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Evidence of Bi-Directional Volatile-Mediated Communication between Drought-Stressed and Well-Watered Grapevines (*Vitis vinifera* L.)

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Abstract: The volatile-mediated interplay between stressed and non-stressed plants has been described in many studies involving both biotic and abiotic stresses as a one-way channel. However, very little is known about the molecular basis and mechanisms by which volatile organic compounds (VOCs) mediate plant communication between drought-stressed 'emitter' plants and non-stressed 'receiver' neighbours for the defence against impending stress challenges. Aiming to address this in grapevine, this study investigated the effect of two-way VOC exchange between stressed and non-stressed *Vitis vinifera* L. cv. Shiraz during drought and recovery using four treatments: isolated well-watered (WW) vines, isolated drought-stressed (DS) vines, and co-located DS 'emitter' and WW 'receiver' vines in a growth room. The results obtained from solid-phase microextraction (SPME) gas chromatography mass spectrometry (GC-MS) analysis showed a synchronised decline in α-pinene concentration in the co-located treatment vines and higher isoprene levels in the DS emitters compared to the isolated DS vines. Targeted gene expression analysis further identified the over-expression of a key gene, allene oxide synthase (AOS), in the jasmonic acid (JA) biosynthesis pathway during peak drought in the DS emitter. Transcript expression of chorismate synthase (CHORS) and α-pinene synthase (VvPNaPin1) showed similar trends in the DS emitter. The results suggest that isoprene and α-pinene may be interplant signalling molecules used by grapevine during drought. To the best of our knowledge, this is the first report of a bi-directional interaction in grapevine between the emitters and receivers under drought stress mediated by the JA and terpenoid biosynthesis pathways.

Keywords: α-pinene; isoprene; plant–plant interactions; VOC priming; VOCs; abiotic stress

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1. Introduction

Plants have evolved a highly varied arsenal of defence mechanisms against a wide range of biotic and abiotic stresses to adapt for their sedentary existence. These mechanisms include the plant's capacity to release and react to volatile organic compounds (VOCs), triggering defence responses, which may confer some degree of tolerance in the plants against the stressors [1–8]. Interestingly, plants have also adapted to 'eavesdrop' VOC signals from their stressed neighbours to modulate their phenotypes against impending stresses [9]. The role of these phytochemical signal molecules in interplant communication has been well-documented and they have been shown to provide fitness benefit to both the emitter and the receiver [10]. The ecological significance of this aerial communication may depend on the ability of a non-stressed plant to perceive these volatile cues from its

stressed neighbours in order to prime for defensive reactions without depleting its fitness by maturing the response before the actual stress is imposed.

The ability of stress-induced VOCs to prime nearby plants that are not under stress to mount a quicker and/or more effective defence to subsequent stress challenges has been demonstrated in several studies involving biotic stress [11–16], with only a few studies being related to abiotic stress [17–21]. The priming effect of stress-induced VOCs, including terpenoids [11,16,20,22], green leaf volatiles (GLVs) such as (*Z*)-3-hexen-1-ol [12–15], and methyl ester forms of jasmonic acid (MeJA) [13,23] and salicylic acid (MeSA) [19,21,24], on the non-stressed receivers have been demonstrated to trigger antixenosis and/or antibiosis resistance mechanisms against herbivory as well as increased tolerance against abiotic stressors. Cold-stress-induced VOCs from tea plants, including nerolidol, geraniol, linalool, and MeSA, were shown to elicit the overexpression of genes for antioxidant enzymes, such as glutathione reductase and ascorbate peroxidase, in the receivers in comparison to control plants [19]. Gene expression of transcription factors associated with cold stress and C-repeat (CRT)-binding factors (CBFs; CBF1 and CBF2) was remarkably upregulated in the receivers [19]. Several studies have also demonstrated that salt-induced VOCs from *Arabidopsis* [17], *Ocimum basilicum* [20], and *V. faba* [18] were significant in priming for salt tolerance in neighbouring receiver plants.

Studies involving constitutive [25], stress-induced [18,21], and exogenously applied [26–29] VOCs have provided evidence of the role of VOCs in decreasing stomatal conductance (g_s) in non-stressed plants. Studies by López-Gresa et al. [29] confirmed the role of GLV esters; (*Z*)-3-hexenyl propionate and (*Z*)-3-hexenyl butyrate; in triggering stomatal closure in tomato as a response to *Pseudomonas syringae* infection. Another study by Rai et al. [25] noted a decrease in g_s in response to monoterpenes from *Prinsepia utilis* in *V. faba*. Studies suggesting that drought- [30] and salt- [18] stress-induced VOCs elicit entrainment behaviour between co-located stressed and non-stressed plants have been documented. In these studies, a synchronised decrease in g_s in both the emitter and receiver plants has been demonstrated. Although this entrainment behaviour has been mostly observed in g_s, it is plausible to assume that VOC-mediated interplant communication may induce entrainment behaviour between co-located stressed and non-stressed plants in other defence-related physiological, metabolic, or molecular responses to biotic and abiotic stressors.

Most studies in VOC-mediated interplant communication, either involving biotic or abiotic stresses, have focused on how stress-induced VOCs modulate responses in the non-stressed plants, often referred to in these studies as the 'receivers'. This one-way channel communication assumes that the so-called 'receivers' have no influence on their stressed neighbours. However, constitutive VOC emissions from non-stressed plants have also been shown to influence responses in stressed and/or susceptible neighbours. In the well-documented ecological concept of VOC-mediated associational resistance (AR), susceptible focal plants have been shown to adsorb defence-related constitutively emitted VOCs from their resistant heterospecific neighbours before re-emitting them against herbivores [15,31]. Similarly, in a drought study by Ormeño et al. [32], it was suggested that *Acer monspessulanum* absorbed constitutively emitted isoprene from neighbouring *Quercus pubescens* to improve its drought tolerance.

Despite the extensive evidence from previous studies which support the concept of plant–plant communication, knowledge of the molecular basis and mechanisms involved in VOC-mediated plant–plant interactions remains scant. Using metabolome, transcriptome, and physiological analyses, this study investigated how drought and recovery affected the two-way VOC exchange between stressed and non-stressed *Vitis vinifera* L. cv. Shiraz. Based on the hypothesis that drought induces VOCs in grapevine that can elicit defence responses in non-stressed receiver vines, this study explored the role played by water stress on VOC emissions, specifically α-pinene, isoprene, MeJA, MeSA, and (Z)-3-hexen-1-ol, as well as how these VOCs induce drought defence responses and stomatal closure in well-watered (WW) receiver vines. The emissions of these VOCs were quantified under well-watered and drought stress conditions. Targeted gene expression (transcript) analysis

was also carried out on genes related to the biosynthesis or signalling pathways of the above-mentioned VOCs.

2. Materials and Methods

2.1. Plant Material and Treatments

Potted own-rooted Shiraz grapevines (*Vitis vinifera* L., Cl. BVRC12) originated from dormant canes taken from mature Shiraz grapevines in the Coombe vineyard at Waite campus, the University of Adelaide (34°58′3.47 S; 138°38′0.43 E), in June 2020. One-node cuttings were prepared from the canes and placed in 50 mL centrifuge tubes that were half-filled with tap water. The cuttings were placed in a glasshouse and allowed to form roots under standard greenhouse conditions (25 °C day and 17 °C night), relative humidity (65%), and 16 h light/8 h dark photoperiod. After six weeks, rootlings were transplanted into 2.8 L pots containing a 50:50 v/v mixture of perlite–vermiculite and University of California (UC) soil mix (61.5 L sand, 38.5 L peat moss, 50 g calcium hydroxide, 90 g calcium carbonate, and 100 g Nitrophoska© (12:5:1, N: P: K plus trace elements per 100 L at pH 6.8) (Incitec Pivot Fertilisers, Southbank, VIC, Australia). Prior to the start of the experiment with one-year-old vines, a transparent plastic film was placed at the base of plant, covering the soil to exclude possible below-ground VOCs.

Four treatments were used in this experiment: (1) well-watered control (WW_c), (2) isolated DS control (DS_c), (3) co-located drought-stressed emitter (DS_e), and (4) co-located well-watered receiver treatment (WW_r). Each treatment had three biological replicates.

2.2. Growth and Stress Conditions

The experiment was conducted in June/July 2021 in a growth chamber (1.8 m^2) at the Australian Plant Phenomics Facility, located at Waite campus. The growth chamber had the following pre-set environmental conditions: max/min temperatures: 22 °C/17 °C; max daylight: 1000 µmol m^{-2} s^{-1}; day/night hours: 16 h/8 h; max relative humidity: 50%; CO_2 400 ppm. Treatment pots were randomised in the growth chamber. Watering was carried out using rainwater and plants were kept at field capacity (FC) (~23% volumetric water content (VWC)) prior to start of experiment. To determine the FC, potted vines were watered to soil saturation capacity and excess water was allowed to drain out from the bottom holes of the pot to ensure the downward redistribution of water. The potted plants were then weighed to establish their individual weights at FC. Well-watered treatment plants, WW_c and WW_r, were kept at FC throughout the experiment. Drought stress conditions were initiated by withholding watering for the drought stress treatment vines until peak drought (PD) was achieved at between 4–5% VWC and stomatal conductance (g_s) was below 50 mmol H_2O m^{-2} s^{-1}. Re-watering to FC was carried out for all treatments after PD measurements were taken.

2.3. Physiological Measurements

Stomatal conductance (g_s) measurements were taken using a SC-1 leaf porometer (METER Group, Pullman, Washington, DC, USA) between 1100–1200 h solar time on three fully expanded mature leaves per vine per treatment. Readings were taken around the same location in all leaves. Soil moisture content (% VWC) was monitored by recording pot weight using an analytical balance (BSCL 15, Bialetti, Coccaglio, Italy) and soil moisture (three measurements per pot) using a TEROS-12 capacitance soil moisture sensor (METER Group, Pullman Washington, DC, USA). Measurements were taken every day until the end of the experiment, except for WW_c, which only had day 1 measurements based on preliminary data (unpublished) that showed a consistent g_s in the well-watered vines upon monitoring for seven days. Tinytag data loggers (TGP-4500, Hastings Data Loggers, Port Macquarie, NSW, Australia) were placed below the vine canopies to monitor ambient temperature and RH in the chamber every 15 min throughout the experiment. These measurements were used to calculate vapour pressure deficit (VPD) according to Murray [33]. The VPD results

were shown as the mean of data collected during the period corresponding to physiological data collection, i.e., between 1100 am–1200 pm solar time.

2.4. VOC Metabolite Analysis

2.4.1. Chemicals and VOC Sampling Materials

Isoprene (≥99%), α-pinene (≥98%), MeJA (≥95%), MeSA (≥99%), and (*Z*)-3-hexen-1-ol (≥98%) were supplied by Sigma-Aldrich (Castle Hill, NSW, Australia) and used as external standards, and d_6-1,8-cineole (CDN Isotopes, Pointe-Claire, QC, Canada) was the internal standard. Stock solutions (1 g L^{-1}) were prepared in redistilled ethanol and subsequently diluted with the same solvent to obtain working solutions for calibration of instrument response. A 2-cm DVB/CAR/PDMS SPME fibre assembly (50/30 µm 24 ga, Supelco, Bellafonte, PA, USA) was used for sampling of volatiles. Prior to use, SPME fibres were pre-conditioned at 270 °C in the gas chromatograph (GC) inlet for 30 min and stored in sterilised glass tubes.

To ensure minimal handling of the plants, volatiles from plant leaves were collected in 1 L fluorinated ethylene polymer (FEP) gas sampling bags (FEP 31C_1 L, HedeTech Co., Ltd., Dalian, Liaoning, China) fitted with a PTFE valve and septum port used for SPME sampling (Figure 1). The concentrations of pre-selected compounds (α-pinene, isoprene, (*Z*)-3-hexen-1-ol, MeSA, and MeJA) were determined in the different treatments at drought initiation, peak drought, and after re-watering (recovery). For each treatment, a leaf was placed in the sampling bag containing a 4.25 cm Whatman filter paper loaded with 20 µL of a 120 µg L^{-1} d_6-1,8-cineole solution. The bag was sealed firmly without detaching from the plant and the SPME fibre inserted into the septum was exposed for 50 min prior to GC-MS analysis.

Figure 1. In situ VOC sampling of *V. vinifera* L. cv. Shiraz potted vine. Volatiles from an intact leaf were collected in a fluorinated ethylene polymer (FEP) gas sampling bag fitted with a PTFE valve and septum port for SPME sampling. Filter paper loaded with d_6-1,8-cineole solution was included in the bag as an internal standard.

2.4.2. GC-MS Instrumentation

After sampling of plant volatiles from an FEP bag, the SPME fibre was manually inserted into the inlet of an Agilent 7890A GC coupled to a 5975C inert XL MS (Agilent Technologies, Santa Clara, CA, USA). Separation was performed with a J&W DB-WAXetr column (30 m × 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies, Santa Clara, CA, USA) using ultrapure helium (BOC Ltd., Mile End South, SA, Australia) as the carrier gas at a constant flow of 1 mL min^{-1}. The GC oven temperature began at 40 °C for 3 min, then increased at 5 °C min^{-1} to 240 °C, and was held for 10 min at 240 °C. The Gerstel CIS inlet was fitted with an ultra-inert SPME liner (0.75 mm i.d., Agilent Technologies) and held at 240 °C throughout the run. Electron ionisation mass spectra were recorded at 70 eV in full scan mode in the range of m/z 35–350. Instrument control and data analysis were performed using Agilent ChemStation software (ver. E.02.02.143).

2.5. Nucleic Acid Analysis

2.5.1. RNA Extraction

The fourth expanded leaf from the shoot apex was collected from each replicate of all treatments. Sampling for control and drought experiments was carried out at drought initiation, peak drought, and 24 h after re-watering/recovery from drought. Samples were placed immediately in liquid nitrogen and stored at −80 °C prior to analysis. Preceding RNA extraction, the snap-frozen leaf tissue was homogenised in a bead beater (2010 Geno/Grinder, SPEX SamplePrep, Metuchen, NJ, USA) at 1500 RPM for 35 s. RNA extraction was performed from the pulverised plant samples using a Spectrum plant total RNA extraction kit (Sigma-Aldrich) following the manufacturer's instructions. An on-column DNase I digestion set (Sigma-Aldrich) was used to remove genomic DNA contamination for 15 min. All RNA samples were quantified and checked for quality using a Nanodrop 1000 Spectrophotometer (ver. 3.8.1, Thermo Fischer Scientific Inc., Adelaide, SA, Australia). Gel electrophoresis was also performed to check RNA quality. The RNA samples were run at 70 V for 40 min in a 2% agarose gel in 0.5× tris-acetate-ethylenediaminetetraacetic acid buffer. Nucleic acids were visualised in a ChemiDoc imaging system (Bio-Rad Laboratories Inc., South Granville, NSW, Australia). Samples were stored at −80 °C until further processing.

2.5.2. cDNA Synthesis and PCR

All RNA samples were reverse transcribed using an iSCRIPT cDNA synthesis kit (Bio-Rad Laboratories) following the manufacturer's instructions. The cDNA was diluted 10-fold with sterile water and then stored at −20 °C until required. A cDNA product quality check was performed using PCR. Reactions consisted of 10 mM forward and reverse primers of VvActin housekeeping gene, 5× Phusion HF buffer, Phusion polymerase, and 1 µL cDNA template. The PCR thermal cycling program was as follows: initial step 98 °C for 30 s, 35 cycles of 98 °C for 5 s, 60 °C for 20 s (annealing), 72 °C for 1 min, followed by 72 °C for 3 min, and held at 15 °C.

2.5.3. Quantitative Real-Time PCR

Quantitative real-time PCR (RT-qPCR) reactions were performed in a QuantStudio 12 K Flex real-time PCR system (Thermo Fisher Scientific Inc.). For each cDNA sample, a 10 µL reaction volume consisting of 10 mM each of forward and reverse primers of the target genes, KAPA SYBR Fast RT-qPCR master mix (KAPA Biosystems), and 1 µL cDNA template was used. The housekeeping genes actin and elongation factor 1 (ELF1) were used as endogenous references for normalisation. The names and the function of the target genes and the primer sequences used for their amplification by qPCR are listed in Table 1.

Thermal cycling was composed of an initial denaturation step for 3 min at 95 °C, 40 cycles at 95 °C for 20 s, 95 °C for 1 s, and 60 °C for 20 s. Relative transcript abundances were quantified using the $2^{-\Delta\Delta Ct}$ method [34], and normalised to the geometric mean of actin and ELF1 [35]. All reactions had three technical replicates for each biological replicate.

Table 1. List of qPCR primers used in this study.

Gene Name/ID	Forward Primer Sequence	Reverse Primer Sequence	References
Allene synthase (AOS)	CTTCGGAGGGATGAAAATCA	GGCTAATTGGGTGTGCAGT	[36]
Chorismate synthase (CHORS)	GCCTTCACATGCAGATGCTA	CTGCAACTCTCCCAATGGTT	[37]
Lipoxygenase (VvLOXD)	ACCCACCAAATCGTCCCACACTATG	ACCTCTTCGTTGTCTGTCCACTCTG	[38]
α-Pinene (VvPNaPin1)	TTGGAGAAGCTTAAGGGAGATG	GGTAGCCATGCTGTCTTAGGAG	[39]

2.6. Statistical Analysis

Statistical tests for plant physiological performance (stomatal conductance), soil moisture (% VWC), and leaf volatile metabolite and gene expression analyses were performed using analysis of variance (ANOVA) and Tukey HSD post-hoc test. Prior to performing ANOVA, data were checked using the Shapiro–Wilk normality test. Data were subjected to two-way ANOVA at $\alpha = 0.05$ using GraphPad Prism software (v.9.0.0, GraphPad Software Inc., San Diego, CA, USA) and represented as mean ± standard error of mean (SEM).

3. Results

3.1. Physiological Analysis

To determine the physiological effect of drought-induced VOCs on co-located WW receiver vines, leaf stomatal conductance (g_s) of all the treatments was monitored at drought initiation (DI, Day 0), peak drought (PD, Day 8), and 24 h after re-watering (Day 9). At DI, all treatment vines were at FC (~23% VWC) and therefore no differences were noted in g_s ($p > 0.05$) (Table 2).

Table 2. Physiological data collected from well-watered and drought-stressed treatment plants on stomatal conductance g_s (mmol H_2O m^{-2} s^{-1}) and soil moisture, VMC (%), $n = 3$. Data were collected at drought initiation (Day 0), peak drought (Day 8), and 24 h after re-watering (Day 9). Values represent the mean ± SEM for all the treatments. Different letters indicate significant differences between treatments using lower case letters for within column comparisons (all treatments per time point) and Greek letters for within row comparisons (each time point per treatment).

Treatments	g_s (mmol H_2O m^{-2} s^{-1})			Soil Moisture (% VWC)		
	Day 1	Day 8	Day 9	Day 1	Day 8	Day 9
WWc	121 ± 25.5 a	-	-	21 ± 0.07 a	-	-
DSc	91 ± 8.3 aα	33 ± 3.9 bβ	70 ± 8.3 aα	21 ± 0.1 aα	4 ± 0.1 aβ	23 ± 0.6 aα
DSe	146 ± 31.7 aα	33 ± 2.4 bβ	63 ± 10.9 aα	24 ± 3.1 aα	4 ± 0.3 aβ	24 ± 2.9 aα
WWr	116 ± 19.9 aα	100 ± 24.79 aα	115 ± 24.0 aα	23 ± 0.9 aα	25 ± 1.5 aα	25 ± 1.5 aα

After eight days of withholding water in the isolated and co-located drought-stressed treatments, DS_c and DS_e, respectively, PD conditions were achieved. Soil moisture was significantly decreased in DS_c from 21 ± 0.1% to 4 ± 0.1%, and in the DS_e from 24 ± 3.1% to 4 ± 0.3%, consequently lowering the g_s of the vines from 91 ± 8.3 mmol H_2O m^{-2} s^{-1} to 33 ± 3.9 mmol H_2O m^{-2} s^{-1} in DS_c and 146 ± 31.7 mmol H_2O m^{-2} s^{-1} to 33 ± 2.4 mmol H_2O m^{-2} s^{-1} in DS_e at PD ($p < 0.0001$) (Table 2). Following re-watering on Day 8, the g_s of both treatments increased on Day 9 to 70 ± 8.3 in DSc and 63 ± 10.9 in DSe. The g_s and soil moisture values of WW_c vines were recorded once at DI and were similar to all treatments at DI. There were no significant differences in g_s of the well-watered receiver (WW_r) vines at any of the time points, indicating that their drought-stressed counterparts (DS_e) did not influence their g_s. Soil moisture was kept at FC in the isolated and co-located well-watered vines and was not significantly different in both treatments. The canopy VPD of DS_c was higher than the VPD recorded for WW_c, being 1.95 kPa and 1.42 kPa, respectively. The co-located treatment vines recorded a VPD of 1.93 kPa, which was similar to that of DS_c ($p = 0.9788$).

3.2. Investigating VOC Signalling Using Volatile Metabolite and Transcript Analyses

3.2.1. Constitutive VOC Emissions

The constitutive emissions of selected VOCs, α-pinene, isoprene, MeJA, MeSA, and (Z)-3-hexen-1-ol, were measured from well-watered control treatment plants. Constitutive emissions of α-pinene and isoprene were recorded from WW_c at 21.3 µg L^{-1} and 24.4 ng mL^{-1}, respectively (Figure 2). Lower concentrations of MeJA (0.4 µg L^{-1}) and MeSA (0.1 µg L^{-1}) were measured, but (*Z*)-3-hexen-1-ol was not detected in any of the samples (Figure 2).

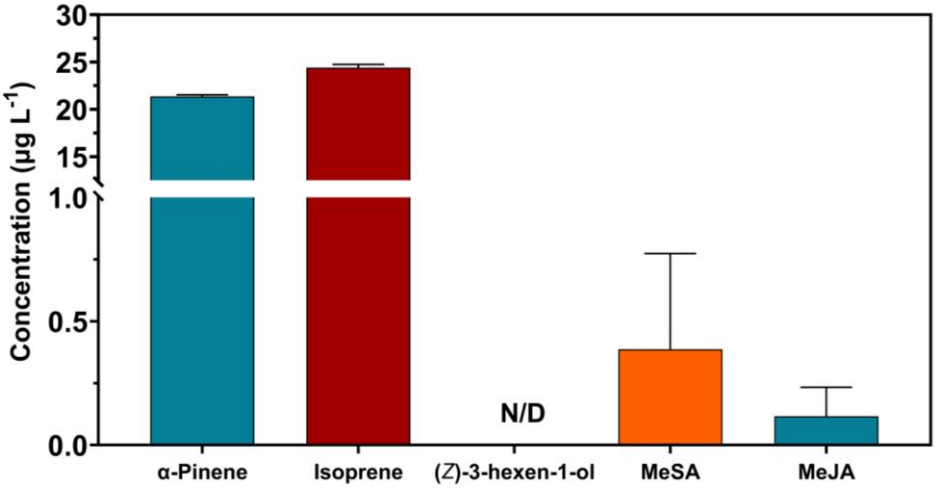

Figure 2. Constitutive VOC mean concentrations of α-pinene, isoprene, (*Z*)-3-hexen-1-ol, methyl SA, and methyl JA emitted from well-watered control treatment plants. All the compounds were emitted constitutively except for (*Z*)-3- hexenol, which was not detected (N/D) in any of the samples. The error bar represents the standard error of the mean (SEM, $n = 3$).

3.2.2. Synchronised Emission of MeJA, MeSA, α-Pinene, and Isoprene in Co-Located Treatment Vines

At DI, detectable levels of MeJA and MeSA were recorded only for the co-located receiver (WW_r) and emitter (DS_e) vines (Figure 3A,B). The concentration of MeJA in WW_r and DS_e was similar at 16.0 µg L^{-1} and 15.9 µg L^{-1}, respectively ($p > 0.05$) (Figure 3A). Likewise, MeSA concentration in DS_e (8.2 µg L^{-1}) and receiver (7.9 µg L^{-1}) were not significantly different at DI (Figure 3B). At PD and recovery, MeJA (16.6 µg L^{-1}) and MeSA levels (8.0 µg L^{-1}) were only detected in DS_c vines (Figure 3A,C).

Expression of the JA biosynthesis-related gene, AOS, was similar across treatments at DI, with a mean relative expression of 1.4 (Figure 3E). At PD, higher relative expression levels of AOS were observed in DS_e (18.7) from an initial expression level of 2.4 observed at DI. This was higher than its WW neighbour, WW_r ($p = 0.005$), whose AOS gene expression level of 1.2 was similar to DI (1.0). AOS expression in DS_c was much lower than DS_e ($p = 0.005$). Both WW treatments (WW_c and WW_r) showed no significant differences at PD ($p > 0.999$). The expression levels of the lipoxygenase gene, LOXD, related to MeJA and (*Z*)-3-hexen-1-ol biosynthesis, were similar between treatments at all time points (Figure 3G). No detectable levels of (*Z*)-3-hexen-1-ol were observed at any time point. There were also no differences in the expression levels of the MeSA-related CHORS gene between all the treatments at all timepoints (Figure 3F).

Significant differences in the isoprene concentration were noted between the treatments at DI ($p < 0.05$) (Figure 3C), with WW_c vines having a higher concentration than WW_r vines, at 24.4 µg L^{-1} and 16.2 µg L^{-1}, respectively ($p < 0.001$). Isoprene concentration in DS_c (19.1 µg L^{-1}) was also higher than DS_e (13.8 µg L^{-1}) at DI ($p = 0.005$). A notable increase in isoprene emission in the DS_e vines was observed at PD (19.5 µg L^{-1}), in contrast

to a decrease in the DS_c vines (13.5 µg L^{-1}). Isoprene levels of WW_r remained relatively constant for the duration of the experiment.

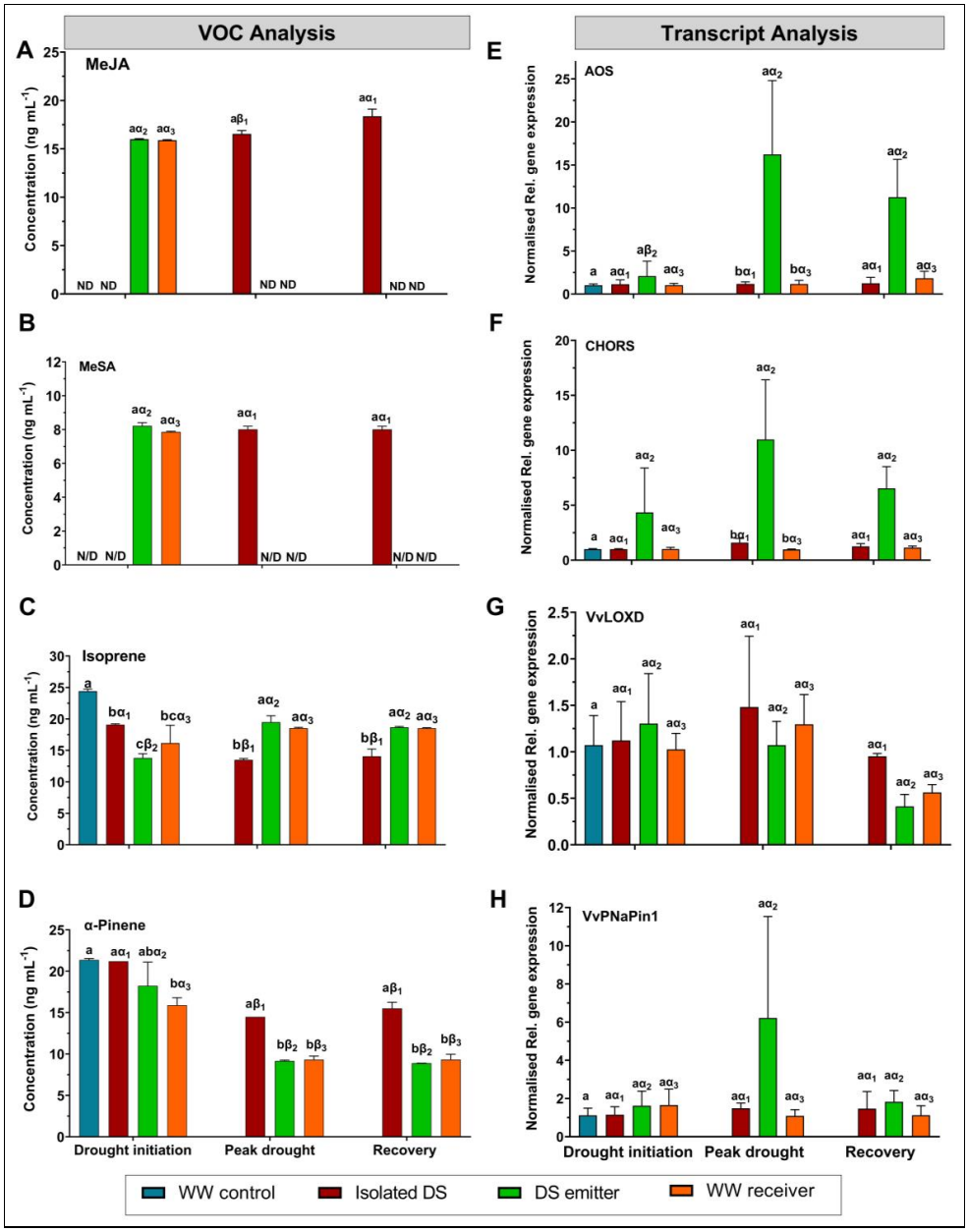

Figure 3. VOC metabolite concentrations (**A**–**D**) and transcripts of key VOC genes (**E**–**H**). VOC concentrations of (**A**) MeJA (methyl jasmonate), (**B**) MeSA (methyl salicylate), (**C**) isoprene, and (**D**) α-pinene in isolated WW control (WW_c), isolated DS (DS_c), co-located DS (DS_e), and well-watered (WW_r) vines during drought initiation, peak drought, and recovery stages. Gene expression values were calculated by applying the $2^{-\Delta\Delta Ct}$ algorithm. Estimated relative quantities were normalised for the expression value of the biosynthesis-related genes of (**E**) MeJA; AOS (allene oxide synthase), (**F**) MeSA; CHORS (chorismate synthase), (**G**) (*Z*)-3-hexen-1-ol; LOXD (lipoxygenase D), and (**H**) α-pinene; VvPNaPin1 (α-pinene synthase) during drought initiation, peak drought, and recovery. Different letters indicate significant differences between the treatments, with lower case letters used for the comparison of treatments within a particular time point and Greek letters used for comparing a treatment across different time points. Significance was set at $\alpha = 0.05$, Tukey test. Error bars indicate the standard error of mean (SEM, $n = 3$).

No significant differences in α-pinene concentration were noted at DI among the treatments, except in the WW_r (15.9 μg L^{-1}) which was significantly lower than the WW_c (21.4 μg L^{-1}) ($p < 0.03$) (Figure 3D). The concentration of α-pinene continued to decrease significantly at PD in the WW_r, yielding 9.3 μg L^{-1} ($p < 0.0001$). The DS_c vines had significantly higher levels of α-pinene (14.5 μg L^{-1}) as compared to 9.2 μg L^{-1} in DS_e at PD ($p = 0.003$). α-Pinene levels in the co-located treatments did not recover to their original levels after re-watering (on Day 9) but remained lower than the DS_c and WW_c. However, the expression of α-pinene synthase, VvPNaPin1, did not correspond to our metabolite data (Figure 3H). There were no significant differences between treatments at any timepoint.

4. Discussion

Aerial communication between plants via volatile signals has been extensively documented for biotic stress, however evidence of such signalling under abiotic stress is very limited. Our study contributes to this limited research, showing that grapevines subjected to drought stress emit volatiles that are detected by non-stressed counterparts. To show this, we had to conduct a combination of physiological, metabolomic, and molecular studies.

4.1. Effect of Drought-Induced VOCs on the g_s of Co-Located WWr

Eight days after withholding water from the drought-stressed treatment vines, both the isolated (DS_c) and co-located (DS_e) Shiraz vines experienced severe drought stress as indicated by the low soil moisture content (~4%) and presented lower g_s at PD compared to the well-watered treatment vines (Table 2). No significant changes were observed in the g_s of the Shiraz receivers throughout the nine-day period of the present experiments. This was in contrast to observations from several other drought recovery experiments involving potted plants in greenhouses that reflected a synchronised decrease in g_s between well-watered and drought-stressed Shiraz grapevines [30,40]. It was hypothesised that the decrease in the g_s of the well-watered vines was elicited by VOCs emitted from the neighbouring drought-stressed vines. It cannot, however, be excluded that changes in environmental conditions such as light, temperature [41], and vapour pressure deficit (VPD) [42] could have accounted for these fluctuations in g_s in the well-watered plants. Parallel to these findings, in a study that investigated the effect of salt-induced VOCs in priming for defence responses in lima beans, Caparrotta et al. [18] reported a similar decline in the g_s in the receiver plants co-located with salt-stressed plants. Their results suggested a possible role of methanol, terpenes, and several GLVs as airborne signalling molecules for communication between plants during salt stress. The effect of VOC exposure of plants in triggering stomatal responses has been demonstrated with several VOCs such as the plant growth regulators MeJA and MeSA [26–28], as well as (*Z*)-3-hexen-1-ol and its esters [29], and several monoterpenes [25]. However, the lack of stomatal responses from the receivers (WW_r) in the current study may be related to the type and severity of stress, and/or the specific composition of the volatile "cocktail" that may have a bearing on the stomatal responses in the receivers.

4.2. Volatile Metabolite and Gene Transcript Analyses

MeJA and MeSA concentrations displayed a similar trend, with no detectable levels of either compound recorded for the WW_c and DS_c controls at drought initiation, as well as for the co-located treatments at peak drought and recovery (Figure 2A,C). However, DS_c had significantly higher MeJA and MeSA levels under PD conditions as compared to both co-located treatments (WW_r and DS_e), which had non-detectable levels. The concentration of MeJA in DS_c continued to increase until recovery, while MeSA remained constant until the end of the experiment. The significant increases in MeJA and MeSA concentrations at PD were consistent with the results from previous studies that found an increase in emissions of both metabolites in response to drought stress [43,44]. In a similar VOC-priming study involving drought stress, tea plants were shown to release MeSA in response to drought [21]. Parallel to these findings, herbivore-induced VOCs were also demonstrated

to induce an upregulation of a subset of JA-inducible genes [16], as well as an increased accumulation of JA and SA in the leaves of receiver plants [11,12], suggesting the key role of JA and SA signalling in mediating defence responses. In the current study, however, the expression of CHORS, the gene corresponding to MeSA production, was not consistent with the metabolite analysis results. There were no significant differences in the expression of the salicylate synthesis gene across all treatments. The MeJA-related AOS gene was significantly upregulated in DS_e. Interestingly, this upregulation was not observed in DS_c vines, suggesting a probable influence of the co-located WW_r on DS_e. Although not decisive, the results suggested that emissions of MeJA and MeSA occur concurrently in grapevine and are synchronised between the emitter and receiver vines. Thus, while inter-vine interactions cannot be concluded based on these results, it was nevertheless evident that MeJA and MeSA were involved in the drought stress response of the emitter but not the receiver vines.

An increase in GLV emissions during drought episodes has been associated with lipid peroxidation as a result of enhanced drought-induced senescence [45]. Although several studies based on drought [46–48] and various biotic [13–15] stresses have shown that (*Z*)-3-hexen-1-ol is emitted in response to stress, no detectable amounts of (*Z*)-3-hexen-1-ol in response to drought were observed in the present work. The lipoxygenase gene, VvLOXD, which is involved in the synthesis of oxylipins and their derivatives such as JA and GLVs, was not significantly modulated in any of the treatments (Figure 2F).

The majority of plant emissions occur through the stomata of leaves and any variations in g_s can differentially impact the rates at which distinct VOCs are emitted from plants under stress [49]. However, isoprene and α-pinene have been observed in several studies to be emitted independently of stomatal regulation [50,51] due to their large Henry's Law constant, which entails that they can sustain an elevated intercellular partial pressure at a specific liquid phase concentration. However, this does not concur with the current results, in which the α-pinene and isoprene concentrations decreased in DS_c at Day 8 (PD) (Figure 2C,D) when both g_s and soil moisture content were low (Table 2). Interestingly, α-pinene concentration decreased in DS_e at PD but not isoprene, with the latter increasing despite the decline in g_s at Day 8 (PD) (Figure 2D). The isoprene levels of WW_r vines were not affected by the presence of the emitter and remained constant throughout the experiment. From these results, it can be proposed that the presence of WW_r influenced an entrainment behaviour in DS_e, resulting in increased isoprene levels compared to the solitary DS_c.

The increase in isoprene emission levels in the DS_e treatment could have arisen from isoprene, adsorbed and re-released by the DS_e from WW_r as a strategy to mitigate drought stress effects. The role of isoprene in improving thermotolerance in drought-tolerant plants under water stress conditions has been well-documented [52–54]. In a study by Ormeño et al. [32], the low isoprene emitter maple plant has been hypothesised to acquire its drought tolerance from its co-existence in nature with a downy oak, a high isoprene emitter. The researchers speculated that the otherwise susceptible maple adsorbs ambient isoprene produced by the downy oak, hence improving its drought tolerance. Based on the evidence from that previous study, which demonstrated increased drought tolerance in maple after isoprene fumigation, it is probable that the co-located DS emitters in the current work may have taken up isoprene from the non-stressed neighbours as a drought defence mechanism. According to Niinemets et al. [55], VOC uptake or emission depends on the direction of the concentration gradient between the leaf intercellular air space and the ambient air, and it has also been observed that isoprenoid-emitting species coexisting with non-emitting species can potentially absorb emitted volatiles from the ambient air [31,56]. It may then be assumed that DS_e has provided a sink for isoprene emitted by the WW_r receivers [55]. Although the well-documented ecological concept of associational resistance mainly describes resistance achieved from neighbouring heterospecifics against herbivory [57,58], the results presented herein may suggest a type of associational resistance based on abiotic stress between conspecifics.

Contrasting results were observed in this study when noting a synchronised decrease in α-pinene concentration between WW_r and DS_e at PD (Figure 2D). Interestingly, α-pinene emissions of WW_r were lower in contrast to those observed in WW_c on Day 1 (DI). The α-pinene levels in both co-located treatments remained significantly lower than DS_c at PD (Day 8) and recovery (Day 9). The general decrease in α-pinene determined for vines in both drought-stressed treatments and that of isoprene in DS_c vines during PD may be a strategy used by the vines to mitigate the effects of oxidative stress, using the isoprenoids as precursors to synthesise non-volatile metabolites such as carotenoids, tocopherols, and zeaxanthin [18,59]. The role of isoprenoids as signalling molecules between plants and other organisms has been well-documented [60], suggesting a possible involvement of these compounds as chemical cues not only within a single plant, but also in interplant communication. α-Pinene and limonene, as well as several other products, arise due to α-pinene synthase (VvPNaPin1), which was also considered [61]. Under the experimental conditions used in this study, however, VvPNaPin1 expression was not modulated in response to drought stress, remaining relatively constant between all treatments and time points (Figure 3H).

5. Conclusions

Evidence of drought-induced VOCs impacting on WW_r was shown as α-pinene decreased in both co-located vines. Although isoprene emission was not altered in the receivers, a significant increase in the compound's emission in DS_e compared to DS_c was noted, suggesting an effect of WW_r on DS_e responses. Evidence of the effect of WW_r on the emitters was also shown by the general trend of the gene expression of targeted genes related to MeJA, MeSA, and α-pinene, i.e., AOS, CHORS, and VvPNaPin1, respectively, which was towards an upregulation in DS_e only. This trend, together with the differential responses in the VOC emissions of isoprene and α-pinene between DS_e and DS_c vines, suggested a bi-directional interaction between the co-located treatments.

This study provides further evidence to that of Balacey [40] of the effect of well-watered plants on drought-stressed plants in grapevine and provides a novel view of bi-directional plant–plant communication between stressed and non-stressed plants, which is worth exploring for other environmental stressors and plant systems. The outcomes also suggest that isoprene and α-pinene may be among the signalling molecules that mediate interplant interactions under abiotic stress. Future studies should investigate the potential of terpenoids to act as priming agents to increase water use efficiency and drought resilience.

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