ECOPHYSIOLOGY, STRESS AND ADAPTATION

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# Intracultivar genetic diversity in grapevine: Water use efficiency variability within cv. Grenache

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## Abstract

The selection of genotypes best adapted to environmental conditions has traditionally focused on agronomic and grape composition parameters. However, to classify the genotypes most adapted to climate change conditions, the aim must be to focus on the ecophysiological responses that will ultimately determine their performance. The variability in water use efficiency of 13 Grenache genotypes over three-seasons was assessed under field conditions at leaf, grape and plant level. Results showed a significant effect of genotype at all three levels, and despite the large interannual variability there was a remarkable consistency among levels. Furthermore, using genotype-specific regressions it was possible to identify significant differences in the intrinsic water use efficiency response of each genotype as a function of the vine water status. The relationship between net photosynthesis and stomatal conductance, as well as carbon isotope discrimination in grapes, were also confirmed as reliable physiological indicators for selecting grapevine genotypes to future environmental conditions. Therefore, the proposed multi-level methodology was useful to quantify the intracultivar variability and the identification of more and less efficient genotypes within Grenache.

## 1 | INTRODUCTION

Viticulture sustainability under arid and semi-arid environments faces water scarcity as its main constraint, and climate change is exacerbating this challenge (Chaves et al. 2007; Medrano et al. 2015a). In this context, the ways to improve the water use efficiency are now a major subject of research, seeking to introduce new agronomic practices to reduce water consumption, but also looking for cultivars with enhanced water use efficiency (WUE; Medrano et al. 2015a; Lavoie-Lamoureux et al. 2017; van Leeuwen et al. 2019). A common way to estimate the WUE is to measure it at the leaf level (Medrano et al. 2002; Flexas et al. 2010) because, from a physiological perspective, the WUE is defined as the quotient between the instantaneous

$CO_2$ assimilation ($A_N$) per unit of transpired $H_2O$, or reducing environmental influences, the quotient among $A_N$ and stomatal conductance ($A_N/g_s$) (intrinsic WUE; $WUE_i$). Carbon isotope discrimination ($\delta^{13}C$) at berry level acts as a surrogate character of $WUE_i$ and has been widely reported as representative of the $WUE_i$ integrated over the time when the organ analysed was built (Santesteban et al. 2015; Bchir et al. 2016). At the agronomic scale, crop WUE ($WUE_c$) is referred as the ratio of biomass to the evapotranspired water, or more specifically considering only the harvested mass portion, that is yield (Flexas et al. 2010; Tomás et al. 2012; Buesa et al. 2017).

Agronomic adaptation to higher water shortage includes modifying viticultural practices (e.g. irrigation, canopy management, training systems, soil management, etc.) and selecting the most resilient

genotypes (e.g. rootstocks, cultivars and clones; van Leeuwen et al. 2019). Some authors have suggested the use of biotechnology and genetic engineering for breeding better adapted cultivars to climate change conditions (Iba 2002; Flexas et al. 2010; Gago et al. 2014). However, developing drought-tolerant grapevine cultivars is complex, and will likely require the integration of multiple traits and physiological processes, something that is currently poorly understood, expensive and slow to address the challenges posed by climate change (Cramer 2010; Gago et al. 2014; Gambetta 2016). Traditional selection and breeding of crop cultivars have focused on productivity and quality traits; however, the response of these traits to climate change will depend on plant ecophysiology, which drives the efficiency of resource use and abiotic stress tolerance (Bartlett & Sinclair 2021). Therefore, in order to select cultivars better adapted to future environmental conditions, the first step is to understand the physiological responses under different conditions of the currently available genetic pool. In this regard, both stomatal regulation and photosynthetic capacity are key in grapevine responses to water stress (Bota et al. 2001; Pou et al. 2008; Hochberg et al. 2013; Martorell et al. 2015). Moreover, differences in respiration among cultivars should be taken into account, as it could have an important effect on the carbon balance in grapevines (Escalona et al. 2012; Hernández-Montes et al. 2018).

In grapevine, there is consistent evidence of variability in WUE depending on the genetic origin (Pou et al. 2012; Coupel-Ledru et al. 2014), both intervariety and interclone (Bota et al. 2001, 2016; Tombesi et al. 2014; Martorell et al. 2015; Medrano et al. 2015a; Tortosa et al. 2016, 2019a, 2020). Recently, Tortosa et al. (2019a) reported that the variance in $WUE_i$ within the Tempranillo cultivar reached up to approximately 80% of the variance found among cultivars. Moreover, exploring grapevine intracultivar variability is particularly interesting because it respects the cultural and social components of winegrape cultivation (Vivin et al. 2017; Tortosa et al. 2020).

Grapevine cultivars have been classified in terms of the degree of stomatal control under water limiting conditions (Chaves et al. 1987; Schultz 2003; Bota et al. 2016). For instance, Tempranillo cultivar has been characterised by a poor stomatal control over evaporative demand or soil moisture (Medrano et al. 2003; Tomás et al. 2012; Martorell et al. 2015). Conversely, cv. Grenache has a reputation for being more adapted to water stress and for preventing fluctuations in vine water status and/or changes in the vapour pressure deficit by a tighter stomatal regulation (Soar et al. 2006; Santesteban et al. 2009; Lavoie-Lamoureux et al. 2017). This might confer a more conservative behaviour of the efficiency in water use under variable environmental conditions to cv. Grenache than to cv. Tempranillo. Indeed, genotype sensitivity to environmental conditions is a very important trait to consider for an efficient clonal selection (Tortosa et al. 2019b), as this will determine whether a genotype can be selected for cultivation in a wide range of environments, or whether it is only adapted to very specific conditions (Gonçalves et al. 2020).

Understanding drought responses among cv. Grenache genotypes is paramount for making vineyards more sustainable all over the world. In this regard, the present study aims (1) to quantify the variability in WUE among Grenache genotypes and over several seasons under a wide range of vine water status at different levels and approaches; (2) to classify genotypes according to their $WUE_i$ at leaf basis, $WUE_c$ at plant level and by surrogate parameters such as $\delta^{13}C$.

## 2 | MATERIALS AND METHODS

### 2.1 | Site description and plant material

The experiment was carried out during three consecutive seasons (2018–2020) in an experimental vineyard located in Miranda de Arga, Navarra, Spain (42°27′50.6''N 1°48′10.6''W, elevation 308 m). The vineyard was planted in 2011 with *Vitis vinifera* (L.) cv. Grenache of different commercial clones 141-W and 143-W (white) and 136, 435, ARA-2, ARA-4, ARA-24, EV-11, EV-13, EV-14, EV-15, RJ-21 and VN-Qualitas (red) grafted onto 110-Richter rootstock at a spacing of $3 \times 1$ m. Vines were pruned to a 10-bud count per vine on a unilateral cordon de Royat and trained to vertical trellis system oriented north–south direction (Figure S1A). The canopy management included manual shoot thinning before bloom and no shoot trimming. Budburst for Grenache in this area usually occurs at the beginning of April, bloom by June, veraison is reached by mid-August with harvest at the beginning of October.

The soil at the site was a Quaternary sedimentary soil with a sandy loam texture, and 8% of active lime, with the presence of pebbles, highly calcareous and of medium fertility (2% organic matter). In the interrow, spontaneous cover crop was maintained and mowed, whereas the crop-line was kept free of vegetation using herbicide. The climate of the area was classified as Continental-Mediterranean, with an average rainfall about 350–400 mm year$^{-1}$ from January to September were 384.5, 242.7 and 363.0 mm in 2018, 2019 and 2020, respectively, and the $ET_o$ for the same period was 982.7, 1128.1 and 931.4 mm for 2018, 2019 and 2020, respectively.

The vineyard was drip irrigated since its establishment through 4 L h$^{-1}$ pressure compensated emitters placed 0.5 m along a single drip line hanging under the vines (Figure S1A). Irrigation was applied from July to September and accounted for approximately 36 mm in 2018 and 48 mm in 2019 and 2020.

### 2.2 | Experimental design

Within the vineyard, 30 plants of each genotype were selected for the experimental determinations. Each genotype was located in a single row, with a row of the same genotype on either side, which were considered as buffers. Each vine was considered as a biological replicate. In 2018 and 2019, plants were maintained under irrigation. In 2020, two water regimes were imposed, 15 vines per genotype were watered as in 2018 and 2019 and the other 15 were not watered during the whole season aiming to ensure comparison among genotypes under different water status. The vines bordering between water regimes were considered as buffers. At every time of measurement, at

least six replicates per genotype and treatment were used to perform all determinations. To balance the effect of the time of day on genotype responses, the determinations were made in two measurement cycles. In addition, the order of measurement was varied on the different dates.

## 2.3 | Water relations

Stem water potential ($\Psi_{stem}$) was determined using two pressure chambers (Model 600, PMS Instruments Company) on bag-covered leaves from each replicate at mid-morning (9:30 and 11:00 solar time). Sun-exposed leaves were bagged using zip-bags covered with aluminium foil for at least 1 h prior to measurement (Santesteban et al. 2011, 2019). Determinations of $\Psi_{stem}$ were performed on day of the year 254 in 2018 ($n \geq 6$); 201 and 241 in 2019 ($n \geq 6$); and in 205 and 239 in 2020 ($n \geq 12$).

## 2.4 | Leaf gas exchange

Stomatal conductance ($g_s$) and net photosynthesis ($A_N$) were measured in fully exposed mature leaves in each replicate ($n \geq 6$) using an infrared open gas exchange analyser system (Li-6400xt, Li-cor Inc.). The $CO_2$ concentration inside the chamber was 400 $\mu$mol $CO_2$ mol$^{-1}$ air, and an air flow of 500 $\mu$mol (air) min$^{-1}$. The chamber used has an area of 6 cm$^2$ exposed to environmental light radiation (Figure S1B), with PAR always above 1500 $\mu$mol m$^{-2}$ s$^{-1}$. All measurements were performed between 8:30 and 13:00 solar time in the same dates as $\Psi_{stem}$. For some dates, however, more plants were measured than those on which the $\Psi_{stem}$ was determined. Intrinsic water use efficiency (WUE$_i$) was calculated as the $A_N$ to $g_s$ ratio.

## 2.5 | Yield components and vegetative growth

Yield was determined in 2019 and 2020 by weighing all the bunches produced in all the experimental vines of each genotype. When harvesting, bunch number per vine was counted, and average bunch mass was calculated as the quotient between yield and number of bunches.

Crop water use efficiency (WUE$_c$) was estimated as the ratio between yield and the amount of total amount of water received by rainfall and irrigation during the growing season (from 1st April to 30th September).

## 2.6 | Carbon isotope ratios

Carbon isotope ratio ($\delta^{13}$C) was determined from samples of 50 berries per genotype randomly collected at harvest. Furthermore, in 2020, berry sampling was carried out separately for irrigated and rainfed vines. Determinations of $\delta^{13}$C were carried out using an Elemental analyser (NC2500, Carlo Erba Reagents) coupled to an Isotopic Mass Spectrometer (Thermoquest Delta Plus, ThermoFinnigan). Carbon isotope ratio was expressed as $\delta^{13}$C = $[(R_s - R_b)/Rb] \times 1000$, where $R_s$ is the ratio $^{13}$C/$^{12}$C of the sample and $R_b$ is the $^{13}$C/$^{12}$C of the PDB (Pee Dee Belemnite) standard (0.0112372). $\delta^{13}$C in 2018 was analysed in 2 mg aliquots of berry powder samples, oven-dried previously (Thermo Flash EA 1112 Series), whereas in 2019 and 2020, determinations were directly performed in centrifuged must.

## 2.7 | Statistical analysis

First, data were checked for normality and when data set were not normal ($P < 0.05$), that is the 3-years WUE$_i$ data, a logarithmic transformation was used. Second, a two-way analysis of variance (ANOVA; $P < 0.05$) was used to evaluate the effects of the factors and their interactions on all the variables measured and calculated. Third, using $g_s$ as reference parameter, the WUE$_i$–$g_s$ relationship was used to assess differences between genotypes by analysing its residuals in respect to the general regression curve following Tortosa et al. (2016). Fourth, the WUE$_i$–$g_s$ regressions obtained specifically for each genotype across years were compared based on differences in their slopes by a one-way analysis of covariance (ANCOVA; $p < 0.05$). Fifth, these specific regressions were used to predict the sensibility of each genotype WUE$_i$ at a fixed range of water stress ($g_s$ between 0.025 and 0.3 mol $H_2O$ m$^{-2}$ s$^{-1}$) following Tortosa et al. (2020). The predicted WUE$_i$ within each range of $g_s$ was the average of that predicted for those threshold limit values. Finally, the ranking of genotypes was presented on an annual and three-year average basis, including an overall mean ranking by averaging the rankings for each level. The ANOVA, ANCOVA and post-hoc tests (Duncan) were performed with the Statgraphics Centurion XVI package (version 16.0.07) (Statgraphics Technologies), and regressions were obtained using SigmaPlot (version 11.0) (Systat Software).

## 3 | RESULTS

### 3.1 | Plant water status

The results presented correspond to two standard years, 2018 and 2020, and to a dryer and warmer one, 2019. Consequently, there was a significant effect of the year on vine water status, as assessed by both leaf stomatal conductance ($g_s$) and stem water potential ($\Psi_{stem}$). The $\Psi_{stem}$ and $g_s$ data are presented in Table 1 seasonally in order to capture the significant effect of the season, although there was no genotype $\times$ season interaction in these parameters. Both $\Psi_{stem}$ and $g_s$ showed a wide range of values across years in all cv. Grenache genotypes. Significant differences in $\Psi_{stem}$ among genotypes and between years were observed ($P < 0.001$), without showing an interactive effect between both factors ($P = 0.82$). The white genotypes (141-W and 143-W) showed significantly less negative $\Psi_{stem}$ values than the red ones. Among the latter, the VN displayed significantly less

**TABLE 1** Seasonal stem water potential ($\Psi_{stem}$), leaf stomatal conductance ($g_s$) and net photosynthesis ($A_N$) for each of the 13 Grenache genotypes in Miranda de Arga, Navarra, Spain

| Genotype | $\Psi_{stem}$ (MPa) | | | $g_s$ (mol $CO_2$ m$^{-2}$ s$^{-1}$) | | | $A_N$ (μmol $CO_2$ m$^{-2}$ s$^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 2018 | 2019 | 2020 | 2018 | 2019 | 2020 | 2018 | 2019 | 2020 |
| 136 | −0.70 ± 0.03 def | −1.17 ± 0.03 abcd | −0.92 ± 0.04 bcd | 0.190 ± 0.018 a | 0.111 ± 0.014 ab | 0.148 ± 0.014 a | 16.7 ± 0.8 bcd | 9.8 ± 0.9 ab | 12.3 ± 0.9 abc |
| 141-W | −0.65 ± 0.03 ef | −0.72 ± 0.02 g | −0.79 ± 0.04 de | 0.205 ± 0.016 ab | 0.231 ± 0.014 c | 0.199 ± 0.014 b | 14.8 ± 0.8 ab | 15.7 ± 0.9 c | 14.7 ± 0.9 c |
| 143-W | −0.47 ± 0.03 g | −0.92 ± 0.03 f | −0.76 ± 0.05 e | 0.208 ± 0.014 ab | 0.144 ± 0.015 b | 0.199 ± 0.015 b | 13.3 ± 0.6 a | 10.9 ± 0.9 b | 14.1 ± 0.9 bc |
| 435 | −0.79 ± 0.03 bc | −1.14 ± 0.03 bcd | −1.04 ± 0.05 ab | 0.212 ± 0.018 abc | 0.102 ± 0.014 ab | 0.150 ± 0.016 a | 17.6 ± 0.8 cd | 9.9 ± 0.9 ab | 12.1 ± 0.9 abc |
| ARA-2 | −0.77 ± 0.03 cd | −1.18 ± 0.03 abc | −1.04 ± 0.05 ab | 0.204 ± 0.018 ab | 0.099 ± 0.014 a | 0.158 ± 0.015 ab | 16.1 ± 0.8 bcd | 9.3 ± 0.9 ab | 11.9 ± 0.9 abc |
| ARA-24 | −0.86 ± 0.03 ab | −1.23 ± 0.02 a | −1.09 ± 0.05 a | 0.285 ± 0.018 d | 0.108 ± 0.013 ab | 0.146 ± 0.015 a | 16.7 ± 0.8 bcd | 9.6 ± 0.9 ab | 10.7 ± 0.9 a |
| ARA-4 | −0.79 ± 0.03 bc | −1.11 ± 0.03 cde | −1.04 ± 0.05 ab | 0.212 ± 0.020 abc | 0.104 ± 0.014 ab | 0.169 ± 0.015 ab | 17.0 ± 0.8 bcd | 10.1 ± 0.9 ab | 11.7 ± 0.9 ab |
| EV-11 | −0.76 ± 0.03 cd | −1.06 ± 0.02 e | −1.05 ± 0.05 ab | 0.232 ± 0.015 abcd | 0.103 ± 0.014 ab | 0.154 ± 0.015 ab | 15.2 ± 0.6 abc | 9.7 ± 0.9 ab | 12.0 ± 0.9 abc |
| EV-13 | −0.81 ± 0.03 bc | −1.23 ± 0.03 a | −1.09 ± 0.05 a | 0.266 ± 0.017 cd | 0.079 ± 0.014 a | 0.138 ± 0.015 a | 16.4 ± 0.7 bcd | 8.3 ± 0.9 ab | 11.2 ± 0.9 ab |
| EV-14 | −0.89 ± 0.03 a | −1.09 ± 0.03 de | −1.07 ± 0.05 a | 0.243 ± 0.017 abcd | 0.084 ± 0.015 a | 0.137 ± 0.016 a | 15.3 ± 0.7 abc | 9.1 ± 0.9 ab | 11.0 ± 0.9 a |
| EV-15 | −0.72 ± 0.02 cde | −1.18 ± 0.03 abc | −0.98 ± 0.05 abc | 0.258 ± 0.017 bcd | 0.081 ± 0.015 a | 0.150 ± 0.015 a | 16.3 ± 0.7 bcd | 7.6 ± 0.9 a | 12.2 ± 0.9 abc |
| RJ-21 | −0.63 ± 0.03 f | −1.22 ± 0.03 ab | −0.99 ± 0.05 abc | 0.277 ± 0.013 d | 0.108 ± 0.014 ab | 0.166 ± 0.016 ab | 17.1 ± 0.8 bcd | 9.1 ± 1.0 a | 12.3 ± 1.0 abc |
| VN | −0.68 ± 0.03 ef | −1.09 ± 0.03 de | −0.85 ± 0.05 cde | 0.232 ± 0.018 abcd | 0.088 ± 0.014 a | 0.173 ± 0.015 ab | 17.8 ± 0.8 d | 9.4 ± 0.9 ab | 13.1 ± 0.9 abc |
| Range | −0.89 to −0.47 | −1.23 to −0.72 | −1.09 to 0.76 | 0.285 to 0.190 | 0.231 to 0.079 | 0.199 to 0.137 | 17.8 to 13.3 | 15.7 to 7.6 | 14.7 to 10.7 |
| Average | −0.73 | −1.10 | −0.98 | 0.233 | 0.111 | 0.160 | 16.2 | 9.9 | 12.2 |

**TABLE 2** Grapevine intrinsic water use efficiency (WUE$_i$) for each of the 13 Grenache genotypes measured in each date of the 3 years of experiment (2018–2020) in Miranda de Arga, Navarra, Spain

| | Year | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **2018** | | **2019** | | | | **2020** | | |
| | **August** | | **July** | | **August** | | **July** | | **August** | |
| **Genotype** | | | | | | | | | | |
| 136 | 88.6 ± 3.3 | e | 99.4 ± 4.2 | bcd | 87.4 ± 5.6 | b | 83.9 ± 3.0 | bcd | 103.0 ± 6.2 | bc |
| 141-W | 73.5 ± 3.3 | bc | 70.8 ± 4.9 | a | 68.8 ± 5.6 | a | 73.9 ± 3.0 | ab | 81.9 ± 6.2 | ab |
| 143-W | 65.7 ± 2.6 | ab | 72.9 ± 6.9 | a | 101.9 ± 3.6 | bc | 68.0 ± 3.3 | a | 81.7 ± 6.0 | ab |
| 435 | 83.7 ± 3.2 | de | 95.9 ± 4.9 | bcd | 104.1 ± 5.4 | bc | 85.0 ± 3.6 | bcd | 87.8 ± 7.2 | abc |
| ARA-2 | 79.8 ± 3.3 | cde | 89.7 ± 4.9 | bc | 111.6 ± 5.0 | cde | 80.1 ± 3.4 | bcd | 94.7 ± 6.7 | abc |
| ARA-24 | 59.2 ± 3.3 | a | 100.8 ± 4.0 | cd | 109.8 ± 5.6 | cd | 80.9 ± 3.3 | bcd | 88.5 ± 6.9 | abc |
| ARA-4 | 81.6 ± 3.7 | cde | 104.2 ± 5.3 | cd | 113.8 ± 5.6 | cdef | 75.1 ± 3.4 | abc | 91.0 ± 6.4 | abc |
| EV-11 | 66.5 ± 2.8 | ab | 104.9 ± 4.2 | cd | 116.4 ± 5.1 | cdef | 86.2 ± 3.3 | cd | 95.6 ± 6.2 | abc |
| EV-13 | 63.6 ± 3.0 | ab | 90.0 ± 4.9 | bc | 128.5 ± 6.3 | ef | 88.2 ± 3.4 | d | 104.7 ± 6.0 | c |
| EV-14 | 63.5 ± 2.9 | ab | 84.5 ± 4.0 | ab | 131.2 ± 5.6 | f | 89.8 ± 3.6 | d | 96.3 ± 6.4 | abc |
| EV-15 | 64.0 ± 3.0 | ab | 92.6 ± 4.9 | bc | 123.0 ± 7.2 | def | 83.2 ± 3.4 | bcd | 90.8 ± 6.1 | abc |
| RJ-21 | 61.7 ± 3.3 | a | 104.8 ± 4.5 | cd | 107.0 ± 7.0 | cd | 80.7 ± 4.0 | bcd | 75.0 ± 7.6 | a |
| VN | 77.0 ± 3.1 | cd | 109.5 ± 5.3 | d | 124.5 ± 5.1 | def | 84.3 ± 3.4 | bcd | 78.7 ± 6.1 | a |
| Range | 59.2–88.6 | | 70.8–109.5 | | 68.8–131.2 | | 68.0–89.8 | | 75.0–104.7 | |
| Average | 71.4 | | 93.8 | | 109.8 | | 81.5 | | 90.0 | |

*Note*: Within each row, mean values and standard errors followed by a different letter are significantly different at $p < 0.05$ (Duncan test).

negative $\Psi_{stem}$ values than ARA-24 and EV-13. On the other hand, the effect of genotype on $g_s$ was not significant, but the white clones (141-W and 143-W) did show a trend towards higher $g_s$ values than the red ones.

## 3.2 | Photosynthesis and WUE$_i$

Net photosynthesis rates ($A_N$) were significantly affected by year but not by genotype or by genotype × season interaction. Seasonal average $A_N$ values ranged from 16.2 to 9.9 µmol $CO_2$ m$^{-2}$ s$^{-1}$ in 2018 and 2019, respectively (Table 1). The values of WUE$_{i,}$ defined as the ratio between $A_N$ and $g_s$, are shown in Table 2 on a monthly basis due to the significant interaction ($p < 0.001$) between genotype and date of measurement for this parameter. The different genotypes exhibited great WUE$_i$ variability across years, ranging from 59.2 to 131.2 µmol $CO_2$ mol$^{-1}$ $H_2O$ on average of the measurement date and clone. The significant differences in WUE$_i$ among the genotypes ($p < 0.0001$) differed according to the date of measurement ($P < 0.0001$). Thus, depending on the measurement date, the coefficient of variation among genotypes ranged between 8 and 16%. Nevertheless, from these field measures it was possible to identify some genotypes that, in most cases, showed a lower than average WUE$_i$ of the clones (141-W and 143-W), while others showed higher than average WUE$_i$ (EV-13 and EV-14).

Moreover, as part of the variability in clonal WUE$_i$ was induced by the variation in vine water status during measurements, each genotype was also evaluated in respect to the general dependence of
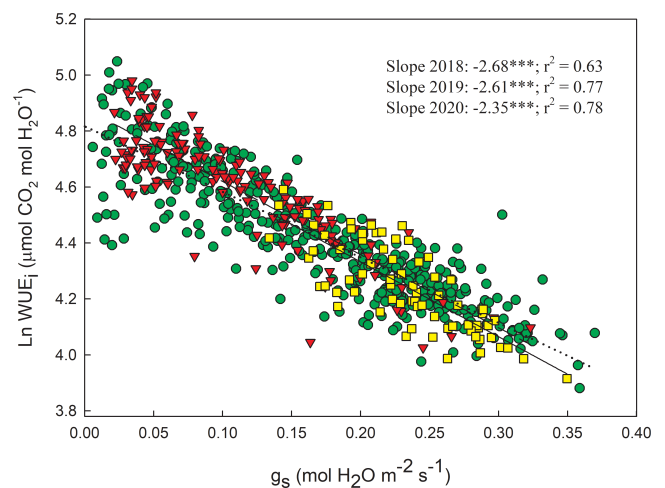


**FIGURE 1** Linearized relationship between intrinsic water use efficiency (WUE$_i$) and stomatal conductance ($g_s$) using the natural logarithm of the WUE$_i$ data measured at leaf basis on the 13 cv. Grenache genotypes in the three experimental seasons (■, 2018; ▼, 2019; ●, 2020) in Miranda de Arga, Navarra, Spain. Fitted regressions for each year are shown as well as the slope for each of them. Asterisks indicate significance of the regression ($P < 0.0001$)

WUE$_i$ over $g_s$. For this purpose, the regressions between Ln WUE$_i$–$g_s$ were calculated including the data of the 13 genotypes on an annual basis (Figure 1). By analysing the residuals of each clone in the general logarithmic WUE$_i$–$g_s$ regression for each year, statistical differences in the WUE$_i$ between genotypes per year could be established
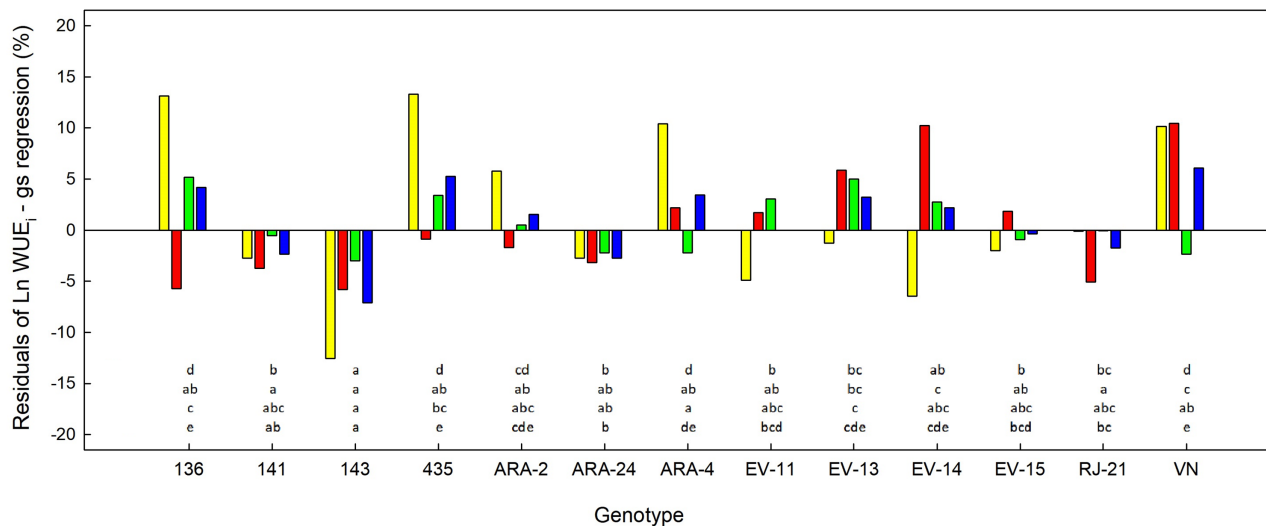
**FIGURE 2**  Average and seasonal deviation of water use efficiency over stomatal conductance (WUE$_i$–$g_s$) on the 13 cv. Grenache genotypes in respect to the common seasonal regression curve WUEi–$g_s$ expressed in terms of percentage. 2018 (yellow), 2019 (red), 2020 (green), and 3-year average (blue). Within each year, different letters mean significantly different at $P < 0.05$ (Duncan test)

**TABLE 3**  Linear regressions between the natural logarithm of intrinsic water use efficiency (WUE$_i$) and stomatal conductance ($g_s$) of all genotypes ("$a$" indicates the slope of the regression; "$r^2$" indicates the correlation coefficients; and "$P$-values" the significance of the regressions

| Genotype | Ln WUE$_i$ − $g_s$ | | | | |
|---|---|---|---|---|---|
| | y | a | | $r^2$ | p value |
| 136 | 4.830 | −2.194 | cd | 0.72 | <0.0001 |
| 141-W | 4.770 | −2.234 | ab | 0.64 | <0.0001 |
| 143-W | 4.751 | −2.277 | a | 0.73 | <0.0001 |
| 435 | 4.830 | −2.135 | d | 0.80 | <0.0001 |
| ARA-2 | 4.866 | −2.649 | bcd | 0.92 | <0.0001 |
| ARA-24 | 4.765 | −2.197 | ab | 0.77 | <0.0001 |
| ARA-4 | 4.814 | −2.357 | bcd | 0.80 | <0.0001 |
| EV-11 | 4.840 | −2.399 | bcd | 0.84 | <0.0001 |
| EV-13 | 4.921 | −2.785 | d | 0.93 | <0.0001 |
| EV-14 | 4.898 | −2.700 | cd | 0.85 | <0.0001 |
| EV-15 | 4.859 | −2.712 | abc | 0.76 | <0.0001 |
| RJ-21 | 4.843 | −2.538 | abcd | 0.89 | <0.0001 |
| VN | 4.847 | −2.420 | bcd | 0.71 | <0.0001 |

*Note*: Within each row, different letters mean significantly different at $P < 0.05$ (Duncan test).

($P < 0.05$). Thus, the relative WUE$_i$ variation for each clone was depicted annually (Figure 2). Across years, the total variability in WUE$_i$ found was ±13%. However, differences in WUE$_i$ between the more extremes genotypes was even bigger in 2018 and 2019. In all years, clone 143-W showed significantly the lower WUE$_i$, followed by ARA-24 and 141-W, respectively. Conversely, genotypes VN, 435 and 136 showed the highest WUE$_i$ in the three-year average (Figure 2).

In a second approach, the Ln WUE$_i$–$g_s$ regression was calculated for each genotype (Table 3) aiming to compare the predicted WUE$_i$ responses against fixed $g_s$. These logarithmic WUE$_i$–$g_s$ regressions were strongly significant for all genotypes ($p < 0.0001$), the correlation coefficient ranging from 0.64 to 0.93. Moreover, there were significant differences between the respective regressions of the genotypes. The slopes ranged from −2.14 in clone 435 to −2.78 in clone EV-13. Predicted WUE$_i$ values are shown for each genotype within the thresholds of severe, moderate and nonwater stress (Table 4). The total variability found under fixed $g_s$ conditions ranged from 15 to 10%, according to the water stress level. Overall, genotypes 143-W, 141-W and ARA-24 showed the lowest WUE$_i$ in the ranking, while EV-13, EV-14, 435 and 136 the highest. Nevertheless, not all genotypes with highest average in predicted WUE$_i$ performed equally in the whole range of $g_s$. For instance, the WUE$_i$ predicted for EV-13 and EV-14 under water stress conditions were the highest, but it was relatively low under nonwater stress. Conversely, genotypes 435 and 136, which were predicted as very high performers under mild and nonwater stress, showed intermediate WUE$_i$ values under severe stress (Table 4). In general, the predicted WUE$_i$ response of the relatively lowest efficient genotypes was more stable across all the $g_s$ range than that of the highly efficient ones.

## 3.3 | WUE$_c$ and yield components

Crop water use efficiency (WUE$_c$) was almost double in 2019 than in 2020 (Figure 3). Differences in WUE$_c$ among genotypes within each season were even greater, ranging from 1.4 and 1.2 kg m$^{-3}$ in RJ-21 to 4.4 and 1.6 kg m$^{-3}$ in 136, in 2019 and 2020 respectively. Across seasons, the coefficient of variation among genotypes was 28%.

**TABLE 4** The intrinsic water use efficiency (WUE$_i$, μmol $CO_2$ mol $H_2O^{-1}$) predicted for each genotype at different vine water status (stomatal conductance, $g_s$, mol $H_2O$ m$^{-2}$ s$^{-1}$)

| Genotype | Severe stress 0.025–0.05 | | Moderate stress 0.075–0.125 | | Nonstress 0.2–0.3 | | Average 0.025–0.3 | |
|---|---|---|---|---|---|---|---|---|
| | WUE$_i$ pred | Rank | WUE$_i$ pred | Rank | WUE$_i$ pred | Rank | WUE$_i$ pred | Rank |
| 136 | 115 | 9 | 101 | 4 | 73 | 2 | 96 | 4 |
| 141-W | 109 | 11 | 94 | 11 | 68 | 9 | 90 | 11 |
| 143-W | 106 | 13 | 92 | 13 | 66 | 13 | 88 | 13 |
| 435 | 116 | 6 | 101 | 3 | 74 | 1 | 97 | 3 |
| ARA-2 | 118 | 3 | 100 | 6 | 68 | 11 | 95 | 7 |
| ARA-24 | 108 | 12 | 94 | 12 | 68 | 8 | 90 | 12 |
| ARA-4 | 113 | 10 | 98 | 10 | 69 | 7 | 93 | 10 |
| EV-11 | 116 | 7 | 100 | 7 | 70 | 4 | 95 | 6 |
| EV-13 | 124 | 1 | 104 | 1 | 69 | 5 | 99 | 1 |
| EV-14 | 121 | 2 | 103 | 2 | 69 | 6 | 98 | 2 |
| EV-15 | 117 | 4 | 99 | 9 | 66 | 12 | 94 | 9 |
| RJ-21 | 115 | 8 | 99 | 8 | 68 | 10 | 94 | 8 |
| VN | 116 | 5 | 100 | 5 | 70 | 3 | 96 | 5 |
| Range | 106–124 | | 92–104 | | 66–74 | | 88–99 | |
| Average | 115 | | 99 | | 69 | | 94 | |

*Note*: The shaded values are simply the ranking of the genotypes for each of the parameters. Its significance is given in the tables in which the raw data are reported.

The significant differences found in WUE$_c$ (yield/rainfall + irrigation) among genotypes were neither significantly related to $\Psi_{stem}$ (data not shown), nor to WUE$_i$ (Figure 3). However, in both seasons, there was a tendency to yield less in genotypes with lower WUE$_i$ (*P*-value 0.15 and 0.06 in 2019 and 2020, respectively). In fact, the relationship between WUE$_i$ and average bunch mass for all the clones did prove to be statistically significant both years (data not shown; *P*-value 0.01 and 0.04 in 2019 and 2020, respectively).

### 3.4 | Carbon isotope discrimination

Differences among genotypes reached up to −3.0‰ within each season, whereas between seasons reached −2.1‰ on average (Table 5). In 2019, the driest year, both the range of variation among genotypes and the $\delta^{13}C$ absolute values were lower than in 2018 and 2020. Overall, the effect of genotype on $\delta^{13}C$ was fairly consistent across years. Genotypes with relatively high, low and intermediate values of $\delta^{13}C$ could be identified. For instance, 136, EV-11, EV-13 and EV-14 showed the most negative $\delta^{13}C$ average values, while 141-W, ARA-24 and VN showed the least negative.

The relationship between $\delta^{13}C$ in grapes and $\Psi_{stem}$ and WUE$_i$ across years were very strong ($r^2 = 0.85$ in both cases; Figure 4A,B). Less negative $\delta^{13}C$ corresponded to more negative $\Psi_{stem}$ and higher WUE$_i$. On the other hand, the WUE$_c$ was also significantly related to $\delta^{13}C$ (Figure 4C). However, this last relationship was clearly weaker ($r^2 = 0.18$) than the ones between $\Psi_{stem}$ and WUE$_i$ and with a slighter slope.
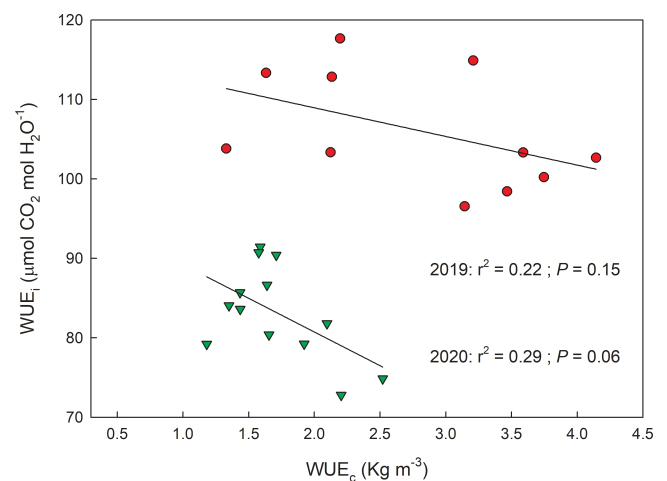


**FIGURE 3** Relationship between seasonal average intrinsic water use efficiency (WUE$_i$) and crop water use efficiency (WUE$_c$) on the 13 cv. Grenache genotypes in two experimental seasons (●, 2019; ▽, 2020) in Miranda de Arga, Navarra, Spain

### 3.5 | Ranking in water use efficiency of genotypes

All genotypes' rankings based on field data (WUE$_i$, WUE$_c$ and $\delta^{13}C$) showed a marked year influence (Table 6). The degree of year-to-year inconsistency was high according to all three levels (leaf, berry and plant level). Classifying genotypes based on WUE$_i$ showed higher interannual variability than WUE$_c$ and $\delta^{13}C$. According to WUE$_i$ and

| Genotype | 2018 | 2019 | 2020 | Average |
|---|---|---|---|---|
| 136 | −24.6 | −23.1 | −24.1 | −23.9 |
| 141-W | −27.0 | −23.1 | −26.6 | −25.6 |
| 143-W | | −22.7 | −26.1 | |
| 435 | | −22.7 | −24.3 | |
| ARA-2 | −24.8 | −23.4 | −25.0 | −24.4 |
| ARA-24 | −24.8 | −23.6 | −25.2 | −24.5 |
| ARA-4 | −24.8 | −23.5 | −24.6 | −24.3 |
| EV-11 | −24.0 | −22.4 | −24.6 | −23.7 |
| EV-13 | −24.2 | −22.5 | −24.5 | −23.7 |
| EV-14 | −24.2 | −22.3 | −24.4 | −23.6 |
| EV-15 | −25.8 | −22.8 | −24.7 | −24.4 |
| RJ-21 | −24.9 | −22.4 | −25.0 | −24.1 |
| VN | −25.2 | −23.1 | −25.2 | −24.5 |
| Range | −27.0 to −24.0 | −23.6 to −22.3 | −26.6 to −24.1 | −25.6 to −23.6 |
| Average | −24.9 | −22.9 | −25.0 | −24.3 |

**TABLE 5**   $\delta^{13}C$ measured in berries of 13 cv. Grenache genotypes in three experimental seasons in Miranda de Arga, Navarra, Spain

$\delta^{13}C$, the relative position of the genotypes was more similar between 2018 and 2020 than with 2019. Moreover, there was a better agreement between $WUE_i$ and $\delta^{13}C$ rankings than with $WUE_c$.

Nevertheless, within each level, some genotypes did stand out as being classified as more or less efficient (better or worse than the average of the genotypes in all three levels; Table 6). This is the case of genotypes 136 and 435 which were found to be the most efficient ones under all three determinations. In contrast, 141-W, 143-W, ARA-24, EV-15 and RJ-21 performed the least efficiently on a fairly consistent year-to-year basis.

# 4 | DISCUSSION

In the context of the increasing concern of genetic improvement for water use efficiency among grapevine's genotypes, the present work explores the existence of such genetic variability among a collection of cv. Grenache genotypes under realistic field conditions along three consecutive years, that is: under different climatic and soil water availability conditions. Across seasons, the range of the vine water status was very wide (Table 1), with values ranging from no water stress to severe stress (Flexas et al. 2002; Cifre et al. 2005; Romero et al. 2010). Under these conditions, the genetic variability within cv. Grenache resulted in significant differences in $A_N$ (Table 1) and also in $WUE_i$ (Table 2). In both parameters, the differences among genotypes were more pronounced in the driest year, 2019, compared to the two wetter years, 2018 and 2020. Differences in $WUE_i$ among genotypes were not fully consistent across seasons (G × Y = 0.0001). This fact can be explained by the dependency of $WUE_i$ on vine water status (i.e. $g_s$ and $\Psi_{stem}$), which was significantly different each season given the year-to-year variability in environmental conditions. Nonetheless, the particular correlations of logarithmic $WUE_i$–$g_s$ within a specific year were quite strong (>0.63; Figure 1). The dispersion of the

data within these seasonal relationships suggests differences in $WUE_i$ response among genotypes. Indeed, it was possible to statistically characterise each genotype by analysing its residuals in respect to the general dependency of $WUE_i$ over $g_s$. This relative comparison among genotypes was made annually since, as explained above, the relative differences were different between years. However, there was a certain annual consistency for most genotypes, so the three-year average $WUE_i$ was calculated as an indicator of the relative response of each genotype.

In a second approach, the physiological assessment of the genotypes was carried out independently for each genotype by predicting their response at fixed ranges of water stress. This method enables to remove the stomatal effect, and thus overlook differences in water status between vines, focusing on genotype photosynthetic capacity (Tortosa et al. 2019b). These genotype-specific linear regressions could be used because it fitted a wide range of $g_s$ values within each genotype and were robust, allowing the ANCOVA to show significant differences among genotypes (Table 3). In general, under conditions of severe water stress ($g_s$ < 0.075 mol $H_2O$ m$^{-2}$ s$^{-1}$), the predicted $WUE_i$ ranged between 100–120 µmol $CO_2$ mol $H_2O^{-1}$ (Table 4), which is in agreement with $WUE_i$ measurements reported by Bota et al. (2016) in this cultivar. Nevertheless, in our experiment, genotypes like EV-13 and EV-14 showed values of predicted $WUE_i$ over 120 µmol $CO_2$ mol $H_2O^{-1}$ that, according to these authors, would be characterised as very efficient under stress conditions. However, the classification of genotypes is not so straightforward, and those that are predicted to exhibit the greatest $WUE_i$ under severe water stress conditions do not have to respond in the same way under moderate or absence of water stress (Table 4). This was already reported by Tortosa et al. (2019a, 2020) in cv. Tempranillo, and it could be also expected in cv. Grenache, insofar as there were differences in the slopes of the Ln $WUE_i$–$g_s$ regressions between genotypes (Table 3). The greater the slope in absolute value, the more sensible the
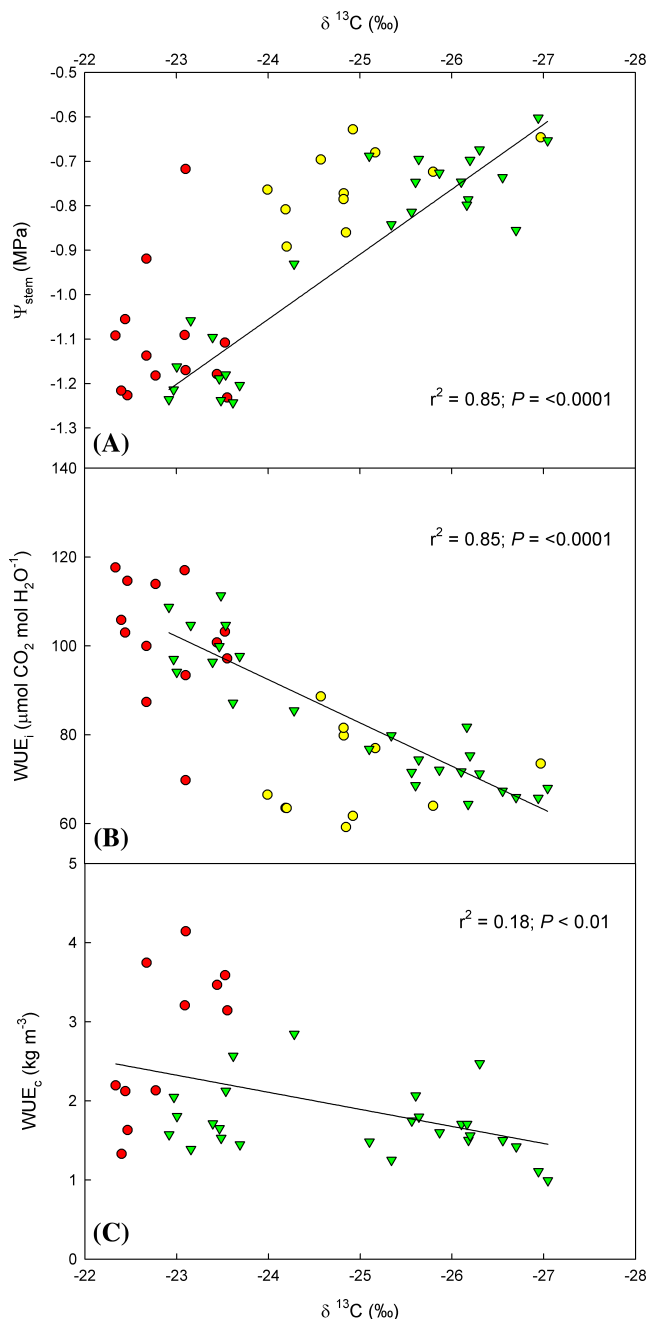
**FIGURE 4** Relationship between carbon isotope discrimination ($\delta^{13}$C) and (A) stem water potential ($\Psi_{stem}$), (B) intrinsic water use efficiency (WUE$_i$) and (C) crop water use efficiency (WUE$_c$) measured on cv. Grenache genotypes in the three experimental seasons (○, 2018; ●, 2019; ▽, 2020) in Miranda de Arga, Navarra, Spain

genotype to vine water status. This is the case of EV-13 and EV-14 genotypes, for which photosynthesis performance varied greatly with vine water status (Table 4), suggesting a tighter stomatal regulation.

Regarding the $\delta^{13}$C in berries of the different Grenache genotypes across seasons it should be noted that, as other authors pointed out, this was a very good surrogate indicator of both vine water status and WUE$_i$ (Figure 4A,B; Tomás et al., 2012, 2014; Santesteban et al. 2015; Bota et al. 2016). Furthermore, this confirms that the

measuring times of gas exchange were representative of the seasons, which is a function of the evolution of the soil water content within the plot. Overall, it was confirmed that these values were in the expected range for Grenache berries at harvest (Table 5; Gaudillère et al. 2002; Bchir et al. 2016). The interannual variability in $\delta^{13}$C can be attributed mainly to meteorological conditions, although there might also be a minor influence of the type of tissue analysed in each year (i.e. whole berries or just must). Even though the differences in $\delta^{13}$C between the analysis of whole berries and must are minimal (de Souza et al. 2005).

In grapevine, it is recognised that WUE$_c$ can be decoupled from WUE$_i$ (Tomás et al. 2012; Merli et al. 2015; Medrano et al. 2015b; Tortosa et al. 2020). This is the caseof ARA-24, for instance, which showed very low WUE$_i$ but yielded relatively high. In our experiment, the seasonal relationships between WUE$_i$ and WUE$_c$ were weak and nonsignificant (Figure 3). This might be attributed to the fact that genetic variability could also affect bud fertility (Guilpart et al. 2014). Nonetheless, mean bunch mass did was significantly related to WUE$_i$. This could be explained by the fact that yield also depends on the number of bunches per vine, which is determined in previous seasons (Levin et al. 2020). Nevertheless, even the relationships between WUE$_i$ and bunch mass were not strong. A possible explanation for this decoupling between produced biomass and the measured carbon exchange could be that improving WUE$_i$ by means of reducing $g_s$ may result in decreased net photosynthesis and yield and, sometimes, in decreased WUE$_c$ (Flexas et al. 2010). But also, because of possible differences between genotypes in carbon partitioning, or even in whole plant carbon balance due to differences in respiration (Escalona et al. 2012; Medrano et al. 2015b; Douthe et al. 2018; Dayer et al. 2020). In this sense, Tomás et al. (2014) suggested that the possible discrepancies between WUE determinations based on the single-leaf level could be due to differences in sunlight interception because of canopy structure. The latter is of particular importance in the case of grapevine for wine making, not only in terms of yield, but also in terms of grape composition (Palliotti et al. 2011; Buesa et al. 2020). In this regard, further efforts are required to study the effects of intracultivar genetic diversity on carbon balance and grape biochemical composition. To this end, the conclusions of the present study on the ecophysiological responses of cv. Grenache genotypes may be very useful.

The classification of genotypes made using the field data integrates different levels of WUE and also surrogated parameters as $\delta^{13}$C (Table 5). The coefficient of variation in WUE among genotypes across seasons was different according to the level of definition, being 13%, 28% and 5% for WUE$_i$ (leaf), WUE$_c$ (plant) and $\delta^{13}$C (grape), respectively. The interannual variability found in ranking genotypes illustrates the importance of addressing genotype selection at different levels and during several seasons. This is due to the fact that genetic variability is associated with plant ecophysiological response, and that variability between levels of WUE and between-year within each level arise due to the different environmental conditions. For instance, the low WUE$_i$ and surrogate $\delta^{13}$C in both white genotypes did not fully match the classification in terms of WUE$_c$, suggesting

**TABLE 6**   Ranking according to the measurements of intrinsic water use efficiency (WUE$_i$, μmol $CO_2$ mol $H_2O^{-1}$), crop water use efficiency (WUE$_i$, kg m$^{-3}$) and δ$^{13}$C (‰) for the different cv. Grenache genotypes grown in Miranda de Arga, Navarra, Spain

| Genotype | WUE$_i$ 2018 | 2019 | 2020 | Average | WUE$_c$ 2018 | 2019 | 2020 | Average | δ$^{13}$C 2018 | 2019 | 2020 | Average | Mean ranking |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 136 | 1 | 10 | 2 | 3 | | 1 | 10 | 1 | 4 | 9 | 1 | 5 | 2 |
| 141-W | 8 | 13 | 8 | 12 | | | 3 | 7 | 11 | 10 | 13 | 13 | 13 |
| 143-W | 13 | 12 | 13 | 13 | | | 4 | 8 | | 6 | 12 | 8 | 11 |
| 435 | 2 | 7 | 1 | 2 | | 2 | 7 | 3 | 5 | 2 | 1 | 1 | 1 |
| ARA-2 | 5 | 8 | 6 | 7 | | 4 | 8 | 4 | 6 | 11 | 9 | 9 | 6 |
| ARA-24 | 10 | 9 | 10 | 11 | | 6 | 5 | 6 | 7 | 13 | 10 | 12 | 9 |
| ARA-4 | 3 | 5 | 11 | 4 | | 3 | 12 | 2 | 5 | 12 | 6 | 7 | 3 |
| EV-11 | 11 | 6 | 4 | 8 | | 9 | 9 | 10 | 1 | 3 | 5 | 3 | 7 |
| EV-13 | 7 | 3 | 3 | 5 | | 10 | 11 | 12 | 2 | 4 | 4 | 4 | 8 |
| EV-14 | 12 | 2 | 5 | 6 | | 7 | 1 | 9 | 3 | 1 | 3 | 2 | 4 |
| EV-15 | 9 | 4 | 9 | 9 | | 8 | 2 | 11 | 10 | 7 | 7 | 10 | 12 |
| RJ-21 | 6 | 11 | 7 | 10 | | 11 | 13 | 13 | 8 | 2 | 8 | 6 | 10 |
| VN | 4 | 1 | 12 | 1 | | 5 | 6 | 5 | 9 | 8 | 11 | 11 | 5 |

*Note:* The shaded values are simply the ranking of the genotypes for each of the parameters. Its significance is given in the tables in which the raw data are reported.

that the genetic differences that originated whites Grenache from its red Grenache parent may have affected not only the loss of berry colour but also physiological characters (Ibáñez et al. 2003; Carbonell-Bejerano et al. 2019). The better agreement found between WUE$_i$ and δ$^{13}$C data than with WUE$_c$ (Figure 4) is expected as the first two are purely physiological parameters, while WUE$_c$ refers to the fraction of carbon that is translocated into yield (Tomás et al. 2012; Merli et al. 2015; Tortosa et al. 2020).

Regardless the mismatches among the three WUE levels and the interannual variability, it was possible to establish a mean ranking of genotypes by WUE with a reasonable degree of consistency (Table 5). This was possible because there were genotypes with relative high photosynthesis rates under nonwater stress, but also an efficient stomatal control under water stress, and others the opposite. This is the case of the genotypes 136 and 435 classified as highly efficient and genotypes 141-W, 143-W, ARA-24, EV-15 and RJ-21 as less efficient by all three levels. However, there were some genotypes that only stood out under particular environmental conditions, as discussed above with respect to the predicted response of EV-13 and EV-14 under water stress conditions. Indeed, predicting genotypes WUE$_i$ responses allows to complement genotypes ranking based on field measurements by focusing on a specific range of water stress. This makes it possible to identify the genotypes best adapted to specific conditions. Furthermore, both approaches to WUE assessment at leaf level, WUE$_i$ and WUE$_i$ predicted, were in good agreement (Figure 2 and Table 4).

## 5 | CONCLUSIONS

The analytical methodology used in this study allowed a fairly consistent evaluation of WUE in 13 cv. Grenache genotypes based on field data at the leaf, grape and plant level. In addition, predicting the WUE$_i$ responses to vine water status using the obtained genotype-specific Ln WUE$_i$–$g_s$ regression proved to be a useful complementary approach. Overall, genetic diversity within this cultivar was shown to confer a variability in WUE$_i$ of 13% on average. There were genotypes that generally showed greater WUE$_i$ than others, but some of them responded differently according to the range of water status. Large differences were found between white and red genotypes, but also within the red ones. Carbon isotope discrimination in berries partially confirmed the differences in WUE$_i$ and WUE$_c$ among genotypes. Moreover, there was a certain consistency between WUE$_i$ and WUE$_c$. Subsequent studies should aim to unravel the physiological processes underlying the observed differences in water use efficiency and stomatal behaviour in order to characterise genotypes in terms of carbon balance and grape biochemical composition.

### CONFLICT OF INTEREST
The authors declare no conflict of interest.

### DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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