The role of monoterpenes in plant physiological and antioxidant responses to drought stress

Zhou, Hao (zhouh5)

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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 Kirsti Ashworth and Prof. Ian Dodd.

During the writing process, I utilised generative AI and AI-assisted technology, specifically ChatGPT, for the purpose of proofreading, language refinement, and suggestions to enhance the clarity and coherence of my written expression. The AI as used strictly for linguistic assistance and did not contribute to the conceptual, analytical, or theoretical development of the research in this thesis. I acknowledge that the responsibility for the originality, accuracy, and academic integrity of the content rested solely with me.

Excerpts of Chapter 2 and 4 of this thesis have been published or in preparation in the following academic publications:

Hao Zhou, Kirsti Ashworth, Ian C Dodd, Exogenous monoterpenes mitigate H_2O_2-induced lipid damage but do not attenuate photosynthetic decline during water deficit in tomato, *Journal of Experimental Botany*, Volume 74, Issue 17, 13 September 2023, Pages 5327–5340, https://doi.org/10.1093/jxb/erad219

Hao Zhou

Lancaster University, UK

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Abstract

Monoterpenes are a group of biogenic volatile organic compounds (BVOCs) known for their role in plant response to environmental stresses. Their composition and emission variation under stress conditions are closely related cellular and intercellular functioning. But several aspects of their function unclear. This thesis firstly investigated the physiological (leaf gas exchange, water status, and redox balance) responses of tomato plants exposed to well-watered conditions and deficit irrigation in combination with **exogenous** monoterpenes (Chapter 2) at 1.25, 2.5 and 5 mM concentrations. **Exogenous** monoterpenes decreased foliar ROS accumulation by enhancing enzymatic antioxidant capacity, thereby reducing oxidative damage. However, this does not appear to protect photosynthetic performance. Whether **endogenous** monoterpenes (Chapter 3 & 4) had similar effects was explored by exposing three transgenic tobaccos (*Nicotiana tabacum*) with upregulated monoterpene precursor genes and individually inserted genes for (-)-α/β-pinene (PG11), myrcene (MG1), and (-)-limonene (LG12) and their wild-type (WT) to drought stress (withholding water) before and during stem elongation. Significant monoterpene emissions were detected in PG11 and LG12 but not in MG1 and WT. The results provided basic information of the impact of overproduction of monoterpene in a non-native emitter and suggested trade-offs in growth and development. The LG12 line was selected to determine whether enhanced **endogenous** limonene emission provides additional antioxidative protection to plants under water deficit. LG12 plants maintained leaf water status under drought, showed increased emission rate as soil moisture declined initially, then decreased dramatically as drought persisted. LG12 plants also had decreased gas exchange and increased lipid peroxidation compared to WT plants. After re-watering, these adverse effects were reversed, with the highest ascorbate peroxidase (APX) activity and lowest lipid damage in LG12, which may benefit plants' long-term recovery from drought. These findings highlight the complexity of monoterpene interactions with plant stress response and oxidative regulation. They suggest a better understanding of the physiological and biochemical significance of monoterpenes in plant drought response might assist efforts in enhancing drought tolerance.

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List of Main Abbreviations

ABA	Abscisic acid
ANCOVA	Analysis of covariance
A_{net}	Net photosynthesis rate to CO_2
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
AsA/Asc	Ascorbic acid/Ascorbate
cv.	Cultivar
DW	Dry weight
Fv/Fm	Maximum quantum efficiency of PSII photochemistry
Fv′/Fm′	Maximum efficiency of PSII photochemistry in the light
FW	Fresh weight
GPP	Geranyl pyrophosphate
g_{sw}	Stomatal conductance to water (H_2O)
H_2O_2	Hydrogen peroxide
iWUE	Intrinsic water use efficiency
MDA	Malondialdehyde
MEP	2-C-methyl-D-erythritol-4-phosphate
MTs	Monoterpenes
NPQ	Non photochemical quenching
P	Turgor pressure
P value	statistical *P* values
PSII	Photosystem II
ROS	Reactive oxygen species
RW	Re-watered
SOD	Superoxide dismutase
WD	Water deficit
WW	Well-watered
ΦPSII	Photosystem II quantum yield

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1 Emission and Function of Monoterpenes in Response to Drought Stress

1.1 Introduction

Environmental changes including extremes of temperature, light conditions and water availability create stresses in plants that reduce growth, development and yield, or even death (Boyer, 1982; Zhang *et al.*, 2023). Plants rapidly sense and have evolved various defence mechanisms to adapt to these abiotic environmental stresses (Lamers *et al.*, 2020). For example, many plants maintain leaf water potential (Ψ_{leaf}) and essential biological processes under stress by regulating physiological processes (e.g. partial stomatal closure to reduce water loss and increase water use efficiency - Nilson and Assmann, 2007; Dodd and Ryan, 2016) and cellular biochemistry (e.g. osmotic adjustment and antioxidants) to mitigate water content decline and oxidative damage (Chen and Jiang, 2010; Foyer and Shigeoka, 2010). Chronic and acute environmental stresses affect plant metabolic functions. Down-regulating primary metabolism results in redox imbalance, photosynthetic limitation, sugar and energy production and transport, eventually reduces plant growth, yield and reproduction (Altmann and Koßmann, 2001; Foyer, 2018). Upregulating secondary metabolism and related signalling pathways seems critical for abiotic stress resistance from leaf level to the whole plant. For instance, defence mechanisms mediated by phytohormones like jasmonic acid (JA), salicylic acid (SA), and abscisic acid (ABA) can regulate plant water status, antioxidants, osmoregulation and protein kinetics (Isah, 2019; Lamers *et al.*, 2020). Some of the secondary metabolites are volatile, comprising a large group of biogenic volatile organic compounds (BVOCs), which respond to environmental stresses and enhance stress resistance.

Plants produce and emit BVOCs throughout their life cycle mostly from flowers, fruits, leaves and roots, with varying compositions and contents across species and developmental stages (Holopainen and Gershenzon, 2010; Bracho-Nunez *et al.*, 2011).

The biosynthesis of BVOCs is genetically controlled and also regulated by abiotic environmental factors such as light, temperature, soil water, ambient CO_2 level, and nutrition (Bertin and Staudt, 1996; Blanch *et al.*, 2009; Carvalho *et al.*, 2016). These chemicals that give plants their distinctive smells are dominated by terpenes, fatty acid-containing derivatives, benzenoids and phenylpropanoids. Others include low molecular weight compounds such as methanol (CH_3OH), ethylene (C_2H_4), formaldehyde (CH_2O), ethanol (C_2H_6O), acetone (C_3H_6O), acetaldehyde (CH_3CHO), and acetic acid (CH_3COOH) (Kreuzwieser *et al.*, 1999), which act as stress indicators or signalling and communication molecules that mediate metabolic and ecological functions within and between plants (Dudareva *et al.*, 2004; Fineschi *et al.*, 2013; Dani and Loreto, 2022).

Terpenes were first recognised for their strong chemical reactivity in the atmosphere after emission (Went, 1960) and their induction by herbivore attacks (Baldwin and Schultz, 1983). Terpene emissions from vegetation include isoprene (C_5H_8), monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$) and oxygenated terpenes. The first two account for more than 60% of natural BVOC emissions, with emission rates expected to increase and/or change in composition in the future (Guenther *et al.*, 2012; Hantson *et al.*, 2017). However, predicting the specific impact of terpenes on atmospheric composition is challenging due to species variation and sensitivity to environmental factors regulating plant emissions (Arneth *et al.*, 2008; Laothawornkitkul *et al.*, 2009). The importance of terpenes in plant biology has become increasingly evident over the past three decades, with initial focus on the emission and functional mechanisms of isoprene (Sharkey and Singsaas, 1995). Plants may benefit from terpenes, some of which maintain or even increase investment in production under stressed conditions, when metabolism and other developmental processes are inhibited (Kesselmeier and Staudt, 1999; Francesco *et al.*, 2011). Stress-induced changes in isoprene emission contribute to plant physiological and biochemical responses, such as photosynthetic protection under thermal and oxidative stresses (Sharkey and Loreto, 1993; Sharkey and Yeh, 2001). Isoprene can directly (e.g., stabilising cellular membranes under heat stress) and indirectly (e.g., regulating heat shock proteins) protect plant cellular and subcellular structures across varying stress levels (Affek and Yakir, 2002; Sharkey *et al.*, 2007b). Monoterpenes have similar effects, although the specific mechanisms of functioning are controversial (Copolovici *et al.*, 2005; Bertamini *et al.*, 2019; Bertamini *et al.*, 2021). Additionally, plant terpene emissions tend to be more sustained with higher internal concentrations, while the emissions of

other stress-induced volatiles are typically transient and depend on the severity of stress and the biosynthetic activity of the compounds involved (Loreto and Schnitzler, 2010; Pichersky and Raguso, 2018). Therefore, terpene emissions play critical ecological roles in plant-plant and plant-insect interactions, below-ground signalling, and enhancing the environmental resilience of ecosystems (Kessler and Heil, 2011; Dudareva *et al.*, 2013; Šimpraga *et al.*, 2016).

This chapter reviews the current knowledge on terpene emissions and their functions in response to abiotic stress, with a particular focus on monoterpenes and drought stress. It examines the processes of terpene biosynthesis at the leaf level, emission mechanisms, and the relationship between physiological and biochemical responses, particularly oxidative stress, along with potential protective mechanisms in plants.

1.2 Terpene biosynthesis and emission mechanisms

1.2.1 Biosynthetic pathway and resource distribution

BVOCs are constitutively produced by plants, stored in specialised storage pools and/or released when exposed to stresses, which can also stimulate direct (*de novo*) synthesis and emissions (Loreto and Schnitzler, 2010; Maffei, 2010). Terpenes are synthesised from the precursor isopentenyl diphosphate (IPP) through two major pathways in plants: the mevalonate (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway (Fig. 1.1). The MVA pathway operates in the cytosol, primarily responsible for sesquiterpene ($C_{15}H_{24}$, SQTs) production. While the MEP pathway, located in plastids, converts IPP into dimethylallyl pyrophosphate (DMAPP), facilitating isoprene and monoterpene biosynthesis (Rohmer, 1999). Some studies suggest that intermediate molecules may be exchanged between these two pathways, and IPP can be transported to mitochondria for alternative sesquiterpene pathway (Okada *et al.*, 2000; Vranova *et al.*, 2013). Isotopic labelling experiments indicate that the MEP pathway may contributes C5 units for cytosolic biosynthesis (Dudareva *et al.*, 2005), in turn, the MVA pathway may also support monoterpene production (Koley *et al.*, 2020), suggesting crosstalk between these two pathways (Laule *et al.*, 2003). However, the relationships between the biosynthesis pathways and other location-specific metabolic and biochemical processes for various terpene compounds requires further study (Dudareva *et al.*, 2013; Fineschi *et al.*, 2013; Rosenkranz and Schnitzler, 2013).

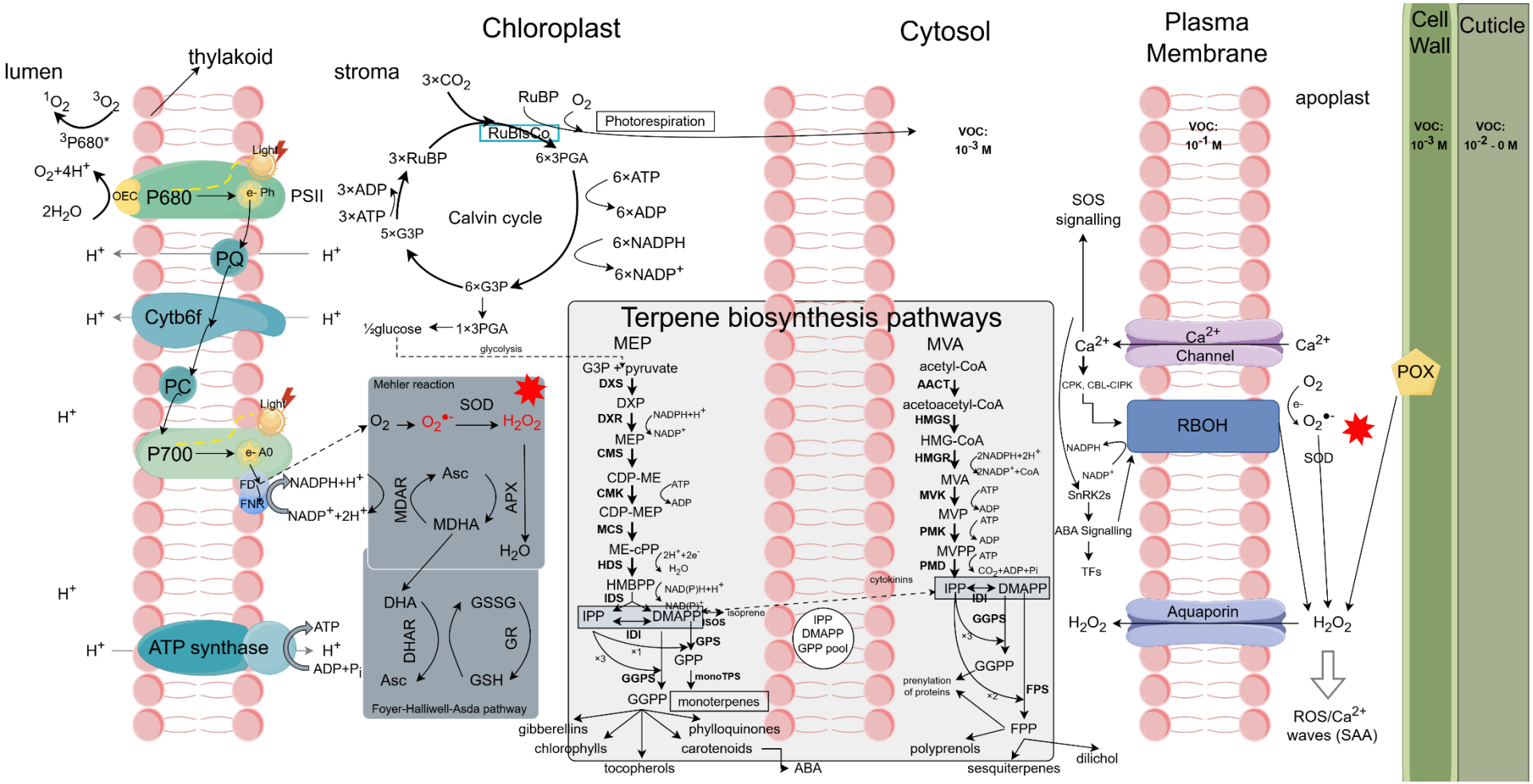
Chloroplast
Cytosol
Plasma Membrane
Cell Wall
Cuticle
lumen
thylakoid
stroma
apoplast
Light
P680
PSII
OEC
e- Ph
PQ
Cytb6f
PC
P700
e- A0
FD
FNR
ATP synthase
Calvin cycle
3×CO2
RuBP
RuBisCo
Photorespiration
3×RuBP
6×3PGA
6×ATP
6×ADP
6×NADPH
6×NADP+
3×ADP
3×ATP
5×G3P
6×G3P
½glucose
1×3PGA
glycolysis
Mehler reaction
SOD
MDAR
Asc
MDHA
APX
DHA
DHAR
GSSG
GR
GSH
Foyer-Halliwell-Asda pathway
Terpene biosynthesis pathways
MEP
G3P + pyruvate
DXS
DXP
DXR
MEP
CMS
CDP-ME
CMK
CDP-MEP
MCS
ME-cPP
HDS
HMBPP
IDS
IPP
DMAPP
IDI
GPS
GPP
GGPS
monoTPS
GGPP
monoterpenes
gibberellins
chlorophylls
phylloquinones
carotenoids
tocopherols
ABA
MVA
acetyl-CoA
AACT
acetoacetyl-CoA
HMGS
HMG-CoA
HMGR
MVK
MVP
PMK
MVPP
PMD
cytokinins
IPP DMAPP GPP pool
prenylation of proteins
FPS
FPP
polyprenols
sesquiterpenes
dilichol
VOC: 10-3 M
VOC: 10-1 M
VOC: 10-3 M
VOC: 10-2 - 0 M
SOS signalling
Ca2+
Ca2+ Channel
CPK, CBL-CIPK
RBOH
NADPH
NADP+
SnRK2s
ABA Signalling
TFs
Aquaporin
H2O2
POX
SOD
ROS/Ca2+ waves (SAA)

Figure 1. 1 Illustrative diagram of the three systems studied in this thesis: photosystem, reactive oxygen species signalling and antioxidation, and terpene biosynthesis pathways. The theoretical BVOC concentration (M) in cellular structures is indicated in the blank space (Widhalm *et al*., 2015). Diagram adapted from Boncan *et al*., 2020. AACT, acetyl-CoA acetyltransferase; ABA, abscisic acid; APX, ascorbate peroxidase; Asc, ascorbate; CBL, calcineurin B-like; CD-ME, 4-(cytidine-5′-diphospho)-2-C-methyl-D-erythritol; CEF, cyclic electron flow; CIPK, CBL-interacting protein kinase; CD-ME2P, 4-(cytidine-5′-diphospho)-2-C-methyl-D-erythritol-2-phosphate; CMK, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; CPK, calcium-dependent protein kinase; Cytb6f, cytochrome b6/f complex; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DMAPP, dimethylallyl pyrophosphate; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose-5-phosphate synthase; FD, ferredoxin; FNR, ferredoxin NADP+ reductase; FPP, farnesyl pyrophosphate; G3P, glyceraldehyde-3-phosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; HDS, 4-hydroxy-3-methylbut-2-enyl- diphosphate synthase; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; IDI, IPP-DMAPP isomerase; IPP, isopentenyl pyrophosphate; MCT, 2-C-methyl-d-erythritol-4-phosphate cytidylyltransferase; MDAR, monodehydroascorbate reductase; MDHA, monodehydroascorbate; MDS, 2-C-methyl-d-erythritol-2,4-cyclodiphosphate synthase; ME-cPP, methylerythritol cyclodiphosphate; MEP, 2-C-methyl-D-erythritol-4-phosphate; MK, mevalonate kinase; MVA, mevalonate/mevalonic acid; MVA, mevalonic acid; MVD, diphosphomevalonate decarboxylase; MVP, mevalonate-5-phosphate; MVPP, mevalonate pyrpophosphate; PEP, phosphoenolpyruvate; Ph, pheophytin; PMK, phosphomevalonate kinase; POX, peroxidase; PQ, plastoquinone; PSI/II, photosystem I/II; PT, prenyl transferase; RBOH, respiratory burst oxidase homolog; RuBisCO, ribulose-1,5-biphosphate carboxylase/oxygenase; RuBP, ribulose-1,5-biphosphate; SAA, systemic acquired acclimation; SOD, superoxide dismutase; SOS, salt overly sensitive; TFs, transcription factors.

The MEP pathway begins with two key precursors: pyruvate and glyceraldehyde-3-phosphate (G3P). These molecules undergo a condensation reaction catalysed by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS), leading to the formation of 1-deoxy-D-xylulose-5-phosphate (DXP). DXP is then reduced by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) to produce 2-C-methyl-D-erythritol 4-phosphate (MEP) (Rodríguez-Concepción, 2006). MEP then undergoes several enzyme-catalysed reactions, eventually leading to the formation of IPP and DMAPP. These two molecules are essential isoprenoid structural units (Zhao *et al.*, 2013; Chatzivasileiou *et al.*, 2019). The critical steps in this process involve a series of intermediates, including 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) and 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate (CDP-MEP), catalysed by specific enzymes such as MEP cytidylyltransferase and MEP kinase (Richard *et al.*, 2002). Isoprene biosynthesis branches from here with isoprene synthase converting DMAPP into isoprene (Sharkey *et al.*, 2005; Vickers *et al.*, 2011). In the MEP downstream, geranyl diphosphate synthase (GPS) catalyses the condensation of one IPP molecule and one DMAPP molecule to form geranyl diphosphate (GPP), which is the central precursor for monoterpene biosynthesis. GPP undergoes cyclisation or rearrangement reactions catalysed by various monoterpene synthases (monoTPS) to produce different monoterpenes, such as limonene, α/β-pinene and myrcene (Mahmoud and Croteau, 2002). The diversity of terpenes arises from the action of specific terpene synthase (TPS) enzymes that modify the GPP structure into distinct monoterpene compounds (Pichersky *et al.*, 2006; Koley *et al.*, 2020). Around 30-40 TPSs are found in *Arabidopsis*, 40 TPSs in tomato, 40-60 TPSs in *Mentha* species, and 50-100 TPS in *Pinus* species (Falara *et al.*, 2011; Tholl and Lee, 2011; Alicandri *et al.*, 2020; Chen *et al.*, 2021). Typically, 10-15 of them processes monoterpene production (Degenhardt *et al.*, 2009; Zhou and Pichersky, 2020; Alicandri *et al.*, 2022). Moreover, some of the monoterpenes (e.g., β-ocimene, β-myrcene, α/β-pinene, sabinene, limonene) showed a tendency to interconvert according to final carbocation intermediate under stressed conditions such as heat (Chen *et al.*, 2011; Jardine *et al.*, 2017).

Foliar terpene emissions are regulated by biosynthetic activity, physiochemical properties of compounds and physiological characteristics of the plant. Chloroplastic production of terpenes via the MEP pathway depends on G3P and pyruvate, which are derived from photosynthetic processes (Lichtenthaler *et al.*, 1997; Perez-Gil *et al.*,

2024). Carbon supplied through the Calvin-Benson cycle (in the form of G3P) is crucial for feeding the MEP pathway. MEP pathway requires substantial amount of ATP, NADPH and electron flux, which are generated during the light dependent reactions of photosynthesis and other biological processes such as photorespiration, glycolysis and the citric acid cycle. For example, from CO_2, 20 ATP and 14 NADPH molecules per isoprene molecule; 40 ATP and 28 NADPH per monoterpene molecule on average (Sharkey and Yeh, 2001). The condensation reactions and subsequent conversions in the MEP pathway (such as the formation of IPP, DMAPP, and GPP) depend on this energy input, with no more than 4% of the total electron flux (typically 1-2%) is used for isoprene and monoterpene production (Niinemets *et al.*, 2002a; Niinemets and Reichstein, 2002; Sharkey *et al.*, 2007b). Consequently, the efficiency of the MEP pathway and, therefore, monoterpene output are regulated by the plant's energy status and metabolic flux allocation. The connection between terpene synthesis and net photosynthesis rate (A_{net}) is further highlighted by activation of *TPS*, which catalyse the conversion of DMAPP into isoprene and GPP into specific monoterpenes, in response to photosynthetic cues such as CO_2, light availability, and photosynthetic electron transport chain (Seemann *et al.*, 2002; Li and Sharkey, 2013). *De novo* production and direct emission of isoprene directly utilise photosynthetically fixed carbon and energy (Delwiche and Sharkey, 1993; Schnitzler *et al.*, 2004). Thus, the rate of isoprene emission is strongly light and CO_2 dependent, with the thermodynamics influences the synthase and substrate activities (Singsaas, 2000; Sharkey *et al.*, 2007b). Unlike isoprene, monoterpenes are not strictly tied to immediate photosynthetic activity, as they are more influenced by storage mechanisms and *de novo* synthesis, which can be triggered by environmental factors, depending on the specific and non-specific storage (Loreto *et al.*, 1996a; Harley, 2013). Consequently, monoterpene emission can occur both continuously and in a delayed, stress-induced manner.

1.2.2 Storage and emission mechanisms

The storage mechanisms vary depending on the plant species and the type of plant tissue involved. Volatile terpenes are primarily stored in glandular trichomes, resin ducts, secretory cavities and oil glands, as well as laticifers (Fineschi *et al.*, 2013; Li and Sharkey, 2013). These structures serve as reservoirs for monoterpenes and regulate emissions in response to environmental stresses. Glandular trichomes are hair-like epidermal structures that are commonly found on the leaf, stem and flower surface.

These trichomes also have biosynthetic capacity, producing and storing essential oil components in a specialised gland at the tip of the structure (Turner *et al.*, 2000). Monoterpenes are synthesized in the secretory cells of the trichomes and accumulated in a storage cavity or subcuticular space, which is formed by separation of the outer cell wall from the cuticle (Schilmiller *et al.*, 2009). Glandular trichomes are particularly important in aromatic plants such as mint (*Mentha* spp.), basil (*Ocimum basilicum*), lavender (*Lavandula angustifolia*) and cannabis (*Cannabis sativa*), which are rich in essential oils (Werker, 1993). Resin ducts are specialised tubular structures constructed by epithelial cells located in the vascular bundles of coniferous plants like pines (*Pinus* spp.) and spruce (*Picea* spp.). These ducts contain a mixture of monoterpenes, sesquiterpenes and diterpenes in resinous form, which solidifies upon exposure to air, creating a physical and chemical barrier against herbivores and pathogens (Trapp and Croteau, 2001). In species like citrus and eucalyptus, monoterpenes are stored in secretory cavities or canals, which allow plants to maintain a large pool of monoterpenes that can be released either passively through volatilisation or actively in response to stress. Laticifers are specialised networks of cells that produce and store latex, a milky fluid containing a mixture of terpenes and other secondary metabolites, enriched in species like rubber tree (*Hevea brasiliensis*) and generally emitted after mechanical damage (Gracz-Bernaciak *et al.*, 2021; Freitas *et al.*, 2024).

After synthesis and storage, BVOCs are released to the atmosphere, with this passive transport critical for maintaining non-toxic levels (no more than 1mM level based on biological mode) of intercellular terpenes (Niinemets *et al.*, 2004; Widhalm *et al.*, 2015; Adebesin *et al.*, 2017; Liao *et al.*, 2023). Stored terpenes accumulate in the intercellular airspace and escape into the ambient atmosphere theoretically through the gas exchange pores, stomata, on the leaf surface. Water vapor and stomatal conductance (g_{sw}) strongly dependent on environmental conditions, such as photosynthetic photon flux density (PPFD), which also diurnally affects biosynthetic activity and emission level (Farquhar and Sharkey, 1982; Guenther *et al.*, 1993). Stomatal opening is usually accompanied by a burst of emissions when the *in vivo* terpene pools stabilise (Niinemets and Reichstein, 2003). Although studies (hypothesising that biosynthesis is not affected) indicate that BVOC emission fluxes are correlated with g_{sw} under more uniform environmental conditions (Kesselmeier et al., 1996; Peñuelas and Llusià, 2002; Centritto et al., 2011; Harley, 2013). ABA-induced stomatal closure significantly reduces the overall emission, with significant variability in the emission rate of specific compounds

(Tingey *et al.*, 1991; Nemecek-Marshall *et al.*, 1995; Gabriel *et al.*, 1999). In contrast, the direct emissions of certain compounds such as isoprene and α-pinene are not regulated by stomata under varying CO_2 concentrations or air vapor pressure deficit (VPD) (Monson and Fall, 1989; Loreto *et al.*, 1996a). Therefore, passive emission (e.g., concentration gradient and diffusion) of these compounds also needs consideration (Kesselmeier and Staudt, 1999; Niinemets *et al.*, 2004; Harley, 2013).

The volatility (i.e., Henry's law constant) and lipid solubility (i.e., octanol/water partition coefficient) determine the physiochemical properties of terpenes (Niinemets and Reichstein, 2003; Niinemets *et al.*, 2004). Their chemical structure determines their volatility, with functional groups (e.g., C=C, phenyl, cycloalkane and epoxide) or the degree of saturation in the terpene molecule altering its vapour pressure and, consequently, its emission characteristics (Guenther, 2013; Possell and Loreto, 2013; Pichersky and Raguso, 2018). For instance, monoterpenes such as limonene and pinene have relatively high vapour pressures compared to sesquiterpenes or diterpenes due to their smaller molecular size, weight and simpler structures. Terpenes are lipophilic, meaning they diffuse through lipid-rich cellular membranes and accumulate in lipid-based storage compartments such as oil glands and trichomes. Studies also suggest that leaf cuticle and intercellular mesophyll tissue can temporarily store monoterpenes in species that lacking specific storage structures(Loreto *et al.*, 1996c; Loreto *et al.*, 2001a; Grote *et al.*, 2013).

Progress has been made in studying relationships and interactions between g_{sw} and gas-aqueous/lipid phase partition, as well as species sensitivities. Short-term stomatal control of emission rate can be achieved when the internal terpene partial pressure is not steady, and its synthesis rate is not affected by the endogenous concentration of the compounds. The equilibrium between emission, diffusion gradient, the synthesis rate and loss rate via metabolism diminishes the sensitivity of stomatal control (Mihaliak *et al.*, 1991; Kesselmeier and Staudt, 1999; Niinemets *et al.*, 2004). Compounds with high constants such as isoprene and monoterpenes have a strong tendency to be in the gas phase and their gas-phase partial pressure is high relative to their concentration in the aqueous phase or in the plant, causing rapid movement from the leaf surface into the atmosphere with gas and liquid pool half-times of a few seconds, and therefore show stomatal insensitivity of emissions (Gabriel *et al.*, 1999; Niinemets and Reichstein, 2003). This may be why soluble volatiles like alcohols, carboxylic acids and other

oxygenated terpenes more sensitive to stomatal control (MacDonald and Fall, 1993; Niinemets *et al.*, 2002b).

Terpene diffusion requires passage through a subcellular barrier and possible transfer/accumulation in lipid structures, with barrier resistance varying by several orders of magnitude and greatest in the cuticle layer (Li and Sharkey, 2013; Boachon *et al.*, 2019). However, achieving effective passive diffusion may require phytotoxic concentrations (over mM level) in plant cells (Widhalm *et al.*, 2015). To avoid self-intoxication, biological trafficking processes may also contribute to volatile emission dynamics by transporting volatile molecules across subcellular structures like membranes, the cytosol and cell walls via ATP-binding cassette transporters (Yazaki, 2006; Demurtas *et al.*, 2023). Despite the limited contribution of these biological mechanisms to the emission of smaller molecules such as isoprene and monoterpenes, we cannot ignore the synergistic effects of all these regulation mechanisms under changing environmental conditions (Effmert *et al.*, 2005; Raza *et al.*, 2023).

1.3 Terpene emissions in changing environment

1.3.1 Emission behavioural responses to abiotic stresses

Abiotic stress factors, including light intensity, temperature, atmospheric composition and water availability change volatile emissions by altering terpene biosynthesis and physiochemical properties, as well as plant physiological and biochemical properties such as gas exchange and redox balance (Kesselmeier and Staudt, 1999). For example, the vapor pressure of terpenes increases with temperature, leading to higher emission rates directly from storage source. Higher temperatures increase the kinetic energy of molecules and facilitate their transition from the liquid phase (or storage pools) to the vapour phase. The lipid octanol/water partition coefficients of monoterpenes are similar ranging from 28000 to 38000 mol mol^{-1}, which is hundred times higher than isoprene (263 mol mol^{-1}), however, the Henry's law constant varies across a few orders of magnitude (Li *et al.*, 1998). For example, 2850 Pa m^3 mol^{-1} for limonene and 13600 Pa m^3 mol^{-1} for α-pinene, suggesting that the passive emission of specifically-stored monoterpene can be more temperature dependent (Guenther *et al.*, 1993; Niinemets and Reichstein, 2002). Although mild heat stress may promote stomatal opening to cool the leaves, while high and persisted heat stress causes stomatal closure to prevent excessive water loss, stomatal control of terpene emission under heat stress is not obvious (Harley,

2013), suggesting the physiological independence of terpene emissions (Yamori *et al.*, 2006; Slot *et al.*, 2019). Biochemically, terpene synthase and substrate activities have a preferred temperature range, the thermostability and catalytic activity of these biosynthetic pathways determines the productivity of endogenous terpenes (Styles *et al.*, 2017). For example, the optimum temperature for β-pinene and limonene synthases activity is 40°C in *Picea abies* and *Quercus ilex L*, below or above which decreases synthase kinetic properties (Fischbach *et al.*, 2000). For *Acer palmatum*, without specialised storage structures, the total monoterpene emission rate (from non-specific storage) increases by about 15% for every 1°C increase in ambient temperature ranging from 20-35°C, which changes monoterpene synthetic capacity in response to heat stress without damage and directly alters the emission behaviour (Mochizuki *et al.*, 2020). For species such as *Pinus sylvestris* L., *Helianthus annuus* and *Liquidambar styraciflua*, with specialised storage structures, foliar terpene emissions remain significantly increased above 40°C, due to increased membrane permeability or/and damage to storage structures (Tingey *et al.*, 1980b; Kleist *et al.*, 2012; Nagalingam *et al.*, 2023). Additionally, stress can trigger the expression of terpene synthetic pathways and co-regulates monoterpene production with the temperature-dependent enzymatic conversion of the substrates, and sometimes irrespective of enzymatic diversity (Degenhardt *et al.*, 2009; Loreto and Schnitzler, 2010). Changes in the terpene composition also show a temperature dependence, with forest-scale β-ocimene emissions increasing by about 4.4 % and α-pinene decreasing by about 0.8 % per degree of elevation in temperature (Chen *et al.*, 2011; Jardine *et al.*, 2017).

Therefore, a wide range of plant intrinsic and extrinsic conditions influence emissions, which vary between plant species and compounds, making it difficult to accurately predict the emission behaviour and fluxes in natural environments under future changing climate (Kesselmeier and Staudt, 1999; Laothawornkitkul *et al.*, 2009). A summarised monoterpene emission behaviour is described in Fig. 1.2, however, most previous research focused on stress-induced isoprene emission and how heat, light and elevated CO_2 concentration affect monoterpenes (Peñuelas and Staudt, 2010). Whereas our understanding of the behaviour and mechanisms of monoterpene emission response in water limited environments is insufficient, possible synergistic effects of drought may have overestimated or underestimated ecosystem monoterpene emissions and inventory when the effects of drought are poorly described (Ormeño *et al.*, 2007; Tiiva *et al.*, 2017; Byron *et al.*, 2022). Since high temperatures usually increase land

evapotranspiration in arid areas, soil water deficits are expected to increase or become more persistent (Naumann *et al.*, 2018). Thus, drought effects on monoterpene emissions and their potential role in modulating physiological and biochemical responses are reviewed.

1.3.2 Monoterpene emissions response to drought and underlying mechanisms

Early drought-induced stomatal closure (caused by physiological and hormonal processes such as ABA regulation) decreases intercellular CO_2 concentration (*Ci*). Concurrently, an increase in mesophyll resistance further limits the photosynthetic process (Possell and Loreto, 2013). Summer decreases in soil moisture (by 26%) increased α-pinene and limonene emissions of *Quercus ilex* L. by approximately 60% and 166% respectively when plants were not heat-stressed (ambient temperature of

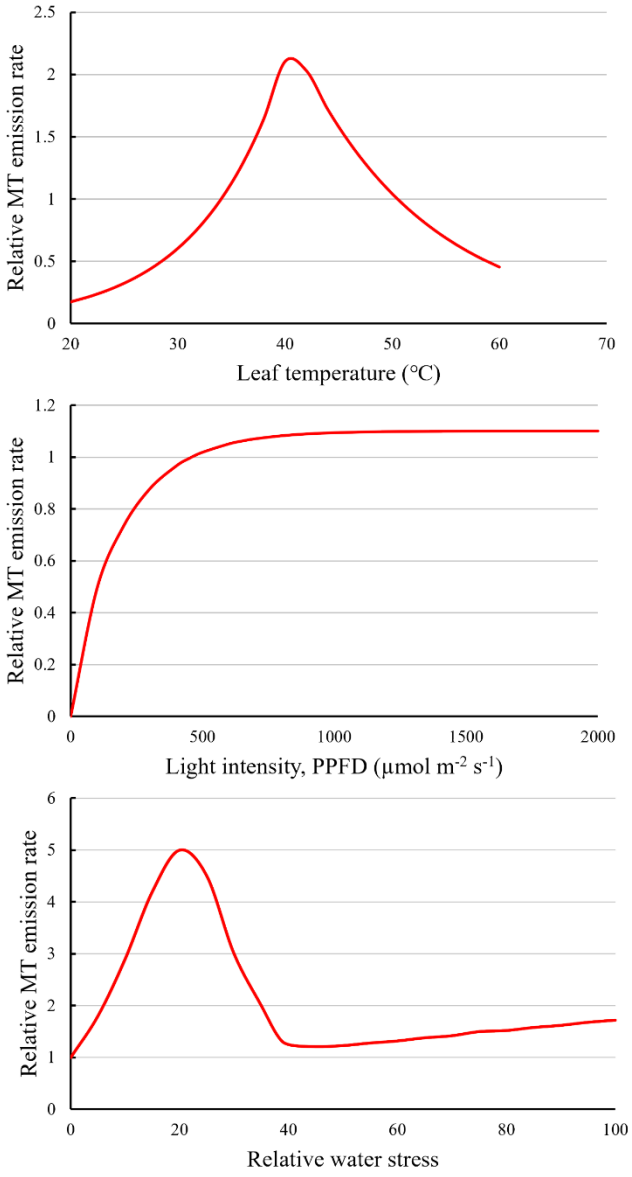

Figure 1. 2 Overall relative monoterpene emission behaviour in response to typical environmental factors including temperature (Loreto *et al.*, 1998c; Niinemets *et al.*, 2010), light intensity (Guenther *et al.*, 1993; Niinemets *et al.*, 2010), and soil water availability (Staudt *et al.*, 2002; Kreuzwieser *et al.*, 2021). Emissions from both specific and non-specific storage emissions. Relative water stress represents the stress level that plants received during drought as literature used different stress indicators such as soil water content or leaf water potential.

26°C). However, a further 5% soil moisture decline as drought persisted into autumn diminished these emission rates by around 20% (Mu *et al.*, 2018). Similarly, total foliar monoterpene emissions in the same species increased by 26% after withholding water for five days, but declined from day 10, eventually reaching a minimum of about 8% of pre-stress levels after 20 days (Šimpraga *et al.*, 2011). Total monoterpene emissions of *Cistus albidus*, *Quercus coccifera*, and *Pinus halepensis* increased 10-fold as leaf water potential (Ψ_{leaf}) declined, but severe water deficits (Ψ_{leaf} < -6 MPa) decreased emissions threefold (Ormeño *et al.* 2007). Generally, initial drought conditions enhance terpene emissions followed by a decline with prolonged drought (Staudt *et al.*, 2002; Peñuelas and Staudt, 2010).

Conversely, other research has indicated that drought at early stages does not consistently affect terpene emission rates. For example, water withholding for eight days did not alter isoprene emissions of *Quercus virginiana* Mill., and a 0.5 MPa decrease of Ψ_{leaf} in *Pinus sylvestris* had no impact on both isoprene and monoterpene emissions under field conditions (Pegoraro *et al.*, 2004; Kreuzwieser *et al.*, 2021). Decreasing stem water potential by 1.4 MPa did not significantly affect total foliar monoterpene emissions from *Quercus robur* L. in an enclosed chamber experiment (Peron *et al.*, 2021). Even when stomata were 90% closed due to drought stress, isoprene emissions from oak were largely unaffected (Tingey *et al.*, 1981). Similarly, isoprene emission was maintained despite substantial reductions in A_{net} and g_{sw} when exogenous ABA was treated to *Populus tremula* leaves (Fall and Monson, 1992). These findings suggest stomata have little effect on emissions with limited water availability.

Under drought conditions, monoterpene emission from the *de novo* synthesis can drop to about 36 % of the total monoterpene emission at the early drought stage (Lüpke *et al.*, 2017). The physicochemical properties of stored compounds and plant's underlying metabolic and photosynthetic processes strongly influence the source and response of monoterpene emissions to soil drying. Resultant increases in leaf temperature as transpiration decreases with soil drying throughout the drought cycle accelerate the diffusion of certain compounds including monoterpenes, by increasing their volatility and enhancing lipid accessibility from storage pools and plant tissues (Niinemets and Reichstein, 2003; Guenther, 2013). Indicating a degree of physiological independence from photosynthetic limitations at this stage. The active *de novo* synthesis when photosynthesis is scarcely affected may be attributed to the sufficiency of endogenous

C3 precursors, such as glyceraldehyde-3-phosphate (G3P) and pyruvate, as well as ATP, NADPH, and electron transport substrates, all of which support continued terpene biosynthesis (Brilli *et al.*, 2007a; Brunetti *et al.*, 2015). Furthermore, plants may utilise alternative carbon sources, such as starch breakdown (Karl *et al.*, 2002; Schnitzler *et al.*, 2004), cytosol and chloroplastic pyruvate, or even carbon re-fixed from xylem transport, respiration and photorespiration processes (Jardine *et al.*, 2014; Srikanta Dani *et al.*, 2017). These alternative carbon resources may compensate for decreased carbon assimilation due to limited intracellular CO_2 (*Ci*), potentially supporting monoterpene synthesis even when photosynthesis is severely restricted during extreme drought (Ormeño *et al.*, 2007). The expression monoterpene synthases may also be regulated by drought conditions (Possell and Loreto, 2013; Kreuzwieser *et al.*, 2021). For example, sabinene synthase is upregulated and cineole synthase is downregulated by drought in *Salvia officinalis* (Radwan *et al.*, 2017).

As drought stress persists and intensifies, further reductions in g_{sw} and A_{net} lead to increased diffusion resistance, limiting energy and substrate availability. This ultimately inhibits the production capacity and overall emission of volatile organic compounds (Sharkey and Loreto, 1993; Marron *et al.*, 2003; Peñuelas and Staudt, 2010). Suppressed metabolic activity and the loss of photosynthetic dependency can diminish emissions under prolonged drought stress. In most cases, plants can still maintain a positive monoterpene emission rate even when stomata are nearly fully closed and net photosynthesis had nearly ceased, with contributions from *de novo* synthesis dropping to 3% when soil moisture is lower than 10% (Šimpraga *et al.*, 2011; Lüpke *et al.*, 2017; Haberstroh *et al.*, 2018). Plants that experience minimal drought-induced emission reductions or retain emissions under extreme stress often have specialised storage structures or high cellular monoterpene concentration, temperature-dependent terpene emissions from both storage and cellular damage may accumulate monoterpenes, maintaining emissions (Delfine *et al.*, 2005; Ormeño *et al.*, 2007). For species (e.g., *Q. coccifera*) lacking such storage structure, increased concentrations of monoterpenes have been observed in the foliar lipid and aqueous phases, suggesting non-specific storage in these temporary pools (Niinemets and Reichstein, 2002; Niinemets *et al.*, 2004). As with monoterpenes stored in specialised structures, this non-specific storage can lead to delayed emission bursts (Staudt and Bertin, 1998). This could explain why some plants have partially increased overall emission rate with changes in the composition at the conclusion of drought experiments following a sharp reduction in

emissions emission, exhibiting a S curve in relation to soil/leaf water status (Ormeño *et al.*, 2007; Šimpraga *et al.*, 2011; Mu *et al.*, 2018). However, oxygenated monoterpenes, such as 1,8-cineole, may depend on photosynthetic activity unrelated to leaf temperature (Lüpke *et al.*, 2017). Hence, plant's monoterpene emission in response to drought is a complex process, with emission rates and compositions varying at different levels of drought. And previous studies do not have a unified measure of water stress (e.g., soil moisture, leaf water potential) due to different degrees of drought tolerance in plants, i.e. differences in the control of terpene synthesis and emission.

1.3.3 The role of terpenes in abiotic stress resistance

Plants produce terpenes as part of their adaptive strategies to abiotic stresses. Earlier terpene research predominantly focused on their ecological and biotic interactions (Kesselmeier and Staudt, 1999; Kessler and Heil, 2011; Pichersky and Raguso, 2018; Boncan *et al.*, 2020). For instance, certain plants release terpenes (e.g., monoterpenes and sesquiterpenes) into the soil, exerting allelopathic effects that inhibit the growth of nearby competing plants (Kong *et al.*, 2019), parasitic plants may also referentially grow toward to plants that emit some specific monoterpenes such as β-phellandrene and β-myrcene (Runyon *et al.*, 2006). This reduces competition for critical resources such as water, light, and nutrients, providing a competitive advantage to the terpene-releasing plant. Additionally, terpenes can function as signalling molecules, whereby plants attacked by herbivorous insects or exposed to abiotic stresses release volatile terpenes as a warning signal to neighbouring plants, triggering pre-emptive defence mechanisms such as priming resistance via hormonal and enzymatic pathways (Kalske *et al.*, 2019; Brosset and Blande, 2021). Terpenes also play an indirect protective role by attracting predatory or parasitic insects that prey on herbivores (War *et al.*, 2012). For example, some terpenes attract parasitic wasps that prey on caterpillars, thereby reducing herbivore pressure (Turlings *et al.*, 1995). Moreover, terpenes are integral to plant reproduction, as they help attract pollinators such as bees, butterflies, and other insects, facilitating successful pollination and reproduction. In addition to these biotic functions, terpenes are increasingly recognised for their role in abiotic stress resistance. The underlying protective mechanisms are hypothesised to involve the following key process:

Acting as photosynthetic and lipid structure stabiliser

Amongst the terpenes, isoprene was first identified as having a thermotolerance function (Siwko *et al.*, 2007; Sharkey, 2013). In *Arabidopsis thaliana*, transgenic plants emitting isoprene demonstrated significantly improved heat resistance in terms of photosynthetic performance, with isoprene gene expression in the MEP pathway upregulated in response to heat treatment at 60°C (Sasaki *et al.*, 2007). Similarly, in wild *Pueraria lobata*, isoprene emission enhanced photosystem II (PSII) fluorescence properties and the maximum rate of photosynthesis, particularly at temperatures exceeding 35°C. In contrast, suppression of endogenous isoprene emission using the chemical inhibitor fosmidomycin made these physiological traits more vulnerable to heat stress (Singsaas *et al.*, 1997). Comparable or slightly weaker physiological optimisations, as in photosynthetic stability and efficiency, were observed in *Quercus* species fumigated with monoterpenes such as α-pinene, sabinene, β-pinene, limonene, and cis-β-ocimene (Loreto *et al.*, 1998c; Delfine *et al.*, 2000; Llusià *et al.*, 2005). Additionally, temperature dependence of monoterpene synthesis and temperature-induced enzymatic conversion in various monoterpenes (Jardine *et al.*, 2017) further establishes that monoterpenes also provide thermal protection for plant leaves and photosynthetic processes, with varying protective capacity between specific compounds (Copolovici *et al.*, 2005; Sasaki *et al.*, 2007). Notably, this thermoprotective function may not extend to plants that do not emit these compounds endogenously (Loreto *et al.*, 1998b).

High temperatures and prolonged drought conditions disrupt cellular processes by decreasing the transmembrane proton gradient, causing proton leakage and reducing ATP production. This cascade of events limits the regeneration of ribulose-1,5-bisphosphate (RuBP), reduces Rubisco activity, and biochemically inhibits photosynthetic function (Bukhov *et al.*, 1999; Crafts-Brandner and Salvucci, 2000). Since membrane stability is critical to delaying these damages, terpenes play an essential role in enhancing membrane integrity. Being highly hydrophobic, terpenes can integrate into the membranes of cellular and subcellular structures, potentially improving their stability by binding to lipid bilayers, photosynthetic subunits, and the protein-membrane interface (Sharkey, 1996; Loreto *et al.*, 1998a; Singsaas, 2000). However, other studies suggest that the incorporation of at least a small percentage of solutes into the lipid membrane is necessary to increase terpene concentration in the bilayer and affect membrane dynamics (Cantor, 1997). Evidence also indicates that the concentrations of terpenes naturally present in plants may be insufficient to drive

efficient passive membrane transport or significantly alter the lipid dynamics of thylakoid membranes (Harvey *et al.*, 2015; Widhalm *et al.*, 2015). As a result, the role of terpenes in stabilising lipid structures remains complex and may require further investigation under various stress conditions.

Acting as *in vivo* signalling molecules facilitating stress response

With further investigation into the first hypothesis, research suggests that alternative protective mechanisms may be more effective and practical for plants under stress conditions. Terpenes likely function as signalling molecules that trigger the expression of stress-responsive genes or interact with membrane-embedded proteins to modulate metabolic dynamics and enhance plant stress tolerance (Peñuelas and Llusià, 2004; Possell and Loreto, 2013; Dani and Loreto, 2022). The MEP pathway intermediate, ME-cPP, has two functions, acting not only in its metabolic role but also triggers plastid to nucleus retrograde signal specific to stresses (Xiao *et al.*, 2012). It plays a crucial role in coordinating light, redox and hormonal signalling pathways, thereby contributing significantly to the plant's adaptive responses to environmental changes (Jiang and Dehesh, 2021). Evidence indicates that both endogenous and exogenous isoprene upregulate ATP synthase activity in the thylakoid membranes of *Arabidopsis thaliana*, maintaining relatively high PSII photochemical efficiency in plants exposed to high temperatures (Velikova *et al.*, 2011). Furthermore, exogenous isoprene (0.02~3 ppm in the ambient air) induce a co-expression network involving phenylpropanoid biosynthetic genes, ERF, and WRKY transcription factors, which promote signalling pathways related to abscisic acid (ABA) and ethylene (Barta and Loreto, 2006; Shang *et al.*, 2010; Harvey and Sharkey, 2016). In addition, isoprene enhances JA and methyl jasmonate (MeJA)-regulated defence and growth responses (Zuo *et al.*, 2019), which are thought to synergise with ABA signalling under drought stress (Wang *et al.*, 2020). In contrast, monoterpenes appear to support systemic acquired resistance. For example, exogenous α/β-pinene (0.35~6 ppm in the ambient air) induces SA-related gene expression, conferring resistance to pathogens and herbivores, and promoting innate immune signalling both within and between plants (Riedlmeier *et al.*, 2017; Wenig *et al.*, 2019). In *A. thaliana*, monoterpene exposure and transformation have been linked to upregulated MeJA-mediated defence factors, ABA-inducible stress proteins, and increased production of fatty acids and waxes under drought stress (Kriegs *et al.*, 2010; Riedlmeier *et al.*, 2017).

It is important to note that the effects of exogenous terpene treatments have concentration limits specific to each species. Prolonged exposure or high concentrations of terpenes can irreversibly damage plants (Brown *et al.*, 1987; Ibrahim *et al.*, 2004). These findings clearly indicate that terpenes play a critical role as signalling molecules involved in membrane proteins, receptor genes, transporter proteins, signalling cascades, and secondary metabolism, while also modulating growth regulation under stress conditions. However, the prevalence and efficacy of these mechanisms, both within individual plants and in neighbouring plants, require further investigation.

Acting as direct or indirect antioxidant and energy dissipator

Plants commonly overproduce reactive oxygen species (ROS), including superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$), in response to abiotic stresses such as heat and drought. Chloroplasts are the primary sites of ROS production, which occur as by-products of electron transport and oxygen metabolism during photosynthesis. These ROS cause oxidative damage to proteins, lipids, and DNA, leading to cellular dysfunction and, ultimately, cell death. During photosynthesis, light energy drives the flow of electrons from photosystem II (PSII) to photosystem I (PSI) via the electron transport chain (Hajiboland, 2014; Foyer, 2018). During this process, molecular oxygen (O_2) is partially reduced, forming $O_2^{\bullet -}$. Under normal conditions, electrons reduce $NADP^+$ to NADPH, which is essential for carbon fixation and other metabolic processes. However, under stress conditions, such as drought or heat, photosynthesis is inhibited, leading to the accumulation of excess NADPH and subsequent electron leakage, which increases ROS production, ATP is produced through photophosphorylation in the chloroplasts, but any imbalance between energy supply and demand exacerbates ROS formation (Møller, 2001; Awad *et al.*, 2015). The Mehler reaction serves as a key pathway for ROS formation and dissipation of electrons in chloroplasts, where O_2 is reduced to $O_2^{\bullet -}$. Superoxide dismutase (SOD) then converts $O_2^{\bullet -}$ to H_2O_2, which is further detoxified into water by catalase (CAT) and ascorbate peroxidase (APX) (Miyake, 2010). Beyond their damaging effects, ROS also function as signalling molecules, modulating gene expression, stomatal behaviour, and stress responses. Retrograde signalling from chloroplasts to the nucleus regulates stress-responsive genes and epigenetic programming, enhancing plant adaptation to abiotic stress (Sierla *et al.*, 2016; Foyer *et al.*, 2017). Enzymatic (SOD, CAT, APX) and non-enzymatic antioxidants (ascorbate, glutathione, carotenoids) maintain redox balance

under normal conditions by scavenging ROS. However, prolonged stress can overwhelm these defences, leading to excessive ROS accumulation and oxidative damage (Alscher *et al.*, 2002; Ramel *et al.*, 2012), which can further damage proteins, lipids, and DNA, ultimately resulting in cellular dysfunction and death (Das and Roychoudhury, 2014).

Since oxidative stress is a common plant response to abiotic stress, terpenes are proposed to enhance oxidative resistance directly or indirectly (Vickers *et al.*, 2009a). Isoprene's specific chemical structure, particularly its conjugated double bonds and reducing capacity, can directly scavenge ROS (Lado *et al.*, 2004; Gonzalez-Burgos and Gomez-Serranillos, 2012). Prolonged drought, acute high temperatures, and intense light can lead to overproduction of ROS, such as $^1O_2$ and $O_2^{\bullet -}$, which directly damage PSII and thylakoid membranes, limiting light energy harvesting, photosynthetic activity, and protein synthesis (Nishiyama *et al.*, 2001; Cruz de Carvalho, 2008; Pospíšil, 2016). For example, in *Phragmites australis*, inhibition of isoprene production by chemically blocking the MEP pathway (inhibited by fosmidomycin, thus, a non-specific response) under high light intensity and temperature led to the accumulation of $^1O_2$ and H_2O_2, causing malondialdehyde (MDA - a lipid peroxidation end product) accumulation and decreasing net photosynthesis (Velikova *et al.*, 2004). Isoprene's ROS scavenging capacity is particularly evident during direct oxidative stress (e.g., ozone exposure). Both endogenous and exogenous isoprene mitigate ozone-induced lipid peroxidation and H_2O_2 accumulation, reducing the decline in stomatal conductance, net photosynthesis rate, and photosynthetic efficiency (Loreto and Velikova, 2001; Vickers *et al.*, 2009b; Ryan *et al.*, 2014). These effects help maintain the integrity of thylakoid membranes and photosynthetic proteins, suggesting that isoprene's membrane stabilization function may be linked to its antioxidant properties. Similar effects have been observed with monoterpenes under heat and drought stress in various plant species, though with compound-specific variability (Foti and Ingold, 2003; Loreto *et al.*, 2004; Nogués *et al.*, 2015a). Furthermore, monoterpene-mediated antioxidant effects may shift toward ascorbic acid-mediated protection as drought and heat stress intensify (Nogués *et al.*, 2015). Additionally, isoprene fumigation (0.01 ppm in the ambient air) delays the depletion of α-tocopherol, ascorbic acid, and β-carotene under increased temperatures, without causing significant photosynthetic inhibition or damage as temperatures rise from 35°C to 45°C (Peñuelas *et al.*, 2005). This suggests coordination between terpene functioning and endogenous antioxidant systems.

Moreover, terpene synthesis pathways may serve as alternative energy dissipation mechanisms, consuming excess ATP and NADPH, diverting energy from the photosynthetic electron transport chain, and reducing photorespiration-induced ROS production when photosynthesis is limited under stress. For instance, while isoprene biosynthesis may consume only 2.7% ATP and 3.4% NADPH, photorespiration requires 20-40% ATP and NADPH (Sharkey *et al.*, 2007b). These proportions in isoprene production increase several-fold during stress when photosynthetic activity is suppressed (Magel *et al.*, 2006; Fortunati *et al.*, 2008). However, the direct ROS scavenging function of isoprene and monoterpenes is questioned. Isoprene oxides (e.g., methyl vinyl ketone) can be more toxic in plants, and the pool of electrons available for isoprene synthesis is larger (Jardine *et al.*, 2012). Some oxygenated monoterpenes exhibit less efficient antioxidant properties (Copolovici *et al.*, 2005), and their accumulation at a slightly lower level can result in phytotoxicity because they are more reactive with functional groups (Ghasemi *et al.*, 2020; Abd-ElGawad *et al.*, 2021). The role of different terpenes in ROS regulation, along with their synergistic or antagonistic effects with endogenous antioxidants and energy dissipation pathways, remains an area requiring further investigation.

1.4 Study focus and thesis structure

This study focuses on the specific roles that monoterpenes (MTs) play in mediating plant responses to drought stress, based on Hypothesis Three presented in Section 1.3.3. By comparing endogenous and exogenous monoterpenes, this project aims to:

a. Determine and quantify the effects of drought stress on the physiology, morphology, and biochemistry of agricultural plants, specifically tomato (*Solanum lycopersicum*) and tobacco (*Nicotiana tabacum*).

b. Investigate the impacts of exogenous and endogenous monoterpenes on plant enzymatic antioxidants (SOD, APX) and photosynthetic responses to abiotic stress, and explore their relationships.

c. Probe the underlying mechanisms by which monoterpenes reduce oxidative stress (e.g., H_2O_2) and damage (e.g., lipid peroxidation, as measured by MDA), thereby protecting plants under water deficit. This objective also includes examining how plants upregulate monoterpene synthesis when exposed to drought, and the implications for responses to other environmental stresses.

The thesis is organised into three experimental chapters:

Chapter 2 examines the effects of applying exogenous monoterpenes at various concentrations to the leaf surface of tomato plants. The study investigates the physiological and antioxidant responses to water stress induced by deficit irrigation. Potential antioxidant effects of exogenous monoterpenes were revealed, and the underlying mechanisms linked to enzymatic antioxidant activities at different concentrations of these compounds elucidated.

Chapter 3 investigates the impact of monoterpene genetic transformation on the growth, physiological responses, and emission characteristics of tobacco plants (*Nicotiana tabacum*) under drought stress. The study assesses morphology, gas exchange, leaf water status, and foliar monoterpene emission at two growth stages — before and during stem elongation — under well-watered and water deficit conditions. The experiment involves three transgenic tobacco lines with upregulated monoterpene precursor genes, specifically engineered to produce (−)-α/β-pinene (PG11), myrcene (MG1), and (−)-limonene (LG12). LG12 was identified as the optimal candidate for further mechanistic investigation.

Chapter 4 explores whether endogenous (−)-limonene emission provides additional antioxidative protection to plants under water deficit, similar to the effects of exogenous monoterpenes in Chapter 2. Using the transgenic tobacco line LG12, the study examines the effects of limonene production in a non-native emitter on monoterpene emission, leaf water status, gas exchange, photosynthetic efficiency, ROS production, and enzyme antioxidant activity in response to drought stress. The findings highlight the complex interplay between water deficit and foliar volatile emissions, offering insights into the role of monoterpenes in plant stress tolerance and their potential applications in developing stress-tolerant crop varieties.

2 Exogenous monoterpenes mitigate H_2O_2-induced lipid damage but do not attenuate photosynthetic decline during water deficit in tomato

Author contributions

Hao Zhou: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization.

Kirsti Ashworth: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition

Ian C. Dodd: Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration

Submission to journal

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Abstract

Although monoterpenes are suggested to mediate oxidative status, their role in abiotic stress responses is currently unclear. A foliar spray of monoterpenes increased antioxidant capacity and decreased oxidative stress of *Solanum lycopersicum* under water deficit stress. The foliar content of monoterpenes increased with spray concentration indicating foliar uptake of exogenous monoterpenes. Exogenous monoterpene application substantially decreased foliar accumulation of hydrogen peroxide (H_2O_2) and lipid peroxidation (malondialdehyde, MDA). However, it appears that monoterpenes prevent the accumulation of reactive oxygen species rather than mitigating subsequent ROS-induced damage. Low spray concentration (1.25 mM) proved most effective in decreasing oxidative stress but did not up-regulate the activity of key antioxidant enzymes (superoxide dismutase and ascorbate peroxidase) even though higher (2.5 and 5 mM) spray concentrations did, suggesting a complex role for monoterpenes in mediating antioxidant processes. Furthermore, soil drying caused similar photosynthetic limitations in all plants irrespective of monoterpene treatments, apparently driven by strong reductions in stomatal conductance as Photosystem II efficiency only decreased in very dry soil. We suggest that exogenous monoterpenes may mitigate drought-induced oxidative stress by direct quenching and/or upregulating endogenous antioxidative processes. The protective properties of specific monoterpenes and endogenous antioxidants require further investigation.

2.1 Introduction

Biogenic volatile organic compounds (BVOCs), specifically terpenes, enhance plant resilience to abiotic stresses such as high temperature and soil drying (Penuelas and Llusia, 2003). The diverse group of compounds known collectively as monoterpenes (MTs, $C_{10}H_{16}$) are the second most important BVOC by global emission rate, surpassed only by isoprene. Their biosynthesis via the methylerythritol phosphate (MEP) pathway (Peñuelas and Staudt, 2010) is affected by environmental conditions such as light, temperature and CO_2 level (Sharkey *et al.*, 2007b), closely related to photosynthetic activity, and regulated by carbon and energy supply. Nevertheless, when environmental stresses limit photosynthesis, biosynthesis of certain terpenes can be maintained, likely by using alternative carbon sources (Brilli *et al.*, 2007a) such as starch breakdown (Karl *et al.*, 2002), cytosolic carbon supply (Fortunati *et al.*, 2008) or precursors such as cytosolic pyruvate (Rosenstiel *et al.*, 2003). While their production and emission are constitutive, abiotic stresses can enhance terpene production dependent on the severity of stress (Peñuelas and Staudt, 2010). Environmental stress can also alter the composition of MTs emitted, likely because different compounds diffuse through the stomata at different rates (Harley, 2013), but possibly also depending on the physiochemical properties of these compounds and cellular lipid structure (Niinemets *et al.*, 2004; Noe *et al.*, 2006).

Terpene biosynthesis and emission are energetically expensive and must therefore provide a net benefit. Under abiotic stresses, plants that sustain terpene production and emissions maintain key functionality (Penuelas and Llusia, 2003; Velikova and Loreto, 2005; Sharkey *et al.*, 2007b), enabling plants to cope with extreme environmental conditions such as heatwaves and drought (Penuelas and Llusia, 2003). Isoprene maintains relatively high photosynthesis and electron transport rate, decreases oxidative status and enhances recovery after stress in plants exposed to high temperatures (>45°C) (Velikova and Loreto, 2005) and drought (Monson *et al.*, 2021). More recently, some MTs (e.g. α-pinene and β-pinene) have been shown to exhibit isoprene-like functionality and are increasingly associated with stress defences of plants exposed to high temperatures (Copolovici *et al.*, 2005; Zuo *et al.*, 2017) and ozone (Loreto *et al.*, 2004). Exogenous MT treatments enhance thermotolerance by maintaining photosynthetic efficiency and decreasing photosynthetic limitation by providing potent antioxidant protection of cell membranes (Loreto *et al.*, 1998c; Delfine *et al.*, 2000;

Peñuelas and Llusià, 2002). However, these protective effects vary between MT compounds (Copolovici *et al.*, 2005) and there is currently no direct evidence that MTs provide similar protection against water deficit.

Global warming is expected to increase the frequency, intensity and duration of soil drying events (Caretta, 2022), increasing drought stress in plants. When plant water losses exceed root water uptake, cellular turgor and leaf water potential (Ψ_{leaf}) decrease, thereby suppressing physiology, growth and development (Lambers *et al.*, 2008). Plants use various signalling processes to maintain leaf water potential during early drought stages by decreasing stomatal conductance to water vapour (Huntenburg *et al.*, 2022). Stomatal closure limits transpiration rate, intercellular CO_2 and net photosynthesis rate (Chaves *et al.*, 2003) but the involvement of MTs in these processes is not clear (Xu *et al.*, 2022).

Leaf water deficit disrupts the transfer of photon energy during photosynthesis. Excess electrons accumulate around the photosystems causing the photoreduction of oxygen molecules (O_2, the Mehler reaction), inevitably producing large quantities of reactive oxygen species (ROS), including singlet oxygen, superoxide ($\bullet O_2^-$), hydroxyl radicals ($HO\bullet$) and hydrogen peroxide (H_2O_2), which are phytotoxic when accumulated in excess (Asada, 2006). Prolonged stress conditions cause increasing photooxidation (Pintó-Marijuan and Munné-Bosch, 2014), and rapid lipid peroxidation, thereby damaging cellular structures and photosynthetic apparatus (Smirnoff, 1993). However, plants have evolved a range of enzymatic and non-enzymatic antioxidant mechanisms to control ROS levels to minimise oxidative damage and maintain the redox balance (Das and Roychoudhury, 2014). The most important enzymatic antioxidation mechanism for the photosystem II (PSII) is the water-water cycle of the Mehler reaction (Asada, 2006). Excess electron flux from the photosystems induces the photoreduction of O_2 producing $\bullet O_2^-$, which is reduced to H_2O_2 by superoxide dismutase (SOD) and is itself detoxified to H_2O by the ascorbate peroxidase (APX)-catalysed ascorbate and monodehydroascorbate radical cycle, using ascorbic acid as a reducer. Ascorbate regeneration also provides an effective dissipation route for electron flow in Photosystem I. The water-water cycle thus efficiently decreases ROS accumulation induced by excess photon energy (Asada, 1999; Miyake, 2010).

Specific terpenes can provide an alternative source of antioxidants for plants (Vickers *et al.*, 2009a; Pollastri *et al.*, 2021). Isoprene- and monoterpene-emitting or fumigated

plants have lower ROS accumulation and lipid peroxidation under heat, ozone and, in the case of isoprene, drought stress (Loreto *et al.*, 2004; Velikova and Loreto, 2005; Vickers *et al.*, 2009b; Ryan *et al.*, 2014). Terpenes are thought to directly quench stress-induced ROS (Loreto *et al.*, 2001b; Vickers *et al.*, 2009b) due to their chemical reducing properties (Graßmann, 2005), or act as a signalling molecules triggering systemic defences (Loreto and Schnitzler, 2010; Zuo *et al.*, 2019). However, MT-mediated ROS scavenging and antioxidative protection may depend on the availability of alternative endogenous antioxidant mechanisms such as photorespiration and ascorbate (Peñuelas and Llusià, 2002; Nogués *et al.*, 2015a). Under high light intensity, terpenes work synergistically with other antioxidants to provide photoprotection (Brilli *et al.* (2022), mitigating oxidative damage by quenching excess energy thereby maintaining photosynthesis (Velikova *et al.*, 2008).

Monoterpenes appear to induce similar effects to isoprene, and their diversity and reducing potential may provide more targeted protection. Exogenous applications of terpinene and β-pinene restored antioxidant enzyme activity (i.e. SOD) and non-enzymatic antioxidants (i.e. carotenoids) under heat stress, possibly by up-regulating downstream reactions of the MEP pathway (Tian *et al.*, 2020). Other exogenous MTs conferred thermal protection to PSII (Loreto *et al.*, 1998c; Delfine *et al.*, 2000). Nevertheless, the antioxidant protection offered by MTs is not always accompanied by photosystem protection (Peñuelas and Llusià, 2002), and high MT concentrations (>2.5 mM) can directly cause oxidative stress and inhibit development (Ibrahim *et al.*, 2004; Singh *et al.*, 2006). It is not clear whether these responses to specific MTs also occur under water deficit conditions or how this relates to endogenous antioxidants.

To investigate whether MTs protect plants grown in drying soil, exogenous MTs were applied to tomato, a high MT-emitting species (Zhou *et al.*, 2022), and exposed to different irrigation treatments. We hypothesised that exogenous MTs maintain PSII photosynthetic activities under water deficit by increasing foliar antioxidative capacity thus decreasing oxidative stress and damage to plants, and that this protective effect would be proportional to the concentration of MTs applied.

2.2 Materials and Methods

2.2.1 Plant Materials and Growth

Tomato (*Solanum lycopersicum* cv. Ailsa Craig) seeds were germinated in John Innes No. 2 compost (Westland Horticulture Ltd, Tyrone, UK) in seed trays (5 x 4.8 x 5 cm cells). Three weeks after sowing, 144 uniform seedlings were selected and transplanted to 2-litre plastic pots (top 14 cm, base 10.5 cm, depth 18.5 cm), filled with John Innes No. 2. Plants were numbered and randomly assigned to one of four 1-m^3 semi-controlled growth chambers constructed with Clear Perspex® Acrylic Sheet, similar to those described by Stockes et al. (1993). Plants were grown for a further four weeks and rotated between chambers every week. At this stage, size of plants were relatively similar, ranging from 20-25cm in height, with 7-8 leaves. Plants were then rotated within each chamber every other day and between chambers every four days. Plants were fed fortnightly with Miracle-Gro® All Purpose Soluble Plant Food at a concentration of 2.5 mL L^{-1} of water, following the manufacturer's recommendation (The Scotts Company Ltd, Surrey, UK).

Growth lamps (Powerstar HQI-BT, 600 W/D daylight, OSRAM, Munich, Germany) provided 400 ± 20 μmol photons m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the level of the sampled leaf for 12 h per day (07:00 to 19:00) throughout the experiment. Day:night temperatures and relative humidity (RH) were maintained at 22 °C:16 °C ± 1.0°C and 40:60 ± 10%, respectively, by pumping air through the chambers at a flowrate of 3.0 ± 0.2 m^{-3} min^{-1}.

2.2.2 Treatments

Three independent, factorial experiments were conducted, each with different watering regimes and MT applications across four growth chambers. Well-watered (WW) plants were irrigated twice a day (08:00 and 18:00) by replacing 100% of daily pot water loss. Water deficit (WD) plants were irrigated once a day (18:00) by replacing 25% of individual daily pot water loss. The water deficit treatment commenced 7 weeks after sowing and was continued for seven days until wilt for all three experiments. The evening before starting the water deficit treatment (at 18:30), the plants received a foliar spray of MT solution to both sides of all leaves to drip point. The spray was then similarly applied twice a day (at 08:30 and 18:30) for the duration of the experiment. Experiments 1 and 2 applied a 1.25 mM MT solution or 5 mM solution respectively,

while Experiment 3 applied a range (1.25 mM, 2.5 mM and 5 mM) of MT solutions. In all three experiments, control plants were sprayed with a 0 mM MT solution.

2.2.3 Monoterpene solutions

The MT compounds included in the exogenous spray were selected based on the composition of MT emissions from well-watered *Solanum lycopersicum*. These were determined in previous experiments under the same growth conditions (following the method described in Zhou et al., 2022) and therefore assumed to reflect the endogenous MTs of *Solanum lycopersicum* cv. Ailsa Crag.

MT solutions were prepared by dissolving 800 µL each of α-pinene, β-pinene, 3-carene, α-terpinene, α-phellandrene, p-cymene, limonene, γ-terpinene and terpinolene (Sigma Aldrich Ltd, Gillingham, UK) in 0.1% (v/v, 10mL) methanol. Milli-Q® water was then added to make up 1 L of 5 mM MT solution. This solution was further diluted with Milli-Q® water to make 2.5 mM and 1.25 mM solutions, as required. The control (0 mM) solution was prepared by adding 990 mL Milli-Q® water to 10 mL methanol (0.1% v/v).

2.2.4 Sampling

Sampling started from Day 0, when the plants were well-watered, for baseline measurements, and was then carried out daily until Day 7. Sampling was conducted before irrigation and spraying of WD treatments and at least two hours after WW plants were irrigated and sprayed. The sampled plants were randomly selected, with the leaflets adjacent to the terminal leaflet on the newest fully developed leaf used for non-destructive physiological measurements, or for destructive measurements such as biochemical assays and leaf water status. Sampled leaflets measured 5.5-7.0 cm length, 2.5-3.0 cm width.

Two plants from each treatment were used for Li-Cor measurements (physiology) and then harvested for leaf water potential and biochemical analysis. One plant was used for destructive measurements only, and Li-Cor measurements were carried out on one further plant which was not harvested, and was measured daily throughout the experiment. A total of three physiological and biochemical replicates were sampled at each timepoint during each experiment, ultimately giving each variable at least six

replicates at 1.25 mM and 5 mM, and three replicates at 2.5 mM per sampling time. Substrate moisture level was measured immediately after sampling using a soil moisture sensor (WET-2, Delta-T Devices Ltd, Cambridge, UK) inserted to a depth of ~8cm from the top of the pot. Measurements and sampling were performed from 10:30 to 18:30, with the first replicates of each treatment completed in treatment order first, then the second replicate and so on. Sections 2.5-2.7 below describe each measurement technique.

2.2.5 Physiology

A LI-6400XT portable photosynthesis system (Li-Cor Ltd, Lincoln, NE, USA) with an integral Leaf Chamber Fluorometer (LCF 6400-40) measured leaf gas exchange and (light-adapted) chlorophyll fluorescence. The leaflet was clamped inside a 1 cm^2 circular sampling cuvette, positioned to avoid the leaf vein. Airflow to the cuvette was set to 500 mmol s^{-1} to provide positive pressure, CO_2 was provided by a CO_2 mixer（Li-Cor 6400-01）and kept at a constant 412 ppm in the cuvette. The cuvette environment was allowed to stabilise for 5-10 mins before readings were logged, every 60 s for 20 mins, to record net photosynthesis rate (*A*; μmol m^{-2} s^{-1}), transpiration rate (*Tr*; mmol m^{-2} s^{-1}), and stomatal conductance (g_{sw}; mol m^{-2} s^{-1}) to understand the fundamental physiological processes of gas exchange and stomatal behaviour. Full detailed settings of the instrument are available in the supplementary information (Supplementary Table S1). Maximum fluorescence under saturating light flash (*Fm'*), photosynthetic steady-state fluorescence (*Fs'*) and minimum fluorescence (F_0') during momentary darkness were also recorded. Operating (*ΦPSII*, Equation 1) and maximum (*Fv'/Fm'*, Equation 2) efficiency of PSII, and PSII efficiency factor (*Fq'/Fv'*, Equation 3) were estimated as described by Murchie and Lawson (2013) to understand photosynthetic performance after light adaption:

$$\Phi PSII = \frac{F_{q'}}{F_{m'}} = \frac{F_{m'} - F_{s'}}{F_{m'}} \quad (1)$$

$$\frac{F_{v'}}{F_{m'}} = \frac{F_{m'} - F_{0'}}{F_{m'}} \quad (2)$$

$$PSII\ factor = \frac{F_{q'}}{F_{v'}} = \frac{F_{m'} - F_{s'}}{F'_m - F'_o} \quad (3)$$

2.2.6 Plant Water Status

Leaf water potential (Ψ_{leaf}, MPa) was measured using the leaf one node below the sampled leaf, using a pressure chamber as described by Boyer (1967). In brief, leaves were cut from the stem using a sharp razor blade, and inserted into the pressure chamber with the petiole protruding from the seal gasket. After sealing the chamber, the pressure was gradually increased at a rate of 0.01 MPa s^{-1} until water exuded from the cut surface, indicating the pressure inside the chamber was equal to that of the xylem, and Ψ_{leaf} read from the chamber gauge.

2.2.7 Biochemical Analysis

The leaflet used for Li-Cor measurements and MT sampling and its corresponding compound leaflet were collected and cut into strips using a razor blade. The strips were placed into separate 2.0 mL Eppendorf tubes, flash-frozen in liquid nitrogen and stored at -80°C. Samples were subsequently used for biochemical analyses, as described below. Assays included foliar MT content and measures of oxidative status, which we define as the balance between 'oxidative stress' and 'enzymatic antioxidative activity'.

In this study, 'oxidative stress' refers specifically to foliar hydrogen peroxide (H_2O_2) content. H_2O_2 is a reactive oxygen species (ROS) indicator that is stable and easy to measure and is often used as a proxy for foliar ROS level (Pintó-Marijuan and Munné-Bosch, 2014). 'Enzymatic antioxidative activity' was measured here as the activities per unit of protein of superoxide dismutase and ascorbate peroxidase, which are routinely sampled chloroplast-related enzymes (Das and Roychoudhury, 2014). In addition, we use malondialdehyde (MDA) equivalents, as a measure of 'oxidative damage to lipids'. MDA is a reactive electrophilic species formed from lipid peroxidation, is generally correlated with oxidative stress and is easy to detect (Hodges *et al.*, 1999).

2.2.7.1 Leaf monoterpene content

Frozen leaf materials were freeze-dried for 48 hours and subsequently ground into fine powder using a Mixer Mill MM 200. Approximately 30 mg of the ground dry leaf materials were transferred to a pre-weighed 10 mL Falcon tube, which was then re-weighed to determine the exact weight of samples. Samples were extracted with 10 mL hexane via ultrasonication at 45 kHz for one hour, followed by a shaking incubation at −4°C overnight. The tubes were then centrifuged at 15,000 rpm for 10 mins at −4°C.

The supernatants were transferred to another 10 ml Falcon tube and concentrated to a volume of less than 0.5 mL, hexane was added to achieve a final volume of 1 mL, and 0.5 mL aliquots were transferred to amber GC vials for MT analysis via gas chromatography-flame ionization detector (GC-FID).

The analysis was performed using an Agilent 7820A gas chromatograph system equipped with an HP-5 non-polar capillary column (30 m x 0.32 mm x 0.25 μm). Hydrogen was used as the carrier gas at constant flow of 1.5 mL min^{-1}. The temperature of injection was 250 °C and injection volume was 1 μL using a split ratio of 1:10 with a split flow of 15 mL min^{-1}. The oven temperature was initially held at 50°C for 1 min, then elevated at a rate of 10°C min^{-1} to 70°C where it was held for 2 mins. The temperature was then increased at a rate of 2°C min^{-1} to 76°C and held for 1.2 min. The oven was finally heated at 30°C min^{-1} to 250°C where it was kept for 2 min, giving a total run of 17 min. The temperature of the FID detector was 300°C with an air flow of 300 mL min^{-1}, H_2 flow of 30 mL min^{-1} and N_2 flow of 30 mL min^{-1}.

Peaks were identified by comparing the retention times (±5%) with standard compounds, in which the supernatant was replaced with 0.5 mL of a solution with the same composition as the spray solution. A calibration curve for quantification was constructed using a range of concentrations of standards (0.5, 1, 2.5, 5, 10, 20, 40, 50, 80, 100 μg/mL). One duplicate was taken for every three samples to ensure the reproducibility of the analysis. Sample compounds not included in the standards were numbered with an MT prefix (e.g. MT1) and quantified using the calibration curve of α-pinene. Data acquisition, identification, and quantification were performed using OpenLAB CDS ChemStation (firmware revision: A.01.18.003; software driver version: 6.03.091). Leaf MT content was then calculated based on the dry weight of samples and expressed as mg g (dry weight)$^{-1}$.

2.2.7.2 ROS and MDA

Aliquots of 100 mg and 40 mg fresh weight of frozen leaves were used for H_2O_2 and MDA assays, respectively. For homogenisation and extraction, leaf materials were firstly ground using pestle and mortar in a liquid nitrogen bath, then transferred to a 2 mL Eppendorf tube containing 0.1% (w/v) trichloroacetic acid (TCA), and vortexed. Subsequently, samples were homogenised in precooled tube blocks using a Mixler Mill (MM200, Retsch Ltd, Hope, UK). The homogenate was centrifuged at 12,000 rpm for

30 mins at 4°C, supernatant was transferred to another Eppendorf tube for further assays.

H_2O_2 was determined as described by Klassen *et al.* (1994) and Velikova *et al.* (2000). In brief, 0.4 mL of the supernatant, generated as described above, was added to 0.4 mL of 10 mM potassium phosphate buffer (pH 7.0) and 0.8 mL of 1 M potassium iodide (KI). The coloured reaction product of H_2O_2 with KI developed within 25 mins. The absorbance of the supernatant at 360 nm was determined, after colour stabilisation for at least 1 hour, using a spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., Waterbeach, UK). A calibration curve was produced by replacing samples with 0.4 mL of H_2O_2 solutions (0, 1, 5, 10, 20, 40, 50, 80 and 100 μM) diluted from commercial H_2O_2 solution (9.8 M, Sigma Aldrich Ltd, Gillingham, UK). H_2O_2 content was calculated from a calibration curve of absorbances of H_2O_2 standard solutions.

MDA content was determined by the thiobarbituric acid-reactive-substances (TBARS) assay (Ryan *et al.*, 2014). In brief, 0.5 mL of the supernatant, generated as described above, was mixed with 1.0 mL of 20% TCA containing 0.5% (w/v) thiobarbituric acid (TBA) and the mixture heated in a water bath for 30 mins at 95°C. The reaction was then immediately stopped in an ice bath and the mixture centrifuged at 10,000 rpm at 4°C for 5 mins. Supernatant absorbance was again determined using a spectrophotometer, here at two wavelengths (532 and 600 nm) to correct for nonspecific turbidity. An absorption coefficient of 155,000 μM^{-1} cm^{-1} was used (Heath and Packer, 1968) to calculate the MDA equivalents content of the samples.

2.2.7.3 Enzymes and total protein

Frozen leaf material (200 mg) was ground to a fine powder in liquid nitrogen, and the powder homogenised in 1.2 mL ice-cold potassium phosphate extraction buffer (pH 7.8, containing 0.1 mM Ethylenediaminetetraacetic acid, EDTA) in a 2 mL Eppendorf tube. Samples were centrifuged at 15,000 rpm for 20 min at 4°C and the supernatant was collected. The pellet at the bottom of the tube was re-suspended in 0.8 mL extraction buffer and then centrifuged at 15,000 rpm for a further 15 min at 4°C. The supernatants were combined as crude leaf enzyme extract and stored on ice, to measure superoxide dismutase (SOD) and ascorbate peroxide (APX) activity based on the total protein content. These assays were only performed in Experiment 3.

Total SOD activity was measured by determining the sample's ability to inhibit the photochemical reduction of nitro-blue tetrazolium chloride (NBT) based on the methodology of Giannopolitis and Ries (1977) as modified by Weydert and Cullen (2010). In short, each 2 mL of reaction mixture contained 100 µL leaf extract, 50 mM phosphate buffer (pH 7.8, 2 mM EDTA), 9.9 mM L-methionine, 55 µM NBT, 0.025% (v/v) Triton-X100, and 1 mM riboflavin. The reaction with NBT was initiated under a lamp providing ~380 µmol m^{-2} s^{-1} PPFD for 10 min. One control and one blank, each without leaf extraction, were illuminated with samples or kept in the dark, respectively, for 10 min to correct for background absorbance. Absorbance was read at 560 nm and SOD activity in units U mg of $protein^{-1}$ was defined as the amount of SOD required to inhibit 50% of NBT photo reduction compared to the control.

APX activity was analysed based on the protocol of Nakano and Asada (1981). Each 1 mL reaction mixture contained 100 µL leaf extract, 50 mM potassium phosphate buffer (pH 7.0), 5 mM ascorbate and 1 mM EDTA. A reaction was initiated by adding 1 mM H_2O_2 and absorbance immediately recorded at 290 nm for 3 min. APX activity was determined using the extinction coefficient of reduced ascorbate (2.8 mM^{-1} cm^{-1}) and expressed as mmol ascorbate min^{-1} $(mg\ protein)^{-1}$. Total protein content of each sample, only used to define enzyme activities, was quantified by the Bradford method (1976) using bovine serum albumin (BSA) as a standard.

2.2.8 Statistical Analysis

All statistical analyses were conducted using R v4.1.0. A general linear model with univariate ANOVA was used to determine significant differences in all independent variables (physiology, biochemistry and Ψ_{leaf}), and two-way and three-way interactions between the main effects (water deficit * exogenous MTs or concentrations). The soil-leaf water relationship was built based on van Genuchten (1980) using R function 'fitsoilwater' in package 'soilphysics' (de Lima *et al.*, 2021). Regression lines were estimated using a linear model to interpret relationships between Ψ_{leaf}, physiological and biochemical responses of plants. Significant differences between physiological and biochemical variables with leaf water potential or each other, as well as their interactions were determined via an ANCOVA. A post-hoc Tukey test with Bonferroni correction was used to compare PSII efficiency variables between and within treatments at different water deficit levels. In all analyses, $p<0.05$ denoted statistical significance.

2.3 Results

2.3.1 Exogenous MTs don't affect soil/plant water status

Leaf water potential (Ψ_{leaf}) declined (by approximately 50%) relatively steadily with soil moisture to ~21.7%, below which further soil drying decreased Ψ_{leaf} more sharply (Fig. 2.1). Neither experiment or treatment ($p>0.05$) significantly affected this relationship, indicating that exogenous monoterpenes did not affect Ψ_{leaf} response to soil drying. A 3-way ANCOVA analysis showed no significant difference in plant responses to treatments between the 3 experiments.

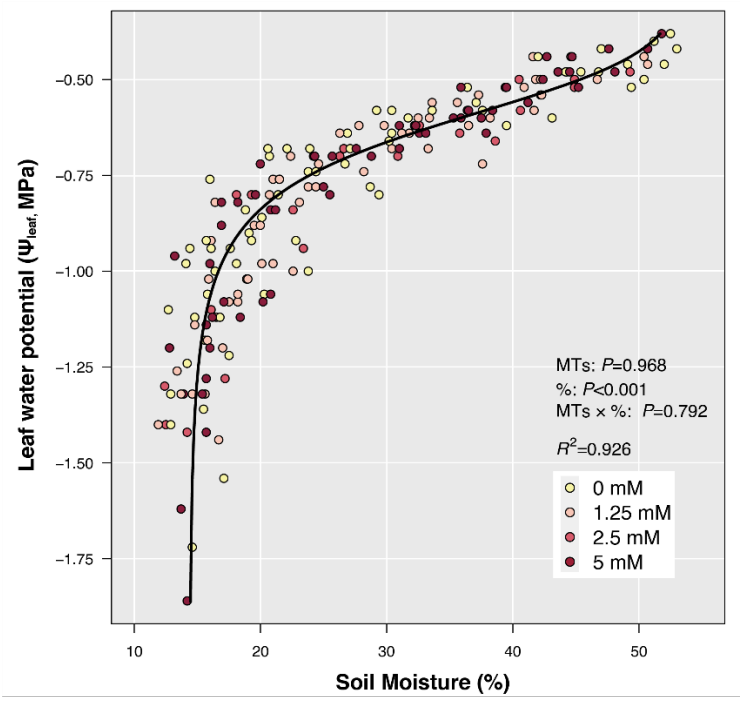

Figure 2. 1. Leaf water potential (Ψleaf) vs soil moisture for tomatoes treated with 0 mM (yellow), 1.25 mM (pink), 2.5 mM (red) and 5 mM (dark red) exogenous MT foliar spray. The response is described by the van Genuchten equation using R package "soilphysics'. Data from all three experiments are included. ANCOVA results (*P* Values reported) for the impact of exogenous MTs, soil moisture (%) and their interaction (MTs*%) are presented.

2.3.2 Exogenous MTs increased foliar MT content

Leaf MT content and composition were analysed on Days 0 (well-watered), 2, 3, and 7 of the experiment to investigate MT content before during and at the end of the drought

regime. Total MT content increased by approximately 2.5-fold on day three in control (0 mM) plants as the soil dried, before decreasing by the end of the experiment (Fig. 2.2). Treating plants with exogenous MTs increased total foliar MT content from Day 2 onwards, by 1.6-fold in plants treated with 1.25 mM and 2.5 mM and by 2.5-fold in plants treated with 5 mM. By the end of the experiment, exogenous MT application was required to sustain total MT content at the maximal levels induced by soil drying.

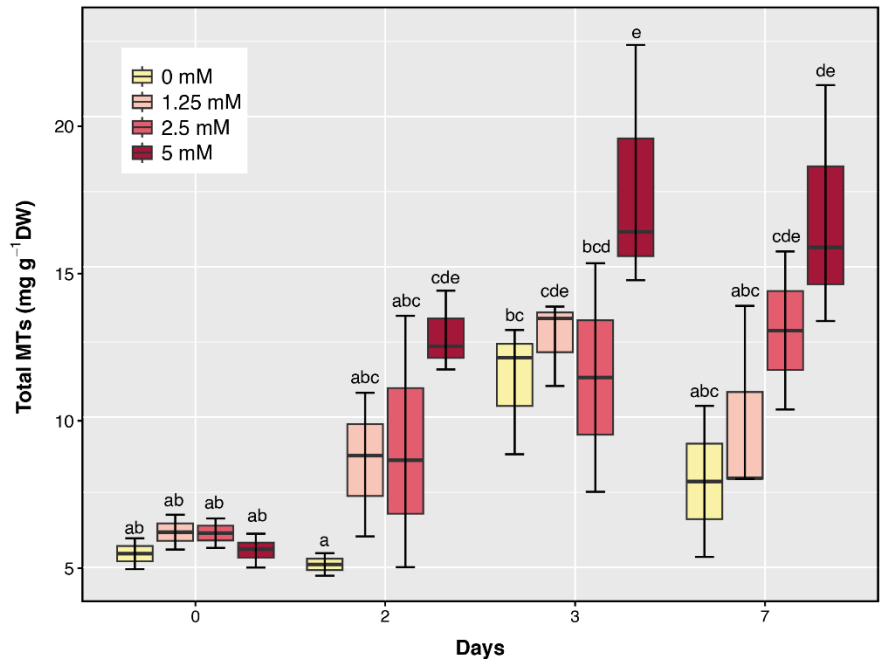

Figure 2. 2. Total foliar MT content of plants treated with 0 (yellow), 1.25 (pink), 2.5 (red) and 5 (dark red) mM exogenous MT spray. Data from experiment three are included. ANOVA results with post hoc test (the significant difference is reported by letters) for the impact of exogenous MTs between treatments and days are reported.

Analysing foliar MT composition revealed 24 compounds, including all of the exogenous MTs applied in the foliar spray, and an additional 15 unidentified MTs within the range of retention times as the standard compounds in the calibration curve. The relative composition of foliar MTs remained nearly constant throughout the experiment. Supplementary Dataset S1 gives a complete list of compounds and their relative contributions and Supplementary Figure S2.1 shows changes in composition over time for each treatment. α-terpinene and MT11, an unidentified compound, were the most abundant in all treatments, each comprising nearly 20% of the total. Both 3-carene and terpinolene each contributed just over 15%. Following application of

exogenous MTs, some compounds that were not present in the leaves prior to the start of spraying were also produced, and the proportion of some endogenous MTs were slightly reduced (by up to 4%, Supplementary Dataset S1 and Supplementary Fig. S2.1). Therefore, exogenous MT not only increased leaf content of the applied MTs but also promoted the production of some endogenous MTs and inhibited the production of others.

2.3.3 Exogenous MTs mitigate oxidative response to water deficit

Oxidative stress, measured by foliar H_2O_2 content, increased linearly across all treatments as Ψ_{leaf} declined (Fig. 2.3A), but exogenous MTs attenuated the effect. In control (0 mM MT) plants, H_2O_2 content increased more than 6-fold at Ψ_{leaf}<-1.75 MPa. This was more than twice the final level of H_2O_2 in plants treated with 1.25 and 2.5 mM MT, which had the lowest H_2O_2 accumulation rate. Interestingly, plants treated with the highest MT concentration (5 mM), accumulated H_2O_2 at a rate intermediate between control and the lower treatment concentrations (1.25 and 2.5 mM), with a 4.7-fold increase. This higher MT concentration seemed less effective in mitigating oxidative stress.

Oxidative damage, measured as foliar MDA content, also increased linearly as Ψ_{leaf} declined (Fig. 2.3B), with MT concentration significantly ($p<0.001$) affecting MDA concentration. Again, control plants showed the greatest MDA accumulation (2.2-fold), while plants treated with 1.25 mM MT had the lowest levels (1.8-fold), with higher MT concentrations inducing an intermediate response. Oxidative damage (foliar MDA content) increased linearly with oxidative stress (foliar H_2O_2 content) similarly across all MT treatments (Fig. 2.3C). Thus, exogenous application of MTs appears not to interfere with ROS-induced lipid peroxidation, but rather mitigates oxidative damage by limiting H_2O_2 accumulation.

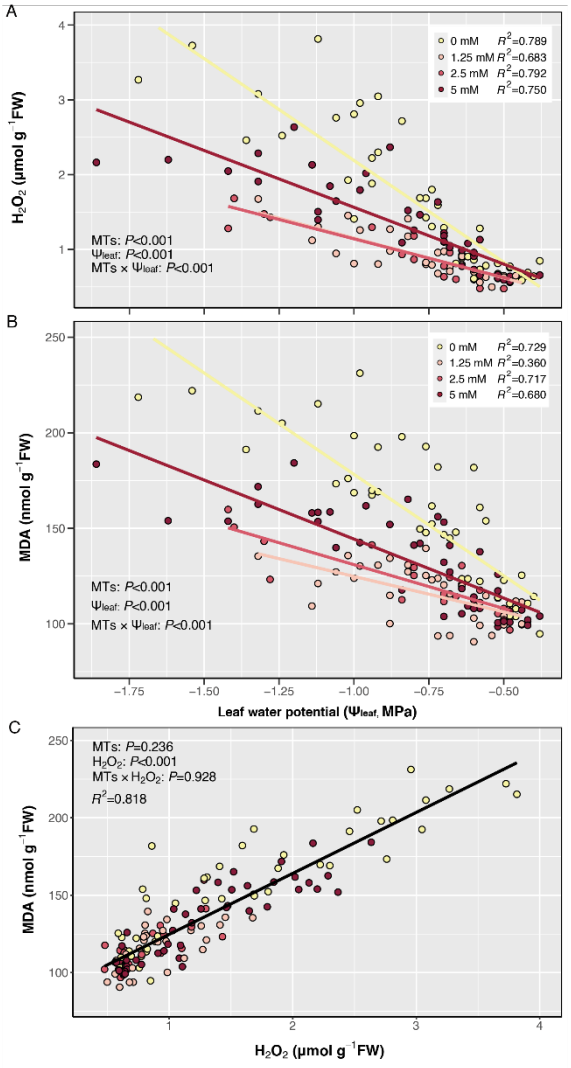

Figure 2. 4 Relationships between foliar H_2O_2 (a) and MDA content (b) and leaf water potential (Ψ_{leaf}, MPa), and between foliar H_2O_2 and MDA content (c) of plants treated with 0 (yellow), 1.25 (pink), 2.5 (red) and 5 (dark red) mM exogenous MT spray. Relationships are described by linear regression lines. Data from all three experiments are included. ANCOVA results (*P* Values reported) for the impact of exogenous MTs, H_2O_2 and leaf water potential (Ψ_{leaf}) and their interaction (MTs*Ψ_{leaf} / H_2O_2) are presented.

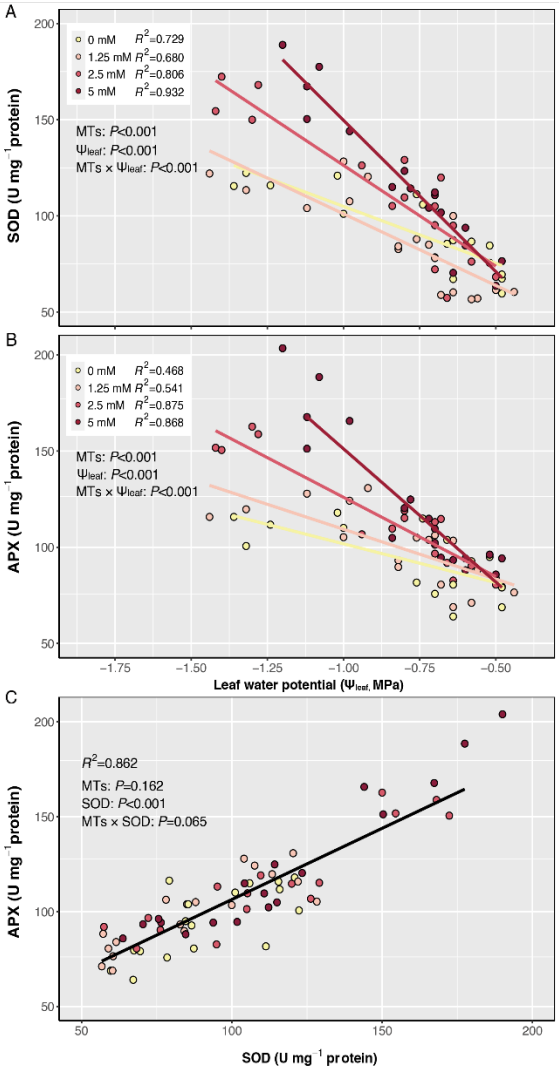

Figure 2. 3 Relationships between foliar SOD (a) and APX activity (b) and leaf water potential, and between foliar SOD and APX activity (c) of plants treated with 0 (yellow), 1.25 (pink), 2.5 (red) and 5 (dark red) mM exogenous MT spray. Relationships are described by linear regression lines. Data are only available from Experiment 3. ANCOVA results (*P* Values reported) for the impact of exogenous MTs and SOD with leaf water potential (Ψ_{leaf}) and their interactions (MTs* Ψ_{leaf} /SOD) are presented.

2.3.4 High exogenous MTs concentrations induce antioxidant enzyme activity

Activities of SOD and APX, the key enzymes involved in forming H_2O_2 from primary ROS and reducing H_2O_2 to H_2O respectively, increased linearly in all treatments as Ψ_{leaf} declined and H_2O_2 increased (Fig. 2.4A, B). At a given Ψ_{leaf}, high MT concentrations promoted SOD and APX activities as indicated by significant interactions ($p<0.001$ for MTs*Ψ_{leaf}). Plants treated with the lowest concentration (i.e. 1.25 mM) showed a similar response to control plants. Therefore, low concentrations of exogenous MTs did not affect enzymatic antioxidants, but high concentrations substantially increased antioxidant enzyme activities as leaf water status declined. All MT treatments showed a similar linear correlation (Fig. 2.4C, $p>0.05$) between SOD and APX activities, indicating that exogenous MTs do not appear to affect the detoxification of ROS to H_2O.

2.3.5 Exogenous MTs do not affect PSII efficiency and gas exchange responses

Leaf water deficit significantly decreased the estimated maximum efficiency (*Fv'/Fm'*, $p<0.001$) and operating efficiency (ΦPSII, $p = 0.005$) of PSII photochemistry in light-adapted tomato leaves, with no effect of exogenous MT treatments (MTs* Ψ_{leaf} $p = 0.68$ and 0.82, Fig. 2.5A&B). Neither water deficit nor exogenous MTs affected PSII efficiency factor (Fig. 2.5C), suggesting that the photochemistry of PSII remains unaffected by water deficit conditions.

Leaf gas exchange (stomatal conductance, g_{sw} and net photosynthesis rate, A_{net}) decreased as Ψ_{leaf} decreased across all treatments (Fig. 2.6A, B), with no apparent effect of MT applications. Likewise, exogenous MT treatments did not affect the relationship between g_{sw} and A_{net} ($p = 0.18$, Fig. 2.6C), with the slope of this line indicating the intrinsic water use efficiency.

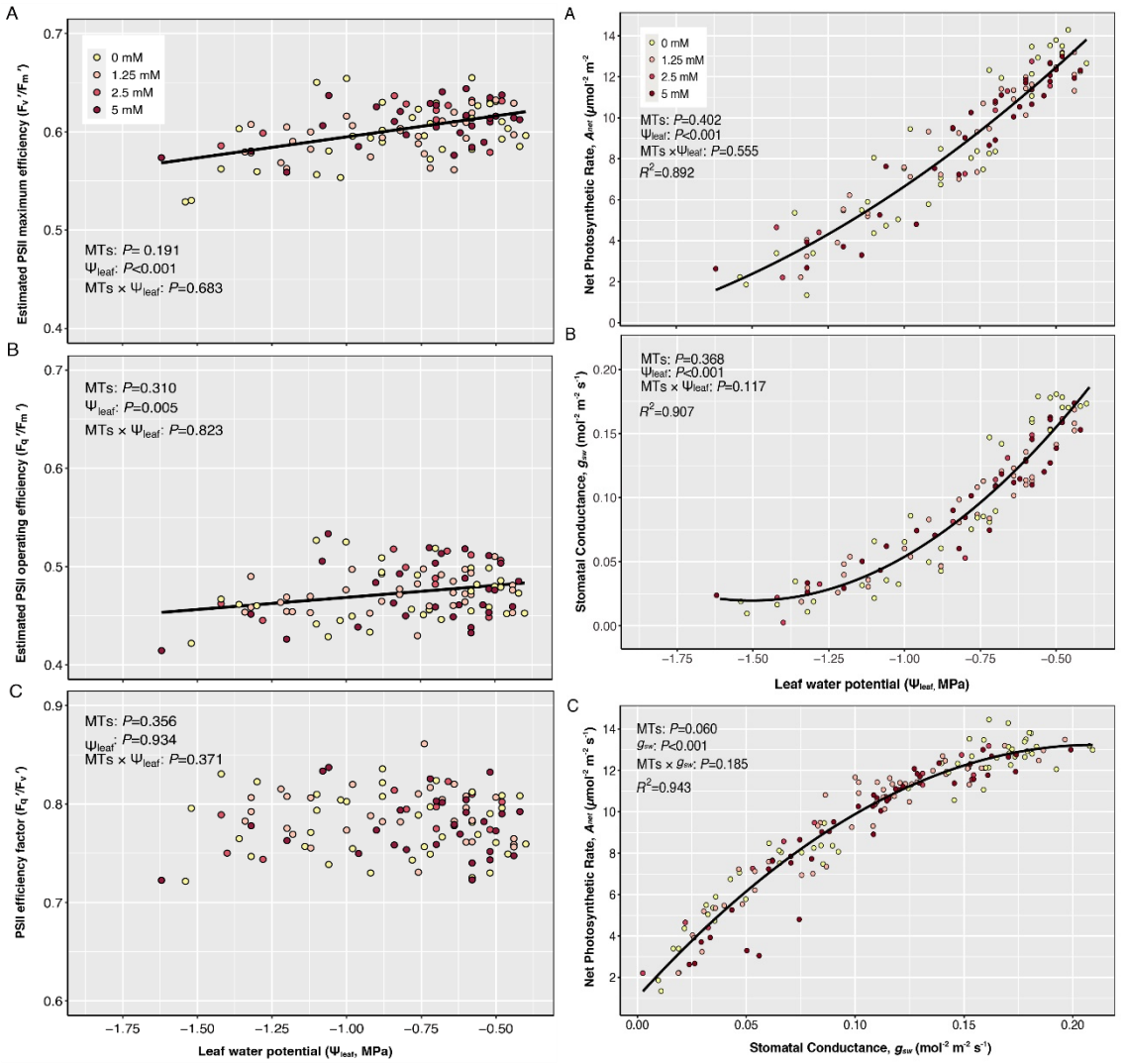

Figure 2. 5 Estimated PSII operating efficiency (a), maximum efficiency (b) and efficiency factor (c) of plants under water deficit with 0 (yellow), 1.25 (pink), 2.5 (red) and 5 (dark red) mM exogenous MT spray. Data from all three experiments are included. ANCOVA results (*P* Values reported) for the impact of exogenous MTs and leaf water potential (Ψ_{leaf}) on PSII with interactions are presented.

Figure 2. 6 Relationships between (a) stomatal conductance (g_{sw}) and (b) net photosynthesis (A_{net}) and leaf water potential and (c) between stomatal conductance (g_{sw}) and net photosynthesis in plants exposed to 0 (yellow), 1.25 (pink), 2.5 (red) and 5 (dark red) mM exogenous MT spray. Data from all three experiments are included. ANCOVA results (*P* Values reported) for the impact of exogenous MTs with leaf water potential (Ψ_{leaf}) and their interactions (MTs* Ψ_{leaf} / g_{sw}) are presented.

2.4 Discussion

Foliar applications of a blend of exogenous MTs, similar in composition to those produced endogenously by tomato (Zhou *et al.*, 2022), increased total foliar MT content (Fig. 2.2) and decreased foliar H_2O_2 and MDA accumulation as the soil dried (Fig. 2.3). Although these MTs enhanced foliar enzymatic antioxidant capacity similarly to isoprene fumigation (Sharkey *et al.*, 2007b), they had no effect on Photosystem II efficiency or net photosynthesis of light adapted plants (Fig. 2.5&6). Despite stimulating enzymatic antioxidant defences (SOD and APX activities – Fig. 2.4), higher concentrations of exogenous MTs induced greater foliar oxidative stress than lower concentrations (Fig. 2.3), although less than control plants, suggesting a threshold MT for maximum protection against oxidative stress. To our knowledge, this is the first time that exogenous MTs have been shown to mitigate drought-induced oxidative stress.

Leaf water status declined with soil water content (Fig. 2.1) as in other studies that fully withheld irrigation from tomato (Živanović *et al.*, 2021), with reduced water transport from roots to leaves (Osakabe *et al.*, 2014) causing Ψ_{leaf} to decline (Lambers *et al.*, 2008). Exogenous MT applications did not affect this relationship. While partial stomatal closure acts to maintain Ψ_{leaf}, exogenous MTs didn't affect stomatal responses to leaf water deficit (Fig. 2.6B), even though increased MT concentrations have been correlated with stomatal closure in other species (Rai *et al.*, 2003; Sancho-Knapik *et al.*, 2017). Although plants exposed to drying soil received an irrigation volume equivalent to 25% of well-watered plant evapotranspiration, this was insufficient to maintain leaf water status (Ψ_{leaf} decreased by 0.23 MPa day^{-1} on average). In contrast, tomato plants grown under similar environmental conditions but receiving 50% ET maintained a Ψ_{leaf} that averaged only 0.1 MPa lower than well-watered plants (Dodd, 2007). While many studies have investigated non-hydraulic signalling causing stomatal closure in tomato (e.g. Dodd, 2007; de Ollas *et al*., 2018), maintenance of Ψ_{leaf} as the soil dries, e.g. by growing plants in large soil volumes (Zhang and Davies, 1989), is most likely to discriminate chemical mechanisms regulating stomatal responses. Although exogenous MTs did not affect stomatal closure as the soil dried, this does not exclude the possibility that endogenous MTs-related hormone interactions (e.g. MEP-ABA biosynthesis) affect stomatal regulation (Barta and Loreto, 2006). Future studies should measure and genetically manipulate endogenous MT production to investigate whether MTs affect stomatal behaviour in drying soil.

2.4.1 Leaves absorb exogenous MTs, which mediate endogenous MT content

Leaf water deficit increased foliar MT content in all plants, suggesting endogenous MTs may mitigate oxidative stress and damage in tomato. However, average total foliar MT content in the exogenous MT treatment remained consistently higher than that of the control plants which received no MTs in the foliar spray (Fig. 2.2), suggesting significant uptake of exogenous MTs and accumulation in the leaves. Likewise, fumigation with a different mix of exogenous volatile MTs increased foliar MT content up to five-fold (Delfine *et al.*, 2000) compared with 2.5-fold in our study, with these differences likely arising from the physiochemical properties of gaseous and aqueous states of the different MTs. These acquired MTs can be stored and even translocated within plant leaves, depending on the concentration and duration of MT application, implying that foliar uptake of MTs is a continuous process that is influenced by the concentrations used in spray.

The primary changes in foliar MT concentrations occurred in 3-carene, α-terpinene, terpinolene, and an unidentified compound designated as MT11 (Supplementary Dataset S1). These not only increased 2 to 3-fold in plants treated with exogenous MT treatments, but also in the untreated (control) plants in response to water deficit. Interestingly, other compounds such as MT1-4 and 12-15, which weren't present in the exogenous MT spray or the well-watered plants on Day 0, were also detected in all sprayed leaves during water deficit treatment. This suggests that oxidative stress induced by water deficit stimulates the biosynthesis and metabolism of certain endogenous MTs, and that exogenous MTs mediate this process. Other components of the foliar spray (e.g. p-cymene), tended to accumulate during the experiment, but to a much lesser extent. These observations support previous findings that both abiotic stresses (e.g. temperature and drought) and exogenous sources (Loreto *et al.*, 1998c; Delfine *et al.*, 2000; Nogués *et al.*, 2015a) change foliar MT concentrations and composition, but the magnitude of changes varies between compounds.

The storage and emission of endogenous MTs and uptake of exogenous MTs by plant leaves vary dramatically between compounds, dependent on the physiochemical characteristics of the individual compounds (Niinemets *et al.*, 2004; Copolovici *et al.*, 2005) and rate of direct uptake through the cuticle (Harley, 2013). For instance, α-terpinene and terpinolene exhibit greater solubility and are more conducive to

intercellular accumulation than α-pinene and limonene, which are more volatile (Copolovici and Niinemets, 2005). Additionally, drought conditions directly (e.g. photosynthetic limitation) or indirectly (e.g. biosynthetic regulation) influence endogenous MT concentrations, which in turn affect leaf uptake driven by concentration gradients (Noe *et al.*, 2006; Noe *et al.*, 2008). However, no information is available about the absolute uptake and consumption of exogenous MTs. For example, while α-terpinene and terpinolene increased in sampled leaves, the foliar content of several compounds, such as α-pinene and α-phellandrene did not appear to change throughout the experiment. Whether this results from differential uptake, the loss of the more volatile MTs by rapid emission or use of the more reactive MTs for direct quenching of ozone, ROS or free hydroxyl radicals is not clear. Further studies are required to determine the extent of uptake for specific MT components and their subsequent impact on biochemical responses, to understand how MTs acquired by the leaves are involved in drought responses.

2.4.2 Exogenous MTs prevent H_2O_2-mediated lipid peroxidation by decreasing H_2O_2 accumulation

Leaf water deficit results in oxidative stress and enhances production of cellular ROS that link signalling pathways and defence mechanisms using H_2O_2 as a secondary messenger (e.g. Cruz de Carvalho, 2008a; Das and Roychoudhury, 2014). As leaf water status decreased, lipid peroxidation (MDA content) was linearly correlated with ROS accumulation (H_2O_2 content), as previously observed (Hasanuzzaman *et al.*, 2020a; Liang *et al.*, 2020), with exogenous MTs not affecting this relationship. Nevertheless, applying exogenous MTs significantly decreased foliar H_2O_2 and hence MDA production under drought stress. Although terpenes can decrease damage from oxidative stress induced by various abiotic stresses (Vickers *et al.*, 2009b; Nogués *et al.*, 2015a; Pollastri *et al.*, 2021), this is the first report that they can mitigate oxidative stress and damage in drought-exposed plants. However, foliar MT treatments at lower concentrations appeared more effective than the higher (5 mM) treatment. Possibly higher concentrations of some terpene compounds perturb the lipid fraction and disrupt protein properties of membranes, due to their low reactivity and high lipophilicity, thus causing lipid peroxidation and solute leakage, as observed with terpinolene (Singh *et al.*, 2009; Agus, 2021). Furthermore, some MTs such as α-pinene can induce ROS accumulation at concentrations >2.5 mM (Singh *et al.*, 2006).

A further question is how exactly MTs act to reduce oxidative stress and damage, with our investigations limited to lipid damage indicated by foliar H_2O_2 and MDA content. Other processes may also lead to photooxidative damage. For example, lipid peroxidation mediated by singlet oxygen ($^1O_2$), which is formed in the energy transfer between excited chlorophyll and O_2 when intercellular CO_2 concentration is decreased by stomatal closure (as in Fig. 2.6) under drought conditions (Cruz de Carvalho, 2008), differs from hydroxide-mediated lipid peroxidation (Triantaphylidès *et al.*, 2008). Although terpenes may stabilise the lipid structure of organelle membranes such as thylakoid membranes (Loreto *et al.*, 2001b), the linear relationship between H_2O_2 and MDA observed across all treatments (Fig. 2.3C) suggested that exogenous MTs did not stabilise lipid structures under oxidative stress. Instead, their protective effects were simply conferred by decreasing accumulation of H_2O_2 under water deficit conditions. The specific terpenes that provide oxidative protection under drought stress may do so by acting as: 1) antioxidants which directly scavenge free radicals and superoxide and/or 2) messengers and indirect antioxidants which enhance signalling pathways and thence both enzymatic and non-enzymatic antioxidant processes (Zuo *et al.*, 2019; Pollastri *et al.*, 2021).

The antioxidative capacity of terpenes generally depends on their specific biochemical properties, in particular their reducing capacity (Graßmann, 2005). For example, the *in vitro* reducing power of phellandrene is approximately twice that of limonene (Lado *et al.*, 2004), while the *in vitro* hydroxyl radical reaction rate, a proxy of reducing power, of myrcene is nearly quadruple that of α-pinene (Atkinson and Arey, 2003; Atkinson *et al.*, 2006), suggesting greater efficacy in decreasing oxidative stress *in vivo*. However, different membrane permeability and uptake, which are determined by the physiochemical properties of MTs and thus their intercellular concentrations, may also result in differences in antioxidative capacity. Despite showing similar radical reaction rates, fumigation by α-pinene moderately restored the heat tolerance of oak leaves in which fosmidomycin had suspended MT biosynthesis, whereas α-terpineol did not. Copolovici *et al.* (2005) ascribe this to the higher volatility of α-pinene, enabling greater uptake. Since the specific antioxidant effects of different terpenes are so variable, we applied a mixture of 9 compounds and cannot confirm whether the observed protective effects are universal or the result of certain individual MTs.

2.4.3 Exogenous MT concentrations differentially affect antioxidative mechanisms

Under oxidative stress, the increased accumulation of ROS stimulates antioxidant processes (such as the SOD and APX enzymes) that are essential to maintain oxidative homeostasis and optimise cell functions and activities (Cruz de Carvalho, 2008). Soil drying increased SOD and APX enzyme activity as H_2O_2 accumulated in all treatments (Fig. 2.3), but exogenous MTs showed dose-dependent effects. Although the 1.25 mM treatment had no detectable effect on the foliar total activity of these key enzymes, higher concentrations (2.5 mM and 5 mM) promoted SOD and APX activity. Applying MTs at low concentration (1.25 mM) may diminish enzyme antioxidant effects by directly acting as an antioxidant or synergistically working with other antioxidants, thereby inhibiting or delaying the activation of endogenous antioxidant defences such as SOD and APX mediated activities.

Nevertheless, the oxidative status was not balanced by promoted enzymatic antioxidative activity. Indeed, higher MT treatments (2.5 mM and 5 mM) seemed to impose oxidative stress via unknown processes. This resulted in higher foliar H_2O_2 and MDA content than in the 1.25 mM treatments, although still lower than the control. In turn, this up-regulated SOD and APX antioxidant enzyme activities to a greater extent in 2.5 mM and 5 mM treatments. While up-regulating SOD converted highly toxic superoxide radicals to the less toxic H_2O_2 more rapidly, a similar up-regulation of APX further detoxified excess H_2O_2 to water at a similar rate (Fig. 2.4C). This water-water cycle which produces H_2O_2 from superoxide in the chloroplast, also provides an alternative pool for electrons from the photosystem, which are required for O_2 photoreduction and ascorbate regeneration, thereby dissipating excess energy (photons) when photosynthesis is limited (Asada, 1999; Miyake, 2010). The Mehler-peroxidase reaction may account for up to 29% of photosynthetic electron flow to avoid over-reduction (Biehler and Fock, 1996). Therefore, higher MT concentrations may trigger enzymatic antioxidant defences to provide an additional energy dissipation process that works together with their own reducing power, thereby mitigating damage compared with the untreated (control) plants.

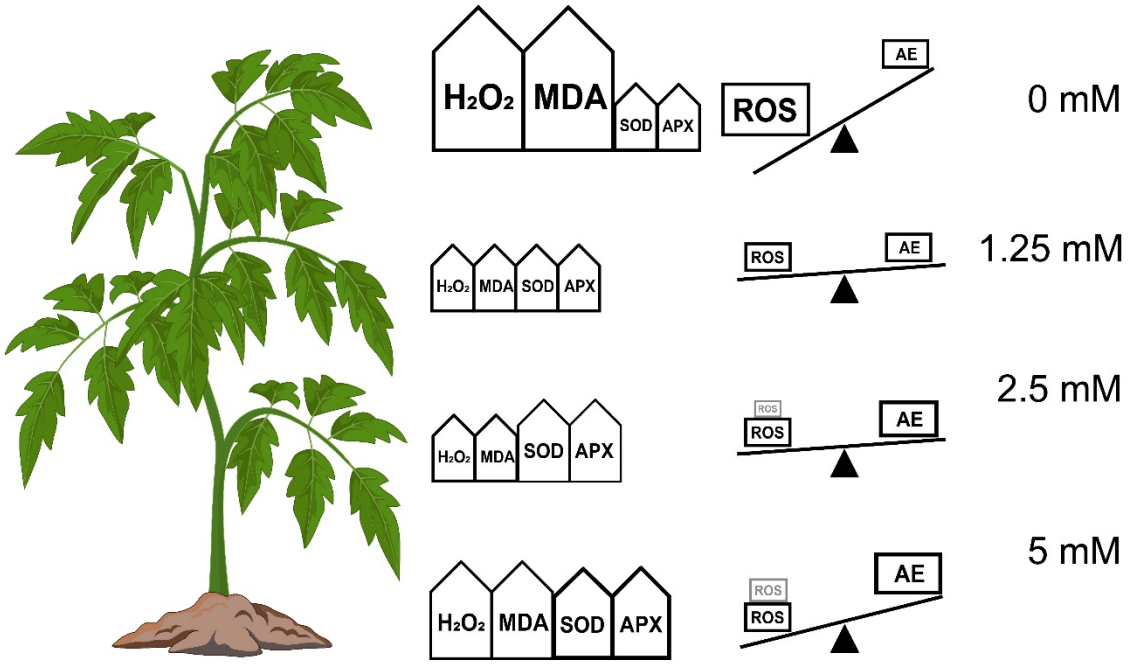

Figure 2. 7 Illustrative sketch of exogenous MTs impact on foliar H_2O_2 (ROS, black frame), MDA content, and SOD and APX activities (antioxidative enzymes, AE). Gray ROS boxes indicate possible toxic effects of exogenous MTs. The rightmost column shows the effect on oxidative status and homeostasis. Based on concepts from Monaghan et al., (2009).

Fig. 2.7 illustrates relationships between ROS (i.e. H_2O_2), antioxidant enzymes (AE) and oxidative status. Under non-stressed conditions, complex antioxidative defence pathways regulate ROS levels, preventing oxidative stress and damage (e.g. lipid peroxidation) to the plant and maintaining oxidative homeostasis, which results from the synergistic cooperation of antioxidant systems (Monaghan *et al.*, 2009). In contrast, leaf water deficit stimulates ROS production that exceeds the capacity of these systems including endogenous terpenoids, leading to oxidative stress and damage as evidenced here by the increased foliar H_2O_2 and MDA content of control plants. Applying 2.5 and 5 mM MT treatments upregulated AE activity, thereby alleviating oxidative stress and improving the redox state. Nevertheless, the oxidative status of plants in 2.5 and 5 mM treatments were not balanced suggesting alternative oxidative stress sources (indicated in grey in Fig. 2.7), possibly directly imposed by exogenous MTs. Notably, in the 1.25 mM treatment, ROS production and oxidative stress reached a near-equilibrium state despite no measurable change in AE activity, implying that exogenous MTs and/or other antioxidant pathways contribute to oxidative homeostasis.

The antioxidative mechanisms in plant are complicated. Although this study measured only basic oxidative status and fundamental enzymatic antioxidative processes, our findings clearly show that MTs have an important role to play in mitigating the effects of drought stress. However, much remains to be done to fully elucidate the mechanisms involved. For example, non-photochemical quenching (NPQ) via the xanthophyll cycle (Cousins *et al.*, 2002) provides another primary chloroplastic energy dissipation pathway to prevent photoreduction when drought suppresses photosynthesis rate. This works synergistically with antioxidative enzymes, providing efficient photoprotection to plants (Beis and Patakas, 2012; Pintó-Marijuan and Munné-Bosch, 2014). It is worth noting that photorespiration also contributes to H_2O_2 production and tends to be more significant when drought stress decreases intercellular CO_2 concentrations, interfering with redox balance and antioxidant status (Noctor *et al.*, 2002). Another detoxification mechanism of ROS involves non-enzymatic antioxidants, especially the scavenging of $^1O_2$ by carotenoids (e.g. β-carotene). This is closely linked to both the chloroplastic photooxidative protection (Ramel *et al.*, 2012) and MEP synthesis pathway (Rodríguez-Concepción, 2010), and has been associated previously with both endogenous terpene production and exogenous MT application (Brilli *et al.*, 2022). Applying increased concentrations of terpinene and β-pinene, which are included in our foliar sprays, to heat-stressed plants increased endogenous carotenoid concentrations (Tian *et al.*, 2020). Although we focus on the key antioxidative enzymes SOD and APX (Fig. 2.4), the decreased oxidative stress and damage to lipids observed under the range of exogenous MT treatment concentrations applied here likely results from a range of oxidant-antioxidant reactions and energy dissipation pathways, which require further investigation.

2.4.4 Monoterpene enhancement of antioxidative protection doesn't affect leaf gas exchange

Both natural isoprene and MT emitters, and plants fumigated with these terpenes, showed higher photosynthesis and PSII efficiency under oxidative stress (Delfine *et al.*, 2000; Vickers *et al.*, 2009b). Exogenous MTs helped to maintain chlorophyll fluorescence, photosynthetic efficiency and net photosynthesis under oxidative stress (Loreto *et al.*, 1998c; Loreto *et al.*, 2004). Furthermore, heat-induced endogenous MTs have been associated with photosynthetic protection (Zuo *et al.*, 2017). While these studies suggest terpenes may protect the photosynthetic apparatus, foliar limonene

application maintained chlorophyll fluorescence in carrot leaves at moderately elevated temperature, but did not sustain photosynthesis (Ibrahim *et al.*, 2004). Similarly, although exogenous MTs improved antioxidative capacity and mitigated drought-induced oxidative damage of tomato, they did not ameliorate declines in photosynthesis or maximum and operating photosynthetic efficiencies (Fig. 2.5&6). Drought-induced decreases in stomatal conductance and intercellular CO_2 concentration likely constrain photosynthesis independent of any non-stomatal responses. Whereas high exogenous MT (>2.5 mM) applications inhibited leaf gas exchange (Ibrahim *et al.*, 2004; Singh *et al.*, 2006; Singh *et al.*, 2009), these concentrations did not affect tomato net photosynthesis (Fig. 2.6), probably because the overall oxidative status and damage were less than the control treatment.

It also appears that stomatal responses to leaf water deficit determined photosynthetic limitation as the soil dried. Nevertheless, PSII operating efficiency declined by ~12% as Ψ_{leaf} declined (Fig. 2.5B). Although statistically significant, this was much lower than the decreases of 35% - 45% in PSII efficiency when water was completely withheld from both tomato and tobacco plants (Mishra *et al.*, 2012; Ryan *et al.*, 2014). Less intense water deficit ($\Psi_{leaf} \geq$-1.0 MPa) does not lead to long-term damage to photosynthetic apparatus and less photochemical quenching, with plants preventing photoinhibition and oxidative damage by upregulating NPQ (Cousins *et al.*, 2002; Beis and Patakas, 2012). Since the antioxidative effect of exogenous MTs did not appear to affect leaf PSII efficiency factor (Fig. 2.5C), it is likely that excess (photon) energy was not dissipated through photochemical quenching. Measuring chlorophyll fluorescence of dark-adapted plants is important in future studies to consider potential electron flow and energy dissipation routes that may affect the production of oxidative products and cellular homogenous, as well as photosynthetic electron flow.

Plant emissions of BVOCs are thought to reflect the important role that these compounds play in plant resilience and tolerance to not only biotic but also abiotic stresses (Brilli *et al.*, 2019). Our findings support this, and suggest that while exogenous MTs, such as limonene, are already used to increase plant resistance to pathogens (Simas *et al.*, 2017), they may have wider agricultural applications. The direct antioxidant properties of MTs and their possible interaction with other plant antioxidant mechanisms, shown here, suggest that applying MTs could mitigate damage from a

wide range of environmental stresses. However, the specific benefits of MTs, both individually and in combination, need further investigation.

2.5 Conclusion

In conclusion, exogenously applied MTs were taken up by tomato leaves, increasing foliar antioxidative capacity as leaf water status declined. Specifically, exogenous MTs decreased oxidative stress (i.e. H_2O_2) thereby mitigating oxidative damage to lipid membranes. Nevertheless, leaf gas exchange and Photosystem II efficiencies declined similarly in all plants as the soil dried, regardless of the concentration of MTs applied. Overall oxidative status depended on the MT concentration applied: 1.25 mM provided the best redox state (i.e. the least H_2O_2 accumulation and lipid peroxidation) likely because MTs directly scavenged ROS and synergistically worked with non-enzymatic antioxidants while higher MT concentrations (2.5 mM and 5 mM) further increased foliar H_2O_2 and MDA concentrations but also upregulated enzyme (SOD and APX) activity, resulting in better oxidative status than untreated (control) plants. This suggests high doses of MTs may have been phytotoxic. Such dose-dependent effects suggest different response mechanisms with MTs also likely interacting with both antioxidants and energy dissipation pathways. These complex relationships and interactions between these various mechanisms require further investigation.

3 Monoterpene production in transgenic tobacco maintains high leaf water potential under drought but reduces biomass

Abstract

This study investigates the role of enhanced monoterpene biosynthesis in three transgenic tobacco (*Nicotiana tabacum*) lines, focusing on their morphology, physiology and their response to drought stress. By genetically engineering the methylerythritol phosphate (MEP) pathway by introducing geranyl diphosphate synthase (*GPS.SSU*) and specific monoterpene synthase genes, production of specific monoterpenes, including (–)-limonene and (–)-α/β-pinene increased. Genetic modification had minimal impact on stomatal development and behaviour, as well as net photosynthesis. Under water-deficit conditions, transgenic lines exhibited significantly increased foliar monoterpene emissions and maintained higher leaf water potential than wild-type (WT) plants when drought was applied during stem elongation. However, overproducing monoterpenes suppressed biomass accumulation, most obviously in PG11 and least in LG1. These findings highlight the potential of terpene biosynthesis pathways as targets for crop improvement in response to environmental stress, with LG12 the most desirable candidate to better understand the role of endogenous monoterpene in plant drought stress responses.

3.1 Introduction

Terpenes are the largest and the most structurally diverse class of plant secondary metabolites (Pichersky and Raguso, 2018; Bergman and Dudareva, 2024). These compounds are essential for a wide range of biological and ecological functions, including growth regulation and defence against biotic and abiotic stressors (Gershenzon and Dudareva, 2007; Dani and Loreto, 2022). Monoterpenes ($C_{10}H_{16}$) form the second-largest class among naturally occurring terpenes and have garnered attention due to their significant roles in tropospheric chemistry (Laothawornkitkul *et al.*, 2009; Peñuelas and Staudt, 2010) and their potential to regulate plant responses to environmental conditions such as drought, high temperatures, and oxidative stress (Peñuelas and Llusià, 2002; Vickers *et al.*, 2009a; Ryan *et al.*, 2014). Emissions of terpenes from vegetation are estimated to account for approximately 0.1-2% of photosynthetically assimilated carbon, with higher emissions (5-20%) observed in species like poplar under environmental stress (Goldstein *et al.*, 1998; Possell and Loreto, 2013; Seco *et al.*, 2015). Thus, it is reasonable to hypothesise that these volatile compounds contribute beneficially to plant biochemistry and ecosystem functioning (Sharkey et al., 2007).

Terpenoid biosynthesis in plants occurs via two independent metabolic pathways: the cytosolic mevalonate (MVA) pathway and the plastidial methylerythritol phosphate (MEP) pathway (Obiol-Pardo *et al.*, 2011; Koley *et al.*, 2020). The MEP pathway, primarily responsible for synthesizing isoprene, monoterpenes, diterpenes, hemiterpenes, and carotenoids, begins with the condensation of pyruvate and glyceraldehyde-3-phosphate (G3P) into 1-deoxy-D-xylulose-5-phosphate (DXP). This precursor undergoes several reactions to produce isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the building blocks for terpene synthesis (Lichtenthaler, 1999; Dudareva *et al.*, 2005). Geranyl diphosphate synthase (GPS), a key enzyme in this process, catalyses the condensation of IPP and DMAPP to form geranyl diphosphate (GPP), the precursor for monoterpenes (Wolff *et al.*, 2003). The structural diversity of monoterpene synthases (*monoTPSs*) enables plants to synthesize a wide array of monoterpene compounds from GPP. This diversity of *monoTPSs* contributes to the remarkable variability of monoterpene emissions observed in plants, with over 1000 distinct compounds identified (Degenhardt *et al.*, 2009; Bergman and Dudareva, 2024). For example, Mentha species (e.g., *Mentha piperita*) are known for high levels of (–)-limonene, menthol, and menthone, which are synthesised by species-

specific *monoTPSs* (Koley *et al.*, 2020). In contrast, coniferous species such as *Pinus halepensis* (Aleppo pine) and *Picea abies* (Norway spruce) produce monoterpenes like α-pinene and β-pinene, which serve as defence compounds against herbivores and pathogens (Trowbridge *et al.*, 2014; Wu *et al.*, 2015; Alicandri *et al.*, 2020).

Not all plants have the capacity to produce monoterpenes. The ability to synthesize these compounds is largely species-dependent and associated with specific evolutionary adaptations. Many plants lack the necessary biosynthetic machinery for monoterpene production due to the absence of key enzymes, such as *monoTPSs*. For instance, most monocots, including grasses and cereals, produce few, if any, monoterpenes (Geron *et al.*, 2000; Sakulyanontvittaya *et al.*, 2008). This absence is often linked to different evolutionary pressures or ecological niches, where other secondary metabolites may fulfil similar protective or communicative roles (Alicandri *et al.*, 2020; Hirose and Satake, 2024). In contrast, plants in the Lamiaceae and Pinaceae families are prolific producers of monoterpenes, which are essential for their survival and interactions within ecological communities (Dudareva *et al.*, 2005; Schmiderer *et al.*, 2023). Previous studies explored the reasons why some plants produce these compounds and the mechanisms of their synthesis.

Factors such as light intensity, temperature, and water availability affect both the biosynthesis rate and the specific profile of monoterpene emissions (Loreto and Schnitzler, 2010; Escobar-Bravo *et al.*, 2023; Bourtsoukidis *et al.*, 2024). The regulation of monoterpene production by light is well-documented, with monoterpene synthase genes being highly expressed in response to increased light intensity. Light regulates the MEP pathway through photosynthesis, providing the necessary energy and precursors for monoterpene biosynthesis (Niinemets *et al.*, 2002c; Sharkey *et al.*, 2007b). Exposure to light (PPFD from 0 to 1000) increase the synthesis of monoterpenes such as menthol and (–)-limonene in most plant species (Niinemets *et al.*, 2002c; Koley *et al.*, 2020). This regulation is closely tied to the chloroplastic localisation of the MEP pathway, where light energy drives the production of intermediates such as 1-deoxy-D-xylulose-5-phosphate (DXP), which are essential for monoterpene biosynthesis (Rodriéguez-Concepcioén *et al.*, 2004; Rodríguez-Concepción, 2006).

Temperature is another critical factor influencing monoterpene production and emission (Bourtsoukidis *et al.*, 2024). Higher temperatures often lead to increased monoterpene

emissions due to enhanced volatilisation and biosynthetic activity (Niinemets *et al.*, 2004; Jardine *et al.*, 2017). For example, in *Pinus sylvestris* (Scots pine) and *Cistus albidus* (rockrose), monoterpene emissions increase several-fold in response to high temperatures and water stress (Nogués *et al.*, 2015a; Byron *et al.*, 2022). Rising temperatures can affect the enzymatic activity of monoTPSs, resulting in elevated biosynthesis of compounds such as α-pinene and β-pinene. High temperature (>40 °C) can suppress the synthase activity (Li and Sharkey, 2013; Trowbridge *et al.*, 2021; Nagalingam *et al.*, 2023). However, the impact of temperature on monoterpene production varies across species and depends on their ecological adaptations. Some species exhibit greater thermal tolerance, enabling them to maintain monoterpene synthesis even under extreme heat (Xu *et al.*, 2022; Bourtsoukidis *et al.*, 2024).

Due to the environmental sensitivity of monoterpene biosynthesis, genetic engineering of the MEP pathway has become a valuable tool to enhance monoterpene production and boost plant stress resilience (Vickers *et al.*, 2011; Rosenkranz and Schnitzler, 2013). By overexpressing key enzymes such as geranyl diphosphate synthase small subunit (*GPS.SSU*) and specific monoterpene synthases, researchers have successfully increased monoterpene production in transgenic plants (Dudareva *et al.*, 2005; Dong *et al.*, 2023). For example, inserting the *Mentha* × *piperita GPS.SSU* gene, along with *Picea abies* and *Pinus sitchensis* monoTPS genes, into tobacco (*Nicotiana tabacum*) increased emissions of (–)-limonene, myrcene, α-pinene, and β-pinene. Monoterpene transformation in tobacco also increased leaf gerbiline concentration and cytokinins, suggesting an upregulated MEP flux and downstream regulation in these genetically modified crops (Yin *et al.*, 2016). The localisation of monoterpene biosynthesis within plant cells further affects their production. While the MEP pathway is plastidial, evidence suggests that some monoTPSs are localised in different cellular compartments, such as the cytosol or endoplasmic reticulum, depending on species and environmental cues (Koley *et al.*, 2020; Zhou and Pichersky, 2020). For instance, in *Lithospermum erythrorhizon* the localisation of GPP synthases in plastids versus the cytosol influenced monoterpene production across different organs (Ueoka *et al.*, 2020). *Nicotiana tabacum* plants engineered with monoterpene synthases targeted to different cellular compartments exhibited varying levels of monoterpene production, depending on enzyme localisation. In plastids, where the MEP pathway operates, limonene production was significantly higher compared to cytosolic localisation and not active in endoplasmic reticulum, emphasising the importance of compartmentalised biosynthesis

(Ohara *et al.*, 2003). Precursors for monoterpene biosynthesis are more readily available from plastidial sources due to their proximity to internal storage pools (Rodríguez-Concepción, 2006), with crosstalk occurring between plastidial and cytosolic sources (Laule *et al.*, 2003).

Collectively, previous research underscores the complex interplay between genetic factors, environmental stimuli, and metabolic pathways in regulating monoterpene production and emission (Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010; Harrison *et al.*, 2013). While the genetic manipulation of the MEP pathway has shown promise in enhancing monoterpene synthesis, these processes are intricately linked to plant growth, development, and environmental context. The changing production of monoterpenes in response to drought, temperature, and light stress suggests that these compounds play a vital role in helping plants adapt to shifting environmental conditions (Possell and Loreto, 2013; Escobar-Bravo *et al.*, 2023).

This study investigates the impact of monoterpene genetic transformation on the growth, physiological responses, and monoterpene emission characteristics of tobacco (*Nicotiana tabacum*) plants, particularly in response to drought stress. This research focuses on key aspects of plant performance, such as morphology, gas exchange, leaf water status, and foliar monoterpene emissions at two growth stages (before and during stem elongation) under two water conditions: well-watered and drought. The study examines three transgenic tobacco lines, each with upregulated monoterpene precursor genes and individually inserted monoterpene synthase genes for the production of specific compounds: (–)-α/β-pinene (PG11), myrcene (MG1), and (–)-limonene (LG12). These transgenic lines are evaluated to understand how enhanced monoterpene biosynthesis influences drought response and physiological performance relative to wild-type plants. This also aims to inform mechanistic studies on the function of monoterpenes.

3.2 Materials and Methods

3.2.1 Plant materials and growth environments

Yin et al. (2016) inserted the *Mentha × piperita geranyl diphosphate synthase small subunit* (*MpGPS.SSU*) (AF182827) gene into 3 different transgenic tobacco (*Nicotiana tabacum* cv. SR1) lines, each of which also contained individual monoterpene synthase

genes, including *Picea abies (−)-limonene synthase* (*PaLimS*), *P. abies myrcene synthase* (*PaMyrS*), and *P. sitchensis (−)-pinene synthase* (*PsPinS*). Both genes were under control of the constitutively expressed *Ubiquitin 10* promoter resulting in increased endogenous monoterpene production and emission. Specifically, the three transgenic lines with enhanced emissions of (−)-limonene (LG12), myrcene (MG1), and (−)-α/β-pinene (PG11) were obtained from the Temasek Life Sciences Laboratory in Singapore.

Wild-type (WT) and transgenic seeds were sown simultaneously and germinated on John Innes NO2 compost in seedling trays (12 cells, each 3.8 × 3.8 × 5 cm; tray size: 17.8 × 14 × 5 cm) covered by transparent domes with humidity control valves. Seed trays were kept in a controlled environment (CE) room illuminated by LED growth lights (Model B150, NS1 spectra, Valoya, Helsinki, Finland) with average bench height PPFD at 500 μmol photons m^{-2} s^{-1}. After four weeks, seedlings were transplanted into 3 L pots (20.5 cm in height, 16 cm at the top, and 12.5 cm at the bottom) filled with 3 kg of premixed sandy substrate (70% garden sand, 30% John Innes No2) and watered to pot capacity. The next day, the pots were transferred into enclosed plant growth chambers as described in Stokes et al. (1993) with improvements. The door and side walls of the chamber were made of 2 mm polycarbonate sheets to enhance the chamber's strength, airtightness, and light transmission (<1.5% difference in the light spectrum). The supporting grid in the chamber was height-adjustable, maintaining a consistent leaf-level light intensity during plant growth at 350 ± 20 μmol photons m^{-2} s^{-1} photosynthetic photon flux density (PPFD). This light intensity was provided by a growth lamp (Powerstar HQI-BT, 600 W/D daylight, Munich, Germany) from 07:00 to 19:00 hours for 12 hours a day. Full light spectrum and chamber specific chamber PPFD distribution and be found in Figure S3.1&2. The temperature and relative humidity inside the chamber were maintained at 22°C:16°C (±2°C) and 40:60% (±5%) during the day and night, respectively. For a comprehensive description of the test chambers, environmental conditions, and leaf-level PPFD, please refer to the supplementary information. Plants were rotated inside the chambers daily and between chambers every two days to prevent the effects of unbalanced environmental conditions. Miracle-Gro® all purpose soluble plant food was applied at 20g per L of water to the substrate every week to ensure the nutritional supply.

3.2.2 Experimental setup

Three factorial experiments were conducted with four lines (LG12, MG1, PG11, and WT) across two water treatments (well-watered and drought) and two growth stages (before elongation and during elongation). Plant pots containing individual plants of the four lines were placed in individual growth chambers. To prevent potential priming effects of volatiles across different lines, chamber doors were opened only for watering and placing measuring instruments. The drought (D) treatment was applied in a separate experiment from the well-watered (WW) treatment due to the limited number of growth chambers available. However, the growth and experimental conditions and setups were identical.

Measurements and harvesting for the before (stem) elongation stage (BE) occurred six weeks after placing seed for germination. The remaining plants were kept in the chambers until the eighth week for measurements during the elongation stage (DE). For the drought experiment, irrigation was stopped in the fifth week, following the same measurement and harvest schedule. This meant that plants experienced one week of drought stress for BE and three weeks of drought for DE. Additionally, four to five spare plants from the two water conditions were kept in the CE room. After DE measurements, the plants under WD conditions were rewatered to pot capacity and all plants were watered daily until the reproductive stage for seed production comparison.

3.2.3 Gas exchange and leaf chlorophyll content

Leaf gas exchange was measured using a LI-6400XT portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA), equipped with an LED light source (model: 6400-02B) and a 2×3 cm leaf cuvette. The system was warmed up, and closely matched the growth chamber environmental conditions: 350 μmol m^{-2} s^{-1} PPFD (10% blue light), 421 μmol mol^{-1} leaf cuvette CO_2 concentration, 40-50% relative humidity. Airflow was 500 μmol s^{-1} for well-watered (WW) conditions and reduced to 300 μmol s^{-1} for water-deficit (WD) conditions to ensure sufficient gas retention time when gas exchange is low under drought stress. To achieve accurate measurements, the LI-6400XT head was placed in the growth chamber for half an hour to equilibrate with the environmental conditions.

Measurements were taken by clipping the leaf cuvette onto the youngest fully developed leaf (the fourth leaf from the stem base before elongation and the sixth leaf from the bottom during elongation). The leaf fully covered the cuvette, and the vein was avoided as much as possible. Instantaneous leaf stomatal conductance (g_{sw}) to water vapor and the net photosynthesis rate (A_{net}) of CO2 assimilation were recorded once ΔCO2 and ΔH2O values stabilized, typically within 1-2 minutes.

3.2.4 Foliar monoterpene emission sampling and analysis

A system blank for foliar monoterpene background was taken after the LI-6400XT warmup. A Carbon Cap™ carbon filtration capsule (Whatman™, Cytiva, Buckinghamshire, UK) was connected to the air inlet on the LI-6400XT console via flexible PTFE tubing to filter any VOCs in the air source. Foliar monoterpene emission was sampled after gas exchange was recorded. Following an additional five-minute stabilization period, a fraction of the air sample from the exhaust below the leaf cuvette was pumped into a stainless-steel sorbent tube (Tenax® TA 35/60 mesh | Carbtrap® 20/40 mesh, 3 1/2 inch long ×1/4 O.D., Markes International Ltd, Llantrisant, UK). This was done via a PTFE T-piece connected to a 20 mm piece of PTFE flexible tubing (Tygon®, 1/4 inch O.D. × 1/8 inch I.D.) with PFA fitting using a Pocket Pump (SKC Ltd., Dorset, UK) at a flow rate of 150 mL/min for ten minutes, yielding a total sample volume of 1.5 L. Tube samples were kept in a 4°C fridge for further GCMS analysis. After the monoterpene sampling, the LI-6400XT was moved to the next randomly selected plant for the same measurements.

For tube sample GCMS analysis, the tubes were initially purged for one minute in the TurboMatrix150 Auto Thermal Desorber (ATD, PerkinElmer Inc., Shelton, CT, USA), followed by a two-stage desorption process with a desorb flow of 80 mL min^{-1} and an outlet split of 40 mL min^{-1}. A hydrogen generator supplied the carrier gas at a constant pressure of 16.5 psi, regulated by the needle valve in the ATD. The heated valve temperature was set to 250°C, and the tube was heated to the same temperature for six minutes. The desorbed compounds were then transferred to a Tenax® TA cold trap, held for one minute, and subsequently injected via a transfer line at 260°C into an AutoSystem XL Gas Chromatographer/TurboMass Gold Mass Spectrometer (GC/MS, PerkinElmer Inc., Shelton, CT, USA) integrated with a Zebron™ ZB-624PLUS capillary column (60 m × 0.25 mm × 1.4 μm, Phenomenex Inc., Macclesfield, Cheshire, UK). The column temperature was ramped from 60°C to 160°C at a rate of 20°C min^{-1}

and held for two minutes. It was then further increased to 280°C at a rate of 35°C min^{-1} and maintained at this temperature for one minute. The transfer line temperature between the GC and MS was set to 220°C, and the ion source of the MS was maintained at 200°C. The electron energy and trap emission (EI) of the ion source were consistently set to 70 eV and 80 eV, respectively, with other parameters being tuned and calibrated before each run. The MS scan range was set to 33-300 m/z for 10 minutes with a one-minute solvent delay.

(−)-limonene, (−)-α/β-pinene, and myrcene were identified based on the mass spectra using TurboMass Software (v.6.1.0, PerkinElmer Inc., Shelton, CT, USA) with the NIST 2020 library. They were quantified by comparing the calibration curve using analytical (-)-limonene, (-)-α-pinene, and myrcene standards (catalog # 62128, 80599, and 64643 Merck Life Science UK Limited, Gillingham, UK). Standard solutions were injected into sorbent tubes in a stream of helium at a flow rate of 100 mL min^{-1} in a calibration solution loading rig (catalog # C-CSLR, Markes International Ltd, Llantrisant, UK) and analyzed alongside the samples in duplicate. The emission rate was calculated based on the leaf area in the LI-6400XT leaf cuvette (6 cm^2) and following Equation 1 (Niinemets et al., 2011):

$$E = \frac{C_{out} - C_{in}}{A} F \quad (1)$$

Where E is the emission rate of monoterpene (pmol m^2 s^{-1}), C_{out} and C_{in} (pmol m^{-3}) are the concentrations of the monoterpene at the air outlet (sampled by sorbent tubes and quantified by GCMS) and air inlet (filtered by carbon filtration capsule, assumed to be zero), respectively. F (m^3 s^{-1}) is the airflow rate into the leaf cuvette, converted according to the volume of gases at STP, and A (m^2) is the leaf area in the cuvette. All samples were corrected based on the system blank in the same measurement.

3.2.5 Morphology, soil and leaf water status

After measuring gas exchange and monoterpene sampling, leaf epidermal impressions were made on the area symmetrical to the central leaf vein (see Fig. 3.1) as described in Matthaeus *et al*. (2020). Briefly, Coltène PRESIDENT light body dental putty (Coltène/Whaledent AG, Alstätten, Switzerland) in the size and thickness of a one-pound coin was pressed onto the upper and lower leaf surfaces of the same spot using a dispenser gun with a mixing tip. Both surfaces were then covered with thick post-it

notes, and pressure was applied evenly on both sides for 5 minutes. The cured putties were removed and placed on a clean lab bench. Quick-drying nail polish was applied on the side that contacted the leaf. After drying, a second coat was applied. Once dried, the double coat was removed and cut into approximately 1×1 cm sections, placed on microscope slides, covered with a cover slip and secured with transparent adhesive tape. The slides were then viewed under an optical microscope mounted with a SPOT CMOS camera (ImSol, Preston, UK). Pictures of the epidermal and guard cells were taken using the imaging software and analysed by ImageJ for stomatal size, density and epidermal cell density. Stomatal index was calculated as the ratio between stomatal and epidermal cell density.

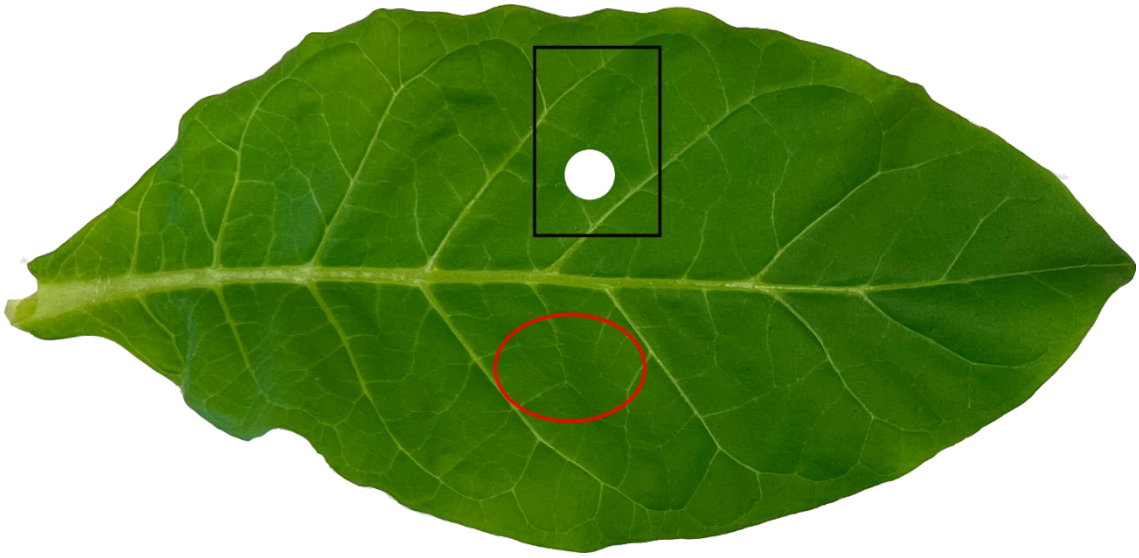

Figure 3. 1 An example of the measured tobacco leaf. The black rectangular (2×3 cm), the circular hole (8 mm) and the red oval are the areas for Li-6400XT measurements, leaf piercing for water potential measurement, and epidermal impression, respectively.

Subsequently, leaf chlorophyll content was measured at the same area used for the LI-6400XT measurement using an MC-100 chlorophyll meter (Apogee Instruments, Inc., Logan, UT, USA). A leaf disc (8 mm diameter) from the same spot was obtained using a sharp cork borer, placed in a clean sample holder, and wrapped in aluminium foil to minimize water loss. The sample holder was then loaded into a C52 psychrometer chamber (Wescor Inc., Logan, UT, USA), located in a stable temperature room alongside a HR-33T dew point microvoltmeter (Wescor Inc., Logan, UT, USA). After a 3-hour incubation at 25°C, the psychrometer chamber was measured using the microvoltmeter, and leaf water potential (Ψ_{leaf}) was calculated using a calibration table

made from standard salt solutions of various concentrations. The plant was then removed from the growth chamber for morphological measurements. Plant height was measured with a ruler from the soil base to the shoot apex, and pot weight, shoot, and leaf biomass were measured with a balance. Plants were then collected and dried in a 50°C oven to determine dry biomass. Soil moisture was measured with a WET-2 sensor (Delta-T Devices Ltd., Cambridge, UK) inserted to a depth of approximately 5 cm from the soil surface.

3.2.6 Data analysis

Statistical analysis and plotting were conducted using R (v. 4.3.1) and R Studio (PBC, Boston, MA, USA). A general linear model (GLM) with univariate ANOVA and post hoc Tukey tests with Bonferroni correction were applied to identify significant ($P< 0.05$) physiological and morphological differences between experimental factors (lines, water treatments and growth stages), as well as their two-way and three-way interactions. Exponential regression lines were fitted to examine the relationship between leaf and soil water status and gas exchange, with the corresponding ANOVA test in the GLM framework.

3.3 Results

3.3.1 Genetic transformation enhanced monoterpene emissions

(−)-α/β-pinene, myrcene and (−)-limonene emissions were not detected from WT plants under well-watered (WW) conditions, but only just detectable under drought (D) conditions (Fig. 3.2). Although pinene emissions were not detected from MG1 plants irrespective of water treatment, drought resulted in detectable limonene emissions from these plants. Genetic transformation with precursor and monoterpenes genes dramatically enhanced leaf-level monoterpene emission of LG12 and PG11 plants under well-watered (WW) conditions, with stable emission rates across different growth stages. In PG11, (−)-α-pinene emission was five-fold higher than (−)-β-pinene emission with little limonene emission, while LG12 showed high limonene emission with barely detectable pinene emission. Drought (WD) doubled and tripled the emission rate of (−)-limonene and pinene in LG12 and PG11 respectively, before elongation. As plants elongated and drought conditions persisted, (−)-limonene emission significantly ($P<$ 0.001) decreased in LG12 compared to those well-watered ones and pinene emissions dropped to the same as WW level. Stimulation of MT emission by drought diminished at later developmental stages.

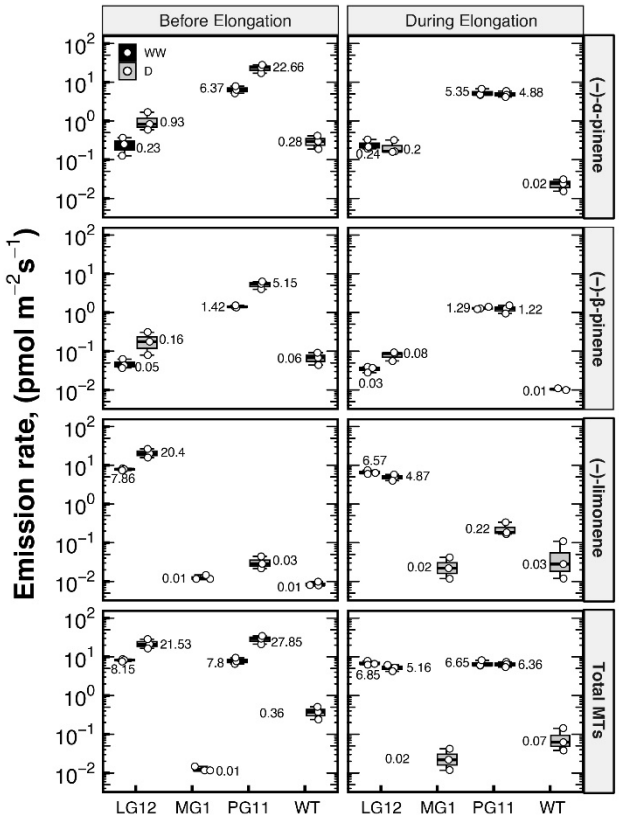

Figure 3. 2 Foliar monoterpene emission rate under well-watered (WW, black bars) and drought (D, grey bars) conditions before (BE) and during (DE) elongation stages. Box plots present the (−)-α-pinene, (−)-β-pinene, (−)-limonene and total monoterpene emission rate of three GM lines and wildtype plants, with mean value under each box. Post hoc Tukey tests results indicate the significant differences between treatment and lines by letters.

3.3.2 Genetic transformation maintained higher leaf water potential under drought

Soil moisture was consistently maintained at just over 30% for all WW plants throughout the experiment. Before elongation, greater soil drying by LG12 and WT plants resulted in approximately 4% lower soil moisture than MG1 and PG11, until all lines reached 6% soil moisture during stem elongation (Fig. 3.3a). Genetic transformation did not affect leaf water potential (Ψ_{leaf}) under WW conditions, although Ψ_{leaf} decreased by over 60% in all lines during stem elongation (Fig. 3.3b). Before stem elongation, soil drying significantly decreased Ψ_{leaf} by ~45% in WT and MG1 plants compared to WW plants, while LG12 and PG11 plants maintained a similar Ψ_{leaf} to WW plants. Prolonged soil drying during stem elongation further decreased Ψ_{leaf}, reaching approximately -2.0 MPa in WT plants–with visible wilting. Leaf water potential decreased exponentially in response to soil moisture (Fig. 3.3c), with transgenic lines maintaining 30% higher Ψ_{leaf} (significant Line × Soil Moisture interaction: P= 0.042) in dry soil. Thus, genetic transformation causing MT emissions attenuated soil drying-induced decreases in Ψ_{leaf}.

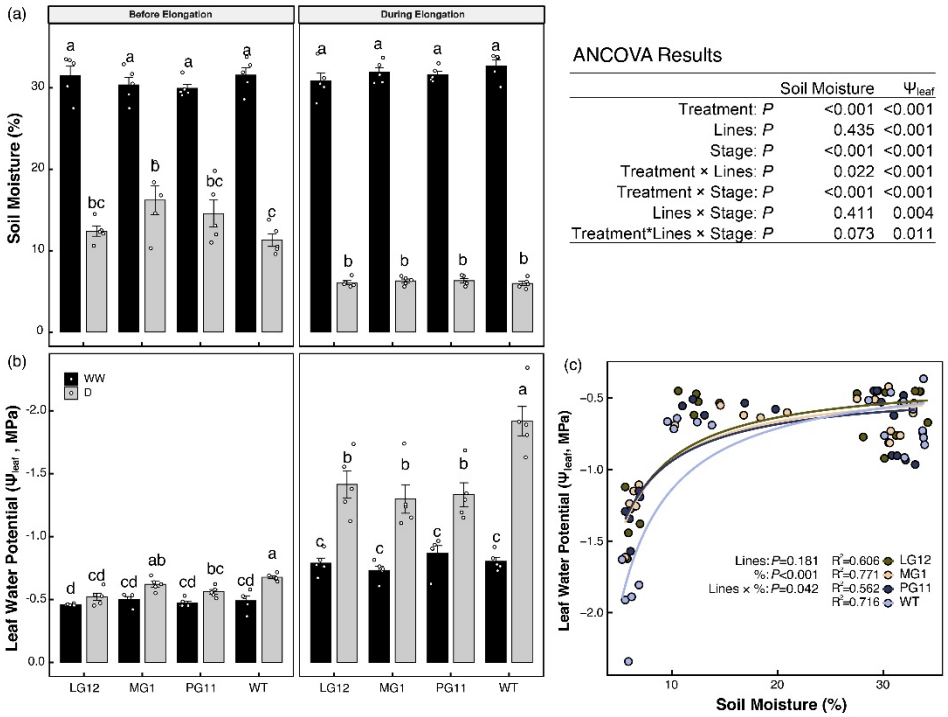

Figure 3. 3 Soil moisture (a) and leaf water potential (b) and their relationship (c) under well-watered (WW) and drought (D) conditions before (BE) and during (DE) elongation stages. Bar plots (±SE) with post hoc Tukey test present the significant differences between treatments and lines by letters. ANCOVA results are presented in the table.

3.3.3 Genetic transformation altered plant morphology and reduced biomass with less variation in stomatal development

Although all seeds germinated simultaneously, and seedlings were transplanted to pots at the same time, leaf and root morphology differed from the seedling stage (Fig. 3.4a). Roots of three-week-old LG12 and MG1 seedlings were significantly ($P< 0.001$) shorter than WT plants by approximately 30%. PG11 had the shortest root length, over 50% shorter than WT plants. Similarly, leaf width of LG12 and MG1 was 11% less than WT plants, and significantly lower ($P< 0.001$) in PG11. Before elongating, well-watered PG11 plants had over 30% less shoot and leaf dry weight and leaf area than WT plants (Fig. 3.4b). Stem elongation amplified adverse effects of transformation, especially with height decreasing by 28% (averaged over all three GM lines) compared to WT, showing a significant Treatment × Lines × Stage interaction (Fig. 3.4c). Leaf area in WT increased by 70% from before to during stem elongation (Fig. 3.4e). Genetic transformation reduced leaf area by over 30% at both stages and leaf area doubled from before to during stem elongation. This suggests a higher stem elongation rate in WT plants and a higher leaf expansion rate in transgenic lines. Although withholding water reduced overall biomass and height across all lines, the genetic variation was much less than in WW plants with significant water treatment and line interactions in shoot and leaf dry weight but not in height and leaf area. Again, PG11 had the lowest shoot dry weight and stem elongation but maintained similar leaf expansion and biomass accumulation.

Genetic transformation had minimal effects on stomatal distribution and development in tobacco plants under WW conditions. Stomatal density was higher on the lower than upper leaf surface, with no difference across all lines and surfaces (Fig. 3.5a). Then increased as plants elongated, which was 14% higher in PG11 than LG12 and MG1 in the lower leaf, without significant difference or interactions (Lines × Stage × Sides: $P = 0.589$). The lower leaf surface maintained a consistent stomatal index and development across lines and stages (Fig. 3.5b). MG1 had a significantly higher stomatal index in the upper leaf compared to LG12 and WT by nearly 27%. As stomatal density and index increased during elongation, stomatal size (width and length) decreased by an average of ~10% across all lines with a significant stage effect ($P< 0.001$), but the stage variation remained consistent with no significant Line × Stage × Sides interactions. Thus, genetic transformation affected stomatal distribution by increasing stomatal index in MG1 and PG11 plants but not size, drought diminished these effects.

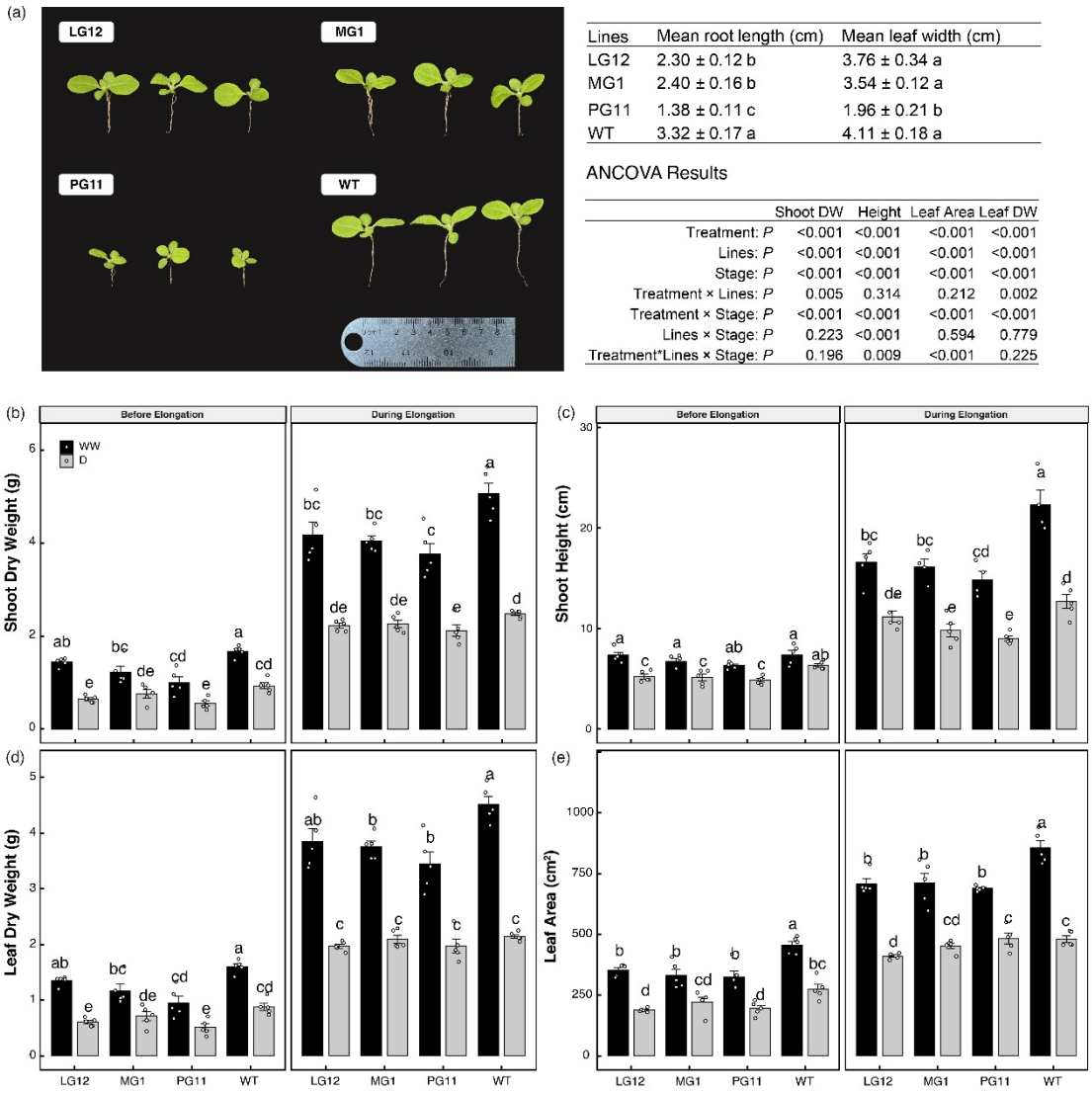

Figure 3. 4 Shoot dry weight (a), height (b), leaf dry mass (c) and area (d), 3-week seedling morphological status under well-watered (WW) and drought (D) conditions before (BE) and during (DE) elongation stages. Bar plots (±SE) with post hoc Tukey test present the significant differences between treatments and lines by letters. Key ANCOVA results are presented in the top table, root length data with t-test reveal the difference in root development.

Table 3. 1 ANCOVA results of stomatal distribution and size. Significant *P* (<0.05) values are highlighted.

	Density	Index	Length	Width
Lines: *P*	0.11	<0.001	0.009	0.006
Stage: *P*	<0.001	0.001	<0.001	<0.001
Sides: *P*	<0.001	0.049	0.012	0.022
Lines × Stage: *P*	0.047	0.97	0.99	0.39
Lines × Sides: *P*	0.42	0.012	0.979	0.98
Stage × Sides: *P*	0.006	0.35	0.504	0.677
Lines × Stage × Sides: *P*	0.59	0.63	0.81	0.54

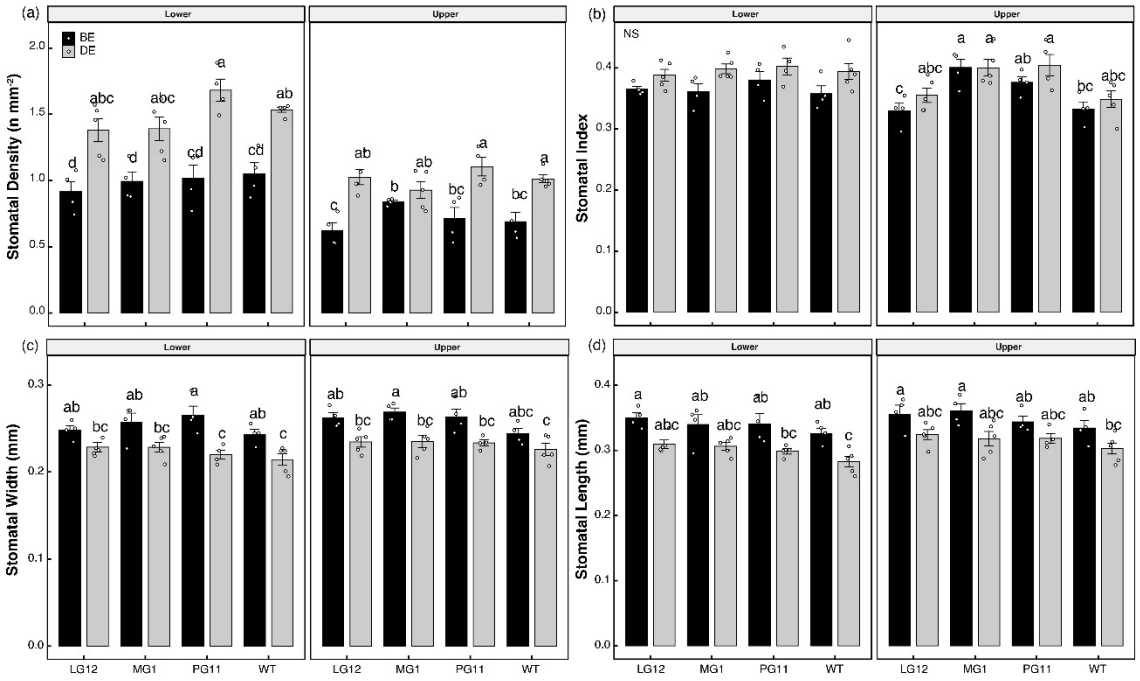

Figure 3. 5 Stomatal distribution and size of lower and upper sides of the leaf under well-watered (WW) condition before (BE) and during (DE) elongation stages. Bar plots (±SE) present the stomatal density (a), index (b), width (c) and length (d) of three GM lines and wildtype plants. Post hoc Tukey tests results indicate the significant differences between treatment and lines by letters.

3.3.4 Genetic transformation altered leaf chlorophyll content and gas exchange, but not intrinsic water-use-efficiency and seed production

Under WW conditions, MG1 and WT plants had the same g_{sw}, which is approximately 40% higher than LG12 and over 30% lower than PG11 (Fig. 3.6a). Drought abolished this variation, with all lines having an average g_{sw} of 0.11 mmol m^{-2} s^{-1}. During plant elongation, g_{sw} also declined under WW conditions to just over 0.21 mmol m^{-2} s^{-1} with no significant difference between lines. Drought nearly fully closed the stomata of all lines. Variations in A_{net} between lines were minimal, with only LG12 showing a significant decline of approximately 20% compared to MG1 and WT before elongation. WD did not significantly affect A_{net} at this stage, except for an 18% reduction in WT plants (Fig. 3.6b). During elongation, WW plants showed no significant genetic variation in A_{net} between lines. However, LG12 and WT plants showed negative CO_2 assimilation (respiration) under WD, significantly lower than those plants of MG1 and PG11, which maintained positive values. The similar relationship between g_{sw} and A_{net} in all lines indicated consistent intrinsic water-use-efficiency (Fig. 3.6c).

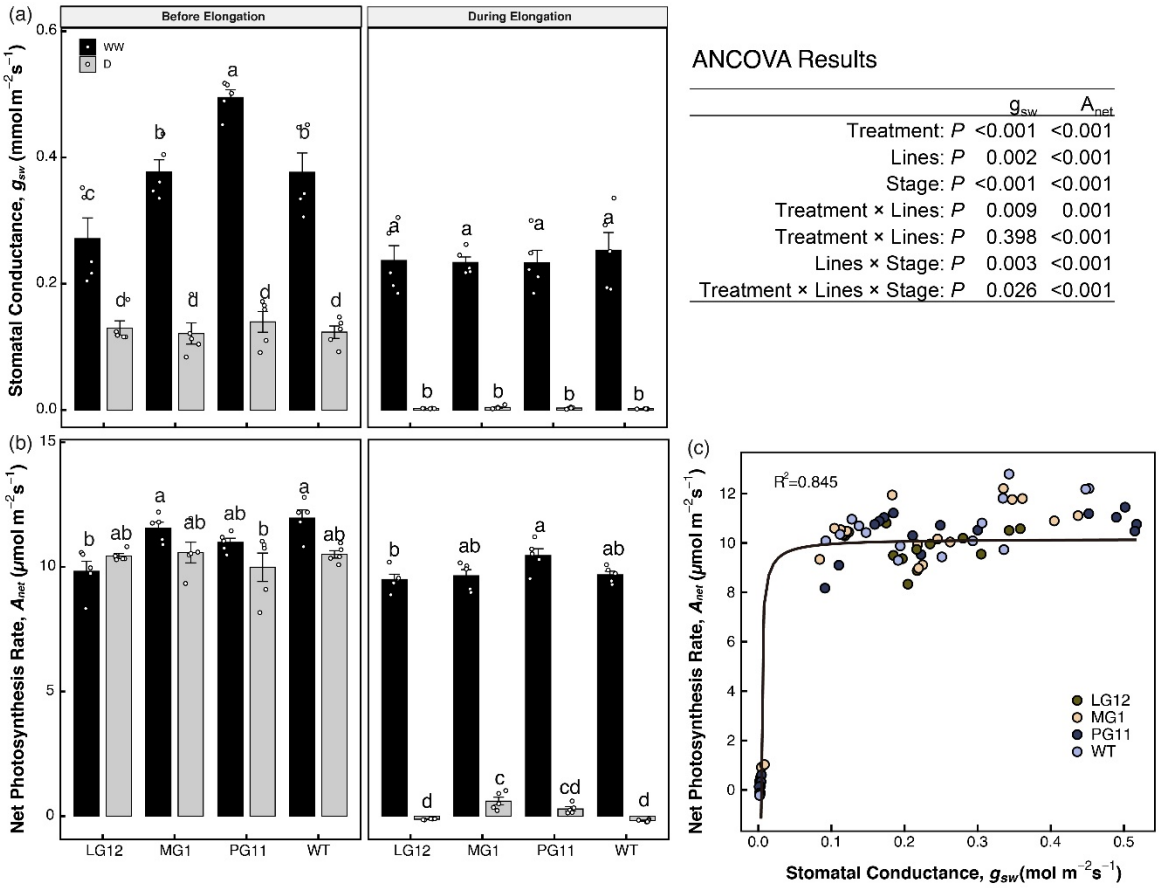

Figure 3. 6 Stomatal conductance (g_{sw}, a), net photosynthesis rate (A_{net}, b) and their relationship (c) under well-watered (WW) and drought (D) conditions before (BE) and during (DE) elongation stages. Bar plots (±SE) with post hoc Tukey test present the significant differences between treatments and lines by letters. ANCOVA in the table reveal the significant effect of lines, stage, side and their interactions.

Before stem elongation, leaf chlorophyll content of well-watered MG1 and WT plants tended to be approximately 10% higher (P>0.05) than LG12 and PG11 plants. This variation was diminished as the stem elongated (Fig. 3.7). Drought increased leaf chlorophyll content, particularly in LG12 and WT plants, the later was significantly higher than MG1 and PG11 by over 20%. This increase continued as plant elongated and drought persisted. Under drought, chlorophyll content of PG11 more than doubled and was approximately 40% higher than that in LG12 and MG1 plants, resulting in a significant Treatment × Lines × Stage interaction (P= 0.047). While this variation was not caused by responses to soil and leaf water status.

While genetic transformation did not affect seed production with no significant Treatment × Lines interaction. Three weeks of withholding water reduced seed yield by an average of 13% in LG12, PG11 and WT plants. While MG1 plants maintained

similar seed production irrespective of drought, resulting a non-significant Treatment × Lines interaction (Fig. 3.8). Thus, genetic transformation had minimal impact on reproduction.

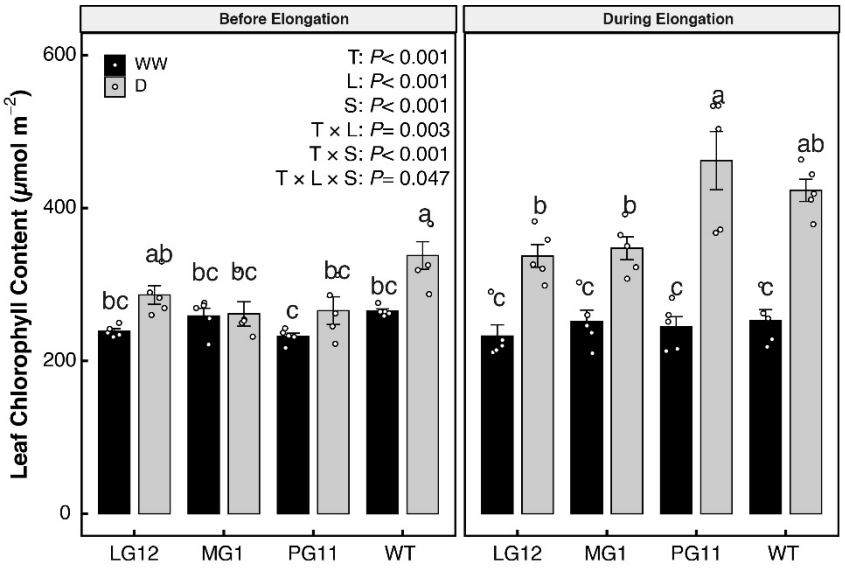

Figure 3. 7 Leaf chlorophyll content under well-watered (WW) and drought (D) conditions before (BE) and during (DE) elongation stages. Spare plants were left under well-watered conditions and their dry weight was measured (b). Bar plots (±SE) with post hoc Tukey test present the significant differences between treatments and lines by letters. ANCOVA in the table reveal the significant effect of lines, stage, side and their interactions. T: Treatment; L: Lines; S: Stages.

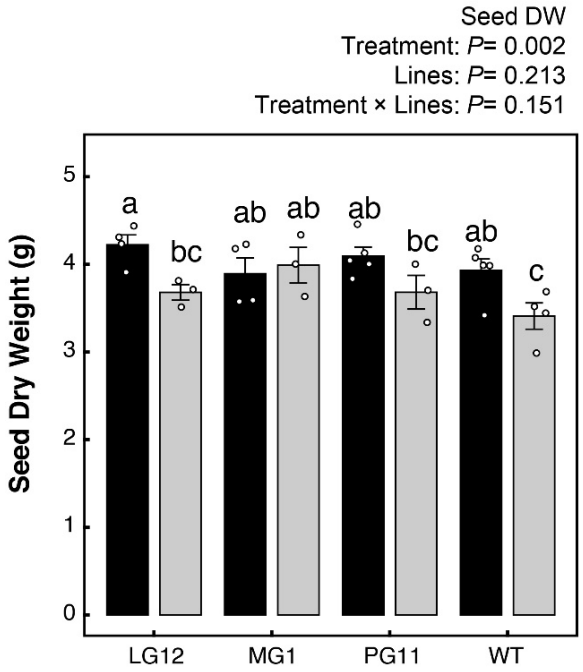

Figure 3. 8 Seed dry weight of plants under well-watered (WW) and water deficit (WD) conditions. Spare plants in WD treatment were rewatered and kept watered with WW treatments for seed harvest. Bar plots (±SE) with post hoc Tukey test present the significant differences between treatments and lines by letters. 2-way ANOVA in the table reveal the significant effect of lines, stage, side and their interactions.

3.4 Discussion

Genetically enhancing volatile organic compound synthesis and emissions can improve plant stress resilience (Dudareva *et al.*, 2013), but such transformations often incur trade-offs in growth and metabolic regulation (Vickers *et al.*, 2009b; Ryan *et al.*, 2014; Yin *et al.*, 2016). Genetic transformation significantly upregulated foliar (−)-limonene and (−)-pinene emissions especially under drought conditions in LG12 and PG11 respectively (Fig. 3.2). These lines maintained a higher leaf water potential than WT plants under drought conditions (Fig. 3.3b&c), perhaps because overproducing monoterpenes suppressed biomass accumulation (Fig. 3. 4). Additionally, PG11 showed higher stomatal density (Fig. 3.5a), which might lead to higher stomatal conductance under well-watered conditions. While LG12 had low stomatal conductance irrespective of stomatal distribution (Fig. 3.6a). Despite these morphological adjustments in the transgenic plants, they maintained seed yield relative to WT plants irrespective of a three-week drought, suggesting a limited impact of monoterpene emission on plant productivity. Overproduction of monoterpenes may confer physiological and biochemical drought resistance in tobacco plants (Vickers *et al.*, 2009a; Pichersky and Raguso, 2018). The physiological and morphological characteristics of these transgenic tobaccos provide us the basics for understanding the mechanistic role of monoterpenes in plants against abiotic stresses.

3.4.1 Transgenic tobaccos were less sensitive to drought

Leaf water potential of all lines decreased similarly during stem elongation under well-watered (WW) conditions (Fig. 3.3). Since the newest fully expanded ones were always measured, the distance between leaf and soil increases, between leaf and lights decreases as the stem elongates. The decline in Ψ_{leaf} during elongation stage probably due to both gravitational gradient (Begg and Turner, 1970) and higher water loss via transpiration in response to light intensity (Hayat *et al.*, 2020). Although there was no difference in leaf water status between lines under well-watered conditions, the transformed lines had less shoot and leaf biomass, especially in PG11 and during the elongation stage (Fig. 3.4b&d). This suggests that diverting the carbon budget to the MEP pathway decreased carbon fixation in plants and therefore biomass. In previous studies, terpene overproduction usually adversely affected development in non-native producers, inhibited growth and productivity from 30-60%, Possibly due to the depletion of IPP resources in the plant (Aharoni *et al.*, 2003; Wu *et al.*, 2006; Lange and Ahkami, 2013). WT plants were taller than transformed lines throughout the experiment (Fig. 3.4c), whereas in the original transformants, tobacco expressing *MpGPS.SSU* had slightly longer stems at the flowering stage with higher foliar gibberellin content (Yin *et al.*, 2016). Presumably, co-expression with *monoTPS* further altered growth-related hormones. Moreover, taller WT plants reached closer to the lights and received higher photon energy per unit leaf area. Hence, increased biomass of WT plants might also be due to higher total photon energy the plant received during the growth cycle.

After a week of drought, soil moisture decreased to one-third in LG12 and WT plants, and to half in MG1 and PG11 plants, then continued to drop to the same level (Fig. 3.3a). Drought may exacerbate biomass reduction of the transgenic lines (Ryan *et al.*, 2014), possibly by increasing the loss of carbon under water stress (Centritto *et al.*, 2011; Huang *et al.*, 2019b). However, drought nearly eliminated biomass differences between lines, especially during elongation (Fig. 3.4). WT and MG1 plants had higher shoot and leaf dry weight than the other lines. As drought conditions persisted, only PG11 showed significantly lower shoot dry weight and height. Decreased leaf water potentials rapidly inhibit growth rates, which can be halved with a 25% reduction in Ψ_{leaf} and may cease with a reduction of more than 50% (Boyer, 1968; Parkash and Singh, 2020). Hence, plants probably stopped growing when Ψ_{leaf} fell below -1.0 MPa, and diminished morphological differences between lines.

After three weeks of withholding water, Ψ_{leaf} dropped to below -1.5 MPa and stomata were fully closed, but re-watering allowed recovery to facilitate flowering and seed production. As expected, leaf water potential decreased exponentially as the soil dries (Fig. 3.3c) independently of stomatal response and distribution. Interestingly, wilting was observed in the WT plants, but was not obvious in transformed lines. Genetically transformed plants had higher leaf water potential under these conditions. This implies that genetic transformation maintains leaf water status under extreme drought conditions by hydraulic regulations or turgor and osmotic adjustment. Leaf water potential at the wilting point determines physiological drought tolerance in plants (Bartlett et al., 2012). Leaves that remain turgid under more negative water potentials tend to maintain structural traits and key physiological processes such as leaf mass per area and leaf hydraulic conductance under drought conditions (Guyot *et al.*, 2012; Maréchaux *et al.*, 2015). Although MG1 and PG11 had slightly higher net photosynthesis than WT and LG12, which may contribute to leaf osmotic adjustment (Boyer, 1968; Shangguan *et al.*, 1999). Therefore, the physiological mechanism of maintained leaf water status in transgenic tobaccos require further investigation.

Stomatal conductance varied between lines under WW conditions before elongation. Lower stomatal conductance in LG12 appeared to drive a lower net photosynthesis rate, and higher values of PG11, presumably mediated by higher stomatal density in this line. (Fig. 3.6). Although the stomatal conductance was much higher in PG11, photosynthesis was similar to MG1 and WT, potentially due to biochemical limitations (Sharkey *et al.*, 2007a). In addition, upregulating the MEP pathway may increase leaf level ABA content, thereby adjusting stomatal conductance and improve drought resistance (Tattini *et al.*, 2014; Pollastri *et al.*, 2023), depending on the relative expression of inserted genes (Ryan *et al.*, 2014), while inhibiting MEP flux causes the opposite response (Barta and Loreto, 2006). Overexpressing substrates and precursors (e.g., DXS, GGPP) of the MEP pathway in plants enhanced the production of other primary and secondary metabolites, such as chlorophylls, carotenoids, and ABA, that are associated with the MEP pathway (Estévez *et al.*, 2001; Jin *et al.*, 2021; Dong *et al.*, 2023), and mediate plant physiological responses. Drought and oxidative stress-induced increases in ROS and antioxidant activity may also trigger retrograde signalling to stimulate relevant physiological responses, including stomatal closure (Noctor *et al.*, 2014; Crawford *et al.*, 2017). Hence, the enhanced stomatal closure during elongation

and long-term water stress may be less related to hormonal regulation. However, PG11 and LG12 showed contradictory responses to upregulated MEP flux if hormone or ROS are the regulators here. The g_{sw} in LG12 plants was only half that of PG11 plants under WW conditions (Fig. 3.6a), suggesting a higher iWUE in LG12 plants. Since these transgenic lines differ in biomass, elongation and leaf area (step down by 5% in LG12, MG1 and PG11) but not seed production (Fig. 3.8), there might be agronomic benefits of overproducing (−)-limonene in tobacco. Further research is needed on the hormone levels of these transgenic tobaccos, seed quality and the effects of transformation on the response of other metabolites to drought.

Although a week of WD had little effect on chlorophyll content (Fig. 3.7), prolonged drought (for another two weeks) increased leaf chlorophyll content by 20% in WT plants, 40% in LG12 and MG1 plants, and 80% in PG11 plants (Fig. 3.7). This suggests that increased flux in the MEP pathway enhanced chlorophyll production or attenuated chlorophyll degradation under long-term drought. Previous study found elevated leaf chlorophyll content under drought stress in tobacco (Gubiš *et al.*, 2007) and other species like tomato (Mäkelä *et al.*, 2000) and barley (Li *et al.*, 2006), presumably due to their drought tolerance via hormonal regulation and antioxidant defence (Killi *et al.*, 2020; Luo *et al.*, 2021). Expression of a single precursor (e.g., GPP) has a limited effect on chlorophyll content, with increased level of carotenoids and tocopherols (Simpson *et al.*, 2016; Jin *et al.*, 2021). This may be related to the coordinated expression of specific MEP pathway components, for example, *GGPPS3* to lutein (Yin *et al.*, 2016) and *GGPPS1-2* to chlorophylls (Dong *et al.*, 2022). Furthermore, transgenic impacts on leaf chlorophyll content only appeared under WD conditions, suggesting that genetic transformation in tobacco altered the metabolic pathway responses to drought stress.

3.4.2 Drought-dependent monoterpene upregulation was unrelated to leaf gas exchange

Wild-type tobacco did not produce any monoterpenes at either growth stage in well-watered plants, as in previous studies of the same tobacco variety (El Tamer *et al.*, 2003; Lücker *et al.*, 2004; Yin *et al.*, 2016). Expressing *Mentha* × *piperita* geranyl diphosphate synthase small subunit (*MpGPS.SSU*) along with *Picea abies (−)-limonene synthase (PaLinS) or P. sitchensis (−)-pinene synthase (PsPinS)* significantly increased the foliar emission of (−)-limonene and (−)-α/β-pinene in LG12 and PG11 lines, respectively irrespective of developmental stage (Fig. 3.2). In the originally constructed

lines, the ratio of relative expression of *MpGPS.SSU* and *monoTPS* in LG12 (3:1) and PG11 (2:3) achieved the most abundant and stable emission of corresponding monoterpenes. The ratio between (−)-α- and (−)-β-pinene emissions in PG11 was also 1:4, as in the original transformants (Yin *et al.*, 2016). The increased metabolic flux to the MEP pathway supports the biosynthesis of monoterpenes in these transgenic lines, and *GPS.SSU* ensured the availability of the GPP pool (Lücker *et al.*, 2004; Vickers *et al.*, 2011). However, MG1 plants, with *MpGPS.SSU* and *P. abies myrcene synthase*, had no monoterpene emission, suggesting a failure of intergenerational inheritance. Monoterpene composition and emission rates may vary with plant growth (Taipale *et al.*, 2020; Byron *et al.*, 2022). This may be attributed to the fact that *Ubiquitin 10* is a constitutive promoter and that emissions were consistently sampled from the newest fully expanded leaves.

Furthermore, the composition of monoterpene emissions slightly changed during the experiment. (−)-α/β-pinene was detected in LG12 under WW conditions. WD induced (−)-α/β-pinene and (−)-limonene emissions in WT plants, and (−)-limonene in MG1 and PG11 plants, though at levels two orders of magnitude lower than those of LG12. This indicates the metabolic diversity of monoterpene biosynthesis products among the lines. However, whether this was due to direct synthesis via feedback mechanisms, or whether the compound structure was altered, could not be confirmed. Synthesis catalysed by a single *TPS* can produce multiple compounds from a single substrate, and is activated by stress conditions (Chen *et al.*, 2011). Moreover, the terpene skeletons may be modified by other active enzymes (e.g., oxidative enzymes), biochemically resulting in structural diversity, particularly under abiotic stress conditions (Pichersky and Raguso, 2018). For example, drought upregulates eighteen *TPS* genes in *Dendrobium catenatum* (Zhan *et al.*, 2022), and enzymatic conversion of six carbocation intermediates of monoterpenes from GPP is temperature-dependent across species (Jardine *et al.*, 2017). Since there is no native monoterpene pathway in this tobacco variety, it is unlikely that a feedback mechanism was responsible for the altered composition.

Aside from composition variations, the emission rate of all compounds doubled in LG12 and tripled in PG11 plants without changes in the emission profile or ratio under drought before elongation, before dropping to WW levels after two weeks of withholding water during elongation. This suggests that a specific level of water stress activates the foliar monoterpene production capacity and alters the emission behaviour

of tobacco plants. Similarly, in native monoterpene emitters such as *Cistus albidus* and *Pinus halepensis*, leaf monoterpene emissions were several times higher under short-term water stress (Ψ_{leaf} decreases > 2-fold) and then abruptly decreased to just detectable levels under long-term (Ψ_{leaf} decreases four-fold) deficit (Ormeño *et al.*, 2007). As tobacco plants are not native monoterpene emitters, they are unlikely to have monoterpene-specific storage pools, thus monoterpene emissions arise from direct biosynthesis irrespective of stomatal conductance and net photosynthesis. Photosynthesis and intercellular CO_2 concentration may regulate instantaneous monoterpene synthesis and emission (Peñuelas and Llusià, 1999; Loreto and Schnitzler, 2010) due to their chloroplastic localisation and delivers the first MEP precursors, but this relationship is lost under long-term drought conditions (Niinemets *et al.*, 2002c). Monoterpene synthesis / emission responds slower to water stress than net photosynthesis (Bertin and Staudt, 1996), and photosynthetic electron transport is insensitive to declines in stomatal conductance (Valentini *et al.*, 1995; von Caemmerer *et al.*, 2004). Thus, direct monoterpene synthesis and emission rate from emitters without specific storage organs (e.g., *Quercus coccifera* L.) may be more related to photosynthetic electron transport (Niinemets *et al.*, 1999; Niinemets *et al.*, 2002c). As photosynthetic electron transport is maintained under mild drought and net carbon assimilation rates are reduced, plants can mitigate possible negative effects such as oxidative stress by diverting electron transport and energy to terpene synthesis (Staudt and Bertin, 1998; Møller, 2001; Niinemets and Reichstein, 2002; Rasulov *et al.*, 2009b). We propose that foliar photosynthetic properties (i.e., upregulation of electron supply for direct terpene biosynthesis) may regulate the increase in MT emission in transgenic tobacco under drought conditions. Although constitutive expression of inserted genes maintained the base level emission, prolonged drought may block terpene biosynthesis metabolic pathways under extreme water stress.

As expected, the monoterpene emissions remained stable due to the constitutive expression of the inserted genes, even when gas exchange was almost completely suppressed. In natural conditions, ecosystem and plant carbon loss as terpenes can be 0.1-4% of assimilated carbon (Kesselmeier *et al.*, 2002; Ryan *et al.*, 2014), with LG12 and PG11 plants losing 1% of carbon from net photosynthesis under well-watered conditions as monoterpenes. This increased to approximately 4% in LG12 and 3% in PG11 after a week of withholding water. Then further rose to 80% in PG11 with additional two weeks. While LG12 plants had the similar emission level as PG11 even

when there was only photorespiration. Under limited net carbon assimilation, plants use alternative carbon from the cytosolic, xylem-allocated, or extraplastidic sources for MEP biosynthesis (de Souza *et al.*, 2018), which has also been demonstrated under drought stress (Funk *et al.*, 2004; Kreuzwieser *et al.*, 2021). Maintaining investment in terpene flux enhances antioxidant properties via direct (e.g., as antioxidants) or indirect (e.g., as signalling molecules) mechanisms (Vickers *et al.*, 2009a; Vickers *et al.*, 2009b), benefits downstream metabolic regulation (Barta and Loreto, 2006; Dudareva *et al.*, 2013), and bring ecological functions such as plant-plant/insect interactions (Cheng *et al.*, 2006; Pichersky and Raguso, 2018; Brosset and Blande, 2021). The emission behaviour of LG12 and PG11 under drought conditions make them effective tools to study the role of emitted compounds in drought stress tolerance in plants.

3.5 Conclusion

Genetic transformation by expressing *geranyl diphosphate synthase small subunit* (*MpGPS.SSU*) and monoterpene synthases enhanced the emission of specific monoterpenes with trade-offs in physiology and morphology, although drought stress diminished these impacts. LG12 has significantly enhanced emission of (–)-limonene with the least adverse effects on physiology and morphology, e.g., similar gas exchange reduction under drought and maintenance of biomass and reproductivity compared to WT plants, and therefore the best candidate for further mechanistic studies to understand the role of (–)-limonene in plants against drought stress.

4 (–)-limonene emitting transgenic tobacco maintains leaf water status but downregulates photosynthesis and APX activity during drought stress

Abstract

This study investigates the physiological and biochemical functions of monoterpenes, specifically (–)-limonene, in genetically transformed tobacco (*Nicotiana tabacum*) plants under drought stress conditions. Growth, leaf water status and gas exchange of monoterpene emitting transgenic tobacco lines (LG12), which overexpress geranyl diphosphate synthase (*MpGPS.SSU*) and (–)-limonene synthase (*PaLimS*), were evaluated under well-watered and water-deficit (drought) conditions. Initially, drought significantly increased (–)-limonene emission, which subsequently declined as water stress intensified. Despite maintaining higher leaf water potential and turgor than wild-type (WT) plants, LG12 plants had lower photosynthesis rates. The production of (–)-limonene altered oxidative stress responses by downregulating ascorbate peroxidase (APX) activity and increasing lipid peroxidation under drought conditions. Foliar hydrogen peroxide (H_2O_2) content and superoxide dismutase (SOD) activity did not significantly differ between genotypes under drought. However, re-watering restored APX activity and reduced oxidative damage. These findings suggest that (–)-limonene modulates drought tolerance by affecting water retention and oxidative stress but may impair photosynthetic efficiency under drought stress. The stress threshold for regulating monoterpene emissions and functioning and interactions with endogenous non-enzymatic antioxidants require further investigation.

4.1 Introduction

Environmental stresses significantly influence the production and emission of volatile organic compounds (BVOCs), including terpenes such as isoprene and monoterpenes, which are closely related to plant physiological and biochemical responses (Laothawornkitkul *et al.*, 2009; Loreto and Schnitzler, 2010). Biotic stresses such as herbivory attack generally promote foliar BVOC emission as a part of the chemical signalling and defence mechanisms, repelling herbivores (Toffolatti *et al.*, 2021). The response of terpenes synthesis and emission to abiotic stresses, however, is more complex, with both increases and decreases observed depending on the severity and duration of the stresses (Alicandri *et al.*, 2020; Bergman and Dudareva, 2024). Previous research mostly focused on isoprene emission and monoterpene production under heat and high drought or synergistic effects. Though isoprene is generally accepted to protect photosynthetic system from high temperatures, the role of monoterpenes is not fully understood (Possell and Loreto, 2013; Tiiva *et al.*, 2017).

Drought stress has effects on monoterpene production and emissions with physiological such as stomatal closure and photosynthetic limitation, and biochemical interactions such as reactive oxygen species signalling and synthase limitation (Niinemets *et al.*, 2004; Li and Sharkey, 2013). Mild drought conditions can stimulate constitutive and *de novo* emission. Constitutive emissions are derived from stored terpenes that accumulate in specialised structures, such as resin ducts or glandular trichomes, where they are readily available for immediate release (Schilmiller *et al.*, 2009; Kreuzwieser *et al.*, 2021; Fitzky *et al.*, 2023). These pools can provide a fast response to stress, such as temperature, drought and mechanical damage, by releasing stored compounds into the atmosphere without the need for new synthesis (Gang *et al.*, 2001; Byron *et al.*, 2022). When storage pools are insufficient or absent, *de novo* emissions involve the synthesis of monoterpenes in response to environmental cues (Fasbender *et al.*, 2018). This form of emission is particularly crucial in species that lack large storage capacities for terpenes (Srikanta Dani *et al.*, 2017; Werner *et al.*, 2020). In such cases, monoterpenes are produced ‘on-demand’ when the plant encounters stress conditions, such as drought. For example, in species like *Quercus ilex* (holm oak) and *Rosmarinus officinalis* (rosemary), drought triggers an increase in *de novo* synthesis of monoterpenes such as α-pinene, β-pinene, and (–)-limonene (Nogués *et al.*, 2015b; Lüpke *et al.*, 2017). Whereas in some species with large storage pools such as *Pinus sylvestris*, the emissions proportion from *de novo* synthesis tends to decrease under drought conditions

(Lüpke *et al.*, 2017). In several plant species, including holm oak and *Pinus halepensis*, drought-induced reductions in stomatal conductance did not significantly affect monoterpene emissions, with continued emission despite limited photosynthetic activity (Blanch *et al.*, 2009; Harley, 2013). This suggests that drought imposes minimal physiological limitations on monoterpene emission.

In severe drought conditions, continued reductions in stomatal conductance (g_{sw}) and photosynthetic assimilation (A_{net}) lead to energy shortages, which inhibit volatile production and *de novo* emission (Staudt *et al.*, 2008; Haberstroh *et al.*, 2018). At this stage, emissions from constitutive pools may still occur, but *de novo* synthesis is severely restricted due to the lack of metabolic resources. However, with prolonged drought, a compensatory increase in *de novo* monoterpene emission can still occur, followed by a significant emission burst upon re-watering (Peñuelas *et al.*, 2009; Guo *et al.*, 2021).

Seasonal and ecosystem-level variations in monoterpene emissions further illustrate the complexity of their regulation under drought (Seco *et al.*, 2015; Mu *et al.*, 2018). For example, monoterpene emissions from Mediterranean shrublands are less suppressed during winter than summer droughts, while woodland ecosystems have exhibited the opposite pattern (Joan Llusià *et al.*, 2008). This variability underscores the importance of understanding both constitutive and *de novo* emission mechanisms in different species and under different environmental conditions. The functional significance of these stress-induced emissions maybe crucial to understand plant stress adaptation and resilience.

Although monoterpenes have not been studied as extensively as isoprene, emerging evidence suggests that they play an important role in protecting plants from heat and oxidative stress (Sharkey *et al.*, 2007b; Jardine *et al.*, 2017; Tian *et al.*, 2020). Monoterpenes are released in higher quantities under elevated temperature conditions. Emissions of specific monoterpenes such as α-pinene, β-pinene, (–)-limonene, and sabinene increased when *Quercus ilex* saplings were exposed to temperatures ranging from 30°C to 55°C (Copolovici *et al.*, 2005). Furthermore, when the plants were fumigated with these monoterpenes, their photosynthesis rates and operating efficiency of PSII were significantly higher than control plants, and lack of emissions decreased physiological performance, suggesting that monoterpenes can protect photosynthesis

under heat stress (Loreto *et al.*, 1998c). The protective role of monoterpenes is not limited to thermal stress but also extends to oxidative stress. Monoterpenes can prevent reactive oxygen species (ROS) accumulation and reduce lipid peroxidation in cellular membranes. For example, exposing non-isoprene-emitting plants to ozone (an oxidative stressor) stimulated monoterpene synthesis (Loreto *et al.*, 2004). However, limiting monoterpene emissions by applying chemical inhibitors such as fosmidomycin rapidly inhibited photosynthesis and increased levels of ROS and malondialdehyde (MDA), a marker of lipid peroxidation (Chen *et al.*, 2008; Tian *et al.*, 2020). This indicates that monoterpenes play a direct role in protecting cellular structures from oxidative damage by acting as antioxidants and mitigating the effects of ROS. Since heat stress is often accompanied by oxidative stress, monoterpenes could act as antioxidants that help maintain membrane integrity during heat.

As a neutral stress biomarker and important signalling molecule, drought also extensively induces ROS accumulation resulting in oxidative damage (Mittler *et al.*, 2022). Thus, monoterpenes, much like isoprene, may serve critical protective functions in plants by mitigating the damaging effects. This study investigates the role of monoterpenes, particularly (–)-limonene, in genetically transformed tobacco (*Nicotiana tabacum*) plants under drought stress. These transgenic lines were engineered to overexpress key enzymes involved in monoterpene biosynthesis, including geranyl diphosphate synthase (*MpGPS.SSU*) and (–)-limonene synthase (*PaLimS*). By evaluating the physiological and biochemical responses of these plants to varying soil moisture conditions, this research aims to elucidate the mechanisms through which constitutive and *de novo* monoterpene emissions influence plant drought tolerance. Specifically, the study will assess leaf water retention, gas exchange, oxidative stress, and antioxidant enzyme activities to understand the role of (–)-limonene in modulating plant responses to drought.

4.2 Materials and Methods

4.2.1 Plant materials and growth environments

Seeds of wild-type (*Nicotiana tabacum* cv. SR1) and the transgenic strain LG12 were procured from the Temasek Life Sciences Laboratory, Singapore. Yin et al. (2016) engineered LG12 to contain two inserted genes: *Mentha × piperita geranyl diphosphate synthase small subunit* (*MpGPS.SSU*) (AF182827) and *Picea abies (−)-limonene*

synthase (*PaLimS*). Previous experiments revealed that LG12 had a marginally slower growth rate (Chapter 3, Fig. 4) than the wildtype (WT). To counteract the impact of plant size on plant physiology, LG12 seeds were sown three days prior to the WT in seedling trays (12 cells size: 3.8 × 3.8 × 5 cm, tray size: 17.8 × 14 × 5 cm) covered by transparent domes with humidity control valve, in John Innes No2 compost. The seeds germinated in a room with a controlled environment, lit by white, blue and red LED growth lights (Model B150, Valoya, Helsinki, Finland). The seedlings were then transplanted to 3 L pots (20.5 cm in height, 16 cm at the top, and 12.5 cm at the bottom) each filled with 2.5 kg pre-mixed John Innes No2 compost, numbered, and cultivated in four enclosed plant growth chambers as described in Stokes et al. (1993). Growth environments as described in Chapter 3.

4.2.2 Drought treatment and experimental setup

Two separate experiments were conducted, each comprising a factorial combination of two genotypes (WT or LG12) and two watering treatments (well-watered or drought), resulting in four genotype × treatment combinations. To prevent potential priming effects of BVOCs across treatments, plants from each combination were placed in individual growth chambers, which were leak-tested with doors closed. All plants were well-watered daily at 08.00 and 18.00 hours, by replenishing the amount of water lost from the pots, by comparing weights at these times versus initial pot weight. Control plants were consistently well-watered (WW) daily throughout the experiment by replacing evapotranspirational losses. Drought was imposed on half the plants of each genotype on the 46th day of growth after seeding, when baseline measurements were made (this is considered day 0 of the drought experiment). After 18 days of soil drying, the remaining plants were re-watered to initial pot weight and maintained well-watered.

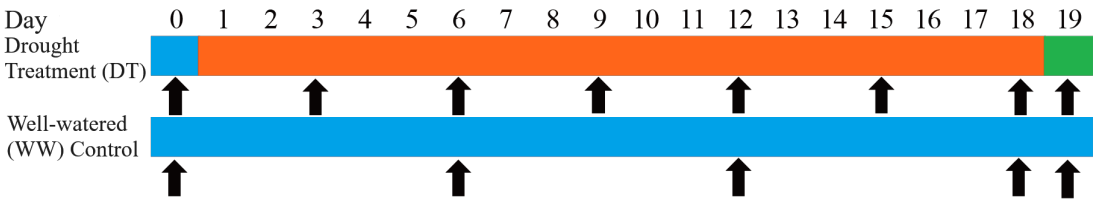

Figure 4. 1 Measurement and sampling schedule (arrows indicated on these days) of both the drought (orange bar) and well-watered control (blue bar) treatments. On Day 19, all remaining droughted plants were re-watered (green bar), then measured.

To test the hypotheses, measurement and sampling including physiology, biochemistry, and foliar limonene emission were carried out. Measurements were conducted every three days for the drought treatments and six days for the control, with additional measurements following re-watering to understand plant recovery (Fig. 4.1). Four plants of each genotype from each treatment were randomly selected using a random number generator for physiology (gas exchange, chlorophyll fluorescence) measurements with a randomised sequence to avoid potential diurnal patterns. Three of those four plants were used for biochemical and foliar limonene emission analyses, the fourth one was only used for physiology measurement. Measurement and sampling details are described below.

4.2.3 Gas exchange, chlorophyll fluorescence measurements

Leaf gas exchange was measured using a LI-6800F portable photosynthesis system (LI-COR Inc., Lincoln, NE, USA) in both experiments. This system was left in the growth chamber for an hour to equilibrate with the environmental conditions, before measurements commenced. The newest fully developed leaf (either the 4th or 5th from the top) was secured in a 6 cm^2 circular leaf cuvette. Once gas exchange reached a steady state (when the real-time plots plateaued for one minute), measurements were taken for the net photosynthesis rate (A_{net}; μmol m^{-2} s^{-1}), stomatal conductance (g_{sw}; mol m^{-2} s^{-1}), and other pertinent physiological and environmental parameters (e.g., transpiration rate, internal CO_2, leaf temperature). These measurements were taken at a PPFD of 350 μmol m^{-2} s^{-1} (colour spectrum r90B40 with 90% red and 10% blue) and a CO_2 concentration of 421 μmol mol^{-1}. The temperature and relative humidity inside the leaf chamber were set to match the leaf-level temperature, which was measured by USB humidity and temperature data loggers with an LCD screen (Lascar Electronics Ltd., Whiteparish, Wiltshire, UK) in the growth chamber prior to the measurement. The airflow rate was initially set at 600 μmol s^{-1} at the start of the experiment and was reduced to 400 μmol s^{-1} in drought treatments from day 9, when g_{sw} was half that of the control.

Chlorophyll fluorescence was recorded at the same time as gas exchange, using a Multiphase Flash fluorometer (6800-01A). A saturating flash of actinic light was applied for 800 ms, with a dark modulation rate of 50 Hz, a light modulation rate of 1 kHz, a flash modulation rate of 259 kHz, and an averaging time of 15s under an automatic (Rectangular or Multiphase) flash type. The steady-state photosynthetic

fluorescence (F_s'), maximum fluorescence (F_m'), and minimum fluorescence (F_o') of the leaf adapted to light were determined during the flash. The maximum fluorescence (F_m) and minimum fluorescence (F_o) of leaves adapted to darkness were measured before the lights were turned on in the morning. Photochemical parameters were calculated as Murchie and Lawson (2013) described, using the equations provided below.

Dark and light-adapted maximum quantum efficiency of PSII

$$\frac{F_v}{F_m} = \frac{F_m - F_o}{F_m} \quad (1)$$

$$\frac{F_v'}{F_m'} = \frac{F_m' - F_o'}{F_m'} \quad (2)$$

Quantum yield of PSII photochemistry (Φ_{PSII})

$$\Phi_{PSII} = \frac{F_q'}{F_m'} = \frac{F_m' - F_s'}{F_m'} \quad (3)$$

Non-photochemical quenching (NPQ)

$$NPQ = \frac{F_m - F_m'}{F_m'} \quad (4)$$

Photochemical quenching (q_p)

$$q_p = \frac{F_q'}{F_v'} = \frac{F_m' - F_s'}{F_m' - F_o'} \quad (5)$$

After measuring leaf gas exchange, the leaf remained in the cuvette until completing the sampling of headspace volatiles.

4.2.4 Limonene emission sampling and analysis

Limonene emission was sampled in experiment two. To ensure the air source to the Li-6800F system is VOC free, a Carbon Cap™ carbon filtration capsule (Whatman™, Cytiva, Buckinghamshire, UK) was connected to the filter covered air inlet on the LI-6800F console via a flexible PTFE tubing and a M5 hosebarb. Foliar volatile sampling was carried out just after the gas exchange measurements as described by Riches et al (2020). In brief, a gas sampling manifold was connected to the SAM subsampling port at the back of the LI-6800F head (as shown in Fig. S4.1). All the fittings of the manifold used were sourced from Swagelok (Swagelok Central UK, Warrington, UK). A zero-

volume reducing union of size 1/16 to 1/8 inch (Part #: SS-200-6-1ZV) was attached to the subsampling port. This was followed by a 20 mm segment of pre-cleaned GC copper tubing (1/8 inch O.D. × 1.65 mm I.D.) which was connected to a brass reducing union of size 1/8 to 1/4 inch (Part #: SS-400-6-2). This was then connected to a 20mm piece of PTFE flexible tubing (Tygon®, 1/4 inch O.D. × 1/8 inch I.D.), which was subsequently connected to a 1/4 inch brass bored-through union tee (Part #: B-400-3). The straight port of the tee is used for volatile sampling with a stainless-steel sorbent tube (Tenax® TA 35/60 mesh | Carbtrap® 20/40 mesh, 3 1/2 inch long ×1/4 O.D., Markes International Ltd, Llantrisant, UK) using a Pocket Pump (SKC Ltd., Dorset, UK). This was connected to the end of the sorbent tube via a soft PTFE tubing (Tygon®, 1/4 inch O.D. × 1/8 inch I.D.) and a PFA fitting. Sampling flow rate of the pump was pre-calibrated with a spare sorbent tube. An air sample of 1.5 L was loaded into the cartridge at a rate of 150 mL min^{-1} for 10 min. Two LI-6800F system blank samples were collected at the beginning and the end of the measurement day. The other port of the tee was sealed with a brass fitting unless sampling was initiated. During the sampling process, the gas flow to the subsampling port was kept at 30 ± 2% of the total flow out of the LI-6800F leaf cuvette. Gas exchange measurements agreed within 1% irrespective of whether the SAM subsampling port was open. Plants were kept in the growth chamber for leaf water status and morphological measurements. Tube samples were kept in a 4°C fridge for further GCMS analysis following the same procedure as in Chapter 3.

4.2.5 Leaf water status and leaf sample collection

Leaf water status was measured in both experiments. After gas exchange measurement and sampling, leaf water potential (Ψ_{leaf}) was measured using a thermocouple psychrometer following the same method in Chapter 3. Subsequently, the leaf disc was removed using tweezers, then wrapped in aluminium foil again and rapidly frozen in liquid nitrogen to disrupt cell membranes. After a brief thawing period, the leaf disc was returned to the sample holder and incubated in the psychrometer chamber for 30 minutes before determining the osmotic pressure (P). The difference between leaf water potential and osmotic potential estimates leaf turgor.

The plant pot was weighed using a balance, and the measured leaf detached from the plant. Leaf strips weighing 0.3 g fresh weight were sliced with a razor blade and stored in three 2 mL Eppendorf centrifuge tubes. Each tube contained 0.1 g of fresh leaf to

analyse oxidative stress and antioxidant enzymes. An extra leaf sample weighing 0.5 g was collected as a backup. All the tubes were immersed into liquid nitrogen to instantly freeze the samples and then stored in a freezer at -80°C. Subsequently, shoot height, leaf and shoot fresh weight, and the number of leaves were measured. Soil moisture was measured by a WET-2 sensor (Delta-T Devices Ltd, Cambridge, UK) inserted to a depth of ~5 cm from the soil surface. Plant morphological measurements followed the same procedure as in Chapter 3.

4.2.6 Oxidative stress and antioxidant enzyme assessment

Wet chemistry analyses were conducted in experiment two. Foliar H_2O_2 and MDA concentrations, as indicators of oxidative stress and damage respectively, were quantified using the Pierce™ Quantitative Peroxide Assay Kit (catalog # 23280, Thermo Fisher Scientific Inc, Waltham, MA, USA) and MDA-TBARs Assay Kit (catalog # KB03016b, BQC Redox Tech, Oviedo Asturias, Spain). The total SOD activity and APX activity were determined using SOD Colorimetric Activity Kit (catalog # EIASODC, Thermo Fisher Scientific Inc, Waltham, MA, USA) and APX Assay Kit (catalog# KB03036, BQC Redox Tech, Oviedo Asturias, Spain), respectively. One unit of SOD is defined as the amount of enzyme causing half the maximum inhibition of the oxidation of 7.5 mM NADH in the presence of EDTA, manganese ions, and mercaptoethanol at 23°C and pH 7.4 over 15 minutes. One unit of APX is defined as the amount of enzyme that catalyses the conversion of one μmol of ascorbic acid to monodehydroascorbate per minute. According to the manufacture, the extraction buffer in the assay kits does not include any additional compounds, such as ascorbic acid, to stabilise the extraction. Samples were mechanically ground using a Cryomill (Retsch GmbH, Haan, Germany) in the presence of liquid nitrogen. The preparation procedures and assay buffers provided in the assay kits were followed, with an additional orbi-shaking process conducted in a cold room (5°C) for an hour. H_2O_2 samples, which came from the same batch as the APX samples, were subjected to a deproteinization protocol using the TCA Deproteinizing Sample Preparation Kit (catalog # ab204708, Abcam, Waltham, MA, USA) to avoid reaction with internal antioxidants. After the extraction process, all samples were stored in a freezer at -80°C, then kept on ice throughout the assay day. The readings were acquired using a FLUOstar Omega microplate reader (BMG Labtech, Ortenberg, Germany) following the manufacturer's instructions.

4.2.7 Data analysis

Raw data from biochemical assays were analysed using the MARS data analysis software (v.5.01, BMG Labtech, Ortenberg, Germany) integrated with the Omega microplate reader. Sample concentrations were calculated by comparing them against standard curves using linear or four-parameter fits. Data handling, graph generation, and statistical analysis were performed using R (v. 4.3.3) and R Studio (PBC, Boston, MA, USA). Interactions and significant differences between variables related to physiology, biochemistry, and leaf water status were assessed using a general linear model (GLM) with univariate 2-way or 3-way ANOVA/ANCOVA. Post hoc Tukey tests were conducted to compare variables after re-watering. Specific details regarding linear or non-linear regressions, sample numbers, and biological replicates are provided in the figure titles. All statistical significances were considered at $P < 0.05$.

4.3 Results

4.3.1 (−)-Limonene-emitting tobacco maintained a higher leaf water status under soil drying

Morphological impacts were minimised (see Fig. S4.2) by sowing LG12 seeds three days later than the WTs. Both leaf water potential (Ψ_{leaf}) and turgor potential (P) significantly ($P<0.001$) declined as soil moisture decreased in both lines (Fig. 4.2). This response was consistent ($P>0.05$) between experiments. Ψ_{leaf} declined exponentially with soil moisture from -0.5 MPa to nearly -2.0 MPa as soil moisture reached a minimum of 10% (Fig. 4.2a), with significant genotypic differences (Geno: $P=0.002$) and nearly significant soil moisture interaction (Geno × %: $P=0.061$). Specifically, LG12 plants had a higher Ψ_{leaf} (by an average of 0.2 MPa) than WT plants within the soil moisture range of ~15 – 35%.

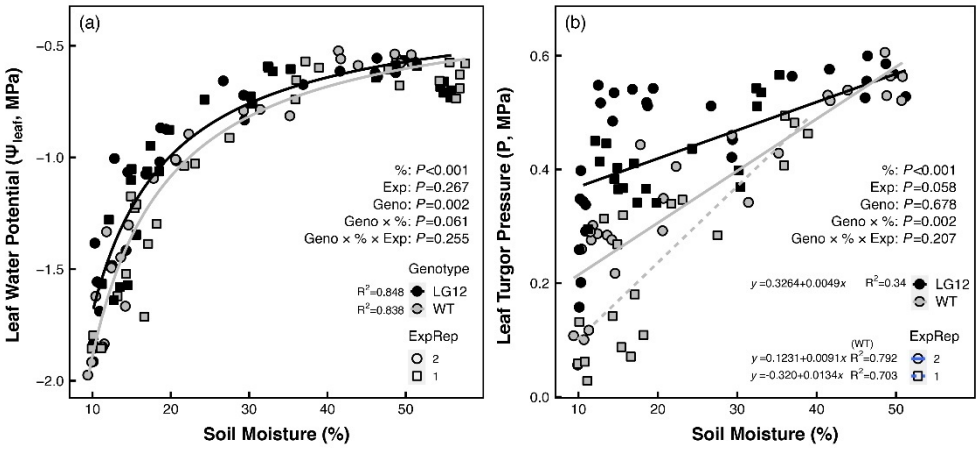

Figure 4. 2 Leaf water potential (a) and turgor pressure (b) responses to soil moisture (%) for LG12 (black) and wild-type (WT, grey) plants from Experiment 1 (square and solid line) and Experiment 2 (circle and dashed line). ANCOVA results (*P*-values) indicate the significant impact of soil moisture (%), experiment (Exp), genotype (Geno) and their interactions. A single regression line was fitted to each genotype when no significant experiment effect occurred.

Under well-watered (WW) conditions, Leaf turgor pressure (P) was approximately 0.58 MPa in both lines. P declined linearly with soil moisture at different rates between the two lines (Fig. 4.2b), as indicated by a significant genotype × soil moisture interaction (Geno × %: P=0.002). As the drought persisted, LG12 plants exhibited an 8% reduction in P for each percentage decline in soil moisture, decreasing to approximately 0.2 MPa by the end. P declined in WT plants at twice the rate of LG12 plants, with experiment one showing nearly 20% slower reduction compared to experiment two. The osmotic potential response was similar between genotypes (data not shown) under drought. This suggests LG12 plants had less sensitive leaf water status to water stress.

4.3.2 Drought altered foliar emission pattern of (−)-Limonene

As anticipated, foliar (−)-Limonene emission was observed in the transgenic line LG12 but not in WT plants. Moreover, (−)-Limonene was not detected in leaf extractions (methods described in the Supplementary Materials) from any plants in either watering condition, indicating the absence of specific storage pools for (−)-Limonene in this tobacco variety. Soil water stress markedly (P< 0.001) altered the emission pattern of (−)-Limonene in LG12 plants, demonstrating an approximately cubic relationship with soil moisture (Fig. 4.3a). Well-watered LG12 plants on Day 0 had a relatively low emission rate of 103 pmol m^{-2} s^{-1}. Withholding water increased this rate 3- to 4-fold compared to Day 0, with maximum E_{Lim} approximately 6-fold higher when soil moisture dropped to just below 20%. The rise of normalised E_{Lim} (the ratio of E_{Lim}

between drought and well-watered plants on the same day) was slower, with a maximum factor of less than 3.

As drought progressed, E_{Lim} subsequently declined dramatically to the same level as day 0, and the emission rate relative to those well-watered plants on the same day continued decreasing (normalised E_{Lim} <1, Fig. 4.3b). After soil water was restored to pot capacity, E_{Lim} remained essentially at the level before withholding water, but was approximately 70% lower than well-watered plants on the same day, so did the normalised E_{Lim} (Fig. 4.3). Thus, plants are unable to sustain high (−)-Limonene emissions with extreme soil water stress (< 20%) but maintain a basal E_{Lim} even when substrate water is almost unavailable (< 10%) and can retain that emission capacity after re-watering (RW).

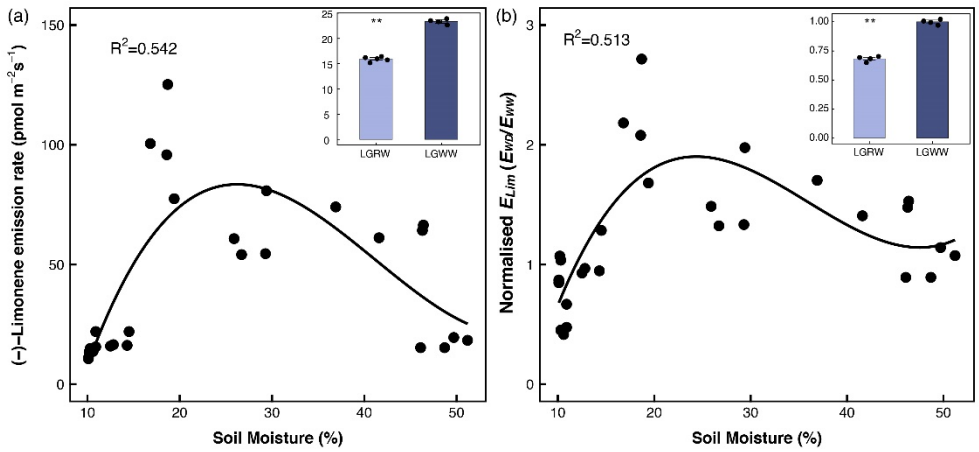

Figure 4. 3 The relationships between soil moisture (%) and (a) (−)-Limonene emission rate (E_{Lim}), (b) normalised (−)-Limonene emission rate with cubic regression, based on data from Experiment 2. Histograms with error bars (±SD) located on the top right corner of each figure present the data for LG12 plants after re-watering (LGRW) and well-watered plants (LGWW) on the same day. ANOVA results (*P*-values) reveal the significance level of the regression model (P<0.001) in both plot and the difference between LGRW and LGWW (**: P< 0.01).

4.3.3 (−)-Limonene transformation altered gas exchange responses to drought but not PSII efficiency

Net photosynthesis rate (A_{net}) and stomatal conductance (g_s) significantly (P<0.001) decreased as Ψ_{leaf} declined in both genotypes (Fig. 4.4) consistently in both experiments (P = 0.27). Well-watered plants showed similar photosynthesis rates (approximately 11 μmol m^{-2} s^{-1}) but variable g_{sw} (0.16 – 0.35 mol m^{-2} s^{-1}). Photosynthesis declined as Ψ_{leaf} decreased (Fig. 4.4a), with LG12 tending to decline more rapidly with leaf water stress (Geno × Ψ_{leaf}: P= 0.056). While A_{net} of WT plants decreased almost linearly (at a rate of

7.5 µmol m^{-2} s^{-1} MPa^{-1}), A_{net} of LG12 plants declined more steeply with Ψ_{leaf} until reaching similar values to the WT. Thus, LG12 plants had lower A_{net} than WT plants at intermediate leaf water status (Ψ_{leaf} between -1.0 – -1.5 MPa), although data were more variable.

While stomatal response to Ψ_{leaf} also varied between genotypes (Geno × Ψ_{leaf}: P= 0.038), this occurred when Ψ_{leaf} was lower than -1.0 MPa with WT plants maintaining slightly higher values by 10-40% (Fig. 4.4b). Furthermore, intrinsic water-use-efficiency (A_{net} /g_{sw}) increased by approximately 4-fold and did not differ (P= 0.47) between genotypes. Following re-watering a day later, A_{net} and g_{sw} showed no genotypic disparity (P= 0.16), both variables regaining 60% and 25% of the WW plants' level on the same day, with (−)-Limonene transformation having no impact on leaf gas exchange recovery after re-watering (Fig. S4.3).

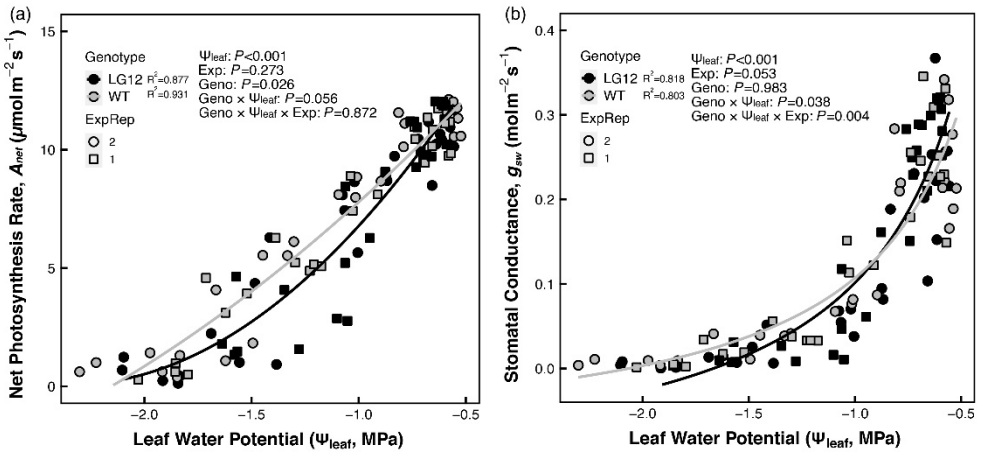

Figure 4. 4 The relationships between leaf water potential (Ψ_{leaf}) and (a) net photosynthesis rate (A_{net}), (b) stomatal conductance (gs) of LG12 (black) and wild-type (WT, grey) plants from Experiment 1 (square) and Experiment 2 (circle). ANCOVA (P-values) results reveal the significant impact of Ψ_{leaf}, experimental replicates (Exp), genotype (Geno) and their interactions on the general linear model (GLM) of corresponding variables. A single regression line was fitted to each genotype when no significant effects of experimental replicates nor interactions were reported.

Although decreased leaf water status markedly (P< 0.001) affected all chlorophyll fluorescence variables except ΦPSII (P= 0.94, Fig. 4.5b), both genotypes responded similarly. F_v'/F_m' decreased by approximately 22% throughout the experiments (Fig. 4.5a). Notably, F_v/F_m increased by more than 10% as Ψ_{leaf} decreased in both genotypes, from 0.7 to just over 0.8 (Fig. 4.5c), which is similar to WW plant throughout the experiment (Fig. S4.4). Suggesting that F_v/F_m may increase as plants age. NPQ increased linearly as Ψ_{leaf} decreased in both genotypes (Fig. 4.5d). Thus, (−)-Limonene

and its precursor transformation do not alter PSII efficiency and other chlorophyll fluorescence variables as leaf water status declines.

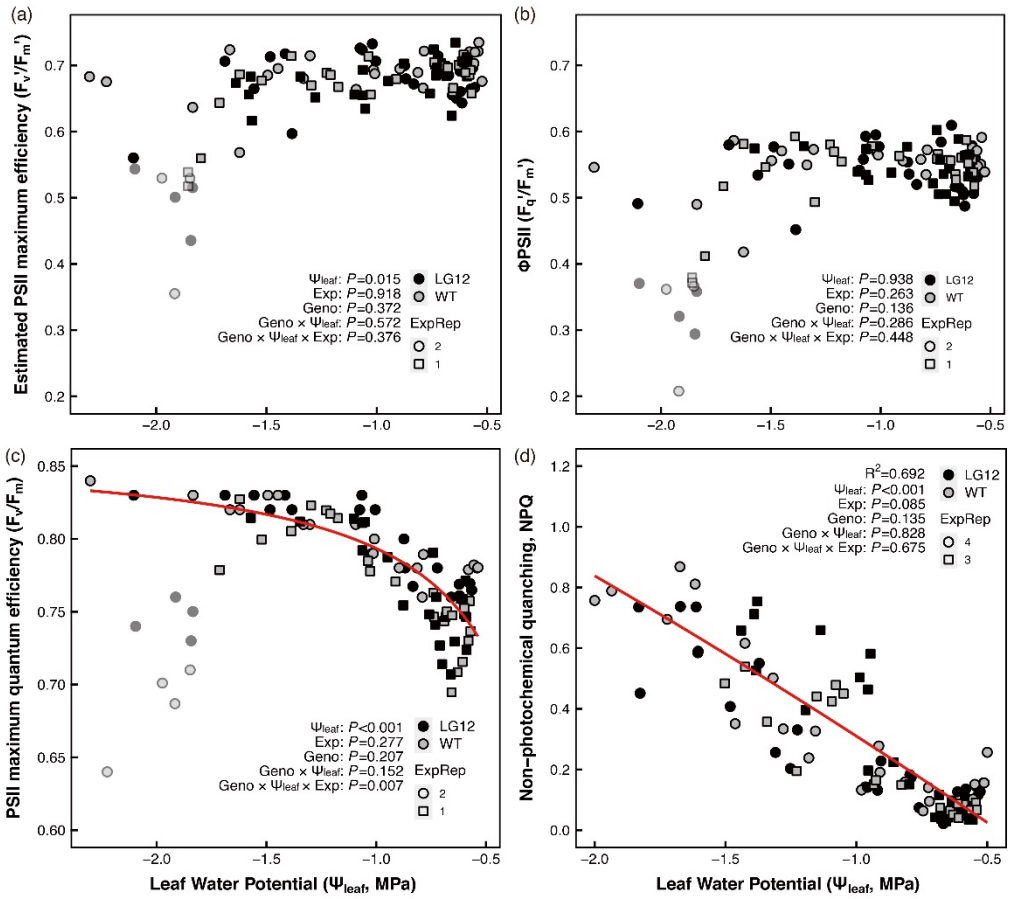

Figure 4. 5 The relationships between leaf water potential (Ψ_{leaf}) and (a) estimated PSII maximum efficiency (F_v'/F_m'), (b) ΦPSII (F_q'/F_m'), (c) PSII maximum quantum efficiency (F_v/F_m), (d) non-photochemical quenching (NPQ). LG12 (black) and wild-type (WT, grey) plants from experiment 1 (square) and experiment 2 (circle). Outliers (faded points) were identified based on the standardised residual larger than three. ANCOVA results (*P*-values) indicate the significant impact of Ψ_{leaf}, experimental replicates (Exp), genotype (Geno) and their interactions on the GLM of corresponding variables.

4.3.4 (−)-Limonene transformation downregulated APX activity, increased lipid peroxidation under drought, which was reversed after re-watering

Drought led to H_2O_2 accumulation and lipid peroxidation and enhanced the activity of enzymatic antioxidants in both LG12 and WT plants (Fig. 4.6). In both genotypes, H_2O_2 content increased 2.5-fold upon withholding water and then stabilised at Ψ_{leaf} < -1.0 MPa (Fig. 4.6a). Foliar MDA content in WT plants increased 3.5-fold before stabilising at Ψ_{leaf} < -1.5 MPa. MDA values were approximately 30% higher in LG12 (Fig. 4.6b),

resulting in a significant (P= 0.029) genotype × Ψ_{leaf} interaction (Fig. 4.6b). Meanwhile, SOD activity increased nearly 7.5-fold, similarly in both genotypes, by the end of drought treatment (Fig. 4.6c) and was positively related with H_2O_2 activity with similar correlation in both genotypes and no significant Geno × Ψ_{leaf} interaction (P= 0.06 in LG12 and 0.43in WT, figure not shown). This suggests that the inserted gene expression and emission of (−)-Limonene did not modify the SOD-catalysed transformation from main ROS (i.e., superoxide radicals) to H_2O_2. Conversely, foliar APX activity approximately doubled in LG12 plants but increased more than 3-fold in WT plants (Fig. 4.6d). Thus, inhibition of APX activity in (−)-Limonene transformed plants could result in higher lipid damage (MDA accumulation) under water stress.

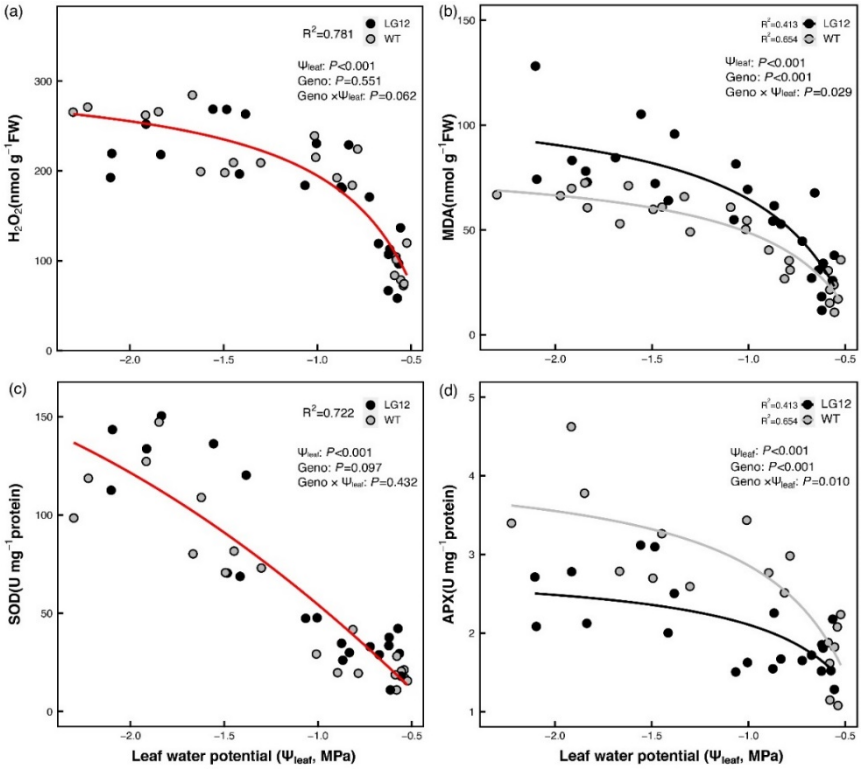

Figure 4. 6 The relationships between leaf water potential (Ψ_{leaf}) and (a) H_2O_2, (b) MDA, (c) SOD, (d) APX of LG12 (black) and wild-type (WT, grey) plants under water deficit, based on data from experiment 2. 2-way ANOVA results (P-values) reveal the significant impact of Ψ_{leaf}, genotype (Geno) and their interactions on the GLM of corresponding variables. A single regression line was applied in cases where no significant effects of experimental replicates or interactions were reported.

Foliar H_2O_2 content and SOD activity were correlated and found to be similar in both genotypes without significant interaction (Geno × SOD: P = 0.52) with respect to Ψleaf (Fig. 4.7a). Under the same levels of APX activity, LG12 plants had significantly higher leaf MDA content than the WT plants, and the MDA content accumulated at a rate twice that of WT (Fig. 4.7b). This suggests that the APX-mediated ROS detoxification

pathway was less effective in transgenic tobacco. Linear correlations were also observed between antioxidant enzymes (Fig. 4.7c), and between H_2O_2 content and lipid damage (Fig. 4.7d). The rate of increase in APX activity relative to SOD activity was almost three times higher in WT than in LG12 plants. This suggests a low APX activity and/or a blocked SOD to APX enzymatic transmission pathway in transgenic tobacco. In addition, the increase in MDA content due to H_2O_2 was 1.5 times higher in LG12 than in WT plants. This indicates an increase in non-H_2O_2-induced lipid peroxidation in transgenic tobacco. Thus, (−)-Limonene transformation resulted in the downregulation of the enzymatic antioxidant pathway and an additional source of lipid damage.

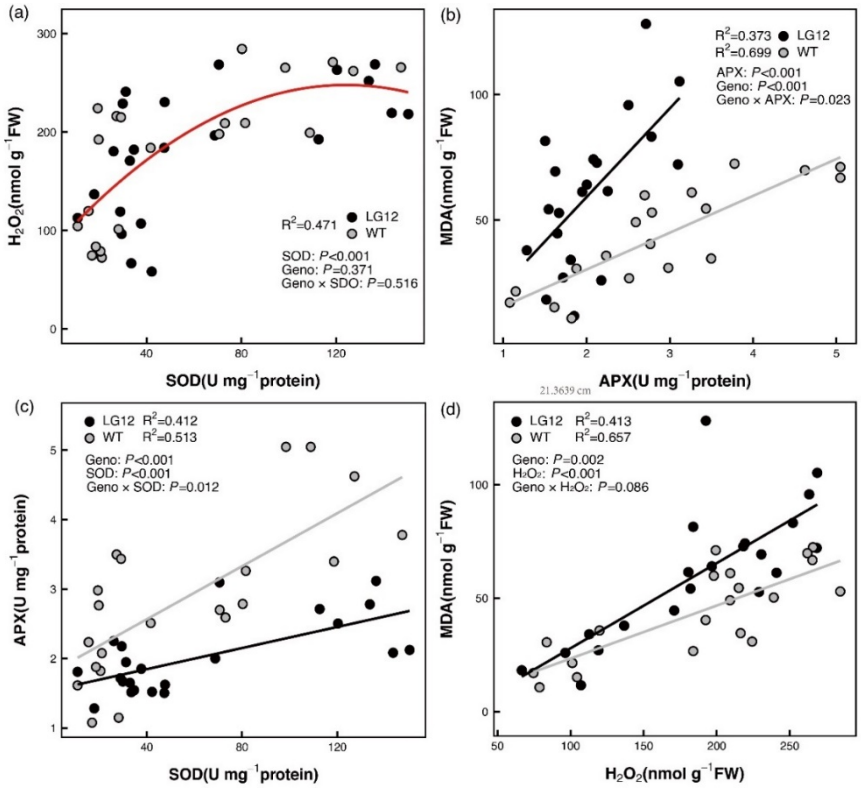

Figure 4. 7 Correlations between (a) H_2O_2 and SOD, (b) MDA and APX, (c) APX and SOD, (d) MDA and H_2O_2 are presented for LG12 (black) and wild-type (WT, grey) plants under water deficit, data and statistical analysis followed Figure 4.6.

After re-watering, foliar lipid damage and enzymatic antioxidant activity varied between genotypes. Rewatering decreased H_2O_2 content similarly (P= 0.84) in both genotypes (Fig. 4.8a) by about half from its peak during drought (Fig. 4.6a). Rewatered plants had lower MDA content than well-watered plants, with LG12 values roughly 60% that of WT plants (Fig. 4.8b). SOD activity remained elevated in WT plants after re-watering and was approximately 25% greater than in other treatments, but LG12 plants had similar SOD activity in well-watered and re-watered plants (Fig. 4.8c). Re-watering significantly (P< 0.001) boosted APX activity in both LG12 and WT plants,

nearly doubling in WT plants and a further 40% higher in LG12 plants, resulting in a significant (P=0.013) interaction (Fig. 4.8d). Thus, (−)-Limonene emission in tobacco enhanced APX activity of re-watered plants and attenuated lipid peroxidation compared to WT plants.

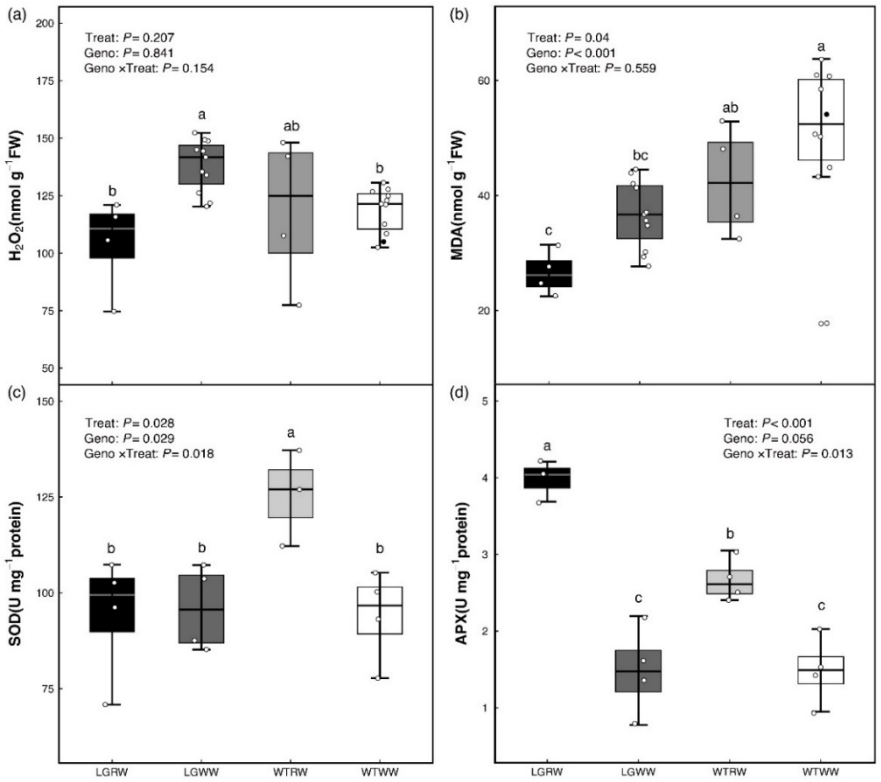

Figure 4. 8 Foliar ROS status and enzymatic antioxidant activity of tobacco plants after re-watering. Boxplots (±SE) present the data for both LG12 and wild-type plants after re-watering (LGRW, WTRW) and well-watered plants (LGWW, WTWW) on the same day, post hoc Tukey test results indicate the significant differences between treatments by letters. 2-way ANOVA results (*P*-values) reveal the significant impact of water treatment (Treat), genotype (Geno) and their interactions on the GLM of corresponding variables.

4.4 Discussion

Monoterpenes can potentially enhance plant physiological and biochemical resilience to abiotic stresses (Vickers *et al.*, 2009a; Nogués *et al.*, 2015a; Bertamini *et al.*, 2021), with exogenous monoterpenes having a potential antioxidative role in drought-stressed plants (Zhou *et al.*, 2023). Leaf water potential (Ψ_{leaf}) of the genetically transformed line LG12, a (−)-limonene emitter, responded less to drying soil than WT plants, potentially was growth limited (Chapter 3, Fig. 3.3). Comparison of the responses of wildtype and

LG12 plants to drought revealed a smaller decline in Ψ_{leaf} in LG12, contributing to better turgor maintenance (Fig. 4.2). Drought affected the emission pattern of (−)-Limonene in LG12 plants, with an initial increase as soil moisture declined, followed by a sharp drop at the end of the drought cycle, exhibiting an approximately cubic relationship with soil moisture (Fig. 4.3). Net photosynthesis rate (A_{net}) and stomatal conductance (g_{sw}) significantly decreased as Ψ_{leaf} declined in both genotypes, with a greater decline in LG12 plants (Fig. 4.4). Drought also induced lipid damage and inhibited APX activity (Fig. 4.6b&d) in (−)-limonene overproducing tobacco plants. After restoring soil moisture to well-watered (WW) levels, (−)-limonene emission rate at a baseline level in LG12 appears to reduce lipid damage and enhance APX activity (Fig. 4.8) but didn't affect gas exchange. The potential improvement in drought resilience of tobacco plants with (−)-Limonene may depend on its emission rates, which influence oxidative damage under varying water conditions by modulating redox balance and enzymatic antioxidant activity.

4.4.1 Foliar (−)-Limonene emission responses to soil moisture

Since emission of other monoterpene compounds was several orders of magnitude lower than that of (−)-limonene (Chapter 3, Fig. 3.1); this study focuses solely on the response and effect of (−)-limonene under drought. *Nicotiana tabacum* (cv. SR1) is not a native producer of monoterpenes (Lücker *et al.*, 2004; Yin *et al.*, 2016), and no foliar monoterpene content was detected in the WT plants. This suggests that the emissions in LG12 plants are not from the storage pool but are likely direct (*de novo*) emission, synthesised and released immediately. Direct emission response of LG12 plants to drought was similar to non-stored emissions and depended on the duration and magnitude of the stress (Chapter 3, Fig. 3.1). The (−)-limonene emission rate (E_{lim}) of LG12 plants under drought exhibited two distinct phases: 1) a smooth increase, where the absolute E_{lim} increased more than fourfold from withholding water to the point where leaf physiological water stress became apparent (i.e., the inflection point of the soil moisture to Ψ_{leaf} relationship curve); and 2) a sharp decrease from the highest E_{lim} level to the basal level, with the rate of Ψ_{leaf} decline under soil drought nearly six times higher than in the previous phase, almost reaching the wilting point of LG12 plants. This response differs from that of transgenic tobacco emitting isoprene, where the emission rate declined continually with soil drying until a lower, stable value is reached (Ryan *et al.*, 2014). Although isoprene emission is less sensitive to water stress than

photosynthesis (Sharkey and Loreto, 1993; Centritto *et al.*, 2011), the dependence of isoprene synthesis/emission on light, photosynthetic carbon, and energy (Delwiche and Sharkey, 1993; Sharkey *et al.*, 2007b) may explain the differing response of monoterpene-emitting transgenic tobacco under temperature-controlled conditions. Although the E_{lim} was two to five-fold higher than previously measured in Chapter three (Fig. 3.1), potentially due to differences in measuring instruments and flow rates in the leaf cuvette, the pattern of (−)-limonene emission in transgenic LG12 plants (Fig. 4.3a) remained consistent under water-deficit conditions and significantly correlated with soil moisture.

A certain degree of drought stress promotes direct monoterpene emissions in controlled environments, especially in plant species without specific storage organs, such as *Rosmarinus officinalis* (rosemary, Ormeño et al., 2007; Nogués et al., 2015) and *Quercus ilex* (Holm oak, Loreto et al., 1996; Staudt and Bertin, 2002), that lack volatilization from storage pools. These studies also linked emission behaviour to various water stress indicators such as soil moisture, leaf water content, and water potential (Ormeño *et al.*, 2007; Wang *et al.*, 2022). Halving relative soil water content increased *de novo* α-pinene emissions from Holm oak by more than 60% (Wu *et al.*, 2015), while a 10%-30% decline in relative leaf water content doubled its total emissions (Blanch *et al.*, 2007; Blanch *et al.*, 2009), with a more rapid response than terpene-storing species under short-term drought conditions. Total monoterpene emissions (including limonene) of rosemary increased by over 20% with a 20% decline in leaf water content (Nogués *et al.*, 2015b). Halving in leaf water potential increased monoterpene emission rates of various species two- to four-fold (Ormeño *et al.*, 2007). At water stress levels reaching the wilting point and with low leaf water content (<50%) or soil water content (<10%), previous studies were almost unanimous in reporting an exponential decrease in emission rates. The decline of non-oxygenated monoterpene (e.g., (−)-limonene and (−)-α-pinene) emissions seems more influenced by severe photosynthetic inhibition and *de novo* biosynthesis due to impaired carbon metabolism (Nogués *et al.*, 2015b; Wu *et al.*, 2015; Kreuzwieser *et al.*, 2021; Byron *et al.*, 2022). Our results suggested that E_{lim} in LG12 plants was more correlated with soil moisture than with Ψ_{leaf} (Fig. 4.3), and the increase in E_{lim} was independent of photosynthetic response. This implies that the emission behaviour of LG12 plants is not C-limited regulation. Production and emission of (−)-limonene from LG12 plants similar to those

native monoterpene emitters without specific storage pools and mechanisms may vary depending on drought stress levels.

Unlike native emitters (Zhan *et al.*, 2022), tobacco doesn't possess the genetic mechanisms for monoterpene biosynthesis under drought stress. Constitutive expression levels of the inserted limonene and GPP synthases, driven by the promoter *Ubiquitin 10*, remained constant throughout the experiment. This suggests a continuous and direct biosynthesis of (−)-limonene without monoterpene cyclase and precursor limitations. Under mild photosynthetic limitations caused by drought stress, the remaining photosynthetic activity is sufficient to drive terpene synthesis in native monoterpene emitters (Brilli *et al.*, 2007b; Nogués *et al.*, 2015a; Tattini *et al.*, 2015). The biosynthesis of monoterpenes is highly influenced by intercellular CO_2 (*Ci*) concentration and photosynthetic electron transport (see review in Chapter one), which provide energy and facilitate sugar production during drought stress (Loreto *et al.*, 1996b; Staudt and Bertin, 1998; Rasulov *et al.*, 2009a; Rasulov *et al.*, 2009b). Monoterpene synthesis generally requires less than 4% of the total electron flux (Niinemets *et al.*, 2002c) and 0.4% of photosynthetically assimilated carbon (Tingey *et al.*, 1980a), alternative carbon sources in plant cells may contribute to increased monoterpene production under drought conditions (de Souza *et al.*, 2018; Kreuzwieser *et al.*, 2021). Presumably, transformed tobacco also has similar feedback mechanisms in resource re-allocation for supporting (−)-limonene overproduction.

Terpene biosynthesis is suggested to act as an energy transfer and dissipation pathway for other metabolic activities such as photosynthesis, driven by its reducing power and energy demands. However, this may not the case as the PSII efficiency and NPQ in this study did not change under drought conditions in MEP pathway enhanced tobacco plants. When harvested photon energy exceeds photosynthetic capacity, plants reallocate carbon and electron transport towards terpene synthesis (Harrison *et al.*, 2013; Li and Sharkey, 2013). Low intercellular CO_2 (*Ci*) limits photosynthesis by decreasing Rubisco activity; excess energy and NADPH are then directed towards MEP flux, inducing terpene production (Sharkey and Loreto, 1993; Perez-Gil *et al.*, 2024). Modelled monoterpene emission rates in response to *Ci* suggest that plants can maintain monoterpene emissions at a basal energy cost when *C*i is higher than 200 μmol mol^{-1}. However, the relative energy cost for monoterpene production and emission dramatically increases when *Ci* falls below 200 μmol mol^{-1}, potentially due to

photoinhibition and increased photorespiration (Niinemets *et al.*, 2002a; Niinemets *et al.*, 2002c). Under drought conditions, although *Ci* decreases due to stomatal closure and CO_2 assimilation declined, electron transport and photosynthetic efficiency are maintained (Fig. 4.5). This increases the supply of electrons for MEP biosynthesis relative to carbon fixation and photosynthetic electron transport, potentially promoting direct synthesis of (−)-limonene. As suggested by modelling studies on *Ci* thresholds, additional electron transport and upstream enzymes (e.g., DAMDP) in the MEP pathway were insufficient to support overproduction of (−)-limonene. Constitutive expression of the inserted genes ensures the essential synthesis of (−)-limonene and its precursor (GPP), resulting in the emission rate returning to basal levels when *Ci* fell below 200 μmol mol^{-1} (Fig. S4.5). Electron transport within the biosynthetic pathway likely regulated the initial burst of E_{lim} as *Ci* decreased, while biochemical limitations of secondary metabolites probably caused the sharp decline of E_{lim} during severe water stress. Nevertheless, the potentially disrupted energy balance did not affect photosynthetic efficiency or alternative energy dissipation pathways (e.g., NPQ), highlighting the remarkable physicochemical plasticity of plants under stress conditions. Further research is required to better understand the importance of photosynthetic energetics and resource allocation in driving monoterpene production mechanisms in these transgenic tobacco plants.

Terpene emissions from *de novo* biosynthesis are less dependent on stomatal control. No direct effect of stomatal decline on (−)-limonene emission (Fig. S4.6) under water stress was observed, particularly during the first phase. Previous studies suggested that terpene concentrations in the leaf's lipid and aqueous phases may serve as temporary storage structures to compensate for emissions under drought in species lacking specific storage pool (Staudt and Bertin, 1998; Niinemets *et al.*, 2004). However, monoterpenes were not detected in the leaves, suggesting they are not stored in any form in transgenic tobacco and are emitted continuously.

In addition, temperature often influences the emission kinetics of monoterpenes (Loreto *et al.*, 1998c; Copolovici *et al.*, 2005; Bourtsoukidis *et al.*, 2024). By causing stomatal closure and restricting transpiration, persistent drought leads to increased leaf surface temperatures (Reynolds-Henne *et al.*, 2010). A moderate temperature increase (20~30°C) contributes to the fraction of photosynthetic electron transport involved in monoterpene synthesis, thereby providing energy and promoting both synthesis and

emission (Loreto *et al.*, 1996b; Bertamini *et al.*, 2021). Leaf temperature also affects the physicochemical properties of monoterpenes and influences their biosynthesis (Guenther *et al.*, 1995; Niinemets and Reichstein, 2003). As temperature increases, Henry's law constant for (−)-limonene rises by 1.65 times per 10°C, while the octanol/water partition coefficient slightly decreases by 1.24 times per 10°C (Copolovici and Niinemets, 2005). This suggests that (−)-limonene becomes more volatile as leaf temperature increases, thereby shifting to the gas phase. Leaf temperatures exceeding 40°C are necessary to inhibit photosynthesis and terpene synthase activity in species like tomato and oak (Staudt and Bertin, 1998; Lehning *et al.*, 1999; Fischbach *et al.*, 2000). Since the maximum leaf surface temperature in the experiment was only 25°C (Fig. S4.7), it is unlikely that temperature inhibited synthase activity thereby causing the sudden emission decline in the second phase. Nevertheless, severe drought-induced ROS disrupts lipid structure, significantly reduces lipid content in leaves (Gigon *et al.*, 2004), and promotes cuticle layer formation in tobacco species (Cameron *et al.*, 2005). This reduces lipid partitioning and permeability and increases the cuticle barrier to volatile emissions (Widhalm *et al.*, 2015). However, E_{lim} and T_{leaf} were not correlated (Fig. S4.8). Thus, downregulated MEP flux during the later stages of water stress likely limits (−)-limonene production thereby significantly decreasing emission, exacerbated by emission pathway constraints caused by reduced lipid-air partitioning of (−)-limonene.

After rewatering, foliar (−)-limonene emission in LG12 plants remained at the same low level observed during the late drought stage, at 75% of WW plants (Fig. 2), similar to photosynthetic activity (Fig. S4.3). Monoterpene emission rates significantly increased after rewatering and recovered more quickly than photosynthesis rate in *Quercus ilex* (Peñuelas *et al.*, 2009). However, *de novo* emissions in some species only recovered to 20-60% of the levels observed under unstressed conditions (Bertin and Staudt, 1996; Lüpke *et al.*, 2017). This limited recovery may be related to the specific degree of water stress (Staudt *et al.*, 2008) and the extent of damage sustained by the plants with continued metabolic imbalance (Pinheiro and Chaves, 2010). Plants change their resource investment strategy for essential activities such as hydraulic repair and starch storage after rewatering (Abid *et al.*, 2018; Guo *et al.*, 2021), but transgenic tobacco may lack the rewatering responses that are present in native emitters.

4.4.2 Transgenic tobacco preserves leaf water status but downregulates gas exchange under drought

LG12 plants had higher leaf water potentials than WT plants within the soil moisture range of approximately 15–35% (Fig. 4.2a) and lost turgor more slowly than WT plants (Fig. 4.2b). By the end of the experiment, WT plants were mostly wilted, while LG12 plants remained turgid. Since sowing time was adjusted to minimise genotypic variations in plant morphology, stomatal closure in LG12 plants may be more sensitive to drought, reducing stomatal conductance during moderate water stress (Ψ_{leaf}: -1.0 to -1.5 MPa, Fig. 4.4b), thereby reducing leaf water loss via transpiration. However, this did not significantly improve intrinsic water use efficiency (iWUE) in LG12 plants. Stomatal closure can be hormonally regulated by abscisic acid (ABA). The MEP pathway is involved in the biosynthesis of ABA in plants (Milborrow, 2001) and MEP-dependent ABA pool responds to environmental stresses rapidly and also regulates stomatal closure (Barta and Loreto, 2006). However, the upregulation of terpene (i.e. isoprene) production in tobacco downregulates leaf ABA concentrations in some lines, and increased stomatal closure under drought is ABA-independent (Ryan *et al.*, 2014) (Ryan et al., 2014). This may be due to the precursor (e.g. IPP and GPP) competition between terpene production and downstream biosynthesis of MEP pathway such as β-carotene, which is metabolised to ABA (Milborrow, 2001). The inserted *MpGPS.SSU* in tobacco provides sufficient GPP but monoterpene production may compete with downstream synthesis for IPP, decreasing foliar β-carotene concentration compared to WT plants, yet simultaneously increasing gibberellin (GA) 3 concentration and upregulated gibberellin 20-oxidase (Yin *et al.*, 2016). Normally downregulation of GA activity prevents plant water loss under drought by promoting stomatal closure, growth regulation and osmotic adjustment (Shohat *et al.*, 2021). However, the hormonal variation in transgenic tobacco under drought is unknown. In addition, we do not know the impact of genetic transformation in LG12 plants on root phytohormone levels, with ABA- and GA-mediated root-to-shoot signalling also important for plant water relations under drought conditions (Dodd, 2005; Gaion and Carvalho, 2021). Plant's foliar anatomical traits also significantly influence hydration status. Palisade cells contribute to water retention and maintain the photosynthetic process, and the water stored in the spongy tissue also facilitates gas exchange (Zhang *et al.*, 2018; Khan *et al.*, 2020). Smaller leaf cells also contribute to water retention under drought conditions (Martínez *et al.*, 2007). The minimum leaf conductance under severe drought stress, which is

determined by cuticular water loss and stomata that are not fully closed, also affects leaf water sensitivity to soil water availability (Duursma *et al.*, 2019). Although transformed tobacco LG12 preserved cell turgor under drought, the specific mechanisms and potential anatomical and hormonal changes resulting from genetic engineering require further investigation.

Maintenance of leaf water status in transgenic tobacco did not translate into photosynthetic benefits; rather net photosynthetic rate decreased more rapidly than WT plants after drought (Fig. 4.4a) and was not correlated with PSII photosynthetic efficiency (Fig. 4.5). Although lower stomatal conductance may reduce the photosynthetic rate by limiting intercellular CO_2 (*Ci*), the extent of photosynthetic downregulation was more pronounced. Thus, stomatal conductance may not be the dominant cause of photosynthetic limitation in LG12 during drought stress. Further CO_2 response experiments are needed to understand more detailed photosynthetic regulation in transgenic tobacco.

The relative energy cost of terpene compound production is higher than that of other metabolic pathways such as photosynthesis and phenolic compound production, and enzyme costs are also substantial and largely independent of other biosynthetic pathways (Gershenzon, 1994). The inserted genes provided sufficient terpene synthase and precursors for (−)-limonene production. However, the constitutive expression of the *de novo* synthesis pathway and the increased emission of (−)-limonene under water stress require additional endogenous resources, such as 3-phosphoglycerate (3-PGA) and ATP, which potentially compete with other metabolic processes (Chatzivasileiou *et al.*, 2019). The Calvin-Benson cycle in C3 plants requires six molecules of NADPH and nine molecules of ATP to fix three molecules of CO2. The fixed CO2 reacts with RuBP to form 3-phosphoglycerate (3-PGA), which is subsequently enzymatically converted to glyceraldehyde 3-phosphate (G3P). The G3P is then used to regenerate RuBP, thereby maintaining the cycle. 3-PGA is converted to pyruvate through the glycolytic pathway, while pyruvate and G3P are initial precursors of the MEP pathway, which requires 12 molecules of fixed CO_2 (Vranova *et al.*, 2013). Furthermore, foliar monoterpene production requires electrons for sugar production and additional photosynthetic electron transport for sugar reduction, which is necessary for the synthesis of specific compounds based on NADPH cost (Loreto *et al.*, 1996b; Zhao *et al.*, 2013). Due to their chemical structure, monoterpenes (e.g., limonene, α/β-pinene) require twice as much

NADPH and ATP for synthesis compared to isoprene (Table 2). Therefore, the imbalance in energy and the consumption of three-carbon molecules between terpene synthesis and photosynthesis is exacerbated in (−)-limonene-producing tobacco. This may explain why isoprene producing tobacco did not show photosynthetic reduction under drought (Ryan *et al.*, 2014). ΦPSII in LG12 plants was not affected by drought (Fig. 4.5b), indicating that total photosynthetic electron transport remained consistent. The model (Niinemets *et al.*, 2002c) suggests that more than a twofold increase in (−)-limonene emission (Fig. 4.3) requires more than a threefold increase in available electron transport. Presumably, these additional substrate and energy requirements further suppressed the normal functioning of the Calvin-Benson cycle under drought conditions, particularly the reduction and Rubisco regeneration processes. Nevertheless, it has also been revealed that photosynthetic products synergise with limonene synthesis in transgenic photosynthetic organs (e.g., cyanobacteria) and increase the non-native MEP flux through photosynthetic upregulation (Wang *et al.*, 2016). More physio-biochemical data are needed to better understand the mechanisms of monoterpene regulation and its effects on photosynthetic activity in transgenic crops under stressed conditions.

Table 4. 1 The average NADPH and ATP (mol mol^{-1}) costs of the fixation of three molecules of CO_2 in Calvin-Benson cycle, isoprene and monoterpene production via MEP pathway (12 molecules of CO_2 are required). terpene data from the average of terpene production in tree species such as *Quercus coccifera* and *Q. ilex* (Niinemets *et al.*, 2002c). Monoterpene production costs more energy and substrate than isoprene.

Compounds	**NADPH (mol mol^{-1})**	**ATP (mol mol^{-1})**
Calvin-Benson (3 CO_2)	6	3
Isoprene	14	20
Monoterpenes (e.g., Limonene α/β-Pinene)	28	40

4.4.3 Genetic transformation increases lipid damage under water stress and APX activity following re-watering

Drought conditions led to the accumulation of H_2O_2 and lipid peroxidation in both LG12 and WT plants. The formation of H_2O_2 catalysed by SOD was not affected by genetic transformation as foliar H_2O_2 content and SOD activity were consistently correlated (Fig. 4.7a), but LG12 plants accumulated more foliar MDA under water

stress (Fig. 4.6), apparently because APX activity was downregulated. APX provides membrane protection in plant drought resistance. Overexpressing cytoplasmic APX in tobacco reduced lipid peroxidation and electrolyte leakage, and improved water use efficiency, accompanied by the upregulation of non-enzymatic antioxidants such as ascorbate and glutathione (Faize *et al.*, 2011). Conversely, knockdown of APX gene expression increased lipid damage and cell death under oxidative stress (Kuo *et al.*, 2020). Plants often have compensatory mechanisms to mitigate the effects of reduced APX activity on oxidative homeostasis. For example, rice lacking APX4 (a peroxisomal ascorbate peroxidase) upregulated photosynthetic activity and associated proteins, along with other oxidative pathways such as the ascorbate/glutathione cycle, to adapt metabolic processes and prevent oxidative stress (Sousa *et al.*, 2015; Sousa *et al.*, 2018). APX activity is also closely related to ascorbate levels in plants. Thylakoid APX is rapidly inactivated when ascorbate levels are insufficient (<20 μM) due to the destabilization of the APX active intermediate in the presence of H_2O_2 in an ascorbate-depleted environment (Miyake and Asada, 1996). Ascorbate also plays a key role in the Mehler reaction by maintaining electron transport under environmental stress and photosynthetic limitation, thereby reducing oxidative damage to the photosynthetic apparatus (Ort and Baker, 2002; Foyer and Shigeoka, 2010). Furthermore, some APX isomers regulate leaf senescence and long-distance root stress signalling (Panchuk *et al.*, 2005; Leng *et al.*, 2021), which may contribute to the morphological differences observed between transgenic and wildtype tobacco. In addition, APX activity increases as the activity of other antioxidant enzymes such as SOD increases (Foyer and Shigeoka, 2010). In the Mehler reaction, the SOD-catalysed conversion of superoxide (O_2^-) to H_2O_2 and the subsequent reduction to water catalysed by APX is a continuous process (Asada, 1999). APX activity in LG12 plants increased with SOD activity under drought significantly less than in WT plants (Fig. 4.7c), suggesting downregulated APX expression and activity and/or reduced enzymatic response, i.e., low O_2^- level. The effects of genetic transformation on oxidative stress and metabolic signalling in tobacco under drought are complex. Whether the reduction in APX activity is due to the downregulation of gene expression or other synergistic effects, and their impact on primary and secondary metabolism, require further investigation.

MDA accumulation in LG12 was higher than in WT plants at the same foliar H_2O_2 level (Fig. 4.7d). Hence, we could not rule out the possibility that (−)-limonene directly induced more toxic ROS causing greater oxidative damage in transgenic tobacco.

Exogenous monoterpenes above 2.5 mM (Ibrahim *et al.*, 2006; Zhou *et al.*, 2023) and endogenous monoterpenes above 1 mM (Widhalm *et al.*, 2015) can be phytotoxic, directly inducing oxidative stress and limiting growth and development. Possibly the higher foliar MDA content in LG12 than WT plants result from reduced detoxification due to downregulated APX activity and toxic effects from high intercellular (−)-limonene concentrations with increased emission rates under drought. Specific ROS species (e.g., hydroxyl radical, O_2^- and singlet oxygen) may also regulate stomatal closure (Sierla *et al.*, 2016). Presumably, increased ROS accumulation due to (−)-limonene production may be one of the reasons for the enhanced stomatal closure in LG12 plants under drought. Nevertheless, there was no genotypic difference in PSII efficiency, which was minimally impacted by drought (Fig. 4.5a & b), or in net photosynthesis rate after recovery (Fig. S4.3). This suggests that the additional lipid damage was insufficient to affect the photosynthetic apparatus in LG12, and that oxidative stress caused similar damage to photosynthesis in both genotypes. Applying limonene and H_2O_2 exogenously may verify the cause of additional lipid damage, and other endogenous ROS components and concentrations in LG12 and WT plants need to be measured.

Conversely, the higher APX activity of LG12 plants likely alleviated H_2O_2 content, lipid peroxidation, and SOD activity during the recovery period following rewatering. The presence of low (−)-limonene emission and/or genetic transformation of the MEP pathway may enhance their antioxidant defences and diminish oxidative damage. In Chapter 2, a low concentration (1.25 mM) of exogenous monoterpenes decreased leaf H_2O_2 accumulation and MDA content without affecting antioxidant enzyme activity, while higher concentrations (2.5 and 5 mM) simultaneously upregulated enzyme activity but had weaker antioxidant benefits. Endogenous (−)-limonene production and emission in tobacco after rewatering significantly decreased SOD activity and H_2O_2 levels compared to WT plants. SOD catalyses primary ROS into less toxic H_2O_2, LG12 plants may have mitigated ROS production at the source or reduced its accumulation by using (−)-limonene production reducing both sink and direct quenching (Graßmann, 2005; Vickers *et al.*, 2009b; Nogués *et al.*, 2015a). But not as an additional energy dissipation pathway due to unaffected PSII efficiency and NPQ. A certain level of H_2O_2 in plants is necessary to maintain growth, development, and signalling (Huang *et al.*, 2019a). At the same time, limonene-emitting tobacco upregulated APX after rewatering

and more efficiently removed H_2O_2 accumulated during drought, thereby preventing further lipid damage. Moreover, LG12 plants under well-watered conditions also showed lower MDA content (Fig. 4.8b), suggesting that (−)-limonene production and emission at certain levels contribute to non-enzymatic antioxidation in plants. Monoterpenes can also stabilise membrane structures (Peñuelas and Llusià, 2002; Loreto *et al.*, 2004).

Other than causing damage, ROS also play an important role in cellular and cell-to-cell signalling in response to stress conditions. In particular, H2O2 has emerged as a key signalling molecule due to its ability to diffuse across membranes through aquaporins and relatively long half-life (milliseconds to seconds compared to microseconds of superoxide radicals), enabling it to coordinate local and systemic stress responses (Petrov and Van Breusegem, 2012; Huang *et al.*, 2019a). Upon perception of drought or other stresses, H2O2 accumulation can activate a cascade of downstream events involving transcription factors, phytohormones (e.g., ABA), and antioxidant enzymes. Thus, plants must balance the dual roles of H2O2, serving as both a detrimental oxidant at high concentrations and a vital signal for stress adaptation at low concentrations (Cruz de Carvalho, 2008; Hu *et al.*, 2017; Foyer, 2018). Since WT and LG12 had similar H2O2 responses under drought stress, the additional endogenous (−)-limonene may also interfere with ROS signalling, resulting in different oxidative stress and enzymatic activities. We propose that a low emission rate of (−)-limonene improves antioxidant capacity in plants during the recovery stage following long-term drought through both direct and indirect antioxidant benefits.

4.5 Conclusion

Out study demonstrates that transgenic LG12 tobacco plants emitting (−)-limonene showed improved leaf water status under drought conditions. However, this enhanced drought tolerance does not extend to improvements in photosynthetic performance. Resource competition between (−)-limonene synthesis and photosynthetic activity may downregulate net photosynthesis. Drought initially boosted (−)-limonene emission but also increased oxidative damage to lipids by downregulating APX activity in LG12 plants. Persistent water stress suppressed (−)-limonene emission rate, which was

retained after rewatering. The low production rate of (−)-limonene may help mitigate oxidative damage and improve antioxidative capacity during recovery, through upregulation of antioxidant enzymes like APX and/or synergistically work with antioxidants. The results indicate that the drought tolerance imparted by (−)-limonene depends on the balance between its production rate and the plant's overall photosynthetic and antioxidant capacity. Future research should focus on elucidating the precise metabolic and hormonal changes in genetically modified plants. as well as the long-term physiological impacts of limonene emissions under varying environmental conditions.

5 General Discussion and Conclusions

This research aims to address the existing knowledge gap by investigating the effects of both exogenously applied MTs and the endogenous production of MTs in genetically modified crop species. The hypotheses and concepts in this thesis are primarily inspired by Vickers *et al.* (2009a), who proposed that terpenes could act as antioxidants in plants exposed to abiotic stress conditions. Chapter 2 hypothesised that the uptake of exogenous MTs by plant leaves could reduce foliar oxidative damage under drought conditions by enhancing enzymatic antioxidative capacity such as superoxide dismutase (SOD) and ascorbate peroxidase (APX), thereby decreasing accumulation of reactive oxygen species (ROS). Mitigation of oxidative damage was expected to be proportional to the concentration of applied MTs. Furthermore, it was hypothesised that the protective effect of exogenous MTs would trigger stomatal closure to prevent water loss during drought and by restricting photosynthetic activity would prevent ROS overproduction in the photosystem thus maintaining photosynthetic efficiency.

Drought typically causes in stomatal closure, reduced intercellular CO_2 concentration, diminished photosynthetic activity, inhibited growth, and increased oxidative stress (Foyer and Shigeoka, 2010; Dodd and Ryan, 2016; Ilyas *et al.*, 2021). Monoterpenes (MTs), a class of biogenic volatile organic compounds (BVOCs), have garnered attention due to their significant natural emissions and their potential role in plant responses to environmental changes (Loreto and Schnitzler, 2010; Peñuelas and Staudt, 2010). Despite advancements in understanding the molecular mechanisms of MT biosynthesis, emission and their ecological roles, particularly in plant-plant and plant-insect communication, their potential to alleviate oxidative damage in stressed plants remains insufficiently investigated (Niinemets and Monson, 2013; Possell and Loreto, 2013; Tholl, 2015). While much of the existing research has focused on MT emissions and their function under stresses such as heat, ozone, and light, the physiological and biochemical pathways by which MTs confer stress tolerance, particularly in response to drought, remain insufficiently explored (Niinemets *et al.*, 2002c; Loreto *et al.*, 2004; Jardine *et al.*, 2017). This knowledge gap has underestimated or overestimated MTs' interactions with other stress factors at larger and more complex feedback response in

ecophysiological scale, as well as in local and global biological and climate modelling (Loreto *et al.*, 2014; Seco *et al.*, 2015).

Applying exogenous MTs at 1.25, 2.5 and 5 mM diminished oxidative stress in tomato leaves, as evidenced by lower levels of foliar hydrogen peroxide (H_2O_2) and lipid damage as in malondialdehyde (MDA) content compared to the control treatment. Despite these antioxidative effects, exogenous MTs did not enhance photosynthetic efficiency or gas exchange under drought conditions. This is consistent with studies showing that MTs scavenge reactive ROS and improve antioxidant capacity, but do not significantly impact photosynthesis under short-term stresses like heat (Peñuelas and Llusià, 2002; Loreto *et al.*, 2004; Ibrahim *et al.*, 2006). Monoterpene protective effects and mechanisms varied were concentration dependent. Specifically, the ROS-scavenging role of MTs was more pronounced at a lower concentration of 1.25 mM, which did not significantly affect native enzymatic antioxidants (SOD and APX, Fig. 2.4) but limited oxidative damage the most (Fig. 2.3). In contrast, higher concentrations of 2.5 and 5 mM MTs increased enzymatic antioxidant activity (Fig. 2.4) but showed slightly weaker oxidative damage mitigation (Fig. 2.3). These findings suggest that the protective mechanism is dependent on the concentration of exogenous MTs and their foliar uptake and accumulation. However, the antioxidative benefits in plants did not extend to physiological level, stomatal conductance (g_{sw}), net photosynthesis rate (A_{net}) and photosynthetic efficiency was not affected by exogenous MT applications.

Since exogenous MTs had potential antioxidative effects under drought stress, a similar function was expected for specific endogenous MTs in plants that do not natively produce these compounds. Therefore, Chapters 3 & 4 utilised transgenic tobacco plants engineered to overproduce MTs, such as (–)-limonene, (–)-α/β-pinene, and myrcene. The energetic costs of overproducing MTs in tobacco plants was hypothesised to cause the trade-offs in growth rate and overall biomass. However, transgenic tobacco plants are expected to exhibit lower levels of foliar ROS and reduced lipid peroxidation (measured as MDA content) under drought conditions. Enhanced MT production and emissions play an antioxidant role under drought stress through distinct mechanisms. First, by directly scavenging ROS, less ROS accumulates in transgenic lines, leading to lower MDA content without altering antioxidant activity. Alternatively, MTs may enhance the enzymatic antioxidant capacity (SOD, APX) of transgenic plants. These mechanisms may vary according to the severity of drought stress.

Chapter 3 examined the morphology, gas exchange, leaf water status, and foliar MT emissions across two growth stages: before and during stem elongation, under two water conditions: well-watered and drought. These experiments were conducted on three transgenic tobacco lines with upregulated MT precursor genes and individually inserted genes for (-)-α/β-pinene (PG11), myrcene (MG1), and (–)-limonene (LG12). While foliar MT emissions were nearly undetectable in wild-type plants, genetic transformation significantly increased (–)-α/β-pinene and (–)-limonene emissions in PG11 and LG12, although no significant increase in myrcene emissions was observed in MG1 (Fig. 3.2). Furthermore, inserting a single MT gene increased emissions of other MTs such as (-)-α/β-pinene in LG12 and (-)-limonene in PG11. Compared to wild-type plants, increased MT production reduced g_{sw} in LG12 and increased in PG11 without significant genotypic difference in A_{net}, the g_{sw} difference was diminished as stem elongate (Fig. 3.6a). MT production also enhanced leaf water potential under drought conditions during the elongation stage, regardless of genotypic differences in stomatal conductance (Fig. 3.3). Although there were minimal impacts on stomatal development compared to wild-type plants at both growth stages (Fig. 3.5), genetic transformation decreased biomass, root length, shoot height, and leaf area. PG11 was most adversely affected and LG12 the least (Fig. 3.4). Therefore, LG12 was selected to test the remaining hypotheses in Chapter 4.

While endogenous (–)-limonene emission initially increased as drought stress developed, emission rates dropped significantly as severe water stress occurred (Fig. 4.3). LG12 plants maintained higher turgor pressure by diminishing leaf water status decline under drought conditions, with significant interactions between genotype and soil moisture (Fig. 4.2). Gas exchange rates were significantly lower in LG12 than wild-type plants (Fig. 4.4), with no observed differences in biomass or PSII photosynthetic efficiency between genotypes (Fig. 4.5). In LG12 plants, (–)-limonene transformation downregulated APX activity, leading to higher lipid damage under drought conditions. However, no significant differences were observed in foliar H_2O_2 content, SOD activity or their relationship between genotypes under drought conditions (Fig. 4.6), indicating SOD-catalysed transformation from superoxide radical to H_2O_2 was not affected. Re-watering maintained a basal emission rate of (–)-limonene, but opposite effects of (–)-limonene transformation on oxidative damage and enzymatic antioxidants were observed. LG12 plants exhibited significantly lower overall lipid damage and double the APX activity under both recovery and control conditions compared to wild-type

plants under the same water treatments (Fig. 4.8). A cubic emission rate response curve to soil moisture decline (Fig. 4.3), aligns with some native MT-emitting plants (Šimpraga *et al.*, 2011; Lüpke *et al.*, 2017; Mu *et al.*, 2018). Moreover, (–)-limonene may function as an antioxidant, working synergistically with native enzymatic antioxidants (e.g., APX) to mitigate residual oxidative stress resulting from short-term drought conditions. However, increased MT emissions under stress conditions may be toxic to plant cells and could directly cause oxidative damage.

Table 5. 1 A comparison of the impact of exogenous and endogenous monoterpenes in plants response to water deficit or drought from Chapter 2 – 4.

	Exogenous	Endogenous
Morphology	No impact	Reduce growth rate, total biomass, leaf biomass and area, stem elongation
Leaf water status	No impact	Improve leaf water potential (Ψ_{leaf}); higher turgor pressure
Leaf chlorophyll	Slight chlorosis at 5mM	Reduce chlorophyll content in LG12 and MG1
Gas exchange	No impact	Reduce net photosynthesis rate and stomatal conductance at Ψ_{leaf} between -1.0 – -1.5 MPa
Photosynthetic efficiency	No impact	No impact
ROS and damage	Reduce H_2O_2 content and lipid peroxidation	No impact on H_2O_2 content, lipid peroxidation increases during drought, and decreases after re-watering
Antioxidant enzymes	Increase SOD and APX activity at 2.5 and 5mM	No impact on SOD activity, APX activity decreases during drought and increases after re-watering

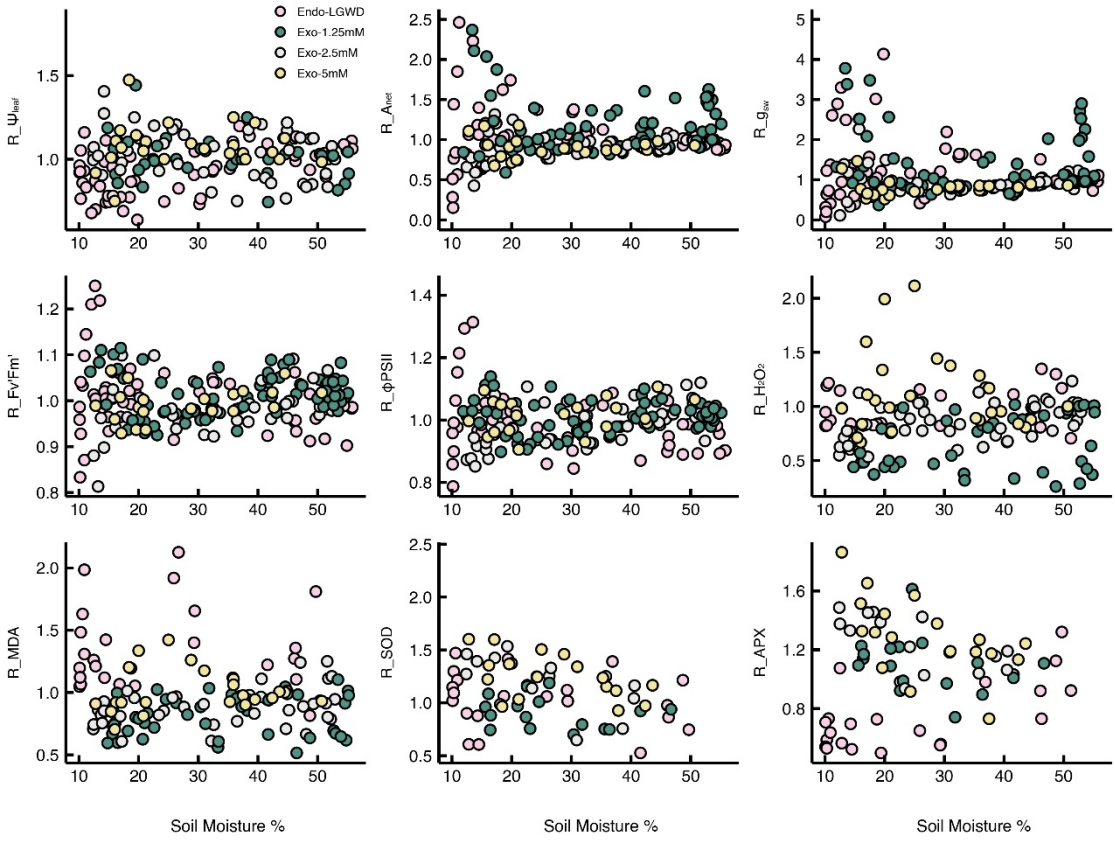

Figure 5. 1 Water-stress responses in tomato and tobacco plants with exogenous and endogenous monoterpenes, evaluating leaf water status, gas exchange, PSII efficiency, oxidative status, and antioxidative enzyme activities. Data are presented as relative values normalised to wildtype tobacco plants for the endogenous (–)-limonene line (LG12), and to 0 mM control plants for exogenous MT applications in tomato plants.

The impacts of exogenous application and endogenous MT are clear as summarised in Table 5.1 and Fig 5.1: exogenous MTs provide short-term oxidative stress relief, but they do not significantly enhance key physiological processes necessary for long-term drought tolerance, such as water-use efficiency and photosynthetic stability (Petrović *et al.*, 2021; Seleiman *et al.*, 2021). By contrast, plants that do not naturally produce MTs but are engineered to produce endogenous MTs can attenuate decline in physiological status such as leaf water potential and oxidative imbalance under intermediate drought stress conditions. For MTs to fully exert their potential antioxidant role, specific cellular concentrations, diffusion conditions, and the physiological state of endogenous MTs in plants are required. Nevertheless, both exogenous and endogenous MTs did not affect the photosynthetic efficiency, suggesting the plasticity of plant photosystems to stress conditions and physiological and biochemical impacts may not affect the status of photosynthetic apparatus. In addition, it is important to note that the Chapters 2 and 4 conducted at different drought regimes. The exogenous MT with tomato plants applied

deficit irrigation by supplying 25% of the daily pot water loss of deficit plants via evaportranspiration, and the endogenous MT with tobacco plants experienced complete **drought** (withheld water). The terms **'deficit irrigation'** will be used for exogenous experiment with tomato and **'drought'** will used for endogenous experiments with tobacco in the rest of this chapter.

5.1 Physiological and oxidative responses of plants with exogenous and endogenous monoterpenes under water stress

Leaf water status and gas exchange responses of plants to drought conditions varied depending on the source of MTs. Tomato Ψ_{leaf} (Fig. 2.1), A, and g_{sw} (Fig. 2.5) remained consistent across exogenous MT treatments under water stress conditions. In contrast, leaf turgor pressure of transgenic tobacco plants over producing MTs endogenously approximately halved compared to wild-type (WT) plants independent of morphology (Fig. 4.2b). Species variation in physiological responses in the presence of MTs may be linked to their drought tolerance. Although both species belong to the Solanaceae family, tobacco seems more physiologically tolerant to water stress than tomato. In drought experiments, tobacco varieties typically maintain photosynthetic activity at lower Ψ_{leaf} ranges (-1.5 – -3 MPa) (Ryan *et al.*, 2014; Rabara *et al.*, 2015; Su *et al.*, 2017; Khan *et al.*, 2020) compared to tomato, which operates within a Ψ_{leaf} range of -1.2 – -1.8 MPa under severe soil moisture reduction (40-60% decrease in relative soil water content) (Rahman *et al.*, 2004; Peco *et al.*, 2023). Similarly, tomato and tomato plants reached Ψ_{leaf} levels as low as -1.7 (Fig. 2.1) and -2 MPa (Fig. 4.2), respectively, before wilting. Notably, tobacco plants required 18 days to reach wilting point under complete water withholding, whereas tomato plants wilted within 7 days under deficit irrigation, probably a function of plant and pot (2L for tomatoes and 3L for tobaccos) sizes.

Tobacco plants, with greater osmotic adjustment capacity, may better retain water in their leaf cells and sustain higher turgor pressure, allowing essential biochemical processes, such as photosynthesis, to continue (Heyser and Nabors, 1981; Binzel *et al.*, 1987; Turner, 2018). For each MPa decline in Ψ_{leaf}, A decreased by approximately 64% in tobacco plants and 85% in tomato plants. Additionally, both species exhibiting near-complete stomatal closure by the experiment's conclusion. Regardless of MT origin, photosynthetic efficiency did not differ between treatments and controls, or between transgenic and wildtype (WT) plants. This suggests that MTs have minimal impact on the plant's photosynthetic apparatus and photochemical processes. Photoinhibition in

both experiments was primarily influenced by physiological and biochemical limitations, depending on the level of stress. Stomatal closure of tomato plants decreased intercellular CO_2 concentration (*Ci*) which limits photosynthesis limitation. In contrast, tobacco exhibited a more gradual shift from physiological to biochemical photosynthetic limitations, with gas exchange more slowly responding to drought. As a result, with potential competition for ATP and NADPH, tobacco plants displayed differences in gas exchange between genotypes under drought conditions. Further measurements about more detailed photosynthetic measurements such as A/Ci curve are needed to better understand the photosynthetic electron transport.

Drought stress triggers the overproduction of ROS, causing oxidative damage of cellular components such as lipid structures (Foyer, 2018). Both exogenous and endogenous MTs demonstrated potential antioxidant effects and phytotoxicity in this study, though the mechanisms differed in their effectiveness and duration. Applying exogenous MTs to tomato plants diminished oxidative stress under deficit irrigation, as indicated by decreased foliar MDA content caused by H_2O_2 accumulation across all concentrations compared to the control (Fig. 2.4A&C). Damage mitigation was most effective at the 1.25 mM concentration and did not significantly impact key antioxidant enzymes, such as SOD and APX. For endogenous MTs, lower foliar MDA content was observed in well-watered plants, which exhibited similar H_2O_2 accumulation and antioxidant enzyme activity as WT plants. After re-watering, when (–)-limonene emission rates returned to well-water levels, APX activity increased, and SOD upregulation was attenuated (Fig. 4.8). Thus, exogenous MTs at a concentration of 1.25 mM and endogenous (–)-limonene at basal emission rate (~20 pmol m^{-2} s^{-1}) potentially could directly quench ROS and mitigate oxidative damage, as observed in both in vivo (Graßmann, 2005; Porres-Martínez *et al.*, 2015) and in vitro (Noacco *et al.*, 2018; Sueishi and Nii, 2018) experiments. Additionally, studies suggest that MTs may have direct (e.g., carotenoids) or indirect (e.g., lutein, ascorbic acid) synergistic effects with other antioxidants (Loreto *et al.*, 2004; Nogués *et al.*, 2015a). Exogenous antioxidants, which can also activate primary antioxidant mechanisms such as the ascorbate–glutathione cycle, may attenuate the upregulation of certain antioxidant enzymes, allowing plants to better manage excess ROS under abiotic stress (Peñuelas *et al.*, 2005; Hasanuzzaman *et al.*, 2020b; Sohag *et al.*, 2020). Future research may focus on measuring changes in leaf content of MT oxides and other non-enzymatic antioxidants to elucidate the specific mechanisms underlying MT effects.

Although H_2O_2 accumulation was lowest in both 1.25 mM and 2.5 mM exogenous MT treatments, at only half the level of the control group, the MDA content increased proportionally with increasing MT concentration (Fig. 2.4B). Similarly, in the endogenous MT experiment with tobacco (a non-native MT producer), increased foliar (–)-limonene emission (two- to three-fold) led to an accumulation of foliar MDA (Fig. 4.6b), indicating direct lipid damage, which is associated with downregulated APX activity in LG12 plants under drought conditions (Fig. 4.7b). The exact cause of the downregulated APX activity remains uncertain. This study measured total APX activity, but APX has multiple isozymes in plant cells, which primarily regulate oxidative stress tolerance in plants (Chen and Asada, 1989). These isozymes exhibit varying sensitivities to stress conditions, for instance, the chloroplast APX isozyme is particularly sensitive to oxidative stress (Kangasjärvi *et al.*, 2008). Limonene transformation in tobacco may not have affected APX expression, as well-watered plants showed no genetic differences in APX activity (Fig. 4.8d). A more plausible explanation is that (–)-limonene production in a non-native producer altered APX effectiveness. APX uses ascorbic acid (AsA) as its specific electron donor to reduce H_2O_2 to water, generating monodehydroascorbic acid (MDAsA), which is then directly reduced back to AsA by NADPH-dependent MDAsA reductase (Asada, 2000; Shigeoka *et al.*, 2002). In conjunction with the AsA-GSH cycle, APX effectively prevents the accumulation of toxic H_2O_2 levels in photosynthetic organisms (Asada, 1992, 1999; Foyer and Shigeoka, 2010). This is particularly important for APX activity in the stroma, microbodies, and cytosol (Shigeoka *et al.*, 2002). Thus, as with the accelerated photosynthetic decline under drought, the decrease in APX activity and H_2O_2 detoxification capacity is likely due to an imbalance of NADPH and ATP caused by limonene overproduction in tobacco plants.

This thesis employed various methodologies for biochemical assays. Chapter 2 analysed H_2O_2 concentration using in-house reagents based on the colorimetric reaction between potassium iodide (KI) and H_2O_2 (Klassen *et al.*, 1994). Although KI has historically been a common reagent for H_2O_2 quantification, it lacks specificity, as it can also react with other ROS and oxidising agents such as chlorine, carbon dioxide, and elemental iodine. This limitation can lead to false-positive results and likely the primary reason for the observed higher foliar H_2O_2 content in tomato compared to tobacco.

Chapters 4 quantified H_2O_2 using the ferrous oxidation-xylenol orange (FOX) assay, based on the oxidation of ferrous ions (Fe^{2+}) to ferric ions (Fe^{3+}) in the presence of xylenol orange (XO). In this reaction, peroxide interacts with sorbitol, generating a peroxyl radical that initiates Fe^{2+} oxidation to Fe^{3+}. In an acidic solution, Fe^{3+} complexes with XO dye to produce a purple product (FOX) with a maximum absorbance at 560 nm. Peroxide levels in test samples were quantified using a standard curve prepared with hydrogen peroxide solutions (Deiana *et al.*, 1999). This method minimizes the potential interference of soluble antioxidants, alternative oxidants, and colour background. However, the FOX assay is sensitive to ascorbic acid in the acidic environment, where ascorbic acid can reduce Fe^{3+} back to Fe^{2+}, diminishing detection sensitivity, particularly when H_2O_2 concentrations exceed 1 μM (Rhee *et al.*, 2010). Consequently, future assays should consider adopting more specific and sensitive detection methods, such as the Amplex Red assay. Additionally, the measurement of enzymatic antioxidant activity was transitioned to commercial assay kits coupled with a microplate reader, which reduced potential background interference and enhanced accuracy. As previously noted, ascorbate peroxidases (APXs) are sensitive to oxidative compounds, which can impact their activity both in vivo and in vitro (Kangasjärvi *et al.*, 2008). APXs are stabilized by ascorbic acid, which serves as an electron donor to prevent oxidative degradation. Adding a small amount of ascorbic acid during the extraction process can help preserve APX activity (Miyake and Asada, 1996; Mano *et al.*, 2001). However, since the assay buffers in commercial kits do not include ascorbic acid, APX activity might be overestimated. Future studies should consider these potential interferences in biochemical assays and adopt optimized methods to ensure accurate and reliable results.

5.2 Monoterpene production and emission under drought stress

Unlike animals, plants cannot move to escape environmental stresses, so they evolved various chemical mechanisms to transmit signals within the organism and initiate metabolic responses to the type and severity of the stress, minimizing potential damage (Possell and Loreto, 2013). The MEP metabolic pathway, i.e., terpenes, exhibits constitutive and induced production and emission at both individual plant and ecological levels (Loreto and Schnitzler, 2010; Li *et al.*, 2023). Constitutive production, storage, and emission, play a role in abiotic stress resistance. Environmentally induced production and emission are species- and condition-specific, with terpene components

and production rates often varying in response to different types and intensities of stress (Holopainen and Gershenzon, 2010; Harrison *et al.*, 2013; Niinemets and Monson, 2013). Since exogenous MTs comprise several compounds, it was difficult to identify the specific antioxidant and oxidative stress components. Additionally, each compound acted at slightly different concentrations. For example, at cellular concentrations below 3 mM, α-pinene exhibits the highest free radical scavenging activity and reducing power compared to limonene and myrcene (Wang *et al.*, 2019). At concentrations above this threshold, α-pinene can induce oxidative stress and inhibit root and shoot growth, with slight varietal differences between species (Ibrahim *et al.*, 2006; Singh *et al.*, 2006). To determine the effectiveness of MTs as antioxidants, it is crucial to establish thresholds for intercellular concentrations, foliar uptake, and constitutive emission rates that result in protective effects or toxicity.

Induced production and emission are critical for mediating both the physiological and biochemical state of plants, and facilitating plant-plant interactions (Kessler *et al.*, 2006; Heil and Silva Bueno, 2007). The priming effect refers to a plant receiving BVOCs from external or neighbouring plants. In many field experiments, plant responses can be induced by exposing them to BVOCs in enclosed environments, testing indirect protective (or toxic) mechanisms and their persistence (Heil and Kost, 2006; Caparrotta *et al.*, 2018). The exogenous MT experiment simulates plant responses to compounds enriched in the natural environment and can be regarded as a theoretical model for understanding the priming effects of MTs. Presumably, plants respond more directly to foliar MT sprays than fumigation as volatile compounds were already dissolved in organic solvent. Boachon *et al.* (2019) describe this as the uptake and transport of volatiles between plant organs through natural fumigation, influencing the specific synthesis and signalling of endogenous terpenes and other metabolites, such as methyl salicylate, to benefit the plant. Although total foliar MT content and emission rates were measured in both Chapter 2 and 4, the physicochemical variation of these MT compounds caused plant leaves to preferentially acquire exogenous β-ocimene for reemission, while α/β-pinene and sabinene accumulated in leaf tissue and were translocated to both upper and lower leaves (Delfine *et al.*, 2000). Volatile emission models from the cellular level to the environment have been developed and verified, however, the mechanisms of volatile uptake via gaseous route remain unclear (Widhalm *et al.*, 2015; Boachon *et al.*, 2019).

Induced emission behaviour of endogenous MTs, such as (–)-limonene in Chapter 4, is commonly observed, with initial increases under drought (Sharkey and Loreto, 1993; Ormeño *et al.*, 2007; Byron *et al.*, 2022), heat (Singsaas, 2000; Tian *et al.*, 2020), and oxidative stress (i.e., ozone) (Loreto *et al.*, 2004; Mochizuki *et al.*, 2017) possibly restricting photosynthetic activity (Niinemets *et al.*, 2002c; Kreuzwieser *et al.*, 2021). Enhanced emissions persist until the stress becomes severe enough to inhibit overall photosynthesis and respiratory metabolism (Ormeño *et al.*, 2007; Niinemets and Monson, 2013; Bertamini *et al.*, 2019). As part of the metabolic tolerance mechanism, it is likely that some terpenes synthesized *in vivo* react with ROS inside the cell and are depleted during stress, causing their actual synthesis rate to be higher than the measured leaf emission rate, as evidenced by comparisons of terpene enzyme activities at varying emission rates (Jardine *et al.*, 2012; Alicandri *et al.*, 2020; Zhan *et al.*, 2022). Additionally, the increased endogenous production and emission rates may be a result of ROS upregulation under stress, as observed in direct oxidative stress experiments with ozone (Loreto *et al.*, 2004; Vickers *et al.*, 2009b; Calfapietra *et al.*, 2013). Additionally, it is unclear whether a legacy effect from relocated and stored MTs exists. The ability of terpenes to detoxify ROS may have a threshold, dependent on the level of ROS accumulation, beyond which oxidative stress signalling pathways are triggered. It is unclear whether the downregulation of photosynthetic metabolism under stress and competition for resources with constitutive emissions enhances ROS accumulation and toxicity (Harrison *et al.*, 2013; Possell and Loreto, 2013).

Other than the toxic effects of ROS, the importance of ROS in plant signalling under stressed conditions is established (Mittler *et al.*, 2022), ROS receptors function as critical regulatory hubs in cellular and subcellular structures, linking ROS signalling to various stress-response pathways and hormones (Chan *et al.*, 2016; Huang *et al.*, 2019c). Additionally, newly identified roles for ROS in organelle-to-organelle and cell-to-cell signalling further underscore its importance in plant stress physiological and biochemical responses (Cruz de Carvalho, 2008; Zou *et al.*, 2015; Hu *et al.*, 2017). Presumably exogenous MT uptake by plant leaves triggers localised apoplastic ROS production, where plasma membrane-localised NADPH oxidases and cell wall peroxidases generate ROS (Huang et al., 2019a). These enzymes, particularly the respiratory burst oxidase homologues, likely respond to increased monoterpene levels in the extracellular space by altering superoxide production and subsequent H_2O_2 formation, noting that this thesis measured H_2O_2 and enzyme activity at the whole leaf

level. Such apoplastic ROS could then influence a variety of physiological processes, including cell wall modification (e.g., lignification), stomatal dynamics (e.g., Ca^{2+} regulated stomatal closure), and even immune-like responses through signalling cascades involving plant receptors (Daudi et al., 2012; Kadota et al., 2015; Sierla et al., 2016).

Since tomato plants already possess endogenous MT biosynthesis pathways, the interplay between exogenous MTs and native metabolic feedback loops may activate or reinforce internal signalling mechanisms in response to both applied MTs and drought stress. Here, potential 'receptors' or sensing components within the plant's existing terpene and stress-response networks may discern exogenous MTs and adjust downstream signalling or gene expression accordingly. These responses might ultimately modulate antioxidative defences, hormone crosstalk, or other aspects of stress tolerance in tomato. In contrast, tobacco plants lacking endogenous MT production and signalling feedback mechanisms, may exhibit different, possibly less orchestrated, responses to additional MT. Especially, how ROS regulates terpene synthesis and their emission remains unclear (Niinemets and Monson, 2013). Tobacco's apoplastic ROS burst in response to stress conditions could be more abrupt or unregulated, potentially leading to a different balance between protective signalling and oxidative stress.

In both endogenous and exogenous terpene experiments, it was not possible to confirm the specific uptake of exogenous MTs, their effect on constitutive or induced production under water stress, or the point at which (domestic defence mechanisms are compromised under stress) a functional shift in endogenous MTs occurred. Consequently, understanding the degree to which native MT production pathways modify ROS signalling — especially in the apoplast — will shed light on the nuanced roles of monoterpenes in stress physiology and may inform crop improvement strategies aimed at enhancing drought resilience.

In summary, stress conditions affect interactions between antioxidant activity from MTs and other antioxidant mechanisms. The intercellular concentration and endogenous production rate of MTs are essential to allow them to protect plants from abiotic stress-induced oxidative damage and regulating photosynthetic responses. MT compounds exhibit diverse antioxidant capacities, and their physiological impact varies according to concentration, foliar uptake, and production rates (Ninkuu *et al.*, 2021; Li *et al.*, 2023).

While MTs can mitigate oxidative damage, excessive concentrations and emissions may induce phytotoxicity, underscoring the need to establish optimal thresholds for their efficacy. The discovery of the upregulated MEP pathway under heat and oxidative stress has spurred interest in the molecular, biochemical, and physiological mechanisms governing the role of terpenes in abiotic stress tolerance (Niinemets and Monson, 2013; Possell and Loreto, 2013; Ninkuu *et al.*, 2021). A key challenge in understanding these protective mechanisms lies in determining how and to what extent plants benefit from allocating resources to secondary metabolism within complex biological systems and varying environmental conditions (Brilli *et al.*, 2019; Boncan *et al.*, 2020).

5.3 Using monoterpenes to improve stress resilience?

Since monoterpenes are proposed to mediate ROS levels by acting as antioxidants, either by directly scavenging ROS or by enhancing the plant's intrinsic antioxidative defence system, they could be used to enhance stress tolerance (Loreto *et al.*, 2014; Brilli *et al.*, 2019). The antioxidant effects of MTs have also been observed in genetically modified crops, which are not native producers, particularly under critical conditions. Classic hypotheses regarding the role of terpenes: stabilizing membranes, functioning as antioxidants, acting as signalling molecules, and serving as safety valves for energy balance under stress (Niinemets and Monson, 2013; Possell and Loreto, 2013), are likely applicable at both cellular and intercellular levels. For instance, terpenes may stabilise phospholipid membrane structures by acting as antioxidants to mitigate oxidative stress and lipid damage (Vickers *et al.*, 2009a; Vickers *et al.*, 2009b); binding with membrane proteins as signalling molecules (Frank *et al.*, 2021; Dani and Loreto, 2022); and partitioning lipid membranes to alter their dynamic properties (Li *et al.*, 1998; Niinemets *et al.*, 2004). While ROS are central to various stress signalling pathways (Sewelam *et al.*, 2016; Mittler *et al.*, 2022), a single biochemical mechanism may not fully account for the diverse functions of terpenes across different stress levels and plant growth stages. Although this study presents new insights into MTs conferring antioxidant capacity to plants under short-term water stress, long-term experiments with different species focusing on various enzymatic and non-enzymatic antioxidants triggered by different sources of ROS are needed to elucidate the specific mechanisms by which MTs contribute to stress resilience and to identify the conditions under which they provide optimal protective benefits.

The key trade-offs observed in this research and in many previous studies are the balance between physiological improvements (such as attenuated leaf water potential decline), biomass retention, and the biochemical cost of producing these compounds. Resource allocation toward defence systems and secondary metabolite production may come at the expense of biomass accumulation (Ryan *et al.*, 2014; Pichersky and Raguso, 2018; Monson *et al.*, 2021). Terpene biosynthetic pathways also involve the expression of key genes such as those related to jasmonic acid, gibberellins, and cytokinins (Dani and Loreto, 2022). These hormones and signalling networks are crucial in influencing plant growth, development, and inducible defence responses (Kazan, 2015; Shohat *et al.*, 2021). For high-yielding crop species such as tomato, tobacco, and rice, metabolic investment in stress resilience may reduce overall yield or fruit quality. Additionally, genetically modified crops engineered to produce terpenes may face challenges in maintaining growth and yield compared to traditional varieties that prioritize rapid biomass accumulation under non-stressed conditions (Dudareva *et al.*, 2013; Brilli *et al.*, 2019; Dwivedi *et al.*, 2021). This research builds on previous studies and suggests a pathway for leveraging MTs to improve plant drought resilience through metabolic engineering, particularly by targeting the MEP pathway. However, recognising the advantages and disadvantages of reshaping terpene emissions and genetic modifications in growth and defence trade-offs is essential for understanding plant adaptation and resilience to stress.

Additionally, the benefits of MTs, particularly in terms of physiological responses such as leaf water status, stomatal conductance, and photosynthesis, appear primarily during specific stages of the plant life cycle and the drought stress period. The most significant physiological improvements were observed during the intermediate stages of drought, while biochemical improvements occurred during the vegetative stage and recovery; however, these effects diminished as stress intensified. Notably, the physiological improvements from MTs did not correlate with increased antioxidant enzyme activity. For instance, exogenous MT treatments mitigated lipid damage, but not enough to prevent photosynthetic decline. MTs may be most effective during transient drought, and their benefits may not extend over the entire life cycle of the plant, reinforcing questions about whether plants truly benefit from MT production and emissions. Although this and many other studies have shown that plants can regulate MT production and emission in response to environmental conditions and that MTs actively regulate stress tolerance and plant-plant communication (Blande *et al.*, 2014; Pichersky

and Raguso, 2018), MTs in the MEP synthetic pathway are likely byproducts or part of a metabolic transfer pathway throughout much of the plant growth cycle (Tian *et al.*, 2016; Karunanithi and Zerbe, 2019).

Another consideration when discussing MTs for stress resilience is the inherent variability, or "noise," in leaf-level responses. Different leaves on the same plant can exhibit varying degrees of stomatal response, photosynthetic efficiency, oxidative stress, MT synthase activity, and emission, with heterogeneous responses within a single plant, and even greater variation at the whole-plant level (Berens *et al.*, 2019). This variability complicates the interpretation of results, such as the uncoupled physiological and biochemical effects mentioned earlier. A significant challenge in scaling from leaf-level responses to whole-plant outcomes is that laboratory results often contradict field observations regarding both physio-biochemical (Poorter *et al.*, 2016; Xu *et al.*, 2019) and emission responses (Mozaffar *et al.*, 2018; Bonn *et al.*, 2019; Bilas *et al.*, 2021). This research mostly measured the water status, gas exchange, photosynthesis, and oxidative stress markers of the newest fully developed leaves, while morphology was assessed at the shoot level. Translating these responses to the whole-plant level can be complex. For example, even though MTs improve leaf water status and antioxidant capacity, these effects may not always enhance shoot physiology, biomass or yield. Additionally, drought tolerance mechanisms at the leaf level might not account for root dynamics and the plant's overall water acquisition capacity (Zia *et al.*, 2021; Moshelion *et al.*, 2024). For example, stomatal regulation under water stress is related to how well roots and soil can move water. However, closing stomata helps reduce water loss but doesn’t improve the plant’s ability to take up water from deeper or drier soil, which depends on the root system. (Westgate and Boyer, 1985; Abdalla *et al.*, 2021). Understanding how MTs influence systemic signalling pathways and how these responses are coordinated across different tissues will be essential for optimising stress tolerance. More research is needed to scale up from leaf-level responses to understand the holistic impact of MTs on plant performance under stressed conditions.

5.4 Future opportunities

This research opens several avenues for future study, particularly in understanding the broader implications and fundamentals of MT production, the advantages and disadvantages of their function in response to drought stress, and their potential applications for improving crop resilience in the field.

MT production and emissions must be understood within the complex framework of climate change, as biotic and abiotic stressors interact in unpredictable ways. One promising area of research involves exploring the diversity and variability of BVOC emitters and species-specific responses (Loreto and Schnitzler, 2010; Li and Sharkey, 2013). Not all plants emit MTs, and their emission levels and behaviour vary widely across species. Therefore, understanding the genetic regulation of MT synthesis and species-specific differences in their drought responses remains a critical area of study. Models for predicting BVOC emissions from vegetation were first developed 30 years ago (Guenther *et al.*, 1993), and recent studies of gene expression and key terpene enzyme activities have provided valuable information for physiological modelling and physicochemical mechanisms (Niinemets *et al.*, 2004; Yin *et al.*, 2016; Zhan *et al.*, 2022). Plant developmental stage is also important as BVOCs are both constitutive and stress-induced, but quantitative models describing how these emissions relate to developmental stages are still in their infancy (Mozaffar *et al.*, 2018). For instance, different stages of plant growth may exhibit varying sensitivities to environmental stressors, which can affect the timing, quantity, and functioning of BVOC emissions (Bracho-Nunez *et al.*, 2011; Zhang *et al.*, 2020). Determining how these factors interact with drought-induced emissions, and how they can be integrated into physiological and biochemical mechanisms to improve plant resilience under fluctuating climatic conditions, is needed.

Additionally, the thresholds for various stressors, such as temperature, water deficit, and herbivory, must be better defined. There is still uncertainty regarding how multitrophic interactions, such as those involving plant-herbivore-predator dynamics, evolve in a changing atmosphere with altered oxidative potential (Holopainen and Gershenzon, 2010; Calfapietra *et al.*, 2013; Possell and Loreto, 2013). Understanding these interactions will be crucial for developing sustainable plant systems that harness the protective properties of MTs and other terpenes. However, predicting and testing accurate terpene excitation pressure thresholds under stress remains challenging (Niinemets, 2010). The dynamics of physiological-biochemical-terpene responses requires an understanding of the complex interplay between various stresses. Future research may explore the long-term, multifactorial biological feedback loops of MTs with other functional compounds and signalling pathways. How these emissions impact overall plant resilience and affect multitrophic interactions over time is a key area of inquiry. Quantifying the role of these emissions under both episodic and chronic stress

conditions will be crucial for understanding their individual and broader ecological functions (Vlot and Rosenkranz, 2022; Escobar-Bravo *et al.*, 2023).

Although advancements have been made in elucidating biochemical pathways, such as the MEP pathway, much remains to be discovered about the regulatory mechanisms governing BVOC production and emissions (Niinemets and Monson, 2013; Possell and Loreto, 2013; Bao *et al.*, 2023; Hirose and Satake, 2024). Understanding how gene expression, enzyme activity, and intercellular biochemical transporters respond to varying environmental conditions across different biological scales—from the cellular level to the canopy—remains a critical knowledge gap. Further investigations into how gene expression and enzyme regulation are modulated under changing climates will be essential to predict plant responses at larger scales and how plants utilize such regulatory mechanisms. Moreover, this research would help develop novel genetically modified plants that overproduce or underproduce MTs in a wide range of species, facilitating an understanding of the impact of specific compounds in model or even commercial crops.

One of the major limitations in current research is the lack of field-based observations capturing MT functioning under real-world conditions. Most studies occurred in controlled environments (Nogués *et al.*, 2015a; Bertamini *et al.*, 2021; Bourtsoukidis *et al.*, 2024). While useful for mechanistic studies, these may not fully represent the complexities of natural ecosystems. Future research may explore high-resolution data collection from field studies to reduce uncertainties in MT emission models, explore the connection to biological mechanisms such as synthase and protein transporter dynamics, and improve experimental methods and using advanced instrument in laboratory investigations. Advances in remote sensing technologies, fast-response sensors, and atmospheric measurement tools will be critical to improve the accuracy of BVOC emission models and validate lab-based findings. Integrating these field-based observations with climate models will help clarify the impact of BVOC emissions on atmospheric chemistry, particularly in relation to climate feedback loops, and enhance our understanding of the role of variable BVOC emissions from individual plants to the ecosystem level.

Applying MTs and other terpenes in agricultural systems offers significant potential for improving crop resilience and productivity under environmental stress (Brilli *et al.*, 2019). MTs have the ability to prime plants to withstand environmental stresses more

effectively (Niinemets, 2010). Priming prepares the plant to respond more efficiently to future stress without immediately activating costly metabolic pathways (Martinez-Medina *et al.*, 2016). This priming effect is particularly relevant for abiotic stress conditions, such as drought. Priming avoids the energetic costs associated with constant defence activation, making it a sustainable crop protection strategy (Buswell *et al.*, 2018). In this context, the use of MTs may reduce the need for synthetic inputs like pesticides, providing a more energy-efficient solution to maintain plant health (van Hulten *et al.*, 2006). Agriculture has used MTs to defend plants against biotic stresses, including pathogens and herbivores. Certain MTs, such as limonene, have effective antifungal properties, inhibiting the growth of harmful fungi like *Botrytis cinerea* (Umagiliyage *et al.*, 2017). This ability to repel or kill pathogens and herbivores without synthetic chemicals aligns with the growing demand for biopesticides in modern agricultural practices (Ninkuu *et al.*, 2021). While one of the key roles of terpenes in plants has been defence, there is increasing evidence that they may also contribute to improved crop productivity. For instance, certain VOCs, including MTs, inhibit the growth of competing plants through allelopathic effects, allowing crops to access more resources such as water, nutrients, and light (Puig *et al.*, 2018; Kong *et al.*, 2019). Furthermore, MTs may act synergistically with other plant hormones, such as cytokinins, to regulate senescence and extend the productive lifespan of leaves and flowers (Dani *et al.*, 2016; Yin *et al.*, 2016). By delaying leaf aging and preventing oxidative damage, sustained MT production may enhance overall plant growth and yield under stress conditions (Woo *et al.*, 2013). This aspect of MT function holds promise for integration into breeding programs to enhance drought tolerance and maintain or even improve productivity.

Despite the clear benefits, several challenges remain to the widespread use of MTs in agriculture. One key limitation is the high reactivity of VOCs like MTs in the environment, which can reduce their effectiveness over time and distance due to interactions with atmospheric components such as ozone and nitrogen oxides (Peñuelas and Staudt, 2010; Blande *et al.*, 2014). Moreover, the variability in MT activity between species and across different developmental stages means that genetically engineering crop MT production is necessary to fully understand their potential and impacts under natural agricultural conditions (Vickers *et al.*, 2011; Dudareva *et al.*, 2013).

5.5 Conclusion and Future work

Monoterpenes play a potential role in enhancing plant resilience to drought stress, primarily by facilitating antioxidative properties if the MTs themselves or via other components such as antioxidant enzymes. Both exogenous application and endogenous production of monoterpenes mitigated oxidative damage by reducing the accumulation of reactive oxygen species and/or mitigating lipid damage and protecting cellular structures, but not necessarily provide benefits to photosystems. Exogenous monoterpenes provided immediate and effective but short-term relief from oxidative stress at low concentrations. While high concentrations may directly impose oxidative stress. The antioxidant property of endogenous monoterpenoids is more dependent on the degree of stress and emission rate, i.e., the recovery phase after watering at the base discharge level. This suggests that genetic engineering and exogenous application to increase monoterpene production or accumulation in crops could be a viable strategy for improving drought tolerance. However, there are growth or metabolic trade-offs.

Nevertheless, to fully exploit the protective roles of monoterpenes for enhancing drought tolerance, future research must address several critical gaps and challenges:

- **Elucidate interactions:**

    1. **Examine signalling crosstalk:** Characterise how monoterpenes integrate into broader stress-response networks — especially those involving ROS — and identify transcription factors or intermediates that link monoterpene biosynthesis with other defence-related pathways.

    2. **Investigate feedback regulation:** Evaluate whether elevated monoterpene levels alter hormonal balance and ROS homeostasis, potentially activating or inhibiting hormone- or ROS-responsive genes in both local and systemic tissues under stressed conditions.

- **Distinguish effective protective components**

    Identify which monoterpene compounds exert the most substantial protective effects, considering their unique biochemical properties and modes of action.

- **Determine stress thresholds**

Establish the thresholds for up- and down-regulating terpene emissions, as well as the concentrations and emission rates that confer phyto-protection versus those that lead to phytotoxicity. These thresholds likely vary between specific monoterpenes and species.

- **Assess stress levels and durations**

  Trial different stress intensities and durations to allow adaptation and reduce the risk of surpassing monoterpene toxicity thresholds. This approach allows clearer evaluation of physiological and biochemical responses.

- **Explore antioxidant mechanisms**

  Investigate in detail how monoterpenes influence different sources and compositions of ROS, antioxidant enzymes, and non-enzymatic antioxidants linked to the MEP pathway.

- **Rational gene editing**

  While gene editing to upregulate drought-tolerant traits may be more efficient, define robust pathways for gene editing that control terpene emission rates to avoid excessive or detrimental monoterpene production.

- **Expand the understanding of MEP pathway upregulation**

  Conduct comprehensive physiological and biochemical analyses of the effects of MEP pathway enhancement on plant growth, reproduction, and overall stress responses. Determine how phytohormones (e.g., abscisic acid, jasmonic acid, salicylic acid, and ethylene) modulate MEP pathway enzymes under both normal and stressed conditions, and how these hormones influence the production, transport, and storage of monoterpenes.

- **Scale from leaf-level to whole-plant**

  Investigate how leaf-level responses alter entire plant physiology. This scaling-up will help in developing practical strategies for using monoterpenes in practice.

6 References

Abd-ElGawad AM, El Gendy AE-NG, Assaeed AM, Al-Rowaily SL, Alharthi AS, Mohamed TA, Nassar MI, Dewir YH, Elshamy AI. 2021. Phytotoxic Effects of Plant Essential Oils: A Systematic Review and Structure-Activity Relationship Based on Chemometric Analyses. Plants **10**, 36.

Abdalla M, Ahmed MA, Cai G, Wankmüller F, Schwartz N, Litig O, Javaux M, Carminati A. 2021. Stomatal closure during water deficit is controlled by below-ground hydraulics. Annals of Botany **129**, 161-170.

Abid M, Ali S, Qi LK, Zahoor R, Tian Z, Jiang D, Snider JL, Dai T. 2018. Physiological and biochemical changes during drought and recovery periods at tillering and jointing stages in wheat (Triticum aestivum L.). Scientific Reports **8**, 4615.

Adebesin F, Widhalm JR, Boachon B, Lefèvre F, Pierman B, Lynch JH, Alam I, Junqueira B, Benke R, Ray S, Porter JA, Yanagisawa M, Wetzstein HY, Morgan JA, Boutry M, Schuurink RC, Dudareva N. 2017. Emission of volatile organic compounds from petunia flowers is facilitated by an ABC transporter. Science **356**, 1386-1388.

Affek HP, Yakir D. 2002. Protection by Isoprene against Singlet Oxygen in Leaves. Plant Physiology **129**, 269-277.

Agus HH. 2021. Chapter 4 - Terpene toxicity and oxidative stress. In: Patel VB, Preedy VR, eds. *Toxicology*: Academic Press, 33-42.

Aharoni A, Giri AP, Deuerlein S, Griepink F, de Kogel W-J, Verstappen FWA, Verhoeven HA, Jongsma MA, Schwab W, Bouwmeester HJ. 2003. Terpenoid Metabolism in Wild-Type and Transgenic Arabidopsis Plants[W]. The Plant Cell **15**, 2866-2884.

Alicandri E, Covino S, Sebastiani B, Paolacci AR, Badiani M, Sorgonà A, Ciaffi M. 2022. Monoterpene Synthase Genes and Monoterpene Profiles in Pinus nigra subsp. laricio. Plants **11**, 449.

Alicandri E, Paolacci AR, Osadolor S, Sorgonà A, Badiani M, Ciaffi M. 2020. On the Evolution and Functional Diversity of Terpene Synthases in the Pinus Species: A Review. Journal of Molecular Evolution **88**, 253-283.

Alscher RG, Erturk N, Heath LS. 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. Journal of Experimental Botany **53**, 1331-1341.

Altmann T, Koßmann J. 2001. Photosynthesis and primary metabolism. Trends in Plant Science **6**, 93-94.

Arneth A, Monson RK, Schurgers G, Niinemets Ü, Palmer PI. 2008. Why are estimates of global terrestrial isoprene emissions so similar (and why is this not so for monoterpenes)? Atmos. Chem. Phys. **8**, 4605-4620.

Asada K. 1992. Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. Physiologia Plantarum **85**, 235-241.

Asada K. 1999. The Water-Water Cycle in Chloroplasts: Scavenging of Active Oxygens and Dissipation of Excess Photons. Annual Review of Plant Physiology and Plant Molecular Biology **50**, 601-639.

Asada K. 2000. The water–water cycle as alternative photon and electron sinks. Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences **355**, 1419-1431.

Asada K. 2006. Production and Scavenging of Reactive Oxygen Species in Chloroplasts and Their Functions. Plant Physiology **141**, 391-396.

Atkinson R, Arey J. 2003. Atmospheric Degradation of Volatile Organic Compounds. Chemical Reviews **103**, 4605-4638.

Atkinson R, Baulch DL, Cox RA, Crowley JN, Hampson RF, Hynes RG, Jenkin ME, Rossi MJ, Troe J, Subcommittee I. 2006. Evaluated kinetic and photochemical data for atmospheric chemistry: Volume II – gas phase reactions of organic species. Atmos. Chem. Phys. **6**, 3625-4055.

Awad J, Stotz HU, Fekete A, Krischke M, Engert C, Havaux M, Berger S, Mueller MJ. 2015. 2-Cysteine Peroxiredoxins and Thylakoid Ascorbate Peroxidase Create a Water-Water Cycle That Is Essential to Protect the Photosynthetic Apparatus under High Light Stress Conditions. Plant Physiology **167**, 1592-1603.

Baldwin IT, Schultz JC. 1983. Rapid Changes in Tree Leaf Chemistry Induced by Damage: Evidence for Communication Between Plants. Science **221**, 277-279.

Bao X, Zhou W, Xu L, Zheng Z. 2023. A meta-analysis on plant volatile organic compound emissions of different plant species and responses to environmental stress. Environmental Pollution **318**, 120886.

Barta C, Loreto F. 2006. The Relationship between the Methyl-Erythritol Phosphate Pathway Leading to Emission of Volatile Isoprenoids and Abscisic Acid Content in Leaves. Plant Physiology **141**, 1676-1683.

Begg JE, Turner NC. 1970. Water Potential Gradients in Field Tobacco. Plant Physiology **46**, 343-346.

Beis A, Patakas A. 2012. Relative contribution of photoprotection and anti-oxidative mechanisms to differential drought adaptation ability in grapevines. Environmental and Experimental Botany **78**, 173-183.

Berens ML, Wolinska KW, Spaepen S, Ziegler J, Nobori T, Nair A, Krüler V, Winkelmüller TM, Wang Y, Mine A, Becker D, Garrido-Oter R, Schulze-Lefert P, Tsuda K. 2019. Balancing trade-offs between biotic and abiotic stress responses through leaf age-dependent variation in stress hormone cross-talk. Proceedings of the National Academy of Sciences **116**, 2364-2373.

Bergman ME, Dudareva N. 2024. Plant specialized metabolism: Diversity of terpene synthases and their products. Current Opinion in Plant Biology **81**, 102607.

Bertamini M, Faralli M, Varotto C, Grando MS, Cappellin L. 2021. Leaf Monoterpene Emission Limits Photosynthetic Downregulation under Heat Stress in Field-Grown Grapevine. Plants **10**, 181.

Bertamini M, Grando MS, Zocca P, Pedrotti M, Lorenzi S, Cappellin L. 2019. Linking monoterpenes and abiotic stress resistance in grapevines. BIO Web Conf. **13**, 01003.

Bertin N, Staudt M. 1996. Effect of water stress on monoterpene emissions from young potted holm oak (Quercus ilex L.) trees. Oecologia **107**, 456-462.

Biehler K, Fock H. 1996. Evidence for the Contribution of the Mehler-Peroxidase Reaction in Dissipating Excess Electrons in Drought-Stressed Wheat. Plant Physiology **112**, 265-272.

Bilas RD, Bretman A, Bennett T. 2021. Friends, neighbours and enemies: an overview of the communal and social biology of plants. Plant, Cell & Environment **44**, 997-1013.

Binzel ML, Hasegawa PM, Rhodes D, Handa S, Handa AK, Bressan RA. 1987. Solute Accumulation in Tobacco Cells Adapted to NaCl 1. Plant Physiology **84**, 1408-1415.

Blanch J-S, Peñuelas J, Llusià J. 2007. Sensitivity of terpene emissions to drought and fertilization in terpene-storing Pinus halepensis and non-storing Quercus ilex. Physiologia Plantarum **131**, 211-225.

Blanch J-S, Peñuelas J, Sardans J, Llusià J. 2009. Drought, warming and soil fertilization effects on leaf volatile terpene concentrations in Pinus halepensis and Quercus ilex. Acta Physiologiae Plantarum **31**, 207-218.

Blande JD, Holopainen JK, Niinemets Ü. 2014. Plant volatiles in polluted atmospheres: stress responses and signal degradation. Plant, Cell & Environment **37**, 1892-1904.

Boachon B, Lynch JH, Ray S, Yuan J, Caldo KMP, Junker RR, Kessler SA, Morgan JA, Dudareva N. 2019. Natural fumigation as a mechanism for volatile transport between flower organs. Nature Chemical Biology **15**, 583-588.

Boncan DAT, Tsang SSK, Li C, Lee IHT, Lam H-M, Chan T-F, Hui JHL. 2020. Terpenes and Terpenoids in Plants: Interactions with Environment and Insects. International Journal of Molecular Sciences **21**, 7382.

Bonn B, Magh RK, Rombach J, Kreuzwieser J. 2019. Biogenic isoprenoid emissions under drought stress: different responses for isoprene and terpenes. Biogeosciences **16**, 4627-4645.

Bourtsoukidis E, Pozzer A, Williams J, Makowski D, Peñuelas J, Matthaios VN, Lazoglou G, Yañez-Serrano AM, Lelieveld J, Ciais P, Vrekoussis M, Daskalakis N, Sciare J. 2024. High temperature sensitivity of monoterpene emissions from global vegetation. Communications Earth & Environment **5**, 23.

Boyer JS. 1968. Relationship of Water Potential to Growth of Leaves 1. Plant Physiology **43**, 1056-1062.

Boyer JS. 1982. Plant productivity and environment. Science **218**, 443-448.

Bracho-Nunez A, Welter S, Staudt M, Kesselmeier J. 2011. Plant-specific volatile organic compound emission rates from young and mature leaves of Mediterranean vegetation. Journal of Geophysical Research: Atmospheres **116**.

Brilli F, Barta C, Fortunati A, Lerdau M, Loreto F, Centritto M. 2007a. Response of isoprene emission and carbon metabolism to drought in white poplar (Populus alba) saplings. New Phytol **175**, 244-254.

Brilli F, Barta C, Fortunati A, Lerdau M, Loreto F, Centritto M. 2007b. Response of isoprene emission and carbon metabolism to drought in white poplar (Populus alba) saplings. New Phytologist **175**, 244-254.

Brilli F, Dani KGS, Pasqualini S, Costarelli A, Cannavò S, Paolocci F, Zittelli GC, Mugnai G, Baraldi R, Loreto F. 2022. Exposure to different light intensities affects emission of volatiles and accumulations of both pigments and phenolics in Azolla filiculoides. Physiologia Plantarum **174**, e13619.

Brilli F, Loreto F, Baccelli I. 2019. Exploiting Plant Volatile Organic Compounds (VOCs) in Agriculture to Improve Sustainable Defense Strategies and Productivity of Crops. Frontiers in Plant Science **10**.

Brosset A, Blande JD. 2021. Volatile-mediated plant–plant interactions: volatile organic compounds as modulators of receiver plant defence, growth, and reproduction. Journal of Experimental Botany **73**, 511-528.

Brown JT, Hegarty PK, Charlwood BV. 1987. The toxicity of monoterpenes to plant cell cultures. Plant Science **48**, 195-201.

Brunetti C, Guidi L, Sebastiani F, Tattini M. 2015. Isoprenoids and phenylpropanoids are key components of the antioxidant defense system of plants facing severe excess light stress. Environmental and Experimental Botany **119**, 54-62.

Bukhov NG, Wiese C, Neimanis S, Heber U. 1999. Heat sensitivity of chloroplasts and leaves: Leakage of protons from thylakoids and reversible activation of cyclic electron transport. Photosynthesis Research **59**, 81-93.

Buswell W, Schwarzenbacher RE, Luna E, Sellwood M, Chen B, Flors V, Pétriacq P, Ton J. 2018. Chemical priming of immunity without costs to plant growth. New Phytologist **218**, 1205-1216.

Byron J, Kreuzwieser J, Purser G, van Haren J, Ladd SN, Meredith LK, Werner C, Williams J. 2022. Chiral monoterpenes reveal forest emission mechanisms and drought responses. Nature **609**, 307-312.

Calfapietra C, Pallozzi E, Lusini I, Velikova V. 2013. Modification of BVOC Emissions by Changes in Atmospheric [CO2] and Air Pollution. In: Niinemets Ü, Monson RK, eds. *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*. Dordrecht: Springer Netherlands, 253-284.

Cameron KD, Teece MA, Smart LB. 2005. Increased Accumulation of Cuticular Wax and Expression of Lipid Transfer Protein in Response to Periodic Drying Events in Leaves of Tree Tobacco. Plant Physiology **140**, 176-183.

Cantor RS. 1997. The Lateral Pressure Profile in Membranes: A Physical Mechanism of General Anesthesia. Biochemistry **36**, 2339-2344.

Caparrotta S, Boni S, Taiti C, Palm E, Mancuso S, Pandolfi C. 2018. Induction of priming by salt stress in neighboring plants. Environmental and Experimental Botany **147**, 261-270.

Caretta MM, A. Arfanuzzaman, M. Betts, RA. Gelfan, A. Hirabayashi, Y. Lissner, TK. Liu, J. Lopez Gunn, E. Morgan, R. Mwanga, Supratid, S. 2022. Water. In: H.-O. Pörtner DCR, M. Tignor, E.S. Poloczanska, K. Mintenbeck, A. Alegría, M. Craig, S. Langsdorf, S. Löschke, V. Möller, A. Okem, B. Rama, ed. *Climate Change 2022: Impacts, Adaptation, and Vulnerability*: Cambridge University Press.

Carvalho SD, Schwieterman ML, Abrahan CE, Colquhoun TA, Folta KM. 2016. Light quality dependent changes in morphology, antioxidant capacity, and volatile production in sweet basil (*Ocimum basilicum*). Frontiers in Plant Science **7**.

Centritto M, Brilli F, Fodale R, Loreto F. 2011. Different sensitivity of isoprene emission, respiration and photosynthesis to high growth temperature coupled with drought stress in black poplar (Populus nigra) saplings. Tree Physiology **31**, 275-286.

Chan KX, Mabbitt PD, Phua SY, Mueller JW, Nisar N, Gigolashvili T, Stroeher E, Grassl J, Arlt W, Estavillo GM, Jackson CJ, Pogson BJ. 2016. Sensing and signaling of oxidative stress in chloroplasts by inactivation of the SAL1 phosphoadenosine phosphatase. Proceedings of the National Academy of Sciences **113**, E4567-E4576.

Chatzivasileiou AO, Ward V, Edgar SM, Stephanopoulos G. 2019. Two-step pathway for isoprenoid synthesis. Proceedings of the National Academy of Sciences **116**, 506-511.

Chaves MM, Maroco JP, Pereira JS. 2003. Understanding plant responses to drought - from genes to the whole plant. Functional Plant Biology **30**, 239-264.

Chen F, Tholl D, Bohlmann J, Pichersky E. 2011. The family of terpene synthases in plants: a mid-size family of genes for specialized metabolism that is highly diversified throughout the kingdom. The Plant Journal **66**, 212-229.

Chen G-X, Asada K. 1989. Ascorbate Peroxidase in Tea Leaves: Occurrence of Two Isozymes and the Differences in Their Enzymatic and Molecular Properties. Plant and Cell Physiology **30**, 987-998.

Chen H, Jiang J-G. 2010. Osmotic adjustment and plant adaptation to environmental changes related to drought and salinity. Environmental Reviews **18**, 309-319.

Chen J-W, Bai K-D, Cao K-F. 2008. Inhibition of monoterpene biosynthesis accelerates oxidative stress and leads to enhancement of antioxidant defenses in leaves of rubber tree (Hevea brasiliensis). Acta Physiologiae Plantarum **31**, 95.

Chen Z, Vining KJ, Qi X, Yu X, Zheng Y, Liu Z, Fang H, Li L, Bai Y, Liang C, Li W, Lange BM. 2021. Genome-Wide Analysis of Terpene Synthase Gene Family in Mentha longifolia and Catalytic Activity Analysis of a Single Terpene Synthase. Genes **12**, 518.

Cheng D-L, Wang G-X, Chen B-M, Wei X-P. 2006. Positive Interactions: Crucial Organizers in a Plant Community. Journal of Integrative Plant Biology **48**, 128-136.

Copolovici LO, Filella I, Llusia J, Niinemets U, Penuelas J. 2005. The capacity for thermal protection of photosynthetic electron transport varies for different monoterpenes in Quercus ilex. Plant Physiology **139**, 485-496.

Copolovici LO, Niinemets Ü. 2005. Temperature dependencies of Henry's law constants and octanol/water partition coefficients for key plant volatile monoterpenoids. Chemosphere **61**, 1390-1400.

Cousins AB, Adam NR, Wall GW, Kimball BA, Pinter Jr PJ, Ottman MJ, Leavitt SW, Webber AN. 2002. Photosystem II energy use,non-photochemical quenching and the xanthophyll cycle in Sorghumbicolor grown under drought and free-air CO2 enrichment(FACE) conditions. Plant, Cell & Environment **25**, 1551-1559.

Crafts-Brandner SJ, Salvucci ME. 2000. Rubisco activase constrains the photosynthetic potential of leaves at high temperature and CO₂. Proceedings of the National Academy of Sciences **97**, 13430-13435.

Crawford T, Lehotai N, Strand Å. 2017. The role of retrograde signals during plant stress responses. Journal of Experimental Botany **69**, 2783-2795.

Cruz de Carvalho MH. 2008. Drought stress and reactive oxygen species: Production, scavenging and signaling. Plant Signal Behav **3**, 156-165.

Dani KGS, Fineschi S, Michelozzi M, Loreto F. 2016. Do cytokinins, volatile isoprenoids and carotenoids synergically delay leaf senescence? Plant, Cell & Environment **39**, 1103-1111.

Dani KGS, Loreto F. 2022. Plant volatiles as regulators of hormone homeostasis. New Phytologist **234**, 804-812.

Das K, Roychoudhury A. 2014. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Frontiers in Environmental Science **2**.

Daudi A, Cheng Z, O'Brien JA, Mammarella N, Khan S, Ausubel FM, Bolwell GP. 2012. The Apoplastic Oxidative Burst Peroxidase in Arabidopsis Is a Major Component of Pattern-Triggered Immunity The Plant Cell **24**, 275-287.

de Lima RP, da Silva AR, da Silva ÁP. 2021. soilphysics: An R package for simulation of soil compaction induced by agricultural field traffic. Soil and Tillage Research **206**, 104824.

De Ollas C, Arbona V, Gómez-Cadenas A, Dodd IC. 2018. Attenuated accumulation of jasmonates modifies stomatal responses to water deficit. Journal of Experimental Botany **69**, 2103-2116.

de Souza VF, Niinemets Ü, Rasulov B, Vickers CE, Duvoisin Júnior S, Araújo WL, Gonçalves JFdC. 2018. Alternative Carbon Sources for Isoprene Emission. Trends in Plant Science **23**, 1081-1101.

Degenhardt J, Köllner TG, Gershenzon J. 2009. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochemistry **70**, 1621-1637.

Deiana L, Carru C, Pes G, Tadolini B. 1999. Spectrophotometric measurement of hydroperoxides at increased sensitivity by oxidation of Fe2+ in the presence of xylenol orange. Free Radical Research **31**, 237-244.

Delfine S, Csiky O, Seufert G, Loreto F. 2000. Fumigation with exogenous monoterpenes of a non-isoprenoid-emitting oak (Quercus suber): monoterpene acquisition, translocation, and effect on the photosynthetic properties at high temperatures. New Phytologist **146**, 27-36.

Delfine S, Loreto F, Pinelli P, Tognetti R, Alvino A. 2005. Isoprenoids content and photosynthetic limitations in rosemary and spearmint plants under water stress. Agriculture, Ecosystems & Environment **106**, 243-252.

Delwiche CF, Sharkey TD. 1993. Rapid appearance of 13C in biogenic isoprene when 13CO2 is fed to intact leaves. Plant, Cell & Environment **16**, 587-591.

Demurtas OC, Nicolia A, Diretto G. 2023. Terpenoid Transport in Plants: How Far from the Final Picture? Plants **12**, 634.

Dodd IC. 2005. Root-To-Shoot Signalling: Assessing The Roles of 'Up' In the Up and Down World of Long-Distance Signalling In Planta. Plant and Soil **274**, 251-270.

Dodd IC. 2007. Soil moisture heterogeneity during deficit irrigation alters root-to-shoot signalling of abscisic acid. Functional Plant Biology **34**, 439-448.

Dodd IC, Ryan AC. 2016. Whole-Plant Physiological Responses to Water-Deficit Stress. *eLS*, 1-9.

Dong C, Qu G, Guo J, Wei F, Gao S, Sun Z, Jin L, Sun X, Rochaix J-D, Miao Y, Wang R. 2022. Rational design of geranylgeranyl diphosphate synthase enhances carotenoid production and improves photosynthetic efficiency in Nicotiana tabacum. Science Bulletin **67**, 315-327.

Dong C, Zhang M, Song S, Wei F, Qin L, Fan P, Shi Y, Wang X, Wang R. 2023. A Small Subunit of Geranylgeranyl Diphosphate Synthase Functions as an Active Regulator of Carotenoid Synthesis in Nicotiana tabacum. International Journal of Molecular Sciences **24**, 992.

Dudareva N, Andersson S, Orlova I, Gatto N, Reichelt M, Rhodes D, Boland W, Gershenzon J. 2005. The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers. Proceedings of the National Academy of Sciences of the United States of America **102**, 933-938.

Dudareva N, Klempien A, Muhlemann JK, Kaplan I. 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytologist **198**, 16-32.

Dudareva N, Pichersky E, Gershenzon J. 2004. Biochemistry of Plant Volatiles. Plant Physiology **135**, 1893-1902.

Duursma RA, Blackman CJ, Lopéz R, Martin-StPaul NK, Cochard H, Medlyn BE. 2019. On the minimum leaf conductance: its role in models of plant water use, and ecological and environmental controls. New Phytologist **221**, 693-705.

Dwivedi SL, Reynolds MP, Ortiz R. 2021. Mitigating tradeoffs in plant breeding. iScience **24**.

Effmert U, Große J, Röse USR, Ehrig F, Kägi R, Piechulla B. 2005. Volatile composition, emission pattern, and localization of floral scent emission in Mirabilis jalapa (Nyctaginaceae). American Journal of Botany **92**, 2-12.

El Tamer MK, Smeets M, Holthuysen N, Lücker J, Tang A, Roozen J, Bouwmeester HJ, Voragen AGJ. 2003. The influence of monoterpene synthase transformation on the odour of tobacco. Journal of Biotechnology **106**, 15-21.

Escobar-Bravo R, Lin P-A, Waterman JM, Erb M. 2023. Dynamic environmental interactions shaped by vegetative plant volatiles. Natural Product Reports **40**, 840-865.

Estévez JM, Cantero A, Reindl A, Reichler S, León P. 2001. 1-Deoxy-d-xylulose-5-phosphate Synthase, a Limiting Enzyme for Plastidic Isoprenoid Biosynthesis in Plants *. Journal of Biological Chemistry **276**, 22901-22909.

Faize M, Burgos L, Faize L, Piqueras A, Nicolas E, Barba-Espin G, Clemente-Moreno MJ, Alcobendas R, Artlip T, Hernandez JA. 2011. Involvement of cytosolic ascorbate peroxidase and Cu/Zn-superoxide dismutase for improved tolerance against drought stress. Journal of Experimental Botany **62**, 2599-2613.

Falara V, Akhtar TA, Nguyen TTH, Spyropoulou EA, Bleeker PM, Schauvinhold I, Matsuba Y, Bonini ME, Schilmiller AL, Last RL, Schuurink RC, Pichersky E. 2011. The Tomato Terpene Synthase Gene Family Plant Physiology **157**, 770-789.

Fall R, Monson RK. 1992. Isoprene Emission Rate and Intercellular Isoprene Concentration as Influenced by Stomatal Distribution and Conductance. Plant Physiology **100**, 987-992.

Farquhar GD, Sharkey TD. 1982. Stomatal conductance and photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology **33**, 317-345.

Fasbender L, Yáñez-Serrano AM, Kreuzwieser J, Dubbert D, Werner C. 2018. Real-time carbon allocation into biogenic volatile organic compounds (BVOCs) and respiratory carbon dioxide (CO2) traced by PTR-TOF-MS, 13CO2 laser spectroscopy and 13C-pyruvate labelling. PLoS One **13**, e0204398.

Fineschi S, Loreto F, Staudt M, Peñuelas J. 2013. Diversification of Volatile Isoprenoid Emissions from Trees: Evolutionary and Ecological Perspectives. *Biology, controls and models of tree volatile organic compound emissions*. Dordrecht ; New York: Springer, xv, 547 p.

Fischbach RJ, Zimmer I, Steinbrecher R, Pfichner A, Schnitzler J-P. 2000. Monoterpene synthase activities in leaves of Picea abies (L.) Karst. and Quercus ilex L. Phytochemistry **54**, 257-265.

Fitzky AC, Kaser L, Peron A, Karl T, Graus M, Tholen D, Halbwirth H, Trimmel H, Pesendorfer M, Rewald B, Sandén H. 2023. Same, same, but different: Drought and salinity affect BVOC emission rate and alter blend composition of urban trees. Urban Forestry & Urban Greening **80**, 127842.

Fortunati A, Barta C, Brilli F, Centritto M, Zimmer I, Schnitzler J-P, Loreto F. 2008. Isoprene emission is not temperature-dependent during and after severe drought-stress: a physiological and biochemical analysis. The Plant Journal **55**, 687-697.

Foti MC, Ingold KU. 2003. Mechanism of Inhibition of Lipid Peroxidation by γ-Terpinene, an Unusual and Potentially Useful Hydrocarbon Antioxidant. Journal of Agricultural and Food Chemistry **51**, 2758-2765.

Foyer CH. 2018. Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. Environmental and Experimental Botany **154**, 134-142.

Foyer CH, Ruban AV, Noctor G. 2017. Viewing oxidative stress through the lens of oxidative signalling rather than damage. Biochemical Journal **474**, 877-883.

Foyer CH, Shigeoka S. 2010. Understanding Oxidative Stress and Antioxidant Functions to Enhance Photosynthesis. Plant Physiology **155**, 93-100.

Francesco S, Antonio C, Livia M, Karthik Mudigere N, Chiara P. 2011. Emission and Function of Volatile Organic Compounds in Response to Abiotic Stress. In: Arun S, Venkateswarlu B, eds. *Abiotic Stress in Plants*. Rijeka: IntechOpen, Ch. 16.

Frank L, Wenig M, Ghirardo A, van der Krol A, Vlot AC, Schnitzler J-P, Rosenkranz M. 2021. Isoprene and β-caryophyllene confer plant resistance via different plant internal signalling pathways. Plant, Cell & Environment **44**, 1151-1164.

Freitas CDT, Demarco D, Oliveira JS, Ramos MV. 2024. Review: Laticifer as a plant defense mechanism. Plant Science **346**, 112136.

Funk JL, Mak JE, Lerdau MT. 2004. Stress-induced changes in carbon sources for isoprene production in Populus deltoides. Plant, Cell & Environment **27**, 747-755.

Gabriel R, Schäfer L, Gerlach C, Rausch T, Kesselmeier J. 1999. Factors controlling the emissions of volatile organic acids from leaves of Quercus ilex L. (Holm oak). Atmospheric Environment **33**, 1347-1355.

Gaion LA, Carvalho RF. 2021. Stomatal response to drought is modulated by gibberellin in tomato. Acta Physiologiae Plantarum **43**, 129.

Gang DR, Wang J, Dudareva N, Nam KH, Simon JE, Lewinsohn E, Pichersky E. 2001. An Investigation of the Storage and Biosynthesis of Phenylpropenes in Sweet Basil. Plant Physiology **125**, 539-555.

Geron C, Rasmussen R, R. Arnts R, Guenther A. 2000. A review and synthesis of monoterpene speciation from forests in the United States. Atmospheric Environment **34**, 1761-1781.

Gershenzon J. 1994. Metabolic costs of terpenoid accumulation in higher plants. Journal of Chemical Ecology **20**, 1281-1328.

Gershenzon J, Dudareva N. 2007. The function of terpene natural products in the natural world. Nature Chemical Biology **3**, 408-414.

Ghasemi G, Fattahi M, Alirezalu A. 2020. A new source of oxygenated monoterpenes with phytotoxic activity: essential oil of Cuminum Cyminum L. from Iran. Natural Product Research **34**, 843-846.

Gigon A, Matos A-R, Laffray D, Zuily-Fodil Y, Pham-Thi A-T. 2004. Effect of Drought Stress on Lipid Metabolism in the Leaves of Arabidopsis thaliana (Ecotype Columbia). Annals of Botany **94**, 345-351.

Goldstein AH, Goulden ML, Munger JW, Wofsy SC, Geron CD. 1998. Seasonal course of isoprene emissions from a midlatitude deciduous forest. Journal of Geophysical Research: Atmospheres **103**, 31045-31056.

Gonzalez-Burgos E, Gomez-Serranillos MP. 2012. Terpene Compounds in Nature: A Review of Their Potential Antioxidant Activity. Current Medicinal Chemistry **19**, 5319-5341.

Gracz-Bernaciak J, Mazur O, Nawrot R. 2021. Functional Studies of Plant Latex as a Rich Source of Bioactive Compounds: Focus on Proteins and Alkaloids. International Journal of Molecular Sciences **22**, 12427.

Graßmann J. 2005. Terpenoids as Plant Antioxidants. In: Litwack G, ed. *Vitamins & Hormones*, Vol. 72: Academic Press, 505-535.

Grote R, Monson RK, Niinemets Ü. 2013. Leaf-Level Models of Constitutive and Stress-Driven Volatile Organic Compound Emissions. In: Niinemets Ü, Monson RK, eds. *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*. Dordrecht: Springer Netherlands, 315-355.

Gubiš J, Vaňková R, Červená V, Dragúňová M, Hudcovicová M, Lichtnerová H, Dokupil T, Jureková Z. 2007. Transformed tobacco plants with increased tolerance to drought. South African Journal of Botany **73**, 505-511.

Guenther A. 2013. Upscaling Biogenic Volatile Compound Emissions from Leaves to Landscapes. In: Niinemets Ü, Monson RK, eds. *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*. Dordrecht: Springer Netherlands, 391-414.

Guenther A, Hewitt CN, Erickson D, Fall R, Geron C, Graedel T, Harley P, Klinger L, Lerdau M, Mckay WA, Pierce T, Scholes B, Steinbrecher R, Tallamraju R, Taylor J, Zimmerman P. 1995. A global model of natural volatile organic compound emissions. Journal of Geophysical Research: Atmospheres **100**, 8873-8892.

Guenther AB, Jiang X, Heald CL, Sakulyanontvittaya T, Duhl T, Emmons LK, Wang X. 2012. The Model of Emissions of Gases and Aerosols from Nature version

2.1 (MEGAN2.1): an extended and updated framework for modeling biogenic emissions. Geosci. Model Dev. **5**, 1471-1492.

Guenther AB, Zimmerman PR, Harley PC, Monson RK, Fall R. 1993. Isoprene and monoterpene emission rate variability: Model evaluations and sensitivity analyses. Journal of Geophysical Research: Atmospheres **98**, 12609-12617.

Guo X, Peng C, Li T, Huang J, Song H, Zhu Q, Wang M. 2021. The Effects of Drought and Re-Watering on Non-Structural Carbohydrates of Pinus tabulaeformis Seedlings. Biology **10**, 281.

Guyot G, Scoffoni C, Sack L. 2012. Combined impacts of irradiance and dehydration on leaf hydraulic conductance: insights into vulnerability and stomatal control. Plant, Cell & Environment **35**, 857-871.

Haberstroh S, Kreuzwieser J, Lobo-do-Vale R, Caldeira MC, Dubbert M, Werner C. 2018. Terpenoid Emissions of Two Mediterranean Woody Species in Response to Drought Stress. Frontiers in Plant Science **9**.

Hajiboland R. 2014. Chapter 1 - Reactive Oxygen Species and Photosynthesis. In: Ahmad P, ed. *Oxidative Damage to Plants*. San Diego: Academic Press, 1-63.

Hantson S, Knorr W, Schurgers G, Pugh TAM, Arneth A. 2017. Global isoprene and monoterpene emissions under changing climate, vegetation, CO2 and land use. Atmospheric Environment **155**, 35-45.

Harley PC. 2013. The Roles of Stomatal Conductance and Compound Volatility in Controlling the Emission of Volatile Organic Compounds from Leaves. In: Niinemets Ü, Monson RK, eds. *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*. Dordrecht: Springer Netherlands, 181-208.

Harrison SP, Morfopoulos C, Dani KGS, Prentice IC, Arneth A, Atwell BJ, Barkley MP, Leishman MR, Loreto F, Medlyn BE, Niinemets Ü, Possell M, Peñuelas J, Wright IJ. 2013. Volatile isoprenoid emissions from plastid to planet. New Phytologist **197**, 49-57.

Harvey CM, Li Z, Tjellström H, Blanchard GJ, Sharkey TD. 2015. Concentration of isoprene in artificial and thylakoid membranes. Journal of Bioenergetics and Biomembranes **47**, 419-429.

Harvey CM, Sharkey TD. 2016. Exogenous isoprene modulates gene expression in unstressed Arabidopsis thaliana plants. Plant, Cell & Environment **39**, 1251-1263.

Hasanuzzaman M, Bhuyan MHMB, Parvin K, Bhuiyan TF, Anee TI, Nahar K, Hossen MS, Zulfiqar F, Alam MM, Fujita M. 2020a. Regulation of ROS Metabolism in Plants under Environmental Stress: A Review of Recent Experimental Evidence. International Journal of Molecular Sciences **21**, 8695.

Hasanuzzaman M, Bhuyan MHMB, Zulfiqar F, Raza A, Mohsin SM, Mahmud JA, Fujita M, Fotopoulos V. 2020b. Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. Antioxidants **9**, 681.

Hayat F, Ahmed MA, Zarebanadkouki M, Javaux M, Cai G, Carminati A. 2020. Transpiration Reduction in Maize (Zea mays L) in Response to Soil Drying. Frontiers in Plant Science **10**.

Heil M, Kost C. 2006. Priming of indirect defences. Ecology Letters **9**, 813-817.

Heil M, Silva Bueno JC. 2007. Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. Proceedings of the National Academy of Sciences **104**, 5467-5472.

Heyser JW, Nabors MW. 1981. Osmotic Adjustment of Cultured Tobacco Cells (Nicotiana tabacum var. Samsum) Grown on Sodium Chloride 1. Plant Physiology **67**, 720-727.

Hirose S, Satake A. 2024. Theoretical analyses for the evolution of biogenic volatile organic compounds (BVOC) emission strategy. Ecology and Evolution **14**, e11548.

Hodges DM, DeLong JM, Forney CF, Prange RK. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta **207**, 604-611.

Holopainen JK, Gershenzon J. 2010. Multiple stress factors and the emission of plant VOCs. Trends in Plant Science **15**, 176-184.

Hu Y, Wu Q, Peng Z, Sprague SA, Wang W, Park J, Akhunov E, Jagadish KSV, Nakata PA, Cheng N, Hirschi KD, White FF, Park S. 2017. Silencing of OsGRXS17 in rice improves drought stress tolerance by modulating ROS accumulation and stomatal closure. Scientific Reports **7**, 15950.

Huang H, Ullah F, Zhou D-X, Yi M, Zhao Y. 2019a. Mechanisms of ROS Regulation of Plant Development and Stress Responses. Frontiers in Plant Science **10**.

Huang J, Forkelová L, Unsicker SB, Forkel M, Griffith DWT, Trumbore S, Hartmann H. 2019b. Isotope labeling reveals contribution of newly fixed carbon to carbon storage and monoterpenes production under water deficit and carbon limitation. Environmental and Experimental Botany **162**, 333-344.

Huang J, Willems P, Wei B, Tian C, Ferreira RB, Bodra N, Martínez Gache SA, Wahni K, Liu K, Vertommen D, Gevaert K, Carroll KS, Van Montagu M, Yang J, Van Breusegem F, Messens J. 2019c. Mining for protein S-sulfenylation in Arabidopsis uncovers redox-sensitive sites. Proceedings of the National Academy of Sciences **116**, 21256-21261.

Huntenburg K, Puértolas J, de Ollas C, Dodd IC. 2022. Bi-directional, long-distance hormonal signalling between roots and shoots of soil water availability. Physiologia Plantarum **174**, e13697.

Ibrahim M, Oksanen E, Holopainen J. 2004. Effects of limonene on the growth and physiology of cabbage (Brassica oleracea L) and carrot (Daucus carota L) plants. Journal of the Science of Food and Agriculture **84**, 1319-1326.

Ibrahim MA, Nissinen A, Prozherina N, Oksanen EJ, Holopainen JK. 2006. The influence of exogenous monoterpene treatment and elevated temperature on growth, physiology, chemical content and headspace volatiles of two carrot cultivars (Daucus carota L.). Environmental and Experimental Botany **56**, 95-107.

Ilyas M, Nisar M, Khan N, Hazrat A, Khan AH, Hayat K, Fahad S, Khan A, Ullah A. 2021. Drought Tolerance Strategies in Plants: A Mechanistic Approach. Journal of Plant Growth Regulation **40**, 926-944.

Isah T. 2019. Stress and defense responses in plant secondary metabolites production. Biological Research **52**, 39.

Jardine K, Chambers J, Alves EG, Teixeira A, Garcia S, Holm J, Higuchi N, Manzi A, Abrell L, Fuentes JD, Nielsen LK, Torn MS, Vickers CE. 2014. Dynamic

Balancing of Isoprene Carbon Sources Reflects Photosynthetic and Photorespiratory Responses to Temperature Stress Plant Physiology **166**, 2051-2064.

Jardine KJ, Jardine AB, Holm JA, Lombardozzi DL, Negron-Juarez RI, Martin ST, Beller HR, Gimenez BO, Higuchi N, Chambers JQ. 2017. Monoterpene 'thermometer' of tropical forest-atmosphere response to climate warming. Plant, Cell & Environment **40**, 441-452.

Jardine KJ, Monson RK, Abrell L, Saleska SR, Arneth A, Jardine A, Ishida FY, Serrano AMY, Artaxo P, Karl T, Fares S, Goldstein A, Loreto F, Huxman T. 2012. Within-plant isoprene oxidation confirmed by direct emissions of oxidation products methyl vinyl ketone and methacrolein. Global Change Biology **18**, 973-984.

Jiang J, Dehesh K. 2021. Plastidial retrograde modulation of light and hormonal signaling: an odyssey. New Phytologist **230**, 931-937.

Jin X, Baysal C, Drapal M, Sheng Y, Huang X, He W, Shi L, Capell T, Fraser PD, Christou P, Zhu C. 2021. The Coordinated Upregulated Expression of Genes Involved in MEP, Chlorophyll, Carotenoid and Tocopherol Pathways, Mirrored the Corresponding Metabolite Contents in Rice Leaves during De-Etiolation. Plants **10**, 1456.

Joan Llusià, Josep Peñuelas, Giorgio A. Alessio, Marc Estiarte. 2008. Contrasting Species-Specific, Compound-Specific, Seasonal, and Interannual Responses of Foliar Isoprenoid Emissions to Experimental Drought in a Mediterranean Shrubland. International Journal of Plant Sciences **169**, 637-645.

Kadota Y, Shirasu K, Zipfel C. 2015. Regulation of the NADPH Oxidase RBOHD During Plant Immunity. Plant and Cell Physiology **56**, 1472-1480.

Kalske A, Shiojiri K, Uesugi A, Sakata Y, Morrell K, Kessler A. 2019. Insect Herbivory Selects for Volatile-Mediated Plant-Plant Communication. Current Biology **29**, 3128-3133.e3123.

Kangasjärvi S, Lepistö A, Hännikäinen K, Piippo M, Luomala E-M, Aro E-M, Rintamäki E. 2008. Diverse roles for chloroplast stromal and thylakoid-bound ascorbate peroxidases in plant stress responses. Biochemical Journal **412**, 275-285.

Karl T, Fall R, Rosenstiel T, Prazeller P, Larsen B, Seufert G, Lindinger W. 2002. On-line analysis of the 13CO2 labeling of leaf isoprene suggests multiple subcellular origins of isoprene precursors. Planta **215**, 894-905.

Karunanithi PS, Zerbe P. 2019. Terpene Synthases as Metabolic Gatekeepers in the Evolution of Plant Terpenoid Chemical Diversity. Frontiers in Plant Science **10**.

Kazan K. 2015. Diverse roles of jasmonates and ethylene in abiotic stress tolerance. Trends in Plant Science **20**, 219-229.

Kesselmeier J, Ciccioli P, Kuhn U, Stefani P, Biesenthal T, Rottenberger S, Wolf A, Vitullo M, Valentini R, Nobre A, Kabat P, Andreae MO. 2002. Volatile organic compound emissions in relation to plant carbon fixation and the terrestrial carbon budget. Global Biogeochemical Cycles **16**, 73-71-73-79.

Kesselmeier J, Staudt M. 1999. Biogenic Volatile Organic Compounds (VOC): An Overview on Emission, Physiology and Ecology. Journal of Atmospheric Chemistry **33**, 23-88.

Kessler A, Halitschke R, Diezel C, Baldwin IT. 2006. Priming of plant defense responses in nature by airborne signaling between Artemisia tridentata and Nicotiana attenuata. Oecologia **148**, 280-292.

Kessler A, Heil M. 2011. The multiple faces of indirect defences and their agents of natural selection. Functional Ecology **25**, 348-357.

Khan R, Ma X, Shah S, Wu X, Shaheen A, Xiao L, Wu Y, Wang S. 2020. Drought-hardening improves drought tolerance in Nicotiana tabacum at physiological, biochemical, and molecular levels. BMC Plant Biology **20**, 486.

Killi D, Raschi A, Bussotti F. 2020. Lipid Peroxidation and Chlorophyll Fluorescence of Photosystem II Performance during Drought and Heat Stress is Associated with the Antioxidant Capacities of C3 Sunflower and C4 Maize Varieties. International Journal of Molecular Sciences **21**, 4846.

Klassen NV, Marchington D, McGowan HCE. 1994. H2O2 Determination by the I3-Method and by KMnO4 Titration. Analytical Chemistry **66**, 2921-2925.

Kleist E, Mentel TF, Andres S, Bohne A, Folkers A, Kiendler-Scharr A, Rudich Y, Springer M, Tillmann R, Wildt J. 2012. Irreversible impacts of heat on the emissions of monoterpenes, sesquiterpenes, phenolic BVOC and green leaf volatiles from several tree species. Biogeosciences **9**, 5111-5123.

Koley S, Grafahrend-Belau E, Raorane ML, Junker BH. 2020. The mevalonate pathway contributes to monoterpene production in peppermint. bioRxiv, 2020.2005.2029.124016.

Kong C-H, Xuan TD, Khanh TD, Tran H-D, Trung NT. 2019. Allelochemicals and Signaling Chemicals in Plants. Molecules **24**, 2737.

Kreuzwieser J, Meischner M, Grün M, Yáñez-Serrano AM, Fasbender L, Werner C. 2021. Drought affects carbon partitioning into volatile organic compound biosynthesis in Scots pine needles. New Phytologist **232**, 1930-1943.

Kreuzwieser J, Schnitzler JP, Steinbrecher R. 1999. Biosynthesis of Organic Compounds Emitted by Plants. Plant Biol (Stuttg) **1**, 149-159.

Kriegs B, Jansen M, Hahn K, Peisker H, Šamajová O, Beck M, Braun S, Ulbrich A, Baluška F, Schulz M. 2010. Cyclic monoterpene mediated modulations of Arabidopsis thaliana phenotype. Plant Signaling & Behavior **5**, 832-838.

Kuo EY, Cai M-S, Lee T-M. 2020. Ascorbate peroxidase 4 plays a role in the tolerance of Chlamydomonas reinhardtii to photo-oxidative stress. Scientific Reports **10**, 13287.

Lado C, Then M, Varga I, Szoke E, Szentmihalyi K. 2004. Antioxidant property of volatile oils determined by the ferric reducing ability. Z Naturforsch C J Biosci **59**, 354-358.

Lambers H, Chapin FS, Pons TL. 2008. Plant Water Relations. *Plant Physiological Ecology*. New York, NY: Springer New York, 163-223.

Lamers J, van der Meer T, Testerink C. 2020. How Plants Sense and Respond to Stressful Environments1 [OPEN]. Plant Physiology **182**, 1624-1635.

Lange BM, Ahkami A. 2013. Metabolic engineering of plant monoterpenes, sesquiterpenes and diterpenes—current status and future opportunities. Plant Biotechnology Journal **11**, 169-196.

Laothawornkitkul J, Taylor JE, Paul ND, Hewitt CN. 2009. Biogenic volatile organic compounds in the Earth system. New Phytologist **183**, 27-51.

Laule O, Fürholz A, Chang H-S, Zhu T, Wang X, Heifetz PB, Gruissem W, Lange M. 2003. Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in Arabidopsis thaliana. Proceedings of the National Academy of Sciences **100**, 6866-6871.

Lehning A, Zimmer I, Steinbrecher R, BrÜggemann N, Schnitzler J-P. 1999. Isoprene synthase activity and its relation to isoprene emission in Quercus robur L. leaves. Plant, Cell & Environment **22**, 495-504.

Leng X, Wang H, Zhang S, Qu C, Yang C, Xu Z, Liu G. 2021. Identification and Characterization of the APX Gene Family and Its Expression Pattern under Phytohormone Treatment and Abiotic Stress in Populus trichocarpa. Genes **12**, 334.

Li C, Zha W, Li W, Wang J, You A. 2023. Advances in the Biosynthesis of Terpenoids and Their Ecological Functions in Plant Resistance. International Journal of Molecular Sciences **24**, 11561.

Li J, Perdue EM, Pavlostathis SG, Araujo R. 1998. Physicochemical properties of selected monoterpenes. Environment International **24**, 353-358.

Li R-h, Guo P-g, Michael B, Stefania G, Salvatore C. 2006. Evaluation of Chlorophyll Content and Fluorescence Parameters as Indicators of Drought Tolerance in Barley. Agricultural Sciences in China **5**, 751-757.

Li Z, Sharkey TD. 2013. Molecular and Pathway Controls on Biogenic Volatile Organic Compound Emissions. In: Niinemets Ü, Monson RK, eds. *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*. Dordrecht: Springer Netherlands, 119-151.

Liang G, Liu J, Zhang J, Guo J. 2020. Effects of Drought Stress on Photosynthetic and Physiological Parameters of Tomato. Journal of the American Society for Horticultural Science J. Amer. Soc. Hort. Sci. **145**, 12-17.

Liao P, Maoz I, Shih M-L, Lee JH, Huang X-Q, Morgan JA, Dudareva N. 2023. Emission of floral volatiles is facilitated by cell-wall non-specific lipid transfer proteins. Nature Communications **14**, 330.

Lichtenthaler HK. 1999. The 1-Deoxy-D-Xylulose-5-Phosphate Pathway of Isoprenoid Biosynthesis in Plants. Annu Rev Plant Physiol Plant Mol Biol **50**, 47-65.

Lichtenthaler HK, Schwender J, Disch A, Rohmer M. 1997. Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway. FEBS Letters **400**, 271-274.

Llusià J, Peñuelas J, Asensio D, Munné-Bosch S. 2005. Airborne limonene confers limited thermotolerance to Quercus ilex. Physiologia Plantarum **123**, 40-48.

Loreto F, Ciccioli P, Brancaleoni E, Cecinato A, Frattoni M. 1998a. Measurement of isoprenoid content in leaves of Mediterranean Quercus spp. by a novel and sensitive method and estimation of the isoprenoid partition between liquid and gas phase inside the leaves. Plant Science **136**, 25-30.

Loreto F, Ciccioli P, Brancaleoni E, Cecinato A, Frattoni M, Sharkey TD. 1996a. Different sources of reduced carbon contribute to form three classes of terpenoid emitted by Quercus ilex L. leaves. Proceedings of the National Academy of Sciences **93**, 9966-9969.

Loreto F, Ciccioli P, Cecinato A, Brancaleoni E, Frattoni M, Fabozzi C, Tricoli D. 1996b. Evidence of the Photosynthetic Origin of Monoterpenes Emitted by Quercus ilex L. Leaves by 13C Labeling. Plant Physiology **110**, 1317-1322.

Loreto F, Ciccioli P, Cecinato A, Brancaleoni E, Frattoni M, Tricoli D. 1996c. Influence of Environmental Factors and Air Composition on the Emission of [alpha]-Pinene from Quercus ilex Leaves. Plant Physiology **110**, 267-275.

Loreto F, Csiky O, Delfine S, Alvino A, Kristiansen T, Seufert G. 1998b. The Effect of Monoterpene Fumigation on Heat-Induced Photosynthesis Inhebition of Quercus. In: Garab G, ed. *Photosynthesis: Mechanisms and Effects: Volume I–V: Proceedings of the XIth International Congress on Photosynthesis, Budapest, Hungary, August 17–22, 1998*. Dordrecht: Springer Netherlands, 2473-2476.

Loreto F, Fischbach RJ, Schnitzler J-P, Ciccioli P, Brancaleoni E, Calfapietra C, Seufert G. 2001a. Monoterpene emission and monoterpene synthase activities in the Mediterranean evergreen oak Quercus ilex L. grown at elevated CO2 concentrations. Global Change Biology **7**, 709-717.

Loreto F, Förster A, Dürr M, Csiky O, Seufert G. 1998c. On the monoterpene emission under heat stress and on the increased thermotolerance of leaves of Quercus ilex L. fumigated with selected monoterpenes. Plant, Cell & Environment **21**, 101-107.

Loreto F, Mannozzi M, Maris C, Nascetti P, Ferranti F, Pasqualini S. 2001b. Ozone quenching properties of isoprene and its antioxidant role in leaves. Plant Physiology **126**, 993-1000.

Loreto F, Pinelli P, Manes F, Kollist H. 2004. Impact of ozone on monoterpene emissions and evidence for an isoprene-like antioxidant action of monoterpenes emitted by Quercus ilex leaves. Tree Physiology **24**, 361-367.

Loreto F, Pollastri S, Fineschi S, Velikova V. 2014. Volatile isoprenoids and their importance for protection against environmental constraints in the Mediterranean area. Environmental and Experimental Botany **103**, 99-106.

Loreto F, Schnitzler J-P. 2010. Abiotic stresses and induced BVOCs. Trends in Plant Science **15**, 154-166.

Loreto F, Velikova V. 2001. Isoprene produced by leaves protects the photosynthetic apparatus against ozone damage, quenches ozone products, and reduces lipid peroxidation of cellular membranes. Plant Physiology **127**, 1781-1787.

Lücker J, Schwab W, Franssen MCR, Van Der Plas LHW, Bouwmeester HJ, Verhoeven HA. 2004. Metabolic engineering of monoterpene biosynthesis: two-step production of (+)-trans-isopiperitenol by tobacco. The Plant Journal **39**, 135-145.

Lücker J, Schwab W, van Hautum B, Blaas J, van der Plas LHW, Bouwmeester HJ, Verhoeven HA. 2004. Increased and Altered Fragrance of Tobacco Plants after Metabolic Engineering Using Three Monoterpene Synthases from Lemon. Plant Physiology **134**, 510-519.

Luo Y, Li W, Huang C, Yang J, Jin M, Chen J, Pang D, Chang Y, Li Y, Wang Z. 2021. Exogenous abscisic acid coordinating leaf senescence and transport of assimilates into wheat grains under drought stress by regulating hormones homeostasis. The Crop Journal **9**, 901-914.

Lüpke M, Leuchner M, Steinbrecher R, Menzel A. 2017. Quantification of monoterpene emission sources of a conifer species in response to experimental drought. AoB PLANTS **9**.

MacDonald RC, Fall R. 1993. Detection of substantial emissions of methanol from plants to the atmosphere. Atmospheric Environment. Part A. General Topics **27**, 1709-1713.

Maffei ME. 2010. Sites of synthesis, biochemistry and functional role of plant volatiles. South African Journal of Botany **76**, 612-631.

Magel E, Mayrhofer S, Müller A, Zimmer I, Hampp R, Schnitzler JP. 2006. Photosynthesis and substrate supply for isoprene biosynthesis in poplar leaves. Atmospheric Environment **40**, 138-151.

Mahmoud SS, Croteau RB. 2002. Strategies for transgenic manipulation of monoterpene biosynthesis in plants. Trends in Plant Science **7**, 366-373.

Mäkelä P, Kärkkäinen J, Somersalo S. 2000. Effect of Glycinebetaine on Chloroplast Ultrastructure, Chlorophyll and Protein Content, and RuBPCO Activities in Tomato Grown under Drought or Salinity. Biologia Plantarum **43**, 471-475.

Mano Ji, Ohno C, Domae Y, Asada K. 2001. Chloroplastic ascorbate peroxidase is the primary target of methylviologen-induced photooxidative stress in spinach leaves: its relevance to monodehydroascorbate radical detected with in vivo ESR. Biochimica et Biophysica Acta (BBA) - Bioenergetics **1504**, 275-287.

Maréchaux I, Bartlett MK, Sack L, Baraloto C, Engel J, Joetzjer E, Chave J. 2015. Drought tolerance as predicted by leaf water potential at turgor loss point varies strongly across species within an Amazonian forest. Functional Ecology **29**, 1268-1277.

Marron N, Dreyer E, Boudouresque E, Delay D, Petit J-M, Delmotte FM, Brignolas F. 2003. Impact of successive drought and re-watering cycles on growth and specific leaf area of two Populus × canadensis (Moench) clones, 'Dorskamp' and 'Luisa_Avanzo'. Tree Physiology **23**, 1225-1235.

Martinez-Medina A, Flors V, Heil M, Mauch-Mani B, Pieterse CMJ, Pozo MJ, Ton J, van Dam NM, Conrath U. 2016. Recognizing Plant Defense Priming. Trends in Plant Science **21**, 818-822.

Martínez JP, Silva H, Ledent JF, Pinto M. 2007. Effect of drought stress on the osmotic adjustment, cell wall elasticity and cell volume of six cultivars of common beans (Phaseolus vulgaris L.). European Journal of Agronomy **26**, 30-38.

Mihaliak CA, Gershenzon J, Croteau R. 1991. Lack of rapid monoterpene turnover in rooted plants: implications for theories of plant chemical defense. Oecologia **87**, 373-376.

Milborrow BV. 2001. The pathway of biosynthesis of abscisic acid in vascular plants: a review of the present state of knowledge of ABA biosynthesis. Journal of Experimental Botany **52**, 1145-1164.

Mishra KB, Iannacone R, Petrozza A, Mishra A, Armentano N, La Vecchia G, Trtílek M, Cellini F, Nedbal L. 2012. Engineered drought tolerance in tomato plants is reflected in chlorophyll fluorescence emission. Plant Science **182**, 79-86.

Mittler R, Zandalinas SI, Fichman Y, Van Breusegem F. 2022. Reactive oxygen species signalling in plant stress responses. Nature Reviews Molecular Cell Biology **23**, 663-679.

Miyake C. 2010. Alternative Electron Flows (Water–Water Cycle and Cyclic Electron Flow Around PSI) in Photosynthesis: Molecular Mechanisms and Physiological Functions. Plant and Cell Physiology **51**, 1951-1963.

Miyake C, Asada K. 1996. Inactivation Mechanism of Ascorbate Peroxidase at Low Concentrations of Ascorbate; Hydrogen Peroxide Decomposes Compound I of Ascorbate Peroxidase. Plant and Cell Physiology **37**, 423-430.

Mochizuki T, Ikeda F, Tani A. 2020. Effect of growth temperature on monoterpene emission rates of Acer palmatum. Science of The Total Environment **745**, 140886.

Mochizuki T, Watanabe M, Koike T, Tani A. 2017. Monoterpene emissions from needles of hybrid larch F1 (Larix gmelinii var. japonica × Larix kaempferi) grown under elevated carbon dioxide and ozone. Atmospheric Environment **148**, 197-202.

Møller IM. 2001. Plant mitochondria and oxidative stress: Electron transport, NADPH turnover, and metabolism of reactive oxygen species. Annual Review of Plant Biology **52**, 561-591.

Monaghan P, Metcalfe NB, Torres R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. Ecology Letters **12**, 75-92.

Monson RK, Fall R. 1989. Isoprene Emission from Aspen Leaves 1: Influence of Environment and Relation to Photosynthesis and Photorespiration. Plant Physiology **90**, 267-274.

Monson RK, Weraduwage SM, Rosenkranz M, Schnitzler J-P, Sharkey TD. 2021. Leaf isoprene emission as a trait that mediates the growth-defense tradeoff in the face of climate stress. Oecologia **197**, 885-902.

Moshelion M, Dietz K-J, Dodd IC, Muller B, Lunn JE. 2024. Guidelines for designing and interpreting drought experiments in controlled conditions. Journal of Experimental Botany.

Mozaffar A, Schoon N, Bachy A, Digrado A, Heinesch B, Aubinet M, Fauconnier ML, Delaplace P, du Jardin P, Amelynck C. 2018. Biogenic volatile organic compound emissions from senescent maize leaves and a comparison with other leaf developmental stages. Atmospheric Environment **176**, 71-81.

Mu Z, Llusià J, Liu D, Ogaya R, Asensio D, Zhang C, Peñuelas J. 2018. Seasonal and diurnal variations of plant isoprenoid emissions from two dominant species in Mediterranean shrubland and forest submitted to experimental drought. Atmospheric Environment **191**, 105-115.

Nagalingam S, Seco R, Kim S, Guenther A. 2023. Heat stress strongly induces monoterpene emissions in some plants with specialized terpenoid storage structures. Agricultural and Forest Meteorology **333**, 109400.

Naumann G, Alfieri L, Wyser K, Mentaschi L, Betts RA, Carrao H, Spinoni J, Vogt J, Feyen L. 2018. Global Changes in Drought Conditions Under Different Levels of Warming. Geophysical Research Letters **45**, 3285-3296.

Nemecek-Marshall M, MacDonald RC, Franzen JJ, Wojciechowski CL, Fall R. 1995. Methanol Emission from Leaves (Enzymatic Detection of Gas-Phase Methanol and Relation of Methanol Fluxes to Stomatal Conductance and Leaf Development). Plant Physiology **108**, 1359-1368.

Niinemets Ü. 2010. Mild versus severe stress and BVOCs: thresholds, priming and consequences. Trends in Plant Science **15**, 145-153.

Niinemets Ü, Hauff K, Bertin N, Tenhunen JD, Steinbrecher R, Seufert G. 2002a. Monoterpene emissions in relation to foliar photosynthetic and structural variables in Mediterranean evergreen Quercus species. New Phytologist **153**, 243-256.

Niinemets Ü, Loreto F, Reichstein M. 2004. Physiological and physicochemical controls on foliar volatile organic compound emissions. Trends in Plant Science **9**, 180-186.

Niinemets Ü, Monson RK. 2013. State-of-the-Art of BVOC Research: What Do We Have and What Have We Missed? A Synthesis. In: Niinemets Ü, Monson RK, eds. *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*. Dordrecht: Springer Netherlands, 509-528.

Niinemets Ü, Monson RK, Arneth A, Ciccioli P, Kesselmeier J, Kuhn U, Noe SM, Peñuelas J, Staudt M. 2010. The leaf-level emission factor of volatile isoprenoids: caveats, model algorithms, response shapes and scaling. Biogeosciences **7**, 1809-1832.

Niinemets Ü, Reichstein M. 2002. A model analysis of the effects of nonspecific monoterpenoid storage in leaf tissues on emission kinetics and composition in Mediterranean sclerophyllous Quercus species. Global Biogeochemical Cycles **16**, 57-51-57-26.

Niinemets Ü, Reichstein M. 2003. Controls on the emission of plant volatiles through stomata: A sensitivity analysis. Journal of Geophysical Research: Atmospheres **108**.

Niinemets Ü, Reichstein M, Staudt M, Seufert G, Tenhunen JD. 2002b. Stomatal Constraints May Affect Emission of Oxygenated Monoterpenoids from the Foliage of Pinus pinea. Plant Physiology **130**, 1371-1385.

Niinemets Ü, Seufert G, Steinbrecher R, Tenhunen JD. 2002c. A model coupling foliar monoterpene emissions to leaf photosynthetic characteristics in Mediterranean evergreen Quercus species. New Phytologist **153**, 257-275.

Niinemets Ü, Tenhunen JD, Harley PC, Steinbrecher R. 1999. A model of isoprene emission based on energetic requirements for isoprene synthesis and leaf photosynthetic properties for Liquidambar and Quercus. Plant, Cell & Environment **22**, 1319-1335.

Nilson SE, Assmann SM. 2007. The Control of Transpiration. Insights from Arabidopsis. Plant Physiology **143**, 19-27.

Ninkuu V, Zhang L, Yan J, Fu Z, Yang T, Zeng H. 2021. Biochemistry of Terpenes and Recent Advances in Plant Protection. International Journal of Molecular Sciences **22**, 5710.

Nishiyama Y, Yamamoto H, Allakhverdiev SI, Inaba M, Yokota A, Murata N. 2001. Oxidative stress inhibits the repair of photodamage to the photosynthetic machinery. The EMBO journal **20**, 5587-5594.

Noacco N, Rodenak-Kladniew B, de Bravo MG, Castro GR, Islan GA. 2018. Simple colorimetric method to determine the in vitro antioxidant activity of different monoterpenes. Analytical Biochemistry **555**, 59-66.

Noctor G, Mhamdi A, Foyer CH. 2014. The Roles of Reactive Oxygen Metabolism in Drought: Not So Cut and Dried Plant Physiology **164**, 1636-1648.

Noctor G, Veljovic-Jovanovic S, Driscoll S, Novitskaya L, Foyer CH. 2002. Drought and Oxidative Load in the Leaves of C3 Plants: a Predominant Role for Photorespiration? Annals of Botany **89**, 841-850.

Noe SM, Ciccioli P, Brancaleoni E, Loreto F, Niinemets Ü. 2006. Emissions of monoterpenes linalool and ocimene respond differently to environmental changes due to differences in physico-chemical characteristics. Atmospheric Environment **40**, 4649-4662.

Noe SM, Copolovici L, Niinemets Ü, Vaino E. 2008. Foliar limonene uptake scales positively with leaf lipid content: "non-emitting" species absorb and release monoterpenes. Plant Biology **10**, 129-137.

Nogués I, Medori M, Calfapietra C. 2015a. Limitations of monoterpene emissions and their antioxidant role in Cistus sp. under mild and severe treatments of drought and warming. Environmental and Experimental Botany **119**, 76-86.

Nogués I, Muzzini V, Loreto F, Bustamante MA. 2015b. Drought and soil amendment effects on monoterpene emission in rosemary plants. Sci Total Environ **538**, 768-778.

Obiol-Pardo C, Rubio-Martinez J, Imperial S. 2011. The methylerythritol phosphate (MEP) pathway for isoprenoid biosynthesis as a target for the development of new drugs against tuberculosis. Curr Med Chem **18**, 1325-1338.

Ohara K, Ujihara T, Endo T, Sato F, Yazaki K. 2003. Limonene production in tobacco with Perilla limonene synthase cDNA. Journal of Experimental Botany **54**, 2635-2642.

Okada K, Saito T, Nakagawa T, Kawamukai M, Kamiya Y. 2000. Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in Arabidopsis. Plant Physiology **122**, 1045-1056.

Ormeño E, Mévy JP, Vila B, Bousquet-Mélou A, Greff S, Bonin G, Fernandez C. 2007. Water deficit stress induces different monoterpene and sesquiterpene emission changes in Mediterranean species. Relationship between terpene emissions and plant water potential. Chemosphere **67**, 276-284.

Ort DR, Baker NR. 2002. A photoprotective role for O2 as an alternative electron sink in photosynthesis? Current Opinion in Plant Biology **5**, 193-198.

Osakabe Y, Osakabe K, Shinozaki K, Tran L-S. 2014. Response of plants to water stress. Frontiers in Plant Science **5**.

Panchuk II, Zentgraf U, Volkov RA. 2005. Expression of the Apx gene family during leaf senescence of Arabidopsis thaliana. Planta **222**, 926-932.

Parkash V, Singh S. 2020. A Review on Potential Plant-Based Water Stress Indicators for Vegetable Crops. Sustainability **12**, 3945.

Peco JD, Pérez–López D, Centeno A, Moreno MM, Villena J, Moratiel R. 2023. Comparison of physiological and biochemical responses of local and commercial tomato varieties under water stress and rehydration. Agricultural Water Management **289**, 108529.

Pegoraro E, Rey A, Greenberg J, Harley P, Grace J, Malhi Y, Guenther A. 2004. Effect of drought on isoprene emission rates from leaves of Quercus virginiana Mill. Atmospheric Environment **38**, 6149-6156.

Peñuelas J, Filella I, Seco R, Llusià J. 2009. Increase in isoprene and monoterpene emissions after re-watering of droughted Quercus ilex seedlings. Biologia Plantarum **53**, 351-354.

Penuelas J, Llusia J. 2003. BVOCs: plant defense against climate warming? Trends in Plant Science **8**, 105-109.

Peñuelas J, Llusià J. 1999. Short-term responses of terpene emission rates to experimental changes of PFD in Pinus halepensis and Quercus ilex in summer field conditions. Environmental and Experimental Botany **42**, 61-68.

Peñuelas J, Llusià J. 2002. Linking photorespiration, monoterpenes and thermotolerance in Quercus. New Phytologist **155**, 227-237.

Peñuelas J, Llusià J. 2004. Plant VOC emissions: making use of the unavoidable. Trends in Ecology & Evolution **19**, 402-404.

Peñuelas j, Llusià j, Asensio d, Munné-bosch s. 2005. Linking isoprene with plant thermotolerance, antioxidants and monoterpene emissions. Plant, Cell & Environment **28**, 278-286.

Peñuelas J, Staudt M. 2010. BVOCs and global change. Trends in Plant Science **15**, 133-144.

Perez-Gil J, Behrendorff J, Douw A, Vickers CE. 2024. The methylerythritol phosphate pathway as an oxidative stress sense and response system. Nature Communications **15**, 5303.

Peron A, Kaser L, Fitzky AC, Graus M, Halbwirth H, Greiner J, Wohlfahrt G, Rewald B, Sandén H, Karl T. 2021. Combined effects of ozone and drought stress on the emission of biogenic volatile organic compounds from Quercus robur L. Biogeosciences **18**, 535-556.

Petrov VD, Van Breusegem F. 2012. Hydrogen peroxide—a central hub for information flow in plant cells. AoB PLANTS **2012**.

Petrović I, Savić S, Gricourt J, Causse M, Jovanović Z, Stikić R. 2021. Effect of long-term drought on tomato leaves: the impact on metabolic and antioxidative response. Physiology and Molecular Biology of Plants **27**, 2805-2817.

Pichersky E, Noel JP, Dudareva N. 2006. Biosynthesis of Plant Volatiles: Nature's Diversity and Ingenuity. Science **311**, 808-811.

Pichersky E, Raguso RA. 2018. Why do plants produce so many terpenoid compounds? New Phytologist **220**, 692-702.

Pinheiro C, Chaves MM. 2010. Photosynthesis and drought: can we make metabolic connections from available data? Journal of Experimental Botany **62**, 869-882.

Pintó-Marijuan M, Munné-Bosch S. 2014. Photo-oxidative stress markers as a measure of abiotic stress-induced leaf senescence: advantages and limitations. Journal of Experimental Botany **65**, 3845-3857.

Pollastri S, Baccelli I, Loreto F. 2021. Isoprene: An Antioxidant Itself or a Molecule with Multiple Regulatory Functions in Plants? Antioxidants **10**, 684.

Pollastri S, Velikova V, Castaldini M, Fineschi S, Ghirardo A, Renaut J, Schnitzler J-P, Sergeant K, Winkler JB, Zorzan S, Loreto F. 2023. Isoprene-Emitting Tobacco Plants Are Less Affected by Moderate Water Deficit under Future Climate Change Scenario and Show Adjustments of Stress-Related Proteins in Actual Climate. Plants **12**, 333.

Poorter H, Fiorani F, Pieruschka R, Wojciechowski T, van der Putten WH, Kleyer M, Schurr U, Postma J. 2016. Pampered inside, pestered outside? Differences and

similarities between plants growing in controlled conditions and in the field. New Phytologist **212**, 838-855.

Porres-Martínez M, González-Burgos E, Carretero ME, Gómez-Serranillos MP. 2015. Major selected monoterpenes α-pinene and 1,8-cineole found in Salvia lavandulifolia (Spanish sage) essential oil as regulators of cellular redox balance. Pharmaceutical Biology **53**, 921-929.

Pospíšil P. 2016. Production of Reactive Oxygen Species by Photosystem II as a Response to Light and Temperature Stress. Frontiers in Plant Science **7**.

Possell M, Loreto F. 2013. The Role of Volatile Organic Compounds in Plant Resistance to Abiotic Stresses: Responses and Mechanisms. *Biology, controls and models of tree volatile organic compound emissions*. Dordrecht ; New York: Springer, xv, 547 p.

Puig CG, Gonçalves RF, Valentão P, Andrade PB, Reigosa MJ, Pedrol N. 2018. The Consistency Between Phytotoxic Effects and the Dynamics of Allelochemicals Release from Eucalyptus globulus Leaves Used as Bioherbicide Green Manure. Journal of Chemical Ecology **44**, 658-670.

Rabara RC, Tripathi P, Reese RN, Rushton DL, Alexander D, Timko MP, Shen QJ, Rushton PJ. 2015. Tobacco drought stress responses reveal new targets for Solanaceae crop improvement. BMC Genomics **16**, 484.

Radwan A, Kleinwächter M, Selmar D. 2017. Impact of drought stress on specialised metabolism: Biosynthesis and the expression of monoterpene synthases in sage (Salvia officinalis). Phytochemistry **141**, 20-26.

Rahman SML, Mackay WA, Nawata E, Sakuratani T, Uddin ASMM, Quebedeaux B. 2004. Superoxide Dismutase and Stress Tolerance of Four Tomato Cultivars. HortScience HortSci **39**, 983-986.

Rai VK, Gupta SC, Singh B. 2003. Volatile Monoterpenes from Prinsepia utilis L. Leaves Inhibit Stomatal Opening in Vicia faba L. Biologia Plantarum **46**, 121-124.

Ramel F, Birtic S, Cuiné S, Triantaphylidès C, Ravanat J-L, Havaux M. 2012. Chemical Quenching of Singlet Oxygen by Carotenoids in Plants Plant Physiology **158**, 1267-1278.

Rasulov B, Copolovici L, Laisk A, Niinemets Ul. 2009a. Postillumination Isoprene Emission: In Vivo Measurements of Dimethylallyldiphosphate Pool Size and Isoprene Synthase Kinetics in Aspen Leaves. Plant Physiology **149**, 1609-1618.

Rasulov B, Hüve K, Välbe M, Laisk A, Niinemets Ul. 2009b. Evidence That Light, Carbon Dioxide, and Oxygen Dependencies of Leaf Isoprene Emission Are Driven by Energy Status in Hybrid Aspen. Plant Physiology **151**, 448-460.

Raza S, Miller M, Hamberger B, Vermaas JV. 2023. Plant Terpenoid Permeability through Biological Membranes Explored via Molecular Simulations. The Journal of Physical Chemistry B **127**, 1144-1157.

Reynolds-Henne CE, Langenegger A, Mani J, Schenk N, Zumsteg A, Feller U. 2010. Interactions between temperature, drought and stomatal opening in legumes. Environmental and Experimental Botany **68**, 37-43.

Rhee SG, Chang T-S, Jeong W, Kang D. 2010. Methods for Detection and Measurement of Hydrogen Peroxide Inside and Outside of Cells. Molecules and Cells **29**, 539-549.

Richard SB, Ferrer J-L, Bowman ME, Lillo AM, Tetzlaff CN, Cane DE, Noel JP. 2002. Structure and Mechanism of 2-C-Methyl-d-erythritol 2,4-Cyclodiphosphate Synthase: AN ENZYME IN THE MEVALONATE-INDEPENDENT ISOPRENOID BIOSYNTHETIC PATHWAY*. Journal of Biological Chemistry **277**, 8667-8672.

Riedlmeier M, Ghirardo A, Wenig M, Knappe C, Koch K, Georgii E, Dey S, Parker JE, Schnitzler J-P, Vlot AC. 2017. Monoterpenes Support Systemic Acquired Resistance within and between Plants. The Plant Cell **29**, 1440-1459.

Rodriéguez-Concepcioén M, Foreés O, Martiénez-Garciéa JF, Gonzaélez V, Phillips MA, Ferrer A, Boronat A. 2004. Distinct Light-Mediated Pathways Regulate the Biosynthesis and Exchange of Isoprenoid Precursors during Arabidopsis Seedling Development. The Plant Cell **16**, 144-156.

Rodríguez-Concepción M. 2006. Early Steps in Isoprenoid Biosynthesis: Multilevel Regulation of the Supply of Common Precursors in Plant Cells. Phytochemistry Reviews **5**, 1-15.

Rodríguez-Concepción M. 2010. Supply of precursors for carotenoid biosynthesis in plants. Archives of Biochemistry and Biophysics **504**, 118-122.

Rohmer M. 1999. The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants. Nat Prod Rep **16**, 565-574.

Rosenkranz M, Schnitzler J-P. 2013. Genetic Engineering of BVOC Emissions from Trees. In: Niinemets Ü, Monson RK, eds. *Biology, Controls and Models of Tree Volatile Organic Compound Emissions*. Dordrecht: Springer Netherlands, 95-118.

Rosenstiel TN, Potosnak MJ, Griffin KL, Fall R, Monson RK. 2003. Increased CO2 uncouples growth from isoprene emission in an agriforest ecosystem. Nature **421**, 256-259.

Runyon JB, Mescher MC, De Moraes CM. 2006. Volatile Chemical Cues Guide Host Location and Host Selection by Parasitic Plants. Science **313**, 1964-1967.

Ryan AC, Hewitt CN, Possell M, Vickers CE, Purnell A, Mullineaux PM, Davies WJ, Dodd IC. 2014. Isoprene emission protects photosynthesis but reduces plant productivity during drought in transgenic tobacco (Nicotiana tabacum) plants. New Phytol **201**, 205-216.

Sakulyanontvittaya T, Duhl T, Wiedinmyer C, Helmig D, Matsunaga S, Potosnak M, Milford J, Guenther A. 2008. Monoterpene and Sesquiterpene Emission Estimates for the United States. Environmental Science & Technology **42**, 1623-1629.

Sancho-Knapik D, Sanz MÁ, Peguero-Pina JJ, Niinemets Ü, Gil-Pelegrín E. 2017. Changes of secondary metabolites in Pinus sylvestris L. needles under increasing soil water deficit. Annals of Forest Science **74**, 24.

Sasaki K, Saito T, Lämsä M, Oksman-Caldentey K-M, Suzuki M, Ohyama K, Muranaka T, Ohara K, Yazaki K. 2007. Plants Utilize Isoprene Emission as a Thermotolerance Mechanism. Plant and Cell Physiology **48**, 1254-1262.

Schilmiller AL, Schauvinhold I, Larson M, Xu R, Charbonneau AL, Schmidt A, Wilkerson C, Last RL, Pichersky E. 2009. Monoterpenes in the glandular trichomes of tomato are synthesized from a neryl diphosphate precursor rather than geranyl diphosphate. Proceedings of the National Academy of Sciences **106**, 10865-10870.

Schmiderer C, Steinborn R, Novak J. 2023. Monoterpene synthases of three closely related sage species (Salvia officinalis, S. fruticosa and S. pomifera, Lamiaceae). Plant Physiology and Biochemistry **196**, 318-327.

Schnitzler Jr-P, Graus M, Kreuzwieser Jr, Heizmann U, Rennenberg H, Wisthaler A, Hansel A. 2004. Contribution of Different Carbon Sources to Isoprene Biosynthesis in Poplar Leaves. Plant Physiology **135**, 152-160.

Seco R, Karl T, Guenther A, Hosman KP, Pallardy SG, Gu L, Geron C, Harley P, Kim S. 2015. Ecosystem-scale volatile organic compound fluxes during an extreme drought in a broadleaf temperate forest of the Missouri Ozarks (central USA). Glob Chang Biol **21**, 3657-3674.

Seemann M, Bui BT, Wolff M, Tritsch D, Campos N, Boronat A, Marquet A, Rohmer M. 2002. Isoprenoid biosynthesis through the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (GcpE) is a [4Fe-4S] protein. Angew Chem Int Ed Engl **41**, 4337-4339.

Seleiman MF, Al-Suhaibani N, Ali N, Akmal M, Alotaibi M, Refay Y, Dindaroglu T, Abdul-Wajid HH, Battaglia ML. 2021. Drought Stress Impacts on Plants and Different Approaches to Alleviate Its Adverse Effects. Plants **10**, 259.

Sewelam N, Kazan K, Schenk PM. 2016. Global Plant Stress Signaling: Reactive Oxygen Species at the Cross-Road. Frontiers in Plant Science **7**.

Shang Y, Yan L, Liu Z-Q, Cao Z, Mei C, Xin Q, Wu F-Q, Wang X-F, Du S-Y, Jiang T, Zhang X-F, Zhao R, Sun H-L, Liu R, Yu Y-T, Zhang D-P. 2010. The Mg-Chelatase H Subunit of Arabidopsis Antagonizes a Group of WRKY Transcription Repressors to Relieve ABA-Responsive Genes of Inhibition The Plant Cell **22**, 1909-1935.

Shangguan Z, Shao M, Dyckmans J. 1999. Interaction of Osmotic Adjustment and Photosynthesis in Winter Wheat Under Soil Drought. Journal of Plant Physiology **154**, 753-758.

Sharkey TD. 1996. Isoprene synthesis by plants and animals. Endeavour **20**, 74-78.

Sharkey TD. 2013. Is it useful to ask why plants emit isoprene? Plant, Cell & Environment **36**, 517-520.

Sharkey TD, Bernacchi CJ, Farquhar GD, Singsaas EL. 2007a. Fitting photosynthetic carbon dioxide response curves for C3 leaves. Plant, Cell & Environment **30**, 1035-1040.

Sharkey TD, Loreto F. 1993. Water stress, temperature, and light effects on the capacity for isoprene emission and photosynthesis of kudzu leaves. Oecologia **95**, 328-333.

Sharkey TD, Singsaas EL. 1995. Why plants emit isoprene. Nature **374**, 769-769.

Sharkey TD, Wiberley AE, Donohue AR. 2007b. Isoprene Emission from Plants: Why and How. Annals of Botany **101**, 5-18.

Sharkey TD, Yeh S. 2001. Isoprene Emission From Plants. Annual Review of Plant Biology **52**, 407-436.

Sharkey TD, Yeh S, Wiberley AE, Falbel TG, Gong D, Fernandez DE. 2005. Evolution of the Isoprene Biosynthetic Pathway in Kudzu Plant Physiology **137**, 700-712.

Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K. 2002. Regulation and function of ascorbate peroxidase isoenzymes. Journal of Experimental Botany **53**, 1305-1319.

Shohat H, Eliaz NI, Weiss D. 2021. Gibberellin in tomato: metabolism, signaling and role in drought responses. Molecular Horticulture **1**, 15.

Sierla M, Waszczak C, Vahisalu T, Kangasjärvi J. 2016. Reactive Oxygen Species in the Regulation of Stomatal Movements Plant Physiology **171**, 1569-1580.

Simas DLR, de Amorim SHBM, Goulart FRV, Alviano CS, Alviano DS, da Silva AJR. 2017. Citrus species essential oils and their components can inhibit or stimulate fungal growth in fruit. Industrial Crops and Products **98**, 108-115.

Šimpraga M, Takabayashi J, Holopainen JK. 2016. Language of plants: Where is the word? Journal of Integrative Plant Biology **58**, 343-349.

Šimpraga M, Verbeeck H, Demarcke M, Joó É, Pokorska O, Amelynck C, Schoon N, Dewulf J, Van Langenhove H, Heinesch B, Aubinet M, Laffineur Q, Müller JF, Steppe K. 2011. Clear link between drought stress, photosynthesis and biogenic volatile organic compounds in Fagus sylvatica L. Atmospheric Environment **45**, 5254-5259.

Simpson K, Quiroz LF, Rodriguez-Concepción M, Stange CR. 2016. Differential Contribution of the First Two Enzymes of the MEP Pathway to the Supply of Metabolic Precursors for Carotenoid and Chlorophyll Biosynthesis in Carrot (Daucus carota). Frontiers in Plant Science **7**.

Singh HP, Batish DR, Kaur S, Arora K, Kohli RK. 2006. α-Pinene Inhibits Growth and Induces Oxidative Stress in Roots. Annals of Botany **98**, 1261-1269.

Singh HP, Kaur S, Mittal S, Batish DR, Kohli RK. 2009. Essential Oil of Artemisia scoparia Inhibits Plant Growth by Generating Reactive Oxygen Species and Causing Oxidative Damage. Journal of Chemical Ecology **35**, 154-162.

Singsaas EL. 2000. Terpenes and the thermotolerance of photosynthesis. New Phytologist **146**, 1-2.

Singsaas EL, Lerdau M, Winter K, Sharkey TD. 1997. Isoprene Increases Thermotolerance of Isoprene-Emitting Species. Plant Physiology **115**, 1413-1420.

Siwko ME, Marrink SJ, de Vries AH, Kozubek A, Schoot Uiterkamp AJM, Mark AE. 2007. Does isoprene protect plant membranes from thermal shock? A molecular dynamics study. Biochimica et Biophysica Acta (BBA) - Biomembranes **1768**, 198-206.

Slot M, Krause GH, Krause B, Hernández GG, Winter K. 2019. Photosynthetic heat tolerance of shade and sun leaves of three tropical tree species. Photosynthesis Research **141**, 119-130.

Smirnoff N. 1993. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytologist **125**, 27-58.

Sohag MAA, Tahjib-Ul-Arif M, Brestič M, Afrin S, Sakil AM, Hossain TM, Hossain AM, Hossain AM. 2020. Exogenous salicylic acid and hydrogen peroxide attenuate drought stress in rice. Plant, Soil and Environment **66**, 7-13.

Sousa RHV, Carvalho FEL, Lima-Melo Y, Alencar VTCB, Daloso DM, Margis-Pinheiro M, Komatsu S, Silveira JAG. 2018. Impairment of peroxisomal APX and CAT activities increases protection of photosynthesis under oxidative stress. Journal of Experimental Botany **70**, 627-639.

Sousa RHV, Carvalho FEL, Ribeiro CW, Passaia G, Cunha JR, Lima-Melo Y, Margis-Pinheiro M, Silveira JAG. 2015. Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory H2O2 induced by CAT deficiency in rice. Plant, Cell & Environment **38**, 499-513.

Srikanta Dani KG, Marino G, Taiti C, Mancuso S, Atwell BJ, Loreto F, Centritto M. 2017. De novo post-illumination monoterpene burst in Quercus ilex (holm oak). Planta **245**, 459-465.

Staudt M, Bertin N. 1998. Light and temperature dependence of the emission of cyclic and acyclic monoterpenes from holm oak (Quercus ilex L.) leaves. Plant, Cell & Environment **21**, 385-395.

Staudt M, Ennajah A, Mouillot F, Joffre R. 2008. Do volatile organic compound emissions of Tunisian cork oak populations originating from contrasting climatic conditions differ in their responses to summer drought? Canadian Journal of Forest Research **38**, 2965-2975.

Staudt M, Rambal S, Joffre R, Kesselmeier J. 2002. Impact of drought on seasonal monoterpene emissions from Quercus ilex in southern France. Journal of Geophysical Research: Atmospheres **107**, ACH 15-11-ACH 15-19.

Styles MQ, Nesbitt EA, Marr S, Hutchby M, Leak DJ. 2017. Characterization of the first naturally thermostable terpene synthases and development of strategies to improve thermostability in this family of enzymes. The FEBS Journal **284**, 1700-1711.

Su X, Wei F, Huo Y, Xia Z. 2017. Comparative Physiological and Molecular Analyses of Two Contrasting Flue-Cured Tobacco Genotypes under Progressive Drought Stress. Frontiers in Plant Science **8**.

Sueishi Y, Nii R. 2018. Monoterpene's multiple free radical scavenging capacity as compared with the radioprotective agent cysteamine and amifostine. Bioorganic & Medicinal Chemistry Letters **28**, 3031-3033.

Taipale D, Aalto J, Schiestl-Aalto P, Kulmala M, Bäck J. 2020. The importance of accounting for enhanced emissions of monoterpenes from new Scots pine foliage in models - A Finnish case study. Atmospheric Environment: X **8**, 100097.

Tattini M, Loreto F, Fini A, Guidi L, Brunetti C, Velikova V, Gori A, Ferrini F. 2015. Isoprenoids and phenylpropanoids are part of the antioxidant defense orchestrated daily by drought-stressed Platanus × acerifolia plants during Mediterranean summers. New Phytologist **207**, 613-626.

Tattini M, Velikova V, Vickers C, Brunetti C, Di Ferdinando M, Trivellini A, Fineschi S, Agati G, Ferrini F, Loreto F. 2014. Isoprene production in transgenic tobacco alters isoprenoid, non-structural carbohydrate and phenylpropanoid metabolism, and protects photosynthesis from drought stress. Plant, Cell & Environment **37**, 1950-1964.

Tholl D. 2015. Biosynthesis and Biological Functions of Terpenoids in Plants. In: Schrader J, Bohlmann J, eds. *Biotechnology of Isoprenoids*. Cham: Springer International Publishing, 63-106.

Tholl D, Lee S. 2011. Terpene Specialized Metabolism in Arabidopsis thaliana. The Arabidopsis Book **2011**.

Tian B, Poulter CD, Jacobson MP. 2016. Defining the Product Chemical Space of Monoterpenoid Synthases. PLOS Computational Biology **12**, e1005053.

Tian Z, Luo Q, Li Y, Zuo Z. 2020. Terpinene and β-pinene acting as signaling molecules to improve Cinnamomum camphora thermotolerance. Industrial Crops and Products **154**, 112641.

Tiiva P, Tang J, Michelsen A, Rinnan R. 2017. Monoterpene emissions in response to long-term night-time warming, elevated CO2 and extended summer drought in a temperate heath ecosystem. Science of The Total Environment **580**, 1056-1067.

Tingey DT, Evans R, Gumpertz M. 1981. Effects of environmental conditions on isoprene emission from live oak. Planta **152**, 565-570.

Tingey DT, Manning M, Grothaus LC, Burns WF. 1980a. Influence of Light and Temperature on Monoterpene Emission Rates from Slash Pine. Plant Physiology **65**, 797-801.

Tingey DT, Manning M, Grothaus LC, Burns WF. 1980b. Influence of light and temperature on monoterpene emission rates from slash pine. Plant Physiology **65**, 797-801.

Tingey DT, Turner DP, Weber JA. 1991. Factors Controlling the Emissions of Monoterpenes and Other Volatile Organic Compounds. In: Sharkey TD, Holland EA, Mooney HA, eds. *Trace Gas Emissions by Plants*. San Diego: Academic Press, 93-119.

Toffolatti SL, Maddalena G, Passera A, Casati P, Bianco PA, Quaglino F. 2021. 16 - Role of terpenes in plant defense to biotic stress. In: Jogaiah S, ed. *Biocontrol Agents and Secondary Metabolites*: Woodhead Publishing, 401-417.

Trapp S, Croteau R. 2001. Defensive Resin Biosynthesis. Annual Review of Plant Biology **52**, 689-724.

Triantaphylidès C, Krischke M, Hoeberichts FA, Ksas B, Gresser G, Havaux M, Van Breusegem F, Mueller MJ. 2008. Singlet Oxygen Is the Major Reactive Oxygen Species Involved in Photooxidative Damage to Plants. Plant Physiology **148**, 960-968.

Trowbridge AM, Adams HD, Collins A, Dickman LT, Grossiord C, Hofland M, Malone S, Weaver DK, Sevanto S, Stoy PC, McDowell NG. 2021. Hotter droughts alter resource allocation to chemical defenses in piñon pine. Oecologia **197**, 921-938.

Trowbridge AM, Daly RW, Helmig D, Stoy PC, Monson RK. 2014. Herbivory and climate interact serially to control monoterpene emissions from pinyon pine forests. Ecology **95**, 1591-1603.

Turlings TC, Loughrin JH, McCall PJ, Röse US, Lewis WJ, Tumlinson JH. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. Proceedings of the National Academy of Sciences **92**, 4169-4174.

Turner GW, Gershenzon J, Croteau RB. 2000. Development of Peltate Glandular Trichomes of Peppermint1. Plant Physiology **124**, 665-680.

Turner NC. 2018. Turgor maintenance by osmotic adjustment: 40 years of progress. Journal of Experimental Botany **69**, 3223-3233.

Ueoka H, Sasaki K, Miyawaki T, Ichino T, Tatsumi K, Suzuki S, Yamamoto H, Sakurai N, Suzuki H, Shibata D, Yazaki K. 2020. A Cytosol-Localized Geranyl Diphosphate Synthase from Lithospermum erythrorhizon and Its Molecular Evolution1 [OPEN]. Plant Physiology **182**, 1933-1945.

Umagiliyage AL, Becerra-Mora N, Kohli P, Fisher DJ, Choudhary R. 2017. Antimicrobial efficacy of liposomes containing d-limonene and its effect on the storage life of blueberries. Postharvest Biology and Technology **128**, 130-137.

Valentini R, Epron D, De Angelis P, Matteucci G, Dreyer E. 1995. In situ estimation of net CO2 assimilation, photosynthetic electron flow and photorespiration in Turkey oak (Q. cerris L.) leaves: diurnal cycles under different levels of water supply. Plant, Cell & Environment **18**, 631-640.

van Genuchten MT. 1980. A Closed-form Equation for Predicting the Hydraulic Conductivity of Unsaturated Soils. Soil Science Society of America Journal **44**, 892-898.

van Hulten M, Pelser M, van Loon LC, Pieterse CMJ, Ton J. 2006. Costs and benefits of priming for defense in Arabidopsis. Proceedings of the National Academy of Sciences **103**, 5602-5607.

Velikova V, Edreva A, Loreto F. 2004. Endogenous isoprene protects Phragmites australis leaves against singlet oxygen. Physiologia Plantarum **122**, 219-225.

Velikova V, Fares S, Loreto F. 2008. Isoprene and nitric oxide reduce damages in leaves exposed to oxidative stress. Plant, Cell & Environment **31**, 1882-1894.

Velikova V, Loreto F. 2005. On the relationship between isoprene emission and thermotolerance in Phragmites australis leaves exposed to high temperatures and during the recovery from a heat stress. Plant, Cell & Environment **28**, 318-327.

Velikova V, Várkonyi Z, Szabó M, Maslenkova L, Nogues I, Kovács L, Peeva V, Busheva M, Garab G, Sharkey TD, Loreto F. 2011. Increased Thermostability of Thylakoid Membranes in Isoprene-Emitting Leaves Probed with Three Biophysical Techniques Plant Physiology **157**, 905-916.

Velikova V, Yordanov I, Edreva A. 2000. Oxidative stress and some antioxidant systems in acid rain-treated bean plants: Protective role of exogenous polyamines. Plant Science **151**, 59-66.

Vickers CE, Gershenzon J, Lerdau MT, Loreto F. 2009a. A unified mechanism of action for volatile isoprenoids in plant abiotic stress. Nature Chemical Biology **5**, 283-291.

Vickers CE, Possell M, Cojocariu CI, Velikova VB, Laothawornkitkul J, Ryan A, Mullineaux PM, Nicholas Hewitt C. 2009b. Isoprene synthesis protects transgenic tobacco plants from oxidative stress. Plant, Cell & Environment **32**, 520-531.

Vickers CE, Possell M, Laothawornkitkul J, Ryan AC, Hewitt CN, Mullineaux PM. 2011. Isoprene synthesis in plants: lessons from a transgenic tobacco model. Plant, Cell & Environment **34**, 1043-1053.

Vlot AC, Rosenkranz M. 2022. Volatile compounds—the language of all kingdoms? Journal of Experimental Botany **73**, 445-448.

von Caemmerer S, Lawson T, Oxborough K, Baker NR, Andrews TJ, Raines CA. 2004. Stomatal conductance does not correlate with photosynthetic capacity in transgenic tobacco with reduced amounts of Rubisco. Journal of Experimental Botany **55**, 1157-1166.

Vranova E, Coman D, Gruissem W. 2013. Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu Rev Plant Biol **64**, 665-700.

Wang C-Y, Chen Y-W, Hou C-Y. 2019. Antioxidant and antibacterial activity of seven predominant terpenoids. International Journal of Food Properties **22**, 230-238.

Wang J, Song L, Gong X, Xu J, Li M. 2020. Functions of Jasmonic Acid in Plant Regulation and Response to Abiotic Stress. International Journal of Molecular Sciences **21**, 1446.

Wang X, Liu W, Xin C, Zheng Y, Cheng Y, Sun S, Li R, Zhu X-G, Dai SY, Rentzepis PM, Yuan JS. 2016. Enhanced limonene production in cyanobacteria reveals photosynthesis limitations. Proceedings of the National Academy of Sciences **113**, 14225-14230.

Wang Y, Lin N, Li W, Guenther A, Lam JCY, Tai APK, Potosnak MJ, Seco R. 2022. Satellite-derived constraints on the effect of drought stress on biogenic isoprene emissions in the southeastern US. Atmos. Chem. Phys. **22**, 14189-14208.

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S, Sharma HC. 2012. Mechanisms of plant defense against insect herbivores. Plant Signaling & Behavior **7**, 1306-1320.

Wenig M, Ghirardo A, Sales JH, Pabst ES, Breitenbach HH, Antritter F, Weber B, Lange B, Lenk M, Cameron RK, Schnitzler J-P, Vlot AC. 2019. Systemic acquired resistance networks amplify airborne defense cues. Nature Communications **10**, 3813.

Went FW. 1960. Blue Hazes in the Atmosphere. Nature **187**, 641-643.

Werker E. 1993. Function of essential oil-secreting glandular hairs in aromatic plans of Lamiacea—a review. Flavour and Fragrance Journal **8**, 249-255.

Werner C, Fasbender L, Romek KM, Yáñez-Serrano AM, Kreuzwieser J. 2020. Heat Waves Change Plant Carbon Allocation Among Primary and Secondary Metabolism Altering CO2 Assimilation, Respiration, and VOC Emissions. Frontiers in Plant Science **11**.

Westgate ME, Boyer JS. 1985. Osmotic adjustment and the inhibition of leaf, root, stem and silk growth at low water potentials in maize. Planta **164**, 540-549.

Weydert CJ, Cullen JJ. 2010. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nature Protocols **5**, 51-66.

Widhalm JR, Jaini R, Morgan JA, Dudareva N. 2015. Rethinking how volatiles are released from plant cells. Trends in Plant Science **20**, 545-550.

Wolff M, Seemann M, Tse Sum Bui B, Frapart Y, Tritsch D, Garcia Estrabot A, Rodriguez-Concepcion M, Boronat A, Marquet A, Rohmer M. 2003. Isoprenoid biosynthesis via the methylerythritol phosphate pathway: the (E)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase (LytB/IspH) from Escherichia coli is a [4Fe-4S] protein. FEBS Lett **541**, 115-120.

Woo HR, Kim HJ, Nam HG, Lim PO. 2013. Plant leaf senescence and death – regulation by multiple layers of control and implications for aging in general. Journal of Cell Science **126**, 4823-4833.

Wu C, Pullinen I, Andres S, Carriero G, Fares S, Goldbach H, Hacker L, Kasal T, Kiendler-Scharr A, Kleist E, Paoletti E, Wahner A, Wildt J, Mentel TF. 2015. Impacts of soil moisture on de novo monoterpene emissions from European beech, Holm oak, Scots pine, and Norway spruce. Biogeosciences **12**, 177-191.

Wu S, Schalk M, Clark A, Miles RB, Coates R, Chappell J. 2006. Redirection of cytosolic or plastidic isoprenoid precursors elevates terpene production in plants. Nature Biotechnology **24**, 1441-1447.

Xiao Y, Savchenko T, Baidoo Edward EK, Chehab Wassim E, Hayden Daniel M, Tolstikov V, Corwin Jason A, Kliebenstein Daniel J, Keasling Jay D, Dehesh K. 2012. Retrograde Signaling by the Plastidial Metabolite MEcPP Regulates Expression of Nuclear Stress-Response Genes. Cell **149**, 1525-1535.

Xu C, Ma Y, Tian Z, Luo Q, Zheng T, Wang B, Zuo Z. 2022. Monoterpene emissions and their protection effects on adult Cinnamomum camphora against high temperature. Trees **36**, 711-721.

Xu X, Yan L, Xia J. 2019. A threefold difference in plant growth response to nitrogen addition between the laboratory and field experiments. Ecosphere **10**, e02572.

Yamori W, Noguchi K, Hanba YT, Terashima I. 2006. Effects of Internal Conductance on the Temperature Dependence of the Photosynthetic Rate in Spinach Leaves from Contrasting Growth Temperatures. Plant and Cell Physiology **47**, 1069-1080.

Yazaki K. 2006. ABC transporters involved in the transport of plant secondary metabolites. FEBS Letters **580**, 1183-1191.

Yin J-L, Wong W-S, Jang I-C, Chua N-H. 2016. Co-expression of peppermint geranyl diphosphate synthase small subunit enhances monoterpene production in transgenic tobacco plants. New Phytologist **213**, 1133-1144.

Zhan X, Qian Y, Mao B. 2022. Metabolic Profiling of Terpene Diversity and the Response of Prenylsynthase-Terpene Synthase Genes during Biotic and Abiotic Stresses in Dendrobium catenatum. International Journal of Molecular Sciences **23**, 6398.

Zhang H, Zhao Y, Zhu J-K. 2020. Thriving under Stress: How Plants Balance Growth and the Stress Response. Developmental Cell **55**, 529-543.

Zhang J, Davies WJ. 1989. Sequential response of whole plant water relations to prolonged soil drying and the involvement of xylem sap ABA in the regulation of stomatal behaviour of sunflower plants. New Phytologist **113**, 167-174.

Zhang S-h, Xu X-f, Sun Y-m, Zhang J-l, Li C-z. 2018. Influence of drought hardening on the resistance physiology of potato seedlings under drought stress. Journal of Integrative Agriculture **17**, 336-347.

Zhang Y, Xu J, Li R, Ge Y, Li Y, Li R. 2023. Plants' Response to Abiotic Stress: Mechanisms and Strategies. International Journal of Molecular Sciences **24**, 10915.

Zhao L, Chang W-c, Xiao Y, Liu H-w, Liu P. 2013. Methylerythritol Phosphate Pathway of Isoprenoid Biosynthesis. Annual Review of Biochemistry **82**, 497-530.

Zhou F, Pichersky E. 2020. The complete functional characterisation of the terpene synthase family in tomato. New Phytologist **226**, 1341-1360.

Zhou H, Ashworth K, Dodd IC. 2023. Exogenous monoterpenes mitigate H2O2-induced lipid damage but do not attenuate photosynthetic decline during water deficit in tomato. Journal of Experimental Botany **74**, 5327-5340.

Zhou H, Beynon-Davies R, Carslaw N, Dodd IC, Ashworth K. 2022. Yield, resource use efficiency or flavour: Trade-offs of varying blue-to-red lighting ratio in urban plant factories. Scientia Horticulturae **295**, 110802.

Zia R, Nawaz MS, Siddique MJ, Hakim S, Imran A. 2021. Plant survival under drought stress: Implications, adaptive responses, and integrated rhizosphere management strategy for stress mitigation. Microbiological Research **242**, 126626.

Živanović B, Milić Komić S, Nikolić N, Mutavdžić D, Srećković T, Veljović Jovanović S, Prokić L. 2021. Differential Response of Two Tomato Genotypes, Wild Type cv. Ailsa Craig and Its ABA-Deficient Mutant flacca to Short-Termed Drought Cycles. Plants **10**, 2308.

Zou J-J, Li X-D, Ratnasekera D, Wang C, Liu W-X, Song L-F, Zhang W-Z, Wu W-H. 2015. Arabidopsis CALCIUM-DEPENDENT PROTEIN KINASE8 and CATALASE3 Function in Abscisic Acid-Mediated Signaling and H2O2 Homeostasis in Stomatal Guard Cells under Drought Stress. The Plant Cell **27**, 1445-1460.

Zuo Z, Wang B, Ying B, Zhou L, Zhang R. 2017. Monoterpene emissions contribute to thermotolerance in Cinnamomum camphora. Trees **31**, 1759-1771.

Zuo Z, Weraduwage SM, Lantz AT, Sanchez LM, Weise SE, Wang J, Childs KL, Sharkey TD. 2019. Isoprene Acts as a Signaling Molecule in Gene Networks Important for Stress Responses and Plant Growth. Plant Physiology **180**, 124-152.

7 Appendices

Appendix 1 Supplementary materials for Chapter 2

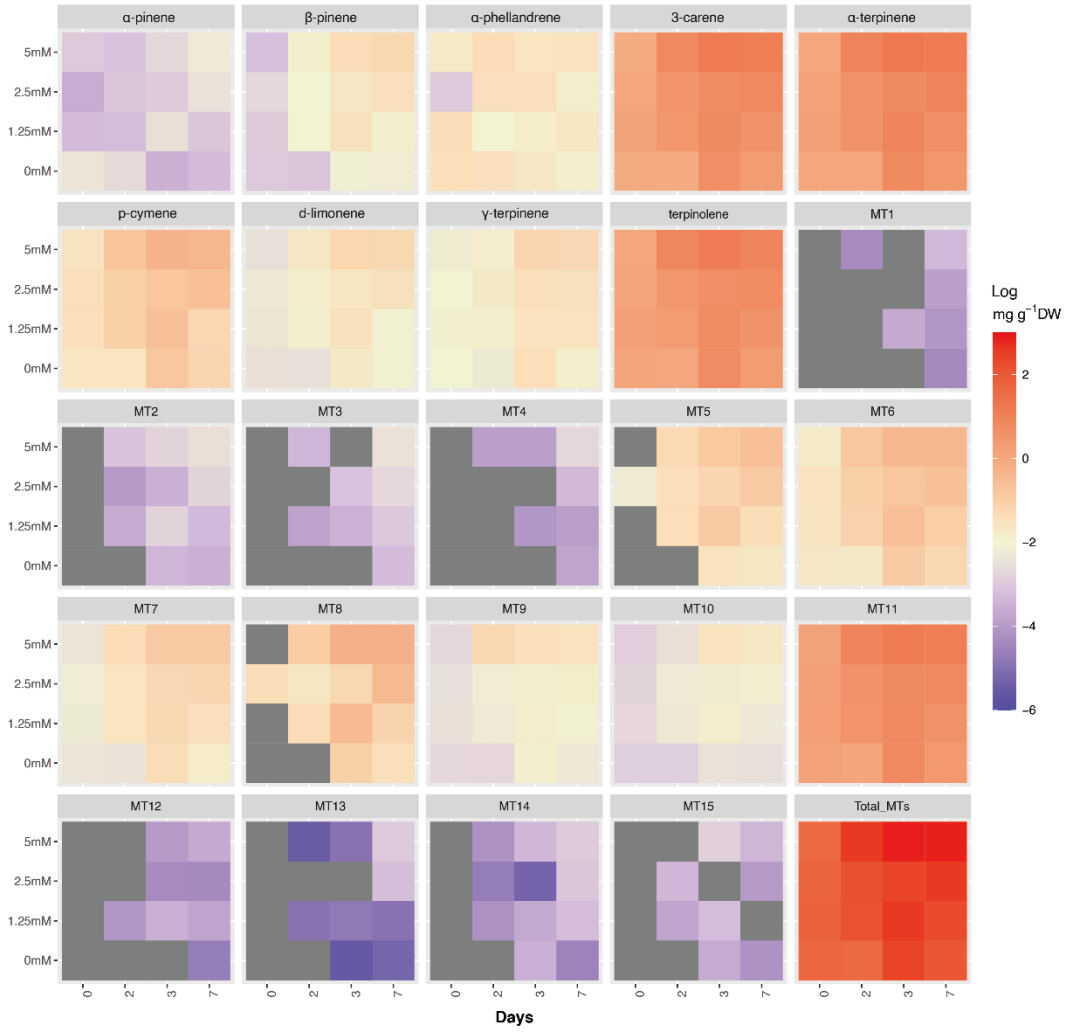

Figure S2. 1 Heatmap of total foliar MT content of plants treated with 0 (yellow), 1.25 (pink), 5 (dark red) mM exogenous MT spray by days. The values are presented on a log scale. Grey boxes indicate zero values. Data from experiment three are included.

Table S2. 1 LI-6400XT specifications.

Settings	Values	
Air flow	500 mmol s^{-1}	
Light intensity	400 µmol photons m^{-2} s^{-1} (10% blue)	
Relative humidity	50 (± 10%)	
Reference CO_2	412 ppm	
Block temperature	22 ℃	
Leaf Chamber Fluorometer		
Measure	Intensity	2
	Modulation	20kHz
	Filter	1
	Gain	10
Flash	Type	Rectangular
	Duration	Adjusted to individual plants
	Intensity	8
	Modulation	20kHz
	Filter	50
Dark	Duration	8 sec
	Far-red intensity	8
	Pre-time	2 sec
	Post-time	4 sec
	Modulation	0.25kHz
	Filter	1

Table S2. 2 A complete list of main foliar monoterpene content (with standard devation) of each compound and relative percentage of four treatments by days.

Day	Treatment	α-pinene			β-pinene			α-phellandrene			3-carene			α-terpinene			p-cymene		
		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD		Mean	SD	
0	0mM	0.092	0.012	1.68%	0.052	0.007	0.95%	0.242	0.033	4.41%	0.951	0.128	17.31%	1.064	0.143	19.38%	0.201	0.027	3.65%
0	1.25mM	0.039	0.005	0.62%	0.054	0.007	0.87%	0.249	0.033	3.99%	1.119	0.150	17.98%	1.253	0.168	20.13%	0.238	0.032	3.82%
0	2.5mM	0.029	0.001	0.47%	0.068	0.004	1.13%	0.053	0.049	0.88%	1.062	0.071	17.62%	1.193	0.080	19.79%	0.224	0.014	3.72%
0	5mM	0.054	0.003	1.04%	0.045	0.002	0.87%	0.180	0.011	3.49%	0.900	0.060	17.46%	1.018	0.068	19.76%	0.205	0.013	3.98%
2	0mM	0.075	0.010	1.47%	0.048	0.004	0.94%	0.222	0.017	4.34%	0.896	0.107	17.47%	1.007	0.127	19.65%	0.197	0.034	3.84%
2	1.25mM	0.041	0.014	0.48%	0.139	0.044	1.61%	0.137	0.088	1.59%	1.520	0.406	17.64%	1.707	0.462	19.80%	0.343	0.091	3.98%
2	2.5mM	0.049	0.018	0.54%	0.135	0.053	1.49%	0.223	0.237	2.45%	1.604	0.732	17.65%	1.799	0.828	19.80%	0.362	0.165	3.98%
2	5mM	0.046	0.047	0.35%	0.156	0.148	1.21%	0.261	0.167	2.02%	2.281	0.234	17.69%	2.527	0.271	19.59%	0.477	0.064	3.70%
3	0mM	0.031	0.028	0.27%	0.124	0.108	1.09%	0.184	0.121	1.62%	1.983	0.477	17.45%	2.225	0.526	19.58%	0.447	0.109	3.93%
3	1.25mM	0.085	0.009	0.66%	0.219	0.034	1.71%	0.162	0.021	1.26%	2.249	0.265	17.50%	2.517	0.291	19.59%	0.508	0.059	3.95%
3	2.5mM	0.054	0.024	0.47%	0.182	0.080	1.59%	0.223	0.064	1.94%	2.031	0.954	17.70%	2.272	1.059	19.80%	0.457	0.210	3.98%
3	5mM	0.073	0.024	0.41%	0.259	0.065	1.44%	0.197	0.103	1.10%	3.196	0.767	17.77%	3.587	0.852	19.95%	0.721	0.170	4.01%
7	0mM	0.039	0.055	0.49%	0.120	0.118	1.51%	0.149	0.025	1.88%	1.395	0.634	17.56%	1.565	0.704	19.69%	0.312	0.142	3.93%
7	1.25mM	0.050	0.048	0.50%	0.154	0.107	1.54%	0.193	0.102	1.93%	1.767	0.580	17.67%	1.976	0.650	19.77%	0.294	0.252	2.94%
7	2.5mM	0.088	0.032	0.67%	0.221	0.092	1.69%	0.160	0.048	1.23%	2.278	0.669	17.43%	2.555	0.754	19.55%	0.516	0.146	3.95%
7	5mM	0.110	0.036	0.65%	0.287	0.079	1.70%	0.213	0.060	1.26%	2.934	0.759	17.37%	3.288	0.839	19.47%	0.662	0.166	3.92%

Day	Treatment	d-limonene			γ-terpinene			terpinolene			Total_MTs	
		Mean	SD		Mean	SD		Mean	SD		Mean	SD
0	0mM	0.086	0.012	1.56%	0.132	0.018	2.41%	1.086	0.146	19.79%	5.491	0.738
0	1.25mM	0.099	0.013	1.60%	0.146	0.020	2.34%	1.244	0.167	19.98%	6.225	0.837
0	2.5mM	0.104	0.006	1.73%	0.135	0.008	2.23%	1.092	0.073	18.13%	6.026	0.440
0	5mM	0.084	0.005	1.63%	0.117	0.007	2.27%	1.023	0.068	19.87%	5.151	0.346
2	0mM	0.082	0.012	1.61%	0.113	0.002	2.21%	1.000	0.087	19.51%	5.127	0.542
2	1.25mM	0.147	0.042	1.71%	0.157	0.037	1.82%	1.429	0.344	16.58%	8.619	2.446
2	2.5mM	0.154	0.072	1.69%	0.173	0.100	1.91%	1.561	0.802	17.17%	9.087	4.282
2	5mM	0.186	0.064	1.44%	0.160	0.138	1.24%	2.405	0.519	18.64%	12.899	1.375
3	0mM	0.169	0.080	1.49%	0.245	0.024	2.15%	2.111	0.054	18.58%	11.365	2.215
3	1.25mM	0.215	0.023	1.68%	0.214	0.028	1.66%	2.043	0.243	15.90%	12.849	1.453
3	2.5mM	0.187	0.087	1.63%	0.226	0.117	1.97%	1.959	0.963	17.07%	11.474	5.487
3	5mM	0.308	0.074	1.71%	0.316	0.073	1.76%	3.039	0.756	16.90%	17.982	4.216
7	0mM	0.128	0.069	1.62%	0.150	0.029	1.89%	1.381	0.370	17.38%	7.946	3.622
7	1.25mM	0.129	0.106	1.29%	0.211	0.058	2.11%	1.723	0.420	17.23%	9.997	3.386
7	2.5mM	0.219	0.068	1.67%	0.220	0.026	1.68%	2.041	0.458	15.62%	13.069	3.789
7	5mM	0.282	0.068	1.67%	0.276	0.062	1.63%	2.617	0.626	15.49%	16.892	4.097

Appendix 2 Supplementary materials for Chapter 3

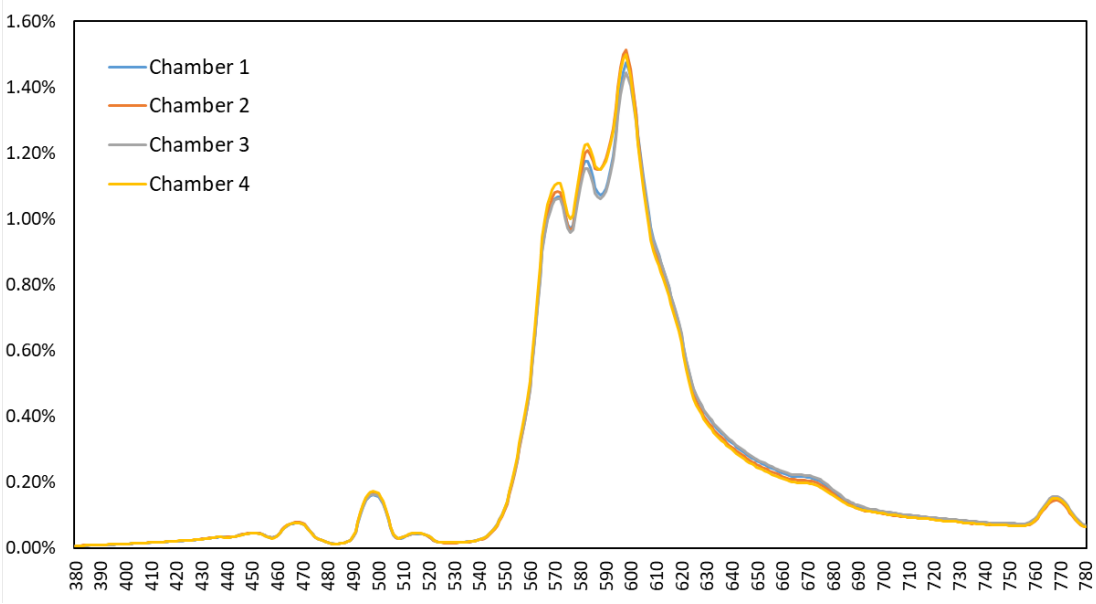

Figure S3. 1 Light spectral distribution (wavelength %) in the growth chambers was measured by Spectral PAR Meter (PG100N, UPRtek, Zhunan, Taiwan).

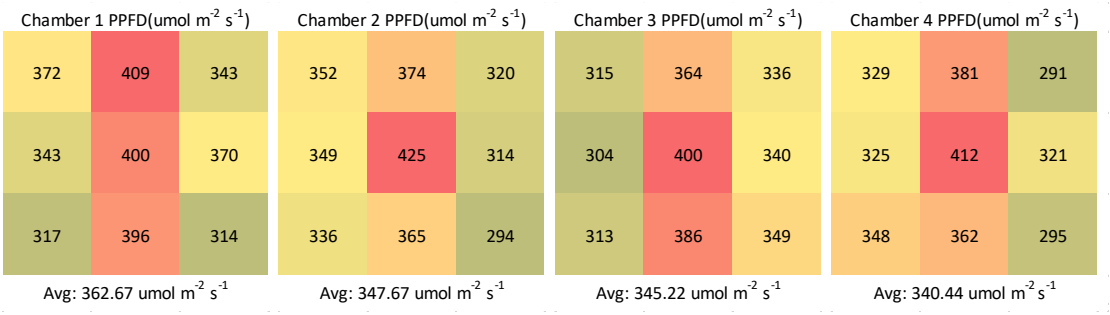

Figure S3. 2 Average light intensity distribution at leaf level in growth chambers. Growth chambers were divided into nine equal parts and light intensity was measured at the leaf level in the centre of each part using a PAR meter.

Threaded steel blind rivets with were fitted to the four aluminium posts, and the supporting grid can be raised every 5 cm from 5-40 cm, supported and secured with aluminium angels and bolts.

Appendix 3 Supplementary materials for Chapter 4

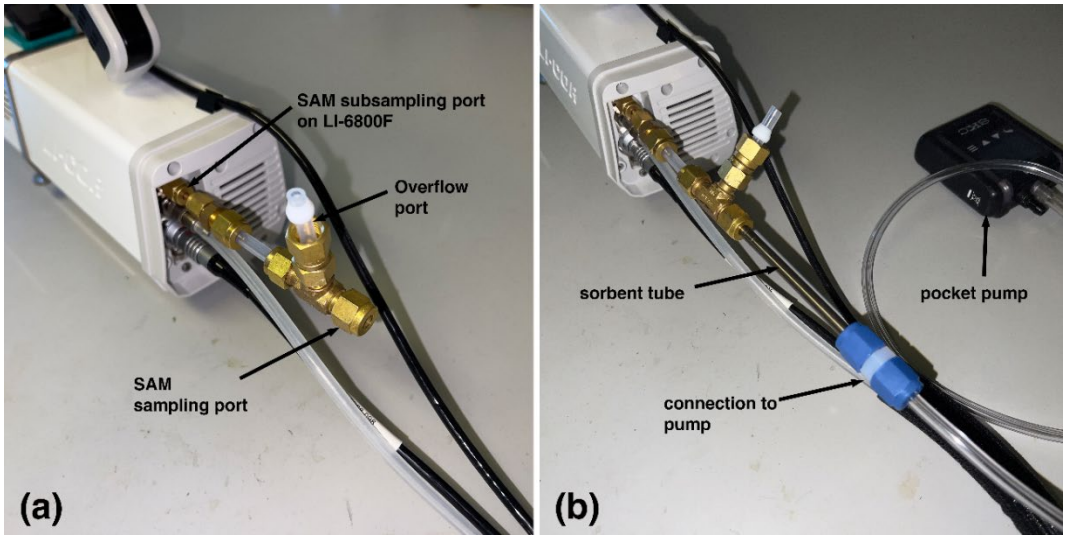

Figure S4. 1 LI-6800F gas sampling manifold.

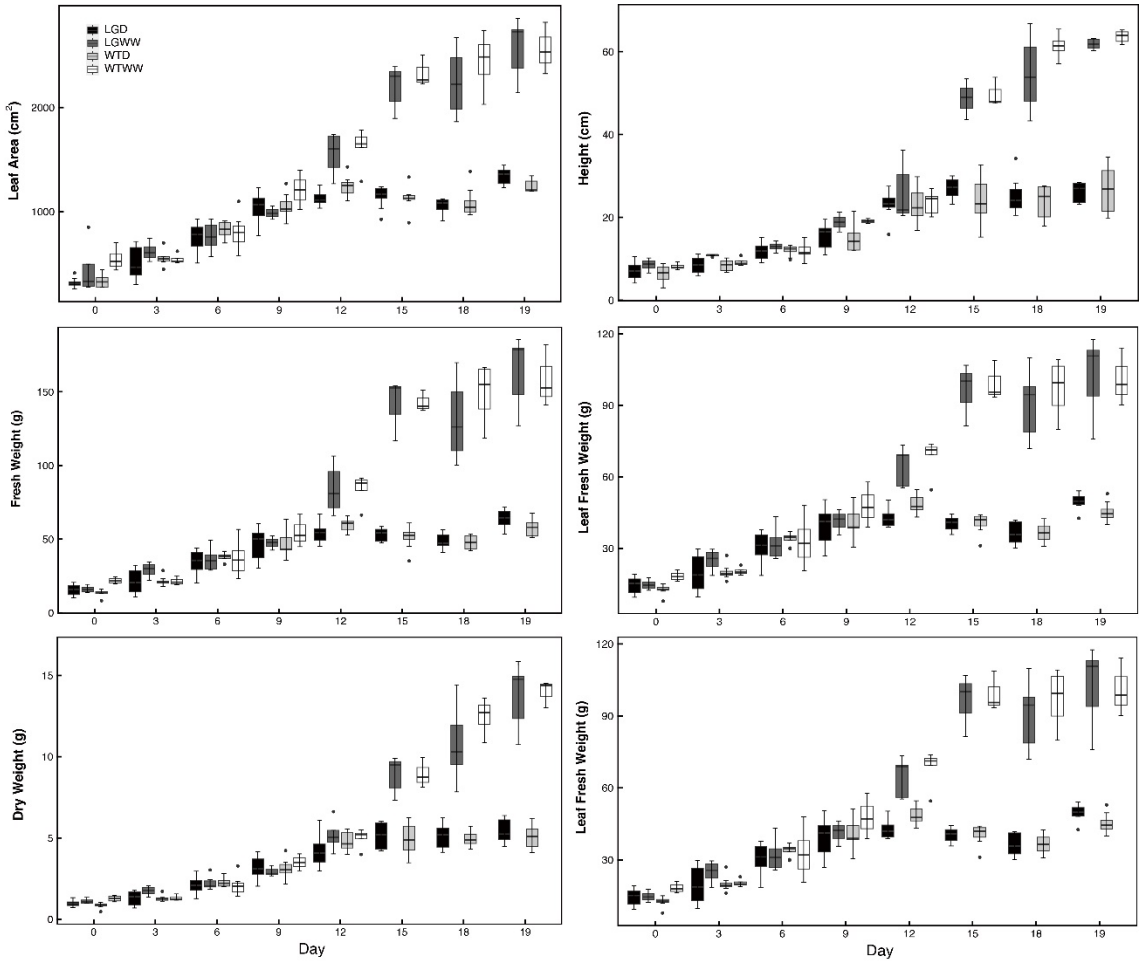

Figure S4. 2 Plant growth and development during experiment. Data from two experiments.

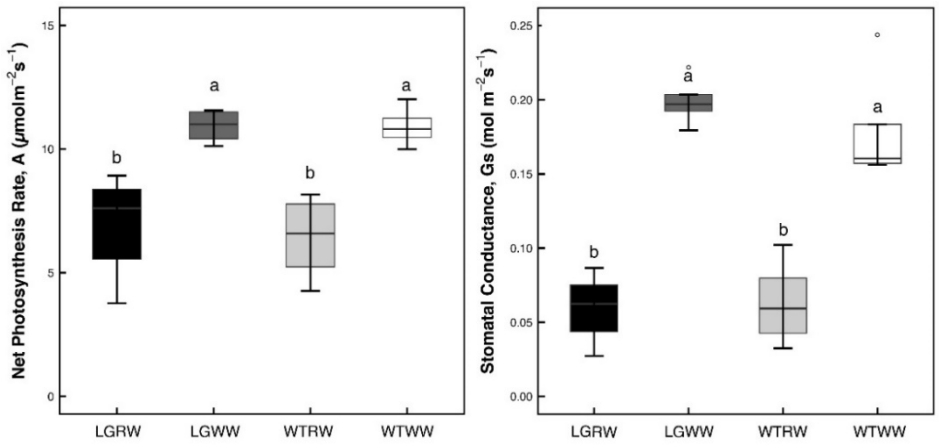

Figure S4. 3 Gas exchange after re-watering. Boxplots (±SE) present the data for both LG12 and wild-type plants after re-watering (LGRW, WTRW) and well-watered plants (LGWW, WTWW) on the same day, post hoc Tukey test results indicate the significant differences between treatments by letters.

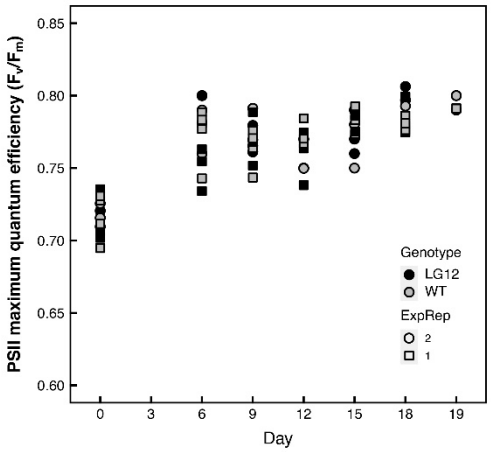

Figure S4. 4 Daily F_v/F_m of well-watered plants during the experiment. Data show an increasing trend of approximately 0.1.

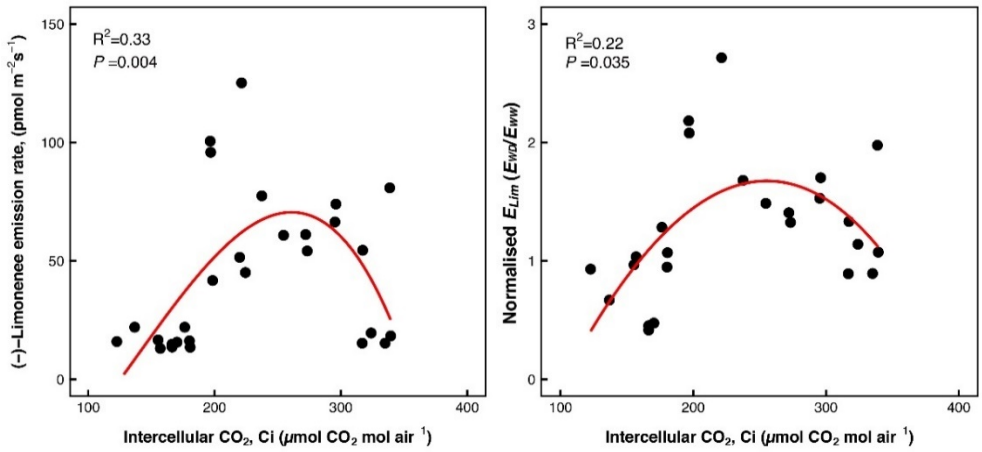

Figure S4. 5 Relationship between LG12 foliar (-)-limonene emission rate, normalised emission rate and intercellular CO2 under water deficit in this study. Each point is an individual plant.

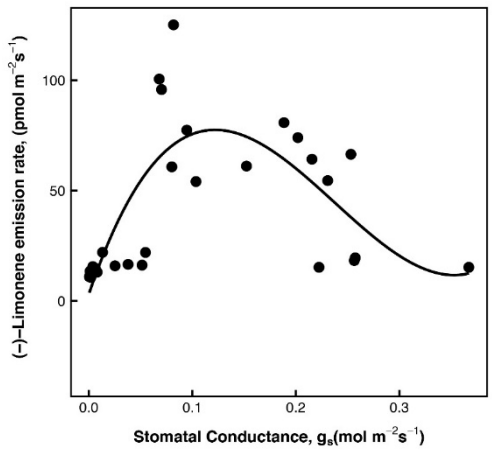

Figure S4. 6 Correlation between (−)-limonene emission rate and stomatal conductance. Followed the similar relationship to Elim vs A.

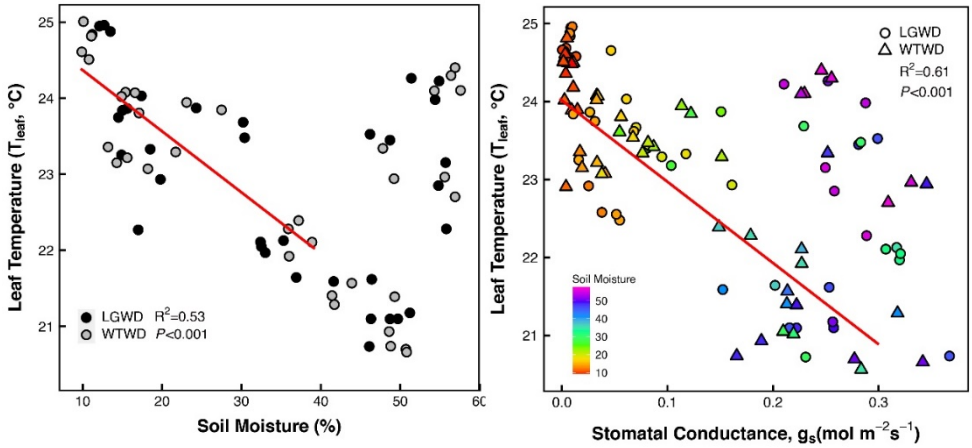

Figure S4. 7 Leaf temperature response to soil moisture (a) and stomatal conductance with colour coded soil moisture level (b) in LG12 and WT under water deficit conditions. Data from two experimental replicates. The data cluster at high soil moisture and leaf temperature is potent. The linear regression lines indicate the response to soil moisture less than 40%.

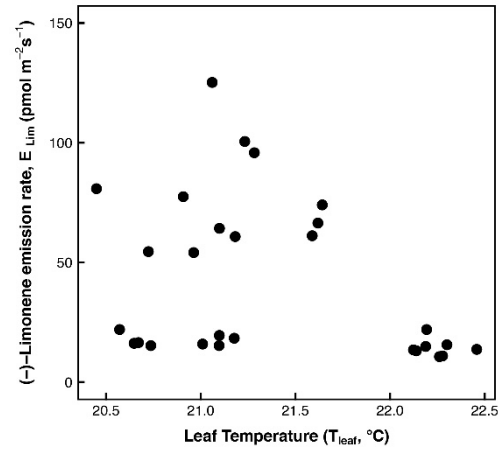

Figure S4. 8 Non-significant relationship between (-)-Limonene emission rate and leaf temperature. Data from measurement in Feb 2024. T_{leaf} varied little as the Li-Cor head preset T_{leaf}.