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Trichoderma spp. volatile organic compounds protect grapevine plants by activating defense-related processes against downy mildew

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Abstract

Volatile organic compounds (VOCs) are produced by soil-borne microorganisms and play crucial roles in fungal interactions with plants and phytopathogens. Although VOCs have been characterized in *Trichoderma* spp., the mechanisms against phytopathogens strongly differ according to the strain and pathosystem. This study aimed at characterizing VOCs produced by three *Trichoderma* strains used as biofungicides and to investigate their effects against grapevine downy mildew (caused by *Plasmopara viticola*). A VOC-mediated reduction of downy mildew severity was found in leaf disks treated with *Trichoderma asperellum* T34 (T34), *T. harzianum* T39 (T39), and *T. atroviride* SC1 (SC1) and 31 compounds were detected by head space-solid phase microextraction gas chromatography–mass spectrometry. Among the *Trichoderma* VOCs annotated, α-farnesene, cadinene, 1,3-octadiene, 2-pentylfuran, and 6-pentyl-2H-pyran-2-one reduced downy mildew severity on grapevine leaf disks. In particular, 6-pentyl-2H-pyran-2-one and 2-pentylfuran increased the accumulation of callose and enhanced the modulation of defense-related genes after *P. viticola* inoculation, indicating an induction of grapevine defense mechanisms. Moreover, 6-pentyl-2H-pyran-2-one activated the hypersensitive response after *P. viticola* inoculation, possibly to reinforce the grapevine defense reaction. These results indicate that *Trichoderma* VOCs can induce grapevine resistance, and these molecules could be further applied to control grapevine downy mildew.

1 | INTRODUCTION

Volatile organic compounds (VOCs) are small molecules with low molecular mass (100–500 Da), high vapor pressure, low boiling point, and a lipophilic character that readily evaporate and diffuse through heterogeneous mixtures of solids, liquids, and gasses, such as the gas- and water-filled pores of the soil (Effmert et al., 2012; Schmidt et al., 2015). VOCs are produced by a large variety of organisms and play crucial roles in the communication among individuals of the same species and life forms of different kingdoms (Werner et al., 2016). In particular, soil-borne microorganisms are prolific producers of VOCs, which play important roles in plant growth and defense against biotic or abiotic stress (Garbeva & Weisskopf, 2020; Li et al., 2016). Some VOCs produced by soil-borne fungi can protect plants by direct

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growth inhibition of phytopathogens (Strobel et al., 2001) or by induction of plant resistance (Li et al., 2016; Werner et al., 2016). Plant resistance can be induced by diverse beneficial microbes, including plant growth-promoting rhizobacteria and soil-borne fungi (e.g., *Trichoderma* spp.), and it is generally associated with an enhanced defense reaction after pathogen inoculation, such as the deposition of callose-rich papillae at the sites of pathogen infection (Segarra et al., 2009). This phenomenon, also known as priming effect, consists of a cost-effective defense mechanism against pathogens, and its targeted activation was proposed as a promising tool for crop protection (Conrath et al., 2015; Martínez-Medina et al., 2016).

Trichoderma spp. are among the most widespread soil microorganisms and have been widely used as biocontrol agents against numerous phytopathogens (Harman et al., 2004). Biocontrol mechanisms of *Trichoderma* spp. are based on induction of plant resistance, production of antimicrobial substances, lytic enzymes, and competition with other microorganisms for nutrients and/or space (Lorito et al., 2010). Moreover, *Trichoderma* spp. are known to produce VOCs (Crutcher et al., 2013; Guo et al., 2020; Hung et al., 2013; Wheatley et al., 1997) that play crucial roles in the inter-kingdom communications and in the biocontrol mechanisms (Guo et al., 2019; Malmierca et al., 2015; Zhang et al., 2014). For example, VOCs produced by *T. asperellum* T1 induced lettuce resistance against leaf spot fungal pathogens (*Corynespora cassiicola* and *Curvularia aeria*) and increased the activity of the cell-wall degrading enzymes (Wonglom et al., 2020). Likewise, VOCs produced by *T. asperellum* T34 and *T. harzianum* T78 primed *Arabidopsis thaliana* and tomato plants for enhanced expression of jasmonic acid (JA)-dependent defense reactions against *Botrytis cinerea* (Martínez-Medina et al., 2017). In particular, 6-pentyl-2H-pyran-2-one was produced by *T. atroviride* P1 and reduced the disease severity on tomato and canola seedlings inoculated with *B. cinerea* and *Leptosphaeria maculans*, respectively (Vinale et al., 2008). The same compound was found in *T. asperellum* IsmT5 and induced resistance against *B. cinerea* and *Alternaria brassicicola* in *A. thaliana* (Kottb et al., 2015). Moreover, VOCs of *T. virens* strains (Tv29.8, Tv10.4, and *Δppt1-1* mutant) included a large number of terpenes (e.g., β-caryophyllene, β-elemene, germacrene D, τ-cadinene, α-amorphene, δ-cadinene, and τ-selinene) and they induced *A. thaliana* resistance against *B. cinerea* (Contreras-Cornejo et al., 2014). Some *Trichoderma* VOCs also showed direct inhibitory effects against phytopathogens (Scarselletti & Faull, 1994; Wonglom et al., 2020; Zhang et al., 2014). For example, 6-pentyl-2H-pyran-2-one (also known as 6-pentyl-α-pyrone produced by *T. harzianum* IMI 288012, *T. harzianum* T23 and *T. atroviride* IMI 206040) inhibited the growth of *Fusarium moniliforme* (El-Hasan et al., 2007) and *Rhizoctonia solani* (Cruz-Magalhães et al., 2019; Scarselletti & Faull, 1994), suggesting broad-spectrum activities and multiple modes of action of *Trichoderma* VOCs against phytopathogens. Likewise, VOCs produced by *T. harzianum* T-E5 inhibited *F. oxysporum* growth, including lignocerane, nerolidol, and verticillol as the most abundant compounds (Zhang et al., 2014), suggesting that *Trichoderma* VOCs have great potential for controlling phytopathogens.

Three *Trichoderma* strains are well documented for their ability to control a broad spectrum of phytopathogens and are used as biofungicides, such as *T. asperellum* T34 (T34), *T. atroviride* SC1 (SC1), *T. harzianum* T39 (T39) (Cotxarrera et al., 2002; Elad et al., 1997; Pertot et al., 2008). In particular, T34 is known for the induction of systemic resistance in cucumber against *Pseudomonas syringae* pv. *lachrymans* (Segarra et al., 2007) and in *A. thaliana* against *Hyaloperonospora arabidopsidis* (Segarra et al., 2009) and *B. cinerea* (Martínez-Medina et al., 2017). SC1 antagonized a broad range of grapevine (*Vitis vinifera*) root (e.g., *Armillaria mellea*) and shoot (e.g., *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum*) pathogens (Longa et al., 2008; Pellegrini et al., 2014; Pertot et al., 2016), while T39 induced systemic resistance against *Plasmopara viticola* by the modulation of defense-related genes and resistance processes (Banani et al., 2014; Perazzolli et al., 2011; Perazzolli et al., 2012). T39-induced resistance was characterized by an enhanced accumulation of callose and reactive oxygen species (ROS) after *P. viticola* infection (Palmieri et al., 2012), and ROS are considered as key signals for hypersensitive response (HR) activation (Kortekamp & Zyprian, 2003). *Plasmopara viticola* is one of the most important phytopathogenic oomycetes (Kamoun et al., 2015) and the causal agent of grapevine downy mildew (Gessler et al., 2011). *Plasmopara viticola* is normally controlled by frequent applications of chemical fungicides (Buonassisi et al., 2017; Gessler et al., 2011), but more sustainable control strategies are needed because of negative impact of pesticides on human health and the environment, emerging pesticide resistance and stricter rules on levels of pesticide residues in agricultural products (Spring et al., 2018). Several studies have highlighted the importance of *Trichoderma* spp. as an alternative to chemical fungicides against grapevine pathogens (Zanotto & Morroni, 2016), but no information is available on the possible biocontrol mechanism mediated by *Trichoderma* VOCs against downy mildew. The aim of this study was to identify and annotate VOCs produced by T34, T39, and SC1 using head space-solid phase microextraction gas chromatography–mass spectrometry (HS-SPME/GC-MS) analysis and to investigate their effects against *P. viticola*. The final goal was to better understand the contribution of *Trichoderma* VOCs to limit downy mildew infection and to provide more information on the VOC-mediated effects of *Trichoderma* spp. against phytopathogens.

2 | MATERIALS AND METHODS

2.1 | Biological material and growth conditions

Vitis vinifera cultivar Pinot Noir (downy mildew-susceptible) and *V. riparia* (downy mildew-resistant) plants were grown in 2.5 L-pots under greenhouse conditions at 25 ± 1°C with a photoperiod of 16 h light and relative humidity (RH) of 70 ± 10% as previously described (Perazzolli et al., 2012).

A *P. viticola* population was collected from an untreated vineyard in the Trentino region (northern Italy) and maintained by subsequent inoculations on Pinot Noir plants under greenhouse conditions, as described by Perazzolli et al. (2012). To obtain the *P. viticola* inoculum,

plants with disease symptoms were incubated overnight in the dark at 95 ± 5% RH, and *P. viticola* sporangia were collected by washing the abaxial leaf surfaces, bearing sporulating lesions, with cold distilled water. The inoculum concentration was then adjusted to 2.5×10^5 sporangia ml^{-1} with a hemocytometer under a light microscope (LMD7000, Leica Microsystems).

The *Trichoderma* strains, T34 (Cotxarrera et al., 2002), T39 (Elad et al., 1997), and SC1 (Pertot et al., 2008), were grown on potato dextrose agar (PDA; Oxoid) for seven days in the dark at 25 ± 1°C. Conidia were scraped gently from the colony surface of each strain with a sterile loop and collected in a sterile 2 ml-tube containing 1 ml of cold (4°C) sterile distilled water. The concentration of the conidial suspension was adjusted to 1×10^7 conidia ml^{-1} for the assessment of direct effects against downy on grapevine plants and to 1×10^4 conidia ml^{-1} for the headspace (HS) VOC analysis, assessment of VOC-mediated effects and callose deposition assay, by counting with a hemocytometer under the light microscope (LMD7000 microscope, Leica Microsystems) (Banani et al., 2014).

2.2 | Assessment of direct effects of *Trichoderma* strains against downy mildew on grapevine plants

Leaves of downy mildew-susceptible plants grown under greenhouse conditions (25 ± 1°C with a photoperiod of 16 h light and 70 ± 10% RH) were sprayed with the conidial suspension of the respective *Trichoderma* strain (1×10^7 conidia ml^{-1}) or treated with water (Control). The abaxial and adaxial surfaces of all leaves were treated three times (1, 2, and 3 days before pathogen inoculation) using a compressed air hand sprayer (20–30 ml for each plant) in order to maximize the *Trichoderma* effects against *P. viticola* (Perazzolli et al., 2008). One day after the last treatment, all leaves of each plant were inoculated with the *P. viticola* suspension (2.5×10^5 sporangia ml^{-1}) using a compressed-air hand sprayer as previously described (Perazzolli et al., 2012), and plants were incubated overnight in the dark at 25 ± 1°C with 95 ± 5% RH to allow *P. viticola* infection (Perazzolli et al., 2012). Inoculated plants were maintained under greenhouse conditions (25 ± 1°C with a photoperiod of 16 h light and 70 ± 10% RH) to allow pathogen development (Perazzolli et al., 2012). Six days post inoculation (6 dpi), plants were incubated overnight in the dark at 25 ± 1°C with 95 ± 5% RH to promote downy mildew sporulation (Perazzolli et al., 2012), and the disease severity of each leaf was assessed visually as the percentage of abaxial leaf area covered by *P. viticola* sporulation, according to the standard guidelines of the European and Mediterranean Plant Protection Organization (EPPO, 2001). The disease severity of each replicate (plant) was calculated as the average of the disease severity of all leaves (Perazzolli et al., 2008). The disease reduction (efficacy) was calculated for each replicate (plant) according to the following formula: (disease severity of control plants—disease severity of *Trichoderma*-treated plants)/disease severity of control plants × 100. Five replicates (plants) were used for each treatment, and the experiment was carried out twice.

2.3 | Head space analysis of volatile organic compounds from *Trichoderma* spp.

For the HS VOC analysis, 5 ml PDA were poured into sterile 20 ml-HS vials (Supelco, Merck), and they were left open in a slanted position under a laminar flow for 2 h at room temperature to avoid condensation (Lazazzara et al., 2017). Each HS vial was inoculated with 20 μl of the conidial suspension of the respective *Trichoderma* strain (1×10^4 conidia ml^{-1}) and left to dry under a laminar flow for 1 h at room temperature (*Trichoderma*-inoculated). Each HS vial was closed with a sterile cotton plug and the rubber strap for aerobic cultivation of *Trichoderma* spp. without oxygen limitation (Stoppacher et al., 2010), and it was incubated at 25 ± 1°C in the dark for 48 or 72 h, as described by Crutcher et al. (2013). The volume (20 μl) and concentration (1×10^4 conidia ml^{-1}) of the conidial suspension were optimized in a preliminary trial in order to allow the complete colonization of the PDA surface of the HS vial by the *Trichoderma* mycelium in 48 h at 25 ± 1°C (data not shown). The time point of 48 h was selected since the VOC production of *Trichoderma* spp. was maximum (Crutcher et al., 2013; Stoppacher et al., 2010). Two independent experiments were carried out (named first and second experiment hereafter), three and five biological replicates were analyzed for each *Trichoderma* strain and time point in the first and second experiment, respectively, due to space limitation in the auto-sampler of the first experiment. For each experiment, three additional HS vials containing non-inoculated PDA (Uninoculated) were used as controls in order to exclude VOCs released from the culture medium in the absence of *Trichoderma* spp. (Kluger et al., 2013).

VOCs produced by *Trichoderma* strains were measured using HS-SPME/GC–MS analysis according to Crutcher et al. (2013). After 48 or 72 h cultivation, each HS vial was purged with synthetic air filtered through a 0.2 μm-politetrafluoroetilene (PTFE) Midisat BV membrane filter (Sartorius) for 30 s, sealed with a sterilized 18 mm-screw metal cap assembled with a 1.3 mm-silicone/PTFE septum (Supelco, Merck) and incubated for 6 h at 25 ± 1°C to accumulate VOCs before analysis (Crutcher et al., 2013, Stoppacher et al., 2010). Each HS vial was then placed in an auto-sampler (MPS2XL, Gerstel) and equilibrated for 15 min at 30°C. For VOC extraction, a polydimethylsiloxane/divinylbenzene fiber (PDMS/DVB 65 μm; Supelco, Merck) was inserted into the HS vial for 30 min at 30°C (Crutcher et al., 2013). The fiber was transferred to the Agilent 6890 N gas chromatograph coupled to a quadrupole mass spectrometer 5975B Mass Selective Detector (MSD; Agilent Technologies), and analytes were desorbed in splitless mode at 250°C for 2 min. A non-polar HP-5MS column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies) was operated at a constant flow of helium (1 ml min^{-1}). The oven temperature consisted of 40°C (hold 2 min), 10°C min^{-1} to 200°C, 25°C min^{-1} to 260°C (hold 5 min) and the transfer line was set at 270°C. Analytes were ionized at 70 eV in the ion source at 230°C and detected in full scan (45–400 amu). Mixed alkane standard solutions for retention index (RI) calibration was included in the sample list to facilitate reliable compound annotation, and three SPME conditions were applied to obtain good peak shapes: (1) 1 μl alkane standard solution from C_5 to C_{10}, with 0.01 min at 90°C for both equilibration and

extraction steps, (2) 10 μl alkane standard solution from C_8 to C_{20}, with 5 min equilibration and 45 min extraction both at 90°C and (3) 40 μl alkane standard solution from C_{21} to C_{40}, with 30 min equilibration and 60 min extraction both at 120°C (Kluger et al., 2013).

Raw data were acquired with an Agilent MSD ChemStation (G1701EA E.02.00.493, Agilent Technologies), and the abundance of each VOC was calculated as the integrated peak area, expressed as counts per scan (cps), using the MetaboliteDetector software (version 3.020151231 Ra-Linux) (Hiller et al., 2009). The mass deconvolution settings were as follows: peak threshold of 5, the minimum peak height of 5, deconvolution width (scans) of 1 and the required number of peaks set at 5. For compound annotation, deconvoluted mass spectra were compared with the NIST14 database (National Institute of Standards and Technology, http://www.nist.gov/) and with an in-house library obtained with authentic reference standards (Crutcher et al., 2013). Compound annotation was achieved imposing a relative deviation of RI value lower than 2% from the reference value published in the literature (http://www.nist.gov/) and according to the highest mass spectrum similarity score, which was set higher than 70% after first successful annotation, in order to include low-abundant substances or substances where the deconvolution process did not lead to a complete elimination of interfering mass signals (Weingart et al., 2012). Chromatograms of not annotated compounds were searched for different types of terpenes using extracted ion current (EIC) chromatograms at a mass/charge ratio (*m/z*) 136 for monoterpenes, *m/z* 202 and *m/z* 204 for sesquiterpenes, and *m/z* 272 for diterpenes (Crutcher et al., 2013). The deconvoluted mass spectra underneath these EIC peaks were inspected manually, and, in the case of typical terpene mass spectrum, the corresponding mass spectra and RI values were included in the data matrix and named as "unknown sesquiterpene" or "unknown diterpene." Deconvoluted mass spectra different from that of terpenes were named "unknown compound" according to their mass spectra and RI values. VOCs were included in the data matrix only if their signal-to-noise ratio (S/N) was greater than 10 (Bu et al., 2016) for at least one time point and *Trichoderma* strain.

2.4 | Standard solutions and pure volatile organic compounds

Alkane standard solutions from C_8 to C_{20} (40 mg L^{-1} each in n-hexane) and C_{21} to C_{40} (40 mg L^{-1} each in toluene) were purchased from Sigma-Aldrich (Merck). A standard solution from C_5 to C_{10} was prepared using pure substances in a ratio resulting in narrow and symmetric peak shapes as described by Weingart et al. (2012). VOCs were selected according to HS-SPME/GC–MS results and pure VOCs were used in functional assays, such as 1,3-octadiene (98%) purchased from ChemSampCo (Dallas, TX, USA); 2-pentylfuran (98%) and 6-pentyl-2H-pyran-2-one (96%) purchased from Sigma-Aldrich (Merck); cadinene (85%, corresponding to a mixture of isomers, such as γ-cadinene, γ-muurolene and δ-cadinene) purchased from BOC Sciences (Shirley), and α-farnesene (corresponding to a mixture isomers, such as (Z,E)-α-farnesene, (E,Z)-α-farnesene, (E,E)-α-farnesene and (Z,Z)-α-farnesene) purchased from SAFC Supply Solutions.

2.5 | Assessment of volatile-mediated effects of *Trichoderma* strains and pure compounds against grapevine downy mildew

Leaf disks (18 mm diameter) were obtained from the greenhouse-grown grapevine plants (from the fourth to the sixth node of downy mildew-susceptible plants) with a cork borer, and they were placed onto two layers of wet sterilized filter paper in Petri dishes (90 mm diameter; five disks for each dish), with the abaxial surface uppermost as previously described (Lazazzara et al., 2018).

To analyze VOC-mediated effects of *Trichoderma* spp. on *P. viticola*, 15 ml PDA was poured into Petri dishes (90 mm diameter), inoculated with 20 μl of the conidial suspension of the respective *Trichoderma* strain (1×10^4 conidia ml^{-1}) and left to dry under a laminar flow for 1 h at room temperature. As control, dishes containing uninoculated PDA were used. The lid of each dish containing grapevine leaf disks was removed, and the dish was covered with the bottom of a *Trichoderma*-inoculated or uninoculated dish (dish sandwich; Figure S1A). The dish sandwich was sealed with Parafilm (Bemis) and incubated at 25 ± 1°C in the dark for 48 h without physical contact with leaf tissues. Each leaf disk was inoculated with five drops (5 μl each) of a *P. viticola* suspension (2.5×10^5 sporangia ml^{-1}; *P. viticola*-inoculated), the dish sandwich was assembled with respective *Trichoderma*-inoculated, or uninoculated, dish and sealed with Parafilm (Bemis). Dish sandwiches were incubated in the dark at 25 ± 1°C overnight, leaf disks were dried under a laminar hood, covered with a dish lid and incubated for 6 days under greenhouse conditions.

In order to verify that leaf disks were not contaminated by *Trichoderma* spp. conidia, control dish sandwiches were prepared. Briefly, the lid of each dish containing 15 ml PDA was removed, and the dish was covered with the bottom of a *Trichoderma*-inoculated dish. The dish sandwich was sealed with Parafilm (Bemis) and incubated at 25 ± 1°C for 72 h. Each *Trichoderma*-inoculated dish was replaced with a dish lid and the absence of *Trichoderma* spp. growth on PDA was verified 6 days after incubation at 25 ± 1°C under greenhouse conditions.

Functional assays of pure VOCs against *P. viticola* were carried out according to Lazazzara et al. (2018). Briefly, each pure VOC was diluted 10-fold in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Merck) and serially diluted in distilled water to obtain the appropriate concentration for each treatment. The respective pure VOC was applied to a filter paper disk (Whatman, Merck) fixed on the lid (without physical contact with the leaf tissues) of a dish containing grapevine leaf disks (Figure S1B). Each VOC was applied at a concentration of 2.5, 5, 10, 15, 20, or 50 mg L^{-1} in air volume (VOC-treated leaf disks), assuming the complete VOC evaporation from the filter paper, and DMSO was applied as control (0 mg L^{-1} in air volume of VOCs; Control leaf disks). Moreover, two blends of the two most efficient VOCs (2-pentylfuran

and 6-pentyl-2H-pyran-2-one) were tested at a concentration of 5 or 10 mg L^{-1} in air volume for each compound. Dishes were sealed with Parafilm (Bemis) and incubated in the dark at 25 ± 1°C under greenhouse conditions for 24 h. Each leaf disk was inoculated with five drops (5 μl each) of a *P. viticola* suspension (2.5×10^5 sporangia ml^{-1}; *P. viticola*-inoculated) or with five drops (5 μl each) of distilled water (mock-inoculated), the respective pure VOC, or VOC blend, was applied again to the filter paper disk in the appropriate concentration. Dishes were sealed with Parafilm (Bemis) and incubated in the dark at 25 ± 1°C overnight. Leaf disks were dried under a laminar hood and dishes were incubated for 6 days under greenhouse conditions.

The downy mildew disease severity was assessed on each leaf disk at 6 dpi as a percentage of the leaf disk surface covered by sporulation (EPPO, 2001), calculated as the sum of the five inoculum drops: 0%, no sporulation; 10%, scarce sporulation; 20%, dense sporulation (Lazazzara et al., 2018). The presence of phytotoxic effect was assessed visually by checking for discolouration, chlorosis, and whitening of leaf disks (EPPO, 2014). The disease severity of each replicate (dishes with five disks each) was calculated as an average of the disease severity of five leaf disks (Lazazzara et al., 2018), five replicates were analyzed for each treatment and the experiment (i.e., VOC-mediated *Trichoderma* effects and functional assay of pure VOCs) was carried out twice.

2.6 | Visualization of callose deposition and *Plasmopara viticola* structures by aniline blue staining

VOC-treated and control leaf disks were collected before inoculation (0 dpi) and at 1, 2, and 6 dpi with *P. viticola*. Samples were stained with aniline blue as reported by Lazazzara et al. (2018) to visualize *P. viticola* structures and callose deposition (Palmieri et al., 2012). 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 LMD7000 microscope (Leica Microsystem) using an A4 filter (BP 360–400 nm excitation, 400 nm dichroic mirror, and 470–400 nm emission). As control of callose deposition (Palmieri et al., 2012), leaf disks were sprayed with T39 conidia (1×10^4 conidia ml^{-1}) and inoculated with *P. viticola* (2.5×10^5 sporangia ml^{-1}) as described above. Five leaf disks were analyzed for each treatment and time point and the experiment was carried out twice.

2.7 | Visualization of grapevine hypersensitive response by lactophenol-trypan blue staining

VOC-treated and control leaf disks were collected before inoculation (0 dpi) and at 1 and 6 dpi with *P. viticola*. Samples were stained with lactophenol-trypan blue as reported by Roetschi et al. (2001) to visualize *P. viticola* structures and dead plant cells of HR (Keogh et al., 1980). VOC-treated and control leaf disks were mock-inoculated with water as an additional control, in order to verify the absence of cell death. For the staining, leaf disks were incubated in lactophenol-trypan blue at 100°C for 2 min and washed with 2.5 g ml^{-1} chloral hydrate for 24 h. Leaf disks were transferred to microscope slides and observed using a light microscope (LMD7000 microscope, Leica Microsystem). As control of the HR activation (Brilli et al., 2018), leaf disk of the downy mildew-resistant grapevine (*V. riparia*) were inoculated with *P. viticola* (2.5×10^5 sporangia ml^{-1}) as described above. Five leaf disks were analyzed for each treatment and time point and the experiment was carried out twice.

2.8 | RNA extraction and gene expression analysis

Plasmopara viticola-inoculated and mock-inoculated leaf disks (25 mm diameter) were collected at 6 dpi from VOC-treated and control samples. This time point was chosen to maximize the grapevine defense reaction (Malacarne et al., 2011; Vrhovsek et al., 2012), according to the lactophenol-trypan blue staining results. Leaf disks were reduced to 18 mm in diameter using a cork borer, in order to eliminate the outlying area where defense responses to injury can occur (Adrian et al., 2017). Samples were immediately frozen in liquid nitrogen, stored at −80°C and crushed using a mixer mill disruptor (MM200, Retsch) at 25 Hz for 45 s with sterile steel jars and beads refrigerated in liquid-N_2. Total RNA was extracted from 100 mg of ground sample 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 first strand cDNA was synthesized from 0.5 μg of total RNA using Superscript III (Invitrogen, Thermo Fisher Scientific) and oligo-dT primer. Genes encoding pathogenesis-related protein 2 (*PR2*), osmotin 1 (*OSM1*), osmotin 2 (*OSM2*), and chitinase 3 (*CHIT3*) were used as markers of grapevine induced resistance (Banani et al., 2014; Perazzolli et al., 2011, 2012) and the HR-related gene (*HSR*) was selected as a marker of grapevine cell death (Lakkis et al., 2019) (Table S1). Quantitative real-time PCR (qPCR) reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific) and specific primers (Table S1) using the Light Cycler 480 (Roche Diagnostics) as previously described (Perazzolli et al., 2012). Briefly, the PCR conditions were: 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 LinReg software version 11.1 was used to calculate reaction efficiencies for each primer pair (Ruijter et al., 2009). The relative expression level (fold-change) of each gene was calculated according to the Pfaffl equation (Pfaffl, 2001), using mock-inoculated control leaf disks as the calibrator. The grapevine *actin* and *VATP16* were used as housekeeping genes for data normalization, because their expression was not affected by *P. viticola* inoculation (Perazzolli et al., 2012; Polesani et al., 2010). Three replicates (dishes with five leaf disks each) were

analyzed for each treatment and the gene expression profiles were confirmed in an independent experimental repetition.

2.9 | Statistical analysis

VOC data were processed using an in-house R-script (R version 3.1.0). Each experimental repetition was analyzed separately, the Kruskal-Wallis test ($P \leq 0.05$) and a fold-change in VOC abundance higher than 1.5 were set as criteria to identify VOCs with significant changes in abundance among the three *Trichoderma* strains for each time point. For each experiment, only VOCs which showed higher mean abundance in *Trichoderma*-inoculated compared to uninoculated HS vials for at least one strain and time point were considered, according to the Kruskal-Wallis test ($P \leq 0.05$) with a fold-change higher than 1.5.

Each experiment was carried out twice and disease severity data were analyzed using the Statistica 13.3 software (TIBCO Software Inc.). Each experimental repetition was analyzed separately and a Kruskal-Wallis test was used to demonstrate equivalent results in the two experiments ($P > 0.05$, non-significant differences between experimental repetitions). Data from the two experimental repetitions were pooled and a Kruskal-Wallis test was used to detect significant differences among treatments ($P \leq 0.05$). Fold change values of the gene expression analysis were transformed using the equation $y = \text{Log}_{10}$ (fold change +1) (Casagrande et al., 2011) and the analysis of variance (ANOVA) with the Fisher's test ($P \leq 0.05$) was carried out to detect significant differences among treatments after validation of normal distribution (Kolmogorov–Smirnov test, $P > 0.05$) and variance homogeneity (Levene's test, $P > 0.05$) of the data.

3 | RESULTS

3.1 | Effect and annotation of *Trichoderma* spp. volatile organic compounds

Treatments with T34, SC1 or T39 conidia reduced downy mildew severity on susceptible grapevine plants compared to control plants (Figure 1A) with an efficacy of 72.1 ± 5.8%, 65.9 ± 9.2%, and 71.1 ± 4.4%, respectively (mean ± standard error values). Moreover, a VOC-mediated reduction of downy mildew severity was found on leaf disks treated with T34, SC1, or T39 colonies in a dish sandwich without contact with the leaf tissues (Figure S1A), with an efficacy of 43.1 ± 4.9%, 46.2 ± 7.2%, and 31.6 ± 4.2%, respectively (Figure 1B). No *Trichoderma* colonies developed on control dish sandwich assembled with a dish containing sterilized PDA covered with the bottom of a *Trichoderma*-inoculated dish (data not shown), confirming that no conidia dropped down and that only VOCs produced by *Trichoderma* spp. were involved in the reduction of downy mildew severity on leaf disks.

In order to characterize VOCs produced by the three *Trichoderma* strains, the HS-SPME/GC–MS analysis was carried out at 48 or 72 h after incubation on PDA and a total of 26 and 21 VOCs were found in the first and second experiment, respectively (Figure 2; Figure S1B; Tables S2 and S3). Annotated *Trichoderma* VOCs include alkenes (e.g., 1,3-octadiene), furanes (e.g., 2-pentylfuran and 2-n-heptylfuran), ketones (e.g., 3-octanone and 2-undecanone), pyrones (lactones, e.g., 6-pentyl-2H-pyran-2-one), and terpenes, such as monoterpenes (α-phellandrene, α-terpinene, limonene, γ-terpinene, and β-phellandrene) and sesquiterpenes ([Z,E]-α-farnesene, γ-cadinene, γ-muurolene, α-curcumene, α-zingiberene, trans-β-farnesene, germacrene A, β-sesquiphellandrene, β-himachalene, β-bisabolene, and δ-cadinene). Moreover, nine VOCs were

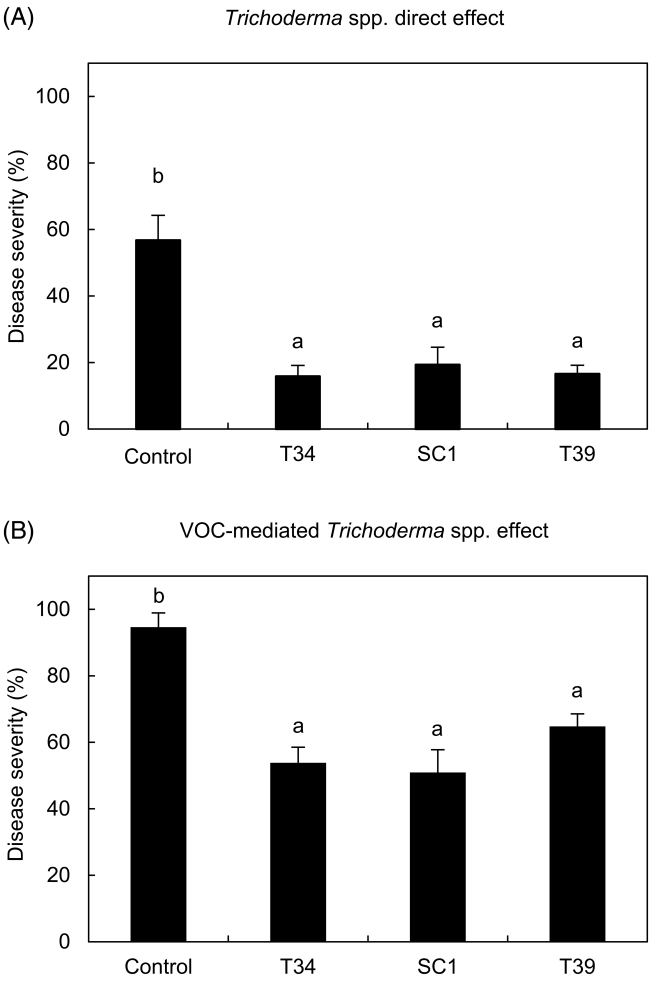

FIGURE 1 Effects of *Trichoderma* strains against downy mildew. Direct effects of *Trichoderma* strains (A) were assessed on greenhouse-grown grapevine plants, sprayed with water (control) and with a conidial suspension of *T. asperellum* T34 (T34), *T. atroviride* SC1 (SC1) or *T. harzianum* T39 (T39). Volatile-mediated effects of *Trichoderma* strains (B) were assessed on grapevine leaf disks, treated with uninoculated potato dextrose agar (PDA) dishes (control) and dishes with PDA-grown T34, SC1 or T39 colonies without contact with leaf tissues. Downy mildew severity was assessed at 6 days post inoculation. Five replicates were assessed for each treatment and each experiment was carried out twice. The Kruskal-Wallis test indicated no significant differences between the two experimental repetitions ($P > 0.05$) and data from the two experiments were pooled. The pooled mean and standard error values of 10 replicates from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments according to the Kruskal-Wallis test ($P \leq 0.05$)

Group	VOC	48h						72h					
		Exp. 1			Exp. 2			Exp. 1			Exp. 2		
		T34	SC1	T39	T34	SC1	T39	T34	SC1	T39	T34	SC1	T39
1	(Z,E)-α-farnesene	b	a	b	b	a	b	a	a	b	a	a	b
	γ-cadinene	a	a	b	a	a	b	a	a	b	a	a	b
	1,3-octadiene							b	a	b	b	a	b
	2-pentylfuran							a	a	b	a	a	b
	unknown diterpene 1							a	a	b	a	a	b
2	γ-muurolene	b	a	b	b	a	b	a	a	b	b	a	c
	unknown sesquiterpene 2	a	a	b	a	a	b	b	a	a	a	a	b
	6-pentyl-2H-pyran-2-one	a	b	a				a	b	b	a	b	b
	α-curcumene	b	a	b	b	a	c	a	a	b	a	a	b
	α-phellandrene	a	b	b	a	a	b	a	a	b	a	a	b
	α-zingiberene	b	a	b	b	a	c	b	a	c	b	a	c
	unknown compound 1				a	a	b	a	a	b	a	a	b
	unknown diterpene 2	b	a	a	a	a	b	a	a	b	a	a	b
	unknown sesquiterpene 1				a	a	b	a	a	b	a	a	b
3	trans-ß-farnesene							a	b	a	a	a	b
	unknown diterpene 3	a	b	a	a	a	b	a	a	b	a	b	c
4	2-n-heptylfuran							a	a	b			
	2-undecanone							a	a	b			
	3-octanone	a	b	c				a	a	b			
	α-terpinene	a	a	b									
	ß-sesquiphellandrene	b	a	a				a	a	b			
	γ-terpinene							a	a	a			
	germacrene A	b	a	b									
	limonene	a	a	b				a	a	a			
	unknown compound 2	a	b	a									
	unknown compound 3	b	a	b				a	a	a			
5	ß-bisabolene				a	a	a				a	a	b
	ß-himachalene				b	a	c				a	a	b
	ß-phellandrene				a	a	b				a	a	b
	δ-cadinene										a	a	b
	unknown sesquiterpene 3										a	a	b

FIGURE 2 Volatile organic compounds (VOCs) produced by *Trichoderma* strains. VOCs analysis was carried out using head space-solid phase microextraction gas chromatography–mass spectrometry (HS-SPME/GC-MS) for *T. asperellum* T34 (T34), *T. atroviride* SC1 (SC1) and *T. harzianum* T39 (T39) grown for 48 h or 72 h on potato dextrose agar (PDA) in two independent experiments (Exp. 1 and Exp. 2). Different letters and color gradients indicate significant differences in VOC abundance among the *Trichoderma* strains for each experiment and time point, according to the Kruskal-Wallis test ($P \leq 0.05$) with a fold change higher than 1.5 (Tables S2 and S3). The letter 'a' was assigned to low VOC abundance (light green cell) or VOC abundance below the limit of detection (white cell). Five metabolite groups were identified according to changes in abundance among the three *Trichoderma* strains: Consistent changes in the two experiments at both time points (group 1) or at one time point (group 2); different changes in the two experiments (group 3); detection in the first (group 4) or in the second (group 5) experiment

annotated as three unknown sesquiterpenes (e.g., unknown sesquiterpene 1, 2, and 3), three unknown diterpenes (e.g., unknown diterpene 1, 2, and 3), and three unknown compounds (unknown compounds 1, 2, and 3), according to the mass spectrum and measured RI (Table S4). VOC emission profiles were mainly consistent in the two experiments and they differed according to the *Trichoderma* strain and time point (Figures 2 and S2). In particular, T39 produced a higher amount of VOCs compared to T34 and

SC1, with 24 and 21 VOCs found in the first and second experiment, respectively. On the other hand, 15 and 9 compounds were found in the case of T34, while 9 and 7 compounds were found in the case of SC1 in the first and second experiment, respectively. Moreover, T39 produced a higher amount of terpenes ([Z,E]-α-farnesene, γ-cadinene, γ-muurolene, α-curcumene, α-phellandrene, α-zingiberene, unknown diterpene 1, 2, and 3, and unknown sesquiterpene 1 and 2) compared to T34 ([Z,E]-α-farnesene, γ-muurolene, α-curcumene, α-zingiberene, and unknown sesquiterpene 2) and SC1 (α-zingiberene and unknown diterpene 3).

VOCs were divided into five metabolite groups according to changes in abundance among the three *Trichoderma* strains (Figure 2; Tables S2 and S3). The first metabolite group included three terpenes ([Z,E]-α-farnesene, γ-cadinene, and unknown diterpene 1), 1,3-octadiene, and 2-pentylfuran, whose changes in abundance were consistent in both experiments and time points (Group 1). More specifically, the abundance of (Z,E)-α-farnesene was higher in T34 and T39 compared to SC1 at 48 h and in T39 compared to T34 and SC1 at 72 h in both experiments. The abundance of γ-cadinene was higher in T39 compared to T34 and SC1 at both time points in both experiments. Moreover, 1,3-octadiene was more abundant in T34 and T39 compared to SC1 at 72 h, 2-pentylfuran and the unknown diterpene 1 were more abundant in T39 compared to T34 and SC1 at 72 h and these three VOCs were not found at 48 h in both experiments. The second metabolite group comprised nine VOCs, whose changes in abundance among the three *Trichoderma* strains were consistent in the two experiments at one time point (Group 2). At 48 h, γ-muurolene was more abundant in T34 and T39 compared to SC1 and the unknown sesquiterpene 2 was more abundant in T39 compared to T34 and SC1 in both experiments. At 72 h, the abundance of 6-pentyl-2H-pyran-2-one was higher in SC1 and T39 compared to T34, while that of α-curcumene, α- phellandrene, α-zingiberene, unknown compound 1, unknown diterpene 2, and uknown sesquiterpene 1 was higher in T39 compared to T34 and SC1 in both experiments. The abundance of two VOCs (trans-β-farnesene and unknown diterpene 3) differed in the two experiments (Group 3). Moreover, 10 VOCs (2-n-heptylfuran, 2-undecanone, 3-octanone, α-terpinene, β-sesquiphellandrene, γ-terpinene, limonene, germacrene A, unknown compounds 2 and 3) and five VOCs (β-bisabolene, β-himachalene, β-phellandrene, δ-cadinene and unknown sesquiterpene 3) were detected only in the first (Group 4) or second (Group 5) experiment, respectively.

3.2 | Efficacy of pure volatile organic compounds against downy mildew on grapevine leaf disks

Five VOCs were selected according to their consistent changes in abundance among the three *Trichoderma* strains in the two experiments and they were tested as a pure compound against *P. viticola* at different concentrations in air volume (Figure S1B). More specifically, (Z,E)-α-farnesene (α-farnesene mixture of isomers), 1,3-octadiene, and 2-pentylfuran were selected, since their changes in abundance were consistent in both experiments and time points (Group 1). Moreover, γ-cadinene and γ-muurolene were tested as a mixture of isomers (namely cadinene) and 6-pentyl-2H-pyran-2-one was used as pure compound, since their changes in abundance were consistent in the two experiments at one time point. The pure VOCs were tested against *P. viticola* and 2-pentylfuran reduced disease severity at dosages of 5, 10, and 15 mg L^{-1} in air volume with no visible phytotoxic effects (Figure 3A). Likewise, 6-pentyl-2H-pyran-2-one reduced downy mildew severity at dosages of 5 and 10 mg L^{-1} in air volume with no visible phytotoxic effects (Figure 3B). However, leaf disks treated with 20 mg L^{-1} in air volume of 2-pentylfuran or with 15 and 20 mg L^{-1} in air volume of 6-pentyl-2H-pyran-2-one showed phytotoxic effects.

Leaf disks treated with 1,3-octadiene, α-farnesene, and cadinene at a concentration of 50 mg L^{-1} in air volume showed a reduction of downy mildew severity with an efficacy of 21.5 ± 4.6% (Figure 4A), 21.6 ± 4.4% (Figure 4B) and 18.4 ± 5.9% (Figure 4C), respectively. At a concentration of 20 mg L^{-1} in air volume, α-farnesene reduced downy mildew severity, but 1,3-octadiene and cadinene did not and these three VOCs were not further used in activity tests due to the low efficacy with high application dosages.

Neither synergistic nor additive effects against downy mildew severity were observed with a blend of the two most efficient VOCs (2-pentylfuran and 6-pentyl-2H-pyran-2-one) at a concentration of 5 and 10 mg L^{-1} in air volume (Kruskal-Wallis test, $P > 0.05$). In particular, the reduction of downy mildew severity on leaf disks treated with the blend of the two most efficient VOCs (77.2 ± 2.1%; at 10 mg L^{-1} in air volume for each compound) was comparable (Kruskal-Wallis test $P > 0.05$) to that on leaf disks treated with 10 mg L^{-1} in air volume of pure 2-penthylfuran (36.2 ± 3.8%) or 6-pentyl-2H-pyran-2-one (82.1 ± 3.3%), therefore this blend was not further used in activity tests.

3.3 | Effects of pure volatile organic compounds on callose deposition and hypersensitive response in grapevine leaf disks

Effects of the two most efficient VOCs were further characterized, using the lowest concentration at which the highest efficacy without visible phytotoxicity was observed (i.e., optimized concentration, namely 10 mg L^{-1} in air volume for 2-pentylfuran and 6-pentyl-2H-pyran-2-one). Aniline blue staining revealed no differences between VOC-treated and control leaf disks before *P. viticola* inoculation (0 dpi, Figure 5A–C), as well as in leaf disks sprayed with T39 conidia (T39 conidia-treated; Figure 5D). At 1 dpi, the pathogen had already penetrated the stomata of control leaf disks and substomatal vesicles were visible (Figure 5E). On the other hand, strong turquoise fluorescence was observed in the stomata of leaf disks treated with 2-pentylfuran or 6-pentyl-2H-pyran-2-one at 1 dpi (Figure 5F,G), indicating intense callose deposition at infection sites, as found in T39 conidia-treated leaf disks (Figure 5H). Thus, the number of zoospores that had successfully entered the stomata at 1 dpi was reduced in leaf disks treated with 2-pentylfuran or 6-pentyl-2H-pyran-2-one.

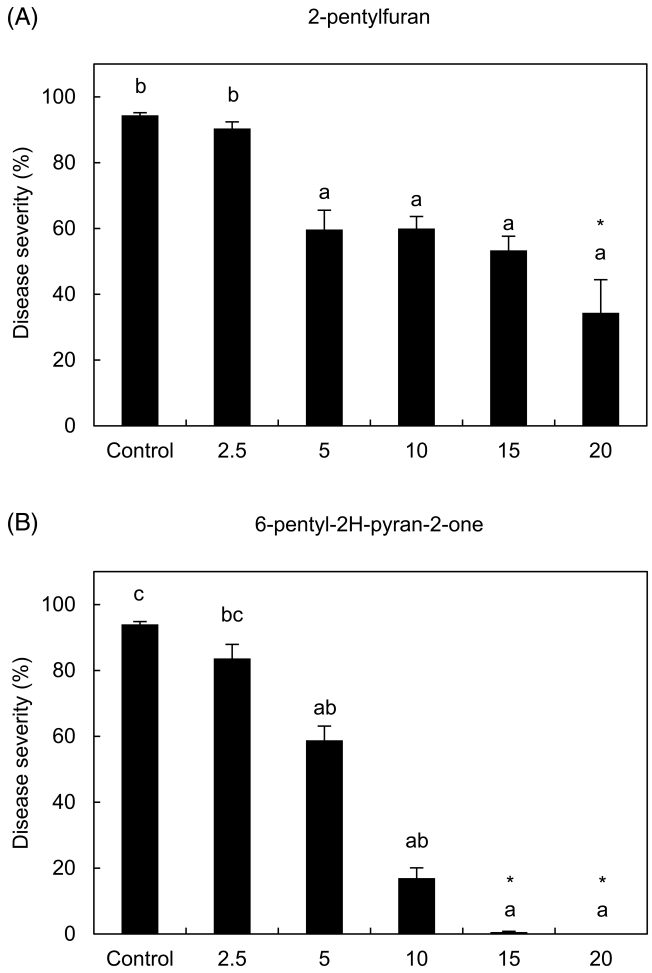

FIGURE 3 Effects of the most efficient pure volatile organic compounds (VOCs) against downy mildew. Grapevine leaf disks were treated with water (control), 2-pentylfuran (A) or 6-pentyl-2H-pyran-2-one (B) at 2.5, 5, 10, 15 and 20 mg L^{-1} in air volume and downy mildew severity was assessed at 6 days post inoculation. Five replicates (dishes with five disks each) were assessed for each treatment and the experiment was carried out twice. For each assay, the Kruskal-Wallis test indicated no significant differences between the two experimental repetitions ($p > 0.05$) and data from the two experiments were pooled. The pooled mean and standard error values of 10 replicates from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments according to the Kruskal-Wallis test ($P \leq 0.05$). Asterisks indicate phytotoxic effects on leaf disks

Elongated and branched hyphae were visible in control leaf disks at 2 dpi (Figure 5I), while hyphae were occasionally visible in leaf disks treated with 2-pentylfuran (Figure 5J) or 6-pentyl-2H-pyran-2-one (Figure 5K). Callose deposition was visible in leaf disks treated with 2-pentylfuran (Figure 5J), 6-pentyl-2H-pyran-2-one (Figure 5K), and T39 conidia at 2 dpi (Figure 5L). At 6 dpi, *P. viticola* mycelium had already spread to the parenchyma and produced sporangiophores in control leaf disks (Figure 5M,Q), while *P. viticola* sporulated areas were reduced in leaf disks treated with 2-pentylfuran (Figure 5N,R), 6-pentyl-2H-pyran-2-one (Figure 5O,S), and T39 conidia (Figure 5P,T).

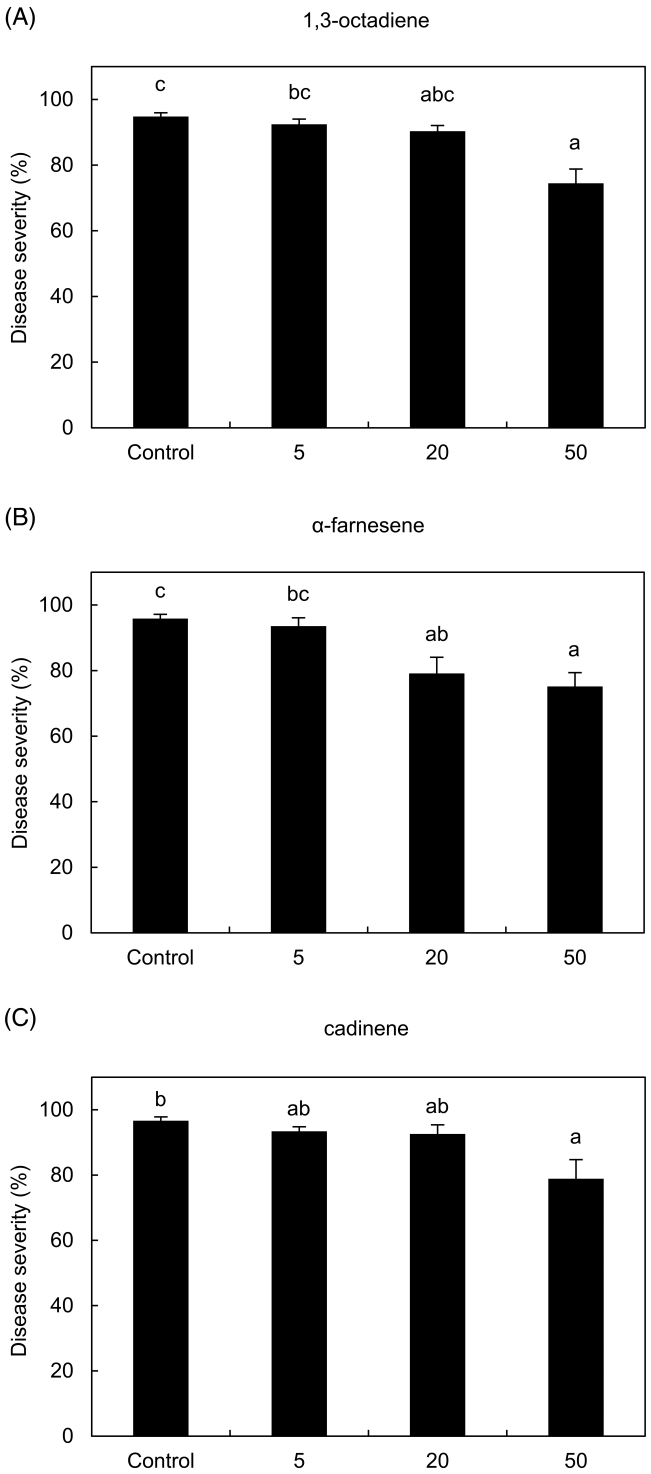

FIGURE 4 Effects of the less efficient pure volatile organic compounds (VOCs) against downy mildew. Grapevine leaf disks were treated with water (control), 1,3-octadiene (A), α-farnesene (B) or cadinene (C) at 5, 20 and 50 mg L^{-1} in air volume and downy mildew severity was assessed at 6 days post-inoculation. Five replicates (dishes with five disks each) were assessed for each treatment and the experiment was carried out twice. For each assay, the Kruskal-Wallis test indicated no significant differences between the two experimental repetitions ($P > 0.05$) and data from the two experiments were pooled. The pooled mean and standard error values of 10 replicates from the two experiments are presented for each treatment. Different letters indicate significant differences among treatments according to the Kruskal-Wallis test ($P \leq 0.05$)

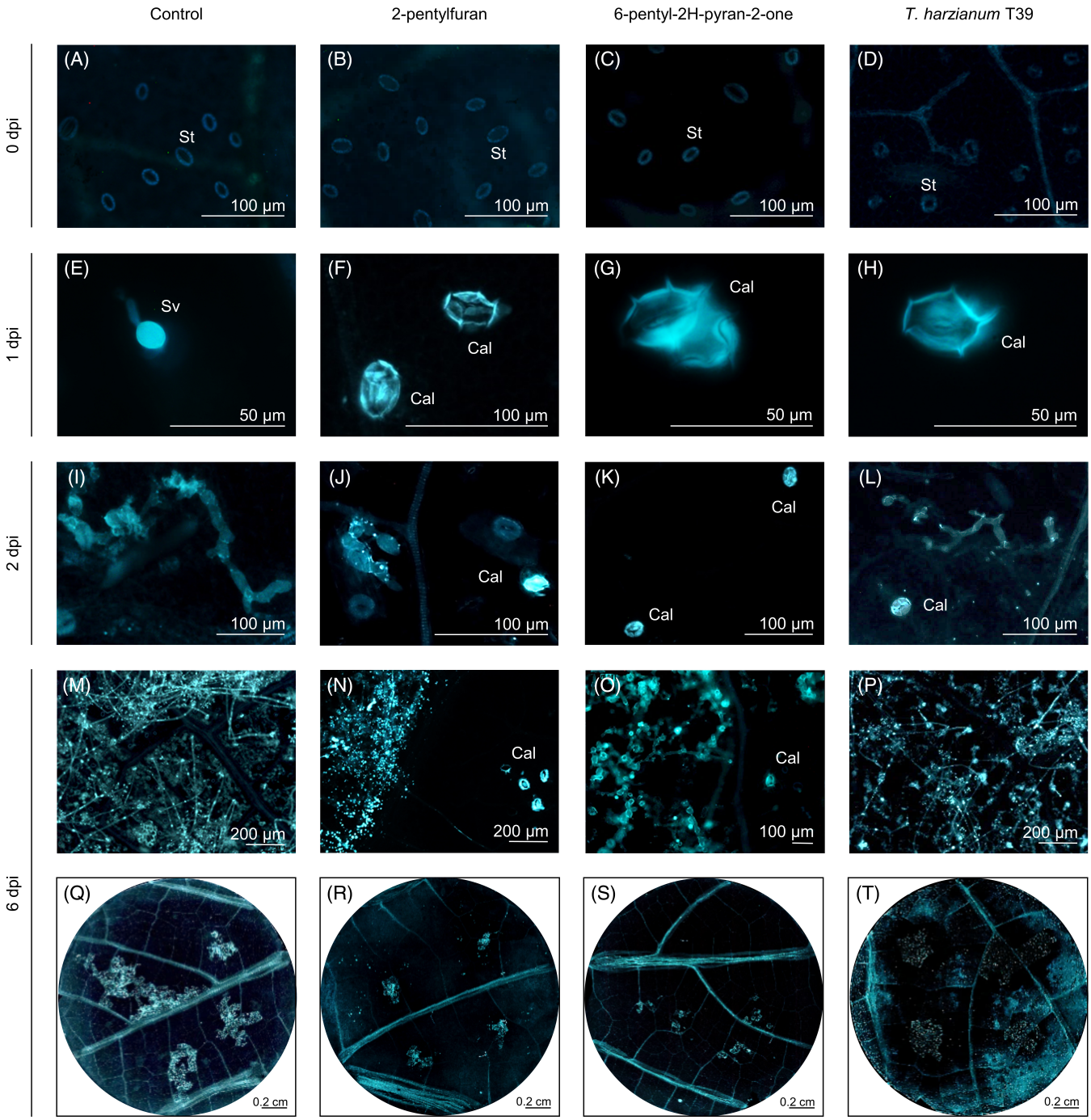

FIGURE 5 Effects of pure volatile organic compounds (VOCs) on callose deposition and downy mildew development. Grapevine leaf disks were treated with water (control) or with 10 mg L^{-1} in air volume of 2-pentylfuran or 6-pentyl-2H-pyran-2-one. Callose deposition and *Plasmopara viticola* development were monitored before inoculation (0 dpi, A–D), at one (E–H), two (I–L) and six (M–T) days post inoculation (dpi) using aniline blue staining. As control of callose deposition, leaf disks were sprayed with *Trichoderma harzianum* T39 conidia and inoculated with *P. viticola* (Palmieri et al., 2012). The experiment was carried out twice and a representative leaf disk of 10 is shown for each treatment. Abbreviations: Cal, callose; Sv, substomatal *P. viticola* vesicle; St, stomata guard cells

Lactophenol-trypan blue staining revealed no pathogen structures nor dead plant cells in control, VOC-treated and *V. riparia* leaf disks at 0 dpi (Figure 6A–D) and 1 dpi (Figure 6E–H). At 6 dpi, blue areas corresponding to *P. viticola* mycelia confirmed the reduction of pathogen growth in 2-pentylfuran-treated (Figure 6J,N) compared to control (Figure 6I,M) leaf disks. Moreover, dark blue-stained dead cells with no mycelial structures were found at *P. viticola* infection sites in 6-pentyl-2H-pyran-2-one-treated leaf disks at 6 dpi (Figure 6K,O), indicating HR activation, as found in downy mildew-resistant (*V. riparia*) leaf disks at 6 dpi (Figure 6L,P). Conversely, no HR response was found in mock-inoculated leaf disks treated with 6-pentyl-2H-pyran-2-one or 2-pentylfuran at 1 and 6 dpi (Figure S3).

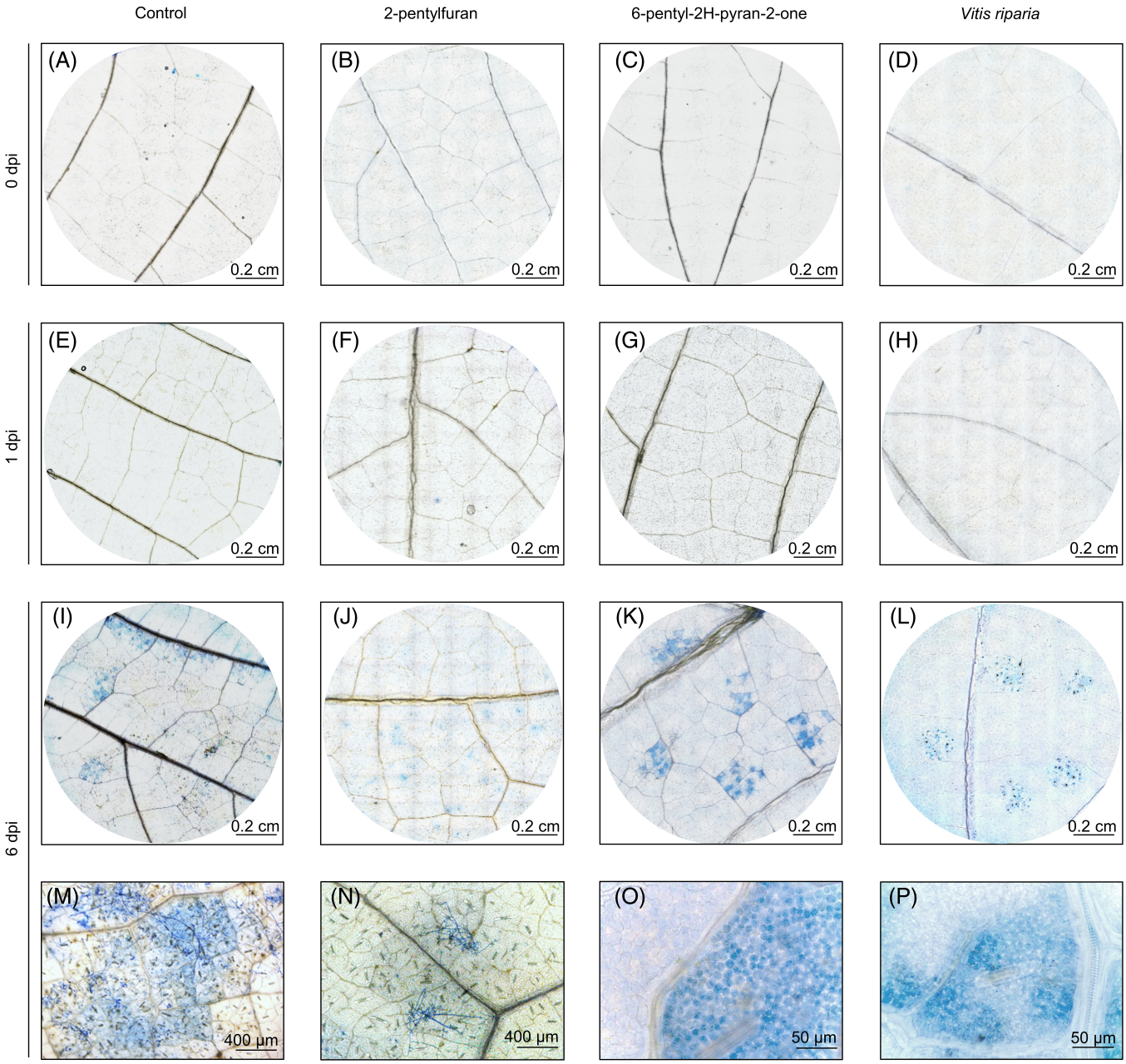

FIGURE 6 Effects of pure volatile organic compounds (VOCs) on grapevine hypersensitive response. Grapevine leaf disks were treated with water (control) or with 10 mg L^{-1} in air volume of 2-pentylfuran or 6-pentyl-2H-pyran-2-one. Hypersensitive response and *Plasmopara viticola* development were monitored before inoculation (0 dpi, A–D), at one (E–H) and six (I–P) days post inoculation (dpi) using lactophenol-trypan blue staining. As positive control, leaf disks of the downy mildew-resistant grapevine (*Vitis riparia*) were inoculated with *P. viticola* (Brilli et al., 2018). The experiment was carried out twice and a representative leaf disk of 10 is shown for each treatment. Infected areas with *P. viticola* sporangiophores were visible in control (I and M) and 2-pentylfuran-treated (J and N) leaf disks at 6 dpi

3.4 | Effects of pure volatile organic compounds on the modulation of defense-related genes in grapevine leaf disks

Plasmopara viticola inoculation upregulated the expression of *PR2*, *OSM1*, *OSM2*, *CHIT3*, and *HSR* at 6 dpi in control leaf disks (Table 1). In mock-inoculated leaf disks, the expression levels of the five defense-related genes were not affected by 2-pentylfuran, but they were induced by 6-pentyl-2H-pyran-2-one. In *P. viticola*-inoculated leaf disks, the expression levels of *PR2*, *OSM2*, and *HSR* were higher in 2-pentylfuran-treated compared to control leaf disks, as a reinforced modulation of defense-related genes after pathogen inoculation. Likewise, the *P. viticola*-dependent upregulation of *PR2*, *OSM1*, *OSM2*, *CHIT3*, and *HSR* was enhanced in 6-pentyl-2H-pyran-2-one-treated compared to control leaf disks.

TABLE 1 Gene expression analysis of defense-related genes in grapevine leaf disks treated with volatile organic compounds and inoculated with *Plasmopara viticola*

Gene name	Abbreviation	Treatment[a]					
		Control		2-pentylfuran		6-pentyl-2H-pyran-2-one	
		Mock	*P. viticola*	Mock	*P. viticola*	Mock	*P. viticola*
Pathogenesis-related protein 2	*PR2*	1.1 ± 0.4a	6.7 ± 0.9b	0.9 ± 0.0a	10.6 ± 1.0c	5.2 ± 0.4b	14.4 ± 0.8d
Osmotin 1	*OSM1*	1.0 ± 0.2a	49.3 ± 9.8b	7.7 ± 1.4a	93.1 ± 39.2c	73.5 ± 13.7b	199.0 ± 2.3d
Osmotin 2	*OSM2*	1.0 ± 0.2a	5.9 ± 0.9b	0.8 ± 0.1a	7.7 ± 2.4b	13.2 ± 1.0c	40.6 ± 3.1d
Chitinase 3	*CHIT3*	1.0 ± 0.1a	3.1 ± 0.4b	1.0 ± 0.2a	5.2 ± 0.6c	9.2 ± 1.4d	12.5 ± 0.6e
HR-related gene	*HSR*	1.4 ± 0.7a	400.4 ± 134.7c	1.4 ± 0.3a	340.4 ± 96.4c	55.0 ± 7.7b	1185.5 ± 84.4d

[a]Grapevine leaf disks (*Vitis vinifera*) were treated with water (Control) or with 10 mg L^{-1} in air volume of 2-pentylfuran or 6-pentyl-2H-pyran-2-one, on a filter paper disk without contact with leaf tissues. Disks were inoculated with *Plasmopara viticola* or water (Mock) and the respective pure VOC was applied again to the filter paper disk. Leaf disks were collected 6 days post-inoculation and the relative expression levels (fold change) were calculated respect to mock-inoculated control leaf disks using *actin* as constitutive gene for normalization (Perazzolli et al., 2012) and comparable results were obtained with VATP16 gene. Mean and standard error values of three replicates (dishes with five leaf disks each) are presented for each treatment. For each gene, different letters indicate significant differences according to Fisher's test ($P \leq 0.05$). Expression profiles were validated by an independent repetition of the experiment.

4 | DISCUSSION

Strains belonging to *T. asperellum*, *T. atroviride*, and *T. harzianum* are known for their biocontrol activity against phytopathogens (Brunner et al., 2005; Inglis & Kawchuk, 2002; Nagaraju et al., 2012; Perazzolli et al., 2008; Segarra et al., 2009; Segarra et al., 2013) and grapevine VOCs are known for their inhibitory activity against downy mildew (Lazazzara et al., 2018), but no information is available on the VOC-mediated effects of *Trichoderma* spp. against *P. viticola*. In this study, T34, T39, and SC1 conidia reduced downy mildew severity on grapevine plants and VOCs produced by the T34, T39, and SC1 colonies reduced downy mildew severity on grapevine leaf disks. Although VOC emission profiles differed according to the *Trichoderma* strain and time point, the VOC-mediated disease reduction was comparable in T34-, T39-, and SC1-treated leaf disks, suggesting possible synergistic interactions among VOCs (Strobel et al., 2001). In particular, 31 compounds were found in the HS-SPME/GC–MS analysis of the three *Trichoderma* strains and they belong to the compound classes of alkenes (e.g., 1,3-octadiene), furanes (e.g., 2-pentylfuran and 2-n-heptylfuran), ketones (e.g., 3-octanone and 2-undecanone), pyrones (lactones, e.g., 6-pentyl-2H-pyran-2-one), and terpenes, such as monoterpenes (e.g., α-phellandrene, α-terpinene, limonene, γ-terpinene and β-phellandrene) and sesquiterpenes (e.g., [Z,E]-α-farnesene, γ-cadinene, γ-muurolene, α-curcumene, α-zingiberene, trans-β-farnesene, germacrene A, β-sesquiphellandrene, β-himachalene, β-bisabolene, and δ-cadinene). In agreement with the previous literature (Contreras-Cornejo et al., 2014; Crutcher et al., 2013; Guo et al., 2019; Nieto-Jacobo et al., 2017; Sridharan et al., 2020; Stoppacher et al., 2010), terpenes dominated the VOC emission profiles of the three *Trichoderma* strains. In particular, T39 produced a higher amount of terpenes compared to T34 and SC1, such as (Z,E)-α-farnesene, γ-cadinene, γ-muurolene, α-curcumene, α-phellandrene, α-zingiberene, unknown diterpene 1, 2, and 3, and unknown sesquiterpene 1 and 2. Strain-specific VOCs were found and the unknown diterpene 1, unknown compound 1, and unknown sesquiterpene 1 were produced by T39 only, but further studies are required to fully elucidate the chemical structure and potential roles of these compounds. Moreover, VOC emission profiles of the three *Trichoderma* strains depended on the time point of sampling, corroborating that VOC production changed according to the incubation time and that it was possibly related to the developmental stage of *Trichoderma* spp. (Crutcher et al., 2013; Guo et al., 2020; Stoppacher et al., 2010). For example among VOCs with consistent changes in abundance in both experiments and time points, the abundance of (Z,E)-α-farnesene was higher in T34 and T39 compared to SC1 at 48 h and in T39 compared to T34 and SC1 at 72 h, while that of γ-cadinene was higher in T39 compared to T34 and SC1 at both time points and that of 1,3-octadiene was higher in T34 and T39 compared to SC1 at 72 h.

Functional assays reported in this study demonstrated that five VOCs (α-farnesene, cadinene, 1,3-octadiene, 2-pentylfuran, and 6-pentyl-2H-pyran-2-one) reduced downy mildew severity on leaf disks when applied in air volume without physical contact with the leaf tissues. In particular, two VOCs (6-pentyl-2H-pyran-2-one and 2-pentylfuran) strongly inhibited downy mildew severity with no synergistic or additive effects when used in a blend. Among them, 6-pentyl-2H-pyran-2-one was more abundant in T39 and SC1 compared to T34 samples at 72 h and it was previously identified as a characteristic VOC of numerous *Trichoderma* spp. (Fadel et al., 2015; Leylaie & Zafari, 2018; Mutawila et al., 2016; Stoppacher et al., 2010). Moreover, 2-pentylfuran was more abundant in T39 compared to T34 and SC1 samples at 72 h and it was previously found also in several *Trichoderma* spp. (Crutcher et al., 2013; Estrada-Rivera et al., 2019; González-Pérez et al., 2018; Lee et al., 2016; Nieto-Jacobo et al., 2017; Stoppacher et al., 2010).

Different modes of action against phytopathogens have been attributed to VOCs, such as induction of plant resistance and direct inhibition of pathogen growth by absorption on cuticular waxes (Camacho-Coronel et al., 2020; Quintana-Rodriguez et al., 2015).

T34 and T39 are well known inducers of systemic resistance in different plant species (Martínez-Medina et al., 2017; Perazzolli et al., 2008; Segarra et al., 2007; Segarra et al., 2009). In particular, T39 has been demonstrated to induce grapevine resistance against *P. viticola* by enhanced callose deposition and modulation of defense-related genes (Banani et al., 2014; Palmieri et al., 2012; Perazzolli et al., 2008, 2011; Perazzolli et al., 2012). In this study, the callose deposition was found at infection sites of leaf disks treated with 2-pentylfuran or 6-pentyl-2H-pyran-2-one, indicating that these *Trichoderma* VOCs induced grapevine resistance against *P. viticola*. The deposition of callose at the sites of pathogen infection is a key defense process against downy mildew (Gindro et al., 2003) that can be enhanced in susceptible grapevine genotypes by chemical resistance inducers, such as β-aminobutiric acid (Hamiduzzaman et al., 2005), benzothiadiazole-7-carbothioic acid S-methyl ester (Palmieri et al., 2012) and sulfated laminarin PS3 (Trouvelot et al., 2008). Furthermore, we found that 6-pentyl-2H-pyran-2-one activated the HR at *P. viticola* infection sites, indicating the VOC-mediated reinforcement of characteristic grapevine defense processes commonly activated in downy mildew-resistant genotypes (Brilli et al., 2018; Gindro et al., 2003). In particular, both callose deposition and HR response were found only after *P. viticola* inoculation in leaf disks treated with 6-pentyl-2H-pyran-2-one, suggesting a priming state activation for enhanced defense reaction upon pathogen infection. Likewise, the *P. viticola*-dependent upregulation of defense-related genes was enhanced by 2-pentylfuran (*PR2*, *OSM2*, and *HSR* genes) and 6-pentyl-2H-pyran-2-one (*PR2*, *OSM1*, *OSM2*, *CHIT3*, and *HSR* genes) treatment, as previously found in T39 conidia-treated grapevine plants (Banani et al., 2014; Perazzolli et al., 2011, 2012). Previous studies showed that VOCs produced by T34 and *T. harzianum* T78 enhanced the JA-dependent defenses of *A. thaliana* and tomato against *B. cinerea* (Martínez-Medina et al., 2017). The ability of *Trichoderma* VOCs to induce plant resistance is known to be related to the upregulation of defense-related genes, such as *PR-1* in 6-pentyl-2H-pyran-2-one-treated *Brassica napus* (Vinale et al., 2008), the activation of defense-related enzyme, such as chitinase and β-1,3-glucanase of lettuce treated with *T. asperellum* T1 VOCs (Wonglom et al., 2020), and the accumulation of defense molecules, such as JA and ROS in *A. thaliana* treated with *T. virens* Tv29.8 VOCs (Contreras-Cornejo et al., 2014). In this study, 6-pentyl-2H-pyran-2-one induced the expression of grapevine defense genes in mock-inoculated leaf disks (*PR2*, *OSM1*, *OSM2*, *CHIT3* and *HSR* genes), suggesting the partial activation of some defense processes also in the absence of the pathogen. This *Trichoderma* VOC is known to induce resistance against *B. cinerea* and *Alternaria brassicicola* in *A. thaliana* (Kottb et al., 2015), against *Erysiphe necator* in *V. vinifera* (Pascale et al., 2017), against *B. cinerea* in tomato seedlings and against *L. maculans* in canola seedlings (Vinale et al., 2008), suggesting a broad spectrum activity against phytopathogens.

Since chemical profiles and functional properties of microbial VOCs differed according to the growth media (González-Pérez et al., 2018; Lazazzara et al., 2017), further studies under natural conditions are required, in order to better evaluate the possible migration of VOCs produced by *Trichoderma* spp. to grapevine tissues and the reduction of downy mildew severity by plant resistance induction. Effects against *P. viticola* can be tested only in the presence of host tissues, due to the obligate biotrophic lifestyle of this pathogen. Thus, possible direct inhibitory effects of *Trichoderma* VOCs against *P. viticola* can also occur on leaf tissues. It was previously reported that some VOCs can be absorbed by the leaf cuticle and can persist on the leaf surface (Himanen et al., 2010), exerting direct inhibitory effects against fungal pathogens (Camacho-Coronel et al., 2020, Quintana-Rodriguez et al., 2015). For example, farnesene can be absorbed by plant cuticular wax layers and persist on plant leaves and to inhibit *Colletotrichum lindemuthianum* (Camacho-Coronel et al., 2020, Quintana-Rodriguez et al., 2015). Moreover, 6-pentyl-2H-pyran-2-one is a well-known compound with antifungal activity against *F. moniliforme* (El-Hasan et al., 2007), *R. solani* (Cruz-Magalhães et al., 2019; Scarselletti & Faull, 1994) and *Sclerotinia sclerotiorum* (Cruz-Magalhães et al., 2019) and it could potentially inhibit *P. viticola*, as well. The antifungal activity of 6-pentyl-2H-pyran-2-one has been proposed to be associated to its hydrophobic nature and the possible mechanical impediment to water absorption by the fungal cells due to the formation of a hydrorepellent film on the cell wall (Scarselletti & Faull, 1994). The same mechanism can be hypothesized against *P. viticola* and it might partially explain the phytotoxic effects observed at high dosages on tomato or oilseed rape seedlings (Vinale et al., 2008) and on grapevine leaf disks, indicating the importance of dose optimization and mode of action investigation for this compound. Likewise, the antifungal effects of 2-pentylfuran were previously reported against *Monilinia fructicola* (Liu et al., 2018), *S. sclerotiorum*, and *F. oxysporum* (Wu et al., 2015), but further studies are required to clarify the mode of action of this compound and the less efficient VOCs (i.e., 1,3-octadiene, α-farnesene, and cadinene) against downy mildew.

5 | CONCLUSIONS

VOCs emission profiles differed in the three *Trichoderma* strains tested and T39 produced higher amounts of terpenes compared to T34 and SC1, indicating genetic determinants of VOC production at strain level. Five *Trichoderma* VOCs (α-farnesene, cadinene, 1,3-octadiene, 2-pentylfuran, and 6-pentyl-2H-pyran-2-one) reduced downy mildew severity on grapevine leaf disks. In particular, 6-pentyl-2H-pyran-2-one and 2-pentylfuran enhanced the accumulation of callose and reinforced the upregulation of defense-related genes after *P. viticola* inoculation, indicating the induction of grapevine resistance. Moreover, 6-pentyl-2H-pyran-2-one upregulated the expression of defense-related genes in mock-inoculated leaf disks and activated HR after *P. viticola* inoculation, as possible reinforcement of the grapevine defense mechanisms against this pathogen. Thus, airborne signals produced by beneficial soil-borne *Trichoderma* spp. can be perceived by plant tissues as possible mediators of fungus-plant communications and as inducers of plant resistance. Although further transcriptomic and functional studies are required to shed light on the mode of

action of *Trichoderma* VOCs in the induction of grapevine defense mechanisms against downy mildew, *Trichoderma* VOCs could open new opportunities to develop biofungicides from natural origin.

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AUTHOR CONTRIBUTIONS

Valentina Lazazzara performed the experiments, analyzed the data and wrote the manuscript. Bianca Vicelli contributed to leaf disk experiments. Christoph Bueschl wrote the R scripts and analyzed the data. Alexandra Parich contributed to chemical analysis. Ilaria Pertot supervised the experiments and revised the manuscript. Rainer Schuhmacher coordinated chemical analysis and helped to draft the manuscript. Michele Perazzolli conceived the study, coordinated the experiments and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supporting information of this article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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