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Plant and leaf responses to cycles of water stress and re-watering of 'Sangiovese' grapevine

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ABSTRACT

The complex relationship between water and grapevine has been examined in several studies. The aim of this study was to understand the impact of water stress on photosynthesis, carbon-13 discrimination in leaves in different positions on the shoot, and the capacity of vines to recover from different levels of water stress intensity. The vine physiological responses to a water stress regimen followed by re-watering for two consecutive cycles was evaluated using potted 'Sangiovese' grapevines. The intensity and the duration of the water limitation affected the emergence and development of new leaves, but did not significantly affect leaf water potential. Leaf stomatal conductance and carbon assimilation during the first water-stress phase were reduced respectively by about 61% and 20%, while after the second water stress cycle both were lower than the initial values by 77% and 21%, respectively. After 1 day of re-watering, only the leaves located in the medial positions on the shoot showed a partial recovery of photosynthesis. After at least 2 days post-re-watering, the leaves located in the distal portion of the shoot showed a recovery of photosynthetic capacity. The results indicated that leaf position along the shoot, i.e., an indicator of leaf age, is an important variable in developing grapevine strategies in response to conditions of limited water availability.

Keywords: carbon isotope, leaf position, photosynthesis, *Vitis vinifera*, water limitation

INTRODUCTION

Water limitation is the major constraint of plant growth and yield. One of the primary vine responses to drought is the reduction in leaf photosynthetic capacity (Lawlor, 1995; Munns, 2002) associated with a decrease in leaf stomatal conductance (g_s) (Lawlor and Cornic, 2002; Flexas et al., 2004a). It is well known that the stomatal regulation under water stress is a complex function that involves chemical signals, such as ABA (Wilkinson and Davies, 2002; Christmann et al., 2005), and hydraulic signals and cavitation of xylem vessels (Christmann et al., 2007). It is also known that upon re-watering, vegetative growth of stressed plants can generally recover to pre-stress levels (Flexas et al., 2004b), indicating a reversibility

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of physiological changes promoted by drought. Although restricted carbon dioxide (CO_2) diffusion into the leaves is likely to be the cause of decreased photosynthesis (P_n) rates under water stress, metabolic impairment may also occur, particularly under severe water stress identified by g_s values below 50 mmol H_2O m^{-2} s^{-1} (Flexas and Medrano, 2002; Cifre et al., 2005). Palliotti et al. (2014) observed reduced photosynthetic efficiency in 'Montepulciano' canopies due to complete stomatal closure with g_s values as low as 32 mmol H_2O m^{-2} s^{-1}. When the strong reduction of photosynthetic activity is primarily due to stomatal closure, P_n may be rapidly reversed upon re-watering (Flexas et al., 2004a). Quick et al. (1992) observed in a study on photosynthetic recovery, using lupines, sunflower, eucalyptus and grapevine that watering the plants in the evening induced full recovery of photosynthesis in the first three species during the night; the grapevine, however, did not respond. This non-recovery may be due to the presence of non-stomatal limitations, such as the inhibition of key metabolic processes, e.g., photophosphorylation (Tezara et al., 1999), the capacity for RuBP regeneration (Escalona et al., 1999), or Rubisco activity (Maroco et al., 2002; Parry et al., 2002; Flexas et al., 2009). Each of these processes has been offered as the main limitation to P_n under water stress.

The grapevine (*Vitis vinifera* L.) has developed various physiological and morphological mechanisms in order to sustain growth and productivity under limited-water conditions (Palliotti et al., 2014), such as reduction in leaf area (Gòmez del Campo et al., 2003) or shoot and internode length, and leaf orientation (Palliotti et al., 2008; Palliotti et al., 2014). Some studies have analysed photosynthetic recovery in plants upon re-watering after a period of water stress (Marron et al., 2002; Pou et al., 2008; Palliotti et al., 2014), suggesting that the recovery is influenced by a) stress severity, b) time of stress occurrence, c) genotype, and d) environmental conditions. However, there is a scarcity of studies analysing the capacity for recovery from different water stress intensities, as well as evaluating the physiological features limiting recovery. In grapevines under mild to moderate water stress, photosynthetic recovery after re-watering is quite fast, and a complete recovery of the maximum P_n occurred after just 1 night post-irrigation, even though previous g_s measurements were below 100 mmol H_2O m^{-2} s^{-1}. In the same species under severe water stress, however, when g_s was lower than 50 mmol H_2O m^{-2} s^{-1}, P_n failed to recover 1 d after irrigation (Quick et al., 1992). Flexas et al. (2004b) showed that, in grapevine growing outdoors during a Mediterranean summer and subjected to very severe water stress, the rate of P_n recovered about 60% only 1 night after irrigation, and that in 4 more days approached full recovery. Marron et al. (2002) obtained similar results, also presenting very low g_s in two different *Populus* × *euramericana* clones subjected to a water stress and re-watering regimen. Castrillo and Calcagno (1989) showed in two cultivars of tomato that Rubisco activity recovered from 50-60% of that of the controls to 100% 4 d after re-watering. It has also been shown that recovery results are quicker and greater after the initial water stress cycle than after subsequent cycles (Flexas et al., 1999). The recovery may also depend on a complex interaction with plant or leaf age, light intensity, and the number of consecutive drying cycles (Flexas et al., 2004b).

In C3 species such as grapevines, g_s is also related to carbon isotope discrimination ($\Delta^{13}C$) via its effect on the C_i/C_a ratio (the ratio between intercellular and atmospheric concentration of CO_2). Carbon isotope discrimination is a measure of the $^{13}C/^{12}C$ ratio in plant tissues compared to the air ratio, as described by the equation $\Delta = a + (b - a) \times (C_i/C_a)$ (Farquhar et al., 1989). Carbon dioxide enters the leaf through the diffusional resistance of the stomatal pores, diminishing to a partial pressure of C_i, and then CO_2 is fixed biochemically into sugar phosphates. In the first step, diffusion discriminates against the slower moving CO_2 containing carbon-13 (^{13}C) with a magnitude of 4.4% (*a*). Then CO_2 reacts with RuBP at the Rubisco enzyme, which discriminates against ^{13}C with a magnitude of 27% (*b*). Carbon isotope composition ($\delta^{13}C$) in samples is calculated with a mass spectrometer as: $\delta = Rp/Rs - 1$, where Rp and Rs are the $^{13}C/^{12}C$ ratio in plant tissue and the standard (PeeDeeBelemnite), respectively. Then, $\Delta^{13}C$ is calculated as: $\Delta = (\delta a - \delta p) / (1 - \delta p)$, where δa is the isotopic composition of the CO_2 in the atmosphere (-8%) and δp is the isotopic composition of the leaf (Farquhar et al., 1989).

The aim of this trial was to investigate the effects of water stress and re-watering cycles on 'Sangiovese' potted grapevines, focusing on morphological and physiological responses relative to leaf position, while also evaluating leaf discrimination capacity against the natural carbon isotope during photosynthesis.

MATERIAL AND METHODS

Plant material and experimental conditions

The study was performed on 1-year-old 'Sangiovese' (*V. vinifera* L.) grapevines grafted onto SO4 rootstock (*Vitis riparia* × *Vitis berlandieri*) and grown in 9-L brown polypropylene pots filled with a mixture of 20 soil : 30 sand : 50 peat (by volume). The vines were maintained outdoors under natural light and ambient temperature conditions, and well watered until the beginning of the trial (DOY 212) when 10 selected plants were divided into two uniform groups to provide a comparison between two different watering treatments: the control and a stress-re-water regimen. Each selected vine featured a single primary shoot, 80-100 cm long and having 20-25 primary leaves. Each pot surface was covered in both treatments with a light-coloured plastic sheet to prevent infiltration of rain water and to minimize losses due to soil evaporation.

One group of 5 vines was kept as the control (C-vines) and continued to be irrigated every evening to maintain the soil at field water capacity.

The second group of 5 treated vines (T-vines) was subjected to two subsequent water stress and re-watering cycles (WS-RW). Water stress levels were defined by leaf stomatal conductance (g_s) (Flexas and Medrano, 2002): moderate water stress condition (150 mmol H_2O m^{-2} s^{-1} > g_s > 100 mmol H_2O m^{-2} s^{-1}) and severe water stress (100 mmol H_2O m^{-2} s^{-1} > g_s > 50 mmol H_2O m^{-2} s^{-1}). Starting from the evening of DOY 213, progressive water stress (WS1) was imposed by replenishing only 25% of C-vines daily water loss (C-DWL). On DOY 214, the water supply was increased to 50% of C-DWL until the evening of DOY 215, when the water replenishment increased to 85% of C-DWL. After 3 days of WS1, the g_s of T-vines indicated a moderate water stress condition. Then, the T-vines were well watered on the late afternoon of DOY 216, and at sunset of DOY 217 a second water stress (WS2) replenishing 60% of C-DWL was established until re-watering to 100% on DOY 220 when leaf g_s values corresponded to the beginning of severe water stress condition. Water consumption for each treatment was determined by weighing the pots using an electronic balance (JC 384 B/L, Life Tool Technologies SpA, Ancona, Italy), and the differences between successive daily irrigations and daily pot weight for each vine were used as the basis for calculations of water loss by the vine. Each vine in both treatments was considered a replicate.

Vine growth

At the end of the experiment, a random sample of leaves from each vine was collected and the area of each leaf measured using a leaf area meter (LI-3100 Area Meter, Li-COR, Inc. Lincoln, NE, USA) to calculate the total leaf area (TLA) per vine. Shoot length was measured, from the beginning of the experiment, from the last unfolding leaf selected at the beginning of the trial to the apex of the shoot.

Gas exchange measurements

On the last day of the trial (DOY 222), the midday leaf water potential ($_{leaf}$) was measured with a Scholander pressure chamber (PMS Instrument Co., Albany, NY, USA) on two fully expanded leaves from distal (young), medial (mature), and basal (old) positions on the shoot, from each vine in each treatment.

Stomatal conductance (g_s), net photosynthesis (P_n), and intercellular CO_2 concentration (C_i) were measured in each treatment for each leaf in different positions, well-exposed to sunlight, using an infrared gas analyser (LCA3 Portable Open-system, Analytical Development Co., BioScientific Ltd., Hoddesdon, Herts, UK). The unit uses a broad leaf chamber with a 6.25 cm^2 window, and all readings were taken at ambient relative humidity with an airflow adjusted to 350 mL min^{-1}. All measurements, taken on alternate days during full WS-RW cycles, were performed in the morning (from 0930 to 1130 hrs) on clear-sky days. The measurements were randomized between treatments and leaf positions in order to avoid g_s and Pn being influenced by the change in sunlight and temperature during the morning. Intrinsic water use efficiency (WUEi, µmol CO_2 / mol H_2O) was calculated as a P_n/g_s ratio. The three basal leaves and the apical leaves not fully expanded, as well as all similarly immature distal leaves were not included in the gas exchange measurements. All the remaining leaves were then classified into three groups by estimated age: old (46-60 d), mature (31-45 d), and young (15-30 d), according to their position on the basal, medial, and distal parts of the shoot, respectively, starting from the base (node 5 or 6) to the apex, including the last leaf of at least 60 cm^2.

Calculation of carbon isotope composition in leaf dry matter

The theoretical values of $\Delta^{13}C$ were calculated using the carbon isotope discrimination formula proposed by Farquhar et al. (1982): $\Delta = a + (b - a) \times (C_i / C_a)$. The C_i values, taken during P_n

measurements, and the C_a value, approximately 375 µmol mol^{-1}, were inserted into that equation. In the formula, *a*, equal to 4.4‰, refers to the discrimination against atmospheric $^{13}CO_2$ during diffusion in the leaf, while *b*, equal to 27‰, refers to the discrimination of the carboxylation enzyme. Then, using the Farquhar et al. (1989) model: $\Delta = (\delta a - \delta p) / (1 - \delta p)$, it is possible to calculate the theoretical values of leaf carbon isotope composition: (δp): $\delta p = (\delta a - \Delta) / (\Delta + 1)$, where the isotopic composition of the CO_2 (δa) in the atmosphere is equal to -8‰. The calculation of $\delta^{13}C$ was carried out for mature distal, medial, and basal leaves on the shoots of each vine on alternate days during the WS-RW cycles, on the same days when the leaf gas exchange measurements were taken. Our aim was to collect information on isotopic signals during the period of the WS-RW cycles.

Statistical analysis

Basic statistics were performed on data related to growth parameters and water use efficiency, testing for homogeneity of variance and subjecting them to analysis of variance (ANOVA). Mean separation test (Student's t test for $p \leq 0.05$) was performed using Statistica (Statistica 4.3; StatSoft, Inc. 1993).

Figures were drawn using Sigma Plot (version 10; SPSS, Chicago, Ill., USA) and data are shown as means ± standard errors (SE).

RESULTS

The environmental conditions during the trial were typical of Mediterranean regions, with mean daytime and maximum temperatures of about 27°C and 37°C, respectively, relative humidity of about 60%, with vapour pressure deficits (VPD) around 2.5 kPa and absence of precipitation. The photosynthetic photon flux density (PPFD) readings ranged from 1600 to 1800 µmol m^{-2} s^{-1}, with a peak at midday typically near 1900 µmol m^{-2} s^{-1}. When all the vines were well watered, the transpiration was similar in both treatments and ranged from 0.5 to 0.6 L per pot per day (Fig. 1). After the WS1 treatment, the transpiration of T-vines gradually decreased to about 0.3 L d^{-1} per pot. On the evening of DOY 216, RW1 (re-watering for 1 day, replenishing 100% of C-DWL) was carried out and the vines increased their transpiration until DOY 218, but after the WS2 treatment finally decreased sharply to below 0.3 L d^{-1} per pot on DOY 219 (Fig. 1). On the following day, RW2 began the process of re-watering all the T-vines until the end of the study (DOY 220 and 221). The effect of the RW2 treatment was noticeable on the last day (DOY 222), with a sharp increase occurring after an abrupt fall to nearly 0.15 L d^{-1} per pot on DOY 221 (Fig. 1). During the experimental period, the C-vines continued to receive enough water to maintain the soil at field capacity, and their transpiration ranged from 0.5 to 0.6 L d^{-1} per pot, except on day 9 when a reduction in their transpiration to 0.4 L d^{-1} per pot was observed (Fig. 1). The midday ψ_{leaf} measured on the last day of the trial (DOY 222) was slightly lower in the T-vines, whose basal

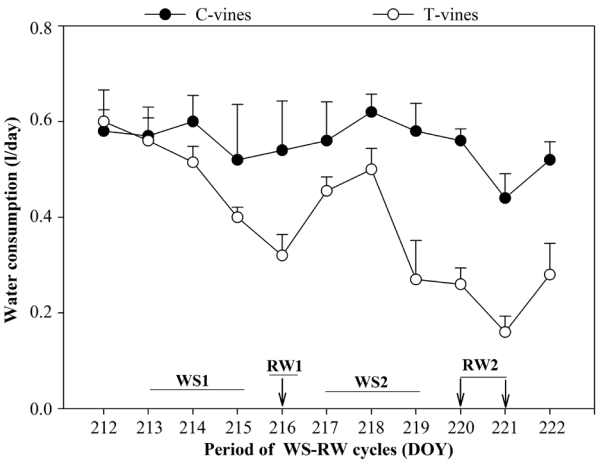

Figure 1. Daily water transpiration in 'Sangiovese' potted well-watered vines (C-vines) and vines subjected to water stress and re-watering cycles (T-vines). The DOY 212 corresponds to all well-watered vines. The figure shows the water stress periods (WS1 and WS2), and the arrows indicate the re-watering times (RW1 and RW2)

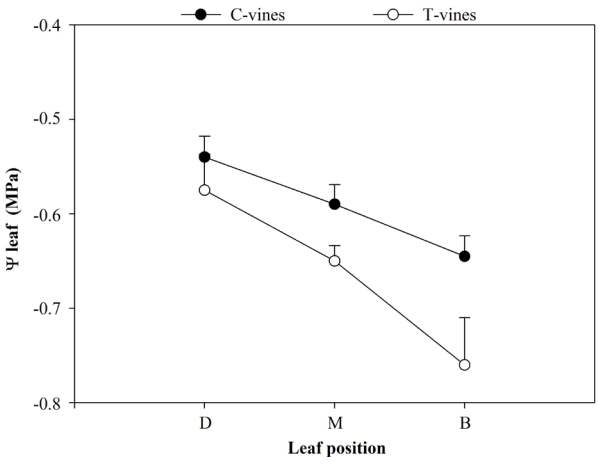

Figure 2. Midday leaf water potential (Ψ_{leaf}) measured at the end of the trial (DOY 222) in distal (D), medial (M), and basal (B) leaves in 'Sangiovese' potted well-watered vines (C-vines) and vines subjected to water stress and re-watering cycles (T-vines). Each point is the mean of 10 leaves (± SE)

Table 1. Growth parameters measured at the end of the trial (DOY 222) in 'Sangiovese' potted well-watered vines (C-vines) and vines subjected to water stress and re-watering cycles (T-vines)

	Shoot elongation (cm)	TLA** (cm^2 per vine) under last marked leaf	New leaves (n)	TLA (cm^2 per vine) in new leaves
C-vines	45 a*	1402.27 a	4 a	15.3 a
T-vines	39 b	1420.45 a	2 b	7.1 b

*Means within a row not followed by the same letter are significantly different at $p \leq 0.05$
**TLA = total leaf area

leaves showed the lowest ψ_{leaf} values approaching -0.8 MPa (Fig. 2). The intensity and duration of the water shortage during the treatment cycles were sufficient to halt the emergence of new leaves, the expansion of young leaves, and shoot elongation (Tab. 1). As expected, the total leaf area (TLA) values at points below the last unfolding leaf were similar in both treatments. However, at the end of the study, a significantly lower TLA was found in new leaves of the T-vines as compared to the C-vines, respectively 7.1 *vs* 15.3 cm^2/vine (Tab. 1). The stomatal conductance (g_s) resulting from the single values detected from all the leaves (distal, median and basal) in both treatments was about 350 mmol H_2O m^{-2} s^{-1} (Fig. 3A). During the WS1 treatment, g_s decreased steeply to about 200 mmol H_2O m^{-2} s^{-1} on DOY 214, while the measurements carried out on DOY 216 showed that g_s fell to nearly 140 mmol H_2O m^{-2} s^{-1}. After applying RW1, a slight increase in g_s was observed, but stomatal closure persisted and, after the WS2 treatment, g_s decreased again to values below 90 mmol H_2O m^{-2} s^{-1} on DOY 220. The daily RW2 regime led to a small delayed increase in g_s observed on the last day of the study (Fig. 3A). Even though the values degraded over time by 28%, the C-vines maintained g_s levels well over 240 mmol H_2O m^{-2} ms^{-1} until the end of the trial, demonstrating steady opening of the stomata to a greater degree (Fig. 3A). As expected, during the treatment periods, both g_s and photosynthesis rate (P_n) proved to be higher in the C-vines (Fig. 3B). Despite the higher g_s values, when all the vines were well watered at the start, the P_n values (the mean of the individual measurements on all leaves) were similar between the treatments, around 5.0-5.5 µmol CO_2 m^{-2} s^{-1} (Fig. 3B). As the study progressed from DOY 214 to 218, the P_n values of the three groups were tracking in close parallel, with values between 4 to 5 µmol CO_2 m^{-2} s^{-1}, and no changes in photosynthetic values after re-watering were noticed. However, the P_n decreased significantly in the T-vines after the onset of WS2, reaching a minimum value of 3.2 µmol CO_2 m^{-2} s^{-1} on DOY 220. It is noteworthy that, simultaneously, g_s also

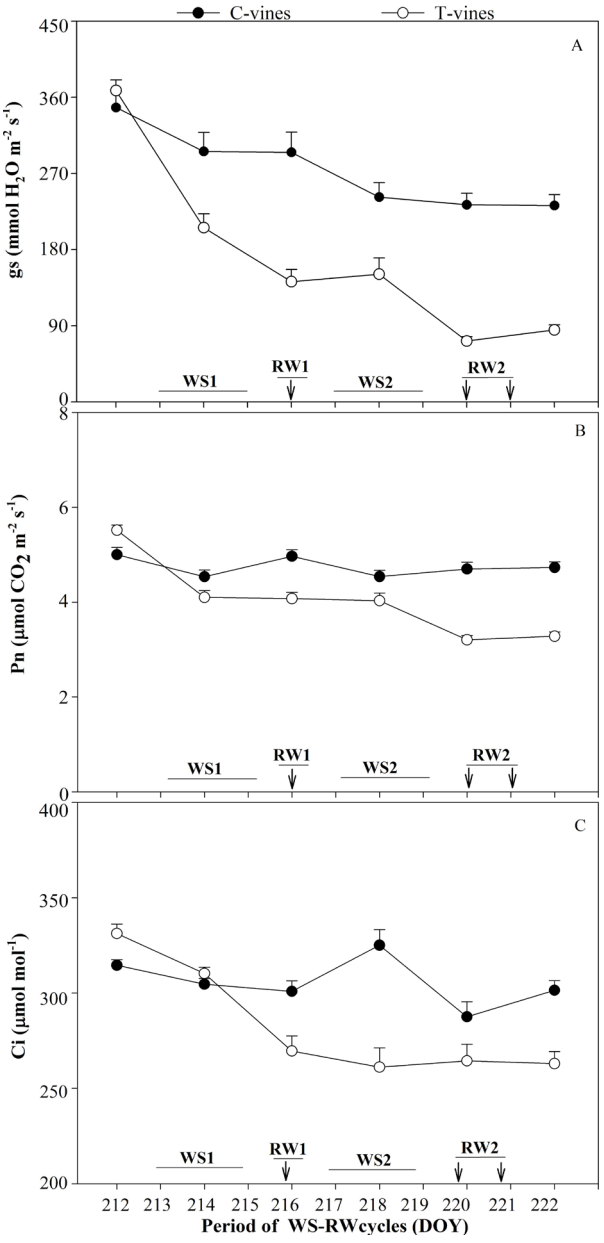

Figure 3. (A) Stomatal conductance (g_s), (B) net photosynthesis (P_n), and (C) internal CO_2 concentration (C_i), in 'Sangiovese' potted well-watered vines (C-vines) and vines subjected to water stress and re-watering cycles (T-vines), during the trial period. Measurements were taken on alternate days in August 2008 between 0900 and 1130 hrs. The DOY 212 corresponds to all well-watered vines. Each point is the mean of 60 leaves (± SE)

Table 2. Intrinsic water-use efficiency (WUEi, μmol CO_2 mol H_2O), in 'Sangiovese' potted well-watered vines (C-vines) and vines subjected to water stress and re-watering cycles (T-vines). The DOY 212 corresponds to all well-watered vines

	DOY 212	DOY 214	DOY 216	DOY 218 (post-RW1)	DOY 220	DOY 222 (post-RW2)
C-vines	14 a*	15 b	17 b	19 b	20 b	20 b
T-vines	15 a	20 a	29 a	27 a	39 a	35 a

*Means within a row not followed by the same letter are significantly different at $p \leq 0.05$

fell substantially, suggesting a relationship. Again, as with g_s, a small increase in P_n after 2 d post-RW2 was detected (Fig. 3A, 3B). The vines subjected to the WS1 treatment showed a quick reduction in C_i to values around 270 μmol CO_2 mol^{-1}, down from the initial value between 300 and 350 μmol CO_2 mol^{-1}, similar in both treatments. After applying RW1 on DOY 218, an additional slight decrease in C_i in the T-vines was measured and then, during RW2 on DOY 220 and 221, C_i remained almost constant, with values near 260 μmol CO_2 mol^{-1}, until the end of the trial. The C_i in the C-vines was characterized by values higher than those measured in the T-vines during the entire experimental period (Fig. 3C). Significant differences in the WUEi (P_n/g_s) between treatments were observed (Tab. 2). When vines were well watered, then the WUEi was similar in both treatments, approximately 15 μmol CO_2 mol H_2O. During the experimental period, the C-vines showed a higher P_n activity, 5-6 μmol CO_2 m^{-2} s^{-1} compared to 3.5-4.0 μmol CO_2 m^{-2} s^{-1} (Fig. 3B). On the second day of WS1, the vines showed an increase in WUEi (+25%) from 15 to 20 μmol CO_2 mol H_2O (Tab. 2) associated with a reduction in g_s (about 200 mmol H_2O m^{-2} s^{-1}) and with a drop in P_n (from 5.5 to 4.0 μmol CO_2 m^{-2} s^{-1}). A further significant increase in WUEi (+48%) was observed on DOY 216 before RW1 was initiated, when the g_s decreased again. On DOY 218, after RW1 and after the application of the WS2 treatment, a reduction in WUEi was detected in the T-vines until bottoming out at 27 μmol CO_2 mol H_2O (Tab. 2), due to a slight increase in g_s at constant values of P_n (Fig. 3A, 3B). During RW2, the WUEi in the T-vines increased markedly, reaching 39 μmol CO_2 mol H_2O on DOY 220, with a slight decrease found on DOY 222; however, the WUEi values were higher in comparison with the values obtained after RW1 (Tab. 2).

Pre-treatment leaf physiological response

Prior to commencing treatment (DOY 212) when T-vines were still well watered, in both treatments the old basal leaves showed a lower P_n (around 5 μmol CO_2 m^{-2} s^{-1}) in comparison with the medial and distal ones (Fig. 4). The g_s values showed a slight difference in the basal leaves, with 319 mmol H_2O m^{-2} s^{-1} in the C-vines and 363 mmol H_2O m^{-2} s^{-1} in the T-vines, while the values for the leaves in the medial and distal shoot portions were very similar between the treatments, respectively around 340 and 390 mmol H_2O m^{-2} s^{-1} (Fig. 5).

Leaf responses to 1st WS-RW cycle

On DOY 214, after the imposition of severe water stress (WS1, replenish 25% of C-DWL), all leaves rapidly suffered the effects of water shortage as evidenced by the reduction in P_n values to near 4 μmol CO_2 m^{-2} s^{-1} (Fig. 4), accompanied by a decrease in g_s, whose values fell to nearly 200 mmol H_2O m^{-2} s^{-1} (Fig. 5). On DOY 216 (after WS1, replenish 50 and 85% of C-DWL), a further reduction in P_n activity was found in younger distal leaves of the T-vines, with values below 4 μmol CO_2 m^{-2} s^{-1}, associated with a slight decline in g_s (from 198 to 177 mmol H_2O m^{-2} s^{-1}), lower than in the C-vines. The WS1 had more impact on the medial and basal leaves, causing a fall in g_s values, respectively around 150 and 97 mmol H_2O m^{-2} s^{-1} (Fig. 5), while the P_n activity of these leaves remained around 4 μmol CO_2 m^{-2} s^{-1}. On DOY 218, after RW1 and 1 d following the start of the WS2 treatment (replenish 60% of C-DWL), a partial P_n recovery of 11% was observed only in the medial leaves. The P_n activity and g_s values increased, reaching respectively about 5 μmol CO_2 m^{-2} s^{-1} and 180 mmol H_2O m^{-2} s^{-1} (Figs 4 and 5). In contrast, the distal and basal leaves showed a low P_n activity, between 3.5 and 4.0 μmol CO_2 m^{-2} s^{-1}. Despite the low P_n values in the basal leaves, the g_s remained around 180 mmol H_2O m^{-2} s^{-1}, while the distal leaves maintained g_s values at 90 mmol H_2O m^{-2} s^{-1} (Fig. 5).

Leaf responses to 2nd WS-RW cycle

On DOY 220, after 3 days of WS2 application, P_n decreased again in all leaves, reaching values between 2.7 and 3.5 μmol CO_2 m^{-2} s^{-1}, accompanied by g_s values near 90 mmol H_2O m^{-2} s^{-1} in the distal

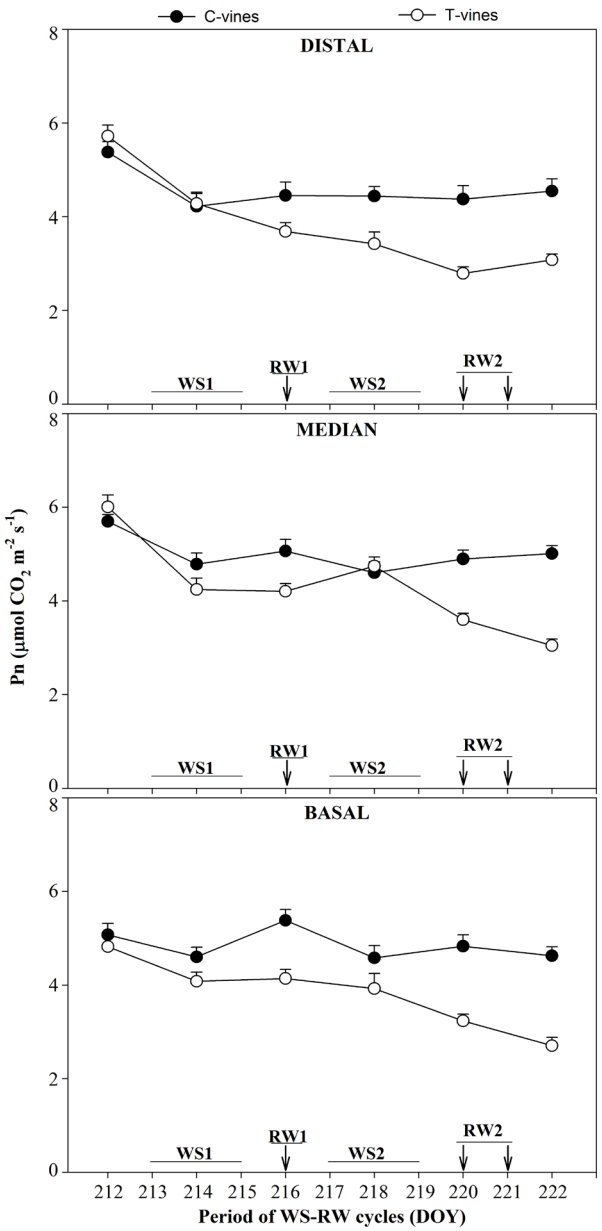

Figure 4. Net photosynthesis (P_n) in distal, medial and basal leaves in 'Sangiovese' potted well-watered vines (C-vines) and vines subjected to water stress and re-watering cycles (T-vines), during the trial period. The DOY 212 corresponds to all well-watered vines. Each point is the mean of 20 leaves (± SE)

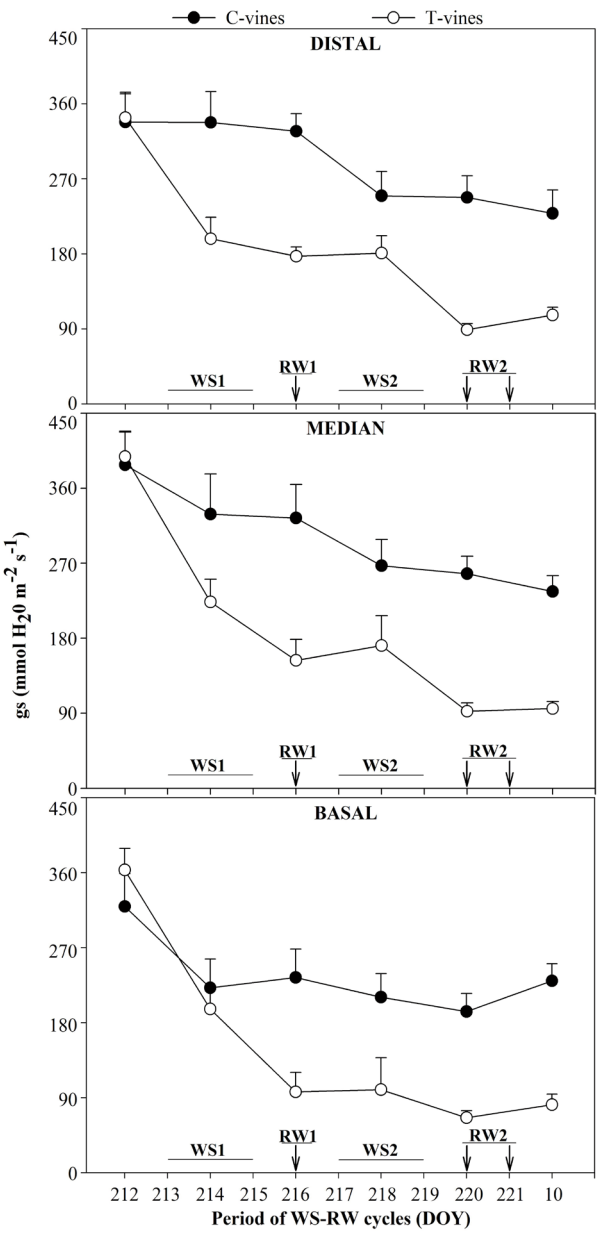

Figure 5. Stomatal conductance (g_s) in distal, medial and basal leaves in 'Sangiovese' potted well-watered vines (C-vines) and vines subjected to water stress and re-watering cycles (T-vines), during the trial period. The DOY 212 corresponds to all well-watered vines. Each point is the mean of 20 leaves (± SE)

and medial leaves, and much lower values (around 70 mmol H_2O m^{-2} s^{-1}) in the basal leaves. During the RW2 period (DOY 220 and 221), the availability of water stimulated young fully expanded distal leaves to partially recover P_n to about 10%, with an increase of 17% in g_s (Figs 4 and 5). During the trial, the C-vines maintained P_n values between 4 and 6 µmol CO_2 m^{-2} s^{-1}, with g_s values always above 190 mmol H_2O m^{-2} s^{-1}, showing a constant water status (Figs 4 and 5).

Effects upon carbon-13 isotope

The evolution of photosynthetic values is closely correlated with carbon isotope discrimination against $^{13}CO_2$ atmospheric ($\Delta^{13}C$) and estimated carbon-13 isotope composition ($\delta^{13}C$) in leaf tissue (Farquhar et al., 1982, 1989). Theoretical $\delta^{13}C$ ($Th\delta^{13}C$) calculations, obtained using the carbon isotope discrimination formula (Farquhar et al., 1982), suggest a different $\Delta^{13}C$ on the leaves in different positions along the shoot (Fig. 6). Before

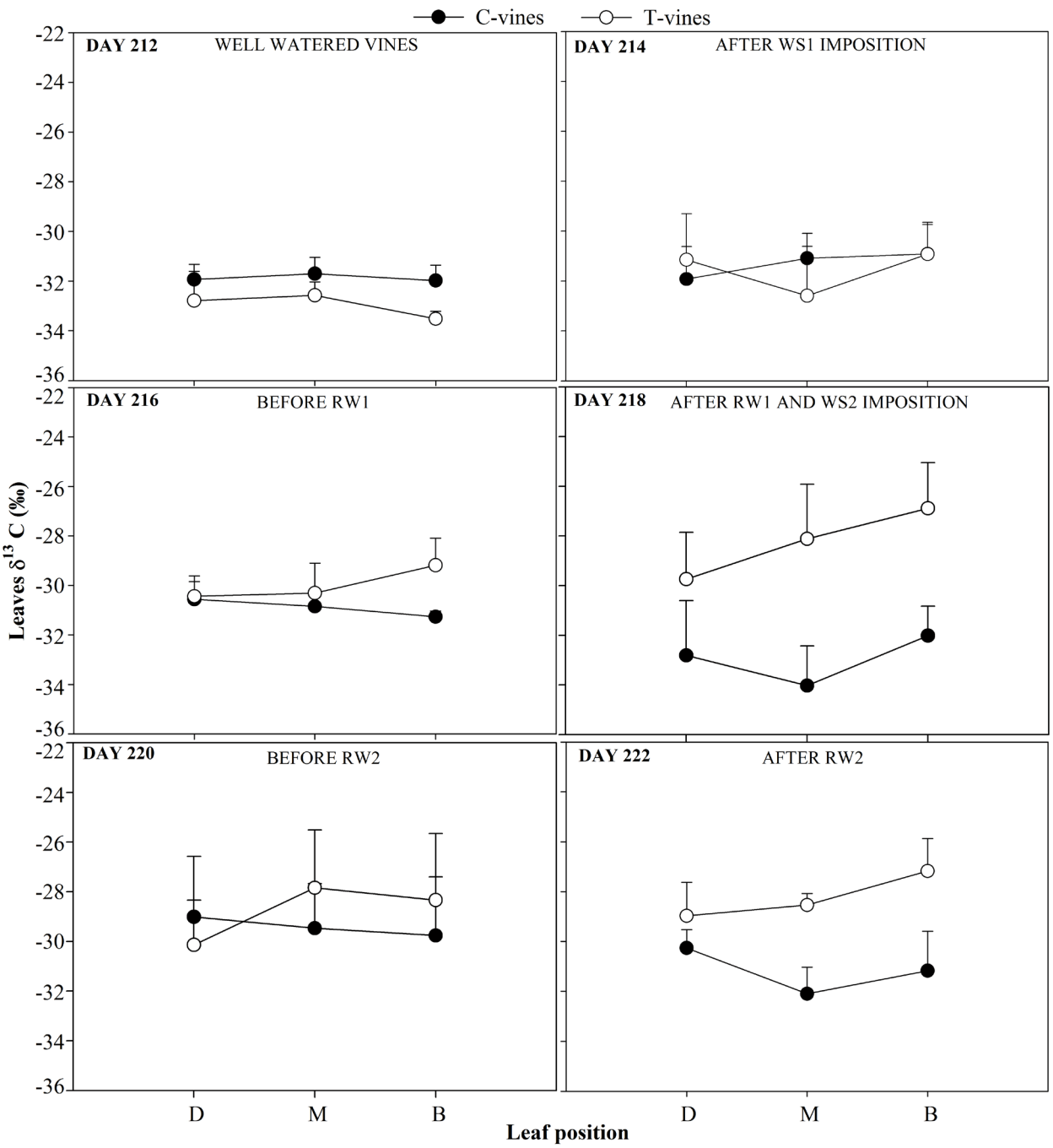

Figure 6. Evolution of the theoretical carbon isotope composition (Thδ^{13}C) in distal (D), medial (M) and basal (B) leaves in 'Sangiovese' potted well-watered vines (C-vines) and vines subjected to water stress and re-watering cycles (T-vines), during the trial period. Each point is the mean of 20 leaves (± SE)

any treatments began, all of the vines were well watered and the Thδ^{13}C ranged from -32 to -34‰ on distal, medial, and basal leaves in both treatments (Fig. 6). On DOY 214, corresponding to the severe water stress of WS1, the Thδ^{13}C on distal and basal leaves decreased to around -31‰. On DOY 216 before RW1 treatment, a reduction in Thδ^{13}C, from -33 to -28‰, was determined in medial leaves, while in distal and basal leaves there was no change. On DOY 218, after RW1 and 1 d of moderate WS2, the water availability generated a decrease in δ^{13}C in medial and basal leaves of the T-vines, respectively from -30‰ to about -28‰ and from -29‰ to about -27‰ (Fig. 6). During the second WS-RW cycle, after the application of WS2, an increase in Thδ^{13}C values in basal leaves to around -28‰ was determined, while the values in both distal and medial leaves showed no change. On the last day of the trial (DOY 222), 2 d after RW2, the Thδ^{13}C values in the T-vines showed a decrease in ^{13}C in the

distal and basal leaf tissue of about 5% relative to prior values, while in the medial leaves the Thδ^{13}C values were maintained around -28‰ (Fig. 6).

DISCUSSION

During the first WS-RW cycle, when WS1 was imposed (from DOY 212 to 215), the transpiration in the T-vines decreased until the application of RW1 on the evening of DOY 216. After re-watering, the transpiration increased and continued to increase even after the WS2 was initiated. This increase in transpiration until DOY 218, during the second water stress regimen (60% of C-DWL), may have been due to our use of 9-L pots, the volume of which allowed a water reserve to form after RW1 in the first cycle, in addition to the fact that the WS2-imposed stress was moderate and characterized by the replenishment of 60% of C-DWL. The vines probably still had extractable water available from RW1 and also sufficient water from the new water stress imposition. During the second WS-RW cycle, the transpiration reached a peak on DOY 218 and decreased thereafter. The minimum transpiration value in both treatments was observed on DOY 221, a result largely due to partly cloudy conditions and, probably, related to a lower vapour pressure deficit demand and lower transpiration in both the C- and T-vines. The intensity and duration of the water shortage was sufficient to reduce shoot elongation and the TLA of new leaves, known to be processes most sensitive to water stress (Boyer, 1970; Hsiao, 1973; Schultz and Matthews, 1993; Schultz, 2000). Furthermore, the vine's response limiting the emergence and development of new leaves to maximize its water resources might also be considered an adaptive strategy to water limitation. The midday ψ_{leaf} measurements, recorded on DOY 222, did not reveal a severe water stress condition in the T-vines, considered to occur when the ψ_{leaf} value is below -1 MPa (Prichard, 2004), although the basal leaves in the T-vines showed the lowest values of ψ_{leaf}, suggesting that they suffered more from water limitation than the leaves developed at the medial and distal positions on the shoot.

The application of water stress, without excessive temperature and light-induced stress in this experiment, reduced both evapotranspiration and shoot growth, suggesting an adaptive response – reduced transpiration by leaf area limitation. The low-water availability negatively influenced g_s and P_n associated with it, and the close relationship between g_s and P_n suggested that the vines were subjected to moderate water stress despite the low g_s values, and that the impact on stressed leaves was ameliorated by stomatal closure, the main factor responsible for a reduction in the net photosynthetic carbon uptake rate, without affecting chloroplast function and photochemical efficiency. In fact, g_s decreased quickly when severe to mild water stress conditions were imposed during the first 4 d of the study and decreased again after another application of moderate water stress. The P_n values showed a similar trend. C_i values in the T-vines remained high (above 250 µmol CO_2 mol^{-1}) throughout the trial period, but a marked decrease was observed in C_i concentration resulting from the WS1 treatment corresponding to progressive water stress from severe to mild. This result suggests that the decrease in P_n in these leaves may be attributed to a decrease in g_s, such as was observed in a similar trial on 'Vignoles' grapevines, where after only 2 d of water stress C_i decreased from values near 200 to below 180 µmol CO_2 mol^{-1} (Lanari et al., 2015) indicating that the stomatal limitations dominated irrespective of any metabolic impairment. Under moderate water stress (WS2), leaf assimilation in grapevine was primarily limited and mostly controlled by partial and reversible stomatal closure with a reduction in g_s to a threshold value of about 75-100 mmol H_2O m^{-2} s^{-1} (Escalona et al., 1999; Flexas and Medrano, 2002). The g_s in the T-vines was reduced to minimum values just below 80 mmol H_2O m^{-2} s^{-1}, showing the beginning of a severe water stress condition (Flexas and Medrano, 2002), but the recovery after re-watering was slower than expected and at the end of the trial period had not been completed. When WS1 was imposed, P_n activity did not show a large reduction, but neither did it show a recovery after re-watering. The available water appeared to be used by the vines to diminish the impact on P_n activity, not achieving a constant level but certainly narrowing the variability. In contrast to these results, Flexas et al. (2004a, 2004b) reported that, in grapevines (*V. vinifera* L.) growing outdoors during summer in a Mediterranean climate and subjected to very severe water stress, a P_n recovery rate of about 60% was observed just 1 night post-irrigation. It took 4 more days to approach full recovery. The severity of water stress and the number of consecutive drying cycles likely influence this discrepancy in the rate of P_n recovery. Moreover, the first drying cycle is most valuable as it typically provides the largest gain in recovery as compared to subsequent cycles (Flexas et al., 1999, 2004b; Larcher et al., 1981). Consistent with earlier reports (Pou et al., 2008; Palliotti et al., 2014), our research confirmed that

water stress induces substantial stomatal closure, which persists several days after re-watering and also drives an increase in WUEi likely due to the greater reduction in g_s compared to P_n. The high values of WUEi obtained in T-vines confirm an adaptation to drought consistent with other results (Bota et al., 2001; Lanari et al., 2015). The highest WUEi values, ranging from 35 to 39 µmol CO_2 mol H_2O, were obtained with g_s values of 72 and 85 mmol H_2O m^{-2} s^{-1}, respectively. With g_s values higher than that level, the WUEi decreased, suggesting a sensitive and responsive stomatal regulation capability. During the first water stress, the increase of WUEi in T-vines was related to a quick reduction in C_i (from 331 to 270 µmol CO_2 mol^{-1}). According to the results found in 'Vignoles' potted vines (Lanari et al., 2015), leaves suffered differently from water stress treatment and were characterized by P_n values determined by the position along the shoot. When severe water stress was imposed on DOY 213 (WS1), all of the leaves rapidly suffered and showed a decrease in P_n activity associated with a reduction in g_s values. The availability of water restored with RW1 positively influenced the P_n activity of the medial leaves, mature and photosynthetically active, where a partial recovery was recorded 2 d after re-watering (and 1 d after the WS2 imposition). Meanwhile, the distal and basal leaves did not show positive changes in P_n activity, indicating that the restored water availability failed to induce any significant recovery. This different behaviour between distal, median and basal leaves may be related to a different capacity for osmotic adjustment related to age. After the imposition of WS2, all of the leaves suffered the water limitation effects again and delivered the lowest P_n and g_s values in comparison with the leaves of the C-vines. Despite good water availability post-RW2, a marked increase in g_s among the leaves in different positions along the shoot was not detected, and only the young fully expanded leaves in the distal positions demonstrated a partial recovery of P_n activity, while the medial and basal leaves showed the lowest P_n values. Our theoretical $\delta^{13}C$ calculations produced different $\delta^{13}C$ values for distal, medial, and basal leaves along the shoot, suggesting a different ^{13}C, as had also been reported by Lanari et al. (2015) in an analogue trial. On DOY 214, after the beginning of WS1, a reduction was observed in P_n activity and in g_s in all T-vine leaves, and the theoretical $\delta^{13}C$ values suggest a decrease in $\delta^{13}C$ in distal and basal leaf tissue. When the vines were re-watered, a decrease in theoretical $\delta^{13}C$ in medial and basal leaves was obtained on DOY 218, consistent with the reduction in C_i due to the water stress imposed during the first days of the experiment. On DOY 222, when all vines were benefiting from re-watering, the Th$\delta^{13}C$ values decreased in young and fully expanded distal leaves and in old basal leaves, while a substantial enrichment with ^{13}C and low discriminatory capacity for $^{13}CO_2$ were observed in medial leaves. According to the inverse relationship between $\delta^{13}C$ and WUE in the discrimination formula put forth by Farquhar et al. (1982, 1989), distal leaves were characterized by an increase in WUE, suggesting a successful adaptation to water shortage conditions.

CONCLUSIONS

The adaptive response by the vines to water stress was to limit water loss through a reduction in transpiring surface area by limiting the emergence and expansion of new leaves, in addition to responding with fast stomatal closure that persisted until after the initial re-watering. In this experiment, the regime of water stress treatment and re-water cycles affected the morphology of the treated 'Sangiovese' grapevines, but in a fairly simple manner. Low water availability also impacted negatively g_s and induced a decrease in P_n associated with stomatal closure. The leaf position appears to be central to the degree of adaptive response. The mature leaves in the medial portion of the shoot responded promptly to water availability, showing a partial P_n recovery after 1 day of re-watering in the first cycle. The youngest fully expanded leaves in the distal positions on the shoot showed a partial P_n recovery also, but needed at least 2 d of re-watering and after moderate water stress to get there.

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AUTHOR CONTRIBUTIONS

All authors contributed equally to all aspects of this manuscript, including the development of the hypothesis, the experimental design and the writing and revisions of the manuscript.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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