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ORIGINAL RESEARCH ARTICLE

Identifying the best parameters to determine genotype capability to retain adequate malic acid at harvest and in final wines

Tommaso Frioni[1]*, Riccardo Collivasone[1], Ginevra Canavera[1], Matteo Gatti[1], Mario Gabrielli[2] and Stefano Poni[1]

[1] Dept. Of Sustainable Crop Production, Università Cattolica del Sacro Cuore, Piacenza, PC, Italy
[2] Dept. Of Sustainable Food Processing, Università Cattolica del Sacro Cuore, Piacenza, PC, Italy

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ABSTRACT

Maintaining optimal grape acidity at harvest is one of the most complicated challenges under climate change pressures, especially in early ripening cultivars. Warming trends are compressing vine phenology and fostering berry malic acid respiration. In this work, over four years, we evaluated yield components and fruit ripening in two local varieties in the Colli Piacentini, Ortrugo (ORT) and Barbesino (BRB). Our goal was to evaluate their ability to maintain satisfying acidity at harvest and understand the limits and features of the genetic control over organic acid degradation during ripening.

The two varieties exhibited comparable yield and grape total soluble solids (TSS) accumulation dynamics, but BRB showed consistently higher acidity during the entire ripening process in any of the four years. BRB's higher acidity was linked to higher malic acid concentrations. ORT had earlier onset of malic acid degradation than BRB and lower maximum malic acid degradation rates. Malic acid degradation rates were lower in ORT also later in the season, until harvest. However, correlations built between malic acid degradation rates and instantaneous malic acid concentration revealed that BRB had a consistently lower malic acid loss for values of malate < 10 g/L.

Our work demonstrates that there is a genetic control over the malic acid degradation rates exhibited at varying malic acid concentrations and higher acidity at harvest can be found in varieties exhibiting low malic acid degradation rates when malic acid is < 10 g/L. Post-veraison berry growth rates could interact with genotype effects. The analysis of the correlation can be used at different scales to identify cultivars retaining higher acidity at harvest.

KEYWORDS: acidity, biodiversity, phenotyping, germplasm, wine quality

*correspondence:
tommaso.frioni@unicatt.it

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INTRODUCTION

Under current climate change pressures, obtaining grapes with adequate acidity at harvest is one of the main challenges for growers, especially if the goal is producing sparkling wines. This issue arises from two main occurrences: i) higher temperatures enhance the degradation of malic acid; ii) grape maturity may occur under suboptimal climatic conditions due to advanced phenology (Palliotti *et al.,* 2014). For this reason, the introduction of new varieties or the reconsideration of minor and insofar neglected cultivars or clones are gaining enormous interest and popularity (Palliotti *et al.,* 2014, Poni *et al.,* 2020, Antolín *et al.,* 2020). However, the criteria for the identification of performing genotypes in terms of 'acidity at harvest' are complex. Late veraison and ripening can be limited traits since they do not necessarily mean that grapes maintain adequate acidity in relation to a satisfying sugar concentration (Poni *et al.,* 2018, Frioni *et al.,* 2020a). Considering the evolution and the destination of organic acids in grapes, the pre-veraison malic acid pool could represent a promising trait. In the same framework, an eventual genetic control over malic acid degradation rates could hint at additional information. The same can be said for the ratio between minimum acidity and pre-veraison malate abundance or for the sugars/acidity ratio. However, any of the above-mentioned parameters seems to underestimate some of the factors involved or, in any case, to provide an incomplete overview of grape ripening.

In this work, we compared the fruit, must, and wine composition of a local, widely grown white grape variety in the Colli Piacentini area (cv. Ortrugo, ORT) with those of a minor autochthonous variety, namely, Barbesino (BRB), over four consecutive seasons. Our goal was to clarify the relationships between the evolution of titratable acidity during ripening and a wide range of parameters concerning organic acids catabolism. We hypothesised that minimum grape malic acid concentration is a genetic trait deriving from malic acid degradation rates displayed at different TA concentrations. The general aim of this study was the identification of the proper parameters to describe the attitude of a genotype to retain satisfying acidity at harvest.

MATERIALS AND METHODS

1. Description of experimental sites

The experiment was carried out over four consecutive seasons (2017-2020) in a germplasm-collection vineyard at Mossi 1558 Estate (Albareto, Ziano Piacentino, Italy, 44° 97' 93'' N 09 40' 99'' E, 270 m asl). Details of the site and vineyard management can be found in Frioni *et al.* (2020a). The plot consists of several local and international varieties (about 40 vines per cultivar), including Ortrugo (ORT) and Barbesino (BRB), which represented the two treatments of this study. The rows are SE-NW oriented in agreement with the soil slope (about 5°) and uniform in terms of vigour and soil physicochemical features. Rows were divided into three uniform sections to maintain three biological replicates along the study. In each replicate, nine vines were tagged as sub-replicates.

Daily maximum average and minimum temperatures and rainfall were collected by a weather station located nearby the weather station. Cumulated Growing Degree Days were calculated according to Winkler (1974).

2. Monitoring fruit composition

In this work, berry mass, grapes sugars (TSS), titratable acidity (TA), malic acid concentration (MA) and MA degradation rates (MAdr) were weekly analysed from pre-veraison to the end of the season. Sampled berries (in a group of 100 berries per replicate, three replicates per cultivar) were brought to the laboratory, weighed, and crushed to obtain juice. Musts were analysed immediately for total soluble solids (TSS) using a temperature-compensated desk refractometer, whereas pH and titratable acidity (TA) were measured by titration with 0.1 N NaOH to a pH 8.2 endpoint and expressed as g/L of tartaric acid equivalents. Grapes organic acids were analysed by HPLC, as reported by Frioni *et al.* (2020a). The TSS/TA and tartaric/malic acid ratios (HT/HM) were then calculated. The malic acid degradation rate was calculated as the difference in malate concentrations between two consecutive sampling dates, divided by the number of elapsed days.

3. Harvest parameters

Nine tagged ORT and BRB vines were harvested when ORT scored TSS of about 20 °Brix. At harvest, test vines were individually picked, the mass of clusters was weighted, and the total cluster number per vine was counted. Concurrently, three representative clusters per vine—usually inserted on basal, median, and apical cane portions—were taken to the laboratory for further subsampling. Fruits were individually weighted, and the main rachis length was measured to calculate the cluster compactness index expressed as cluster mass-to-rachis length ratio. From each of the three clusters, a 50-berry sub-sample was taken by carefully cutting each berry at the pedicel with small sharp scissors and then crushing, and the obtained must was then used for technological maturity determinations.

4. Winemaking and wines composition

In 2020, about 30 kg of grapes per replicate for each treatment (ORT, BRB, three replicates per two treatments) were hand-harvested, and each grape sample was destemmed and gently pressed with a hydraulic press to obtain approximately 20 L of juice for each batch. The juices were moved separately to 30-litre stainless steel vats, 50 mg/L potassium metabisulphite (Sigma-Aldrich, St. Louis, MO, USA) was added, and the juices were inoculated with Saccharomyces cerevisiae at 30 g/hL (L'Enotecnica, Nizza Monferrato, Italy). The fermentations were performed at 17 ± 1 °C and monitored daily by measuring wine density until the end of the process (constant density for three consecutive days). At the end of the alcoholic fermentation, the wines were racked, added to potassium metabisulphite at 40 mg/L, bottled in 330 mL glass crown-capped bottles, and stored at 8 °C for two months prior to analyses. The ethanol content, pH, titrable acidity (TA), volatile acidity (VA), free, combined, and total SO2 were determined according to the official methods (OIV, 2009). A kit K-FRUGL 11/05 for the determination of D-fructose and D-glucose was purchased from Megazyme International Ireland Ltd (Megazyme International Ltd, Wicklow, Ireland). Organic acids were analysed, as reported by Izquierdo-Llopart *et al.* (2020). All analyses were performed in triplicate.

5. Statistical analysis

Data collected over four years were subjected to a two-way ANOVA (treatment, year), and the evolution of parameters assessed over multiple samplings during the season was analysed using the function repeated measures ANOVA in IBM SPSS Statistics 24.0 (SPSS Inc., Chicago, IL, USA). Furthermore, wine composition data were subjected to a one-way ANOVA, and means were separated according to Student's *t*-test at $p < 0.05$.

RESULTS

1. Weather evolution and phenology

In the experiment site, 2017 and 2018 were considerably hotter than the two following seasons (Supplemental figure 1). In particular, in the first two vintages, 2022 and 2058 GDD were cumulated from 1 Apr to 31 Oct, vs the 1892 and 1886 GDD recorded in 2019 and 2020.

Even if no significant difference between ORT and BRB was observed in terms of the date of occurrence of main phenological stages, in 2017 and 2018, full bloom and veraison were anticipated as compared to 2019 and 2020 (Supplementary Table 1).

2. Yield components and shoot fruitfulness

No difference in vine yield was observed between treatments in any of the four years of study (Table 1). The yield was affected by the season due to changes in cluster weight and shoot fruitfulness, but no interactive effect between year and treatment was found. ORT had a significantly lower number of clusters per vine (–7 clusters/vine), in agreement with the shoot fruitfulness observed in spring (1.33 inflorescences/shoot in BRB vs 0.71 inflorescences/shoot in ORT). However, ORT also showed a notably higher cluster size (291 g vs 162 g in BRB), resulting in a vine yield comparable to BRB. Interestingly, BRB had looser clusters than ORT (–45 %) due to a lower number of berries per cluster (data not shown), while berry mass was similar between the two varietals. Data were consistent between the three replicates, and standard errors were similar between the two varietals.

Berry mass pre-veraison was similar between the two varieties (Supplemental Figure 2). Then, during ripening, BRB showed an anticipated and higher berry growth, especially in 2017 and 2019, even if at harvest, no difference between ORT and BRB was found (Table 1).

3. Grape ripening and malic acid degradation rates

ORT and BRB showed a very similar grapes TSS accumulation pattern in any of the four years of study (Figure 1).

The onset of TSS accumulation differed according to the season, but both varietals started accumulating sugars concomitantly in all the years. Final TSS was higher in 2017 and 2020 than in 2018 and 2019. Conversely, TA was significantly higher in BRB from preveraison to the end of the season in all the years (Figure 1). Pre-veraison differences in TA were higher in 2017 (+ 9.5 g/L in BRB) and lower in 2020 (+ 3.2 g/L in BRB). Then BRB maintained higher TA until the end of the season with final differences scoring + 2.2 g/L in 2017, + 2.7 g/L in 2018, + 2.3 g/L in 2019 and + 4 g/L in 2020. As a result, data pooled over the four years demonstrate that BRB exhibits higher TA independently by the TSS concentration (Supplementary Figure 3). Differences in grape malic acid during ripening tracked those in grape malic acid concentration (Figure 2A–D). In any of the four years of study, the differences between BRB and ORT peaked right after veraison, and, despite a quite steady reduction of the gap later in the season, in all the years, the final grape malic acid concentration was higher in BRB.

TABLE 1. Yield, shoot fruitfulness, and cluster morphology in grapevines cv. Ortrugo (ORT) and cv. Barbesino (BRB) over four seasons (2017–2020).

	Yield (kg/vine)	Cluster weight (g)	Clusters/vine (n)	Shoot fruitfulness (Infl./shoot)	Cluster compactness (g/cm)	Berry mass (g)
ORT[1]	2.71	291	9	0.71	12.2	2.02
BRB	2.52	162	16	1.33	19.1	1.90
V	ns[2]	***	***	***	***	ns
Y	**	*	**	*	ns	ns
VxY	ns	ns	ns	ns	ns	ns

[1] ORT = Ortrugo, BRB = Barbesino, V = Variety, Y = Year.

[2] *,** and *** denote significant difference per $p < 0.05$. ns = no significant difference.

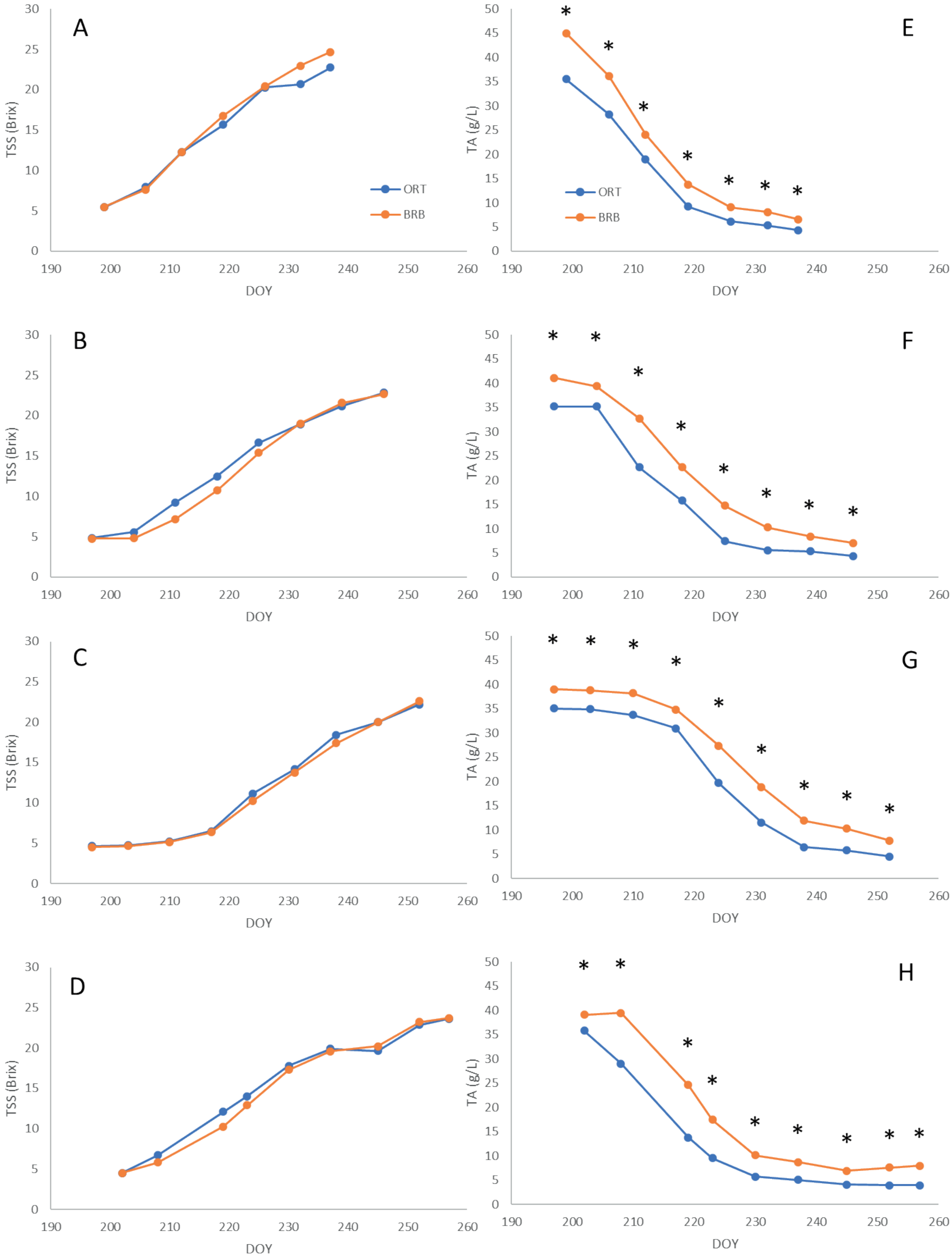

FIGURE 1. Seasonal dynamic of grapes total soluble solids (TSS, A–D) and titratable acidity (TA, E–H) in grapevines cv. Ortrugo (ORT) and cv. Barbesino (BRB) in 2017 (A,E), 2018 (B,F), 2019 (C,G) and 2020 (D,H). DOY= Day Of the Year. Asterisks denote significant difference within date between the two treatments per $p < 0.05$.

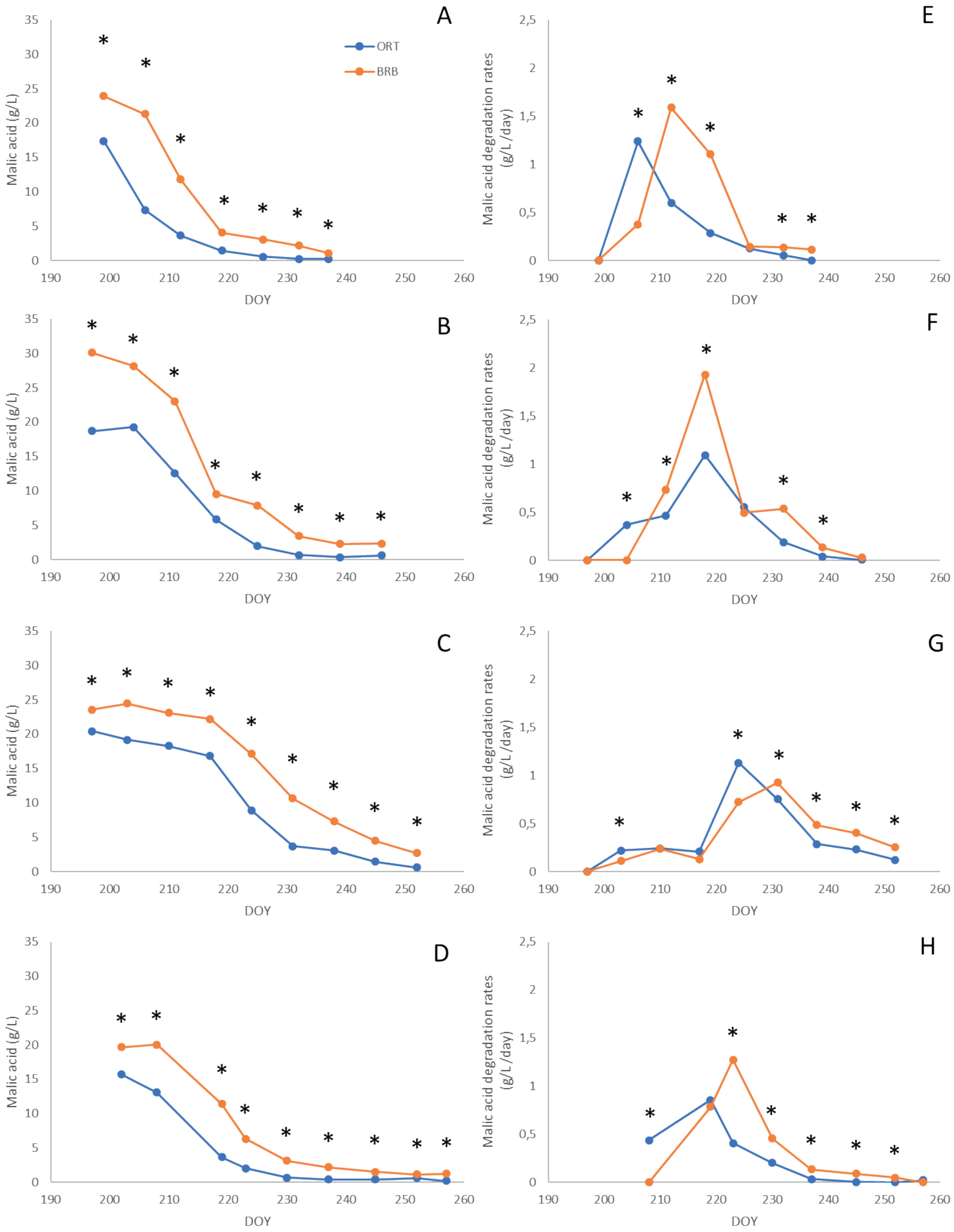

FIGURE 2. Seasonal dynamic of grapes malic acid concentration (A–D) and malic acid degradation rates (E–H) in grapevines cv. Ortrugo (ORT) and cv. Barbesino (BRB) in 2017 (A,E), 2018 (B,F), 2019 (C,G) and 2020 (D,H). DOY= Day Of the Year. Asterisks denote significant difference within date between the two treatments per $p < 0.05$.

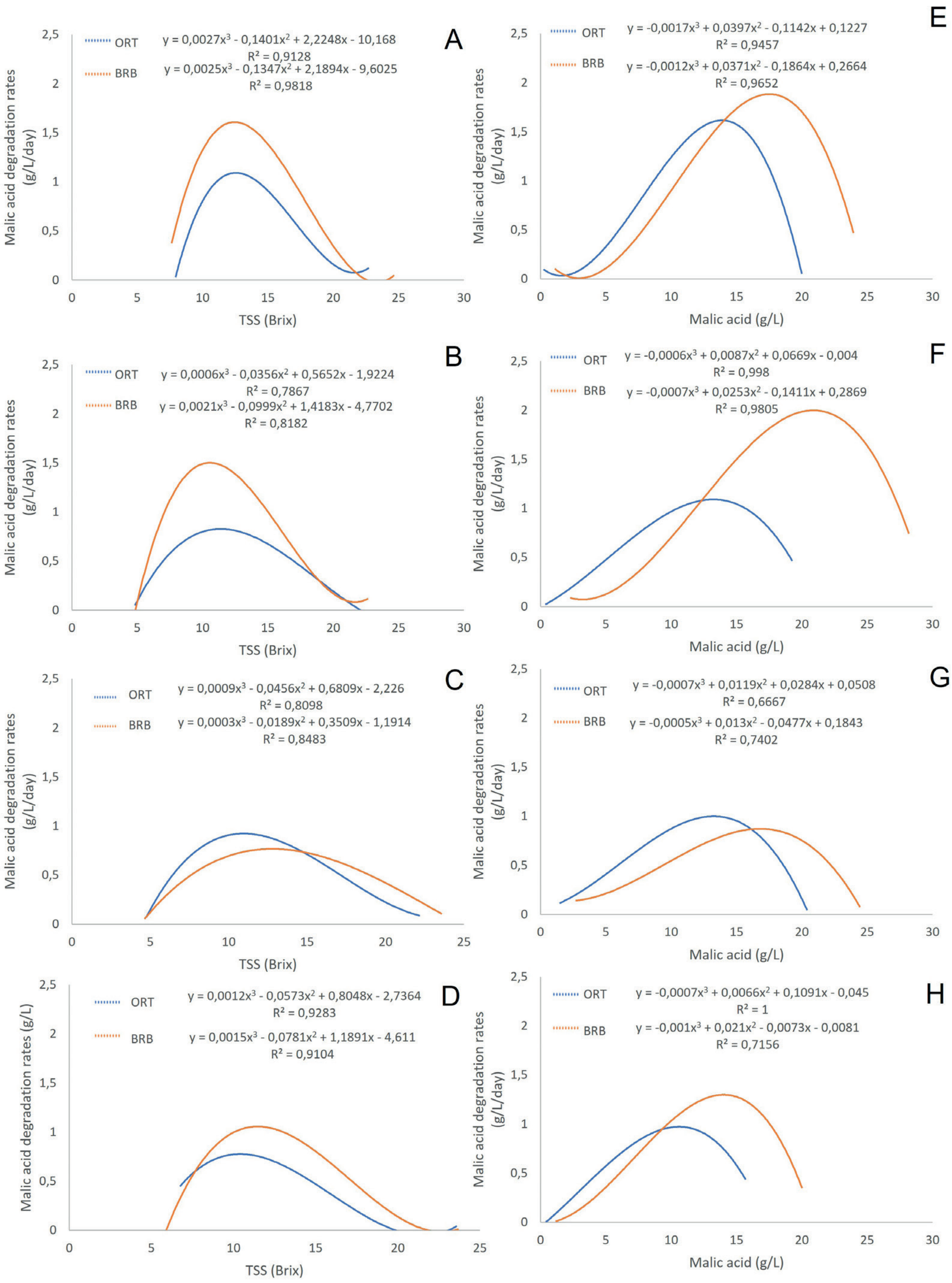

FIGURE 3. Correlation (A–D) between grapes total soluble solids concentration (TSS) and malic acid degradation rates in grapevines cv. Ortrugo (ORT) and cv. Barbesino (BRB) in 2017 (A), 2018 (B), 2019 (C) and 2020 (D). Correlation (E–H) between grapes malic acid concentration and malic acid degradation rates in grapevines cv. Ortrugo (ORT) and cv. Barbesino (BRB) in 2017 (E), 2018 (F), 2019 (G) and 2020 (H). All correlations were significant per $p < 0.05$.

Both varietals exhibited slower sugar accumulation rates on the lower part of the parcel, while no difference in TA decrease was observed within the different sections of the rows, independently by the cultivar.

The analysis of malic acid degradation rates (Figure 2 E–H) reveals that in any of the four years, ORT starts losing malic acid earlier than BRB. Maximum malic acid degradation rates were achieved earlier by ORT in 2017, 2019 and 2020, while peaks coincided in 2018. However, maximum malic acid degradation rates were higher in BRB in 2017, 2018 and 2020. In these three years, BRB showed higher rates also later in the season until the last sampling dates, with a few exceptions. In 2019 only, maximum malic acid degradation rates were higher in ORT, but later in the season, BRB showed higher rates until the end of the season, such as in 2017, 2018 and 2020.

Polynomial third-grade correlations were fitted between grapes TSS and malic acid degradation rates in both varietals for all the years (Figure 3). Analysis revealed that the relationship between TSS and malic acid degradation rates were significantly affected by the season, with BRB showing: i. higher malic acid degradation rates at TSS ranging from 8 °Brix to 15 °Brix, except for 2019; ii. Higher malic acid degradation rates at the TSS interval 15–20 °Brix, except for 2018 when curves were coinciding for TSS > 18 °Brix.

Significant polynomial third-grade correlations were also built for the correlation between instantaneous grapes malic acid concentrations and malic acid degradation rates displayed in the period (Figure 3). BRB showed consistently higher malic acid degradation rates for malic acid concentration > 15 g/L, while the rates were consistently lower when malic acid was lower than 10 g/L.

4. Fruit composition at harvest and final wine characteristics

Picking grapes at TSS = 20 °Brix, no difference was found in TSS or tartaric acid concentration between treatments, whereas BRB showed significantly lower pH (–0.14) and higher TA and malic acid concentration (+ 4.09 g/L and + 2.43 g/L, respectively), independently by the year (Table 2). The tartaric acid on malic acid ratio was significantly higher in ORT due to the low malic acid final concentration.

Table 3 shows the oenological parameters of ORT and BRB wines produced in 2020. All wines were regularly fermented to dryness (residual sugars < 0.80 g/L) within 10 days. No differences were found in ethanol content. ORT wines showed a higher pH (3.20) and a TA of 3.91 g/L, while BRB had a significantly higher TA (5.04 g/L) and lower pH (3.00). Tartaric acid concentrations ranged from 3.71 to 3.80 g/L, with no difference due to the variety. On the other hand, the differences in titratable acidity were mainly due to malic

TABLE 2. Grapes composition and organic acids concentration in grapevines cv. Ortrugo (ORT) and cv. Barbesino (BRB) over four seasons (2017–2020).

	TSS (Brix)	pH	Titratable acidity (g/L)	Malic acid (g/L)	Tartaric acid (g/L)	HT/HM[1]
ORT[1]	21.2	3.15	5.02	0.52	6.52	12.5
BRB	20.9	3.01	9.11	2.95	7.81	2.65
V	ns[2]	**	***	***	ns	***
Y	*	***	**	***	ns	**
VxY	ns	ns	ns	ns	ns	ns

[1] ORT = Ortrugo, BRB = Barbesino, V = Variety, Y = Year, HT/HM = Tartaric acid/malic acid ratio

[2] *,** and *** denote significant difference per $p < 0.05$. ns = no significant difference.

TABLE 3. Oenological parameters and organic acids profile of white wines obtained by cv. Ortrugo (ORT), cv. Barbesino (BRB) in 2020.

	Attributes	ORT	BRB	V[1]
General parameters	Ethanol (% v/v)	10.17	12,50	ns
	Glucose (g/L)	0.06	0.06	ns
	Fructose (g/L)	0.71	0.70	ns
	pH	3.20	3.00	**
	Titratable Acidity (g/L of sulfuric acid)	3.91	5.04	***
	Volatile acidity (g/L of acetic acid)	0.31	0.40	**
	Free SO_2 (mg/L)	17	12	**
	Total SO_2 (mg/L)	82	87	ns
Organic acids	L-(+) Tartaric acid (g/L)	3.90	3.81	ns
	L-(-) Malic acid (g/L)	1.02	2.96	***
	Lactic acid (g/L)	0.19	0.17	ns
	Acetic acid (g/L)	0.16	0.29	***

[1] *,** and *** denote significant difference per $p < 0.05$, $p < 0.01$, $p < 0.005$, respectively. ns means no difference. V = Variety.

acid, which was significantly higher in BRB wines (2.76 g/L vs 1.02 g/L in ORT wines).

DISCUSSION

Maintaining adequate acidity in grapes is one of the main current issues for producing white and sparkling wines in warm climates (Palliotti *et al.,* 2014, Poni *et al.,* 2018). While managing grape sugars is quite easier due to their dependence to leaf to fruit ratio and crop load (Kliewer and Dokoozlian, 2005), controlling organic acids is definitely more complicated since they respond mainly to air temperatures and genetic traits (Famiani *et al.,* 2014; Ford, 2012). For this reason, the most efficient strategies to increase grape acidity consist of the so-defined long-term adaptation strategies, encompassing, for instance, site selection, altitude, and varietal choice (Palliotti *et al.,* 2014). In this framework, many research groups and wine districts are re-considering old neglected cultivars and clones (Arrizabalaga-Arriazu *et al.*, 2018; Arrizabalaga-Arriazu *et al.*, 2020; Arrizabalaga-Arriazu *et al.*, 2021, Frioni *et al.,* 2020a, 2020b, Antolín *et al.,* 2020, 2022, Torres *et al.,* 2018, Medrano *et al.,* 2018), or identifying *quantitative trait loci*s codifying for acidity to breed new varieties coping with warming trends (Bigard *et al.,* 2018; Bigard *et al*., 2020).

In this context, the definition of the best parameters to describe the attitude of a genotype in maintaining satisfying acidity at the final stages of ripening is a key factor. In our work, ORT had lower acidity at harvest than BRB in four consecutive years, with an average difference of 4.07 g/L (Table 2). Such a consistent and stable behaviour was accompanied by a lower pre-veraison TA and malic acid concentration (Figures 1 and 2A–D). However, no significant correlation was found between the seasonal pre-veraison malic acid concentration and its final minimum abundance when pooling the two varietals together, nor considering them separately (data not shown). Therefore, seasonal final malic acid concentration depended on other factors.

After veraison, malic acid is mainly lost due to berry respiration, which, in turn, is driven by temperatures and substrate abundance (Ford, 2012). In grapes, this process leads to a drastic reduction of the initial malate pool from 20–40 g/L to 0–4 g/L. In white/sparkling wines, optimal technological ripening consists of the balance between TA and TSS (Poni *et al.,* 2018). However, Figure 2E–H demonstrates that time of achievement of maximum malic acid degradation rates changes according to the genotypes, even if those exhibit an identical TSS accumulation pattern (Figure 1). In detail, ORT started degradation of malic acid earlier in all four years, then rates peaked earlier than BRB in 2017, 2019 and 2020 (Figure 2E,G,H), whereas in 2018, the two cultivars achieved maximum malic acid degradation rates concomitantly (Figure 2F). Data suggest that a strong varietal factor drives timings in malic acid degradation rates, which can significantly vary between genotypes independently by the TSS accumulation onset time and later trend. This confirms that TSS dynamics and timings cannot be taken as a representative parameter to define varietal TA and malic acid loss trends.

Additionally, other factors leading to malic acid loss is dilution due to berry growth and volume increase. In our work, BRB and ORT started from similar pre-veraison berry weight levels and exhibited no difference at harvest (Supplementary Figure 2, Table 1). Therefore, higher final malic acid concentrations in BRB seem to be unrelated to a different berry volume expansion from veraison to harvest.

Surprisingly, in three years out of four, BRB had higher maximum post-veraison malic acid degradation rates. In 2019 only, ORT scored higher maximum rates (Figure 2G). The 2019 season was the coolest among the four examined, displaying delayed veraison and TA loss as compared to the others, and this can be one of the reasons for such occurrence. In detail, ORT maximum malic acid degradation rates ranged between 0.89 g/L (2020) and 1.24 g/L in 2017, whereas in BRB, rates ranged between 0.92 g/L (2019) and 1.93 g/L (2018). Overall, our data suggest that maximum malic acid degradation rates depend on the pre-veraison malic acid abundance, and that varieties exhibiting higher initial malate pool are also those more sensitive to weather evolution in terms of seasonal changes in maximum malic degradation rates.

After veraison, malic acid concentration and its degradation rates were consistently higher in BRB in all the years examined (Figure 2). Therefore, low malic acid degradation rates during ripening do not mean higher final acidity in grapes. Conversely, our data demonstrate that a simple analysis of malic acid loss rates can be misleading since the varietals displaying lower post-veraison malic acid degradation rates are those having the lower final TA and malate concentration.

As expected, correlations between TSS and TA or malic acid depict higher TA in BRB for any TSS value (data not shown), in agreement with Frioni (Frioni *et al.* 2020a, Frioni *et al.*, 2020b). In Figure 3, a polynomial correlation between grapes TSS and malic acid degradation rates was built for the two genotypes. Interestingly, in any of the four years, BRB showed higher rates for TSS >15 °Brix, even if in 2018 (Figure 3B), correlations of the two varietals overlapped for TSS > 18 °Brix. Considering the above-mentioned link between malic acid degradation rates and its abundance, higher rates at TSS > 15 °Brix mean retaining higher TA and malic acid at TSS, approaching optimal values for harvest.

In Figure 3E–H, correlations between instantaneous malic acid concentration and malic acid concentration rates are reported. Data reveal that higher malic acid degradation rates reported for BRB in Figure 2E–H are essentially a smokescreen: even if rates are higher in BRB for malic acid concentrations > 15 g/L, ORT displays higher malic acid degradation rates for malate < 10 g/L in all the years examined. Additionally, despite a certain seasonal variability in terms of peaks and their occurrence, the transition from higher rates in BRB to higher rates in ORT was something consistent and steady in all four years. This transition occurred at 13 g/L in 2017, at 12.5 g/L in 2018, at 16 g/L in

2019 and at 10 g/L in 2020. Our data demonstrate that there is a strict genetic control over malic acid degradation rates at varying malic acid concentrations and that this control easily overcomes seasonal effects, which instead affect a simple analysis of TSS, TA, veraison times, and, particularly, malic acid degradation rates.

Notably, BRB exhibited an anticipated berry growth right after veraison. This could have contributed to the high malic acid degradation rates found in BRB at malic acid concentration rates >10 g/L. Therefore, we cannot exclude a possible role in malic acid degradation rates due to different timing in berry volume expansion.

Wine analytic data confirms that if malo-lactic fermentation is not undertaken, wines tend to preserve the relative differences in TA at harvest (Table 3). Although the pH and titratable acidity of wines is an essential parameter for ensuring wine quality, the organic acids (i.e., tartaric acid and malic acid) and low molecular weight play a crucial role in maintaining the chemical and microbiological stability of the wine, its colour, and taste (Mato *et al.*, 2005).

Finally, when evaluating genotypes for their adaptability to climate change, productivity cannot be exchanged for better fruit composition. In our work, BRB demonstrated a comparable yield to ORT, with potentially lower susceptibility to rots, due to its lower cluster compactness (Table 1).

In conclusion, our work demonstrates that when evaluating genotypes of grape acidity, malic acid degradation rates itself can be a misleading parameter since rates mainly respond to malic acid abundancy, and varietals having higher malic acid pool pre-veraison or during ripening exhibit higher rates, no matter what the final TA or malic acid concentration is. Conversely, our work highlights the potentialities of the analysis of relationships between instantaneous malic acid concentration and malic acid degradation rates. The polynomial correlations built allow us to identify the genotypes that, in the given environment, can reduce the loss of acidity when most of the pre-veraison malic acid pool is already depleted and maintain a final high must acidity. The approach can be of relevance for the determination of varieties maintaining higher acidity at different scales: i. In farm, when establishing new vineyards; ii. In wine districts revising appellations according to local or international germplasm; iii. At the research level, for phenotyping or breeding new varieties or for selecting accessions within progenies.

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