Agronomic and physiological impacts of irrigation frequency on green basil (*Ocimum basilicum* L.)

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Abstract

Water scarcity is a major factor restricting agricultural production and irrigation globally, with sustainable agricultural development calling for less irrigation water use and more production per unit of water applied. Improved understanding of plant physiological responses to water stress, and the effect of irrigation frequency on plant biomass production and quality, may help to optimize irrigation scheduling. Glasshouse-grown basil (*Ocimum basilicum* L.) received three different irrigation strategies: well watered, WW (daily irrigation with full crop evapotranspiration, as control), sustained deficit irrigation, SDI (daily irrigation with 75% full crop evapotranspiration) and infrequent drought and re-watering, DRW (applying the same volume of water as SDI but every 6 days). Leaf water potential (Ψ_{leaf}) and shoot xylem sap ABA concentration ($[ABA]_{xyl}$) were correlated with decreased stomatal conductance (g_s) under both deficit irrigation treatments. While the relationship between g_s and Ψ_{leaf} depended on irrigation frequency, g_s consistently declined as $[ABA]_{xyl}$ increased, in both intact plants (under both irrigation frequency treatments) and detached shoots fed synthetic ABA via the transpiration stream. Thus ABA played a dominant role in mediating stomatal closure in response to soil water deficit. Both SDI and DRW increased plant water use efficiency (WUE), and significantly increased the foliar phenolic composition (caffeic acid by 9% and 12%, and rosmarinic acid by 6% and 10%, respectively). Compared to WW plants, SDI increased biomass production (by 8% and 18% in leaf area and dry weight) but negatively affected quality (an undesirable peppery taste, with a rubbery texture during chewing). Although DRW decreased biomass production (by 12% for both leaf area and dry weight), quality was improved (traditional taste and flavor with a slight sweetness). To summarise, basil can be cultivated with less irrigation, but with different effects on either yield or quality according to irrigation frequency.

Keywords

soil water content, shoot water potential , transpiration rate, relative humidity

Declaration

I declare that this work has been originally produced by myself for the present dissertation and it has not previously been submitted for the award of a higher degree at any other institution.

Lancaster, UK, September 2015

Introduction

Water is an increasingly scarce resource on a global scale due to the demands of an expanding human population, and the urban, industrial, and environmental sectors (Fereres and Soriano, 2007). Irrigation of agricultural lands is a major consumer of water, accounting for over two thirds of total fresh water usage worldwide (Fereres and Evans, 2006). Water scarcity is a major factor restricting agricultural production and the use of irrigation all over the world (Turner, 1986, Martin et al., 1989). In some regions, naturally available water supplies are insufficient to satisfy full crop water requirements. In other locations, regulation of water supplies for irrigation results in insufficient irrigation to maximize agricultural productivity (Martin et al., 1989). For sustainable agricultural development, irrigation strategies should be built on the more efficient use of an often limited water resource (Condon et al., 2004, Fereres and Soriano, 2007). Thus improving crop water use efficiency (WUE) should be a key issue for research (Costa et al., 2007).

WUE is defined as the ratio between crop yield (total harvestable biomass or marketed yield) and either applied irrigation volume or total growing season evapotranspiration (De Wit, 1958, Taylor et al., 1983). Growing crop genotypes with increased WUE (Condon et al., 2004), adopting drip irrigation and better irrigation scheduling are all techniques that improve WUE (Chaves et al., 2003). Although drip irrigation and protected cultivation can improve WUE by decreasing water runoff and limiting evapotranspiration losses respectively (Stanghellini et al., 2003, Jones, 2004, Kirnak and Demirtas, 2006), their use may be restricted by the high infrastructure costs of installation. Appropriate irrigation scheduling that increases WUE is needed (Costa et al., 2007), but successful application of these techniques likely needs a sound understanding of plant physiological responses to water deficit.

Water deficit and plant physiological responses

Plant water deficit occurs when the rate of transpiration exceeds the rate of water uptake, which induces stomatal closure and inhibits leaf growth (Chaves et al., 2002, Ache et al., 2010) and in severe cases, wilting, damage to cell membranes and death by dehydration (Bray, 1997, Wilkinson and Davies, 2010). Since stomatal closure is a primary response to limited water availability, induced by soil drying or high

atmospheric evaporative demand, many studies have sought to understand its regulation.

Stomatal closure may be triggered by decreased leaf water potential (Ψ_{leaf}) and turgor (Comstock and Mencuccini, 1998, Mencuccini et al., 2000), since stomatal conductance and leaf water relations can decline in parallel as the soil dries. Furthermore, experiments that increased leaf water status by applying pneumatic pressure to the roots of plants in drying soil showed that stomata could be made to reopen (Saliendra et al., 1995, Fuchs and Livingston, 1996, Comstock and Mencuccini, 1998), suggesting that leaf water status directly regulated stomatal conductance (g_s).

However, several experiments have demonstrated stomatal closure without any changes in, or even increased, leaf water status. After water was withheld from apple trees in the field, low g_s was associated with higher Ψ_{leaf} (Jones et al., 1983). Furthermore, root pressurization of wheat and sunflower plants grown in drying soil failed to re-open the stomata even though the leaves were at full turgor (Gollan et al., 1986). When plants were grown with roots split between two compartments, withholding water from one compartment (while the other remained well-watered) did not decrease $\Psi_{leaf,}$ but decreased g_s of maize (Blackman and Davies, 1985) and inhibited apple leaf growth (Gowing et al., 1990). Re-watering the dry soil or excising the roots in the drying compartment resulted in leaf growth recovery. These studies suggested that root-sourced chemical signalling regulated shoot physiological responses to soil drying.

Abscisic acid (ABA) has been suggested to play a pivotal role in root to shoot communication of water stress (Wilkinson and Davies, 2002, Christmann et al., 2006) and can significantly promote stomatal closure (Jones and Mansfield, 1970). The combination of ABA and its receptors (with external and internal loci in plasma membranes) in the stomatal guard cells induces an internal signal transduction cascade (usually involving increases in both externally and internally sourced cytoplasmic calcium), which eventually reduces guard cell osmotic potential to cause stomatal closure (McAinsh et al., 1997, Assmann and Shimazaki, 1999). Increased

xylem ABA concentration ($[ABA]_{xyl}$) was correlated with decreased g_s in several species (Zhang and Davies, 1990, Tardieu and Davies, 1992, Tardieu et al., 1996, Borel et al., 2001) but it can be difficult to be certain whether increased $[ABA]_{xyl}$ causes, or is a response to, stomatal closure. Decreased transpiration rate could increase $[ABA]_{xyl}$ if loading of ABA into the xylem remains unchanged as the soil dries (Dodd, 2005, Dodd et al., 2008), yet ABA delivery (the product of concentration and flow rate) to the shoot can be increased by soil drying (Jokhan et al. 1996). In white lupins (Loveys, 1984) and maize (Zhang and Davies, 1991), feeding synthetic ABA to detached leaves via the transpiration stream induced stomatal closure, while the antitranspirant activity of maize xylem sap was eliminated by removing its ABA content (Zhang and Davies, 1991). These observations provide a strong case for xylem-borne ABA acting as a regulatory signal to close the stomata.

However, supplying synthetic ABA to detached wheat leaves (at the concentrations detected *in vivo*) via the transpiration stream failed to promote stomatal closure, and removing ABA from wheat xylem sap had no impact on its antitranspirant effect (Munns and King, 1988), suggesting the existence of other xylem-borne antitranspirants. Soil drying raised the pH of the xylem sap, which correlated with stomatal closur (Wilkinson et al., 1998), and xylem sap alkalinisation increased the stomatal sensitivity to xylem ABA (Schurr et al., 1992). In addition, different species may show different chemical signalling responses: soil drying increased $[ABA]_{xyl}$ in tobacco and promoted stomatal closure (Borel et al., 2001), while the small changes in $[ABA]_{xyl}$ detected in apricots failed to induce stomatal closure (Loveys et al., 1987). Understanding species variation in different physiological responses to soil drying may provide basic information for improving irrigation practice and improving crop use efficiency (Wilkinson and Hartung, 2009). Partial stomatal closure can improve leaf water use efficiency (the ratio of photosynthesis to g_s or transpiration) if transpiration is decreased while photosynthesis is unchanged (Davies et al., 1978, Jones, 1992, Liu et al., 2005), and is an intended aim of several irrigation strategies that aim to limit crop water use and/or induce other physiological responses.

Deficit irrigation effects on water use efficiency, crop yield and quality.

Although sufficient irrigation can maintain yield and improve plant quality (Schultz,

2000), applying less water than full crop-water requirements (termed "deficit irrigation" - DI) can improve crop quality, and sometimes economic yield, while decreasing water use (English, 1990, Fereres and Soriano, 2007). In cucumber crop, irrigation at 70% crop evapotranspiration (70% ETc) increased crop yield and WUE compared to full irrigation (100% ETc) (Rahil and Qanadillo, 2015). DI (irrigation at 50% ET_c) promoted long-term WUE compared with fully-irrigated grapevines (100% ET_c), and increased berry anthocyanin and total phenol concentrations (de Souza et al., 2005). In addition, DI can control excessive vegetative growth and optimize fruit production and quality (Goodwin and Jerie, 1992). DI practices may also decrease leaching of nutrients and pesticides into groundwater (Teviotdale et al., 2001). Finding a favourable tradeoff between WUE, crop yield and quality is imperative for the successful application of DI in irrigated agriculture.

Deficit irrigation may be simply imposed by decreasing irrigation frequency. Increased irrigation frequency increased yield of rose (Katsoulas et al., 2006), summer squash (Ertek et al., 2004), and melon (Sensoy et al., 2007). However, decreasing irrigation frequency decreased cracking of radishes thereby improving crop quality (Wan and Kang, 2006). These studies indicated that optimizing irrigation frequency could improve crop production and quality.

DI has had most success when applied to grapevine and several fruit tree crops, while other crops like vegetables tended have not adapted so well, showing decreased yield and quality (Costa et al., 2007). Relatively few studies have considered the impacts of DI on leafy crops such as lettuce (Yazgan et al., 2008) and fresh herbs (Bekhradi et al. 2015). Moreover, many studies of irrigation frequency have examined the effects of different irrigation intervals on crop yield and quality, with few able to discriminate the effects of irrigation volume and frequency (Qian and Fry, 1996). Taken together, varying irrigation volume and frequency may provide more efficient irrigation strategies to cope with water scarcity.

Deficit irrigation effects on Basil

Basil (*Ocimum basilicum* L.) is widely grown for its leaves and seeds, fresh leaves for cooking, dry leaves for spice industries, and as an ornamental (Topalov, 1962,

Simon et al., 1990). It is a source of aroma compounds used for medical treatments (Charles et al., 1990), the success of which are attributed to the essential oils and soluble phenolic fractions (Thorsen and Hildebrandt, 2003, Politeo et al., 2007, Surveswaran et al., 2007). These include rosmarinic acid, the most prevalent phenolic in basil, which at high concentrations contributes to antioxidant, antibacterial, anti-inflammatory and anti-HIV activities (Mazumder et al., 1997, Javanmardi et al., 2002, Petersen and Simmonds, 2003). Furthermore, caffeic acid (another constituent of basil) inhibited oxidation of low density lipoprotein (LDL) *in vitro*, which may provide protection from cardiovascular disease (Laranjinha et al., 1994, Nardini et al., 1995, Olthof et al., 2001). While higher foliar concentrations of phenolic compounds may improve human health and crop nutritive value, it is not clear to what extent irrigation management alters concentrations of these constituents.

Basil is commercially produced in field, greenhouse and hydroponic growing systems (Craker, 2003), and black plastic (cover trickle irrigation tubes) is widely used in raised-bed basil culture for high quality (Loughrin and Kasperbauer, 2001). In purple basil, water stress decreased plant height and yields, while positively affecting the essential oil content (Ekren et al., 2012) and anthocyanin and proline content (Alishah, 2006). In contrast, applying 75% of field water capacity (FWC) on both sweet basil and american basil obtained the highest yield of herb and essential oil concentrations compared with other irrigation treatments (50% and 100% of FWC) (Khalid, 2006). Additionally, in purple and green Iranian cultivars and Genovese variety of basil, deficit irrigation failed to maintain sensory quality due to leaf darkening, but showed better aroma and increased antioxidant capacity than fully irrigated plants (Bekhradi et al., 2015). Although cultivating basil under deficit irrigation can improve both quality and WUE while reducing the water supplied, no generic recommendations emerge from the literature, suggesting that irrigation scheduling needs to be optimised for the specific production system.

Project aims

Increasing demand for basil in European markets has increased its cultivated area in Mediterranean countries (Putievsky and Galambosi, 1999), where water availability is a major restriction. Therefore decreasing irrigation volumes to basil, while

maintaining yield and quality, are considered desirable (Bekhradi et al., 2015). **Thus** the impact of irrigation frequency (at the same irrigation volume) on physiological (root-to-shoot signalling and crop water relations) and agronomic (water use efficiency, yield, production quality assessed by a consumer panel and measurements of chemical composition) variables of basil was investigated. This research proposes two hypotheses below:

Frequency of irrigation under deficit irrigation may manipulate root-to-shoot signalling and affect crop WUE.

Frequency of irrigation under deficit irrigation may maintain (even enhance) basil yield and quality.

Three different irrigation strategies were applied: WW (daily irrigation with full crop evapotranspiration, as control), sustained deficit irrigation, SDI (daily irrigation with 75% full crop evapotranspiration) and infrequent drought and re-watering, DRW (applying the same volume of water as SDI but once every 6 days). Since both ABA and leaf water potential were correlated with stomatal closure at both irrigation frequencies, further studies sought to resolve their relative importance in determining stomatal responses by imposing soil drying on plants grown at two relative humidities, and feeding synthetic ABA to detached basil shoots.

Materials and methods

Experiment 1: Effects of different irrigation frequency

Plant material and culture

Basil (*Ocimum basilicum* L. cv. Genovese) seeds (Moles Seeds, Colchester, UK) were sown in cylindrical 1.05 L (13 cm diameter x 11.3 cm high) pots (Pöppelman TEKU®, Germany), filled with a well-watered peat-based substrate (Levington's M3, Scotts Company Ltd, UK) with three seeds in each pot. During germination and subsequent growth, all pots were placed on a saucer and maintained in a naturally lit greenhouse compartment (5 m x 3 m) at the Lancaster Environment Centre, with supplementary lighting (Osram Plantastar sodium lamps, Augsburg, Germany) over a 14 h photoperiod (06:00 h to 20:00 h). Lights were suspended 1.6 m above bench height and provided an average photosynthetic photon flux density (*PPFD*) of 325 μ mol m^{-2} s^{-1} at bench height. The day/night temperature was 25/17 °C (the average temperature was 21.6 °C), the day/night relative humidity was 33/44 %, and the average CO_2 concentration was 490 ppm. After the first set of true leaves emerged (2 weeks), extra seedlings were removed to retain only one seedling in each pot, ensuring that all seedlings were of similar size. During subsequent growth and throughout the whole experiment, the position of all the pots in the greenhouse was re-randomized daily when the plants were watered (17:00h daily). A total of 100 plants were grown per irrigation treatment, with 4 plants per irrigation treatment measured per day for each of variables described below (from April to May).

Irrigation treatments

After another 2 weeks, to minimize evaporative losses from the substrate, the surface of all the pots was covered with black tape around the shoot, leaving an area of approximately 15.6 cm^2 (11% of the pot surface area). At this time, all pots were watered (150 mL) until drainage was visible from the bottom of the pot. Plants were left to freely drain for 24 h，at which point all pots were weighed using a balance with 0.1 g resolution (Scout Pro Portable balance, Ohaus, Switzerland) as a reference weight (Day 0). Plants were then randomly allocated to one of three treatments:

- A well watered control of 100% ET, where plants received 100% of the previous day's

mean evapotranspiration (determined gravimetrically) via daily irrigation;

- SDI (Sustained Deficit Irrigation) where plants received 75% of the previous day's mean evapotranspiration of control plants (determined gravimetrically) via daily irrigation
- DRW (Drying and Re-watering), where plants received no irrigation for 6 days, then received 75% of the accumulated ET for the 100% ET treatment at the end of the 6th day (Table 1).

Thus the SDI and DRW treatments received the same irrigation volume but the frequency varied (daily *versus* every 6 days). Plants from all irrigation treatments were sampled for physiological measurements and harvested every 2 days.

Table 1. Sustained irrigation treatments (well watered = 100% ET *versus* deficit = 75% ET) versus infrequent drying and re-watering (DRW) treatments. Six days comprised one irrigation cycle, with 3 cycles throughout the experiment.

Volume of Water added (Evapotranspiration)	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Total water added**
100% ET (WW)	V_1	V_2	V_3	V_4	V_5	V_6	V_T
75% ET (SDI)	$75\%V_1$	$75\%V_2$	$75\%V_3$	$75\%V_4$	$75\%V_5$	$75\%V_6$	$75\%V_T$
DRW	0	0	0	0	0	0	**Re-watering** $75\%V_T$

Soil measurements

A theta probe (ML2x, Delta-T Devices, Cambridge, UK) connected to a HH2 Moisture Meter (Delta-T Devices, Cambridge, UK) was used to measure the volumetric soil moisture content twice in each pot (12 plants, three times per day, at 09:00h, 11:00h and 16:00h). The black tape covering the pots was first removed before measurements, the theta probe vertically inserted into the soil to a depth of 6 cm, then the probe was removed and the tape replaced.

Whole pot soil gravimetric water content (GWC) was determined (daily, following the shoot water potential measurements, 4 pots for each treatment) by collecting the entire soil volume (including roots) weighing it, (Scout Pro SPU6001 6000g Cap Digital Scale Balance, OHAUS, USA), drying it in an oven for 7 d at 60 °C and then

re-weighing. GWC was calculated as the weight of water divided by the dry weight of soil.

Physiological measurements

Stomatal conductance (g_s) was routinely measured on the abaxial side of the youngest fully expanded leaf (either side of the mid-rib) with a porometer (Model AP4, Delta-T Devices, Cambridge, UK) immediately following the soil moisture measurement (three times per day, at 09:00h, 11:00h and 16:00h).

Following measurement of g_s, leaf water potential (Ψ_{leaf}) of the same leaf was measured by thermocouple psychrometry as previously described (Dodd and Davies, 1996). These measurements were made every 2 days (11:30h, excepting day 20). An 8 mm diameter of leaf disc (from the same part of the leaf as g_s measurement) was removed, placed immediately on a clean sample holder and then wrapped in aluminum foil to minimize water loss. After 12 discs had been collected (approximately 10 min), they were unwrapped and then loaded into C52 sample chambers (Wescor Inc., Logan, UT, USA), incubated for 2 h then voltages were read with a microvoltmeter (Model HR-33T; Wescor Inc., Logan, UT, USA). Voltages were converted into water potentials based on calibration with salt solutions of known osmotic potential.

After sampling for Ψ_{leaf} measurement, the apical 4 cm of the youngest fully expanded leaf (0.0174 g average dry weight, DW) was excised (every 2 days), placed into a pre-weighed and labeled Eppendorf (1.5 ml), weighed (Precisa 125A SCS Digital Analytical Balance, Scale Model 300-9205H, PARTS) and frozen in liquid nitrogen. Samples were stored at –20 ℃ prior to determination of foliar ABA concentration ($[ABA]_{leaf}$).

Shoot water potential (Ψ_{shoot}) was determined using a Scholander-type pressure chamber (Model 3000F01 Plant Water Status Console; Soil Moisture Equipment Corp. Santa Barbara, CA, USA). The chamber was lined with moistened filter paper, and measurements were made between 11:30h and 14:00h (daily). The shoot of the plant was excised 2 cm above the cotyledons and transported in a sealed plastic bag to

minimize transpiration, and placed in the pressure chamber within 15 s of excision. Once in the chamber, the cut surface of the shoot was cleaned with deionised H_2O and filter paper. Pressure was raised in the chamber at a rate of 0.02 MPa s^{-1}, and Ψ_{shoot} recorded when xylem sap collected on the surface of the cut shoot. Following measurement of Ψ_{shoot}, an overpressure of 0.4 MPa was applied to the shoot for 60-120 s, to collect sufficient xylem sap for analysis (daily). The initial droplets of sap were discarded, then sap samples were frozen in liquid nitrogen and stored at 20 ℃ prior to determination of shoot xylem ABA concentration ($[ABA]_{xyl}$).

Following measurement of Ψ_{shoot} and xylem sap collection, whole plant leaf area was recorded (every 2 days) with a Li-3100 Area Meter (Li-Cor Inc., Lincoln, Nebraska, USA), and all the leaves was collected to determine the leaf fresh weight for each treatment (every 2 days), then transferred to an oven for 7 d at 60 °C, and re-weighing to record the leaf dry weight (every 2 days).

Abscisic acid analysis

Prior to measuring ABA, all leaf samples were freeze-dried (48 h), dry weight was measured, and samples were finely ground using dissecting scissors. Samples were then diluted with deionized water (ddH_2O, 1:50 g/mL extraction ratio), and placed on a mechanical shaker in a cold room (4°C) overnight to extract ABA.

Leaf tissue and shoot xylem sap ABA concentration was measured by competitive radioimmunoassay (RIA) (Quarrie et al., 1988), using radiolabelled ABA (DL-cis/trans [3^H] ABA) and the antibody MAC 252. Samples were centrifuged for 4 min to remove any plant fragments held in suspension, which may interfere with the assay. The RIA was undertaken by the following protocol:

200 μL 50% phosphate buffered saline (PBS; 50 mM sodium dihydrogen phosphate, 50 mM disodium hydrogen phosphate and 100 mM sodium chloride adjusted to pH 6) was added to each tube, along with 50 μL ABA standards (0, 62.5, 125, 250, 500, 1000, 2000 pg 50 μL^{-1} and 3 mM ABA) or 50 μL samples (leaf tissue extract or shoot xylem sap). Sequentially, 100 μL of ABA and 100 μL of MAC 252 were added. All tubes were centrifuged for 1 min, then replaced in sequential order in the foam rack

and refrigerated for 45 min at 4 °C. Saturated ammonium sulphate solution (0.5 mL) was added to precipitate the ABA-antibody complex. Samples were mixed by turning over the capped tubes in the rack 6 times, and were then incubated for 30 min in the dark at room temperature. Afterwards, all tubes were centrifuged for 4 min to precipitate the pellet. The remaining supernatant was discarded, and any excess liquid was removed by gently placing the rack upside down on tissue paper. 1.0 mL 50 % ammonium sulphate (50 mL saturated ammonium sulphate and 50 mL ddH_2O) was then added as a second wash to remove excess unbound radioactivity, and then the pellet re-suspended. All tubes were then centrifuged for 5 mins to reform the pellet, after which any excess supernatant was discarded. 100 μL ddH_2O was added to the pellet, which was re-suspended via gentle vibration from a bench-top whirl-mixer. Finally, 1.5 mL of Ecoscint H was added to all tubes to allow radioactivity to be visualized as fluorescence by a scintillation counter (Packard TriCARB 1600TR Liquid Scintillation Analyser; Canberra, CT, USA).

The ABA concentration from samples was calculated by referencing to the standard curve, which was used to convert readings from counts per minute (CPM) to ABA concentrations.

Transpiration bioassay of detached shoots

Well-watered plants (3 weeks old, irrigated at 100% daily evapotranspiration (ET)) were kept in a dark room overnight. Prior to starting the transpiration bioassay, the whole shoot of 30 plants was removed by a razor blade (2 cm above the cotyledons), then recut (5 mm) under deionized water (ddH_2O) to prevent embolism of the xylem. The shoot was instantly transferred to a 15 mL glass vial, containing 15 mL artificial xylem sap solution, and placed in dark room to stabilize for 2 h. The artificial xylem sap solution comprised 1 mM KH_2PO_4, 1 mM K_2HPO_4, 1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 3 mM KNO_3, and 0.1 mM MnSO4 (Dodd et al., 2003). The top of the glass vial was sealed by parafilm with a small hole to allow the shoot to be inserted in the artificial sap solution, whilst reducing evaporative losses. After 2 h, all shoots were transferred to glass vials containing 15 mL artificial xylem sap with different ABA concentrations (0, 10, 50, 100, 500, 1000 nM). Shoots were then randomized within a controlled environment growth chamber (Fig. 1).

Environmental conditions in the growth chamber were an average temperature of 24 °C, a relative humidity of 41.3±0.2% and a vapour pressure deficit (VPD) of 1.75±0.01 kPa. Metal halide lights (HR5005H, Siemens, Munich, Germany), suspended 118 cm above bench height, provided an average photosynthetic photon flux density (PPFD) of 236 µ mol m^{-2} s^{-1} at bench height. Each vial (with shoot) was weighed initially by a four point analytical balance (METTLER TOLEDO, BB 2440 BasBal), then re-weighed every hour over a 5 hour period. At the end of the assay, stomatal conductance (g_s) and leaf water potential (Ψ_{leaf}) were measured by porometry and thermocouple psychrometry respectively. Finally, total leaf area of each shoot was measured as described above, to normalise transpiration rate for leaf area.

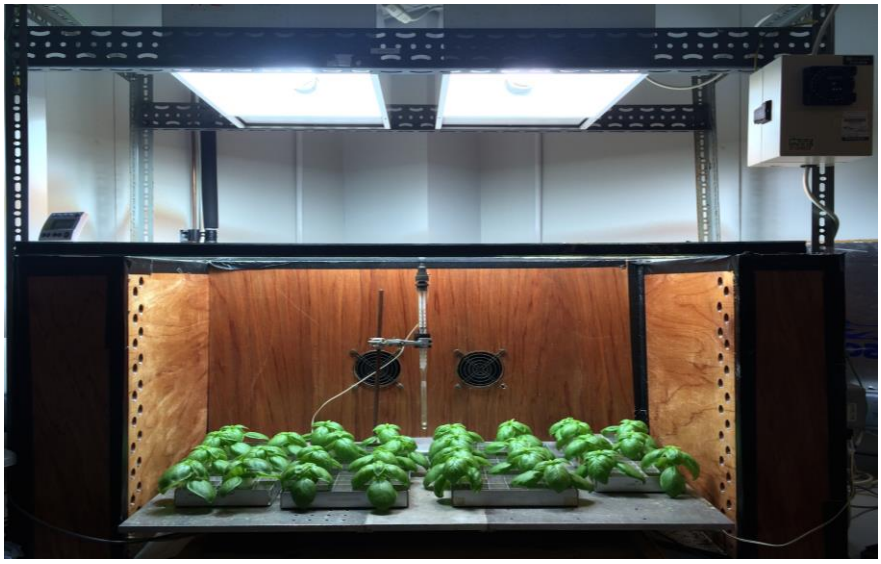


Figure 1. Detached shoots within controlled environment growth chamber.

Experiment 2: Effects of varying relative humidity on responses to soil drying

As stomata response to changes in the evaporative conditions in the atmosphere, high air humidity correlated with stomata open (Lange et al., 1971), low air humidity resulted in stomatal closure (Hall and Kaufmann, 1975). Maintaining leaf water potential by high relative humidity to detect the stomata response to drought stress, further to resolve relative importance (leaf water potential or ABA) in determining stomatal responses by imposing soil drying on plants grown at two relative humidities.

Plants (4 weeks old) were watered (150 mL) until drainage was visible from the bottom of the pot, and were left to freely drain for 24 h, at which point all pots (covered with black tape around the shoot) were weighed using a balance with 0.1 g resolution as a reference weight (Day 0). Plants were then randomized between two separate environment-controlled growth cabinets (Snijder Microclima 1750, Snijder Scientific, Tilburg The Netherlands) which had a 14 h photoperiod (06:00 h to 20:00 h) with day/night temperature of 26/20°C (**Appendix**, Fig. 15). Artificial lighting (Phillips daylight and red/far red fluorescent bulbs), suspended 108 cm above bench height, provided an average photosynthetic photon flux density (*PPFD*) of 295 µ mol m^{-2} s^{-1} at bench height. One cabinet was set to a high relative humidity of 92-95% to moderate effects of soil drying on leaf water potential (Ψ_{leaf}), while the second cabinet was set to a low relative humidity of 50% similar to greenhouse condition. The mean values of VPD were 0.26 kPa and 0.12 kPa for day and night in 92-95% humidity cabinet, 1.48 kPa and 1.16 kPa for day and night in 50% humidity cabinet.

Two treatments (lasting for 12 days) were imposed in each cabinet: 100% ET (WW) and drying without re-watering (Drying) in the 92-95% humidity cabinet. In the 50% humidity cabinet, the treatments were 100% ET (WW) and drying and watering (DRW, received no irrigation for 6 days, then received accumulated ET for the 100% ET treatment at the end of the 6^{th} day) treatments. Re-watering was necessary in the 50% RH cabinet, as the plants had dried the soil considerably (to the threshold of wilting) after 6 days. Plants from all treatments were sampled for physiological measurements daily and harvested after 12 days to measure leaf dry weight. Stomatal conductance (g_s) and leaf water potential (Ψ_{leaf}) were measured (11:00h to 11:30h), then leaf tissues collected for foliar ABA ($[ABA]_{leaf}$) determination (11:00h to 11:30h). Afterward, shoot water potential (Ψ_{shoot}) were measured, then shoot xylem sap were collected to determine shoot xylem ABA concentration ($[ABA]_{xyl}$) (11:30h to 14:00h). Whole pot soil gravimetric water content (GWC) was determined (daily) by measuring whole pot soil fresh weight and dry weight (entire soil with roots was dried in the oven for 7 d at 60 °C). Throughout the whole experiment, the position of all pots in each cabinet was re-randomized daily when the irrigation treatments were applied (17:00h daily).

Sensory evaluation and quality analysis

Taste panel

Sensory evaluation of basil plants grown under three different irrigation treatments (WW, SDI and DRW) were assessed by 4 trained panelists (R & G Fresh Herbs) using a Hedonic test. Panelists were asked to grade the color, appearance, aroma, taste and texture of the leaf samples on an ascending scale from 1 to 5, indicating increasing quality. Basil leaf quality was quantitatively evaluated as the average of all grades, and qualitatively by the comments of panelists (**Appendix**, Taste Panel Survey).

Foliar Quality analysis by HPLC

Chemicals

Caffeic acid (≥98.0%, HPLC, Sigma-Aldrich Company Ltd. UK), rosmarinic acid (≥98.0%, HPLC, Sigma-Aldrich Company Ltd. UK), ethanol (Analytical reagent grade, Fisher Scientific, UK), methanol (HPLC gradient grade, Fisher Scientific, UK), trifluoroacetic acid (HPLC gradient grade, Fisher Scientific, UK), acetonitrile (ACN, HPLC gradient grade, Fisher Scientific, UK) and water (Milli Q grade) were used in HPLC. All solvents were carefully degassed before use.

Plant materials

Leaf samples (from an additional 3 plants of each treatment in which leaf area wasn't measured, to ensure sufficient dry leaf material for sample extraction) were collected for HPLC to determine the concentrations of rosmarinic acid and caffeic acid, only on Day 0 and at the end of each drying cycle (every 6 days). Fresh leaves (at least 50 g fresh weight) were collected into 50 mL tubes (while aiming to minimize leaf damage), then were freeze-dried (48 h) and stored in sealed plastic bags before chemical analysis.

Preparation of stock and working solutions

Caffeic acid and rosmarinic acid standards (each 10 mg) were weighed into separate volumetric flasks (5 mL), and dissolved in methanol (filtered a 0.2 μm PTFE Syringe Filter) to give 2 mg/mL stock solutions of each. These were diluted with water to give 1mg/mL solutions of each standard. Caffeic acid and rosmarinic acid (0.5 mL each at 1mg/mL) were mixed to provide a standard stock solution. Then, the mixed standard

was sequentially diluted with water (0.1mg/ml, 0.05mg/ml, 0.01mg/ml, 0.005mg/ml, 0.0025mg/ml and 0.001mg/ml), to provide a calibration curve.

Plant sample extracts and sample solution preparations

Basil leaves (7 g dry weight, DW) were accurately weighed and placed in a conical flask, then ultrasonically extracted (15 min at 100W and 30℃) with 70 mL of 80% ethanol solution (the ratio of material to liquid was 1:10), extraction was repeated 3 times. The extracts were filtered by a Buchner funnel and the filtrates combined (following repetitive extractions). The filtrate was concentrated by rotary evaporator to dryness (rotational temperature not higher than 60℃), adding a certain amount (20 mL, 3 times) of 25% methanol to dissolve it (ultrasonically aided), then transferring a fixed volume into a 50 mL volumetric flask.

Sample extracts (filtered by 0.2 μm PTFE Syringe Filter) were transferred (50 μL) to Eppendorf (1.5 mL) tubes, then 950 μL methanol added to give 1mL solutions for each plant sample (the dilution factor was 20 times) for injection into the HPLC.

HPLC Conditions

The Dionex ICS-3000 liquid chromatograph system is comprised of vacuum degasser (purge with Helium), dual Pump, auto sampler, thermostatted column compartment, and diode array detector. By Kinetex F5 (100 mm length x 2.1 mm diameter, 2.6 μm) chromatographic column, mobile phase A is 0.1% Trifluoroacetic acid (TFA), B phase is Acetonitrile (ACN), gradient elution order: 0.00 mins to 5.00 mins 90% A to 20% A gradient, 5.00 mins to 15.00 mins 20% A Isocratic, 15.00 mins to 20.00 mins 20% A to 90% A gradient, 20.00 mins to 25 mins 90% A Isocratic; The ultraviolet (UV) detection wavelength was 325 nm, flow rate of 0.4 mL/min, column temperature of 30℃, injection volume was 10 μL.

Separation of caffeic acid and rosmarinic acid in standards (Marker check)

The negligible peak area responses of water in UV spectra suggested the interference of water could be omitted in this gradient condition (**Appendix**, Fig. 16a). Comparing the retention times and UV spectra of separate caffeic acid and rosmarinic acid

standards with that of the mixed caffeic acid and rosmarinic acid standard, the single peak with the shorter retention time (**Appendix,** Fig. 16b) should be the first peak in the mixed standard UV spectra (**Appendix,** Fig. 16d), which was caffeic acid with the same retention time (6.185 min). Similarly, the second peak in the UV spectra of mixed standard was rosmarinic acid with the same retention time (6.753 min) (**Appendix,** Fig. 16c,d). In addition, clear separation of caffeic acid and rosmarinic acid was achieved from 3.5 to 9.5 min, and the rest of the gradient condition ensured efficient column washing (**Appendix**, Fig. 16).

The retention times and UV spectra of caffeic acid and rosmarinic acid in reference standards were compared with that of caffeic acid and rosmarinic acid in basil sample extracts to confirm their chromatographic peaks (**Appendix**, Fig. 17a,b).

The series of standard working solutions were injected into HPLC to obtain the peak area responses. A calibration curve was constructed by plotting the concentrations of standard working solution *versus* peak area. Quantification was carried out from integrated peak areas of the samples by the corresponding calibration curve (determined by linear regression, **Appendix**, Fig. 17c,d). According to the linear regression equations (**Appendix**, Fig. 17c,d), the concentrations (mg/mL) of caffeic acid and rosmarinic acid in basil samples were measured, then the percent content of caffeic acid and rosmarinic acid in basil leaves (dry weight) calculated from the equations below:

$$CA = \frac{C[CA]\,mg/ml \times 20 \times 50\,ml}{Mg}$$

$$RA = \frac{C[RA]\,mg/ml \times 20 \times 50\,ml}{Mg}$$

CA ~ the content of caffeic acid (mg g^{-1} DW, per unit leaf dry weight)
RA ~ the content of rosmarinic acid (mg g^{-1} DW, per unit leaf dry weight)
C[CA] ~ the concentration of caffeic acid in basil samples

C[RA] ~ the concentration of rosmarinic acid in basil samples

20 ~ the dilution factor was 20 times

50 ml ~ the total volume of basil sample extracts

M ~ the dry weight of basil leaves used for extraction (the unit was g)

Statistics

The irrigation frequency experiment was repeated twice, and data from a representative experiment illustrated. Effects of different irrigation treatments on any measurement occasion were evaluated by analysis of variance (ANOVA) at $p<0.05$ using SPSS Statistics 19 (IBM), with means discriminated using *Tukey's* multiple comparisons test. Effects of irrigation treatment (SDI *versus* DRW) on relationships between plant and soil variables were determined via ANCOVA (statistically similar x-variable when comparing the 2 irrigation frequencies with a restricted x-axis range represented by the red dashed box). Effects of high and low relative humidity (92-95% RH *versus* 50% RH) on relationships between plant and soil variables were determined via ANCOVA (all the data are chosen within the same restricted range of whole pot soil gravimetric water content, $0.5< GWC<1.7$ g g^{-1}). P Values from ANCOVA for each entire data set are shown in **Appendix** (Table 7-14), while effects of irrigation frequency data are compared within a restricted range (not including WW plants) in Figures 5-7.

Results

Experiment 1: Effects of different irrigation frequency

As the plants grew over the course of the experiment, irrigation volumes supplied to all irrigation treatments increased by 70%. The total irrigation volumes supplied to the WW, SDI and DRW plants by the end of the experiment were 2267, 1700 and 1700 mL respectively (Fig. 2a).

The average GWC of WW plants (2.7 ± 0.03 g g^{-1}) was consistently higher than SDI and DRW plants throughout the experiment. In the SDI and DRW treatments, GWC decreased over time (Fig. 2b), with GWC of the SDI treatment diverging from the WW treatment after 2 days of treatment. During DRW cycles, the GWC decreased from Days 0 to 6, then sharply rose after re-watering (but not to the value of WW plants) and reached the highest point on the first day of the next cycle, before declining again from the second day. DRW plants had a lower GWC than the SDI plants except on Day 0, and up to 3 days after re-watering (Fig. 2b).

The average evapotranspiration (ET) of SDI and DRW plants were 8% and 39% lower than WW plants (129 ± 3.4 mL day^{-1}) over the course of the experiment. In plants exposed to DRW cycles, ET decreased (by 56%) until the first day after re-watering, then increased over the next 2 days (reaching the values attained by SDI plants) before decreasing again as soil moisture decreased (Fig. 2c). Notably, ET of DRW plants remained low the day after re-watering.

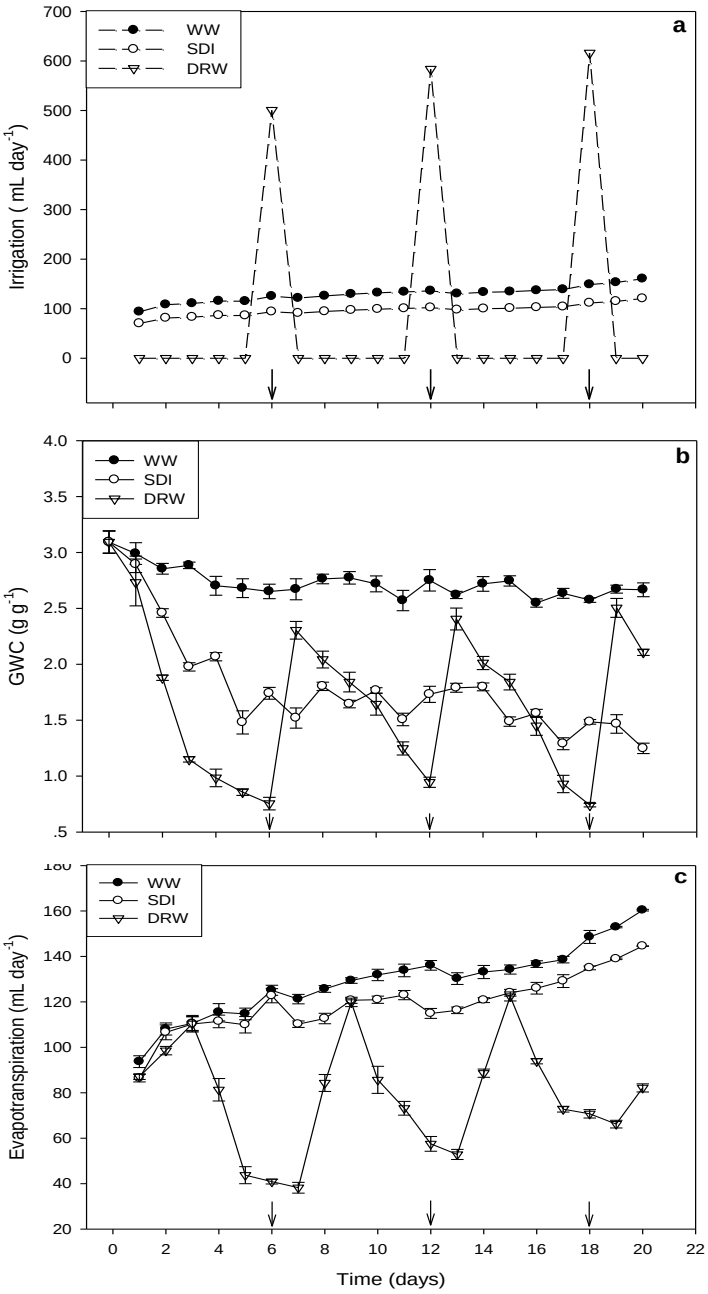


Figure 2. (a) Irrigation, (b) whole pot soil gravimetric water content (GWC), and (c) evapotranspiration of basil plants supplied with 100% ET daily (WW, filled circles), and 75% ET either supplied daily (SDI, hollow circles) or the accumulated volume every 6 days (drying and re-watering, hollow triangle). Data are means ± SEM (n=4). Arrows on x-axis indicate the day of re-watering for DRW plants.

No significant differences in leaf area or leaf dry weight were found between WW and SDI irrigation treatments during the first 12 days of the experiment. By the end of the experiment, SDI plants had the largest leaf area and dry weights (Fig. 3a,b), 8% and 18% higher than the WW plants respectively. Plants exposed to DRW had reduced leaf area and dry weight compared to plants irrigated at SDI throughout the experiment (by 17% and 34% respectively) (Fig. 3a,b).

Total plant water use (accumulated evapotranspiration) increased over the experimental period in all plants (Fig. 3c). Although accumulated ET did not differ between WW and SDI plants over the experiment, it was lower in plants exposed to DRW (by 35%) (Fig. 3c).

There was no statistically significant difference between WW and DRW plants for applied water use efficiency (calculated as leaf dry weight divided by water applied) throughout the experiment. The applied WUE of SDI plants were 25% higher than the other two treatments (Fig. 3e).

Intrinsic water use efficiency (calculated as leaf dry weight divided by water used) showed no statistically significant difference between different irrigation treatments (except after Cycle 3). After Cycle 3, SDI and DRW plants had significantly higher intrinsic WUE (by 19%) than WW plants (Fig. 3f).

Despite increases in applied water use efficiency at SDI, irrigation frequency had no effect on intrinsic water use efficiency ($p_{Frequency\ x\ ET} > 0.05$, Fig. 3d). While the two deficit irrigation strategies may allow more efficient plant water use, only SDI maintains similar leaf area and leaf dry weight to well watered controls (WW plants).

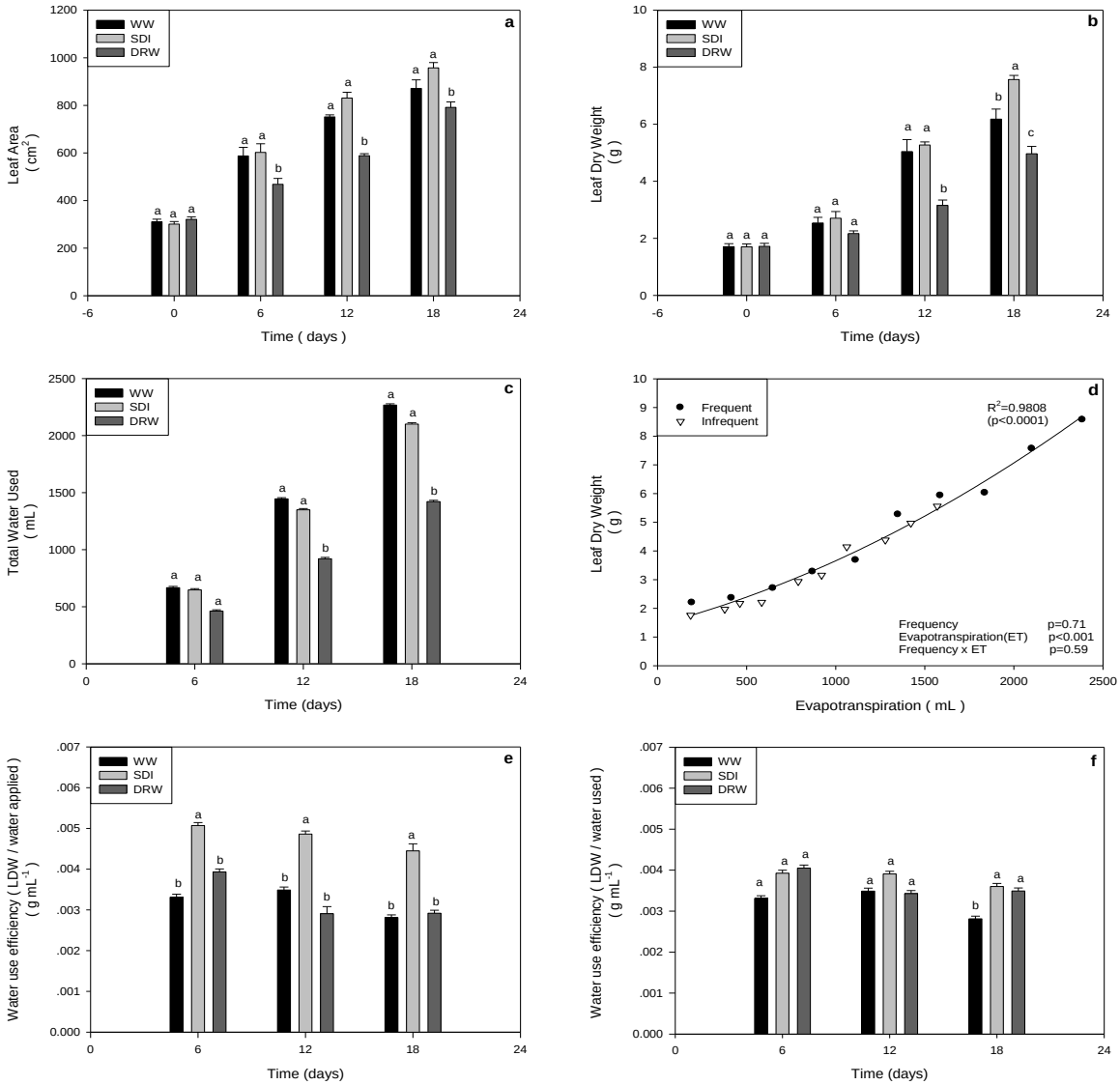


Figure 3. Leaf area (a), leaf dry weight (b), total water used (accumulated ET) (c) and water use efficiency calculated as leaf dry weight divided by water applied (e) and leaf dry weight divided by water used (f) respectively, at the end of each drying and re-wetting cycle (the black rectangle was WW, the light-grey rectangle was SDI, the dark-grey rectangle was Drying and re-watering). Data are means±SEM (n=4). Different letters in a panel indicate significant differences between each irrigation treatment on each day according to an ANOVA (p<0.05). Panel (d) plots leaf dry weight *versus* ET every 2 days, with effects of irrigation frequency at 75% ET indicated by ANCOVA (*P* values reported).

Despite differences in GWC, stomatal conductance (g_s) did not differ between WW and SDI plants (except on day 3 and day 6, **Appendix,** Table 5). Over the entire experiment, g_s of SDI plants was 9% lower than WW. In DRW cycles, g_s decreased from Days 0 to 6, then sharply rose after re-watering and reached the highest point on the second day of the next cycle, but then decreased again thereafter (Fig. 4a).

Whilst GWC decreased under SDI and DRW, generally there were no significant differences in Ψ_{leaf} between the irrigation treatments (except on Days 4, 8, 10, 12, 14 and 18, **Appendix,** Table 6). While there was no consistent pattern in Ψ_{leaf} throughout the experiment, on Days 2, 4 and 14 it was lower in the SDI treatment, on Days 8 and 10 it was higher in the DRW treatment, on Day 18 it was lower in DRW treatment. Across the entire experiment, Ψ_{leaf} averaged -0.55 ± 0.03 MPa and decreased under all irrigation treatments (by 0.16 MPa) as the experiment duration increased (Fig. 4b).

From Day 3, Ψ_{shoot} was significantly lower in plants irrigated at SDI (by 0.04 MPa) than WW plants (Table 5), a difference that was maintained (or increased) throughout the experiment. Under DRW, Ψ_{shoot} decreased from Days 0 to 6, and had the lowest value at the end of each drying cycle. In response to re-watering, Ψ_{shoot} recovered and reached the highest point on the third day of next cycle, but still remained lower (at least 0.08 MPa) than WW plants (Fig. 4c).

The average foliar ABA concentration ($[ABA]_{leaf}$) of WW plants (594 ± 70 ng g^{-1}DW) was consistently lower than SDI (758 ± 91 ng g^{-1}DW) and DRW (1779 ± 95 ng g^{-1}DW) plants throughout the experiment. DRW plants had higher $[ABA]_{leaf}$ than the SDI plants, except on Day 2. During DRW cycles, the $[ABA]_{leaf}$ increased from Days 0 to 6, then sharply dropped after re-watering and reached the lowest point on the second day of the next cycle, but increased again thereafter (Fig. 4d), all the while remaining higher than in SDI plants.

On any measurement occasion, shoot xylem sap ABA concentration ($[ABA]_{xyl}$) did not statistically differ between WW and SDI plants (except Day 18, **Appendix,** Table 6). Nevertheless, $[ABA]_{xyl}$ of SDI plants (53 ± 8 nM) was 3-fold higher than WW plants (17 ± 6 nM) averaged over the experiment. DRW plants had higher $[ABA]_{xyl}$

than SDI plants throughout the experiment (except Day 0). Under DRW, the $[ABA]_{xyl}$ increased from Days 0 to 6, then sharply dropped after re-watering and reached the lowest point on the second day of the next cycle, but generally increased again from the second day (Fig. 4e). After re-watering, DRW and SDI plants transiently showed similar $[ABA]_{xyl.}$.

In DRW cycles, soil drying decreased g_s and Ψ_{shoot} progressively until the end of each cycle, then both variables sharply rose after re-watering (but were still lower value than in WW and SDI plants). Both $[ABA]_{leaf}$ and $[ABA]_{xyl}$ had opposite trends to g_s. Although $[ABA]_{leaf}$ of DRW plants remained higher than WW and SDI plants after re-watering, $[ABA]_{xyl}$ was more responsive to fluctuations in soil moisture in DRW plants (Fig. 4).

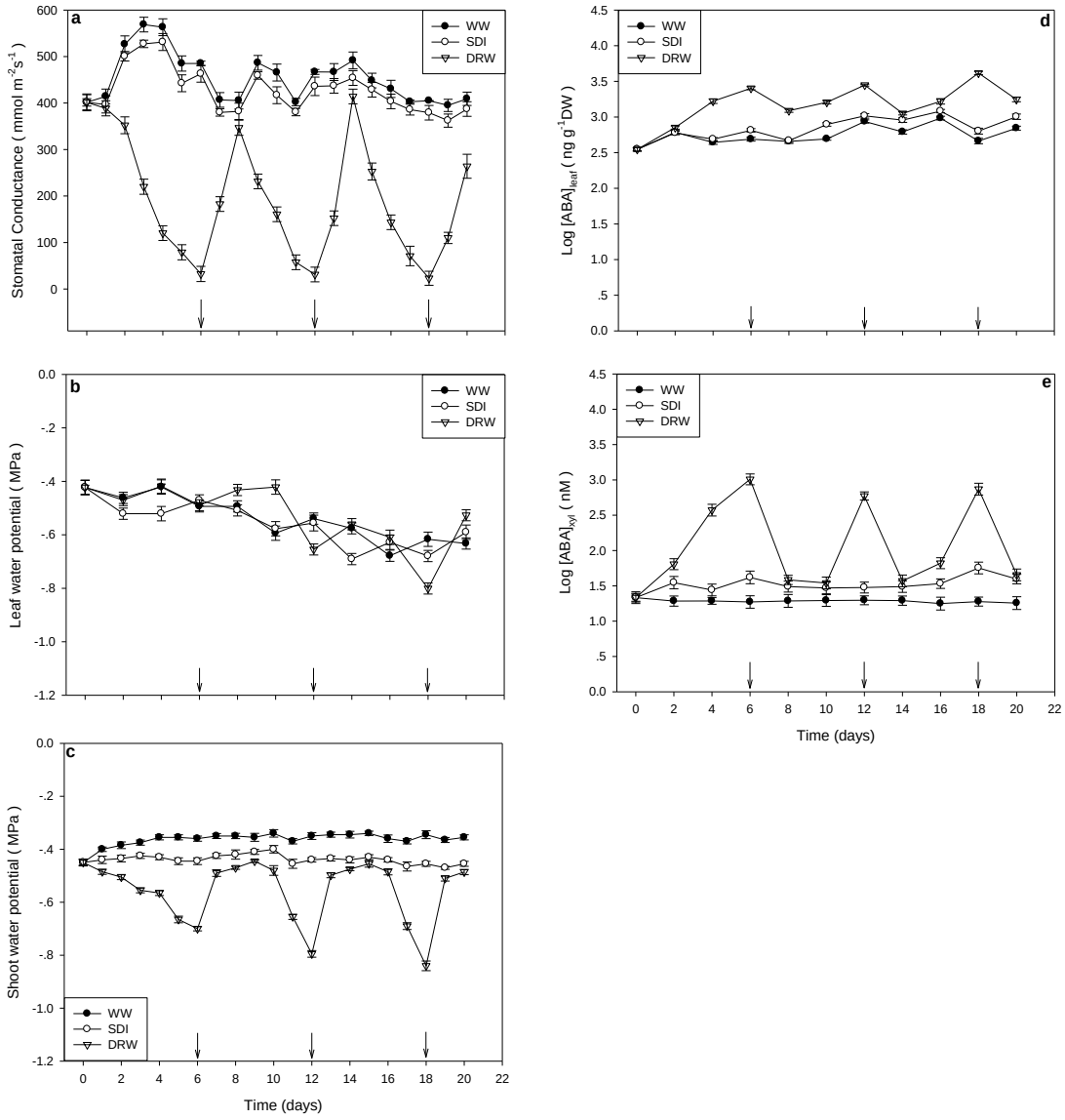


Figure 4. Stomatal conductance was measured at 11:00 h (a), leaf water potential (b), shoot water potential (c), foliar (d) and shoot xylem sap (e) ABA concentration under WW (filled circles), SDI (hollow circles), drying and re-watering (hollow triangle) treatments over time. Data are means±SEM (n=4) for each treatments, arrows on x-axis indicate the day of re-watering (at the end of the 6th day in each cycle).

Leaf water potential (Ψ_{leaf}) decreased as Ψ_{shoot} diminished, but to a greater extent with infrequent irrigation (significant Frequency x Ψ_{shoot} interaction) across the entire data set and when the range of Ψ_{shoot} was restricted ($-0.6 < \Psi_{shoot} < -0.3$ MPa – to compare both irrigation frequencies across a similar Ψ_{shoot} range). Ψ_{leaf} was generally lower (by 0.13 MPa on average) than Ψ_{shoot} in the experiment (Fig. 5a, **Appendix,** Table 7).

Foliar ABA concentration ($[ABA]_{leaf}$) increased as shoot xylem sap ABA concentration ($[ABA]_{xyl}$) increased under both irrigation frequency treatments (no significant Frequency x $[ABA]_{xyl}$ interaction) across the entire data set and when the range of $Log[ABA]_{xyl}$ was restricted ($0.5 < Log[ABA]_{xyl} < 2.0$ nM – to compare both irrigation frequencies across a similar $[ABA]_{xyl}$ range) (Fig. 5b, **Appendix**, Table 8).

As both Ψ_{leaf} and Ψ_{shoot}, and $[ABA]_{leaf}$ and $[ABA]_{xyl}$ were correlated, the following sections focus on Ψ_{leaf} and $[ABA]_{xyl}$, in explaining the effects of irrigation treatment (SDI *versus* DRW) on relationships between plant and soil variables.

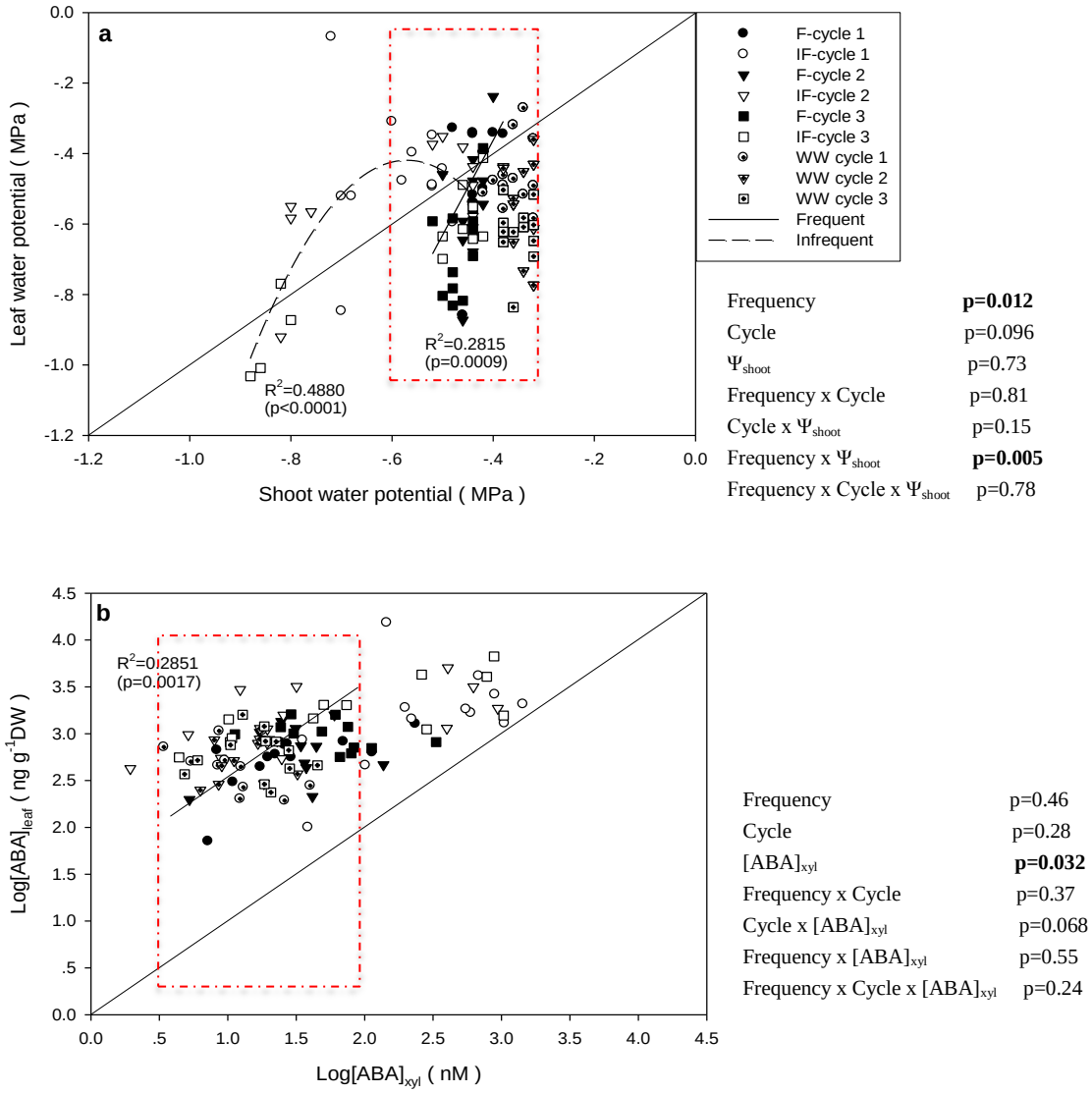


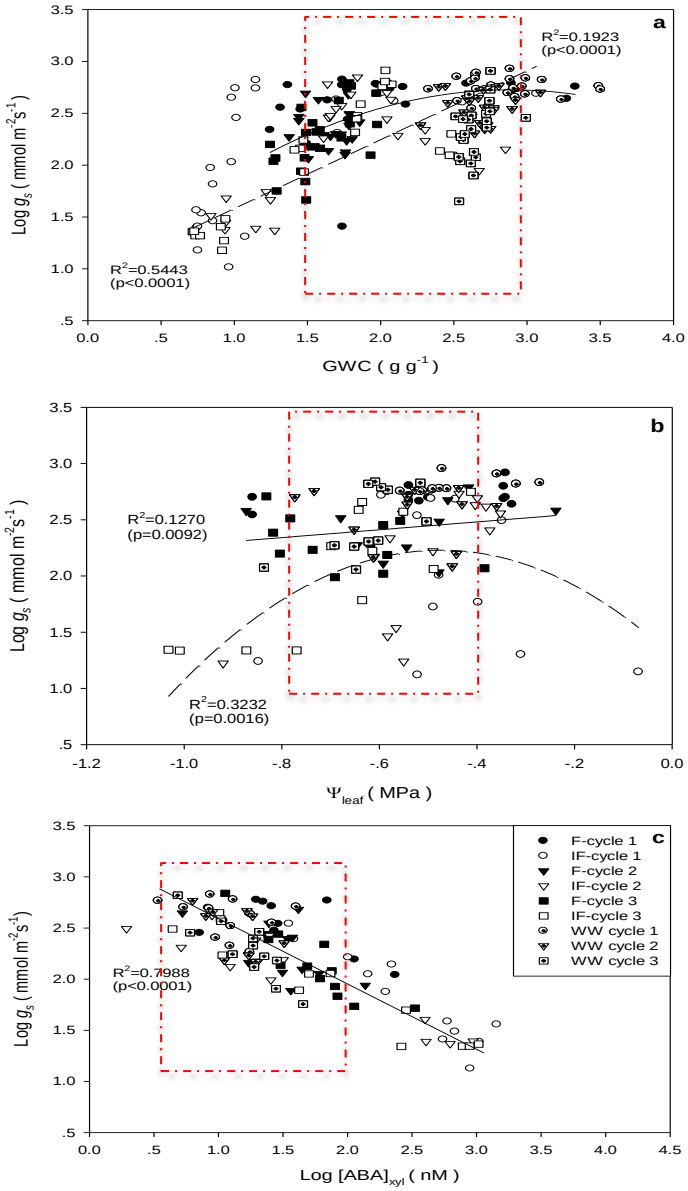
Figure 5. Shoot water potential and leaf water potential (a), foliar and shoot xylem sap ABA concentration (b) for plants grown under frequent (SDI, filled point) and infrequent (DRW, hollow point) irrigation in Cycles 1 (circle), 2 (triangle) and 3 (square). Plants under WW irrigation treatment in Cycles 1 (crossed circle), 2 (crossed triangle) and 3 (crossed square). Each point represents a single plant and regression lines were fitted to frequent (solid line) and infrequent (dashed line) treatments when significant ($P<0.05$). The 1:1 relationship is also indicated. P values determined by ANCOVA for each main effect (frequency, cycle and x-variable) and their interaction are reported for a restricted x-axis range represented by the dashed box (statistically similar x-variable when comparing the 2 irrigation frequencies).

Stomatal conductance (g_s) decreased as GWC diminished, but this response was accentuated by infrequent irrigation (significant Frequency x GWC interaction) across the entire data set and even when the range of GWC was restricted (1.5 < GWC< 3.0 g g^{-1} – to compare both irrigation frequencies across a similar GWC range). Decreased irrigation frequency results in lower g_s at the same GWC, and a tighter relationship (r^2 = 0.54 compared with r^2 = 0.19 for frequent irrigation) between g_s and GWC (Fig. 6a, **Appendix,** Table 9).

Stomatal conductance also decreased as Ψ_{leaf} decreased, and decreased irrigation frequency increased the sensitivity of g_s to Ψ_{leaf} (significant Frequency x Ψ_{leaf} interaction). Decreased irrigation frequency resulted in a lower stomatal conductance at the same Ψ_{leaf}, even if Ψ_{leaf} explained only 17% (frequent irrigation) and 25% (infrequent irrigation) of the variations in g_s (Fig. 6b, **Appendix,** Table 10). However, when the range of Ψ_{leaf} was restricted ($-0.8 < \Psi_{leaf} < -0.4$ MPa – to compare both irrigation frequencies across a similar Ψ_{leaf} range), there were no significant effects of frequency, cycle ot Ψ_{leaf} (and their interactions) on g_s (Fig. 6b). Nevertheless, g_s was still lower (by 6%) under infrequent irrigation within this Ψ_{leaf} range.

Stomatal conductance decreased as shoot xylem sap ABA concentration ($[ABA]_{xyl}$) increased, but irrigation frequency did not affect the sensitivity of g_s to $[ABA]_{xyl}$ (no significant Frequency x $[ABA]_{xyl}$ interaction) across the entire data set and when the range of Log $[ABA]_{xyl}$ was restricted (0.5 < Log $[ABA]_{xyl}$ < 2.0 nM). The impact of $[ABA]_{xyl}$ on g_s became more pronounced as the experiment duration increased, indicated by a significant Cycle x $[ABA]_{xyl}$ interaction (Fig. 6c).

Decreased GWC and Ψ_{leaf} correlated with diminished g_s, but increased $[ABA]_{xyl}$ correlated with decreased g_s. While g_s declined similarly with increasing $[ABA]_{xyl}$ under both irrigation frequency treatments (at least at higher values of $[ABA]_{xyl}$, the relationship between g_s and Ψ_{leaf} differed substantially according to irrigation frequency (Fig. 6).



Frequency	**p=0.003**
Cycle	p=0.52
GWC	p=0.62
Frequency x Cycle	p=0.062
Cycle x GWC	p=0.24
Frequency x GWC	**p=0.005**
Frequency x Cycle x GWC	p=0.085

Frequency	p=0.90
Cycle	p=0.72
Ψ_{leaf}	p=0.22
Frequency x Cycle	p=0.74
Cycle x Ψ_{leaf}	p=0.75
Frequency x Ψ_{leaf}	p=0.75
Frequency x Cycle x Ψ_{leaf}	p=0.71

Frequency	p=0.77
Cycle	p=0.066
$[ABA]_{xyl}$	**p<0.001**
Frequency x Cycle	p=0.75
Cycle x $[ABA]_{xyl}$	**p=0.001**
Frequency x $[ABA]_{xyl}$	p=0.43
Frequency x Cycle x $[ABA]_{xyl}$	p=0.77

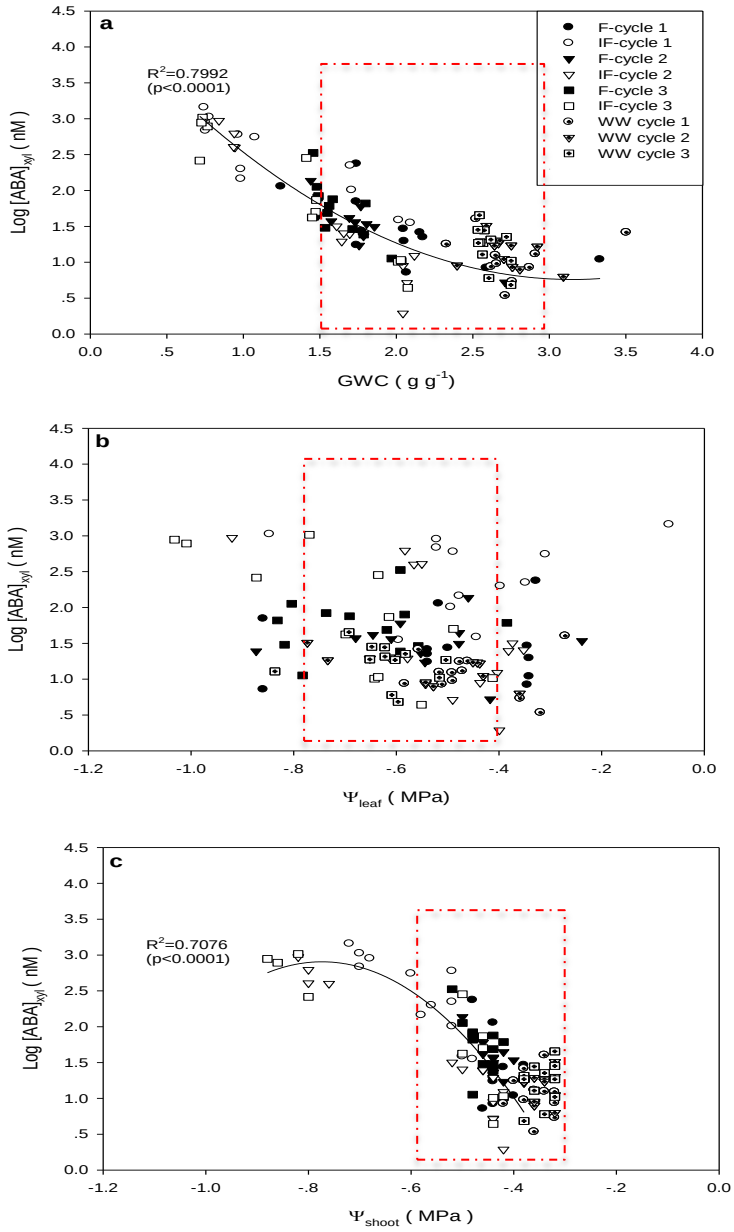
Figure 6. Relationships between stomatal conductance and (a) whole pot soil gravimetric water content, (b) leaf water potential, (c) shoot xylem sap ABA concentration for plants grown under frequent (SDI, filled point) and infrequent (DRW, hollow point) irrigation in Cycles 1 (circle), 2 (triangle) and 3 (square). Plants under WW irrigation treatment in Cycles 1 (crossed circle), 2 (crossed triangle) and 3 (crossed square). Each point represents a single plant and regression lines were fitted to frequent (solid line) and infrequent (dashed line) treatments when significant (P<0.05). P values determined by ANCOVA for each main effect (frequency, cycle and x-variable) and their interaction are reported for a restricted x-axis range represented by the dashed box (statistically similar x-variable when comparing the 2 irrigation frequencies).

As expected, shoot xylem sap ABA ($[ABA]_{xyl}$) increased as the GWC decreased under both irrigation treatments (no significant Frequency x GWC interaction; Fig. 7a, **Appendix,** Table 12) across the entire data set and when the range of GWC was restricted ($1.5 < GWC < 3.0$ g g^{-1} – to compare both irrigation frequencies across a similar GWC range). However, decreasing GWC had a more pronounced effect on $[ABA]_{xyl}$ as the experiment duration increased (significant Cycle x GWC interaction; Fig. 7a).

There was no significant relationship between leaf water potential (Ψ_{leaf}) and $[ABA]_{xyl}$ at either irrigation frequency (Fig. 7b, **Appendix,** Table 13).

Decreased Ψ_{shoot} correlated with increased $[ABA]_{xyl}$ under both irrigation frequency treatments (no significant Frequency x Ψ_{shoot} interaction) across the entire data set and when the range of Ψ_{shoot} was restricted ($-0.6 < \Psi_{shoot} < -0.3$ MPa – to compare both irrigation frequencies across a similar Ψ_{shoot} range) (no significant Frequency x Ψ_{shoot} interaction; Fig. 7c, **Appendix,** Table 14).

In summary, decreased GWC and Ψ_{shoot} correlated with increased $[ABA]_{xyl}$. However, there was no significant relationship between Ψ_{leaf} and $[ABA]_{xyl}$ at either irrigation frequency (Fig.7).



Frequency	p=0.053
Cycle	**p<0.001**
GWC	**p<0.001**
Frequency x Cycle	**p=0.002**
Cycle x GWC	**p<0.001**
Frequency x GWC	p=0.075
Frequency x Cycle x GWC	**p=0.006**

Frequency	p=0.75
Cycle	p=0.57
Ψ_{leaf}	p=0.32
Frequency x Cycle	p=0.32
Cycle x Ψ_{leaf}	p=0.57
Frequency x Ψ_{leaf}	p=0.82
Frequency x Cycle x Ψ_{leaf}	p=0.28

Frequency	p=0.95
Cycle	p=0.19
Ψ_{shoot}	p=0.59
Frequency x Cycle	p=0.61
Cycle x Ψ_{shoot}	p=0.23
Frequency x Ψ_{shoot}	p=0.99
Frequency x Cycle x Ψ_{shoot}	p=0.66

Figure 7. Relationships between shoot xylem sap ABA concentration and (a) whole pot soil gravimetric water content, (b) leaf water potential, (c) shoot water potential for plants grown under frequent (SDI, filled point) and infrequent (DRW, hollow point) irrigation in Cycles 1 (circle), 2 (triangle) and 3 (square). Plants under WW irrigation treatment in Cycles 1 (crossed circle), 2 (crossed triangle) and 3 (crossed square). Each point represents a single plant and regression lines were fitted to frequent (solid line) and infrequent (dashed line) treatments when significant (P<0.05). P values determined by ANCOVA for each main effect (frequency, cycle and x-variable) and their interaction are reported for a restricted x-axis range represented by the dashed box (statistically similar x-variable when comparing the 2 irrigation frequencies).

The transpiration rate (TR) of detached shoots decreased as the ABA concentrations in artificial xylem sap increased (Fig. 8a), by the end of the bioassay, TR decreased by 22%, 29%, 38%, 54%, and 65% when fed with 10 nM, 50 nM, 100 nM, 500 nM and 1000 nM ABA respectively, compared with 0 nM ABA (Fig. 8c). Moreover, as the ABA concentrations supplied increased, TR declined more rapidly, with significant differences from control (0 nM ABA) shoots detected after 60 min for 100 nM, 500 nM and 1000 nM ABA, 120 min for 50 nM ABA, and 180 min for 10 nM ABA respectively (Fig. 8a, **Appendix,** Table 15). After supplying different ABA concentrations to the detached shoots for 5 hours, g_s significantly differed between treatments, with g_s decreased by 44%, 55%, 66%, 87%, and 90% for 10 nM, 50 nM, 100 nM, 500 nM and 1000 nM ABA respectively, compared with 0 nM ABA (Fig.8b). Thus direct measurements of g_s more sensitively detected stomatal closure than gravimetric measurement of transpiration The relationship between relative g_s (g_s%) and endogenous xylem ABA concentration *in vivo* in drying soil was similar to that of relative detached shoot g_s (g_s%) and the ABA concentration supplied via the transpiration stream to the detached shoots (Fig. 8d). In addition, ABA concentration had no effect on Ψ_{leaf} at the end of the transpiration assay (data not shown), with Ψ_{leaf} equaling (the average value was -0.46 ± 0.03 MPa).

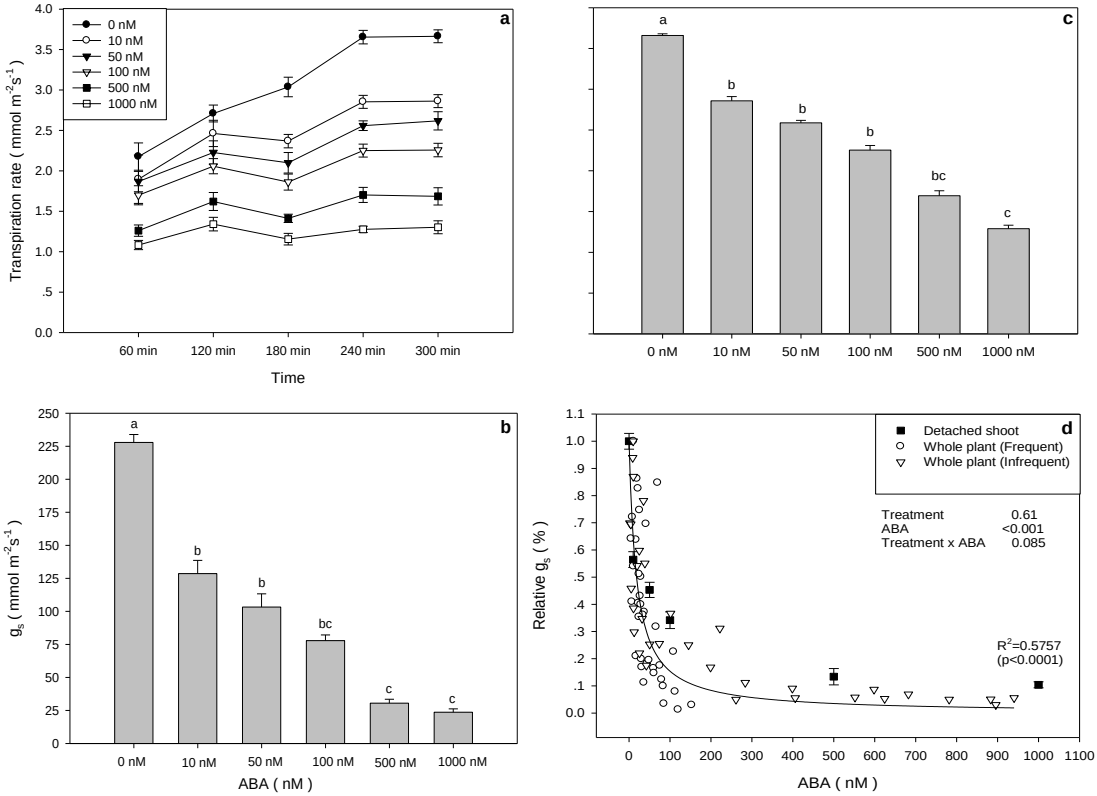


Figure 8. (a) Transpiration rate of detached shoots fed artificial xylem sap with ABA concentrations at 0 nM (filled circles), 10 nM (hollow circles), 50 nM (filled triangle), 100 nM (hollow triangle), 500 nM (filled square), 1000 nM (hollow square). (b) Mean stomatal conductance of detached shoots after transpiration bioassay. (c) Mean transpiration rate from 240 min to 300 min. (d) ABA concentration and relative g_s (the maximum g_s for each treatment as 100%) under different treatments. The maximum value of g_s was 280 mmol $m^{-2}s^{-1}$ in detached shoots (filled square), 690 mmol $m^{-2}s^{-1}$ in whole plants with frequent (SDI, hollow circles) and 445 mmol $m^{-2}s^{-1}$ in whole plants with infrequent (DRW, hollow triangle) irrigation. Data are means±SEM (n=5). Significant differences are indicated by different letters within a panel according to ANOVA (p<0.05).

Experiment 2: Effects of varying relative humidity on responses to soil drying

In Experiment 2, The controlled environment conditions altered humidity (RH) at the similar temperature, the average temperature was 22.9±0.1 ℃ and 23.2±0.1 ℃ in high relative humidity (92-95% RH) cabinet and low relative humidity (50% RH) cabinet respectively (**Appendix**, Fig. 15a,b).

The total irrigation volumes applied to the WW and Drying plants were 726 mL and 0 mL under high RH, while the WW and DRW plants under low RH received 2156 mL and 873 mL (Fig. 9a).

There was no statistically significant difference in GWC between high and low RH conditions in WW plants, with average values of 2.9±0.01 g g^{-1} and 2.8±0.02 g g^{-1} under high and low RH respectively (Fig. 9b, **Appendix.** Table 16). In high RH, the GWC of Drying plants decreased over time by 71% throughout the experiment. Under low RH, during DRW, GWC declined from Days 1 to 6 by 76%, then sharply rose after re-watering and reached the highest point on Day 7, but decreased again from Day 8. From Days 1 to 6, Drying plants with high RH had a higher GWC than DRW plants with low RH, while Drying plants under high RH maintained a lower GWC than DRW plants from Day 7. Finally on Day 12, Drying and DRW plants had a similar GWC (Fig. 9b, **Appendix,** Table 16).

For WW plants, the evapotranspiration (ET) increased over time in both RH treatments, but average values at low RH (180±11 mL day^{-1}) were significantly higher than at high RH (60±6 mL day^{-1}) (Fig. 9c). From Days 1 to 7, the ET of DRW plants decreased by 76%, then increased over the next 2 days following re-watering before decreasing again from Day 10 as soil moisture declined. In high RH, ET of Drying and WW plants were similar from Days 1 to 7, but thereafter ET of Drying plants decreased throughout the experiment (Fig. 9c).

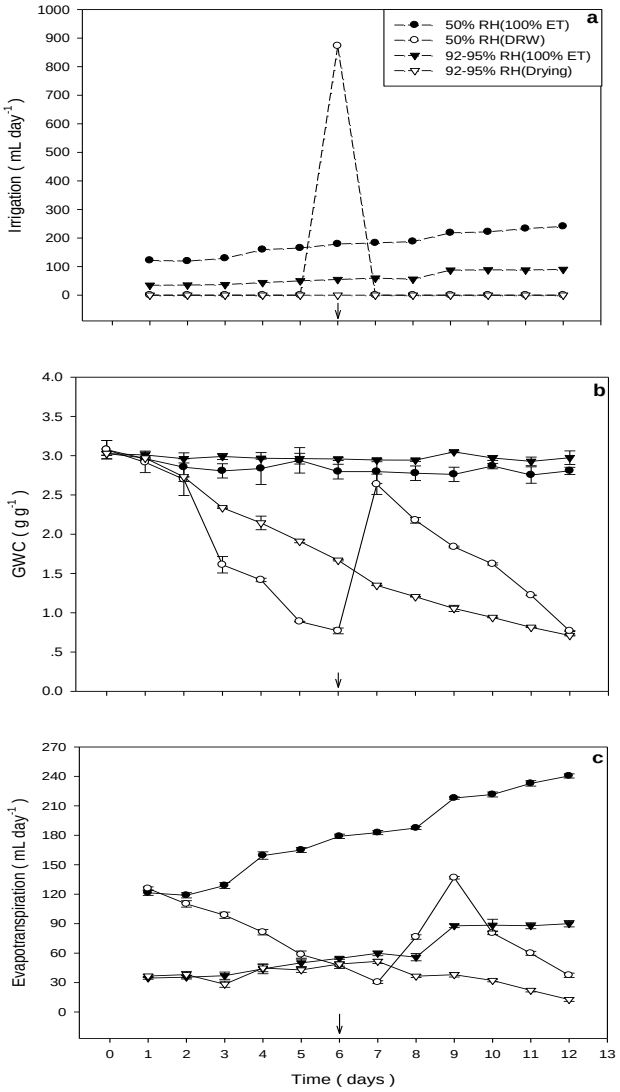


Figure 9. (a) Irrigation, (b) whole pot soil gravimetric water content (GWC), and (c) evapotranspiration of basil plants supplied with 100% ET either daily (WW, filled circles) or the accumulated volume on day 6 days (DRW, hollow circles) with low relative humidity, 100% ET daily (WW, filled triangle) and Drying (stop watering, hollow triangle) with high relative humidity over time. Data are means±SEM (n= 3). Arrows on x-axis indicate the day of re-watering for DRW plants with 50% RH. Plants were transferred to cabinets on day 0.

The stomatal conductance (g_s) for WW plants under high RH was consistently higher (47%) than under low RH, with significant differences throughout the experiment (Fig. 10a, **Appendix,** Table 17). Drying plants showed decreases in g_s (from Day 4) in high RH conditions, with a 92% decrease by the end of experiment. They maintained a higher g_s than WW plants in low RH conditions (from Days 0 to 7), but from Day 8, showed a rapid decrease as soil moisture declined (Fig. 10a, **Appendix,** Table 17). While g_s of DRW plants decreased from Days 0 to 6, it sharply rose after re-watering, but decreased again on Day 10. Generally, g_s of DRW plants with low RH maintained a lower value than g_s of Drying plants with high RH (Fig. 10a, **Appendix,** Table 17).

Leaf water potential (Ψ_{leaf}) of WW plants was significantly higher at high RH (−0.40±0.01 MPa) than low RH (−0.55±0.00 MPa) (Fig.10b, **Appendix,** Table 17). Although there was no statistically significant difference in Ψ_{leaf} between Drying and WW plants in high RH (from Days 0 to 7), Ψ_{leaf} of Drying plants declined rapidly from Day 8 as soil moisture decreased. Also, Ψ_{leaf} of Drying plants with high RH maintained a higher Ψ_{leaf} (0.16 MPa) than WW plants with low RH (expect on Day 10, Day 11 and Day 12) (Fig. 10b, **Appendix,** Table 17). DRW plants with low RH showed a lower Ψ_{leaf} than Drying plants with high RH (except on Day 11 and Day 12) (Fig.10b, **Appendix,** Table 17).

Shoot water potential (Ψ_{shoot}) of WW plants in high RH was significant higher than at low RH, a difference that was maintained throughout the experiment, with average values of Ψ_{shoot} of −0.39±0.00 MPa and −0.33±0.01 MPa respectively (Fig. 10c, **Appendix,** Table 17). At high RH, Ψ_{shoot} decreased over time (by 85%) in Drying plants, and maintained a lower Ψ_{shoot} (0.07 MPa) than WW plants in low RH (from Day 3) (Fig.10c, **Appendix,** Table 17). Under DRW with low RH condition, Ψ_{shoot} decreased from Days 0 to 6, and recovered in response to re-watering, but decreased again from Day 10. DRW plants in low RH remained a lower Ψ_{shoot} than Drying plants in high RH (expect on Day 8, Day 9, Day 10 and Day 11, as re-watering treatments applied) (Fig. 10c, **Appendix,** Table 17).

There was no statistically significant difference in foliar ABA concentration

($[ABA]_{leaf}$) for WW plants between high (213±3 ng g^{-1}DW) and low (267±8 ng g^{-1}DW) RH (expect from Days 9 to 12, Fig. 10d, **Appendix,** Table 18), both $[ABA]_{leaf}$ were consistently lower than Drying plants (from Day 5) in high RH and DRW plants (from Day 3) in low RH as experiment duration increased (Fig. 10d, **Appendix,** Table 18). The $[ABA]_{leaf}$ of DRW plants (1299±246 ng g^{-1}DW) in low RH increased more rapidly than Drying plants (1066±258 ng g^{-1}DW) in high RH, except from Days 7 to 10 in response to re-watering, finally on Day 11 and Day 12, both of them reached the similar point as soil moisture decreased (Fig. 10d, **Appendix,** Table 18).

Shoot xylem sap ABA concentration ($[ABA]_{xyl}$) of WW plants did not differ between high (29±1.1 nM) and low (42±1.3 nM) RH (except from Days 8 to 12, Fig.10e, **Appendix,** Table 18). Nevertheless, $[ABA]_{xyl}$ of Drying plants (143±47.1 nM) increased over time and reached 5-fold higher than WW plants. At low RH, $[ABA]_{xyl}$ of DRW plants (287±68.9 nM) reached 7-fold higher than WW plants at the end of the experiment. DRW plants with low RH showed a more rapid increase in $[ABA]_{xyl}$ than Drying plants with high RH (from Days 0 to 7), but then sharply dropped after re-watering with lower $[ABA]_{xyl}$ (at least 117 nM) than Drying plants in high RH (except on Day 12) (Fig. 10e, **Appendix**, Table 18).

In summary, WW plants in high RH maintained a higher g_s, Ψ_{leaf} and Ψ_{shoot}, but a lower $[ABA]_{leaf}$ and $[ABA]_{xyl}$ than at low RH conditions (Fig. 10). High RH increased Ψ_{leaf} and Ψ_{shoot} by 0.1 ~ 0.15 MPa and delayed the soil drying induced decline in Ψ_{leaf} (but not Ψ_{shoot}) (Fig. 10b,d), however, the increase of $[ABA]_{leaf}$ and $[ABA]_{xyl}$ in response to the GWC rapidly declined (Fig.10d, Fig.10e). Under low RH conditions, soil drying decreased g_s, Ψ_{leaf} and Ψ_{shoot} progressively until Day 6, but both variables sharply rose after re-watering (reached the similar value with WW plants). Both $[ABA]_{leaf}$ and $[ABA]_{xyl}$ had opposite trends to g_s. Although $[ABA]_{leaf}$ of DRW plants never achieved the values of WW plants after re-watering, this occurred for $[ABA]_{xyl}$ (Fig. 10).

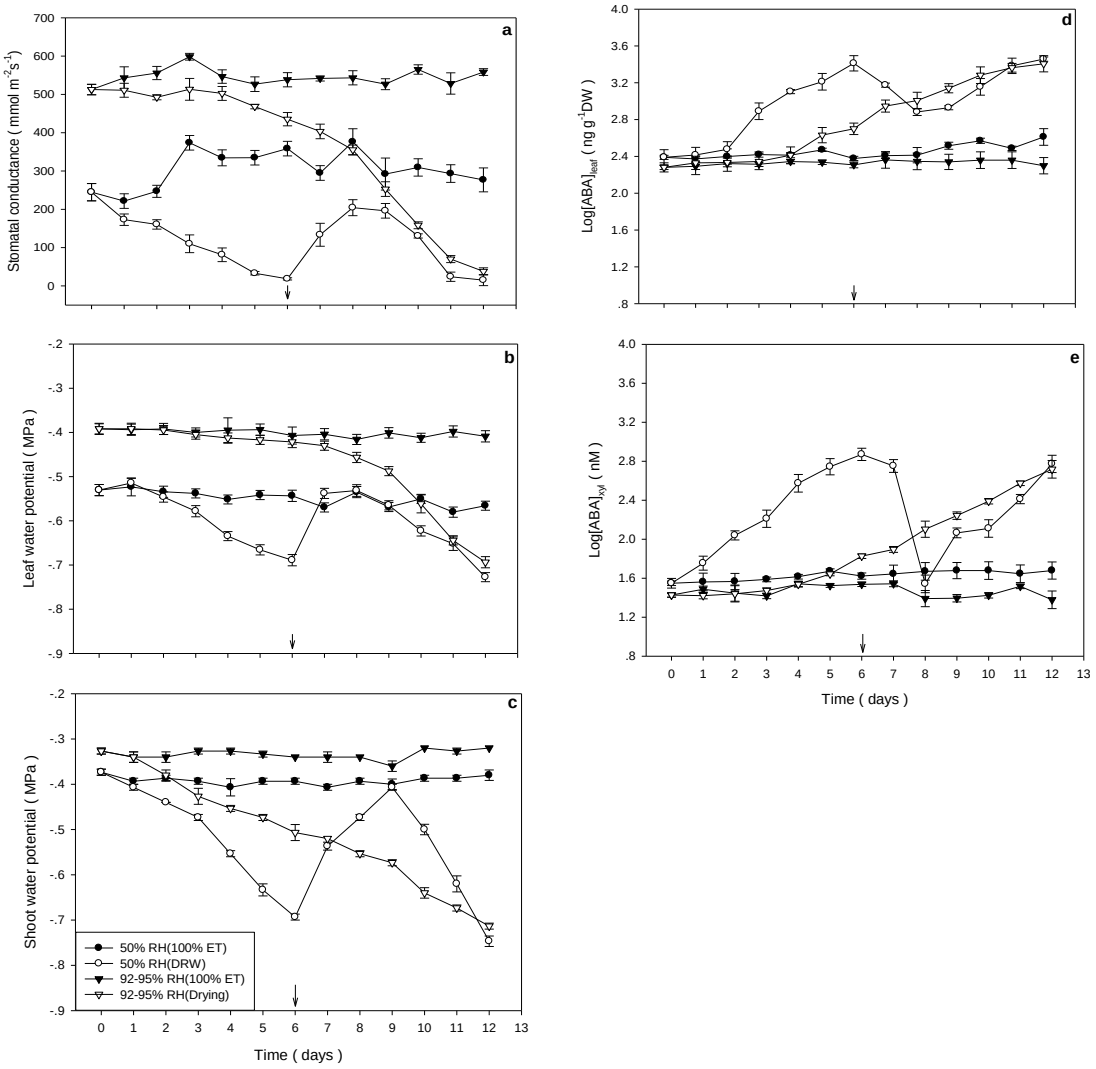


Figure 10. Stomatal conductance measured at 11:00 h (a), leaf water potential (b), shoot water potential (c), foliar (d) and shoot xylem sap (e) ABA concentration under 100% ET either daily (WW, filled circles) or the accumulated volume on day 6 days (DRW, hollow circles) with low relative humidity (50% RH), 100% ET daily (WW, filled triangle) and Drying (stop watering, hollow triangle) with high relative humidity (92-95% RH) over time. Data are means±SEM (n= 3) for each treatments, arrows on x-axis indicate the day of re-watering for the DRW plants with 50% RH.

Leaf water potential (Ψ_{leaf}) decreased as Ψ_{shoot} diminished, but to a greater extent with high RH (significant RH x Ψ_{shoot} interaction) across the similar GWC range. High RH altered Ψ_{leaf} with generally higher value (0.06 MPa) than Ψ_{shoot} in similar GWC range (significant RH effect, Fig. 11a).

However, foliar ABA concentration ($[ABA]_{leaf}$) increased similarly with increasing shoot sap ABA concentration ($[ABA]_{xyl}$) under both RHs (no significant RH x GWC interaction, Fig. 11b). Also, high RH failed to alter $[ABA]_{leaf}$ at the similar $[ABA]_{xyl}$ range (no significant RH effect) (Fig. 11b).

In all, high RH significant increased Ψ_{leaf} and delayed the soil drying induced declined in Ψ_{leaf} (but not in Ψ_{shoot}), while failed to altered the $[ABA]_{leaf}$ (Fig. 11).

As both Ψ_{leaf} and as Ψ_{shoot}, $[ABA]_{leaf}$ and $[ABA]_{xyl}$ are correlated, the following sections focus on the Ψ_{leaf} and $[ABA]_{xyl}$, to show the effects of relative humidity (92-95% RH *versus* 50% RH) on relationships between plant and soil variables.

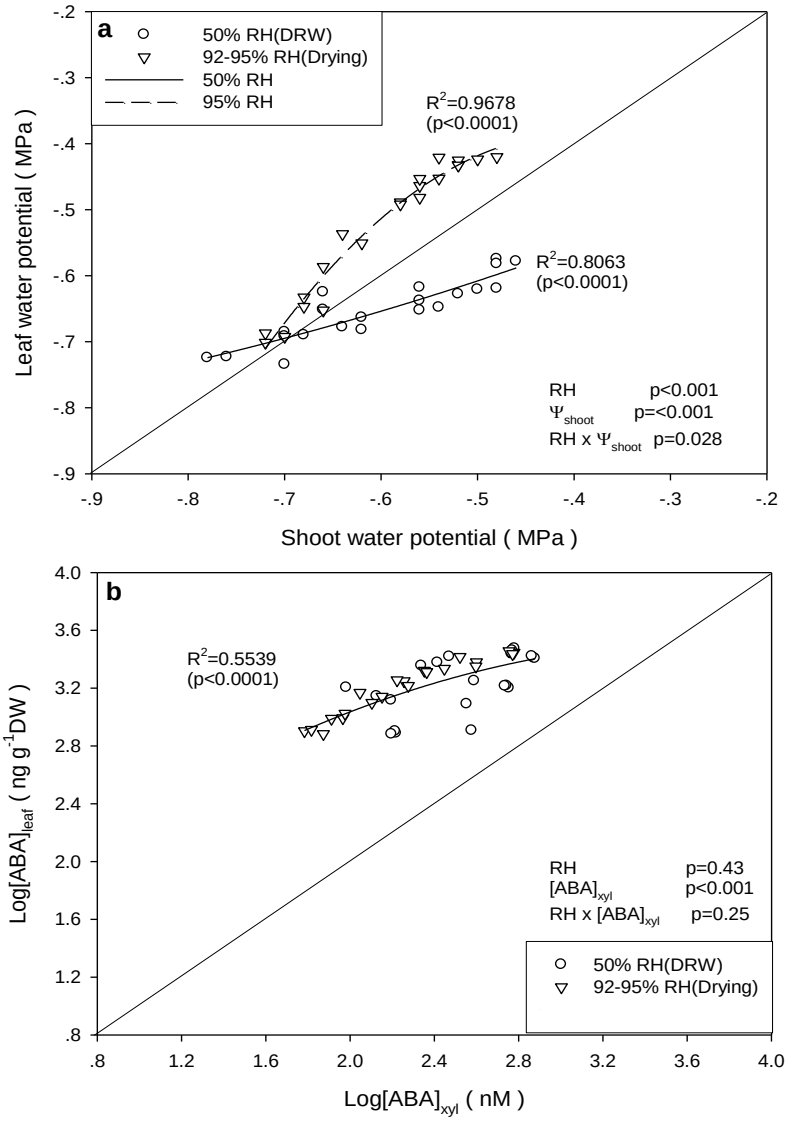


Figure 11. Relationships between shoot water potential and leaf water potential (a), foliar and shoot xylem sap ABA concentration (b) for Drying plants grown under high relative humidity (92-95% RH, hollow triangle), and DRW plants grown under low relative humidity (50% RH, hollow circle). All the data are chosen within the restricted range of whole pot soil gravimetric water content ($0.5 < GWC < 1.7$ g g^{-1}), to ensure high/low relative humidity are compared across the similar GWC range. Each point represents a single plant and regression lines were fitted to high and low relative humidity conditions. *P* values determined by ANCOVA for each main effect (relative humidity and x-variable) and their interaction were reported in each panel respectively. The 1:1 relationship is also indicated.

Stomatal conductance (g_s) decreased under both high and low RH, and was correlated with diminished GWC (Fig. 12a). However, RH affected the relationship between g_s and GWC (with significant RH x GWC interaction), with the g_s of plants under high RH generally higher (265 mmol $m^{-2}s^{-1}$) than under low RH (significant RH effect, Fig. 12a) at the similar GWC.

Decreased leaf water potential (Ψ_{leaf}) correlated with decreased g_s, but high and low RH differed in the response of g_s to Ψ_{leaf} (significant RH x Ψ_{leaf} interaction). Thus high RH resulted in higher (73%) g_s than low RH at the same Ψ_{leaf} (Fig. 12b).

Also, high and low RH differed in the response of g_s to shoot xylem sap ABA concentration ($[ABA]_{xyl}$), with a higher g_s at high RH (significant RH effect, RH x $[ABA]_{xyl}$ interaction, Fig. 12c) at the similar $[ABA]_{xyl}$ range. However, different relative humidity altered the sensitivity of g_s to $[ABA]_{xyl}$ (the slopes comprising 0.001 mmol $m^{-2}s^{-1}$ $Log(nM)^{-1}$ for low RH and 0.002 mmol $m^{-2}s^{-1}$ $Log(nM)^{-1}$for high RH) (Fig. 12c).

In summary, high RH increased g_s, and decreased Ψ_{leaf} compared to low RH. Moreover, g_s declined more sensitively in response to the decreased GWC (steeper slope of the response) and increased $[ABA]_{xyl}$, under high RH compared to low RH (Fig. 12).

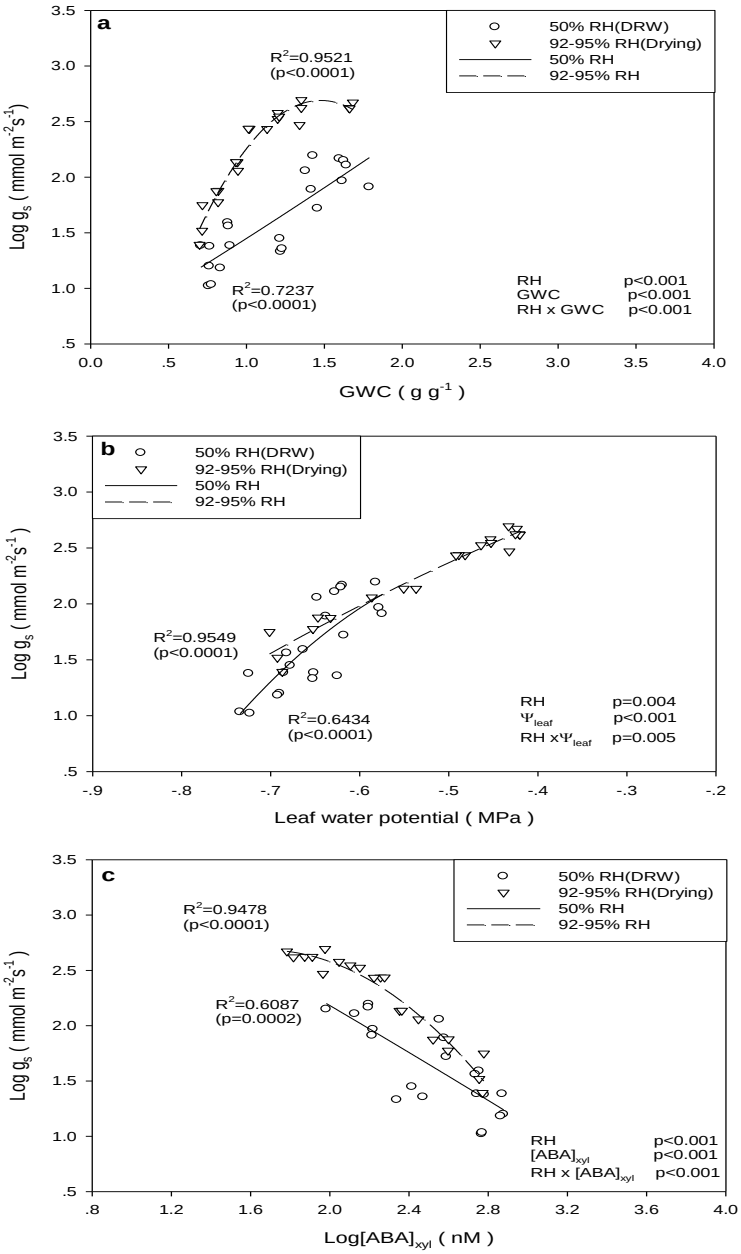


Figure 12. Relationships between stomatal conductance and (a) whole pot soil gravimetric water content, (b) leaf water potential, (c) shoot xylem sap ABA concentration for Drying plants grown under high relative humidity (92-95% RH, hollow triangle), and DRW plants grown under low relative humidity (50% RH, hollow circle). All the data are chosen within the restricted range of whole pot soil gravimetric water content ($0.5 < GWC < 1.7$ g g^{-1}), to make sure compare high/low relative humidity with 21 points across the similar GWC range. Each point represents a single plant and regression lines were fitted to high and low relative humidity conditions. *P* values determined by ANCOVA for each main effect (relative humidity and x-variable) and their interaction were reported in each panel respectively.

Shoot xylem ABA concentration $[ABA]_{xyl}$ increased similarly with the decreasing GWC under both RH treatments (no significant RH x GWC interaction, Fig. 13a). $[ABA]_{xyl}$ significantly increased as leaf water potential (Ψ_{leaf}) decreased, more sensitively at low RH (with significant RH x Ψ_{leaf} interaction, Fig. 13b). In contrast, $[ABA]_{xyl}$ increased as shoot water potential (Ψ_{shoot}) declined, but more sensitively at high RH (significant RH x $[ABA]_{xyl}$ interaction). At low Ψ_{leaf} and Ψ_{shoot} following prolonged soil drying, relative humidity failed to alter $[ABA]_{xyl}$ (Fig. 13c).

In summary, decreased GWC was significantly correlated with decreased $[ABA]_{xyl}$ (significant GWC effect, RH x GWC interaction). High relative humidity resulted in higher Ψ_{leaf} and significantly decreased $[ABA]_{xyl}$ (Fig. 13).

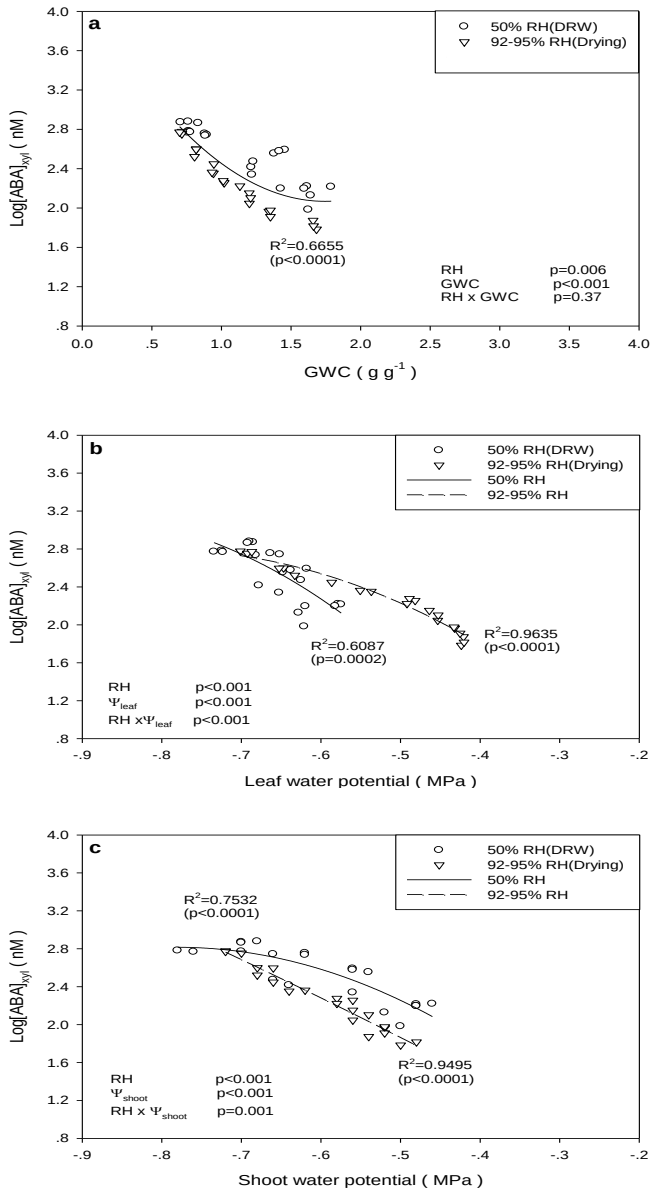


Figure 13. Relationships between shoot xylem sap ABA concentration and (a) whole pot soil gravimetric water content, (b) leaf water potential, (c) shoot water potential for Drying plants grown under high relative humidity (92-95% RH, hollow triangle), and DRW plants grown under low relative humidity (50% RH, hollow circle). All the data are chosen within the restricted range of whole pot soil gravimetric water content ($0.5 < GWC < 1.7$ g g^{-1}), to make sure compare high/low relative humidity with 21 points across the similar GWC range. Each point represents a single plant and regression lines were fitted to high and low relative humidity conditions. *P* values determined by ANCOVA for each main effect (relative humidity and x-variable) and their interaction were reported in each panel respectively.

Sensory evaluation and quality analysis

Reduced irrigation frequency significantly darkened the leaf colour (Table 2), but there were no significant treatment differences for aroma, taste, texture and consistency (Table 2). Nevertheless, panelists preferred the DRW plants according to their comments. DRW plants were said to have the best aroma, a traditional taste and flavor (with a slight sweetness), and had the softest and best textured leaves. In contrast, WW plants were pale in color, had no aroma, were hard and brittle during chewing, and had a strong, peppery taste. Furthermore, plants irrigated at SDI had a strong, peppery taste, a rubbery texture during chewing, and a gentle aroma. In summary, DRW plants showed the best quality and more traditional taste.

Table 2. Sensory evaluation results for three different irrigation treatments. The score was given on a scale from 1 to 5, indicating increasing quality. Data are means ± SE of 6 replicates for each treatment, values without a common letter within a row are significantly different according to a one-way ANOVA (P<0.05).

Score	**Treatments**		
Quality characteristics	**WW**	**SDI**	**DRW**
Color and appearance	1.5 ± 0.3 b	1.5 ± 0.3 b	3 ± 0.4 a
Aroma	2.5 ± 0.6 a	3.25 ± 0.3 a	3.5 ± 0.6 a
Taste	2.25 ± 0.3 a	2.75 ± 0.5 a	3 ± 0.4 a
Texture and consistency	1.25 ± 0.3 a	2 ± 0.6 a	2.5 ± 0.3 a

While concentrations of rosmarinic acid (7.80 mg g^{-1} DW) were obviously higher (56-fold) than caffeic acid (0.14 mg g^{-1} DW) in WW plants, concentrations of both constituents increased as the experiment duration increased (Table 3). Caffeic acid concentrations significantly increased in SDI and DRW plants leaves (from Days 12 to 20) compared to WW plant, with 0.18 mg g^{-1} DW and 0.19 mg g^{-1} DW (from Days 12 to 20) on average respectively, but there was no effect of irrigation frequency (Table 3). Similarly, rosmarinic acid concentrations of SDI and DRW plants significantly increased, with the average content of 12.0 mg g^{-1} DW and 12.8 mg g^{-1} DW (from Days 18 to 20), but no effect of irrigation frequency.

In summary, both frequent (SDI) and infrequent (DRW) irrigation treatments obviously increased the content of caffeic acid by 9% and 12%, and rosmarinic acid by 6% and 10%, respectively, compared with WW plants (Table 3). However, irrigation frequency had no statistically significant effect on both caffeic acid and rosmarinic acid concentrations (Table 3).

Table 3. The content of caffeic acid and rosmarinic acid in basil leaves (mg g^{-1} DW, in dry weight). Data are means±SE of 3 replicates for each treatment, values without a common letter within a row are significantly different according to a one-way ANOVA (P<0.05).

The UV spectra of basil sample is shown in **Appendix** (Fig. 17b).

Content (mg g^{-1} DW)		**Treatments**		
	Time	**WW**	**SDI**	**DRW**
Rosmarinic acid	**Day 0**	6.60 ± 0.4 a	6.60 ± 0.4 a	6.60 ± 0.4 a
	Day 6	7.22 ± 0.3 a	8.08 ± 0.4 a	8.18 ± 0.3 a
	Day 12	7.97 ± 0.4 a	**8.94 ± 0.5 b**	**9.34 ± 0.5 b**
	Day 18	8.21 ± 0.4 a	**11.10 ± 0.5 b**	**12.00 ± 0.4 b**
	Day 20	9.00 ± 0.3 a	**13.00 ± 0.2 b**	**13.60 ± 0.4 b**
Caffeic acid	**Day 0**	0.13 ± 0.04 a	0.13 ± 0.01 a	0.13 ± 0.07 a
	Day 6	0.14 ± 0.07 a	0.15 ± 0.02 a	0.15 ± 0.03 a
	Day 12	0.14 ± 0.05 a	0.16 ± 0.03 a	0.17 ± 0.05 a
	Day 18	0.15 ± 0.05 a	**0.18 ± 0.05 b**	**0.20 ± 0.04 b**
	Day 20	0.15 ± 0.03 a	**0.20 ± 0.02 b**	**0.21 ± 0.06 b**

Discussion

Stomatal responses to soil drying

The primary adaptive response of basil plants to soil drying was stomatal closure (Fig. 6a), which decreased transpiration under both irrigation frequency treatments (Fig. 8d). Plants that were less frequently irrigated showed greater stomatal closure, as reported previously in the ornamental plant *Pelargonium x hortorum* (Boyle et al., 2015). In *Pelargonium*, that response was attributed to decreased Ψ_{leaf} and enhanced $[ABA]_{xyl}$, which together may interact to sensitise the stomata (Tardieu and Davies 1992). In basil, decreased stomatal conductance was correlated with decreased shoot water relations (Ψ_{leaf} and Ψ_{shoot}) and increased ABA ($[ABA]_{leaf}$ and $[ABA]_{xyl}$) status (Fig. 6), thus it is important to resolve the importance of the individual signalling mechanism(s) involved.

Stomatal closure was correlated with decreased Ψ_{leaf} under both irrigation frequency treatments, and decreased irrigation frequency increased the sensitivity of g_s to Ψ_{leaf} (Fig. 6b). In contrast, in *P. x hortorum*, stomatal closure was correlated with decreased Ψ_{leaf} only when irrigation was withheld, while stomatal closure was associated with higher Ψ_{leaf} under daily irrigation at 75%ET (Boyle et al., 2015). These species differences indicate that *P. x hortorum* is more isohydric than basil, and suggest that decreased Ψ_{leaf} may mediate stomatal closure in response to drying soil in basil.

To further examine this question, Ψ_{leaf} was altered by growing plants at different relative humidities (50% and 92-95% RH), since high RH increases Ψ_{leaf} (Lange et al., 1971). In well watered plants, higher g_s was correlated with increased Ψ_{leaf} at 92-95% RH, yet Ψ_{leaf} decreased with g_s similarly at both relative humidities as the soil dried (Fig. 12b). While RH clearly influenced maximum g_s, soil drying induced parallel decreases in Ψ_{leaf} and increases in ABA (Fig. 14), both of which could regulate stomatal closure (Boyle et al., 2015).

The primary role of ABA in long distance chemical signalling of soil drying has been well documented (Gowing et al., 1990, Davies and Zhang, 1991, Sauter et al., 2001, Dodd, 2005), with $[ABA]_{xyl}$ increasing as the soil dried (Correia and Pereira, 1995, Jarvis and Davies, 1997) and under both irrigation frequency treatments

(Fig.7a). This effect became more pronounced as the experiment duration increased (significant Cycle x GWC interaction; Fig. 7a, **Appendix,** Table 12), which may reflect hysteresis in the soil moisture release curve in response to drying and re-wetting cycles.

Furthermore, g_s decreased in response to increased $[ABA]_{xyl}$ under both irrigation frequency treatments (with no significant Frequency x $[ABA]_{xyl}$ interaction) across the entire data set and the restricted $[ABA]_{xyl}$ range ($0.5 < \text{Log } [ABA]_{xyl} < 2.0$ nM) (Fig. 6c). In contrast, foliar ABA concentration was accentuated by infrequent irrigation (**Appendix,** Fig. 6e). Stomatal closure was better correlated with increased $[ABA]_{xyl}$ (explaining 49-84% of the variance in g_s according to irrigation frequency) than with $[ABA]_{leaf}$ (explaining 17-24% of the variance in g_s), as in other studies (Tardieu and Davies, 1993, Heilmeier et al., 2007), likely since much of the ABA present in the leaf is compartmentalized in chloroplasts of mesophyll cells (Loveys, 1977) and unavailable to receptors on the guard cell plasmalemma or in the cytosol. Alkalisation of the xylem sap can result in more xylem-delivered ABA reaching the guard cells and less being compartmentalized in the mesophyll (Wilkinson and Davies, 1997).

This raises the question of whether there is sufficient ABA in the xylem stream to elicit stomatal closure. Supplying synthetic ABA to detached shoots via the transpiration stream (at the same concentrations detected in plants exposed to drying soil) showed the same stomatal response (Fig. 8b) as observed *in vivo* under different irrigation frequency treatments (Fig.8d). This suggests the relationship is causal in basil. Nevertheless, there was variation in the relationship between g_s and $[ABA]_{xyl}$ according to relative humidity (significant RH x $[ABA]_{xyl}$ interaction, Fig. 12c). High RH increased the sensitivity of g_s to $[ABA]_{xyl}$ (Fig.12c), as previously reported in cotton (Barbour and Farquhar, 2000). While the mechanism is not clear, it is possible that high RH may have altered the concentrations of other phytohormones which sensitized the ABA response.

Although both chemical and hydraulic signals can regulate stomatal closure in response to water deficit (Wilkinson and Davies, 2010), distinguishing their effects

can be challenging, especially since ABA has been reported to decrease leaf hydraulic conductance (Pantin et al., 2013). While ABA appears to play an important role in mediating stomatal closure of basil in response to soil water deficit, its effectiveness as an antitranspirant seems to depend on environmental conditions (Fig. 12c).

Water use efficiency, yield and quality under different irrigation frequencies

It has been widely accepted that mild water deficit induces partial stomatal closure that can decrease transpiration without limiting photosynthesis, thereby increasing water use efficiency (WUE) (Davies et al., 1978, Turner, 1997, Tardieu, 2005).

Irrigation frequency altered water use efficiency (Fig. 3e) while having different effects on basil yield (leaf area and dry weight)(Fig. 3a,b). Daily irrigation at 75% ET (SDI) had higher yield (8% and 18% increase in leaf area and dry weight, respectively), while infrequent irrigation (DRW) had lower yield (12% decrease in both leaf area and dry weight, respectively) compared with control plants (WW, Fig.3a,b). Similarly, increased irrigation frequency (irrigation quantities based on pan evaporation) increased yield in summer squash (Ertek et al., 2004) and melon (Sensoy et al., 2007) under field conditions. While this suggests potential to improve WUE and biomass production of basil, impacts on quality (bioactive compounds and taste) need to be assessed before recommending such irrigation to growers.

Leaf water deficits can induce protective mechanisms involving the synthesis and accumulation of phenolic compounds (de Abreu and Mazzafera, 2005, Hura et al., 2008), which can limit the excitation of chlorophyll during conditions unfavourable for the photosynthetic apparatus (Nogués and Baker, 2000). Rosmarinic acid has been consistently reported as the predominant phenolic acid in basil (Javanmardi et al., 2002, Hakkim et al., 2007, Kwee and Niemeyer, 2011), with 7.80 mg g^{-1} DW in control plants (WW, Table 3) similar to previous studies in other cultivars (Kwee and Niemeyer, 2011, Nguyen et al., 2010). Basil has lower concentrations of caffeic acid (0.14 mg g^{-1} DW, Table 3) than rosmarinic acid (Kwee and Niemeyer, 2011), as reported here (WW, Table 3). Both frequent (SDI) and infrequent (DRW) irrigation treatments significantly increased caffeic acid (by 9% and 12%) and rosmarinic acid (by 6% and 10%) contents compared with WW plants (Table 3). The similar increases

in caffeic acid and rosmarinic acid contents independent of irrigation frequency, despite differences in ABA and Ψ_{leaf}, suggest that other factors (such as the generation of reactive oxygen species, ROS) may have upregulated production of these phenolic compounds.

WUE versus quality: A favourable tradeoff ?

Taken together, sustained deficit irrigation (SDI, daily irrigation with 75% full crop evapotranspiration) and infrequent drought and re-watering (DRW, applying the same volune of water as SDI but once every 6 days) strategies could allow more efficient plant water use (Fig. 3e) and significantly enhance foliar phenolic composition (caffeic acid and rosmarinic acid, Table 3) in basil (Fig. 14). However, only SDI increased biomass production (highest leaf area and dry weight, Fig. 3a,b), but had negative effects on quality characteristics (an undesirable peppery taste, with a rubbery texture during chewing, Table 2). In contrast, DRW reduced the biomass production (Fig. 3a,b), but had the highest foliar phenolic content, Table 3) with positive effects on quality (best aroma, traditional taste and flavor with a slight sweetness, Table 2) (Fig. 14). Taken together, this suggests that basil as a popular fresh herb can be cultivated with less water (improved water use efficiency, WUE), but the choice of irrigation strategy depends on grower/consumer requirements. If used as a culinary herb and flavoring agent for the food industry (De Masi et al., 2006), DRW is recommended, since better quality is more desirable for human health. In contrast, if used for pharmaceutical and cosmetic preparations (Javanmardi et al., 2002, Kiferle et al., 2011), SDI is recommended as it produced more biomass (Fig.14).

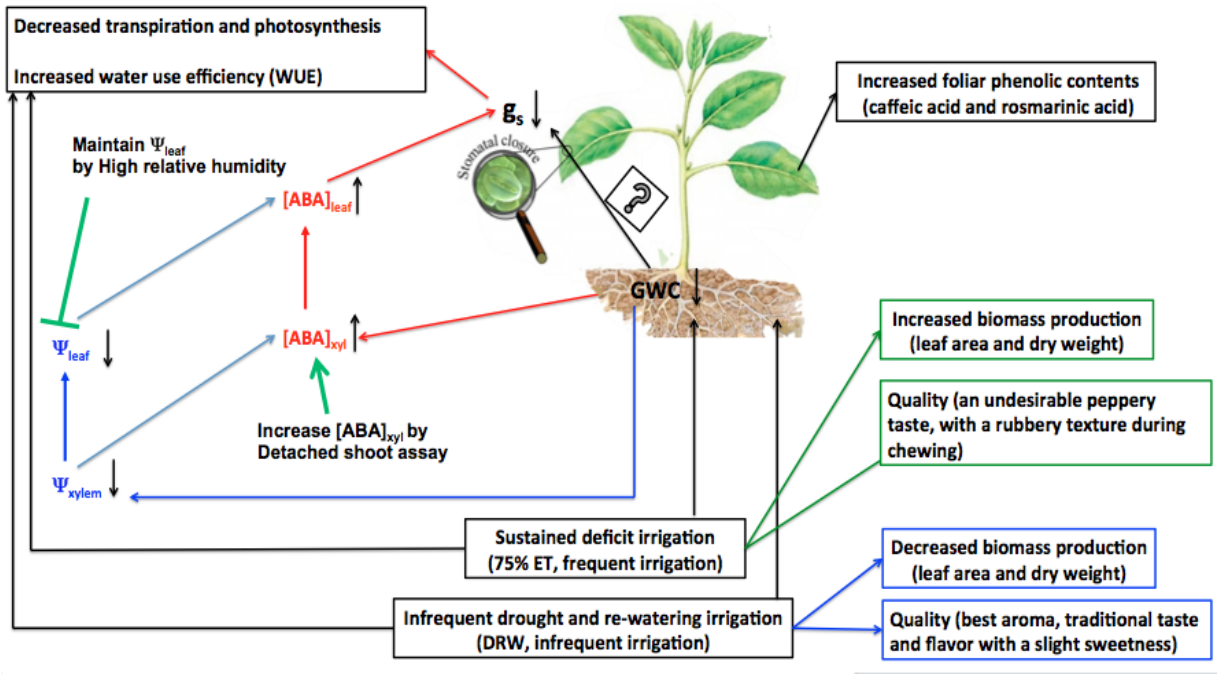


Figure 14. Schematic diagram for agronomic and physiological impacts of irrigation frequency on green basil.

Conclusions

Basil (*Ocimum basilicum* L.) was sensitive to water deficit, but irrigation frequency altered relationships between stomatal conductance, leaf water status and ABA status. Infrequent irrigation resulted in lower g_s at any Ψ_{leaf} and $[ABA]_{xyl}$, while high relative humidity increased g_s at any Ψ_{shoot}, $[ABA]_{xyl}$ or $[ABA]_{leaf}$. Paradoxically, high RH sensitised stomatal conductance to these variables, while there was a single relationship between g_s and Ψ_{leaf} irrespective of relative humidity. While this suggests that Ψ_{leaf} is the principal factor regulating stomatal conductance, a consistent relationship between g_s and $[ABA]_{xyl}$ in both detached shoots fed synthetic ABA via the xylem and plants exposed to different irrigation frequencies indicates that ABA may play a dominant role in mediating stomatal closure of basil in response to soil water deficit. Further experiments are needed to decouple leaf water status and ABA status in basil, to test the relative importance of these variables. These physiological changes may be implicated in increasing the water use efficiency and foliar phenolic composition (caffeic acid and rosmarinic acid) in basil. While frequent irrigation increased biomass but decreased quality, infrequent irrigation limited biomass production with improved quality. Basil can be cultivated with 25% less water without incurring significant yield penalties, but the desirable irrigation frequency will depend on the intended use of the crop. DRW is better to be used as culinary herb and flavoring agent for the food industry, while SDI is better to be used in pharmaceutical and cosmetic preparations.

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Appendix

Table 4. Significant differences between irrigation treatments according to a one-way ANOVA ($p<0.05$) of Whole pot soil gravimetric water content (GWC) and Evapotranspiration (ET) on each day throughout the experiment for each irrigation treatment (as with Fig.2). Differences between irrigation treatments on each day are indicated by different letters according to *Tukey's* multiple comparisons test. " - " represents days where no data was collected.

Sig.	Treatments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20
GWC	WW	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	SDI	a	ab	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
	DRW	a	b	c	c	c	c	c	a	ab	b	b	c	c	a	c	c	b	c	c	a	c
ET	WW	-	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	SDI	-	a	a	a	a	a	a	b	b	b	b	b	b	b	b	b	a	a	b	b	b
	DRW	-	a	a	a	b	b	b	c	c	b	c	c	c	c	c	b	b	b	c	c	c

Table 5. Differences between irrigation treatments of stomatal conductance and shoot water potential on each day throughout experiment (as with Fig.4) were evaluated according to *Tukey's* multiple comparisons test (significant different $p<0.05$) by different letters.

Sig.	Treatments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20
g_s	**WW**	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	SDI	a	a	a	b	a	a	b	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	DRW	a	a	b	c	b	b	c	b	a	b	b	b	b	b	a	b	b	b	b	b	b
Ψ_{shoot}	**WW**	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a	a
	SDI	a	a	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b	b
	DRW	a	b	c	c	c	c	c	c	c	b	c	c	c	c	b	b	c	c	c	c	b

Table 6. Differences between irrigation treatments of leaf water potential, foliar and shoot xylem sap ABA concentration on every two days day throughout experiment (as with Fig.4) were evaluated according to *Tukey's* multiple comparisons test (significant different $p<0.05$) by different letters.

Sig.		Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12	Day 14	Day 16	Day 18	Day 20
Ψ_{leaf}	**WW**	a	a	a	a	a	a	a	a	a	a	a
	SDI	a	a	a	a	a	a	a	b	a	b	a
	DRW	a	a	b	a	b	b	b	a	a	c	a
$Log[ABA]_{leaf}$	**WW**	a	a	a	a	a	a	a	a	a	a	a
	SDI	a	a	a	b	a	b	a	b	b	a	b
	DRW	a	a	b	c	b	c	b	b	c	b	c
$Log[ABA]_{xyl}$	**WW**	a	a	a	a	a	a	a	a	a	a	a
	SDI	a	a	a	a	a	a	a	a	a	b	a
	DRW	a	a	b	b	a	a	b	a	a	c	a

Table 7. Effects of irrigation frequency, cycle, Ψ_{shoot} and their interactions on leaf water potential. **Table 8.** Effects of irrigation frequency, cycle, $[ABA]_{xyl}$ and their interactions on $[ABA]_{leaf}$ (*P* values are presented, as with Fig.5).

Table 7.	**Frequent vs Infrequent**
Effect or Interaction	**All Data**
Ψ_{shoot} - Ψ_{leaf}	
Frequency	0.065
Cycle	0.93
Ψ_{shoot}	**0.002**
Frequency x Cycle	0.58
Cycle x Ψ_{shoot}	0.86
Frequency x Ψ_{shoot}	**0.037**
Frequency x Cycle x Ψ_{shoot}	0.60

Table 8.	**Frequent vs Infrequent**
Effect or Interaction	**All Data**
Log $[ABA]_{xyl}$ – Log$[ABA]_{leaf}$	
Frequency	0.38
Cycle	0.46
$[ABA]_{xyl}$	**< 0.001**
Frequency x Cycle	0.73
Cycle x $[ABA]_{xyl}$	0.89
Frequency x $[ABA]_{xyl}$	0.10
Frequency x Cycle x $[ABA]_{xyl}$	0.80

Table 9. Effects of irrigation frequency, cycle, GWC and their interactions on g_s. **Table 10.** Effects of irrigation frequency, cycle, Ψ_{leaf} and their interactions on g_s. **Table 11.** Effects of irrigation frequency, cycle, $[ABA]_{xyl}$ and their interactions on g_s (*P* values are presented, as with Fig.6).

Table 9	**Frequent vs Infrequent**
Effect or Interaction	
GWC - Log g_s	**All Data**
Frequency	**0.013**
Cycle	**0.012**
GWC	**< 0.001**
Frequency x Cycle	0.35
Cycle x GWC	0.051
Frequency x GWC	**0.003**
Frequency x Cycle x GWC	0.40

Table 10	**Frequent vs Infrequent**
Effect or Interaction	
Ψ_{leaf} - Log g_s	**All Data**
Frequency	0.076
Cycle	0.070
Ψ_{leaf}	0.007
Frequency x Cycle	**0.001**
Cycle x Ψ_{leaf}	**0.035**
Frequency x Ψ_{leaf}	**0.004**
Frequency x Cycle x Ψ_{leaf}	**0.008**

Table 11	**Frequent vs**
Effect or Interaction	**Infrequent**
Log $[ABA]_{xyl}$ - Log g_s	**All Data**
Frequency	0.95
Cycle	0.086
$[ABA]_{xyl}$	**< 0.001**
Frequency x Cycle	**0.026**
Cycle x $[ABA]_{xyl}$	0.27
Frequency x $[ABA]_{xyl}$	0.37
Frequency x Cycle x $[ABA]_{xyl}$	**0.019**

Table 12. Effects of irrigation frequency, cycle, GWC and their interactions on $[ABA]_{xyl}$. **Table 13.** Effects of irrigation frequency, cycle, Ψ_{leaf} and their interactions on $[ABA]_{xyl}$. **Table 14.** Effects of irrigation frequency, cycle, Ψ_{shoot} and their interactions on $[ABA]_{xyl}$ (*P* values are presented, as with Fig.7).

Table 12	Frequent vs Infrequent
Effect or Interaction GWC- Log $[ABA]_{xyl}$	All Data
Frequency	0.15
Cycle	**0.022**
GWC	< 0.001
Frequency x Cycle	0.15
Cycle x GWC	**0.001**
Frequency x GWC	0.16
Frequency x Cycle x GWC	0.15

Table 13	Frequent vs Infrequent
Effect or Interaction Ψ_{leaf} - Log $[ABA]_{xyl}$	All Data
Frequency	0.074
Cycle	**0.018**
Ψ_{leaf}	**0.002**
Frequency x Cycle	**0.024**
Cycle x Ψ_{leaf}	0.052
Frequency x Ψ_{leaf}	**0.029**
Frequency x Cycle x Ψ_{leaf}	0.13

Table 14	Frequent vs Infrequent
Effect or Interaction Ψ_{shoot} - Log $[ABA]_{xyl}$	All Data
Frequency	0.61
Cycle	0.94
Ψ_{shoot}	**< 0.001**
Frequency x Cycle	0.95
Cycle x Ψ_{shoot}	0.98
Frequency x Ψ_{shoot}	0.49
Frequency x Cycle x Ψ_{shoot}	0.95

Table 15. Differences of transpiration rate for detached shoot fed artificial xylem sap with variable ABA concentrations were evaluated according to *Tukey's* multiple comparisons test (significant different $p<0.05$) by different letters (as with Fig.8).

Sig. ABA content	60 min	120 min	180 min	240 min	300 min
0 nM	a	a	a	a	a
10 nM	a	a	b	b	b
50 nM	a	b	b	c	b
100 nM	b	b	b	d	c
500 nM	c	c	c	e	d
1000 nM	c	c	c	f	e

Table 16. Significant differences between irrigation treatments according to a one-way ANOVA ($p<0.05$) of Whole pot soil gravimetric water content (GWC) and Evapotranspiration (ET) on each day throughout the experiment for each irrigation treatment under high and low relative humidity (as with Fig.9). Differences between irrigation treatments on each day are indicated by different letters according to *Tukey's* multiple comparisons test. " - " represents days where no data was collected.

Sig.	Relative Humidity	Treatments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
GWC	High (92-95% RH)	WW	a	a	a	a	a	a	a	a	a	a	a	a	a
		Drying	a	a	a	b	b	b	b	b	b	b	b	b	b
	Low (50% RH)	WW	**a**	**a**	**a**	a	a	a	a	a	a	a	a	a	a
		DRW	**a**	**a**	**a**	c	c	c	c	a	c	c	c	c	b
ET	High (92-95% RH)	WW	-	a	a	a	a	a	a	a	a	a	a	a	a
		Drying	-	a	a	a	a	a	a	a	b	b	b	b	b
	Low (50% RH)	WW	-	b	b	b	b	b	b	b	c	c	c	c	c
		DRW	-	b	b	c	c	a	a	c	d	d	a	d	d

Table 17. Differences between irrigation treatments of stomatal conductance, leaf water potential and shoot water potential on each day throughout experiment for each irrigation treatment under high and low relative humidity (as with Fig.10), were evaluated according to *Tukey's* multiple comparisons test (significant different $p<0.05$) by different letters.

Sig.	Relative Humidity	Treatments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
	High	**WW**	a	a	a	a	a	a	a	a	a	a	a	a	a
	(92-95% RH)	**Drying**	a	a	b	b	b	b	b	b	b	b	b	b	b
gs															
	Low	**WW**	**b**	**b**	**c**	a	c	c	c	c	b	b	c	c	c
	(50% RH)	**DRW**	**b**	**b**	**d**	c	c	c	c	c	c	c	b	d	b
	High	**WW**	a	a	a	a	a	a	a	a	a	a	a	a	a
	(92-95% RH)	**Drying**	a	a	a	a	a	a	a	a	b	b	b	b	b
Ψ_{leaf}															
	Low	**WW**	**b**	**b**	**b**	b	b	b	b	b	c	c	b	c	c
	(50% RH)	**DRW**	**b**	**b**	**b**	c	c	c	c	b	c	c	c	b	b
	High	**WW**	a	a	a	a	a	a	a	a	a	a	a	a	a
	(92-95% RH)	**Drying**	a	a	b	b	b	b	b	b	b	b	b	b	b
Ψ_{shoot}															
	Low	**WW**	**b**	**b**	**b**	**b**	c	c	c	c	c	c	c	c	c
	(50% RH)	**DRW**	**b**	**b**	**c**	**c**	d	d	d	b	d	c	d	d	b

Table 18. Differences between irrigation treatments of foliar and shoot xylem sap ABA concentration on each day throughout experiment for each irrigation treatment under high and low relative humidity (as with Fig.10), were evaluated according to *Tukey's* multiple comparisons test (significant different $p<0.05$) by different letters.

Sig.	Relative Humidity	Treatments	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12
	High	**WW**	a	a	a	a	a	a	a	a	a	a	a	a	a
	(92-95% RH)	**Drying**	a	a	a	a	a	b	b	b	b	b	b	b	b
Log[ABA]$_{leaf}$															
	Low	**WW**	**a**	**a**	**a**	a	a	a	a	a	a	c	c	c	c
	(50% RH)	**DRW**	**a**	**a**	**a**	b	b	c	c	c	b	d	b	b	b
	High	**WW**	a	a	a	a	a	a	a	a	a	a	a	a	a
	(92-95% RH)	**Drying**	a	a	a	a	a	a	b	b	b	b	b	b	b
Log[ABA]$_{xyl}$															
	Low	**WW**	**a**	**a**	**a**	a	a	a	a	a	c	c	c	c	c
	(50% RH)	**DRW**	**a**	**a**	**b**	b	b	b	c	c	a	d	d	b	b

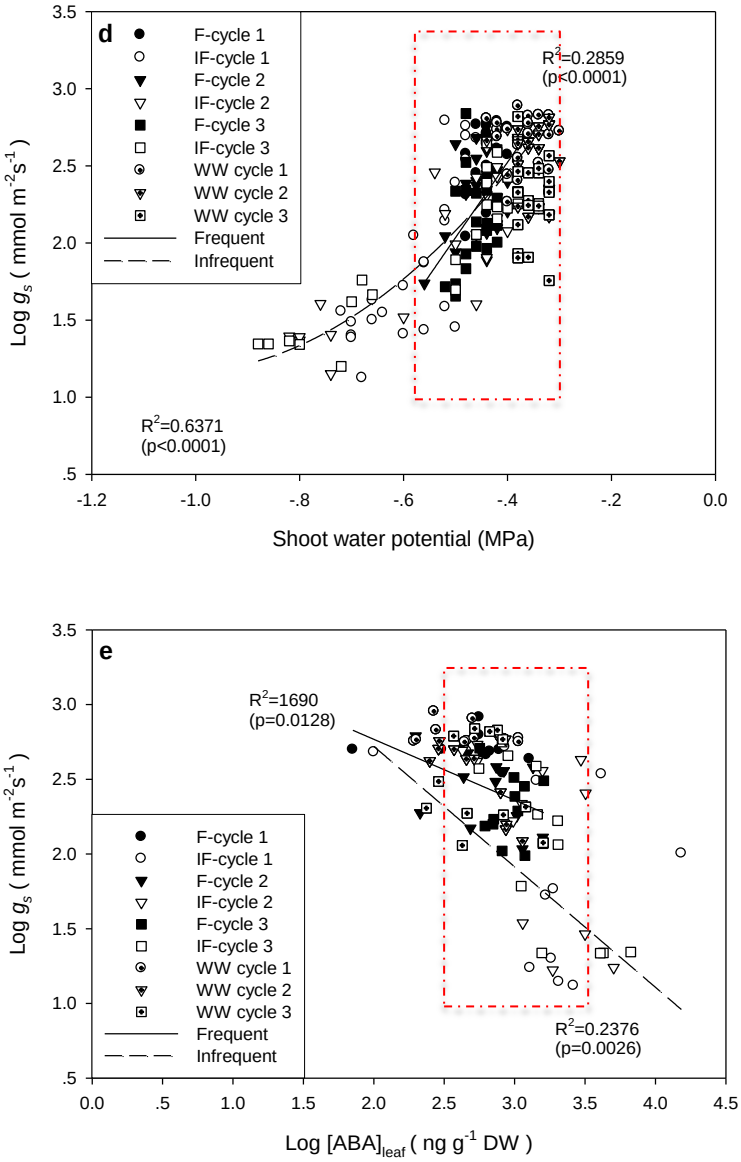

Figure 6. Relationships between stomatal conductance and (d) shoot water potential, (e) foliar ABA concentration for plants grown under frequent (SDI, filled point) and infrequent (drying and re-watering, hollow point) irrigation in Cycles 1 (circle), 2 (triangle) and 3 (square). Plants under WW irrigation treatment in Cycles 1 (crossed circle), 2 (crossed triangle) and 3 (crossed square). Each point represents a single plant and regression lines were fitted to frequent and infrequent treatments. P values determined by ANCOVA for each main effect (frequency, cycle and x-variable) and their interaction were reported in Table 19 and Table 20.

Table 19. Effects of irrigation frequency, cycle, Ψ_{shoot} and their interactions on g_s.

Table 20. Effects of irrigation frequency, cycle, $[ABA]_{leaf}$ and their interactions on g_s (*P* values are presented, as with Fig. 6d,e).

Table 19 **Effect or Interaction** **Ψ_{shoot} - Log g_s**	**Frequent vs Infrequent** **All Data**	**Frequent vs Infrequent** **Restricted** **($-0.6<\Psi_{shoot}<-0.3$ MPa)**
Frequency	0.77	0.17
Cycle	0.45	**0.041**
Ψ_{shoot}	**< 0.001**	**< 0.001**
Frequency x Cycle	0.45	0.077
Cycle x Ψ_{shoot}	0.62	0.43
Frequency x Ψ_{shoot}	0.74	0.16
Frequency x Cycle x Ψ_{shoot}	0.40	**0.042**

Table 20 **Effect or Interaction** **Log $[ABA]_{leaf}$ - Log g_s**	**Frequent vs Infrequent** **All Data**	**Frequent vs Infrequent** **Restricted** **(2.5 < Log $[ABA]_{leaf}$ < 3.5 ng g^{-1} DW)**
Frequency	0.065	**0.013**
Cycle	0.39	0.65
$[ABA]_{leaf}$	**0.003**	**< 0.001**
Frequency x Cycle	0.57	0.34
Cycle x $[ABA]_{leaf}$	0.38	0.69
Frequency x $[ABA]_{leaf}$	**0.042**	**0.009**
Frequency x Cycle x $[ABA]_{leaf}$	0.74	0.27

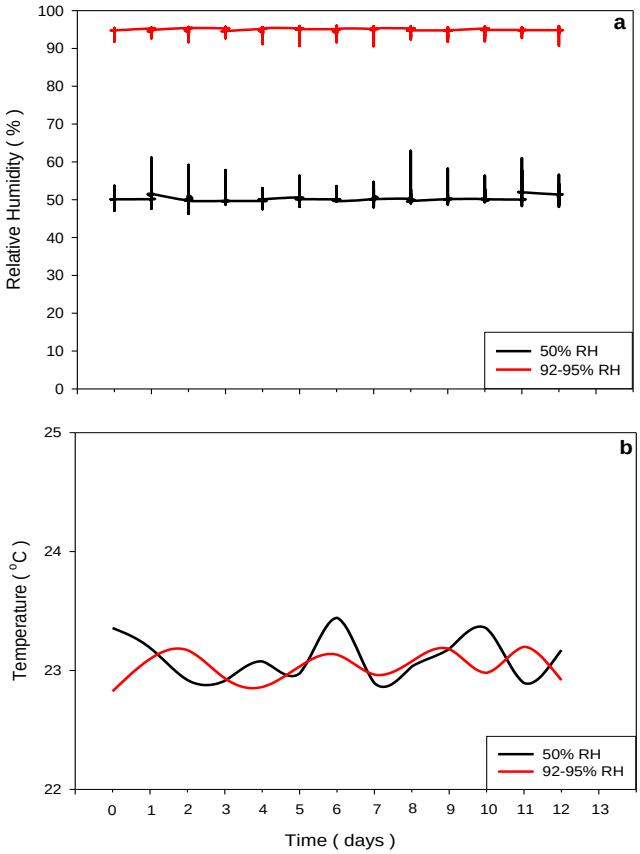

Figure 15. Controlled environment conditions of (a) high/low relative humidity and (b) temperature of growth cabinets recorded every 30 min over the whole experimental period, the red lines represent high relative humidity (92-95% RH) and temperature, the black lines represent low relative humidity (50% RH) and temperature.

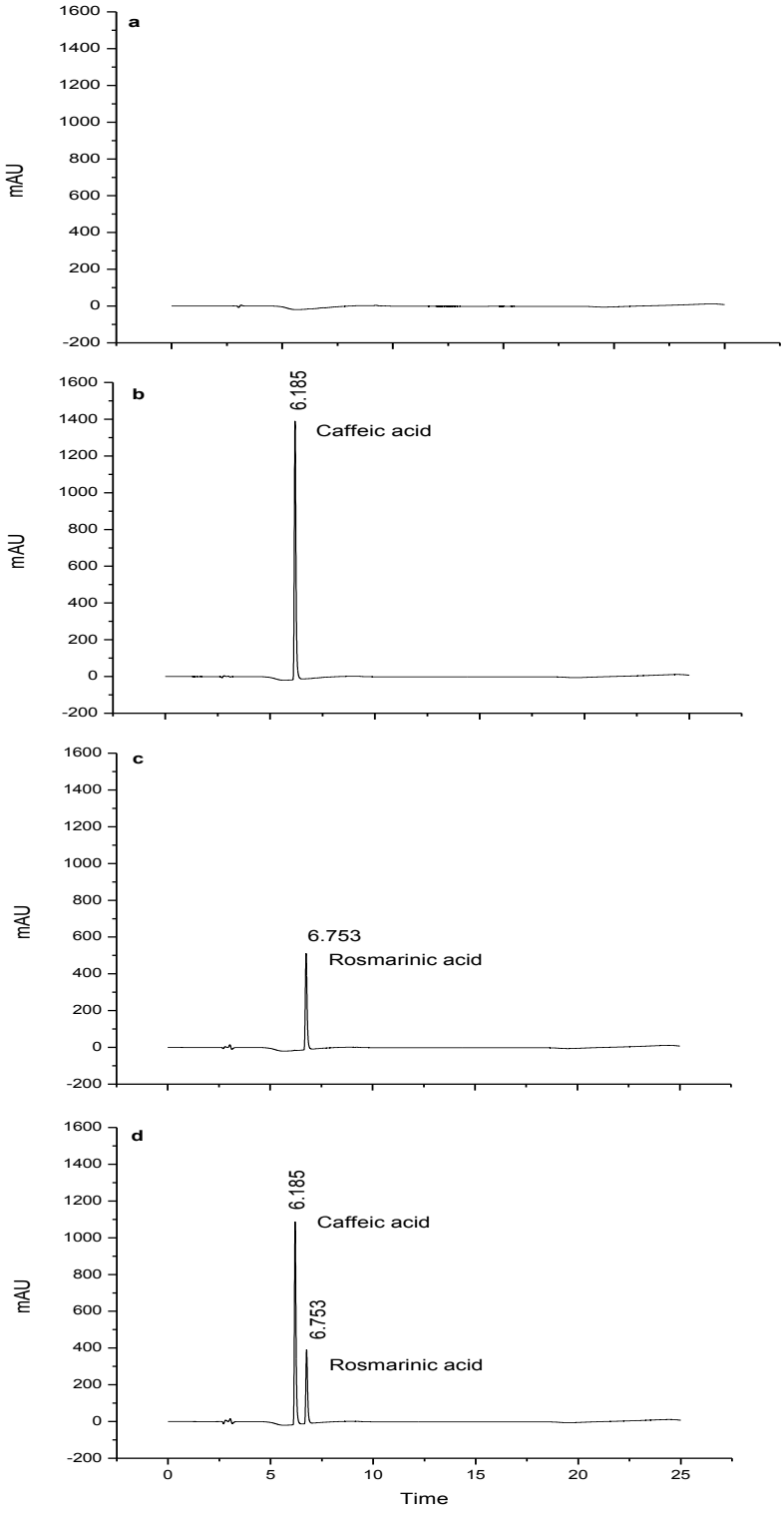

Figure 16. Chromatogram of **(**a) water, (b) caffeic acid (0.05 mg/mL), (c) rosmarinic acid (0.05 mg/mL) and (d) a mix of caffeic acid and rosmarinic acid (0.05 mg/mL). Peak identification with the components and retention times are indicated.

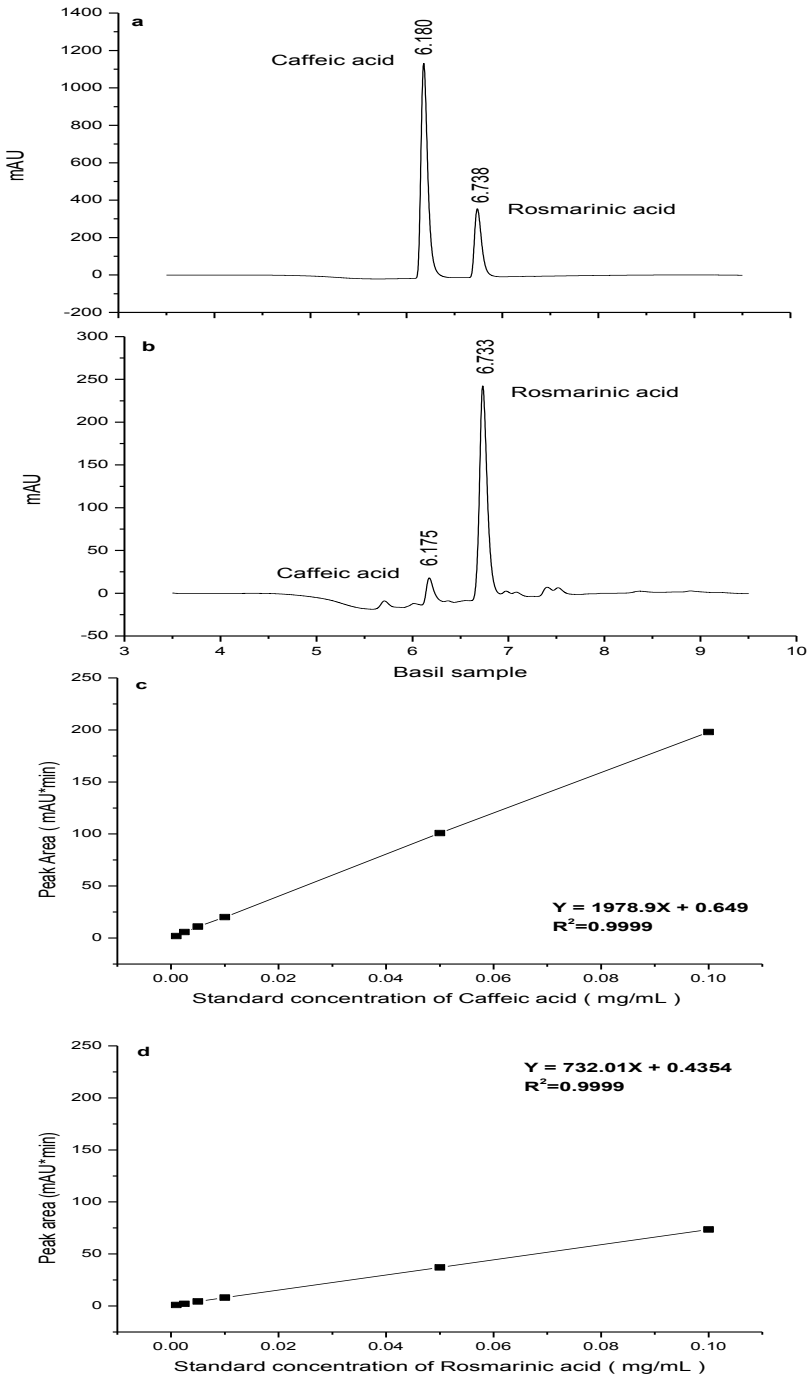

Figure 17. Chromatogram of **(**a) mix standard of caffeic acid and rosmarinic acid (0.05 mg/mL), (b) one extract from basil leaves from WW plants). Calibration curves of (c) caffeic acid and (d) rosmarinic acid, constructed by plotting their concentrations of standard working solution versus peak area, separately.

Taste Panel Survey for Basil under different irrigation frequencies

Current research for my Masters degree aims to investigate the effect of irrigation frequency on the production of green basil. The goal of this survey is therefore to establish whether altering irrigation frequency can improve basil quality, defined by different characteristics as described below. Plants have been divided into groups by irrigation frequency (labeled A, B and C). Please can you evaluate the leaf samples on a scale from 1 to 5 (5 being the highest and 1 the lowest) under the different categories below. Finally, it would be useful if you can provide some further comments in sections 3-5.（Group A with orange labeled, Group B with green labeled, Group C with white labeled.

1. Which leaves have the most attractive color and appearance?

	5	4	3	2	1
Group A	☐	☐	☐	☐	☐
Group B	☐	☐	☐	☐	☐
Group C	☐	☐	☐	☐	☐

Why do you rank them this way?

_____

_____

2. Which leaves have the most favourable aroma?

	5	4	3	2	1
Group A	☐	☐	☐	☐	☐
Group B	☐	☐	☐	☐	☐
Group C	☐	☐	☐	☐	☐

Is there any big difference in aroma among the three groups? Why do you rank them this way?

_____

_____

3. Which leaves have the most favourable taste?

	5	4	3	2	1
Group A	☐	☐	☐	☐	☐
Group B	☐	☐	☐	☐	☐
Group C	☐	☐	☐	☐	☐

Why do you rank them this way?

Could you define the groups as either sweet or bitter?

_____

_____

4. Which leaves have the best texture and consistency?

	5	4	3	2	1
Group A	☐	☐	☐	☐	☐
Group B	☐	☐	☐	☐	☐
Group C	☐	☐	☐	☐	☐

Is there any big difference in texture and consistency among the three groups?

_____

_____

5. How would you rate the leaves overall? Could you please provide some additional comments relating to any of the samples that have not come up in previous sections?

_____

_____

_____

_____

_____