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Metabolomics Insight into the Variety-Mediated Responses to *Aspergillus carbonarius* Infection in Grapevine Berries

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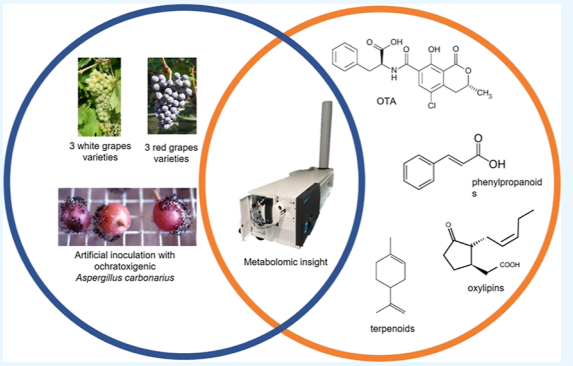
ABSTRACT: Limited knowledge regarding the susceptibility of grape varieties to ochratoxin A (OTA)-producing fungi is available to date. This study aimed to investigate the susceptibility of different grape varieties to *Aspergillus carbonarius* concerning OTA contamination and modulation at the metabolome level. Six grape varieties were selected, sampled at early veraison and ripening, artificially inoculated with *A. carbonarius*, and incubated at two temperature regimes. Significant differences were observed across cultivars, with Barbera showing the highest incidence of moldy berries (around 30%), while Malvasia and Ortrugo showed the lowest incidence (about 2%). OTA contamination was the lowest in Ortrugo and Malvasia, and the highest in Croatina, although it was not significantly different from Barbera, Merlot, and Sauvignon Blanc. Fungal development and mycotoxin production changed with grape variety; the sugar content in berries could also have played a role. Unsupervised multivariate statistical analysis from metabolomic fingerprints highlighted cultivar-specific responses, although a more generalized response was observed by supervised OPLS-DA modeling. An accumulation of nitrogen-containing compounds (alkaloids and glucosinolates), phenylpropanoids, and terpenoids, in addition to phytoalexins, was observed in all samples. A broader modulation of the metabolome was observed in white grapes, which were less contaminated by OTA. Jasmonates and oxylipins were identified as critical upstream modulators in metabolomic profiles. A direct correlation between the plant defense machinery and OTA was not observed, but the information was acquired and can contribute to optimizing preventive actions.



INTRODUCTION

According to FAO statistics (2019), grapes are among the most critical fruits produced worldwide (55,853,000 t), ranking fifth after bananas, watermelons, apples, and oranges. Most grapes come from *Vitis vinifera*, which covers about 94% of the world's grape-bearing area. At the same time, hybrids and other species accounted for the remaining 6%. *V. vinifera* typically colonizes temperate areas, while other species (*Vitis amurensis* and *Muscadinia rotundifolia*) or fruiting hybrids are better adapted to extreme zones (very cool or very warm areas).[1] About 10,000 *V. vinifera* subsp. vinifera varieties are recorded in the most extensive database (http://www.vivc.de/), and the genetic variability is quite significant in terms of morphology, phenology, vegetative, qualitative behavior, and resistance/susceptibility to biotic or abiotic stresses.[2]

Fungal infection and mycotoxin contamination are reported in grapes, and their severity varies depending on weather conditions, agricultural, harvest, and post-harvest practices, and the cultivar considered.[3−5] Among others, filamentous fungi, *Aspergillus carbonarius*, and *Aspergillus niger*, are the most frequently reported species among *Aspergillus* strains isolated from grapes in vineyards.[6] These species are also the main ones responsible for the presence of ochratoxin A (OTA) in grapes and wine.[7,8] Currently, limited information on the processes underlying *Aspergillus* infection in grape bunches is available. However, the physical and chemical characteristics of berries have been proposed as key factors determining differences in *Aspergillus* incidence and OTA production across varieties.[4,9] Among others, total sugars, acidity, and phenolic compounds have been proposed to affect the incidence of filamentous fungi.[4] Still, no accurate information is available to date. This can be ascribed to the multilayered complex biochemical processes at the plant-pathogen interface, mediated by pathogen-associated molecular pattern-triggered immunity,[10] and effector-triggered immunity.[11] The subsequent signaling cascade involves several metabolic hubs that cooperatively regulate systemic acquired resistance (SAR), a heightened immune state that plays a pivotal role in determining resistance/susceptibility to pathogen challenges.[12] Despite involving the complete hormone profile, the elicitation of

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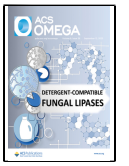

SAR primarily consists of the accumulation of salicylate complemented by methyl salicylate and jasmonate (JA),[12,13] and a subordinate accumulation of pathogenesis-related (PR) proteins, reactive oxygen species (ROS), and a chemically diverse class of specialized defense metabolites commonly known as phytoalexins. Considering that the biosynthesis of phytoalexins and the related regulatory networks are still largely unknown, different cultivars, transgenic plants, or omics approaches have been proposed to investigate their production and specific role(s) in plant defense.[14] Due to its inherent untargeted nature, metabolomics has been proposed as an effective tool to unravel the multifaceted biochemical response of plants to pathogen infection and plant-pathogen interaction.[15−19] Metabolomics fingerprinting, defined as the high-throughput and qualitative screening of metabolites, can provide a hypothesis-free signature of plant secondary metabolism, thus allowing the comparison and discrimination of samples efficiently. The effectiveness of metabolomics in covering plant-pathogen responses has also been reported for grapevine, including the investigation of induced resistance and cultivar-specific responses.[20−22] Notably, the ability of metabolomics to cover cultivar-mediated differences in susceptibility to *Plasmopara viticola* has been highlighted in previous reports.[23]

On these bases, and given the limited knowledge regarding the susceptibility of grape varieties to ochratoxin A (OTA)-producing fungi, the aim of this study was to comparatively investigate the response of grapes to *A. carbonarius* infection. Specifically, susceptibility, OTA production, and modulation at the metabolome level were considered functions of the variables used. Unraveling the mechanisms underlying cultivar-mediated *Aspergillus* infection in grape berries may pave the way toward adopting better management of this phytopathogen in the framework of food security and in line with more sustainable agriculture.

MATERIALS AND METHODS

Grape Inoculation. Six wine grape varieties [Merlot, Sauvignon Blanc, Barbera, Croatina, Malvasia di Candia aromatica (mentioned as Malvasia), and Ortrugo] were sampled at early veraison and at harvest time in 2019 in commercial vineyards located very close to each other in the same estate of north Italy (Piacenza province). The varieties were cultivated under the same pedoclimatic conditions and managed with the same cultural practices.

Five bunches were collected for each variety at early veraison and ripening. Harvest time was done under different sugar levels according to the enological destination of the varieties, from fizzy, sweet, low-alcohol wines like Malvasia to wines to be aged like the red ones. At each sampling time, 18 groups of 20 berries were prepared by randomly detaching berries from each bunch. Berries were homogeneous in size and color, with an intact pedicel and without visible damage or fungal growth on their skin. The berries were surface disinfected using sodium hypochlorite (2% v/v) for 2 min, then ethanol (80% v/v) for 2 min, and rinsed in sterile distilled water for 5 min.

Two strains of *A. carbonarius* were used for inoculation. These fungi were isolated in 2001 from bunches sampled in Italian vineyards and are known to produce OTA. They are stored in the fungal collection of the Institute of Sciences of Food Production (ISPA-CNR) (http://server.ispa.cnr.it/ITEM/Collection/) with the following code numbers: ITEM 5000 and ITEM 5012.

Three treatments were considered: (i) untreated control, (ii) inoculation with *A. carbonarius* ITEM 5000, and (iii) inoculation with *A. carbonarius* ITEM 5012. The inoculum was prepared by growing the two strains separately in Petri dishes filled with potato dextrose agar (PDA; Biolife, Milano, Italy) for 7 days at 25 °C. At the end of incubation, Petri dishes were washed with 10 mL of sterile distilled water, and conidia were counted using a Burker chamber. The suspensions were adjusted to a concentration of 10^5 conidia/mL. Aluminum vessels (14.0 × 11.5 cm, 4.5 cm depth), with damp paper (15 mL of sterile water) on the bottom and a plastic grid, were prepared and sterilized at 120 °C for 20 min to be used as humid chambers. The previously prepared berry groups were dipped for 5 min into the conidial suspension or sterile water for the untreated treatment. Berries were then placed into humid chambers, put into a plastic bag, and incubated for 7 days at 15 or 25 °C. The experiments were conducted in triplicate.

Fungal Quantification. At the end of incubation, from each sample of 20 berries, those showing visible growing molds were counted, and their incidence was reported as a percentage. Then, each sample was homogenized with a mixer (Bagmixer 400, Interscience, Paris, France), and the juice obtained was used to quantify colony-forming units per mL of juice (cfu/mL). 1 mL of juice was added to 9 mL of 1% peptone−water mixed with a vortex. The solution obtained was used for serial dilutions from 10^{-1} to 10^{-5}, plated on Potato Dextrose Agar (PDA, Biolife, Milano, Italy), and incubated at 25 °C for 7 days (12 h dark/12 h light). The trial was replicated three times.

OTA Quantification and Total Solids Quantification. Grape juice was extracted with an equal volume of ethanol (70% v/v). The commercial ELISA kit AgraQuant Ochratoxin A (RomerLabs, Getzersdorf, Austria) was used for OTA quantification. The analyses were performed according to the procedure described in the Agra-Quant Assay kit manual. Absorption in microwells was measured with a microwell reader (Sirio, Radim, Italy) using a 450 nm absorbance filter. The results were reported as μg/L using the standard curve developed according to the kit's instructions.

At the end of incubation, grape berries were crushed, and the juice was collected; 1 mL of juice was put in an Automatic Refractometer SMART-1 (ATAGO CO., LTD., Tokyo, Japan) to determine the soluble solids of the berries and reported as °Brix.

Untargeted Metabolomics Analysis. The untargeted metabolomics analysis was directly conducted on grape juice following centrifugation (8000*g* for 15 min) and filtration (cellulose membrane, 0.22 μm) into amber glass vials. Analysis was done using ultra-high-pressure liquid chromatography coupled to a quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS) from Agilent Technologies (Santa Clara, CA, USA), as previously reported.[24] Briefly, the chromatographic separation was achieved using a Pursuit 3 pentafluorophenyl (PFP) column (2.0 × 100 mm, 3 μm) from Agilent Technologies (Santa Clara, CA, USA) and a binary gradient from 6 to 94% acetonitrile (LC−MS grade, VWR, Milan, Italy) in 33 min with a flow rate of 200 μL min^{-1}. The samples were injected randomly, with an injection volume of 6 μL, and SCAN acquisition was adopted (100−1000 m/z, 30,000 fwhm, 1 Hz). The raw data were processed using Agilent Profinder B.06 software, as previously described,[25] to putatively annotate compounds based on isotopic spacing and

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Table 1. Results of the Analysis of Variance Run for the Incidence of Moldy Berries (%), Colony Forming Units (cfu/mL), and Ochratoxin A Content in Grape Berries Belonging to 6 Grape Varieties, Collected at Two Different Growing Stages (Veraison and Ripening) and Artificially Inoculated with *A. carbonarius*[a]

factor		incidence of moldy berries (%)		colony forming units (cfu/mL)		ochratoxin A (μg/L)	
grape variety (A)		**		**N.S.**		**	
	Malvasia	1.7	C	$2.5*10^3$		1.091	AB
	Ortrugo	0.8	C	$6.0*10^2$		0.582	B
	Sauvignon blanc	6.5	B	$2.5*10^4$		2.619	AB
	Barbera	16.1	A	$3.5*10^4$		2.355	A
	Croatina	13.5	A	$8.5*10^4$		2.921	A
	Merlot	7.8	B	$2.5*10^3$		1.853	AB
growth stage (B)		**		**N.S.**		**N.S.**	
	veraison	3.19	B	$1.5*10^4$		1.654	
	ripening	12.27	A	$3.5*10^4$		2.153	
treatment (C)		**		**		**	
	untreated	0.00	B	$3.5*10^3$	B	0.827	B
	ITEM 5000	12.15	A	$2.4*10^4$	A	1.774	AB
	ITEM 5012	11.04	A	$4.7*10^4$	A	3.109	A
temperature (D)		**		**		**	
	15°C	6.94	B	$8.5*10^2$	B	1.179	B
	25°C	8.52	A	$4.9*10^4$	A	2.627	A
AxB		**		N.S.		N.S.	
AxC		**		N.S.		*	
AxD		**		N.S.		N.S.	
BxC		**		N.S.		N.S.	
BxD		**		N.S.		N.S.	
CxD		**		N.S.		*	
AxBxC		**		N.S.		N.S.	
AxBxD		**		N.S.		N.S.	
AxCxD		*		N.S.		N.S.	
BxCxD		**		N.S.		N.S.	
AxBxCxD		N.S.		*		**	

[a]Different letters indicate significant differences according to the Tukey Test ($p \leq 0.01$).

ratio according to the "find-by-formula" algorithm (5 ppm mass accuracy for monoisotopic mass and 0.05 min for retention time alignment). The PlantCyc 12.6 database[26] was used as a reference, and level 2 confidence was achieved concerning the COSMOS Metabolomics Standards Initiative.[27] The Metabolomics data were deposited into the EMBL-EBI MetaboLights database with the identifier MTBLS7841.

Statistical Analysis and Data Interpretation. Data on fungal incidence were arcsine transformed. Data on cfu/mL and OTA (μg/L) content were ln transformed before statistical analysis.[28,29] All data obtained were subjected to univariate analysis of variance (ANOVA) using the generalized linear model procedure, and significant differences between means were confirmed using Tukey's test. Data correlation was evaluated using Pearson's correlation test ($p \geq 95\%$). The statistical package IBM SPSS Statistics 27 (IBM Corp., Armonk, NY, USA) was used for data analysis.

Metabolomics data were interpreted in Mass Profiler Professional B.12.06 (Agilent Technologies, Santa Clara, CA, USA) following mass and retention time alignment, normalization, and baselining.[24] Fungal strain and temperature were not considered classification factors in the metabolomics interpretations to provide broader responses and more generalizable results. Unsupervised hierarchical cluster analysis (HCA, Euclidean distance, and Ward's linkage rule) was conducted to naively describe patterns from the fold-change (FC)-based heatmap compared to the median in the dataset. Supervised statistics were used to maximize predictive variability at the expense of orthogonal variability. To this aim, orthogonal projection to latent structures discriminant analysis (OPLS-DA) was performed in SIMCA 16 (Umetrics, Malmo, Sweden). CV-ANOVA was used to validate the supervised OPLS-DA model ($P < 0.01$), overfitting was excluded by the permutation test ($n = 100$), and outliers were investigated using Hotelling's T2 (95 and 99% confidence limits for the suspect and strong outliers, respectively). The compounds annotated in at least 75% of replicates within at least one treatment were subjected to a volcano plot analysis by combining an analysis of variance (ANOVA; $p < 0.05$, Bonferroni multiple testing correction) and FC analysis (FC ≥ 1.2, compared to control) to identify differential metabolites. Statistically significant and differentially modulated metabolites were uploaded as a smart table, with compound name and log FC values, in the Omic Viewer Pathway Tool of PlantCyc (https://www.plantcyc.org/; Stanford, CA, USA), for pathway analysis.[30] The different biosynthetic pathways were plotted with the sum of the log FC values of each metabolite involved in the biosynthetic pathway. Metabolites are grouped into different biosynthetic pathways based on their biochemical roles. Finally, a Venn analysis was performed to investigate the similarities and dissimilarities of the metabolomic response across the different varieties.

RESULTS

Fungi and Ochratoxin A Quantification. Mold was only found in inoculated berries and was absent in control. All

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Table 2. Results of the Analysis of Variance Run for the Incidence of Moldy Berries (%), Colony Forming Units (cfu/mL), and Ochratoxin A Content in Grape Berries Belonging to 6 Grape Varieties, Collected at Full Ripening, Artificially Inoculated with *A. carbonarius*[a]

factor		incidence of moldy berries (%)		colony forming units (cfu/mL)		ochratoxin A (μg/L)	
grape variety (A)		**		**N.S.**		*	
	Malvasia	2.2	C	$2.8*10^3$		0.99	C
	Ortrugo	1.7	C	$1.0*10^3$		0.24	C
	Sauvignon blanc	10.8	B	$4.2*10^4$		2.50	AB
	Barbera	29.7	A	$6.8*10^4$		2.69	AB
	Croatina	14.4	B	$9.0*10^4$		4.49	A
	Merlot	14.7	B	$3.3*10^3$		2.01	AB
treatment (B)		**		**		*	
	untreated	0	B	$7.0*10^3$	B	0.94	B
	treated	18.4	A	$4.9*10^4$	A	2.76	A
AXB		**		N.S.		*	

[a]Different letters indicate significant differences according to the Tukey Test ($p \leq 0.01$).

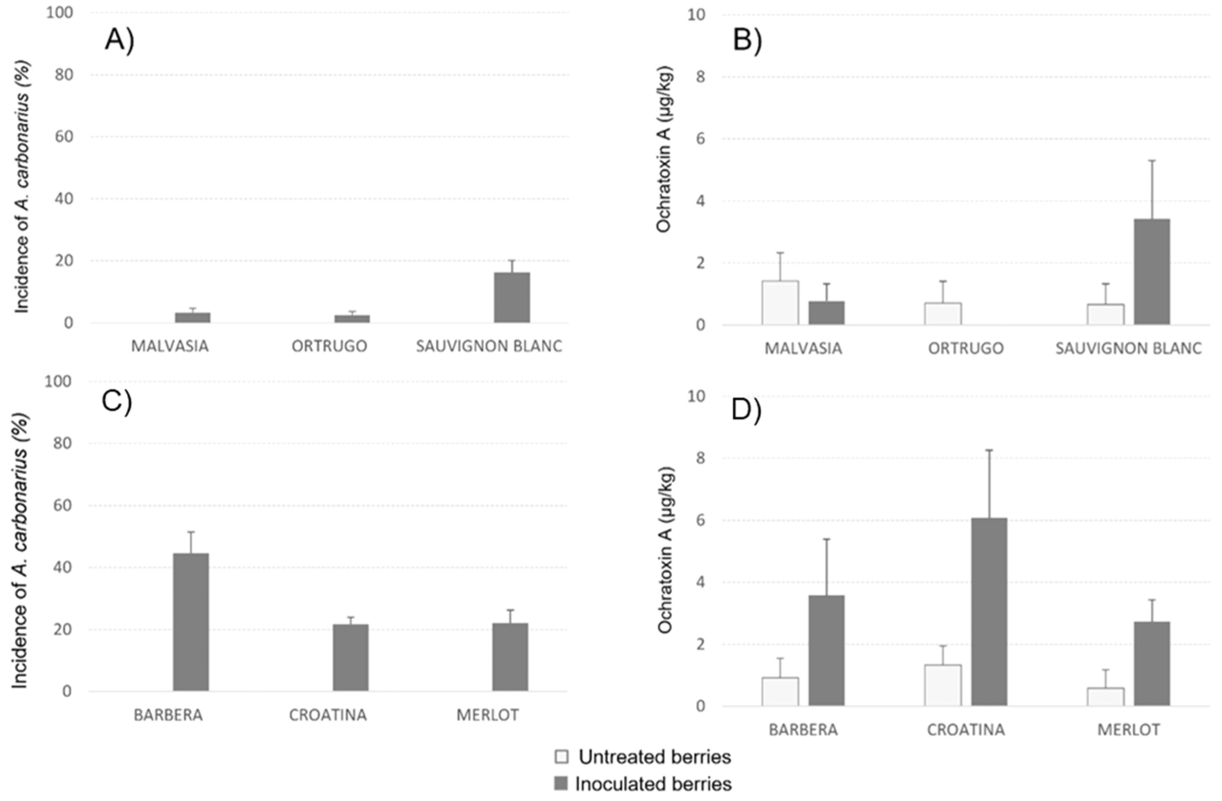

Figure 1. Mean incidence of *A. carbonarius* (%) and ochratoxin A content in untreated and artificially inoculated grape berries of white varieties (A,B) and red varieties (C,D) at the growth stage of fully ripening. Bar plots represent the average number obtained from the 3 experimental replicates considered in the study. The error standard is reported on each bar.

berries with growing mold showed black mold, as all colonies were observed in the UFC/mL count. Based on the mold/colony color and morphology, they were attributed to *A. carbonarius*. Therefore, all the results reported in this study refer to *A. carbonarius*.

Soluble solids at ripening were as follows: 15.1 °Brix for Malvasia, 16.9 °Brix for Ortrugo, 16.9 °Brix for Sauvignon Blanc, 18.2 °Brix for Barbera, 19.4 °Brix for Croatina, and 20.0 °Brix for Merlot (Table S1). The factor "grape variety" was shown in the ANOVA (Tables 1 and 2) to play a significant role in determining the incidence of moldy berries and OTA contamination ($P \leq 0.01$).

The inoculation treatment and the temperature regime during incubation significantly impacted fungal growth and OTA synthesis. Significant differences regarding the grape growth stage at infection were found only in the incidence of infected berries ($P \leq 0.01$), with a significantly higher incidence in ripening berries compared to those collected at veraison (12.27 vs 3.19%, respectively). No significant differences were found between the two *A. carbonarius* strains used for inoculation concerning the incidence of moldy berries or fungal cfu/mL. However, *A. carbonarius* ITEM 5012 produced more significant amounts of OTA (Table 1). The ANOVA was re-run using only data on grape sampling at ripening to simplify the interpretation of the results. At the same time, the two strains and the two temperatures were not considered factors (Table 2).

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ACS Omega 2023, 8, 32352−32364

The grape varieties considered in this study had different susceptibilities at ripening to *A. carbonarius* infection ($P \leq 0.01$) (Table 2). Around 30% of the berries had visible black mold in Barbera. In contrast, the incidence of berries with mold was very low in Malvasia and Ortrugo, at 2.2 and 1.7%, respectively (Table 2). As expected, the incidence of moldy berries was significantly influenced by the artificial inoculation, with 18.4 vs 0% in untreated berries.

The number of cfu/mL was significantly influenced by the inoculation treatment ($P \leq 0.01$; Table 2). Fungal contamination in the different grape varieties ranged from 10^3 to 10^4 cfu/mL without significant differences. Grape variety and inoculation treatment significantly affected the OTA content in grape berries ($P \leq 0.01$; Table 2). The lowest OTA contamination was detected in Ortrugo, comparable to Malvasia, and the highest in Croatina, which was not significantly different from Barbera, Merlot, and Sauvignon Blanc (Figure 1B,D). The inoculated berries were significantly more contaminated than those untreated for fungal presence and OTA content ($P \leq 0.05$; Figure 1A,C).

Considering the possible impact of soluble solids on grape infection by *A. carbonarius*, Pearson's correlation was applied to the dataset. Significant positive correlations were found between soluble solids and OTA content ($P \leq 0.05$), while no correlations were found with fungal incidence or cfu/mL (Table 3). However, *A. carbonarius* incidence was positively correlated with *A. carbonarius* (cfu/mL) and OTA (μg/L) quantity ($P \leq 0.05$), and *A. carbonarius* quantity was positively correlated with the OTA content in grapes ($P \leq 0.01$; Table 3).

Table 3. Pearson's Correlation Analysis Run Considering Soluble Solids (°Brix), the Incidence of Moldy Berries, Colony Forming Units (cfu/mL), and Ochratoxin A Content in Berries Belonging to Different Varieties

factor	incidence of moldy berries (%)	colony forming units (cfu/mL)	ochratoxin A (μg/L)
°Brix	0.336	−0.014	0.442[a]
incidence of moldy berries (%)		0.394[a]	0.398[a]
colony forming units (cfu/mL)			0.452[b]

[a] $p \leq 0.05$. [b] $p \leq 0.01$.

Metabolomic Profiling. The metabolic profiles of the six grape varieties inoculated and not inoculated with *A. carbonarius* were determined using an untargeted metabolomic approach through UHPLC/QTOF-MS. The cultivar and the inoculation were considered interpretation factors, whereas the samples from different fungal strains (*A. carbonarius* ITEM 5000 or ITEM 5012) and different temperatures (15 °C + 25 °C) were pooled. The untargeted metabolomics analysis putatively annotated more than 3500 metabolites; the comprehensive list of metabolites is provided as Supporting Information (Table S3), including ontology classification, pathways, abundances, and composite mass spectra.

An unsupervised multivariate analysis (HCA; Figure 2A), produced by FC values, was used to naively describe relatedness across treatments. The red and white grape varieties were distinguished by HCA as the hierarchically prevalent factor. As expected, the second level of clustering within each main cluster represented variety. Notably, grapes inoculated with *A. carbonarius* had a distinctive metabolic profile within each variety compared to the non-inoculated ones. Therefore, this unsupervised analysis indicated that the inoculation resulted in a shift in metabolite signatures, although with variety-specific traits.

Based on the HCA outcomes, a subsequent supervised OPLS-DA analysis was conducted for red and white varieties. Inoculated vs non-inoculated samples were modeled to better focus on the specific effects of *A. carbonarius* inoculation. OPLS-DA score plots for white and red grapes are provided in Figure 2B,C, respectively. As reported, both OPLS-DA models had a clear separation of inoculated (ITEM) and non-inoculated (TEST) samples by the contribution of the first latent vector $t[1]$. The goodness of fit and prediction ability of these two models were $R^2Y = 0.968$ and $Q^2Y = 0.453$ for the white grapes (Figure 2B) and $R^2Y = 0.976$ and $Q^2Y = 0.577$ for the red grapes (Figure 2C), respectively. Both models were cross-validated (CV-ANOVA, *P*-value ≤ 0.01), inspected for outliers (by Hotelling's Test), and overfitting can be excluded (Table S1).

Afterward, variable importance in projection (VIP) markers were selected for both OPLS-DA models to select the most discriminant grape metabolites in response to *A. carbonarius* infection for the red and white grape varieties. Overall, 141 (Table S4) and 143 metabolites (Table S5) were selected for the white and red varieties, respectively. The entire list of VIP marker compounds is provided as Supporting Information, including the ontology, individual VIP scores, standard error of the score, and log FC values for inoculated vs control treatments. VIP compounds could be ascribed mainly to secondary metabolites, such as phenylpropanoids, alkaloids, terpenoids, and phytoalexins, followed by fatty acids, lipids, and hormones. Fatty acids, alkaloids, sesquiterpenes, phenylpropanoids, and terpenoids showed the highest discrimination potential.

Effect of *A. carbonarius* Infection on White Grape Varieties. The effect of *A. carbonarius* inoculation on Malvasia, Ortrugo, and Sauvignon Blanc was investigated by combining ANOVA ($p \leq 0.05$) and FC analysis (FC ≥ 1.2). The resulting list, consisting of 132 compounds, is reported as Supporting Information (Table S6) and was interpreted in terms of the biochemical processes involved, as shown in Figure 3. A different trend in the biosynthesis pathways could be observed among the three varieties, indicating a higher modulation in secondary metabolism and hormone biosynthesis (Figure 3A). Malvasia had a reduction in secondary metabolites, compared to Ortrugo and Sauvignon Blanc, involving phenylpropanoids (flavonol derivatives and anthocyanin glycosides) and phytoalexins already reported in grapes (oryzalexin C and D, and oryzalide A). In contrast, monocyclic and benzylisoquinoline alkaloids, together with terpenoid metabolites, accumulated in response to inoculation. Regarding Sauvignon Blanc, an accumulation of defense compounds was observed, while inoculation seemed to have a comparatively lower effect in Ortrugo. Sauvignon Blanc accumulated different nitrogen-containing compounds following inoculation, namely alkaloids ((*S*)-colchicine, gamma-coniciene, coniine, and sanguinarine-related compounds) and glucosinolates derived from homomethionine (Figure 3B and Table S6). Interestingly, the indole-3-carbinonium ion, a precursor for the biosynthesis of indole-3-carbinol and indol-3-ylmethyl-L-cysteine, phytoalexins derived from glucosinolate degradation, was significantly modulated in the Sauvignon Blanc and Ortrugo varieties. Regarding hormones (Figure 3C), Sau-

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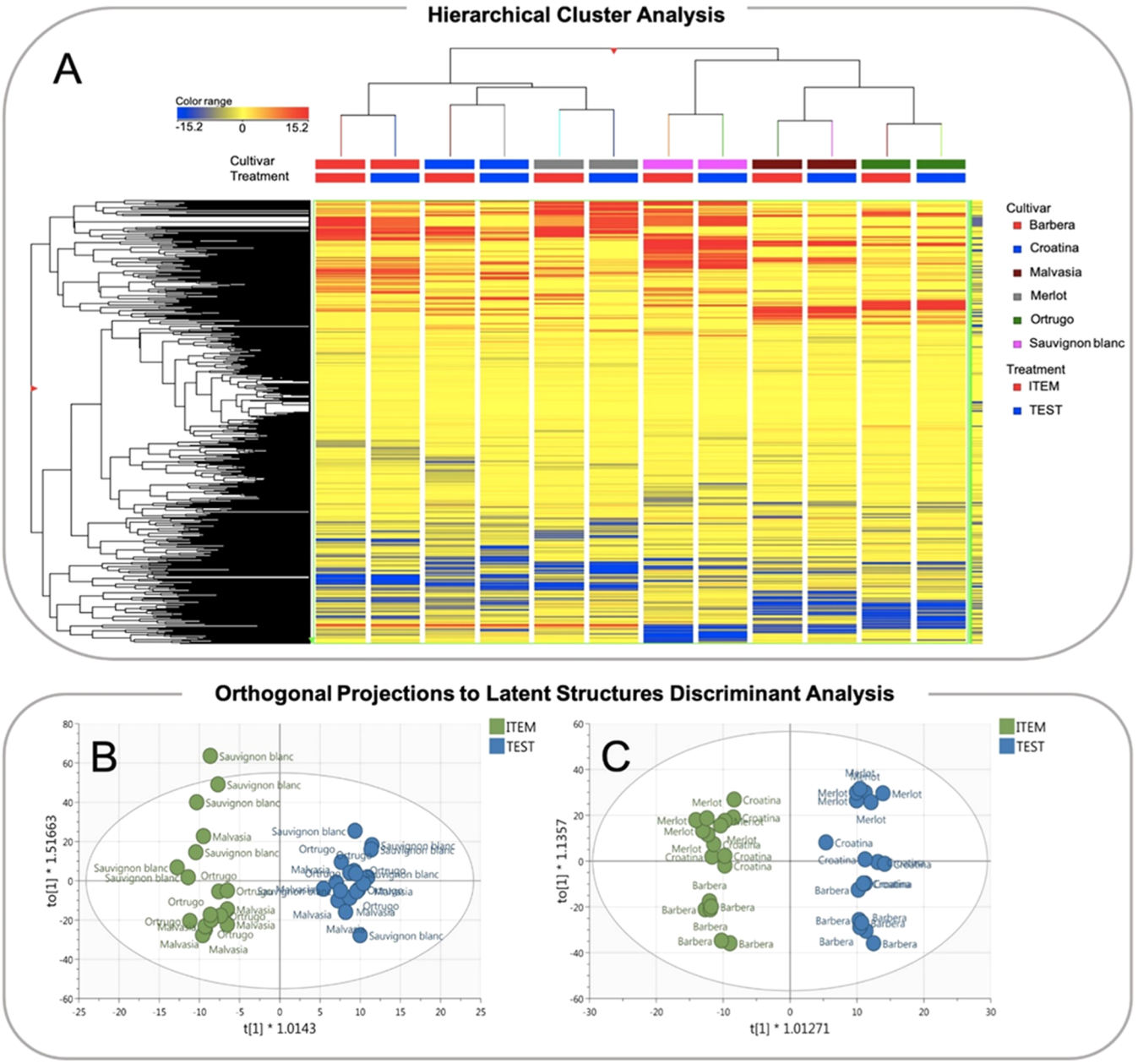

Figure 2. Multivariate statistical elaboration of metabolomic profiles of grape juices following bunch inoculation with *A. carbonarius* (ITEM, as a pool of two strains and two different temperatures), compared to non-inoculated samples (TEST). Unsupervised hierarchical cluster analysis (HCA, Euclidean distance) obtained from the FC heatmap (A) and supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) for white (B) and red grape varieties (C).

vignon Blanc presented a significant modulation of gibberellins, auxin conjugates, JAs, and cytokinins. Among others, the recruitment of methyl jasmonate in response to infection is worth mentioning, especially in Sauvignon Blanc.

Effect of *A. carbonarius* Infection on Red Grape Varieties. The metabolomic responses of Barbera, Croatina, and Merlot to *A. carbonarius* inoculation resulted in a final list of 112 differential metabolites (Table S7), as summarized in Figure 4. As was the case for the white varieties, secondary metabolites (Figure 4B), and hormones (Figure 4C) were the most affected classes, with similar trends between Croatina and Merlot, differentiating only for the degree of modulation. In particular, the accumulation of nitrogen-containing compounds, phenylpropanoids, terpenoids, and phytoalexins could be highlighted. Specifically, alkaloids, phenolics, and the phytoalexin malonyldaizin were higher in both Croatina and Merlot varieties following inoculation.

Concerning Barbera, a comparatively lower remodeling of secondary metabolism could be observed following infection, including a down-accumulation of phenylpropanoids and phytoalexins (Figure 4B). Among the hormones, a slight modulation of brassinosteroids, cytokinins, gibberellins, and JA was observed (Figure 4C). Considering brassinosteroids, brassinolide-related compounds (e.g., teasterone, 26-hydroxybrassinolide, and 26-hydroxycastasterone) were the most involved. Moreover, degradation products of abscisic acids were also found in all varieties, whereas the adenine-type cytokinin glucoside dihydrozeatin-9-*N*-glucoside accumulated in response to *A. carbonarius* inoculation.

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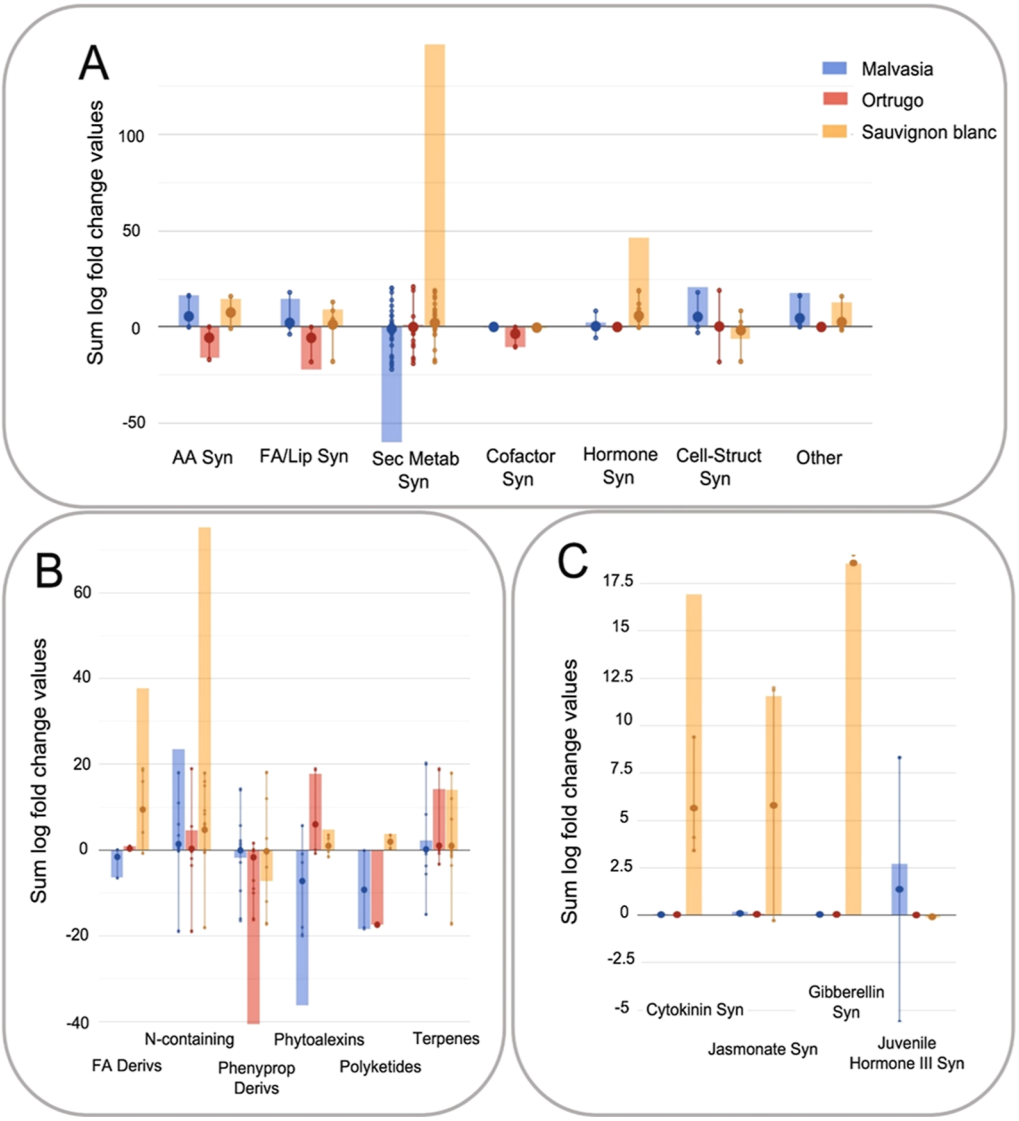

Figure 3. Pathway analysis of white grape varieties (Malvasia, Ortrugo, and Sauvignon Blanc) conducted on the significantly and differentially modulated metabolites (ANOVA p-value < 0.05; FC ≥ 1.2) following inoculation with *A. carbonarius* (ITEM, as a pool of two strains and two different temperatures), compared with non-inoculated samples (TEST). Differential metabolites were interpreted in terms of biosynthesis pathways (A), secondary metabolites (B), and hormone biosynthesis (C). The bars represent the sum of the log FC values of each metabolite involved in the biosynthetic pathway. The different dots within each vertical bar indicate the log FC value for each metabolite belonging to the biosynthetic pathway, while the larger dots indicate the median value. Abbreviations: AA: amino acids; FA/Lip: fatty acids and lipid; Sec Metab: secondary metabolites; Cell-Struct: cell-structure; Phenylprop Derivs: phenylpropanoid and derivatives; syn: synthesis.

Variety-Specific Responses to *A. carbonarius* Infection. Pairwise comparisons of inoculated vs control samples within each variety considered were performed through a volcano plot by combining ANOVA and FC analysis ($p \leq 0.05$ with FC ≥ 1.2). The comprehensive differential compound list is shown in the Supporting Information (Table S8). The differential metabolites were next subjected to Venn analysis to identify commonly shared and specific responses to infection separately for white (Figure 5A,B) and red grape varieties (Figure 5C,D), formerly on up-accumulated and down-accumulated metabolites, respectively.

Overall, it was indicated in the Venn analysis that white grape varieties showed a more distinct response to fungal infection than red ones. Concerning up-accumulated metabolites, only 24 common compounds were shared among the three white grape varieties (Figure 5A). Most of the standard compounds were shared between Malvasia and Ortrugo (29 metabolites), suggesting a more similar response. Among the 24 common metabolites, isoprenoids and their precursors, such as abieta-7,13-diene-18-al (involved in synthesizing abieta-7,13-diene-18-oate) and glycyrrhetinate, may be found (Table S8). Moreover, indol-3-ylmethyl-L-cysteine, an indole-phytoalexin derived from glucosinolate degradation, was identified, along with other plant defense-related metabolites such as alkaloids, polyphenols, and glucosinolates. Interestingly, the hormones gibberellin A_{20} and methyl jasmonate were also commonly accumulated in white grape varieties.

Regarding red grape varieties, a more generalized response to fungal infection was suggested in the Venn analysis (Figure 5C), with a higher number of common compounds (i.e., ref 38). Among these common metabolites, polyphenols, glucosinolates, alkaloids, terpenoids, phytoalexins, glutathione, and hormone-related compounds were the most represented (Table S8). Among polyphenols, anthocyanin glycosides,

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ACS Omega 2023, 8, 32352−32364

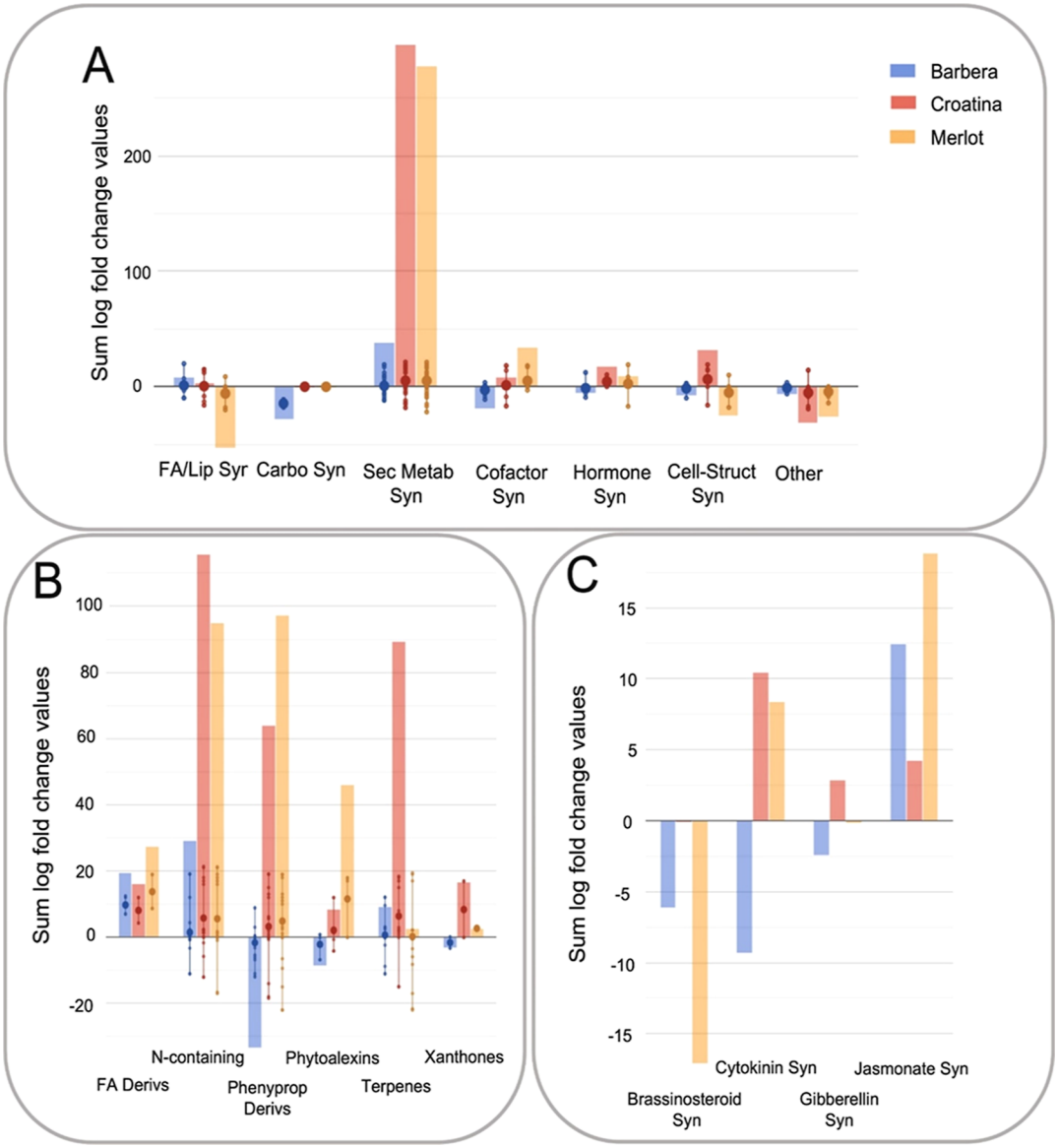

Figure 4. Pathway analysis of red grape varieties (Barbera, Croatina, and Merlot) conducted on the significantly and differentially modulated metabolites (ANOVA p-value < 0.05; FC ≥ 1.2) following inoculation with *A. carbonarius* (ITEM, as a pool of two strains and two different temperatures), compared to non-inoculated samples (TEST). Differential metabolites were interpreted in terms of biosynthesis pathways (A), secondary metabolites (B), and hormone biosynthesis (C). The bars represent the sum of the log FC values of each metabolite involved in the biosynthetic pathway. The different dots within each vertical bar indicate the log FC value for each metabolite belonging to the biosynthetic pathway, while the larger dots indicate the median value. Abbreviations: FA/Lip: fatty acids and lipids; Carbo: carbohydrates; Sec Metab: secondary metabolites; Cell-Struct: cell-structure; Phenylprop Derivs: phenylpropanoid and derivatives; syn: synthesis.

flavones, and other low-molecular-weight phenolics were detected. Regarding nitrogen- and sulfur-containing compounds, alkaloids, and aliphatic glucosinolates were engaged in response to inoculation. Moreover, the biosynthesis of terpenoids was also involved, including mono-, di-, and sesquiterpenes, such as manoyl oxide, dehydroabietate, and abietatriene. Other defense-related compounds commonly modulated by the inoculum were jasmonic acids, phytoalexins, and the glutathione-related detoxification metabolite (*R*)-*S*-lactoylglutathione. Specifically, two precursors of jasmonic acid biosynthesis, namely (9*Z*,13*S*,15*Z*)-12,13-epoxyoctadeca-9,11,15-trienoate and 9-(*S*)-HPOTE, accumulated in response to *A. carbonarius*. The JA precursor (9*Z*,13*S*,15*Z*)-12,13-epoxyoctadeca-9,11,15-trienoate and the diterpene abieta-7,13-diene-18-al were up-accumulated in all varieties in both white and red grape varieties.

Regarding the down-accumulated metabolites, Venn analysis confirmed a variety-dependent response to fungal infection (Figure 5B,D and Table S8). Among the 12 common metabolites detected in white grape varieties (Figure 5B), dehydroabietadienal, an upstream precursor of the diterpene abieta-7,13-diene-18-al biosynthesis, was identified. Concerning red grape varieties (Figure 5D), the common metabolites[17] among grape varieties were higher than variety-specific metabolites (8, 11, and 15 for Barbera, Croatina, and Merlot, respectively). Among the 17 common metabolites, precursors of plant cell wall polysaccharides, flavin electron carriers, long-chain fatty acids, CMP- and UDP- sugars, and chlorophyll

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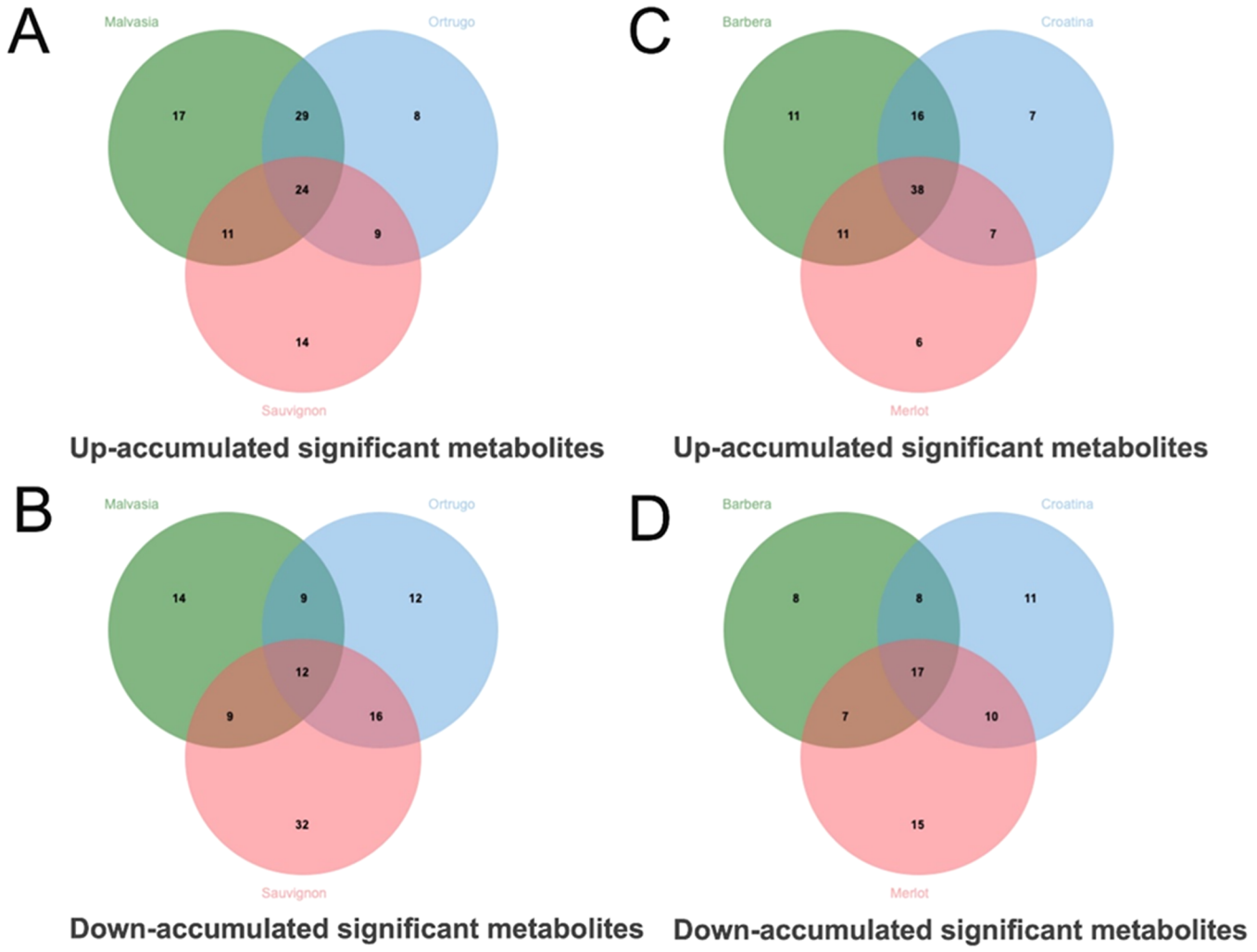

Figure 5. Venn analysis conducted from the lists of differential metabolites that passed ANOVA and FC analysis (p-value < 0.05, FC ≥ 1.2) following inoculation with *A. carbonarius* for (A,B) white grape varieties (Malvasia, Ortrugo, Sauvignon Blanc) and (C,D) red grape varieties (Barbera, Croatina, and Merlot) as up-accumulated metabolites and down-accumulated metabolites, respectively.

were all down-accumulated compared to the non-infected control.

DISCUSSION

The grape varieties included in this study are commonly grown in Northern Italy and have never been considered for their susceptibility to OTA-producing fungi. Regarding resistance/susceptibility to other relevant fungi, Sauvignon Blanc is reported to be very susceptible to gray mold and trunk diseases, susceptible to powdery mildew and low susceptibility to downy mildew. On the other hand, Merlot is known to be very susceptible to downy mildew, a little susceptible to gray mold, and little susceptible to powdery mildew and trunk diseases. The other varieties, native to Italy, are not well characterized for their susceptibility to fungal plant pathogens.

Berries contaminated by black Aspergilli showed fungal colonies and low OTA contamination. Although the absence of symptoms is not always evidence of health in grape berries,[31,32] artificially infected berries had a significantly higher incidence of infected berries than the non-inoculated control. The effect of grape variety changed depending on the variable considered, being irrelevant when *A. carbonarius* was quantified but significant for the incidence of moldy berries. White varieties had fewer moldy berries than red ones, particularly Malvasia and Ortrugo vs Barbera and Croatina. At harvest, the incidence of infected berries was highest in Barbera. The significant difference was limited to Ortrugo vs Barbera and Croatina when OTA was quantified. As observed in previous studies, this confirms that grape susceptibility to fungal infection and OTA contamination are not necessarily related.[33] Sugar content is higher in red varieties compared to white varieties. This is due to the different wines produced by these kinds of grapes. In particular, Malvasia, which had one of the lowest fungal contaminations, ripens at a lower sugar content because its enological destination is to produce fizzy, sweet, and low-alcohol wine. Of course, the level of sugars can contribute to fungal metabolism, but it is not sufficient per se to explain these differences.[34−39]

Significant differences were observed across varieties considering metabolomic signatures, as expected. A distinct metabolomic profile was observed for Sauvignon Blanc compared to the other two white varieties. There was an accumulation of defense compounds and significant modulation of gibberellins, auxin conjugates, JAs, and cytokinins. It is interesting to note that white grapes seem more susceptible to *A. carbonarius* infection than red grapes when considering metabolomics shifts in response to fungal contamination. This

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ACS Omega 2023, 8, 32352−32364

is possible because of the higher amounts of phenolics in the latter varieties. A higher number of metabolites was implicated in the response of Malvasia and Ortrugo to infection, with both varieties showing similar behavior in terms of fungal incidence. However, significant differences can be noted in OTA occurrence, which is slightly higher in Malvasia than in Ortrugo. Interestingly, within secondary metabolism, these two varieties showed opposite responses regarding phytoalexins, which generally were higher in the former than in the latter.

Red varieties, on the contrary, showed more generalized biochemical modulation, although Barbera had a comparatively lower remodeling of secondary metabolism. Barbera was the only red variety showing a down-accumulation of phenylpropanoid derivates, phytoalexins, and xanthones. It was noteworthy that Barbera also presented the highest fungal contamination among red grapes. However, Croatina and Merlot had similar behavior after *A. carbonarius* infection, characterized by the accumulation of nitrogen-containing compounds, phenylpropanoids, terpenoids, and phytoalexins. Fungal occurrence in red grapes did not follow the detected OTA levels, with Croatina being the most contaminated by OTA. The greatest differences found in Croatina compared to Barbera and Merlot were due to an evidently higher accumulation of terpenes and a complete absence of brassinosteroids. Interestingly, Merlot had the lowest OTA infection among red grapes and was characterized by the most increased production of phytoalexins and the lowest accumulation of brassinosteroids.

Despite showing distinct metabolomic responses to infection as a function of the cultivar considered, more generalized responses could be highlighted thanks to the OPLS-DA supervised modeling for white and red grape varieties. A common response to infection across all cultivars involves eliciting JA biosynthesis. The accumulation of JA precursors, such as (9*Z*,13*S*,15*Z*)-12,13-epoxyoctadeca-9,11,15-trienoate and 9(*S*)-HPOTE, is consistent with the well-recognized pivotal role of JA in orchestrating plant adaptive responses following interaction with pathogens, including fungi, as well as in triggering the expression of defense-related genes.[40−42] By playing a significant role in several developmental responses, JA and the related oxylipins are also essential in eliciting secondary metabolites, including sesquiterpenes, phenolics, and glucosinolates.[43−46] Consistently, these classes of compounds were also elicited in our experiments, although with individual differences across the red and white varieties.

Phenylpropanoids are known phytoalexins reported across different plant families,[47] whose biosynthesis is induced by fungal infection.[48] In grapes, nitrous oxide-induced accumulation of phenylpropanoids has been reported to enhance resistance to *Botrytis cinerea*, significantly reducing the diameter and incidence of lesions during post-harvest.[49] With direct antifungal activity, phenylpropanoids may also regulate ROS levels during infection by necrotrophic pathogens[50] rather than the modulation of hormonal profile to support a more effective response to the pathogen.[51] The interplay between phenylpropanoids, ROS, and phytohormones could also be observed in our experiments, where epoxy-, oxo-, and hydroxy fatty acids, all related to oxidative imbalance, together with JA, cytokinins, brassinosteroids, and gibberellin-related metabolites, were differentially accumulated following fungal infection.

Still, among defense compounds, glucosinolates and their methylsulfonyl derivatives represented a common response to fungal infection. These secondary metabolites are a broad class of compounds featuring a thioglucose moiety and a sulfonated oxime, with variable aglycone side chains derived from amino acids. These metabolites are hydrolyzed upon fungal infection to produce thiocyanates and other downstream defense intermediates.[52] In crucifers, glucosinolates have also been connected to phytoalexin biosynthesis.[53] Infection by *A. carbonarius* also elicited the accumulation of oryzalexins and abietane-related compounds, diterpene phytoalexins, with antifungal activity in higher plants.[47,54] Consistent with our findings, fungal-related non-biotic elicitors, like the exopolysaccharide chitosan, were shown to elicit the accumulation of di- and triterpene compounds.[55] It is interesting to observe that diterpenoid oryzalexins and isoflavones (malonyldaizin) seem to be variety-related, with diterpenoids present in white grape varieties and isoflavones present in red grape varieties. The biosynthesis of most phytoalexins and their upstream and downstream regulatory networks in crops are primarily unknown.[14]

Nonetheless, the complex crosstalk between phytoalexins, such as flavonoids and other phenylpropanoids, oxooctadecadienoic acids, ROS, and JAs, has been postulated by several authors.[14,56,57] In contrast to earlier studies in which the involvement of stilbenes was reported in Vitaceae resistance to *P. viticola* and *Erysiphe* necator,[14,58,59] we could not observe the modulation of stilbenes in response to infection by *A. carbonarius*. This might be related to either the pathogen or the limited coverage of this class by the metabolomic approach adopted.

Nevertheless, a broad modulation of biochemical processes was indicated by differences in metabolomic signatures, starting from the cascade triggered by JA and lipoxygenase-mediated oxylipins. These latter compounds mediate the SAR response through longer-distance signaling of lipid-based compounds.[60] Their direct antimicrobial potential by interacting with pathogen membranes has been postulated as well.[61,62] JAs and oxylipins can also alter the expression of genes related to the plant regulatory network, with synergistic and antagonistic effects on other phytohormones.[63] Thereafter, the production of specialized secondary metabolites is induced by JA signaling,[42] including phenylpropanoids and glucosinolates.[64,65] Indeed, a signaling cascade involving JAs, phenylpropanoids, and glucosinolates could also be observed in our experiments. Interestingly, Sauvignon Blanc, the cultivar with the highest accumulation of methyl JA, also had an increased incidence of *A. carbonarius* and high OTA contamination, considering JA and its cascade. This situation has already been reported, even for *Fusarium verticillioides* and fumonisin contamination in maize.[66] This may indicate that, at least under our experimental conditions, JA may represent a response to infection rather than a protection factor at the time of our metabolomic investigation.

Regarding the JA-induced cascade, it is noteworthy that a decrease in chlorophyll has been linked to flavonoid accumulation as part of plant resistance to fungi.[67] This is consistent with our findings, where a general down-accumulation of chlorophyllide can be observed across all cultivars.

Finally, concerning the link between plant defense machinery and OTA accumulation, a direct relationship between mycotoxin levels and severity of infection could not be observed, as already reported in many studies,[68,69] and recently discussed by a panel of experts.[70] However, not much

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ACS Omega 2023, 8, 32352−32364

is known to date regarding the factors behind mycotoxin production, a process arising from a complex ecological perspective that should consider the pathogen, the host, and the whole microbiota of the host.[71] Both oxylipins and antioxidant compounds are essential in determining the actual level of mycotoxins,[72] even though the dynamics of metabolomic changes following infection are also worth considering.

CONCLUSIONS

Susceptibility to infection from *A. carbonarius*, OTA contamination, and modulation at the biochemical level were investigated in different grape cultivars using an untargeted metabolomics approach. The results highlighted significant differences across cultivars, with Barbera showing the highest incidence and Malvasia and Ortrugo the lowest. The highest OTA contamination was observed in Croatina, and mycotoxin levels were not directly correlated with the level of infection.

Supervised statistics allowed the highlighting of common responses among cultivars, although metabolomic fingerprints were, instead, cultivar-specific. Overall, the JA and oxylipin signaling cascade elicited the accumulation of nitrogen-containing compounds (e.g., alkaloids and glucosinolates), phenylpropanoids, and terpenoids, in addition to phytoalexins.

In conclusion, broad metabolomic reprogramming was observed at the secondary metabolism level, involving several defense-related compounds that supported the differences in infection that we observed. Nonetheless, the levels of OTA accumulation did not directly reflect the remodeling of plant defense, which is not surprising given that the role of OTA in the fungus−plant interaction is not well known. Even if our results can support resilience to infection, further studies are advisable on the mechanisms underlying OTA accumulation to contribute to preventive actions and address food safety issues.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c01381.

Soluble solids of grape berries were collected at full ripening and incubated at different temperatures (15 and 25 °C) considering different theses (e.g., untreated and artificially inoculated with *A. carbonarius*); dataset of compounds identified in grape wine samples conducted from the untargeted metabolomics approach using UHPLC-ESI/QTOF-MS; discriminant metabolites identified in white grape samples using the variable importance in the projection (VIP) selection method following supervised OPLS-DA modeling; compounds provided with ontology classification, VIP scores (a measure of the importance of variables in the OPLS model), and log FC values obtained by pairwise comparison against non-inoculated samples; discriminant metabolites identified in red grape samples using the variable importance in projection (VIP) selection method following supervised OPLS-DA modeling; compounds provided with ontology classification, VIP scores (a measure of variable importance in the OPLS model), and log FC values obtained by pairwise comparison against non-inoculated samples; volcano analysis ($p < 0.05$; fold change cut-off = 1.2) carried out from the UHPLC-ESI/QTOF-MS metabolites dataset, comparing inoculated and non-inoculated white grape berries with *A. carbonarius*; volcano analysis ($p < 0.05$; fold change cut-off = 1.2) carried out from the UHPLC-ESI/QTOF-MS metabolites dataset, comparing inoculated and non-inoculated red grape berries with *A. carbonarius*; metabolites significantly up- and down-accumulated from Venn analysis (p-value < 0.05, FC ≥ 1.2) following the inoculation of different grape varieties with *A. carbonarius*; validation of the OPLS-DA models carried out from the metabolomic datasets; permutation test ($n = 100$) used to exclude overfitting in the white and red grape variety models; and Hotelling's T2 test (95 and 99% confidence limit for suspect and strong outliers, respectively) used to exclude outliers in the white and red grape varieties datasets (XLSX)

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Notes

The authors declare no competing financial interest.

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