Research Article

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The effect of terroir on volatilome fingerprinting and qualitative attributes of non-irrigated grapes reveals differences on glycosylated aroma compounds

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Abstract

BACKGROUND: 'Xynisteri' is considered as the reference white grape cultivar in Cyprus with remarkable adaptation to adverse edaphoclimatic conditions and appreciable oenological properties that renders it as an appropriate cultivar for studies within a global context due to climate change. To this aim, two distinct non-irrigated plots with different climatic conditions, soil properties and levels of rainfall were selected; Koilani [KO, altitude 800 m, 76% calcium carbonate ($CaCO_3$) content, pH 7.97, average temperature: 16.5 °C, rainfall: 229 mm] and Kyperounda (KY, altitude 1200 m, $CaCO_3$-free soil, pH 6.47, average temperature: 14.9 °C, rainfall: 658 mm). An array of physiological, biochemical and qualitative indices during successive developmental stages (BBCH 75–89) were determined. During the advanced on-vine developmental stages (BBCH 85–89), the aromatic profile of grapes was assessed with the employment of gas chromatography–mass spectrometry (GC–MS). Such analysis was complemented with non-destructive chemometric analyses.

RESULTS: Berry ripening process substantially differed on the examined plots; BBCH 89 stage reached at 267 and 303 Julian days for KO and KY, respectively. Results indicated that berry weight, soluble solids content (SSC) and α-amino nitrogen were higher in KO than in KY, with exception made for ammonium nitrogen content. A total of 75 compounds, including aliphatic alcohols, benzenic compounds, phenols, vanillins, monoterpenes and C13-norisoprenoids were identified and quantified. The variations of mesoclimatic conditions affected the volatile organic compound (VOC) profiles at the fully-ripe stage, showing a considerable rise in glycosylated aroma compounds, especially monoterpenes and benzenic compounds. In particular, the higher amount of glycosylated aroma compounds were obtained in KY berries up to mid-ripe, whereas KO showed higher glycosylated aroma compounds at fully-ripe stage. Results reported herein indicate that aroma profile of 'Xynisteri' grapes varied substantially in the examined terroirs. Interestingly, the limited rainfall in KO non-irrigated vine did not compromise qualitative and aromatic properties of berries.

CONCLUSIONS: The present study aimed at dissecting the impact of terroir on bush-trained, non-irrigated grapevines of a cultivar appropriate for extreme climate change scenarios. The volatilome fingerprint was highly variable among the

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examined plots; such results can be further exploited at vinification level towards production of single vineyard premium end products.

Supporting information may be found in the online version of this article.

Keywords: volatile organic compounds (VOCs); GC–MS; E-nose; FT-NIR; indigenous cultivar; soil properties; altitude

INTRODUCTION

The secondary metabolism compounds like aromas regulate the nutraceutical and organoleptic properties of grape berries or wines, including flavour or odour.[1] The aromas of grapes are volatile organic compounds (VOCs) or glycosylated VOC precursors belonging to terpenoids (monoterpenes, C13-norisoprenoids, and sesquiterpenes), aliphatic C6–C9 volatile compounds (alcohols and aldehydes), shikimate pathway derivatives (benzenic derivatives, volatile phenols, vanillins), methoxypyrazines, as well as volatile thiols (or mercaptans).[2]

Grape cultivars do not produce the same aromas and flavours due to the presence or absence of specific compounds as well as due to the variations in the ratios of the compounds that constitute the grape's aroma profile.[3] Terpenoids, in the form of glycoside conjugates, comprise some of the most significant aroma compounds of grape berries.[4] Thus, terpenoids, and primarily monoterpenes, have been extensively studied.[5] The aroma content of grapes can be enhanced by using different approaches, including agronomic practices such as leaf removal, irrigation, foliar fertilization, bunch thinning, canopy training systems, as well as exogenous compound applications.[1,6,7]

Vine growth, physiology and vigour are influenced by several factors such as cultivar, climate, soil composition and vine-growing practices, which are collectively called the 'terroir'[8-11] and greatly affect berry composition.[12] Vine development and production are highly dependent on climate and topography of the vines and especially the temperature, which can alter the quality of the vintage every year.[13] Altitude, which is considered the main factor in terroir, can have an effect on the ripening and its quality.[14] The texture, the depth and the pH of the soil affect the root depth, the water and nutrient holding ability which in turn determine the water and nutrients the vine accesses.[15] Soil properties may significantly affect the vine vigour and the quality of the wine.[16] Furthermore, soil physiological properties can also determine the uniqueness of the composition of berries when vines are cultivated in a specific climate. VOCs as an integral part of wine quality are heavily influenced by the cultivar, the methods of cultivation, the weather, the location and soil properties, the condition of the plant and the wine-making practices.[17] In general, water deficit and high temperatures influence the growth of vines and the size of the berry, the latter largely determining the quality of the wine produced.

Cyprus is one of the very few phylloxera-free areas in the world with its own rooted vineyards. In addition, the vast majority of vineyards are non-irrigated. 'Xynisteri' is the reference white-fleshed Cypriot cultivar, used both for the production of premium white dry wines and for 'Commandaria', a Protected Designation of Origin (PDO) dessert wine, originating from dehydrated grapes.[18] Despite its considerable societal and economic impact, 'Xynisteri' has been scarcely characterized regarding its secondary metabolism with scarce information regarding the varietal VOCs governing its aroma.[19-21] Thus, the aim of the current study was to evaluate the impact of terroir cv. 'Xynisteri' cultivar with the employment of an array of high-throughput analytical approaches.

MATERIALS AND METHODS

Plant material, vineyard cultivation protocol and experimental set up

In 2020, field experiments were conducted in two 50-year old commercial vineyards of own-rooted cv. 'Xynisteri' grapevines (*Vitis vinifera* L.) in Koilani (KO) (34°50′36.3″ N 32°51′13.3″ E; 800 m and sandy clay loam soil) and Kyperounda (KY) (34°55′42″ N 32°58′09″ E; 1200 m and sandy clay loam soil), both located at Limassol district, Cyprus. Soil profile analysis was quantified based on particle size, organic matter, pH, electrical conductivity and total/active calcium carbonate ($CaCO_3$) analyses. Air temperature (°C), rainfall (in millimetres) and relative humidity (RH, %) were recorded by meteorological station installed in the vicinity of the study sites.

The KO vineyards were spaced 2 m between rows and 2 m within the row, trained as highbush vines at 0.75 m, using the spur pruning system. The KY vineyards were spaced 1.5 m between rows and 1.8 m within the row, trained as low bush vines at 0.65 m, using the spur pruning system. In both locations, winter pruning took place during February and no fertilization was applied. Soluble and dustable sulphur and *Bacillus thuringiensis* formulations were applied during April–May and in June–August to combat powdery mildew and *Lobesia botrana*, respectively. Additionally, the soil in KO was ploughed two times using a rotary hoe at 25 cm, in March–May, shoots were tipped during bloom in May, while weeds between the plants were mechanically removed. In contrast, the soil in KY had not been ploughed for years, shoots were tipped during bloom in June and weeds between the plants were removed by hand. In addition, the Huglin index (HI) and growing degree-days (GDD) equation were calculated to define the climate characteristics.[22]

The experiment setup was based on a completely randomized design, consisting of three plots (four grapevines per plot) per location. Each experimental plot was considered as one biological replication. To determine the grapevine developmental stages, the Biologische Bundesanstalt, Bundessortenamt, Chemische Industrie (BBCH) scale was used. Leaf and berry samples were collected at advanced on-vine developmental stages, spanning from BBCH 75 up to BBCH 89. Leaf, berry and juice were sampled and analysed for an array of physiological, biochemical, qualitative indices and analytical approaches as described later. For biochemical analyses, leaves (ten per plot) and berries (50 per plot) were flash frozen in liquid nitrogen in the vineyard, ground into powder in the laboratory, and subsequently stored at −80 °C until use. The schematic overview of the experimental setup and the

handling procedure for further experimentation of leaves and grapes at different developmental stages are depicted in Supporting Information Fig. S1.

Soil analysis

Soil profile description was conducted after preparation of soil pits and soil sampling after the definition of three soil horizons. The soil samples were air-dried for 2–4 days depending on moisture and texture. After drying, the soil samples were passed through a different sieve and 1 kg for each sample was used for particle size analysis, organic matter, pH, electrical conductivity and total/active $CaCO_3$ determination.

Particle size analysis was conducted using the hydrometer method as described elsewhere.[23] Briefly, after dispersion of 40 g of soil with 50 mL of 5% sodium hexa-metaphosphate using a commercial type blender, the suspension was transferred to a 1 L volumetric cylinder and measurements were taken with an ASTM 152H hydrometer at 40 s and 2 h for the estimation of sand and clay fractions, respectively. The soil pH was measured as described according to Thomas.[24] Soil pH was measured in 1:1 soil water ratio using a combined electrode connected to a digital pH meter (CRISON GLP 21). Organic matter (OM) was measured using the wet oxidation method of Walkley–Black.[25] Briefly, 1 g of soil was ground to pass a 0.5 mm sieve, subsequently transferred to a 500 mL volumetric flask and 10 mL of 1 N potassium dichromate ($K_2Cr_2O_7$) were added followed by 20 mL of concentrated sulphuric acid. The mixture was left standing for 30 min. After adding 3–4 drops of *o*-phenanthroline indicator, the excess 1 N $K_2Cr_2O_7$ was titrated with 0.5 N iron sulphate ($FeSO_4$). A correction factor '*f*' of 1.3 was used to estimate total organic carbon and a factor of 2.0 was used to convert organic carbon to OM content.

Soil salinity was measured in a saturated extract. Briefly, after the saturated paste was formed it was left to stand for at least 2 h and then the extract was obtained after vacuum filtration. Specific electrical conductivity was measured with a conductivity meter.[26] The amount of $CaCO_3$ was measured with a digital calcimeter (FOGL, bd INVENTIONS). For the determination of active $CaCO_3$, 1 g of soil was shaken with 25 mL of 0.1 M ammonium oxalate for 2 h. After centrifugation, 10 mL of ammonium oxalate were titrated with potassium permanganate to determine the unreacted ammonium oxalate.[27]

Physiological measurements, photosynthetic pigment analysis and cellular damage indicators

Stomatal conductance, stem water potential (SWP) and soil plant analysis development (SPAD) measurements were taken from four leaves per plot between 12 p.m. and 2 p.m at different developmental stages (BBCH 75–89) from both locations. To measure stomatal conductance, ΔT-Porometer AP4 (Delta-T Devices, Cambridge, UK) was used, following the manufacturer's instructions. SWP values were measured on the leaves, as well as measurement of leaf SWP after enclosure in dark plastic bags for 60 min to allow water potential equilibration. SPAD measurements were conducted in four leaves per plot with a hand-held chlorophyll meter SPAD-502Plus (Konica Minolta Inc., Tokyo, Japan) following the manufacturer's instructions.

Photosynthetic pigments were extracted with dimethyl sulphoxide (DMSO) from four leaves per plot at different developmental stages (BBCH 75–89) and were measured spectrophotometrically (Infinite 200® PRO; TECAN, Männedorf, Switzerland) at 661, 643, 470, and 534 nm, respectively.[28] The concentrations of chlorophylls (Chla, Chlb and total) and carotenoids were calculated using the equations proposed by Misra and Dey,[29] whereas anthocyanins concentrations were quantified using the equations reported by Nikiforou *et al.*[30]

Lipid peroxidation, hydrogen peroxide (H_2O_2) and nitrite-derived nitric oxide (NO) (cellular damage indicators) were determined in leaves for successive on-vine developmental stages, spanning from BBCH 75 to BBCH 89. Lipid peroxidation was assessed through malondialdehyde (MDA) content resulting from the thiobarbituric acid (TBA) reaction using the Lambert–Beer law, with extinction coefficient of 155 L $mmol^{-1}$ cm^{-1}.[31] The H_2O_2 content was evaluated based on the oxidation of iodide ion (I^{-1}) to iodine (I), after the reaction of H_2O_2 with potassium iodide.[32] The NO content was measured using the Griess reagent as described elsewhere described.[33]

Total nitrogen and stable isotope ratios

Total nitrogen was determined with the Kjeldahl method at different developmental stages (BBCH 75–89) from KO and KY as described by Bremner.[34] Briefly, 5 g of leaves were digested with 10 mL of concentrated sulphuric acid and 3.5 g of potassium sulphate-catalyst mixture for at least 5 h. The digest was distilled with 10 mol L^{-1} sodium hydroxide (NaOH) in a Buchi B-324 distillation unit. The distillate was titrated with 0.05 N standard sulphuric acid using 2% boric-acid indicator. Stable isotope ratios $\delta^{13}C$ and $\delta^{15}N$ were determined at developmental stage BBCH 89 with a SERCON HS 2022 analyzer coupled with a EuroVector EA 3000 Elementary Analyser.

Qualitative attributes

Two hundred berries per plot were sampled in order to isolate the must with a commercial juicer on a weekly basis, spanning from end of veraison until fully-ripe stage (BBCH 85–89 for KO and BBCH 81–89 for KY). Soluble solids content (SSC), titratable acidity (TA), ripening index (RI), pH, ammonium nitrogen, α-amino nitrogen, malic and tartaric acid were the quality attributes of must assessed, as described elsewhere.[20] Weight and number of all bunches of grapes were measured from four grapevines per plot at fully-ripe stage. It should be noted that the four grapevines per plot used to compute yield indicators, CIELab parameters and flesh firmness (FF), were different from the other four grapevines per plot used for all other measurements. All relevant measurements were performed as stated by Georgiadou *et al.*[21]

Must from the tissue of berries (BBCH 75, BBCH 85, BBCH 87, BBCH 89) was used for sugar determination as described elsewhere.[35] Total soluble sugars (TSS), sucrose and glucose were measured spectrophotometrically,[36] while fructose was determined according to Edewor–Kuponiyi protocol.[37]

Analysis of berry aroma compounds

Solid-phase extraction (SPE) was used for the analysis of berry glycosylated aroma compounds (aliphatic alcohols, benzenic compounds, phenols, vanillins, monoterpenes, C13-norisoprenoids).[1] Each sample was extracted from 100 fresh berries (for each plot) at BBCH 85–89 for KO and BBCH 81–89 for KY, respectively.

For the preparation of grape samples, the skins of 100 berries were extracted and used for each replicate with 20 mL of methanol for 1 h, while flesh and juice were placed in a glass containing 100 mg of sodium metabisulphite, whereas seeds were disposed. After 1 h, the skins were placed together with the flesh and juice, to which 150 mL of the pH 3.2 tartaric buffer solution (2 g L^{-1} sodium metabisulphite, 5 g L^{-1} tartaric acid and 22 mL L^{-1} NaOH 1 N) had been added and with the use of an immersion blender,

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they were homogenized (Ultra-Turrax; IKA, Staufen, Germany). After centrifugation at 5000 × *g* for 5 min, the supernatant was collected in a flask, and the pellet was washed with an additional 100 mL of pH 3.2 tartaric buffer solution. After a further centrifugation, the supernatant was added to the first one, the volume adjusted to 400 mL by adding further tartaric buffer solution, and stored at −20 °C. To the extract was added a pectolytic enzyme (Vinozym FCEG) and incubated overnight at room temperature to make it limpid. The day after it was centrifuged, just before the SPE procedure.

For the extraction of aroma compounds, a total of 200 µL of 1-heptanol (40 µg mL^{-1}) was added to these extracts as an internal standard and eluted through a 5 g C18 cartridge (Mega Bond Elut; Agilent, Santa Clara, CA, USA), which had been activated with 20 mL of methanol and 50 mL of water. The cartridge was washed with 100 mL of water and then with 30 mL of dichloromethane so as to recover the fraction containing free compounds dehydrated with sodium sulphate anhydrous and concentrated to 200 µL before the analysis. The glycosylated compounds were eluted with 30 mL of methanol. Under vacuum the methanol was evaporated and the residue solubilized in 5 mL of a phosphate–citrate buffer [0.1 mol L^{-1} disodium hydrogen phosphate (Na_2HPO_4) and 50 mmol L^{-1} citric acid; pH 5]. The glycosidically-bound fraction was hydrolysed with 600 µL of a glycosidic enzyme with strong glycosidase activity (CYTOLASE M102; Ferrari) and kept at 40 °C overnight (16 h). Next, 200 µL of 1-heptanol (40 µg mL^{-1}) were added as an internal standard. The mixture, containing the aglycones released by enzymatic hydrolysis, was then centrifuged and eluted through a 1 g C18 (Mega Bond Elut; Agilent) which had been previously activated with 5 mL of methanol and 10 mL of water. The fraction containing the aglycones was eluted with 6 mL of dichloromethane, dehydrated with sodium sulphate anhydrous, and concentrated to 200 µL before analysis.

Chromatographic analysis was performed as described by D'Onofrio and coworkers[1,3] using an Agilent 7890A gas-chromatograph coupled with an Agilent 5975C quadrupole mass spectrometer (Agilent, Waldbron, Germany). Helium was the carrier gas at a constant flow rate of 1 mL min^{-1}. The capillary column was an HP-Innowax [30 m length, 0.25 mm inner diameter (i.d.), 0.25 mm film thickness] from Agilent. The temperature programme of the column oven started at 30 °C, then was increased at 30 °C min^{-1} to 60 °C for 2 min, at 2 °C min^{-1} to 190 °C, and at 5 °C min^{-1} to 230 °C for 10 min. The mass spectrometry (MS) detector scanned within a mass range of *m/z* 30–450. There were tentatively identified volatile compounds by comparing the mass spectra with those available in the data system library (NIST 08, National Institute of Standards and Technology, Gaithersburg, MD, USA; 2008) and using published retention indices.[1,3] A positive characterization was achieved when a volatile compound was identified with a probability of > 70%, and when possible, the identity of the compounds were further confirmed by comparison of the retention times with 30 authentic standards. Calibration curves of some of these authentic standards were chosen to quantify the compounds of the same class sub-group (arranged by functional moiety) whose standards were not available (Supporting Information Table S2). For gas chromatography–mass spectrometry (GC–MS) analysis, matrices of the original component data (metabolite concentrations *versus* treatment and developmental stages) were standardized in order to present (via a hierarchical clustering analysis heatmap) differences in the relative metabolite content using the gplots version 3.0.1 (heatmap.2 command; R Foundation for Statistical Computing, Vienna, Austria).

Non-destructive detections and chemometric analysis

Must samples were extracted from 200 fresh berries (for each plot) from BBCH 85–89 for KO and BBCH 81–89 for KY were used for E-nose (electronic nose) measurements and Fourier-transform near-infrared (FT-NIR) spectra detections. The samples were centrifuged at 5500 rpm for 5 min at 4 °C and the supernatant was used for the analysis. The method described by Santonico *et al.*,[38] slightly adapted, was followed for E-nose detections. Briefly, 20 mL of must for each replicate (three biological replicates per treatment) were incubated in 50 mL glass vials at 25 °C for 20 min. After the equilibration, the headspace generated into the capped vials was extracted by a constant flow of filtered air through the perforable septum, then pumped into the E-nose device to be exposed to the sensor cell. Measured data were calculated as the resonant frequency shift between the signal of the sensors exposed to pure nitrogen (used as reference signals) and that obtained from the sample. The employed E-nose, a prototype device designed and assembled at the University of Rome Tor Vergata, Rome, Italy, is based on an array of eight quartz microbalances (QMBs) which are electromechanical resonators whose resonant frequency changes proportionally according to the mass adsorbed onto the sensor surfaces. Sensors were constructed with AT-cut quartz plates oscillating in the thickness-shear mode at a resonance frequency of 20 MHz. QMBs were functionalized by solid-state layers of metalloporphyrins (TPPs).[39] Samples were analysed in triplicate. The dedicated software (TEN NET, University of Rome Tor Vergata) detected the E-nose signals coming from the array of eight-sensor and representing the characteristic

Table 1. Particle size analysis, organic matter, pH, electrical conductivity and total/active calcium carbonate of soil from KO and KY

Location	Particle size analysis (%)			Organic matter (%)	pH	Electrical conductivity (mS/cm)	Total calcium carbonate (%)	Active calcium carbonate (%)
	Sand	Silt	Clay					
Koilani (KO)	46.00 ± 12.06 a	19.60 ± 6.11 a	34.40 ± 6.43 a	1.18 ± 0.36 a	7.97 ± 0.03 a	0.43 ± 0.11 a	75.97 ± 8.63	13.67 ± 0.88
Kyperounda (KY)	48.00 ± 4.16 a	26.93 ± 2.40 a	25.07 ± 2.40 a	1.10 ± 0.54 a	6.47 ± 0.22 b	0.28 ± 0.06 a	nd	nd

Data are the means of three replications ± standard error; nd, not detectable. Similar letters indicate no statistically significant differences between the two locations examined.

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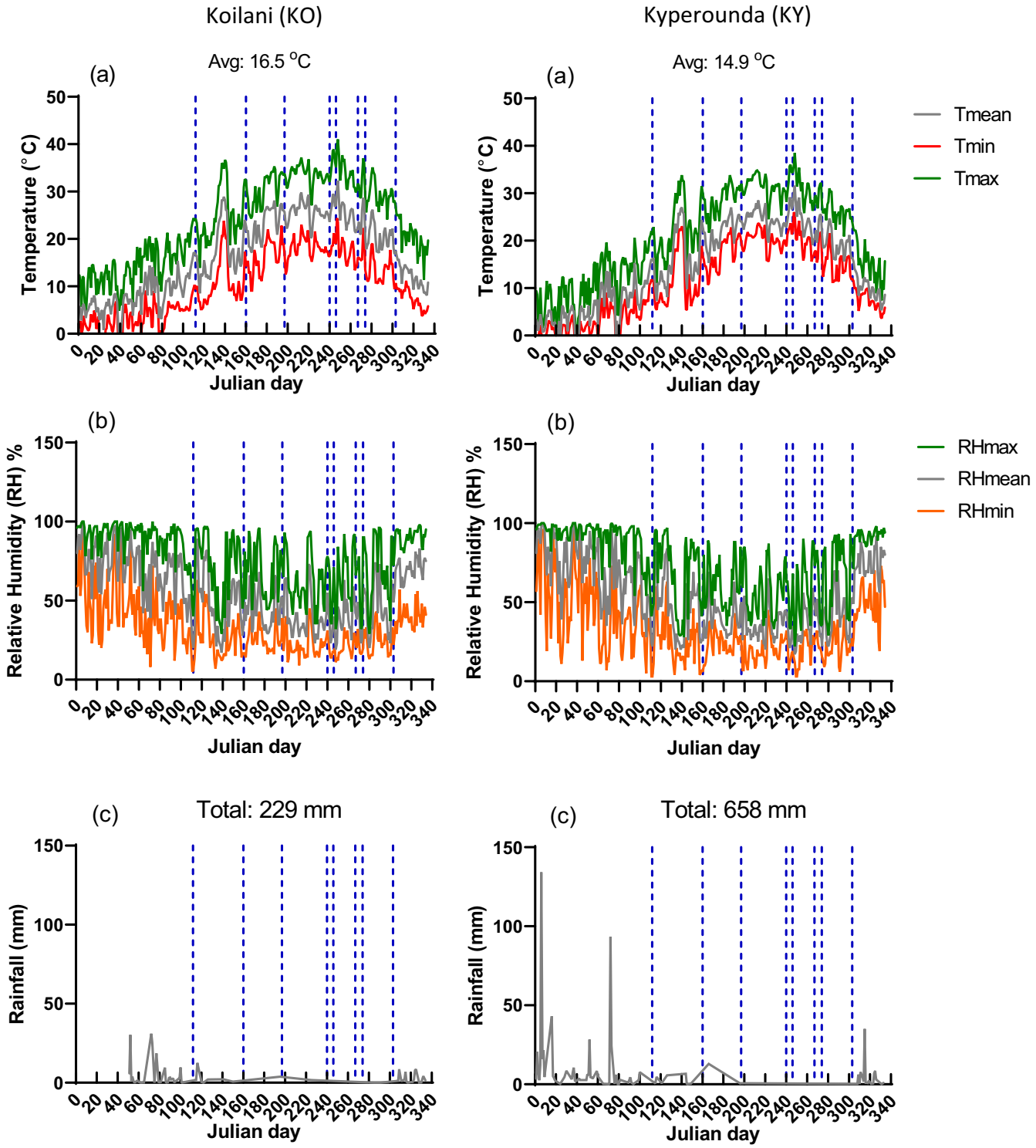

Figure 1. Changes in (a) daily air temperature (Tmax, Tmean, Tmin), (b) daily relative humidity (RHmax, RHmean, RHmin) and (c) daily rainfall (mm) during berry development in the experimental vineyards from Koilani (KO) and Kyperounda (KY). For KO, the 112, 160, 197, 240, 246 and 267 Julian days correspond to BBCH 07, BBCH 65, BBCH 75, BBCH 85, BBCH 87 and BBCH 89 as shown with blue dotted lines. For KY, the 112, 160, 197, 240, 246, 267, 274 and 303 Julian days correspond to BBCH 07, BBCH 65, BBCH 75, BBCH 81, BBCH 83, BBCH 85, BBCH 87 and BBCH 89 as shown with blue dotted lines

aromatic patterns of each must sample. Original data were then used in statistical pretreatments and chemometric calculations which were performed by statistical reprocessing software as reported later. FT-NIR spectra were transformed from transmittance to absorbance (log 1/*T*), then autoscaled and employed as X-block variables for principal component analysis (PCA), and principal component regression (PCR) computations. In the latter, selected quality attributes (i.e., SSC, TA, tartaric and

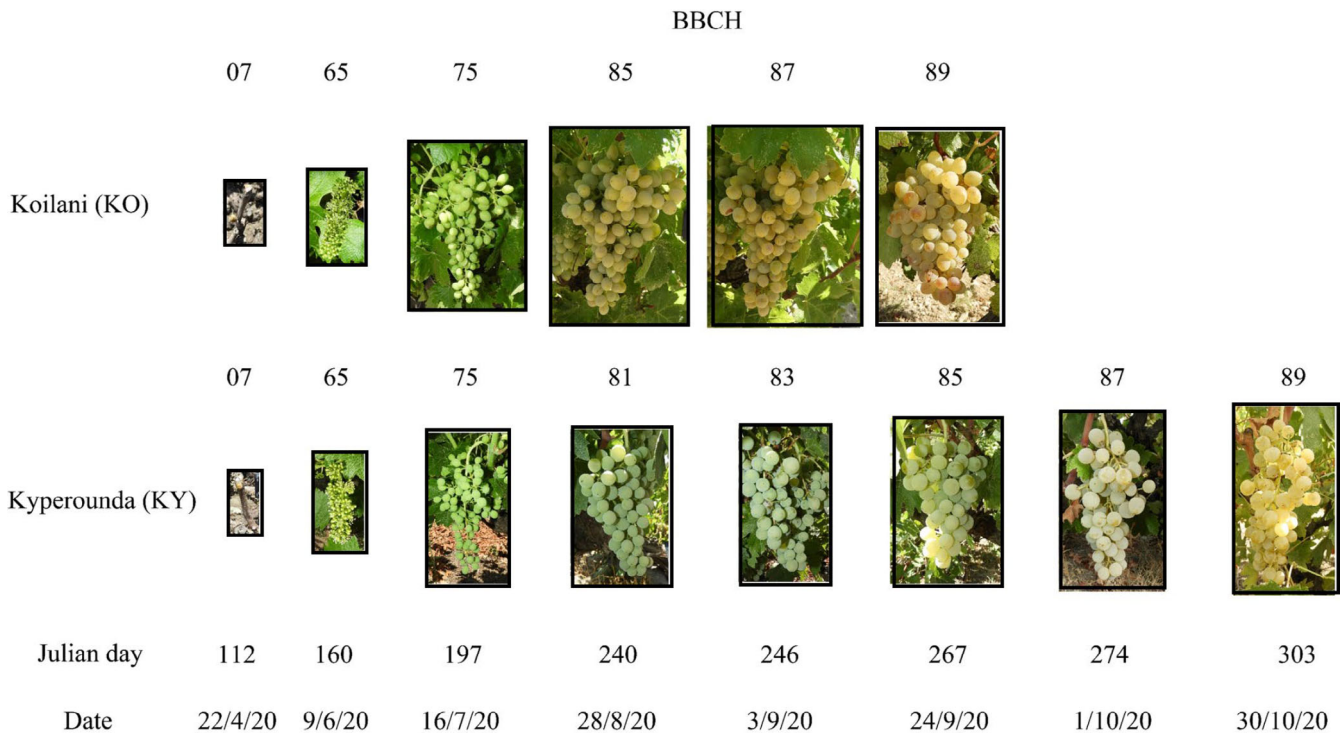

Figure 2. Phenological stages of Koilani (KO) and Kyperounda (KY) brunches according to the BBCH scale.

malic acid content, and pH) represented the dependent variables (Y-block).

Must samples (BBCH 85–89 for KO and BBCH 81–89 for KY) prepared as described earlier were placed in a 10 mm cuvette and analysed by FT-NIR spectra using an FT-NIR NeoSpectra OEM module (NeoSpectra™ by Si-Ware) equipped with external light source and detector. The transmittance method of detection was used to perform the measurements. Acquisition was conducted in the 1300–2600 nm range, with 8 nm wavelength increments and five spectra per average, which represented the spectral measurement of a single sample. Collected spectra were manipulated and employed for chemometric calculations. Data belonging to the E-nose patterns were centred (mean centred) then used for cluster analysis (by Ward's method) computation based on PCA iteration. For all chemometric calculations, in both cases of FT-NIR spectra and E-nose data, a cross-validation by 'leave-one-out' method was used. All multivariate computations, for both E-nose and FT-NIR data, were performed by using Matlab R2013a (MathWorks, Natick, MA, USA), and PLS Toolbox (Eigenvector Research, Inc., Manson, WA, USA). The results were graphically reported and interpreted as scoreplots, scatterplots and loadingplots.

Statistical analysis

The one-way analysis of variance (ANOVA) following Duncan's multiple way test with a significance level of 5% ($P \leq 0.05$) and significant difference at 5% ($P \leq 0.05$) with *t*-test: independent two sample for means were performed out using the SPSS version 25.0 (SPSS, Chicago, IL, USA) software package. Figures were created using Prism 8.3.1 (GraphPad, La Jolla, CA, USA). Statistical analysis of GC–MS, E-nose and FT-NIR analysis are referred to the respective sections earlier.

RESULTS AND DISCUSSION

Soil properties and mesoclimatic conditions of the examined plots

Soil analysis showed considerable differences among the examined plots (Table 1). Striking differences were detected in terms of total (76%) and active (14%) $CaCO_3$ levels with high levels in KO area and non-detectable levels in KY area. KO vineyard was characterized by higher OM content, pH and electrical conductivity compared to KY, and KO has considerable contents in terms of total and active $CaCO_3$, that was not the case for KY plot. Intriguingly and despite the differences in soil pH between the two areas, the nitrogen uptake capacity, along with that of many other minerals, remains within the optimal range. This suggests that the vines in both soils can effectively absorb nitrogen with the same capacity, despite the pH variation. However, it is important to note that further investigation is required to draw definitive conclusions about the impact of these soil pH differences. Conducting studies under similar climate conditions would provide a more controlled environment to better understand how soil pH affects the uptake of nitrogen and other nutrients, as well as overall plant physiology. Such research would ensure that other variables do not confound the results, leading to more reliable conclusions. Since higher amounts of $CaCO_3$ were detected, the soil probably presents also higher concentration of calcium. High levels of calcium enhance the structure of soil and as a result roots penetrate easier, the soil warms quicker in spring and the internal drainage improves.[40]

KO has slightly higher temperature and considerably lower rainfall compared with KY (Fig. 1). Such differences may account for the fact that the developmental stages differed greatly between the two areas; indicatively, the fully ripe stage (BBCH 89) was 36 calendar days later in the KY region (Fig. 2), primarily due to

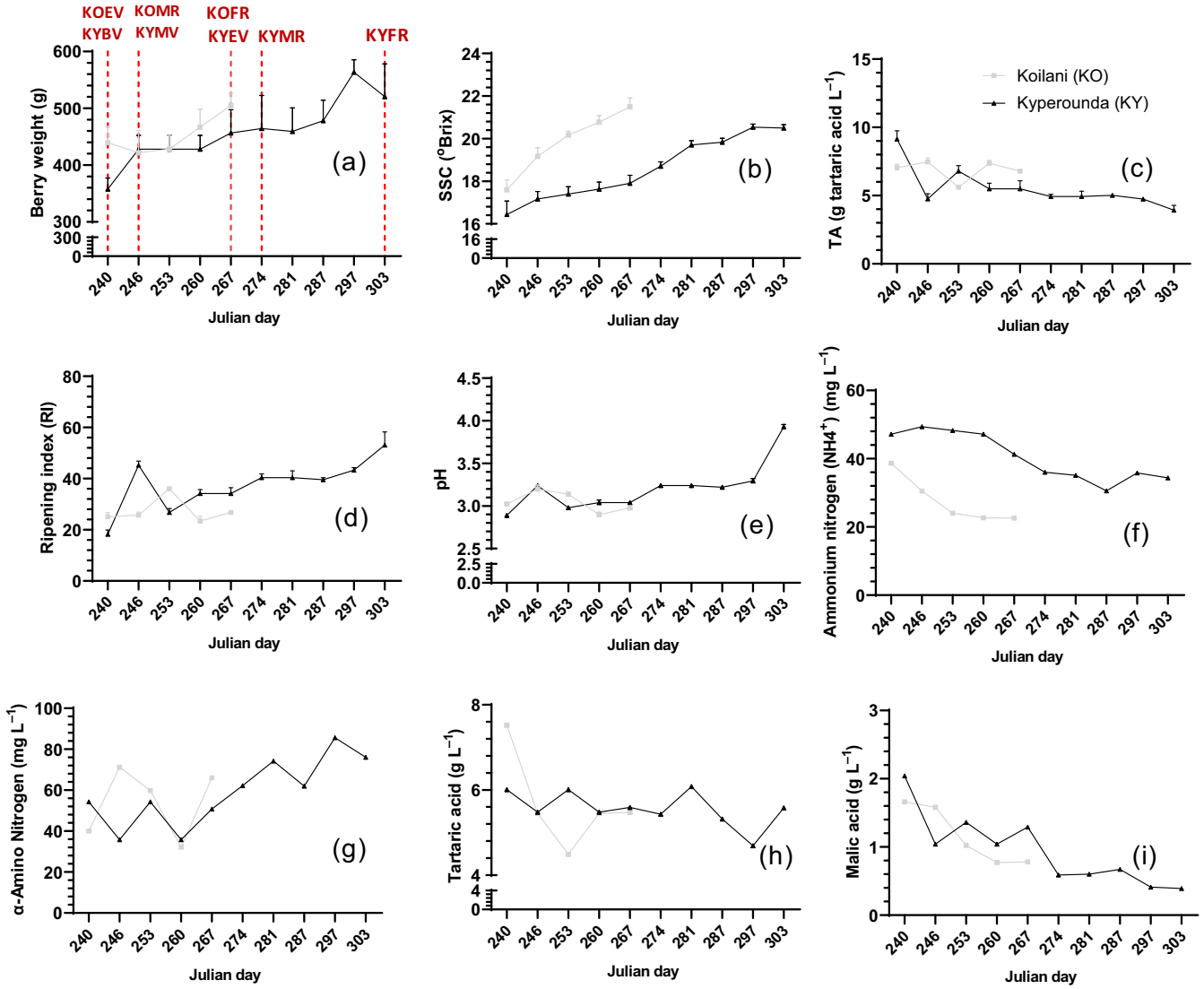

Figure 3. Berry weight (a), soluble solids content (SSC; b), titratable acidity (TA; c), ripening index (RI; d), pH (e), ammonium nitrogen (NH^{4+}; f), α-amino nitrogen (g), tartaric acid (h), malic acid (i) of Koilani (KO) and Kyperounda (KY) during berry development. For KO, the 240, 246 and 267 Julian days correspond to BBCH 85, BBCH 87 and BBCH 89 as shown with red dotted lines in (a). For KY, the 240, 246, 267, 274 and 303 Julian days correspond to BBCH 81, BBCH 83, BBCH 85, BBCH 87 and BBCH 89 as shown with red dotted lines in (a). Results are the mean ± standard error (SE; $n = 3$).

the different altitude of the two areas. The HI was 2800 (indicative of a very warm class limit) and 2473 (indicative of a warm class limit) for KO and KY vineyards, respectively. The GDD value was 2492 for KO and 2385 for KY that for both locations corresponds to region V in Winkler index that typically is considered as appropriate for extremely high production, reasonable quality table wine or table grape cultivars destined for early season consumption are grown.

Physiological measurements, and cellular damage indicators

Due to the high rainfall in KY soil compared to KO region, there is a significant leaching of calcium, potentially leading to a deficiency in this essential nutrient. This deficiency may contribute to a decrease in physiological parameters and photosynthetic pigments, as well as an increase in stress marker parameters such as MDA and H_2O_2, indicating heightened environmental stress (Table S1). Calcium plays a crucial role as a signalling molecule in reducing oxidative stress and mitigating environmental pressures. Therefore, the observed elevation in stress markers could be attributed to calcium deficiency in the region, highlighting the importance of addressing this nutrient imbalance for improved agricultural resilience.[41,42]

Total nitrogen and stable isotope ratios

Nitrogen is a significant nutrient in grapevines as it has an impact in vine vigour, crop level, berry size, and in the primary metabolites (sugar, organic acids) as well as secondary metabolites (phenolic compounds, aromas and aroma precursors) of the grape. For these reasons, the level of nitrogen was investigated in both areas.[40] Total nitrogen was higher at grapes originating from the vineyard with the higher altitude and that received substantially higher rainfall (KY) (Table S1). Similarly, the stable isotope ratio $\delta^{15}N$ ($^{15}N/^{14}N$) at BBCH 89 for KY was 0.67 ± 0.27 and for KO was 0.32 ± 0.16 that can be attributed to the highest amount of rainfall received by grapes grown at the higher altitude (KY).

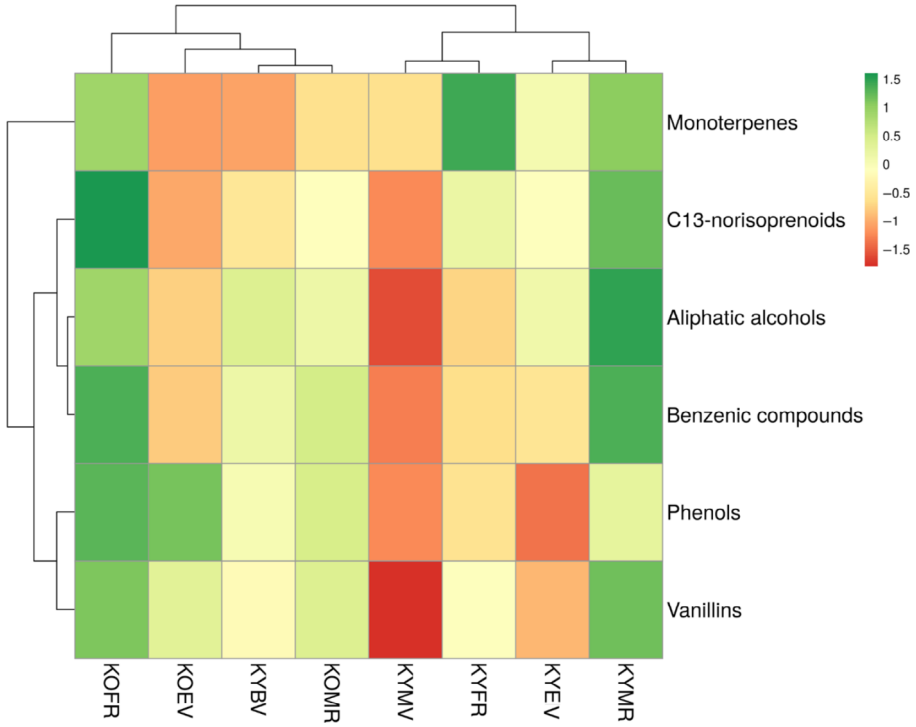

Figure 4. Heat map representing differences in the concentrations of six groups of glycosylated aromatic compounds (aliphatic alcohols, benzenic compounds, phenols, vanillins, monoterpenes, C13-norisoprenoids) at BBCH 81–89 for Koilani (KO) and Kyperounda (KY). The data were standardized as described in Materials and Methods section. Up-regulation is indicated in green; down-regulation is indicated in red. A scale of colour intensity is presented as a legend. Concentrations of the six groups of aroma compounds are shown in Supporting Information Table S2. Notation: KO, Koilani; KY, Kyperounda; BV, BBCH 81 (beginning of veraison); MV, BBCH 83 (mid-veraison); EV, BBCH 85 (end of veraison); MR, BBCH 87 (mid-ripe); FR, BBCH 89 (fully ripe).

Therefore, the highest amount of rainfall allows more nitrogen to be absorbed by the vines that greatly affects the yield, the vigour and the grape composition at the ripening stage.[43,44] Finally, the stable isotope ratio $\delta^{13}C$ ($^{13}C/^{12}C$) at BBCH 89 for KY was −26.17 ± 0.44 and for KO was −27.4 ± 0.15. The calcium concentration in leaf petioles, a variable correlated with $\delta^{13}C$, had a strong influence on (−)-rotundone accumulation.[44,45]

Effect of terroir on qualitative attributes

Several qualitative attributes (SSC, TA, RI, pH, ammonium nitrogen, α-amino nitrogen, malic and tartaric acid) were measured in grape must of cv. 'Xynisteri' of KO and KY. It was observed that the berry weight, SSC and α-amino nitrogen were higher in KO than in KY, ammonium nitrogen was lower, whereas the remaining quality attributes where similar in both locations. In addition, ammonium nitrogen, tartaric acid and malic acid contents were lower during ripening, while α-amino nitrogen was increased during ripening (Fig. 3).

Phenotypic representation at BBCH 89 presented that KY bunches had an apparent green colouration, while this was not the case for KO bunches that were characterized by a slightly brownish colour, partially due to polyphenol light oxidation (Fig. S2). At BBCH 89, yield indicators showed bigger and more berries per bunch in KO and also higher seed percentage and more seeds. In addition, KO berries were characterized by higher *L* and *b** and chroma values compared with KY berries, suggesting a more vivid colouration. Hue angle was higher in KO berries than in KY berries deriving from a more yellow colouration. In addition, FF was higher in KO berries (Fig. S2).

The total soluble solids and particularly the glucose and fructose contents increased during berry ripening. Fructose was the most abundant type of sugar, followed by glucose, while sucrose was in trace amounts. In particular, fructose constituted 42.68–50.95% of the total sugar, while glucose accounted for 42.13–46.80% and KO generally showed higher contents compared to KY (Fig. S3). The ripening of grapes in very warm conditions greatly affects sugar accumulation.[46,47] In the current study, the differences among the two plots were more evident in the mid-ripe stage. A screening study of 18 grape varieties showed that fructose, glucose, and sucrose accounted for practically the total sugar content.[48]

Effect of terroir on the glycosylated aroma compounds in berries

Berry glycosylated aroma compounds at BBCH 81–89 are provided in detail in Table S2. A total of 75 compounds were identified and quantified, including aliphatic alcohols, benzenic compounds, phenols, vanillins, monoterpenes, and C13-norisoprenoids. The total amount of glycosylated aroma compounds in the berries obtained in KY was higher by 28% in BBCH 85 (end of veraison) and 30% in BBCH 87 (mid-ripe) compared with KO, while in BBCH 89 (fully ripe) it was lower by 11% (Table S2). The two classes of aroma that contributed mostly to these differences were monoterpenes and benzenic compounds. In particular, monoterpenes in KY were 75–79% higher than KO up to mid-ripe but only 14% higher at fully ripe (Fig. 4). All other classes of aroma compounds had lower concentration at fully ripe in KY.

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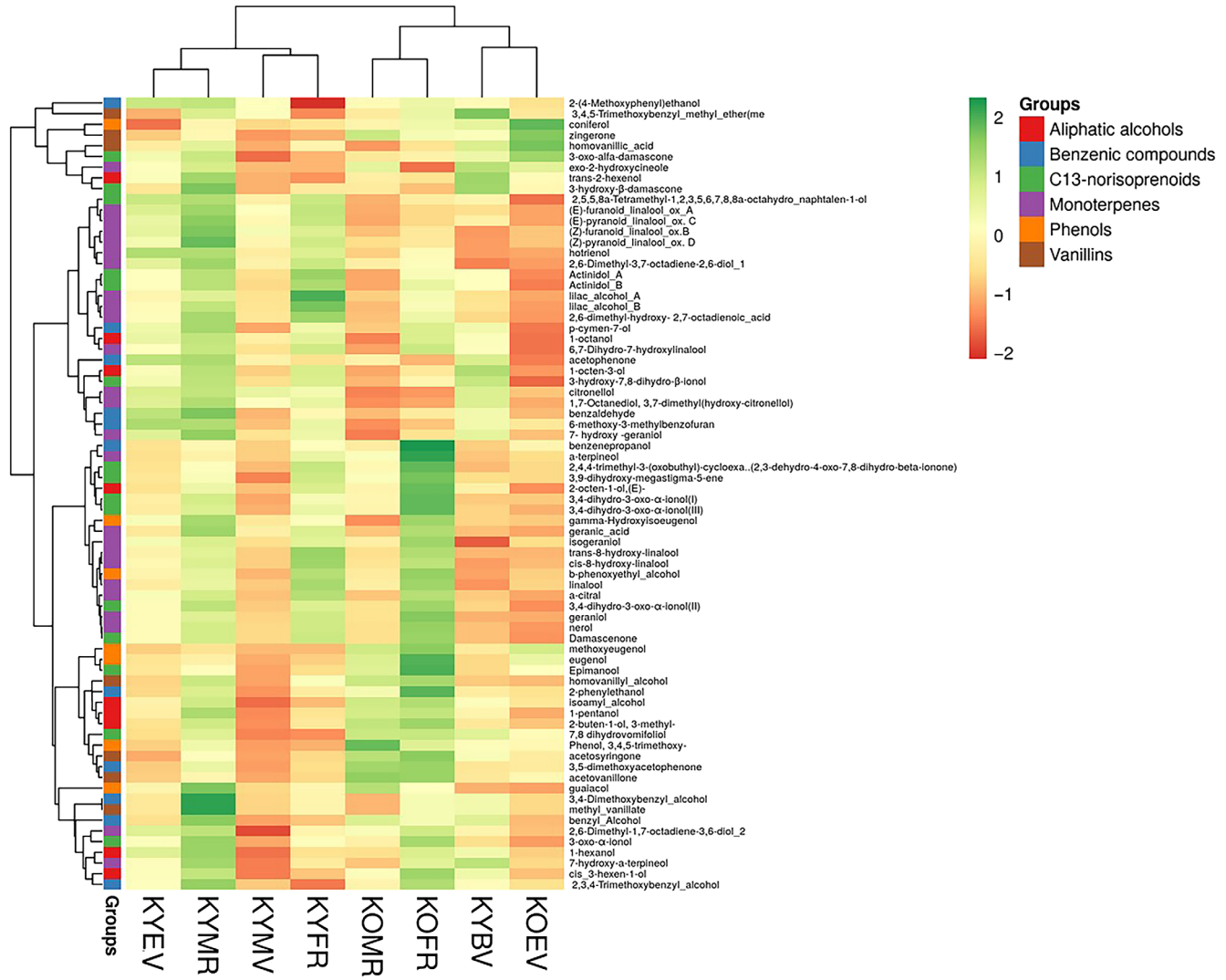

Figure 5. Heat map representing differences in the concentrations of 75 glycosylated aromatic compounds from the six groups (aliphatic alcohols, benzenic compounds, phenols, vanillins, monoterpenes, C13-norisoprenoids) at BBCH 81–89 for Koilani (KO) and Kyperounda (KY). Data were standardized as described in Materials and Methods section. Up-regulation is indicated in green; down-regulation is indicated in red. A scale of colour intensity is presented as a legend. The group of each aroma compound is indicated using a colour box next to the compound name. Concentrations of the 75 aroma compounds from the six groups are shown in Supporting Information Table S2. Abbreviated terms are explained in the caption to Fig. 4.

Twenty-five monoterpene compounds were detected in both locations. Particularly, grapes at end of veraison (BBCH 85) and mid-ripe (BBCH 87) were characterized by enhanced concentrations of 2,6-dimethyl-hydroxy-2,7-octadienoic acid in KY, both showing a 3.5-fold change compared to KO. Furthermore, in the mid-ripe grapes another nine monoterpenes were in higher concentration in KY than in KO, yet not always statistically different (Fig. 5 and Table S2). Among the detected benzenic compounds, benzaldehyde at KY exhibited the largest concentration increase of 4.2- and 3.9-fold change at end of veraison (BBCH 85) and mid-ripe (BBCH 87), respectively (Fig. 5 and Table S2).

Interestingly, all aroma compounds except for monoterpenes were higher at KO than KY during advanced on-vine developmental stage (BBCH 89). In particular, benzenic compounds, which had the highest overall concentration at this stage, were 39% higher at KO (Fig. 5 and Table S2). Among them, benzenepropanol and 2-phenylethanol registered the highest difference at 124% and 37%, respectively. Therefore, higher temperatures decrease monoterpene concentrations and in particular linalool and geraniol that is additionally linked with a decrease in linalool synthase gene expression. Belancic *et al*. showed that high temperature decreases monoterpene concentrations in berries.[49] In contrast, other studies presented a positive effect of increased GDD on monoterpenes, and specifically linalool, in 'Riesling' and other varieties.[50,51] Therefore, the distribution pattern of single monoterpenes depends on temperature.[50] These results suggest an important effect of temperatures in the two areas of cultivation.

The lower average temperature (14.9 °C) and the higher average rainfall (658 mm) at KY compared with KO (with average temperature of 16.5 °C and average rainfall of 229 mm) (Fig. 1) appeared particularly favourable for the monoterpene accumulation, but until the mid-ripening. Subsequently, these differences appeared attenuated in fully ripe grapes, probably as an effect of the increase of thermal accumulation in the KY following a

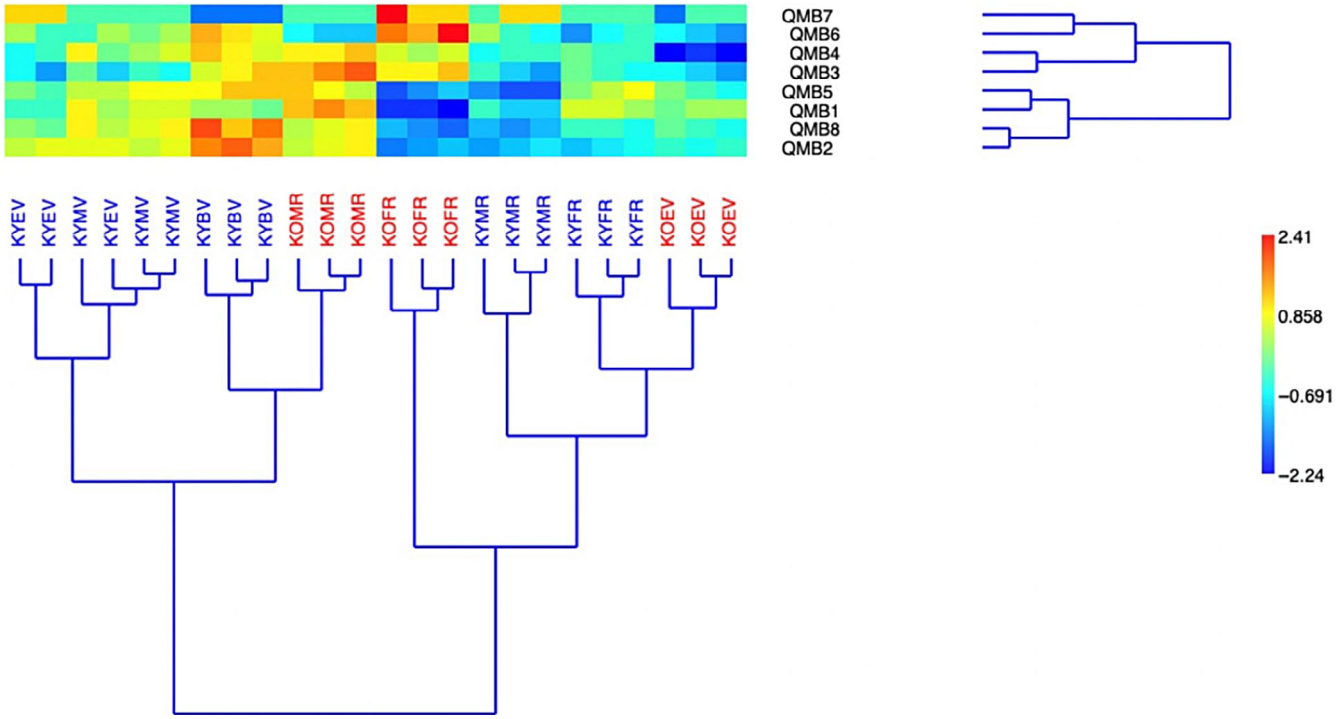

Figure 6. Graphical representation of the two-way cluster analysis (by Ward's method) performed on E-nose measurements. In detail, a hierarchical dendrogram of the sample scores (bottom, left), a hierarchical dendrogram of the loadings [the eight quartz microbalances (QMBs) coupled to metalloporphyrins (TPPs) on the top, right], and a heat-map of the loading influence on the score clustering (top, left) are reported. PCR and cluster analysis were computed on FT-NIR spectra and E-nose measurements which were performed on grape musts derived from Koilani (KO) and Kyperounda (KY) at BBCH 81–89. Abbreviated terms are explained in the caption to Fig. 4.

36-day harvest delay. Interestingly, Giordano *et al.*[52] reported higher terpineol levels in irrigated vines whereas other studies found that monoterpene concentrations rise when there is water shortage.[51]

Non-destructive detections and chemometric analysis

Figure S4(a) provides the scoreplot derived from PCR calculation performed on FT-NIR spectra. PCR is a statistical multivariate method in which a supervising approach is combined to a regressive one. Four principal components (PCs) were requested for covering the 95% of the explained variance, so minimizing the residual one under the 5%. The graph clearly shows how the greatest variability associated to the first PC (PC1, 49.33%) is influenced by KY scores belonging to the fully ripe stage (KYFR), which are well segregated from others. The blue dotted line ending with the arrow follows the movement of KY scores on the second PC (PC2, 22.82%) starting from the beginning of veraison stage (KYBV) and moving, from bottom to top, through the mid-veraision (KYMV), the end of veraison (KYEV), and up to the middle stage of ripening (KYMR). Detected spectra for those must samples (scores) look progressively and slightly clustering on PC2, while KYFR more significantly segregated, as reported earlier, on PC1. This observation seems to show how the fully ripened musts sampled at the KY area significantly differ from all others. Within the scores referring to the KO area, scores relative to the end of veraison stage (KOEV) are quite well segregated both on PC1 and PC2 from the others, and those samples are included in the same quadrant of KYBV, KYMV, and KYEV. Scores relative to middle and final ripening stages, KOMR and KOFR respectively, are practically unsegregated from them, and they look to be very close to KYMR ones.

Figure S4(b)–(d) are the scatterplots representing the regressions associated to the chemometric computation for the three main quality attributes (e.g., SSC, TA, and pH) of wine grapes significantly affecting PCR results. In particular, for SSC and pH content (Fig. S4(b),(c)), significant correlations (R^2 in calibration and in cross-validation) were obtained (0.879, 0.859, and 0.810, 0.715, respectively), as well as quite low errors performed by the models in calibration and cross-validation [root mean standard error in calibration (RMSEC) and root mean standard error cross-validation (RMSECV)] were observed. Slightly less significant are the results obtained for TA model in terms of correlation (R^2 equal to 0.691 and 0.524, for calibration and cross-validation, respectively) and an estimated error in prediction (RMSECV) of 0.92 g L^{-1}. Limitations in terms of statistical efficiency of the obtained models are for sure significantly affected by the low numbers of samples, even into a set of well spread data. In any case, current results act as useful tools to demonstrate the feasibility of FT-NIR spectra in identifying samples coming from different areas of cultivation, and related to different stage of maturity, even for being correctly associated to chemical parameters describing the ripening behaviour as well as defining the technological ripening.

Figure 6 represents the graphical results associated to the two-way cluster analysis performed on the signals detected by the eight-sensor array of the E-nose device. In detail, hierarchical dendrogram associated to the sample scores (bottom, left) shows the presence of two main clusters well segregated between them. Into the first one, KOFR samples are segregated from a subcluster

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including KYMR scores which, in turn, cluster from a second subcluster where KYFR and KOEV scores are grouped. In the second big cluster, two subgroups are included. The first one where KYBV and KOMR scores are linked, while in the second one KYEV and KYMV are grouped. However, the heat-map (top, left) and a second smaller dendrogram (top, right) demonstrate the influence of the eight QMBs sensors on the observed clustering of scores (loading influence). QMB2 and QMB8 appear to be combined and they mark the segregation of KYBV scores, while QMB1 and QMB5 are grouped and look to slightly affect KOMR ones into the same first cluster of loadings. In the second and last one, QMB3 and QMB4 are combined, and they affect KOMR scores while, finally, the combination of QMB6 and QMB7 shows to have a slight influence on KOFR ones.

Previous published studies, employing the same QMB-based E-nose device, tested the affinity of each single sensor equipped by specific TPPs for single groups of volatile compounds.[53-55] These authors reported an affinity of QMB3 (Sn-TPP) for esters, of QMB5 (Co-p-TPP), QMB7 (Co-TPP), and QMB8 (Ru-TPP) for aldehydes, esters and alcohols, while QMB4 (Rh-TPP) has been associated to lactones and volatile phenols.[55] In general, the E-nose must be considered as a sensor-based device whose discriminative capacity, related to the perception of an aromatic profile, is closer to the human nose action rather than to the VOC identification and quantification by GC–MS.[56] Thus, the E-nose device is being exploited for revealing aromatic fingerprints characterizing different samples rather than single volatile molecules or groups.

CONCLUSIONS

The present study reached the conclusion that different mesoclimatic conditions, soil properties and altitude had an impact on the composition of berries obtained from non-irrigated 'Xynisteri' cultivar. Specifically, different mesoclimatic conditions had an effect on the VOC profiles during the fully-ripe stage with a significant increase being observed in glycosylated aroma compounds, especially for monoterpenes and benzenic compounds. The total amount of glycosylated aroma compounds was higher in the berries obtained in KY up to mid-ripe, whereas KO have higher glycosylated aroma compounds at fully ripe. Moreover, KO had higher berry weight, sugars and α-amino nitrogen than in KY, but lower ammonium nitrogen, whereas the rest of the quality attributes were similar in both locations. To what extent the striking differences monitored in the two different growing areas of 'Xynisteri' are a major result of altitude, rainfall and/or distinct soil properties needs to be further dissected by providing deeper mechanistic insight through the employment of modern molecular tools such as transcriptomic and proteomic platforms in future experiments.

AUTHOR CONTRIBUTIONS

Egli C. Georgiadou: conceptualization, methodology, formal analysis, investigation, data curation, visualization, project administration, writing – original draft. Minas Mina: conceptualization, supervision, writing – review and editing. Nicolas Valanides: methodology, investigation. Anna-Maria Taliadorou: investigation. Stefanos Koundouras: methodology, writing – review and editing. Andrea Bellincontro: methodology, formal analysis, data curation, writing – review and editing. Claudio Donofrio: methodology, formal analysis, data curation, writing – review and editing. Fabio Mencarelli: methodology, formal analysis, data curation, writing – review and editing. Nikolaos Barbayiannis: methodology. Vasileios Fotopoulos: supervision, writing – review and editing. George A. Manganaris: conceptualization, supervision, writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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