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# Variability in Water Use Efficiency of Grapevine Tempranillo Clones and Stability over Years at Field Conditions

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**Abstract:** One way to face the consequences of climate change and the expected increase in water availability in agriculture is to find genotypes that can sustain production at a lower water cost. This theoretically can be achieved by using genetic material with an increased water use efficiency. We compared the leaf Water Use Efficiency (WUE<sub>i</sub>) under realistic field conditions in 14 vine genotypes of the Tempranillo cultivar (clones), in two sites of Northern Spain for three and five years each to evaluate (1) if a clonal diversity exists for this traits among those selected clones and (2) the stability of those differences over several years. The ranking of the different clones showed significant differences in WUE<sub>i</sub> that were maintained over years in most of the cases. Different statistical analyses gave coincident information and allowed the identification of some clones systematically that had a higher WUE<sub>i</sub> or a lower WUE<sub>i</sub>. These methods also allowed the identification of the underlying physiological process that caused those differences and showed that clones with a higher WUE<sub>i</sub> are likely to have an increased photosynthetic capacity (rather than a different stomatal control). Those differences could be useful to orientate the decision for vines selection programs in the near future.

**Keywords:** Grapevine; vitis; WUE; clonal selection; intra-cultivar; breeding; photosynthesis; stomatal conductance

## 1. Introduction

Agriculture is one of the largest water consumers on the planet. In many semi-arid areas, dryland viticulture is becoming increasingly more challenging and sometimes is reconverted into irrigated crops, thus there is concern about the water scarcity increase linked to climatic change. Intergovernmental Panel on Climate Change (IPCC) predicts an increase of average temperatures and the frequency of extreme drought and/or warm events [1]. So, for most viticulture areas, improving the water use efficiency (WUE) of the grape is becoming more and more important to secure the sustainability of vineyards [2,3]. There are different ways to improve grape WUE, and the most immediate way is to adjust irrigation dosage and schedule [4]. However, to explore the genetic variation inside grapevine varieties and clones, and to identify the genetic material characterised by a better WUE, is also a promising way to improve the vineyard WUE [5,6].

The WUE can be measured at different spatial and temporal scales [7,8]. From an agronomic approach, the crop WUE ( $WUE_{crop}$ ) refers to the final yield and total water consumed. At the leaf level, intrinsic WUE ( $WUE_i$ ) reflects the balance between carbon gain ( $A_n$ ) and stomatal conductance ( $g_s$ ). Several studies on grapevines have intended to link the different scales and results are sometimes contradictory, showing good or bad correspondences [8–12]. Fortunately, for the wine industry the main concern is not only to reach a high productivity, but rather a higher quality of grapes. Grape quality components are largely dependent on an efficient water deficit, which can be achieved by adjusting irrigation but also the plant density or pruning management, [13–16].

In either case, to classify genotypes' WUE, the measurements of leaf gas exchange have been recognised as a useful tool because it is feasible to screen a large quantity of genotypes grown under field conditions and to characterize their behaviour under different water statuses [17,18]. This allows the revealing of a genetic variability of drought tolerance and  $WUE_i$  between several vine cultivars [17–19]. In the same way, some progress has also been made by using other techniques such as the  $\delta^{13}C$  [20–22].

In our research group, after having identified differences in  $WUE_i$  at the cultivar scale [17–19,23], and considering the narrow rules of wine regions to introduce new varieties, the next step has consisted of an intra-cultivar variability evaluation [24]. First, our study showed such variability in a Tempranillo clonal collection. With respect to a wide cultivar collection, the intra-cultivar variability was at least 80% of the shown variability among cultivars [25]. In more recent work, it has been shown that an intra-cultivar genetic variability of  $WUE_i$  exists among several clones of the Tempranillo cultivar [26].

It is important to note that there are several ways to estimate the WUE of a given genotype under realistic field conditions. Most of the studies showed average gas exchange values under different water conditions. As mentioned above, the  $WUE_i$ , or photosynthetic water use efficiency, is obtained from the quotient between net photosynthesis and stomatal conductance, ( $A_N/g_s$ ) and is largely used to qualify drought resistance or water scarcity responses in plants [27,28]. However, there is a strong mathematical influence of  $g_s$  upon  $WUE_i$ , because the soil water depletion is followed by a progressive reduction in  $g_s$  thus WUE is largely dependent on the soil water availability for the plant [26,29,30]. This implies that the comparison of the  $WUE_i$  should be done under a similar range of  $g_s$ . To overcome this dependency and compare under the whole range of  $g_s$  samples, we have used a method proposed by Tortosa et al. [25]. This consists of establishing a general  $WUE_i$ - $g_s$  relationship (log transformed for linearity) based on the data of all genotypes that, in general, presents a high correlation coefficient. Then, an average of the residuals of each genotype (observed-predicted value) is calculated (and expressed in percentage), to rank the given genotype among the rest.

As part of a wide program to identify Tempranillo clones with enhanced WUE, the main objective of the present study was to evaluate whether, under realistic field conditions, the previously observed clonal differences in  $WUE_i$  within Tempranillo cultivars were affected by environmental complex variations such as the “year effect,” commonly reported for grapevine harvest and quality.

## 2. Material and Methods

### 2.1. Experimental Sites and Plant Material

The experiments were conducted in two experiment sites, both in Northern Spain. The first one in the experimental field of the ICVV (Instituto de las Ciencias de la Vid y el Vino, Logroño, La Rioja, Spain), called “La Grajera.” In this site, five clones (232, 807, 1048, 1052, 1084) were measured during five consecutive years. The second site was located at the Roda estate (Bodegas Roda, Haro, La Rioja, Spain), where nine clones (6, 44, 78, 109, 121, 155, 215, 260, 463) were measured during three consecutive years. In both sites, plants were grafted onto 110-Richter rootstock, trained as a double cordon system in La Grajera, and head-trained bush system in Haro. The vine density in La Grajera were 2600 plants  $Ha^{-1}$  and in Haro 3300 plants  $Ha^{-1}$ .

Climatic conditions of each site were characterized. Data were collected from the 1st of May to the 1st of October, for the same years as gas exchange parameters were measured. Growing Degree Days

(in °C day<sup>-1</sup>) from 1 May to 1 October were calculated as  $T_{\text{mean}} - T_{\text{base}}$  (only positive values) for each day, using  $T_{\text{base}} = 10$  °C. Also, accumulated standard evapotranspiration ET<sub>0</sub> (mm), and accumulated precipitation (mm) were recorded [31].

## 2.2. Gas Exchange Measurements

Leaf net photosynthesis ( $A_n$ ) and stomatal conductance ( $g_s$ ) were measured in a fully exposed mature leaf (one per plant,  $n = 4\text{--}6$  per clone). All measurements were performed between 10:00 h and 13:00 h (local time) using an infrared open gas analyser system (Li-6400xt, Li-cor, Inc., Lincoln, NE, USA). The CO<sub>2</sub> concentration inside the chamber was 400 μmol CO<sub>2</sub> mol<sup>-1</sup> air, photosynthetic active radiation (PAR) was always above saturation levels. WUE<sub>i</sub> was calculated as the ratio between  $A_n$  and  $g_s$ .

## 2.3. Characterization of the Differences in WUE<sub>i</sub>

We used three different ways to estimate differences in WUE<sub>i</sub>. The first consists of averaging all the values of a given genotype. However, because of the strong influence of  $g_s$  upon the WUE<sub>i</sub>, the method developed by Tortosa et al. [22] was applied in order to overcome this effect. Following this method, first, a general relationship between WUE<sub>i</sub> and  $g_s$  is obtained. Provided such a relationship shows a high regression coefficient, the WUE<sub>i</sub> expected for each  $g_s$  value is calculated and compared to the observed value for a determined clone obtaining the residual value as percentage ( $\text{residual}_{\text{clone}}/\text{predicted}_{\text{clone}}$ ). The third approach is applied to study in detail differences between two specific clones. For this, we compared their respective slopes and intercepts on their particular WUE<sub>i</sub>- $g_s$  relationship.

## 2.4. Yield Estimations

Average yield was provided from Roda site for seven consecutive years. Those data were based on the average plant yield of 8–10 vines per given clone. These data were extrapolated to get an estimated yield in t Ha<sup>-1</sup>, considering a vine density of 3300 plants Ha<sup>-1</sup> (1.5 × 2 m).

## 2.5. Statistical Analysis

All statistical analyses were performed using R [32]. First, a global Two-Ways ANOVA was performed with Genotype × Years as main effect and their interaction, within each site. Then, a separated One-Way ANOVAs was performed within each year to check in which year the Genotype effect was significant. When significant, a Post-Hoc test ('agricolae' package, [33]) was applied to determine which were different from each other, and so to estimate a ranking. The WUE<sub>i</sub>- $g_s$  relationship was compared (ANCOVA from the 'car' package [34] of some specific clones, using the cld analysis from the 'emmeans' package [35]). Any differences were accepted with  $p$ -value < 0.05.

# 3. Results

## 3.1. Experimental Fields Comparison and Year Effect

We compared the WUE of different Tempranillo clones in two experimental sites located in La Rioja (Spain); one located in Logroño belonging to the ICVV Research Institut ("La Grajera" experimental field), and a second one in Haro belonging to the commercial winery Roda. These two locations have a typical Mediterranean climate, with high temperatures and low precipitation in summer. However, slight differences were observed between experimental years and sites (Table 1). The growing degree days were always higher (almost 15%) in La Grajera than in Roda. Related to this, the accumulated ET<sub>0</sub> is likewise higher and the total rainfall is slightly lower in La Grajera than in Roda.

**Table 1.** Climatic conditions of the two experimental sites. Data are the sum of each year, since 1 May to 1 October [31].

Field Year	La Grajera			Roda		
	GDD ( $^{\circ}\text{C day}^{-1}$ )	ET0 (mm)	P (mm)	GDD ( $^{\circ}\text{C day}^{-1}$ )	ET0 (mm)	P (mm)
2015	1482.2	775.6	112.7			
2016	1456.9	759.2	83.2	1247	719	105
2017	1516.3	768.8	174.7	1291	740	191
2018	1469.8	699.1	267.9			
2019	1485.4	779.5	184.5	1232	739	171

GDD: Growing Degree-days, considering  $T_{\text{base}} = 10^{\circ}\text{C}$  ET0 is the daily accumulated standard evapotranspiration and P the total rainfall over the period.

The water plant status, the main determinant of WUE, was indirectly estimated by the stomatal conductance following Medrano and Flexas 2002 [36]. To compare the impact of the effect of climatic conditions on plant water status, all years and genotypes  $g_s$  values were averaged (Table 2). In La Grajera,  $g_s$  varied in average between 0.08 and 0.09  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$  in four over the five studied years, showing values typical of moderate to severe water deficit. The year 2016 showed the largest  $g_s$  values reaching 0.13  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ . In the case of  $\text{WUE}_i$ , the range of variation was between 98 and 124  $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ . Interestingly, we noticed a remarkable increase of  $\text{WUE}_i$  in 2015 compared to 2017 (+25% higher) but at similar  $g_s$  values (average 0.09  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ ). In parallel we noted an increased net assimilation rate ( $A_n$ ) in 2015 compared with 2017 (9.6 and 8.6  $\mu\text{mol CO}_2 \text{m}^{-2} \text{s}^{-1}$ , respectively). The same effect was found when compared 2018 and 2019.

**Table 2.** Monthly averages of  $g_s$  and water use efficiency ( $\text{WUE}_i$ ) in the two experimental fields.

Year	La Grajera		Roda	
	$g_s$ ( $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ )	$\text{WUE}_{i\text{int}}$ ( $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ )	$g_s$ ( $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ )	$\text{WUE}_{i\text{int}}$ ( $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ )
2015	0.09 $\pm$ 0.015 b	123.6 $\pm$ 6.2 a		
2016	0.130 $\pm$ 0.012 a	98.1 $\pm$ 3.9 c	0.393 $\pm$ 0.014 a	51.0 $\pm$ 1.5 c
2017	0.09 $\pm$ 0.004 b	99.1 $\pm$ 1.8 c	0.132 $\pm$ 0.007 c	86.2 $\pm$ 1.8 a
2018	0.082 $\pm$ 0.006 b	103.5 $\pm$ 2.9 bc		
2019	0.084 $\pm$ 0.007 b	115.3 $\pm$ 2.7 ab	0.303 $\pm$ 0.014 b	67.2 $\pm$ 2.1 b

Two-Way ANOVA: Year \*\*\*, Field \*\*\*, Year  $\times$  Field \*\*\*

Different letters indicate statistical differences within each field by Tukey test ( $p < 0.05$ ). \*\*\*  $p$ -value  $< 0.001$ .

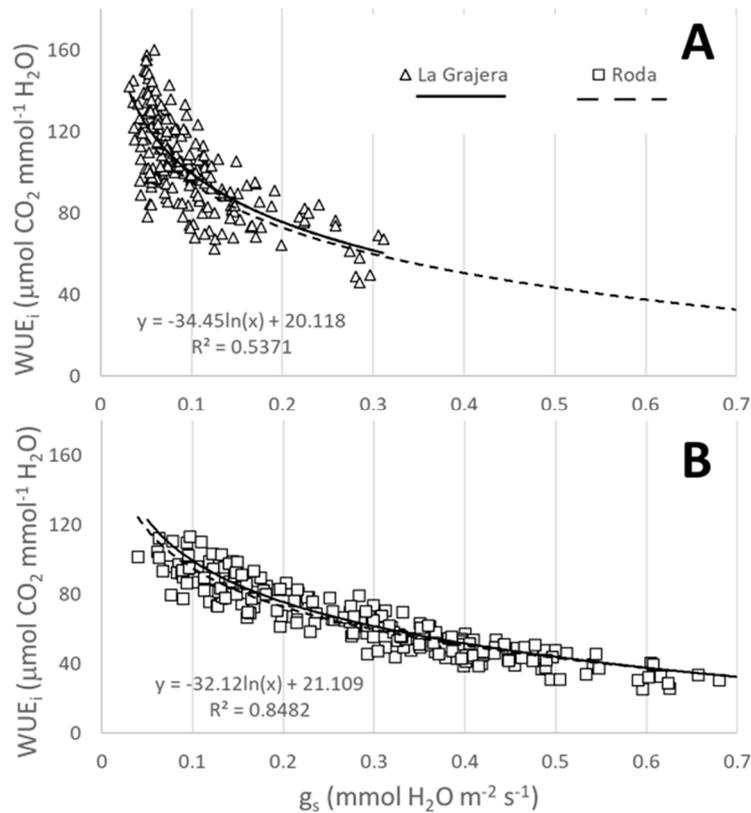
In the Roda field, the  $g_s$  values were clearly higher than in La Grajera, ranging between 0.13 and 0.39  $\text{mol H}_2\text{O m}^{-2} \text{s}^{-1}$ , which is between a mild to moderate water deficit. The corresponding range of variation in  $\text{WUE}_i$  in this site was lower than in La Grajera ( $p < 0.001$ , Table 2) and was between 51 and 86  $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ .

Despite the differences between experimental fields in the  $g_s$  range, when the two general  $\text{WUE}_i$ - $g_s$  relationships were compared, there were no differences in either slope or intercept (Figure 1).

### 3.2. Genotypic Variability of $\text{WUE}_i$ and Stability over Years

Significant Genotype and Year effects (and their interaction) in both La Grajera and Roda sites were found ( $p < 0.001$  in both, Tables 3 and 4 respectively). In La Grajera, extreme values were reached by clones 1084 and 807 (with 87.7 and 108.5  $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$ , respectively, all year confounded). When each year was analysed separately, the genotype effect was significant within each year in Roda, and in three out of five years in La Grajera. Moreover, some repetitive patterns were present, like that of clone 1084, showing systematically the lowest values of  $\text{WUE}_i$ . A systematic genotype effect within each year was also present in Roda, with some clones (260, 109) showing the lowest values ( $-55 \mu\text{mol}$

CO<sub>2</sub> mol<sup>-1</sup> H<sub>2</sub>O) and others showing repetitively the highest WUE<sub>i</sub> values (clones 463, 44, 6) around 75 μmol CO<sub>2</sub> mol<sup>-1</sup> H<sub>2</sub>O.



**Figure 1.** Correlations between WUE<sub>i</sub> and stomatal conductance ( $g_s$ ) in the two experimental sites; La Grajera (A) and Roda (B). Continuous line shows the relation for the La Grajera genotypes and dashed lines shows the same relation for Roda genotypes.

**Table 3.** Variation in WUE<sub>i</sub> (μmol CO<sub>2</sub> mol<sup>-1</sup> H<sub>2</sub>O) per genotype and year in La Grajera field (values are means ± SE).

Genotype	2015	2016	2017	2018	2019	Average
232	123.7 ± 9.4 a	113.8 ± 8.3	106.4 ± 4.9	95.5 ± 9.2 b	116.4 ± 2.8 b	110.9 ± 3.9 a
807	129.4 ± 13.4 a	102.1 ± 8.1	97.4 ± 2.7	122.2 ± 3.9 a	122.6 ± 3.6 ab	108.5 ± 2.6 a
1048	143.1 ± 4.5 a	90.9 ± 7.8	101.3 ± 3.1	105.9 ± 3.1 ab	128.4 ± 2.9 a	107.5 ± 3.4 a
1052	139.6 ± 12.9 a	94.7 ± 5.7	97.7 ± 2.7	102.7 ± 1.4 b	113.2 ± 3.9 b	103.3 ± 3.6 a
1084	79.6 ± 12.1 b	81.5 ± 9.2	92.1 ± 4.8	91.8 ± 9.8 b	93.1 ± 2.4 c	87.7 ± 3.6 b

Two-Way ANOVA: Year \*\*\*, Genotype \*\*\*, Year × Genotype \*\*\*

Different letters indicate statistical differences within each year by Tukey test ( $p < 0.05$ ). \*\*\*  $p$ -value < 0.001.

**Table 4.** Variation in WUE<sub>i</sub> (μmol CO<sub>2</sub> mol<sup>-1</sup> H<sub>2</sub>O) per genotype and year in Roda field (values are means ± SE).

Genotype	2016	2017	2019	Average
121	49.9 ± 3.0 abc	98.3 ± 4.2 a	88.4 ± 2.8 a	78.1 ± 4.4 a
6	56.7 ± 4.5 ab	96.6 ± 2.7 a	83.7 ± 7.1 a	75.1 ± 4.6 ab
463	55.0 ± 5.0 ab	95.6 ± 6.0 a	76.1 ± 3.8 ab	74.0 ± 4.4 ab
44	58.5 ± 4.7 ab	90.6 ± 6.6 ab	71.8 ± 6.9 abc	70.0 ± 4.2 abc
78	56.8 ± 5.7 ab	76.2 ± 4.8 bc	64.6 ± 1.5 bcd	64.0 ± 3.0 abc
155	61.2 ± 3.1 a	69.5 ± 3.2 c	57.3 ± 2.5 cd	62.0 ± 1.9 abc
215	43.3 ± 1.0 bc	86.6 ± 2.3 abc	52.4 ± 3.4 d	59.3 ± 4.2 bc
109	44.0 ± 4.4 abc	76.4 ± 4.2 bc	48.9 ± 1.2 d	53.5 ± 3.3 c
260	34.0 ± 2.0 c	81.9 ± 2.6 abc	50.6 ± 1.9 d	52.8 ± 4.4 c

Two-Way ANOVA: Year \*\*\*, Genotype \*\*\*, Year × Genotype \*\*\*.

Different letters indicate statistical differences within each year by Tukey test ( $p < 0.05$ ). \*\*\*  $p$ -value  $< 0.001$ .

To overcome the WUE<sub>i</sub> variability induced by the range of variation of  $g_s$ , each genotype was characterised following its residuals of a general WUE<sub>i</sub>- $g_s$  relationship (see Introduction and Material and methods sections), expressed as a percentage (Tables 5 and 6). Thus, by doing so, we found, globally, the same pattern as the previous comparison (see above). In this case, Roda clones showed more variability with a significant genotype effect in the three measured years in comparison with only two out of five measured years in La Grajera. In Roda, the same genotypes were identified as less (clones 260, 215, 109) or more (44, 463) efficient in terms of WUE<sub>i</sub>. Moreover, some genotypes were more constant through the years than others. We estimated a Year effect for each clone separately, and the clones 260 and 463 (two extremes) were seen as the most constants over the years (no Year effect).

**Table 5.** Variation in percentage respect to *predicted value* per genotype and year in La Grajera field.

Genotype	2015	2016	2017	2018	2019	Average
1048	2.5%	1.7%	0.0%	-0.8% b	2.2% a	1.1% ± 2
1052	1.7%	0.6%	1.4%	0.1% b	-1.8% ab	0.4% ± 1.7
232	1.7%	2.1%	0.5%	-16.2% c	6.5% a	0.3% ± 2.4
807	-4.3%	-3.7%	-1.9%	17.4% a	0.5% a	0.1% ± 2.3 *
1084	-2.1%	2.1%	0.7%	-3.8% bc	-8.2% b	-0.9% ± 2.1

Two-Way ANOVA: Genotype \*\*\*, Year × Genotype \*\*\*

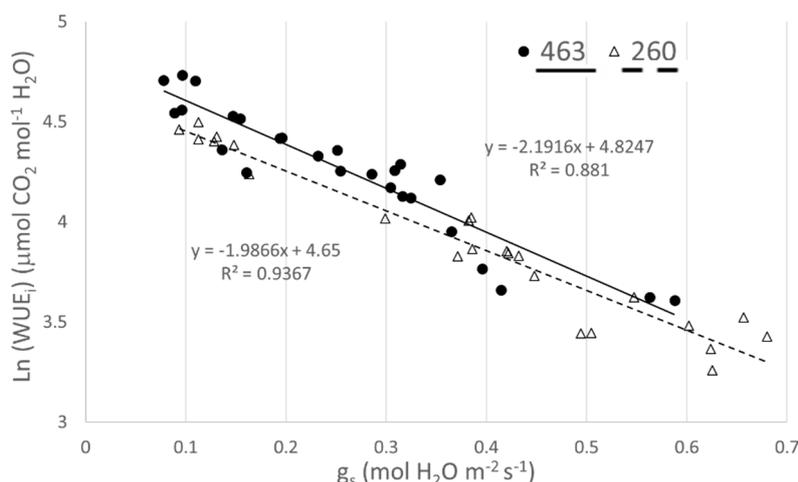
Different letters indicate statistical differences within each year by Tukey test ( $p < 0.05$ ). \* means significant differences between year for each genotype ( $p$ -value  $< 0.05$ ), \*\*\*  $p$ -value  $< 0.001$ .**Table 6.** Variation in percentage respect to *predicted value* per genotype and year in Roda field.

Genotype	2016	2017	2019	Average
44	13.5% a	10.7% a	-4.0% bc	8.6% ± 2.5 a *
463	8.0% abc	7.2% a	2.3% bc	4.6% ± 2.1 ab ~
155	9.5% ab	0.3% ab	-0.5% abc	4.0% ± 1.6 ab **
6	2.2% abcd	8.1% a	-1.8% bc	2.2% ± 1.9 ab
121	-2.7% bcd	0.0% ab	4.7% ab	0.3% ± 1.7 bc
78	-4.3% bcd	-1.7% ab	0.7% abc	-1.5% ± 1.7 bc
109	-4.8% cd	-10.2% b	6.5% a	-1.9% ± 2.0 bc ***
260	-7.1% d	-4.9% ab	-6.5% c	-6.1% ± 1.6 c ~
215	-9.2% d	-3.1% a b	-6.1% c	-6.2% ± 1.2 c

Two-Way ANOVA: Genotype \*\*\*, Year × Genotype \*\*\*

Different letters indicate statistical differences within each year by Tukey test ( $p < 0.05$ ). Asterisk or tilde mean significant differences between year for each genotype (~  $p < 0.1$ ; \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ).

The two extreme clones (260, less efficient and 463, more efficient) were tested in more detail (Figure 2). These clones were revealed to have different  $WUE_i$ - $g_s$  relationships, with similar slopes but a higher intercept for 463 (values of 4.65 and 4.82 respectively). The 463 clone presents a constant higher  $WUE_i$  of 10%, over the whole range of  $g_s$  compared to 260. Those data were based on all years confounded.



**Figure 2.** Relationship between the natural logarithm of the  $WUE_i$  against  $g_s$ , all years confounded, in the two more contrasting genotypes.

### 3.3. Yield Variations between Clones and over Years

Total yield variation between clones is reported for the Roda site (Table 7). This variation is also shown when comparing total production within different years. Unfortunately, yield data were not compiled during the same years in which gas exchange measurements were performed. However, we used seven consecutive years (from 2003 to 2009) from the same experimental site of Roda to estimate the variability in yield of the same nine tested clones, thus avoiding the potential effect of differential experimental conditions. From the data collected by the company, a huge variability in total production was present between clones, varying from 1.3 to 13.3 t Ha<sup>-1</sup> (all years and clones confounded). Because only the average clone data were available for each Genotype\*Year, separated ANOVAs of genotype along different years and year comparison was done. The Genotype effect was significant ( $p < 0.001$ ) but not the Year effect. The total production varied from 9.7 to 3.3 t Ha<sup>-1</sup> for clones 463 and 155, respectively. Although any general relationship between yield and  $WUE_i$  was found, the clone 463 was the most productive in terms of yield and one of the more efficient in terms of  $WUE_i$ .

**Table 7.** Production (t Ha<sup>-1</sup>) of Roda genotypes each year.

Genotype	2003	2004	2005	2006	2007	2008	2009	Gen. av.
6	5.0	8.0	8.3	10.7	4.7	8.7	3.7	7.0 ± 1.0 ab
44	4.0	3.7	2.7	4.7	2.0	3.3		3.4 ± 0.4 c
78	7.7	8.0	9.3	10.7	8.7	7.3	9.4	8.7 ± 0.5 a
109	7.3	7.0	8.0	13.3	8.0	7.7	10.5	8.8 ± 0.9 a
121	5.0	4.3	4.7	5.3	3.3	4.0	4.4	4.4 ± 0.3 bc
155	5.7	4.0	1.3	2.0	2.7	3.3	3.8	3.3 ± 0.6 c
215	6.7	4.3	3.3	6.7	2.0	4.7	5.5	4.7 ± 0.7 bc
260	7.7	3.7	4.0	7.3	1.3	6.0	8.6	5.5 ± 1.1 bc
463			7.0	11.3	10.0	9.3	10.8	9.7 ± 0.8 a
Year av.	6.3 ± 0.5	5.0 ± 0.7	5.0 ± 1.0	7.7 ± 1.3	4.8 ± 1.2	5.7 ± 0.8	7.6 ± 1.1	

Gen. av.: Genotype average; Year av: Year average. Different letters indicate statistical differences within each genotype by Tukey test ( $p < 0.05$ ).

#### 4. Discussion

The present data show, as expected, a wide variability in the estimated WUE among Tempranillo clones in field conditions at both locations. An important component of this variability was clearly due to the “year effect,” a complex integral of differences in climatic conditions along the growing period, which is also largely reported for most agronomic characteristics of grapevine crops including yield and grape quality [37,38].

In the present work, we first analysed the absolute values of leaf  $WUE_i$  (or photosynthetic WUE) to compare different Tempranillo clones between each other. This method presents the disadvantage of including a large variability of  $WUE_i$  related to soil water availability variations which will be reflected in  $g_s$  values, and this is the case when plants are measured under realistic field conditions which means a different water status [39–41]. Even taking into account this fact, the two-ways ANOVA revealed a clear Genotype effect, confirming the existence of a clonal variability of  $WUE_i$  within the Tempranillo cultivar. This confirms the results of Tortosa et al. [26]. Moreover, in this work we highlight that those differences are also maintained through the years, thus including the “year effect” as another important factor to be considered under realistic field conditions. This suggests that those differences are truly fixed at a genetic level, because they resulted in being independent of variations in climatic conditions between years. Moreover, the same patterns were repetitively encountered—the same groups of clones were systematically the most efficient, and others were systematically less efficient.

We also used a ranking based on residuals of the general  $WUE_i$ - $g_s$  relationship [25]. This method gave the same conclusion as above, resulting in the same groups of clones having systematically the higher or lower  $WUE_i$ . The advantage of ranking is to consider the whole range of  $g_s$  and to be able to determine the distance of each data within the general relationship WUE vs  $g_s$ . This method also has the advantage of removing the stomatal effect. This implies that, if differences are revealed, they come from differences in photosynthetic capacity, and not from different stomatal control. This was the case in our study (especially for Roda site), where there was a good correspondence between the ranking from absolute  $WUE_i$  values and the percentage analysis. Unfortunately, we do not have data on the photosynthetic capacity of those clones, but it will be very interesting in the future to characterize it.

Finally, we tested another approach to compare two extreme clones identified with the other methods. By comparing their intercepts and slopes of  $WUE_i$ - $g_s$ , we found that the slopes were identical, but not the intercepts. This means that for a given  $g_s$ , the more efficient clone had a systematically higher  $A_N$  and confirms again the influence of a superior photosynthetic capacity to achieve a greater  $WUE_i$ , among Tempranillo clones. Even if we did not characterise the photosynthetic capacity of each clone, we can expect that this could be related to differences in leaf  $CO_2$  diffusion capacity (not  $g_s$  but mesophyll conductance), or in biochemical properties of the leaf (higher RubisCO content or more efficient RubisCO), as mentioned by Gago et al. [42] among others [43,44].

A main objective of this work was to evaluate whether the  $WUE_i$  range position of the different clones was maintained through years. In the large majority of cases, we found the same pattern, with the same clones being the most/less efficient throughout several years of measurements. This stability was encountered by calculating differences based on absolute values and also percentages. The differences were weaker in La Grajera since clonal differences were identified three years over five (when comparing absolute values of  $WUE_i$ ) but only two years over five when using percentages. We identified as possible causes of this, that the standard errors are much larger in La Grajera, limiting statistical significance for clonal effect. This could be due to two possible reasons: (1) more variability in the field conditions (soil composition, slope, water availability, etc.) that induce different plant water status/nutrient availability; (2) because the climatic conditions (vapor pressure deficit) also induced lower  $g_s$  (below  $0.1 \text{ mol H}_2\text{O m}^{-2} \text{ s}^{-1}$ ) that induces a larger variability of the  $WUE_i$ . On the other hand, in Roda, each year (using absolute values or percentage) showed a clear Genotype effect, reinforcing those clonal differences.

As an agronomic reference, the analysis of the grape yield data for the nine evaluated clones in Roda (extrapolated to yield production in Tones/Hectare) also showed large variations even though

the climatic conditions variations were in the range of the expected for this location. As for  $WUE_i$ , some clones showed a capacity to maintain the same ranking in yield compared to other ones (some clones are the most productive, independently of yield variations due to years' differences).

Nevertheless, the results showed clear differences among genotypes, in general without a clear correspondence with the ranking in  $WUE$  showing the complex relations among environmental conditions and yield.

## 5. Conclusions

With the present results, we confirm the existence of significant clonal variability in  $WUE_i$  within the Tempranillo cultivar. Those results were shown in two different sites, with two different sets of clones, and across several years of measurements. Clonal differences were apparently due more to differences in photosynthetic capacity than to a more efficient control of water loss. This finding opens new ways for future research which would be focused on the physiological and biochemical basis responsible for the variations in  $WUE_i$ .

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## References

1. IPCC. *Climate Change 2014: Impacts, Adaptation, and Vulnerability, Part A: Global and Sectoral Aspects; Contribution of Working Group II to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*; Cambridge University Press: Cambridge, UK; New York, NY, USA, 2014.
2. Medrano, H.; Tomás, M.; Martorell, S.; Escalona, J.M.; Pou, A.; Fuentes, S.; Flexas, J.; Bota, J. Improving water use efficiency of vineyards in semi-arid regions. A review. *Agron. Sustain. Dev.* **2015**, *35*, 499–517. [[CrossRef](#)]
3. Zarrouk, O.; Costa, J.; Francisco, R.; Lopes, C.; Chaves, M. Drought and water management in Mediterranean vineyard Grapevine. In *A Changing Environment: A Molecular and Ecophysiological Perspective*; Delrot, S., Chaves, M., Gerós, H., Medrano, H., Eds.; Wiley-Blackwell: Chichester, UK, 2016; pp. 38–67.
4. Cifre, J.; Bota, J.; Escalona, J.M.; Medrano, H.; Flexas, J. Physiological tools for irrigation scheduling in grapevine (*Vitis vinifera* L.): An open gate to improve water-use efficiency? *Agric. Ecosyst. Environ.* **2005**, *106*, 159–170. [[CrossRef](#)]
5. Flexas, J.; Galmés, J.; Gallé, A.; Gulías, J.; Pou, A.; Ribas-Carbo, M.; Tomas, M.; Medrano, H. Improving water use efficiency in grapevines: Potential physiological targets for biotechnological improvement. *Aust. J. Grape Wine Res.* **2010**, *16*, 106–121. [[CrossRef](#)]
6. Costa, J.M.; Escalona, J.M.; Egipto, R.; Lopes, C.; Medrano, H.; Chaves, M.M. Modern viticulture in southern Europe: Vulnerabilities and strategies for adaptation to water scarcity. *Agric. Water Manag.* **2016**, *164*, 5–18. [[CrossRef](#)]
7. Medrano, H.; Tomás, M.; Martorell, S.; Flexas, J.; Hernández, E.; Rosselló, J.; Pou, A.; Escalona, J.M.; Bota, J. From leaf to whole-plant water use efficiency (WUE) in complex canopies: Limitations of leaf WUE as a selection target. *Crop J.* **2015**, *3*, 220–228. [[CrossRef](#)]
8. Douthe, C.; Medrano, H.; Tortosa, I.; Escalona, J.M.; Hernández-Montes, E.; Pou, A. Whole-plant water use in field grown grapevine: Seasonal and environmental effects on water and carbon balance. *Front. Plant Sci.* **2018**, *9*, 1540. [[CrossRef](#)]

9. Tomás, M.; Medrano, H.; Pou, A.; Escalona, J.M.; Martorell, S.; Ribas-Carbó, M.; Flexas, J. Water-use efficiency in grapevine cultivars grown under controlled conditions: Effects of water stress at the leaf and whole-plant level. *Aust. J. Grape Wine Res.* **2012**, *18*, 164–172. [[CrossRef](#)]
10. Medrano, H.; Pou, A.; Tomás, M.; Martorell, S.; Gulias, J.; Flexas, J.; Escalona, J.M. Average daily light interception determines leaf water use efficiency among different canopy locations in grapevine. *Agric. Water Manag.* **2012**, *114*, 4–10. [[CrossRef](#)]
11. Poni, S.; Bernizzoni, F.; Civardi, S.; Gatti, M.; Porro, D.; Camin, F. Performance and water-use efficiency (single-leaf vs. whole-canopy) of well-watered and half-stressed split-root Lambrusco grapevines grown in Po Valley (Italy). *Agric. Ecosyst. Environ.* **2009**, *129*, 97–106. [[CrossRef](#)]
12. Tarara, J.M.; Peña, J.E.P.; Keller, M.; Schreiner, R.P.; Smithyman, R.P. Net carbon exchange in grapevine canopies responds rapidly to timing and extent of regulated deficit irrigation. *Funct. Plant Biol.* **2011**, *38*, 386–400. [[CrossRef](#)]
13. Keller, M.; Romero, P.; Gohil, H.; Smithyman, R.P.; Riley, W.R.; Casassa, L.F.; Harbertson, J.F. Deficit irrigation alters grapevine growth, physiology, and fruit microclimate. *Am. J. Enol. Vitic.* **2016**, *67*, 426–435. [[CrossRef](#)]
14. Chaves, M.M.; Zarrouk, O.; Francisco, R.; Costa, J.M.; Santos, T.; Regalado, A.P.; Rodrigues, M.L.; Lopes, C.M. Grapevine under deficit irrigation: Hints from physiological and molecular data. *Ann. Bot.* **2010**, *105*, 661–676. [[CrossRef](#)] [[PubMed](#)]
15. Smart, R.E.; Dick, J.K.; Gravett, I.M.; Fisher, B.M. Canopy management to improve grape yield and wine quality—principles and practices. *S. Afr. J. Enol. Vitic.* **1990**, *11*, 3–17. [[CrossRef](#)]
16. Romero, P.; García, J.G.; Fernández-Fernández, J.I.; Muñoz, R.G.; Saavedra, F.A.; Martínez-Cutillas, A. Improving berry and wine quality attributes and vineyard economic efficiency by long-term deficit irrigation practices under semiarid conditions. *Sci. Hortic.* **2016**, *203*, 69–85. [[CrossRef](#)]
17. Tomás, M.; Medrano, H.; Escalona, J.M.; Martorell, S.; Pou, A.; Ribas-Carbó, M.; Flexas, J. Variability of water use efficiency in grapevines. *Environ. Exp. Bot.* **2014**, *103*, 148–157. [[CrossRef](#)]
18. Bota, J.; Tomás, M.; Flexas, J.; Medrano, H.; Escalona, J.M. Differences among grapevine cultivars in their stomatal behavior and water use efficiency under progressive water stress. *Agric. Water Manag.* **2016**, *164*, 91–99. [[CrossRef](#)]
19. Martorell, S.; Diaz-Espejo, A.; Tomàs, M.; Pou, A.; El Aou-ouad, H.; Escalona, J.M.; Vadell, J.; Ribas-Carbó, M.; Flexas, J.; Medrano, H. Differences in water-use-efficiency between two *Vitis vinifera* cultivars (Grenache and Tempranillo) explained by the combined response of stomata to hydraulic and chemical signals during water stress. *Agric. Water Manag.* **2015**, *156*, 1–9. [[CrossRef](#)]
20. Santesteban, L.G.; Miranda, C.; Barbarin, I.; Royo, J.B. Application of the measurement of the natural abundance of stable isotopes in viticulture: A review. *Aust. J. Grape Wine Res.* **2015**, *21*, 157–167. [[CrossRef](#)]
21. Bchir, A.; Escalona, J.M.; Gallé, A.; Hernández-Montes, E.; Tortosa, I.; Braham, M.; Medrano, H. Carbon isotope discrimination ( $\delta^{13}\text{C}$ ) as an indicator of vine water status and water use efficiency (WUE): Looking for the most representative sample and sampling time. *Agric. Water Manag.* **2016**, *167*, 11–20. [[CrossRef](#)]
22. Gaudillere, J.P.; Van Leeuwen, C.; Ollat, N. Carbon isotope composition of sugars in grapevine, an integrated indicator of vineyard water status. *J. Exp. Bot.* **2002**, *53*, 757–763. [[CrossRef](#)]
23. Tomás, M.; Medrano, H.; Brugnoli, E.; Escalona, J.M.; Martorell, S.; Pou, A.; Ribas-Carbo, M.; Flexas, J. Variability of mesophyll conductance in grapevine cultivars under water stress conditions in relation to leaf anatomy and water use efficiency. *Aust. J. Grape Wine Res.* **2014**, *20*, 272–280. [[CrossRef](#)]
24. Ibáñez, J.; Carreño, J.; Yuste, J.; Martínez-Zapater, J.M. Grapevine breeding and clonal selection programmes in Spain. In *Grapevine Breeding Programs for the Wine Industry*; Woodhead Publishing: Cambridge, UK, 2015; pp. 183–209.
25. Tortosa, I.; Escalona, J.M.; Bota, J.; Tomas, M.; Hernandez, E.; Escudero, E.G.; Medrano, H. Exploring the genetic variability in water use efficiency: Evaluation of inter and intra cultivar genetic diversity in grapevines. *Plant Sci.* **2016**, *251*, 35–43. [[CrossRef](#)] [[PubMed](#)]
26. Tortosa, I.; Escalona, J.M.; Douthe, C.; Pou, A.; Garcia-Escudero, E.; Toro, G.; Medrano, H. The intra-cultivar variability on water use efficiency at different water status as a target selection in grapevine: Influence of ambient and genotype. *Agric. Water Manag.* **2019**, *223*, 105648. [[CrossRef](#)]
27. Fracasso, A.; Trindade, L.M.; Amaducci, S. Drought stress tolerance strategies revealed by RNA-Seq in two sorghum genotypes with contrasting WUE. *BMC Plant Biol.* **2016**, *16*, 115. [[CrossRef](#)] [[PubMed](#)]

28. Puangbut, D.; Jogloy, S.; Vorasoot, N. Association of photosynthetic traits with water use efficiency and SPAD chlorophyll meter reading of Jerusalem artichoke under drought conditions. *Agric. Water Manag.* **2017**, *188*, 29–35. [[CrossRef](#)]
29. Pou, A.; Medrano, H.; Tomàs, M.; Martorell, S.; Ribas-Carbó, M.; Flexas, J. Anisohydric behaviour in grapevines results in better performance under moderate water stress and recovery than isohydric behaviour. *Plant Soil* **2012**, *359*, 335–349. [[CrossRef](#)]
30. Negin, B.; Moshelion, M. The evolution of the role of ABA in the regulation of water-use efficiency: From biochemical mechanisms to stomatal conductance. *Plant Sci.* **2016**, *251*, 82–89. [[CrossRef](#)]
31. Gobierno de la Rioja. Available online: <https://www.larioja.org/agricultura/es/informacion-agroclimatica/red-estaciones-agroclimaticas-siar> (accessed on 15 October 2019).
32. Team, R.C. *A Language and Environment for Statistical Computing*; R Foundation for Statistical Computing: Vienna, Austria, 2014.
33. De Mendiburu, F.; Simon, R. *Agricolae—Ten Years of an Open Source Statistical Tool for Experiments in Breeding, Agriculture and Biology (No. e1748)*; PeerJ PrePrints: London, UK, 2015.
34. Fox, J.; Weisberg, S. Multivariate linear models in R. In *An R Companion to Applied Regression*; SAGE Publications, Inc.: Los Angeles, CA, USA; Thousand Oaks, CA, USA, 2011.
35. Lenth, R.; Lenth, M.R. Package ‘lsmeans’. *Am. Stat.* **2018**, *34*, 216–221.
36. Flexas, J.; Medrano, H. Drought-inhibition of photosynthesis in C3 plants: Stomatal and non-stomatal limitations revisited. *Ann. Bot.* **2002**, *89*, 183–189. [[CrossRef](#)]
37. Medrano, H.; Escalona, J.M.; Cifre, J.; Bota, J.; Flexas, J. A ten-year study on the physiology of two Spanish grapevine cultivars under field conditions: Effects of water availability from leaf photosynthesis to grape yield and quality. *Funct. Plant Biol.* **2003**, *30*, 607–619. [[CrossRef](#)]
38. Schultz, H.R.; Jones, G.V. Climate induced historic and future changes in viticulture. *J. Wine Res.* **2010**, *21*, 137–145. [[CrossRef](#)]
39. Medrano, H.; Escalona, J.M.; Bota, J.; Gulías, J.; Flexas, J. Regulation of photosynthesis of C3 plants in response to progressive drought: Stomatal conductance as a reference parameter. *Ann. Bot.* **2002**, *89*, 895–905. [[CrossRef](#)] [[PubMed](#)]
40. Flexas, J.; Bota, J.; Escalona, J.M.; Sampol, B.; Medrano, H. Effects of drought on photosynthesis in grapevines under field conditions: An evaluation of stomatal and mesophyll limitations. *Funct. Plant Biol.* **2002**, *29*, 461–471. [[CrossRef](#)]
41. Manzoni, S.; Vico, G.; Katul, G.; Fay, P.A.; Polley, W.; Palmroth, S.; Porporato, A. Optimizing stomatal conductance for maximum carbon gain under water stress: A meta-analysis across plant functional types and climates. *Funct. Ecol.* **2011**, *25*, 456–467. [[CrossRef](#)]
42. Gago, J.; Douthe, C.; Florez-Sarasa, I.; Escalona, J.M.; Galmes, J.; Fernie, A.R.; Flexas, J.; Medrano, H. Opportunities for improving leaf water use efficiency under climate change conditions. *Plant Sci.* **2014**, *226*, 108–119. [[CrossRef](#)]
43. Ren, T.; Weraduwage, S.M.; Sharkey, T.D. Prospects for enhancing leaf photosynthetic capacity by manipulating mesophyll cell morphology. *J. Exp. Bot.* **2018**, *70*, 1153–1165. [[CrossRef](#)]
44. Silva-Pérez, V.; De Faveri, J.; Molero, G.; Deery, D.M.; Condon, A.G.; Reynolds, M.P.; Evans, J.R.; Furbank, R.T. Genetic variation for photosynthetic capacity and efficiency in spring wheat. *J. Exp. Bot.* **2019**. [[CrossRef](#)]

