

Carbon isotope discrimination and water use efficiency in interspecific *Prunus* hybrids subjected to drought stress

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ABSTRACT

In C_3 plants, carbon isotope composition ($\delta^{13}C$) is influenced by isotopic effects during diffusion from the atmosphere to the chloroplasts and carboxylation reactions. This work aimed to demonstrate if $\delta^{13}C$ of leaf soluble carbohydrates ($\delta^{13}C_{leaves}$) and of dry matter from new-growth shoots ($\delta^{13}C_{shoots}$) of *Prunus* plants subjected to a period of water deficit was related to water use efficiency (WUE). For this purpose, three interspecific *Prunus* hybrids rootstocks (6–5, 7–7 and $G \times N$) were gradually subjected to drought and then rewatered. Soil water content (SWC) decreased from 26.1 to 9.4% after 70 days of water shortage, when plants reached values of predawn leaf water potential (LWP) ranging from -3.12 to -4.00 MPa. Gas exchange, particularly net photosynthetic and transpiration rates, differed among the three hybrids, leading to different values of WUE. After 70 days of drought, a significant $\delta^{13}C$ increase of 5.86, 4.28 and 4.99‰ was observed in 6–5, 7–7 and $G \times N$, respectively. Significant correlations between $\delta^{13}C$ and other parameters (substomatal CO_2 /atmospheric CO_2 ratio, stomatal conductance and stem water potential) were found in all hybrids. The rewatering phase caused a recovery of the physiological status of the plants. The isotope composition of $\delta^{13}C_{shoots}$ was correlated with the average WUE measured during the whole experiment. $\delta^{13}C_{leaves}$ and $\delta^{13}C_{shoots}$ were positively related ($r = 0.87$; $p < 0.001$). The isotopic signature was a reliable screening tool to identify *Prunus* genotypes tolerant to drought stress. The results suggest the possibility of using $\delta^{13}C$ as an integrated indicator of level of drought stress in plants subjected to prolonged stress conditions.

1. Introduction

Water use efficiency (WUE), defined as the ratio between fixed carbon and transpired water, is an important physiological trait relevant for plant adaptation to drought (Walker et al., 2015; Avramova et al., 2019; Castillo-Argaez et al., 2020; Diefendorf et al., 2021). During drought stress, plants with a higher WUE are expected to be more tolerant and, possibly, to achieve higher productivity, either in terms of biomass produced per unity of surface or per plant (Scartazza et al., 1998; DaMatta et al., 2003; Cernusak et al., 2013; Ingwers et al., 2021). Using genotypes with higher WUE may increase the efficiency of irrigation under well-watered conditions and that of rainfall use under rainfed environments (Cabuslay et al., 2002). Genotypic variation in WUE has

been reported in many tree species, such as *Eucalyptus* (Osorio et al., 1998), *Castanea* (Lauteri et al., 1997), *Quercus* (Ponton et al., 2001), *Pistacia* (Esmailpour et al., 2016), *Pinus* (McDowell et al., 2010), *Populus* (Maier et al., 2019), *Picea* (Duan et al., 2011), apple (Liu et al., 2012), redbay tree and avocado (Castillo-Argaez et al., 2020), and *Juniperus* (Diefendorf et al., 2021). However, the use of WUE as a physiological index is hampered by the difficulty in taking measurements, especially under field conditions. On the contrary, an indirect measure of WUE, based on plant carbon isotope composition ($\delta^{13}C$), makes it possible to easily analyze a large number of plant samples and well before fruit production (Brugnoli et al., 1998; Cabuslay et al., 2002; Bchir et al., 2016).

The carbon in atmospheric CO_2 is composed by about 1.1% of the

Abbreviations: $\delta^{13}C$, carbon isotope composition; C_a , ambient CO_2 concentration; C_i , substomatal CO_2 concentration; g_s , stomatal conductance; E , transpiration rate; LWP_{PD} , pre-dawn leaf water potential; PAR, photosynthetic active radiation; P_N , net photosynthetic rate; RH, relative humidity; SWP, stem water potential; VPD, vapour pressure deficit; WUE, water use efficiency.

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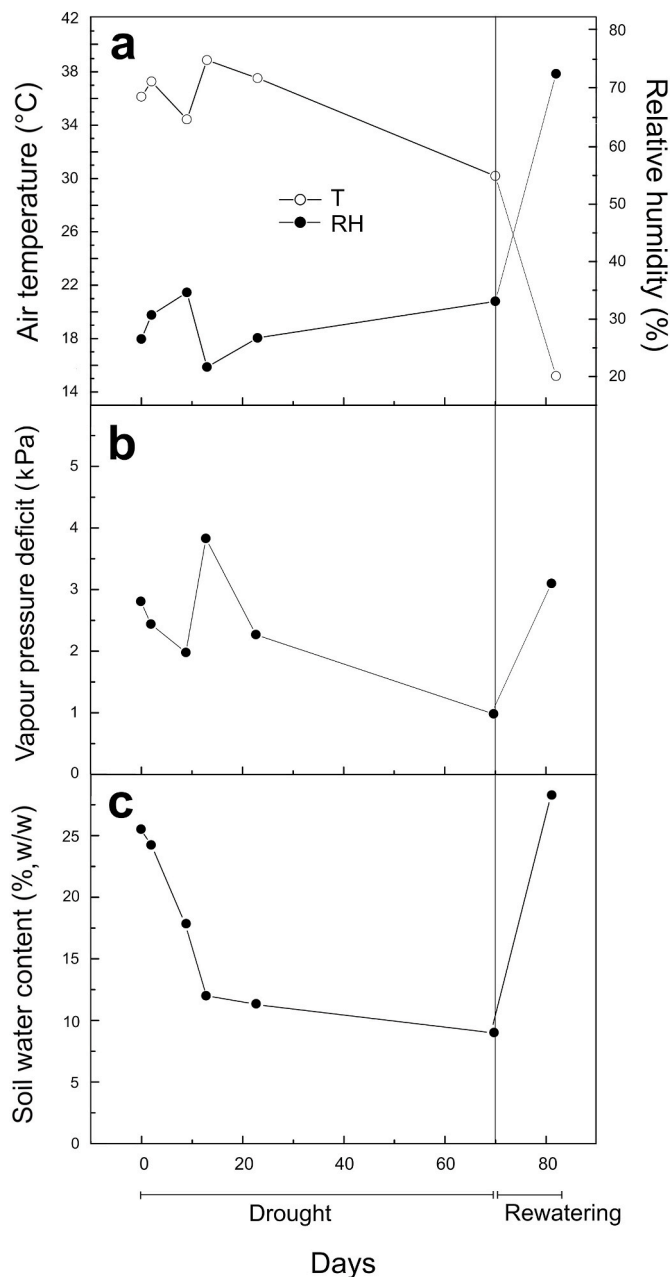


Fig. 1. (a) Air temperature (T) and relative humidity (RH), (b) vapour pressure deficit, and (c) soil water content (SWC) measured before the drought period (day 0), during the drought period (days 2, 9, 13, 23 and 70), and at the end of the rewating period (day 84). Each value of T, RH and VPD represents the mean ($n = 8$) from eight measurements carried out between 9:00 and 11:00 h, each value of SWC represents the mean of 12 measurements ($n = 12$).

heavier isotope ^{13}C and 98.9% of the isotope ^{12}C . During C fixation, plants discriminate against ^{13}C , causing the carbon isotope composition of plant material to be more negative than atmospheric CO_2 (Marion and O'Leary, 1981). $\delta^{13}\text{C}$ is influenced by isotopic effects during diffusion from the atmosphere to the chloroplasts and carboxylation reactions. This process is affected, amongst other factors, by the stomatal control of leaf gas exchange and by the ratio of substomatal (C_i) to ambient (C_a) CO_2 concentrations (Farquhar et al., 1989; Ehleringer, 1991; Scartazza et al., 1998; Brugnoli et al., 1988; Bielsa et al., 2018). Therefore, there is an independent correlation between $\delta^{13}\text{C}$ and the WUE of the whole plant.

It has been demonstrated that $\delta^{13}\text{C}$ in soluble carbohydrates

extracted from leaves gives a short-term integration of C_i/C_a over a diurnal course (Brugnoli et al., 1988). Since $\delta^{13}\text{C}$ positively correlates with WUE in several species, the C isotope composition or discrimination has been suggested as a selection criterion in breeding for improved WUE and possibly high yield under drought conditions (Farquhar et al., 1988; Brugnoli et al., 1998). However, the application of stable isotopes in breeding programs has not been as successful as expected and conflicting results have often been reported. For instance, various relationships have been found between biomass, productivity, and $\delta^{13}\text{C}$, depending on the species and experimental conditions (Brugnoli et al., 1988; Bchir et al., 2016). Thus, the use of $\delta^{13}\text{C}$ in breeding programs should be species-specific.

The variation of $\delta^{13}\text{C}$ may result from changes in carboxylation efficiency or stomatal conductance (g_s) or both. When changes of the C_i/C_a ratio result from variation of stomatal conductance, a high $\delta^{13}\text{C}$ will lead high WUE but low productivity. Conversely, when changes of the C_i/C_a ratio are caused by carboxylation efficiency, a high $\delta^{13}\text{C}$ will lead to high WUE and increased productivity (Farquhar et al., 1988; Brugnoli et al., 1998; Bchir et al., 2016).

Stone fruit crops, such as peach (*Prunus persica* L.), plum (*Prunus cerasifera* L. and *Prunus domestica* L.), almond (*Prunus dulcis* L.), apricot (*Prunus armeniaca* L.) and cherry tree (*Prunus avium* L.) are economically important cultures mainly localized in Mediterranean regions, where they are often subjected to drought and to high temperatures. Productive stone fruit trees are grafted plants with a lower part, the rootstock, and an upper grafted part, which is the genotype of the commercial variety and is genetically different from the rootstock. Plum genotypes have been often used as rootstocks for other *Prunus* species, and confer various genetic traits, such as resistance to drought or pathogens (Lecouls et al., 2004; Sofo et al., 2005).

The responses to water deficit of these species are well-documented (Torrecillas et al., 1996; Esparza et al., 2001; Klein et al., 2001) but only a few studies highlighted the application of $\delta^{13}\text{C}$ analysis for studying the physiological responses of *Prunus* species to drought conditions (Arndt et al., 2000; Arndt and Wanek, 2002; Bielsa et al., 2018), and very little is known about the relationship between $\delta^{13}\text{C}$ and WUE in this genus.

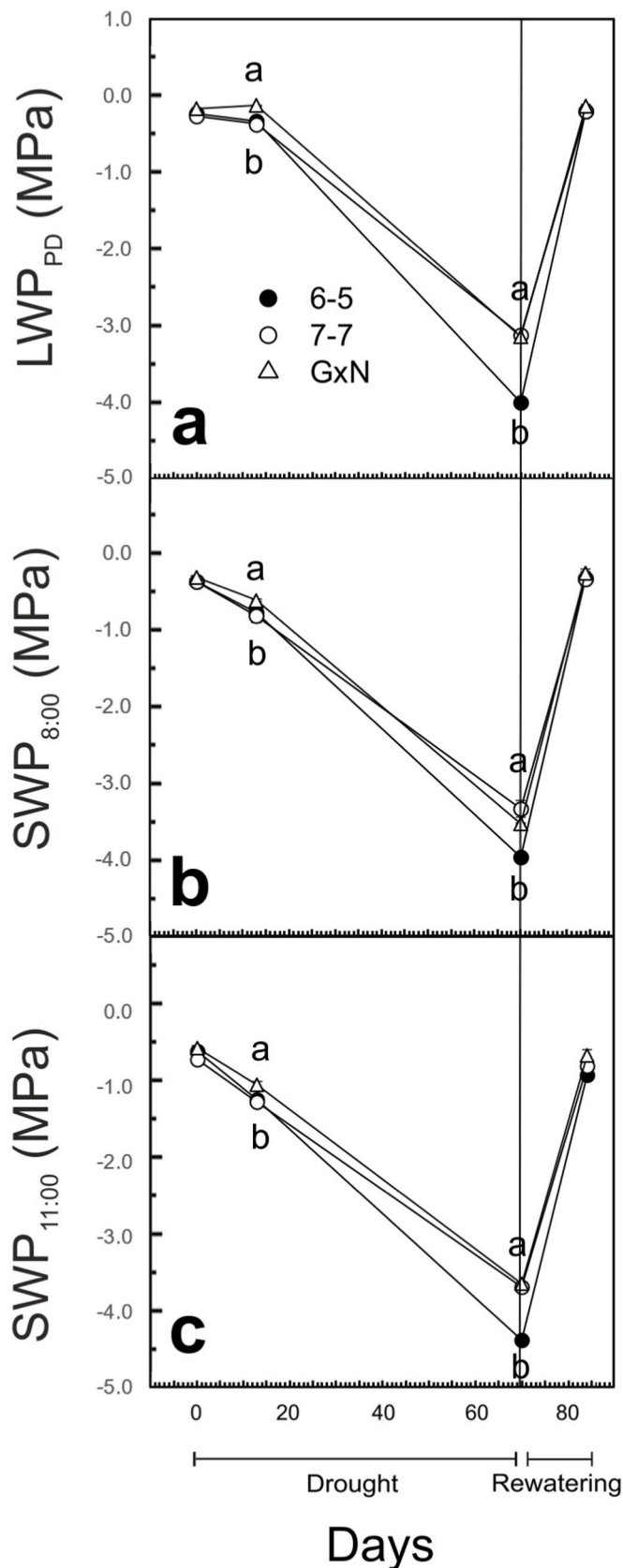
This work aimed to demonstrate if: a) carbon isotope composition of soluble carbohydrates of *Prunus* plants subjected to a period of water deficit is related to WUE; b) $\delta^{13}\text{C}$ can be used as a screening tool for the selection of interspecific *Prunus* hybrids having a high WUE under drought conditions.

2. Materials and methods

2.1. Study site and plant material

The study was conducted in an experimental field located at the 'University of Basilicata' in Potenza (Italy, Basilicata Region; N 40° 39', E 15° 47'). The soil was a silty-clay having a water content of 32% at the field water capacity and of 13% at the wilting point. In the last ten years, the value of mean rainfall was 650 mm and the mean yearly air temperature was 14 °C. Trials were conducted on virus-free plant material obtained from the breeding programs of INRA Bordeaux and SIA Zaragoza (EU funded project FAIR-6-CT-98-4139). The material included three interspecific hybrids named '6-5' (*Prunus cerasifera* L. 'P2175' x *Prunus amygdalus* L. 'Garfi'), '7-7' (*Prunus cerasifera* L. 'P2175' x *Prunus davidiana* L.), and 'G x N' (*Prunus amygdalus* L. 'Garfi' x *Prunus persica* L. 'Nemared').

The experimental scheme was carried out using one-year-old rootstocks planted in spring, spaced at 2 m in the row with 2 m between rows. Trees were irrigated with drip emitters per plant discharging 3 L h^{-1} . Soil water content was maintained at a constant value of around 85% of the field water capacity by integrating the amount of water lost through transpiration during the day. Plants were fertilized at 25-day intervals throughout vegetative growth with 3.5 g of slow-release



(caption on next column)

Fig. 2. Pre-dawn leaf water potential (LWP_{PD}) and stem water potential measured at 8:00 h (SWP_{8:00}) and at 11:00 h (SWP_{11:00}) in three interspecific *Prunus* hybrids (6-5, 7-7 and G × N) measured before the drought period (SWC₀), at two days of drought (SWC₁₃ and SWC₇₀), and at the end of the rewatering period (SWC₈₄). Each value (±SE) represents the mean (n = 9) from three plants. The values followed by different uppercase letters are statistically different (p ≤ 0.01) within rows for each level of SWC, whereas the values followed by different lowercase letters are statistically different (p ≤ 0.01) within rows independently from the level of SWC.

nitrogen complex fertilizer Nitrophoska Gold - BASF-15N-9P-15K+2Ca+17.5 Mg (Compo Agricoltura, Cesano Maderno, Milan, Italy).

2.2. Experimental design and environmental parameters

The experimental period started on July 1 and ended on October 24, 2002. Initially, plants were maintained in an optimal soil water condition (85% of the field water capacity). Successively, plants were subjected to a water shortage period of 70 days (from July 12 to September 18) with no irrigation. The soil was covered with plastic film to avoid rainfall infiltrations and evaporation from the soil surface. After this 70 day-period of drought, stressed plants were subjected to a rewatering treatment of 14 days (from September 19 to October 2).

Measurements of air temperature and relative humidity (RH) were recorded between 9.00 and 11.00 h at 0, 2, 9, 13, 23, and 70 days of drought and at the end of the rewatering period by a Tinytag Ultra 2K data logger (Maeco, Cranleigh, Surrey, UK) located inside the experimental plot. Vapour pressure deficit (VPD) was calculated from the values of air temperature and RH measured at 11.00 h, according to Goudriaan and van Laar (1994).

Soil samples were taken at a depth of 0–60 cm randomly in different points of the soil and the values of soil humidity at 0, 2, 9, 13, 23, and 70 days of drought and at the end of the rewatering period were determined from the weight differences of soil samples before and after drying at 105 °C for 18 h and expressed as percentages of water (w/w) on soil dry matter.

2.3. Water status and leaf gas exchange

At 0, 9, 13, 23 and 70 days of drought and at the end of the rewatering period, predawn leaf water potential (LWP_{PD}) was measured at 04.00 h whereas stem water potential (SWP) was measured at 08:00 and 11:00 h. The measurements were carried out on three fully expanded leaves selected from each plant along the median segment of new-growth shoots using a Scholander pressure chamber (PMS Instrument Co. Corvallis, OR, USA), according to Turner (1981). The leaves used for SWP measurement were placed in a plastic envelope, to allow leaves to reach water equilibrium, and covered with an aluminium foil, to avoid leaf heating (Garnier and Berger, 1985). At 0, 13 and 23 days of drought, the measurements of water potential were carried out throughout the day (LWP_{PD} at 4:00 h, and SWP at 8:00, 11:00, 14:00 and 17:00 h).

Three plants from each hybrid were chosen to measure leaf gas exchange. Measurements were taken at 11:00 h, on days 0, 9, 13 and 70 after start of drought treatment and at the end of the rewatering period on four fully expanded leaves selected from each plant along the median segment of new-growth shoots and marked at the beginning of the experiment. The measurements of net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s) and substomatal CO₂ concentration (C_i) were carried out using the portable photosynthesis system LCA-4 (Analytical Development Company, Hoddesdon, UK) operated at 200 μmol s⁻¹ flow rate, under field conditions (photosynthetic active radiation, PAR >1400 μmol m⁻² s⁻¹) at 10:00 h. At 0, 13 and 23 days of drought, the measurements of P_N and E were carried out throughout the day (at 8:00, 11:00, 14:00 and 17:00 h).

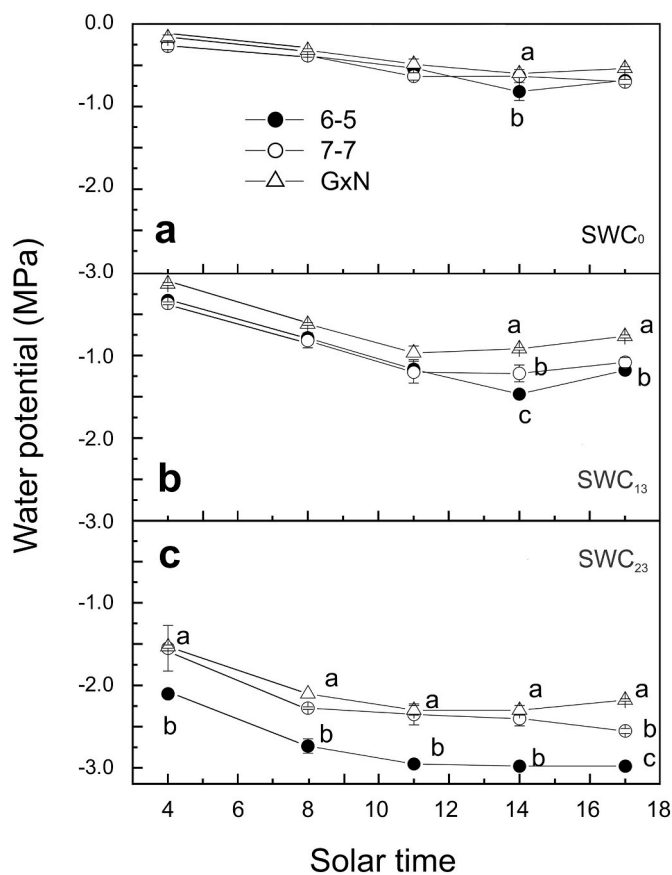


Fig. 3. Daily trend of water potential (LWP_{PD} at 4:00 h, and SWP at 8:00, 11:00, 14:00 and 17.00 h) in three interspecific *Prunus* hybrids (6-5, 7-7 and G × N) measured (a) before the drought period (SWC₀) and (b, c) at two days of drought (SWC₁₃ and SWC₂₃). Each value (±SE) represents the mean ($n = 9$) from three plants. The values followed by different letters are statistically different ($p \leq 0.01$) for each level measuring day.

2.4. Tissue sampling

Three plants having similar LWP_{PD} values were selected from each hybrid for tissue sampling. Leaves were collected at 18:00 h on days 0, 23 and 70 after the beginning of drought. Each sample contained three fully expanded leaves taken on similar branches, at the same height from the soil and at the same distance from the median axis of the plant, and fully exposed to sunlight ($\text{PAR} > 1400 \mu\text{mol m}^{-2} \text{s}^{-1}$). After 14 days of rewatering (October 2), three new shoots per plant grown during the experimental period were sampled at 18:00 h. To identify these new-growth portions, an elasticized ribbon was applied to the last apical node on the day on which the irrigation was stopped.

Both leaf and shoot samples were washed with distilled water, dried with filter paper, temporarily covered with an aluminium foil and a plastic envelope, immediately put in liquid nitrogen (to block carbohydrate respiration) and then lyophilized and finely ground to powder.

2.5. Carbon isotope composition

A 150 mg aliquot of dry powder of leaves was used for the extraction and the purification of soluble carbohydrates. The extraction was carried out by dissolving the powdered leaves in distilled water, according to Brugnoli et al. (1988). The supernatant obtained after centrifugation was purified by using the ion exchange resins Dowex 50X8-100 hydrogen form; Sigma-Aldrich, St. Louis, MI, USA) to remove amino acids and anions, and Dowex 1X2-100 chloride resins (Sigma-Aldrich) to

separate organic acids and cations.

Soluble carbohydrates extracted from leaves collected at 0, 23 and 70 days of drought, and the dry matter of new-growth shoots sampled at the end of the rewatering period were used for the following analysis. Carbon isotope composition was determined by a dual-inlet mass spectrometer with a purification system and cryogenic traps (SIRA II, VG Isotech, Middlewich, UK), following the method described by Lauteri et al. (1993). The reference gas was calibrated with the international standard Vienna-PeeDee Belemnite (V-PDB). The control of eventual fractionations was carried out using sucrose (Sigma Chemicals, USA) calibrated against V-PDB as a standard with a $\delta^{13}\text{C} = -25.09 \pm 0.06$.

The natural abundance of ^{13}C was determined as:

$$\delta^{13}\text{C} = [(R_S/R_{V\text{-PDB}}) - 1] \times 1000 \quad (1)$$

where R_S and $R_{V\text{-PDB}}$ are the values of $^{13}\text{C}/^{12}\text{C}$ ratio of the sample and of the international standard, respectively.

2.6. Statistical analysis

The values of gas exchange parameters were represented as means of 12 measurements ($n = 12$) from three selected plants (four measurements per plant and three replications of each measurement), whereas the values of LWP_{PD} and SWP were represented as means of nine measurements ($n = 9$) from three selected plants (three measurements per plant and three replications of each measurement). The values of carbon isotope discrimination were expressed as means of three measurements ($n = 9$) from three plants having a similar value of LWP_{PD} (three measurements per plant and three replications of each measurement). Standard error (SE) was used as an index of statistical dispersion. Statistical analysis was performed using a two-way analysis of variance (ANOVA) with randomized blocks by the software CoStat (Analysis Statistics Software of Monterey, California, USA). LSD test was used for statistical comparisons at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$.

3. Results and discussion

3.1. Environmental parameters and soil water content

During the experiment, the air temperature fluctuated between 38 and 16 °C (Fig. 1a), while the air relative humidity ranged between 16 and 74% (Fig. 1b). The minimum VPD value (1.3 kPa) was recorded on September 18, while the maximum value (3.8 kPa) on July 23, 13 days after the interruption of irrigation. The soil water content (SWC) decreased by over 70% in the first 13 days of drought (Fig. 1c).

Soil water content decreased from 26.1% (SWC₀, at day 0 of drought), to 24.0% (SWC_{2d}, at day 2 of drought) and 12.5% (SWC₁₃, at day 13 of drought), up to 9.4% (SWC_{70d}, at day 70 of drought) (Fig. 1c). The rapid loss of water that occurred in the first 13 days of the drought period was followed by a long period in which strong reductions in plant water potential corresponded to small fluctuations in SWC (a decrease of only 3.1% between SWC₁₃ and SWC₇₀) (Fig. 1c). After 70 days of drought, the plants showed clear visual signs of suffering, with clear stress symptoms such as loss of leaf turgidity in the midday, and chlorosis of the basal leaves. At the end of the rewatering period (SWC₈₄), SWC recovered completely to the values found at the beginning of the drought period (Fig. 1c).

3.2. Water potentials

During the progressive drought and the consequent decrease of SWC (Fig. 1c), a significant reduction of LWP_{PD} and SWP occurred in all hybrids (Fig. 2). This confirms the scarce ability of *Prunus* spp. to compensate for water losses during the day by the maintenance of water uptake (Garnier and Berger, 1985, 1987; Arndt and Wanek, 2002; Avramova et al., 2019). At the minimum soil moisture (SWC₇₀), the

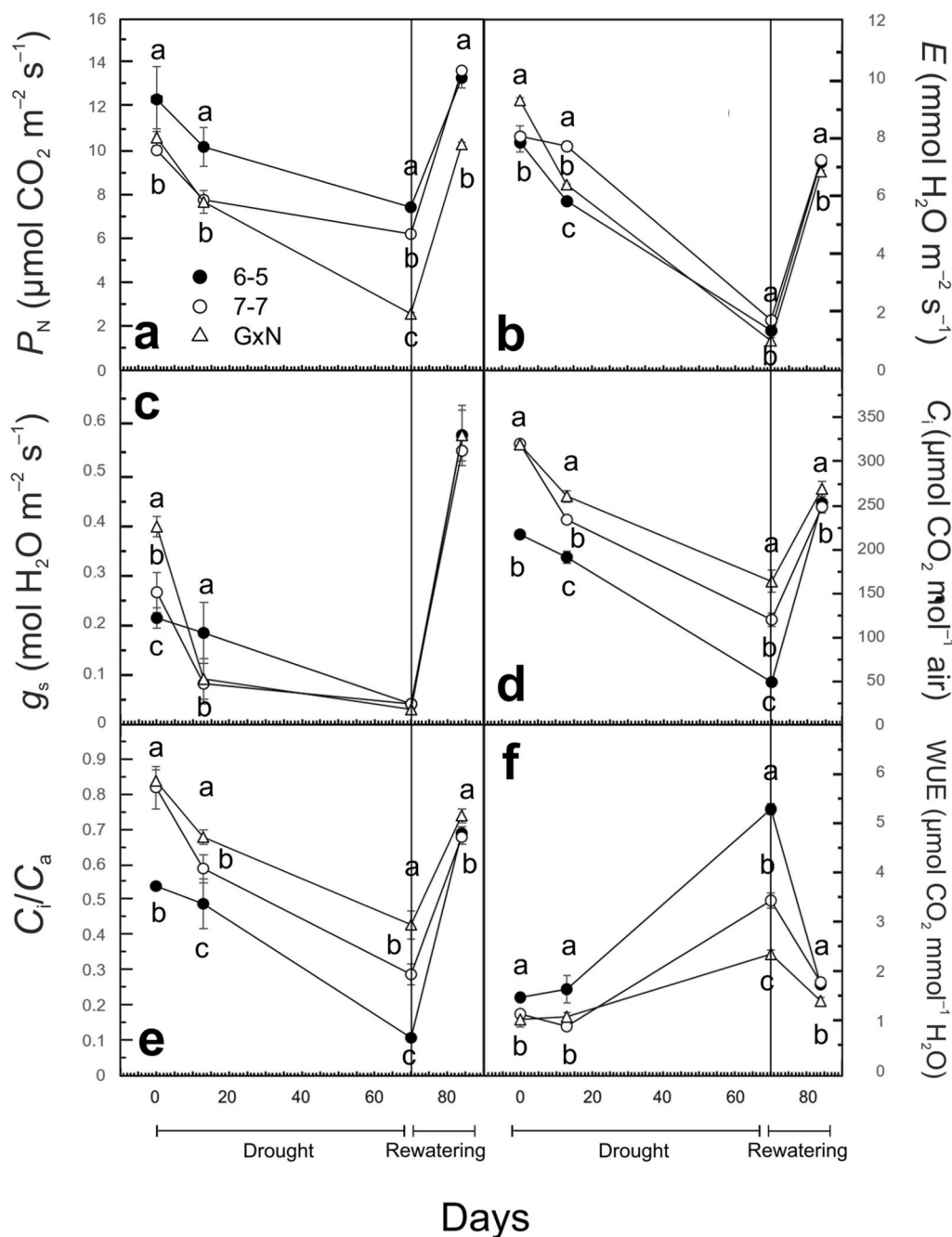


Fig. 4. Net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s), substomatal CO_2 concentration (C_i), substomatal/atmospheric CO_2 concentration (C_i/C_a), and instantaneous water use efficiency ($\text{WUE} = P_N/E$) measured at 11:00 in three interspecific *Prunus* hybrids (6-5, 7-7 and $G \times N$) measured before the drought period (SWC_0), at two days of drought (SWC_{13} and SWC_{70}), and at the end of the rewatering period (SWC_{84}). Each value ($\pm \text{SE}$) represents the mean ($n = 9$) from three plants. The values followed by different letters are statistically different ($p \leq 0.01$) for each measuring day.

hybrid 6-5 reached LWP_{PD} of -4.00 MPa, with a difference ($\Delta\Psi_{\text{LWP}}$) of -3.77 MPa compared to the LWP_{PD} measured at SWC_0 , that is about 32% lower than the $\Delta\Psi_{\text{LWP}}$ of 7-7 and about 26% lower than that of $G \times N$ (Fig. 2). The $G \times N$ maintained LWP_{PD} values significantly higher than the other two hybrids, both at SWC_0 (-0.17 MPa) and SWC_{13} (-0.13 MPa), whereas LWP_{PD} at SWC_{70} (-3.15 ± 0.03 MPa) was not significantly different from that of 7-7, and significantly higher by 21% compared to that of 6-5 (Fig. 2). The values of LWP_{PD} at SWC_{70} (Fig. 2) can be considered extreme values for *Prunus* spp. Other authors (Arndt and Wanek, 2002) found in *P. persica* values of -2.0 MPa in non-irrigated plants and after a dry period of about 100 days. In *P. domestica*, McCutchan and Shackel (1992) observed a drop in water potential in trees not irrigated from the beginning of the growing season, with plants in August reaching values close to -3.0 MPa. Romero et al. (2004) found a LWP_{PD} of -2.52 MPa in *P. dulcis* plants subjected to

controlled water deficit for 60 days, where only 20% of the evapotranspiration was returned with irrigation. The rapidity and extent of the fall of water potentials within the *Prunus* genus can also be related to the conformation and morphology of the root systems, which determine the quantity and speed with which water is taken up and made available for the transpiratory flow and the depth of available water reservoirs. On this basis, other authors (Arndt and Wanek, 2002; Bielsa et al., 2018) concluded that *Prunus* spp. has a poor ability to compensate for water losses by maintaining water uptake. The ability of plants to establish a high gradient of water potential between leaves, roots and soil plays an important role in determining its ability to take up water from the soil and therefore to overcome or tolerate dry periods.

The values of SWP measured at 8:00 h ($\text{SWP}_{8:00}$) and 11:00 h ($\text{SWP}_{11:00}$) confirmed the differences of LWP_{PD} observed among the hybrids (Fig. 2). However, SWP values, and particularly $\text{SWP}_{11:00}$,

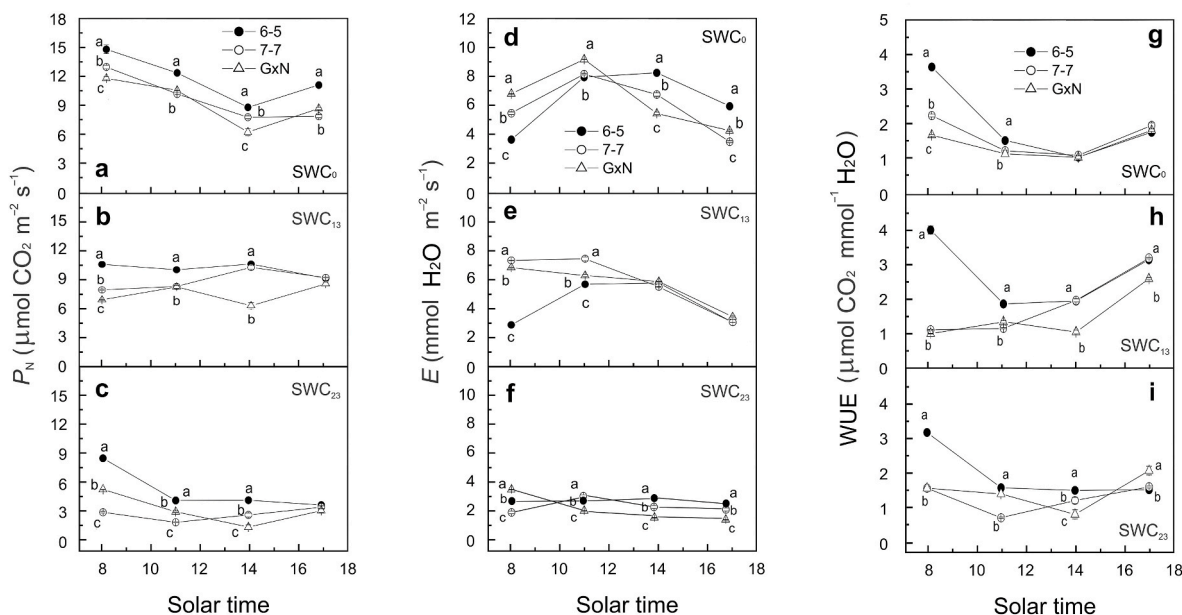


Fig. 5. (a, b, c) Daily trend of net photosynthetic rate (P_N), (d, e, f) transpiration rate (E), and (g, h, i) instantaneous water use efficiency ($\text{WUE} = P_N/E$) in three interspecific *Prunus* hybrids (6-5, 7-7 and $G \times N$) measured before the drought period (SWC_0) and at two days of drought (SWC_{13} and SWC_{23}). Each value ($\pm\text{SE}$) represents the mean ($n = 9$) from three plants. The values followed by different letters are statistically different ($p \leq 0.01$) for each measuring hour.

changed more promptly than LWP_{PD} , in particular at SWC_{13} (Fig. 2). This confirms the higher sensitivity of SWP for estimating plant water status related to the variation of soil water content (Garnier and Berger, 1985; McCutchan and Shackel, 1992; Cernusak et al., 2013; Gaudillere et al., 2002; Bchir et al., 2016). Indeed, the $\Delta\Psi_{\text{LWP}}$ measured at SWC_0 and SWC_{13} in the hybrids 6-5 and 7-7 was -0.10 MPa, while in the $G \times N$ hybrid it was slightly lower (-0.04 MPa). On the other side, $\Delta\Psi_{\text{SWP}}$ measured at 11:00 at SWC_0 and SWC_{13} were -0.64 , -0.57 and -0.49 MPa, for 6-5, 7-7 and $G \times N$, respectively. These values are in agreement with what found by other authors in peach trees with similar stress levels (Garnier and Berger, 1985; McCutchan and Shackel, 1992; Bielsa et al., 2018). The most negative $\text{SWP}_{11:00}$ at SWC_{70} was found in 6-5 (-4.38 MPa), which was significantly lower by 26% and 22% than the values found for the hybrids 7-7 and $G \times N$, respectively, confirming the trend observed for LWP_{PD} (Fig. 2). After 14 days of rewatering (SWC_{84}), all the three hybrids completely recovered plant water status, showing LWP_{PD} values statistically equal or even higher than those of well-watered plants (SWC_0) (Fig. 2).

From the analysis of the daily trends of water potentials (LWP_{PD} at 4:00 h, and SWP at 8:00, 11:00, 14:00 and 17.00 h), it appears that at optimal water availability (SWC_0), the three hybrids showed no differences, except for the measurements at 14:00 h, in which 6-5 continued to lose water and achieved a water potential of about 30% lower than that of the other two hybrids (Fig. 3a). Therefore, the hybrid 6-5 seems to adopt a non-conservative mechanism (little or no fast stomatal closure with decreasing SWC) in response to reduced SWC, contrary to what has been observed for the other two hybrids. The same behavior of 6-5 was found in olive by other authors (Xiloyannis et al., 1999; Dichio et al., 2009) and could be partly because of active osmotic adjustment, one of the primary mechanisms of adaptation of plants to water stress. Avramova et al. (2019) and Arndt and Wanek (2002) and Bielsa et al. (2018) found that in peach trees under water scarcity, a certain osmoregulation occurs, allowing to support cellular turgor, delaying phenomena of incipient plasmolysis and helping to determine high gradients of water potentials between soil, roots and leaves.

The daily trends of water potential during drought (SWC_{13} and SWC_{23}) (Fig. 3b and c) confirm that the values in 6-5 showed the highest reduction. The low water potentials likely allowed this hybrid to

withstand dehydration processes by improving water uptake from the soil, so feeding the transpiration flow. This mechanism is typical of species that resist to drought, and which continue to maintain high levels of transpiration even in conditions of limited soil water availability (Dichio et al., 2006, 2013; Sofò et al., 2008).

3.3. Leaf gas exchange

Compared to the conditions at the beginning of the drought period (SWC_0), the values of P_N at SWC_{13} decreased by 18, 23 and 28% in 6-5, 7-7 and $G \times N$, respectively, and by 40, 38 and 76%, respectively at SWC_{70} (Fig. 4a). At every level of SWC, 6-5 had the highest P_N , which was 23, 31 and 20% higher than 7-7 at SWC_0 , SWC_{13} and SWN_{70} , respectively, and 16, 33 and 188% higher than $G \times N$ at SWC_0 , SWC_{13} and SWC_{70} , respectively (Fig. 4). Also comparing the daily trend of P_N , 6-5 had higher values in all three sampling dates (Fig. 5a-c). At 8:00 h, the hybrid 6-5 showed values of P_N higher than 1.97, 3.22 and 5.91 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, compared to those of 7-7, at SWC_0 , SWC_{13} and SWC_{23} , respectively, and values of P_N higher than 3.23, 4.49 and 3.41 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, compared to those of $G \times N$, at SWC_0 , SWC_{13} and SWC_{23} , respectively (Fig. 5a-c).

The three hybrids had significant differences in E values at all the levels of SWC, while for g_s values significantly different were found at SWC_0 and SWC_{13} but not at SWC_{70} (Fig. 4). In all hybrids, the values of g_s were drastically reduced by the decrease of SWC and, at maximum stress, stomata were completely closed reaching values down to 0.03 $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ in $G \times N$ (Fig. 4). Similar values have been found by Garnier and Berger (1985) on peach trees, in correspondence to LWP_{PD} of about -2.0 MPa. The values of g_s measured at SWC_0 and SWC_{13} in the hybrid 6-5 (0.21 and 0.15 $\text{mol H}_2\text{O m}^{-2} \text{ s}^{-1}$, respectively) were significantly lower compared to those in 7-7 and $G \times N$ (Fig. 4). The hybrids 7-7 and $G \times N$ at SWC_{13} showed prompt control of the stomatal openings, with a reduction in g_s by 69 and 77%, respectively, compared to the values at SWC_0 . However, while this reduction of g_s in $G \times N$ was followed by a reduction by 31% of E , in 7-7 no significant differences were found between E values measured at SWC_0 and SWC_{13} (Fig. 4). In contrast, 6-5 showed an initial insensitivity to the reduction of g_s measured at SWC_0 and SWC_{13} (Fig. 4). At SWC_{70} , a significantly lower E

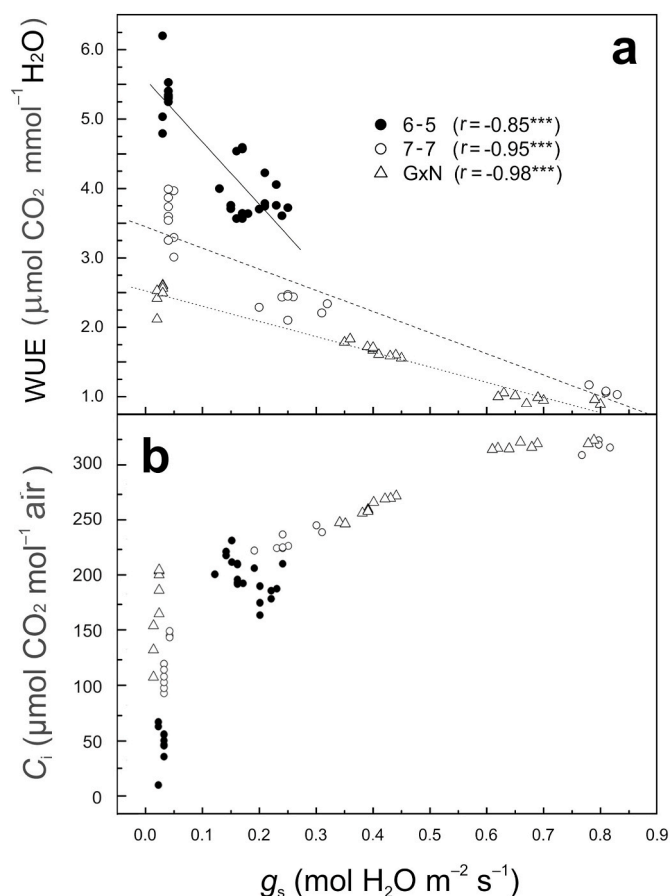


Fig. 6. (a) Trends of water use efficiency ($WUE = P_N/E$) and stomatal conductance (g_s), and (b) trends of substomatal CO_2 concentration (C_i) and stomatal conductance (g_s) in three interspecific *Prunus* hybrids (6–5, 7–7 and $G \times N$) during the period of drought stress. The values of gas exchange were measured at 11.00 h. Each value represents a single measurement. Regression coefficients are reported in the figure ($n = 36$; *** = $p \leq 0.001$).

Table 1

Carbon isotope composition ($\delta^{13}C$) of leaf soluble carbohydrates in three interspecific *Prunus* hybrids (6–5, 7–7 and $G \times N$) measured before the drought period (SWC_0) and at two days of drought (SWC_{13} and SWC_{70}), and isotope composition ($\delta^{13}C$) of the dry matter of new-growth shoots measured at the end of the rewetting period (SWC_{84}). Each value ($\pm SE$) represents the mean ($n = 9$) from three plants. The values followed by different uppercase letters are statistically different ($p \leq 0.01$) within rows for each level of SWC, whereas the values followed by different lowercase letters are statistically different ($p \leq 0.01$) within rows independently from the level of SWC.

		$\delta^{13}C$ (‰)		
		6–5	7–7	$G \times N$
Well-watered	SWC_0	–26.59 \pm 0.06	–26.50 \pm 0.06	–27.42 \pm 0.06
	Af		Af	Bg
Drought	SWC_{13}	–25.57 \pm 0.06	–24.33 \pm 0.05	–25.68 \pm 0.06
	Be		Ad	Be
	SWC_{70}	–20.73 \pm 0.05	–22.22 \pm 0.05	–22.43 \pm 0.05
	Aa		Bb	Cc
Rewetting	SWC_{84}	–26.20 \pm 0.03	–27.12 \pm 0.05	–27.78 \pm 0.03
	Af		Ag	Bg

was found in $G \times N$, and it was 24 and 40% lower than the values of the hybrids 6–5 and 7–7, respectively (Fig. 4). At SWC_{70} , The $G \times N$ hybrid reduced its E by 89% compared to SWC_0 , while the genotypes 6–5 and 7–7 had reductions of 82 and 78%, respectively. Regarding the daily trends of E , at SWC_0 and SWC_{23} the hybrid 6–5 had the lowest E at 8:00 h,

while in the rest of the day showed an increasing E (Fig. 5df). This clarifies the sharp drop in water potential observed in 6–5 during the hottest hours (Fig. 3).

At 13 days from the beginning of drought (SWC_{13}), a high value of VPD (5.5 kPa) was recorded (Fig. 1b) and this, together with the decrease in SWC (Fig. 1c), resulted in the drop in gas exchange in the three hybrids (Fig. 4). Noteworthy, the values of E and g_s of Fig. 4 reflected the variations in VPD values, confirming the influence of VPD in conditioning the water losses of the leaf and therefore in determining the gradients of water potentials which represent the main driving force of water movements, from the soil to the atmosphere, through the plant.

The differences of gas exchange observed among the three genotypes in conditions of good water availability (SWC_0) (Fig. 4 and Fig. 5ad) were presumably related to the genetic differences, and therefore to the different sensitivity of the three hybrids to regulate the stomatal openings with the levels of atmospheric VPD (Fig. 1c). Generally, the better response of gas exchange to water deficit observed in 6–5 (Fig. 4) was probably linked both to a higher capacitance, that is to greater elasticity of the tissues which therefore transfer water to the transpiration flow, but also to a higher ability of the root apparatus to feed the xylem flow, taking water from the deeper layers of the soil. Indeed, the hybrid 6–5 maintained the highest P_N and E values with the progressive drought, in particular in the post-meridian hours (Fig. 5bcef), when it was more necessary for the plant to ensure the cooling of the leaf, according to what found in *Coffea canephora* by DaMatta et al. (2003) and in olive by Dichio et al. (2013).

In the hybrid 6–5 at SWC_{70} , despite the limited g_s ($0.04 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$), a P_N of $7.42 \text{ } \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$ was measured (Fig. 4). This was accompanied by a decrease of C_i to values of about $48.10 \text{ } \mu\text{mol CO}_2 \text{ mol}^{-1}$, with a decrease of 78%, compared to SWC_0 (Fig. 4), so indicating the absence of non-stomatal factors on the control of photosynthetic activity. Such low values of C_i are not compatible with the rate of photosynthesis observed, as $48.10 \text{ } \mu\text{mol CO}_2 \text{ mol}^{-1}$ should be very close or even lower than the compensation point. In addition, with strong stomatal closure, determination of accurate C_i may be not possible due to occurrence of patchy stomatal aperture (i.e., heterogeneity in stomatal aperture over the leaf surface). Comparing SWC_0 and SWC_{70} , the $G \times N$ hybrid at a g_s value of $0.03 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$, a value similar to that of 6–5, had a reduction of P_N by 76% and a decrease of C_i by only 49%. This was probably related to the onset of non-stomatal inhibition factors on photosynthesis and hence on the variations of C_i . The lower C_i observed in 6–5 (Fig. 4) was likely determined by higher photosynthetic efficiency. This result can be the consequence of greater Rubisco content or activity, increased capacity of the electron transport in the thylakoidal membranes or of a higher conductance of the mesophyll, which makes CO_2 more available at the carboxylation sites. The same decreases of C_i were also found for substomatal/atmospheric CO_2 concentration (C_i/C_a) (Fig. 4). The $G \times N$ hybrid, unlike the 6–5, simultaneously reduced water potentials (Fig. 3) and gas exchange (Fig. 5). In correspondence with the maximum level of stress (SWC_{70}), $G \times N$ was more sensitive to water deficit, and it completely blocked the assimilation activity, probably because of the down regulation or inhibition of the photosynthetic systems or to CO_2 levels inside the chloroplast below the compensation point, so that photorespiration become prevailing.

At the end of the rewetting phase, all the three hybrids fully recovered the values of gas exchange, showing statistical differences among hybrids only in some parameters of $G \times N$ (Fig. 4). Such rapid recovery, would show that any down regulation of photosynthesis was not irreversible and probably due to adjustment of the system to the CO_2 availability.

3.4. Water use efficiency

The WUE, calculated as the instantaneous ratio between P_N and E , significantly increased with decreasing SWC in all the *Prunus* hybrids (Fig. 4), as expected as a general plant response to limited water

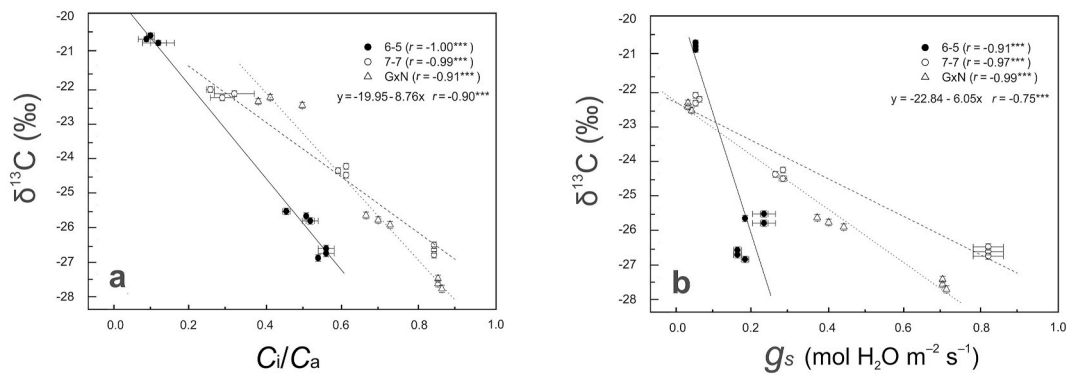


Fig. 7. (a) Trends of carbon isotope composition ($\delta^{13}\text{C}$) and sub-stomatal CO_2 /atmospheric CO_2 (C_i/C_a) in three interspecific *Prunus* hybrids (6–5, 7–7 and G \times N) during the period of drought stress. (b) Trends of carbon isotope composition ($\delta^{13}\text{C}$) and stomatal conductance (g_s) in three interspecific *Prunus* hybrids (6–5, 7–7 and G \times N) during the period of drought stress. The values of gas exchange were measured at 11.00 h. Each value (\pm SE) represents the average ($n = 4$) from each plant. Regression coefficients are reported in the figure ($n = 9$; $*** = p < 0.001$).

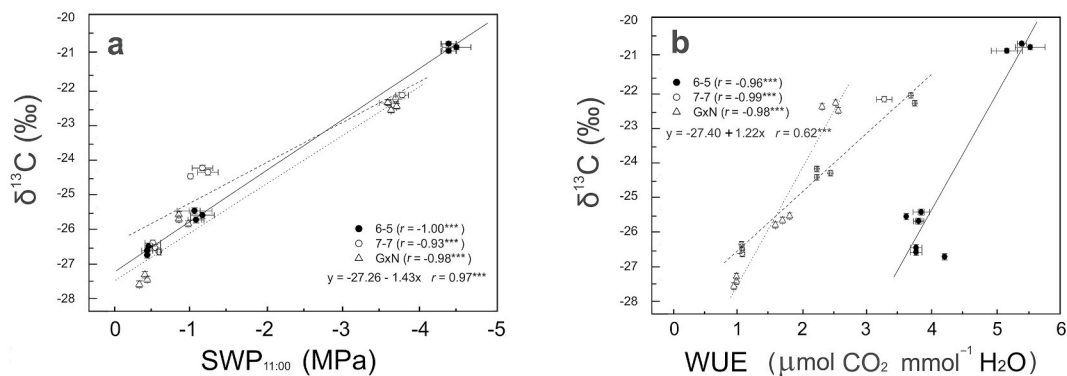


Fig. 8. (a) Trends of carbon isotope composition ($\delta^{13}\text{C}$) and stem water potential measured at 11:00 (SWP_{11:00}) in three interspecific *Prunus* hybrids (6–5, 7–7 and G \times N) during the period of drought stress. (b) Trends of carbon isotope composition ($\delta^{13}\text{C}$) and instantaneous water use efficiency (WUE = P_N/E) in three interspecific *Prunus* hybrids (6–5, 7–7 and G \times N) during the period of drought stress. The values of WUE were measured at 11.00 h. Each value (\pm SE) represents the average ($n = 4$) from each plant. Regression coefficients are reported in the figure ($n = 9$; $*** = p < 0.001$).

availability and confirming what observed in other species of the genus *Prunus* (Arndt et al., 2000; Bielsa et al., 2018). Moreover, the WUE was negatively correlated to the g_s in all the clones, and the effect of g_s variation in determining changes in the WUE of the hybrid 6–5 ($r = -0.85$; $y = 5.57 - 8.99x$; $p < 0.001$) was very strong (Fig. 6a). The hybrid 6–5 showed the highest WUE at each level of SWC, with a maximum of $5.34 \mu\text{mol CO}_2 \text{ mmol}^{-1} \text{H}_2\text{O}$ at SWC₇₀ (Fig. 4). In the hybrids 6–5 and 7–7, the increase in WUE with decreasing SWC was mainly related to stomatal control on E reduction and to a lesser extent of P_N (Fig. 4 and Fig. 5ef), unlike what happened in the G \times N hybrid, in which, particularly at the maximum level of stress, the effect of non-stomatal factors prevailed.

The better performance of 6–5 during drought is also clear in WUE daily trends, where WUE decreased with decreasing SWC but remained generally higher in 6–5 (Fig. 5g–i). The reduction of the WUE, observed in the first 23 days from the interruption of irrigation (Fig. 5g–i) was likely linked to a decrease in g_s that limited water losses and the availability of CO_2 in the sub-stomatal chamber, and therefore to the carboxylation sites.

3.5. Carbon isotope composition

Isotope compositions of leaf soluble sugars, which represents a significant proportion (89–90%) of the most recently fixed carbon, during the decreasing SWC reflected the trends of leaf water potential (Figs. 2 and 3) and gas exchange (Fig. 4 and Fig. 5a–f). At SWC₇₀, a significant increase of C isotope composition ($\delta^{13}\text{C}$) of 5.86, 4.28 and 4.99‰ was

observed in 6–5, 7–7 and G \times N, respectively, compared to the values recorded at SWC₀ (Table 1). At SWC₇₀, the hybrid 6–5 showed the highest value of $\delta^{13}\text{C}$ ($-20.73\text{‰} \pm 0.05$), while its correspondent value at SWC₀ was not statistically different from that of 7–7 and significantly higher than G \times N (Table 1). Arndt and Wanek (2002) found soluble sugars $\delta^{13}\text{C}$ of -26.4‰ in *Prunus persica* and this value increased by 2.0‰ with drought stress that reduced LWP_{PD} from -0.4 MPa to -2.0 MPa. At the maximum level of drought stress (SWC₇₀), the greater P_N found in 6–5 likely resulted in a greater deficit of CO_2 at the carboxylation sites (Fig. 4), causing its higher $\delta^{13}\text{C}$ (Table 1). In the G \times N hybrid at SWC₇₀, there was a sharp reduction in P_N (Fig. 4), resulting in a low turnover of soluble carbohydrates and consequently a more limited effect on $\delta^{13}\text{C}$ (Table 1).

The values of $\delta^{13}\text{C}$ resulted to be negatively and significantly ($p \leq 0.001$) correlated with C_i/C_a ($r = -1.00$, -0.99 and -0.99 in 6–5, 7–7 and G \times N, respectively) (Fig. 7a), and with g_s ($r = -0.91$, -0.97 and $r = -0.99$ in 6–5, 7–7 and G \times N, respectively) (Fig. 7b). Moreover, significant correlations have also been found between $\delta^{13}\text{C}$ and SWP_{11:00} ($r = -1.00$, -0.93 and -0.98 in 6–5, 7–7 and G \times N, respectively), and between $\delta^{13}\text{C}$ and WUE ($r = 0.96$, 0.99 and 0.98 in 6–5, 7–7 and G \times N, respectively). All these correlations confirm what has been observed by other authors in peach (Arndt et al., 2000), and in other tree species (Gaudillère et al., 2002; DaMatta et al., 2003; Bielsa et al., 2018; Maier et al., 2019; Castillo-Argaez et al., 2020; Diefendorf et al., 2021). Grouping the three hybrids together and comparing all the values of $\delta^{13}\text{C}$ with those of C_i/C_a (Fig. 7a), g_s (Fig. 7b), and SWP_{11:00} (Fig. 8a), robust linear correlations were found, confirming what observed on the

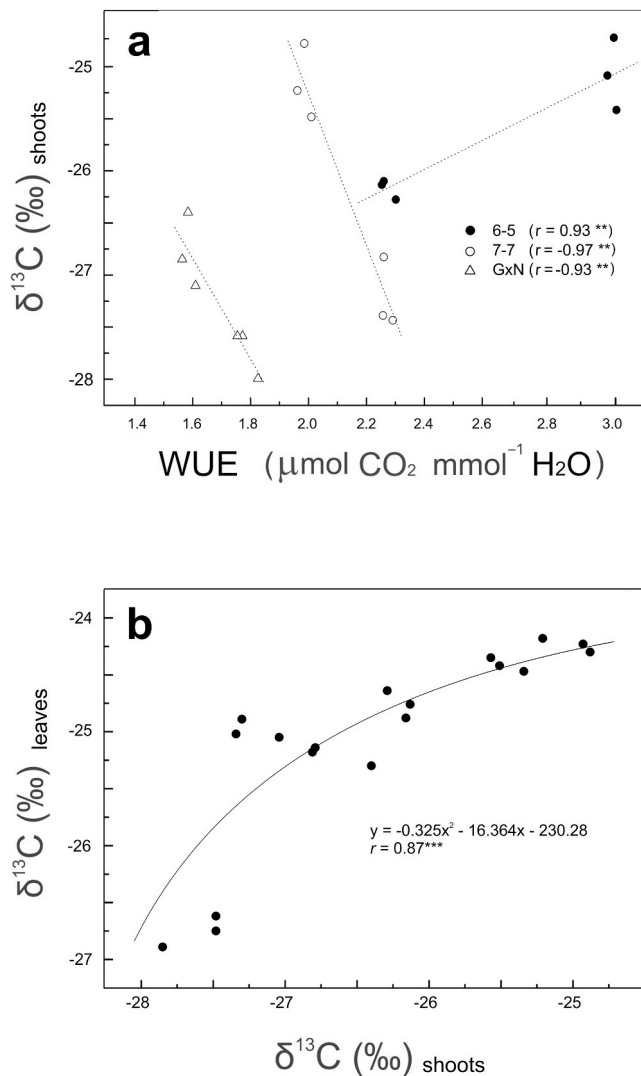


Fig. 9. (a) Correlation between instantaneous water use efficiency ($WUE = P_N/E$) and carbon isotope composition measured in the dry matter of new-growth shoots ($\delta^{13}C_{shoots}$) ($n = 9$; $** = p < 0.01$). (b) Correlation between carbon isotope composition measured in the dry matter of new-growth shoots ($\delta^{13}C_{shoots}$) and in leaf soluble carbohydrates ($\delta^{13}C_{leaves}$) ($n = 9$; $*** = p < 0.001$).

single individual hybrids (Figs. 7 and 8). By the contrary, the correlation between $\delta^{13}C$ and WUE was non-significant at $p \leq 0.001$ (Fig. 8b). The hybrid 6-5 showed the highest WUE values compared to the other two hybrids and reached higher $\delta^{13}C_{leaves}$, while no significant differences were found between the hybrids 7-7 and $G \times N$ at the same stress level (Fig. 8b).

The isotope composition of the dry matter of new-growth shoots ($\delta^{13}C_{shoots}$), sampled at the end of the rewatering period, was linearly correlated with the average WUE measured during the whole experiment (Fig. 9a). This result confirms that a relevant contribution to the formation of new shoots derives from carbohydrates produced during the period of 84 days of drought/rewatering. Noteworthy, in the hybrid 6-5, $\delta^{13}C_{shoots}$ was positively correlated to WUE, unlike what was found in the other two hybrids (Fig. 9a). The opposite relationship between $\delta^{13}C_{shoots}$ and WUE in the three hybrids confirms both the strong genetic control on physiological responses and, ultimately, the application difficulties of using carbon isotopes as a screening tool among hybrids. The relationship between $\delta^{13}C_{shoots}$ and the integrated value of WUE for the whole experiment exalts the genetic differences between the three hybrids. The hybrid 6-5 was the only one that, with the drought-dependent

increase in WUE simultaneously increased $\delta^{13}C_{shoots}$ (Fig. 8b).

The isotope composition of leaf soluble carbohydrates ($\delta^{13}C_{leaves}$) measured during the drought period was closely related ($r = 0.87^{***}$) to $\delta^{13}C_{shoots}$, according to a polynomial equation (Fig. 9b). These parameters are also related according to a linear regression, with $r = 0.83$ (data not shown). This result suggests the possibility of using the $\delta^{13}C_{shoots}$ in *Prunus* species as an integrated indicator of physiological responses under prolonged stress conditions. This provides greater application advantages, higher cost-effectiveness of the analyses, and reliability of the results, since the latter are indicators of plant physiological activity in a broad period, and therefore less influenced by daily environmental and physiological variations. The close relationship between $\delta^{13}C_{leaves}$ and $\delta^{13}C_{shoots}$ (Fig. 9b), also shows that the fraction of carbon translocated to the vegetative apices from other parts of the plant is negligible. Therefore, the isotopic signature of a sprout that has grown during or right after water stress can effectively reflect its physiological history (Brugnoli et al., 1998; 1998).

The observed increase in $\delta^{13}C$ was mainly related to stomatal control, which represented the main factor able to avoid water losses, according to what was observed in peach by Arndt et al. (2000). However, reductions in stomatal conductance at the same time limited the availability of CO_2 in the substomatal cavity (C_i) and therefore to the sites of carboxylation. As g_s decreases, the availability of CO_2 in the substomatal cavity and the discrimination against ^{13}C was reduced. On the contrary, decreases in photosynthetic capacity correspond to increases in C_i/C_a and therefore to decreases of $\delta^{13}C$ (and increased discrimination) (O'Leary, 1981). A higher g_s in hybrid 6-5 at SWC₁₃ could explain its significantly higher photosynthetic activity (P_N), and at the same time, the lower enrichment in ^{13}C compared to the hybrid 7-7, despite its significantly lower C_i/C_a ratio (Fig. 7a). Possible differences in mesophyll conductance might also be invoked to explain the observed results.

In each hybrid, the $\delta^{13}C$ of leaf soluble sugars resulted closely and negatively correlated to SWP (Fig. 8a), confirming what observed by Arndt and Wanek (2002) in *Prunus persica* and Bielsa et al. (2018) in other *Prunus* genotypes. However, the correlation between WUE and $\delta^{13}C$ of leaf soluble sugars, considering all hybrids together, was not significant, suggesting that at moderate levels of stress (SWC₁₃) the genetic differences prevail over the mechanisms that determine an increase in $\delta^{13}C$ during the onset of drought stress. Finally, as stated above, the observed differences in $\delta^{13}C$ among the three hybrids could also be related to the conductance of the mesophyll (g_m), which is the ability to move CO_2 from the substomatal cavity to the carboxylation sites, and is related to processes occurring partly in the gaseous phase and partly in the liquid phase. The flux of CO_2 in the intercellular spaces is strictly and linearly related to the assimilation rates and therefore to the extent of C discrimination in different herbaceous and tree species (Scartazza et al., 1998; Brugnoli et al., 1998; Le Roux et al., 1996; Bchir et al., 2016).

4. Conclusions

The isotope composition of leaf soluble sugars in the studied interspecific hybrids of the *Prunus* genus subjected to progressive drought stress was closely and positively related to the WUE. From the results obtained, the application of stable carbon isotopes in selection programs of interspecific *Prunus* hybrids cannot be applied under moderate levels of drought because of the species-specificity of the responses, although it may represent a valid selection tool at high levels of water deficit.

Indeed, a selection made at the intermediate level of stress (SWC₁₃) solely based on the isotope composition of soluble sugars would have chosen the hybrid 7-7 as the most efficient under drought conditions and would have hidden the highest WUE of the hybrid 6-5. At intermediate levels of water deficit, the species-specificity response can flatten or hide the genetic differences in maximizing WUE, leading away from the aim of selection. At the extreme level of drought (SWC₇₀), the highest values of WUE corresponded to higher values of $\delta^{13}C$ of soluble sugars, as observed in the responses of the single hybrids. Thus, the $\delta^{13}C$

discrimination can be used to reveal drought tolerance or high WUE at high levels of water deficit.

The close relationship between $\delta^{13}\text{C}$ and the physiological parameters showing the water status of the plant suggests the possibility of using the isotopic signature as a screening tool among clones/hybrids/genotypes to identify genotypes tolerant to drought stress. Furthermore, the relationship observed between $\delta^{13}\text{C}_{\text{shoots}}$ and $\delta^{13}\text{C}_{\text{leaves}}$ indicates a significant advantage for the application of the isotopic technique, as it allows the use of plant material which reflects its physiological history. The analysis of newly developed shoot is much easier, cheaper and less time consuming compared to the analysis of purified soluble carbohydrates. This facilitates the study of the response of each genotype on an intermediate time scale, with a high reduction of experimentation costs.

Author contributions

ANM and ACT designed and carried out the research; EB carried out the isotope analyses; AS and BD analyzed the data; BD obtained funds for the research; AS and ACT wrote the draft manuscript; AS wrote the final manuscript. All authors read and approved the manuscript.

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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