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Distinctive phytohormonal and metabolic profiles of Arabidopsis thaliana and Eutrema salsugineum under similar soil drying

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	as more stress tolerant than its close relative Arabidopsis thaliana, their responses to soil water deficit have not been compared. To ensure a similar rate of soil drying for the two species, daily soil water depletion was controlled to 5-10 % of the soil water content. While partial stomatal closure occurred earlier in Arabidopsis (Day 4) than Eutrema (from Day 6 onwards), thereafter both species showed similar stomatal sensitivity to drying soil. Nonetheless, both targeted and untargeted metabolite analysis showed larger responses in Arabidopsis in both early/mild drought (Days 1, 3, 5 – no significant change in leaf relative water content, RWC) and late/severe drought (Day 12 – circa 80 % decrease in leaf RWC). Arabidopsis (but not Eutrema) showed early peaks in foliar abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) contents. Arabidopsis showed greater metabolic adjustment than Eutrema during both early drought stress (428 versus 35 variables significantly changing) and severe drought (607 versus 171 variables significantly changing). Different sugar profiles between species were accompanied by opposing patterns in the bioactive cytokinin profile responses. The distinctive metabolic responses of each species during early drought, which occurred prior to leaf water status declining, were apparently independent of later stomatal closure in response to drought. These biochemical differences can have implications in regulating transpiration, since Eutrema reduced whole plant water use very early (Day 3) while this occurred later (Day 6 onwards) in Arabidopsis. Arabidopsis provides a promising model to evaluate the mechanisms responsible for stress-induced growth inhibition under the mild/moderate soil drying that crop plants are typically exposed to.
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Distinctive phytohormonal and metabolic profiles of *Arabidopsis thaliana* and *Eutrema salsugineum* under similar soil drying

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Running title: **Arabidopsis and Eutrema responses to soil drying**

Abstract

Although plants perceive and respond to soil drying via a series of concurrent physiological and molecular events, drought tolerance differs greatly within the plant kingdom. While *Eutrema salsugineum* (formerly *Thellungiella salsuginea*) is regarded as more stress tolerant than its close relative *Arabidopsis thaliana*, their responses to soil water deficit have not been compared. To ensure a similar rate of soil drying for the two species, daily soil water depletion was controlled to 5-10 % of the soil water content. While partial stomatal closure occurred earlier in Arabidopsis (Day 4) than Eutrema (from Day 6 onwards), thereafter both species showed similar stomatal sensitivity to drying soil. Nonetheless, both targeted and untargeted metabolite analysis showed larger responses in Arabidopsis in both early/mild drought (Days 1, 3, 5 – no significant change in leaf relative water content, RWC) and late/severe drought (Day 12 – circa 80 % decrease in leaf RWC). Arabidopsis (but not Eutrema) showed early peaks in foliar abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA) contents. Arabidopsis showed greater metabolic adjustment than Eutrema during both early drought stress (428 *versus* 35 variables significantly changing) and severe drought (607 *versus* 171 variables significantly changing). Different sugar profiles between species were accompanied by opposing patterns in the bioactive cytokinin profile responses. The distinctive metabolic responses of each species during early drought, which occurred prior to leaf water status declining, were apparently independent of later stomatal closure in response to drought. These biochemical differences can have implications in regulating transpiration, since Eutrema reduced whole plant water use very early (Day 3) while this occurred later (Day 6 onwards) in Arabidopsis. Arabidopsis provides a promising model to evaluate the mechanisms responsible for stress-induced growth inhibition under the mild/moderate soil drying that crop plants are typically exposed to.

Keywords: bioactive cytokinins; rewatering; redox state; stomatal conductance; unsupervised multivariate analysis

Introduction

In view of climate change, a major goal for the plant biology community is to understand the mechanisms that allow some plants to withstand drought or hot weather. Knowledge of how plants survive and reproduce in challenging environmental conditions can allow novel targets to be tested in crop-breeding programs. The well-known model species *Arabidopsis thaliana* provides information that can be applied to crop systems (Piquerez *et al.*, 2014; Gilliham *et al.*, 2017). Using the Columbia accession (Col-0) and its mutants has allowed many stress regulatory and responsive pathways to be deciphered (Koornneef and Meinke, 2010; Osakabe *et al.*, 2014), although its stress resilience has not been fully established. Despite wide ecotypic variation (Montesinos-Navarro *et al.*, 2011; Clauw *et al.*, 2016), Arabidopsis is not expected to cope well in extreme environments (Zhu *et al.*, 2015). Instead, Arabidopsis relatives such as *Eutrema salsugineum* have been proposed as stress-tolerant models (Orsini *et al.*, 2010; Zhu *et al.*, 2015). Eutrema seems prepared for stress, as its stress-related genes are upregulated in comparison to Arabidopsis even when grown under optimal conditions (Taji *et al.,* 2004; Gong *et al.,* 2005). As in *Arabidopsis*, *Eutrema salsugineum* ecotypes from different geographical regions show significant genetic variation (Lee *et al.,* 2016). However, physiological and metabolic responses of Arabidopsis and its stress tolerant relatives to soil water deficit have not been directly compared.

Physiological responses to water deficit are modulated by the intensity, duration, and rate of progression of imposed drought (Pinheiro and Chaves, 2011). Extensive research on the stomatal regulation of water loss demonstrates a trade-off between carbon assimilation, efficient water use and leaf cooling capacity (Chaves *et al.*, 2016). Plants can be grouped according to whether they avoid heat (keeping their stomata open for longer) or use water efficiently (closing their stomata sooner, a typical drought-avoidance strategy). However, if plants can avoid the deleterious effects of heat by keeping their stomata open for longer, while maintaining a favourable water status by extracting more water (e.g. by having deep roots), this strategy benefits carbon uptake in addition to the cooling effect. Under drought, Arabidopsis Col-0 closes its stomata at higher soil moisture levels than other Arabidopsis genotypes (Meyre *et al.,* 2001). The two well-studied ecotypes of Eutrema, Shandong and Yukon, can grow under limited soil water availability (Xu *et al.,* 2014; Macleod *et al.,* 2015), but their drought performance, relative to Arabidopsis, is unknown.

The two plant species seemingly have distinct water consumption strategies, although it may

be difficult to separate species *versus* accession variation. Arabidopsis (Col-0) had relatively higher total transpiration than Eutrema (Shandong) under non-challenging conditions, which was related to its higher relative growth rate (Orsini *et al.*, 2010). Salinity decreased transpiration to a larger extent in Arabidopsis than Eutrema. In addition to these different water consumption strategies, Eutrema and Arabidopsis also had different biochemical composition under non-challenging growth conditions, with foliar sucrose and glucose content higher in Eutrema, while the hormones salicylic acid (SA) and jasmonic acid (JA) were higher in Arabidopsis (Arbona *et al.*, 2010; Pilarska *et al.*, 2016). Furthermore, Eutrema expressed more stress and defence genes than Arabidopsis under non-challenging conditions, which is described as stress priming (e.g. Zhu *et al.*, 2015; Lee *et al.*, 2016). It is uncertain whether these biochemical differences regulate differences in transpiration, and consequently different rates of soil water depletion.

However, the metabolic features associated with the initial stages of soil drying are not clear. In Arabidopsis, soil drying partially closes the stomata well before any decrease in carbon assimilation rate (Hummel *et al.*, 2010; Bechtold *et al.*, 2016) or any significant increase in foliar abscisic acid (ABA) content (Bechtold *et al.*, 2016). ABA is described as the main driver controlling plant performance under limited water availability since it induces stomatal closure, but more comprehensive recent studies demonstrated that most of the plant hormones are involved in stress signalling (Müller and Munné-Bosch, 2015). In addition, during the very early stages of water limitation, effects on carbon metabolism (CO_2 assimilation, and sucrose and starch formation and allocation) may be decoupled from stomatal closure (Pinheiro *et al.*, 2011; Bechtold *et al.*, 2016). Many players are involved in stress perception and signal transduction leading to large alterations in carbon metabolism, and in the transcription program (Golldack *et al.*, 2014; Urano *et al.*, 2017). The metabolic balance between several molecules triggers adjustment mechanisms, and when several thresholds are achieved, physiological responses to drought occur (Pinheiro *et al.*, 2011). The integration of multiple environmental signals by sugars, hormones, and reactive oxygen species (ROS) adjusts plant growth and determines whether plants survive or perish under given environmental conditions (Pinheiro and Chaves, 2011; Osakabe *et al.*, 2014). The precise chain of events is not yet defined, and although some pathways and interactions are understood, others are more elusive (Rivas-San Vicente and Plasencia, 2011; Munné-Bosch and Müller, 2013; Ruan, 2014; Considine and Foyer, 2014; de Ollas and Dodd, 2016). Although recent reports highlight that stomatal closure is one of the initial events in response

to soil drying, many other metabolic adjustments also take place.

This work aimed to explore the metabolic adjustments prior to significant stomatal closure in Arabidopsis (Col-0) and Eutrema (Shandong) responding to slowly-imposed soil water deficit. Our working hypothesis is that Arabidopsis and Eutrema show distinctive responses to progressive and slow soil drying. In addition, untargeted analysis of LC-MS metabolite data was used to detect similarities and differences between these two species.

Materials and Methods

Arabidopsis thaliana (Col-0) and *Eutrema salsugineum* (Shandong) seeds were soaked and stratified at 4 ºC for 4 or 14 days, respectively. *Eutrema salsugineum* is the current designation of *Thellungiella salsuginea* (Integrated Taxonomic Information System on-line database, www.itis.gov; The International Plant Names Index, www.ipni.org). Seeds were then transferred to pots (300 mL) containing a 1:1 mixture of coarse sand and peat (Shamrock). Plants were grown under controlled conditions, under a 12 h photoperiod, temperatures ranging from 20 to 24 ˚C, with a 60-70 % relative humidity and photosynthetically active radiation (PAR) of 250-300 µmol m^{-2} s^{-1} (SON-T Agro 400w, Phillips). Plants were watered every day with demineralized water to 85 % of soil water content (SWC). SWC was monitored daily and is defined as: SWC = [(pot weight – pot weight with totally dried substrate)] / [(pot weight at drained capacity – pot weight with totally dried substrate)] x 100. Drought stress treatments were imposed when plants had 8 to 10 fully expanded leaves (40 days for Eutrema and 36 days for Arabidopsis) and had covered the surface of the pots (thereby minimising evaporation from the soil). During the experiment plant growth increased, on average by 2.9 g fresh weight (FW) for Arabidopsis and 2.5 g FW for Eutrema, corresponding to less than 0.8 % error in estimating SWC (Fig. 1).

Preliminary drought experiments, in which water was withheld, showed faster soil water depletion and more rapid stomatal closure in Arabidopsis (supplementary Fig. S1). Similarly, higher transpiration rates of Arabidopsis were previously reported (Orsini *et al.* 2010). Stomatal conductance of the two species was differentially sensitive to soil drying (supplementary Fig. S1B and C). Within the 45-55 % SWC range, Arabidopsis showed greater stomatal closure than Eutrema, but below 40 % SWC both species showed similar stomatal sensitivity to soil water deficit and were severely affected by drought. However,

analysis of covariance demonstrating no significant species x SWC interaction, both species showed a similar relationship between % gs *vs* soil water content, with (supplementary Fig S1C).

To compare stress duration and intensity effects on plant responses, the rate of soil water depletion was controlled to 5-10 % of the SWC per day by pre-dawn irrigation (Fig. 1). Even when controlling the SWC, Arabidopsis consumed more water than Eutrema, as indicated by the greater divergence between SWC measured at maximum soil water deficit (symbols) and the SWC to which the pot was re-turned to pre-dawn ("stress" line in Fig 1). This greater water use of Arabidopsis was most prominent between Days 3 and 7.

Plants were harvested 0 (last day of watering), 1, 3, 5 and 12 days after the beginning of the assay, corresponding to 75 %, 66 %, 45 %, and 12 % SWC, respectively. Samples were also taken the day after re-watering (1 d). Six biological replicates were obtained at each time-point, except for Day 1 controls for which there were only five biological replicates, providing 65 samples of each plant species. At the beginning of the assay, the most recently expanded two-three leaflets were identified and used for physiological and water status measurements. For the biochemical analysis, and when analysing severe drought and early rewatering, only non-senescent leaflets were used, i.e. the younger leaflets. Samples for biochemical (hormone, carbohydrate, pigment and oxidative status) analysis were immediately frozen in liquid nitrogen and kept at -80 ºC until further extraction and analysis. Samples for osmotic potential and for RWC were then collected.

Leaf conductance, water status and osmotic adjustment

Stomatal conductance was measured 2-3 h after the beginning of the photoperiod in five plants per treatment using a portable gas exchange photosynthesis system coupled to a 6400-15 chamber (1 cm^2 diameter cuvette, Li-6400, Li-Cor, Lincoln, Nebraska, USA). Three to five measurements were made per plant on the most recently expanded leaf.

Leaf and root samples were taken 4 h after the beginning of the photoperiod. Leaf discs (3 mm diameter) and total roots were weighed to obtain fresh weight (FW), placed in darkened petri dishes containing distilled water for 2 h to fully hydrate, then re-weighed to obtain turgid weight (TW), and then dried at 80 ºC for 48 h to obtain dry weight (DW). Leaf (LRWC) and root (RRWC) relative water content were calculated as: RWC = [(FW – DW) x 100 / (TW – DW)].

Leaf osmotic potential (ψs) was evaluated from leaf disks (8 mm, n = 5–6), frozen and stored at -80 ºC. The leaf osmotic potential was measured with an HR-33T dew point microvoltmeter and C-52 sample chambers (Wescor, Inc., Logan, UT, USA). The osmotic potential was adjusted to the LRWC to calculate the osmotic potential at full turgor (OP100), and the osmotic adjustment was calculated as previously described (Turner *et al.*, 2007).

Phytohormone quantification via LC-MS targeted analysis

Freeze-dried shoots (50 mg) were used to extract and quantify the following hormones (Müller and Munné-Bosch, 2011): auxin (indole-3-acetic acid: IAA), gibberellins (GA1, 4, 9, 19, 20, 24), cytokinin (CK) compounds (trans-zeatin: Z; trans-zeatin riboside: ZR; 2-isopentenyl adenine: 2iP; isopentenyl adenosine: IPA; dihydrozeatin: DHZ; dihydrozeatin riboside: DHZR), and stress-related phytohormones (ABA; JA; SA; and ethylene precursor 1-amino-cyclopropane-1-carboxyic acid: ACC).

Extraction was performed in methanol solutions containing 1 % glacial acetic acid, using the following standards: d_5-IAA, d_6-2-isopentenyl adenine (d_6-2iP), d_6-IPA, d_6-ABA, d_5-JA, d_4-SA, d_4-ACC, d_2-GA_1, d_2-GA_4, d_2-GA_9, d_2-GA_{19}, d_2-GA_{20} and d_2-GA_{24}; d_5-Z and d_5-ZR were used as standards for Z, DHZ, ZR, and DHZR. After adding 170 µL of the extraction solution and 30 µL of a solution containing 100 ppm of the standards in the same solvent, the materials were mixed in a vortex mixer for 5 s and exposed to ultrasound for 30 min, followed by centrifugation at 9,500 *g* for 10 min. The supernatant was removed and the residue was washed twice with 100 µL of the solvent solution. The supernatant and washes were combined and filtered through PTFE 0.22 µm filter paper (Waters, Milford, MA) and 5 µL aliquots were analysed using a UPLC-ESI-MS/MS (Acquity UPLC System from Waters, Milford, MA) and tandem MS/MS experiments were performed on an API 3000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ont., Canada) using a HALO™ C18 column (2.1 × 75 mm, 2.7 µm) (Advanced Materials Technology, Inc. Wilmington, DE) and a binary mobile phase system composed of (A) water modified with 0.05 % glacial acetic acid and (B) acetonitrile modified with 0.05 % glacial acetic acid. Quantification was performed by preparing a calibration curve including each of the analysed compounds and calculating the compound/standard ratio using Analyst™ software (Applied Biosystems, Inc., Foster City, CA). The results were expressed on a dry weight (DW) basis.

Ascorbate oxidative status

Ascorbate reduced and oxidized forms were determined by a plate-reader method (Queval and Noctor, 2007) with slight modifications. Briefly, lyophilised leaves (20 mg DW) were placed in a microcentrifuge tube with two tungsten balls and ground under liquid nitrogen in a Retsch MM300 Bead Mill Cell Disrupter (Retsch GmbH & Co Haan Germany). Subsequently, 1 mL of extraction buffer (6 % meta-phosphoric acid) was added, vortexed for 1 min and clarified by centrifugation at 10,000 *g* (10 min, 4ºC). Finally, extracts were neutralized and adequately diluted before spectrophotometric readings on a 96 well quartz microplate (Hellma Hispania SL, Badalona, Spain). The levels of ascorbate (AscA) (reduced) and dehydroascorbate (DHA) (oxidized) were determined using ascorbate oxidase (AO) and dithiothreitol (DTT), respectively (Foyer *et al.*, 1983). AO specifically oxidizes all AscA in the sample. Therefore, the decrease in O.D. at 265 nm is related to AscA content. Alternatively, when the samples are incubated with DTT, DHA is reduced to AscA and the increase in O.D. is proportional to the initial DHA content. The ascorbate oxidative status was estimated as DHA/(DHA + AscA).

Photosynthetic pigments quantification

For pigment extraction, lyophilised leaf samples (15 mg DW) were placed in a microcentrifuge tube with two tungsten balls, ground under liquid nitrogen in a Retsch MM300 Bead Mill Cell Disrupter (Retsch GmbH & Co Haan Germany), and extracted with ice-cold 80 % acetone (v/v). After centrifuging at 6,500 *g* for 10 min at 4 °C, the supernatant was collected and the pellet was re-extracted with the same solvent until it was colourless. Then, supernatants were pooled and analysed spectrophotometrically. Specific absorption coefficients in 80 % acetone previously reported were used to quantify chlorophyll a, chlorophyll b and carotenoids (Lichtenthaler and Buschmann, 2001).

Extraction of water soluble carbohydrates and starch

Water-soluble carbohydrates were extracted from freeze-dried leaf material following a chloroform:methanol method previously described (Antonio *et al.*, 2008). Briefly, 50 mg DW of leaf material was ground in liquid nitrogen and extracted with 250 µL ice-cold chloroform: methanol (3:7, v/v), vortex-mixed and incubated at -20 ºC for 2 h. After incubation, samples were extracted twice with ice-cold water, and after centrifugation at 17,900 *g* at 4 ºC for 10 min, the upper phases were collected and pooled. The combined supernatants containing the water-soluble carbohydrates were evaporated to dryness using a centrifugal concentrator (Savant SpeedVac Plus SC110A, Thermo Electron Corporation, Runcorn, UK). Samples

were reconstituted in 100 µL water and centrifuged at 6,800 *g* at 20 ˚C for 30 min, followed by LC-MS analysis.

For starch analysis, the pellet resulting from the chloroform:methanol extraction was washed twice with water. Ten volumes of water were added to the pellet, boiled for 3 min, and autoclaved at 130 ˚C for 1 h. After cooling, samples were incubated with 6 U amyloglucosidase (Roche Applied Science, Amadora, Portugal) for 2 h at pH 4.8 and 60 ˚C. Starch was quantified in the supernatant using a starch enzymatic quantification kit (nº 10207748035, R-Biopharm Aktiengesellschaft, Darmstadt, Germany) and by making use of the Hatterscheid and Willenbrink modification as previously described (Pinheiro *et al.*, 2001).

Untargeted LC-MS analysis of the water-soluble carbohydrate fraction

Arabidopsis and Eutrema samples were analysed as separate cohorts. In each case, samples were randomized and run in batches of eight or nine with the injection of a pooled sample between batches for quality control (QC). LC-MS analyses were performed on a Dionex U3000 2D HPLC system coupled to a Bruker maXis UHR-Q-TOF MS with an ESI interface. Analytes were detected in the negative ion mode using the following MS parameters: capillary voltage, 4500 V; nebulizer gas, 2 Bar; drying gas, 8.0 L/min; drying temperature, 200 ºC, and collision energy, -10.0 eV. Mass spectra were acquired over the scan range *m/z* 50-1000. Chromatographic separation was carried out using a porous graphitic carbon (PGC) Hypercarb™ column (5 µm, 100 mm × 4.6 mm; Thermo Electron, Runcorn, Cheshire, UK) at a flow rate of 600 µL min^{-1}. All samples were reconstituted with 500 µL deionised water with a further 50-fold dilution in deionised water to prevent signal saturation and to minimise matrix effects. The sample injection volume was 20 µL and the PGC column was used at ambient temperature (25 ºC). The binary mobile phase was composed of (A) water modified with 0.1 % (v/v) formic acid (FA) and (B) acetonitrile modified with 0.1 % FA. The gradient elution was as follows: 0-4 min maintained at 2 % B; 4-7 min, 2 to 8 % B; 7-10 min 8-25 % B and maintained for 3 min, followed by column regeneration and re-equilibration: 13-19 min, 25 to 40 % B; 19-19.5 min, 40 to 50 % B held for 1 min; 20.5-21 min 50 to 99 % B held for 2 min; 23-25 min 99 to 2 % B and maintained for 10 min. All solvents were purchased from Fisher Scientific except FA, which was purchased from Sigma Aldrich.

Statistical analysis

Raw LC-MS data were pre-processed using Progenesis QI (Nonlinear Dynamics, Newcastle Upon Tyne, UK). Mass spectra were aligned by retention time and normalized to the same total ion count before peak picking was performed to provide a matrix of potential metabolites for each observation, annotated by the accurate mass (*m/z* between 50 and 1000) and retention time (between 1 and 30 min) of the corresponding peak. In total, 53208 and 33032 peaks were recorded for Arabidopsis and Eutrema, respectively, and were used as variables in multivariate and univariate analyses.

When the Eutrema data were scaled to unit variance to allow smaller variables to contribute to the analysis, differences between batches became apparent, with the last two batches differing substantially from the rest (supplementary Fig. S2A). Liquid chromatography-mass spectra are often acquired batch-wise to allow necessary calibrations and cleaning of the instrument. However, this may introduce further sources of variation, such as differences in the conditions under which data for individual batches is acquired. Quality control (QC) samples are frequently employed to both judge and correct for this variation.

However, batch correction using the QC observations increased inter-batch variation as the change in observations between batches was often not well-represented by the change in corresponding QCs. Therefore, background correction for each variable was performed (Rusilowicz *et al.*, 2016; Wehrens *et al.*, 2016). This method identifies a background trend, using experimental observations as well as the QCs, with which to adjust the intensities. The run order for data collection was randomized, but by chance a disproportionate number of early-stress observations occurred in batch 3 and several late-stress observations in batch 4. With the exception of these two batches, which were combined, we used a separate trend for each batch, obtained as a moving median with a window width of 5 observations. The effectiveness of batch correction was assessed using the Bhattacharrya distance (Wehrens *et al.*, 2016). In addition, an outlier that dominated the variance after scaling was removed before calculating the trend. Control correction was also performed on each variable to remove differences due to growth. For each day of harvest, this was achieved by subtracting the median over the six control replicates from the corresponding variable in the water-stressed observations for that day. The Arabidopsis data showed no obvious differences between batches (supplementary Fig. S3), and therefore, batch correction was deemed unnecessary but control correction was performed to prevent differences due to growth from masking early-stress characteristics.

Principal components analysis (PCA) was used for unsupervised multivariate analysis with both unscaled data and after scaling to unit variance to prevent high content metabolites dominating the analysis. To identify patterns in metabolites over time, k-means cluster analysis was performed with the control-corrected time-series for both datasets. The obtained initial clusters were filtered using the sum of squared values to remove the time-series for metabolites that did not differ appreciably between drought and control observations, i.e. where all values in the control-corrected time-series were close to zero. Cluster analyses of the remaining time-series (with various values of k) showed the largest cluster to consist of less interesting time-series and an iterative filtering process was used to reduce the number of time-series, leaving only those with the most interesting patterns. In each iteration, k-means clustering with k = 15 was performed and the largest cluster removed before the next analysis. After four iterations, 46 time-series remained and were clustered using k-means with k = 9.

Univariate analyses were performed using the non-parametric Mann-Whitney U-test with Benjamini-Hochberg correction for multiple comparisons (Benjamini and Hochberg, 1995). Three-way group comparisons were carried out (early stress/late stress/rewatered and Days 1, 3 and 5 for each species) with one-way ANOVA and Tukey's honest significant difference (HSD) correction for multiple pairwise testing. Data correction methods were implemented using C code written in-house and statistical analyses were performed in the R platform, version 2.13.1 (R Core Team, 2016) or in Matlab (The MathWorks Inc., Natick, MA, USA).

Analysis of covariance (ANCOVA) discriminated possible species difference in stomatal sensitivity to drying soil.

Results and discussion

Stomatal sensitivity to drying soil and plant water status

Under well-watered conditions, stomatal conductance (gs) of both species exceeded 0.11 mol m^{-2} s^{-1} (supplementary Fig S4A). Since gs of well-watered plants varied from day to day, gs of plants in drying soil was normalised according to the average well-watered values of each species. As the soil dried (Fig. 2), partial stomatal closure of Arabidopsis and Eutrema was detected on Days 4 and 6, respectively (Fig. 2). Within the 45-55 % SWC range, Arabidopsis showed greater stomatal closure than Eutrema, but below 40 % SWC both species showed

similar stomatal sensitivity to soil water deficit and were severely affected by drought. Stomatal conductance responded sluggishly to re-watering, with limited recovery (supplementary Fig S4A). Across the entire experiment, both species showed a similar relationship between % gs *vs* soil water content, with analysis of covariance demonstrating no significant species x SWC interaction (supplementary Fig S4B). Thus, both species showed similar stomatal sensitivity to drying soil.

Initial stomatal closure was not associated with decreased leaf water status, i.e. lower cell volume did not trigger early stomatal closure (Sack *et al.*, 2018). On imposing soil water deficit, no significant differences in Eutrema leaf (and root) RWC were detected until Day 5 (supplementary table S2). In Arabidopsis, leaf RWC transiently decreased on Day 3 (supplementary table S1). Although statistically significant (at the 95% confidence level), its small magnitude (~4%) could be within the method error or due to daily fluctuations.

In contrast to plant water status, the water consumption patterns changed very early on, but were not temporally correlated with stomatal closure. Compared with its well-watered control, Eutrema started to lose less water from Day 3 onwards (3 days before any significant stomatal closure), as indicated by the slope of the soil RWC% line for plants in drying soil (Fig. 1). In contrast, Arabidopsis started to use less water on Day 6 onwards (two days after partial stomatal closure occurred). This suggests that earlier growth inhibition of Eutrema, decreased whole plant water loss independent of changes in plant water status.

By Day 12, leaf RWC of both species had declined to very low values (< 20 %) and leaflets selected for water status measurements (those most recently expanded at the onset of the assay) were severely wilted and exhibited senescence symptoms. Lower leaf chlorophyll fluorescence (Fv/Fm) and lower chlorophyll a content indicated photo-inhibition and/or leaf senescence (Kalaji *et al.*, 2016).

Despite the severity of the stress imposed, root water status of both species recovered within 24 h of re-watering. Root RWC of Eutrema was similar to those of the well-watered controls, while the root RWC of Arabidopsis was ~90 % of that of the controls. However, leaf RWC remained low, only ~50 % and ~40 % of the well-watered control values in Eutrema and Arabidopsis respectively (supplementary tables S1 and S2). In addition, Fv/Fm tended to increase in Eutrema, but values were unaffected in Arabidopsis (supplementary tables S1 and S2).

Traditionally, it has been argued that only resurrection plants can survive such severe drought, i.e. recover from leaf RWC values below 20% (Dinakar and Bartels, 2013). Since leaf RWC was determined in the most recently expanded leaves at the beginning of the assay (see Material and Methods), these older leaves were severely wilted and senescent after 12 days, while younger leaves visually maintained turgor. Several reports indicate that Arabidopsis Col-0 plants are able to recover from severe drought, with 30% of Col-0 plants surviving exposure to 15% SWC and severe wilting (Sun et al. 2013) while 20% of severely wilted Col-0 plants survived SWCs < 20% (Zhao *et al.* 2016). Moreover, Col-0 plants with 40-50% leaf RWC recovered from drought (Meyre *et al.* 2001; Tran *et al.* 2007; Kosma *et al.* 2009; Koffler *et al.* 2014) while some plants recovered from 20% leaf RWC although the survival percentage was very low (Lü *et al.* 2012; Nguyen *et al.* 2016). In contrast to Arabidopsis, Shandong was able to recover from drought if the leaf RWC declined to 50%, but not 30% (Dedrick 2007). Since our measurements were made only 1 Day after rewatering and no plants were available to evaluate long-term recovery, irreversible damage cannot be ruled out.

As small changes in soil water content (10-15 %) affect not only leaf conductance but also plant metabolism (Davies *et al.*, 1990; Pinheiro *et al.*, 2011), both untargeted metabolite analysis and targeted metabolite/biochemical analysis aimed to determine the impact of gradually declining soil water availability on leaf metabolism. This approach aimed to detect species differences in metabolism when plant water status was not affected (up to Day 5).

Untargeted metabolite analysis

The responses to soil water depletion in Arabidopsis and Eutrema were analysed via untargeted LC-MS, making use of the water-soluble fraction. After batch correction of the Eutrema data, PCA of the control corrected and scaled data grouped according to drought-stress duration for both species (Fig. 3). Moreover, PCA of unscaled data showed that most of the variance is due to large differences between early-stress (Days 1, 3 and 5) and late-stress (Days 12 and 13) observations. Statistical separation of late-stress effects was not related to differing sample water content, since comparable dry weights were used and the resulting data normalised before statistical analysis.

When considering only the early-stress observations, the PCA scores plot shows clear

grouping of the observations by stress duration for both plant species (Fig. 4). Distinctive metabolic signatures were obtained even for early days with limited soil drying (< 20 % change in SWC at Day 3). The PCA loadings identified variables that most influenced the separation between Days 1, 3 and 5, and early- and late-stress observations. In addition, an iterative k-means algorithm filtered out the largest clusters to leave those comprising more unusual, and potentially more informative, patterns (supplementary Fig. S5). Hierarchical clustering with the 46 time-series selected by the k-means analysis (Fig. 5; supplementary Fig. S6) allowed the similarities (or differences) between the associated metabolites to be visualised.

Severe drought causes larger metabolic alterations in Arabidopsis than in Eutrema

Late-stress markers for both Arabidopsis and Eutrema included peaks that were identified as the carbohydrates sucrose and raffinose, by comparison with authentic standards of these molecules. Sucrose significantly increased and raffinose significantly decreased ($p < 0.001$) in late stress (Day 12) and on re-watering (Day 13). A significant decrease was found for features with *m/z* values of 341 and 387, most probably a hexose disaccharide. A feature with *m/z* 711, also decreasing significantly, is tentatively assigned to stachyose, known to co-elute with raffinose (Antonio *et al.*, 2008). Sucrose (detected in a range of ionic forms) observed at high levels in the leaf blade tissue (Table 1) was not unexpected. Drought stress upregulated sucrose synthesis in different resurrection plants (Peters *et al.*, 2007; Whittaker *et al.*, 2007; Gechev *et al.*, 2013) as well as in herbaceous and woody plants (e.g Antonio *et al.*, 2008; Pinheiro and Chaves, 2011; Granda and Camarero, 2017). Although stress downregulated raffinose synthesis in several resurrection plants (Muller *et al.*, 1997; Moyankova *et al.*, 2014), raffinose is typically described as being upregulated under stress (Elsayed *et al.*, 2014), including in Arabidopsis (Taji *et al.*, 2002). Soil water deficit significantly ($p < 0.00001$) decreased two co-eluting features (with *m/z* 191 and *m/z* 405) in both plant species The feature with *m/z* 191 was assigned to citric acid, following tandem mass spectrometry (MS^2) analysis and comparison of the fragmentation pattern in both METLIN (www.metlin.scripps.edu) and PRIMe (www.prime.psc.riken.jp) metabolomics databases. The co-eluting feature at *m/z* 405.0019 on MS^2 produced a single fragment at *m/z* 191.0185, that was tentatively assigned as the $[2M-2H+Na]^-$ charge-sharing dimer of citric acid (accurate mass 405.0287). Univariate analyses (after multiple test correction) indicated that

607 variables significantly ($p < 0.0001$) differed between late-stress observations and controls in Arabidopsis, in comparison to just 171 in Eutrema, suggesting that Arabidopsis more extensively adjusts its metabolism. An alternative view is that larger changes in Arabidopsis indicate less active metabolism, since metabolites accumulate because the plant has no capacity to use them.

In the cluster analysis, three clusters tend to decrease over time, including the response of raffinose (supplementary Fig. S5C, S5D, S5G), which was more extreme in Arabidopsis than Eutrema, therefore occurring in a different cluster. Severe drought stress decreased the levels of raffinose in lupins, although this was preceded by a transient increase during early stress (Antonio *et al.*, 2008; Pinheiro *et al.*, 2011). Although the different ionic forms of citric acid from both Arabidopsis and Eutrema group together in cluster D, a difference in the trend between the two different plant species can be seen, with Eutrema showing an early increase before the overall decrease. Citric acid decreased in response to late and severe drought, as previously observed in lupin and Eutrema (Pinheiro *et al.*, 2004; MacLeod *et al.*, 2015). The final two clusters (Fig. S5H and S5I) show the response profiles of (unknown) compounds that are significantly greater than or lower than the controls throughout the time-series, notably all from Arabidopsis, and are good candidates for further studies.

In contrast, four clusters tended to increase rapidly in late drought; the scale of the response accounts for the difference between these four clusters. They mostly comprise the differing ionic forms of sucrose. In Arabidopsis, unknown compounds with *m/z* 133 and *m/z* 288 exhibited a very similar pattern to sucrose (supplementary Fig. S5A, S5B, S5E and S5F). The most extreme responses result in separate clusters consisting of just one or two observations (Fig. S5E-F). For each sucrose ionic species, the response for Days 12 and 13 is more extreme for Arabidopsis than for Eutrema. In both plant species, a further unknown with *m/z* 195 also clusters with sucrose, and re-watering causes a greater response than during late stress.

Severe drought causes larger biomass reduction in Eutrema than in Arabidopsis

Although severe drought decreased the biomass of both species, Arabidopsis (22% decrease) was less sensitive than Eutrema (38% decrease) (supplementary tables S1 and S2). The mechanisms that limit biomass accumulation are poorly understood (Pinheiro and Chaves,

2011; Skirycz *et al.*, 2011). Under drought, the higher availability of sugars indicates that CO_2 assimilation is not limited as much as growth, with carbon being available but plants unable to use it, termed "sink limitation" (Wiley and Helliker 2012; Granda and Camarero, 2017). This also supports the concept of passive accumulation. On the other hand, higher sugar content may reflect their use in osmoregulation, maintaining cell integrity and providing readily available carbon to resume growth (active reserve storage concept; Granda and Camarero, 2017). Although osmotic adjustment was detected under severe drought and rewatering in Eutrema (supplementary table S2), it was only detected in Arabidopsis on rewatering (supplementary table S1). The growth reduction was accompanied by starch remobilization, supporting the hypothesis of carbon reserve reallocation. A regulatory mechanism that integrates carbon availability and its use within the plant (Smith and Stitt, 2007; Pinheiro and Chaves, 2011) partitions photoassimilates to other biochemical pathways (than growth) to withstand severe drought and/or resume growth whenever possible. To determine whether sucrose accumulation represents an active or passive process, it would be necessary to determine if and how carbon limits growth (Wiley and Helliker 2012). A better understanding of these mechanisms is crucial to select genotypes with more stable growth under stress. In that sense, a favourable ideotype will depend on where the plant is to be grown. Higher survival, largely due to conservative water consumption can be the most relevant selection criterion in arid or semi-arid regions. On the other hand, in moderate climates with milder droughts, plant production can be boosted if stress has little impact on growth (Skirycz *et al.*, 2011; Tardieu, 2012). Higher stomatal conductance is a positive trait when plants are under moderate/mild drought since it favors growth maintenance (Tardieu, 2012). In that scenario, Arabidopsis can provide a suitable model for biomass production under moderate drought.

Severe drought decreased ascorbate content of both species

Under severe stress, some parameters, including ascorbic acid (AscA), leaf chlorophyll fluorescence (Fv/Fm) and chlorophyll a (Chla), have similar patterns in the two species. Lower Fv/Fm indicates a lower photochemical harvest, with a larger proportion of photons being diverted to non-photochemical quenching (heat and ROS production). Although two hormones (IAA and SA) are proposed to up-regulate ROS production (Rivas-San Vicente and Plasencia, 2011; Considine and Foyer, 2014; Khokon *et al.*, 2017), severe drought

significantly decreased SA content but did not alter IAA content. Thus, these hormones are unlikely to promote ROS accumulation. Water deficit has variable effects on carotenoid content (Koffler *et al.*, 2014; Uarrota *et al.*, 2018), but we did not detect quantitative alterations. However, decreased chlorophyll a content indicates that chlorophyll degrades faster than carotenoids (Lichtenthaler and Buschmann, 2001). A higher carotenoid to chlorophyll content can favour photo-protection per amount of light received. On the other hand, non-photochemical quenching via carotenoids requires ascorbate for correct function (Koffer et al. 2014). Severe drought significantly decreased ascorbate content (43% in Eutrema; 24% in Arabidopsis), which could have compromised such quenching. Noctor *et al.* (2014) state that ascorbate content is only significantly decreased by drought when stress-induced senescence processes are activated. Although the sampled leaves did not show visible symptoms of senescence, their ascorbate levels suggest senescence programs were already activated. Under drought, both Arabidopsis Col-0 and Col-0 mutants with lower ascorbate content show senescence symptoms (Koffler *et al.*, 2014), with some mutant leaves showing necrosis. Rewatering further decreased the ascorbate content (55% in Eutrema; 52% in Arabidopsis), indicating the senescence program is still active. Changes in ascorbate reduction status can also modulate signalling pathways, affecting stress-response mechanisms (Considine and Foyer, 2014).

Arabidopsis and Eutrema are metabolically distinct

To characterize in more detail the responses to soil water depletion in Arabidopsis and Eutrema, various biochemical parameters (Table 2) were measured during early drought. Some metabolites showed minimal (< two-fold) differences between species, while starch, JA and ZR were more abundant in Arabidopsis, and IAA and DHA were more abundant in Eutrema (supplementary tables S1 and S2). Distinctive phyothormone profiles n under non-challenging conditions were previously reported (Arbona *et al.*, 2010; Pilarska *et al.*, 2016).

To remove the effects of differential growth between species, data were control-corrected by subtracting the median control value from the measurements for each day. PCA analysis with all biochemical parameters for both species (supplementary Fig. S7) showed the greatest source of variance to be the separation of late/severe drought and re-watered (RW) observations, as in the untargeted analyses. Without variable scaling, loadings plots showed a large influence of the variables with the greatest mean values (leaf RWC, osmotic potential

(OP) and starch) in the total variance. After scaling to unit-variance, the separation of late stress/re-watered observations is still seen along the first principal component, although accounting for far less of the total variance. In Arabidopsis, variables from re-watered samples were closer to those from early-day observations. In Eutrema, the difference between late stress and re-watering is only apparent along the second component, which represents less variance and more similar metabolic status. These findings suggest: 1) Arabidopsis responds faster to soil water availability; and/or 2) Eutrema requires prolonged stimulus to reprogram its metabolism. However, whole plant water use of Eutrema is more conservative than Arabidopsis (with soil drying decreasing water use after 3 and 6 days respectively – Fig 1) in spite of an opposite stomatal response (with soil drying decreasing gs after 4 and 6 days in Arabidopsis and Eutrema respectively – Fig. 2). Thus vegetative growth and stomatal closure seem independently regulated, with Eutrema reacting faster to soil water availability and Arabidopsis requiring prolonged stimulus. The "optimistic" strategy of Arabidopsis Col-0 maintains biomass production under mild stress and/or under deficit irrigation (Skirycz *et al.*, 2011). An alternative hypothesis could be that Eutrema slows its metabolism much earlier while Arabidopsis reprograms metabolism in a different way. It will be important to determine whether growth is maintained, both above and below ground, and if reserves are reallocated.

Eutrema responds with small changes to early drought

In both Arabidopsis and Eutrema, inspection of the PCA loadings showed that many variables contribute to the separation of each of the early stress days (Fig. 4). Thus metabolic separation between sampling dates is due to the cumulative changes arising from small contributions of many metabolites. However, the two species react differently to similar decrease in the soil water availability. More metabolites responded to early drought stress in Arabidopsis, with 428 variables showing statistically significant differences between Days 1, 3 and 5 ($p < 0.01$; 36 with $p < 0.001$) in comparison to 35 in Eutrema ($p < 0.01$; 4 with $p < 0.001$). However, none of the variables that consistently differed between the early days corresponded to those identified as late-stress markers (such as sucrose), showing different metabolism during early and late drought.

The larger changes in Arabidopsis suggest different metabolic strategies to deal with the progressive decline in soil water availability. This is consistent with greater stomatal closure

of Arabidopsis than Eutrema on Day 5 (Fig. 2), supporting a drought avoidance strategy for Arabidopsis. Partial stomatal closure can limit carbon assimilation and carbon availability for respiration and growth (Pinheiro and Chaves, 2011). Acclimation of Arabidopsis to optimise carbon use (Smith and Stitt, 2007) would increase carbon storage (e.g. via changes in starch synthesis and turnover) and adjust the growth rate. It could be that the changing conditions did not achieve the threshold required to induce the acclimatory response as, although stomata were starting to close, plant water status was not yet affected. A similar rationale can be used for Eutrema although its perception as a drought avoider (MacLeod *et al.*, 2015), suggests a different soil water availability threshold for acclimation. Transpiration data indicate more conservative water use in Eutrema than Arabidopsis (Fig. 1) although Arabidopsis had greater and stomatal sensitivity to drying soil (Fig. 2). Together, these data suggest species differences in regulating water consumption, implying distinct integration of environmental signals to limit vegetative growth in Eutrema and induce stomatal closure in Arabidopsis

Species-dependent hormonal responses during early stress

ABA, JA, SA and GA profiles clearly discriminated the plant species between Days 1 and 5 (Table 3; Fig. 5 and supplementary Fig. S8). Despite daily irrigation to ensure a similar rate of soil drying in the two species, soil water deficit increased foliar ABA content of Arabidopsis, but not Eutrema, on Day 5 (Fig. 6). Although daily irrigation increases leaf water status and decreases ABA accumulation of plants exposed to reduced soil water availability (Puértolas *et al.*, 2017), significantly higher water consumption of Arabidopsis between Days 3 and 7 (Fig. 1) enhanced foliar ABA accumulation, potentially mediating stomatal closure. Nevertheless, temporal decoupling of foliar ABA accumulation from stomatal closure (Pinheiro *et al.*, 2011; Bechtold *et al.*, 2016) suggests that ABA quantification at the guard cell level (Harris and Outlaw, 1991) is needed to better understand the regulation of stomatal conductance. Alternatively, direct hydraulic regulation of stomatal conductance (that was not detected in RWC measurements), or water-deficit stimulation of localised foliar ABA accumulation (that was not detected in bulk leaf ABA measurements) provide alternative hypotheses for stomatal closure.

Also, increased JA content may stimulate stomatal closure (Daszkowska-Golec and Szarejko, 2013), either independently or in association with ABA accumulation (de Ollas and Dodd,

2016). Foliar JA content transiently increased on Day 3 only in Arabidopsis, preceding increased ABA accumulation on Day 5 (Fig. 6, supplementary Fig. S8). Transient increases in foliar JA accumulation preceded ABA accumulation in *Solanum lycopersicum* leaves (Muñoz-Espinoza *et al.*, 2015) and Arabidopsis (Arbona *et al.*, 2010). Thus, transient JA accumulation may be necessary to induce ABA accumulation thereby contributing to stomatal closure.

Similarly to JA, SA content transiently increased on Day 3 in Arabidopsis (Fig. 6). In Arabidopsis, SA promotes ROS production and accumulation, and activates S-type anion channels in guard cells involved in stomatal closure (Khokon *et al.*, 2017). SA-mediated stomatal closure is considered to be ABA-independent, but a positive cross-talk is also accepted (Rivas-San Vicente and Plasencia, 2011; Miura and Tada, 2014). On the other hand, SA has also been proposed to modulate redox homeostasis, by regulating antioxidant enzymes (Rivas-San Vicente and Plasencia, 2011).

In Eutrema, changes in leaf RWC and ABA occurred after Day 5, with foliar ABA accumulation in Eutrema occurring below 45 % SWC. Species differences could be associated with the osmotic potential (OP) and the redox state regulation, as significant changes were observed in Eutrema, but not in Arabidopsis (Fig. 7 and supplementary Fig. S8). Decreased OP in Eutrema at Day 3 may maintain turgor, thereby removing the stimulus for ABA synthesis (Sack *et al.*, 2018). The opposing trends seen in AscA and DHA for Days 3 and 5 in Eutrema may induce signalling patterns that prevented ABA accumulation. In Arabidopsis, ABA increased at Day 5, but there were no significant changes in AscA or DHA until Day 5. Diminished stomatal closure of Eutrema may be related to its limited metabolic response to soil drying (Fig. 2), since hormonal activation of stomatal closure may require specific metabolic thresholds to be achieved. On the other hand, since Eutrema limits whole plant water consumption earlier than Arabidopsis, its limited metabolic response can be related to slower metabolism, reflecting stress avoidance (Tardieu, 2012). Altered GA metabolism also supports the hypothesis that Arabidopsis responds differently than Eutrema to soil water availability. Two precursors of the bioactive GA4 (GA24, GA9; Fig. 6&7, Table 3) showed altered profiles in Arabidopsis but not in Eutrema; with increased GA24 and GA9 contents at Day 5 indicating GA4 deactivation, a growth inhibitory signal.

Despite similar stomatal response to drying soil, phytohormonal responses substantially differed between the two species. The multiplicity of possible stomatal effectors suggests that

the most parsimonious stomatal regulation is via leaf hydraulics (Tardieu, 2016), yet until Day 5 there was scant evidence that soil drying altered leaf water status, at least in Eutrema. Redundancy in stomatal regulation between closely-related species may confer an evolutionary advantage under specific environmental conditions.

Severe drought and rewatering induce opposite ACC and CK profiles in the two species

With the exception of SA, hormone responses to soil drying are quite distinct in the two species (Table 2). Severe drought increased content of the ethylene precursor ACC by 70 % in Eutrema, but had no effect in Arabidopsis, suggesting ethylene-independent stomatal closure as both species showed similar stomatal sensitivity to drying soil. In contrast, re-watering Eutrema returned ACC levels to well-watered values, while profoundly increasing ACC content in Arabidopsis. Enhanced foliar ethylene emission following re-watering has been attributed to additional root ACC export (Gomez-Cadenas *et al.*, 1996) and may be involved in citrus leaf abscission. Moreover, assuming these increases in foliar ACC content are coincident with enhanced ethylene emission, they may also be involved in growth regulation.

Several CK species including ZR and 2-iP, long distance translocation forms of CKs (Kieber and Schaller, 2014), as well as the 2-iP precursor IPA accumulated in Arabidopsis but not in Eutrema during late stress (Fig. 8; supplementary Fig. S9). In contrast, re-watering returned content of these CKs to well-watered values in Arabidopsis, while stimulating their accumulation in Eutrema. IPA and 2-iP are precursors of Z, one of the most active CK forms (Jameson, 2017). However, the mobilization (metabolism and/or transport) of these CKs in Arabidopsis was not reflected in higher Z levels. Decreased levels of bioactive CKs due to severe and prolonged drought stress have been associated with better performance under drought, in mutants with decreased levels of bioactive CKs achieved via overexpression of *CKX* genes or by inactivating *IPT* genes (Ha *et al.*, 2012). Since these mutant lines show reduced growth under optimal conditions, it can be argued that their water requirements are lower than those of the WT. However, while lower evapotranspiration is described for some *CKX* mutants (Farber *et al.*, 2016), *ipt* mutants show similar water consumption (Nishiyama *et al.*, 2011). On the other hand, senescence-induced *IPT* overexpression, maintained bioactive CK content as the soil dries (Rivero *et al.*, 2007; Xu *et al.*, 2017), without reducing growth (Rivero *et al.*, 2007). Nevertheless, re-watering increased bioactive CKs in these

drought-tolerant transgenics (Rivero *et al.*, 2007). Although differential CK profiles may mediate plant drought stress responses, the two species showed similar stomatal sensitivity to soil drying and re-watering, suggesting CK-independent stomatal regulation.

Conclusions

Arabidopsis and Eutrema responded physiologically (stomatal closure) and metabolically with temporal differences to slowly imposed drought. Following soil drying (Days 1 to 5), Arabidopsis rapidly increased JA and SA content (Day 3) and later increased ABA levels (Day 5), whereas in Eutrema, ABA levels did not change until Day 5. Untargeted metabolite analysis demonstrated larger responses in Arabidopsis than in Eutrema. In contrast, greater soil drying was needed to initiate partial stomatal closure in Eutrema (Day 6), although decreased water use (when compared to controls) was observed earlier than in Arabidopsis. We hypothesise that growth inhibition is a first response to water deficit in Eutrema. With severe and prolonged drought, conserved metabolic responses (increased sucrose and decreased raffinose and citric acid in both species) co-occurred with near-complete stomatal closure in both species. The different physiological and metabolic responses and their timing allow these species to utilise alternative pathways to physiologically adjust to soil drying, likely reflecting adaptations to their respective niches.

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Figure Legends

Figure 1. Soil water content (SWC, %) after imposing water deficit and on re-watering (shaded area). SWC is shown before (symbols) and after (dotted lines) partial water replacement (to regulate the rate of soil water depletion). Data show the means ± standard error of 6 pots (except Day 1 with 5 pots). For pre-irrigation SWC, significance levels were calculated using the Mann–Whitney U test. Data differing significantly from Day 0 are denoted by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Figure 2. Leaf stomatal conductance (as a % of the control plants) plotted against SWC. Mean values (of 3 to 5 biological replicates) are shown with only positive standard errors for clarity. Significant results, as determined by Mann-Whitney U test, are denoted by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). ANCOVA for each main effect (treatment and species) and their interaction is presented in supplementary Fig S4B.

Figure 3. PCA plots showing the scores for the first two principal components obtained for the untargeted metabolomic analysis coloured by experimental group and the day of harvest, for (A) Arabidopsis and (B) Eutrema. For both plant species, the data have been scaled to unit variance and control corrected. In the case of Eutrema only, batch correction has also been performed.

Figure 4. PCA scores plots for the first two principal components obtained from scaled early-stress observations (Days 1, 3 and 5) in the untargeted analysis after control correction for (A) Arabidopsis and (B) Eutrema. The observations are coloured according to the day of harvest, showing that the clustering of observations is related to drought duration.

Figure 5. Dendrogram obtained from hierarchical clustering of the 46 time-series selected by the iterative k-means analysis of the metabolite data. The clusters are coloured and annotated A-I according to the clusters identified in the k-means analysis (supplementary Fig S5). Metabolites within clusters are labelled as follows: S= sucrose; R = raffinose; St = stachyose; CA = citric acid; U = unassigned hexose disaccharide.

Figure 6. Biochemical parameters with a statistically significant change in early drought stress in Arabidopsis but not in Eutrema. The mean difference from well-watered plants for leaf RWC and the hormones ABA, JA, SA and GA24 are shown with error bars representing the standard error. The mean values after control correction (i.e. the mean value for the controls has been subtracted) are represented. Arabidopsis: dark grey; Eutrema: light grey. Significant results are shown in Table 3.

Figure 7. Biochemical parameters with a significant change in early stress in Eutrema but not in Arabidopsis. The mean measurement for osmotic potential, DHA, AscA, 2iP and GA9 are shown with error bars representing the standard error of the observations. The mean values after control correction (i.e. the mean value for the controls has been subtracted) are represented. Arabidopsis: dark grey; Eutrema: light grey. Significant results are shown in Table 3.

Figure 8. Cytokinins during early (Days 1, 3, 5) and late (Day 12) stress and on re-watering (Day 13). Mean values and ± standard error of 6 biological replicates (except for Day 1 where n=5). The mean values after control correction (i.e. the mean value for the controls has been subtracted) are represented. In Arabidopsis, ZR, 2iP and IPA peak at late stress and decrease on re-watering. However, in Eutrema, these hormones show a slight decrease in late stress and increase dramatically on re-watering. Arabidopsis: dark grey; Eutrema: light grey. Significant results are shown in Table 2.

Supplementary table S1. Biochemical parameters for both control (WW) and stressed (WD) observations in Arabidopsis. Data are the means ± standard error of 6 biological replicates, except for day 1 (n = 5). Asterisks in the third row show parameters with a significant difference between WW and WD for a particular day (obtained using Mann-Whitney tests). Asterisks in the final column for each day show days that are significantly different from earlier days (using Tukey's HSD test) with the specific days given in parentheses. Here, asterisks denote *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Supplementary table S2. Biochemical parameters for both control (WW) and stressed (WD) observations in Eutrema. Data are the means ± standard error of 6 biological replicates, except for day 1 (n = 5). Asterisks in the third row show parameters with a significant difference between WW and WD for a particular day (obtained using Mann-Whitney tests). Asterisks in the final column for each day show days that are significantly different from earlier days (using Tukey's HSD test) with the specific days given in parentheses. Here, asterisks denote *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Supplementary table S3. Biochemical parameters for both control (WW) and stressed (WD) observations in Arabidopsis. Samples were control-corrected (see Methods section). Data shown are the means ± standard error of 6 biological replicates, except for day 1 (n = 5).

Supplementary table S4. Biochemical parameters for both control (WW) and stressed (WD) observations in Eutrema. Samples were control corrected (see). Data shown are the means ± standard error of 6 biological replicates, except for day 1 (n = 5).

Supplementary Figure S1. Preliminary drought assay. **A** - Soil water content (SWC, %) progression during the assay for Eutrema and Arabidopsis; **B** - Leaf stomatal conductance (% of the control gs) as a function of the SWC. For controls, percentage gs was calculated relative to day 0; for treatments, percentage gs was calculated relative to the control for the same day. The 80 % gs level was achieved on different days: by day 4 in Arabidopsis and by day 6 in Eutrema; **C** - Regression line fit % gs vs soil water content. Each point represents a single measurement and p-values were determined by ANCOVA for each main effect (treatment and species) and their interaction (ns: not significant; #: $p < 0.1$; ***: $p < 0.001$).

Supplementary Figure S2. PCA plots showing the scores for the first two principal components obtained for the Eutrema data after scaling to unit variance with the observations coloured by batch. **A**- Before batch correction, clustering within batches can be seen and, in particular, batches 7 and 8 cluster separately. **B**- After batch correction, differences between batches are no longer apparent.

Supplementary Figure S3. PCA scores for the first two principal components obtained for the Arabidopsis data after scaling to unit variance. The observations are coloured by data collection batch and no obvious differences between batches can be seen, so that batch correction is not necessary.

Supplementary Figure S4. **A** - Leaf stomatal conductance of Arabidopsis and Eutrema after imposing water deficit and on re-watering (shaded area); **B** - Regression line fitting % gs vs soil water content. Each point represents a single measurement and p-values were determined by ANCOVA for each main effect (treatment and species) and their interaction (ns: not significant; ***: $p < 0.001$).

Supplementary Figure S5. The nine clusters obtained with k-means analysis of the 46 time-series remaining after iterative filtering of the metabolite data. Cluster A and cluster B include several sucrose species. Cluster C includes raffinose and cluster D includes citric acid.

Supplementary Figure S6. Heatmap showing the similarity of the 46 time-series selected by iterative k-means analysis of the metabolite data. Metabolites are labelled as follows: S= sucrose; R = raffinose; St = stachyose; CA = citric acid; U = unassigned hexose disaccharide.

Supplementary Figure S7. PCA plots of the biochemical parameters for both control (WW) and treatment (WD) observations in Arabidopsis and Eutrema after control correction. (A) unscaled variables; (B) scaled variables.

Supplementary Figure S8. Line plots showing physiological and biochemical parameters in early-drought stress (days 1, 3 and 5) after control correction. Error bars show the standard error between observations (n = 6 biological replicates, except for day 1, n = 5). Dark grey: Arabidopsis; light grey: Eutrema. ANOVA results are presented in Table 3.

Supplementary Figure S9. Bar charts showing physiological and biochemical parameters in

early- (days 1, 3 and 5) and late-drought stress and on re-watering (day 13) after control correction. Error bars show the standard error between observations (n = 6 biological replicates, except for day 1, n = 5). Dark grey: Arabidopsis; light grey: Eutrema. ANOVA results are presented in Table 2.

Figure1

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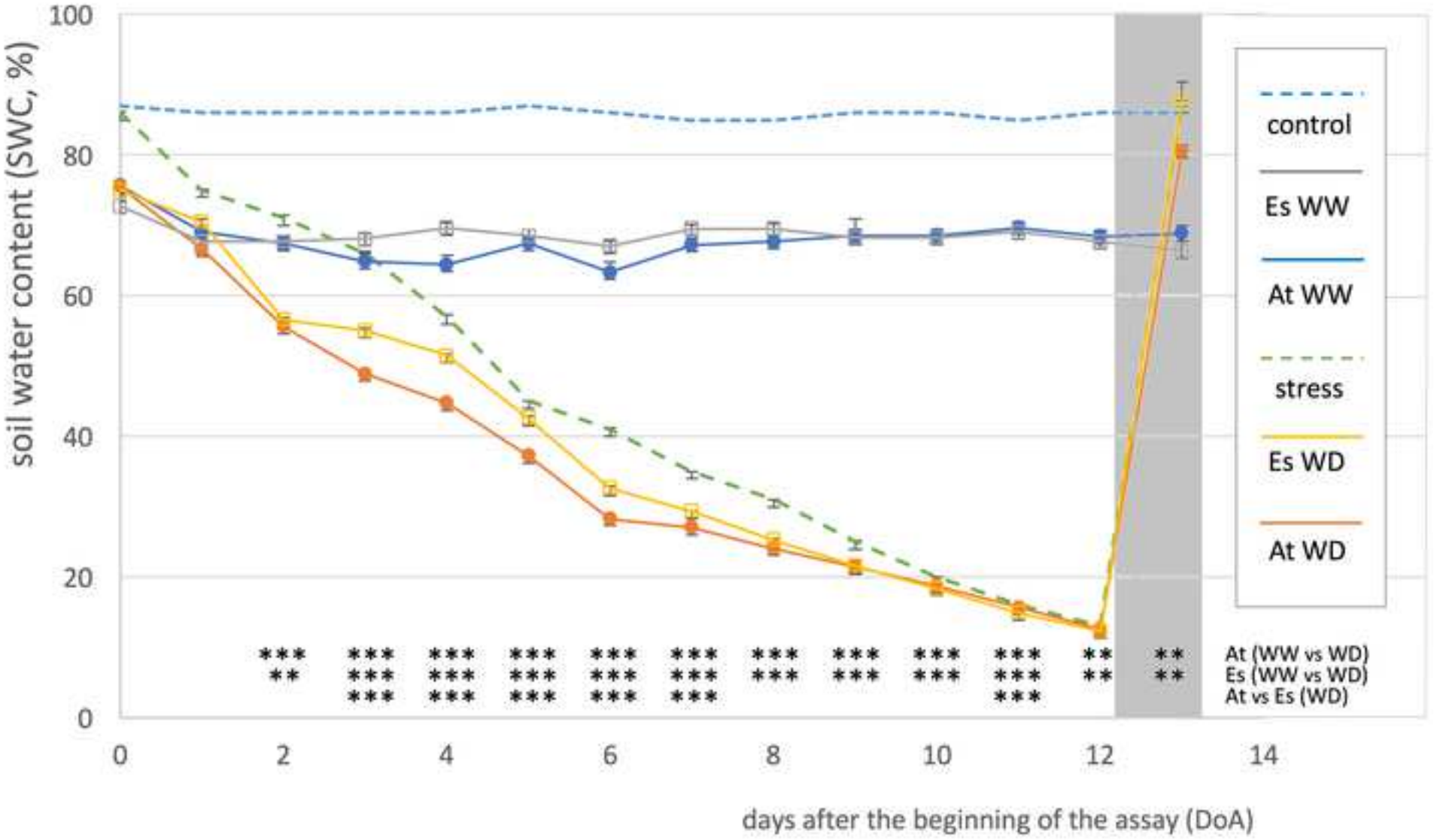

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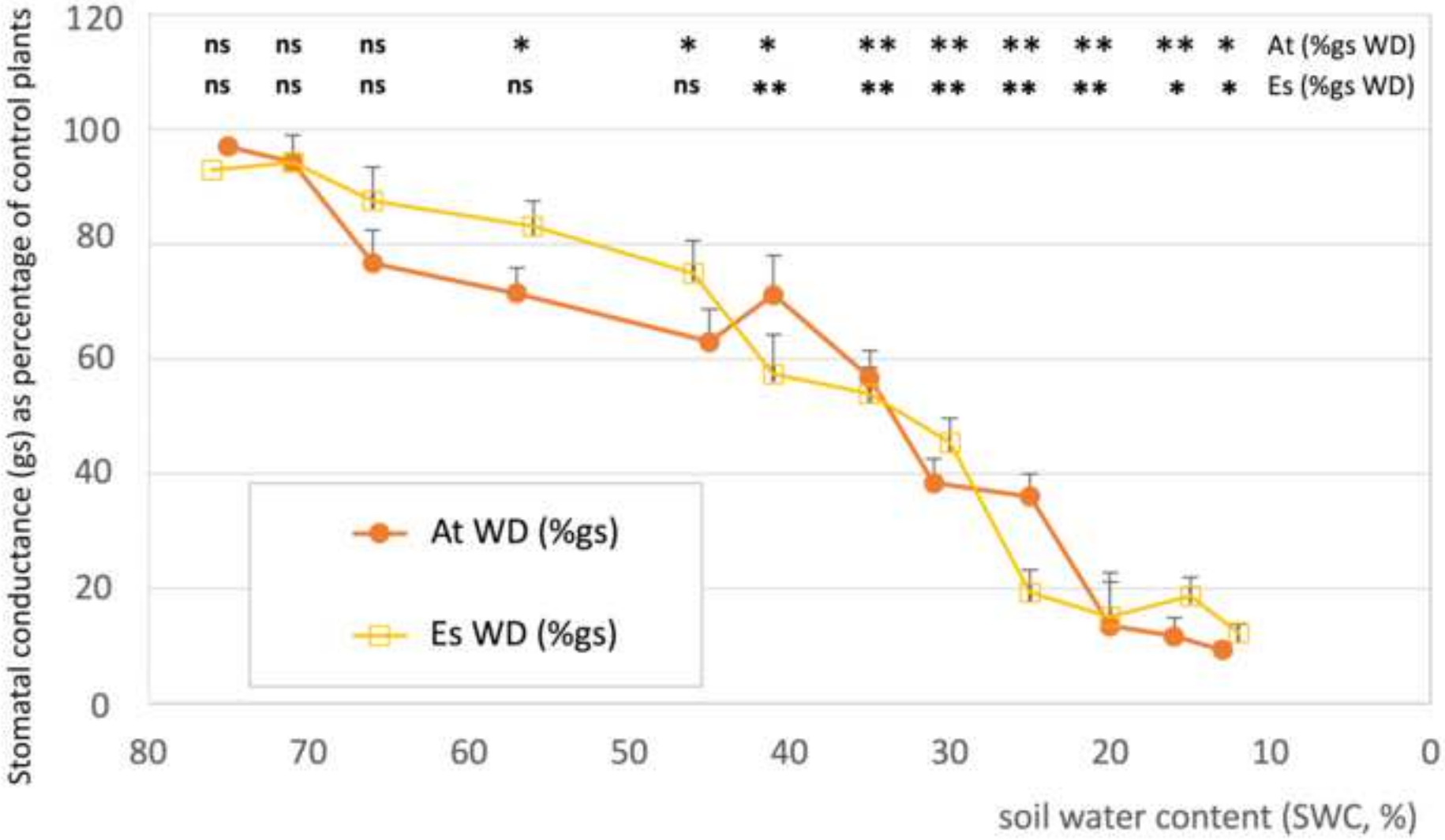

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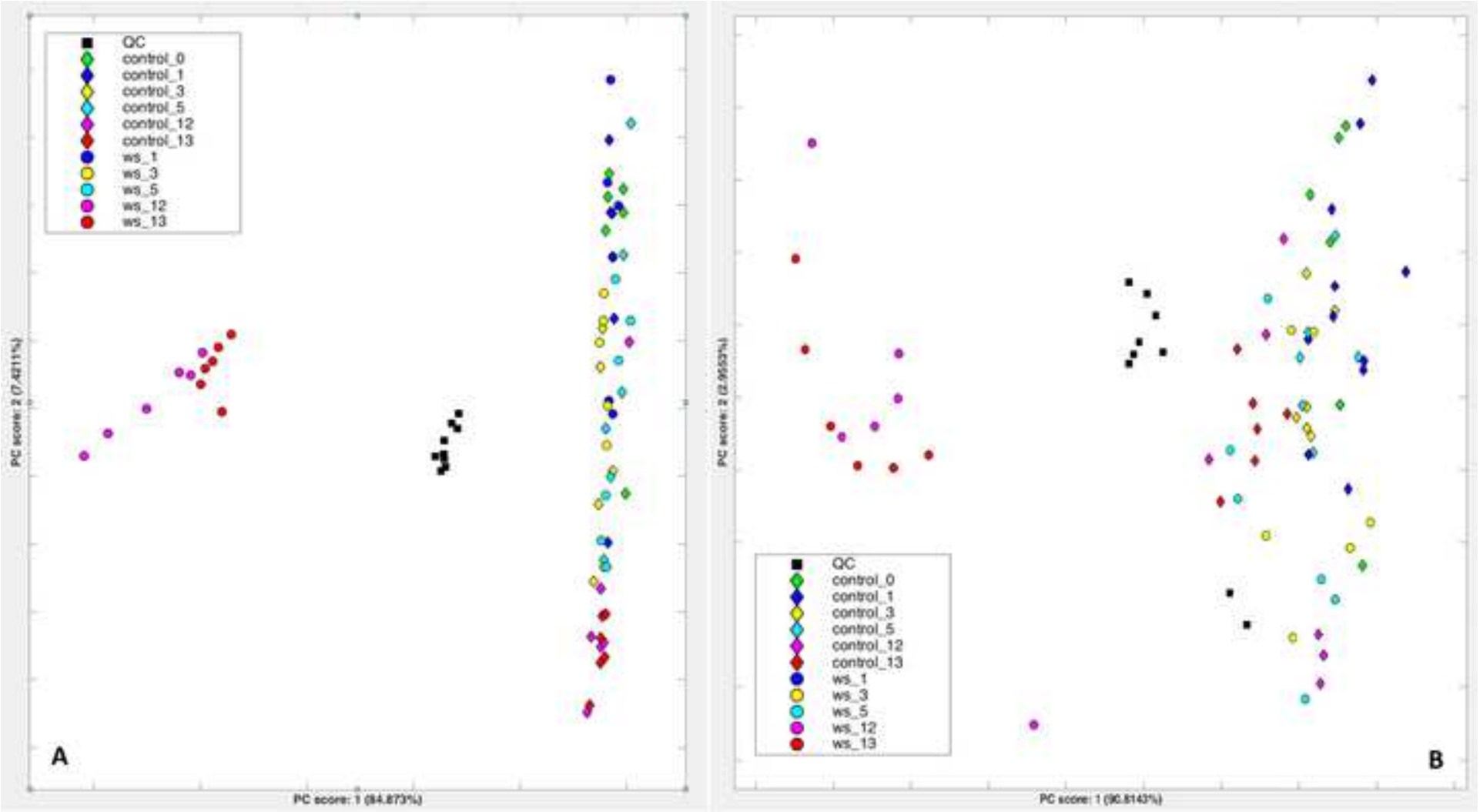

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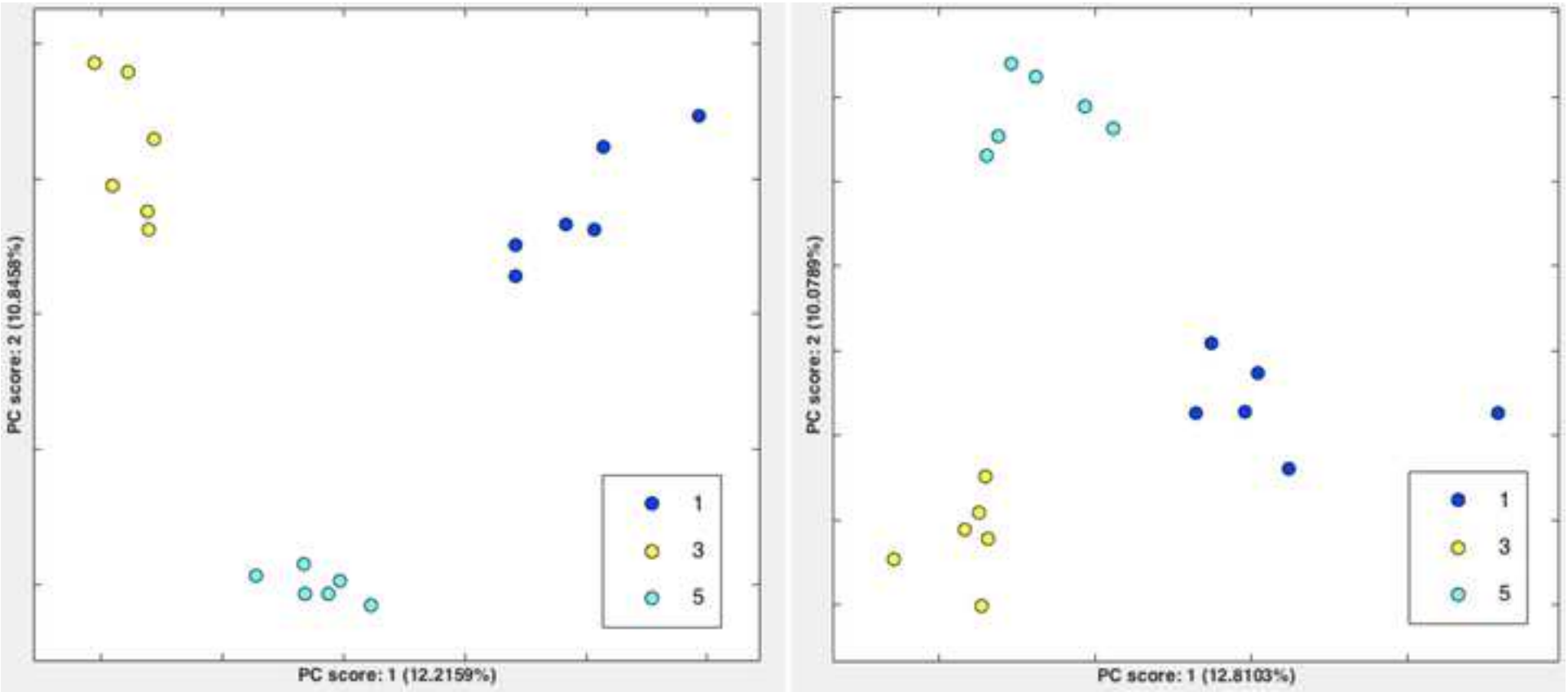

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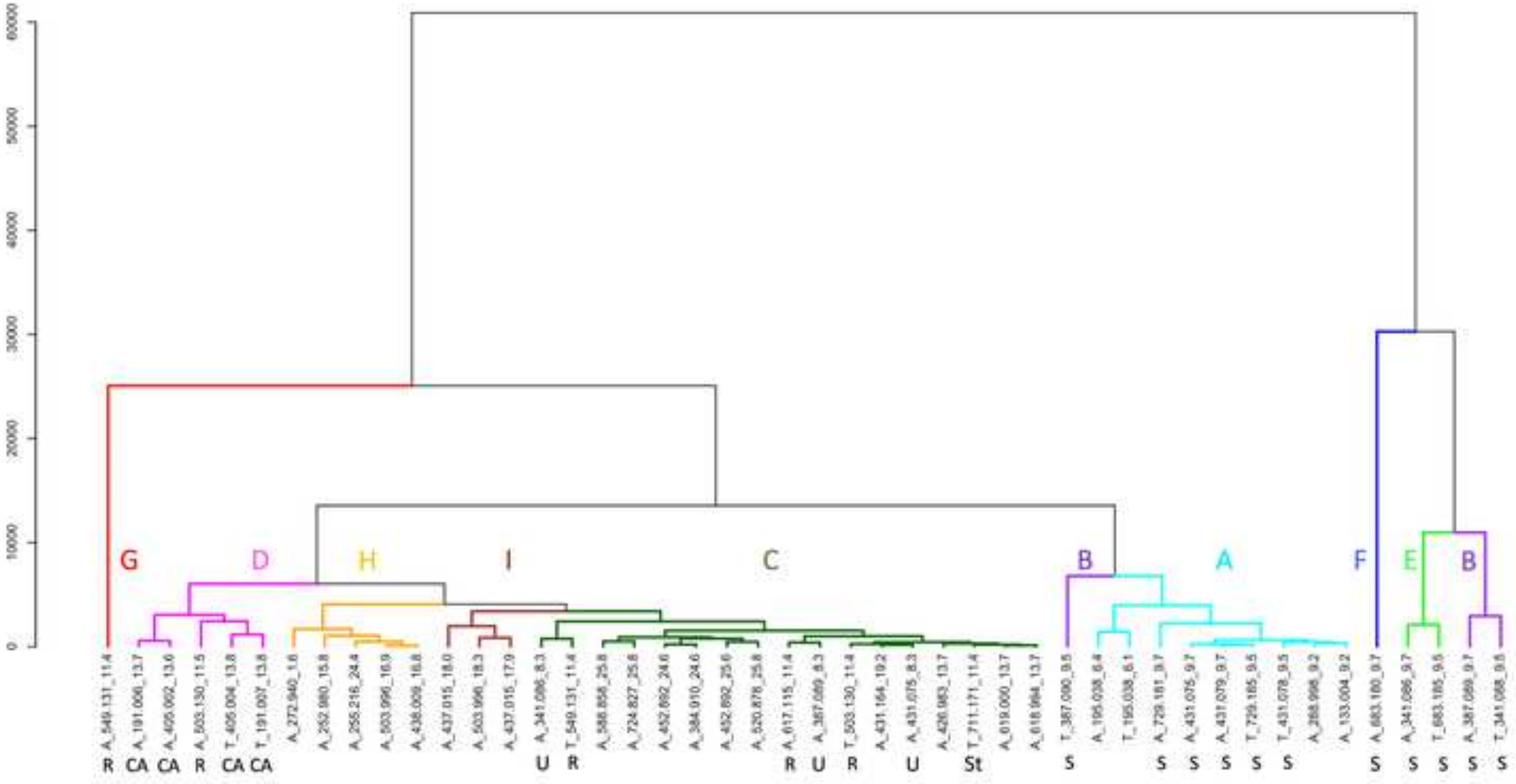

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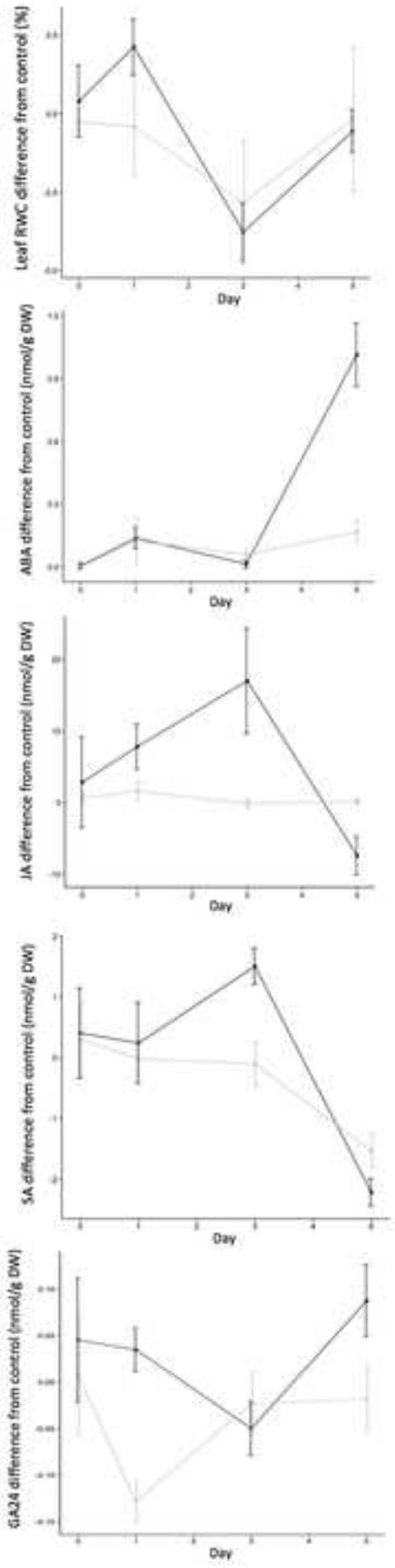

Figure7

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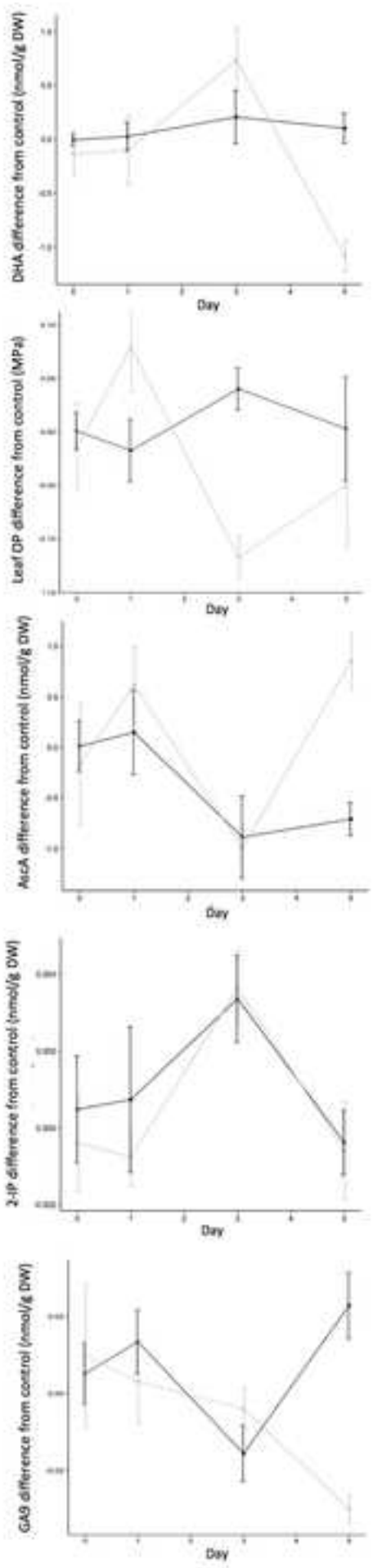

Figure8

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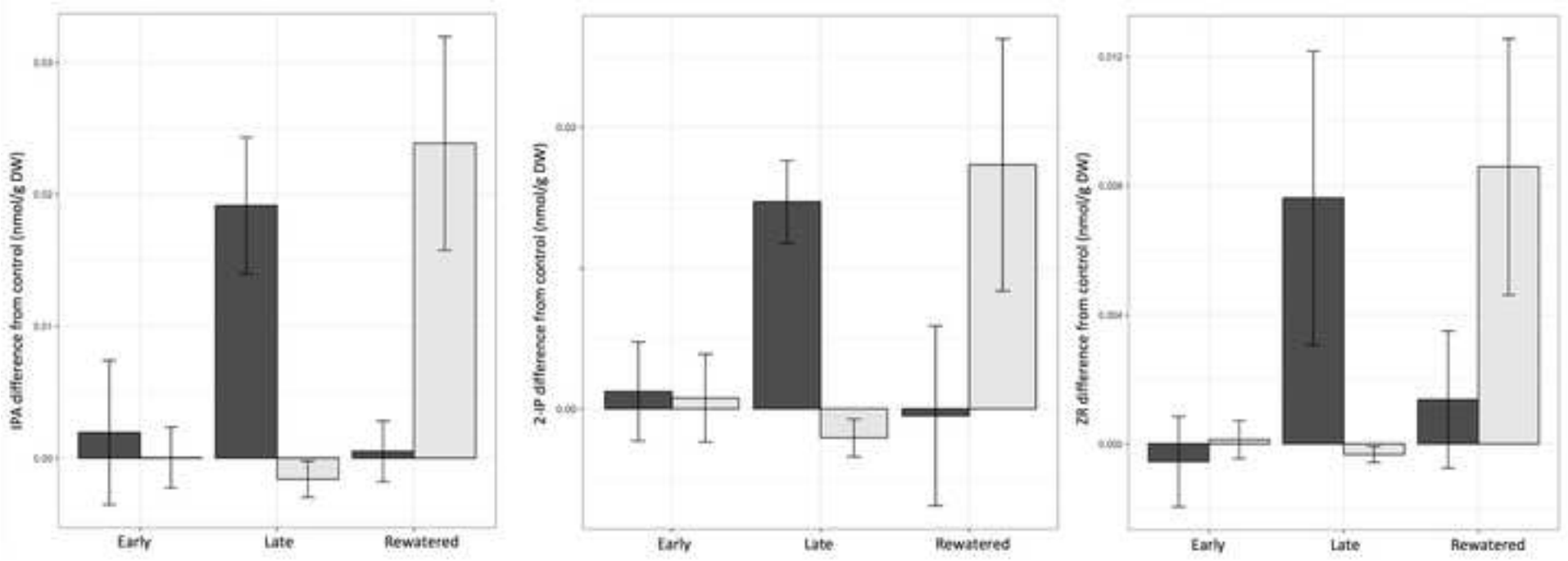

Table 1: Molecular forms assigned to sucrose, raffinose and citric acid that were significantly different ($p < 0.001$) between late drought-stressed and rewatering observations (days 12 or 13) and the corresponding controls.

Compound	**Molecular form**	***m/z***
sucrose	$[M-H]^-$	341.087
sucrose	$[M+HCOO]^-$	387.092
sucrose	$[M-2H+2Na+HCOO]^-$	431.092
sucrose	$[2M-H]^-$	683.181
sucrose	$[2M-HCOO]^-$	729.186
raffinose	$[M-H]^-$	503.126
raffinose	$[M-HCOO]^-$	549.131
raffinose	$[M+HCOONa+HCOO]^-$	617.119
citric acid	$[M-H]^-$	191.006
citric acid	$[2M-2H+Na]^-$	405.002

Table2

Table 2. Representation of ANOVA results after Tukey's HSD correction for pairwise testing between early stress (combined days 1, 3 and 5), late stress (day 12) and re-watered (day 13) observations. Separate ANOVA models were obtained for each species. Light green represents $p < 0.05$, mid-green $p < 0.01$ and dark green $p < 0.001$. White cells indicate no significant difference at the 95 % confidence level. Carotenoids, DHA, IAA, DHZ DHZR, GA19, GA20 and GA24 were measured, but omitted from the table as no significant difference between groups was found for either plant species. Arrows denote an increase (↑) or decrease (↓) in measurement.

Variable	Species	Early - late	Late - rewatered
Biomass	At	↓	
	Th	↓	
Root RWC	At	↓	↑
	Th	↓	↑
Leaf RWC	At	↓	↑
	Th	↓	↑
Starch	At	↓	
	Th	↓	↑
OP	At	↓	
	Th	↓	
OA	At		↓
	Th	↑	↓
OP100	At		↓
	Th	↑	↓
PSII	At	↓	
	Th	↓	↑
Chla	At	↓	↑
	Th	↓	
Chlb	At		
	Th		↓
AscA	At	↓	↓
	Th	↓	
ABA	At		
	Th	↑	
IAA	At		
	Th		
IPA	At	↑	↓
	Th		↑
2-IP	At	↑	↓
	Th		↑
ZR	At	↑	↓
	Th		↑
Z	At		
	Th		↑
DHZ	At		
	Th		
JA	At	↓	
	Th	↓	
SA	At	↓	↓
	Th	↓	↓
ACC	At		
	Th	↑	↓

Table 3. Representation of ANOVA results after Tukey's HSD correction for pairwise testing between early stress observations. Light green represents $p < 0.05$, mid-green $p < 0.01$ and dark green $p < 0.001$. White cells indicate no significant difference at the 95% confidence level. Variables that were measured, but for which no significant difference between groups was found for either plant species were omitted from the table. Arrows denote an increase (↑) or decrease (↓) in measurement. Plots are shown in Fig. 7 and 8 and supplementary Fig. S7.

Variable	Species	Day 1 - day 3	Day 3 - day 5
Leaf RWC	At	↓	↑
	Th		
ABA	At		↑
	Th		
JA	At		↓
	Th		
SA	At		↓
	Th		↓
OP100	At	↑	
	Th		
OP	At		
	Th	↓	
DHA	At		
	Th		↓
AscA	At		
	Th	↓	↑
2-IP	At		
	Th	↑	↓
GA24	At		↑
	Th		
GA9	At	↓	↑
	Th		

Supplementary Material_SF2

Supplementary Material_SF3

Supplementary Material_SF5

Supplementary Material_SF6

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