Oregano essential oil vapour prevents *Plasmopara viticola* infection in grapevine (*Vitis Vinifera*) and primes plant immunity mechanisms

Markus Rienth, Julien Crovadore, Sana Ghaffari, François Lefort

1 Changins, HES-SO University of Applied Sciences and Arts Western Switzerland, Nyon, Switzerland, 2 Plants and Pathogens Group, Institute Land Nature and Environment, Hepia, HES-SO University of Applied Sciences and Arts Western Switzerland, Jussy, Geneva, Switzerland

Abstract

The reduction of synthetic fungicides in agriculture is necessary to guarantee a sustainable production that protects the environment and consumers’ health. Downy mildew caused by the oomycete *Plasmopara viticola* is the major pathogen in viticulture worldwide and responsible for up to 60% of pesticide treatments. Alternatives to reduce fungicides are thus utterly needed to ensure sustainable vineyard-ecosystems, consumer health and public acceptance. Essential oils (EOs) are amongst the most promising natural plant protection alternatives and have shown their antibacterial, antiviral and antifungal properties on several agricultural crops. However, the efficiency of EOs highly depends on timing, application method and the molecular interactions between the host, the pathogen and EO. Despite proven EO efficiency, the underlying processes are still not understood and remain a black box. The objectives of the present study were: a) to evaluate whether a continuous fumigation of a particular EO can control downy mildew in order to circumvent the drawbacks of direct application, b) to decipher molecular mechanisms that could be triggered in the host and the pathogen by EO application and c) to try to differentiate whether essential oils directly repress the oomycete or act as plant resistance primers. To achieve this a custom-made climatic chamber was constructed that enabled a continuous fumigation of potted vines with different EOs during long-term experiments. The grapevine (*Vitis vinifera*) cv Chasselas was chosen in reason of its high susceptibility to *Plasmopara viticola*. Grapevine cuttings were infected with *P. viticola* and subsequently exposed to continuous fumigation of different EOs at different concentrations, during 2 application time spans (24 hours and 10 days). Experiments were stopped when infection symptoms were clearly observed on the leaves of the control plants. Plant physiology (photosynthesis and growth rate parameters) were recorded and leaves were sampled at different time points for subsequent RNA extraction and transcriptomics analysis. Strikingly, the *Oregano vulgare* EO vapour treatment during 24h post-infection proved to be sufficient to reduce downy mildew development by 95%. Total RNA was extracted from leaves of 24h and 10d treatments and used for whole transcriptome shotgun sequencing (RNA-seq). Sequenced reads were then mapped onto the
V. vinifera and P. viticola genomes. Less than 1% of reads could be mapped onto the P. viticola genome from treated samples, whereas up to 30% reads from the controls mapped onto the P. viticola genome, thereby confirming the visual observation of P. viticola absence in the treated plants. On average, 80% of reads could be mapped onto the V. vinifera genome for differential expression analysis, which yielded 4800 modulated genes. Transcriptomic data clearly showed that the treatment triggered the plant’s innate immune system with genes involved in salicylic, jasmonic acid and ethylene synthesis and signaling, activating Pathogenesis-Related-proteins as well as phytoalexin synthesis. These results elucidate EO-host-pathogen interactions for the first time and indicate that the antifungal efficiency of EO is mainly due to the triggering of resistance pathways inside the host plants. This is of major importance for the production and research on biopesticides, plant stimulation products and for resistance-breeding strategies.

Introduction

Global food supply is highly dependent on industrial agriculture, which in turn would not be possible without the intensive use of pesticides against fungal diseases and other pests. Responding to consumers’ increasing demands for a sustainable food production implies developing alternatives to conventional synthetic plant protection products. Long-term fungicide applications have consequently led to increased resistances of pathogens and detrimental impacts on ecosystems and humans [1], followed by a decreasing acceptance by consumers [2]. This is particularly true for grapevine (Vitis vinifera L.), which is highly sensitive to fungal diseases such as downy mildew caused by the obligate biotrophic pathogenic oomycete Plasmopara viticola (Berk. & M.A. Curtis) Berl. & De Toni, (1888) which is natural to North America. This organism was accidentally introduced in Europe via infected cuttings at the end of the 19th century and is one of the most devastating diseases of viticulture worldwide [3], which explains that the application of relatively large amounts of pesticides in viticulture, when compared to other crops, is necessary to guarantee yield and quality of grape production.

The infection cycle of P. viticola starts with zoomeiospores, which are released by mature zoosporangia germinating from oospores which are the only source of primary inoculum. Encysted zoomeiospores germinate to form a germ tube, which will penetrate the leaf through a stoma. A substomatal vesicle then develops and gives rise to the intercellular mycelium with its many haustoria penetrating cell walls of the mesophyll. The incubation time until visible symptoms appear may last from 4 to approximately 18 days depending on temperature. After this time span, oil-spot lesions appear on the adaxial surface. If the leaf is subsequently incubated in conditions of high humidity, hyphal coils grow into the sub-stomatal cavity and give rise to sporangiophores emerging from the stoma. Incubated in the laboratory at 14–28°C and high humidity (above 90%), the sporangiophores and zoosporangia develop overnight, before liberating zoospores [4].

During the vegetative cycle, growers usually need to treat between 3 to 15 times against downy mildew with systemic or organic fungicides. This represents a fundamental problem regarding the sustainability of ecosystems, biodiversity, consumer health and acceptance as well as long term efficiency of systemic fungicides, along with an increased development of resistances [5, 6].

To reduce synthetic pesticides, organic production is one alternative to conventional farming but still highly dependent on copper (Cu), the oldest and still a very efficient treatment
against downy mildew. It remains, however, a heavy metal accumulating in vineyard soils. When compared to the overall average Cu concentration of 16.85 mg kg\(^{-1}\) in soils, vineyards have the highest mean soil Cu concentration (49.26 mg kg\(^{-1}\)) of all land categories, followed by olive groves and orchards [7, 8]. Cultivation of disease resistant varieties is certainly one of the most ecological solutions to reduce pesticides and, due to tremendous efforts of international public breeding programmes, a large choice of disease-resistant grape cultivars is nowadays available to growers [9–12]. However, the organoleptic quality is still often inferior to the one of traditional cultivars, making them less attractive to consumers and thus producers. For these last reasons, access of organic wines to the wine market is still difficult. Furthermore, the durability of resistance factors is often not stable, in particular in monogenetic cultivars [13]. Genetically modified organisms are not a solution either, since they are so far neither premised nor authorised in most producing countries. Alternative plant protection strategies are thus utterly needed to guarantee a sustainable viticulture that is both environment- and consumer-friendly.

It is now widely acknowledged that plants possess two forms of innate immune system responses against pathogens. Molecular expression patterns induced by microbial molecules, i.e. Pathogen, Microbial or Damage-associated molecular pattern (PAMPs, MAMPs or DAMPs) lead to pattern triggered immunity (PTI), which represents the first line of defense of plants against pathogens. PTI leads to a cascade, which is marked by common signaling events, such as ion fluxes, protein phosphorylation cascades, accumulation of reactive oxygen species (ROS), induction of defense genes and cell-wall reinforcement by callose deposition [14, 15].

The second innate immune defense response is the effector-triggered immunity (ETI), where the plant’s response is triggered by pathogen effectors. ETI results from the highly specific, direct or indirect interaction of pathogen effectors and the products of plant disease resistance (R) genes, which leads to a strong local defense response often associated with programmed cell death (PCD) as a part of hypersensitive response (HR) that stops pathogen growth [16].

Inducing resistance to pathogens by activating the plant’s innate immunity through application of natural products which trigger PTI and/or ETI, could thus represent an alternative strategy to protect plants against diseases [17].

Plant phytochemicals have been investigated for decades, and it has been demonstrated that specific plant volatile organic compounds (VOCs) have antifungal, anti-bacterial, as well as repulsive effects on insect pests. It is however not known to what extent VOCs have direct effects on the pathogen or induce stimulation of the host plant’s defense mechanisms.

During the last decade, several different resistance inducers have been tested for their ability to induce defense responses of the susceptible \(V.~vinifera\) against \(P.~viticola\), such as beta-amino-nobutyric acid [18] chitosan [19], laminarin, sulfated laminarin [20, 21], Frutogard\(^\text{R}\) and other plant extracts [22, 23].

As natural products, EOs have shown antifungal properties against several pathogens such as \(P.~viticola\), \(Botrytis~cinerea\) Pers. 1794 and \(Fusarium~sp\). [24, 25]. The chemical composition and activities of members of the EO-producing family \(Lamiaceae\) have been widely studied, and the antioxidant activity could mainly be attributed to carnosic acid, carnosol, rosmarinic acid, and other phenolic compounds, whereas the fungicidal activity seems to be due to molecules such as carvacrol, thymol, and \(p\)-cymene [26]. The use of EOs to control plant pathogens has been evaluated on different species with different pathogens. In such studies, the timing of EO applications seems to be a crucial point to increase the efficiency of pathogen control. For instance, \(Origanum~vulgaris\) L. EO had preventive effects against \(B.~cinerea\) development on tomato leaves, with the highest efficiency when applied 24h post-infection. This suggested an effect on early fungal development such as spore germination, germ tube growth and/or
appressorium formation, since light- and electron-scanning microscope observations revealed alterations of hyphal morphology when exposed to EOs [27, 28].

Studies conducted on grapevines, infected with downy mildew and treated with sage (Salvia officinalis L.) EO showed a 94% reduction in disease severity. However, due to the very low rainfastness and degradation of EOs, the efficiency in rainy years was much less important [29]. This is probably the reason why other field studies on the variety Merlot, using EOs of Corymbia citriodora (Hook.), Syzygium aromaticum (L.), O. vulgare and Thymus vulgaris L. did not show any efficiency in field trials against downy mildew, whereas thyme and oregano EOs were very efficient in inhibiting fungal growth in Petri dishes. This highlights that a major problem of EO efficiency against pathogens is due to degradation by light, heat, oxygen and humidity [30], application time and bad rainfastness.

Several studies indicate that the vapour phase of EO is more fungitoxic than the contact liquid phase, although this has only been shown for Botrytis in Petri dishes [28, 31, 32]. Thus, a continuous fumigation of plants with EO vapour could possibly circumvent these drawbacks. For a sustainable agricultural production, using EO vapour as a direct treatment with diffusers or in the form of co-plantations of EO-VOCs emitting plants could be considered in integrated systems, able to control fungal diseases.

The mechanisms underpinning the effect of antifungal EO-VOCs are not well understood so far. Some VOCs seem to have a direct effect on pathogens, while others seem to elicit the plant innate immune system with its complex mechanism. Several authors have found genes involved in the biosynthesis of phytoalexins, pathogenesis-related (PR) proteins and cell wall proteins when VOCs were applied. [33]. Understanding the mechanism involved in EO efficacy against fungi could thus provide very valuable information when developing natural fungicides, plant defense stimulation products, as well as providing genetic targets for the breeding of resistant varieties.

The aims of the present study were thus to investigate if the vapour phase, applied by a continuous fumigation of different EOs, could inhibit the development of downy mildew on grapevine leaves and, in case of proven efficiency, to study and reveal the induced transcriptomic changes by RNA-sequencing in an attempt to elucidate the underlying molecular interactions.

**Material and methods**

**Experimental set up**

Two customised vapourisation systems (control and treatment) were built to enable a continuous flow of EO vapour during several weeks. Inside a basic climatic chamber (CLF Plant Climatics, Model L-66LL VL), an air-tight Plexiglas chamber was fitted top and bottom with connections for the vapourisation hose (supplemental S1 File). EOs were put in a Petri dish, inside two plastic boxes, which could optionally be heated to 35˚C. The two Plexiglas chambers (control and treatment) were connected to a continuously running custom-made compressor. This way, vapour was distributed from the plastic boxes containing Petri dishes, with or without EO, inside the climatic chamber by the bottom tube and extracted by the exhaustion tube connected to the top of the chamber. Plants were put on an alveoled platform to ensure a homogenous distribution of vapour inside the chamber.

Because, according to literature, the most efficient EOs against bacteria and fungi come from the species belonging to the Origanum and Thymus genera [28, 34], these were chosen to be tested during the experiment.

Commercially available standard EOs were purchased from Compagnie des sense© (France). Components of these EOs were analysed by a gas chromatography (Agilent 7890B;
Capillary column: 60m, 0.25mm ID, 1.4 μm, Rtx-1301, vector gas: hydrogen, flow 4mL.min⁻¹, injector temperature 100˚C, Gradient: 5 min at 40˚C then 3˚C.min⁻¹ until 240˚C, total run-time 71.67 min) with Flame Ionization Detector (FID) (250˚C, Air flow 400mL.min⁻¹, H2 fuel flow 30mL.min⁻¹). Composition of EOs are provided in supplemental S1 Table.

The EO vapour concentration inside the chamber during evaporation was also assessed by GC-FID. For this purpose, active charcoal was placed at different spots inside the control and vapour chamber, and subsequently extracted by dichloromethane and injected in the GC-FID system.

**Plant material and experimental conditions**

Each experiment was carried out with two-year-old cuttings of cv Chasselas (V. vinifera) at a 12 to 15 leaf stage. All leaves of the 12 plants were artificially infected with P. viticola, using a suspension containing 10⁵ sporangia.mL⁻¹, which was sprayed on the lower site of each leaf. Inoculated plants were subsequently split into two groups of 6 plants for control and treatment, then put inside their respective climatic chamber. Infection was performed at the end of the day so as to provide optimal infection conditions for P. viticola, i.e. overnight in the dark and in presence of a humidifier.

The appearance of oil spots on control plants (untreated) indicated that incubation of P. viticola was completed. Plants were then moistened in the evening to provoke sporulation during the night and allow better visual assessment and easy identification of infected leaf tissue for RNA-seq sampling. Disease severity (DS) was assessed the day after sporulation was triggered, via a visual estimation of all leaves of each plant. For each leaf, DS was expressed as the proportion (percentage of 0–100%) of the abaxial leaf area covered with white P. viticola spores in relation to the total leaf area, and a mean value was calculated for each plant. Disease reduction in treated plants was calculated as [(DS in control plants–DS in treated plants) / (DS in control plants)] × 100%.

Two series of experiments were conducted with each EO, resulting in a total of 4 sets of 10d experiments.

The two first series consisted in a continuous treatment with each EO vapour (T. vulgaris and O. vulgaris) for a duration of 10d starting straight after infection. Plants in the control chamber were infected with P. viticola but not treated with EO vapour, however a continuous air flow with the same debit as in the treatment was applied throughout the whole period.

For the second set of experiments, plants were also treated immediately after infection with P. viticola, but evaporation was maintained only for 24h. Plants were subsequently maintained in the chamber for a total of 10d (without treatment), until oil spots eventually appeared on the control plants. Photosynthesis (A) was measured on 1 adult leaf of each of the 6 plants in each chamber with a Ciras 3 (PP systems on three), USA Environmental parameters inside the chambers were...
Photosynthetically Active Radiation (PAR) of 500 mmol.m$^{-2}$.s$^{-1}$, a temperature of 26/20˚C (respectively day/night) and a relative humidity of 50%.

RNA extraction

Total RNA was extracted from three entire adult leaves situated between position 3 and 10 on the main stem, from three different vines for non-EO treated controls respectively for the EO-treatment. The three leaves from each vine were pooled during N2 grinding to constitute one replicate per vine. For the non-EO-treated control, leaves that showed a maximum number of spores after induction of sporulation (assessed by visual inspection). Leaf samples were drawn after 10d of continuous fumigation in the first set of experiments and 24h post-inoculation coinciding with the end of the vaporisation in the second set of experiments.

RNA extraction was performed according to the protocol described by Rienth, Torregrosa [35]. Briefly, one gram of leaf matter was ground to powder under liquid nitrogen. Subsequently, 5 ml of extraction buffer (6 M guanidine-hydrochloride, 0.15 M tri-sodium-citrate, 20 mM EDTA and 1.5% CTAB) was added. Cell debris were removed by centrifugation. After chloroform extraction, one volume of isopropanol was added to the resulting aqueous phase to precipitate RNAs. Samples were kept at—20˚C for at least two hours. Precipitated RNAs were separated by centrifugation and cleaned with 75% ethanol. The pellet was re-suspended in the RLC buffer of RNAeasy$^{11}$ Kit (Qiagen, Switzerland) and an additional chloroform-cleaning step was applied. The successive washing steps and the DNase treatment were performed following the manufacturer’s recommendations. Optical densities were measured at 260 and 280 nm with a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Switzerland).

Transcriptome sequencing

Total RNA samples were qualitatively and quantitatively controlled using a Bioanalyser 2100 (Agilent, Switzerland) and a Qubit$^{12}$ 3.0 Fluorometer (Thermo Fisher Scientific, Switzerland). Libraries were created with the TruSeq Stranded mRNA sample preparation kit (Illumina, Switzerland) following the manufacturer’s recommendations. The libraries’ quality was then checked with the Fragment Analyser (AATI–Agilent, Switzerland). Transcriptome-sequencing was carried out within one Illumina MiniSeq run at 2 x 151 bp paired-end read length, using a MiniSeq High Output kit (Illumina). Total sequencing yielded 12.4 Gbp and generated between 0.73 Gbp and 1.49 Gbp per sample. Reads were automatically trimmed for adaptor removal and demultiplexed using the BaseSpace Sequence Hub (Illumina). The quality of reads was performed with FastQC version 0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). A final reads trimming (Illumina adapters removal) and filtering step was performed using Trimmomatic software version 0.36 with a specified average quality cut-off of 30 (Phred score) and a minimum read length of 40 bp.

The raw sequences reads of the twelve samples have been made publicly available as fastq files, in the Sequence Read Archive (SRA) database of the National Centre for Biotechnology Information (NCBI) [36], under the following accessions SRR8439286, SRR8439287, SRR8439284, SRR8439285, SRR8439288, SRR8439289, SRR8439290, SRR8439291, SRR8439292, SRR8439293, SRR8439294, SRR8439295

RNA sequences analysis

Cleaned reads were first mapped against predicted mRNAs (PN40024 12X v2 grape reference transcriptome) obtained from the gene prediction version 2.0 of NCBI. Reads were also mapped to the P. viticola genome available at DDBJ/ENA/GenBank under the accession MTP100000000 [37], to check presence or absence of the fungi inside the leaves.
Mapping was performed using HISAT 2 [38] followed by read counting with HTSeq [39]. Differential expression analysis was performed using the DEseq 2 package for R [40]. A principal component analysis was performed with R on all normalised reads. Transcripts were considered as differentially expressed (DEG) when adjusted p-value was $< 0.01$ and log2-ratio was $> 0.5$.

Hierarchical clustering by heatmap was performed with DEseq2 on log transformed DEGs. To have an overview of similarities and dissimilarities among samples, the count data were used to perform heatmap analysis with hierarchical clustering and principal component analysis (PCA) with DESeq2 R package. Venn diagrams were drawn with tools provided by the Center of Plant Systems Biology at Ghent University [http://bioinformatics.psb.ugent.be/webtools/Venn/]. Gene annotation was derived from Grimplet et al., 2012. Functional Categories of transcripts up- and downregulated and allocated to different clusters were analysed with FatiGO [41], to identify significant enrichment of functional category. Categories were derived from Grimplet et al (2012) [42] and Fisher’s exact test was carried out to compare the genes list with non-redundant transcripts from the grapevine genome. Significant enrichment was considered in case of p value $< 0.05$ and illustrated as fold change. For the illustration of the phenylpropanoid pathway, transcripts that were significantly and concomitantly modulated (fc $> 2$, $p<0.05$) in either both green or both ripe stages were mapped using VitisNet networks through cytoscape v 2.8.3 s.

**Results & discussion**

**EO vapour impedes *Plasmopora viticola* development**

Terpene composition of EOs as determined by GC-FID (Gas Chromatography with Flame-Ionization Detection) analysis, showed that the main constituents were carvacrol (21–60%), p-cymene (6–20%), γ-terpine (9–26%) and thymol (9–26%) in EO of *O. vulgare* and thymol (24–45%), p-cymene (15–37%) and g-terpinene (6–24%) in *T. vulgaris* (supplemental S1 Table). These compositions are congruent with the commonly reported values in literature for *O. vulgare* [43–45] and *T. vulgaris* [46]. Still, huge variations can occur depending on the species, the collecting season, the geographical position, the collected plant organ and the oil extraction method [34].

Concentrations of oil vapour inside the chamber varied for *O. vulgare* between 0.023 and 0.015% (heated and non-heated respectively) between 0.1 and 0.06% for *T. vulgaris*. Concentration of vapour without heating was considered sufficiently high, thus experiments were carried out without the heating system.

In the first series of experiments with *O. vulgare* and *T. vulgaris*, vines were treated continuously for 10d after *P. viticola* infection. The visual assessment after sporulation induction (Fig 1) showed that both oils were highly efficient in inhibiting *P. viticola* development on leaves and reduced disease severity by up to 98%. Antifungal efficiency of both oils was comparable and not significantly different. Unfortunately, after a 10d exposure to essential oil vapour (of both oils), vines expressed symptoms of an acute phytotoxicity consisting of browning of young leaves, a decline in growth velocity as well as a reduction in photosynthesis (Fig 2) and leaf-nitrogen content. The effects of different essential oils on vine physiology had been previously tested in the same chamber with the same process of application but without mildew inoculation (Furet-Gavallet et al., 2018) and had shown a slight phytotoxic effect of different essential oils when the application was longer than 2 days. For this reason, the treatment with *P. viticola* was minimized.

A second set of experiments was carried out to test an EO treatment time reduced to 24h and still resulting in an antifungal effect, with the advantage of reducing EO quantity and
eventually phytotoxicity. For this second test, EO vapour treatments were maintained for only 24h after infection. Infected plants were then kept for 10d in the chamber to assess disease severity after induction of sporulation, as for the first experiments. Interestingly, the efficiency of the EO treatment against downy mildew was only slightly but not significantly lower, compared to the 10d treatment with a 95% decrease in disease severity in treated plants (Fig 1). Additionally, oregano EO showed a slightly higher efficiency than thyme EO, and for this reason only the oregano EO modality was used for transcriptomics.

This strongly suggests that the antifungal effect of EOs acts during the early stages of the infection cycle or even prior to infection. However, it remains unclear whether this is because of a direct lethal effect either on the zoospores before producing haustoria or on the haustoria growth before entering the stomata or else due to the inhibition of mycelium growth inside the leaf. An alternative hypothesis is that EO vapour stimulates innate plant immunity by activating PTI and/or ETI, which would impede entrance of the haustoria through the stomata and/or limit mycelium growth inside the leaf.

This ability of different EO against downy mildew has been highlighted for crops other than grapevine. For example in cucumber, castor and clove oils significantly reduced the severity of downy mildew [47]. Similarly, sage EO applied at a concentration of around 1% reduced disease severity of downy mildew on cucumber by almost 100% in greenhouses but only 70%
in the field (40). This illustrates the problem of rainfastness and degradability of EO in field conditions, thereby justifying the vaporisation approach.

Several studies on grapevine have confirmed that EOs could be an efficient alternative treatment against *P. viticola*. Dagostin et al. (2011) used *Salvia officinalis* EO at concentration of 50 mL/L on potted *Pinot gris* vines in greenhouses, and on *Carbernet Sauvignon* in field trials (29). The efficiency was here also much lower in the field due to previously mentioned reasons.

La Torre et al. (2014) used clove and tea-tree oil on leaf discs and in the field on cv. *Malvasia di Candia* against downy mildew and showed that both EOs controlled the development of downy mildew both in situ and *in vivo* (41). An *in vitro* study of direct and vapour phase
application of EOs on Chardonnay leaves, the EOs being of cinnamon, *Eucalyptus globulus* Labill., marjoram, tee-tree, peppermint, oregano and thyme, on *P. viticola* showed very good efficiency of all oils, with cinnamon and *Eucalyptus globulus* EOs as the most fungitoxic [32]. Applying only the vapour phase, has, up to now, never been tested in vivo on *V. vinifera*. Studies on other plants showed that EOs, encapsulated in mesoporous silica and subsequently slowly released as vapour, had direct antifungal properties on *Aspergillus niger* [31]. Similar results were obtained by Soylu et al., (2007, 2010), where the vapour phase generally showed a higher efficacy against *Botrytis cinerea* [27].

This direct antimicrobial or antifungal activity of EOs might mainly be caused by the properties of their terpenes/terpenoids, that—due to their highly lipophilic nature and low molecular weight—are capable of disrupting the cell membrane, causing cell death or inhibiting the sporulation and germination of fungi [48]. Antifungal properties are generally linked to cell membrane disruption, alteration and inhibition of cell wall formation, dysfunction of the fungal mitochondria, inhibition of efflux pumps and / or ROS production. More specifically, the antifungal activity of carvacrol and thymol have often been attributed to their cell membrane damaging effect, because of their interaction with membrane sterols, in particular with ergosterol. Carvacrol would be able to bind with the sterols of the fungal plasma membrane, which would result in damage to the membrane, conducing to the death of the fungus [49]. Thymol seems to affect mycelium morphology, with changes in the localisation of chitin within the hyphae [48, 50].

An exhaustive analysis of scientific literature up to the most recent, does not help to clarify whether EOs trigger the innate immune system of host plants or only have a direct effect on pathogens. To test the hypothesis of EOs being potential primers of plant immunity a transcriptomic analysis was carried out; it is presented in the subsequent chapter.

**Global transcriptomic reprogramming induced by *O. vulgare* vapour treatment**

RNA-seq was carried out on the experiments with *O. vulgare*, where the 24h and the 10d samples were sequenced and analysed for differentially expressed genes (DEGs).

A total number of 94 million reads were sequenced from all samples. Read alignment to the *V. vinifera* genome resulted in 70 to 90% of reads that could be mapped onto the grapevine genome (Fig 3). A second alignment of reads was performed to the *P. viticola* genome, which has been recently published [37], to check the presence or absence of *P. viticola* genes. For the 10d treatment between 25 to 35% of reads from control samples mapped to the *P. viticola* genome, while only 1–4% of reads from the treated plants mapped to the *P. viticola* genome. This strongly demonstrates the absence of *P. viticola* when plants were treated with EO vapour (Fig 3). Interestingly, no difference in mapped reads was found between control and treatment after 24h, where in both cases only a very low number of reads mapped to the *P. viticola* genome, indicating a very low presence of the oomycete inside the host leaves at this early stage of infection, even in control plants. It can thus not be concluded from these present data whether EO treatment had a direct or indirect inhibitory effect on the Oomycete development, since its development even in control plants was too reduced to detect *P. viticola* genes for differential expression analysis.

Principal component analysis on normalised gene expression showed a good correlation of biological replicates with the two first Principal Components (PC), explaining 90% of variance between samples (Fig 4). PC1, explaining 80.33% of the variability in gene expression, separated the EO treatment from the control of the 24h experiment, whereas the second PC accounting only for a 9.47% variation, separated the EO treatment from the control of the 10d
samples. This indicates that EO induced a more important transcriptomic programming during the first 24h of vapour exposure than after the 10d treatment.

DEG analysis of mapped transcripts on the *V. vinifera* genome yielded a total of 4800 DEGs for EO treatments after 24h and 10d (supplemental S2 Table). For the 24h treatment, 1061 DEGs were induced and 1189 transcripts were repressed by *O. vulgare* EO, whereas for the 10d treatment 1210 DEGs were upregulated and 807 DEGs were downregulated, respectively (Fig 5). Interestingly, the number of concomitantly deregulated genes at 24h and 10d was very low. Only 40 genes were downregulated, while 37 genes were upregulated, being common to 24h and 10d treatments. In the meantime, respectively 63 and 37 genes were inversely expressed between the 24h and 10d treatments. This highlights a brief early response of the plant to EO vapour, which is only maintained during a limited time span (0 to > 24h after treatment) and is then followed by an adaptation period, where many early elicited genes return to the pre-treatment expression and a somehow long-term adaptation takes over. This early transcriptomic reprogramming conditioned by EO vapour might thus base its main effect on an eventual plant innate immunity priming.

This is in some way confirmed by analysing the enriched functional categories (FC) within DEGs (Fig 6A and 6B), where for the 10d treatment, no significant enriched FC could be
detected. This indicates that the long-term (10d) treatment did trigger genes that are not, or less, collectively regulated within the main metabolic pathways. It would thus be likely that the observed gene deregulation by EO in the 10d treatment is somehow random. However, for the 24h treatment several categories were significantly and highly enriched in upregulated genes (Fig 6A) as well as in downregulated genes (Fig 6B). This observation is discussed in subsequent sections below.

Hierarchical clustering of DEGs, as illustrated by the heatmaps in Fig 7A and 7B, showed the higher number of upregulated genes for each treatment, in both 24h and 10d experiment conditions (supplemental S3 Table). The stringent grouping of genes detected in biological triplicates highlighted the rightness and strong significance of these results. Functional enrichment analysis of genes within each cluster, shown in Fig 8, confirmed the results obtained on total up- and downregulated transcripts as discussed above, since no significant FCs could be detected within the 10d clusters.

_O. vulgare_ vapour triggers innate plant defense mechanism controlled by hormonal signaling leading to the stimulation of phenylpropanoids

It is well established that plant responses to biotic and abiotic stress stimuli are mediated by defense-related phytohormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), which act as primary signals in the regulation of plant defense [17]. However, the crosstalk of hormones and downstream-induced responses are still far from being completely elucidated [51]. The current state of knowledge is that SA-signaling mediates resistance to biotroph and hemibiotroph pathogens, whereas JA- and ET-related pathways activate resistance against necrotrophs, with SA-JA crosstalk being the backbone of the plant’s immune signaling network [52].

The overview of enriched functional categories of upregulated genes, given in Fig 6A and 6B, unambiguously indicated that the innate plant immune system was primed by 24h EO
vapour treatment with several highly activated metabolic pathways linked to the above-mentioned hormonal defense regulation (JA- and ET-mediated signaling), biotic stress response and secondary metabolism related to phenolic compound synthesis.

Jasmonic acid

Amongst defense-related phytochromes, JA is thought to play an essential role in response to tissue wounding, by regulating gene expression to redirect metabolism towards producing defense molecules and repairing damage [53].

JA biosynthesis starts with the release of α-linolenic acid from galacto- and phospholipids localised on the chloroplast membrane by the action of phospholipases, which are subsequently oxidised by Lipoygenase (LOX) leading to 13-hydroperoxy-9,11,15-octadecatrienoic acid (13HPOT). Two different enzyme families, termed Allene Oxyde Synthase (AOS) and Allene Oxyde Cylase (AOC), successively convert 13-HPOT into the stable cis(+)-oxophytodienoic

---

Fig 5. Venn diagram of differentially expressed genes (DEGs, pval>0.01, lfc<0.5) upon O. vulgare vapour treatment: 24h_up and 24h_down: up- respectively downregulated genes after 24h treatment; 10d_up and 10d_down: up- respectively downregulated genes after 10d treatment.

https://doi.org/10.1371/journal.pone.0222854.g005
A

Protein transport. Tethering factors
Phosphate carrier
Phosphate: H+ Symporter
Protein kinase
Calcium sensors and Signaling
Jasmonate-mediated Signaling pathway
Ethylene-mediated Signaling pathway
Biotic stress response
WRKY family transcription factor
NAC family transcription factor
Anthocyanin-glycoside biosynthesis
HSP-mediated protein folding
Glycerolipid catabolism
Glutathione metabolism
Amino sugar metabolism
Cell death

fold change

B

Oxidase-dep Fe2+ Transporter
a-Type channels.Major Intrinsic Protein
Light Signaling
Auxin-mediated Signaling pathway
GRF family transcription factor
Fatty acid biosynthesis
Photosystem I
Photosynthetic-chain phosphorylation
Antenna proteins
Chlorophyll biosynthesis
Cell wall structural protein
Xyloglucan modification

fold change
acid (cis-OPDA) intermediate. This latter is reduced by an Opda Reductase (OPR) and then undergoes three rounds of \( \beta \)-oxidation by Acyl-CoA Oxidase (ACX) enzymes leading to the production of JA [54].

We showed here that EO vapour treatment triggered the expression of 3 key enzymes of the JA pathway, with LOX (VIT_14s0128g007901), being the key enzyme, followed by AOX (VIT_18s0001g11630) and ACX3 (VIT_12s0028g02660). This points out that JA biosynthesis was highly induced by the EO treatment. Other studies reported a similar induction of LOX leading to a stimulation of induced resistance against *P. viticola* upon thiamin treatment of grapevines [20, 55].

Several downstream regulated genes of the JA pathway, putatively mediated by JA-signaling, were highly upregulated in EO treated plants. Amongst these latter, several isogenes of Enhanced Disease Susceptibility (EDS1; VIT_17s0000g07400, VIT_17s0000g07420, VIT_17s0000g07370) also known as Nonexpressor of PR Genes 1 (NPR1), or Non-inducible Immunity 1 (NIM1) or Salicylic Acid Insensitive 1 (SAI1), which is an important redox sensitive transcriptional regulator of SA response and a mediator of SA-JA crosstalk [56]. This NPR1 gene is thought to be a central immune regulator for systemic-acquired resistance (SAR) and is considered as crucial for the regulation of Pathogenesis Related (PR) genes expression [56–58].

Consequently, several *PR Proteins 1* (PR1) transcripts (VIT_03s0088g00700, VIT_03s0088g00750, VIT_03s0088g00710), as well as several members of the JASMONATE-ZIM DOMAIN (JAZ) protein family (VIT_10s0003g03790, VIT_10s0003g03800, VIT_11s0016g00710, VIT_09s0002g00890) were induced. This latter JAZ family has been recently discovered and characterised [59] as a family of proteins orchestrating the crosstalk between JA and other hormone-signaling pathways such as ET, SA, gibberellin, and auxin. JAZs are transcriptional regulators that target different *bHLH* TFs, such as MYCs [60], which appeared as upregulated in the present data, too (VIT_02s0012g01320). This MYC branch of the JA-signaling pathway is typically activated upon wounding or feeding by herbivorous insects. The latter branch is furthermore thought to be antagonistically regulated to the...
ethylen-responsive transcription factor (ERF) branch [61]. However, this has not been confirmed by the present study since ERFs are also highly activated as discussed in the ET section. Other important regulators of the interaction between the SA and JA pathways have been identified and belong to the WRKY and TGA TF families [62]. In O. vulgare EO treated plants a very high number of WRKYs such as WRKY 6; 11; 23; 33; 40; 47;48; 51; 53; 55; 65; 70; 72 and 75, were concomitantly upregulated with 2 WRKY33 isogenes (VIT_08s0058g00690 and VIT_06s0004g07500) being amongst the highest upregulated ones (28 and 11 fold, respectively).
In general, WRKY TFs are a large family of regulatory proteins that are involved in various plant processes, but most notably in coping with diverse biotic and abiotic stresses. WRKY33 functions as a positive regulator of resistance toward the necrotrophic fungi such as Alternaria brassicicola and Botrytis cinerea, [63] and was found to be induced by EO treatment in the present study.

In Arabidopsis thaliana L., WRKY70 (2 upregulated isogenes: VIT_08s0058g01390, VIT_13s0067g03140) acts at a convergence point determining the balance between SA- and JA-dependent defense pathways while required for R-gene mediated resistance [64] and, together with WRKY53 (VIT_02s0025g01280), positively modulate SAR [65]. Moreover, SA biosynthesis and expression of NPR1 also appear to be regulated by WRKY TFs [66]. Whether WRKY70 is indispensable for JA- and SA-signaling has, however, recently been questioned [66]. This has not been confirmed by the present data, where both signaling pathways are highly activated concomitantly with WRK70.

**Salicylic acid**

SA biosynthesis is triggered during PTI and ETI upon recognition of PAMPs or effectors of pathogens. SA is a phenolic compound that can be synthesised from the primary metabolite chorismate via two distinct enzymatic pathways, one involving Phenylalanine Ammonia Lyase (PAL) and the other Isochorismate Synthase (ICS/SID2). Diverse PAL coding isogenes (VIT_16s0039g01280, VIT_16s0039g01120, VIT_16s0039g01240, VIT_16s0039g01100, VIT_11s0016g01520, VIT_11s0016g01660, VIT_11s0016g01640, VIT_16s0039g01170) were found amongst the highest induced genes upon EO treatment. Other studies showed that a high induction of the phenylpropanoid pathway and downstream reactions also triggers, besides SA production, the production of many phytoalexins. For example, a high PAL activation, leading to an enhanced phenolic compound accumulation, provided a fair protection against leaf stripe caused by Drechsiara graminea, as reported on barley treated with aqueous leaf extract of Azadirachta indica Juss. [67].

Besides enzymes that directly contribute to SA biosynthesis (ICS and PAL), other proteins have been identified as participating in pathogen-induced SA accumulation. These include EDS1/NPR1 (as already discussed above for JA) and PAD4 (Phytoalexin Deficient 4; VIT_07s0031g02390), highly upregulated by EO vapour [68]. Similar results were observed when A. thaliana was treated with Gaultheria procumbens L. EO, inducing an SA-mediated defense response and resistance to Colletotrichum higginsianum Sacc. [69]. However, these authors demonstrate that this effect is mainly due to the methyl salicylate present in the used EO, which triggered the SA-mediated defense response. Another microarray study was carried out on A. thaliana plants treated with different commercial herbal plant preparations, and showed that SA- and JA defense responses could be induced together [70]. As far as we know, the induction of an SA-JA-mediated defense response triggered by the vapour phase of O. vulgare has never been reported previously.

Other SA-related enzymes, which play an important role in plant-pathogen interactions are Glutathione S-Transferases (GSTs). In the present study, several tau class GSTs, GSTUs (GSTU 1; 8; 9; 10; 20; 22) and a very high number of GSTU 25 isogenes were the most upregulated transcripts, particularly a VIT_19s0015g02730, a GSTU 25, which were induced more than 128 times. Elevated GST activities have often been observed in plants treated with such beneficial microbes (bacteria and fungi), resulting in the induction of a systemic resistance response to subsequent pathogen infections. Their confirmed roles in biotic and abiotic stress tolerance are due to a detoxification capacity by conjugation with glutathione, the attenuation of oxidative damage and their contribution in hormone transport. The exact metabolic functions and their contribution to disease resistance in plants, however, remain to be elucidated [71].
Ethylene and Ethylene-mediated signaling

ET is a principal and pleiotropic modulator of many aspects of plant life, including various mechanisms by which plants react to pathogen attacks. ET leads to a cascade of transcription factors (TF) consisting of primary Ethylene-insensitive (EIN) 3-like regulators and downstream ERF (Ethylene Response Factor)-like TF, controlling the expression of various effector genes involved in various aspects of systemic-induced defense responses. Moreover, as discussed above, a significant cross-talk occurs with other defense response pathways controlled by SA and JA, eventually resulting in a differentiated response to disease that is not very well understood so far.

ET biosynthesis involves the conversion of S-adenosyl-Met (SAM) to 1-aminocyclopropane-1-carboxylate (ACC) and methylthioadenosine, performed by the enzyme ACC synthase (ACS) and a further step where ACC is converted to ET, CO₂, and cyanide by 1-aminocyclopropane-1-carboxylate oxidase (ACO), whereas conversion of AdoMet to ACC by ACS is generally considered the rate-limiting step [51].

In our study, upon *O. vulgare* EO treatment, several ACOs (VIT_02s0012g00400, VIT_05s0049g00410, VIT_01s0011g05650, VIT_12s0059g01380) and one ACS 1 (VIT_02s0025g00360) were induced, indicating that ET synthesis was activated by the EO treatment. Interestingly, a recently discovered TF, MBF1c (VIT_11s0016g04080), which acts upstream of ET and SA, and was thought to be only involved in abiotic stress responses such as heat stress in *A. thaliana* [72, 73] and grapevine [74–77], was found to be upregulated here.

Accordingly, a cascade of ethylene-controlled ERFs was highly upregulated, notably an *Apetala2* gene (AP2)/ERF (VIT_11s0016g00660), which was overall the second most upregulated transcript in the 24h treatment. The AP2/ERF superfamily plays a pivotal role in adaptation to biotic stresses [78], emphasising that the innate immune response of the plant is highly active. This was confirmed by other highly induced ERFs, notably an ERF109 (VIT_03s0063g00460), which plays an important role in abiotic stress adaptation, as reported for salt tolerance [79]. Two other ERFs related to AP2 (VIT_18s0072g00260 and VIT_11s0016g00670) were simultaneously upregulated up to 50 times by the EO treatment. Interestingly, ERF 105 VIT_16s0013g00980 seems to play a complex role in biotic and abiotic regulation in different tissues, just as it has been shown, for example, to be highly heat stress responsive [76], as well as circadianly regulated in grape berry tissue [75].

In general, ERFs act as a key regulatory hub and integrate ET, ABA, JA, and redox signaling in the plant response to a number of abiotic and biotic stresses, such as those caused by pathogens, wounding, cold and heat stress, UV light, drought, and salinity [78]. Their upregulation in the present experiment have given an indication of their role within the regulation of the innate immune system upon exogenous elicitors and pointed out their important role within plant defense mechanism to biotic stresses. The set of target genes regulated by each ERF has not yet been completely elucidated, and the present gene expression data might contribute to the understanding of complex coregulations. ERFs activate the transcription of basic type defense-related genes, such as pathogenesis-related (PR) genes, osmotins, chitinase and β-1,3-glucanase genes. This also happened in the present study where several PRs putatively controlled by ERF, such as β-1,3-glucanases (VIT_06s0061g00120, VIT_08s0007g06060, VIT_08s0007g06040), chitinases (VIT_05s0094g00220, VIT_16s0050g02230) and osmotins (VIT_02s0025g04280, VIT_02s0025g04250), were simultaneously upregulated.

These latter are grouped in different PR families. Chitinases, belonging to the PR-3 protein group, are hydrolytic enzymes that break down glycosidic bonds in chitin, which is a major component of the cell wall of pathogenic fungi. Chitinases make the fungi inactive without any negative impact on the plants and can thus enhance the plant’s defense system [80]. The fact
that *P. viticola* is an oomycete and, as such, does not contain chitin, highlights that the triggered plant immune response is unspecific to the pathogen and might thereby impede infection with other pathogens as well. The \( \beta-1,3\)-glucanases are grouped in the PR-2 family of PR proteins and are involved in plant defense by hydrolysing the cell walls of fungal pathogens most commonly in combination with chitinases. *In vitro* analysis has shown that \( \beta-1,3\)-glucanases directly act on fungal pathogens by degrading \( \beta-1,3/1,6\)-glucans and that chitinases act by attacking the bond between the C1 and C4 of two consecutive N-acetylglucosamines of chitins in the fungal cell wall [81].

It was shown that they also indirectly act as defense mechanisms elicitors by releasing \( \beta-1,3\)-glucan and chitin oligosaccharides [81]. Glucans have been shown to play a major role in grapevine immunity against *P. viticola* and could be elicited by treatment with sulfated laminarin [82]. We here show the possibility that these defense PRs could be triggered by EO treatments.

Osmotins are PR-5s proteins and have been shown to play an important role in response to biotic and abiotic stresses in plants, which role is mainly activated by MAPK (Mitogen-activated protein kinases) pathways. Osmotin proteins enter the fungal plasma membrane and activate the defense system. They are also involved in the initiation of apoptosis and PCD [81].

Their expression was shown to be induced by a number of different signals as, for instance, by SA, ABA, auxin, ET, salinity, drought conditions, UV light, wounding, desiccation, cold, fungal infection, oomycetes, bacteria and viruses. The present data suggests that osmotin synthesis would also be triggered by *O. vulgare* vapour which, as far as we know, has never been reported in literature before.

**Mitogen-activated protein kinases (MAPKs) and CA-signaling induction**

MAPKs are important regulators of plant immunity that generally transduce extracellular stimuli into cellular responses. These stimuli include the perception of PAMPs by host transmembrane pattern recognition receptors, which leads to PTI. In the A. thaliana model, molecular genetic evidence implicates a number of MAPK cascade components in PAMP signaling, and in responses to immunity-related phytohormones such as ET and SA [83]. In *Arabidopsis*, MPK3, MPK6, MPK4, and MPK11 are rapidly activated during PTI [84] but also in ETI, where MPK3/MPK6 seem to play an essential role [85].

In response to *O. vulgare* EO treatment, several MAPK cascade members, such as a MPK3 (*VIT_06s0004g03540*) and a MPK13 (*VIT_06s0004g03620*) were upregulated. Shoresh et al. (2006) Shoresh, Gal-On (86) Shoresh, Gal-On (86) Shoresh, Gal-On (86) Shoresh, Gal-On (86) showed that MPK3 orthologues conferred immunity to cucumber plants against *Pseudomonas syringae pv. lachrymans* [86] and their overexpression created autoimmune phenotypes characterised by dwarfism associated with spontaneous cell death, accumulations of ROS and SA, and modification of phytoalexin metabolism [87].

In addition to MAPK cascade signaling, PAMP perception was shown to induce Ca\(^{2+}\) dependent kinases (CDPKs) by regulating Ca\(^{2+}\) influx channels [88]. Recent findings indicate that Ca\(^{2+}\)-ATPases regulate Ca\(^{2+}\) efflux as well as innate immune defenses [89, 90]. Our results pointed in the same direction with an upregulation of Fcs related to calcium sensors and Ca-signaling (Fig 6) upon *O. vulgare* EO treatment. Within these categories several *Calmodulin* iso- genes were found to be highly overexpressed, as well as Ca\(^{2+}\)-ATPases (*VIT_05s0020g04300, VIT_07s0129g00180, VIT_07s0129g00110*) and several transcripts, coding for Calcium-binding proteins (*VIT_01s0010g02950, VIT_08s0056g00290 VIT_01s0026g02590*).

Hypersensitive Response (HR) is associated with the innate immune response of plants and commonly regarded as a feature of ETI. This particular response involves programmed cell
death (PCD) and occurs at the point of pathogen entry, resulting in an efficient containment of the pathogen [91]. Reactive Oxygen Species (ROS) have been well established as an integral aspect of plant immunity in a process generally described as the oxidative burst involved in PCD and thus in HR [92]. In the present study, the apoptosis associated FC was highly enriched in EO-triggered genes, pointing out that this part of the innate immune response was activated along with a Respiratory Burst Oxidase Protein F (RBOHF) transcripts (VIT_02s0025g00510), known to be regulated by abscisic acid (ABA), whose biosynthetic enzyme 9-cis-epoxycarotenoid dioxygenase (NCED) VIT_19s0093g00550 was induced as well. This highlighted a contradiction sometimes encountered in transcriptomic literature, where JA/ET are often reported to be antagonistically regulated, notably in abiotic stress studies [93]. Other genes related to ROS production, such as dicyanin, dicyanin blue copper protein, glutaredoxin, as well as several peroxidases and an alternative oxidase (VIT_02s0033g01380, 18 fold upregulation), which are crucial in the early generation of mitochondrial ROS and precede fungal elicitor-induced HR or a fungal toxin-induced cell death [94], were found upregulated, which highlighted that the oxidative stress response was triggered by EO vapour. This was concomitant with the upregulation of several Heat Shock TFs, which can function as molecular sensors that directly sense ROS and control the expression of oxidative stress response genes during oxidative stress (Miller and Mittler, 2006).

**Phenylpropanoid synthesis is highly activated by O. vulgaris vapour**

Phytoalexins are defined as low-weight antimicrobial secondary metabolites that are synthesized and accumulate in plants after pathogen attacks, but can be activated by PTI [95] as well. Many reported phytoalexins represent phenylalanine-derived phenylpropanoids. As previously discussed, this latter pathway regulated by the key enzyme PAL was highly activated upon EO treatment. Downstream of PAL, the rate-limiting enzyme of flavonoid/isoflavonoid biosynthesis, chalcone synthase (CHS; VIT_16s0100g00860), was highly induced as well, indicating an enhanced synthesis of those phenolic compounds. Besides being part of the plant developmental programme, the CHS gene expression was reported to be induced in plants under stress conditions such as drought, UV light, bacterial or fungal infection [96]. CHS expression causes accumulation of flavonoid and isoflavonoid phytoalexins and is involved in the SA defense pathway [95, 97]. Respectively a flavanol synthase coding transcript (VIT_18s0001g03430), as well as a Quercetin 3-O-methyltransferase (VIT_10s0042g00890; VIT_10s0042g00870) were amongst the top ten induced genes, two Leucoanthocyanidin dioxygenase (LAR) coding isogenes (VIT_11s0078g00360) and several genes related to Anthocyanin biosynthesis, were concomitantly induced by the EO treatment. Studies on cucumbers showed a similar increase of PAL activity and other genes of the flavonoid pathway, after application of Milsana®), a resistance inducer, also known for its direct antimicrobial effect [98].

Amongst the most important representative of phenolpropanoids-derived phytoalexins are stilbenes, notably resveratrol, which accumulates following infection in several phylogenetically unrelated species, including grapevine [99]. The biosynthesis of this secondary metabolite requires the presence of only one unique enzyme, stilbene synthase (STS), which has thus become a feasible molecule for metabolic engineering. For example, heterologous expression of the grapevine STS gene VST1 under control of its native promoter in tobacco (Nicotiana tabacum) has led to pathogen-inducible biosynthesis of resveratrol, which correlated with enhanced disease resistance to B. cinerea. Upon O. vulgaris treatment, 9 isogenes coding for STSs were upregulated to very high levels such as VIT_10s0042g00890 and VIT_10s0042g00870 with a respectively 42- and 32-fold induction. In addition, several Myb14 TF coding isogenes (VIT_07s0005g03340,
VIT_12s0134g00480, VIT_07s0005g03340) were highly upregulated (20 to 22 fold). The latter is, together with MYB15, the transcriptional regulator of stilbene biosynthesis in grapevine [100]. Enhanced accumulation of stilbenic phytoalexins has been shown to be implicated in the resistance of grapevine cultivars to three major fungal pathogens, B. cinerea, P. viticola and Erysiphe necator (powdery mildew) [10], and could thus be amongst the main reasons why P. viticola development was inhibited under O. vulgare treatment. An overview of deregulated transcripts within the phenylpropanoid pathway is given as a supplemental in S1 Fig.

Stimulation of the phenylpropanoid pathway and notably stilbene synthesis is highly regulated by ET and JA, as shown by exogenous application of JA or ET in several studies [101–103], which also seems to be the case in the present experiment.

O. vulgare impedes the photosynthetic machinery and cell wall synthesis related processes

Visual observation as well as photosynthesis (PS) measurements (Fig 2) during EO vaporisation showed a phytotoxic effect of EOs on vine physiology, which correlated with gene expression data.

Several FC related to PS are enriched in repressed genes, as shown in Fig 6B (antenna proteins, light signaling, PSI, PS chain-reaction, Chlorophyll biosynthesis). Within these categories, 18 light-harvesting complex (LHCs) coding transcripts were repressed by EO treatment after 24h. Interestingly, some were no longer repressed or even induced after a 10d treatment, as is the case of LHCB6 (VIT_12s0055g01110). A similar observation can be made regarding genes involved in PSI (Photosystem I reaction center subunit genes), and PSII genes linked to photosynthetic-chain phosphorylation and chlorophyll biosynthesis, that were systematically repressed after a 24h treatment but no longer after 10d. This would indicate that the primary reactions transiently disappear with an increased duration of treatment and suggest some kind of adaptation to EO vapour.

A direct phytotoxic effect of EO has previously been reported in several studies [104] and mainly attributed to carvacrol and thymol [105–107], which are also the main terpenes of the applied EO in the present study.

However, downregulation of PS and PS-related processes could also be part of the innate immune plant response, which has been described in several studies. In particular, upregulation of MPK6, as discussed above, has been associated with an altered expression of photosynthesis-related genes and inhibition of photosynthesis [85].

Conclusion

The need for sustainable alternatives to replace or reduce the use of synthetic pesticides in agriculture is of utmost urgency. EOs that were shown to possess antifungal properties in many previous studies, could potentially represent a natural strategy to replace or at least reduce the use of synthetic fungicides. However, their adhesiveness and stability on the plant is very bad, when applied in their liquid phase. The present study is, as far as we know, the first one to test and confirm that the vapour phase of EOs is in vivo highly efficient against the major pathogen P. viticola, the causing agent of downy mildew. This would offer new alternatives in the development of innovative plant protection strategies that would involve fumigation systems in greenhouses or dispensers in field-grown crops, using volatile organic compounds from plant EOs.

Even more significantly, the present study provides important information regarding the underlying mechanisms in the host-pathogen-EO interaction and shows clearly that the O. vulgare vapour triggered a multilayered immune system of the plants. Gene expression analysis revealed a complex activation of hormonal crosstalk involving JA, ET, and SA biosynthesis.
and their signaling cascades. This led to the activation of different immune mechanisms involving PR genes activation, flavonoids and stilbene synthesis, as well as transcriptional networks in relation to PCD and apoptosis. Not only does O. vulgare vapour, or one or several of its constituents, seem to act as PAMPs to trigger PTI, but also as an elicitor that transiently triggers ETI. This complex interaction is confirmed by numerous studies that have shown that ETI, basal defense and PTI use a common set of signaling components including multiple regulatory proteins, reactive oxygen intermediates (ROIs), as well as the phytohormones salicylic acid (SA), ethylene (ET) and jasmonic acid [17]. Starting from these results a next step would be to select between 5–10 marker genes of the plant immune system, for which expression was enhanced, in order to allow for a quick and easier follow-up of EOs application experiments though qRT-PCR amplification of these genes.

To what extent the inhibitory effect of EO vapour on P. viticola development was due to a direct toxicity of the EO vapour on the pathogens or to the stimulation of the innate immune system of vines, could not be clearly elucidated with the applied methodology.

The study, however, provides important molecular data, such as target genes, gene networks and metabolic pathways involved in the innate immune system of the plant and therefore important for future genetics studies and resistance-breeding programmes [108–110]. The long term objective is to understand better the effects of EOs and to identify, if possible, which of their terpene components, are the most effective and in the mean time to develop systems that would allow for EOs application to reduce the need of pesticide applications. Among the potential strategies to be tested, some sort of field dispensers similar to pheromone dispenser already used against the grape berry moth, and continuously dispersing effective EOs or EOs components could be tested in a quite close future, in fields or greenhouses. Though it is not expected that such systems completely inhibit infections, they probably could lower the disease pressure and help to reduce systemic fungicides. Testing EO emitting plants in co-plantation in greenhouses or in the fields, should also be explored, particularly in organic production systems.

Supporting information

S1 File. Picture of the custom-made climatic chamber and vaporisation system.
(PDF)

S1 Fig. Cytoscape graph of affected transcripts within the phenylpropanoid synthesis pathway. Blue: repressed, red: induced transcripts by 24h EO vapour treatment. Parallelograms: RNA; Round rectangles: proteins; Ellipse: simple molecules; Diamonds: state transitions, transcription and translation.
(PDF)

S1 Table. Oil composition and concentration inside the chamber.
(XLSX)

S2 Table. Differentially expressed genes with annotations and clusters.
(XLSX)

S3 Table. Genes allocated to clusters of Fig 7A and 7B.
(XLSX)

Acknowledgments

The authors are grateful to the HealthFood Research Programme of the HES-SO University of Applied Sciences and Arts Western Switzerland for funding this study. The authors also
gratefully acknowledge Marylin Cléroux for the GC-FID analysis, Arnaud Pernet for constructing the vaporisation system, Jean-Philippe Burdet for fruitful discussions and suggestions, Raphael Gonzales and Eric Remolif for support with plant cutting preparations and Romain Chablais for technical assistance during sequencing work. Furthermore, authors would like to thank the COST (European Cooperation in Science & technology)—action CA17111 –INTEGRAPE that permitted valuable exchange with another scientist on data analysis and integration and the Swiss Government excellence scholarship for financing the postdoctoral position of Dr. Sana Ghaffari.

Author Contributions

Conceptualization: Markus Rienth, François Lefort.
Data curation: Markus Rienth.
Formal analysis: Markus Rienth, Julien Crovadore, Sana Ghaffari.
Funding acquisition: Markus Rienth, François Lefort.
Investigation: Markus Rienth, Sana Ghaffari.
Methodology: Markus Rienth, Julien Crovadore.
Project administration: Markus Rienth.
Resources: Markus Rienth, François Lefort.
Software: Markus Rienth.
Supervision: Markus Rienth.
Validation: Markus Rienth.
Visualization: Markus Rienth.
Writing – original draft: Markus Rienth, François Lefort.
Writing – review & editing: Markus Rienth, François Lefort.

References

1. Gisi U, Sierotzki HJEJoPP. Fungicide modes of action and resistance in downy mildews. European Journal of Plant Pathology. 2008; 122(1):157–67. https://doi.org/10.1007/s10658-008-9290-5
2. Kim K-H, Kabir E, Jahan SA. Exposure to pesticides and the associated human health effects. Science of The Total Environment. 2017; 575:525–35. https://doi.org/10.1016/j.scitotenv.2016.09.009 PMID: 27614863
3. Gessler C, Pertot I, Perazzolli M. Plasmopara viticola: a review of knowledge on downy mildew of grapevine and effective disease management. Phytopathologia Mediterranea. 2011; 50(1).
4. Burrano S. The life-cycle of Plasmopara viticola, cause of downy mildew of vine. Mycologist. 2000; 14:179–82.
5. Furuya S, Mochizuki M, Saito S, Kobayashi H, Takayanagi T, Suzuki S. Monitoring of QoI fungicide resistance in Plasmopara viticola populations in Japan. Pest management science. 2010; 66 (11):1268–72. Epub 2010/08/28. https://doi.org/10.1002/ps.2012 PMID: 20799246.
6. Chen W-J, Delmotte F, Cervera SR, Douence L, Greif C, Corio-Costet M-F. At Least Two Origins of Fungicide Resistance in Grapevine Downy Mildew Populations. Appl Environ Microbiol. 2007; 73 (16):5162–72. https://doi.org/10.1128/AEM.00507-07 J Applied and Environmental Microbiology. PMID: 17586672
7. Ballabio C, Panagos P, Lugato E, Huang J-H, Orgiazzi A, Jones A, et al. Copper distribution in European topsoils: An assessment based on LUCAS soil survey. Science of The Total Environment. 2018; 636:282–98. https://doi.org/10.1016/j.scitotenv.2018.04.268 PMID: 29709848
25. Soylu EM, Kurt Ş, Soylu S. In vitro and in vivo antifungal activities of the essential oils of various plants against tomato grey mould disease agent Botrytis cinerea. International Journal of Food Microbiology. 2010; 143(3):183–9. https://doi.org/10.1016/j.ijfoodmicro.2010.08.015 PMID: 20826030

26. Pina-Vaz C, Goncalves Rodrigues A, Pinto E, Costa-de-Oliveira S, Tavares C, Salgueiro L, et al. Antifungal activity of Thymus oils and their major compounds. J Eur Acad Dermatol Venereol. 2004; 18 (1):73–8. Epub 2003/12/18. PMID: 14679356.
27. Soylu S, Yigitbas H, Soylu EM, Kurt S. Antifungal effects of essential oils from oregano and fennel on Sclerotinia sclerotiorum. Journal of applied microbiology. 2007; 103(4):1021–30. Epub 2007/09/28. https://doi.org/10.1111/j.1365-2672.2007.03310.x PMID: 17897206.

28. Soylu EM, Kurt S, Soylu S. In vitro and in vivo antifungal activities of the essential oils of various plants against tomato grey mould disease agent Botrytis cinerea. Int J Food Microbiol. 2010; 143(3):183–9. Epub 2010/09/10. https://doi.org/10.1016/j.ijfoodmicro.2010.08.015 PMID: 20826038.

29. Dagonis S, Formolo T, Giovannini O, Pertot I, Schmitt A. Salvia officinalis Extract Can Protect Grape-Vine Against Plasmopara viticola. Plant Disease. 2010; 94(5):575–80. https://doi.org/10.1094/PDIS-94-5-0575 PMID: 30754462

30. Turek C, Stintzing FC. Stability of Essential Oils: A Review. 2013; 12(1):40–53. https://doi.org/10.1111/1541-4337.12006

31. Janatova A, Bernards A, Smid J, Frankova A, Lhotkova L, et al. Long-term antifungal activity of volatile essential oil components released from mesoporous silica materials. Industrial Crops and Products. 2015; 67:216–20. https://doi.org/10.1016/j.indcrop.2015.01.019.

32. Oliveira Fialho R, Stradioto Papa MdF, Rodrigo Panosso A, Rodrigues Cassiolato A. Fungitoxicity of essential Oils on Plasmopora viticola, causal agent of grapevine downy mildew. Rev Bras Frutic., 2017; 39(4).

33. Burketova L, Trda L, Ott PG, Valentova O. Bio-based resistance inducers for sustainable plant protection against pathogens. Biotechnol Adv. 2015; 33(6 Pt 2):994–1004. Epub 2015/01/27. https://doi.org/10.1016/j.biotechadv.2015.01.004 PMID: 25617462

34. Sakkas H, Papadopoulou C. Antimicrobial Activity of Basil, Oregano, and Thyme Essential Oils. Journal of microbiology and biotechnology. 2017; 27(3):429–38. Epub 2016/12/21. https://doi.org/10.4014/jmb.1608.08024 PMID: 27994215.

35. Rienhardt M, Torregrosa L, Ardisson M, De Marchi R, Romieu C. Versatile and efficient RNA extraction protocol for grapevine berry tissue, suited for next generation RNA sequencing. Australian Journal of Grape and Wine Research. 2014; 20(2):247–54. https://doi.org/10.1111/agw.12077

36. Leinonen R, Sugawara H, Shumway M. The sequence read archive. Nucleic Acids Res. 2011; 39(Database issue):D19–21. Epub 2010/11/11. https://doi.org/10.1093/nar/gkq1019 PMID: 21062823; PubMed Central PMCID: PMC3013647.

37. Yin L, An Y, Qu J, Li X, Zhang Y, Dry I, et al. Genome sequence of Plasmopara viticola and insight into the pathogenic mechanism. Scientific Reports. 2017; 7:46553. https://doi.org/10.1038/srep46553

38. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat Methods. 2015; 12(4):357–60. Epub 2015/03/10. https://doi.org/10.1038/nmeth.3317 PMID: 25516281

39. Al-Shahrour F, Diaz-Uriarte R, Dopazo J. FatigOG: a web tool for finding significant associations of Gene Ontology terms with groups of genes. Bioinformatics. 2004; 20(4):578–80. Epub 2004/03/03. https://doi.org/10.1093/bioinformatics/btg455 PMID: 14990455.

40. Grimplet J, Van Hemert J, Carbonell-Bejerano P, Diaz-Riquelme J, Dickerson J, Fennell A, et al. Comparative analysis of grapevine whole-genome gene predictions, functional annotation, categorization and integration of the predicted gene sequences. BMC Research Notes. 2012; 5(1):213. https://doi.org/10.1186/1756-0500-5-213 PMID: 22554261

41. Bish D, Chanotiya CS, Rana M, Serwai M. Variability in essential oil and bioactive chiral monoterpene compositions of Indian oregano (Origanum vulgare L.) populations from northwestern Himalaya and their chemotaxonomy. Industrial Crops and Products. 2009; 30(3):422–6. https://doi.org/10.1016/j.indcrop.2008.07.014.
46. Boruca O, Jianu C, Misca C, Golet I, Gruia AT, Horhat FG. Thymus vulgaris essential oil: chemical composition and antimicrobial activity. Journal of medicine and life. 2014;7 Spec No. 3:56–60. Epub 2014/01/01. PMID: 25870697; PubMed Central PMCID: PMC4391421.

47. Mohamed A., Hamza A., A D. Recent approaches for controlling downy mildew of cucumber under greenhouse conditions. 52:1–9. Plant Protect Sci. 2016; 52:1–9.

48. Nazzaro F, Fratianni F, Coppola R, Feo VD. Essential Oils and Antifungal Activity. Pharmaceuticals (Basel, Switzerland). 2017; 10(4):86. https://doi.org/10.3390/ph10040086 PMID: 29099084.

49. Nobrega RO, Teixeira AP, Oliveira WA, Lima EO, Lima IO. Investigation of the antifungal activity of carvacrol against strains of Cryptococcus neoformans. Pharmaceutical biology. 2016; 54(11):2591–6. Epub 2016/05/27. https://doi.org/10.3109/13880209.2016.1172319 PMID: 27225838.

50. Chavan PS, Tupe SG. Antifungal activity and mechanism of action of carvacrol and thymol against vineyard and wine spoilage yeasts. Food Control. 2014; 46:115–20. https://doi.org/10.1016/j.foodcont.2014.05.007.

51. Broekaert WF, Delaure SL, Bolle MFCD, Cammue BPA. The Role of Ethylene in Host-Pathogen Interactions. 2006; 44(1):393–416. https://doi.org/10.1146/annurev.phyto.44.070505.143440 PMID: 16602950.

52. Pieterse CMJ, Does DVd, Zamioudis C, Leon-Reyes A, Wees SCMV. Hormonal Modulation of Plant Immunity. 2012; 28(1):489–521. https://doi.org/10.1146/annurev-cellbio-092910-154055 PMID: 22559264.

53. Santino A, Taurino M, De Domenico S, Bonsegna S, Poltronieri P, Pastor V, et al. Jasmonate signaling in plant development and defense response to multiple (a)biotic stresses. Plant Cell Rep. 2013; 32(7):1085–98. Epub 2013/04/16. https://doi.org/10.1007/s00299-013-1441-2 PMID: 23584548.

54. Larrieu A, Vernoux T. Q&A: How does jasmonate signaling enable plants to adapt and survive? BMC biology. 2016; 14:79–. https://doi.org/10.1186/s12915-016-0308-8 PMID: 27643853.

55. Boubakri H, Wahab MA, Chong J, Bertsch C, Mliki A, Soustre-Gacougnolle I. Thiamine induced resistance to Plasmopara viticola in grapevine and elicited host-defense responses, including HR like-cell death. Plant Physiol Biochem. 2012; 57:120–33. Epub 2012/06/16. https://doi.org/10.1016/j.plaphy.2012.05.016 PMID: 22698755.

56. Pajerowska-Mukhtar KM, Emerine DK, Mukhtar MS. Tell me more: roles of NPRs in plant immunity. Trends Plant Sci. 2013; 18(7):402–11. Epub 2013/05/21. https://doi.org/10.1016/j.tplants.2013.04.004 PMID: 23683896.

57. Spoel SH, Koornneef A, Claessens SMC, Korzelius JP, Van Pelt JA, Mueller MJ, et al. NPR1 Modulates Cross-Talk between Salicylate- and Jasmonate-Dependent Defense Pathways through a Novel Function in the Cytosol. 2003; 15(3):760–70. https://doi.org/10.1105/tpc.009159 J The Plant Cell. PMID: 12615947.

58. Backer R, Naidoo S, van den Berg N. The NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and Related Family: Mechanistic Insights in Plant Disease Resistance. Front Plant Sci. 2019; 10(102). https://doi.org/10.3389/fpls.2019.01022 PMID: 30815005.

59. Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, et al. The JAZ family of repressors is the missing link in jasmonate signalling. Nature. 2007; 448(7154):666–71. Epub 2007/07/20. https://doi.org/10.1038/nature06006 PMID: 17637675.

60. Kazan K, Manners JM. JAZ repressors and the orchestration of phytohormone crosstalk. Trends in Plant Science. 2012; 17(1):22–31. https://doi.org/10.1016/j.tplants.2011.10.006 PMID: 22123886.

61. Proietti S, Caarls L, Pieterse CMJ, Van Wees SCM. Genome-wide association study reveals novel players in defense hormone crosstalk through a Novel Function in the Cytosol. 2003; 15(3):760–70. https://doi.org/10.1105/tpc.009159 J The Plant Cell. PMID: 12615947.

62. Caarls L, Pieterse CMJ, Van Wees SCM. How salicylic acid takes transcriptional control over jasmonic acid signaling. Frontiers in plant science. 2015; 6:170–. https://doi.org/10.3389/fpls.2015.00170 PMID: 25859250.

63. Zheng Z, Qamar SA, Chen Z, Mengiste T. Arabidopsis WRKY33 transcription factor is required for resistance to necrotrophic fungal pathogens. Plant J. 2006; 48(4):592–605. Epub 2006/10/25. https://doi.org/10.1111/j.1365-313X.2006.02901.x PMID: 17059405.

64. Li J, Brader G, Kariola T, Palva ET. WRKY70 modulates the selection of signaling pathways in plant defense. Plant J. 2006; 46(3):477–91. Epub 2006/04/21. https://doi.org/10.1111/j.1365-313X.2006.02712.x PMID: 16623907.

65. Wang D, Amornsiripanitch N, Dong X. A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathog. 2006; 2(11):e123. Epub 2006/11/14. https://doi.org/10.1371/journal.ppat.0020123 PMID: 17096590; PubMed Central PMCID: PMC1635530.
67. Paul PK, Sharma PD. Azadirachta indica leaf extract induces resistance in barley against leaf stripe disease. Physiological and Molecular Plant Pathology. 2002; 61(1):3–13. https://doi.org/10.1006/pmpp.2002.0412.

68. Gao Q-M, Zhu S, Kachroo P, Kachroo A. Signal regulators of systemic acquired resistance. Frontiers of plant science. 2015; 8:628–. https://doi.org/10.3389/fpls.2015.00228 PMID: 25918514.

69. Vergnes S, Ladouce N, Fournier S, Ferhout H, Attia F, Dumas B. Foliar treatments with Gaultheria pro-cumbens essential oil induce defense responses and resistance against a fungal pathogen in Arabidopsis. 2014; 5(477). https://doi.org/10.3389/fpls.2014.00477 PMID: 25295045.

70. von Rad U, Mueller MJ, Dumer J. Evaluation of natural and synthetic stimulants of plant immunity by microarray technology. New Phytol. 2005; 165(1):191–202. Epub 2005/02/22. https://doi.org/10.1111/j.1469-8137.2004.01211.x PMID: 15720633.

71. Gullner G, Komives T, Kiraly L, Schroder P. Glutathione S-Transferase Enzymes in Plant-Pathogen Interactions. Front Plant Sci. 2018; 9:1836. Epub 2019/01/10. https://doi.org/10.3389/fpls.2018.01836 PMID: 30622544; PubMed Central PMCID: PMC6308375.

72. Suzuki N, Sejima H, Tam R, Schlauch K, Mittler R. Identification of the MBF1 heat-response regulon of Arabidopsis thaliana. The Plant Journal. 2011; 66:844–51. https://doi.org/10.1111/j.1365-313X.2011.04550.x PMID: 21457365.

73. Suzuki N, Koussevitzky S, Mittler R, Miller G. ROS and redox signalling in the response of plants to abiotic stress. Plant Cell Environ. 2012; 35(2):259–70. Epub 2011/04/14. https://doi.org/10.1111/j.1365-313X.2011.04301.x PMID: 21463035.

74. Rienth M, Torregrosa L, Sarah G, Ardisson M, Brillouet J-M, Romieu C. Temperature desynchronizes sugar and organic acid metabolism in ripening grapevine fruits and remodels their transcriptome. BMC Plant Biology. 2016; 16(1):164. https://doi.org/10.1186/s12870-016-0850-0 PMID: 27439426.

75. Rienth M, Torregrosa L, Kelly MT, Luchaire N, Pellegrino A, Grimplet Jrm, et al. Is Transcriptomic Regulation of Berry Development More Important at Night than During the Day? PLoS One. 2014; 9(2):e88844. https://doi.org/10.1371/journal.pone.0088844 PMID: 24551177.

76. Rienth M, Torregrosa L, Luchaire N, Chatbangton R, Lecourtieux D, Kelly M, et al. Day and night heat stress trigger different transcriptomic responses in green and ripening grapevine (vitis vinifera) fruit. BMC Plant Biology. 2014; 14(1):108. https://doi.org/10.1186/1471-2229-14-108 PMID: 24774299.

77. Torregrosa L, Rienth M, Romieu C, Pellegrino A. The microvibe, a model for studies in grapevine physiology and genetics. OENO One. 2019; 53(3). https://doi.org/10.10870/oeno-one.2019.53.3.2409.

78. Mizoi J, Shinozaki K, Yamaguchi-Shinozaki K. AP2/ERF family transcription factors in plant abiotic stress responses. Biochimica et Biophysica Acta (BBA)—Gene Regulatory Mechanisms. 2012; 1819(2):86–96. https://doi.org/10.1016/j.bbagrm.2011.08.004.

79. Bahieldin A, Atef A, Edris S, Gadalla NO, Ali HM, Hassan SM, et al. Ethylene responsive transcription factor ERF109 retards PCD and improves salt tolerance in plant. 2016; 16(1):216. https://doi.org/10.1186/s12870-016-0908-z PMID: 27716054.

80. Kumar M, Brar A, Yadav M, Chawade A, Vivekanand V, Pareek N. Chitinases—Potential Candidates for Enhanced Plant Resistance towards Fungal Pathogens. 2018; 8(7):88. https://doi.org/10.3390/agriculture8070088.

81. Hakim, Ullah A, Hussain A, Shaban M, Khan AH, Alariqi M, et al. Osmotin: A plant defense tool against biotic and abiotic stresses. Plant Physiology and Biochemistry. 2018; 123:149–59. https://doi.org/10.1016/j.plaphy.2017.12.012 PMID: 29245030.

82. Gauthier A, Trouvelot S, Kelloniemi J, Frettinger P, Wendehenne D, Daire X, et al. The Sulfated Laminarin Triggers a Stress Transcriptome before Priming the SA- and ROS-Dependent Defenses during Grapevine’s Induced Resistance against Plasmopara viticola. PLOS ONE. 2014; 9(2):e88145. https://doi.org/10.1371/journal.pone.0088145 PMID: 24516597.

83. Rasmussen M, Roux M, Petersen M, Mundy J. MAP Kinase Cascades in Arabidopsis Innate Immunity. 2012; 3(169). https://doi.org/10.3389/fpls.2012.00169 PMID: 22837762.

84. Tsuda K, Mine A, Bethke G, Igarashi D, Botanga CJ, Tsuda Y, et al. Dual regulation of gene expression mediated by extended MAPK activation and salicylic acid contributes to robust innate immunity in Arabidopsis thaliana. PLoS Genet. 2013; 9(12):e1004015. Epub 2013/12/19. https://doi.org/10.1371/journal.pgen.1004015 PMID: 24348271; PubMed Central PMCID: PMC3861249 adherence to all PLoS Genetics policies on sharing data and materials.

85. Su J, Yang L, Zhu Q, Wu H, He Y, Liu Y, et al. Active photosynthetic inhibition mediated by MPK3/MPK6 is critical to effector-triggered immunity. PLOS Biology. 2018; 16(5):e2004122. https://doi.org/10.1371/journal.pbio.2004122 PMID: 29723186.
86. Shorosh M, Gal-On A, Leibman D, Chet I. Characterization of a Mitogen-Activated Protein Kinase Gene from Cucumber Required for Trichoderma-Conferred Plant Resistance. Plant Physiol. 2006; 142(3):1169–79. https://doi.org/10.1104/pp.106.082107 J Plant Physiology. PMID: 16950863

87. Genot B, Lang J, Berriri S, Garmier M, Gilard F, Pateyron S, et al. Constitutively Active Arabidopsis MAP Kinase 3 Triggers Defense Responses Involving Salicylic Acid and SUMM2 Resistance Protein. Plant Physiology. 2017; 174(2):1238–49. https://doi.org/10.1104/pp.17.00378 PMID: 28400495

88. Kwaiatal M, Huisman R, Maintz J, Reinstädler A, Panstruga R. Ionotropic glutamate receptor (iGlur)-like channels mediate MAMP-induced calcium influx in Arabidopsis thaliana. 2011; 440(3):355–73. https://doi.org/10.1042/BJ20111112 J Biochemical Journal. PMID: 21848515

89. CyanoBase.

90. Zhu X, Caplan J, Mamillapalli P, Czymmek K, Dinesh-Kumar SP. Function of endoplasmic reticulum calcium ATPase in innate immunity-mediated programmed cell death. Embo j. 2010; 29(5):1007–18. Epub 2010/01/16. https://doi.org/10.1038/emboj.2009.402 PMID: 20075858; PubMed Central PMCID: PMC2837167.

91. Lam E, Kato N, Lawton M. Programmed cell death, mitochondria and the plant hypersensitive response. Nature. 2001; 411(6839):848–53. Epub 2001/07/19. https://doi.org/10.1038/35081184 PMID: 11459068.

92. Stael S, Kmiecik P, Willems P, Van Der Kelen K, Coll NS, Teige M, et al. Plant innate immunity—sunny side up? Trends in plant science. 2015; 20(1):3–11. Epub 2014/10/29. https://doi.org/10.1016/j.tplants.2014.10.002 PMID: 25457110.

93. Anderson JP, Badruzsaufari E, Schenk PM, Manners JM, Desmondo OJ, Ehlert C, et al. Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in Arabidopsis. Plant Cell. 2004; 16(12):3460–73. https://doi.org/10.1105/tpc.104.025833 PMID: 15548743.

94. Vanlerberghe GC. Alternative oxidase: a mitochondrial respiratory pathway to maintain metabolic and signaling homeostasis during abiotic and biotic stress in plants. Int J Mol Sci. 2013; 14(4):6805–47. Epub 2013/03/28. https://doi.org/10.3390/ijms14046805 PMID: 23531539; PubMed Central PMCID: PMC3645666.

95. Piasecka A, Jedrzejczak-Rey N, Bednarek P. Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. New Phytol. 2015; 206(3):948–65. Epub 2015/02/11. https://doi.org/10.1111/nph.13325 PMID: 25659829.

96. Scholasch T, Rienth M. Review of water deficit mediated changes in vine and berry physiology; Consequences for the optimization of irrigation strategies. OENO One. 2019; 53(3). https://doi.org/10.20870/oeno-one.2019.53.3.2407.

97. Mierziak J, Kostyn K, Kulma A. Flavonoids as important molecules of plant interactions with the environment. Molecules. 2014; 19(10):16240–65. Epub 2014/10/14. https://doi.org/10.3390/molecules191016240 PMID: 25310150.

98. Fofana B, McNally DJ, Labbé C, Boulanger R, Benhama N, Séguin A, et al. Milsana-induced resistance in powdery mildew-infected cucumber plants correlates with the induction of chalcone synthase and chalcone isomerase. Physiological and Molecular Plant Pathology. 2002; 61(2):121–32. https://doi.org/10.1006/pmpp.2002.0420.

99. Richard T, Abdelli-Belhad A, Vitrac X, Waffo-Téguo P, Méronillon J-M. Vitis vinifera canes, a source of stilbenoids against downy mildew. OENO One. 2016; 50(3). https://doi.org/10.20870/oeno-one.2016.50.3.1178.

100. Höll J, Vannonzi A, Czemmel S, D’Onofrio C, Walker AR, Rausch T, et al. The R2R3-MYB Transcription Factors MYB14 and MYB15 Regulate Stilbene Biosynthesis in Vitis vinifera. 2013; 25(10):4135–49. https://doi.org/10.1105/tpc.113.117127 J The Plant Cell. PMID: 24151295.

101. Belhadj A, Telef N, Saïgne C, Cluzet S, Barrieu F, Hamd Si, et al. Effect of methyl jasmonate in combination with carbohydrates on gene expression of PR proteins, stilbene and anthocyanin accumulation in grapevine cell cultures. Plant Physiol Biochem. 2008; 46:493–9. https://doi.org/10.1016/j.plaphy.2007.12.001 PMID: 18594857.

102. D’Onofrio C, Cox A, Davies C, Boss P. Induction of secondary metabolism in grape cell cultures by jasmonates. Funct Plant Biol. 2009; 36:323–38.

103. Garde-Cerdan T, Gutiérrez-Gamboa G, Pé A, P., Rubio-Bretón P. Foliar application of methyl jasmonate to Graciano and Tempranillo vines: effects on grape amino acid content during two consecutive vintages. OENO One., 2019; 53(1). https://doi.org/10.20870/oeno-one.2019.53.1.2163.

104. La Torre A, Mandalá C, Pezza L, Caradon F, Battaglia V. Evaluation of essential plant oils for the control of Plasmopara viticola. Journal of Essential Oil Research. 2014; 26(4):282–91. https://doi.org/10.1080/10412905.2014.889049
105. Kordali S, Cakir A, Ozer H, Cakmakci R, Kesdek M, Mete E. Antifungal, phytotoxic and insecticidal properties of essential oil isolated from Turkish Origanum acutidens and its three components, carvacrol, thymol and p-cymene. Bioresource Technology. 2008; 99(18):8788–95. https://doi.org/10.1016/j.biortech.2008.04.048 PMID: 18513954

106. de Almeida LFR, Frei F, Mancini E, De Martino L, De Feo V. Phytotoxic activities of Mediterranean essential oils. Molecules (Basel, Switzerland). 2010; 15(6):4309–23. https://doi.org/10.3390/molecules15064309 PMID: 20657443.

107. Amri IS, Hamrouni L, Hanacac M, Jamoussi B. REVIEWS ON PHYTOTOXIC EFFECTS OF ESSENTIAL OILS AND THEIR INDIVIDUAL COMPONENTS: NEWS APPROACH FOR WEEDS MANAGEMENT. International Journal of Applied Biology and Pharmaceutical Technology. 2012:96–114.

108. Duchêne E, Dumas V, Jaegli N, Merdinoglu D. Genetic variability of descriptors for grapevine berry acidity in Riesling, Gewürztraminer and their progeny. Australian Journal of Grape and Wine Research. 2014; 20(1):91–9. https://doi.org/10.1111/ajgw.12051

109. Merdinoglu D, Schneider C, Prado E, Wiedemann-Merdinoglu S, Mestre P. Breeding for durable resistance to downy and powdery mildew in grapevine. 52(3), 203–209. OENO One. 2018; 52(3):103–209. https://doi.org/10.20870/oenone-one.2018.52.3.2116.

110. Torregrosa L, Bigard A, Doligez A, Lecourieux D, Rienth M, Luchaire N, et al. Developmental, molecular and genetic studies on grapevine response to temperature open breeding strategies for adaptation to warming. OENO One. 2017; 51(2):155–65. https://doi.org/10.20870/oenone-one.2017.51.2.1587.