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Impact of Japanese beetles (*Popillia japonica* Newman) on the chemical composition of two grape varieties (Nebbiolo and Erbaluce) grown in Italy

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ABSTRACT

The effect of Japanese beetles (*P. japonica*) on critical quality indicators of Nebbiolo and Erbaluce grapes, specifically on their phenolic and volatile composition, was assessed. Adult beetle symptoms include extended skeletonization of leaves. Leaves are frequently left with their mid-vein intact but, when severely damaged, quickly turn brown. However, the plant tends to recover by generating a new leaf apparatus and the grapes reach ripeness. It emerged that the phenolic content of grapes produced by plants attacked by *P. japonica* (396 and 550 mg/kg, Nebbiolo and Erbaluce respectively) was generally higher when compared to healthy plants (266 and 188 mg/kg, Nebbiolo and Erbaluce respectively). Similarly, in the (red) Nebbiolo cultivar, the anthocyanin content was significantly lower in grapes produced with healthy plants. The influence of *P. japonica* on the volatile composition of Nebbiolo and Erbaluce grapes showed a total volatile fraction of affected grapes (433 and 439 μg/kg, respectively) significantly higher than the one related to healthy grapes (391 and 386 μg/kg, respectively). In response to the attack by *P. japonica* the plant significantly increases the content of some volatile compounds such as hexanal, *(E)*-2-hexenal, 1-hexanol, *(E)*-2-hexen-1-ol and phenyl ethyl alcohol.

1. Introduction

The Japanese beetle, *Popillia japonica* Newman, is a highly polyphagous parasite that is indigenous to Japan and northern China. It is a member of the Scarabaeidae family (Hammons, Kurtural, & Potter, 2008). This parasite is regarded as one of the most dangerous from an agricultural standpoint because it may damage more than 300 plant kinds (Potter & Held, 2002).

It arrived in North America around the start of the 20th century, when it quickly expanded in new areas due to its ecological adaptability and caused significant harm. More than 460 million dollars are spent yearly in the USA to manage and minimize the harm caused by the pest. It does not cause severe infestations in Japan because natural enemies keep their levels under control (Hammons et al., 2009).

Due to the climate and topography in Europe being favorable for *P. japonica* colonization, it was first discovered there in the 1970 s in Portugal and the Azores Islands. In 2014, a reported outbreak in Italy's Ticino Natural Park, between the Piedmont and Lombardy regions, and in 2017 in Switzerland were also confirmed. The afflicted area in Italy has been growing, and it now covers a surface area of around 8,000 km^2, with millions of specimens spread throughout the country (Marianelli et al., 2019).

This beetle has been added to the list of priority pests of the European Union (Commission Delegated Regulation (EU) 2019/1702) and quarantine pests (regulation EU 2016/2031) due to the harm it does to crops, pastures, and meadows, with subsequent economic and environmental impact (EPPO/CABI, 1997; EPPO, 2006; PM 9/21(1), 2016).

The majority of *P. japonica* life cycle is spent in the soil as a grub. Grubs do significant harm to turf grasses, hayfields, soccer fields, and golf courses at this stage and are vulnerable to biological control agents

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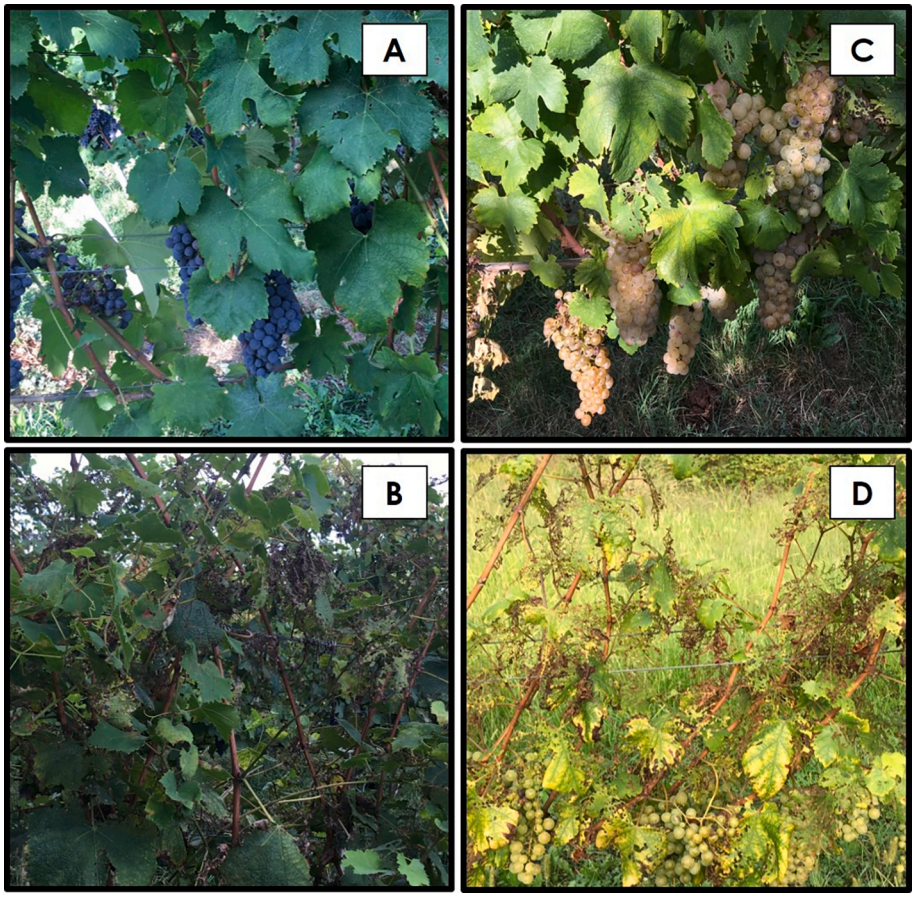

Fig. 1. The damage caused by *P. japonica* in the vineyards. A) Healthy Nebbiolo plant. B) Damaged Nebbiolo plant. C) Healthy Erbaluce plant. B) Damaged Erbaluce plant.

in the soil. The bug is currently spreading throughout Italy, preying on vines, fruit trees, forest trees, crops, vegetables, ornamental plants, and untamed plant species (EPPO, 2014). Adult beetle symptoms include eating holes in the host plants that extend to the skeletonization of leaves in cases of high population density (Potter & Held, 2002). Leaves are frequently left with their mid-vein intact. The severely damaged leaf quickly turns brown. They might fall off or hold on. The beetles consume oddly shaped parts on some plants with thin leaves and fine venation, as well as on the petals of flowers, like to many Lepidoptera. The gregarious adults typically start eating foliage at the top of a plant and work their way down, according to reports published in the USA (Mercader & Isaacs, 2003).

Phenolic compounds are the most important compounds found in grapes affecting the quality. These compounds play an important role, especially in the colour, bioactive properties and sensory characteristics of grapes and corresponding wines. Among phenolic compounds, the most important are anthocyanins and proanthocyanidins (Giacosa et al., 2021; Bordiga et al., 2011). Anthocyanins are the colour pigments in black grapes. These compounds are natural colourants that give grapes and wines their distinctive red, blue, and purple hues and are less soluble in water and soluble in alcohol (Ribéreau-Gayon et al., 2021).

Aroma compounds, generally, are another important quality parameter that affects consumer preferences. The synthesis of aroma substances in grapes increases with maturity of grapes. Esters, terpene compounds, higher alcohols, carbonyl compounds and nitrogenous compounds, are the main aroma compounds determined in grapes. The intensity and types of aroma compounds differ according to grape varieties (Selli et al., 2004, Bordiga et al., 2013; Bordiga et al., 2014).

The main purpose of this study was to evaluate the impact of Japanese beetles (*P. japonica*) on important quality parameters of Nebbiolo and Erbaluce grapes, namely on its phenolic and volatile composition.

2. Material and methods

2.1. Reagents, standards, and materials

All chemicals and reagents were of analytical quality grade. HPLC grade acetonitrile (ACN), ethanol (EtOH), and formic acid (FA) were obtained from Fischer Scientific (Loughborough, UK). Ultrapure water (18 MΩ cm) was obtained from a Milli-Q® system (Millipore, Bedford, MA, USA). Internal standard (IS) 4-nonanol and sodium chloride (NaCl, 99.5 %) were obtained from Sigma-Aldrich (Madrid, Spain), whereas GC carrier gas, helium of purity 5.0 was purchased from Air Liquide, Portugal. The SPME fibre was a 1-cm, 50/30-μm film thickness DVB/Carboxen/PDMS Stableflex SPME fiber (Supelco); a SPME holder for manual sampling and glass vials were purchased from Supelco (Bellefonte, PA, USA). The *n*-alkane series, C8 to C20, with a concentration of 40 mg/L in *n*-hexane used to determine the Kovats index (KI) was supplied from Fluka (Buchs, Switzerland).

2.2. Samples

Nebbiolo (NG, red grapes) and Erbaluce (EG, white grapes) are the *V. vinifera* L. cultivars selected for this study. These were harvested in three different sampling points (N1, N2 and N3, and E1, E2 and E3), during the last phase of berry development (vintage 2020), from the vineyard located in the Northern part of the Piedmont Region (Briona – Italy; 225 m a.s.l.). Samples collection was conducted from August 26th to September 9th, including both healthy and *P. japonica*-affected samples (Fig. 1). Groups of 50 berries, randomly selected from the collected

Table 1
Evolution of phenolic compounds identified in healthy and infected Nebbiolo and Erbaluce grapes (relative standard deviation lower than 8%).

Phenolics	Acronym	RT[a] (min)	Family	UV λ^{max} (nm)	[M−H] −(*m/z*)	MS/MS (*m/z*)	Concentration (mg/Kg) NHG[c]	NIG[d]	EHG[e]	EIG[f]
Gallic acid	*P*1	13.84	PA	276	169	125	2.9	2.6	6.9	43.7
Protocatechuic acid-O-hexoside	*P*2	16.70	PAD	296	315	153, 109	2.7	4.4	2.2	4.9
2-S-glutathionyl-caffeoyltartaric acid	*P*3	18.53	PAD	330	616	484, 440, 272	12.9	20.9	10.8	11.5
Protocatechuic acid	*P*4	17.10	PA	294	153	109	26.4	42.6	21.1	47.6
Hydroxy-caffeic acid dimer isomer	P5	22.61	PAD	315	373	305, 193	2.5	4.0	1.9	4.7
(E)-Caftaric acid	*P*6	25.24	PAD	328	311	179, 149	6.2	8.2	8.6	73.7
(E)-Coutaric acid	P7	31.19	PAD	314	295	163	4.0	4.4	1.6	19.0
(Z)-Fertaric acid	P8	27.67	PAD	322	325	193, 149	14.8	20.2	17.0	24.6
(E)-Fertaric acid	P9	35.01	PAD	328	325	193, 149	40.5	35.1	31.7	62.8
Syringic acid	P10	43.66	PA	272	197	182, 167, 153	21.8	33.2	3.5	6.9
p-Coumaric acid	P11	45.14	PA	310	163	119	17.9	26.1	3.7	14.8
Procyanidin dimer	P12	23.27	FLA-3-OL	280	577	289, 245	18.4	48.4	25.6	80.0
Catechin	P13	28.31	FLA-3-OL	280	289	245, 175	52.9	78.2	13.7	70.3
Epicatechin	P14	36.25	FLA-3-OL	280	289	245, 175	19.0	30.4	14.0	35.4
Tyrosol	P15	28.39	STLBN	275	137	93	4.5	6.6	1.2	5.9
Dihydrokaempferol 3-O-β-d-glucoside	P16	46.58	FLVN	290	449	287	0.5	0.3	0.2	0.4
Rutin	P17	47.71	FLVN	355	609	301, 271, 255, 179	2.7	3.6	1.9	3.2
Quercetin-3-O-galactoside	P18	47.83	FLVN	353	463	301, 300, 179	3.1	3.7	1.8	3.2
Quercetin-3-O-glucoside	P19	48.30	FLVN	356	463	301, 300, 255, 179	5.2	6.6	7.70	18.6
Quercetin-3-O-glucuronide	P20	48.49	FLVN	355	477	301, 179	6.3	13.5	11.5	25.5
Isorhamnetin-O-hexoside	*P*21	52.69	FLVN	356	477	315, 301, 300, 299	1.6	1.34	1.39	1.75
Quercetin	P22	63.33	FLVN	355	301	151	0.4	0.69	0.08	0.48
Total content							267	395	188	559

[a] RT: Retention time; [b] UV λ^{max}: Ultraviolet maximum wavelength; [c] NGH: Nebbiolo healthy grapes; [d] NIG: Nebbiolo infected grapes; [e] EHG: Erbaluce healthy grapes; [f] EIG: Erbaluce infected grapes.

samples of each sampling point, were used for the analysis.

In the two vineyards studied in this work, it was estimated that about 15 % of the plants were significantly damaged, in particular the outermost plants of the vineyard. Of this percentage, it must be reported that only half of the plants showed loss greater than 50 % of damaged leaf area. As further information, the white cultivar (Erbaluce) is the one that recorded an average greater damage than the other red cultivar (Nebbiolo).

2.3. Extraction of phenolic compounds

Phenolic constituents were extracted according to the method by Kelebek, Selli & Sevindik (2020) with slight modifications. A 5 g grape berry sample was placed into a centrifuge tube. It was mixed for one minute on a vortex with 10 mL methanol–water (80:20, v/v) and then the tube was centrifuged at 6500 rpm for 15 min. The solution was filtered using a membrane filter with a 0.45 μm pore size (Whatman Inc., Clinton, NJ, USA) and held at −20 °C until the analyses. All the extractions were carried out in triplicate.

2.3.1. Analysis of phenolic compounds by LC-MS/MS

Phenolic compounds were analyzed based on the method reported by Kelebek & Selli, (2011) using LC-DAD-ESI-MS/MS with negative and positive ionization modes. High-performance liquid chromatography equipment (Agilent 1260 HPLC; Agilent Tech., Palo Alto, California, USA) was utilized with a diode array detector (G1351D 1260 DAD VL) which comprised a binary pump (G1312 B, 1260 Bin pump), a degasser (G1322 A, 1260 Degasser) and an autosampler (G1367 E, 1260 HIP ALS). A Phenomenex Luna reversed-phase C-18 column with 4.6 cm × 250 mm × 5 μm size (Torrance, California, USA) was employed in the analyses. Two mobile phases were used: solvent A (water/formic acid; 99:1; v/v) and solvent B (acetonitrile/solvent A; 60:40; v/v). Standard curves were obtained by using pure standards at concentrations that exist in the extracts (nearly 1–100 mg/L) and obtaining R^2 values greater than 0.995. In case of the absence of the reference compound, the calibration of similar substances was employed by using the molecular weight correction factor. The limits of quantification (LOQ) and limits of detection (LOD) were computed by utilizing the S/N values (signal to noise) of 10 and 3, respectively (Guclu et al., 2021).

2.4. Extraction of grape volatiles by SPME

After the separation of the seed, the extraction of volatiles from grapes was performed using a well-known technique, solid phase microextraction (SPME), in headspace mode (HS) with the addition of 2 mL of saturated NaCl. The SPME fibre coating was 50/30 μm DVB/Carboxen/PDMS. Fibres were exposed to the headspace of a 20 mL capped vial, which contained 3 g of pulp and skins, 2 mL of juice, and 5 μL of internal standard (4-nonanol in EtOH, 8.3 μg/100 mL). The fibre was supplied by Supelco (Bellefonte, Pennsylvania, USA) and was conditioned by keeping them in the GC injector following instructions from the manufacturer. The extraction conditions were set as follows: extraction temperature: 60 °C, extraction time: 15 min, agitator on time: 5 s, agitator off time: 1 s, agitator speed: 250 rpm. After extraction, fibers were desorbed into the injector at 250 °C for 5 min (in splitless mode 0.8 min).

2.4.1. Analysis of grape volatiles by GC–MS

Gas chromatography-mass spectrometry (GC–MS) data were obtained from an Agilent 7890B GC equipped with an Agilent 7010B Network Mass Selective Detector (EI) (electron energy = 70 eV) over a mass range of 20–550 amu. Volatile compounds were separated on a DB-Wax (60 m × 0.25 mm × 0.25 μm thickness; J&W Scientific, Folsom, CA). The flow rate of carrier gas (helium) was 1.5 mL/min. The oven temperature was first increased from 50 to 200 °C at a rate of 5 °C/min and then to 260 °C at 8 °C/min with a final hold at 260 °C for 5 min. The mass detector was operated in scan mode, with an electronic impact ionization energy of 70 eV. The GC–MS interface and ionization source temperatures were set at 250 and 180 °C, respectively. Identification and quantification were performed in full scan mode scanning a mass range of *m/z* 30–300 at 2.0 scan/s. The compounds were identified by comparing their mass spectra with those in Wiley 9, NIST 14 mass spectral data libraries and an in-house library created with the use of alkane standards.

2.5. Statistical analysis

Data processing was done using the web-based application MetaboAnalyst 5.0, developed by the University of Alberta, Canada. Previous

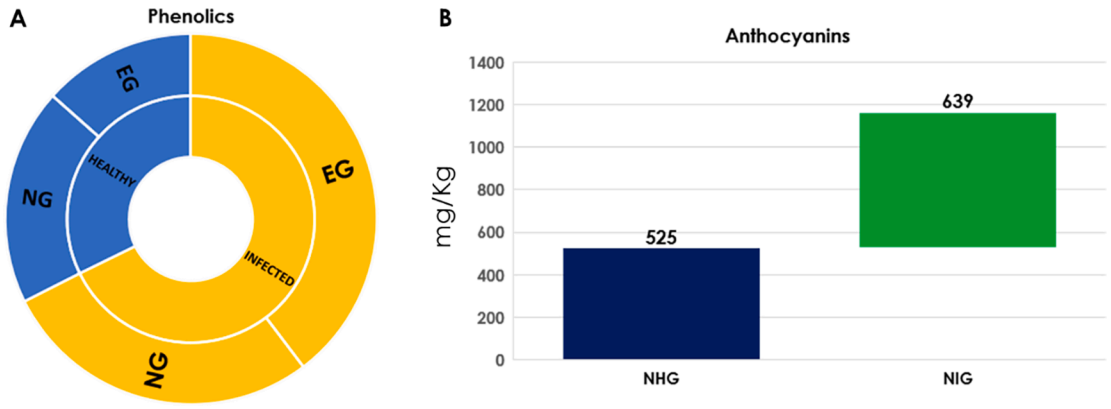

Fig. 2. Effect of the *P. japonica* in the phenolic and anthocyanins (mg/kg) composition of Nebbiolo (NG) and Erbaluce (EG) grapes (NHG: Nebbiolo healthy grapes; NIG: Nebbiolo infected grapes).

to statistical analysis, the data matrix was pre-processed to eliminate VOCs, phenolics and anthocyanins with missing values (MV), followed by the normalization using cubic root and data scaling by auto-scaling. After normalization, principal component analysis (PCA) was applied to visualize the group organization, using the whole data obtained (VOCs, phenolic, anthocyanins). Additionally, to investigate the difference in VOCs, phenolics and anthocyanins concentration, a partial least squares-discriminant analysis (PLS-DA) was carried out to identify the target analytes responsible for the grape discrimination based on their state, infected *versus* healthy. Only the target analytes that showed variable importance of projection (VIP) scores higher than 1.5 were considered putative markers. The efficiency and feasibility of the PLS-DA model were evaluated based on the goodness fit (R^2), predictive ability (Q^2) and a permutation test (1000 permutation). The significant difference in the model was evaluated by the determination of *p*-values achieved from the cross-validation analysis. For hierarchical cluster analysis (HCA), the squared Euclidean distance through the Ward agglomeration method was applied in the organization of the cluster.

3. Results and discussion

3.1. Phenolic compounds

A total of 22 phenolic compounds were identified and quantified by LC-DAD-ESI-MS/MS in grape samples regarding the collected data with main fragment ions in MS/MS, λ^{max} in the ultraviolet region, retention indexes and molecular ions. As listed in Table 1 most of the phenolics present in the grape samples were phenolic acids and their isomers (gallic acid, protocatechuic acid-o-hexoside, 2-S-glutathionyl-caffeoyl-tartaric acid, protocatechuic acid, hydroxy-caffeic acid dimer isomer, (*E*)-caftaric acid, (*E*)-coutaric acid, (*Z*)-fertaric acid, (*E*)-fertaric acid, syringic acid, p-coumaric acid) followed by flavanols, and flavonols. Although the number of identified phenolic acids is quite high, the most dominant phenolic compounds were found to be catechin and epicatechin in grape samples as expected. On the other hand, the total concentrations of phenolic compounds varied depending on the sample and so the affected grapes from the Japanese beetle showed a different phenolic profile. The highest total phenolic concentrations were found in both samples of grapes produced by plants attacked by *P. japonica* 395 (Nebbiolo) and 559 (Erbaluce) mg/kg, respectively. Grapes obtained from healthy plants showed lower values (266 and 188 mg/kg, respectively).

The influence of the effect of *P. japonica* in the phenolic composition of Nebbiolo and Erbaluce grapes is shown in Fig. 2**A**.

3.1.1. Phenolic acids

Among all samples evaluated in the present study, Erbaluce infected grapes (EIG) showed higher amounts of phenolic acids (314 mg/kg) when compared to other samples having 152 mg/kg for Nebbiolo healthy grapes (NGH), 201 mg/kg for Nebbiolo infected grapes (NIG) and 109 mg/kg for Erbaluce healthy grapes (EHG), respectively. However, these values represent approximately 50 to 60 % of the total concentration of phenolics detected in all the grape samples.

Considering the whole data collected for the phenolic acids, a massive difference in gallic acid concentration between NIG (2.6 mg/kg) and EIG (43.7 mg/kg) samples were observed. This compound is mostly presented naturally in grapes, particularly in seeds, stems, pomace, and wines (Kaur et al., 2009; Bordiga, Travaglia & Locatelli, 2019). It's generally found as an ester form attached to procyanidins or in free form. Liu et al., (2013) already mentioned the richness of grape seed extracts using gallic acid and its high potential of restraining Alzheimer's disease and amyloid fibril inhibitory effect. It must be reported that only the Erbaluce grape showed almost 6 times higher values of gallic acid in the plants attacked by *P. japonica.* Unlike Nebbiolo grapes which do not show significant variations between healthy and attacked plants.

As given in Table 1, the most abundant phenolic acids were (*E*)-caftaric acid, (*Z*)-fertaric, (*E*)-fertaric, protocatechuic and syringic acids and their amount varied significantly between samples. These remarkable compounds found in grape samples were previously detected in Chardonnay grapes (de Bruijn et al., 2009), Albanian (Topi et al., 2021), and Italian wines (Minussi et al., 2003).

Interestingly, the content of these molecules tends to increase in grapes obtained from damaged plants. For example, (*E*)-caftaric and (*E*)-coutaric acids increased by about nine times in the Erbaluce variety, reaching values of 74 and 19 mg/Kg, respectively. The content of (*Z*)- and (*E*)-fertaric acids doubles in both varieties following the attack.

Syringic acid, which possesses several functionalities in the biomedical sector with its high antioxidant, anti-cancer, antimicrobial, anti-inflammation, and anti-diabetic activities, is found in high concentrations in NHG and NIG samples (ranging from 21.8 to 33.2 mg/kg) when compared to the literature data (Srinivasulu et al., 2018).

Another important phenolic acid detected in grape samples was protocatechuic acid which has several bioactivities and health potential due to its high antioxidant content (Ferreira et al., 2009) and strong anticarcinogenic, antimicrobial and neuroprotective (Alves et al., 2013) bioactivities. Protocatechuic acid showed similar behavior in both of the studied varieties. Its content essentially doubled once the plant was attacked by the *P. japonica* reaching values of about 43 (NIG) and 48 (EIG) mg/Kg, respectively. Although NHG and EHG grape samples possessed a much lower amount of protocatechuic acid concerning extant literature data, their protocatechuic acid content is still higher than some red (Montepulciano, Barbera, Sicily Merlot and Syrah) and white wines (Pino Grigio, Greco di Tufo and Chardonnay) (Teissedre &

Table 2
Anthocyanins (mg/Kg) identified in Nebbiolo grapes (healthy and infected) by LC-DAD-ESI-MS/MS, maximum wavelength and major MS fragments (RSD < 12 %). The anthocyanins levels correspond to the average values of the sampled zones (N1-N3 and E1-E3).

Anthocyanins	Acronym	RT[a] (min)	UV λ^{max} (nm) [b]	[M +] [c]	[M +] -MS2 Mass loss	[MS2] [d]	NHG[e]	NIG[f]
Delphinidin-3-O-glucoside	A1	21.37	277, 298(sh), 346, 440(sh), 524	465	−162	303	6.6	10.7
Cyanidin-3-glucoside	A2	26.14	280, 292(sh), 325(sh), 380(sh), 440(sh), 517	449	−162	287	27.2	86.2
Petunidin-3-glucoside	A3	28.27	276, 298(sh), 348, 440(sh), 527	479	−162	317	11.9	15.3
Peonidin-3-glucoside	A4	33.51	280, 292(sh), 325(sh), 380(sh), 440(sh), 518	463	−162	301	292.4	361.6
Malvidin-3-glucoside	A5	34.83	276, 298(sh), 348, 440(sh), 528	493	−162	331	115.3	89.2
Delphinidin-3-O-acetylglucoside	A6		280, 298(sh), 346, 440(sh), 526	507	−204	303	ND	ND
Cyanidin-3-O-acetylglucoside	A7	42.65	283, 313, 440(sh), 522	491	−204	287	2.1	5.2
Petunidin-3-O-acetylglucoside	A8	43.16	269, 298(sh), 348, 440(sh), 528	521	−204	317	1.6	1.5
Peonidin-3-O-acetylglucoside	A9	45.38	280, 292(sh), 325(sh), 380(sh), 440(sh), 529	505	−204	301	26.6	25.7
Malvidin-3-O-acetylglucoside	A10	45.70	277, 298(sh), 348, 440(sh), 529	535	−204	331	11.0	7.4
Delphinidin-3-O-p-coumaroylglucoside	A11		282, 298(sh), 316(sh), 440(sh), 530	611	−308	303	ND	ND
Cyanidin-3-O-coumaroylglucoside	A12	47.85	283, 313, 440(sh), 522	595	−308	287	2.8	5.7
Petunidin-3-O-p-coumaroylglucoside	A13	48.57	282, 298(sh), 316(sh), 440(sh), 531	625	−308	317	4.2	3.3
Peonidin-3-O-p-coumaroylglucoside	A14	51.22	283, 313, 440 (sh), 521	609	−308	301	17.3	22.8
Malvidin-3-O-p-coumaroylglucoside	A15	51.48	283, 298(sh), 316(sh), 440(sh), 532	639	−308	331	6.3	4.6
Total content							525	639

[a] RT: Retention time; [b] UV λ^{max}: Ultraviolet maximum wavelength; [c] [M +]: mass of molecular ion; [d] [MS2]: molecular mass of the second most abundant fragment ion; [e] NGH: Nebbiolo healthy grapes; [f] NIG: Nebbiolo infected grapes.

Landrault, 2000; Minussi et al., 2003; La Torre et al., 2006).

3.1.2. Flavanols

Flavanols, a group of phenolic compounds member of flavonoids and found in high quantity in grape flesh, skin, and seeds. Among this group of compounds, also responsible for the astringent taste, catechin, epicatechin, and dimer procyanidins are dominant in grapes and wines (Topi et al., 2021). Besides the taste, catechin and epicatechin are considered quite important phenolic compounds in reducing the risk of cardiovascular diseases and carcinogenic structures due to their high antioxidant capacity and amphipathic characteristic (Takanashi et al., 2017). Thanks to these characteristics, it is at least interesting to note that also in this case, in both cultivars the plant responded with an overproduction of these compounds following the attack by *P. japonica.* Erbaluce infected grapes (EIG) showed higher amounts of flavanols (185 mg/kg) when compared to other samples reaching 156 mg/kg for NIG, 90 mg/kg for NHG and 53 mg/kg for EHG, respectively. Among grape samples, NIG and EIG samples showed the highest catechin content (78.2 and 70.3 mg/kg respectively) (Table 1). Compared to grapes produced from healthy plants, the previously reported values almost double for the Nebbiolo cultivar and quadruple for the Erbaluce.

3.1.3. Flavonols

Flavonols, another subclass of flavonoids, are found in high amounts after flavanols and phenolic acids in grape samples. These remarkable compounds provide grapes with important health-promoting activities, and they also stabilize the anthocyanins especially in young red wines via copigmentation (Vernhet et al., 2020). In the present study, dihydrokaempferol 3-*O*-*β*-*d*-glucoside, rutin, quercetin-3-*O*-galactoside, quercetin-3-*O*-glucoside, quercetin-3-*O*-glucuronide, isorhamnetin-*O*-hexoside and quercetin were identified flavonols in grape samples. Among these compounds, quercetin-3-*O*-glucoside and quercetin-3-*O*-glucuronide were the major flavonols quantified in grapes and the EIG sample was found to be the sample with the highest concentration of quercetin-3-*O*-glucuronide (25.5 mg/kg). This compound was already detected for example in Spanish Petit Verdo *cv.* grapes (Castillo-Muñoz et al., 2009) and Greek Roditis *cv.* (Makris et al., 2008). Besides, quercetin-3-*O*-glucoside showed the highest content in EIG samples (about 19 mg/kg). It must be reported that only the Erbaluce grape showed almost 3 times higher values of this compound in the plants attacked by *P. japonica.* Conversely, Nebbiolo grapes do not show significant variations between healthy and attacked plants.

3.1.4. Anthocyanins

A total of 13 anthocyanin compounds were determined in grapes, five of them glycosides, four of them acetyl-glucosides, and four of them in the form of coumaryl-glucosides (Table 2). The total amount of these compounds varied between 525 (NHG) and 639 (NIG) mg/kg, respectively. Also, in this case, the highest anthocyanins content was detected in grapes produced from plants damaged by *P. japonica.* Peonidin-3-glucoside and its acetyl and coumaroyl forms constituted a significant part of the total anthocyanins (336 (NHG) and 410 (NIG) mg/kg, respectively. These compounds were followed quantitatively by malvidin-3-glycoside and its acetyl and coumaryl forms. Unlike peonidin-3-glucoside and cyanidin-3-glucoside, malvidin-3-glucoside exhibited reverse behavior. The damaged plants produced grapes with a lower content of malvidin-3-glucoside than the healthy ones. Normally, malvidin-3-glucoside and its derivatives are expected to be the predominant anthocyanin in *Vitis vinifera* grapes, however the Nebbiolo cultivar is characterized by a higher content of peonidin (Locatelli et al., 2016).

The influence of the effect of *P. japonica* towards the anthocyanin's composition of Nebbiolo and Erbaluce grapes is shown in Fig. 2**B**.

3.2. Volatile composition of healthy and infected Nebbiolo and Erbaluce grapes

Volatile compounds are secondary metabolites that play a critical role in grape quality for enological properties as it strongly affects consumer preferences. These compounds are one of the most important factors in elucidating wine types and quality. Terpenes, C13-norisoprenoids, and alcohols are the most dominant aroma groups in different grape cultivars (Rocha et al., 2010). Berry health is one of the most important parameters that affect grape quality and grape products. The volatile compounds identified in healthy and infected grape samples and their linear retention indexes on the DB-Wax column were provided in Table 3.

Eight chemical groups of volatiles were identified during the development of Nebbiolo and Erbaluce grape berries including, aldehydes, alcohols, esters, terpenes, C13-norisoprenoids, benzene derivates, naphthalene derivates and acids.

3.2.1. Higher alcohols

Alcohols were the main volatiles in Nebbiolo grape berries which accounted for the highest proportion during the whole fruit development. Among all volatile chemical groups, the total concentration of the volatile alcohols was dominant. The highest total alcohol concentrations

Table 3
Volatile composition Nebbiolo and Erbaluce healthy and infected grapes obtained by HS-SPME/GC–MS analysis. The relative peak areas correspond to the average values of the sampled zones (N1-N3 and E1-E3).

RT[a] (min)	Acronym	LRI[b]	Identified VOCs	Concentration ± SD[g] (μg/kg) NHG[c]	NIG[d]	EHG[e]	EIG[f]	Identification Mode
Alcohols								
17.58	V4	1220	1-Pentanol	ND	2.4 ± 0.2	1.8 ± 0.4	1.0 ± 0.3	LRI,MS,STD
24.04	V15	1361	1-Hexanol	138.6 ± 21.4	139.8 ± 28.4	132.0 ± 34.7	91.2 ± 24.7	LRI,MS,STD
25.16	V18	1376	(Z)-3-Hexen-1-ol	5.8 ± 1.5	3.4 ± 1.2	7.5 ± 2.4	21.1 ± 5.5	LRI,MS,STD
26.01	V21	1380	(E)-2-Hexen-1-ol	31.7 ± 3.4	16.2 ± 4.6	33.0 ± 9.2	50.9 ± 8.8	LRI,MS,STD
26.30	V22	1382	(Z)-2-Hexen-1-ol	3.6 ± 0.3	1.0 ± 0.4	1.6 ± 0.4	1.5 ± 0.3	LRI,MS,STD
27.69	V26	1445	1-Octen-3-ol	3.1 ± 0.6	3.3 ± 0.8	3.2 ± 1.1	3.7 ± 0.6	LRI,MS,STD
27.90	V27	1450	1-Heptanol	5.8 ± 0.3	2.6 ± 0.8	1.9 ± 0.5	2.0 ± 0.3	LRI,MS,STD
29.13	V31	1505	2-Ethyl-1-hexanol	6.4 ± 0.6	5.6 ± 1.1	5.9 ± 1.9	6.3 ± 0.6	LRI,MS,STD
29.78	V33	1510	3-Ethyl-4-methylpentan-1-ol	5.5 ± 0.3	9.5 ± 2.8	2.0 ± 0.2	1.6 ± 1.1	LRI,MS,tent
31.43	V38	1561	Octanol	4.2 ± 0.7	5.8 ± 1.4	3.9 ± 0.9	4.2 ± 0.6	LRI,MS,STD
33.57	V41	1590	(Z)-2-Octen-1-ol	ND	ND	1.5 ± 0.4	1.7 ± 0.2	LRI,MS,STD
42.52	V55	1866	Benzyl alcohol	2.6 ± 1.2	2.3 ± 0.4	2.3 ± 0.5	1.5 ± 0,5	LRI,MS,STD
43.50	V57	1923	Phenylethyl alcohol	3.7 ± 0.5	8.6 ± 2.9	2.9 ± 1.0	1.7 ± 0.6	LRI,MS,STD
Aldehydes								
11.93	V1	1080	Hexanal	20.2 ± 1.4	27.2 ± 12.7	15.6 ± 4.4	13.7 ± 5.0	LRI[h],MS[i],STD[j]
14.32	V2	1212	3-Hexenal	5.9 ± 1.1	1.4 ± 0.4	0.8 ± 0.4	0.6 ± 0.3	LRI,MS,tent[l]
16.50	V3	1214	Heptanal	ND[m]	ND	0.9 ± 0.3	2.2 ± 0.2	LRI,MS,STD
17.86	V5	1228	(E)-2-Hexenal	65.9 ± 12.9	45.8 ± 15.8	54.9 ± 11.6	63.7 ± 10.9	LRI,MS,STD
21.23	V7	1267	Octanal	3.2 ± 0.3	3.1 ± 0.9	2.5 ± 1.0	3.3 ± 0.5	LRI,MS,STD
22.63	V8	1273	(E)-2-Heptenal	2.2 ± 0.8	2.4 ± 0.3	3.8 ± 0.6	3.7 ± 1.1	LRI,MS,STD
22.68	V9	1278	(Z)-2-Heptenal	ND	1.8 ± 0.2	1.1 ± 0.2	0.7 ± 0.3	LRI,MS,STD
25.61	V19	1378	Nonanal	17.5 ± 1.4	15.7 ± 2.9	16.1 ± 0.8	15.9 ± 1.1	LRI,MS,STD
26.86	V23	1416	(E)-2-Octenal	4.4 ± 0.4	3.7 ± 0.5	4.3 ± 0.8	5.2 ± 1.3	LRI,MS,STD
29.05	V30	1497	(E,E)-2,4-Heptadienal	11.9 ± 2.0	10.1 ± 1.1	9.6 ± 0.9	8.5 ± 0.9	LRI,MS,STD
29.48	V32	1508	Decanal	4.9 ± 1.2	2.7 ± 0.6	3.0 ± 0.9	2.8 ± 1.1	LRI,MS,STD
29.99	V34	1512	Benzaldehyde	6.8 ± 1.8	7.7 ± 1.3	3.9 ± 1.6	4.1 ± 1.9	LRI,MS,STD
30.60	V36	1542	(E)-2-Nonenal	4.6 ± 0.5	3.3 ± 0.8	2.7 ± 0.6	3.6 ± 1.2	LRI,MS,STD
33.84	V42	1606	β-Cyclocitral	ND	ND	0.7 ± 0.2	1.0 ± 0.3	LRI,MS,STD
34.38	V43	1618	Phenylacetaldehyde	9.0 ± 1.2	10.5 ± 0.8	3.5 ± 2.0	5.4 ± 1.3	LRI,MS,STD
38.48	V50	1698	β-Cyclocitral	ND	3.8 ± 1.1	1.0 ± 0.3	0.9 ± 0.2	LRI,MS,STD
39.08	V51	1710	2,4-Dimethylbenzaldehyde	2.4 ± 0.8	2.8 ± 0.7	1.6 ± 0.4	1.9 ± 1.0	LRI,MS,tent
Esters								
20.57	V6	1234	Hexyl Acetate	1.8 ± 0.8	2 ± 0.7	0.9 ± 0.1	1.1 ± 0.1	LRI,MS,STD
23.03	V11	1325	(E)-2-Hexenyl acetate	1.5 ± 0.4	ND	1.3 ± 0.3	1.1 ± 0.1	LRI,MS,STD
23.16	V12	1327	(Z)-2-Hexenyl acetate	9.2 ± 3.2	6.6 ± 1.1	8.1 ± 2.5	9.4 ± 1.6	LRI,MS,STD
27.26	V24	1420	Ethyl octanoate	5.1 ± 1.1	4.9 ± 0.4	3.9 ± 1.2	4.2 ± 1.5	LRI,MS,STD
27.99	V28	1459	2-Butoxyethyl acetate	0.8 ± 0.2	4.2 ± 0.8	5.2 ± 1.4	5.9 ± 0.8	LRI,MS,tent
34.83	V45	1633	Ethyl decanoate	1.4 ± 0.3	1.4 ± 0.5	2.8 ± 1.3	1.8 ± 0.1	LRI,MS,STD
35.83	V47	1660	3-Phenylpropyl acetate	2.3 ± 0.5	2.5 ± 1.1	1.8 ± 0.2	2.1 ± 0.5	LRI,MS,STD
Terpenes								
23.75	V14	1351	3,7-Dimethyl-6-octen-1-yl-3-ol	2.3 ± 0.7	1.2 ± 0.4	2.2 ± 0.4	2.3 ± 0.6	LRI,MS,tent
27.44	V25	1433	Linalool oxide	ND	ND	0.2 ± 0.05	1.5 ± 0.2	LRI,MS,STD
31.02	V37	1552	Linalool	1.7 ± 0.6	2.7 ± 1.1	2.1 ± 0.1	6.0 ± 0.9	LRI,MS,STD
33.12	V40	1578	4-Terpineol	1.5 ± 0.3	2.4 ± 0.8	1.0 ± 0.3	3.3 ± 0.7	LRI,MS,STD
34.99	V46	1656	Pulegone	3.2 ± 0.9	2.4 ± 0.7	2.9 ± 0.7	4.5 ± 1.4	LRI,MS,STD
37.05	V49	1692	α-Terpineol	1.2 ± 0.4	0.9 ± 0.1	1.2 ± 0.3	4.6 ± 0.9	LRI,MS,STD
41.86	V53	1855	Geraniol	4.1 ± 0.9	3.2 ± 0.5	3 ± 1.1	3.9 ± 0.4	LRI,MS,STD
42.14	V54	1862	Geranyl acetone	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	1 ± 0.2	LRI,MS,tent
Benzene derivates								
22.81	V10	1283	p-Cymene	2.2 ± 0.7	1.1 ± 0.1	1.8 ± 0.1	2.7 ± 1.1	LRI,MS,STD
24.23	V16	1362	4-Ethyl-o-xylene	4.2 ± 1.0	2.5 ± 0.4	3.8 ± 1.0	4.4 ± 1.6	LRI,MS,tent
24.54	V17	1364	2-Ethyl-p-xylene	3.9 ± 0.5	3.2 ± 0.6	3.2 ± 0.2	3.5 ± 0.6	LRI,MS,tent
25.82	V20	1379	1-Methyl-3,5-diethylbenzene	7.5 ± 1.2	4.8 ± 0.4	6.1 ± 2.3	8.6 ± 1.4	LRI,MS,STD
28.56	V29	1481	4-Ethylstyrene	1.5 ± 0.3	1.2 ± 0.3	1.4 ± 0.1	4.6 ± 1.6	LRI,MS,tent
C13 Norisoprenoids								
34.57	V44	1625	α-Ionol	0.1 ± 0.01	0.1 ± 0.04	0.4 ± 0.06	2.7 ± 0.7	LRI,MS,STD
41.16	V52	1752	β-Damascenone	5.0 ± 0.4	6.1 ± 0.4	2.2 ± 0.7	0.9 ± 0.2	LRI,MS,STD
44.41	V58	1951	(E)-β-Ionone	0.5 ± 0.1	0.7 ± 0.2	0.6 ± 0.3	0.7 ± 0.1	LRI,MS,STD
Acid								
30.17	V35	1516	m-Hydroxybenzoic acid	ND	7.7 ± 2.2	5.2 ± 1.3	5.3 ± 0.6	LRI,MS,tent

[a] RT: Retention time; [b] LRI: Linear retention index; [c] NGH: Nebbiolo healthy grapes; [d] NIG: Nebbiolo infected grapes; [e] EHG: Erbaluce healthy grapes; [f] EIG: Erbaluce infected grapes; [g] SD: Standard deviation; [h] LRI: Identification based on linear retention index; [i] MS: Identification based on comparison of mass spectra with NIST library; [j] STD: Identification based on mass spectra comparison with pure standard; [l] tent: Compound tentatively identified; [m] ND: Not detected.

were found in both samples of grapes produced by healthy plants (211 (Nebbiolo) and 199 (Erbaluce) μg/kg, respectively. Grapes obtained from plants attacked by *P. japonica* showed lower values (200 and 188 μg/kg, respectively). 1-hexanol was found to be the most abundant volatile compound in whole grape samples, reaching mean values around 130 μg/kg. It must be reported that only the Erbaluce grape showed almost a 40 % reduction of this compound in the plants attacked by *P. japonica.* Conversely, Nebbiolo grapes do not show variations between healthy and attacked plants.

This specific volatile compound was previously found in several grape varieties such as Muscat and Merlot providing generally herbaceous and green notes to the grapes and wines (Song et al., 2012).

Apart from 1-hexanol, several C_6 volatile compounds such as (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, (*Z*)-2-hexen-1-ol and 2-ethyl-1-hexanol were also identified in grape samples. Selli et al., (2003) have detected these C6 volatiles in the Emir white grape variety of Turkey, while Fang and Qian (2012) found these remarkable aroma contributors in Pinot Noir grapes. It has to be noted that the content of (*Z*)-3-hexen-1-ol increased 3 times in grapes produced by attacked plants. Moreover, it is at least interesting to note that (E)-2-hexen-1-ol showed two opposite behaviors based on the cultivar considered. The samples of Nebbiolo grapes, it has halved their content following the attack. On the contrary, in the samples of Erbaluce, it almost doubled its content if compared to grapes produced by healthy plants, reaching a value of about 50 μg/kg. Finally, phenylethyl alcohol doubled its content in the Nebbiolo grape after the attack of *P. japonica.*

3.2.2. Aldehydes

A total of seventeen aldehydes were determined in grape samples and (*E*)-2-hexenal, hexanal, nonanal and (*E, E*)-2,4-heptadienal were found to be the most abundant aldehydes. The total amount of aldehydes ranged between 126 and 158 μg/kg and among all aldehydes, (*E*)-2-hexenal showed the highest concentration ranging between 45 and 66 μg/kg followed by another C_6 aldehyde which is hexanal having a concentration between 14 and 27 μg/kg. Interestingly, (E)-2-hexenal showed two opposite behaviours based on the cultivar considered. The samples of Nebbiolo grapes, it has almost halved their content following the attack. On the contrary, in the samples of Erbaluce, its content increased from 55 to 64 μg/kg if compared to grapes produced by healthy plants. These C_6 aldehydes are known to be responsible for green, fatty and vegetable scents and were previously found in high amounts in Muscat berry skins and pulp (Sánchez-Palomo et al., 2005), Pinot Noir grape juice (Fang & Qian, 2012) and many other varieties. It is a well-known fact that the odor threshold values of many aldehydes are generally low and therefore they have a considerable effect on the overall aroma profile of grapes and grape products (Smit et al., 2009). It must be reported that only the Nebbiolo grape showed almost a 50 % reduction of decanal in the plants attacked by *P. japonica.* Conversely, Erbaluce grapes do not show variations between healthy and attacked plants. The same behavior also for the 3-hexenal which reduces its content from almost 6 to 1.5 μg/kg in the Nebbiolo grape samples only.

3.2.3. Esters

Among hundreds of grape and wine volatiles, although higher alcohols are considered the dominant group in terms of concentration, esters play a crucial role in the formation of the overall aroma character of a grape and so wines (Ferreira et al., 2000). According to results provided in Table 3, a total of seven esters were identified and quantified in grape samples including hexyl acetate, (*E*)-2-hexenyl acetate, (*Z*)-2-hexenyl acetate, ethyl octanoate, 2-butoxyethyl acetate, ethyl decanoate, 3-phenylpropyl acetate. The total esters concentrations were found substantially comparable in all samples (both healthy and infected), ranging from 21 to 25 μg/kg, respectively. Among these esters, (*Z*)-2-hexenyl acetate was found to be the most dominant compound in most of the grape samples assayed in this study (ranging from 6.6 to 9.4 μg/kg). (*Z*)-2-Hexenyl acetate is known as an acetate ester resulting from the formal condensation of the hydroxy group of (*Z*)-hex-2-en-1-ol with the carboxy group of acetic acid. In the extant literature, this compound was also found in Riesling and Muscat (Wu et al., 2020) varieties. These C_6 esters are also essential volatile compounds of wine-making grape varieties such as Cabernet Sauvignon and Merlot providing them with sweet-floral and fruity notes (Fan et al., 2010). Besides (*Z*)-2-hexenyl acetate, ethyl octanoate, the well-known wine volatile responsible for the fruity odors like ananas, pear and fresh notes, was identified in most of the *Vitis vinifera* L. varieties including Cabernet Sauvignon, Grenache, Merlot, Tempranillo, Riesling, Corvina, Rondinella, Kalecik karasi and Okuzgozu (Tetik et al., 2018; Fedrizzi et al., 2011; Selli et al., 2004). It must be reported that only the Nebbiolo grape showed almost a 50 % increase of 2-butoxyethyl acetate in the plants attacked by *P. japonica.* Conversely, Erbaluce grapes do not show particular variations between healthy and attacked plants.

3.2.4. Terpenes

Terpenes are known to be the most investigated odoriferous chemicals in *V. vinifera* and in particular, terpenoids are fundamental volatiles of the sensory expression of the grape and wine aroma. As one of the most important groups of aroma compounds, terpenes, both their volatile free forms and non-aromatic precursors (especially glycosylated compounds) have been previously found in grapes in numerous studies (Wu et al., 2020).

In the present work, 3,7-dimethyl-6-octen-1-yl-3-ol, linalool oxide, linalool, 4-terpineol, pulegone, α-terpineol, geraniol and geranyl acetone were the terpenes determined in grape samples. Monoterpenes, especially the monoterpene diols, have been determined among grape volatiles and there have been identified more than 50 monoterpenoids in grapes up to now (Mateo & Jiménez, 2000). It has been observed by

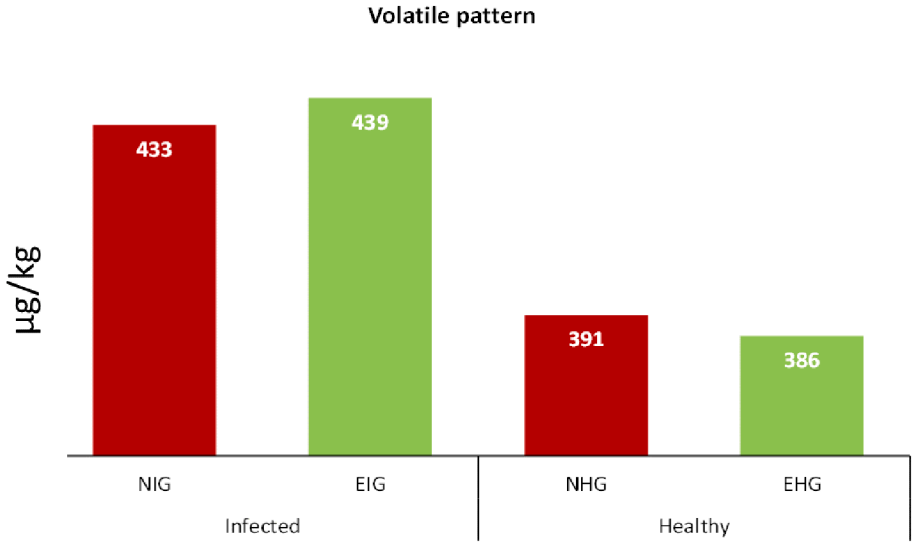

Fig. 3. Effect of the *P. japonica* in the volatile pattern (μg/kg) of Nebbiolo (NG) and Erbaluce (EG) grapes (NHG: Nebbiolo healthy grapes; NIG: Nebbiolo infected grapes).

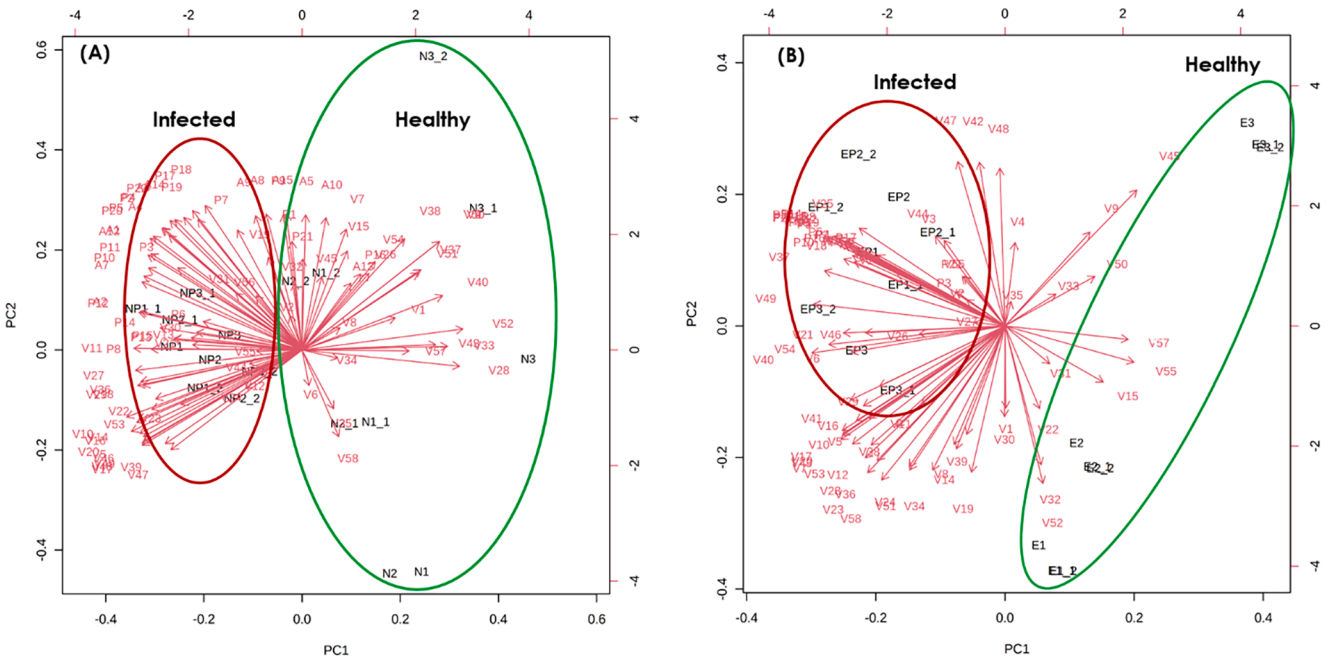

Fig. 4. Biplots of all analyte targets identified in red (A) and white (B) grapes (attribution of the peak is shown in Tables 1-3) V corresponds to VOCs, P to phenolics and A to anthocyanins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

several researchers that the most abundant monoterpene alcohols are known to be geraniol, linalool and α-terpineol, also identified in the present study. Although these compounds are found in low concentrations concerning other grape volatiles, they are indeed dominant contributors to the characteristic aroma due to their low odor threshold values. Among monoterpenes, geraniol was the most abundant found in all samples, showing the highest concentration ranging between 3.0 and 4.1 μg/kg in EHG and NHG, respectively. Most of the terpenes, especially acetyl-CoA-derived volatiles such as geraniol and terpinene-4-ol, detected in the present study also existed in other grape varieties such as Fiano, Muscat, among others, providing floral and herbaceous odors (Sanchez-Palomo et al., 2005; Fenoll et al., 2009). Apart from these compounds, linalool-derived compounds such as linalool oxide are other terpenes presented in the studied grape samples. Interestingly, this compound was detected only in the Erbaluce samples. Its content increased about three times in the grapes attacked by *P. japonica*. The same increase was also recorded for linalool in the same samples. These terpenes were previously found in Pinot Noir, Chardonnay and Fernão-Pires (Fang & Qian, 2006) and lastly identified by Wang et al., (2022) in the "Shine Muscat" variety. Generally, the samples of grapes produced by attacked plants showed higher values of terpenes. Between the two types of grapes, Erbaluce showed a greater difference when compared to grapes obtained from healthy plants (for example, 4-terpineol and α-terpineol).

3.2.5. *Benzene derivates and C_{13} norisoprenoids*

4-Ethyl-o-xylene, 2-ethyl-p-xylene and 1-methyl-3,5-diethylbenzene were the main benzene derivates determined in grape samples. Of the five compounds belonging to this group shown in Table 3, the total content varies from 12 to 24 μg/kg, however, showing an inverse trend for the two cultivars. Nebbiolo grapes showed the highest values in grapes produced from healthy grapes, vice versa for the Erbaluce. Only the Erbaluce grape showed almost a fourfold increase of 4-ethylstyrene in the plants attacked by *P. japonica.* Conversely, Nebbiolo grapes do not show particular variations between healthy and attacked plants.

Among C_{13} norisoprenoids, *β*-damascenone was found in higher amounts, especially in Nebbiolo grape samples with values between 5 and 6 μg/kg in NHG and NIG, respectively. *β*-damascenone is a remarkable C_{13} norisoprenoid providing a complex flower, stewed apple and floral scents to the grapes and wines deriving from the tetraterpenes (C_{40}) in megastigmane forms. For example, this specific compound was previously found in Grenache (Sabon et al., 2002) and Muscat grapes (Bordiga et al., 2013). Another important norisoprenoid found in the present study was (*E*)-*β*-ionone. This specific compound is known to be responsible for a pleasant violet odor (Fan et al., 2010).

Summarizing, the influence of *P. japonica* on the volatile composition of Nebbiolo and Erbaluce grapes is shown in Fig. 3. As can be observed the total volatile fraction of infected grapes (NIG and EIG) is higher than the total volatile fraction of healthy (NHG and EHG) grapes.

3.3. *Statistical analysis*

The data matrices constituted by 93 and 80 analytical variables (58 VOCs, 22 phenolics, 13 anthocyanins) identified in red and white grapes, respectively, were submitted to PCA analysis. Fig. 4 shows the biplot of the two first principal components (PC1 vs PC2) for red and white grapes, which explains 55.4 and 60.0 % of the total variability of the data set, allowing us to categorize the *Vitis vinifera* L. grapes based on their state (infected and healthy), as a function of PC axis.

The infected red grapes projected in PC1 negative are mainly characterized by (*E*)-2-hexenyl acetate (V11), 1-heptanol (V27), (Z)-fertaric acid (P8), syringic acid (P10), procyanidin dimer (P12), cyanidin-3-glucoside (A2) and cyanidin-3-O-acetylglucoside (A7), whereas the healthy red grapes placed in PC1 positive is by 2-butoxy ethyl acetate (V28), 3-ethyl-4-methylpentane-1-ol (V33) and geraniol (V53). Fertaric acid and its derivatives belong to the class of coumaric acids. They are involved in the browning reactions of must and wine, and they are precursors of volatile phenols with antimicrobial and antioxidant activities. In general, the amount of fertaric acid was found to be higher in grapes produced by damaged plants. Proanthocyanidin dimers are a specific type of proanthocyanidin, which are a class of flavanoids. They are oligomers of flavan-3-ols and increasingly reactive with proteins and, therefore, have a more important astringent character. Its molecular size could also affect bitterness since monomers are more bitter than oligomers and polymers. The amount of procyanidin dimer (P12) varied between 18 and 80 mg/kg and was found to be significantly higher in

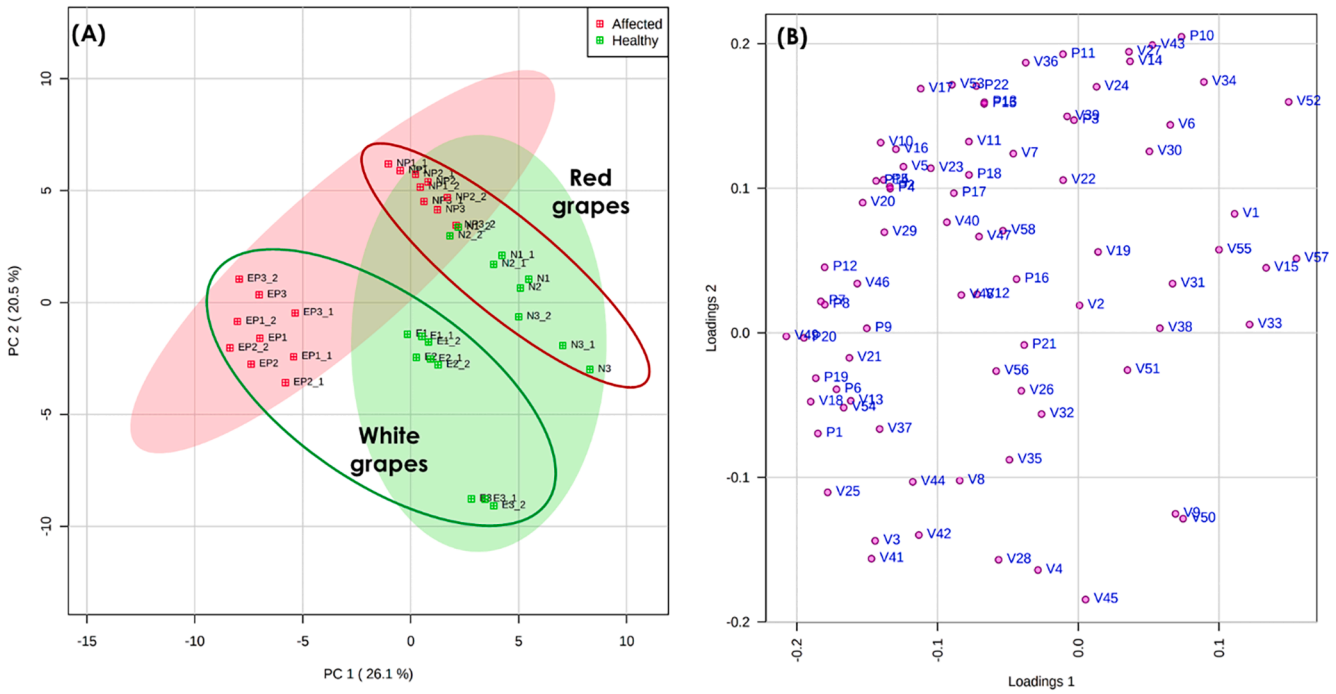

Fig. 5. PCA score plot (A) and loading weight plot (B) of *V. vinifera* L. grapes (attribution of the peak is shown in Tables 1-3).

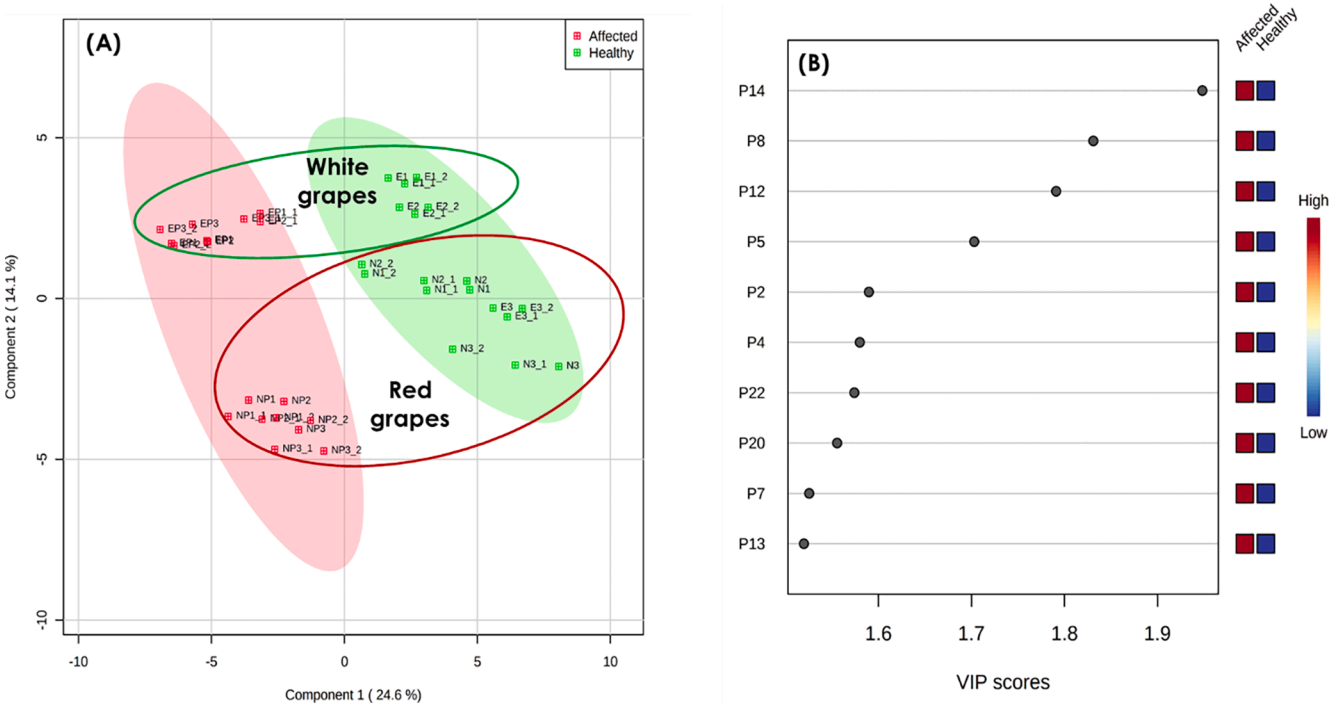

Fig. 6. PLS-DA of target analytes identified in *V. Vinifera* L. grape investigated. (A) Score plot and (B) VIP scores.

grapes produced by damaged plants. Syringic acid is one of the important phenolic acids with a hydroxybenzoic structure. Various studies have found syringic acid to exhibit useful pharmaceutical properties such as antioxidant, anti-microbial, anti-inflammation, anti-cancer, and anti-diabetic. It has been reported that the amount of syringic acid in Italian wines varies between 0.2 and 0.4 mg/100 mL (Minussi et al., 2003). Cyanidin-3-glucoside and its derivatives are anthocyanin compounds that are partially effective on the colour of *Vitis vinifera* grapes and wines. It is noteworthy that these anthocyanins increase in grapes obtained by damaged plants.

Related to infected white grapes projected in PC1 negative are mainly characterized by linalool (V37), α-terpineol (V49), (*E*)-coutaric acid (P7), quercetin-3-O-galactoside (P18), quercetin-3-O-glucuronide (P20), and quercetin (P22), whereas the healthy placed in PC1 positive by decanal (V32), linalool (V37), ethyl decanoate (V45), β-damascenone (V52), benzyl alcohol (V55) and phenylethyl alcohol (V57). Coutaric acid is an ester formed from coumaric acid and tartaric acid. Its content was found to be higher in grapes produced with plants attacked

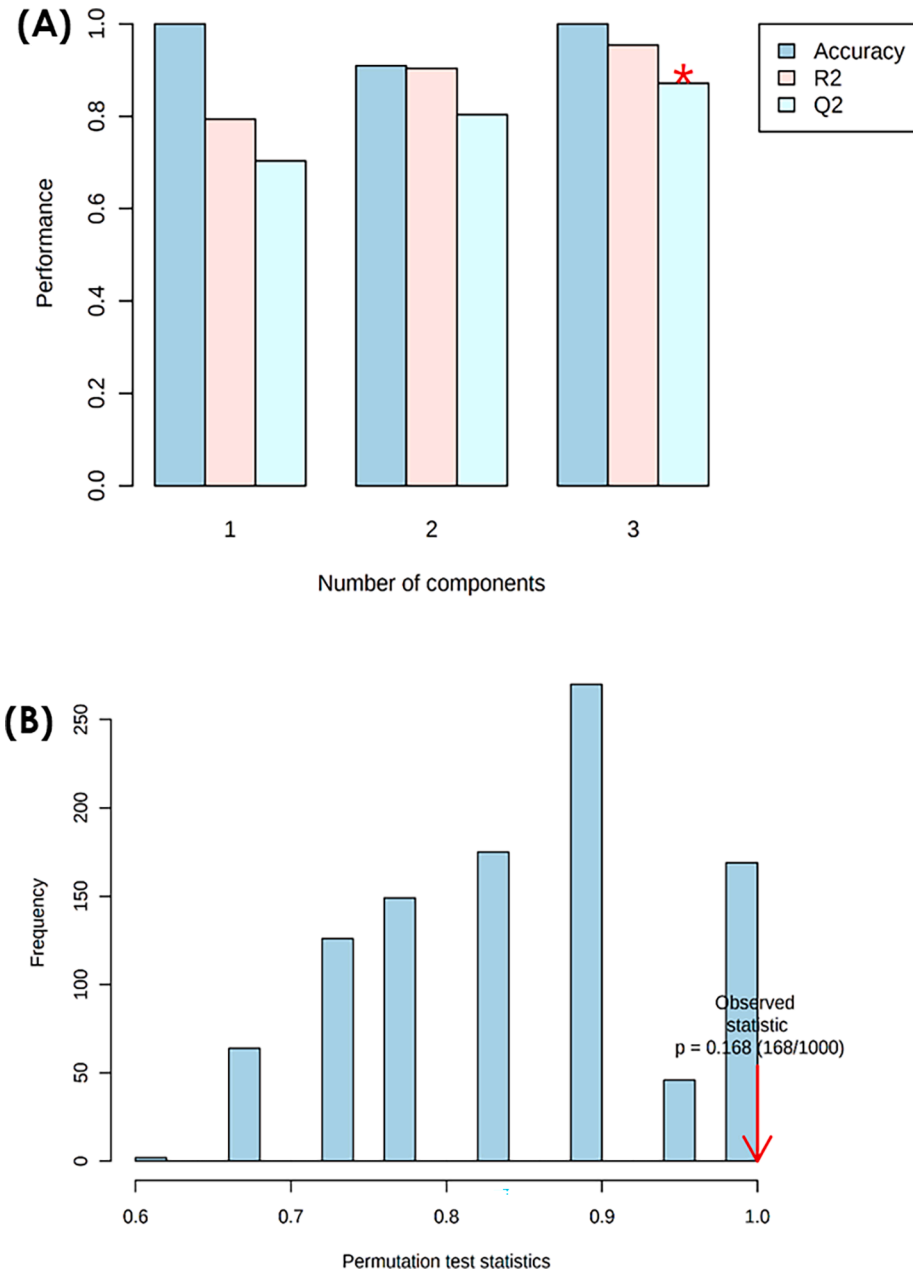

Fig. 7. 10-Fold cross-validation performance of PLS-DA model (A) and model validation by permutation test based on 1000 permutations of 80 target analytes identified in red and white grapes for a different healthy state. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by *P.* japonica. Quercetins are mainly located in the skins of white and red grapes. In our study, the amounts of quercetin-3-O-galactoside (P18) and quercetin-3-O-glucuronide (P20) varied between 2 and 4 mg/kg and 6–25 mg/kg, respectively, and the amount of these compounds increased if the plant is attacked by the *P. japonica*.

A subsequent PCA was performed using the concentration of 80 analytical variables (58 VOCs and 22 phenolics) identified in both red and white grapes. The PCA score plot and loading plot from *V. vinifera* grapes analyzed are shown in Fig. 5. The variance of PC1 and PC2 were 26.1 and 20.5 %, respectively, representing 46.6 % of the total variability of the data. To further understand the difference between healthy and affected states, a PLS-DA model was built. A clear separation was observed between infected and healthy grapes independently of grape variety (Fig. 6**A**). From the data set used, 10 phenolics were identified with VIP scores higher than 1.5, namely protocatechuic acid-O-hexoside (*P*2), protocatechuic acid (*P*4), hydroxy-caffeic acid dimer isomer 1 (P5), (*E*)-coutaric acid (P7), (*Z*)-fertaric acid (P8), procyanidin dimer (P12), catechin (P13), epicatechin (P14), quercetin-3-O-glucuronide (P20), and quercetin (P22), which are the most significant analytes to explain the discrimination between affected and healthy grapes (Fig. 6**B**). Three significant components described 0.955 of the goodness of fit (R^2) and 0.875 of predicted ability (Q^2) based on cross-validation (Fig. 7**A**). The difference between R^2 and Q^2 was 0.08, indicating that the model was not overfitting and has a good predictive ability to distinguish affected from healthy grapes, independently of the grape variety. Additionally, the robustness of the model was evaluated by a random permutation test with 1000 permutations (Fig. 7**B**).

Providing intuitive visualization of the dataset, and it is often applied to identify samples or features that are unusually high or low (Fig. 8). An analogous color tone to the heat map indicates the area, a group of samples, considering the concentration of the target analyte is similar. However, apart from grape variety, other factors like vineyard location,

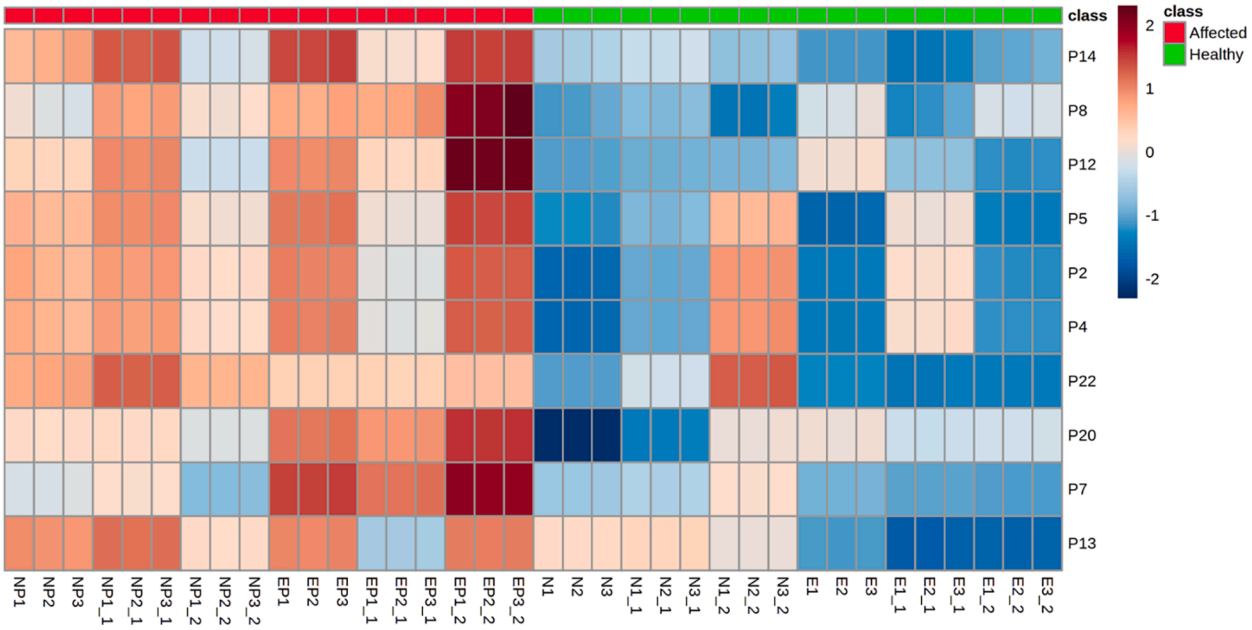

Fig. 8. Hierarchical cluster analysis (HCA). The heat maps of the phenolic compounds identified in *V. vinifera* L. grapes were generated by average algorithm and Pearson distance analysis (attribution of the abbreviation is shown in Table 1-3).

vine cultivation practices, and ripeness, could also affect the grape composition.

4. Conclusions

Considering the obtained data, it can be highlighted that the different considered parameters have rather similar trends. This trend is better understood when looking at the HPLC data. Considering the same sampling point, it was evident that the response of the plant significantly increased the content of some molecules such as gallic acid, (E)-caftaric acid, (Z)-fertaric acid, (E)-fertaric acid, catechin, epicatechin and quercetin-3-O-glucuronide. In the same way, if we consider the Nebbiolo samples, the anthocyanins also followed this trend (peonidin-3-O-glucoside, malvidin-3-glucoside and cyanidin-3-O-glucoside especially). From these results, it can be deduced that the plant when damaged, while decreasing the leaf apparatus and the yield of the bunch, tends to protect its survival by producing and transferring to the fruit higher levels of polyphenols with a consequent increase of antioxidant properties. The plant, therefore, responds significantly to this stressful situation. Even if with the due proportions, it partially recalls the effect that winegrowers are looking for using the technique of defoliation. The increase in grape flavanols and anthocyanins concentration as an outcome of defoliation is due to sunlight-driven upregulation biosynthesis of polyphenols. An increase in sun exposure leads to temperature elevation and more pronounced fluctuations in daytime berry temperature in fully exposed bunches.

The response put in place by the plant also included the volatile composition of Nebbiolo and Erbaluce grapes. In line with what was reported above, considering the same sampling point, in response to the attack by *P. Japonica* the plant significantly increased the content of some volatile compounds such as hexanal, (E)-2-hexenal, 1-hexanol, (E)-2-hexen-1-ol and phenyl ethyl alcohol.

CRediT authorship contribution statement

Serkan Selli: Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Rosa Perestrelo:** Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Hasim Kelebek:** Conceptualization, Visualization, Writing – original draft. **Onur Sevindik:** Conceptualization, Visualization, Writing – original draft. **Fabiano Travaglia:** Conceptualization, Visualization, Writing – review & editing. **Jean Daniel Coïsson:** Investigation, Methodology, Writing – review & editing. **José S. Câmara:** Formal analysis, Investigation, Methodology, Validation, Writing – review & editing, Funding acquisition. **Matteo Bordiga:** Project administration, Resources, Funding acquisition, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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