from Days 2 – 6, net photosynthesis *A* (Treat: 10.0 ± 1.9 to 2.6 ± 0.7 µmol m^{-2} s^{-1}, WD+MT: 12.6 ± 0.3 to 5.3 ± 1.1 µmol m^{-2} s^{-1}) and stomatal conductance *Gs* (Treat: 0.109 ± 0.044 to 0.014 ± 0.005 mol m^{-2} s^{-1}, WD+MT: 0.147 ± 0.017 to 0.036 ± 0.007 mol m^{-2} s^{-1}) were both significantly reduced, and at the same rate, in both WD and WD+MT until re-

watering. However, iWUE did not increase in the same way as wild-type WD (lower than 50 %).

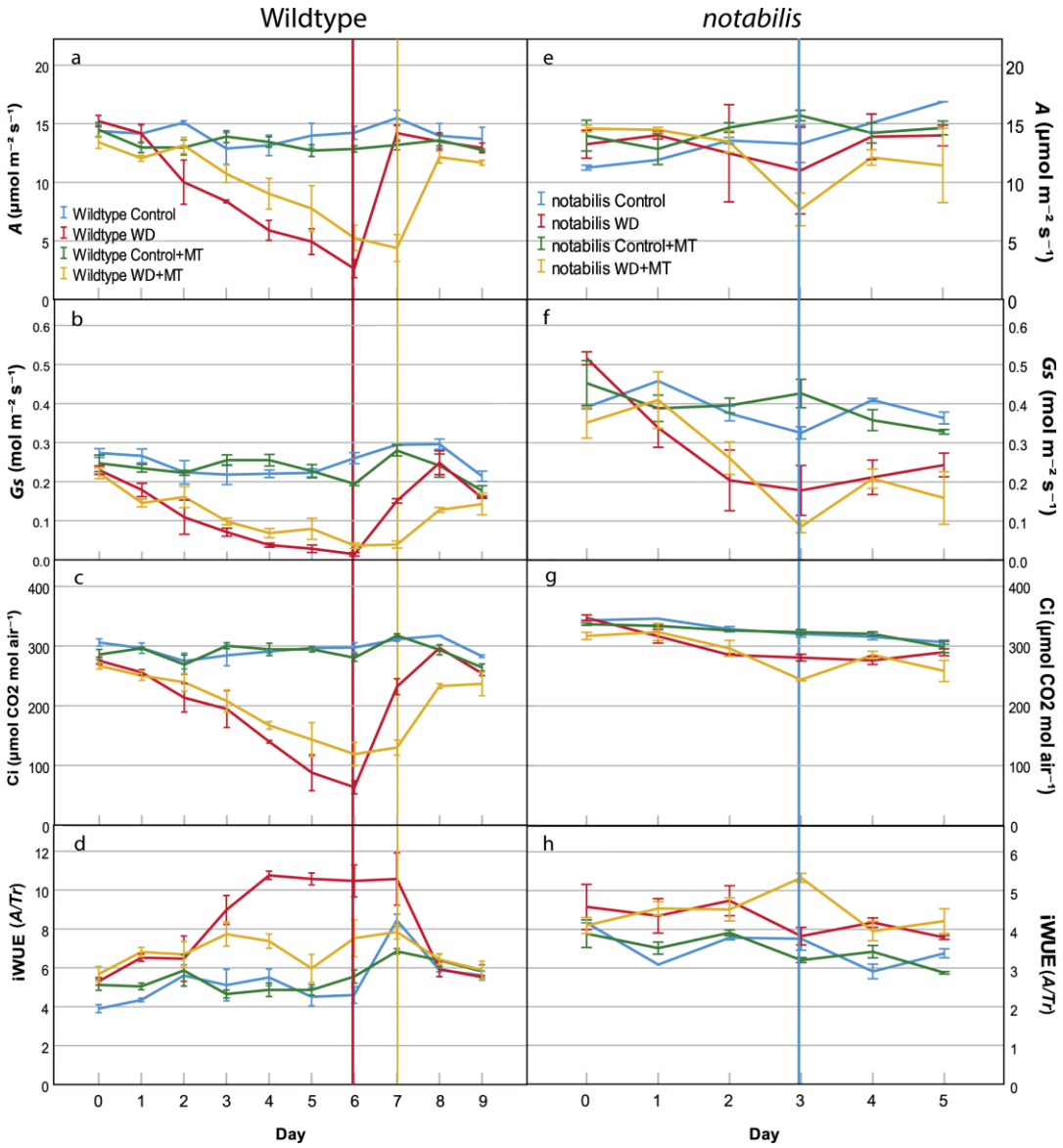

Figure 3.4 Time series of leaf level CO_2 assimilation (A), stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and intrinsic water-use-efficiency (iWUE, µmol CO_2 mmol H_2O^{-1}) of wild-type (a, b, c, d) and *not* (e, f, g, h). Treatments: control (blue lines), WD (red lines), control with MT spray (green lines) and WD with MT spray (yellow lines). The red and yellow vertical lines on wild-type plots indicate re-watering days for WD (Day 6) and WD+MT (Day 7). The blue vertical line on *not* plots indicates re-watering day for both WD and WD+MT. Data are means (± SE) of at least n = 3 replicates.

While elevated VPD maintained the *Tr* and iWUE increment it had no effect on the reduction of *A* under drought stress. The timing of decreases in *A* and *Gs* correspond with changes in soil moisture rather than changes in VPD. Hence, we conclude that the significant decreases observed in *A* and *Gs* were the result of

water deficit conditions rather than chamber VPD. Accordingly, iWUE increased with soil drying in wild-type Treat, but not in wild-type WD+MT due to the chamber effect. The remainder of the results will therefore focus on the relationships between measured variables and soil moisture, rather than time.

By contrast in *not*, responses of gas exchange and stomatal conductance were not affected by either chamber VPD or MT spray treatment. Although the differences in *A* between genotypes were not statistically significant, *not* tended to maintain photosynthesis better than wild-type under drought treatment. The two genotypes had similar initial values, but *A* only dropped by 30-39% in *not* compared with 59-83% in wild-type (Fig 3.4e). As expected, *Gs* was about one third higher in *not* control or well-watered treatments than wild-type . Somewhat unexpectedly, however, stomatal conductance responded to water deficit more rapidly in *not* than wild-type , although the absolute value of *Gs* at wilting point was over 10 times higher in *not* than wild-type . As a result, *Ci* (> 250 μmol CO_2 mol air) and *Tr* (> 2.0 mmol m^{-2} s^{-1} data not shown) remained high in *not* under drought stress, leading to significantly less enhancement of iWUE ($P < 0.001$, Table 3.1) than wild-type . This resulted in higher water loss and significantly lower Ψ_{leaf} in *not* over time. Over the drought treatment period, wild-type treatments had similar decreasing rates of net photosynthesis rate and stomatal conductance, which reduced to approximately 70 – 90 % of well-watered plants. In *notabilis,* stomata closure under drought stress further reduced *A* by up to 50 %. Exogenous monoterpene had no impact on *Gs* or *A* in either genotype.

Monoterpene spray promoted the initial decrease of net photosynthesis under drought stress

By comparing variables under different stress levels, the impacts of drought and exogenous monoterpene application on photosynthesis and stomatal conductance of the two genotypes can be determined. *A* was similar across chambers and genotypes under well-watered conditions (Fig 3.5a, c). In wild-type Treat, there was no significant reduction of *A* under moderate drought stress compared with WW control ($P = 0.927$), but severe drought significantly inhibited *A* in both wild-type WD and wild-type WD+MT treatments in comparison with their WW controls ($P < 0.001$). On re-watering, *A* recovered to control or WW levels ($P >$

0.05). However, a significant decline (18 % of WW) in *A* was observed in WD+MT under moderate drought with a further decrease under severe drought. *A* then fully recovered after re-watering ($P < 0.001$; Fig 3.5a). the differences in *A* are not significant when considering MT spray treatment alone ($P = 0.078$), but under moderate drought stress, MT treatment significantly increased the rate at which *A* initially decreased ($P = 0.029$, Fig 3.5e).

The significant differences observed in *Gs* and its response to water deficit in wild-type corresponded with those of *A* (Fig 3.5b, d). Applying exogenous monoterpenes changed wild-type photosynthetic and stomatal response of wild-type to deficit irrigation. wild-type plants maintained net photosynthesis rate until the onset of severe drought, at which point *A* decreased rapidly. Photosynthesis was also maintained at this value until severe drought (Fig 3.5e), from which point *A* and *Gs* fell at the same rate in wild-type WD and wild-type WD+MT until re-watering.

By contrast, there were no significant differences in net photosynthesis rate and stomatal conductance in *not* WD compared with WD+MT under either water deficit or re-watering. The responses of *not* to deficit irrigation were the same as in wild-type WD+MT, i.e. *A* dramatically declined under moderate drought and was significantly further reduced under severe drought ($P < 0.001$; Fig 3.5c). Again, stomatal conductance responded in the same way as net photosynthesis (Fig 3.5d). However, after re-watering *A* recovered to WW value, but *Gs* only recovered to ~half of WW, very close to its value under moderate drought stress (Fig 3.5d).

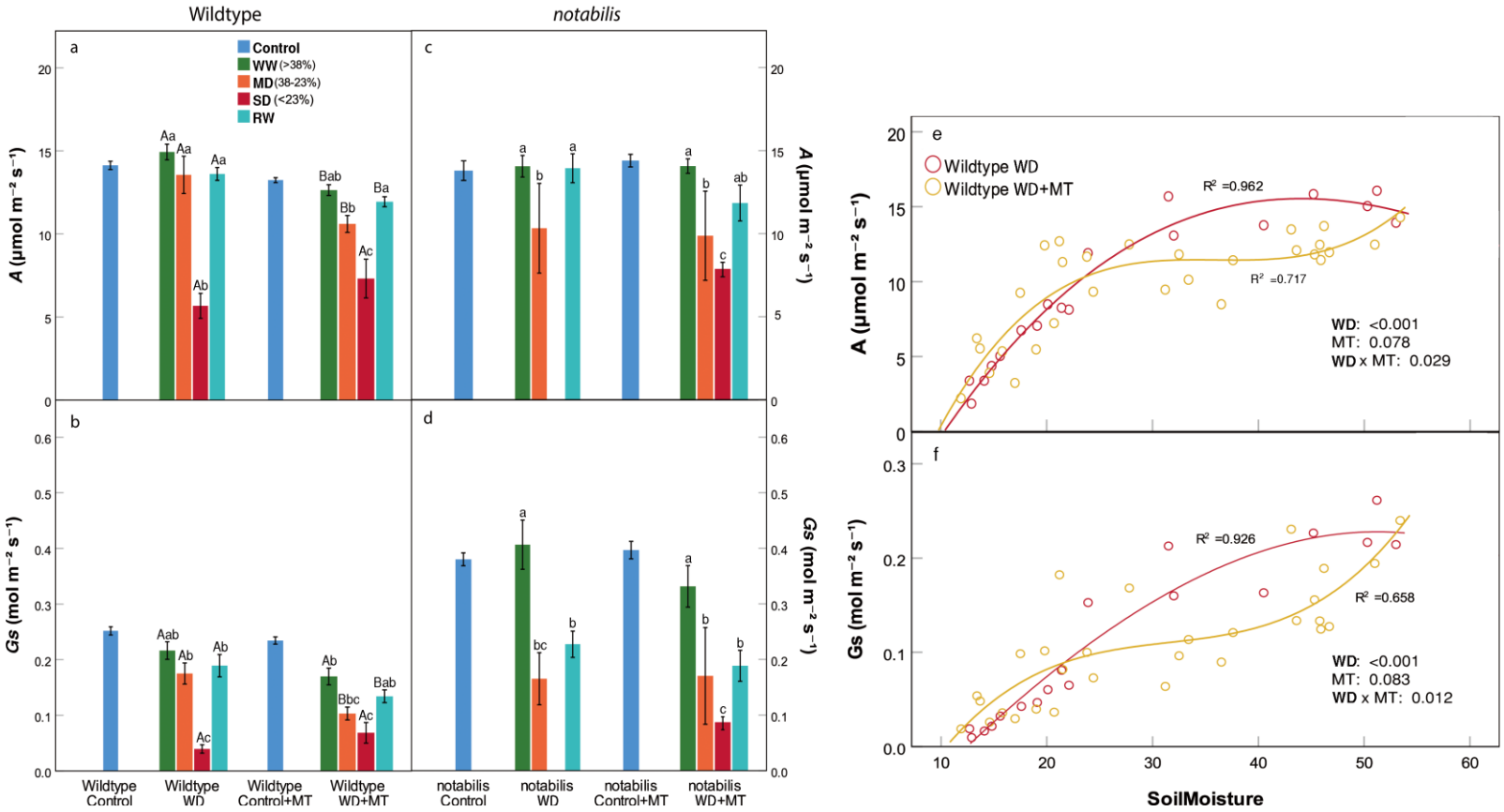

Figure 3.5 CO_2 assimilation rate (*A*) and stomatal conductance (*Gs*) under different water stress levels for wild-type (a, b) and *not* (c, d). Bars are mean values (± SE) of at least n = 3 replicates in control (blue), well-watered (SM > 38%, green), moderate drought (23% < SM < 38%, orange), severe drought (SM < 23%, red) and re-watered (cyan). Uppercase letters indicate significant differences between treatments at the same stress level; lowercase letters indicate significant differences within a treatment (compared with its control). Correlations in the right-hand panels (e, f) show the relationship between (e) leaf CO_2 assimilation rate (*A*) and (f) stomatal conductance (*Gs*) with soil moisture for wild-type without MT spray (Treat; red) and with MT spray (WD+MT; yellow). Each symbol is an individual plant. Data only includes drought treatments, and R^2 is the regression coefficient. *P* values and interactions were determined by two-way ANOVA

3.3 Soil moisture and MT effects on foliar ABA concentration

Water deficit increased foliar ABA content in wild-type *but not notabilis, exogenous monoterpenes reduced foliar ABA content under severe drought in wildtype but not notabilis*

The foliar ABA content of wild-type control treatments remained constant at 1977 ± 84 ng g^{-1} DW throughout the experiment (Fig 3.6a, c) but increased in drought treatments when soil moisture decreased below 30% from Day 3 onwards (Fig 3.1). Drought stress significantly (and differentially) increased ABA concentration with drought severity in both wild-type treatments (Fig 3.6c). From moderate drought, foliar ABA then continued to accumulate more rapidly in wild-type WD than wild-type WD+MT until, at the point of re-watering, foliar ABA content in wild-type WD was almost twice that in wild-type WD+MT (Fig 6a). Under severe drought stress, applying exogenous monoterpene spray (WD+MT) significantly reduced (by 39 %) ABA content compared with unsprayed (Treat) plants ($P < 0.001$). Although the differences under MD were not significant ($P > 0.05$), WD+MT still had lower absolute mean values than WD (by up to 10.2 %). As already seen in the timeseries, WD+MT had significantly higher ABA content after re-watering than WD ($P = 0.001$). The concentration of ABA in wild-type WD fully recovered to well-watered levels almost immediately following re-watering, but although ABA content dropped in WD+MT it remained elevated by approximately 46 % above control treatments after re-watering.

There were no significant differences in foliar ABA concentrations between any of the treatments in *not*, with levels remaining relatively constant throughout the experiment irrespective of drought severity (Fig 3.6b).

However, as described above, differences in average ABA content in wild-type were significant under SD ($P < 0.001$) and following re-watering, suggesting that exogenous monoterpene spray does alter foliar ABA responses to deficit irrigation and re-watering, and the lack of statistical significance may simply be due to the low number of replicates. Although slight fluctuations in ABA content in *notabilis* were observed under the influence of drought and monoterpene spray, changes were not significant (*P:* MT = 0.113, WD < 0.001, MTxWD = 0.318) suggesting *not* remains ABA-deficient under drought conditions and that it does not respond to exogenous

monoterpene spray. In spite of the temporal differences in ABA concentrations, MT spray had no effect on soil drying-induced ABA accumulation, i.e. there is no significant drought severity WD x MT interaction (P = 0.256). Thus, monoterpenes appeared to have no significant effect on leaf ABA content under water deficit conditions (Fig 3.6e).

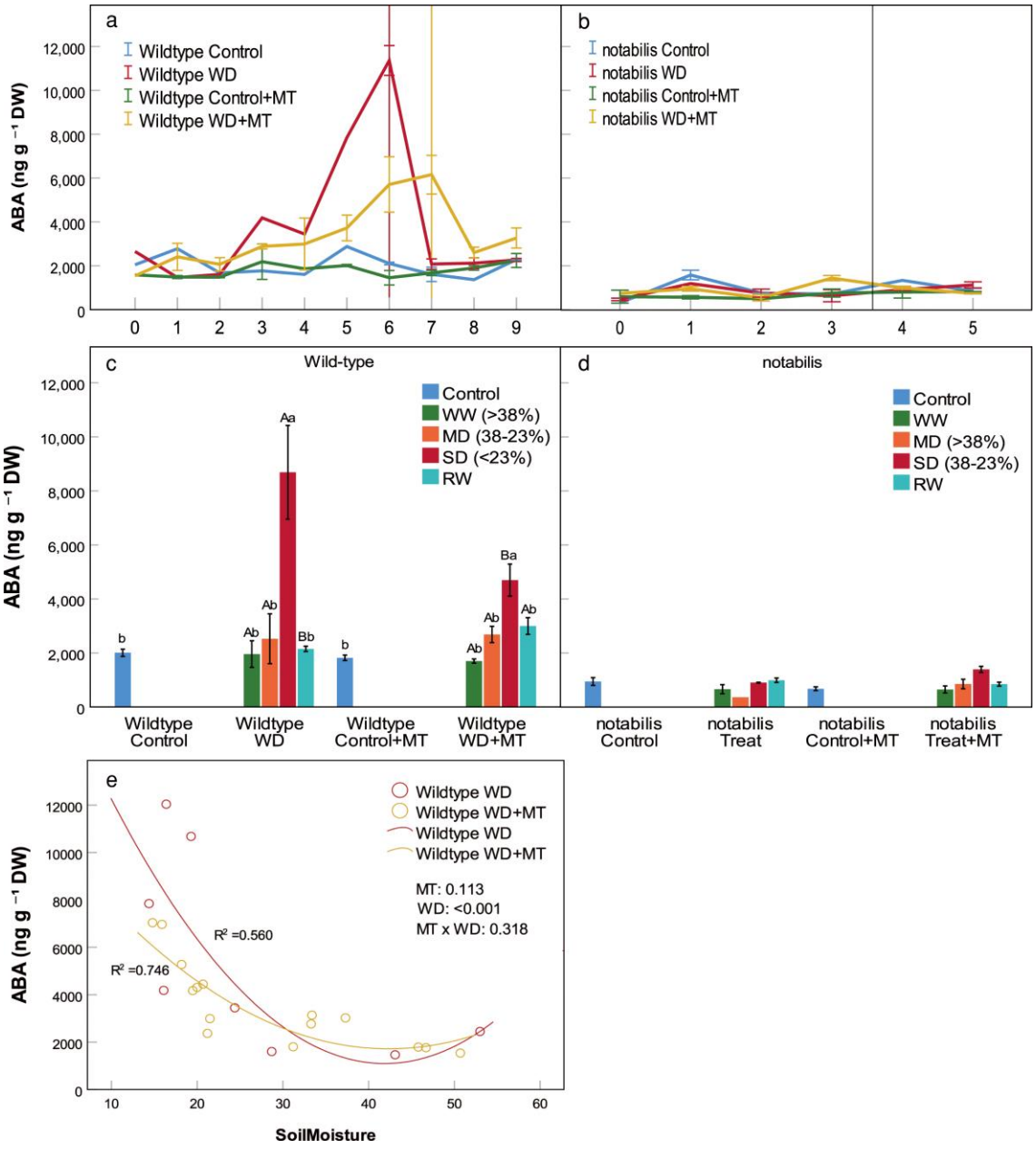

Figure 3.6 Timeseries of foliar ABA content in wild-type (a) and *notabilis* (b). Red, yellow and blue vertical lines indicate re-watering days as before. Data are mean (± SE), with n = 1, 2 in drought treatment, and n = 2, 3 in WD + MT treatment. Foliar ABA content separated by drought severity for wild-type (c) and *notabilis* (d). (e) Regression analysis between foliar ABA content and SM % of without MT spray (Treat; red) and with MT spray (Treat+MT; yellow) for WT treatments. Each symbol is an individual plant. Data only includes the drought period. *P* values and interactions were determined by two-way ANOVA.

3.4 PSII efficiency under drought stress

Drought reduced photosystem II efficiency in wild-type

In general, the response of PSII maximum efficiency (F_v'/F_m') and operating efficiency (φPSII) correlated with net photosynthesis in both wild-type and *not* plants (Fig 3.7a). Compared to control, plants at well-watered condition (SM > 38%) showed no differences. Although WW and MD conditions had positive impacts on F_v'/F_m' (by 7 % in average) in non-spray plants, they were not significant (P = 0.080). Under severe water deficit (SM < 23%), F_v'/F_m' and φPSII rapidly and significantly declined in wild-type WD (P = 0.003 and P < 0.001 , respectively) with a total reduction of approximately 14.5 % in F_v'/F_m' and 10 % in φPSII in comparison with WW. After re-watering, both maximum and operating efficiencies recovered above WW, which did not significantly differ from Control (P = 0.372, P = 0.441; Fig 3.7a). For wild-type WD+MT, spray treatment reduced F_v'/F_m' (by up to 4%) under MD compared with both Control (P = 0.012) and WD MD plants (P = 0.001). The mean value (0.64) of F_v'/F_m' remained similar under SD (P = 0.002; Fig 3.7a). The changes in F_v'/F_m' were accompanied with reduced φPSII in WD+MT plants but neither difference was significant when compared with either its WW treatment or control (P > 0.05; Fig 3.7b).

There was no significant difference in F_v'/F_m' or φPSII in *not* Control or WW treatments when compared to corresponding wild-type treatments. Only φPSII under SD in *not* WD+MT was significantly lower than the same condition in wild-type (P = 0.012; not shown). Mean values indicated MD and SD stresses reduced PSII efficiencies by a steady gradient across drought severities in *not* WD+MT, but there were no significant differences comparing with *not* Treat, resulting in significant D x MT x G differences (Table 3.1), although it should be noted that no data were available for SD in *not* Treat.

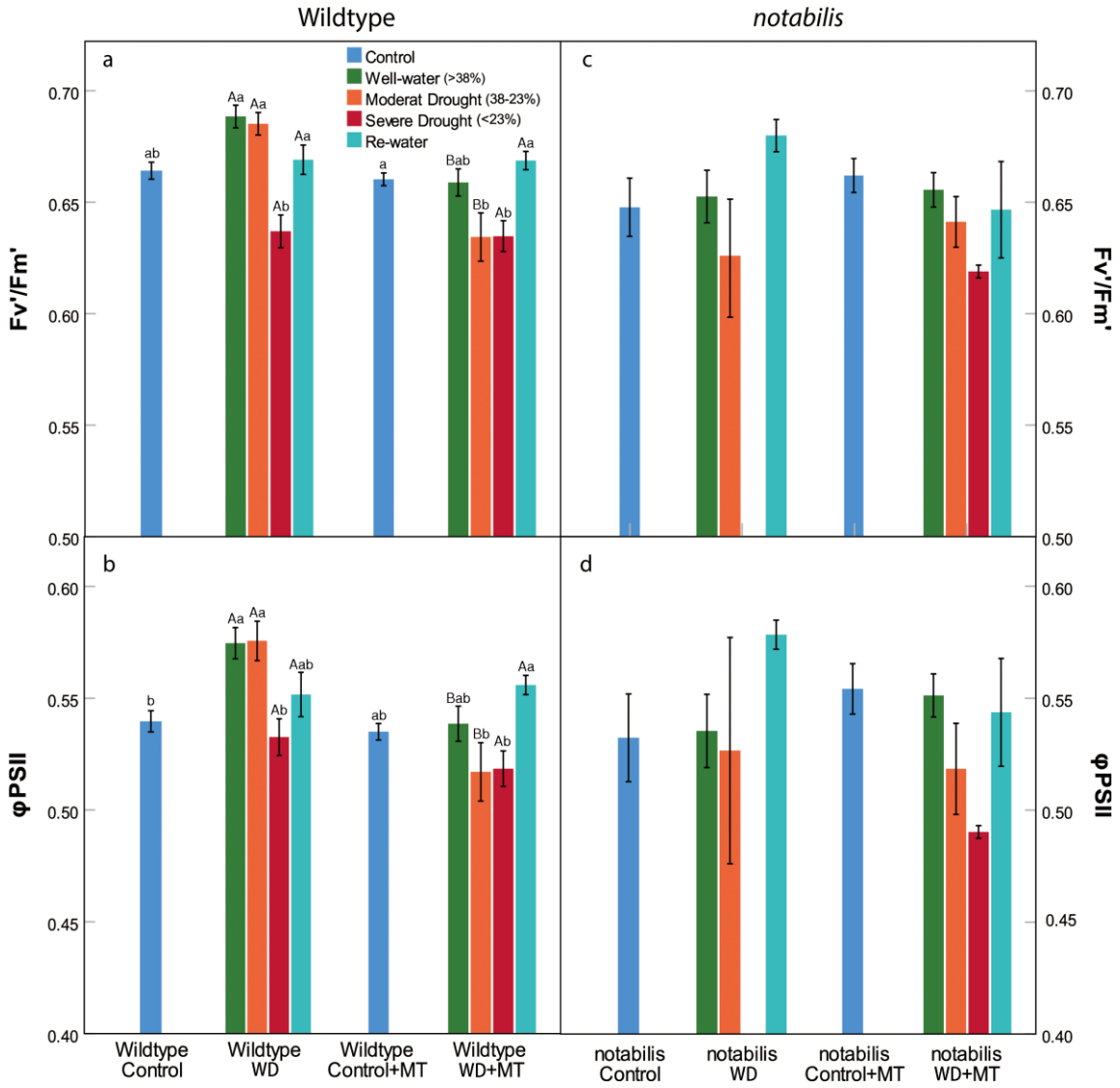

Figure 3.7 Estimated photosystem II (PSII) maximum efficiency (Fv'/Fm', a, c) and operating efficiency (φPSII, b, d) by different drought severity for wild-type (a, b) and *not* (c, d). Bars are mean values (± SE) of n = 2, 3 replicates in control (blue), well-watered (SW > 38%, green), moderate drought (23% < SW < 38%, orange), severe drought (SM < 23%, red) and re-watered (cyan). Differences within and between treatments are indicated by lowercase letters (including control) and uppercase letters respectively.

3.5 Leaf oxidative stress (H_2O_2) and oxidative damage (MDA)

Exogenous monoterpene spray mitigated oxidative damage under all drought stress

In spite of some day-to-day fluctuations, both the timeseries (Fig 3.8) and drought severity plots (Fig 3.9) show clear differences in leaf H_2O_2 and lipid peroxidation product (MDA) between control and drought treatments in wild-type . H_2O_2 content (indicative of total ROS level) increased immediately on application of water deficit in wild-type Treat, while wild-type WD+MT took 4 days to respond (Fig 3.8a). All wild-type treatments showed the same ROS content from Day 6 (re-watering) to the end of the experiment. Leaf MDA content (indicative of oxidative damage) increased ~50% in wild-type WD under deficit irrigation but recovered to levels similar to the Control plants following re-watering (Fig 3.8b). By contrast MDA content in wild-type WD+MT tended to decrease from Day 1 to Day 5 (Fig 3.8b), although total changes are relatively small.

H_2O_2 content was much higher in *not* than wild-type with concentrations in *not* control over twice that in wild-type control. Concentrations accumulated rapidly from Day 2 of deficit irrigation in both *not* WD and WD+MT, peaking at 1.5-2 times the initial levels on the first day following re-watering. Levels then dropped but remained 10 – 50 % higher than ROS levels in Control (Fig 3.8c). MDA content appeared to show fluctuations, some of which were large (e.g. an initial rapid rise in *not* WD on Day 1 or the unexpected increase of *not* Control on Day 5) rather than a consistent upward or downward trend (Fig 3.8d).

Under deficit irrigation, H_2O_2 accumulation in *not* increased with severity of drought. Although the elevation of H_2O_2 under MD in WD and WD+MT was not significant (P = 0.201), mean values were still higher than WW plants in both treatments (increment of 20 % in wild-type WD and 27 % in WD+MT; Fig 3.9a). More substantial increases (by up to 72 %) were seen under SD when concentrations in WD were significantly higher than WD+MT plants (P = 0.05). Re-watering had no effect on H_2O_2 content in Treat, but significantly increased H_2O_2 levels in WD+MT to values higher than under any other drought stress level (P < 0.001).

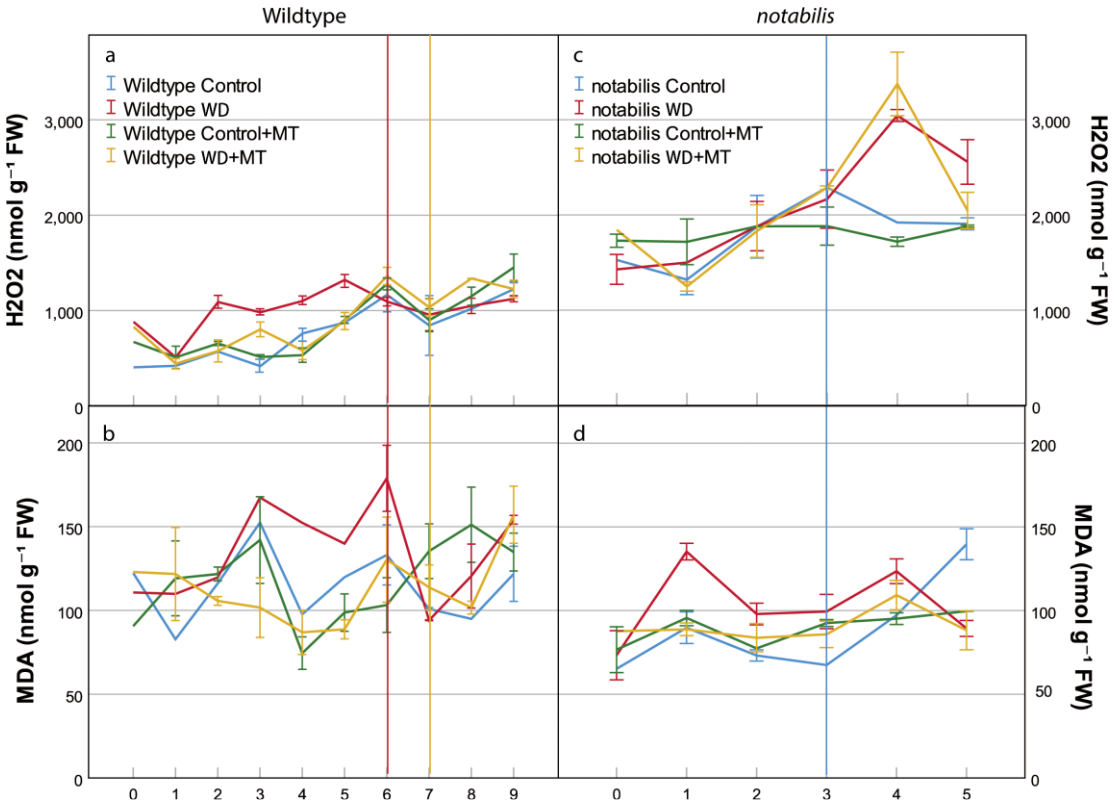

Figure 3. 8 Timeseries of foliar content of hydrogen peroxide (H_2O_2; a, c) and malondialdehyde equivalents (MDA; b, d) of wild-type (a, b) and *not* (c, d) during drought and re-watering. Treatments: control (blue), WD (red), control with MT spray (green) and WD with MT spray (yellow). Red, yellow and blue vertical lines indicate re-watering days as before. Data are means (± SE) of n = 1, 2 replicates for WD and n = 2, 3 replicates for WD+MT.

MDA increased substantially (increment of 41 %) and significantly ($P < 0.001$) under MD in wild-type WD but was significantly inhibited (by up to 18 %) in wild-type WD+MT when comparing against their respective Control ($P < 0.001$). Under SD, wild-type WD accumulated even higher levels of MDA. Although it remained significantly lower in wild-type WD+MT than wild-type Treat, it was higher than under MD. After re-watering, MDA concentrations in WD were reduced to the levels of Control and WW. By contrast, MDA levels rose on re-watering in WD+MT and the difference was significantly higher when compared with WW condition (Fig 3.9b).

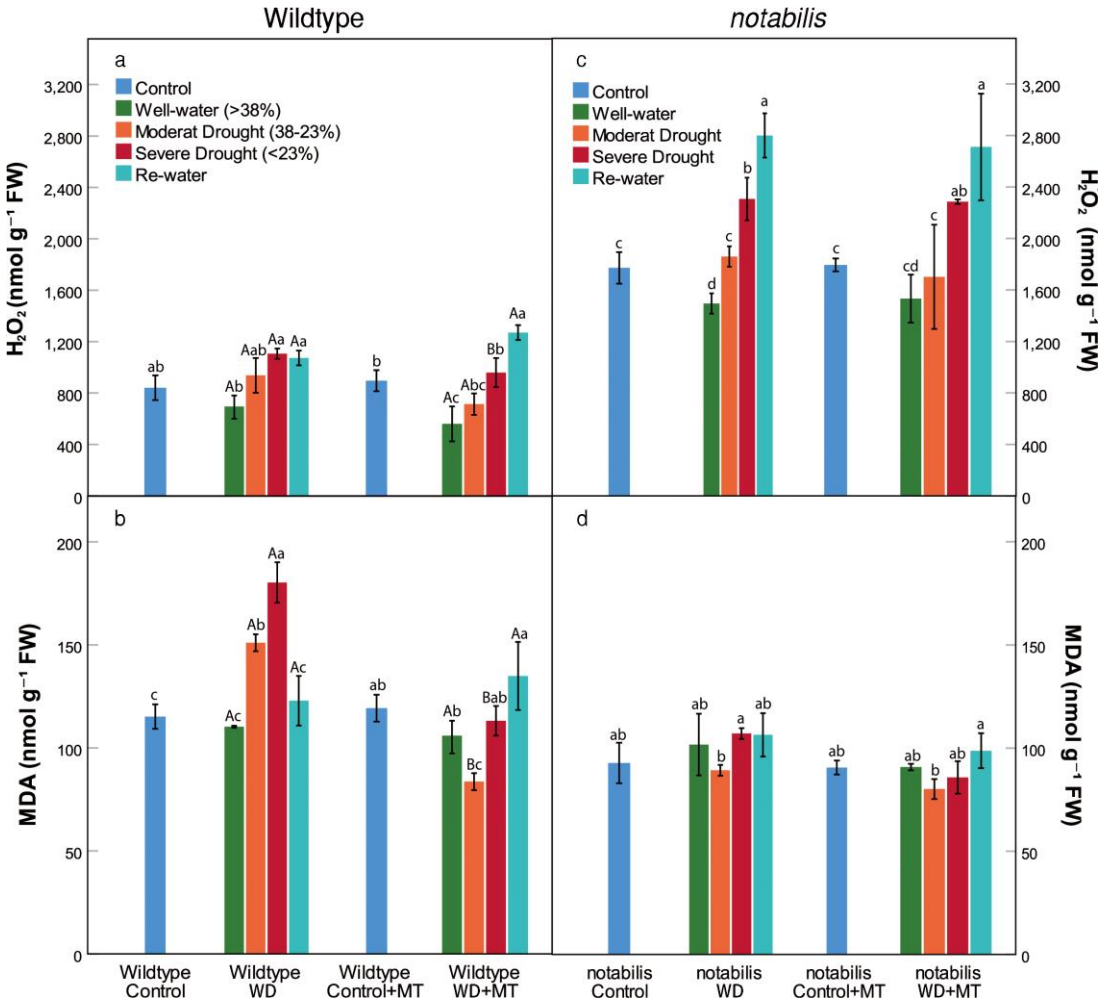

Figure 3. 9 Foliar H_2O_2 (a, c) and MDA (b, d) content by different drought severity for wild-type (a, b) and *not* (c, d). Differences within or between treatments are indicated by lowercase letters (compared with control) and uppercase letters respectively. Data are means (± SE) of n = 1, 2 replicates for no MT water deficit (WD) treatment and n = 2, 3 replicates for +MT treatment.

The absolute levels of H_2O_2 were significantly higher in *not* than wild-type (by up to 117 %) throughout the experiment (Table 3.1) but similar incremental patterns of changes in H_2O_2 content were observed in *not* as previously described for wild-type under increasing severity of drought stress (Fig 3.9c). However, re-watering did not reduce leaf H_2O_2 content in *not* but, rather, significantly increased it in comparison with all other stress conditions ($P < 0.001$). MDA concentrations in *not* showed no significant changes under any treatments (Fig 3.9d).

Exogenous monoterpenes did not alter the increase of H_2O_2 under drought stress in wild-type (P for MT = 0.691, WD x MT = 0.408; Fig 3.10a) but did significantly influence the accumulation of MDA (P for MT < 0.001, Fig 3.10b). wild-type WD+MT had significantly lower (by 35 – 40%) leaf MDA content during the entire drought period ($P = 0.008$, Fig 3.10b)

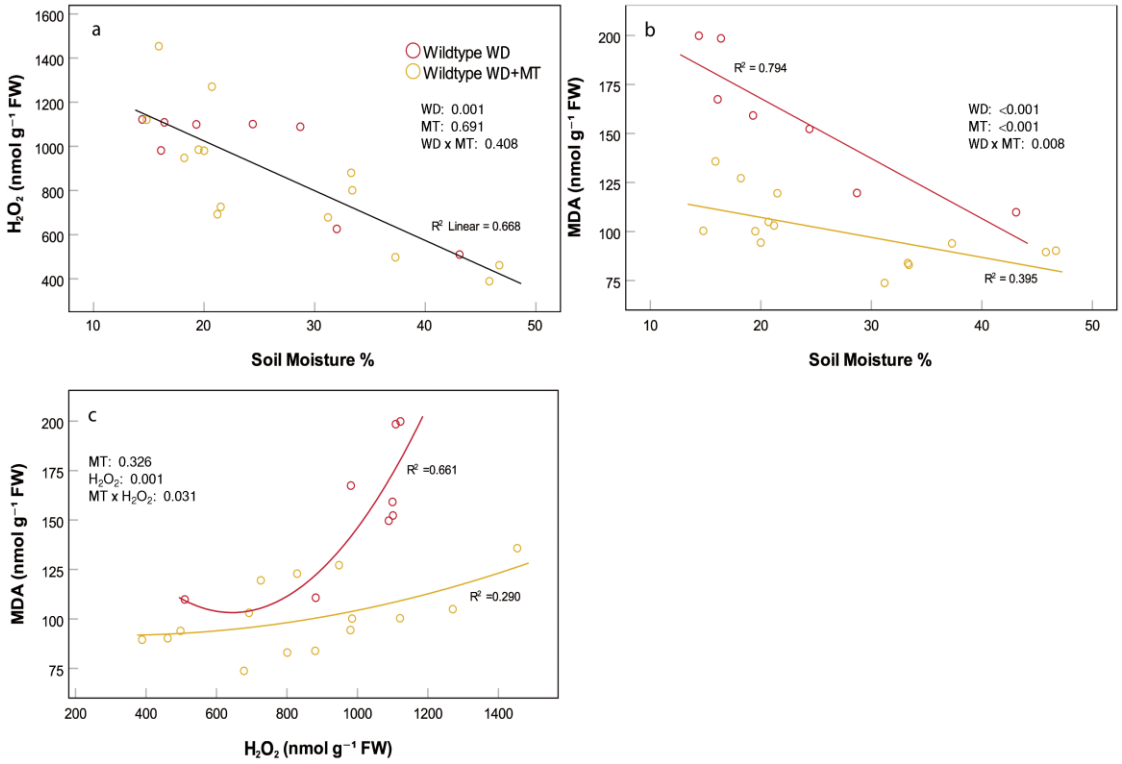

Figure 3.10 The relationship between soil moisture and (a) leaf ROS (hydrogen peroxide, H_2O_2; , (b) oxidative damage (malondialdehyde equivalents, MDA; for wild-type for plants not sprayed with MTs (Treat; red) and sprayed (WD+MT; yellow). Each symbol is an individual plant. Data only includes droughted plants. R^2 represents the regression coefficient that fitted to WD (red line), WD+MT (yellow line) and both treatments (black line). *P* values and interactions were determined by two-way ANOVA.

Monoterpene application affected the increase of MDA as H_2O_2 concentration increased, as indicated by a significant MT x H_2O_2 interaction (P = 0.031, Fig 3.10c). Whereas MDA increased exponentially as H_2O_2 increased in Treat, the increase became close to linear following MT application. Thus, there was a much larger increase in MDA in WD (66.7 %) than WD+MT (9.5 %) over similar increases in H_2O_2. MDA levels were generally substantially lower for WD+MT for a given H_2O_2 concentration, suggesting that exogenous monoterpenes significantly reduced the oxidative damage (MDA content) that resulted from the increase of ROS (H_2O_2 content) under deficit irrigation in wild-type .

3.6 Link between ABA and oxidative stress, PSII efficiency and photosynthesis

Exogenous monoterpene inhibited ABA-H_2O_2 responses

Both oxidative stress (H_2O_2) and damage (MDA) are significantly ($P \leq 0.001$) correlated with foliar ABA content in both wild-type WD and WD+MT. Exogenous monoterpene application affected the increase of H_2O_2 as ABA concentration increased, as indicated by a significant MT x ABA interaction ($P = 0.004$; Fig 3.11a). Leaf H_2O_2 content was not different at low ABA concentrations in both treatments, but for ABA concentrations above ~6000 ng g FW^{-1}, H_2O_2 increased much more rapidly in wild-type WD+MT than wild-type WD. In contrast, although MDA concentration significantly ($P = 0.004$) increased as ABA concentration increased, MT application did not affect the relationship (no significant MT x MDA interaction - $P = 0.710$). As shown in Fig. 3.11b, ABA increased linearly with MDA under both WD and WD+MT and at very similar rates. In summary, exogenous monoterpene spray changed the relationship between H_2O_2 and ABA under drought stress.

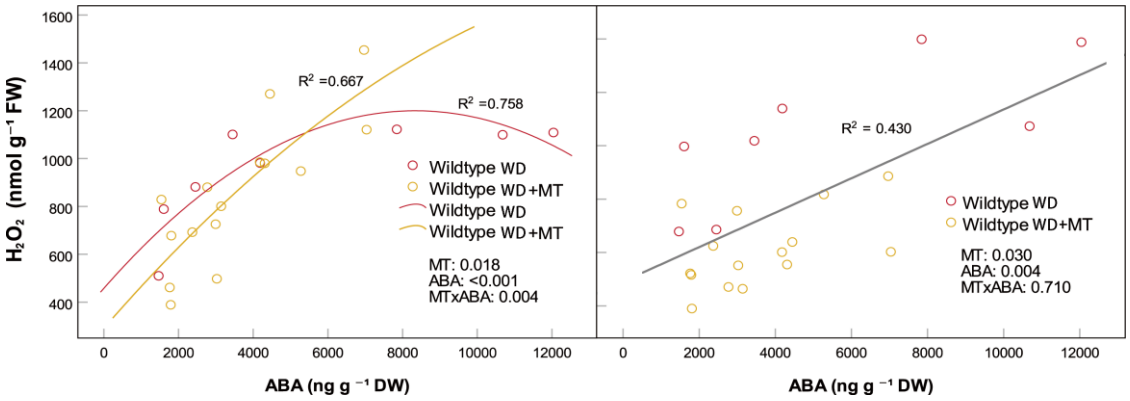

Figure 3.11 The relationship between foliar ABA and (a) H_2O_2 and (b) MDA content of wild-type plants. Each point represents an individual plant; cubic and linear lines were fitted to H_2O_2 and MDA respectively for both treatments. P values and interactions were examined by two-way ANOVA for exogenous monoterpene treatment (MT), oxidative stress (H_2O_2) and damage (MDA).

Table 3.2 Significance of the effect of water deficit (WD), leaf ABA content and monoterpene spray (MT) on leaf H_2O_2 content. *P*-values and interactions were examined by two-way ANOVA. *, $P = 0.05$; **, $P = 0.001$; ***, $P < 0.001$.

Factors	*P*-value ABA
WD	**
MT	**
ABA	***
WD x MT	*
WD x ABA	*
MT x ABA	**
WD x MT x ABA	*

Exogenous monoterpene spraying mitigated the impact of photosystem damage on net photosynthesis

In addition to the stomatal limitation (relationship between *A* and *Gs*), non-stomatal regulation of net photosynthesis was also observed under drought stress. *A* declined with both PSII maximum (F_v'/F_m') and operating (φPSII) efficiency factors (Fig 3.12) in both wild-type treatments, but responses were significantly different following exogenous MT application. For similar levels of PSII efficiency, net photosynthesis was higher in plants treated with exogenous monoterpene spray than those left unsprayed, suggesting MTs enhanced net photosynthesis in droughted plants, but this effect was not significant when considering water deficit impact (Appendix 1).

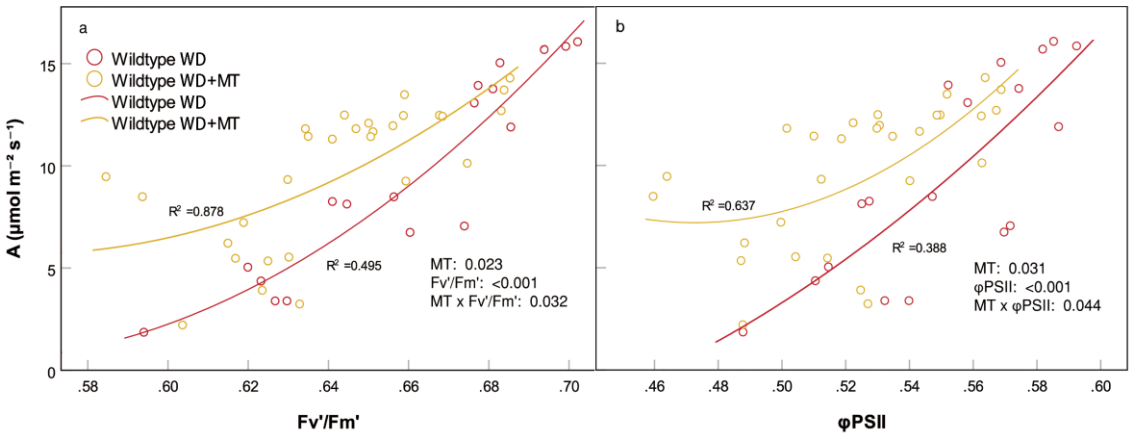

Figure 3.12 The relationship between CO_2 assimilation rate (*A*) and (a) PSII maximum (*Fv'/Fm'*) and (b) operating (φPSII) efficiency of wild-type plants. Each point represents an individual plant; cubic lines were fitted to *A vs. Fv'/Fm'* and *A vs.* φPSII respectively for both treatments. P values and interactions were examined by two-way ANOVA for exogenous monoterpene treatment and PSII efficiency factors.

4 Discussion

Few previous studies have focused on the response of monoterpene emissions to drought and these have variously reported both suppressed and increased MT emissions, most likely linked to the severity and duration of the drought (Staudt et al., 2002; Ormeno et al., 2007; Peñuelas et al., 2009; Peñuelas and Staudt, 2010). Few studies have demonstrated the potential role for MTs in mitigating abiotic stress impacts, and these have mostly considered heat stress. There is a fundamental gap in our knowledge of the effects of MTs as limited research has specifically investigated plant physiological and biochemical responses under water deficit conditions (Peñuelas et al., 2005; Dani et al., 2014), or investigated interactions between terpenoids and drought-induced hormones, such as ABA, which share a common biosynthetic pathway (Vranova et al., 2013; Marino et al., 2017). Nevertheless, the antioxidative photoprotection role of monoterpenes has been documented for other abiotic stresses such as heat (Loreto et al., 1998; Copolovici et al., 2005).

In this study we investigated the physiological and biochemical response of two tomato genotypes (wild-type *(Ailsa Craig)* and *notabilis*) to drought stress. Although we do not have the final monoterpene emissions data, preliminary tests suggest that no differences in emission rate, components or behaviour between wild-type and *notabilis*. Thus, the hypothesis that ABA and monoterpenes compete for a pool of carbon precursors was rejected. Monoterpene emissions from tomato depend on the severity of drought stress, with moderate stress suppressing MT emissions, which recovered under severe drought, then fully recovered with small burst after re-watering (data not shown). These behaviours are similar to previous observations on *Q. ilex* seedlings under short drought and re-water treatment (Peñuelas et al., 2009). Recent evidence shows that the MT emissions may decrease because of the reduction of photosynthesis rate and stomatal conductance (Sharkey and Loreto, 1993; Schnitzler et al., 2004). Further studies are necessary to find out the mechanisms whether emission rates coupled with photosynthesis during severe drought stress and re-watering.

Foliar spray of exogenous monoterpenes determined the direct impacts of MTs on plant responses to drought stress. We found that exogenous monoterpenes did not contribute to maintain photosynthesis or photosynthetic efficiencies under drought stress, but it reduced foliar MDA concentration without influence in foliar H_2O_2.

In wild-type, drought stress significantly decreased stomatal conductance (*Gs*) (Fig 3.4b), intercellular carbon dioxide (*Ci*) (Fig 3.4c), and photosynthesis (*A*) (Fig 3.4a) by 90%, 72% and 63% respectively, as soil moisture dropped from well-watered (WW) to severe drought (SD) conditions. These variables recovered immediately on re-wetting (RW) (Fig 3.4), suggesting that photosynthesis was mainly limited by stomatal closure decreasing *Ci*. Unexpectedly, stomatal conductance was also significantly decreased in *not*, although the reduction percentage (52 %) was dramatically lower than the WT, and *Ci* was not significantly reduced. Hence, photosynthesis rate only decreased by up to 38 % before re-wetting (Fig 3.4; 3.5). previous studies show inconsistent leaf gas exchange responses when comparing *not* and wild-type plants. Although *not* and wild-type plants showed similar stomatal closure in response to water stress, photosynthetic decline was greater in *not*, perhaps related to grater leaf water deficit (Yuan et al., 2010). However, salinity-induced stomatal closure (30-120mM NaCl) was attenuated in *not* (Mulholland et al., 2003; Ntatsi et al., 2014), consistent with the results presented here. These genotypic differences in response likely depend on the magnitude of foliar ABA accumulation, and the relative importance of stomatal and non-stomatal limitation of photosynthesis.

MT spray decreased stomatal conductance and photosynthesis (by up to 15 %) in WT tomato under moderate drought stress (Fig 3.5). Thus, exogenously applied MTs can direct affect guard cells and/or can be taken up by foliage to interact with plant processes, requiring further studies to distinguish these possibilities. Photosynthesis and stomatal conductance were similar under severe drought stress, and then recovered after re-watering in both treatments, but MT spray decreased the recovery effects. These results differ from previous studies where exogenous MTs (α-pinene, sabinene, β-pinene, ocimene, myrcene, limonene) had no impact on photosynthesis of unstressed plants (*Q. ilex*) (Loreto et al., 1998; Delfine et al., 2000). Nevertheless, no studies have examined exogenous MTs effect on gas exchange under drought

stress specifically, hence, further studies are needed to determine the mechanism(s) of action

In *notabilis*, exogenous MTs did not significantly affect the response of *A* to water deficit as there were no stomatal responses to MTs spray (Fig 3.4; 3.5). Nevertheless, soil drying still decreased stomatal conductance of *not*. independent of MT spray, soil drying decreased *A* by 23 % from WW to MD. At wilting point, which occurred at ~-1.3 MPa for both WT and *not*, the stomata were nearly closed in WT plants (*Gs* was 10 % of WW) whereas in *not*, the stomata were still partly open (*Gs* was 32 % of WW). This means the ABA-deficient tomato was less able to control transpiration to avoid water loss. There was no difference in stomatal response when exogenous MT was applied to *not*, suggesting that exogenous MTs are acting via an ABA-dependent mechanism.

As an ABA-deficient mutant, *notabilis* had significantly lower ABA concentrations than wild-type (by 50 – 60 %) under WW conditions, and ABA accumulation was not significantly promoted by deficit irrigation. This shows that *not* remained ABA deficient under drought stress, which is consistent with some observations of its ABA production under stress conditions (Thompson et al., 2004; Secchi et al., 2012), but not others (Mulholland et al., 2003; Yuan et al., 2010). Partial stomatal closure of drought-stressed *not* implies that ABA is not the only factor that reduced *Gs* under drought; other hormones like jasmonic acid or signalling molecules like Ca^{+2} and ROS may induce stomatal control in ABA-deficient plants (Ackerson and Krieg, 1977; McAinsh et al., 1996; Pei et al., 2000). Future investigations into stomatal control in *notabilis* may need to consider effects other than ABA signalling, however, this is not the focus in this research.

Drought significantly stimulated foliar ABA accumulation in WT tomatoes, and re-watering returned ABA content to well-watered levels. There was no effect of MT spraying observed under well-watered and moderate drought conditions, but ABA accumulation was attenuated (by up to 55 %) compared to unsprayed plants (Fig 3.6c). Nevertheless, the ABA-soil moisture response curve of wild-type WD+MT also had no significant differences compared to wild-type WD (MT x Drought: P = 0.256; Fig 3.6e). Applying exogenous MTs to plants may reduce the synthesis of endogenous MTs via the MEP pathway, limiting the production precursors of foliar ABA synthesis (Delfine et al., 2000). These results suggest monoterpenes have no

significant effect on foliar ABA accumulation under short or moderate water stress but do interfere with ABA accumulation when the drought becomes severe.

Future investigations, we need to analyse and compare the response of MT synthesis and emissions under drought and with MT spray, to understand how exogenous MTs affect the biosynthesis of MEP pathway when photosynthesis is limited by drought, and by determining the endogenous MT emissions and ABA accumulation in wild-type plants. By conducting reciprocal grafting experiments with ABA-deficient plant (i.e. Ntatsi et al., 2014), the effects of root-to-shoot ABA transport on foliar ABA content and MT emissions can then be determined (Finkelstein, 2013; Manzi et al., 2015). Alternatively, exogenous ABA can be applied on leaves to assess the monoterpene response ABA or using carbon labelling techniques.

By measuring chlorophyll fluorescence of light-adapted leaves, the maximum and operating efficiency of PSII can be estimated (Kate Maxwell, 2000) to assess the status of photosynthetic apparatus under stress conditions (E.H. Murchie, 2013). In this study, only severe drought stress significantly reduced PSII maximum (*Fv'/Fm'*) and operating (φPSII) efficiency in wild-type without MT application. This is consistent with previous observations (Mishra et al., 2012; Conti et al., 2019). Severe drought stress (minimum soil moisture of 15 % and Ψ_{leaf} of -1.30 MPa) greatly decreased photosynthesis (by 63%) of WT plants at which point photosynthetic functioning was significantly inhibited with *Fv'/Fm'* (Fig 3.7a) and φPSII (Fig 3.7b) decreased by up to 15 % and 8 % respectively. This suggests PSII efficiency does affect net photosynthesis under drought stress (Wang et al., 2018). When MT spray was applied, PSII efficiency factors were also reduced and significantly lower than wild-type drought treatment under moderate drought (by up to 4 %). This also correlates with the observed changes of net photosynthesis, further supporting the suggested relationship between PSII efficiencies and photosynthesis. On the other hand, these results imply a similar impact of MTs on PSII efficiency as on net photosynthesis, i.e. that monoterpenes induced an initial decrease of *Fv'/Fm'* and φPSII but do not help the recovery of the photosynthetic system after re-watering. For now, we conclude that exogenous monoterpenes do not have a substantial positive impact on either photosynthesis rate or photosynthetic system.

Determining the relationships between *A* and PSII efficiency factors of WT plants produced some interesting findings, which we cannot as yet explain. When

exogenous MTs were applied, the responses of net photosynthesis to changes in PSII maximum (*Fv'/Fm'*) and operating (φPSII) efficiencies were significantly altered (Fig 3.12). Monoterpene application attenuated the decrease in net photosynthesis rate with the reduction of PSII efficiency factors ($P = 0.032$, and 0.044 respectively). However, when considering the interaction with water deficit, these relations were insignificant, implying the drought response of wild-type tomatoes is principally driven by stomatal limitations and that the impact of monoterpenes on photosynthetic apparatus still does not substantially contribute to net photosynthesis.

Drought stress increases ROS (measured as H_2O_2 in this study) consistently in all treatments, although not always significantly (Fig 3.9 a, c), and MT spray had no significant effect on H_2O_2 concentration. In *notabilis*, H_2O_2 continued to increase almost linearly to a value nearly twice higher than in well-watered plants, and significantly higher than (up to 118 %) the wild-type under the same stress conditions (Fig 3.9 c). Accelerating foliar ROS production due to water stress induced stomatal closure and photosynthesis limitation was already reported (Asada, 2006; Miller et al., 2010). Increased foliar H_2O_2 in WT and *notabilis* during drought stress is consistent with previous findings, however, they found no significant differences between genotypes (Yuan et al., 2010). Moreover, the H_2O_2–soil moisture response curve showed no differences between spray and unspray treatments. These findings indicate drought stress accelerates the production of reactive oxygen species, while exogenous MT does not affect the response.

Differences in MDA content indicates the extent of lipid peroxidation in response to the changes in oxidative stress (ROS content) (Apel and Hirt, 2004; Miller et al., 2010; Sharma et al., 2012). MDA content increased progressively as the severity of drought stress increased (Figure 3.9), consistent with reports of elevated MDA content by leaf fresh weight under drought and heat induced oxidative stress in tomato or tobacco (Ryan et al., 2014; Li et al., 2015). Whereas with exogenous monoterpene spray, foliar MDA content did not increase under drought stress, and in fact was significantly decreased by 17 % compared to WW tomatoes under moderate drought (Fig 3.9b). Monoterpene spray significantly reduced MDA accumulation under drought stress. By comparing the relationship between foliar H_2O_2 and MDA content, we further demonstrated exogenous monoterpene reduced oxidative damage and protected membrane structure, by enhancing wild-type

antioxidative capacity. This protective function of selective monoterpenes under drought is similar to the role of isoprene in drought protection (Ryan et al., 2014), and monoterpenes in heat protection (Velikova and Loreto, 2005).

By integrating the results of photosynthesis and PSII efficiency with oxidative stress, we found that although, monoterpenes protected cell membranes from lipid oxidation under drought stress, they did not confer positive impacts on photosynthetic activities. We conclude that selected monoterpenes enhance antioxidative capacity of plants but cannot essentially promote net photosynthesis or PSII efficiency factors. This partially supported our fourth hypothesis: "*Monoterpene synthesis and emissions maintain photosynthetic system efficiency, protect the photosynthetic apparatus, and sustain photosynthesis rate by limiting ROS damage resulting from drought*".

The antioxidative property of some monoterpenes, such as α-pinene and ϒ-terpinene, and their inhibition of lipid peroxidation have been demonstrated (Foti and Ingold, 2003a; Lado et al., 2004). In this research, several protection mechanisms of monoterpenes are possible. Firstly, as demonstrated in previous studies, monoterpenes may directly act on photosynthetic apparatus, enhancing photochemical properties and electron transport (Loreto et al., 1998; Delfine et al., 2000). Secondly, monoterpenes may act as antioxidants, quenching reactive oxygen species preventing lipid peroxidation, and thereby maintaining membrane stability under drought stress (Loreto and Velikova, 2001; Foti and Ingold, 2003b; Lado et al., 2004). Thirdly, monoterpenes may trigger biosynthetic pathways inducing the production of antioxidants such as carotenoids, ascorbic acid and promoting enzyme activities (Obiol-Pardo et al., 2011; Zhao et al., 2013). Future investigations are necessary to elucidate which of these mechanisms are acting, although the first mechanism is rejected in this research (Fig 3.5; 3.7). Many more parallel physiological and biochemical replicates are needed under drought stress and/or monoterpene treatments to link ROS levels and oxidative damage (MDA) with net photosynthesis, PSII efficiency and photochemical electron transport rate. Assays of superoxide dismutase (SOD) and antioxidative enzymes such as catalase and glutathione reductase are essential to quantify the possible monoterpene enhancement of antioxidant status.

To conclude, exogenous monoterpene spray in wild-type tomato did not maintain photosynthesis when photosynthetic efficiency was reduced under moderate to severe drought stress. Exogenous monoterpenes significantly reduced oxidative damage that resulted from drought-induced oxidative stress (H_2O_2); since lipid peroxidation and its product (MDA) was considerably and significantly lower. Monoterpene spray limited foliar ABA accumulation under severe stress, possibly by inhibiting MEP synthesis pathway and thereby reducing production of ABA precursors. The ABA-deficient mutant *notabilis* did not show the same response and protection mechanism; exogenous monoterpenes did not alter the response of *notabilis* to drought stress, suggesting a possible non-ABA stomatal regulation when foliar ABA accumulation is significantly suppressed. We speculate that hydrogen peroxide prompted the initial reduction of stomatal conductance, either directly or via Ca^{2+} signalling, which decreased *Gs* and *A* under moderate drought. This mechanism would account for ABA-deficient tomatoes having similar stomatal regulation as wild-type plants with high ABA content. The impact of monoterpenes on this possible mechanism remains unclear, and further studies are necessary to examine the roles of other antioxidants, enzymes, and hormones.

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6 Appendices

Appendix 1. Effect of water deficit (WD), monoterpenes (MT), PSII maximum (Fv'/Fm') and operating (φPSII) efficiency on net photosynthesis rate (A)

P values and interactions examined by two-way ANOVA for exogenous monoterpene treatment and oxidative stresses or damage. *, P = 0.05; **, P = 0.001.

Factors	A	Factors	A
WD	0.018*	Drought	0.081 ^{ns}
MT	0.208^{ns}	MT	0.507 ^{ns}
Fv'/Fm'	<0.001**	φPSII	0.001**
WD x MT	0.181 ^{ns}	D x MT	0.422 ^{ns}
WD x *Fv'/Fm'*	0.027*	D x φPSII	0.149 ^{ns}
MT x *Fv'/Fm'*	0.176 ^{ns}	MT x φPSII	0.412 ^{ns}
WD x MT x *Fv'/Fm'*	0.152 ^{ns}	D x MT x φPSII	0.353 ^{ns}