Current Plant Biology 35–36 (2023) 100298

Contents lists available at ScienceDirect

Current Plant Biology

journal homepage: www.elsevier.com/locate/cpb

Volatile linalool activates grapevine resistance against downy mildew with changes in the leaf metabolome

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ARTICLE INFO

Keywords:
Volatile organic compounds
Induced systemic resistance
Vitis vinifera
Downy mildew
Untargeted metabolomics analysis
Gene expression

ABSTRACT

Volatile organic compounds (VOCs) are produced by plants in response to biotic and abiotic stimuli. In grapevine, volatile terpenoids are triggered by downy mildew infection (caused by *Plasmopara viticola*), suggesting their involvement in plant defense responses. In particular, linalool was detected in leaves of downy mildew-resistant genotypes, but no information is available on its involvement in the defense mechanisms against *P. viticola*. The aim of this study was to investigate the defense mechanisms activated by linalool in grapevine leaves against *P. viticola* and to identify metabolic changes associated with linalool-induced resistance. Linalool treatment reduced downy mildew severity on leaf disks of susceptible grapevines (cultivar Pinot noir) and stimulated callose deposition at the sites of *P. viticola* infection. Moreover, the upregulation of defense-related genes was found in linalool-treated leaf disks, indicating the activation of grapevine defense mechanisms of salicylic acid and jasmonic acid pathways. Linalool treatment caused changes in the leaf metabolome of mock-inoculated and *P. viticola*-inoculated samples at one and six days post inoculation, as revealed by ultra-high pressure liquid chromatography-electrospray ionization-high-resolution quadrupole time of flight-mass spectrometry. Pathway analysis of annotated features with significant increases and decreases in abundance revealed the reprogramming of amino acid, phenylpropanoid, and terpenoid metabolisms in response to linalool treatment and *P. viticola* inoculation. In particular, features with significant increases in abundance in linalool-treated samples mainly belonged to putative phenylpropanoids, putative terpenoids, putative lipids, and lipid-like compounds, including molecules possibly associated with plant defense against pathogens, such as 2-phenylethanol, 2,4-heptadienal, α-terpineol, citral, and geraniol. These results demonstrated that linalool induces grapevine resistance against downy mildew, acting as a signaling molecule for plant resistance induction.

1. Introduction

Plants are constantly exposed to abiotic and biotic stresses and have evolved intricate ways of interacting with neighboring organisms through communications mediated by volatile organic compounds (VOCs) [1]. VOCs are signaling molecules produced in response to biotic and abiotic stimuli and are involved in both intra- and inter-plant communications, providing fitness benefits to the emitter and neighboring receiver tissues [2]. Plant VOCs are small molecules with low molecular mass (100–500 Da) and high vapor pressure (0.01 kPa or higher at 20 °C) that can cross cellular membranes and evaporate into the atmosphere to reach their biological targets [3,4]. VOCs can be distinguished according to their biosynthetic pathway (e.g., plastidic methylerythritol phosphate pathway, cytosolic mevalonic acid pathway, shikimate pathway, phenylalanine pathway, and lipoxygenase pathway), and they can be classified into amino acid derivatives, benzenoids, fatty acid derivatives, phenylpropanoids, and terpenoids [5]. Terpenoids constitute the largest and most diverse class of plant secondary metabolites that include volatile terpenes (e.g., hemiterpenes, homoterpenes, monoterpenes, and sesquiterpenes) and volatile carotenoid derivatives (e.g., C_{13}-norisoprenoids) [5]. Terpenoids are mainly synthesized from the condensation of two five-carbon precursors, such

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https://doi.org/10.1016/j.cpb.2023.100298
Received 13 July 2023; Received in revised form 11 September 2023; Accepted 23 September 2023
Available online 25 September 2023

as isopentenyl diphosphate, or its allylic isomer, and dimethylallyl diphosphate [5].

Specific emission patterns of volatile terpenoids are known to be triggered by plants in response to herbivore insect feeding, egg deposition, abiotic stresses, and pathogen infections [6]. Although the production and roles of plant VOCs in response to mechanical wounding, herbivore insects, and abiotic stresses were largely investigated, their involvement in defense mechanisms against pathogens was less studied [7]. For example, the production of terpenoids (e.g., 1,8-cineole, β-caryophyllene, β-copaene, and γ-muurolene) in *Chrysanthemum morifolium* leaves was associated with the resistance against *Alternaria tenuissima* [8]. Likewise, volatile terpenoids (e.g., α-terpinolene and β-caryophyllene) and green leaf volatiles can be triggered by mechanical wounding in barley, and they can contribute to plant resistance against fungal infection (*Blumeria hordei*) [9]. Moreover, the VOC profile of tomato leaves inoculated with avirulent *Pseudomonas syringae* was characterized by terpenoids (e.g., 4-terpineol, α-terpineol, and linalool) [10], suggesting the involvement of this VOC class in resistance mechanisms against pathogens. Two possible modes of action against pathogens have been attributed to plant VOCs, such as direct inhibition of pathogen growth and induction of plant resistance [11]. For example, some volatile terpenoids (e.g., carvacrol, caryophyllene, farnesene, limonene, and linalool) can directly inhibit the growth of plant pathogens [11–15]. Moreover, volatile terpenoids (e.g., β-cyclocitral, caryophyllene, ionone, camphene, isoprene, limonene, linalool, and pinene) can induce defense-related processes against pathogens in different plant species [6,11,16–21]. Thus, volatile terpenoids can be responsible for disease reduction in distal parts of locally attacked plants (systemic resistance) or in neighboring plant receivers (plant–plant communication) [22,23], but deeper knowledge is required on metabolic responses associated with VOC-induced resistance mechanisms activated against pathogens in receiver tissues of crop plants.

Grapevine (*Vitis vinifera*) is one of the most widely cultivated fruit crops and VOCs are well-documented determinants of berry and wine aroma, such as aldehydes, esters, terpenoids, and thiols [24]. Specific VOC emission patterns can be stimulated in grapevine by abiotic (e.g., heat stress, water stress, light, growth regulators, and resistance inducers) and biotic factors (e.g., pathogens and beneficial microorganisms), suggesting the involvement of these signaling molecules in grapevine responses to exogenous stimuli [7]. However, scarce information is available on the role of grapevine VOCs produced in response to pathogen infection [7]. In particular, the involvement of grapevine VOCs in defense responses against *Plasmopara viticola* (the causal agent of downy mildew) was hypothesized by the higher abundance of volatile alcohols, aldehydes, and terpenoids in leaves of downy mildew-resistant genotypes compared to susceptible genotypes after *P. viticola* inoculation [25,26]. The production of volatile terpenoids was found in different downy mildew-resistant grapevine genotypes, such as α-muurolene, α-eudesmol, cadinene, caryophyllene, linalool, selinene, epizonarene, and ledol in BC4, Kober 5BB, SO4, or Solaris [26]; 1,6-octadien-3-ol, 3,7-dimethyl-, 2-aminobenzoate (also known as linalyl anthranilate), 2(*4H*)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl- (dihydroactinidiolide), 3,7,11-trimethyl-1,6,10-dodecatrien-3-ol (nerolidol), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol), 3-buten-2-one-4-(2,6,6-trimethyl-1-cyclohexen-1-yl) (β-ionone), 4-(2,6,6-trimethylcyclohexa-1,3-dienyl)but-3-en-2-one (dehydro-β-ionone), 5,9-undecadien-2-one, 6,10-dimethyl-(*E*)- (geranyl acetone), 6,10-dimethyl-2-undecanone, farnesene, and *p*-menth-1-en-8-ol (terpineol) in Bianca and/or Mgaloblishvili [27]; α-ionone, α-terpinolene, β-ciclocitral, β-ionone, β-ionone-epoxido, citral, dihydroactinidolide, farnesene, geranic acid, geraniol, geranylacetone, isogeraniol, linalool, and nerol in Bianca [28] and a genotype resulting from the Bianca × SK77–4/5 cross [29]; α-farnesene, α-muurolene, α-terpineol, ß-cyclocitral, ß-myrcene, ß-ocimene, γ-muurolene, caryophyllene, citronellol, copaene, geranylactone, humulene, limonene, linalool, menthol, and *p*-cymene in Croatian cultivars (Malvazija istarska, Ranfol, and/or Teran) [30]. Thus, linalool was found in several downy mildew-resistant genotypes, such as Bianca [28,31], Teran [30], Kober 5BB and SO4 [26], BC4 and Solaris [26,31] and pyramided resistant genotypes [29,31], but no information is available on its involvement in the defense mechanisms against *P. viticola*. In other pathosystems, linalool can inhibit the growth of *Botrytis cinerea* [32], *Colletotrichum lindemuthianum* [11], *Fusarium graminearum* [33], *F. oxysporum* f. sp. *radicis-lycopersici* [34], and it can reduce the virulence of *P. syringae* pv. *tomato* DC3000 [35]. Moreover, linalool can induce defense mechanisms of rice against *Xanthomonas oryzae* [36], tomato against *Fusarium oxysporum f.* sp. *radicis-lycopersici* [34], and Arabidopsis against *Plutella xylostella* [37]. Thus, VOC applications on crops were proposed to limit pathogen infection and to reduce the use of chemical fungicides, but further studies on the mode of action, application dosage, and formulation are required [23,38]. In particular, the possible roles of linalool in plant–plant communication and plant resistance induction were previously proposed [22,23], but no information is available on the physiological and biochemical responses activated by linalool in receiver tissues. The aim of this study was to provide better information on defense mechanisms activated by linalool in grapevine leaves against downy mildew and to identify metabolic changes associated with linalool-induced resistance.

2. Materials and methods

2.1. Biological material

Grapevine plants (*V. vinifera* cultivar Pinot Noir; downy mildew-susceptible) were grown in 2.5 L-pots containing a mixture of peat and pumice (3:1; GR Intensivo, Tercomposti) under greenhouse conditions at 25 ± 1 °C with a 16 h light / 8 h dark photoperiod and 70 ± 10% relative humidity (RH) of [39]. A *P. viticola* population was collected from an untreated vineyard in the Trentino region (northern Italy) and maintained on *V. vinifera* Pinot Noir plants by subsequent inoculations under greenhouse conditions, as previously described [40]. To obtain the *P. viticola* inoculum, plants with disease symptoms were incubated overnight in the dark at 95 ± 5% RH to promote pathogen sporulation, and sporangia were collected by washing the abaxial leaf surfaces bearing freshly sporulating lesions with cold (4 °C) distilled water. The inoculum concentration was then adjusted to 2.5×10^5 sporangia mL^{-1} using a hemocytometer under a light microscope (LMD7000, Leica Microsystems), as previously described [39].

2.2. Assessment of linalool effects against downy mildew

Leaf disks (25 mm diameter) were obtained from the greenhouse-grown grapevine plants (from the fourth to the sixth node) with a cork borer, and they were placed randomly on two layers of wet filter paper in dishes (90 mm diameter; six leaf disks for each dish) with the abaxial surface uppermost [39]. Treatments with linalool (CAS No. 78–70–6, Sigma-Aldrich, Merck) on leaf disks were carried out according to Lazazzara, et al. [26]. Briefly, linalool was 10-fold diluted in dimethyl sulfoxide (Sigma-Aldrich, Merck) and the stock solution (50 μl linalool and 450 μl DMSO) was serially diluted in distilled water to obtain the appropriate concentration for each treatment. Treatments were applied to a filter paper disk (Whatman, Merck) fixed on the lid (without physical contact with the leaf tissue) of a dish containing grapevine leaf disks (dish chamber; Fig. S1). In particular, linalool was applied to the filter paper disk at a concentration of 10, 25, or 50 mg L^{-1} of air volume in the dish chamber (linalool-treated leaf disks), assuming the complete VOC evaporation from the filter paper, and water was applied as control (0 mg L^{-1} of air volume of linalool in the dish chamber; control leaf disks). Dishes were sealed with Parafilm (Beims) and incubated in the dark at 25 ± 1 °C for 16 h. Each leaf disk was inoculated with five 5 drops (5 μl each) of a *P. viticola* suspension (*P. viticola*-inoculated) or treated with five drops (5 μl each) of distilled water (mock-inoculated). The respective treatment (linalool or water) was applied again to the

filter paper disk, dishes were sealed with Parafilm (Beims) and incubated in the dark at 25 ± 1 °C for 16 h. Leaf disks were dried under a laminar hood and incubated for six days under greenhouse conditions.

Downy mildew severity was assessed on each leaf disk at six days post inoculation (dpi) as a percentage of the leaf disk surface covered by sporulation [41], calculated as the sum of the five inoculum drops scored as follows: 0%, no sporulation; 10%, scarce sporulation; 20%, dense sporulation [39]. The disease severity of each replicate (dish) was then calculated as the average of the disease severity of leaf disks contained in the dish. The disease reduction (efficacy) was calculated for each replicate according to the following formula: (disease severity of control leaf disks — disease severity of linalool-treated leaf disks) / disease severity of control leaf disks × 100. The presence of phytotoxic effects was assessed visually by checking for discoloration, chlorosis, and whitening of leaf disks [42].

For disease assessment, at least five replicates (dishes with six leaf disks each) were assessed for each treatment and the experiment was carried out twice. Disease severity data were analyzed using Past 4.03 software (https://www.nhm.uio.no/english/research/resources/past/) and a Kruskal-Wallis test was used to demonstrate equivalent results in the two experiments ($P > 0.05$). Data from the two experiments were pooled and a Kruskal-Wallis test with Dunn's post hoc test was used to detect significant differences among treatments ($P \leq 0.05$).

2.3. Visualization of callose deposition and Plasmopara viticola structures by aniline blue staining

Linalool-treated and control leaf disks were collected at 1 and 6 dpi from *P. viticola*-inoculated and mock-inoculated samples and stained with aniline blue to visualize *P. viticola* structures and callose deposition [26]. Briefly, leaf disks were incubated in 1 M KOH at 95 °C for 15 min and stained with 0.05% aniline blue (Sigma-Aldrich, Merck) in 0.067 M K_2HPO_4 at pH 8 for 15 min. Leaf disks were observed under a fluorescent microscope (LMD7000, Leica Microsystems) using an A4 filter (360 ± 40 nm excitation, 400 nm dichroic mirror, and 470 ± 40 nm emission). Five leaf disks were analyzed for each treatment and time point, and the experiment was carried out twice.

2.4. Sample collection for gene expression and metabolomic analysis

Linalool-treated and control leaf disks were collected at 1 and 6 dpi from *P. viticola*-inoculated and mock-inoculated samples. These time points were chosen to analyze the grapevine defense reactions [40,43,44] and leaf disks were reduced to 18 mm in diameter to eliminate areas where defense responses related to wounding are possible [45]. Six replicates were collected for each treatment and time point, and each replicate comprised ten leaf disks. Samples were immediately frozen in liquid nitrogen, crushed to a fine powder using a mixer mill disruptor (MM200, Retsch) at 25 Hz for 60 s with 2 mL-tubes and 6 mm-beads refrigerated in liquid nitrogen, and stored at − 80 °C until further use.

2.5. RNA extraction and gene expression analysis

Total RNA was extracted from 100 mg of leaf disk powder using the Spectrum Plant Total RNA kit (Sigma-Aldrich, Merck) with an on-column DNase treatment with the RNase-Free DNase Set (Qiagen). RNA was quantified by Qubit RNA Broad Range Assay Kit (Thermo Fisher Scientific) and the effectiveness of the DNase treatment was confirmed by running PCR with grapevine *actin* primers (Supplementary Table S1) in the absence of retro-transcription, and no amplification signals were detected. The first strand cDNA was synthesized from 1.0 μg of total RNA using Superscript III (Invitrogen, Thermo Fisher Scientific) and oligo-dT primer. Genes encoding chitinase 3 (*CHIT-3*), lipoxygenase 9 (*LOX-9*), osmotin 2 (*OSM-2*), pathogenesis-related protein 2 (*PR-2*) were used as markers of grapevine induced resistance against downy mildew [40,46]. In particular, *PR-2* is known as a marker of the salicylic acid (SA) defense pathways [47], while *PR-4* and *LOX-9* are markers of the jasmonic acid (JA) defense pathways [48]. The hypersensitive response-related gene (*HSR*) was used as a marker of cell death [49] and VOC-induced resistance [39], while stilbene synthase gene (*STS*) was used as a marker of the phenylpropanoid pathway [49]. Quantitative real-time PCR (qPCR) reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific) and specific primers (Supplementary Table S1) using the Light Cycler 480 (Roche Diagnostics), as previously described [40]. Briefly, the PCR conditions were as follows: 50 °C for 2 min and 95 °C for 2 min as initial steps, followed by 50 cycles at 95 °C for 15 s and at 60 °C for 1 min. Each sample was examined in three technical replicates and dissociation curves were analyzed to verify the specificity of each amplification reaction.

The Light Cycler 480 SV 1.5.0 software (Roche) was used to extract Ct values based on the second derivative calculation and the reaction efficiency (Eff) was calculated with the LinRegPCR 11.1 software for each gene [50]. The expression level of each gene was calculated according to the Hellemans equation [51], using *actin* and *VATP16* as housekeeping genes for normalization [26]. Briefly, relative quantities (RQ) were calculated according to the formula: $RQ = Eff^{(Ct-Ct')}$, where Ct is the threshold cycle and Ct' is the average Ct of all the treatments analyzed. Normalized relative quantities (NRQ) were then calculated by dividing the RQ by the normalization factor based on the RQ values of the two housekeeping genes [51]. Six replicates (pool of ten leaf disks each) were analyzed for each treatment and time point. Values of the gene expression analysis were Log_{10}-transformed [39] and a Kruskal-Wallis test with Dunn's post hoc test was used to detect significant differences among treatments for each time point ($P \leq 0.05$) using the Past 4.03 software.

2.6. Ultra-high pressure liquid chromatography-electrospray ionization-high-resolution quadrupole time of flight-mass spectrometry (UHPLC-ESI-Q-TOF-MS) analysis

An aliquot of each sample (30 mg of leaf disk powder) was supplemented with an internal standard mixture (30 μl) consisting of 1 g L^{-1} caffeine-(trimethyl-d9) (Sigma-Aldrich, Merck), 1 g L^{-1} DL-aspartic acid-2,3,3-d3 (Sigma-Aldrich, Merck), and 1 g L^{-1} choline chloride-(trimethyl-d9) (Sigma-Aldrich, Merck) to validate the extraction efficiency. Metabolite extraction was carried out as reported by Billet, et al. [52] with slight modifications. Briefly, samples were extracted in 1 mL of methanol:water (80:20; v v^{-1}) with sonication for 15 min (Ultrasonic Cleaners, VWR) and shaking for 15 min (Thermomixer, Eppendorf) at room temperature. Samples were centrifuged at 20000 × *g* for 15 min at 4 °C, the supernatant was collected and stored at 4 °C overnight. Before chromatographic analyses, sample extracts were centrifuged again at 20000 × *g* for 15 min at 4 °C, and each vial was prepared by mixing an aliquot of the sample extract (235 μl) with an additional internal standard mixture (15 μl) consisting of (+)-catechin-2,3,4-$^{13}C_3$ (Sigma-Aldrich, Merck; 500 mg L^{-1}), (±)-catechin-2,3,4-$^{13}C_3$ gallate (Sigma-Aldrich, Merck; 500 mgL^{-1}), and gallocatechin-2,3,4-^{13}C (Sigma-Aldrich, Merck; 500 mgL^{-1}), in order to monitor instrument performance and signal stability. As quality control (QC) samples, equal aliquots of each sample were homogenized to assess technical variability. Samples were measured in a randomized complete block design and a QC sample was analyzed every five samples.

Ultra-high pressure liquid chromatography-electrospray ionization-high-resolution quadrupole time of flight-mass spectrometry (UHPLC-ESI-Q-TOF-MS) analysis was carried out using an Ultimate 3000 UHPLC (Thermo Scientific) coupled with an Impact HD Q-TOF (Bruker) mass spectrometer. A Waters Acquity HSS T3 C18 column (150 × 2.1 mm, 1.8 μm) was used to separate the analytes with a flow rate of 0.4 mL min^{-1} at 55 °C and the injection volume was 5 μl. The mobile phase was made of 0.1% formic acid (Sigma-Aldrich, Merck) in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B, Sigma-Aldrich, Merck). The

chromatographic separation was performed using an 18-min linear gradient from 5% to 50% solvent B as reported by Billet, et al. [52] followed by washing and reconditioning of the column for 17 min. The mass spectrometric conditions were set according to Gorfer, et al. [53] as follows: mass to charge ratio (m/z) range, 50–1500; capillary voltage, 4500 V in positive electrospray ionization (ESI) mode and 2500 V in negative ESI mode; nebulizer gas (nitrogen) pressure, 3 bar; dry gas (nitrogen), 12 L min^{-1}; dry temperature, 230 °C. In addition, to validate annotated compounds with authentic reference standards, MS/MS analysis was carried out with the collision energy of 10 eV. A sodium formate solution (10 mM) was used as a calibrator to maintain mass accuracy [53,54].

2.7. Data processing and selection of features with significant changes in abundance

Chromatograms (in the range from 0 to 18 min), obtained in positive and negative ESI modes, were converted into the mzML format and pre-processed with the xcms R package [55–57] using an in-house R script designed for peak picking and alignment [58]. The extraction efficiency was confirmed by calculating the extraction yield of internal standards added in each sample powder (greater than 90%). The signal stability was confirmed by the analysis of relative standard deviations of internal standards added in each sample extract (lower than 5%). Six replicates (pool of ten leaf disks each) were analyzed for each treatment and time point and missing values of a feature in a sample (abundance below the detection limit of the instrument) were replaced by half of the minimum value for that feature found in the dataset [59].

A principal component analysis (PCA) was carried out on detected features using the MetaboAnalyst online platform (version 5.0; http://www.metaboanalyst.ca/) [60] with interquartile range (IQR) data filtering on peak areas, data normalization according to QCs, Log_{10} transformation, and Pareto scaling [54,61]. The clustering of QCs samples was visually confirmed in the PCA (data not shown). Features with significant changes in abundances were selected imposing an adjusted P-value of t-test lower than 0.05 (5% false discovery rate) and minimum Log_2-transformed fold change (FC) of one (increases or decreases in abundance) [62] in four pairwise comparisons for each time point, such as between i) linalool-treated mock-inoculated and control mock-inoculated samples; ii) linalool-treated *P. viticola*-inoculated and control *P. viticola*-inoculated samples; iii) control *P. viticola*-inoculated and control mock-inoculated samples; iv) linalool-treated *P. viticola*-inoculated and linalool-treated mock-inoculated samples. Features with significant increases and decreases in abundance were grouped for each time point according to Venn diagrams obtained with a web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

2.8. Compound annotation

All features were submitted to the MassTrix 3 server (http://masstrix3.helmholtz-muenchen.de/masstrix3/) [63,64] and putative chemical names, elemental formulas, and database entry codes were obtained (annotated features) using *V. vinifera* as reference organism [45], considering possible adducts $[M+H]^+$ and $[M+Na]^+$ for positive ESI data and $[M-H]^-$ for negative ESI data with a maximum mass error acceptance of 3 ppm [65]. A frequency histogram of eight combinations (CHO, CHON, CHNOP, CHONS, CHOP, CHOS, CHN, and CH) of six chemical elements, such as carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P), and sulfur (S), was built according to the putative elemental formula of annotated features [45]. Putative chemical classes of annotated features were obtained with the van Krevelen diagram according to the hydrogen/carbon (H/C) and oxygen/carbon (O/C) ratios of the elemental formula, such as putative carbohydrates (O/C from 0.6 to 1.2 and H/C from 1.5 to 2.2), putative lipids (O/C from 0 to 0.3 and H/C from 1.3 to 2.2), putative amino acids (O/C from 0.1 to 0.5 and H/C from 1.3 to 2.2) and putative polyphenols (O/C from 0.2 to 0.7 and H/C from 0.4 to 1.4) according to Onzo, et al. [66]. Pathway analysis of annotated features with significant changes in abundance was carried out using the pathway analysis tool of MetaboAnalyst 5.0 (https://www.metaboanalyst.ca) [59]. Briefly, pathways were analyzed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, using the hypergeometric test for pathway enrichment analysis and the out-degree centrality for pathway topology analysis with KEGG pathway library of *Arabidopsis thaliana* as reference [67].

Feature annotation was further improved with a manually curated annotation by searching annotated features with significant increases in abundance in the PubChem (http://pubchem.ncbi.nlm.nih.gov/) [68], ChEBI (https://www.ebi.ac.uk/chebi/) [69], KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/kegg2.html) [70], Lipid Maps (http://www.lipidmaps.org) [71] and human metabolome (https://hmdb.ca/) [72] databases, in order to retrieve the exact mass, InChI code, and InChIKey code. The reference mass spectrum was obtained for each annotated feature with the CFM-ID 4.0 web server (Competitive Fragmentation Modeling for Metabolite Identification; https://cfmid.wishartlab.com) according to InChI code search [73,74]. Reference mass spectra were then visually compared with the experimental mass spectra of the respective annotated features, to select the most probable compound annotation, molecular ions, and database entry codes (annotated compounds). Annotated compounds were classified into ten putative chemical classes (benzenoids, carbohydrates and conjugates, carbonyl compounds, carboxylic acids and derivatives, indoles and derivatives, lipids and lipid-like compounds, nucleosides and nucleotides, phenylpropanoids, terpenoids, and unknown) according to a manually curated annotation based on the classification obtained with the ClassyFire web-based application (https://cfb.fiehnlab.ucdavis.edu/) by InChI code search [75].

Authentic reference standards were selected to validate annotated compounds with significant increases in abundance and belonging to putative benzenoids and terpenoids, such as 2-phenylethanol (Acros Organics, Thermo Fisher), geraniol (Sigma-Aldrich, Merck), linalool (Sigma-Aldrich, Merck), and linalyl oxide (Sigma-Aldrich, Merck). Authentic reference standards were analyzed at the concentration of 10 mg L^{-1} in methanol:water (80:20; v v^{-1}) by UHPLC-QTOF-MS analysis as described above. For compound identification, mass spectra and retention times of authentic reference standards and annotated compounds were compared.

3. Results

3.1. Linalool treatment reduces downy mildew severity

Linalool was tested against *P. viticola* at different concentrations, and the concentration of 10 mg L^{-1} of air volume in the dish chamber reduced downy mildew severity on grapevine leaf disks with an efficacy of 74.5 ± 4.2% and no visible phytotoxic effects (Fig. 1 and Supplementary Fig. S2). Conversely, leaf disks treated with linalool at the concentration of 25 and 50 mg L^{-1} of air volume in the dish chamber showed phytotoxic effects and a disease severity comparable to leaf disks treated with 10 mg L^{-1} of air volume in the dish chamber (Supplementary Fig. S2). Thus, the lowest concentration of linalool that reduced downy mildew symptoms with no visible phytotoxic effects (10 mg L^{-1} of air volume in the dish chamber) was selected for further analyses.

3.2. Linalool treatment stimulates callose deposition and the expression of defense-related genes

Aniline blue staining revealed marked differences between linalool-treated and control leaf disks at 1 and 6 dpi with *P. viticola* (Fig. 2). At 1 dpi, the pathogen had already penetrated the stomata of control leaf disks and substomatal *P. viticola* vesicles were visible (Fig. 2A). Moreover, *P. viticola* mycelium spread to the parenchyma and produced

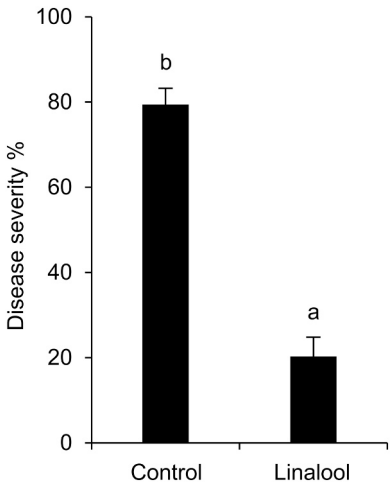

Fig. 1. Effect of linalool against downy mildew. Grapevine leaf disks were treated with water (Control) or linalool at 10 mg L^{-1} of air volume in the dish chamber (Linalool), applied on a filter paper disk without contact with leaf tissues. Disks were inoculated with *Plasmopara viticola* and downy mildew severity was assessed at six days post inoculation. Ten replicates (dishes with six leaf disks each) were assessed for each treatment and the experiment was carried out twice. The Kruskal-Wallis test indicated no significant differences between the two experiments ($P > 0.05$), and data from the two experiments were pooled. Mean and standard error values of 20 replicates from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments according to the Kruskal-Wallis test with Dunn's post hoc test ($P \leq 0.05$).

sporangiophores in control leaf disks at 6 dpi (Fig. 2B). On the other hand, strong turquoise fluorescence was observed in the stomata of linalool-treated leaf disks at 1 dpi (Fig. 2C), indicating callose deposition at infection sites. Thus, the number of zoospores that had successfully entered the stomata at 1 dpi was reduced in linalool-treated compared to control leaf disks, and *P. viticola* sporulated areas were limited in linalool-treated leaf disks at 6 dpi (Fig. 2D). However, no differences between control (Fig. 2E, F) and linalool-treated (Fig. 2G, H) leaf disks were observed in mock-inoculated samples at both time points.

Plasmopara viticola inoculation upregulated the expression of *HSR* at 1 dpi and *CHIT-3*, *HSR*, *LOX-9*, *OSM-2*, *PR-2*, *PR-4*, and *STS* at 6 dpi in control leaf disks (Table 1). Linalool treatment upregulated the expression of six genes (*CHIT-3*, *HSR*, *LOX-9, OSM-2*, *PR-2*, and *PR-4*) and two genes (*HSR* and *OSM-2*) in mock-inoculated leaf disks at 1 and 6 dpi, respectively. In *P. viticola*-inoculated leaf disks, the expression level of *CHIT-3*, *HSR*, *LOX*-9, *OSM-2*, *PR-2*, and *PR-4* was higher in linalool-treated compared to control leaf disks at 1 dpi, as a reinforced upregulation of defense-related genes after pathogen inoculation.

3.3. Linalool treatment modifies the grapevine leaf metabolome

A total of 8591 features were detected in grapevine leaf disks by UHPLC-ESI-Q-TOF-MS analysis and 1367 of them were annotated with the MassTrix 3 server (annotated features; Supplementary Table S2). The PCA on detected features discriminated grapevine samples according to the time point in the first component (23.1% of the total variance) and according to the linalool treatment in the second component (15.6% of the total variance; Fig. 3). Annotated features mainly showed a CHO and CHNO composition according to their elemental formulas (Supplementary Fig. S3A), and they were assigned to putative amino acids, lipids, polyphenols, and sugars in the van Krevelen diagrams of features detected in control mock-inoculated, control *P. viticola*-inoculated, linalool-treated mock-inoculated, and linalool-treated *P. viticola*-inoculated samples at 1 and 6 dpi (Supplementary Fig. S3B and C).

Features with significant increases and decreases in abundance were found (adjusted *P*-value of *t*-test lower than 0.05 and minimum Log_2-transformed FC of one) in the pairwise comparisons at 1 dpi (257 and 232, respectively) and 6 dpi (463 and 321, respectively), and they were grouped according to Venn diagrams for each time point (Supplementary Fig. S4 and Supplementary Table S3). At 1 dpi, features with increased and decreased abundance in linalool-treated compared to control leaf disks were 51 and 34 in mock-inoculated samples, 50 and 117 in *P. viticola*-inoculated samples, and 152 and 77 in both inoculation conditions, respectively (Supplementary Fig. S4A and B). Moreover, four and two features showed increases and decreases in abundance in *P. viticola*-inoculated compared to mock-inoculated leaf disks in control samples at 1 dpi, respectively (Supplementary Fig. S4A and B). At 6 dpi, 44, 74, and 148 features showed increases in abundance (72, 144, and

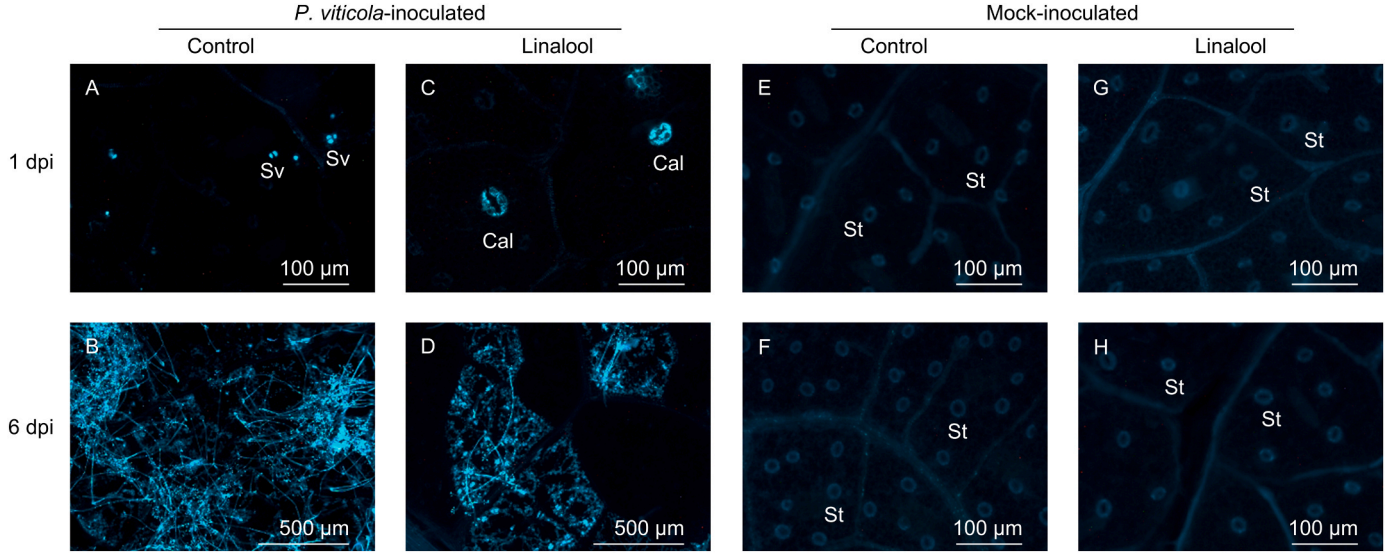

Fig. 2. Effects of linalool on downy mildew development and callose deposition. Grapevine leaf disks were treated with water (Control) or linalool at 10 mg L^{-1} of air volume in the dish chamber (Linalool), applied on a filter paper disk without contact with leaf tissues. Disks were inoculated with *Plasmopara viticola* (*P. viticola*-inoculated; A-D) or treated with water (Mock-inoculated, E-H). Pathogen development and callose deposition were visualized at one and six days post inoculation (dpi) using aniline blue staining. The experiment was carried out twice and a representative leaf disk of ten is shown for each treatment. Abbreviations: Cal, callose; Sv, substomatal *P. viticola* vesicle; St, stomata guard cells.

Table 1
Gene expression analysis of defense-related genes in grapevine leaf disks treated with linalool and inoculated with *Plasmopara viticola*.

Gene name[a]	1 dpi								6 dpi							
	Control				Linalool				Control				Linalool			
	Mock		*P. viticola*		Mock		*P. viticola*		Mock		*P. viticola*		Mock		*P. viticola*	
Chitinase 3 (*CHIT-3*)	1.2 ± 0.2	a	1.9 ± 0.7	a	17.5 ± 4.1	b	14.3 ± 3.2	b	0.3 ± 0.1	a	3.4 ± 0.4	c	0.6 ± 0.1	ab	1.5 ± 0.4	bc
Hypersensitive response-related gene (*HSR*)	0.4 ± 0.1	a	1.6 ± 0.1	b	52.3 ± 14.8	c	199.0 ± 59.6	d	0.1 ± 0.0	a	37.3 ± 7.2	c	2.0 ± 1.0	b	14.6 ± 4.9	bc
Lipoxygenase 9 (*LOX-9*)	2.3 ± 0.7	a	2.2 ± 0.5	a	25.4 ± 5.4	b	41.0 ± 9.0	b	0.4 ± 0.1	a	4.3 ± 0.9	c	0.6 ± 0.1	a	1.9 ± 0.3	bc
Osmotin 2 (*OSM-2*)	0.7 ± 0.1	a	1.2 ± 0.3	a	58.2 ± 4.9	b	282.8 ± 145.8	c	0.4 ± 0.1	a	21.1 ± 7.8	c	2.0 ± 0.9	b	17.6 ± 7.6	c
Pathogenesis-related protein 2 (*PR-2*)	1.4 ± 0.2	a	2.3 ± 0.3	ab	13.6 ± 3.1	bc	56.1 ± 18.4	c	0.3 ± 0.1	a	5.6 ± 0.3	b	0.7 ± 0.2	a	3.1 ± 0.9	b
Pathogenesis-related protein 4 (*PR-4*)	0.7 ± 0.1	a	1.7 ± 0.3	ab	11.3 ± 1.5	bc	24.5 ± 4.6	c	1.4 ± 0.2	a	7.4 ± 0.7	b	2.3 ± 0.1	ab	5.5 ± 2.3	b
Stilbene synthase *(STS)*	1.5 ± 0.4	a	3.3 ± 0.7	ab	3.8 ± 0.9	ab	11.4 ± 5.7	b	0.4 ± 0.1	a	17.8 ± 3.8	c	1.1 ± 0.4	ab	8.5 ± 1.6	bc

[a] The relative expression level (normalized relative quantities) of each gene was calculated using the Hellemans equation [51], with *actin* and *VATP16* as housekeeping genes. Grapevine leaf disks were treated with water (Control) or linalool at 10 mg L^{-1} of air volume in the dish chamber (Linalool), applied on a filter paper disk without contact with leaf tissues. Disks were inoculated with *Plasmopara viticola* (*P. viticola*) or treated with water (Mock), and they were collected at one and six days post inoculation (dpi). Mean and standard error values of six replicates (pool of ten leaf disks each) are presented for each treatment and time point. For each gene and time point, different letters indicate significant differences according to the Kruskal-Wallis test with Dunn's post hoc test ($P \leq 0.05$).

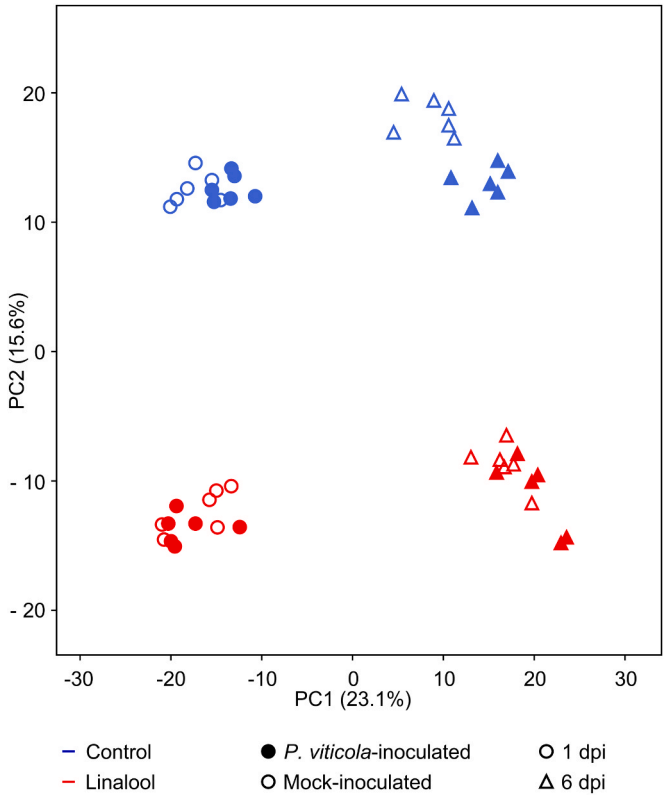

Fig. 3. Principal component analysis (PCA) of metabolomic data. Grapevine leaf disks were treated with water (Control; blue) or linalool at 10 mg L^{-1} of air volume in the dish chamber (Linalool; red), applied on a filter paper disk without contact with leaf tissues. Disks were inoculated with *Plasmopara viticola* (*P. viticola*-inoculated; solid symbols) or treated with water (Mock-inoculated; open symbols). Six replicates (pool of ten leaf disks each) for each treatment were analyzed at one (circles) and six (triangles) days post inoculation (dpi). PCA was obtained with MetaboAnalyst on 8591 features detected in grapevine samples by ultra-high pressure liquid chromatography-electrospray ionization-high-resolution quadrupole time of flight-mass spectrometry.

20 features showed decreases in abundance) in linalool-treated compared to control leaf disks in mock-inoculated samples, in *P. viticola*-inoculated samples, and in both inoculation conditions, respectively (Supplementary Fig. S4C and D). Moreover, 105, 28, and 22 features showed increases in abundance (60, three, and zero features showed decreases in abundance) in *P. viticola*-inoculated compared to mock-inoculated leaf disks in control samples, in linalool-treated samples, and in both treated conditions, respectively (Supplementary Fig. S4C and D).

Amino acid (phenylalanine, tyrosine and tryptophan), galactose, phenylpropanoid (flavone, flavonol, and flavonoid), purine, and terpenoid metabolisms were mainly found by pathway analysis of annotated features with significant increases in abundance in linalool-treated compared to control leaf disks in mock-inoculated samples and *P. viticola*-inoculated samples at 1 and 6 dpi (Supplementary Fig. S5A-F). Annotated features with significant decreases in abundance in linalool-treated compared to control leaf disks were related to amino acid (arginine, phenylalanine, proline, tyrosine, and tryptophan), glutathione, phenylpropanoid (flavone, flavonol, and flavonoid), chlorophill and sulfur metabolisms at 1 and 6 dpi (Supplementary Fig. S5G-J). Moreover, five (amino acid, anthocyanin, glucosinolate, pantotenate, and selenocompound) and three (galactose, indole alkaloid and purine) metabolisms were mainly found by pathway analysis of annotated features with significant increases and decreases in abundance in control *P. viticola*-inoculated compared to control mock-inoculated samples at 6 dpi, respectively (Supplementary Fig. S5L and M).

3.4. Linalool treatment and Plasmopara viticola inoculation increase the abundance of putative phenylpropanoids, terpenoids, and lipids

The manually curated annotation of features with significant increases in abundance and annotated with the MassTrix 3 server allowed the classification of 159 annotated compounds into ten putative chemical classes (Fig. 4 and Supplementary Table S4). In particular, annotated compounds with increased abundance in linalool-treated compared to control leaf disks were nine in mock-inoculated samples (Group 1), 13 in *P. viticola*-inoculated samples (Group 2), and 37 in both inoculation conditions (Group 3) at 1 dpi (Fig. 4A). These annotated compounds mainly belonged to putative terpenoids (e.g., α-terpineol, α-tocopheronic acid, α-tocopheronolactone, bornanedione, boschniakine, castelanone, citral, geraniol, linalool, linalyl oxide, and picrocrocin), putative phenylpropanoids [e.g., piceid, epimedoside C,

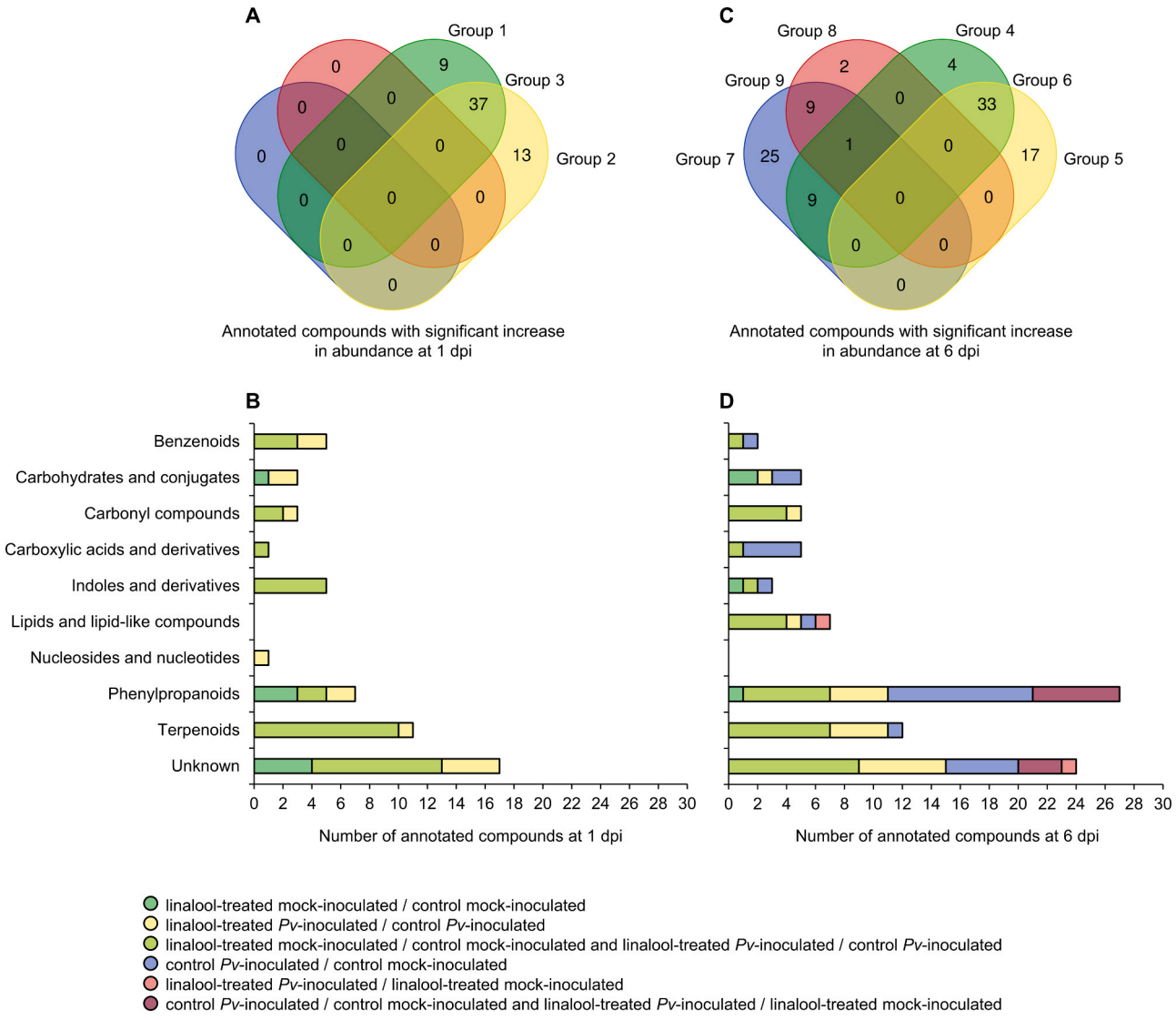

Fig. 4. Annotated compounds with significant increases in abundance. Grapevine leaf disks were treated with water (Control) or linalool at 10 mg L^{-1} of air volume in the dish chamber (Linalool), applied on a filter paper disk without contact with leaf tissues. Disks were inoculated with *Plasmopara viticola* (*Pv*-inoculated) or treated with water (Mock-inoculated) and analyzed at one and six days post inoculation (dpi). Venn diagrams summarize the distribution of annotated compounds with significant increases in abundance in at least one of the pairwise comparisons between linalool-treated mock-inoculated and control mock-inoculated samples (green), linalool-treated *Pv*-inoculated and control *Pv*-inoculated samples (yellow), control *Pv*-inoculated and control mock-inoculated samples (blue), linalool-treated *Pv*-inoculated and linalool-treated mock-inoculated (pink) samples at one (A) and six (C) days post inoculation (dpi). Numbers of annotated compounds with significant increases in abundance at 1 dpi (B) and 6 dpi (D) are reported for each chemical class according to the groups reported on the Venn diagrams, such as compounds affected only in the comparison between linalool-treated mock-inoculated and control mock-inoculated samples at 1 dpi (Group 1) or 6 dpi (Group 4); only in the comparison between linalool-treated *Pv*-inoculated and control *Pv*-inoculated samples at 1 dpi (Group 2) or 6 dpi (Group 5); in both comparisons (linalool-treated mock-inoculated and control mock-inoculated samples, as well as linalool-treated *Pv*-inoculated and control *Pv*-inoculated samples) at 1 dpi (Group 3) or 6 dpi (Group 6); only in the comparison between control *Pv*-inoculated and control mock-inoculated samples at 6 dpi (Group 7), only in the comparison between linalool-treated *Pv*-inoculated and linalool-treated mock-inoculated samples at 6 dpi (Group 8), or in both comparisons (control *Pv*-inoculated and control mock-inoculated samples, as well as linalool-treated *Pv*-inoculated and linalool-treated mock-inoculated samples) at 6 dpi (Group 9).

laricitrin, lilaline, malvidin 3-gentiotrioside, quercetin 3-(2''-galloyl-α-L-arabinopyranoside), and rhododendrin], and unknown compounds (Fig. 4B, Supplementary Fig. S6, and Supplementary Table S4). As expected, linalool (feature FTP0703) was mainly found in linalool-treated mock-inoculated and linalool-treated *P. viticola*-inoculated samples at 1 dpi (Supplementary Fig. S6, and Supplementary Table S4). At 6 dpi, the abundance of four, 17, and 33 annotated compounds increased in linalool-treated compared to control leaf disks in mock-inoculated samples (Group 4), in *P. viticola*-inoculated samples (Group 5), and in both inoculation conditions (Group 6), respectively (Fig. 4C). Annotated compounds affected by linalool at 6 dpi mainly belonged to putative phenylpropanoids (2-*O*-caffeoylglucarate, apigenin 4′-(2''-feruloylglucuronosyl)-(1->2)-glucuronide, aureusidin 6-*O*-beta-glucoside, gallocatechin-(4α->8)-epigallocatechin, kaempferol 5-glucoside, plantaginin, pseudobaptigenin 7-*O*-laminaribioside, rhododendrin, schisantherin A, and serpyllin), putative terpenoids [e.g., α-tocopheronic acid, α-tocopheronolactone, (-)-fusicoplagin A, (+)-isodihydrocarvone, bornanedione, citral, geranic acid, geraniol, jolkinol B, safranal, and valerosi datum], putative lipids and lipid-like compounds [e.g., 9-hydroxy-decanoic acid, 10-hydroxydecanoic acid, β-(acetylthio)estra-1,3,5(10)-trien-3-ol acetate, cyclic-3,20-bis(1,2-ethanediylacetal)− 11α-(acetyloxy)− 5α,6α-epoxypregnane-3,20-dione, and fisetin], and unknown compounds (Fig. 4D, Supplementary Fig. S7, and Supplementary Table S4).

Plasmopara viticola inoculation increased the abundance of 25

annotated compounds in control samples (Group 7), two annotated compounds in linalool-treated samples (Group 8), and nine annotated compounds in both conditions (Group 9; Fig. 4C). Annotated compounds affected by *P. viticola* mainly belonged to putative phenylpropanoids (e.g., 3′-hydroxyflavanone, abruquinone B, calopogoniumisoflavone B, canaliculatol, capillarin, *cis*-ε-viniferin, delphinidin 3-*O*-glucoside, leucadenone, methylophiopogonone, piceid, pinocembrin 7-*O*-benzoate, resveratrol, *trans*-δ-viniferin, *trans*-ε-viniferin, *trans*-ω-viniferin, and variabiloside) and unknown compounds (Fig. 4D, Supplementary Fig. S7 and Supplementary Table S4). Moreover, two compounds belonging to putative nucleosides and nucleotides were found at 1 dpi (adenosine 5′-diphosphate and thymidine 3′,5′-cyclic monophosphate), while no compounds belonging to this class were found at 6 dpi. The analysis of authentic reference standards confirmed the identification of 2-phenylethanol, geraniol, linalool, and linalyl oxide (Supplementary Fig. S8).

4. Discussion

Linalool is a ubiquitous VOC that can be responsible for plant-plant communication and plant resistance induction in model systems [22,23]. In grapevine, linalool is known to be emitted by downy mildew-resistant genotypes [26,28–31], but no information is available on its involvement in the defense mechanisms against *P. viticola*. Here, we showed that linalool treatment reduced downy mildew severity on leaf disks of a susceptible grapevine cultivar, indicating its possible application against *P. viticola* in the vineyard. Downy mildew is normally controlled by frequent use of chemical fungicides [76,77], and linalool has great potential to be further tested under field conditions to develop a sustainable product for downy mildew control. Callose deposition was found in linalool-treated leaf disks only after *P. viticola* inoculation at the sites of pathogen infection, but no differences were observed between control and linalool-treated leaf disks in mock-inoculated samples, indicating an enhanced defense reaction upon pathogen infection. The deposition of callose is a key defense process against downy mildew that can be stimulated by *Trichoderma* VOCs [39] and resistance inducers, such as β-aminobutiric acid [48], benzothiadiazole-7-carbothioic acid S-methyl ester [78], and sulfated laminarin PS3 [79]. In addition, linalool treatment induced the expression of six genes (*CHIT-3*, *HSR*, *LOX-9, OSM-2*, *PR-2*, and *PR-4*) and two genes (*HSR* and *OSM-2*) in mock-inoculated samples at 1 and 6 dpi, respectively. Moreover, the expression level of six genes (*CHIT-3*, *HSR*, *LOX*-9, *OSM-2*, *PR-2*, and *PR-4*) was higher in linalool-treated compared to control leaf disks also in *P. viticola*-inoculated samples at 1 dpi. These genes are markers of induced resistance (*CHIT-3*, *LOX*-9, *OSM-2*, *PR-2*, and *PR-4* genes) [39,40,46] or markers of defense processes related to hypersensitive response (*HSR* gene) [49], and their modulation in linalool-treated samples indicated the induction of grapevine resistance mechanisms against *P. viticola*. In particular, the expression levels of defense-related genes were higher in linalool-treated compared to control leaf disks in both mock-inoculated and *P. viticola*-inoculated samples, with stronger effects at 1 dpi than 6 dpi, suggesting an early activation of defense-related processes also in the absence of the pathogen. In particular, grapevine *PR-2* is a marker of the SA defense pathways [47], *PR-4* and *LOX-9* are markers of the JA defense pathways [48] and their upregulation in linalool-treated samples indicated the activation of multiple defense pathways. Similarly, linalool can induce defense mechanisms in Arabidopsis, rice, and tomato, with the involvement of both SA- and JA-mediated processes [34,36,37]. Other volatile terpenoids are known to induce transcriptional reprogramming in plants, such as pinene on SA-related genes in Arabidopsis [17], β-ionone on defense-related genes in barley [21], and β-cyclocitral on defense-related genes in rice [18] and tomato [19], indicating the VOC-mediated induction of plant resistance mechanisms. However, VOC effects can be tested against downy mildew only in the presence of host tissues, due to the obligate biotrophic lifestyle of *P. viticola*. Thus, possible inhibitory properties of linalool against *P. viticola* can not be excluded. In other pathosystems, linalool showed inhibitory activity against *B. cinerea* [32], *C. lindemuthianum* [11], *F. graminearum* [33], and *F. oxysporum f.* sp. *radicis-lycopersici* [34], indicating broad-spectrum efficacy of this volatile terpenoid against plant pathogens. In particular, linalool can be sequestered by epicuticular waxes, suggesting that it can partially persist on the leaf surface and exert direct inhibitory effects against microbial invaders [13].

The whole metabolome of grapevine leaf disks was affected by linalool treatment, indicating the activation of plant defense responses. Moreover, the metabolic content of grapevine leaves differed according to the time point, confirming that grapevine leaf metabolome is influenced by the developmental stage of downy mildew, as found in previous studies [28,29,31,43,45]. In particular, KEGG pathway analysis of annotated features with significant changes in abundance revealed a major reprogramming of amino acid, phenylpropanoid, and terpenoid metabolisms in response to linalool treatment and *P. viticola* inoculation. In the metabolomic data, linalool showed higher abundance in linalool-treated mock-inoculated and linalool-treated *P. viticola*-inoculated leaf disks compared to the respective control samples at 1 dpi, but not at 6 dpi, suggesting a possible degradation and/or metabolization of this VOC. Moreover, a linalool-derived compound (linalyl oxide) showed increased abundance in linalool-treated compared to control leaf disks in *P. viticola*-inoculated samples at 1 dpi, indicating a possible conversion of the applied linalool into linalyl oxide. Linalyl oxide derives from linalool oxidative metabolism mediated by the cytochrome P450 in Arabidopsis [80], suggesting that grapevine leaves could partially metabolize the exogenous linalool. However, further metabolomic analyses of plants treated with ^{13}C-labeled linalool are required to better understand the metabolic fate of the applied VOC.

Annotated compounds with increased abundance in linalool-treated compared to control leaf disks mainly belonged to putative phenylpropanoids, putative terpenoids, putative lipids and lipid-like compounds at 1 and 6 dpi. Phenylpropanoids [28,43,45], terpenoids [25–30], and lipids [31,81,82] have been classified as markers of grapevine resistance against grapevine downy mildew. Some linalool-induced terpenoids (e.g., α-terpineol, citral, and geraniol), benzenoids (e.g., 2-phenylethanol), and carbonyl compounds (e.g., 2,4-heptadienal) found in our study were previously detected in downy mildew-resistant grapevines [26,29], indicating that linalool can stimulate in a susceptible genotype the accumulation of compounds known to be implicated in the defense response of downy mildew-resistant genotypes. In particular, 2-phenylethanol, citral, and geraniol are known to accumulate in downy mildew-resistant grapevines upon *P. viticola* inoculation [26,29] and to display inhibitory activities against grapevine pathogens, such as *P. viticola* [26], *Pseudocercopora vitis*, and *Sphaceloma ampelinum* [83]. Likewise, some compounds found in linalool-treated samples showed antifungal activities against other pathogens, such as 2-phenylethanol against *Phytophthora infestans* [84], 2,4-heptadienal against *Magnaporthe oryza*e [85], citral and α-terpineol against *Geotrichum candidum var. citri-aurantii* [86], geraniol against *X. oryzae* pv. *oryzae* [87], citral against *Penicillium italicum* [88], *M. grisea* [89], and *Botryosphaeria dothidea* [90], indicating that linalool treatment can stimulate the accumulation of defense-related compounds in grapevine leaf disks. In agreement with our findings, α-farnesene, and β-ocimene treatment can alter the metabolite content of tea leaves, increasing the abundance of compounds belonging to benzenoids (e.g., methyl gallate and 2,3,4,5-tetrahydroxy-6-oxohexyl gallate) and carbohydrates and conjugates (e.g., 1,3,4,5,6,7-hexahydroxyheptan-2-one) [91]. Likewise, VOCs released from wounded barley plants increased the abundance of benzenoids (e.g., hordatines), lipids and lipid-like compounds (e.g., linolenic acid and linolenate-conjugated lipids) in receiver plants [9], suggesting overlapping metabolic responses to VOC treatments. Moreover, some compounds previously found in susceptible grapevine cultivars [92], such as putative epicatechin (feature number: FTN0482, FTN1675, FTP1341, FTP3881, and FTP4915), leucocyanidin

(FTP1341, FTP1478, and FTP1479), and caffeic acid (FTP0391 and FTP0392) derivatives, showed decreased abundance in linalool-treated compared to control leaf disks in *P. viticola*-inoculated samples, suggesting a possible disorder of susceptibility-related processes.

In agreement with previous findings [28,29,31,43,45,52], annotated compounds with increased abundance in *P. viticola*-inoculated compared to mock-inoculated leaf disks mainly belonged to putative phenylpropanoids (e.g., *cis*-ε-viniferin, piceid, resveratrol, *trans*--δ-viniferin, *trans*-ε-viniferin, and *trans*-ω-viniferin). Phenylpropanoids are known to be synthesized during grapevine response against *P. viticola* with specific profiles in downy mildew-susceptible and resistant genotypes, indicating the involvement of these compounds in grapevine defense mechanisms [31,43,93–95]. However, a large fraction of unknown compounds, uncharacterized compounds, and features not annotated by MassTrix 3 server was found in our study, indicating that further metabolomic and physiological studies are required to better identify the chemical formula and functional roles of features with increased abundance in linalool-treated and *P. viticola*-inoculated samples.

5. Conclusions

Linalool treatment reduced downy mildew severity by the activation of grapevine defense mechanisms, such as the deposition of callose and the upregulation of defense-related genes of SA and JA defense pathways. Linalool-induced resistance was associated with a reprogramming of the grapevine metabolome and with increases in the abundance of compounds possibly associated with plant defense, such as phenylpropanoids, terpenoids, lipids, benzenoids, and carbonyl compounds. Although further studies on formulation and efficacy under controlled and field conditions are required, the low toxicity of linalool in humans [96] indicates a great potential for this compound to be developed as a sustainable product for downy mildew control.

Funding

This research was funded by the University of Trento and Laimburg Research Centre (Project 'Understanding of grapevine communication mediated by volatile organic compounds for the resistance against downy mildew'). Laimburg Research Centre is funded by the Autonomous Province of Bozen-Bolzano.

CRediT authorship contribution statement

S.A. and V.L. carried out the experiments and qPCR analyses. S.A. carried out the microscopic analysis, metabolomic analysis, and metabolite annotations. S.A, V.L, P.P., M.O., and M.P. contributed to data interpretation. M.P. and P.P. conceived the study, designed the experiment, and coordinated all research activities. S.A and M.P. wrote the manuscript. All the authors revised and approved the final manuscript.

Declaration of Competing Interest

The authors declare no competing interests.

Data Availability

All data are reported in the manuscript.

Acknowledgments

We thank Pietro Franceschi (Fondazione Edmund Mach) for the in-house R script for peak picking and alignment, Tobia Minuzzo and Alberto Zanini for their contribution in qPCR experiments.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2023.100298.

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