From plant resistance response to the discovery of antimicrobial compounds: the role of volatile organic compounds (VOCs) in grapevine downy mildew infection

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Abstract

The discovery of new mechanisms of resistance and natural bioactive molecules could be two of the possible ways to reduce fungicide use in vineyard and assure an acceptable and sustainable protection against *Plasmopara viticola*, the grapevine downy mildew agent. Emission of volatile organic compounds (VOCs), such as terpenes, norisoprenoids, alcohols and aldehydes, is frequently induced in plants in response to attack by pathogens, such as *P. viticola*, that is known to cause an VOCs increment in cultivars harboring American resistance traits. In this study, the role of leaf VOCs in resistance mechanism of two resistant cultivars (Mgaloblishvili, a pure *Vitis vinifera* cultivar, and Bianca, an interspecific hybrid) and the direct antimicrobial activity of four selected VOCs have been investigated. The leaf VOC profiles, analyzed through solid-phase microextraction gas chromatography-mass spectrometry analysis, as well as the expression of six terpene synthases (TPSs), were determined upon pathogen inoculation. In both cultivars, the expression pattern of six TPSs increased soon after pathogen inoculation and an increment of nine VOCs has been detected. While in Mgaloblishvili, VOCs were synthesized early after *P. viticola* inoculation, in Bianca, they constituted a late response to pathogen. All the four terpenes (farnesene, nerolidol, ocimene and valencene), chosen according to the VOC profiles and gene

expression analysis, caused a significant reduction (53-100 %) in *P. viticola* sporulation. These results support the role of VOCs into defense mechanisms of both cultivars and suggest their potential role as a natural and eco-friendly solution to protect grapevine from *P. viticola.*

Keyword: volatile organic compounds, monoterpenes, sesquiterpenes, disease containment, *Plasmopara viticola*, *Vitis vinifera*, sustainable crop production

1. Introduction

Plants are exposed to different environmental and biological stresses and they have the ability to thrive against threats through various pathways, including the production of secondary metabolites. Secondary metabolites are synthesized by different plant species not only as a defense mechanism against biotic and abiotic stresses but also for reproducibility and dissemination of their offspring (Algarra Alarcon et al., 2015). These bioactive metabolites can be alkaloids, flavonoids, saponins, tannins, terpenes and others.

In particular, terpenes are the largest and most investigated class of secondary metabolites that plants produce. They derive from the condensation of two or more isoprenic units, the precursor isopentenyl pyrophosphate (C5) and its allylic isomer dimethylallyl pyrophosphate, to form mono- (C10), sesqui- (C15) and diterpene (C20) precursors, through two alternative pathways: the mevalonate pathway and the methylerythritol phosphate pathway (MEP). The MEP pathway, localized in the plastids, leads to the biosynthesis of hemiterpenes, monoterpenes and diterpenes, while the cytosol-localized mevalonate pathway leads to sesquiterpene biosynthesis. The last step of the pathway catalyzes the conversion of each precursor to the primary representatives of each class by a large family of enzyme known as terpene synthases. Finally, some terpenes are formed by oxidation, dehydrogenation, acylation, and other reaction types (Dudareva et al., 2004).

Terpenoids thus synthesized, together with alkanes, alkenes, alcohols, esters and acids, belong to the class of volatile organic compounds (VOCs). In the plant defense systems, secondary metabolites having antifungal properties are synthetized immediately after pathogen infection (Brilli et al., 2019). Among these secondary metabolites, VOCs can act against pathogens and herbivores either by a direct, as defense metabolites, or indirect mechanisms, mediating the

signals between different parts of the same plant, from plant to plant and other organisms (Pierik et al., 2014).

The effectiveness of VOC-mediated induced resistance has been demonstrated in several plant pathosystems, such as: tobacco and *Ralstonia solanacearum* (Dorokhov et al., 2012); *Arabidopsis thaliana* and *Trichoderma* spp. (Estrada-Rivera et al., 2019). Furthermore, numerous studies reported the ability of leaf VOCs to inhibit spore germination and mycelial growth of fungal pathogens. For instance, citral, carvacrol, and *trans*-2-hexenal showed an inhibitory activity against *Monilinia laxa* (Neri et al., 2007).

The involvement of VOCs in response to pathogens, such as the oomycete *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, has been demonstrated in grapevine, as well. *P. viticola* is the causal agent of downy mildew, one of the most destroying diseases affecting the Eurasian grapevine cultivars (*Vitis vinifera*). It originated in North America, where autochthonous species, such as *V. labrusca, V. aestivalis, V. riparia*, have been developed resistance traits due to the co-evolution with the pathogen. At the end of 19th century, *P. viticola* reached Europe, leading to substantial quantitative and qualitative damages due to the high susceptibility of the *V. vinifera* species. It has been demonstrated that *P. viticola* infection is inhibited in leaf tissues by some VOCs (2-ethylfuran, 2-phenylethanol, β-cyclocitral or *trans*-2-pentenal) (Lazazzara et al., 2018). On the other hand, non-*vinifera* resistant genotypes (Kober 5BB, SO4) showed to emit specific VOC profiles in response to *P. viticola* infection (Algarra Alarcon et al., 2015; Lazazzara et al., 2018).

Mgaloblishvili is a *V. vinifera* cultivar native to Georgia (Caucasus, the first grapevine domestication center), showing unique resistance traits against *P. viticola* (Silvia Laura Toffolatti et al., 2018; Toffolatti et al., 2016). This cultivar shows a limitation of *P. viticola* growth and sporulation (up to 80 % in comparison to the susceptible *V. vinifera* cultivar Pinot noir) and an overexpression of genes related to the synthesis of antimicrobial enzymes and compounds such as terpenes (Silvia Laura Toffolatti et al., 2018; Toffolatti et al., 2020). In particular, two genes showed a remarkable expression pattern: valencene synthase and a cytochrome P450 (CYP72A219 element). Valencene synthase is a terpene synthase, involved in the biosynthesis of (+)-valencene, a sesquiterpene, and its isomer (-)-7-epi-α-selinene, by using farnesyl diphosphate as a substrate (Lücker et al., 2004).

In this study the role of VOCs in the resistance mechanism of grapevine to *P. viticola* has been investigated. To this purpose, the VOC profile and biosynthetic pathway of two resistant varieties, Bianca (an interspecific hybrid obtained by crossing American species with *V. vinifera*) and Mgaloblishvili (*V. vinifera*), experimentally inoculated with *P. viticola* has been investigated, as well as the inhibitory effect of some VOCs against *P. viticola* infection.

2. Material and Methods

2.1 Plant material and experimental inoculation with *P. viticola*

The study of VOC biosynthesis in response to *P. viticola* inoculation was carried out on leaves of Mgaloblishvili (the Georgian *V. vinifera* cultivar showing unique resistance behavior against *P. viticola* (Silvia Laura Toffolatti et al., 2018) and Bianca (a *Vitis* interspecific hybrid variety), artificially inoculated with *P. viticola*. Mgaloblishvili and Bianca plants were four-years old, maintained in greenhouse (24 °C, 16-h photoperiod) at the Department of Agricultural and Environmental Sciences (University of Milan, Italy) in 5 L pots filled with sand-peat mixture (7:3 v/v), regularly drip watered. The plants were regularly treated against powdery mildew with azole fungicides and did not show any other disease symptoms.

Two strains belonging to the two different *P. viticola* genetic populations (one from the Western and the other from the Eastern population) identified in Italy (Maddalena et al., 2020) were mixed and used for the experimental inoculations. Recent studies (Maddalena et al., 2020) showed that two genetically different *P. viticola* populations, separated over an East-West gradient, are present in Italy: it was chosen to mix two strains belonging to these two populations to achieve a plant response that is representative of the genetic variability of the Italian population of *P. viticola*. *P. viticola* strains were isolated from single sporangia (obtained from serial dilutions of a single sporangiophore) of two populations sampled in Northern Italy, namely Lombardy (S. Maria della Versa, western location) and Friuli (Casarsa della Delizia, eastern location), and routinely propagated on the underside of detached leaves of grapevine (cv Pinot noir). The inoculated leaves were placed in Petri dishes (9 cm diameter) containing moistened filter paper and incubated in growth chamber at 22 °C with a 12/12 photoperiod (Toffolatti et al., 2012). After 7 days of incubation, sporangia were collected with sterile distilled water and counted in Kova chambers to estimate the number of sporangia contained in one mL of water.

Three plants per variety and one shoot per plant were used in the experimental procedure. Three leaves per shoot were inoculated with *P. viticola* and one leaf per shoot was not inoculated (airbrushed with sterile distilled water). Experimental inoculations were carried out by airbrushing a suspension of 2.5 x 10^4 *P. viticola* sporangia per leaf on the underside of three leaves located between the second and the fifth leaf starting from the apex of each shoots. Inoculated shoots were covered with transparent plastic bags to keep a high percentage of humidity. Three leaf disks (15 mm in diameter) were excised with a cork borer from a single inoculated leaf per shoot at 1, 2 and 3 days post inoculation (dpi) and incubated, as previously described, in Petri dishes to assess the disease occurrence through the estimation of the area covered by sporulation at 7 dpi. The remaining leaf material was stored at -80 °C until VOCs and gene expression analysis. The percentage of sporulating area (PSA) was estimated by visually assigning a class from 0 (absence of sporulation) to 7 (75-100% of the leaf disc covered by sporulation) to each leaf disc and using the following formula $PSA = \frac{\sum(n \times v)}{7 \times N} \times 100$ where n = number of leaf discs in each class, v = numerical value of each class and N = total number of leaf discs in the sample (Toffolatti et al., 2012). Experimental inoculation was performed on Pinot noir (a *V. vinifera* variety susceptible to downy mildew) as well, to evaluate the level of resistance of the two cultivars, Mgaloblishvili and Bianca.

2.2 Volatile compound determination

Free VOCs from inoculated and non-inoculated leaf tissues, collected at 0, 1, 2 and 3 dpi, were assessed by gas chromatography coupled with mass spectrometry using solid-phase microextraction technique (SPME-GC/MS) following the procedure reported by Griesser et al. (2015) with some modifications. Inoculated and non-inoculated leaves were homogenized with liquid nitrogen and 100 mg of tissue were placed in a glass-vial that was immediately hermetically closed. Leaf samples were added with 5 µl of 1-heptanol (12.5 µg 20 ml^{-1} in 10 % ethanol; Sigma-Aldrich, Germany), as internal standard. The fiber was a carboxen-polydimethylsiloxane-divinylbenzene (CAR-PDMS-DVB; 50/30 µm x 1 cm) (Supelco, Bellefonte, PA, USA). The SPME was carried out with an autosampler (HTA autosampler, Brescia, Italy) set at the following conditions: incubation for 30 min at 90 °C without agitation; extraction for 60 min; desorption for 20 min. The GC/MS equipment was a Perkin Elmer Autosystem XL Gas Chromatograph coupled with a Turbomass Mass Spectrometer (Perkin

Elmer, Italy). The separation was achieved by a Stabilwax-MS column (30 m x 0.250 mm x 0.25 μm) (Restek, Bellefonte, PA, USA) and using helium as carrier gas at 1 mL min^{-1} flow rate. The oven temperature was initially set at 40 °C and held for 5 min, ramped at 5 °C min^{-1} up to 220 °C and held for 5 min. The transfer line temperature was set at 230°C and the source temperature at 250 °C. The MS detector registered the m/z in the range from 33 up to 350 Da. The ions used for identification of target metabolites were chosen according to the National Institute of Standards and Technology (NIST) MS Search 2.0 library. Only ions showing a fixed fitting value (R) of 90 % to the library spectra were recorded with the exception of valencene. The latter compound was confirmed by the analysis of pure standards (Pub Chem SID 24901709, Sigma-Aldrich, St. Louis, MO, USA). Semi-quantitative data (referred to μg ml^{-1} of internal standard) were revealed considering the ratio between the peak area of each identified compound and the peak area of internal standard and referred to the internal standard. For the latter, a 5-point calibration curve was obtained in the range 0-125 μg 20 ml^{-1} using a leaf sample in order to exclude any possible matrix effect. Data were expressed as μg 100 mg^{-1} of leaf.

2.3 RNA extraction and quantitative real-time RT-PCR (qPCR)

Total RNA was extracted from 100 mg of three leaf samples non-inoculated (0 dpi) and inoculated with *P. viticola* (1, 2 and 3 dpi). The samples were ground with liquid nitrogen into a fine powder using mortar and pestle and RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) and then digested with Amplification Grade DNase I (Sigma-Aldrich), according to manufacturer's instructions. Quantity and quality of RNA was verified by NanoDrop Spectrophotometer (Thermo Scientific, MA) and agarose gel electrophoresis. For samples showing a 260/230 ratio lower than 1.8, a lithium-chloride purification was performed (Silvia Laura Toffolatti et al., 2018).

Candidate genes belonging to monoterpene and sesquiterpene biosynthetic pathway were selected according to their expression profile in mature grape leaves, as reported in literature (Matarese et al., 2013; S. L Toffolatti et al., 2018). Six candidates, respectively *VvGwECar2* ((E)-β-caryophyllene synthase), *VvGwaBer* ((E)-α-bergamotene synthase), *VvCSaFar* ((E, E)-α-farnesene synthase), *VvCSbOciM* ((E)-β-ocimene synthase), *VvTer* ((-)-α-terpineol synthase) and *VvVal* (valencene synthase), were consequently chosen and their expression investigated through the technique of quantitative real-time reverse transcriptase (RT)-PCR (qPCR). Primers of the

first five candidate genes were obtained from Matarese et al. (Matarese et al., 2013), while the ones for the amplification of *VvVal* gene were designed on the available sequence of Mgaloblishvili variety, using the Primer3Plus webtool (https://primer3plus.com/cgi-bin/dev/primer3plusPackage.cgi). Ubiquitin (Fujita et al., 2007) and actin (Reid et al., 2006) genes were used as references for data normalization. Table 1 reports forward and reverse primer sequences.

Total RNA (500 ng) was reverse transcribed with SuperScript®IV Reverse Transcriptase (Thermo Fischer) using oligo(dT)20 and following manufacturer's instructions. Real-time PCR was carried out on QuantStudio® 3 Real-Time PCR Systems (Thermo Fischer). Each reaction was carried out in a volume of 20 µL, using 10 µL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems), 4 µL of cDNA diluted 1:10, 500 nM of each primer and water up to the final volume of reaction. Each reaction was performed in triplicate. Thermal cycling conditions were obtained from Matarese et al. (2013). Each thermal cycle was followed by a melting curve stage, with temperatures ranging from 60 °C to 95 °C. The specificity of gene amplification per each sample was evaluated comparing the melting curves. Geometric average of ubiquitin and actin was used to normalize the Ct (cycle threshold) values of all analysed samples. The expression of each gene in different varieties and treatments was calculated $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4 Efficacy test of pure terpene solutions against *P. viticola* under laboratory conditions

The efficacy of pure terpene solutions against *P. viticola* infection was evaluated on Pinot noir leaf disks experimentally inoculated with a sporangia suspension. Grapevine leaves (3^{rd}-5^{th} leaf starting from the shoot apex) were detached from three five-years healthy plants of Pinot noir, grown in greenhouse, as above mentioned. Leaf disks (25 mm diameter) were excised from each leaf with a cork borer, soaked in terpene solutions for 2 minutes and, then, placed, with their abaxial surface upwards, in Petri dishes (9 cm diameter) containing moistened filter paper. Four concentrations (0.01, 0.1, 1 and 5 g l^{-1}) of farnesene (mixture of isomers; Pub Chem SID: 24901903, Sigma-Aldrich), nerolidol (mixture of *cis* and *trans*; Pub Chem SID: 24895721, Sigma-Aldrich), ocimene (mixture of isomers; Pub Chem SID: 329830629, Sigma-Aldrich) and valencene (Pub Chem SID: 57652542, Sigma-Aldrich) were tested. Each terpene was diluted to reach a concentration of 50 g l^{-1} with 2 % DMSO (Sigma-Aldrich) and serially diluted with

sterile, distilled water to obtain the appropriate concentration for each treatment. A negative control (distilled water only) and a DMSO control (distilled water with 0.2 % g l^{-1} of DMSO) were included in each assay. Three repetitions were prepared per treatment. The experimental inoculations were carried out airbrushing 0.2 ml per disk of a suspension of *P. viticola* sporangia ($5x10^4$ sporangia ml^{-1}) on the leaf disk surface, as above mentioned. After inoculation, the plates were incubated at 22 °C under light with a 12-h photoperiod. The percentage of sporulating area (PSA) was estimated at 7 dpi, as previously described (Toffolatti et al., 2012). Sporangia produced on six leaf disks at 0.01 g/L of each terpene were collected in 1 ml water-glycerol (20 %) and counted in a KOVA counting grid (KOVA International Inc., USA) following the manifacturer's instruction, to determine the sporangia concentration (sporangia ml^{-1}). Microscopy observations were performed the same leaf disks by staining with cotton blue dye (Wick, 2009). Leaf disks were fixed in absolute ethanol and cleared as described by Alexander et al. (2005) with some modifications: samples were boiled in 85 % (v/v in water) ethanol for 10 minutes, and incubated in pre-warmed lactic acid at 70 °C for 30 minutes. Reagents were purchased from Sigma-Aldrich. Samples were observed under an EasyLab CX40 (Olympus) optical microscope equipped with Primo Cam HD5 camera (Tiesselab, Milano, Italy).

2.5 Data analysis

2.5.1 Statistical analysis to evaluate disease severity

In order to estimate the existence of significant differences in the disease severity among accessions (Mgaloblishvili, Bianca and Pinot noir), one way ANOVA and post-hoc test (REGWF) were carried out on transformed PSA values ($arcsin\sqrt{PSA}/100$). Statistical analysis was carried out with SPSS v. 26 (IBM Statistics Italia, Milano).

2.5.2 Statistical analysis to determine volatile compounds

VOCs profiles were subjected to Levene's test to assess homogeneity of variance and tested for statistical significance through a GLS (generalized least squares) model, accounting for inhomogeneity of variance, with *nlme* R package (Pinheiro et al., 2020). *p*-values were obtained through post-hoc test carried out with *multcomp* R package (Hothorn et al., 2008). Graphs were generated using IBM SPSS Statistic v.21 software. Principal component analysis (PCA) and

clustered heatmap were carried out by *ggbiplot* (https://github.com/vqv/ggbiplot) and *gplots* (Warnes et al., 2014) R packages, respectively.

2.5.3 Statistical analysis to determine gene expression levels of six terpene synthases

Gene expression values were subjected to Levene's test to assess homogeneity of variance and tested for statistical significance through a GLS model, using *nlme* R package. *p*-values were obtained through post-hoc test carried out with *multcomp* R package. Graphs were generated using IBM SPSS Statistic v.21 software.

2.5.4 Statistical analysis to determine efficacy test of pure terpene solutions against *P. viticola*

Statistical analysis (ANOVA with multiple comparison REGW post-hoc test) was performed on transformed PSA percentages ($\mathrm{asin}(\sqrt{\%/100})$) to establish if the treatment with terpenes or DMSO caused a significant reduction in disease severity compared to untreated control. Kruskal-Wallis and Dunn's multiple comparison post-hoc tests, with Bonferroni correction was performed on sporangia concentration to establish if the treatment with terpenes caused a significant reduction in in sporangia production compared to untreated control. Non-parametric correlation tests (Kendall's Tau and Spearman's Rho) were performed to evaluate the existence of correlation between I%I and sporangia concentration on the overall data obtained at 0 and 0.01 g l^{-1} of each terpene.

3. Results

3.1 Disease severity evaluation

Leaf disks of Mgaloblishvili and Bianca were inoculated with a suspension of *P. viticola* sporangia and the disease severity (PSA) was evaluated at 7 dpi. Pinot noir leaf disks were inoculated as positive control. No disease symptoms were observed on Bianca, where numerous necrotic areas were present as a consequence of the hypersensitive response (Figure 1A). A few areas with sporulation, covering 22 % of the leaf disks on average, were observed on Mgaloblishvili samples (Figure 1B). While, a uniform presence of sporulation, covering 84 % of the leaf disks on average, was observed in Pinot noir (Figure 1C). Statistical analysis showed a

significant four-times reduction of PSA in Mgaloblishvili compared to Pinot noir ($F=148.9$; df=2,6; $P<0.001$) (Figure 2).

3.2 VOCs detection in leaves inoculated with *P. viticola*

In total, 54 VOCs were identified during SPME-GC/MS analysis of 24 Mgaloblishvili and Bianca leaf samples collected at 0, 1, 2 and 3 dpi with *P. viticola*. This dataset was filtered for those compounds identified in all the three biological replicates. The final dataset accounted for 33 VOCs. Based on the biochemical features, the VOCs were categorized into three main groups: alcohols (6 compounds), aldehydes (11 compounds), terpenes (10 compounds). A fourth group of 6 compounds included alkenes and esters. Table 2 reports the amount (μg/100 mg of leaf sample) of each VOC per cultivar and treatment. Most of the 33 VOCs were detected in both cultivars and overall the treatments, except for 1-hexanol, 2-ethyl- and phenylethyl alcohol among alcohols, benzeneacetaldehyde and dodecanal among aldehydes, farnesene and *p*-menth-1-en-8-ol among terpenes and 1-octadecene, 1-(4-bromobutyl)-2-piperidinone and *trans*-2-(2-pentenyl)furan among other VOCs. The highest amount of total VOCs was detected at 2 dpi and 3 dpi for Mgaloblishvili and Bianca, respectively. In both cultivars, the amount of some VOCs increased (such as benzyl alcohol) and some other decreased (such as farnesene) as the time after inoculation increased (Table 2).

Bianca showed statistically significant values at 1 dpi for the amount of other VOCs and the total VOCs, at 2 dpi for the amount of aldehydes, terpenes, other VOCs and total VOCs, at 3 dpi for the amount of aldehydes, terpenes and total VOCs. At 1 dpi, a statistically significant increase for hexanal, 2-n-octylfuran, *trans*-2-(2-pentenyl)furan and methylhydrazine was detected. At 2 dpi, Bianca showed a statistically significant increase for 2-hexenal, hexanal and farnesene. While at 3 dpi, 3-hexen-1-ol, 2-hexenal, hexanal, farnesene, 3-buten-2-one-4-(2,6,6-trimethyl-1-cyclohexen -1-yl), 4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one, 2-n-octylfuran and methylhydrazine showed a statistically significant increase (Table 2).

In comparison to the 0 dpi samples, Mgaloblishvili showed statistically significant abundances at 1 dpi for the amount of other VOCs and at 2 dpi for the amount of terpenes, other VOCs and total VOCs. At 1 dpi, Mgaloblishvili showed a statistically significant increase for 2-undecanone, 6,10-dimethyl, 1-(4-bromobutyl)-2-piperidinone and methylhydrazine. At 2 dpi, statistically significant increase was detected for 4-(2,6,6-trimethylcyclohexa-1,3-dienyl)but-3-

en-2-one, 1-(4-bromobutyl)-2-piperidinone and methylhydrazine. Farnesene was detected at 2 (the highest amount) and 3 dpi. None of the VOC amount significantly increased at 3 dpi (Table 2).

PCA was performed to detect significantly influenced VOC categories after *P. viticola* inoculation. The first two principal component (PC) explained about the 85 % of total variance (Figure 3). Bianca and Mgaloblishvili samples differentiated mainly along the PC2. Bianca samples collected at 0 dpi were differentiated from 1 dpi samples and 2 and 3 dpi samples. 1 dpi Bianca samples appeared differentiated based on other VOCs variable, while 2 and 3 dpi samples for alcohol and aldehyde variables. Mgaloblishvili samples appeared more homogeneous, with slightly differentiation of 1 and 2 dpi samples from 0 and 3 dpi ones. Mgaloblishvili samples were differentiated based on other VOCs (mainly) and terpene (less) variables.

Figure 4 represents the accumulation pattern of volatile compounds during *P. viticola* infection clustered by hierarchical cluster analysis. Tree well distinct clusters (Cluster 1, Cluster 2 and Cluster 3) have been highlighted. Cluster 1 grouped Bianca samples collected at 2 and 3 dpi, Cluster 2 grouped Bianca samples collected at 0 dpi and Mgaloblishvili samples collected at 3 dpi, while Cluster 3 grouped 1 dpi Bianca samples and 1 and 2 dpi Mgaloblishvili samples. Cluster 1 showed a positive correlation with the amount of alcohols, aldehydes and terpenes and a negative correlation with the amount of other VOCs. Cluster 2 showed mainly a negative correlation with all the four VOC categories. Cluster 3 showed a positive correlation with the amount of other VOCs and a negative correlation with the amount of alcohols and aldehyde.

3.3 Relative expression of terpene synthases in leaves inoculated with *P. viticola*

The expression pattern of six genes involved in the biosynthesis of monoterpenes and sesquiterpenes (*VvGwaBer*, *VvGwECar2*, *VvCSaFar*, *VvCSbOciM*, *VvTer* and *VvVal*) were investigated in leaf samples of Bianca and Mgaloblishvili collected at 0, 1, 2 and 3 days after inoculation with *P. viticola*. Supplementary Figure 1 reports the gene melt curve plots. Both varieties showed a similar pattern of expression, characterized by an increase in the expression level in response to the pathogen inoculation (Figure 5). For every gene and variety, apart from *VvTer* in Mgaloblishvili, the highest expression level was obtained at 1 dpi, followed by a drop at 2 dpi and another minor increase at 3 dpi. Overall, Mgaloblishvili appeared to show a greater increment in the gene expression of candidate genes compared to Bianca, with double or triple

values. Compared to the other genes, *VvVal* in Mgaloblishvili exhibited a remarkably high increase in its expression level at 1 dpi, with a value equal to 120 times the non-inoculated sample value (0 dpi). A similar difference in the gene expression is shared, at the same time point and in the same variety, by *VvGwaBer* (80 times the 0 dpi sample value). The only exception to this behaviour is *VvGwECar2*, in which the Mgaloblishivili gene expression resulted lower than Bianca and it showed a decrease throughout the time points.

3.4 Efficacy of pure terpene solutions in containing *P. viticola* infections

Based on the VOC profiles and gene expression data, the efficacy of pure solutions of farnesene, nerolidol, ocimene and valencene in reducing *P. viticola* infection on Pinot noir leaves were evaluated (Supplementary Figure 2). The average I%I of the untreated control was 62 % (Table 3). No significant differences were found between the I%I recorded on the untreated and DMSO controls ($F=1.6$; df=1-4; $P=0.28$). Significant reduction in *P. viticola* sporulation (I%I) was observed following treatment with each terpene starting from 0.01 g l^{-1} ($F>4.9$; df=4-10; $P<0.018$). Indeed, the I%I showed a significant 3-, 4-folds reduction until 17-26 % between 0.01 and 1g l^{-1} and a further significant decrease to 0 % at 5 g l^{-1} of nerolidol and ocimene (Table 3). No further reductions in I%I values occurred between 0.01 and 5 g l^{-1} of farnesene and valencene (Table 3). It must be pointed out that some signs of phytotoxicity, visible as brown spots, were visible at 5 g l^{-1} of ocimene (Supplementary Figure 2). All terpenes at 0.01 g/l caused a significant reduction in sporangia concentration compared to the control ($KW=11.33$, df=4, $P=0.023$) (Table 4). A significant, positive correlation was found between PSA and sporangia concentration with both Kendall's Tau ($\tau=0.516$; N=15; $P=0.01$) and Spearman's Rho ($\rho=0.61$; N=15; $P=0.016$) tests. The observation of pathogen structures at the microscope showed a regular development of the vegetative structures of the pathogen (hyphae and haustoria) in untreated samples (Figure 6a-b). The vegetative structures of the pathogen in terpene-treated samples did not morphologically differed from those of the untreated samples. Alterations in reproductive structures were, on the contrary, visible in all terpene treated samples (Figure 6e) compared to the untreated control (Figure 6c-d). In Figure 6e is reported the sporangiophore morphology observed in the leaf tissues treated with of 0.01 g/L of nerolidol, as an example. In the untreated sample, the sporangiophore showed a tree-like morphology, constituted by a single moopodial branch (septate) showing an apical ramification in branches and branchlets at the top

of which sterigma were differentiated (Figure 6c). Sporangia were formed at the end of each sterigma (Figure 6d). In terpene treated samples, short and ramified sporangia with no apical branching, nor sporangia production, were seen (Figure 6e).

4. Discussion

4.1 The VOCs biosynthesis in response to *P. viticola* is cultivar-specific

VOCs play a crucial role in the plant-pathogen interaction mechanism (Brilli et al., 2019). In grapevine, their biosynthesis was associated to resistance against *P. viticola* infection (Algarra Alarcon et al., 2015; Lazazzara et al., 2018). Their role in grapevine defense mechanism against downy mildew was confirmed by the detection of high amount of VOCs in resistant genotypes, harboring the American species background, in comparison to the susceptible ones following the pathogen infection (Lazazzara et al., 2018). Transcriptomic data on the *V. vinifera* cultivar Mgaloblishvili leaves inoculated with *P. viticola* revealed the overexpression of genes involved in the biosynthesis of terpenoids, such as several cytochrome P450s and valencene synthase (S. L Toffolatti et al., 2018; Toffolatti et al., 2020).

In response to the pathogen infection, the two cultivars analyzed in this work showed a different behavior (Figure 3 and 4). In both cultivars, the increased accumulation of VOCs was found in the inoculated samples, but with a different timing: Mgaloblishvili showed the highest amount at 2 dpi and Bianca at 3 dpi (Table 2). The detection of a highest amount of VOCs in response to *P. viticola* inoculation suggested that their biosynthesis can be related to the plant-pathogen interaction mechanism as proposed for other resistance cultivars (Lazazzara et al., 2018). Indeed, the plant response in Mgaloblishvili occurs at 1 dpi, as demonstrated by the high transcriptomic changes, but the damages to *P. viticola* structures are visible starting from 3 dpi (Silvia Laura Toffolatti et al., 2018). At 1 and 2 dpi, regular hyphae and haustoria were observed in Mgaloblishvili. The results obtained in this study showed that genes encoding for VOCs are overexpressed at 1 dpi, as highlighted by the transcriptomic data, while the antifungal molecules, that lead to the alterations of the vegetative structures observed at 3 dpi (Silvia Laura Toffolatti et al., 2018), are synthesized at 2 dpi. On the contrary, Bianca transcriptome showed greater changes in its transcriptome between 1 (when hypersensitive response, HR, is observed) and at 3

dpi (Silvia Laura Toffolatti et al., 2018), but the VOCs accumulation at 3 dpi could indicate that they constitute a late response of plant to pathogen inoculation.

In Mgaloblishvili, the VOC class mostly affected by the inoculation at 2 dpi was the class of terpenes (Table 2, Figure 3 and 4). Terpenes are recruited to a number of ecological roles in plants. Many of these substances have antimicrobial and anti-herbivore properties, suggesting their role in defending the most important parts of the plant (Li et al., 2020). The increase of terpenes was mainly due to the detection of farnesene, that was not detected in the not inoculated samples (0 dpi) and the 1 dpi samples (Table 2). Farnesene is a sesquiterpenes, being one of the principal compounds of some essential oils, extracted from seeds, fruits, flowers, leaves or roots, showing a good antimicrobial activity: for example, *Vitex agnus-castus* essential oil is active against *Streptococcus mutans* (Gonçalves et al., 2017). Among the other VOCs showing a statistically significant increase after the *P. viticola* inoculation, 1-(4-bromobutyl)-2-piperidinone and 4-(2,6,6-trimethylcyclohexa-1,3-dienyl)but-3-en-2-one (β-ionone) are noteworthy for their proved antimicrobial activities. Indeed, the synthesis of 1-(4-bromobutyl)-2-piperidinone in *Trichoderma asperellum* has been correlated to the biocontrol of *Fusarium oxysporum* (Wu et al., 2017). While, the antimicrobial activity of β-ionone has been ascertained against some organisms, such as *Microcystis aeruginosa* (Shao et al., 2011).

Alcohols and aldehydes, on the contrary, were the two classes mostly discriminating the Bianca response to *P. viticola* infection (Table 2, Figure 3 and 4). Alcohols and aldehydes arise from fatty acid metabolism and are commonly referred to as “green leaf volatiles”, synthesized in plant green organs in response to wounding (Dudareva et al., 2006, 2004). Among them, 3-hexen-1-ol and hexenal are two compounds known to be involved in the plant-pathogen interactions. The first has a key role in insect repelling and deterring (Wei and Kang, 2011), while, the latter is a molecule with remarkable antimicrobial properties against *Aspergillus flavus* (Gardini et al., 2001).

4.2 The expression of terpene synthases correlates with the pathogen colonization

The biosynthesis of VOCs occurs in every grapevine organ, though each organ shows a different VOC profile, and basically terpene synthase (TPSs) genes are expressed in all organs, while only some showed an organ-specific expression pattern (Matarese et al., 2013). *VvGwaBer*, *VvGwECar2*, *VvCSaFar*, *VvCSbOciM*, *VvTer* and *VvVal* genes were selected because they

showed a gene expression in grapevine leaves at juvenile and mature stage (Matarese et al., 2013; S. L Toffolatti et al., 2018). Our real-time RT-PCR data revealed that all the analysed TPSs had detectable transcripts in both not inoculated and inoculated samples (Figure 5), confirming their involvement in response to *P. viticola* infection, by producing metabolites that act as antifungal compounds (Dudareva et al., 2006, 2004). In both cultivars, the TPSs showed a peak of expression at 1 dpi, except for *VvTer*. This peak of expression, already described in previous transcriptomic studies, can be correlated to the timing of infection. At 1 dpi, *P. viticola* produces the first haustorium and activates the plant response (Perazzolli et al., 2012; Polesani et al., 2010; S. L Toffolatti et al., 2018; Toffolatti et al., 2020, 2012).

The gene expression patterns were consistent with the highest quantity of VOCs being detected at 2 and 3 dpi (Table 2). Unfortunately, it was not able to correlate analysed TPSs gene expression with metabolites extracted from leaves, apart from farnesene, the main product of *VvCSaFar*, and nerolidol (3,7,11-trimethyl-1,6,10-dodecatrien-3-ol), one of the *VvGwaBer* products. The inability to detect the products of some TPSs is not unusual (Falara et al., 2011; Matarese et al., 2013), and it can be due to the extremely sensitive methods required to detect the compounds present at very low concentration, or a low level of compounds, or further conversion to other metabolites.

The genes showing the highest expression level were *VvGwaBer* and *VvVal* (Figure 5). *VvGwaBer* was identified as the functional gene responsible for the biosynthesis of α-bergamotene as a major product, and nerolidol as minor product (Martin et al., 2010). The antimicrobial activity of this compound was widely demonstrated (Chan et al., 2016). In grapevine, nerolidol was synthetized following inoculation with *Phaeoacremonium parasiticum*, as well as the increase of VvPNLinNer1 transcripts, gene responsible of (E)-nerolidol biosynthesis (Escoriaza et al., 2019). A biosynthesis of nerolidol was also found in grape leaves (*Vitis labrusca*) attacked by *Popillia japonica* (Loughrin et al., 1997). Nerolidol was detected in our experimental conditions, in both not inoculated and inoculated samples, although the concentrations detected did not statistically change after inoculation (Table 2).

VvVal catalyzes the conversion of farnesyl diphosphate to valencene, a sesquiterpene with antimicrobial activity (Manter et al., 2006). In *V. vinifera*, this gene was only expressed in flower buds and no transcripts were detected in the vegetative tissues of young leaves (Lücker et al., 2004; Matarese et al., 2013). Our results demonstrated that some *VvVal* transcripts were detected

in Mgaloblishvili and Bianca leaves not inoculated and they increased after the inoculation with *P. viticola* (Figure 5). Nevertheless, neither valencene nor its isomer (-)-7-epi-α-selinene were detected in inoculated samples.

4.3 New natural bioactive molecules against *P. viticola* infection

The identification of natural bioactive molecules is a key point in developing a sustainable crop production. Due to their antimicrobial activity, natural VOCs can be a valid eco-friendly strategy to implement green agricultural practices and limiting the use of synthetic molecules, representing to date the most common disease management strategy (Brilli et al., 2019). Indeed, the efficacy of Oregano essential oil and other molecules, such as 2-ethylfuran, 2-phenylethanol, β-cyclocitral, *trans*-2-pentenal, in reducing the development of grapevine downy mildew symptoms has been already demonstrated (Lazazzara et al., 2018; Rienth et al., 2019). In this work, the efficacy of four terpenes (farnesene, nerolidol, ocimene and valencene) that are specifically synthesized by Mgaloblishvili upon pathogen inoculation, in counteracting *P. viticola* was proved in *ad hoc* experimental inoculations where the disease severity and pathogen sporulation were significantly hampered compared to the untreated control, confirming their role as bioactive compounds in the resistance mechanism. The positive significant correlation between disease severity and sporangia concentration indicates that an increase in disease severity is directly related with an higher sporulation by the pathogen. Aniline blue staining allowed to observe that terpene mainly act on the pathogen reproductive structures, that appeared short and sterile. Analogous alterations were observed during the interaction of *P. viticola* with resistant grapevine varieties such as Mgaloblishvili (Toffolatti et al., 2018) and in case of abiotic stress caused by light irradiation (Rumbolz et al., 2002). Overall, these results indicate that terpenes mainly have an antisporulant activity that could be related to their volatile nature.

However, the direct involvement of these terpenes in the resistance mechanism has to be further established through more deep investigations, *e.g.* by coupling microscopic observations, sporangia production and *P. viticola* quantification at different time points, to assess if these terpenes possess a fungistatic effect, as well as their effectiveness to enhance plant defenses in the field. Furthermore, the possibility of using mixtures of VOCs other protocols for the terpene application could be evaluated. It was demonstrated that VOCs work in blend rather than alone in inhibiting the pathogen infection, by acting in an additive or synergistic way with different

plant secondary metabolites, such as phenolics and terpenoids, in resistance establishment (Henriquez et al., 2012). Thus, the discovery of new antimicrobial molecules and the availability of a wide range of bioactive molecules are crucial to set up new blends able to effectively contain the disease in the field in an eco-friendly and sustainable way.

5. Conclusions

In this study, the investigation of the resistance mechanism of two grapevine cultivars characterized by different genetic background (American and Eurasian) demonstrated that VOCs biosynthesis increased in leaves following the infection with *P. viticola*, although we cannot exclude that a fraction of detected VOCs was emitted by the pathogen. Moreover, the results obtained on the antifungal activity of four selected VOCs confirmed that farnesene, ocimene, nerolidol and valencene are indeed able to reduce disease severity in *in vitro* conditions. Further investigation is needed to establish the mode of action of these molecules and their toxicity profile. The identification of compounds biologically active against *P. viticola*, such as those reported here, opens new perspectives for a sustainable viticulture. Cultural practices, in fact, are scarcely effective in reducing downy mildew incidence, whereas fungicide treatments more efficiently protect grapevine against the disease. In the next few years, the use of some synthetic substances active against *P. viticola* will be banned or strictly regulated in Europe due to the application of the regulation concerning the placement on the market and the use of pesticides (Regulation (EC) No 1107/2009; Directive 2009/128/EC). To assure an acceptable protection against the pathogen, the discovery of new bioactive molecules is, therefore, strictly needed. In this view, the exploitation of molecules that are naturally produced by the plant in response to the pathogen, could be one of the possible to accomplish with this need.

6. Acknowledgments

The research was supported by University of Milan, DiSAA, Research Support Plan 2018, Linea 2 A, project “Dal phenotyping al genome editing: strategie per limitare i danni da peronospora e legno nero in vite (ResVite)”. The authors wish to thank Stefania Prati and Andrea Giupponi for the plant management in greenhouse and Dr. Davide Sordi of Vivai Cooperativi Rauscedo for providing plants. The manuscript is dedicated to the memory of Annamaria Vercesi.

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List of tables

Table 1. Forward and reverse primers sequences of two reference (actin and ubiquitin) genes and six terpene synthases (E)-β-caryophyllene synthase, (E)-α-bergamotene synthase, (E,E)-α-farnesene synthase, (E)-β-ocimene synthase, (-)-α-terpineol synthase and valencene synthase) genes involved in the biosynthesis of terpenes in grapevine leaves.

Gene	Sequence 5'-3'	Reference
Actin	F: CTTGCATCCCTCAGCACCTT	Reid et al. (2006)
	R: R: TCCTGTGGACAATGGATGGA	Reid et al. (2006)
Ubiquitin	F: TCTGAGGCTTCGTGGTGGTA	Fujita et al. (2007)
	R: AGGCGTGCATAACATTTGCG	Fujita et al. (2007)
(E)-β-caryophyllene synthase	F: TGCCTCAGCTGTTGAATGCT	Matarese et al. (2013)
	R: TGAGGACGGTCATCGGAACA	Matarese et al. (2013)
(E)-α-bergamotene synthase	F: CCTAGCATTTGGGGCAATAC	Matarese et al. (2013)
	R: CCGTTGAACTGCATCGATAA	Matarese et al. (2013)
(E,E)-α-farnesene synthase	F: GGGTGCACGTTGCTTCTAGT	Matarese et al. (2013)
	R: TGGCATCAGCACTGGTGTAG	Matarese et al. (2013)
(E)-β-ocimene synthase	F: GGAACATCACTGGATGAGTTGA	Matarese et al. (2013)
	R: ATCTCCATGCTGATACATGCAC	Matarese et al. (2013)
(-)-α-terpineol synthase	F: AGAGTCTCCATTCCCTGAAACA	Matarese et al. (2013)
	R: GGGCTCAACGAGTAATGACAA	Matarese et al. (2013)
Valencene synthase	F: AGTTGTGGATGCATGGAAGG	The present work
	R: TTTGGTCATGCGATAGGGTG	The present work

Table 2. VOCs accumulation (μg 100 mg^{-1} leaf sample) in Bianca and Mgaloblishvili leaves at 0, 1, 2, and 3 days post inoculation (dpi). Statistical analysis was performed on subtotal and total amounts per each cultivar. Values followed by '*' significantly differ from the values recorded at 0 dpi, according to gls method (*** *P*=0.000; ** *P*=0.001; * *P*=0.01). n.d. = not detected.

VOC ID	VOC	Bianca				Mgaloblishvili			
		0 dpi	**1 dpi**	**2 dpi**	**3 dpi**	**0 dpi**	**1 dpi**	**2 dpi**	**3 dpi**
	Alcohols								
VOC1	1-Hexanol, 2-ethyl-	16.19±3.10	6.63±1.46 *	12.59±4.18	8.24±2.11 *	n.d.	n.d.	n.d.	n.d.
VOC2	1-Nonol	5.33±1.51	3.30±1.73	3.51±1.69	2.95±1.62	8.35±2.28	4.69±0.36 *	6.67±3.89	5.31±2.34 *
VOC3	4,8-Dimethyl-1,7-nonadien-4-ol	2.12±0.47	1.95±0.46	2.39±0.53	2.73±0.66	5.61±1.60	4.27±0.65	4.38±0.91	2.27±0.77 *
VOC4	3-Hexen-1-ol	3.98±0.88	6.68±1.44	8.07±2.39	9.98±2.19 *	1.94±0.84	1.51±1.02	2.19±1.72	3.89±1.54
VOC5	Benzyl alcohol	18.73±4.33	9.96±1.31 *	14.74±3.76	10.56±3.59	11.13±2.46	9.55±4.47	6.75±2.57 **	10.69±1.03
VOC6	Phenylethyl alcohol	16.10±6.67	5.47±3.70 *	19.50±1.98	16.21±5.12	n.d.	n.d.	n.d.	n.d.
	Subtotal	**62.45±21.23**	**33.98±11.23**	**60.80±10.09**	**50.68±13.76**	**27.03±9.45**	**20.02±4.87**	**19.99±4.09**	**22.16±1.89**
	Aldehydes								
VOC7	1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-	6.05±0.34	6.11±0.63	6.97±1.14	7.97±1.47	11.64±1.93	11.54±0.49	12.05±1.91	6.34±1.17 *
VOC8	2-4 Heptadienal, (E,E)-	n.d.	n.d.	n.d.	n.d.	3.12±0.15	4.87±1.32	5.12±1.89	1.91±0.40 *
VOC9	2-Hexenal	199.96±19.52	112.11±17.56 *	340.56±26.38 ***	353.10±44.73 ***	169.93±25.10	142.45±22.53	119.33±14.88 *	201.40±23.42
VOC10	2,4-Hexadienal	18.86±2.01	19.57±7.37	30.54±1.46	21.84±14.94	27.43±5.77	23.84±5.84	16.07±6.95	14.73±11.73
VOC11	Benzeneacetaldehyde	n.d.	20.12±2.44	24.29±7.78	22.55±4.95	n.d.	n.d.	n.d.	n.d.
VOC12	Benzaldehyde	14.80±1.58	6.47±1.63 *	9.57±1.72	12.31±1.08	10.48±0.51	11.08±2.13	4.90±0.29 *	7.53±2.23
VOC13	Decanal	4.99±0.31	4.18±0.64	2.30±1.97	3.59±1.73	17.42±5.85	13.41±0.17	11.85±2.06	7.87±1.91 **
VOC14	Dodecanal	n.d.	n.d.	n.d.	n.d.	18.88±3.81	9.61±3.47 *	13.87±2.94	9.94±5.37
VOC15	Furfural	10.79±3.64	12.66±1.73	11.72±3.03	7.32±1.04	14.16±4.25	7.23±1.90	7.25±1.39	5.57±3.34
VOC16	Hexanal	12.62±6.23	26.48±5.74 *	39.46±4.38 **	27.45±7.40 *	24.95±7.32	17.67±4.57	11.10±2.00 **	16.27±2.84
VOC17	2-Furancarboxaldehyde,5-(hydroxymethyl)	18.36±8.6	20.79±6.36	9.97±2.09 *	11.43±7.13	8.50±6.45	4.14±1.25	4.13±1.12	1.67±1.73 **
	Subtotal	**286.42±24.68**	**228.49±35.90**	**475.38±19.47 *****	**467.55±46.02 *****	**306.52±55.36**	**245.85±24.89**	**205.66±27.68**	**273.24±27.31**

	Terpenes								
VOC18	Farnesene	8.26±4.78	22.40±6.83	86.88±11.04 **	115.91±6.81 **	n.d.	n.d.	108.67±18.03	34.11±13.32
VOC19	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	75.23±9.58	64.60±31.54	20.29±1.54 *	25.34±2.41 *	81.72±8.61	61.87±7.76	77.53±14.38	84.20±14.53
VOC20	*p*-Menth-1-en-8-ol	2.71±0.53	4.22±2.33	4.93±1.91	4.42±1.03	n.d.	n.d.	n.d.	n.d.
VOC21	3-Buten-2-one-4-(2,6,6-trimethyl-1-cyclohexen -1-yl)	48.64±15.36	75.08±29.20	78.53±25.37	112.40±23.60 **	100.82±12.62	68.87±5.92	79.71±24.55	68.25±26.52
VOC22	1,6-Octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate	1.14±0.28	1.53±0.27	2.44±1.03	2.00±0.08	3.21±0.38	3.36±0.69	3.57±1.55	2.18±0.45
VOC23	5,9-Undecadien-2-one, 6,10-dimethyl-(E)-	24.92±4.28	37.40±7.47	23.07±2.92	35.77±12.35	13.08±5.35	34.83±11.66	18.65±4.33	8.52±3.10
VOC24	3,7,11-trimethyl-1,6,10-dodecatrien-3-ol	4.11±0.27	5.75±0.15	5.50±1.79	4.83±0.95	7.95±1.15	7.78±2.51	3.11±1.74	3.60±1.86
VOC25	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	3.54±0.73	9.12±4.90	8.31±3.52	13.86±4.53 *	7.27±1.72	6.17±0.37	14.18±3.56 *	5.68±1.71
VOC26	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-	13.75±5.48	12.16±2.01	11.97±2.96	14.52±3.77	18.49±6.27	16.22±1.92	16.44±1.85	8.16±4.41
VOC27	2-Undecanone, 6,10-dimethyl	7.57±2.78	6.69±2.28	4.16±2.85	5.70±1.87	11.33±4.27	49.15±11.91 **	10.19±2.67	9.04±2.35
	Subtotal	**189.85±23.24**	**238.95±24.75**	**246.10±37.58 ***	**334.75±54.27 *****	**243.88±34.99**	**248.25±25.82**	**332.05±57.07 ****	**223.74±41.91**
	Other VOCs								
VOC28	1-Octadecene	n.d.	21.63±6.51	16.19±5.31	13.89±4.25	n.d.	15.70±6.05	11.04±3.20	8.16±3.71
VOC29	1-(4-Bromobutyl)-2-piperidinone	n.d.	4.13±0.94	4.96±0.63	7.72±0.62	3.21±0.42	34.87±7.69 ***	36.14±7.02 ***	n.d.
VOC30	Decanoic acid, ethyl ester	5.32±1.01	4.62±1.63	6.46±3.93	3.67±2.33	4.89±1.03	9.75±8.63	5.93±2.94	6.88±2.44
VOC31	2-n-Octylfuran	7.32±3.44	19.46±5.54 *	14.38±2.82	17.75±2.53 *	37.35±5.81	18.44±5.95 *	17.13±6.59 *	39.25±10.98
VOC32	*trans*-2-(2-Pentenyl)furan	0.84±0.11	8.46±3-87 **	0.71±0.17	11.04±3.77 **	n.d.	n.d.	n.d.	n.d.
VOC33	Methylhydrazine	197.57±31.39	292.10±31.63 ***	76.27±7.02 ***	110.49±10.02	158.46±33.78	253.79±34.95 **	226.91±15.34 *	165.54±51.04
	Subtotal	211.05±34.51	350.40±23.02 ***	118.98±16.72 ***	164.56±4.04	203.90±36.31	332.55±44.69 **	297.14±18.11 *	219.82±60.84
	Total	749.78±66-45	851.83±56.26 **	901.26±31.29 ***	1017.54±38.32 ***	781.34±41.3	846.68±75.74	854.85±20.79 *	738.96±39.87

Table 3. Average disease severity (I%I) ± standard deviation recorded on Pinot noir leaves infected with *P. viticola* and untreated (0) and treated with DMSO (0.2 %) and farnesene, nerolidol, ocimene and valencene at four different concentrations. Untreated and treated with DMSO leaves were considered as controls. Letters indicate statistically different PSA values (P<0.05) following ANOVA and multiple comparison REGW post-hoc test.

			Concentration (g l^{-1})			
Treatment	**0**	**DMSO**	**0.01**	**0.1**	**1**	**5**
Farnesene	62±10 a	57±12 a	19.0±11 b	16.7±15 b	14.3±19 b	11.7±11 b
Nerolidol			16.3±4 b	26.3±4 b	14±7 b	0±0 c
Ocimene			14±12 b	11.7±8 b	9.3±8 b	0±0 c
Valencene			16.7±11 b	21.3±7 b	16.7±8 b	12±9 b

Table 4. Average sporangia concentration (sporangia/ml) ± standard deviation recorded on Pinot noir leaf discs infected with *P. viticola* and untreated (0) or treated with farnesene, nerolidol, ocimene and valencene at 0.01 g/l concentration. Letters indicate statistically different sporangia/ml values ($P<0.05$) following Kruskal-Wallis and Dunn's multiple comparison post-hoc tests.

Treatment	Average sporangia/ml
Untreated	122±53 a
Farnesene	66±79 b
Nerolidol	50±13 b
Ocimene	35±25 b
Valencene	98±58 ab

List of figures

Figure 1. *P. viticola* sporulation (in white) on the inoculated leaf disks of Bianca (A), Mgaloblishvili (B), Pinot noir (C) at seven days after inoculation. Brown spots in Bianca correspond to necrotic areas, where hypersensitive response (HR) occurred.

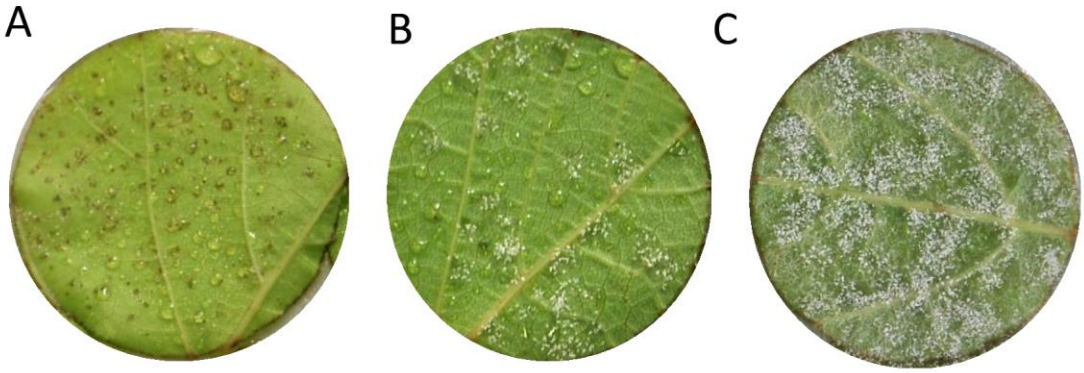


Figure 2. Box plot distribution of the percentages of sporulating area (PSA) estimated 7 days post inoculation with *P. viticola* on Bianca, Mgaloblishvili and Pinot noir leaf disks and results of statistical analysis (different letters correspond to a significant difference among mean PSA values for $P<0.001$).

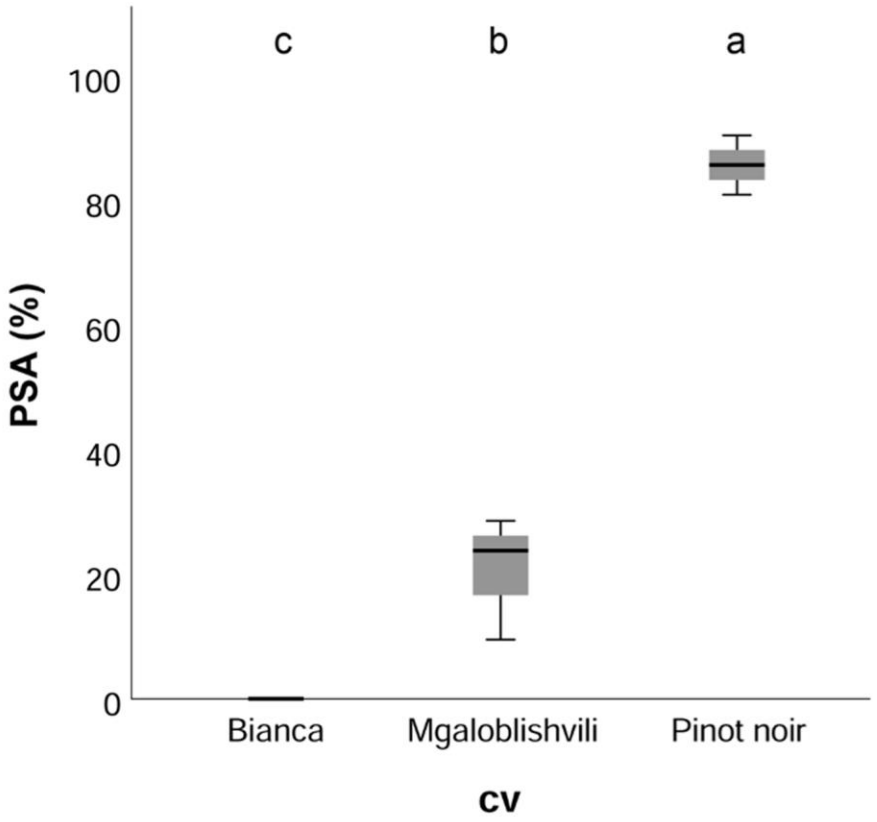


Figure 3. Principal Component Analysis (PCA) along the first two components (PC) obtained using the amount of volatile metabolites (alcohols, aldehydes, terpenes and other VOCs) detected in Mgaloblishvili and Bianca leaves collected at 0, 1, 2 and 3 days post inoculation (dpi) with *P. viticola*.

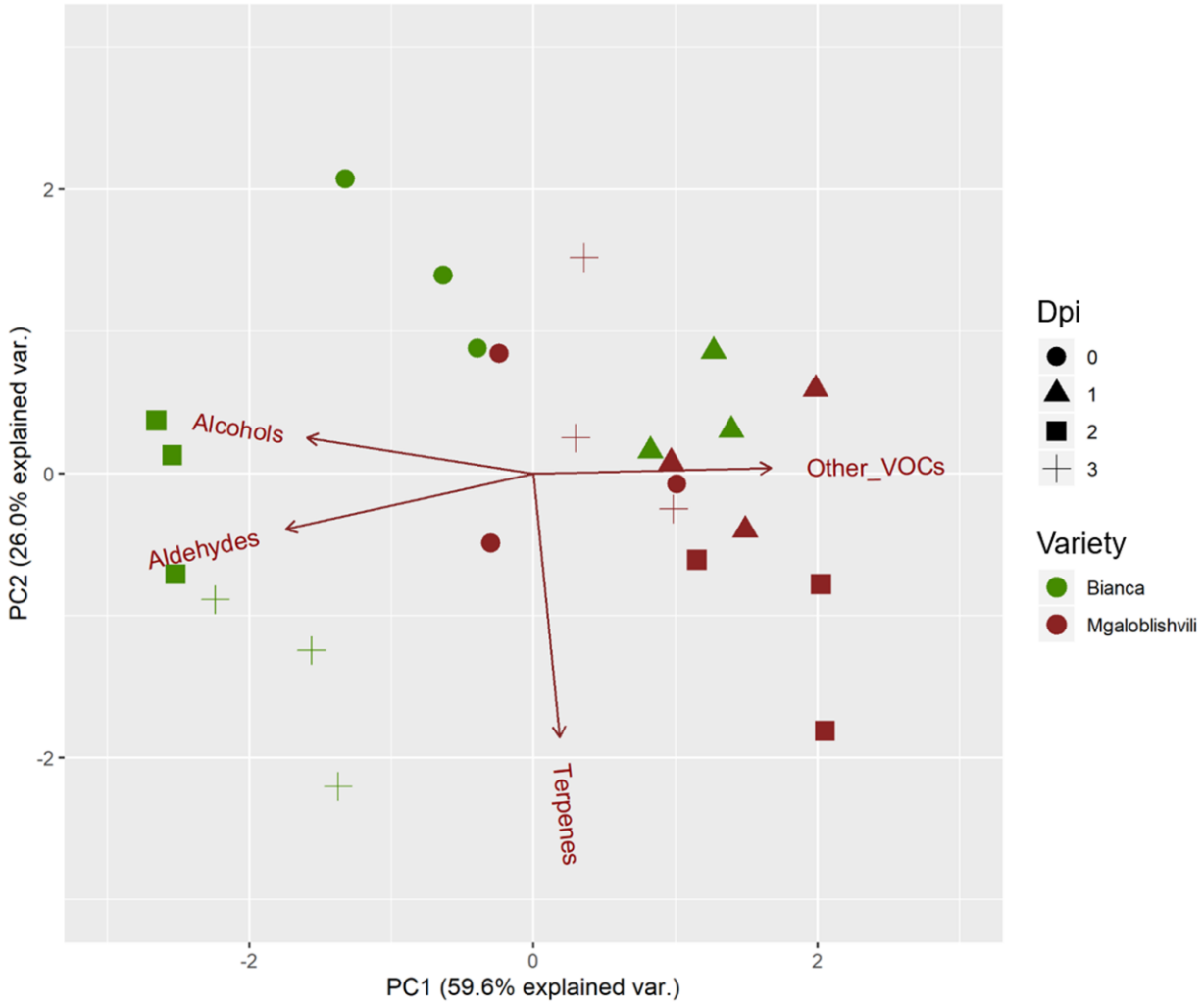


Figure 4. Hierarchical clustering and heatmap visualization for volatile metabolites (alcohols, aldehydes, terpenes and other VOCs) detected in Mgaloblishvili (M) and Bianca (B) leaves collected at 0 (0day), 1 (1day), 2 (2days) and 3 (3days) days post inoculation with *P. viticola*.

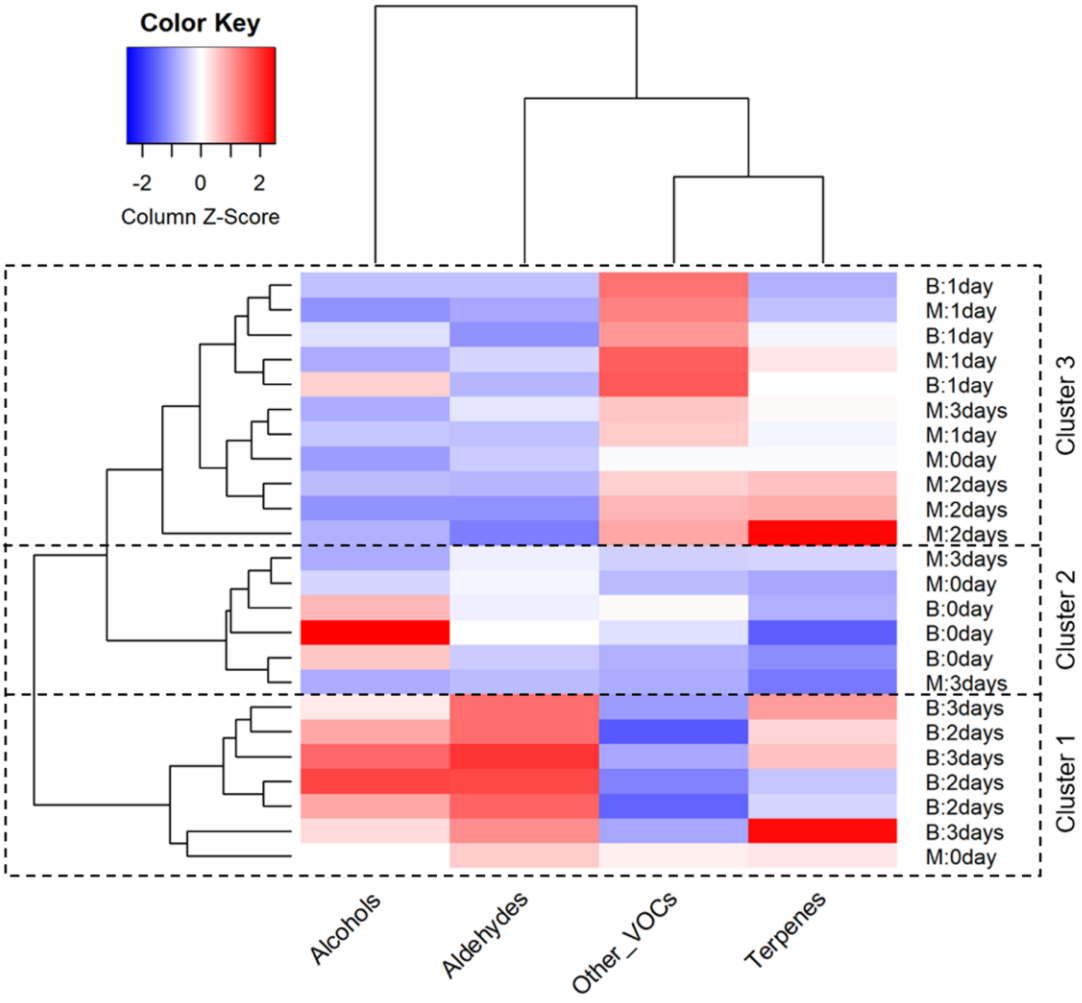


Figure 5. Expression level of genes involved in the biosynthesis of terpenes in Mgaloblishvili (violet bars) and Bianca (green bars) leaves at 0, 1, 2 and 3 days post inoculation with *P. viticola*. The expression of each gene has been normalized using the gene expression values of actin at each time point. The relative gene expression has been determined based on the $2^{-\Delta\Delta Ct}$ method. Standard error bars are visualized. Bars followed by asterisks indicate significant differences from the values recorded at 0 day after inoculation, according to gls test (* *P*= 0.01; ** *P*=0.001; *** *P*= 0.000). *VvGwECar2*: (E)- β-caryophyllene synthase; *VvGwaBer*: (E)-α-bergamotene synthase; *VvCSaFar*: (E,E)-α-farnesene synthase; *VvCSbOciM*: (E)-β-ocimene synthase; *VvTer*: (-)-α-terpineol synthase; *VvVal*: valencene synthase.

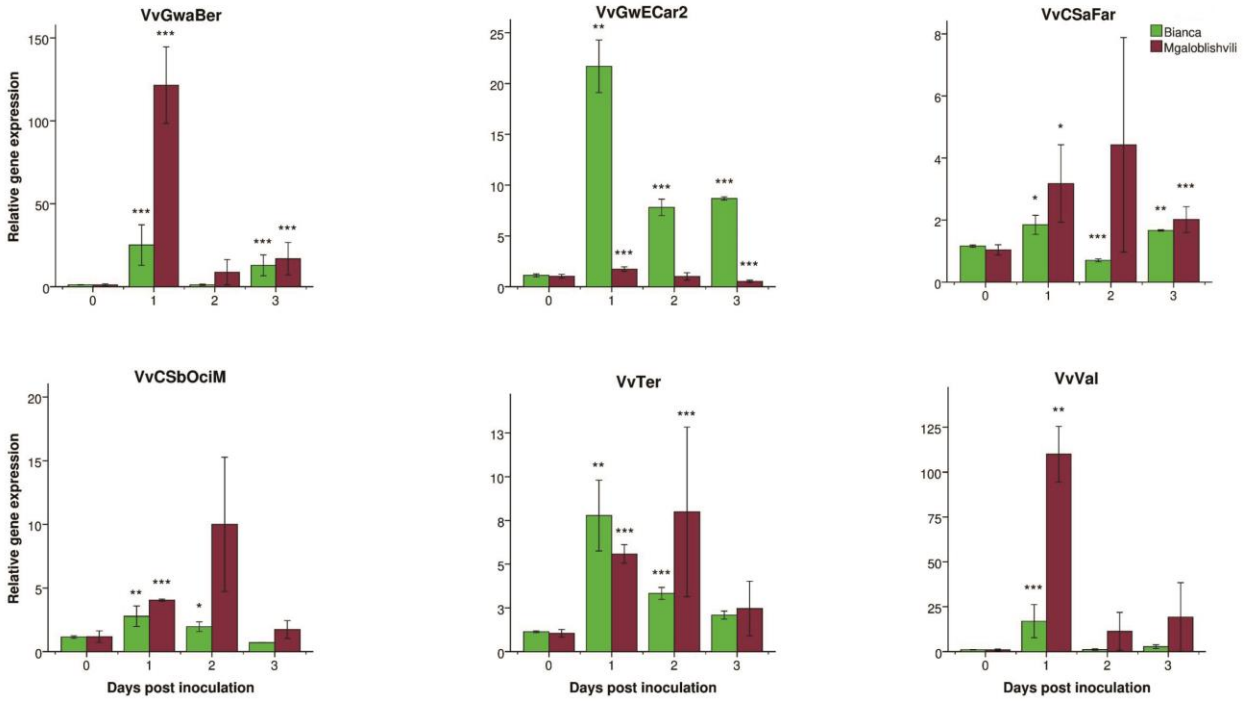
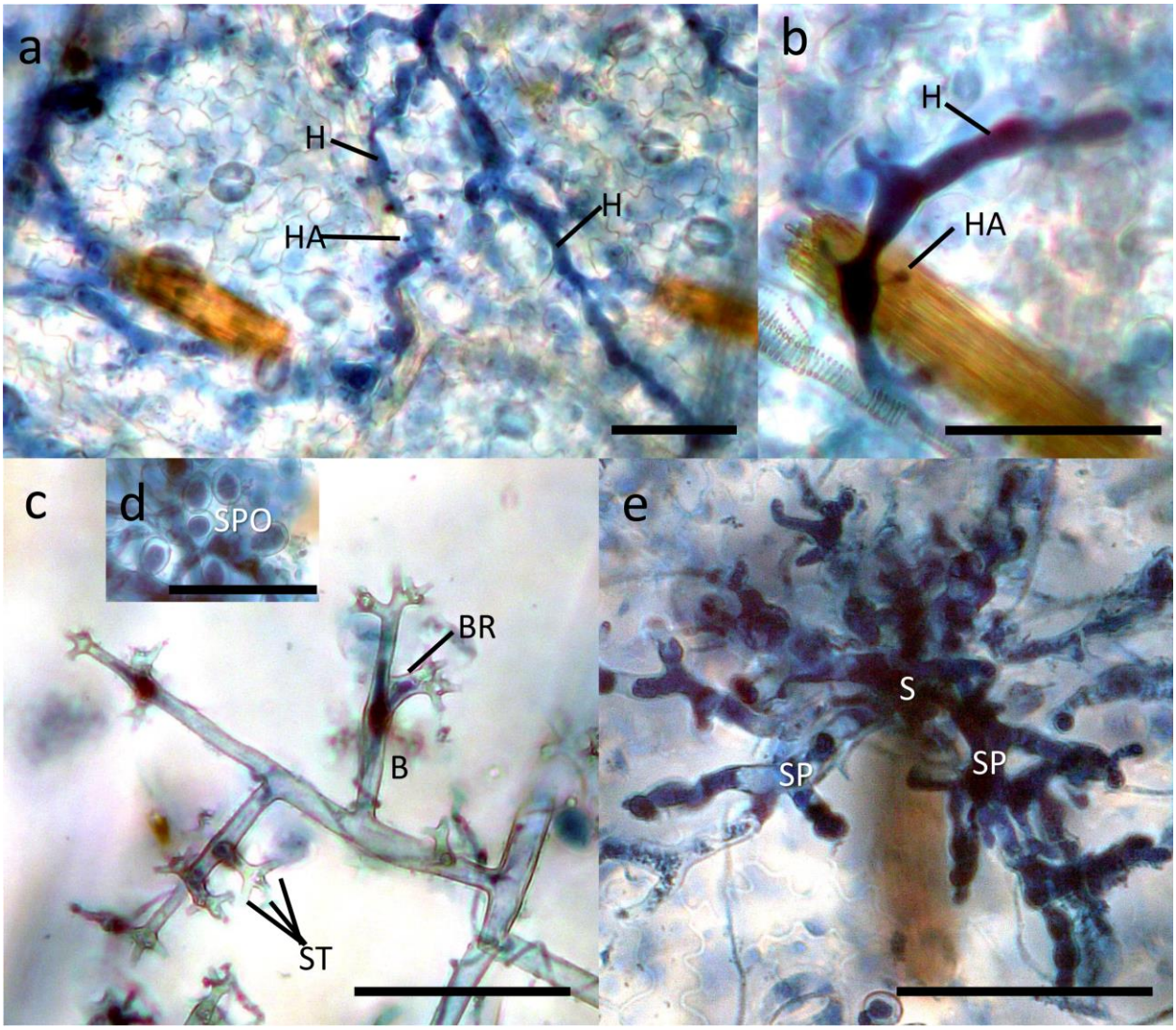
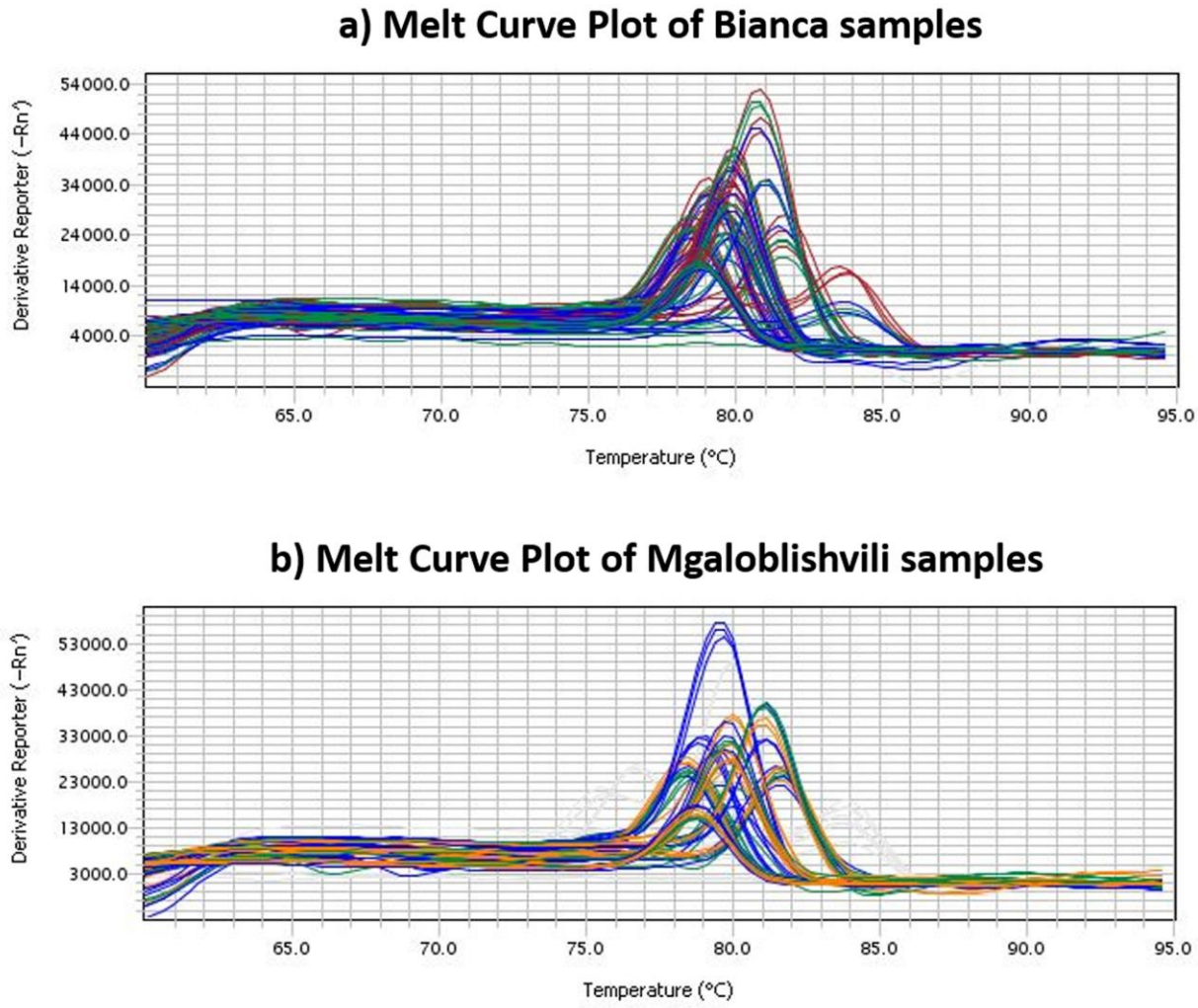


Figure 6. *P. viticola* structures, visible in dark blue colour, in (a-d) untreated and (e) nerolidol-treated (0.01 g l^{-1}) leaf tissues. a) Hyphae with haustoria developing in the mesophyll cells. b) Detail of an hypha with haustoria; c) detail of the apex of a regular sporangiophore showing branches, branchlets and sterigma. d) Sporangia formed at the end of each sterigma. e) Short, hyperbranched and sterile sporangiophores emerging from the stoma. S=stoma; H=hypha; HA=haustorium; SP=sporangiophore; SPO=sporangia; B=branch of the sporangiophore; BR=branchlet; ST=sterigma. Scale bar: 50 µm

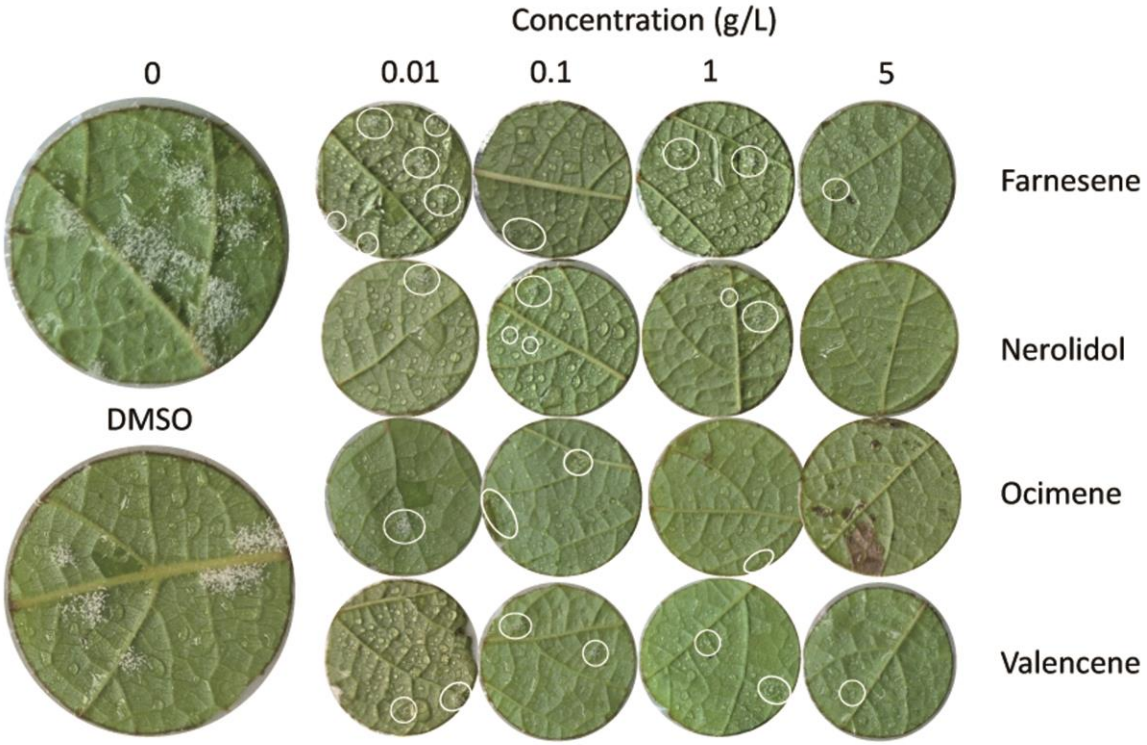

a
H
HA
H
b
H
HA
c
d
SPO
BR
B
ST
e
S
SP
SP

List of supplementary material

Supplementary Figure 1. Example of melt curve plot of Bianca (a) and Mgaloblishvili (b) samples for the six terpene synthases ((E)-β-caryophyllene synthase, (E)-α-bergamotene synthase, (E,E)-α-farnesene synthase, (E)-β-ocimene synthase, (-)-α-terpineol synthase and valencene synthase)) plus two reference genes (ubiquitin and actin) analyzed in this work.



Supplementary Figure 2. Pictures of the leaf disks inoculated with *P. viticola* and covered by white sporulation at 7 days post inoculation. White circles indicate the presence of sporulation on the leaf disks treated with increasing concentrations of farnesene, nerolidol, ocimene and valencene. Leaf disks untreated (0) and treated with DMSO (0.2 %) were considered as controls.


Concentration (g/L)
0
0.01
0.1
1
5
DMSO
Farnesene
Nerolidol
Ocimene
Valencene