

# Mitigating negative effects of long-term treated wastewater irrigation: Leaf gas exchange and water use efficiency response of avocado trees (*Persea americana* Mill.)

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## ABSTRACT

Declining performance of avocado orchards growing in a clayey soil irrigated with treated wastewater (TWW) for more than 5 years has been observed in Israel. Measures studied to mitigate this were freshwater (FW), blended TWW:FW in a 1:1 ratio (MIX), low-frequency TWW-irrigation (LFI), TWW irrigated tuff trenches (TUF) and TWW as the control treatment. This study reports on the response of avocado leaf gas exchange, intrinsic water use efficiency, leaf water potential and leaf hydraulic conductance ( $K_{leaf}$ ) to the mitigation measures applied to a 'Hass' avocado orchard (*Persea americana* Mill.) previously irrigated with TWW for 7 years. Stomatal conductance ( $g_s$ ) of FW, MIX, TUF and LFI was greater than that for TWW on most measurement dates, which increased net leaf  $CO_2$  assimilation rate ( $A_{leaf}$ ) and intercellular  $CO_2$  concentration ( $C_i$ ). Increased  $g_s$  reduced intrinsic water use efficiency [WUE, determined from either  $^{13}C$  discrimination (iWUE) or gas exchange (WUEi)] as compared to TWW. Although the WUE in TWW was higher than that in mitigation treatments on most measurement dates, the lower assimilation rates at leaf ( $A_{leaf}$ ) and whole tree ( $A_{tree}$ ) levels observed in TWW indicate that TWW irrigation had negative effects on tree physiological performance. A strong negative relationship between WUEi ( $A_{leaf}/g_s$ ) and  $g_s$  was found in all treatments, with highest WUEi and lowest  $g_s$  in TWW. Leaf hydraulic conductance was also lowest for TWW, which may indicate that lower gas exchange was linked to lower water availability to the leaves. Overall, the increased  $g_s$ ,  $A_{leaf}$ ,  $A_{tree}$  and  $K_{leaf}$  in FW, MIX and TUF relative to TWW indicate that these mitigation measures are good candidates for improving orchard performance negatively affected by long-term irrigation with TWW in clayey soils. Comparing all treatments, FW was the most effective.

## 1. Introduction

Avocado plantations in Mediterranean regions are important for the grower's economy and fresh fruit requirement of society and since avocados are healthy and nutritional they are increasing in demand. According to a recent market research report, the global avocado market valued at US\$13.64 bn in 2018 may reach a US\$21.56 bn by 2026 ([www.transparencymarketresearch.com/avocado-market.html](http://www.transparencymarketresearch.com/avocado-market.html)). Estimated total water requirements of avocado are ~650 mm during the rainless summer provided through irrigation, plus 500–600 mm supplied from winter rain (Carr, 2013; Lahav and Whiley, 2002). Peak water use in the rainless Mediterranean summer is 3–5 mm day<sup>-1</sup> (Carr, 2013) and most

cultivars are sensitive to both water deficits and excess soil water caused by poor drainage (Menzel and Le Lagadec, 2014). For instance, regulated deficit irrigation and water-stress caused significant yield reduction in avocado in Israel (Silber et al., 2019) compared with non-stressed. Chartzoulakis et al. (2002) reported decreased photosynthesis and  $g_s$ , and changes in leaf anatomy of avocado grown in water stress (water deficit). Similarly, moderate or severe water stress decreased net  $CO_2$  assimilation during periods of avocado fruit growth that would have probably caused fruitlet abscission (Schaffer and Whiley, 2003; Silber et al., 2012).

In many parts of the world, especially in arid and semiarid climates, water demand exceeds the water supply from fresh water sources (FAO,

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2012; UN Environment, 2019). As a consequence, inter-regional and international conflicts, sometimes called ‘water wars’, can occur (Per-eira et al., 2002). On the other hand, the production of large quantities of wastewater and its disposal to surrounding areas is becoming a critical environmental and health hazard. Thus, the scarcity of fresh water (FW) coupled with the need to manage ever-growing quantities of wastewater from municipal sewage treatment plants, increased the need to use treated wastewater (TWW) for irrigation. Long-term application of TWW to an avocado orchard is detrimental to plant water relations and sap flow (Nemera et al., 2020) and leaf photosynthesis (Bernstein et al., 2004) and can reduce yield by 20–40% (Assouline and Narkis, 2013). Avocado is a highly salinity sensitive fruit crop (Carr, 2013) and irrigation with marginal quality water of high salinity and other undesirable characteristics reduces productivity. Bernstein et al. (2004) reported that avocado roots are more sensitive to salinity than shoots. In California, Oster et al. (2007) speculated that avocado yield declines by 65% for each unit increase of irrigation water electrical conductivity (EC,  $\text{dS m}^{-1}$ ) above a threshold level of  $0.57 \text{ dS m}^{-1}$ . Avocado growers in Israel are advised not to use irrigation water containing more than 150 and  $250 \text{ mg l}^{-1}$  chloride for Mexican and Antillean rootstocks, respectively (Carr, 2013).

Poor soil aeration reduces oxygen supply, leading to reduced root absorption of nutrients and water, resulting in reduced tree performance (Kozłowski, 1997). High sensitivity of avocado trees to low oxygen levels in the soil was reported in several investigations (Ferreira et al., 2010; Valoras et al., 1964). Lower oxygen levels in avocado tree root zones in clay soils irrigated with TWW were noted compared with those found in freshwater (FW) irrigated trees (Assouline and Narkis, 2013; Yalin et al., 2017). In both publications the conclusion was that low oxygen levels in the root zone was a major factor that caused yield reduction. In general, the reduction in tree growth and yield following long-term irrigation with marginal quality water may be a result of the deterioration of soil physicochemical properties which reduce plant physiological performance.

Climatic changes may also induce or worsen abiotic stresses like water deficit and heat stress (Hatfield and Dold, 2019). Specifically, increased global warming and drought are expected in the Mediterranean basin climate, which is mostly conducive for growing avocado. Therefore, elucidating the combined effects of abiotic stresses on plants caused by irrigation with poor quality water (in our case) and climate variables governing gas exchange and plant water relations is important to anticipate potential problems. Schaffer and Whitley (2003) suggested that leaf conductance is a more reliable early indicator of water stress in avocado than leaf water content, leaf water potential and growth variables. However, Moreno-Ortega et al. (2019) did not find significant differences in gas exchange of avocado trees under different regimes of water supply.

Mitigating detrimental effects of long-term salinity and poor aeration in avocado orchards requires measures that will provide sufficient water to plants and minimize salt accumulation at least in the upper profile of the soil where most roots are located. We recently reported the effect of the following four mitigation measures on avocado tree water relations: freshwater (FW), blended water (MIX), low-frequency irrigation (LFI), and tuff trenches (TUF) along with control (TWW) (Nemera et al., 2020). We found that tree water use increased in all mitigation measures. Treatment ranking from highest to lowest sap flow, canopy conductance, and whole plant hydraulic conductance was  $\text{FW} > \text{TUF} > \text{MIX} > \text{LFI} > \text{TWW}$ . The observed differences can result from changes in leaf water relations or from changes in canopy structure, i.e., reduced leaf area. Previous work on the response of citrus to treated wastewater showed that the reduction in tree water use resulted from poorer leaf water relations and reduced leaf conductance, which was associated with changes in leaf specific hydraulic conductance and root activity (Paudel et al., 2018). Changes in root hydraulic conductivity were also associated with aquaporin gene downregulation (Paudel et al., 2017), indicating that TWW can impact the whole water transport

system.

Here we report on the impact of the mitigation measures on avocado leaf water relations, gas exchange and water use efficiency. Following our previous findings of increased water use of mitigation measures, we ask to what extent leaf level processes were affected. In particular (i) was stomatal conductance influenced and to what degree did mitigation measures affect gas exchange? (ii) was water use efficiency influenced? (iii) is there a relationship between the water use efficiency determined from gas exchange and  $^{13}\text{C}$  discrimination? (iv) is the change in leaf gas exchange influenced by climatic variables? and (v) are plant and soil water status (water potential) and leaf hydraulic conductance affected?

## 2. Materials and Methods

### 2.1. Experimental platform and treatment description

The experimental setup and treatments are described by Nemera et al. (2020). In brief, the experiment was conducted in a mature commercial fruit-bearing ‘Hass’ avocado orchard (*Persea americana* Mill.) grafted on Degania 117 rootstock at Kibbutz Yasur, the Western Galilee, Israel. The Orchard was established in 2009 and was irrigated with secondary TWW until 2016 when the study commenced. The experiment consisted of four mitigation measures and a control with six replications in a randomized design. Treatments were: (1) Irrigation with freshwater (FW), using 2 drip lines for each row of trees. (2) Irrigation with a 1:1 ratio of TWW and FW (MIX) using 4 drip lines; due to health regulations, the mix of the two water sources was obtained by irrigation with two drip lines side by side, one pipe for each type of water on both sides of the tree rows. (3) Low-frequency irrigation with TWW (LFI) obtained by adding a third drip line to each row of trees. (4) Tuff trenches (TUF), 0.3 m deep and 0.3 m wide, that were dug about 0.3 m away from the tree trunks on both sides of the tree rows and filled with volcanic tuff of particle size ranging from 0 to 8 mm; one drip lateral was laid on the tuff trenches on each side of the tree rows and irrigated with TWW. (5) Treated wastewater (TWW, i.e., control), irrigation using 2 drip lines for each row of trees about 0.3 m from each side of the tree trunks. Irrigation frequency varied among the treatments; TWW, FW, and MIX treatments were irrigated three times a week; LFI was irrigated twice a week; TUF trenches were irrigated every day from June 2016 to July 2018 and thereafter ten times a day. All treatments were irrigated with the same amount of water per week determined from reference  $\text{ET}_0$  and crop factors. Each drip lateral consisted of a set of drippers with emitters of 0.7 or  $1.6 \text{ l h}^{-1}$  discharge (UNIRAM, Netafim) spaced 0.3 m apart. Each plot comprised three rows with at least seven trees in each row. Tree spacing was 3.5 m and 6 m in and between rows, respectively. The average textural composition of the soil in the orchard was 58% clay, 19% silt, and 23% sand and the main physical properties, reported by Russo et al. (2020) are: saturated water content ( $\Theta_s$ ),  $-0.88 \text{ m}^3 \text{ m}^{-3}$ , residual water content ( $\Theta_r$ )  $-0.147 \text{ m}^3 \text{ m}^{-3}$ , saturated hydraulic conductivity ( $K_s$ )  $-0.108 \text{ m day}^{-1}$ . The main chemical properties of the soil are:  $\text{CaCO}_3$  100–240  $\text{g kg}^{-1}$ , soil organic matter 25  $\text{g kg}^{-1}$ , CEC 30–35  $\text{cmol}_c \text{ kg}^{-1}$ . Some physical properties of the tuff, reported by Wallach et al. (1992), are: particle size ranging from 0 to 8 mm, bulk density - 1091  $\text{kg m}^{-3}$ , porosity  $-0.587 \text{ m}^3 \text{ m}^{-3}$ ,  $\Theta_s - 0.55 \text{ m}^3 \text{ m}^{-3}$ ,  $\Theta_r - 0.08 \text{ m}^3 \text{ m}^{-3}$ ,  $K_s - 105.4 \text{ m day}^{-1}$ . The CEC of tuff at pH 7 is 28.5  $\text{cmol}_c \text{ kg}^{-1}$  (Silber et al., 1994). The tuff does not contain measurable  $\text{CaCO}_3$  or organic matter.

### 2.2. Climate data

The climatic parameters measured were wind speed ( $\text{m s}^{-1}$ ), solar radiation ( $\text{W m}^{-2}$ ), temperature ( $^{\circ}\text{C}$ ), and relative humidity (%) about 1 m above the treetops. Temperature and relative humidity were measured with a HMP35C sensor (Campbell Sci., Logan, UT), solar radiation with a pyranometer (CM5, Kipp and Zonen, Delft, Holland), and wind speed with a cup anemometer (014 A, Campbell Sci.). The

instruments used for measuring weather data were connected to a CR1000 data logger and a AM25 Tmultiplexer (Campbell Sci.). Vapor pressure deficit (VPD) was determined following Allen et al. (1999).

$$VPD = es - ea \quad (1)$$

$$es = \frac{e^0(T_{max}) + e^0(T_{min})}{2} \quad (2)$$

$$ea = \frac{e^0(T_{min}) \left( \frac{RH_{max}}{100} \right) + e^0(T_{max}) \left( \frac{RH_{min}}{100} \right)}{2} \quad (3)$$

Where  $es$  is the saturation vapor pressure at air temperature  $T$  (kPa),  $ea$  is the actual vapor pressure at air temperature  $T$  (kPa),  $e^0(T_{min})$  and  $e^0(T_{max})$  are the saturation vapor pressure at daily minimum temperature and maximum temperature (kPa), respectively, and  $RH_{max}$  and  $RH_{min}$  are maximum and minimum relative humidity (%), respectively.

### 2.3. Gas exchange measurements

Leaf gas exchange was measured on four to eight randomly selected, mature, current season sunlit leaves from four to eight trees for each treatment between 9:30 am and 14:30 pm using a portable photosynthesis system (LI-6400XT, LI-COR, Lincoln, Nebraska, USA) equipped with a LED-light source (6400-02B), coupled to a sensor head/IRGA, and with a CO<sub>2</sub> mixer (6400-01) to modify the incoming air's CO<sub>2</sub> concentrations. Leaf gas exchange was measured on June 27, July 25, and August 28, in 2017, and June 4, August 26, and September 12 in 2018. The measured parameters were net CO<sub>2</sub> assimilation rate ( $A_{leaf}$ , in  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), stomatal conductance ( $g_s$ , in  $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ), intercellular CO<sub>2</sub> concentration ( $C_i$ , in  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ ), and transpiration rate ( $E$ , in  $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ). These measurements were made by maintaining photosynthetic photon flux density (PPFD) of about 1000  $\mu\text{mol m}^{-2} \text{ s}^{-1}$  with 10% blue light and a CO<sub>2</sub> concentration of 400  $\mu\text{mol mol}^{-1}$  in the chamber during measurement. Air temperature and humidity in the chamber were set to match environmental conditions, where the leaf temperature was between 24 and 32.4 °C.

### 2.4. Water use efficiency

Water use efficiency (WUE), the amount of carbon assimilated per unit of water used is determined from measurements of gas exchange and carbon isotope ratios of leaf dry matter. In this study the focus is on an integrative intrinsic water use efficiency and instantaneous water use efficiency. The short term intrinsic water use efficiency (WUE<sub>i</sub>) was calculated as net photosynthetic rate ( $A_{leaf}$ ) divided by stomatal conductance ( $g_s$ ) (Hatfield and Dold, 2019). The gas exchange based WUE determined from CO<sub>2</sub> and water exchange at the leaf level on a short temporal scale is labor intensive and requires accessing leaves of tall trees (Yi et al., 2019). Therefore, we analyzed the <sup>13</sup>C discrimination derived intrinsic water use efficiency (iWUE) as an indicator of long-term trends in the internal regulation of carbon uptake and water loss of plants (Seibt et al., 2008).

Leaf discrimination against <sup>13</sup>C ( $\Delta^{13}\text{C}$ ) was calculated from the carbon isotope signature ( $\delta^{13}\text{C}$ ) results and expressed in delta notation as parts per thousand (‰) deviations from the international carbon isotope standard following (Leonardi et al., 2012).

$$\Delta^{13}\text{C} = \frac{\delta^{13}\text{Ca} - \delta^{13}\text{Cp}}{1 + \frac{\delta^{13}\text{Cp}}{1000}} \quad (4)$$

where  $\delta^{13}\text{Ca}$  and  $\delta^{13}\text{Cp}$  are the respective isotopic ratios of atmospheric CO<sub>2</sub> and of plant material.

The  $\delta^{13}\text{C}$  measurement method has been described previously (see Nemera et al., 2020; Uni et al., 2020). In brief, samples were oven-dried at 60 °C for 48 h and ground to dust by mortar and pestle in liquid

nitrogen. Measurements were performed using a <sup>13</sup>CO<sub>2</sub> cavity ring down spectroscopy analyzer (CRDS) (Picarro G2131i, Picarro, Santa Clara, CA, USA) with Combustion module equipped with an auto-sampler (ECS 4010, Costech Analytical, Valencia, CA, USA). International standards (IAEA-CH-3, Cellulose, International Atomic Energy Agency, Vienna, Austria) were used for calibration.

The  $\Delta^{13}\text{C}$  derived iWUE can provide a time-integrated measure of WUE over the course of the growing season because of the seasonal storage of CO<sub>2</sub> assimilated during the growing season (Belmecheri et al., 2014; Frank et al., 2015). The iWUE was determined from  $\Delta^{13}\text{C}$  as described by Leonardi et al. (2012).

$$iWUE = 0.625 * \frac{Ca(b - \Delta^{13}\text{C})}{(b - a)} \quad (5)$$

where  $Ca$  is the atmospheric CO<sub>2</sub> concentration in ppm,  $\Delta^{13}\text{C}$  is the leaf discrimination against <sup>13</sup>C, 0.625 is the ratio between the stomatal conductance to CO<sub>2</sub>, and water vapor,  $a$  is a constant for the leaf-level discrimination against <sup>13</sup>C in diffusion through the stomata (4.4‰), and  $b$  is a constant for the enzymatic carbon fixation by Ribulose 1,5-bisphosphate carboxylase (27‰).

### 2.5. Tree scale carbon assimilation rate

Tree-scale carbon assimilation rate ( $A_{tree}$ ) was determined from  $\Delta^{13}\text{C}$  derived intrinsic water use efficiency (iWUE) (Eq. (5)) and  $g_s$  (Klein et al., 2016) as follows:

$$A_{tree} = \frac{iWUE}{g_s} \quad (6)$$

The  $g_s$  was determined from measured individual tree transpiration rate ( $T$ ) and VPD following (Beer et al., 2009; Klein et al., 2016).

$$g_s = \frac{T}{VPD} \quad (7)$$

### 2.6. Leaf water potential and leaf hydraulic conductance

Midday stem water potential ( $\Psi_{stem}$ ) measurements were made on fully matured leaves taken from five trees for each mitigation treatment using a pressure chamber (Arimad, M.R.C Ltd., Israel).  $\Psi_{stem}$  was measured on June 14, July 6 and 19, August 7 and 21 in 2017 and on May 21, June 11, June 25, July 9 and August 6 in 2018. Additionally, predawn water potential ( $\Psi_{pd}$ ),  $\Psi_{stem}$ , and leaf water potential ( $\Psi_{leaf}$ ) were measured on July 23, 2018 from fully-grown leaves taken from trees with active sap flow sensors. The leaf samples for  $\Psi_{pd}$ ,  $\Psi_{stem}$ , and  $\Psi_{leaf}$  measurement on July 23, 2018 were taken from the same branches used for gas exchange measurement.  $\Psi_{stem}$  was measured on fully-grown shaded leaves near the main stem enclosed in aluminum and covered plastic bags for about two hours to stop transpiration and allow leaf water potential to equilibrate with stem water potential.  $\Psi_{leaf}$  was measured on fully-grown sunlit leaves.  $\Psi_{stem}$  and  $\Psi_{leaf}$  were measured at about the same times, at midday between 11:30 am and 2:30 pm.  $\Psi_{pd}$ , considered as a proxy for soil water potential, was measured between 4:00 and 5:30 am, before sunrise. All water potential measurements were made on at least 6 leaves per treatment from 6 different trees in the orchard.

Leaf hydraulic conductance ( $K_{leaf}$ ,  $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1} \text{ MPa}^{-1}$ ) was determined from leaf transpiration and the water potential gradient between the stem and leaf (Sperry and Pockman, 1993) as:

$$K_{leaf} = \frac{E}{\Psi_{stem} - \Psi_{leaf}} \quad (8)$$

where  $E$  is transpiration ( $\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ),  $\Psi_{stem}$  is stem water potential (MPa) and  $\Psi_{leaf}$  is leaf water potential (MPa).

## 2.7. Statistical analysis

The statistical analysis was performed with JMP® pro statistical software version 15.1.0 (SAS Institute Inc., USA). Differences in studied parameters among treatments were evaluated by analysis of variance (ANOVA). Differences between mitigation measures were considered significant at the 5% probability level. Prior to ANOVA, normality and variance homogeneity assumptions were tested by using the Shapiro-Wilk W and the Levene's test, respectively. When significant differences among means were found, Tukey's honestly significant difference (HSD) test was used to compare mean values.

## 3. Results

### 3.1. Climate variables

There was much variability in climatic conditions between measurement years and dates (Fig. 1). The highest mean midday (10:00–15:00) VPD, 2.2 kPa, air temperature, 31.3 °C and RH, 65% were recorded in August 2017, September 2018, and June 2018, respectively. The lowest mean midday (10:00–15:00) VPD, 1.27 kPa, air temperature, 27.0 °C, and RH, 51%, were recorded in June 2018, June 2018, and August 2017, respectively.

### 3.2. Leaf gas exchange

Differences in leaf gas exchange between treatments for different measurement periods were not always consistent.  $A_{\text{leaf}}$  in FW and MIX was significantly higher than TWW in July 2017, June 2018, August 2018 and September 2018 ( $p < 0.05$ ; Fig. 2A). The  $A_{\text{leaf}}$  in TUF was significantly higher than TWW in July 2017, June 2018, and August 2018 (Fig. 2A). The values of  $A_{\text{leaf}}$  in LFI were intermediate between TWW and the other three remediation measures. The values of  $A_{\text{tree}}$  were consistently higher in all mitigation measures than in TWW, particularly FW, MIX and TUF, but differences were not statistically significant. The ranges of values of  $A_{\text{tree}}$  for FW, MIX, TUF, LFI and TWW were 229–476, 204–468, 214–440, 195–439, and 178–412 g C per tree day<sup>-1</sup> (Fig. 2B), respectively.

The  $g_s$  in FW was significantly higher than TWW on all measurement dates except August 2017 (Fig. 3A). The  $g_s$  in TWW was significantly lower than MIX in June 2018, August 2018, and September 2018, and TUF in July 2017, June 2018 and August 2018 (Fig. 3A). The  $C_i$  in FW was significantly higher than TWW in June 2017 and August 2018 (Fig. 3B).  $C_i$  values in MIX and TUF were significantly higher than TWW only in August 2018 (Fig. 3B). The  $E$  was significantly higher in FW and MIX than in TWW on all measurement dates except June 2017 for FW and June 2017 and July 2017 for MIX (Fig. 3C). The values of  $E$  in TUF were significantly higher than in TWW on all measurement dates except

in June 2017 and September 2018 (Fig. 3C).

### 3.3. Water use efficiency

The integrative intrinsic water use efficiency (iWUE) determined from  $\Delta^{13}\text{C}$  was significantly higher in TWW than in FW, MIX and TUF in 2018 ( $p < 0.05$ ; Fig. 4). Values of gas exchange measurement based instantaneous intrinsic water use efficiency (WUEi) were not consistent between measurement dates. The WUEi was significantly higher in TWW than in FW in June 2017, August 2018 and September 2018 ( $p < 0.05$ ; Fig. 5) and in TUF in August 2018 ( $p < 0.05$ ; Fig. 5). The relationship between WUEi and  $A_{\text{leaf}}$  showed inconsistent trends for all treatments (Fig. 6B). Yet in general, an inverse type relationship was noted between WUEi and  $g_s$  showing that an increase in  $g_s$  led to a decrease in WUEi (Fig. 6A). Regression analysis of WUEi vs.  $A_{\text{leaf}}$  and WUEi vs.  $g_s$  are shown in Table 1. A high correlation was found between iWUE and WUEi in the three sampling dates in 2018 with slopes very close to 1.0 in August and September 2018 (Fig. 7B and 7C).

### 3.4. Leaf water potential and leaf hydraulic conductance

The seasonal stem water potential ( $\Psi_{\text{stem}}$ , MPa) measurements show that there was significantly higher  $\Psi_{\text{stem}}$  in FW on May 21, 2018, June 11, 2018, and August 6, 2018 ( $p < 0.05$ ; Fig. 8). The values of the  $\Psi_{\text{stem}}$  in TUF were significantly lower than TWW on June 25, 2018 ( $p < 0.05$ ; Fig. 8). The determination of water potential gradient revealed that the soil water potential ( $\Psi_{\text{soil}}$ ) ( $p < 0.05$ ; Fig. 9A) determined based on pre-dawn leaf water potential was significantly higher in FW than TWW with no significant differences between mitigation measures and the control in  $\Psi_{\text{stem}}$  (Fig. 9B) and  $\Psi_{\text{leaf}}$  (Fig. 9C).

Leaf hydraulic conductance ( $K_{\text{leaf}}$ ) determined from leaf transpiration rate and the leaf water potential gradient showed that  $K_{\text{leaf}}$  was significantly higher in FW and TUF than in TWW ( $p < 0.05$ ; Fig. 9D).

## 4. Discussion

The results of our study demonstrate that the mitigation measures, FW, MIX and TUF increased leaf gas exchange, and leaf hydraulic conductance, and decreased water use efficiency of mature avocado trees relative to TWW on most measurement dates. Specifically,  $g_s$  was significantly higher in FW on five out of six measurement days, and in both MIX and TUF in three out of six measurements as compared to TWW ( $p < 0.05$ ; Fig. 3A).

The significantly lower  $g_s$  and  $C_i$  in TWW relative to FW with no significant reduction in  $A_{\text{leaf}}$  and  $E$  in June 2017 shows the onset of stress. From our data it seems that  $g_s$  is the most sensitive gas exchange trait affected by the long-term irrigation with treated wastewater. Stomatal closure or the reduction of stomatal opening in avocado is an early

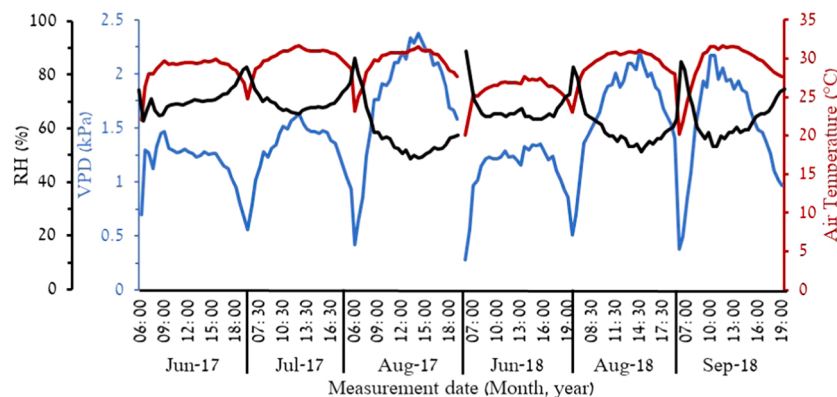
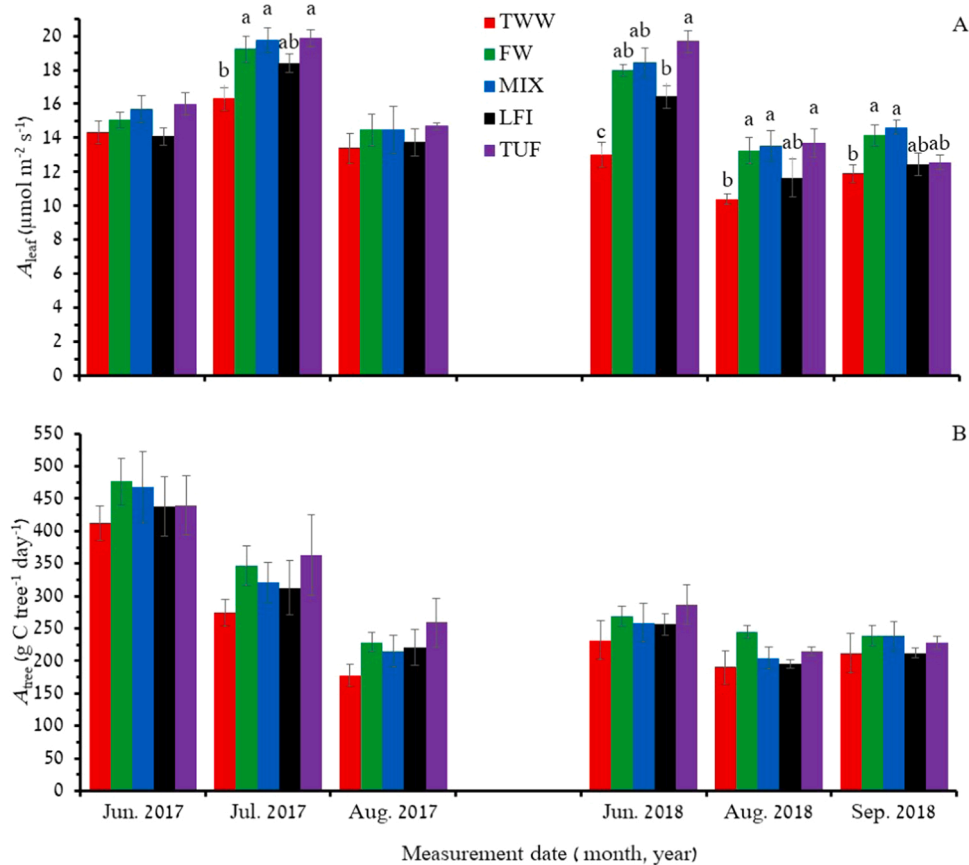


Fig. 1. Day time (6:00–19:00) courses of half hourly values of air temperature (red line), relative humidity (RH) (black line) and vapor pressure deficit (VPD) (blue line) on gas exchange measurement dates.





**Fig. 2.** Courses of net CO<sub>2</sub> assimilation rate of leaves ( $A_{leaf}$ , A) and trees ( $A_{tree}$ , B). Data are means  $\pm$  SE ( $n = 4-8$  for  $A_{leaf}$  and  $n = 6-9$  for  $A_{tree}$ ). Different lowercase letters indicate significant differences ( $p < 0.05$ ) between treatments for a given measurement date. The gap separates between 2017 and 2018.

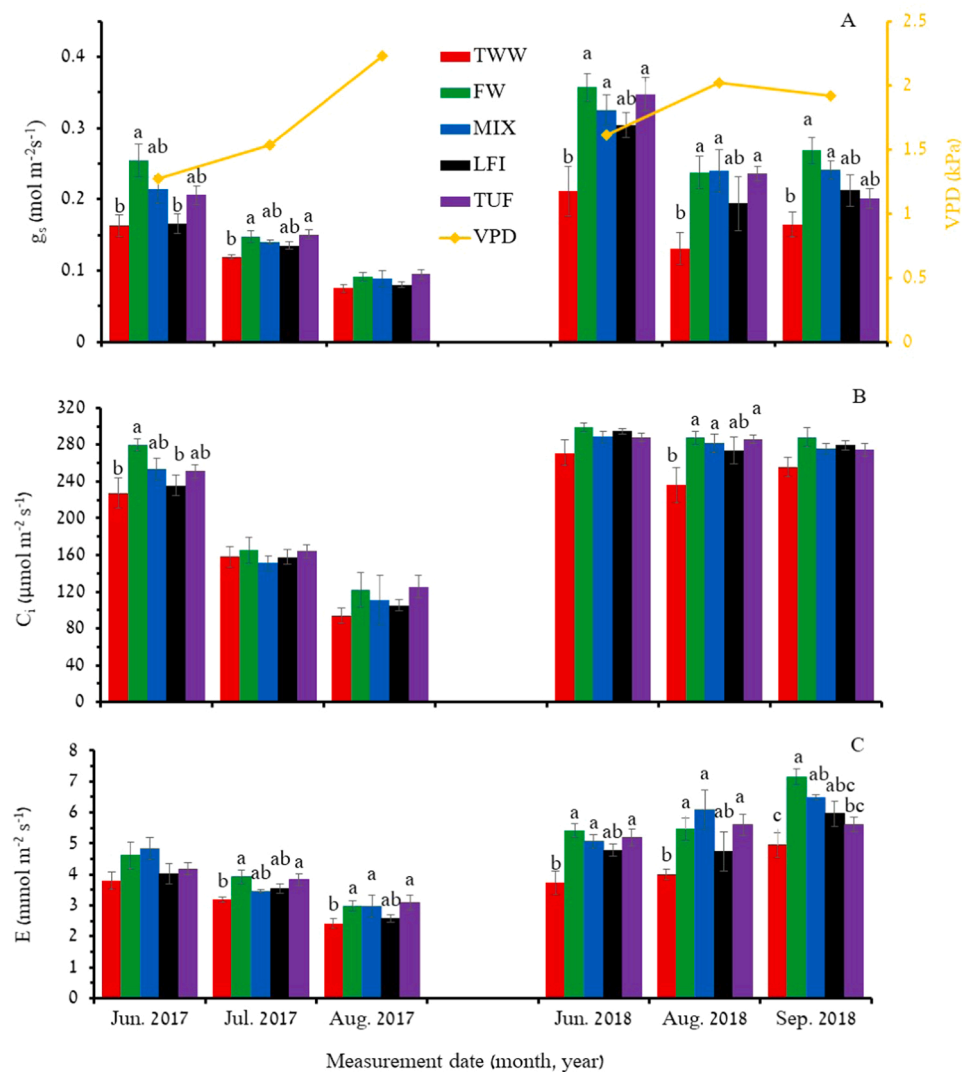
indicator of water stress (Carr, 2013). Plaguezuelo et al. (2018) also indicated that stomatal closure in avocado in a Mediterranean climate is rapid and is an early indicator of water stress associated with changes in leaf anatomy restricting the diffusion of CO<sub>2</sub>. The  $A_{leaf}$  in TWW was also significantly lower than both FW and MIX in four out of six measurements and TUF in three out of six measurements (Fig. 2A).

The lower  $A_{leaf}$  and  $g_s$  indicate that trees in TWW might be under water stress from various reasons discussed elsewhere (Assouline and Narkis, 2013; Paudel et al., 2018, 2017, 2016a; Rahav et al., 2017; Soda et al., 2017). In addition to the plant response observed in this study, long-term irrigation with TWW deteriorates soil aggregate stability (Levy et al., 2011) and increases soil repellency to water (Nadav et al., 2013; Schacht and Marschner, 2015; Wallach et al., 2005) leading to non-uniform distribution of water in the rhizosphere. Under such a heterogeneous conditions water availability to the plant uptake may be limited. Moreover, the higher salinity in TWW might have impacted the osmotic potential gradient of the soil-root junction particularly compared to FW and MIX. Reduced plant water uptake in TWW relative to FW, MIX and TUF was found in the same orchard from measurements of whole tree sap flow, whole plant hydraulic conductance and <sup>13</sup>C signature (Nemera et al., 2020). Similar reductions in water uptake were predicted by a numerical simulation of TWW irrigation using soil parameters of an avocado orchard on clay soil (Russo et al., 2020). A study on the effect of TWW on citrus trees (Paudel et al., 2018, 2017, 2016b) also found that tree water uptake is reduced in TWW relative to FW. Furthermore, significantly lower  $\Psi_{soil}$  (determined from pre-dawn leaf water potential) ( $p < 0.05$ ; Fig. 9A) strengthens the speculation that long-term irrigation with TWW negatively affects plant water uptake. In mitigation measures which improved water quality (FW and MIX), the higher leaf gas exchange observed in this study and the increased water uptake reported by Nemera et al. (2020) could result from the combined

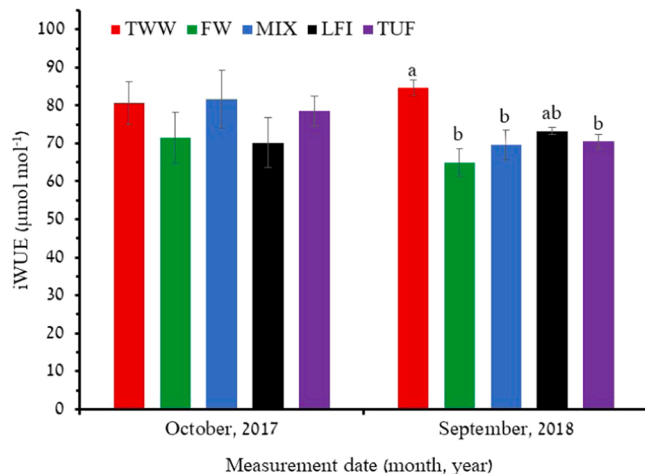
effect of improvement in soil physicochemical characteristics such as reduced salinity, reduced concentration of toxic ions (Na, and Cl), reduced sodium adsorption ratio (SAR), reduced exchangeable sodium percentage (ESP) and the increase in soil aggregate stability in FW (Nemera et al., 2021), in agreement with the response of a citrus orchard (Paudel et al., 2018, 2016b) to long-term irrigation with treated wastewater in Israel. The increased gas exchange in TUF relative to TWW seems to be due to increased soil aeration (Nemera et al., 2021) and higher hydraulic conductivity of the TUF. The increased soil aeration is expected to improve tree root function.

Plants in dry environment experience stress from climate variables like high temperature and irradiance that would lead to higher vapour pressure deficit (VPD) and evapotranspiration which make the effects of water deficit more severe and favors salinity build up. The numerical differences in  $A_{leaf}$ ,  $g_s$ ,  $C_i$  and  $E$  observed between measurement dates irrespective of mitigation measures in our study show a change that was related to atmospheric water demand expressed by VPD (Fig. 3A). The lower  $A_{leaf}$  and  $g_s$  were observed in August 2017 when highest VPD, air temperature and lowest RH were recorded (Fig. 1). The high evaporative demand of the atmosphere during the irrigation seasons also decreases water use efficiency of plants. Notably, the lowest  $g_s$ ,  $C_i$  and  $E$  were observed at higher VPD irrespective of the treatments, evidence for the important role of VPD in regulating gas exchange.

The above-discussed stressors (soil quality, water quality and climate variables) might lead to stomatal and/or nonstomatal limitation to photosynthesis. The significantly lower  $A_{leaf}$  (Fig. 2A) in TWW relative to FW, MIX and TUF was observed when there was no significant difference in  $C_i$  (July 2017, August 2017, June 2018 and September 2018) (Fig. 3B). This can indicate that the poor-quality irrigation water might also cause non-stomatal limitation to photosynthesis. At the time of linear increase of CO<sub>2</sub> assimilation with stomatal conductance, WUEi



**Fig. 3.** Courses of  $g_s$  (stomatal conductance) (A),  $C_i$  (intercellular  $CO_2$  concentration) (B), and  $E$  (transpiration rate) (C). Data are means  $\pm$  SE (n = 4 – 8) and different lowercase letters indicate significant differences ( $p < 0.05$ ) between treatments for a given measurement month. The gap separates between 2017 and 2018.

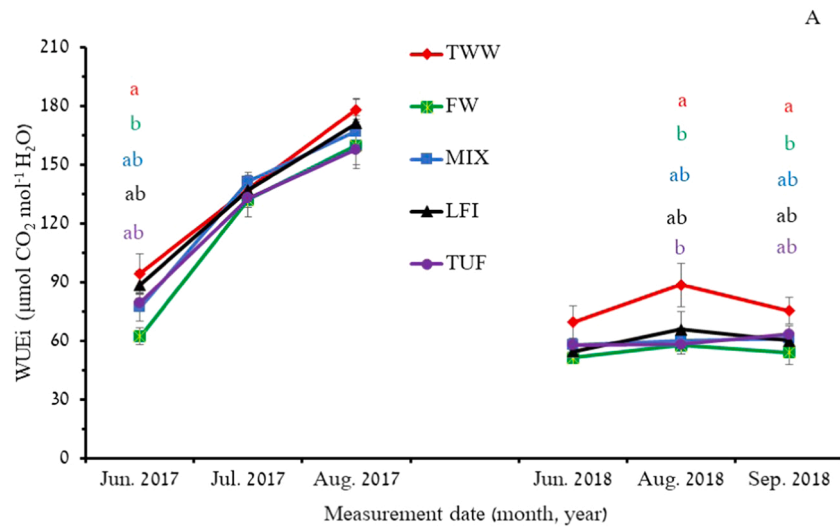


**Fig. 4.**  $\Delta^{13}C$  derived intrinsic water use efficiency (iWUE) in October 2017 and September 2018. The data are means  $\pm$  SE (n = 5–6 per treatment). Different lowercase letters indicate significant differences ( $p < 0.05$ ) between at least two treatments for a given measurement date.

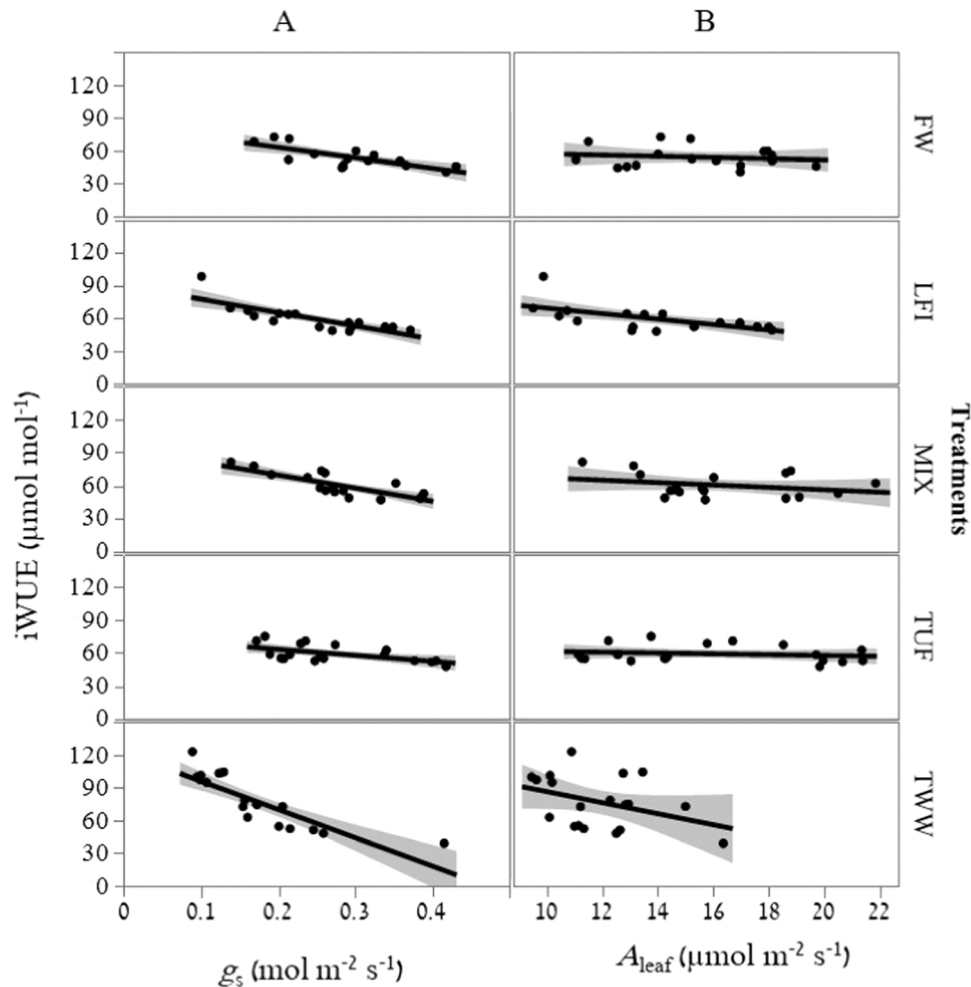
increases with  $g_s$  because the slope of net  $CO_2$  assimilation is steeper than the slope of transpiration.

The significant reduction in  $g_s$  led to a larger increase in  $WUE_i$  in TWW than in FW and TUF on two (August and September 2018) and one (August 2018) measurement dates, respectively ( $p < 0.05$ ; Fig. 5). The regression analyses performed to evaluate the relationship between the  $WUE_i$ ,  $A_{leaf}$  and  $g_s$  show that there was a strong decreasing relationship between  $WUE_i$  and  $g_s$  (Fig. 6A). This indicates that water use efficiency decreased with increasing  $g_s$  more sharply in TWW than in the other mitigation treatments. Except for LFI, there is no relation between  $WUE_i$  and  $A_{leaf}$  (Fig. 6A and Table 1). The relationship between  $WUE_i$  and  $g_s$  shows that  $g_s$  plays a crucial role in controlling not only photosynthesis rate but also the intrinsic water use efficiency of the plant.

From our data and the literature, it is evident that  $WUE$  determined from instantaneous gas exchange measurements changes temporally with climate. The iWUE showed significantly higher values in TWW compared with FW, MIX and TUF in the 2018 irrigation season ( $p < 0.05$ ; Fig. 4). The  $\Delta^{13}C$  derived intrinsic  $WUE$  (Fig. 4), which is a more temporally integrative variable, was more stable but lower than the values of gas exchange derived intrinsic  $WUE_i$  (Fig. 5). According to Klein et al. (2016) this difference in  $WUE$  is expected as the gas exchange measurements performed under saturating light conditions favors higher  $A_{leaf}$  and  $WUE_i$  than under ambient conditions which is the case



**Fig. 5.** Courses of intrinsic water use efficiency (WUEi) determined from gas exchange measurements in the 2017 and 2018 irrigation seasons. Data are means  $\pm$  SE ( $n = 4-8$ ). Means accompanied by different lowercase letters indicate significant differences ( $p < 0.05$ ) between treatments for the given measurement date. The gap separates between 2017 and 2018.



**Fig. 6.** Relationship between WUEi and  $g_s$  (A) and WUEi and  $A_{\text{leaf}}$  (B) for the treatments on the three measurement dates in 2018. The leaner relationship between WUEi and  $g_s$  was significant for all treatments. That between WUEi and  $A_{\text{leaf}}$  was not significant for all treatments.

**Table 1**

Linear regression results for water use efficiency (WUEi) vs stomatal conductance ( $g_s$ ) and WUEi vs leaf  $CO_2$  assimilation rate ( $A_{leaf}$ ) on 2018 gas exchange measurement dates (June, August and September). In the equations for the least-square regression lines, y is WUEi and x is either  $g_s$  or  $A_{leaf}$ .

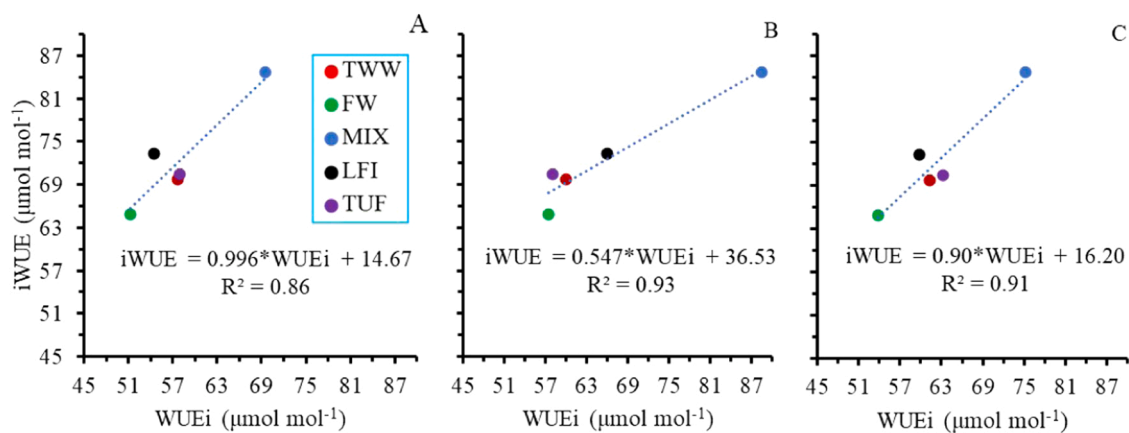
Regression parameters	Treatments	Equation	R <sup>2</sup>	P-Value
WUEi vs $g_s$	TWW	$WUEi = -389.7 * g_s + 141.6$	0.85	< 0.0001
	FW	$WUEi = -94.6 * g_s + 81.8$	0.53	0.001
	MIX	$WUEi = -116.8 * g_s + 93.4$	0.65	< 0.0001
	LFI	$WUEi = -122.9 * g_s + 89.6$	0.65	< 0.0001
	TUF	$WUEi = -66.7 * g_s + 78.2$	0.39	0.003
WUEi vs $A_{leaf}$	TWW	$WUEi = -3.20 * A_{leaf} + 116.2$	0.05	0.41
	FW	$WUEi = -0.45 * A_{leaf} + 62.38$	0.02	0.57
	MIX	$WUEi = -1.31 * A_{leaf} + 82.3$	0.12	0.17
	LFI	$WUEi = -2.53 * A_{leaf} + 94.46$	0.39	0.006
	TUF	$WUEi = -0.62 * A_{leaf} + 70.03$	0.07	0.27

in the iWUE. Nemera et al. (2020) reported that the bulk leaf tissue  $\delta^{13}C$  of avocado trees changed gradually in the mitigation measures, and that it was significantly lower in FW, MIX, and TUF by an order of magnitude compared with TWW. This suggest that the TWW had higher intrinsic water use efficiency, indicative of reduced stomatal conductance. In

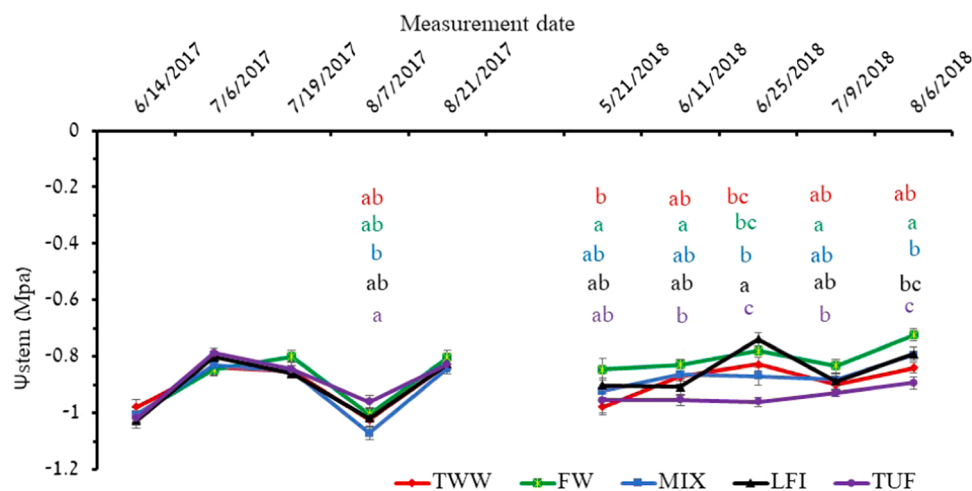
contrast to our finding, Acosta-Rangel et al. (2019) reported that the intrinsic WUE of "Hass" avocado scions grafted on different rootstocks was lower under higher salinity. Increased iWUE under stress in our case (salinity, water deficit or poor rhizosphere aeration) is the result of reduced stomatal conductance, which in turn causes a reduction of  $CO_2$  concentration at the site of carboxylation, forcing Rubisco to assimilate more  $^{13}CO_2$  (Acosta-Rangel et al., 2019). The evidence for the larger contribution of reduced stomatal conductance as compared with the increase in carbon assimilation rate to increasing iWUE in TWW is the lower  $CO_2$  assimilation rate per tree (Fig. 2B) and the lower plant trunk growth than in the mitigating treatments in the same avocado orchard (Nemera et al., 2020). Thus, high intrinsic WUE indicated that stomata closed, and tree activity was decreased, which had negative effects on tree physiological response, growth, and productivity. The correlation analysis of the iWUE (Long-term) and WUEi (short-term) showed a strong positive correlation with the highest value in TWW and lowest in FW.

## 5. Conclusions

Long-term irrigation of orchards with TWW in clayey soil causes degradation of soil physicochemical properties and orchard performance. This ill effect of TWW requires introduction of remediation measures to enhance sustainable TWW use with minimal detrimental

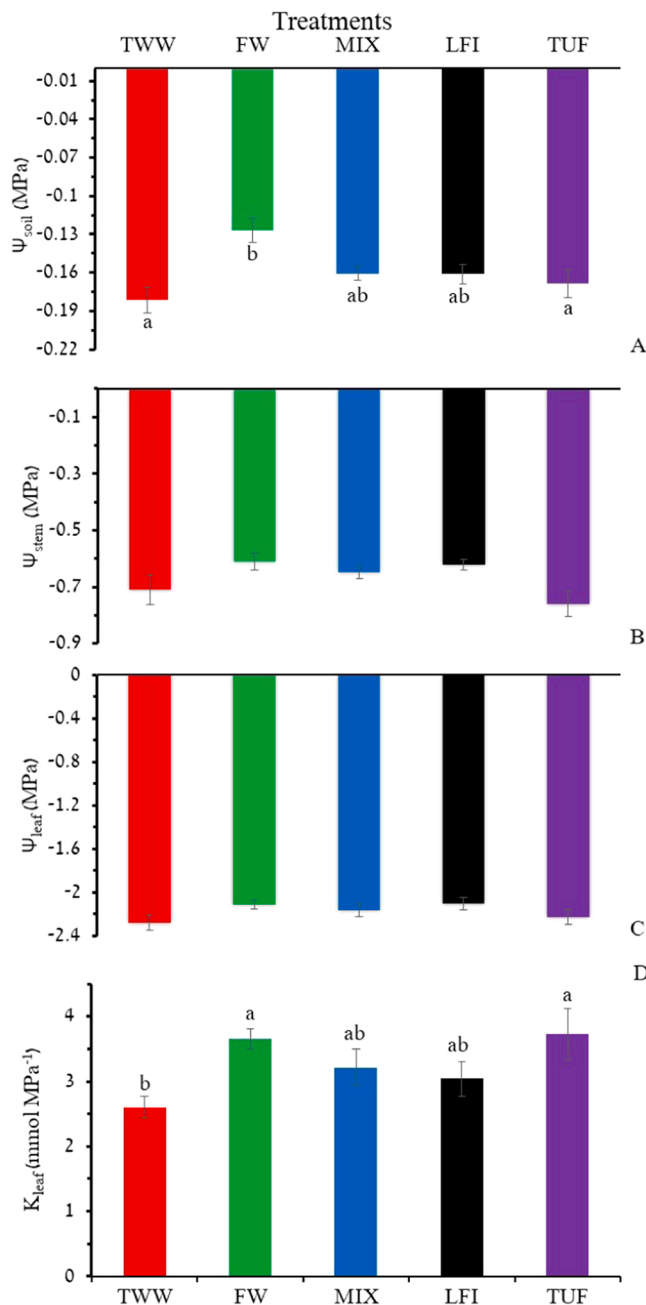


**Fig. 7.** The relationship between  $\delta^{13}C$  derived integrative intrinsic water use efficiency (iWUE) and instantaneous gas exchange derived intrinsic water use efficiency (WUEi) in June 2018 (A), August 2018 (B), and September 2018 (C). Treatments are indicated by different colors. The values are average per treatment.



**Fig. 8.** Courses of stem water potential ( $\Psi_{stem}$ ). Bars indicate two standard errors. Different lowercase letters indicate significant differences ( $p < 0.05$ ) between treatments for the given measurement date. The gap separates between seasons 2017 and 2018.





**Fig. 9.** Effects of the mitigation measures on  $\Psi_{\text{soil}}$  (A),  $\Psi_{\text{stem}}$  (B),  $\Psi_{\text{leaf}}$  (C), and  $K_{\text{leaf}}$  (D). Bars indicate two standard errors. Different lowercase letters indicate significant differences ( $p < 0.05$ ) between treatments. Measurement was made on July 23, 2018.

effects on orchard performance. This study investigated avocado leaf gas exchange, water use efficiency, and leaf hydraulic conductance in four mitigation measures together with TWW (control) in one comprehensive study in the same orchard. The results revealed that  $g_s$  and  $A_{\text{leaf}}$  were significantly increased in FW, MIX and TUF on most measurement dates relative to TWW. In addition, FW and TUF significantly increased leaf hydraulic conductance. As expected, increased  $g_s$  significantly reduced  $\Delta^{13}\text{C}$  derived and gas exchange intrinsic water use efficiency; and  $i\text{WUE}$  and  $\text{WUE}_i$  exhibited a strong positive correlation. The higher intrinsic WUE in TWW indicates that stomata close, and consequently tree activity decreases. Although the WUE in TWW was higher than in mitigation treatments on most measurement dates, the lower assimilation rates at leaf ( $A_{\text{leaf}}$ ) and whole tree ( $A_{\text{tree}}$ ) level observed in TWW indicate

that TWW irrigation had a negative effect on tree physiological performance. Overall, the increased  $g_s$ ,  $A_{\text{leaf}}$ ,  $A_{\text{tree}}$  and  $K_{\text{leaf}}$  in FW, MIX and TUF relative to TWW indicate that these mitigation measures are good candidate treatments for improving orchard performance negatively affected by long-term irrigation with TWW. Therefore, we conclude that FW, MIX and TUF treatments improved avocado leaf gas exchange, and leaf hydraulic conductance relative to TWW.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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