Decreased irrigation volume, not irrigation placement, promotes accumulation of multiple hormones in cotton leaves during partial rootzone drying

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Highlights:

- Partial rootzone drying (PRD) with adequate irrigation caused stomatal closure
- Stomatal closure coincided with ABA accumulation but not perturbed water status
- Prolonged PRD with limited sap flux from dry soil allowed stomatal re-opening
- cis-OPDA, IAA and SA accumulated with inadequate irrigation, but later than ABA
- PRD with inadequate irrigation caused earlier stomatal closure

Abstract: While ABA is often assumed to mediate partial stomatal closure as the soil dries, other plant hormones and hydraulic signals may also be involved. We tested whether irrigation volume (% of crop evapotranspiration, ET) and placement (partial rootzone drying [PRD] or deficit irrigation [DI], which irrigate part or all of the rootzone respectively) affect this signalling by measuring stomatal conductance (g_s), leaf and shoot water potential (Ψ_{leaf}, Ψ_{shoot}), shoot xylem sap ABA concentration ($[X-ABA]_{shoot}$) and various foliar hormones (ABA, IAA, SA, JA, JA-Ile and cis-OPDA) in cotton plants exposed to different irrigation volumes (100%ET or 50%ET) and placements (DI or PRD). Partial rootzone drying caused stomatal closure coincident with sustained foliar ABA accumulation and minimal changes in Ψ_{shoot}, but continued soil drying of the dry compartment reversed partial stomatal closure (with g_s of 100%ET PRD plants sometimes greater than well-watered plants). With 100%ET PRD, partial stomatal closure correlated with decreased soil moisture of the dry compartment and increased $[ABA]_{leaf}$, but neither Ψ_{leaf} nor $[X-ABA]_{shoot}$. Irrespective of irrigation placement, 50%ET significantly decreased g_s, Ψ_{leaf} and Ψ_{shoot}, but significantly increased $[ABA]_{leaf}$, $[X-ABA]_{shoot}$, $[SA]_{leaf}$, $[IAA]_{leaf}$ and $[cis-OPDA]_{leaf}$, with stomatal closure of 50%ET PRD plants occurring earlier than 50%ET DI plants. While stomatal closure at 50%ET correlated with foliar accumulation of multiple plant hormones, foliar ABA dynamics best explained transient stomatal closure at 100%ET PRD but not stomatal re-opening with prolonged soil drying. Thus, stomatal sensitivity to drying soil (and putative regulatory signals such as ABA) depended on irrigation volume and placement.

Keywords: foliar phytohormones, stomatal conductance, water use efficiency, xylem ABA

1 Introduction

Cotton (*Gossypium hirsutum*) contributes 80% of the global natural fiber production (Townsend, 2020). It is predominantly cultivated in subtropical monsoon and temperate continental climates, to take advantage of both a long growing period and permissive temperatures. However, with a changing climate, increasing temperatures and lack of rainfall mean that water scarcity restricts cotton yields. Thus, cotton cultivation often requires irrigation (Tang et al., 2005; Chapagain & Orr, 2009), with more efficient irrigation strategies needed to optimize crop water use efficiency and mitigate effects of regional water scarcity.

Deficit irrigation (DI) is a practice that applies lower irrigation volumes than crop evapotranspiration requirements to the entire rootzone, in aiming to maintain yields (Kirda et al., 2007; Dodd, 2009) and enhance water use efficiency (WUE) (Kang & Zhang, 2004). In contrast, partial rootzone drying (PRD) utilizes the same irrigation volume applied to only one part of the root system. Allowing the other part of the root system to dry the soil stimulates roots to produce chemical signals that are transported to the shoot, where they reduce vegetative growth, induce stomatal closure (Zhang & Davies, 1990), and restrict luxury transpiration. Typically, the wet and dry sides of the root system are regularly alternated to ensure continued transmission of these signals in the transpiration stream (Stoll et al., 2000; Dodd et al., 2015), and to enhance root growth (Mingo et al., 2004). Both DI and PRD can enhance root-to-shoot chemical signalling to varying degrees (Dodd et al., 2008), causing more immediate physiological responses such as decreased stomatal conductance (Dodd, 2009; Dodd et al., 2015) and longer-term morphological responses such as decreased leaf area (Santos et al., 2003; Melgar et al., 2010) and increased root-to-shoot ratio (Mingo et al., 2004).

When compared to fully irrigated field-grown cotton plants, PRD plants receiving 30% less irrigation (70%PRD) maintained leaf water potential, decreased stomatal conductance and restricted leaf area, plant height and yield (by no more than 16%), but higher quality fibers were harvested earlier with alternate PRD (Tang et al., 2005). Although 50%PRD decreased stomatal conductance thereby enhancing WUE of pot-grown cotton plants compared to full irrigation, cotton yield and quality (fiber strength, length and fineness) also decreased (Iqbal et al., 2021), perhaps because there were limited benefits of enhanced root foraging within a finite soil volume. With sufficient irrigation, not alternating the wet and dry sides of the root system allowed PRD plants to maintain whole plant gas exchange at similar levels to fully irrigated plants (Puértolas & Dodd, 2022). These contrasting agronomic and physiological responses suggests that more work is required to understand how PRD alters root to shoot signalling.

Most investigations of PRD effects on such signalling have focused on xylem-borne abscisic

acid ($[ABA]_{xylem}$) as the main regulator of stomatal closure when plants are exposed to progressive soil drying (Zhang & Davies, 1990). DI and PRD can differentially affect $[ABA]_{xylem}$ (Dodd, 2007; Wang et al., 2012) and $[ABA]_{leaf}$ (Iqbal et al., 2021) according to total soil water availability, with alternation of wet and dry sides of the root system enhancing $[ABA]_{xylem}$ in some (Dodd et al., 2006; Topcu et al., 2007) but not all (Pérez-Pérez & Dodd, 2015; Pérez-Pérez et al., 2020) studies. During PRD, drying soil stimulates root ABA accumulation in the dry side of the root system (Stoll et al., 2000; Pérez-Pérez et al., 2018), but continued soil drying progressively restricts sap flow from these roots which diminishes root-to-shoot ABA signalling (Dodd et al., 2008). The relative soil moisture thresholds of ABA accumulation within, and export of ABA from, those roots determine the dynamics of $[ABA]_{xylem}$ during PRD (Dodd et al., 2008). During alternate and fixed PRD with irrigation volumes less than transpirational requirements, PRD has variable effects on $[ABA]_{xylem}$ according to when xylem sap is collected, yet typically induces stomatal closure.

This raises the possibility that other plant hormones such as indole acetic acid (IAA), salicylic acid (SA) and jasmonic acid (JA) are involved in regulating stomatal closure during PRD, either by directly affecting stomatal behavior or by interacting with ABA (de Ollas & Dodd, 2016; Tan et al., 2017). While IAA can promote stomatal opening and antagonize ABA-induced stomatal closure (Snaith & Mansfield, 1982; Salehin et al., 2019), SA and JA are considered antitranspirants (Hao et al., 2011; Luo et al., 2019). Both DI and PRD decreased tobacco (*Nicotinia tabacum*) leaf IAA levels by 16%, coincident with stomatal closure (Liu et al., 2022). Although PRD initially decreased foliar IAA levels of apple (*Malus x domestica*) trees by 23-38% compared to full irrigation, it caused higher IAA levels later in the season as shoot growth slowed (Ghafari et al., 2020). PRD irrigation also increased leaf ABA and SA concentrations by 40-90%, which coincident with stomatal closure (Ghafari et al., 2020). A tomato mutant with compromised drought-induced accumulation of jasmonates also accumulated less foliar ABA and had lower stomatal sensitivity to drying soil (de Ollas et al., 2018), but ultimately showed similar stomatal closure as wild-type plants likely due to considerable accumulation of the potent antitranspirant and JA precursor cis-(+)-oxo-phytodienoic acid concentration (cis-OPDA) (Savchenko et al., 2014). Quantifying various hormones in relation to soil moisture and leaf water potential during PRD seems necessary to better understand the mechanisms of stomatal regulation in response to soil moisture heterogeneity.

To understand how PRD might affect long-distance signalling of soil water deficit in cotton, several experiments were conducted. Initially, to establish how soil water deficit affects cotton physiological responses, water was withheld from pot-grown plants to measure the dynamics

of ABA accumulation and shoot water status. Then plants were grown in split-pots to impose PRD to understand the dynamics of stomatal responses and ABA accumulation in fully irrigated plants that were watered on both (controls) or only one (PRD) sides of the pot during a drying and re-wetting cycle. Lastly, a factorial experiment varied irrigation volume (full and deficit irrigation) and placement (watering both or only one side of the pot) while measuring foliar accumulation of a range of plant hormones (ABA, IAA, SA, JA, JA-Ile, and cis-OPDA) to determine their possible impacts on stomatal regulation. We hypothesized that 1): soil moisture dynamics regulate shoot xylem ABA concentration to mediate stomatal responses to PRD; 2): other plant hormones sustain stomatal closure should ABA concentration diminish to the levels of well-watered plants.

2. Materials and methods

2.1 Plant material and experimental setup

Three experiments were performed sequentially in a climate-controlled greenhouse at Lancaster Environment Centre, with supplementary high-pressure sodium (HPS) lamps providing 400-600 µmol photons $m^{-2}s^{-1}$ at 1.5 m above pot height during a 13 h photoperiod (between 7:00-20:00). Set points for day-night temperatures were 25/20 °C and relative humidity was 50%, with actual data reported in Supplementary Fig. 1. All experiments used a fertilized organic loam from the same manufacturer (John Innes No.2; J. Arthur Bowers, UK), although different batches of the same substrate were used, with soil water holding capacity of 52% in Experiment 1 and 45% in Experiments 2 and 3, respectively. Cotton seeds (*Gossypium hirsutum L cv.* Shaan No. 518) were soaked in distilled water for 5-6 h and then pre-germinated on damp filter paper in petri dishes. The dishes were placed in a dark environment at room temperature (24°C ± 5%) for 12 h.

Experiment 1

(July-September 2021)

Pre-germinated cotton seeds were sown into 84 cylindrical 2 L pots (17 cm in height and 12.5 cm in diameter) filled with 1.2 kg of organic loam. To minimize soil evaporation, a 1 cm layer of perlite was applied to cover the soil surface at 26 days after sowing. All pots were well-watered every two days by replacing evapotranspiration (100%ET) during the first 33 days. Four plants were harvested at 33 days and the treatments initiated at 34 days after sowing, respectively. Half of the seedlings were irrigated daily (between 8:00-8:40) with 100%ET as well-watered plants (W1), and water withheld from the remainder (D) to ensure progressive soil drying for 10 days until stomatal conductance (g_s) declined to 10% of the well-watered plants. Treatments were randomly arranged within the greenhouse and rotated daily to minimize

the effects of environmental gradients on plant growth. Four plants of each treatment were harvested daily.

Experiment 2

(October-December 2021)

Pre-germinated cotton seeds were sown individually into 50 mL Arabidopsis net pots (TekuTM, Pöppelmann Plastiques, Pöppelmann, France) of 50 mm diameter × 50 mm deep with 5 mm × 7 mm pores in the side, each filled with 0.1 kg organic loam. At two-leaf stage, the net pots were placed into 95 cylindrical 2 L pots (17 cm in height and 12.5 cm in diameter) that were evenly divided into two vertical compartments by a plastic divider, which was glued to the pot walls to prevent water exchange between the two compartments. The top of each pot had a 50 mm gap in the plastic divider to allow each "net pot" to be inserted while minimizing seedling disturbance. To minimize soil evaporation, a 1 cm layer of perlite was applied to cover the soil surface 10 days after transplanting. All pots were well-watered every 2 days (100%ET) during first 18 days after transplanting, with irrigation evenly applied to both sides of the pot. Five plants were harvested the day before imposing two treatments (each comprising 45 plants) at 18 days after transplanting, with plants receiving either 100%ET applied evenly to two compartments (W2), and partial rootzone drying (PRD) with 100%ET applied to one compartment and water withheld from the other compartment to allow soil drying. Irrigation occurred between 7:30-10:00. The irrigated and drying sides of PRD plants were alternated 23 days after the treatments were established. Five plants of each treatment were harvested 5, 7, 8, 12, 13, 14, 21, 27 and 28 days after imposing the irrigation treatments, respectively.

Experiment 3

(February-May 2022)

Plants were prepared as in Experiment 2, with four irrigation treatments (each comprising 20 plants) applied (between 7:30-10:00) 45 days after transplanting:
- well-watered plants received 100%ET evenly to two compartments (W3-100%ET)
- partial rootzone drying supplied 100%ET to one compartment with the other allowed to dry the soil (PRD3-100%ET)
- deficit irrigation supplied 50%ET evenly to two compartments (W3-50%ET)
- partial rootzone drying supplied 50%ET to one compartment with other allowed to dry the soil (PRD3-50%ET)

After 5 plants were harvested immediately before imposing the treatments, five plants of each treatment were harvested 49, 52, 58 and 59 days after transplanting.

2.2 Soil water content determination

177   An electronic balance measured pot weight daily between 8:00-8:40, 7:30-10:00, and 7:00-
178   10:00 in Experiments 1, 2 and 3, respectively, before and after irrigation had been applied,
179   allowing evapotranspiration to be calculated. Simultaneously, volumetric soil water content
180   (SWC_{vol}) of the upper 6.5 cm of each pot in Experiment 1, and each soil compartment in
181   Experiments 2 and 3, was measured using a theta probe (Delta-T Devices, UK) before and after
182   irrigation, respectively. The decline in soil water content with soil drying over consecutive days
183   was used to calculate the fraction of sap flow from roots in dry soil (Pérez-Pérez & Dodd, 2015).

184 **2.3 Plant water relations measurements**

185   Stomatal conductance (g_s) was determined daily after irrigation between 9:00-10:00, 10:00-
186   11:30 and 10:00-11:30 in Experiments 1, 2 and 3, respectively, with a transient time porometer
187   (Model AP4, Delta-T Devices, UK) on the youngest fully expanded leaf. On selected days, leaf
188   water potential (Ψ_{leaf}) of the same leaf was measured using a Scholander type pressure chamber
189   (Model 3000; Soil Moisture Equipment. Corp., California, USA). After measuring Ψ_{leaf}, the leaf
190   was placed into a 1.5 mL centrifuge tube, frozen in liquid nitrogen, then placed at -80°C to
191   subsequently measure leaf ABA concentration. Then, the entire shoot was severed below the
192   cotyledons (about 1 cm from the soil surface), and placed in a pressure chamber to measure
193   shoot water potential (Ψ_{shoot}). Shoot water potential was correlated (R^2=0.75, 0.13 and 0.77 in
194   Experiments 1, 2 and 3, respectively) with leaf water potential measured in the same plants
195   (Supplementary Fig. 2). Additional pressure ($\approx$0.5 MPa) was applied after measuring Ψ_{shoot} to
196   collect xylem sap, which was stored at -80 °C to subsequently measure xylem sap ABA
197   concentration.

198 **2.4 ABA quantification**

199   Leaf samples were freeze-dried for 48h, finely ground and weighed, then extracted with
200   deionized, distilled water in the ratio of 1:50 (sample: water) in a 1.5 mL tube for 12 h at 4 °C.
201   After centrifuging the extract, the supernatant was used to measure leaf ABA concentration
202   ($[ABA]_{leaf}$), while xylem sap was used directly to measure shoot xylem sap ABA concentration
203   ($[X-ABA]_{shoot}$), using a radioimmunoassay (Quarrie et al., 1988) with the monoclonal antibody
204   AFRC MAC 252 (provided by Dr. Geoff Butcher, Babraham Institute, Cambridge, UK). Xylem
205   ABA concentrations measured with this immunological technique were correlated (R^2=0.67,
206   0.14 and 0.47 in Experiments 1, 2 and 3, respectively) with leaf ABA concentrations measured
207   in the same plants with the same technique (Supplementary Fig. 3). Leaf ABA concentrations
208   measured with this immunological technique were highly correlated (R^2=0.81) with those
209   determined by the physico-chemical technique described in Section 2.5 (Supplementary Fig. 4)
210   in Experiment 3.

2.5 Foliar phytohormones extraction and quantification

Experiment 3 collected the youngest fully expanded leaves (about 250 mg fresh weight). Leaf samples were freeze-dried for 48h, finely ground and approximately 30 mg was weighed into 2.0 mL Sarstedt vials. The sample was extracted with 1.5 mL methanol containing 60 ng D_6-abscisic acid (Toronto Research Chemicals, Toronto, Canada), 60 ng of D_6-jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany), 60 ng D_4-salicylic acid (Santa Cruz Biotechnology, Santa Cruz, U.S.A) and 12 ng D_6-jasmonic acid-isoleucine conjugate (HPC standards GmbH, Cunnersdorf, Germany) and 60 ng of D_5-IAA (OlChemIm s.r.o., Olomouc, Czech Republic) as an internal standards (Dávila-Lara et al., 2021). Foliar ABA, SA, JA, JA-Ile and cis-OPDA analyses were conducted by LC-MS/MS as previously described (Heyer et al., 2018) on an Agilent 1260 series HPLC system (Agilent Technologies, Böblingen, Germany) with the modification that a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany) was used. Foliar IAA was quantified using the LC-MS/MS system with the same chromatographic conditions but using positive mode ionization with an ion spray voltage at 5500 eV. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion to product ion fragmentations as follows: m/z 176 ->130 (collision energy (CE) 19 V; declustering potential (DP) 31 V) for IAA; m/z 181 ->133+ m/z 181 ->134+m/z 181 ->135 (CE19V; DP 31V) for D5-IAA.

2.6 Plant growth and water use efficiency

At each harvest, plant height (H) and leaf area (LA) was determined with a ruler and portable leaf area meter (Model LI-3100, LI-COR, USA), respectively. Shoot biomass (DM_{shoot}) was measured after drying at 75°C in an oven to constant weight. Evapotranspiration (ET) was calculated from initiating the irrigation treatments (using gravimetric measurements). Water use efficiency (WUE) was calculated as the ratio of DM_{shoot} to ET.

2.7 Statistical analysis

One-way ANOVA determined the effects of withholding water and partial rootzone drying in Experiments 1 and 2, respectively. Two-way ANOVA determined the effects of irrigation volume and partial rootzone irrigation in Experiment 3, using SPSS version 23.0 (IBM, Corporation, New York, USA), to discriminate significant ($P < 0.05$) differences. Relationships between plant and soil variables were fitted by linear regression.

3. Results

3.1 Soil water content

In Experiment 1 (Fig. 1A), volumetric soil water content (SWC_{vol}) at the soil surface of well-watered plants (W1), prior to daily irrigation, was sustained at 35% throughout the experiment,

and steadily declined with progressive soil drying to about 13% at the end of the experiment (Fig. 1A). In Experiment 2 (Fig. 1B) with a different batch of substrate, average volumetric soil water content (SWC_{vol}) of the two soil compartments of well-watered plants (W2) was maintained about 26%. In the plants exposed to PRD, SWC_{vol} of the irrigated compartment slowly increased to 41% while SWC_{vol} of the dry compartment decreased to 9%. At this time (23 days after PRD application), the irrigated and drying compartments alternated, with differential irrigation returning SWC to comparable values after a week. In Experiment 3 (Fig. 1C), the well-watered plants (W3-100%ET) maintained an average SWC_{vol} of around 25% throughout the experiment, with the deficit irrigated plants (W3-50%ET) progressively drying to reach an average SWC_{vol} of 4% at the end of the experiment. In the plants exposed to PRD (PRD3-100%ET), SWC_{vol} of the irrigated compartment slowly increased to 37% while SWC_{vol} of the dry compartment decreased to 15%. In contrast, smaller irrigation volumes applied to PRD plants (PRD3-50%ET) resulted in SWC_{vol} of the irrigated compartment slowly decreasing to 8% while SWC_{vol} of the dry compartment decreased to 2%. Thus, PRD ensured differential soil moisture between the two soil compartments, but overall soil moisture declined if less irrigation was applied.

3.2 Stomatal conductance, and sap flow from drying roots

Whereas stomatal conductance (g_s) of well-watered plants averaged 0.39 mol m^{-2}s^{-1} throughout Experiment 1, progressive soil drying decreased g_s by 17% within 3 days of withholding water and by 81% at the end of the experiment (Fig. 2A). Stomatal closure in response to partial rootzone drying (PRD) was delayed (occurring 8 days after treatment application) and limited (averaging 19% between Days 8 to 14, with a maximal inhibition of 30% on Day 12) in Experiment 2 (Fig. 2B). This stomatal closure occurred when SWC_{vol} of the dry compartment decreased to between 19 and 14% (Fig. 1B). However, further soil drying resulted in no difference between well-watered plants (W2) and PRD treatments between Days 15 to 20, although g_s of W2 plants had decreased by 23% compared to values at the beginning of the experiment. Correspondingly, the fraction of sap flow from drying roots (F_{dry}) decreased to < 10% from Days 15 to 20 (Fig. 3A). Meanwhile, stomatal conductance of 100% PRD plants decreased with SWC_{vol} of the dry compartment declining to 15% but increased with further soil drying (Fig. 3B). Since g_s of PRD plants exceeded that of well-watered plants on Days 21 to 23 by 16% (Fig. 2B), the wet and dry compartments were alternated. Additional stomatal closure occurred on Days 25 and 26 coincident with SWC_{vol} of the dry compartment decreasing to between 10 and 15% (Fig. 3B). Almost complete cessation of sap flow from the dry side of the root system (Days 27 to 29) with SWC_{vol} declining to *circa* 10% (Fig. 3A) allowed stomata of

PRD plants to re-open again with their g_s exceeding that of 100% well-watered plants (Fig. 3B). Thus, PRD plants only showed stomatal closure when SWC_{vol} of the dry compartment was within a certain soil moisture range, as limited or excessive soil drying of this compartment resulted in a similar g_s of PRD and well-watered plants.

PRD plants receiving 100%ET in Experiment 3 showed comparable stomatal and sap flow changes as Experiment 2, with g_s 15% lower than well-watered plants (Fig. 2C), when SWC_{vol} of the dry compartment decreased to $\leq 20\%$ between Days 6 to 9 while F_{dry} decreased to $< 10\%$ (Fig. 3A). While g_s decreased with soil drying during this period, further soil drying tended to re-open the stomata (Fig. 3B), although g_s did not significantly differ between the W3 and PRD3-100%ET treatments at the end of the experiment (Fig. 2C). When plants were irrigated with 50%ET, PRD plants had lower g_s by 26% than deficit irrigated plants between Days 3 to 8, however, these differences became non-significant thereafter. At the end of the experiment, g_s of 50%ET plants was only 10% of well-watered plants. Thus, lower irrigation volumes significantly decreased g_s compared to well-watered plants, but earlier in PRD plants (from Day 3) than deficit irrigated plants (from Day 6) causing significant effects of irrigation placement (Table 1).

3.3 Plant water relations

Progressive soil drying gradually decreased leaf water potential (Ψ_{leaf}) and shoot water potential (Ψ_{shoot}) in Experiment 1 (Fig. 4A, B) by 0.05 and 0.15 MPa within 3 and 4 days respectively of withholding water, and by 0.56-0.58 MPa at the end of the experiment. However, partial rootzone drying (PRD) only occasionally (Day 12 for Ψ_{leaf}, Days 7 and 8 for Ψ_{shoot}) decreased plant water status (by 0.10-0.14 MPa) compared to well-watered plants in Experiment 2 (Fig. 4C, D). Similarly, in plants receiving 100%ET, PRD had no effect on Ψ_{leaf} and Ψ_{shoot} in Experiment 3 (Fig. 4E, F; Table 3). Compared to well-watered (100%ET) plants, 50%ET decreased Ψ_{leaf} by 0.84-1.42 MPa on Days 14 and 15, respectively. Shoot water potential (Ψ_{shoot}) of 50%ET plants decreased much earlier on Day 5 by 0.18 MPa and by 1.38 MPa on Day 15 (Fig. 4F; Table 3). Early in Experiment 3, DI and PRD plants had similar water status but Ψ_{leaf} and Ψ_{shoot} of PRD plants was higher than deficit irrigated plants on Day 14, although the opposite effect occurred for Ψ_{shoot} on Day 15. When 100%ET was supplied, PRD generally maintained leaf water status but it declined with less irrigation volume.

3.4 Endogenous hormone levels

Progressive soil drying gradually increased leaf ABA concentration ($[ABA]_{leaf}$) and shoot xylem sap ABA concentration ($[X-ABA]_{shoot}$) in Experiment 1. $[ABA]_{leaf}$ significantly increased by 15% and 35% within 3 and 5 days of withholding water respectively (Fig. 5A),

while $[\text{X-ABA}]_{\text{shoot}}$ increased by 64% within 4 days of withholding water (Fig. 5B). At the end of the experiment, $[\text{ABA}]_{\text{leaf}}$ and $[\text{X-ABA}]_{\text{shoot}}$ had increased by 2-fold and 6-fold respectively. Seven and 12 days of partial rootzone drying at 100%ET significantly increased $[\text{ABA}]_{\text{leaf}}$ by 37% and 56% respectively in Experiment 2 (Fig. 5C), but had no significant effect on $[\text{X-ABA}]_{\text{shoot}}$ (Fig. 5D). Although PRD did not alter $[\text{X-ABA}]_{\text{shoot}}$ in Experiment 2 (Fig. 5D), it significantly increased $[\text{X-ABA}]_{\text{shoot}}$ by 29% at 8 days in Experiment 3 (Fig. 5F). Compared to well-watered (100%ET) plants, PRD at 50%ET significantly increased $[\text{ABA}]_{\text{leaf}}$ by 37% at 8 days in Experiment 3 (Fig. 5E), but values were similar to deficit irrigated plants at this and subsequent days. Both 50%ET treatments increased $[\text{ABA}]_{\text{leaf}}$ by 64% at the end of the experiment. Although $[\text{X-ABA}]_{\text{shoot}}$ of 50%ET PRD plants was 12% higher than 50%ET DI plants on Day 5, $[\text{X-ABA}]_{\text{shoot}}$ of both treatments was similar throughout the remainder of Experiment 3, significantly increasing by 63% after 5 days (Fig. 5F) and by 3-fold at the end of the experiment. Although withholding water or 50%ET irrigation volume significantly enhanced ABA status, 100%ET PRD caused foliar ABA accumulation on some occasions.

Although decreased irrigation volume did not affect concentrations of jasmonates (JA, JA-Ile and OH-JA-Ile) in the leaves (Supplementary Table 2, 3), it increased leaf salicylic acid concentration ($[\text{SA}]_{\text{leaf}}$), leaf indole acetic acid concentration ($[\text{IAA}]_{\text{leaf}}$) and leaf cis-(+)-oxo-phytodienoic acid concentration ($[\text{cis-OPDA}]_{\text{leaf}}$) on Days 14 and 15 of Experiment 3 (Table 2, 3). Maximal SA, IAA and cis-OPDA accumulation was 1.3-, 3.4- and 1.7-fold higher than well-watered plants at the end of the experiment. Except for Day 2 when PRD plants had 29% higher $[\text{IAA}]_{\text{leaf}}$ than well-watered plants (Table 2, 3), irrigation placement (PRD) did not affect foliar hormone levels.

3.5 Stomatal regulation

Soil drying decreased stomatal conductance (g_s) in all 3 experiments, with average SWC_{vol} (across both soil compartments in split-root plants) accounting for 70% and 60% of variation in g_s in Experiments 1 (Supplementary Fig. 5A) and 3 (Fig. 6A), respectively. In contrast, average SWC_{vol} explained relatively little of the variation (2%) in g_s in Experiment 2 when PRD was applied at 100% ET (Supplementary Fig. 5E). However, SWC_{vol} of the dry soil compartment was significantly correlated with g_s during the early (but not late) stages of each drying cycle in Experiments 2 and 3 when PRD was applied at 100%ET (Supplementary Fig. 6B, D). The fraction of sap flow from the dry side of the root system declined as SWC_{vol} of the dry soil compartment decreased in both experiments (Fig. 3A), such that g_s of 100%ET PRD plants initially declined, but later increased to values exceeding 100%ET WW plants (Fig. 3B). Stomatal conductance declined with decreased leaf water potential in Experiments 1 and 3

(Supplementary Fig. 5B, H) although these variables were not related in Experiment 2 (Supplementary Fig. 5F). Although substantial soil drying caused stomatal closure by decreasing soil and leaf water status, g_s of 100%ET PRD plants was best explained by considering the dynamics of water uptake from the dry soil compartment.

Additionally, g_s decreased linearly with increased $[ABA]_{leaf}$ in all Experiments (Fig. 6B, C; Supplementary Fig. 5C). Although g_s declined linearly with $[X-ABA]_{shoot}$ in Experiments 1 and 3 (Supplementary Fig. 5D, I), g_s and $[X-ABA]_{shoot}$ weren't related in Experiment 2 (Supplementary Fig. 5G). In Experiment 3, g_s declined linearly as $[SA]_{leaf}$, $[IAA]_{leaf}$ and $[cis-OPDA]_{leaf}$ increased (Fig. 7A, B, C), accounting for 14, 45 and 23% of the variation in g_s respectively. While leaf water status and multiple phytohormones strongly correlated with stomatal closure in Experiment 3, g_s was only weakly correlated with soil moisture and foliar ABA concentration in Experiment 2 when all plants received 100% ET.

3.6 Phytohormone regulation

$[ABA]_{leaf}$ increased linearly as SWC_{vol} and Ψ_{leaf} declined in Experiments 1 and 3 (Fig. 8A, B, E, F), while variation in SWC_{vol} and Ψ_{leaf} could not explain changes in $[ABA]_{leaf}$ of 100%ET PRD plants in Experiment 2 (Fig. 8C, D). In Experiment 3, $[SA]_{leaf}$, $[IAA]_{leaf}$ and $[cis-OPDA]_{leaf}$ increased as SWC_{vol} (Fig. 9A, C, E) and Ψ_{leaf} (Fig. 9B, D, F) decreased, irrespective of variation in irrigation volume (100%ET *versus* 50%ET) or placement (deficit irrigation versus PRD). Variation in both soil and leaf water status was associated with changes in foliar phytohormone concentrations.

3.7 Plant morphology and water use efficiency

In Experiment 1, progressive soil drying decreased plant height (H), leaf area (LA), shoot biomass (DM_{shoot}) and water use (WU) by 6%, 14%, 35% and 41%, respectively compared to well-watered plants (W1), but water use efficiency (WUE) was similar between treatments (Table 4). Partial rootzone drying at 100%ET didn't significantly affect H, LA, DM_{shoot}, WU and WUE in Experiments 2 and 3 (Table 4), even though evapotranspiration of 100%PRD plants in Experiment 2 was consistently less than well-watered plants (Supplemental Fig. 7B). Irrespective of irrigation placement, deficit irrigation (50% ET) decreased H, LA and WU by 6%, 16% and 64%, respectively, compared to well-watered plants, and increased WUE by 2.7-fold (Table 4), but didn't affect DM_{shoot}.

4. Discussion

4.1 Effects of PRD on plant water relations and WUE

Partial rootzone drying aims to exploit root-to-shoot signalling of drying soil to enhance crop water use efficiency (WUE) by partially closing the stomata while sustaining photosynthesis

and leaf water status (Dry et al., 2000; Kang et al., 1997; Stoll et al., 2000). Alternating the irrigated and drying parts of the rootzone was considered necessary to maintain root-to-shoot signalling (Stoll et al., 2000), but applying 70% of the irrigation volume of well-watered plants to field-grown cotton via alternate and fixed furrow irrigation (wetting half the soil volume) elicited similar stomatal closure and comparable WUE (Tang et al., 2005). Supplying half the irrigation volume of well-watered plants to cotton seedlings grown in split pots, while alternating the irrigated and drying parts of the rootzone every 3 days, approximately doubled WUE (Li et al., 2017a), but these gains are caused by lower irrigation volumes and not variation in irrigation placement (Table 4). Indeed, applying PRD at 100%ET scarcely affected (a non-significant 10% increase) WUE (Table 4), probably since soil drying elicited only transient stomatal closure between Days 8 and 14, and Days 25 and 26 after alternation (Fig. 2B) in Experiment 2, and between Days 6 and 9 (Fig. 2C) in Experiment 3, respectively. Likewise, substantial stomatal closure caused by withholding water from the entire rootzone (Fig. 2A) scarcely affected (a non-significant 10% increase) WUE (Table 4), probably because these plants spent little time (at most a day) above the inflection point in the photosynthesis versus g_s curve (Wong et al., 1979) where stomatal closure decreases water use but has negligible effects on photosynthesis. Indeed, withholding water substantially decreased cotton biomass accumulation, probably as decreased leaf water status limited leaf expansion (Table 4) and photosynthesis. Harvesting only shoot biomass (Table 4) may obscure treatment effects on WUE, as soil drying and especially PRD (Mingo et al., 2004) promotes root biomass accumulation. Nevertheless, as root biomass comprises less than 20% of total cotton biomass (Tang et al., 2010), understanding how irrigation volume and placement affects leaf water status seems necessary to interpret these effects on WUE.

Withholding irrigation or supplying 50%ET decreased leaf and shoot water potential (Fig. 4A, B, E, F; Table 3), but 100%ET rarely affected shoot water status (Fig. 4C, D). Although 30% less irrigation tended to decrease Ψ_{leaf} of field-grown cotton by no more 0.1 MPa, these differences were not significant (Tang et al., 2005) and only substantial soil moisture depletion (> 40%) decreased Ψ_{leaf} of cotton grown in large pots (Devi & Reddy, 2020). In both studies, stomatal closure in response to soil drying maintained cotton Ψ_{leaf}, as in 100%ET plants (Fig. 4C, E). However substantial soil drying in Experiments 1 and 3 (Fig. 1A, C) caused stomatal closure to correlate with decreased Ψ_{leaf} (Supplementary Fig. 5B, H). Even strongly isohydric plants such as maize that maintain Ψ_{leaf} with drying soil when field-grown (Tardieu & Simonneau, 1998) are unable to prevent Ψ_{leaf} decreasing when water is withheld from small soil volumes (Li et al., 2017b). Since cotton plants exposed to PRD had a lower g_s at the same Ψ_{leaf}

than well-watered plants (Tang et al., 2010), and because 100%ET PRD plants sustained stomatal closure for several days (Fig. 2B, C) despite maintaining Ψ_{leaf} (Fig. 4C, E) non-hydraulic signalling was implicated.

4.2 ABA-mediated stomatal regulation during PRD

Irrespective of irrigation placement, withholding irrigation or supplying 50%ET increased leaf and shoot xylem ABA concentration (Fig. 5A, B, E, F; Table 3) as total soil water availability and Ψ_{leaf} declined (Fig. 8A, B, E, F). Thus, substantial soil drying caused stomatal closure associated with increased $[ABA]_{leaf}$ and $[X\text{-}ABA]_{shoot}$ (Fig. 6C; Supplementary Fig. 5C, D, I), supporting the suggestion that drought stress induced ABA accumulation mediated partial stomatal closure (Zhang & Davies, 1990; Davies & Zhang, 1991; Dodd et al., 2006). However, 50%ET PRD plants decreased g_s earlier than deficit irrigated plants (Fig. 2C) despite similar overall soil water availability, as in field-grown cotton plants (Du et al., 2006). With reduced irrigation volume, PRD plants had higher shoot xylem sap ABA concentration than deficit irrigated plants (Days 5, 8 in Fig. 5F; Dodd et al., 2008), as in PRD-grown cotton plants receiving half the irrigation volume that had 2-fold higher foliar ABA accumulation (Iqbal et al., 2021). This differential ABA accumulation cannot always be attributed to variation in shoot water status.

With sufficient irrigation (100%ET), PRD increased leaf and shoot xylem ABA accumulation only occasionally early in the drying cycle, as in apple seedlings where foliar ABA accumulation was limited and not physiologically significant (Einhorn et al., 2012). Thereafter, well-watered and PRD plants had similar $[ABA]_{leaf}$ (Fig. 5C, E) and $[X\text{-}ABA]_{shoot}$ (Fig. 5D, F). Stomatal closure occurred when SWC_{vol} of the dry compartment declined to 21-15% (from Days 8-14) in Experiment 2 (Fig. 1B; 2B) and 20%-18% (from Days 6-9) in Experiment 3 (Fig. 1C; 2C) with such PRD-induced stomatal closure in Experiment 2 accompanied by increased $[ABA]_{leaf}$ (Fig. 5C), with g_s inversely related to $[ABA]_{leaf}$ (Fig. 6B). That leaf water potential was maintained during this period (Fig. 4C) supports the hypothesis that PRD-induced leaf ABA accumulation mediates stomatal responses, with mobile signalling molecules moving in the transpiration stream from the roots to the leaves to modulate ABA accumulation (Takahashi et al., 2018). Maintaining water uptake from roots in dry soil was necessary to transport these signals to the shoot (Dodd et al., 2008), but continued drying of part of the rootzone restricted sap flow and limited upward transmission of signalling molecules. Thus, g_s of PRD plants recovered to (or even exceeded) the levels of well-watered plants (Fig. 2B, C) when < 10% of total sap flow was sourced from roots in dry soil (Fig. 3A), with PRD and well-watered treatments having similar xylem ABA concentrations (Fig. 5D, F) as reported previously

(Puértolas et al., 2015). Maintaining sap fluxes from roots in very dry soil seems necessary to sustain ABA-induced stomatal closure of PRD plants, if leaf water relations are not perturbed with sufficient irrigation.

Lower g_s of 100%ET PRD plants than well-watered plants (Fig. 2B, C) despite similar $[ABA]_{leaf}$ and $[X\text{-}ABA]_{shoot}$ (Fig. 5E, F) might be attributed to other phytohormones. Indeed, roots exposed to drying soil had not yet accumulated ABA but had increased ACC concentrations (Pérez-Pérez et al., 2020), with root-to-shoot ACC signalling affecting temporal dynamics of foliar ethylene evolution in plants exposed to PRD. However, further soil drying (when SWC_{vol} of the dry compartment decreased to between 10-15%, Fig. 1B) resulted in similar g_s, $[ABA]_{leaf}$ and $[X\text{-}ABA]_{shoot}$ between 100%ET PRD and well-watered plants, analogous to the comparable whole plant gas exchange of these treatments in tomato (Puértolas & Dodd, 2022) and in grapevine (Stoll et al., 2000). Likewise, when SWC_{vol} of the dry compartment < 10% (Fig. 1C), g_s of PRD plants even exceeded well-watered plants, yet variation in foliar ABA concentrations couldn't explain these changes. Thus, several phytohormones were measured in trying to account for stomatal dynamics of plants exposed to PRD in Experiment 3.

4.3 Stomatal regulation during PRD by other hormones

PRD increased leaf IAA concentration by 29% on Day 2 while deficit irrigation (50%ET) approximately doubled foliar IAA accumulation after 14 days (Table 2, 3). In field-grown apple trees, PRD decreased foliar IAA levels at earlier growth stages but significantly increased IAA levels at a later growth stage (Ghafari et al., 2020). Soil-drying induced changes in IAA levels may be ABA-mediated, as soil drying greatly upregulated auxin-related (synthesis, distribution and response) gene expression of wild-type (WT) tomato roots, whereas few changes occurred in the ABA-deficient *notabilis* mutant (Zhang et al., 2021). Exposing olive (*Olea europea*) roots to drying soil during PRD increased their IAA concentration by up to 25% compared to those roots in wet soil, but PRD-induced foliar IAA accumulation (especially at decreased irrigation volumes) did not reflect root ABA increments (Abboud et al., 2021), suggesting long-distance signalling was not regulating foliar IAA homeostasis, but instead leaf water deficits.

Stomatal closure with increased $[IAA]_{leaf}$ (Fig. 7B), agrees with observations that low IAA levels promote ion influx to sustain stomatal opening but higher IAA levels inhibit ion channels to promote stomatal closure (Assmann & Armstrong, 1999; Hajihashemi. 2019). Comparing the temporal dynamics of IAA accumulation and stomatal closure provides limited support for IAA-induced stomatal movement early in the PRD cycle but later IAA accumulation results from water restriction to the shoot (Fig. 7B). Although g_s of 100%ET PRD plants was higher than well-watered plants on Days 13-15 (Fig. 2C), $[IAA]_{leaf}$ did not differ, suggesting other

483   hormones regulate stomatal re-opening.

484   Irrigation volume (Munne-Bosch & Penuelas, 2003), frequency (Puértolas et al., 2020), and

485   placement (Ghafari et al., 2020) can all affect foliar SA concentration, although variable

486   experimental designs in these studies make it difficult to resolve the individual effects. Field-

487   grown apple trees that received 25-50% less irrigation via PRD every 2 days maintained

488   elevated leaf SA concentrations (42-97% higher than control plants) throughout the irrigation

489   season (Ghafari et al., 2020), while mild soil water deficit (75%ET irrigated daily) only

490   transiently stimulated SA concentration in pot-grown tomato (Puértolas et al., 2020). Dry soil

491   stimulated root and leaf SA accumulation in ABA-deficient *flacca* tomato (Munoz-Espinosa et

492   al., 2015), indicating that SA accumulation was independent of ABA accumulation. Co-

493   occurrence of root and shoot SA accumulation (Munoz-Espinosa et al., 2015) might be

494   explained by shoot export of SA to the roots, as stem girdling of soybean caused foliar SA

495   accumulation without root SA accumulation (Castro-Valdecantos et al., 2021). That decreased

496   irrigation volume rather than irrigation placement (Table 3) stimulated foliar SA accumulation,

497   coincident with decreased leaf and shoot water status (Fig. 4E, F), suggests leaf water deficit

498   regulated foliar SA accumulation.

499   While exogenous SA applications at concentrations up to 1 mM scarcely affect g_s, and

500   Arabidopsis mutants impaired in SA biosynthesis and signalling responded normally to

501   exogenous ABA (Zamora et al., 2021), the lower stomatal aperture of the *siz1-2* mutant (with

502   almost 30-fold higher SA concentrations) increased its drought survival (Miura et al., 2013),

503   suggesting ROS priming associated with enhanced SA production contributes to stomatal

504   closure, which might be independent of ABA signalling. While both [SA]$_{leaf}$ (Fig. 7A) and

505   [ABA]$_{leaf}$ (Fig. 6C) inversely correlated with g_s, the earlier increase in ABA concentration (Day

506   8) than SA (Day 14) suggests that ABA initiates stomatal closure prior to SA accumulation as

507   the soil dries.

508   Jasmonates (jasmonic acid [JA] and jasmonic acid-isoleucine conjugate [JA-Ile]) and their

509   precursor in the oxylipin pathway (cis-(+)-oxo-phytodienoic acid or cis-OPDA) have also been

510   associated with stomatal closure (Savchenko et al., 2014). While PRD stimulated root and foliar

511   JA accumulation in field-grown olive trees (Abboud et al., 2021) and osmotic stress applied to

512   part of the root system of hydroponically grown cotton caused foliar JA and JA-Ile

513   accumulation (Luo et al., 2019), these analytes were not affected by either irrigation volume

514   and placement (Supplementary Table 2, 3), perhaps because JA accumulation can be transient

515   (Wang et al., 2020; Puértolas et al., 2020). Root and leaf JA accumulation co-occurred in ABA-

516   deficient *flacca* tomato (Munoz-Espinosa et al., 2015), indicating JA accumulation

independently of ABA accumulation. That stem girdling substantially decreased (but did not abolish) soil drying-induced root (and to a lesser extent shoot) JA accumulation highlights the importance of shoot-to-root jasmonate export (Castro-Valdecantos et al., 2021). Here, the 50%ET treatments significantly enhanced leaf cis-OPDA accumulation (Table 2, 3), as in tomato plants exposed to soil water deficit (de Ollas et al., 2018). Reciprocal grafting experiments with wild-type (WT) and a jasmonate-compromised mutant (*def-1*) demonstrated that foliar JA, JA-Ile and cis-OPDA accumulation of WT scions exposed to water deficit was rootstock-independent (de Ollas et al., 2018), suggesting leaf water deficit stimulated cis-OPDA accumulation.

Stomatal bioassays indicate that cis-OPDA is a more potent antitranspirant than other jasmonates (Savchenko et al., 2014). Furthermore, the *def-1* mutant that accumulates cis-OPDA has lower g_s than WT plants (de Ollas et al., 2018), and g_s was inversely correlated to increased [cis-OPDA]$_{leaf}$ (Fig. 7C). While cis-OPDA might act independently of JA and in co-operation with ABA (Savchenko et al., 2014; de Ollas & Dodd, 2016; Merlaen et al., 2020) in regulating stomata of plants exposed to soil drying, its delayed accumulation relative to ABA (that increased almost a week earlier) suggests it may be more important in maintaining, than initiating, stomatal closure.

However, complex signal networks regulating stomata during environmental stresses involve multiple hormones. Strigolactones (SL) are root-derived singals (Bouwmeester et al., 2003) that mediate stomatal development and function independently or by interacting with other hormones (Huntenburg et al., 2022). SL biosynthesis and signalling impaired mutants has an ABA-insensitive phenotype and increased stomatal density, indicating that SL plays regulatory role at least in part through ABA signalling (Ha et al., 2013). Although foliar ABA, IAA, SA and cis-OPDA concentration correlated with stomatal closure during prolonged soil drying (Fig. 6B, C; Fig. 7), they couldn't explain stomatal re-opening of plants grown under PRD, suggesting that future studies of stomatal responses to PRD should consider other potential regulators.

5. Conclusions

While restricting irrigation (50%ET) decreased leaf water status causing foliar accumulation of multiple hormones (ABA, SA, IAA and cis-OPDA), imposing 100%ET PRD attenuated (Ψ, ABA) or abolished (SA, IAA and cis-OPDA) these effects. Nevertheless, drying a single soil compartment transiently decreased g_s before prolonged drying allowed stomatal re-opening, with the latter apparently independent of altered foliar or xylem ABA dynamics. Further investigating foliar volatile emissions such as ethylene (Pérez-Pérez et al., 2020) and xylem sap

composition seems necessary to account for non-ABA mediated stomatal responses of plants exposed to PRD.

Acknowledgements

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784 **Figure Legends**

785 **Fig. 1.** Average volumetric soil water content of the upper 6.5 cm of each compartment (PRD plants) or pot (deficit-irrigated or well-watered plants) in Experiments 1 (A), 2 (B) and 3 (C)

prior to irrigation being applied each day. Black vertical dotted line indicates when irrigated and drying compartments were alternated in (B). Points are means ± SE of 4, 5 and 6 replicate pots in Experiments 1, 2 and 3, respectively. Evenly irrigated pots (W2, W3-100% ET and W3-50% ET) averaged the soil water content of the two compartments.

Fig. 2. Stomatal conductance in Experiments 1 (A), 2 (B) and 3 (C), respectively. Black vertical dotted line indicates when irrigated and drying compartments were alternated in (B). Points are means ± SE of 4, 5 and 6 replicate plants in Experiments 1, 2 and 3, respectively. *, ** and *** indicates statistical significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$ in (A) and (B). Table 1 tabulates two-way ANOVA data for (C).

Fig. 3. Fraction of sap flow from the dry soil compartment (F_{dry}) (A) and stomatal conductance (g_s) ratio of 100%ET PRD plants to 100%ET well-watered (WW) plants (B) in Experiments 2 and 3 *versus* the soil water content of the dry side of the root system of 100%ET PRD plants. Black horizontal dotted line indicates when PRD and WW plants had the same stomatal conductance in (B). Each point is the mean value of 5 and 6 replicate plants in Experiments 2 and 3, respectively, with SE omitted for clarify.

Fig. 4. Leaf water potential (Ψ_{leaf}) and shoot water potential (Ψ_{shoot}) in Experiments 1 (A, B), 2 (C, D) and 3 (E, F), respectively. Black vertical dotted line indicates when irrigated and drying compartments were alternated in (C, D). Points are means ± SE of 4, 5 and 6 replicate plants in Experiments 1, 2 and 3, respectively. Table 3 tabulates two-way ANOVA data.

Fig. 5. Leaf ABA concentration ([ABA]$_{leaf}$) and shoot xylem sap ABA concentration ([ABA]$_{shoot}$) in Experiments 1 (A, B), 2 (C, D) and 3 (E, F), respectively. Black vertical dotted line indicates when irrigated and drying compartments were alternated in (C, D). Points or bars are means ± SE of 4, 5 and 6 replicate plants in Experiment 1, 2 and 3, respectively. Table 3 tabulates two-way ANOVA data.

Fig. 6. Relationships between stomatal conductance (g_s) and gravimetric soil water content in Experiment 3 (A) and leaf ABA concentration in Experiments 2 (B) and 3 (C), respectively. Each point is an individual plant, with linear regressions fitted when significant: *** indicates statistically significance at $P < 0.001$.

Fig. 7. Relationships between stomatal conductance (g_s) and leaf salicylic acid concentration (A), indole acetic acid concentration (B) and leaf cis-(+)-oxo-phytodienoic acid concentration (C) in Experiment 3. Each point is an individual plant, with regression lines fitted when significant: *** indicates statistical significance at $P < 0.001$.

Fig. 8. Relationships between leaf ABA concentration ([ABA]$_{leaf}$) and soil water content (A, C and E) and leaf water potential (B, D and F) in Experiments 1 (A and B), 2 (C and D) and 3 (E

821 and F), respectively. Each point is an individual plant, with regression lines fitted when

822 significant: *** indicates statistical significance at $P < 0.001$, ns indicates not significant.

823 **Fig. 9.** Relationships between leaf salicylic acid concentration ($[SA]_{leaf}$) (A, B), leaf indole

824 acetic acid concentration ($[IAA]_{leaf}$) (C, D), leaf cis-(+)-oxo-phytodienoic acid (E, F) and soil

825 water content (A, C, E) and leaf water potential (B, D, F) in Experiment 3, respectively. Each

826 point is an individual plant, with regression lines fitted when significant: *** indicates statistical

827 significance at $P < 0.001$.

828 **Table 1** Output of two-way ANOVA (P Values reported) for daily stomatal conductance (g_s) as

829 affected by irrigation volume (100% or 50% ET), placement (PRD or DI) and their interaction

830 in Experiment 3.

831 **Table 2** Leaf salicylic acid concentration ($[SA]_{leaf}$), leaf indole acetic acid concentration

832 ($[IAA]_{leaf}$) and leaf cis-(+)-oxo-phytodienoic acid concentration ($[cis\text{-}OPDA]_{leaf}$) in Experiment

833 3. Data are means ± SE of 6 replicate plants. Different letters within a column indicate

834 significant ($P < 0.05$) treatment differences on that day of measurement.

835 **Table 3** Output of two-way ANOVA (P values reported) for leaf water potential (Ψ_{leaf}), shoot

836 water potential (Ψ_{shoot}), leaf ABA concentration ($[ABA]_{leaf}$), shoot xylem sap concentration ($[X\text{-}$

837 $ABA]_{shoot}$), leaf salicylic acid concentration ($[SA]_{leaf}$), leaf indole acetic acid concentration

838 ($[IAA]_{leaf}$) and leaf cis-(+)-oxo-phytodienoic acid concentration ($[cis\text{-}OPDA]_{leaf}$) as affected by

839 irrigation volume (100% or 50% ET), placement (PRD or DI) and their interaction in

840 Experiment 3.

841 **Table 4** Plant height (H), leaf area (LA), shoot biomass (DM_{shoot}), plant water use (WU) and

842 water use efficiency (WUE) in Experiments 1, 2 and 3, respectively. Data are means ± SE of 4,

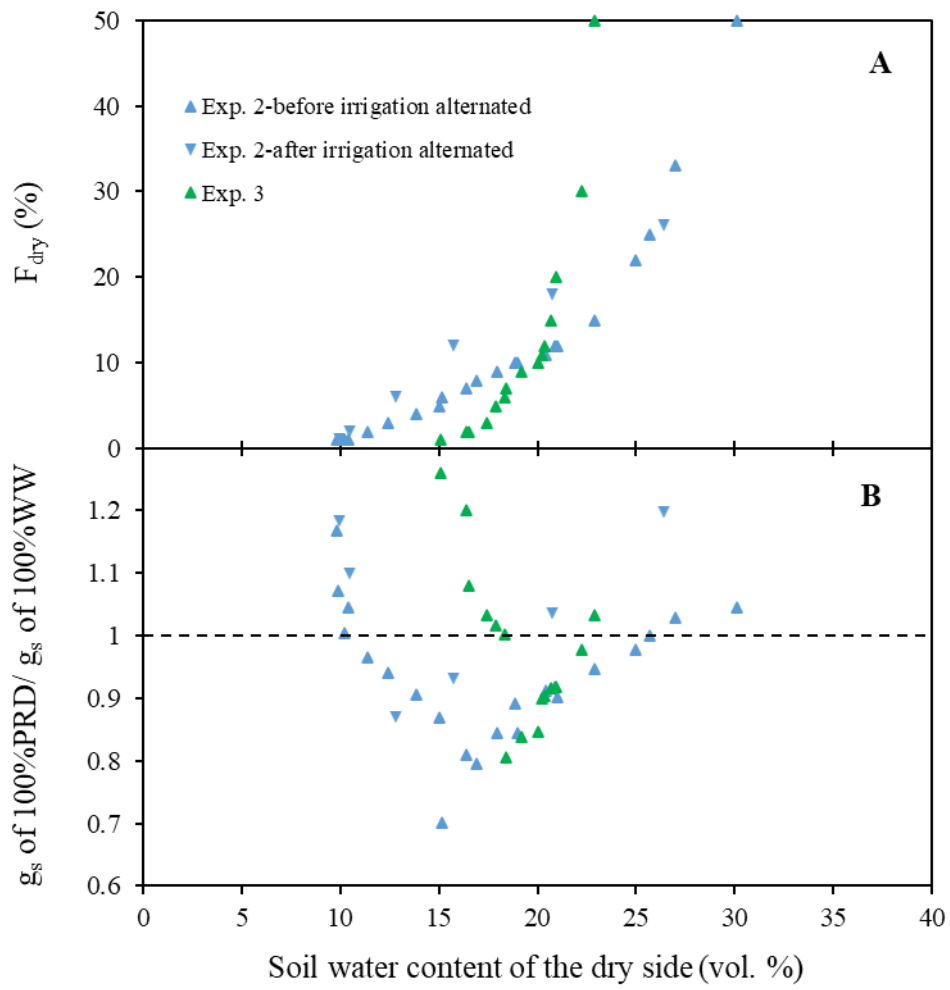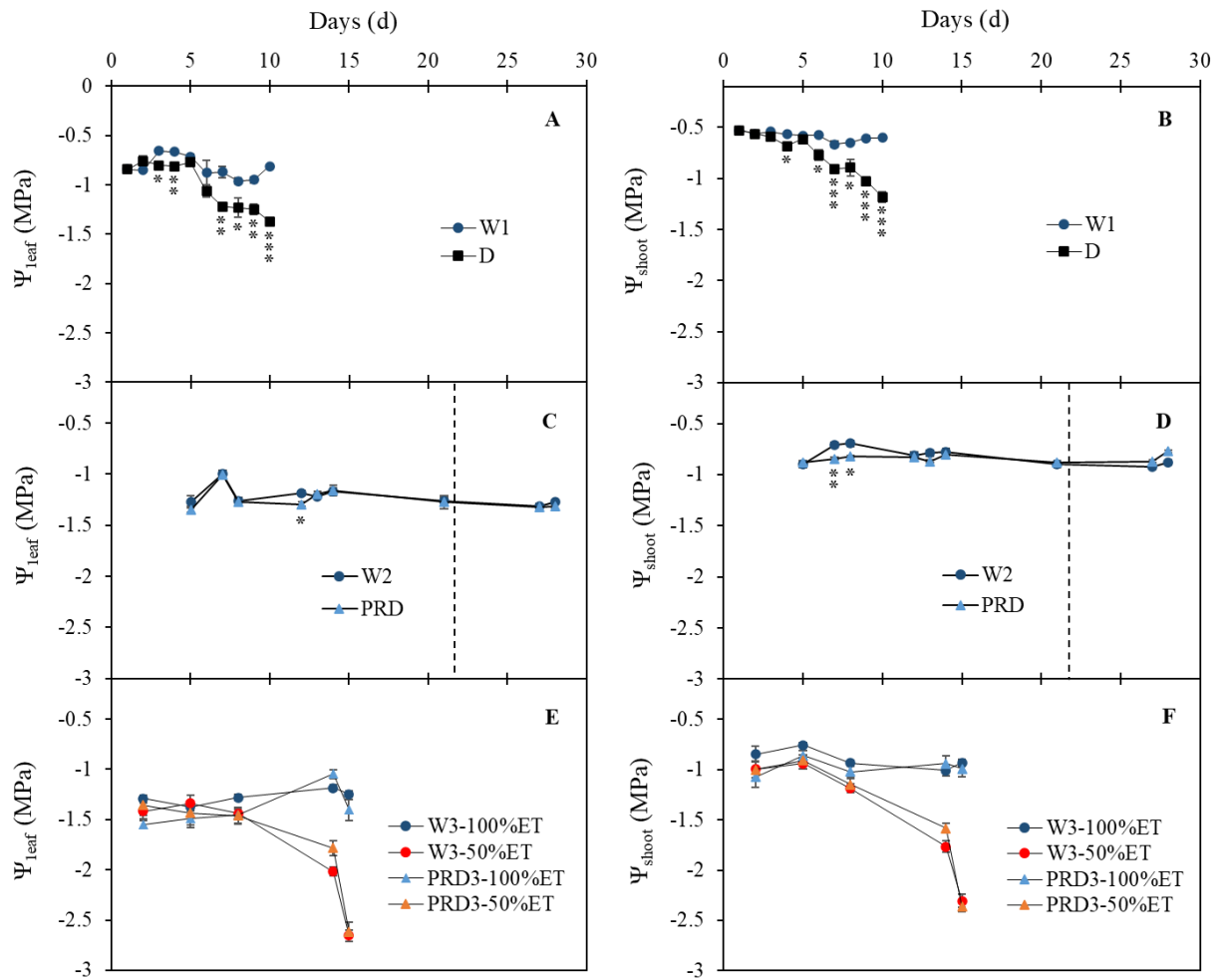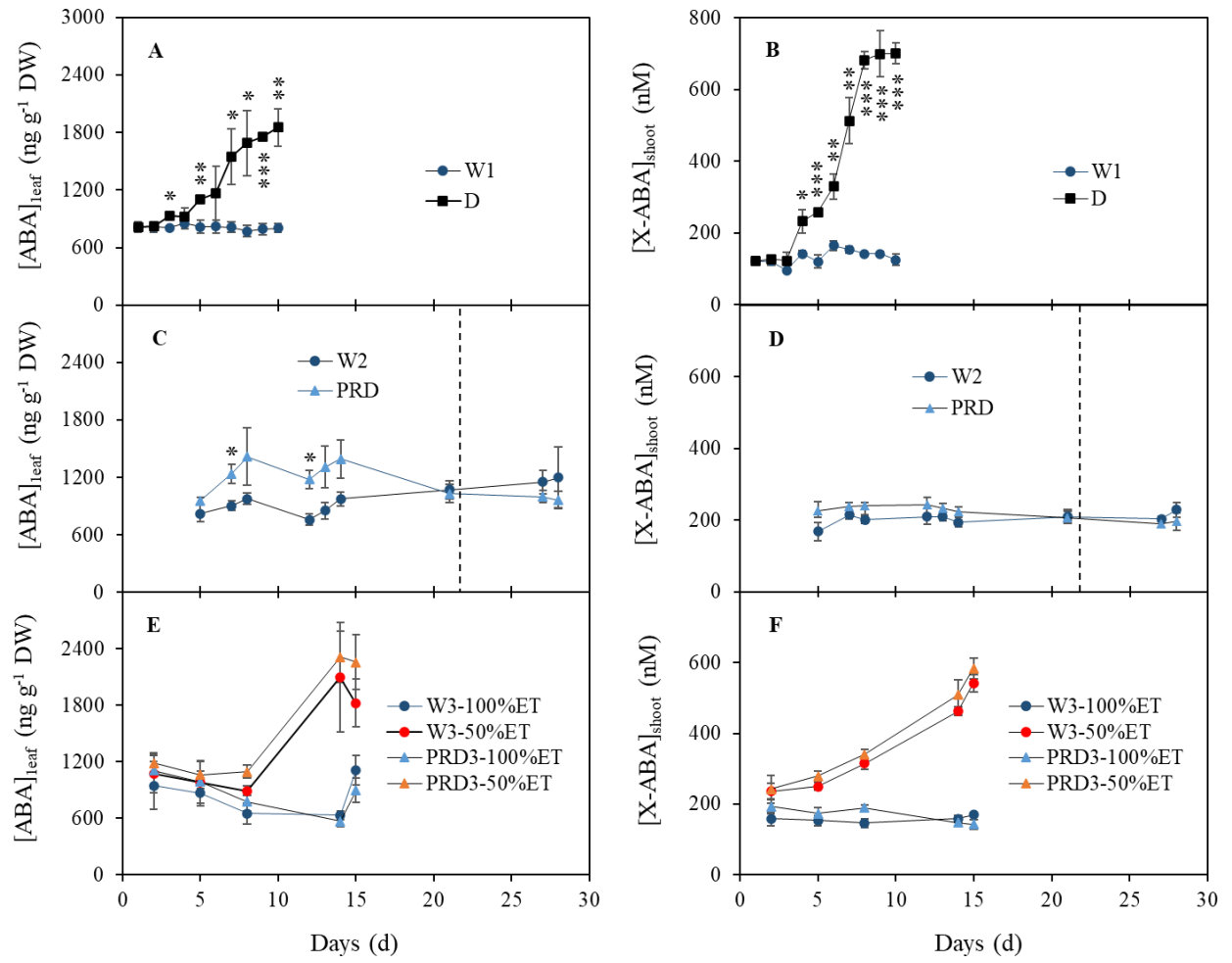843 5 and 6 replicate plants in Experiments 1, 2 and 3, respectively.
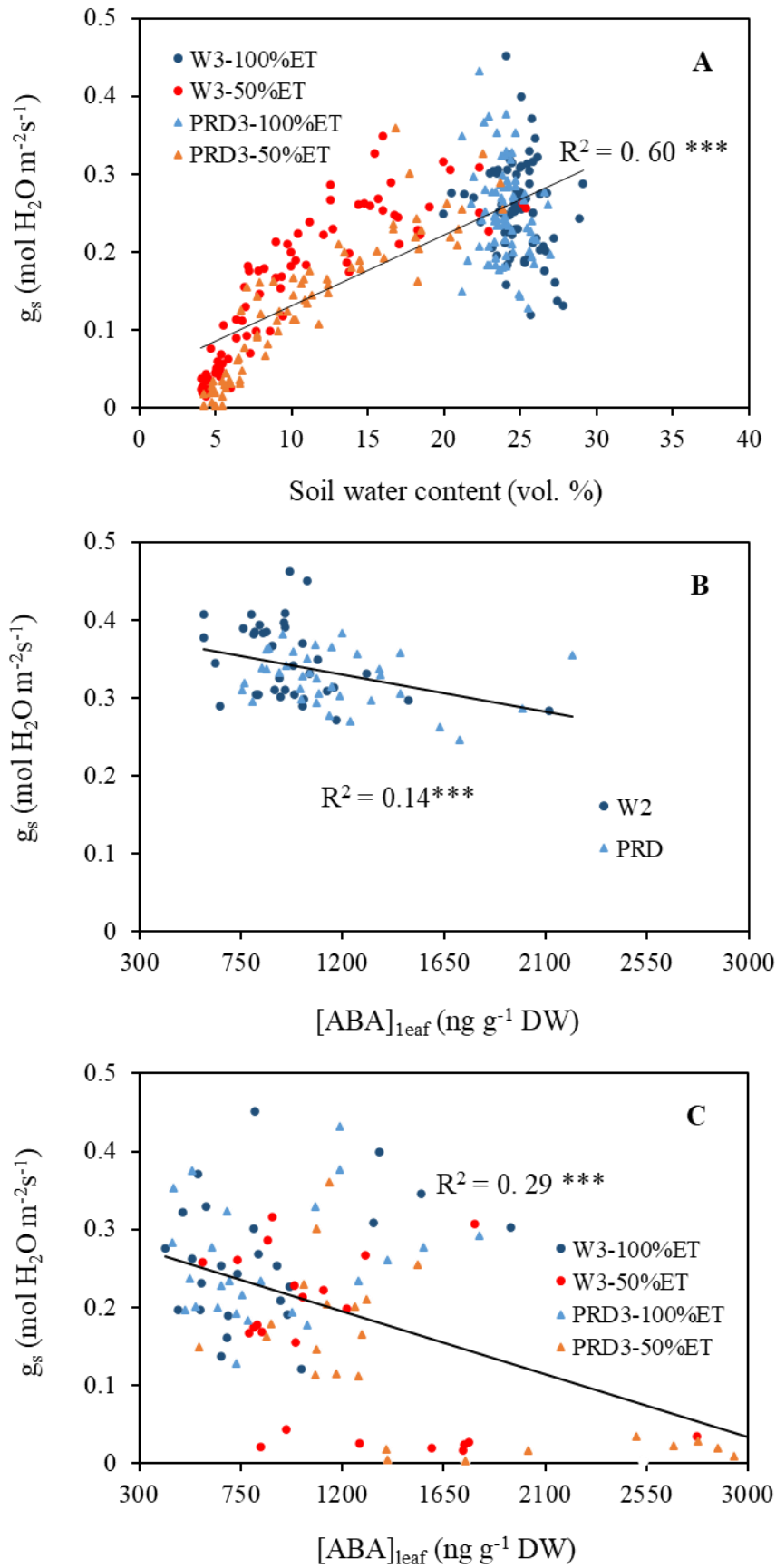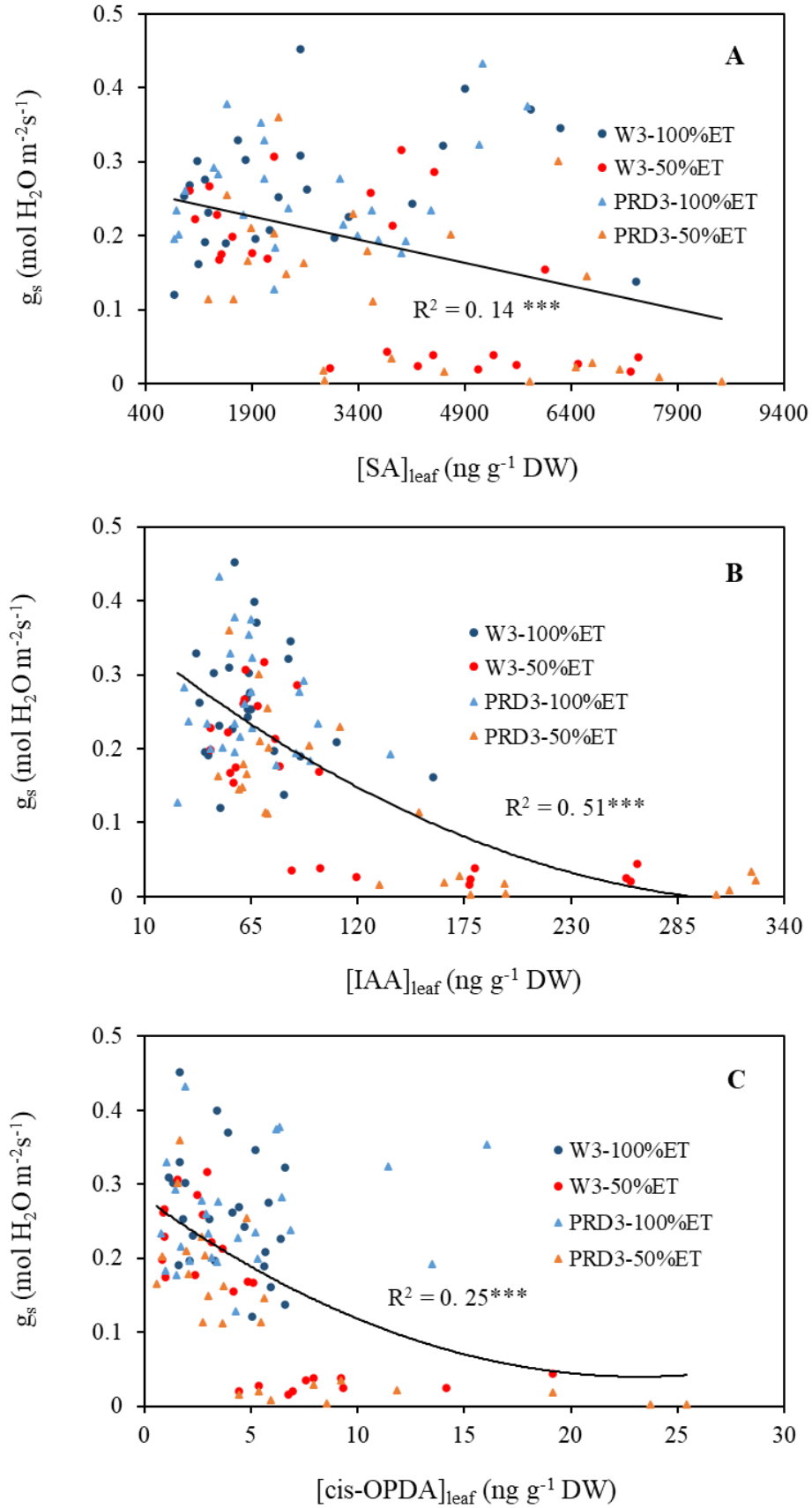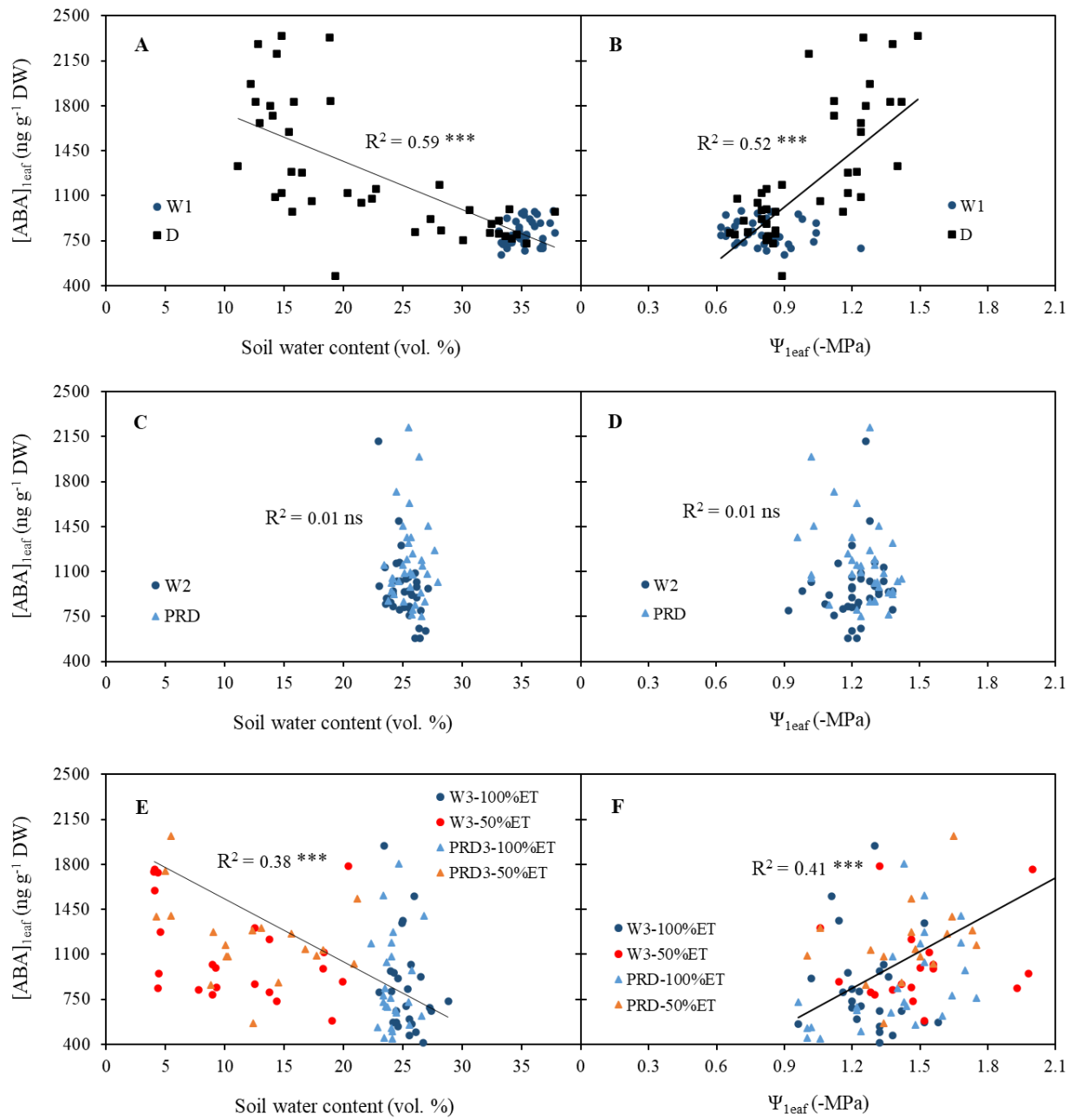
844 **Fig. 1**
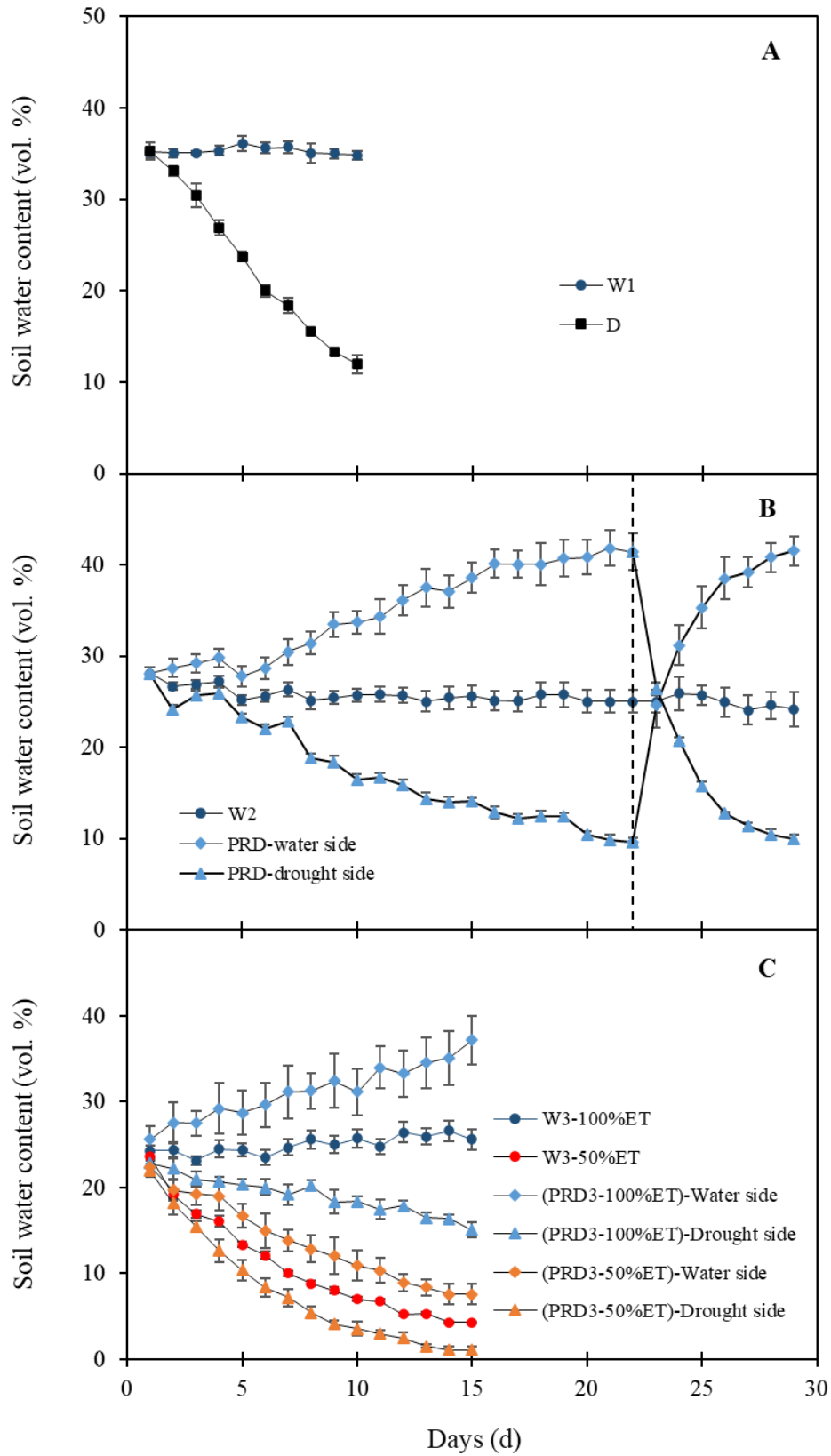
845 **Fig. 2**

846   **Fig. 3**

847   **Fig. 4**

848   **Fig. 5**

849   **Fig. 6**

850   **Fig. 7**

851   **Fig. 8**

852   **Fig. 9**
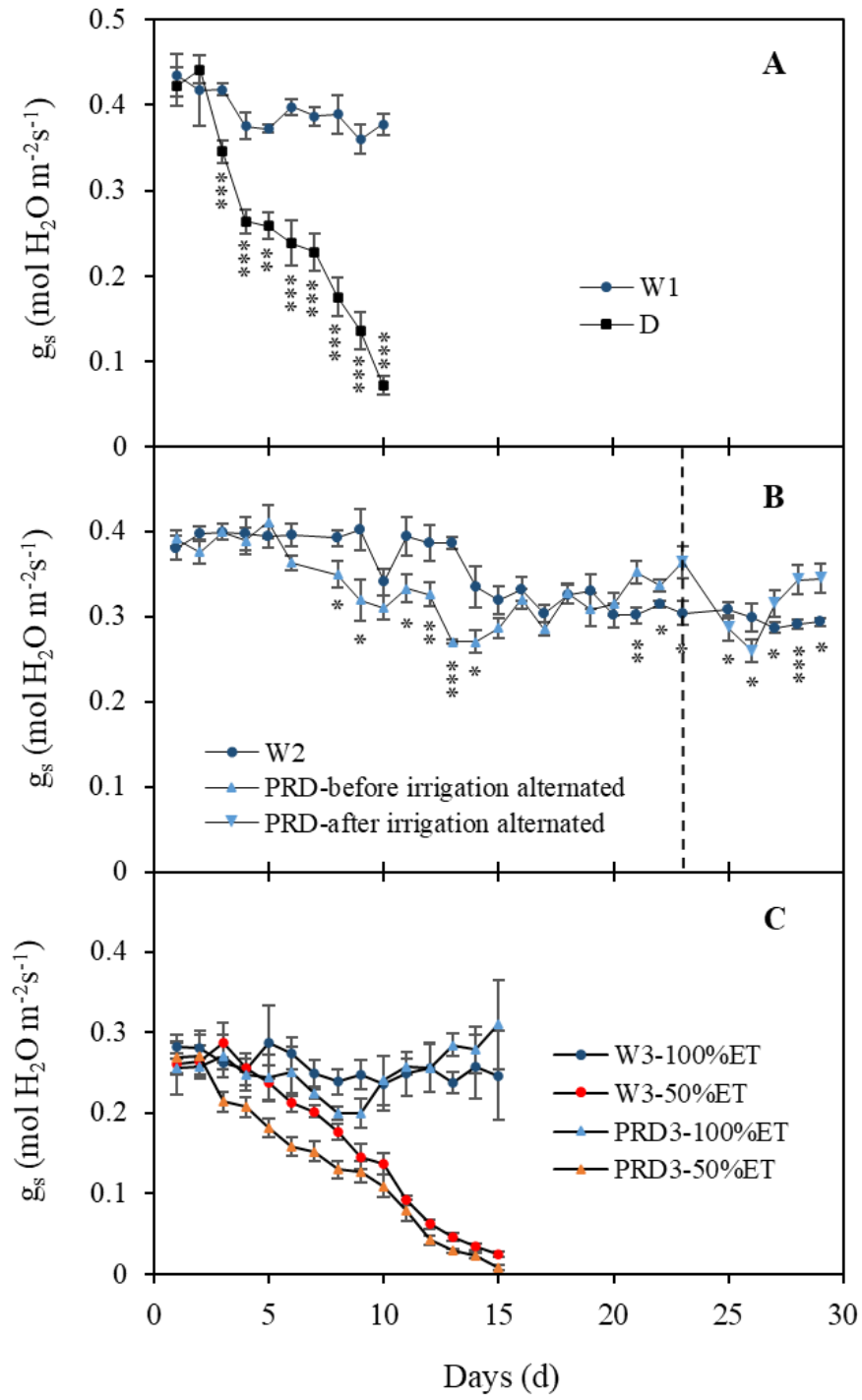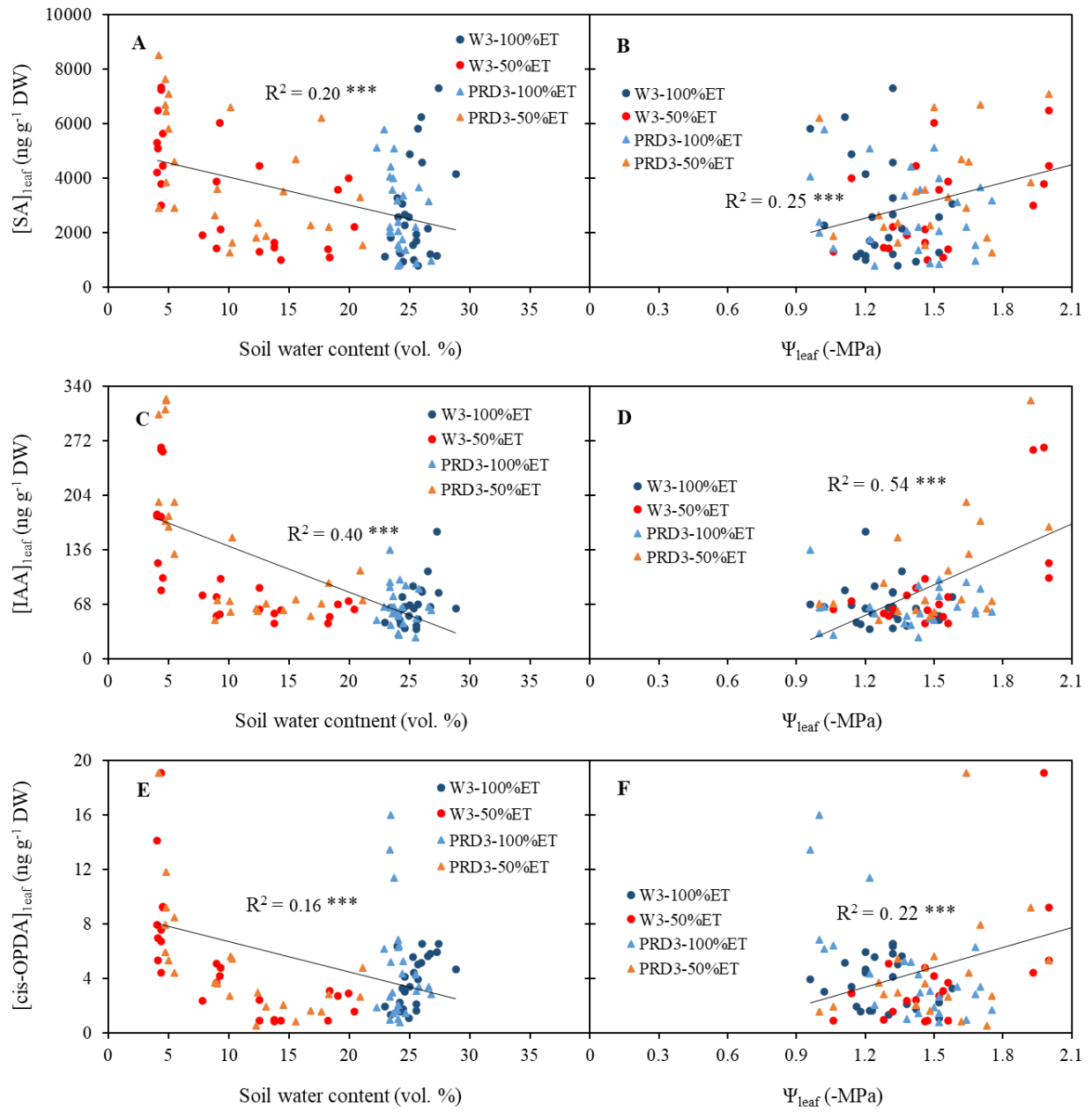
853 **Table 1**

	1d	2d	3d	4d	5d	6d	7d	8d	9d	10d	11d	12d	13d	14d	15d
Volume	0.85	0.99	0.44	0.24	0.08	**0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
Placement	0.65	0.65	0.11	0.11	0.11	0.07	**0.01**	**0.002**	0.06	0.63	0.90	0.66	0.16	0.83	0.55
Interaction	0.44	0.54	**0.05**	0.20	0.84	0.45	0.31	0.73	0.38	0.53	0.54	0.65	**0.01**	0.54	0.33

854

Table 2

Treatments	$[SA]_{leaf}$ (ng g^{-1} DW)	$[IAA]_{leaf}$ (ng g^{-1} DW)	$[cis\text{-}OPDA]_{leaf}$ (ng g^{-1} DW)
2d			
W3-100%ET	1739 ± 364	57.4 ± 7.1	2.6 ± 0.6
W3-50%ET	2458 ± 575	59.9 ± 5.1	2.3 ± 0.4
PRD3-100%ET	2120 ± 556	74.3 ± 8.5	2.5 ± 0.5
PRD3-50%ET	3112 ± 826	80.4 ± 10.1	2.7 ± 0.6
5d			
W3-100%ET	1722 ± 355	53.2 ± 3.4	1.7 ± 0.2
W3-50%ET	1975 ± 632	62.6 ± 7.2	1.2 ± 0.3
PRD3-100%ET	1926 ± 404	71.5 ± 9.2	1.5 ± 0.2
PRD3-50%ET	2862 ± 550	65.5 ± 2.7	1.7 ± 0.4
8d			
W3-100%ET	2055 ± 238	63.7 ± 12.7	4.2 ± 0.7
W3-50%ET	3072 ± 848	73.4 ± 8.5	4.0 ± 0.5
PRD3-100%ET	2740 ± 605	65.2 ± 8.3	3.5 ± 0.6
PRD3-50%ET	3153 ± 956	80.9 ± 18.3	4.2 ± 0.6
14d			
W3-100%ET	3452 ± 907	92.9 ± 17.2[B]	5.4 ± 0.5[B]
W3-50%ET	4608 ± 605	185.0 ± 34.2[B]	9.2 ± 2.6[A]
PRD3-100%ET	3001 ± 683	65.7 ± 19.2[B]	5.4 ± 1.9[B]
PRD3-50%ET	5030 ± 807	197.0 ± 33.0[A]	10.9 ± 2.6[A]
15d			
W3-100%ET	4505 ± 1147	67.9 ± 7.1	5.3 ± 0.6[B]
W3-50%ET	5906 ± 608	232.5 ± 63.5	9.0 ± 1.4[AB]
PRD3-100%ET	3816 ± 829	48.1 ± 6.4	4.8 ± 0.8[B]
PRD3-50%ET	6269 ± 958	263.2 ± 31.3	15.1 ± 4.0[A]

Table 3

	Ψ_{leaf}	Ψ_{shoot}	$[ABA]_{leaf}$	$[X\text{-}ABA]_{shoot}$	$[SA]_{leaf}$	$[IAA]_{leaf}$	$[cis\text{-}OPDA]_{leaf}$
				Significance: 2d			
Volume	0.65	0.68	0.61	**0.03**	0.18	0.59	0.91
Placement	0.22	0.19	0.49	0.46	0.40	**0.03**	0.65
Interaction	0.06	0.20	0.90	0.60	0.82	0.82	0.68
				Significance: 5d			
Volume	0.63	**0.02**	0.56	**0.003**	0.25	0.79	0.73
Placement	0.27	0.45	0.56	**<0.001**	0.29	0.11	0.72
Interaction	0.92	0.15	0.89	**0.002**	0.50	0.24	0.28
				Significance: 8d			
Volume	0.26	**0.001**	**0.004**	**<0.001**	0.33	0.33	0.64
Placement	0.17	0.59	0.07	**0.02**	0.60	0.73	0.74
Interaction	0.28	0.20	0.66	0.52	0.68	0.82	0.51
				Significance: 14d			
Volume	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.05**	**0.001**	0.60
Placement	**0.01**	**0.05**	0.82	0.46	0.99	0.79	0.21
Interaction	0.40	0.35	0.66	0.22	0.57	0.48	0.21
				Significance: 15d			
Volume	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**0.05**	**<0.001**	**0.01**
Placement	0.45	0.32	0.63	0.74	0.86	0.88	0.22
Interaction	0.24	0.99	0.16	0.12	0.57	0.49	0.14

857 **Table 4**

	Treatment	Height (cm)	Leaf Area (cm^2)	DM$_{shoot}$	ET (L plant^{-1})	WUE (g L^{-1})
Experiment 1	W1	26.5 ± 0.5	322 ± 12	2.0 ± 0.1	0.6 ± 0.0	3.3 ± 0.2
	D	25.0 ± 0.5	276 ± 10	1.3 ± 0.1	0.4 ± 0.0	3.7 ± 0.3
Output of one-way ANOVA		**0.03**	**0.004**	**0.003**	**<0.001**	0.34
Experiment 2	W2	29.8 ± 0.8	396 ± 24	6.0 ± 0.4	2.0 ± 0.1	2.9 ± 0.2
	PRD	29.6 ± 0.8	383 ± 25	6.0 ± 0.4	1.9 ± 0.0	3.2 ± 0.2
Output of one-way ANOVA		0.88	0.70	0.81	0.06	0.37
Experiment 3	W3-100%ET	39.2 ± 1.2^A	405 ± 12^A	7.6 ± 0.2	1.1 ± 0.0^A	3.5 ± 0.1^A
	W3-50%ET	36.9 ± 0.8^{AB}	341 ± 15^C	7.4 ± 0.1	0.4 ± 0.1^B	9.6 ± 1.1^B
	PRD3-100%ET	38.6 ± 0.7^A	369 ± 15^{AB}	7.5 ± 0.2	1.1 ± 0.1^A	3.8 ± 0.3^A
	PRD3-50%ET	35.9 ± 0.8^B	342 ± 15^C	7.4 ± 0.2	0.4 ± 0.0^B	10.4 ± 0.6^B
Output of two-way ANOVA						
	Volume	**0.01**	**0.002**	0.35	**<0.001**	**<0.001**
	Placement	0.37	0.22	0.93	0.56	0.37
	Interaction	0.86	0.20	0.82	0.86	0.67

Supplemental data

859 **Supplementary Table 1** Important dates in the three experiments.

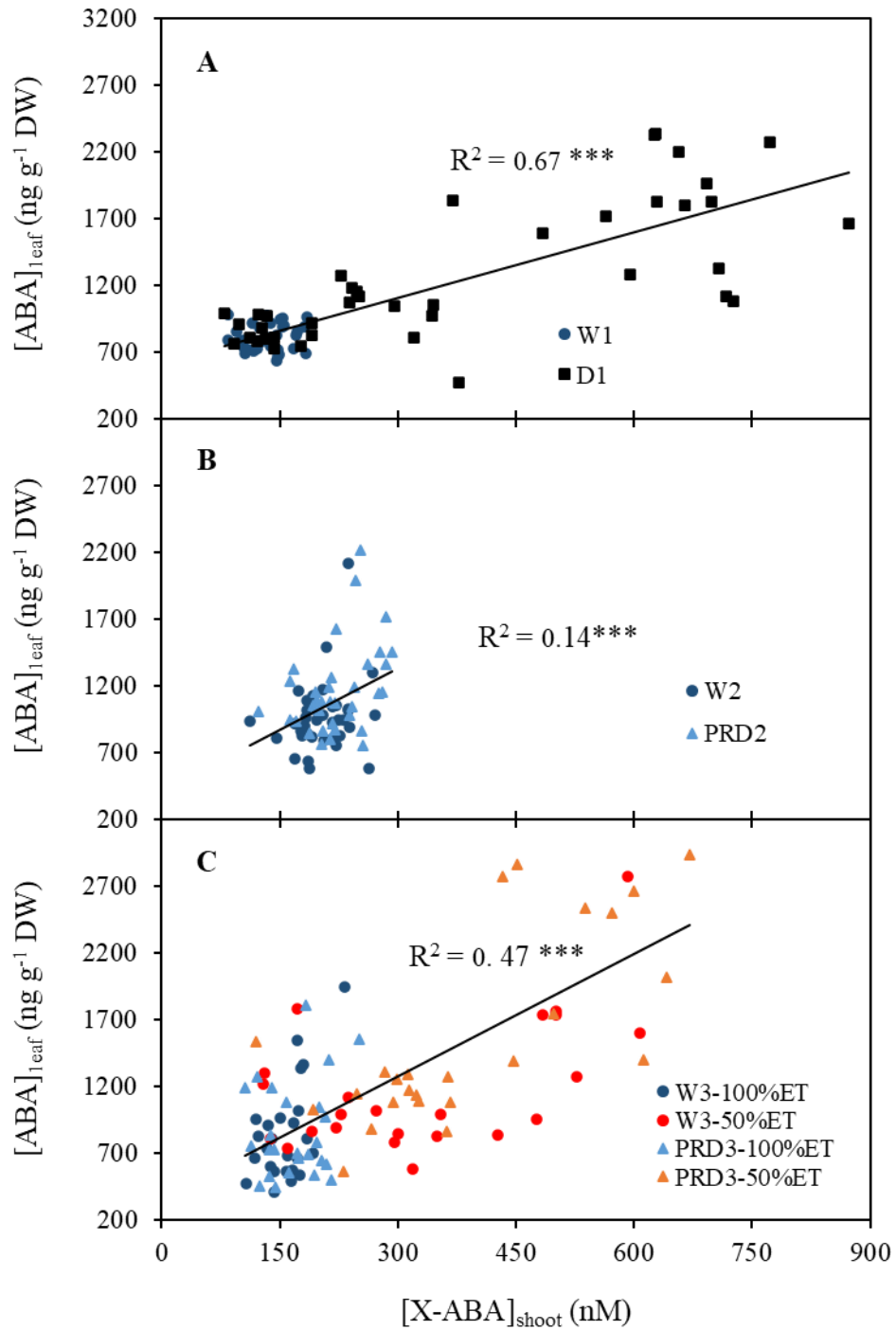| Dates	Experiment 1	Experiment 2	Experiment 3
Sowing	26th July, 2021	17th October, 2021	24th February, 2022
Transplanting	–	28th October, 2021	12th March, 2022
Perlite applied	19th, July, 2021	20th October, 2021	18th March, 2022
Pre-treatment harvest	28th August, 2021	14th November, 2021	24th April, 2022
Starting treatments	29th August, 2021	15th November, 2021	25th April, 2022
Alternating irrigation	–	7th December, 2021	–
Post treatment harvests	29th, 30th, 31st, August, 2021; 1st, 2nd, 3th, 4th, 8th, 9th, 10th, September, 2021	20th, 22nd, 23rd, 27th, 28th, 29th November, 2021; 6th, 12th, 13th, December, 2021	26th, 29th, April, 2022; 2nd, 8th, 10th, May, 2022

860 **Supplementary Fig. 1.** Daily maximum (T$_{max}$) and minimum (T$_{min}$) temperatures, daily

861 maximum (RH$_{max}$) and minimum (RH$_{min}$) relative humidity and daily maximum (VPD$_{max}$) and

862 minimum (VPD$_{min}$) vapor pressure deficit in the greenhouse during the treatment period in

863 Experiments 1, 2 and 3, respectively.
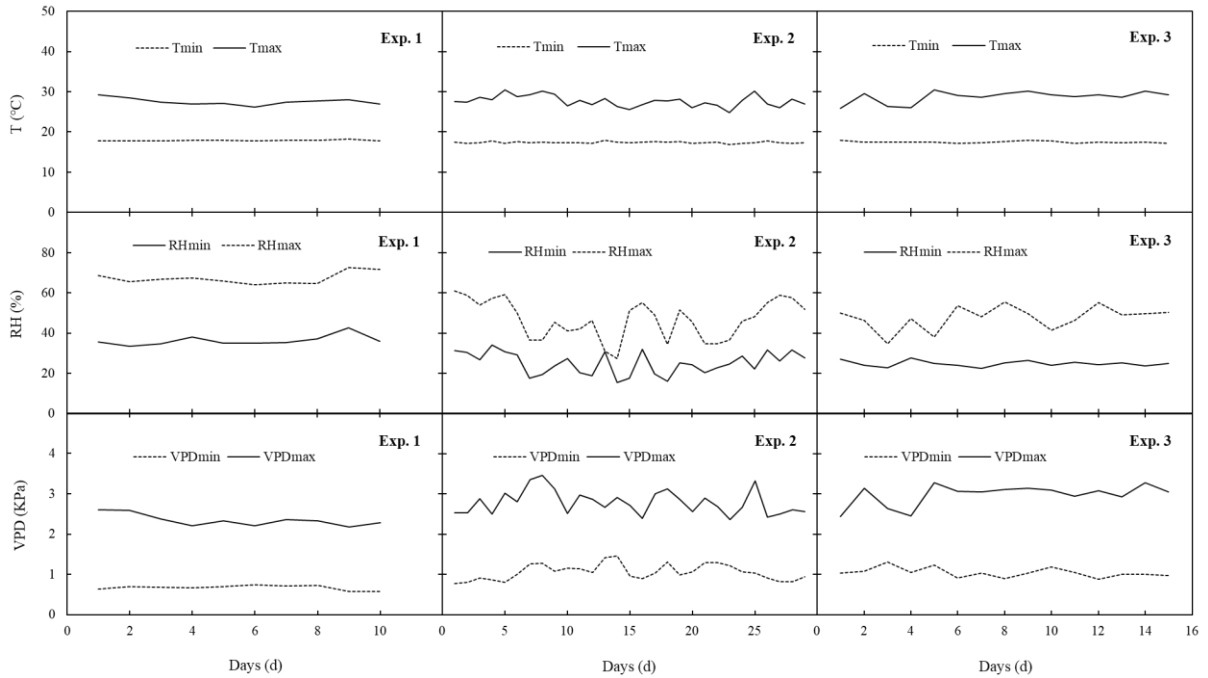
Supplementary Fig. 2. Relationships between leaf water potential (Ψ_{leaf}) and shoot water

potential (Ψ_{shoot}) in Experiments 1 (A), 2 (B) and 3 (C), respectively. Each point is an individual

plant, with regression lines fitted when significant: *** indicates statistical significance at $P <$

0.001.
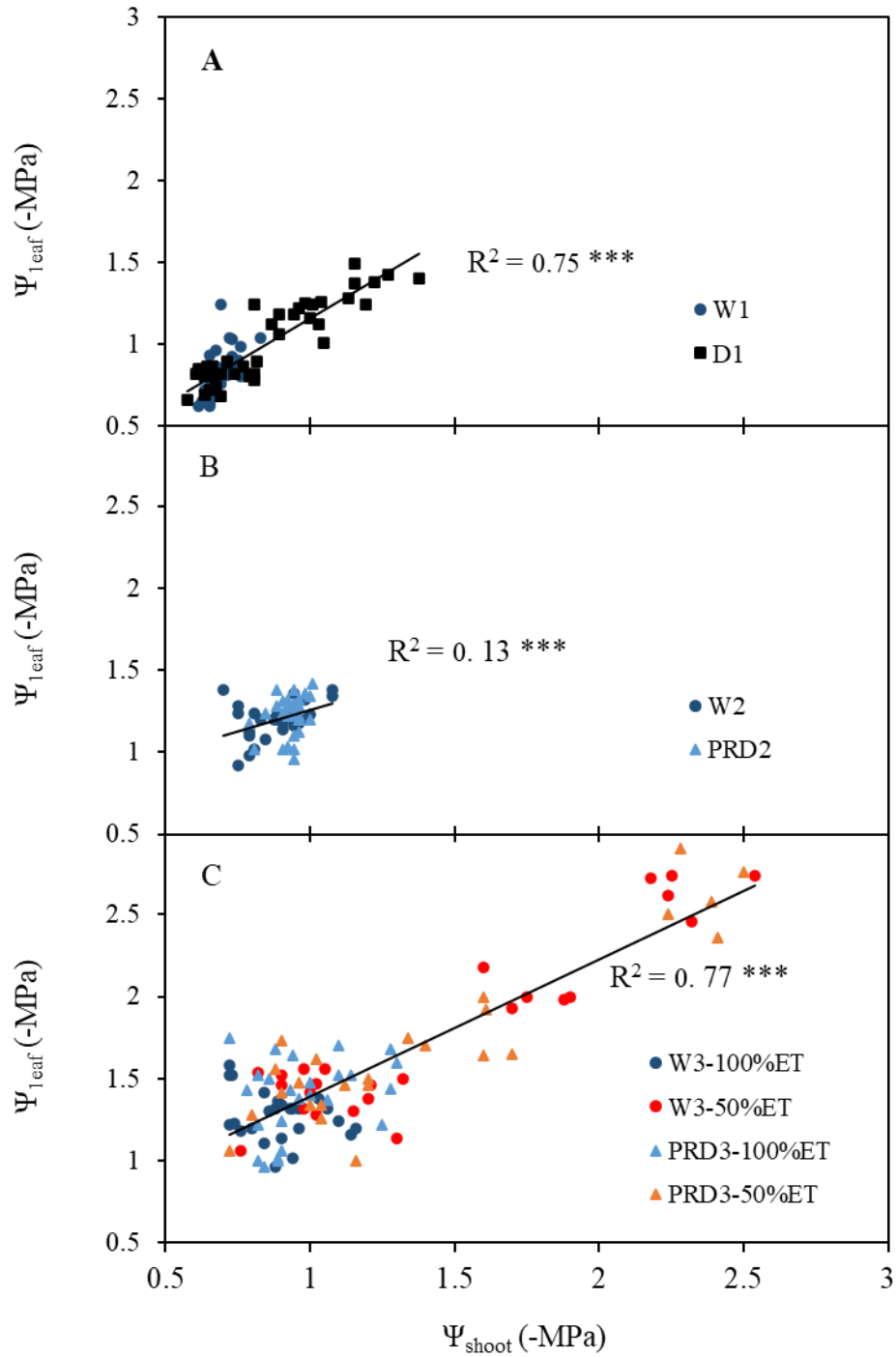
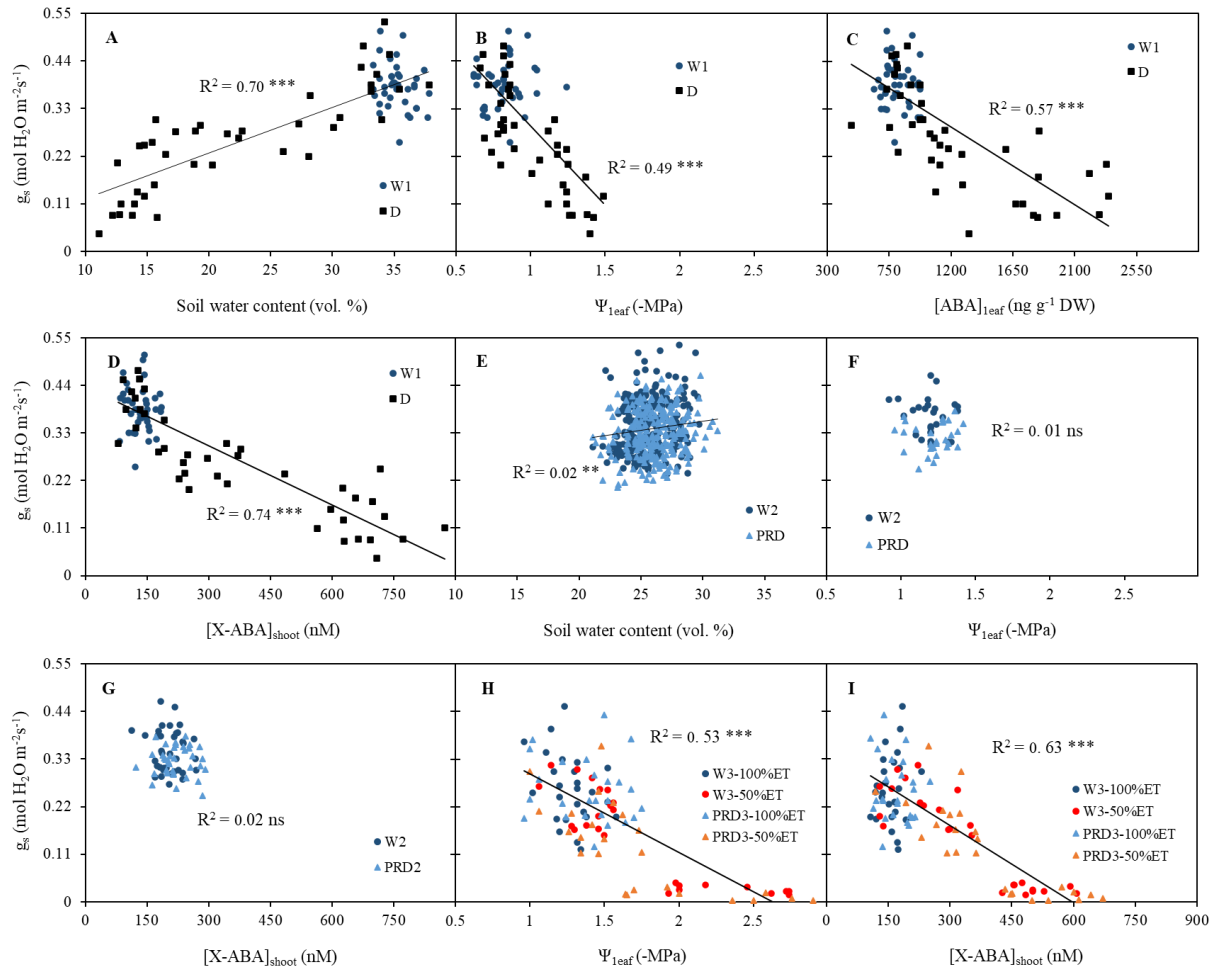**Supplementary Fig. 3.** Relationships between leaf ABA concentration ($[ABA]_{leaf}$) and shoot

water potential ($[X\text{-}ABA]_{shoot}$) in Experiments 1 (A), 2 (B) and 3 (C), respectively. Each point

is an individual plant, with regression lines fitted when significant: *** indicates statistical

significance at $P < 0.001$.

872 **Supplementary Fig. 4.** Relationships between leaf ABA concentration measured in UK via
873 radioimmunoassay (RIA) and in Germany via LC-MS-MS of samples from Experiment 3. Each
874 point is an individual plant, with regression lines fitted when significant: *** indicates statistical
875 significance at $P < 0.001$.
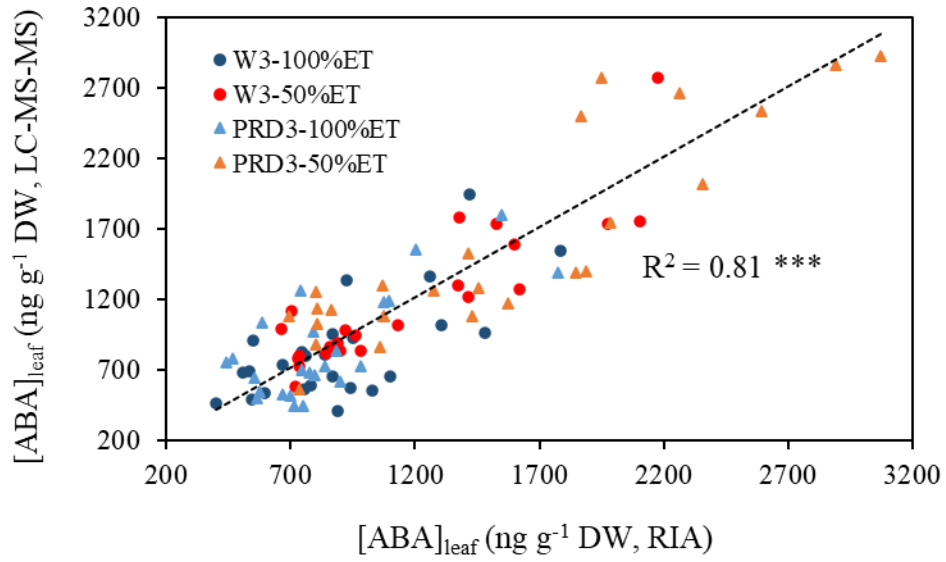
Supplementary Fig. 5. Relationships between stomatal conductance (gs) and gravimetric soil
water content (A, E), leaf water potential (B, F, H), leaf ABA concentration (C) and shoot xylem
sap ABA concentration (D, G, I) in Experiments 1 (A-D), 2 (E-G) and 3 (H-I), respectively.
Each point is an individual plant, with linear regressions fitted when significant: ** and ***
indicates statistically significance at $P < 0.01$ and $P < 0.001$, ns indicates not significant.
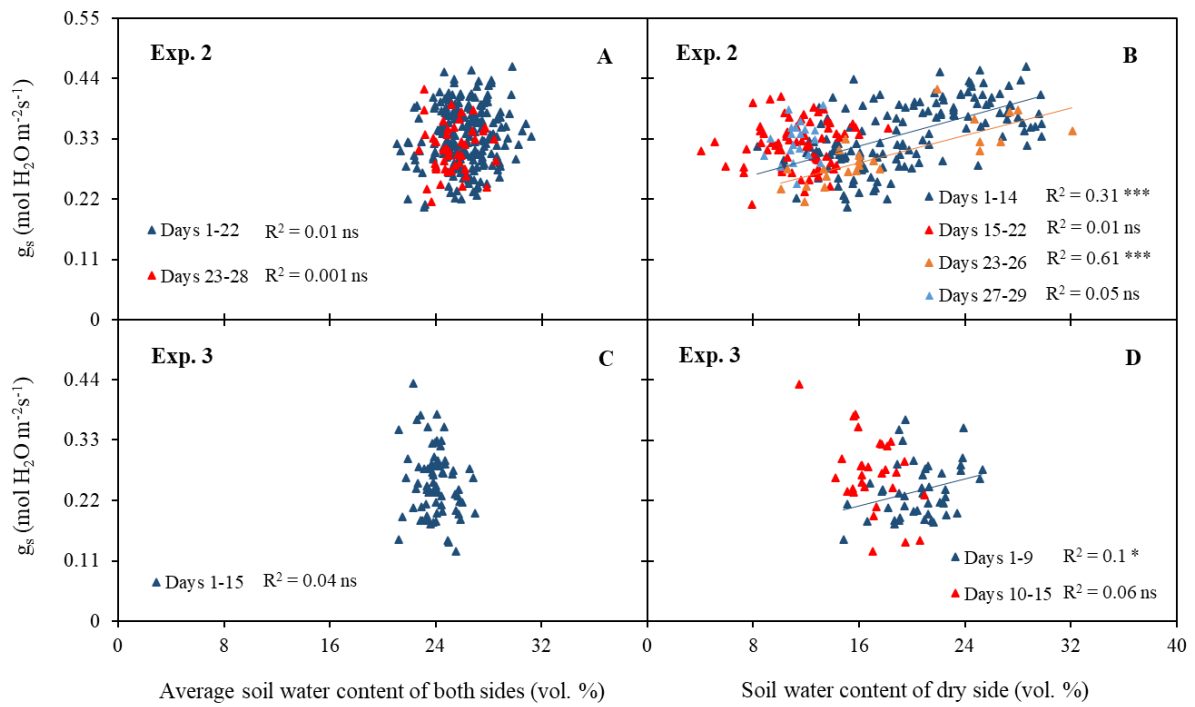
Supplementary Fig. 7. Evapotranspiration of the treatments in Experiments 1 (A), 2 (B) and 3

(C), respectively. Black vertical dotted line indicates when irrigated and drying compartments

were alternated in (B). Each point is the mean value of 4, 5 and 6 replicate plants in Experiments

1, 2 and 3, respectively.
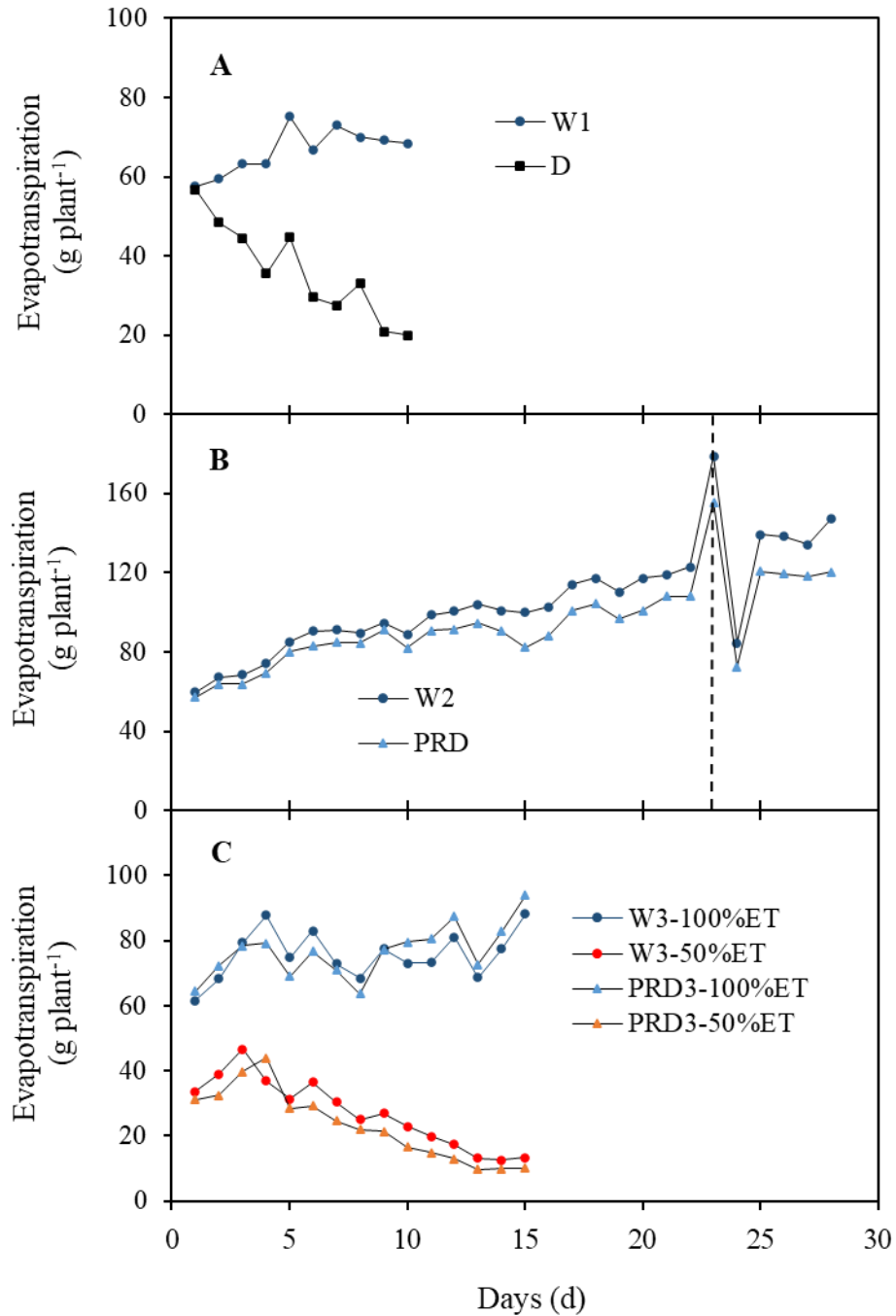
Supplementary Table 2 Leaf jasmonic acid concentration ([JA]$_{leaf}$), leaf jasmonic acid

892 isoleucine concentration ([JA-Ile]$_{leaf}$) and leaf OH-JA-Ile concentration ([OH-JA-Ile]$_{leaf}$) in

893 Experiment 3. Data are means ± SE of 6 replicate plants.

Treatments	[JA]$_{leaf}$ (ng g^{-1} DW)	[JA-Ile]$_{leaf}$ (ng g^{-1} DW)	[OH-JA-Ile]$_{leaf}$ (ng g^{-1} DW)
	2d		
W3-100%ET	32.5 ± 3.7	2.1 ± 0.1	12.8 ± 2.6
W3-50%ET	30.7 ± 3.2	2.1 ± 0.5	13.5 ± 1.4
PRD3-100%ET	33.0 ± 3.9	2.7 ± 0.3	14.9 ± 2.2
PRD3-50%ET	36.9 ± 3.5	2.2 ± 0.6	13.0 ± 4.4
	5d		
W3-100%ET	31.3 ± 3.1	2.0 ± 0.8	14.3 ± 1.9
W3-50%ET	22.0 ± 2.4	2.8 ± 0.3	16.4 ± 3.6
PRD3-100%ET	28.4 ± 4.7	3.4 ± 1.3	15.9 ± 5.0
PRD3-50%ET	27.4 ± 5.7	3.0 ± 0.9	14.1 ± 2.0
	8d		
W3-100%ET	37.0 ± 6.9	2.0 ± 0.6	13.2 ± 3.1
W3-50%ET	35.7 ± 3.3	1.8 ± 0.4	11.6 ± 1.4
PRD3-100%ET	36.3 ± 3.5	2.3 ± 0.4	14.5 ± 2.1
PRD3-50%ET	37.0 ± 2.3	2.1 ± 0.5	13.0 ± 1.3
	14d		
W3-100%ET	39.0 ± 4.1	2.7 ± 0.3	13.3 ± 0.4
W3-50%ET	37.3 ± 6.7	3.2 ± 0.4	8.5 ± 2.2
PRD3-100%ET	35.3 ± 1.2	2.4 ± 0.9	9.2 ± 3.3
PRD3-50%ET	34.2 ± 4.7	4.3 ± 1.5	13.6 ± 2.6
	15d		
W3-100%ET	42.4 ± 3.4	2.3 ± 1.1	15.3 ± 2.7
W3-50%ET	32.3 ± 6.2	3.2 ± 0.4	6.4 ± 1.2
PRD3-100%ET	34.1 ± 2.3	2.2 ± 0.5	11.9 ± 3.2
PRD3-50%ET	35.0 ± 4.5	4.5 ± 0.6	15.2 ± 6.3

894 **Supplementary Table 3** Output of two-way ANOVA (P values reported) for leaf JA
895 concentration ($[JA]_{leaf}$), leaf JA-Ile concentration ($[JA-Ile]_{leaf}$) and leaf OH-JA-Ile concentration
896 ($[OH-JA-Ile]_{leaf}$) as affected by irrigation volume (100% or 50%ET), placement (PRD or DI)
897 and their interaction in Experiment 3. Bold text indicated significant ($P < 0.05$) effects.

	$[JA]_{leaf}$	$[JA-Ile]_{leaf}$	$[OH-JA-Ile]_{leaf}$
Significance: 2d			
Volume	0.28	0.50	0.83
Placement	**0.04**	0.07	0.78
Interaction	0.65	0.10	0.66
Significance: 5d			
Volume	0.25	0.39	0.97
Placement	0.77	0.51	0.91
Interaction	0.34	0.83	0.58
Significance: 8d			
Volume	0.96	0.69	0.47
Placement	0.95	0.61	0.51
Interaction	0.83	0.97	0.97
Significance: 14d			
Volume	0.76	0.20	0.94
Placement	0.94	0.65	0.07
Interaction	0.47	0.48	0.84
Significance: 15d			
Volume	0.31	0.35	0.48
Placement	0.53	**0.03**	0.49
Interaction	0.23	0.40	0.13