Detection, identification and functional characterisation of plant and microbial volatile organic compounds with inhibitory activity against two plant pathogens

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To my family…

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List of abbreviations

AMDIS	Automated Mass spectral Deconvolution and Identification System
CAR	carboxene
CI	chemical ionisation
CoA	coenzyme A
DAHP	3-deoxy-D-arabinoheptulosonate-7 phosphate
DMAPP	dimethylallyl pyrophosphate
DVB	divinylbenzene
EI	electron ionisation
EIC	extracted ion current
Ery4P	erythrose 4-phosphate
FID	flame ionisation detector
FPP	farnesyl pyrophosphate
GAP	D-glyceraldehyde 3-phosphate
GC	gas chromatography
GGPP	geranylgeranyl pyrophosphate
GLVs	green leaf volatiles
GPP	geranyl pyrophosphate
HS	headspace
IPP	isopentenyl pyrophosphate
IT	ion trap
LLE	liquid-liquid extraction
LOX	lipoxygenases
LTPRI	linear temperature programmed retention index
LTPs	lipid transfer proteins
m/z	mass-to-charge ratio
MA-HD	microwave-assisted extraction hydrodistillation
MEP	methylerythritol phosphate
MS	mass spectrometry
MS^2	tandem mass spectrometry
MVA	mevalonic acid
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NIST	national institute of standards and technology
NPP	neryl pyrophosphate
PA	polyacrylate
PDMS	polydimethyl siloxane
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
PTR	proton-transfer reaction

QqQ	triple quadrupole
RT	retention time
SFE	supercritical fluid extraction
SPME	solid phase microextraction
TIC	total ion current
ToF	time of flight
TPSs	terpene synthases
VOCs	volatile organic compounds

Aims and structure of the thesis

Numerous devastating plant diseases are caused by pathogenic oomycetes. For example, the oomycetes *Plasmopara viticola*, which is the causal agent of grapevine downy mildew, and *Phytophthora infestans*, which is the causal agent of potato late blight, cause severe damages to economically important crops. In conventional agriculture, plant diseases are controlled by frequent applications of chemical fungicides, but concerns about the impact of fungicide overuse to the environment and human health and the development of resistant populations of these plant pathogens have sparked off crescent interest in the developing of alternative approaches in pest management. For this reason, more environmental-friendly solutions are being increasingly investigated, such as development of new cultivars trough breeding programs between resistant and susceptible plant genotypes and the use of biocontrol agents. For instance, resistance traits have been identified in wild grapevine species (*Vitis riparia*, *V. rupestris*, *V. amurensis* and *Muscadinia rotundifolia*) and breeding programs have been already used in order to combine the quality traits of European cultivars (*V. vinifera*) with the downy mildew resistance traits of wild grapevine species. Grapevine genotypes such as BC4, Kober 5BB, SO4 and Solaris are resistant to downy mildew and may therefore be used as resources to study resistance mechanisms and to develop alternatives to chemical treatments, such as the development of new molecules for downy mildew control. In this respect, the accumulation of antimicrobial compounds is one of the most important defence mechanisms of resistant grapevine genotypes against *P. viticola*. Among the plant secondary metabolites, volatile organic compounds (VOCs) seem to have essential ecological roles in the communication of plants with other organisms, but scarce information is available regarding the role of grapevine VOCs in defence mechanisms against *P. viticola* infection. Moreover, biocontrol agents are promising alternatives to chemical pesticides and the bacterial genus *Lysobacter* encompasses species with auspicious antagonistic features against oomycetes. Although the production of non-volatile antimicrobial compounds by these bacteria has been extensively characterised, the chemical composition and the toxic properties of the *Lysobacter* VOCs against *P. infestans* have been poorly investigated until now.

Motivated by the concept that plant- and microbial-derived VOCs may play a key role in plant protection against economically important pathogens, the following

objectives have been defined. The specific goals of this PhD project were the detection, identification and functional characterization of VOCs produced from susceptible and resistant grapevines during the interaction between *P. viticola*. In particular, the role of plant VOCs and their effect as toxic molecules against downy mildew were characterised. Moreover, the formation of VOCs by biocontrol *Lysobacter* spp. was investigated in order to identify active molecules to control *P. infestans*. The final aim of this PhD thesis was to better understand the role and relevance of plant- and microbe-derived VOCs in plant-microbe and microbe-microbe communications in order to identify new active molecules from natural origin, which show the potential to control plant pathogenic oomycetes. To this end, an interdisciplinary approach has been followed. Headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME/GC-MS) and proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS) analysis have been used for the detection, annotation/identification of VOCs. Moreover, grapevine plants grown *in vitro* or under greenhouse conditions and *Lysobacter* type strains grown *in vitro* have been used. The experiments have been carried out in cooperation between the Center for Analytical Chemistry, IFA-Tulln (Austria) and the Foundation Edmund Mach (FEM, Italy).

During my PhD studies, I have contributed to the design of the experiments together with my supervisors, Ao. Univ. Prof. Dr. Rainer Schuhmacher (IFA-Tulln) and Dr. Michele Perazzolli (FEM). My own responsibility was the preparation and execution of all the experiments which have been carried out both at FEM and IFA-Tulln. I personally prepared and measured all the samples and I was responsible for the literature study, data evaluation, metabolite identification, biological interpretation of results and manuscripts writing. I also contributed to the maintenance of the laboratory instrumentations.

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The presented thesis is divided in three main parts. PART I describes the background of my research topic with a main focus on the state of the art of VOCs emitted by plants and soil microorganisms, in particular by *Vitis* spp. and *Lysobacter* spp., and the associated sampling techniques for the measurement with both HS-SPME/GC-MS and PTR-ToF-MS analyses. As a mid- to long-term perspective, the expected outcome of my PhD thesis may be helpful for the further development of novel products for controlling plant diseases. PART II consists of the publications, which have resulted from my PhD studies. For each publication, a brief motivation of the study and personal contribution are shortly summarised. PART III gathers my curriculum vitae and a list of scientific publications and contributions.

The following publications form an integral part of the presented thesis:

1. “Emission of volatile sesquiterpenes and monoterpenes in grapevine genotypes following *Plasmopara viticola* inoculation *in vitro*”
Alberto Algarra Alarcon*, **Valentina Lazazzara***, Luca Cappellin, Pier Luigi Bianchedi, Rainer Schuhmacher, Georg Wohlfahrt, Ilaria Pertot, Franco Biasioli and Michele Perazzolli
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Abstract

Volatile organic compounds (VOCs) play crucial ecological roles in interactions among organisms. For example, plant VOCs can act as a powerful deterrent of herbivore insects and pathogens or they can act as resistance inducers to stimulate plant defences. Likewise, bioactive VOCs can be emitted by beneficial microorganisms and they may potentially act as key molecules in the microbe-microbe and plant-microbe communications. However, scarce information is available concerning the role of VOCs produced by grapevine (*Vitis vinifera*) plants and beneficial bacteria belonging to the *Lysobacter* genus in defence mechanisms against two important phytopathogenic oomycetes, namely *Plasmopara viticola* and *Phytophthora infestans*, which are the causal agents of grapevine downy mildew and potato late blight, respectively.

The major objectives of this PhD thesis were the detection, identification and the functional characterization of VOCs from *Vitis* spp. and *Lysobacter* spp., in order to better understand their role in plant-microbe and microbe-microbe communications and to identify new active molecules from natural origin to control phytopathogens. In particular, VOCs from resistant and susceptible grapevine genotypes were identified following *P. viticola* inoculation and their effect as toxic molecules against downy mildew was explored (publications 1 and 2). Likewise, VOCs produced by *Lysobacter* spp. were identified and characterised, in order to identify microbial VOCs able to inhibit *P. infestans* growth (publication 3).

In order to reach these goals, a headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME/GC-MS) and proton transfer reaction time of flight-mass spectrometry (PTR-ToF-MS) have been used. Two downy mildew resistant hybrids (SO4 and Kober 5BB) and the susceptible *V. vinifera* cultivar Pinot noir were analysed *in vitro* using PTR-ToF-MS. We found that *P. viticola* inoculation resulted in a significant increase monoterpene and sesquiterpene emission by resistant genotypes (SO4 and Kober 5BB) and not by the susceptible cultivar (*Vitis vinifera* Pinot noir; publication 1). Grapevine VOCs were further identified by HS-SPME/GC-MS using greenhouse-grown plants. The four resistant genotypes tested (BC4, Kober 5BB, SO4 and Solaris) showed significantly increased production of VOCs after *P. viticola* inoculation under greenhouse conditions. Conversely, no significant emission of volatile terpenes was detected from Pinot noir plants after *P. viticola* inoculation, suggesting that VOCs of resistant genotypes could play an important role in grapevine

resistance against downy mildew. The chemical structures of *P. viticola*-induced VOCs were identified by retention index and the GC-MS spectrum evaluation and VOCs potentially involved in the grapevine resistance were selected according to their emission profiles. Pure compounds were tested against *P. viticola* by leaf disk assays and different experiments were set up, in order to elucidate the efficacy of pure VOCs both in a liquid suspension of *P. viticola* sporangia and after application via the gas phase. These experiments revealed six (2-phenylethanol, β-caryophyllene, β-selinene, trans-2-pentenal, 2-ethylfuran, and β-cyclocitral) and four VOCs (2-phenylethanol, trans-2-pentenal, 2-ethylfuran, and β-cyclocitral) which impaired downy mildew symptoms after direct application of liquid suspension and after treatment with VOC enriched air (without direct contact with the leaf tissue), respectively. With these results we demonstrated that VOCs produced by resistant grapevine genotypes are related to post-infection mechanisms and may contribute to grapevine resistance against *P. viticola* by inhibition of pathogen development (publication 2).

In the second part of the PhD project, the volatilome of *Lysobacter* spp. was characterised for its inhibitory activity against the soil pathogen *P. infestans* (publication 3). The effect of VOCs emitted by *Lysobacter* strains was demonstrated *in vitro* by dual-culture assay and profiles were characterised by HS-SPME/GC-MS and PTR-ToF-MS analysis. Interestingly, the biocontrol activity and VOC profiles of *Lysobacter* spp. depended on the bacterial growth media. In particular, VOCs with inhibitory properties (pyrazines, pyrrole and decanal) were mainly emitted by *Lysobacter* type strains grown on a protein-rich medium, demonstrating the importance of the culture medium composition to optimise the biocontrol efficacy of *Lysobacter* spp. against plant pathogens.

In summary, the presented thesis showed that both analytical chemistry techniques used (PTR-ToF-MS and HS-SPME/GC-MS) can be employed synergistically to detect and identify VOCs from different biological matrixes such as leaf tissue or bacterial cultures. The presented thesis also suggested that VOCs contribute to grapevine resistance and they can effectively be used to control economically important plant pathogens such as *P. viticola.* Furthermore, results generated in this work indicate that nutrient availability may affect the aggressiveness of *Lysobacter* spp. in the soil to maximise biocontrol efficacy against *P. infestans*. However, further metabolomic and transcriptomic analyses are required to investigate the VOC-mediated plant defence

mechanisms and to characterize metabolic changes and VOC emissions of *Lysobacter* spp. grown in soil conditions.

Zusammenfassung

Flüchtige organische Substanzen (VOC´s) spielen eine wichtige Rolle in der Interaktion zwischen Lebewesen. VOC´s, die von Pflanzen gebildet werden, können beispielsweise als Abwehrstoffe gegen herbivore Insekten und mikrobielle Schadorganismen fungieren oder sie können zum Beispiel auch die Pflanzenabwehr stimulieren. Biologisch aktive VOC´s können aber nicht nur von Pflanzen sondern auch von (nutzbringenden) Mikroorganismen wie zum Beispiel Bakterien der Gattung *Lysobacter* gebildet werden und dabei eine zentrale Rolle in der molekularen Kommunikation mit anderen Mikroorganismen oder auch mit Pflanzen spielen. Trotz dieser allgemeinen Kenntnis über die weitreichende ökologische Bedeutung flüchtiger organischer Substanzen, ist deren genaue Funktion in der Wechselwirkung zwischen miteinander in direktem Kontakt stehenden Organismen vielfach unbekannt. So kennt man bisher zum Beispiel von den durch Weinreben (*Vitis vinifera*) in Kontakt mit phytopathogenen Eipilzen (Oomyceten) gebildeten und abgesonderten VOC's weder die genaue chemische Zusammensetzung noch die Bedeutung, welche diese flüchtigen Substanzen für die Wechselwirkung mit dem jeweiligen Schadorganismus haben. Im Rahmen der vorliegenden Arbeit wurde dieser Aspekt der Wechselwirkung zwischen der Weinrebe und dem Verursacher des Falschen Mehltaus (*Plasmopara viticola*) genauer untersucht.

Die wichtigsten Ziele dieser Dissertation waren der Nachweis, die Identifikation und funktionale Charakterisierung der von Pflanzen der Gattung *Vitis* und Bakterien der Gattung *Lysobacter* um gebildeten VOC's um deren Rolle in der Kommunikation Pflanze-Mikroorganismus bzw. Mikroorganismus-Mikroorganismus besser verstehen zu können und um neue aktive Substanzen natürlichen Ursprungs zu finden, die gegen phytopathogene Mikroorganismen eingesetzt werden können. Im Rahmen der vorgelegten Dissertation konnten zunächst die VOC´s, welche von unterschiedlich Mehltau-resistenten *Vitis*-Arten nach Behandlung mit *P. viticola* gebildet werden, erfolgreich identifiziert werden. Im Anschluss daran wurde die biologische Aktivität einiger dieser flüchtigen Substanzen näher charakterisiert und es konnte gezeigt werden, dass manche VOC's eine toxische Wirkung gegen den Falschen Mehltau besitzen (Publikation 1 und 2).

In gleicher Weise wurden von *Lysobacter* spp. gebildete VOC´s mit dem Ziel identifiziert und charakterisiert, Substanzen zu finden, die das Wachstum des

Schaderregers der Kartoffelfäule *Phytophthora infestans* inhibieren können (Publikation 3).

Um diese Ziele zu erreichen wurden die flüchtigen Substanzen mittels Gaschromatographie-Massenspektrometrie (GC-MS) nachgewiesen. Die Analysen der Pflanzen- und Pilzproben wurden mit zwei unterschiedlichen Techniken durchgeführt: Headspace-Festphasen Mikroextraktion/GC-MS (HS-SPME/GC-MS) und Protonen-Transfer-Reaktions-Flugzeit-Massenspektrometrie (PTR-ToF-MS). Zunächst wurden zwei gegen Falscher Mehltau resistente *Vitis* Hybride (SO4 und Kober 5BB) und die anfällige *V. vinifera* Sorte Pinot noir in vitro mittels PTR-ToF-MS analysiert auf die Bildung flüchtiger Substanzen. Wir fanden, dass die Behandlung der Blätter mit *P. viticola* bei den Pflanzen der resistenten Genotypen (SO4 und Kober 5BB) zu einer signifikant erhöhten Emission an Monoterpenen und Sesquiterpenen führte, was bei der anfälligen Sorte *Vitis vinifera* Pinot noir nicht der Fall war (Publikation1). Die unter Infektionsbedingungen von den Weinreben gebildeten VOC-Klassen und deren Vertreter wurden zusätzlich an Hand von im Glashaus kultivierten Pflanzen mittels HS-SPME/GC-MS chromatographisch aufgetrennt und auf Basis ihres GC-MS-Spektrums und Retententionsindex annotiert / identifiziert. Dabei zeigte sich für die vier getesteten resistenten Genotypen (BC4, Kober 5BB, SO4 und Solaris) nach *P. viticola* Inokulation erneut ein signifikanter Anstieg in der Produktion der VOC´s. Im Gegensatz dazu konnten bei den Pinot noir Pflanzen nach *P. viticola* Inokulation keine flüchtigen Terpene nachgewiesen werden, was die Vermutung nahe legt, dass die durch die resistenten Genotypen gebildeten VOC´s eine wichtige Rolle in der Resistenz von Weinreben gegen falschen Mehltau spielen können. Daher wurden die am interessantesten erscheinenden, potentiell an der Resistenz der Weinreben beteiligten VOC´s ausgewählt und weiter untersucht. Die Reinsubstanzen wurden in verschiedenen zum Teil im Rahmen dieser Arbeit entwickelten Testformaten auf ihre Wirkung gegen *P. viticola* getestet. Neben Tests an kreisförmig ausgestanzten Blattstücken wurde die Wirksamkeit der reinen VOC´s sowohl direkt gegen in flüssiger Suspension auf der Blattoberfläche vorliegende *P. viticola* Sporenbehälter getestet als auch nach deren Einwirkung über die Gasphase untersucht. Mit Hilfe dieser Experimente konnten die sechs Substanzen 2-Phenylethanol, β-Caryophyllen, β-Selinen, trans-2-Pentenal, 2-Ethylfuran, und β-Cyclocitral gefunden werden, welche die Symptome des falschen Mehltaus nach direkter Anwendung der flüssigen Suspension vermindern. Vier weitere Substanzen (2-Phenylethanol, trans-2-Pentenal, 2-Ethylfuran, und β-Cyclocitral)

zeigten eine *P. viticola* inhibierende Wirkung nach Behandlung mit VOC-angereicherter Luft ohne direkten Kontakt mit dem Blattmaterial. Unsere Ergebnisse legen nahe, dass die von den resistenten Reben-Genotypen gebildeten VOC´s in direktem Zusammenhang mit der Pathogenabwehr nach erfolgter Infektion stehen und dass diese flüchtigen Substanzen durch Inhibierung der Pathogenentwicklung zur Resistenz gegen *P. viticola* beitragen können (Publikation 2).

Im zweiten Teil des Dissertationsprojektes wurde das Profil flüchtiger Substanzen (Volatilom) von Bakterien der Gattung *Lysobacter* spp hinsichtlich ihrer wachstumshemmenden Wirkung gegen den Schadorganismus *Phytophthora infestans* charakterisiert (Publikation 3). Die Wirksamkeit der durch verschiedene *Lysobacter* spp Stämme abgegebenen VOC´s wurde in vitro mit Hilfe eines dualen Kulturassays untersucht und die gebildeten Substanzen wurden mittels nachgewiesen und HS-SPME/GC-MS und PTR-ToF-MS und hinsichtlich ihrer chemischen Struktur charakterisiert. Interessanterweise stellte sich heraus, dass sowohl die Biokontrollaktivität als auch die VOC-Profile des *Lysobacter* spp. von der Zusammensetzung des für die Kultivierung von *Lysobacter* verwendeten Wachstumsmediums abhängig ist. Insbesondere hemmende Eigenschaften besitzende VOC's wie beispielsweise Pyrazin, Pyrrol und Decanal wurden hauptsächlich von auf proteinreichem Medium kultivierten *Lysobacter*-Stämmen emittiert. Diese Ergebnisse unterstreichen die Bedeutung der Medienzusammensetzung für die Wirksamkeit von *Lysobacter* spp. gegen Pflanzenpathogene.

Zusammenfassend konnte mit der vorliegenden Arbeit gezeigt werden, dass die beiden verwendeten chemisch-analytischen Techniken (PTR-ToF-MS and HS-SPME/GC-MS) ergänzend eingesetzt werden können um die Bildung von VOC´s unterschiedlicher biologischer Systeme wie Pflanzenblätter oder Bakterienkulturen zu detektieren und zu identifizieren. Die vorliegende Arbeit legte nahe, dass VOC´s zur Resistenz von Weinreben gegen das Pflanzenpathogen *P. viticola* beitragen und dass die biologisch aktiven Substanzen auch verwendet werden können, um wirtschaftlich wichtige Pflanzenkrankheiten wie Falscher Mehltau wirkungsvoll zu kontrollieren. Weiters zeigen die im Rahmen der der Arbeit durchgeführten Experimente, dass die Nährstoffverfügbarkeit die Aggressivität von *Lysobacter* spp. gegenüber *P. infestans* stark beeinflussen kann. Diese Ergebnisse deuten daher darauf hin, dass durch die Beeinflussung der Nährstoffzusammensetzung des Bodens die Biokontroll-Wirksamkeit dieser Bakterien gegen den bodenbürtigen Schadorganismus *P. infestans*

gezielt verbessert werden kann. Weitere Forschungsarbeiten auf diesem interessanten Gebiet werden dazu beitragen die komplexen Wechselwirkungen zwischen lebenden Organismen noch besser zu verstehen um in Zukunft verbesserte Methoden im biologischen Pflanzenschutz entwickeln zu können.

Part I

1 Introduction to VOCs

1.1 *Definition of VOCs*

Volatile organic compounds (VOCs) play an important role in nature as messenger compounds to transmit information between and within organisms (Herrmann, 2010). VOCs are generally based on a hydrocarbon skeleton which may additionally contain oxygen, nitrogen or sulphur as part of their molecular structure. Usually, these molecules are lipophilic, with low molecular weight (less than 300 Da) and high vapour pressure (0.01 kPa or higher at 20°C) that permit them to travel from their point of origin through the air, porous soils and liquids to reach their putative biological targets (Wenke*, et al.*, 2012, Bitas*, et al.*, 2013, Peñuelas*, et al.*, 2014). Such common chemical and physical properties make VOCs ideal signalling molecules for mediating both short- and long-distance interactions, playing essential ecological and biological roles both above- and belowground (Effmert*, et al.*, 2012, Bitas*, et al.*, 2013). Various type of organisms such as microbes, plants, humans and animals emit VOCs that affect their environments and each other (Schulz, 2007, Baldwin, 2010, Effmert*, et al.*, 2012).

1.2 *Functions of VOCs*

VOCs perform numerous functions, as so-called "semiochemicals", "infochemicals" or "pheromones". For example, plant VOCs are notably involved in the attraction of pollinators (Raguso, 2008, Das*, et al.*, 2013) and seed dispersers, above- and below-ground defence against herbivore insects (Unsicker*, et al.*, 2009, Das*, et al.*, 2013, Heil, 2014), plant-plant and within-plant signalling (Baldwin*, et al.*, 2006, Heil & Bueno, 2007, Heil & Karban, 2010), or even, as flavours and fragrances or smell to humans (Cappellin*, et al.*, 2013). Likewise, microbial VOCs appear to be involved in antagonism, mutualism, intra- and interspecific regulation of cellular and developmental processes, and modification of their surrounding environments (Schulz, 2007, Bitas*, et al.*, 2013, Davis*, et al.*, 2013).

1.2.1 Functions of plant VOCs

Plants release a large variety of VOCs into the surrounding atmosphere, and the release of these substances is frequently associated with resistance to a range of biotic and abiotic stress factors such as oxidative stresses. Particularly, isoprenoids can increase the stability of membrane bilayers and they can interact with oxidants in order to protect plants from oxidative damage (Loreto & Velikova, 2001). Plant VOCs can also contribute to resistance against other abiotic stress factors, such as high temperature and light intensity (Schuh*, et al.*, 1997), and water stress (Sharkey & Loreto, 1993). Plant VOCs are also involved in communication both within plants and with other organisms, such as animals (Dudareva*, et al.*, 2013). For example, floral volatiles are essential to help insects to discriminate among plant species and even among individual flowers within a single species. Volatiles are also emitted from roots and they can contribute to a belowground defence system by acting as antimicrobial or anti-herbivore substances, or by attracting enemies of root-feeding herbivores (Rasmann*, et al.*, 2005). Furthermore, floral volatiles attract pollinators and seed disseminators in order to ensure reproductive and evolutionary success (Dudareva*, et al.*, 2013). For example, VOCs emitted by fruits determine their aroma and taste and thus have a role in attraction of animal seed dispersers (Goff & Klee, 2006). Moreover, the roles of plant VOCs in response to mechanical wounding and herbivore insect attack have been largely studied (Dudareva*, et al.*, 2006, Heil, 2014). However, little information is available concerning the role of plant VOCs in defence mechanisms against phytopathogens (Heil, 2014).

As of today, plant VOCs can act in three possible modes of action against plant pathogens. The first mode consists in direct inhibition of microbial growth. For example, green leaf volatiles (GLVs) (Nakamura & Hatanaka, 2002) and β-caryophyllene (Huang*, et al.*, 2012) which directly inhibited bacterial growth. Neri and collaborators (Neri*, et al.*, 2007), as well as Fallik and colleagues (Fallik*, et al.*, 1998) demonstrated that trans-2-hexenal reduced the germination of *Monilinia laxa* and *Botrytis cinerea*, respectively. Two monoterpenes such as limonene and β-linalool, together with nonanal and methyl jasmonate inhibited the germination of *Colletotrichum lindemuthianum* (Quintana-Rodriguez*, et al.*, 2015). The second mode of action of plant VOCs against plant pathogens consists of induced resistance. In this case, plant VOCs can contribute to disease reduction on systemic parts of a locally attacked plant or in neighbouring plant receivers by stimulating the activation of plant

defence reactions (Quintana-Rodriguez*, et al.*, 2015). For example, a mixture of two monoterpenes (α-pinene and β-pinene) promoted systemic acquired resistance within and between *Arabidopsis thaliana* plants (Riedlmeier*, et al.*, 2017). Moreover, Bate and Rothstein (Bate & Rothstein, 1998) demonstrated that gaseous treatment of trans-2-hexenal induced defence genes in *A. thaliana* plants. Finally, the third mode of action of plant VOCs against phytopatogens is called associational resistance and it consists of the adsorption of VOCs from an emitter plant to the cuticle of a receiver plant (Quintana-Rodriguez*, et al.*, 2015). This accumulation of VOCs on plant surfaces could serve as a direct defence mechanism against pathogens, as reported for *C. lindemuthianum* (Quintana-Rodriguez*, et al.*, 2015).

Although the role of plant VOCs has been characterised in various pathosystems, scarce information is available concerning the role of VOCs emitted by grapevine. Tasin and colleagues (Tasin*, et al.*, 2006) found out that three terpenoids [(E)-β-caryophyllene, (E)-β-farnesene and (E)-4,8-dimethyl-1,3,7-nonatriene] were released by grapes in a specific blend ratio that attracted females of the grapevine moth *Lobesia botrana*. More recently, increasing attention has been paid to the role of VOCs in the grapevine/downy mildew pathosystem. For example, the emission of a sesquiterpene [(E,E)-α-farnesene] was associated with the resistance induced by a sulphated laminarin against downy mildew, indicating a possible role of this sesquiterpene as a biomarker of elicitor of induced resistance (Chalal*, et al.*, 2015). Furthermore, the emission of benzaldehyde (Chitarrini*, et al.*, 2017) was found to be more pronounced in resistant grapevine genotypes following *Plasmopara viticola* inoculation, indicating its involvement as a putative biomarker of downy mildew infection.

My contribution to this research topic consisted of two papers. In the first paper (publication 1), the emission of volatile sesquiterpenes and monoteprenes was found to be higher in downy-mildew resistant than in susceptible grapevine genotypes, indicating that these VOC classes could contribute to the grapevine resistance against downy mildew. In the second paper (publication 2), the role of grapevine VOCs was investigated against downy mildew. VOCs including terpenoids, alcohols, aldehydes and heterocyclic compounds were found to play a role in defence mechanisms of resistant grapevines against *P. viticola*.

1.2.2 Functions of bacterial VOCs

As of today, more than 1000 bacterial VOCs have been described, although this number is an underestimation due to the huge diversity of bacterial environmental niches (Audrain, *et al.*, 2015). Bacterial VOCs play an important role in interaction with the environment and several studies demonstrated the involvement of bacterial VOCs in modulation of bacterial responses to different stress, such as the exposure to antibiotics. Trimethylamine, a molecule produced by *Escherichia coli* and many other Gram-negative bacteria in animal intestines and infected tissues, increases resistance to tetracycline in *E. coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus subtilis* (Létoffé, *et al.*, 2014). Kim and colleagues (Kim, *et al.*, 2013) found that 2,3-butanedione and glycoxylic acid emitted by *B. subtilis* mediated global changes in gene expression related to antibiotic resistance in *E. coli*. Aerial exposure to several bacterial VOCs affected negatively *E. coli* (1-butanol) and *P. aeruginosa* (indole, 2-butanone, and acetoin) motility, while acetoin increased *P. aeruginosa* motility (Létoffé, *et al.*, 2014). Furthermore, VOCs emitted from *B. subtilis* negatively affected the swarming of *E. coli*, *Burkholderia glumae*, *P. aeruginosa* and *Paenibacillus polymyxa* (Kim, *et al.*, 2013). Other bacterial VOCs are able to influence the formation of bacterial biofilms. For example, indole inhibits biofilm formation in aerially exposed *E. coli* and *P. aeruginosa*, whereas it stimulates *S. aureus* biofilm formation (Létoffé, *et al.*, 2014).

Bacterial VOCs play essential ecological roles acting as regulators of plant growth and inducers of plant resistance. For instance, exposure of *Arabidopsis thaliana* plants to VOCs (and in particular to 2,3-butanediol) from *B. subtilis* and *B. amyloliquefaciens* resulted in significant growth promotion (Ryu, *et al.*, 2003), and *B. subtilis* emitting 2,3-butanediol contributes to induced systemic resistance in *A. thaliana* against *Erwinia carotovora* subsp. *carotovora* (Ryu, *et al.*, 2004). Bacterial VOCs influence differentiation and growth of other bacteria (Kai, *et al.*, 2009, Effmert, *et al.*, 2012, Wenke, *et al.*, 2012). For example, the albaflavenone (a volatile sesquiterpene) emitted by *Streptomyces* spp. exhibits antibiotic activity against *B. subtilis* (Gürtler, *et al.*, 1994) and dimethyl disulphide from *P. fluorescens* and *Serratia plymuthica* showed bacteriostatic activity against the plant pathogens *Agrobacterium tumefaciens* and *A. vitis* (Dandurishvili, *et al.*, 2011). Numerous studies assessed that bacterial VOCs exhibit properties for growth inhibition of fungi responsible for major crop losses in agriculture (Weisskopf, 2013). Benzothiazole, citronellol and 1-octen-3-ol produced by

P. polymyxa BPM-11 inhibited *Rhizoctonia solani* and *Phytophthora capsici* mycelial growth (Zhao*, et al.*, 2011). Similarly, 1-undecene from *Pseudomonas* strains inhibited *P. infestans* mycelium growth (Hunziker*, et al.*, 2015). Likewise, bacteria belonging to genus *Lysobacter* spp. included species which produce non-volatile antimicrobial compounds, and for this reason, these bacteria may be potential candidates for biological control of crop diseases (Hayward*, et al.*, 2010). However, there is a limited number of studied about the possible contribution of *Lysobacter* spp. VOCs in inhibitory activities against plant pathogens. These studies described VOCs emitted by *L. gummosus* KCTC 12132 and *L. enzymogenes* ISE13 which inhibited mycelial growth on nematicidal fungi (*Paecilomyces lilacinus* and *Pochonia chlamydosporia*) (Zou*, et al.*, 2007) and phytopathogenic microorganisms (*Colletotrichum acutatum* and *P. capsici*) (Sang*, et al.*, 2011), respectively.

My personal contribution to this topic was to elucidate the biocontrol potential of *Lysobacter* spp. by studying the volatilome of four *Lysobacter* type strains grown on a sugar-rich and a protein-rich medium. *Lysobacter* volatilome differed according to the growth medium. Moreover, the application via gas phase of volatile pyrazines, pyrrole and decanal exhibited strong inhibitory activity against *P. infestans* (publication 3).

2 Biosynthesis of VOCs

VOCs have diverse chemical structures and arise from the activities of several biochemical pathways (Choudhary*, et al.*, 2008). Biosynthesis of secondary metabolites including VOCs depends on the availability of carbon, hydrogen, oxygen, nitrogen and sulphur, as well as energy which are both provided by primary metabolism (Dudareva*, et al.*, 2013). In this thesis, plant and bacterial VOCs have been studied, thus biosynthesis of these organisms will be presented in the following chapters in more detail.

2.1 Biosynthesis of plant VOCs

Plants are perhaps the most prolific producers of VOCs (Loreto*, et al.*, 2008, Baldwin, 2010). Plant VOCs are produced by a range of physiological processes in many different plant tissues (Peñuelas & Llusià, 2004), using as much as 20% of their fixed CO_2 (Baldwin, 2010). Based on their structure and biosynthetic pathways, plant VOCs can be divided into four major classes such as terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives and amino acid derivatives (Figure 1) (Heil & Bueno, 2007, Heil & Karban, 2010). Biosynthesis of a wide array of VOCs relies on a few major biochemical pathways, and various forms of enzymatic modifications such as hydroxylations, acetylations and methylations are also included, resulting in enhanced volatility or changed olfactory properties at the final step of plant VOCs formation (Dudareva*, et al.*, 2004)

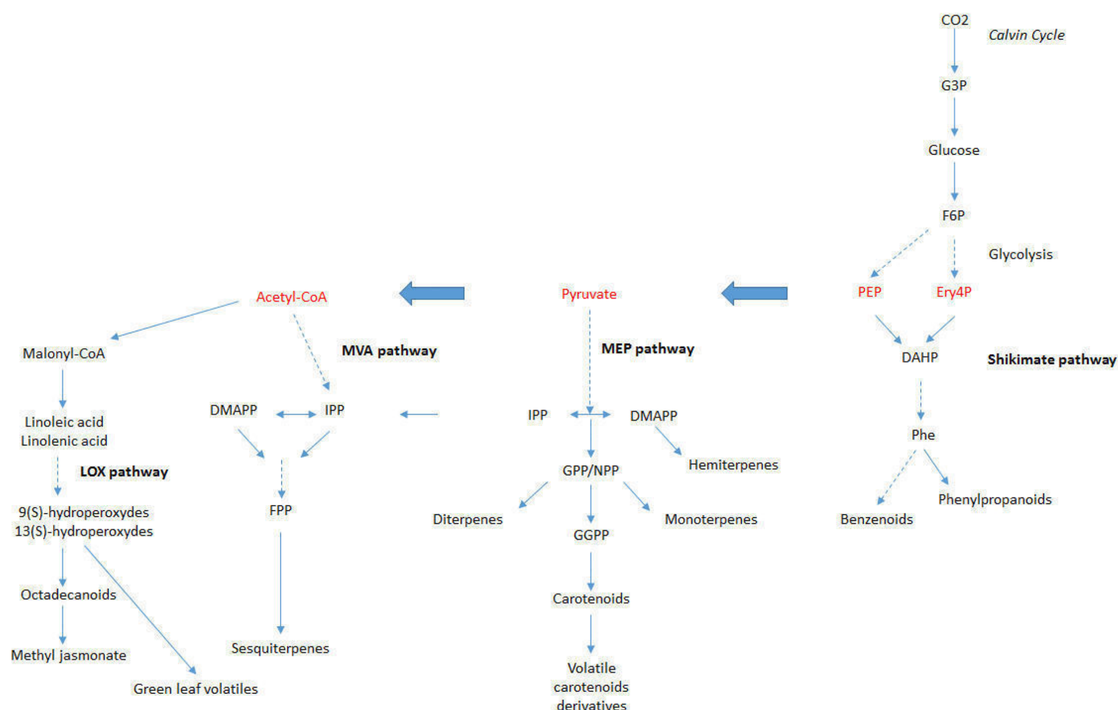


Figure 1. The four main biosynthetic pathways (showed in bold) leading the emission of plant VOCs. Precursors of plant VOCs originate from primary metabolism (showed in red). Dotted arrows indicate multiple enzymatic reactions. Abbreviations: DAHP, 3-deoxy-D-arabinoheptulosonate-7 phosphate; DMAPP, dimethylallyl pyrophosphate; Ery4P, erythrose 4-phosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, isopentenyl pyrophosphate; NPP, neryl pyrophosphate; PEP, phosphoenolpyruvate; Phe, phenylalanine.

2.1.1 Terpenoids

Terpenoids compose the largest class of plant secondary metabolites, including many VOCs (Dudareva*, et al.*, 2006, Dudareva*, et al.*, 2013). All terpenoids originate from two C_5-isoprene building units as precursors: isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). Both IPP and DMAPP are substrates for short-chain prentyltransferases, which produce prenyl diphosphate precursors [geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphate (GGPP)] for various terpene cyclases and terpene synthases enzymes (Dudareva*, et al.*, 2013).

In plants, two independent and compartmentally separated pathways are responsible for the formation of IPP and DMAPP (Dudareva*, et al.*, 2013). These are the mevalonic acid (MVA) and the methylerythritol phosphate (MEP) pathways.

The subcellular localization of the MVA pathway is still unclear, however, the MVA pathway produces only IPP and generates only sesquiterpenes (C_{15}) (Dudareva,

et al., 2013), and it consists in six enzymatic reactions starting from a condensation of three molecules of acetyl-CoA to 3-hydroxy-3-methylglutaryl-CoA, which forms MVA and IPP, as final product (Lange*, et al.*, 2000).

By contrast, the MEP pathway is exclusively plastidic (Hsieh*, et al.*, 2008). This pathway provides both IPP and DMAPP precursors and generates hemiterpenes (C_5), monoterpenes (C_{10}), and diterpenes (C_{20}). The MEP pathway involves seven enzymatic steps starting from the condensation of D-glyceraldehyde 3-phosphate (GAP) and Pyruvate to produce 1-deoxy-D-xylulose 5-phosphate which forms MEP and thus IPP and DMAPP as final products (Dudareva*, et al.*, 2013).

Both C5-isoprene building precursors (IPP and DMAPP), but also GPP and FPP facilitate the metabolic crosstalk between the compartmentally separated MVA and MEP pathways by acting as connecting metabolites(Schuhr*, et al.*, 2003), although this metabolic crosstalk is mediated by an undefined metabolite transporter (Bick & Lange, 2003).

The huge diversity of volatile terpenoids in plants is due to the action of terpene synthases (TPSs) which can synthesise multiple products from a single prenyl diphosphate substrate (Degenhardt*, et al.*, 2009), while other TPSs are able to accept more than one substrate (Tholl*, et al.*, 2006).

2.1.2 Phenylpropanoids/benzenoids

Phenylpropanoids and benzenoid compounds constitute the second largest class of plant VOCs (Knudsen*, et al.*, 2006), however, little is known about the biosynthesis of these class of compounds (Dudareva*, et al.*, 2006, Dudareva*, et al.*, 2013). This class of compounds originates from the aromatic amino acid phenylalanine in plastids through the shikimate/phenylalanine biosynthetic pathways (Maeda & Dudareva, 2012) and further converted to volatile compounds outside this organelle (Dudareva*, et al.*, 2013). The prior reaction involved in the shikimate pathway starts with the synthesis of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) through the DAHP synthase from the precursors phosphoenolpyruvate and D-erythrose 4-phosphate (Tzin*, et al.*, 2012). DAHP is further converted to phenylalanine through the shikimic acid pathway. Outside the plastids, the first step of Phe-biotransformation in the majority of phenylpropanoids/benzenoids is catalysed by L-phenylalanine ammonia-lyase which deaminates L-phenylalanine to trans-cinnamic acid (Dudareva*, et al.*, 2013). Then, a

variety of hydroxycinnamic acids, aldehydes and alcohols are formed via a series of transformations including hydroxylation, methylation and decarboxylation reactions. For example, benzenoid compounds originate from trans-cinnamic acid as a side branch of the phenylpropanoid pathway via a CoA-dependent-β-oxidative pathway, a CoA-independent-non-β-oxidative pathway, or via a combination of both. The first one is analogous to the β-oxidation of fatty acids and proceeds through the formation of four CoA-ester intermediates, while the second pathway involves hydrogenation of the trans-cinnamic acid to 3-hydroxy-3-phenylpropionic acid and side change degradation via a reverse aldol reaction leading to benzaldehyde, which is oxidised to benzoic acid by an $NADP^+$-dependent aldehyde dehydrogenase (Dudareva*, et al.*, 2006). Conversely, the biosynthesis of volatile phenylpropanoids (C_6-C_2) such as 2-phenylethanol, occurs via phenylacetaldehyde, and is in competition with trans-cinnamic acid synthesis for phenylalanine utilisation (Boatright*, et al.*, 2004).

2.1.3 Volatile fatty acid derivatives

Biosynthesis of volatile fatty acid derivatives, such as C_6 and C_9 aldehydes or methyl jasmonate, relies on a plastidic pool of acetyl-CoA generated from pyruvate, the final product of glycolysis (Dudareva*, et al.*, 2013). Volatile fatty acid derivatives arise from C_{18} unsaturated fatty acids, linoleic or linolenic acids (Dudareva*, et al.*, 2013). In the lipoxygenase pathway, linoleic and linolenic acids undergo deoxygenation in a reaction catalysed by lipoxygenases (LOX) (Feussner & Wasternack, 2002). These enzymes can catalyse the oxygenation of polyenoic fatty acids at C_9 or C_{13} positions (9-LOX and 13-LOX enzymes), yielding two groups of compounds, 9-hydroperoxy and 13-hydroperoxy intermediates. 9(S)-hydroperoxy linolenic acid and 13(S)-hydroperoxy linolenic acid can be further metabolised by an array of enzymes such as allene oxide synthase and 13-hydroperoxyde lyase which represent the two branches of the lipoxygenase pathway yielding methyl jasmonate and green leaf volatiles such as hexanal, respectively (Song*, et al.*, 2005, Dudareva*, et al.*, 2013). Other enzymes such as alcohol dehydrogenase can also metabolise the 9(S)-hydroperoxy linolenic acid intermediate in other GLVs such as 3-nonenol (Dudareva*, et al.*, 2013).

2.1.4 *Non-aromatic amino acid derivatives*

Plant VOCs can also be synthesised from non-aromatic amino acids such as alanine, valine, leucine, isoleucine and methionine or their intermediates and may also contain nitrogen and sulphur (Dudareva*, et al.*, 2006). These amino acid volatiles are highly abundant in floral scents and fruit aromas (Knudsen*, et al.*, 2006). Similarly to yeast or bacteria (Tavaria*, et al.*, Dickinson*, et al.*, 2000), biosynthesis of non-aromatic amino acid plant volatiles starts from deamination or transamination, leading to the formation of the corresponding α–ketoacid (Dudareva*, et al.*, 2006). These α–ketoacids can be further subjected to other enzymatic reactions such as reduction or esterification leading to the formation of volatile aldehydes, alcohols, or esters, respectively.

2.2 *Biosynthesis of bacterial VOCs*

Bacteria produce and emit highly diverse inorganic and organic volatile compounds, and they use different catabolic pathways to synthesize VOCs such as glycolysis, proteolysis and lipolysis (Peñuelas & Llusià, 2004, Schulz, 2007). Biosynthesis of the wide array of different bacterial VOCs belongs to a few primary metabolic pathways, and these biosynthetic pathways are aerobic heterotrophic carbon metabolism, fermentation, amino acid degradation, terpenoid biosynthesis and sulphur reduction (Peñuelas & Llusià, 2004). Several microbial VOCs are released as intermediate or end products of fermentative and respiratory (aerobic or anaerobic) microbial metabolic pathways (Peñuelas & Llusià, 2004).

Bacterial VOCs consist of inorganic and organic VOCs (Effmert*, et al.*, 2012) and they include several classes such as fatty acid derivatives, aromatic compounds, nitrogen-containing compounds, volatile sulphur compounds, terpenoids and other compounds (Schulz, 2007).

2.2.1 *Inorganic compounds*

Carbon dioxide is the major inorganic volatile produced by all heterotrophic living organisms (Effmert*, et al.*, 2012), but bacteria also emit nitric oxide synthesised by nitric oxide synthases from L-arginine (Mattila & Thomas, 2014), hydrogen sulphide produced by degradation of L-cysteine, ammonia which is produced from the metabolism of peptide and amino acid (L-aspartate catabolism) (Bernier*, et al.*, 2011),

or hydrogen cyanide that is catalysed by hydrogen cyanide synthase (Audrain*, et al.*, 2015).

2.2.2 *Fatty acid derivatives*

Linear-chained hydrocarbons derive from products of the fatty acid biosynthetic pathway via elongation-decarboxylation or head-to-head condensation pathways (Ladygina*, et al.*, 2006). Decarboxylation of fatty acids is responsible of the formation of methyl ketones, while long–chain aliphatic alcohols are produced through β- or α-oxidation of fatty acid derivatives (Audrain*, et al.*, 2015). Acids are the products of anaerobic metabolism and they are usually formed during bacterial fermentation of carbohydrates (Audrain*, et al.*, 2015). Acids are less abundant than ketones and alcohols in bacteria volatilome and mainly consist of acetic, propionic and butyric acids (Schulz, 2007).

2.2.3 *Aromatic compounds*

Aromatic compounds are less common in bacteria than in plants, and are generated by the shikimate pathway or by degradation of the aromatic acids L-phenylalanine and L-tyrosine (Schulz, 2007, Peñuelas*, et al.*, 2014).

2.2.4 *Nitrogen-containing compounds*

The simplest nitrogen-containing compound is ammonia although it is very difficult to detect because of its high volatility and low molecular mass (Schulz, 2007). Pyrazines appear to constitute one of the major classes of volatiles released by bacteria and, due to their strong odour, they are used as important flavouring compounds (Peñuelas*, et al.*, 2014). The biosynthesis of pyrazines is not well established, although methyl and ethyl pyrazines seem to be synthesised not enzymatically via dihydropyrazines, while higher alkyl pyrazines require enzymatic activity and amino acids as precursors (Schulz, 2007). Indole production has been described in 85 bacterial species (Audrain*, et al.*, 2015) and is synthesised enzymatically from amino acids (i.e. tryptophan) and other compounds found widely in nature (Davis*, et al.*, 2013).

2.2.5 *Volatile sulphur compounds*

The biogenesis of sulphur volatile compounds play an important role in the global biogeochemical cycle of sulphur (Peñuelas*, et al.*, 2014). This class of compounds originates from the degradation of L-methionine either by direct cleavage of the amino acid by L-methionine gamma-lyase or by its transamination to alpha-keto-gamma-methylbutyric acid and subsequent reductive demethylations (Peñuelas*, et al.*, 2014).

2.2.6 *Terpenoids*

Terpenoids are biosynthetically derived from the universal terpene building blocks dimethyallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP), which can arise either on the mevalonate pathway or on the deoxyxylulose phosphate pathway (Schulz, 2007). Only monoterpenes (C10) and sesquiterpenes (C15) and their derivatives or degradation products have been reported in bacterial volatile blends (Schulz, 2007).

2.2.7 *Other compounds*

Several other compounds are produced by bacteria. Halogenated compounds, volatile selenium and tellurium compounds and volatiles derived from other metal or metalloids are part of this class (Schulz, 2007). Moreover, iodine compounds are important in the global biochemical cycle of iodine, although little information is available about the production of volatile iodine compounds by bacteria (Schulz, 2007). The formation of selenium and tellurium containing volatiles by bacteria and the role of other metals or metalloid containing volatiles is poorly understood (Schulz, 2007).

2.3 *Mechanisms of VOCs emission*

Besides VOCs biosynthesis mechanisms, in the past decade, increasing attention has been paid to understand VOCs regulation, especially concerning the plant system (Dudareva*, et al.*, 2013). Indeed, VOCs production and release are finely regulated and depend on biotic and abiotic factors. The biosynthesis of VOCs in nongreen tissues, such as flowers or roots, occurs predominantly in epidermal cells. In vegetative organs, VOCs are often synthesised in the secretory cells of glandular trichomes on the leaf

surface (Wang*, et al.*, 2008) and stored until mechanical disruption (Iijima*, et al.*, 2004). If trichomes are not involved in vegetative VOC production, volatiles are produced in mesophyll cells (Köllner*, et al.*, 2013) and released through stomata (Kesselmeier & Staudt, 1999), mechanical disruption or emission through cuticle (Niinemets*, et al.*, 2002). However, while mechanical disruption provides releasing of VOCs directly to the atmosphere, it remains unclear how VOCs cross plasma membrane, hydrophilic cell walls and, in some cases, the cuticle to exit spontaneously the cells (Widhalm*, et al.*, 2015). In the last decade, it is largely presumed that VOCs passively diffuse from cells to the environment, however, Widhalm and collaborators (Widhalm*, et al.*, 2015) demonstrated, by Fick's law that VOCs emission rates would need toxic levels to passively diffuse in cellular membranes and can increase membrane permeability, which leads to disruption of proton and ion gradients that are responsible for the homeostasis of the cells (Sikkema*, et al.*, 1995). Moreover, the VOCs are primarily nonpolar compounds, making spontaneous diffusion into aqueous cellular compartments very slow. For these reasons, alternative emission paths (Figure 2) have been proposed by Widhalm and collaborators (Widhalm*, et al.*, 2015).

Regarding VOCs synthesised in the cytosol, they can favourably reach the plasma membrane via endoplasmic reticulum (ER) membrane or via vesicle trafficking processes associated with the ER, Golgi and/or vacuole. Regarding VOC synthesised in other organelles such as terpenes in plastids, volatiles could be delivered to the ER via interorganellar membrane hemi-diffusion. Alternatively, VOCs trafficking to the plasma membrane could be mediated by soluble carrier proteins with hydrophobic pockets.

From the plasma membrane to the apoplast, VOCs have to cross a lipophilic layer and reach an aqueous environment. One solution could be the possibility of plasma membrane-localised transporters involved in the export of VOCs out of the cell, and recently, extracellular lipid transfer proteins (LTPs) have been suggested to transport VOCs from the plasma membrane to neighbouring cells, intercellular spaces or the cuticle, which imposes the largest resistance to VOCs emission and needs further investigations.

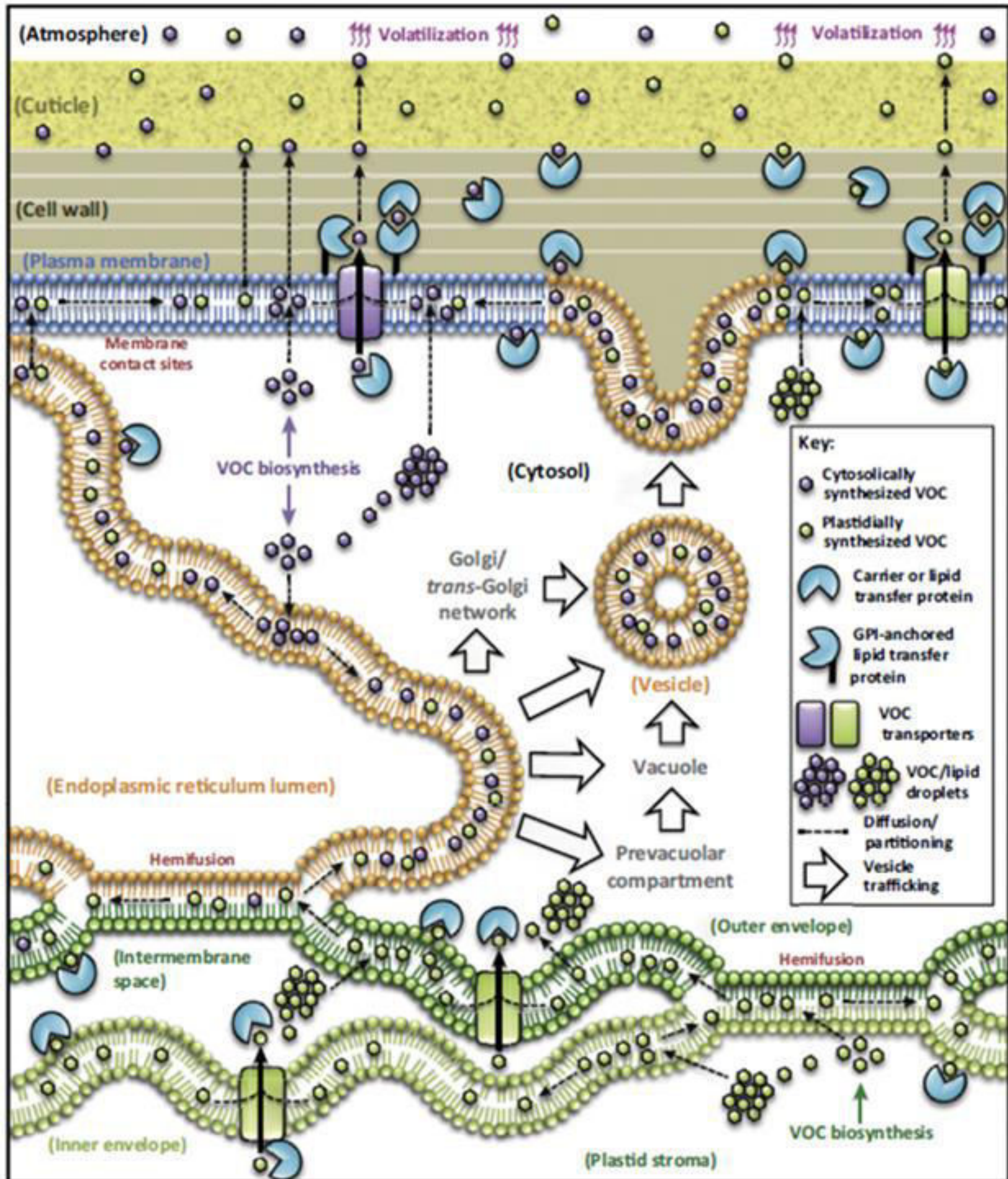


Figure 2. Proposed models for VOC trafficking in plant cells. Figure from (Widhalm, et al., 2015). VOCs may diffuse through the cell membrane or by unknown mechanisms similar to other hydrophobic compounds. Possible vesicles can transport the VOCs through the cytosol, and lipid transfer proteins (LTPs) or other types of carrier proteins may contribute to guide VOCs through the plastid stroma, cytosol, and/or cell wall. Abbreviation: GPI, glycosylphosphatidylinositol.

3 Measurement of VOCs

The volatile fraction of a biological sample is generally defined "as a mixture of volatiles that can be sampled because of their ability to vaporise spontaneously and/or under suitable conditions or by employing appropriate techniques" (Herrmann, 2010). The most important task in the measurement of VOCs is to detect, identify and, when necessary, quantify the volatile component(s) that are marker(s) of the investigated biological phenomenon (Hayward*, et al.*, 2010). This requires analytical methods and technologies to be adopted that are sensitive enough to detect variations in the composition of volatiles, and to enable the dynamics of the reaction of a living organism to be monitored when its metabolism is altered (Hayward*, et al.*, 2010). Advances on automated analysis of VOCs have allowed the monitoring of fast changes in VOC emissions and facilitated *in vivo* studies of VOC biosynthesis (Tholl*, et al.*, 2006). Indeed, automated VOC analysis systems have become indispensable for monitoring fast changes of volatile profiles during development of a biological system under investigation or under experimentally controlled stress conditions (Aylor*, et al.*, 2001). The approach to the measurement of VOCs needs several important steps in order to obtain the highest level of information from the samples investigated. These steps consist of sample handling, sample extraction, VOCs determination (which includes VOCs separation and detection), VOCs annotation/identification, and data analysis.

3.1 Sample handling

One of the most important parts of VOCs measurement consists of a correct sample handling. After a thorough analysis of the biological experiment, samples must be taken and processed for the VOCs measurement. VOCs can be sampled by various techniques grouped in two main categories. The first category of VOCs sampling encompasses the non-invasive methods which allow the investigation of the living organisms without influencing their biological system (i.e. the bacterial cultures or *in vitro* plantlets used in this thesis). The second category of VOCs sampling includes the invasive procedures which permit sampling of parts of the biological sample for investigation of the desirable target (i.e. foliar VOCs, as described in this thesis). The first step of the latter procedures is quenching biological samples in order to stop instantly the metabolism by inhibiting the endogenous enzymes upon harvesting. To be useful and effective,

quenching must be very fast in order to avoid any metabolic change of the sample. During sampling, the damage of cells must be minimised and the technique itself must not lead to any change in terms of chemical properties. After quenching, samples shall be usable for further sample treatments. There are two main quenching techniques reported in literature and they are based on rapid modification of sample conditions, usually pH or temperature (Álvarez-Sánchez*, et al.*, 2010). In this thesis, both sampling categories have been used. As in the case of grapevine leaves from greenhouse (publication 2), an invasive method has been used and grapevine samples have efficiently been quenched in liquid nitrogen in order to literally freeze the metabolic state of the leaves at the time point of sampling. This is the most simple and rapid procedure that can be applied after harvesting (Kim & Verpoorte, 2010). Conversely, the *in vitro* grapevine plants (publication 1) were as well as bacterial cultures reported in publication 3 sampled in a non-invasive way.

Once the quenching procedure is successfully carried out, samples shall be analysed as fast as possible or shall be stored properly until their preparation and analysis. For example, as reported for publication 2, after quenching, samples were immediately stored at -80°C in order to avoid any degradation of VOCs. Conversely, no storage has been used for publications 1 and 3 because we measured VOCs from living plant and bacteria, respectively. Sample quenching is generally followed by sample preparation which is defined as all sample manipulation steps between quenching and instrumental analysis (Weingart*, et al.*, 2013). Freeze-drying is frequently used as further sample stabilization measure in metabolomics, but was not used here because the aim of this thesis is the investigation of VOCs and freeze-drying could lead to a high loss of VOCs (Aprea*, et al.*, 2011). Sample homogenisation is the next sample preparation step prior to extraction. Homogenisation can be done with mortar and pestle, vibration mill, ultrasonic bath of thermomixer and when possible, this step should be performed under cooled conditions in order to not only prevent sample defrosting (Álvarez-Sánchez*, et al.*, 2010), but also to avoid loss of VOCs. As reported in publication 2, leaf samples were homogenised with a ball mill using both milling containers and stainless balls previously cooled in liquid nitrogen. Immediately after the homogenisation step, samples were weighed into 20 mL headspace vials and analysed.

3.2 Extraction of VOCs

In the past decade, volatile analysis has improved by the design of relatively inexpensive but sensitive bench-top instruments for GC-MS (Tholl, *et al.*, 2006). The development of headspace techniques for collection of volatiles has significantly improved our understanding of the biosynthesis and ecology of plant and bacterial VOCs (Tholl, *et al.*, 2006). Methodology commonly used for airborne volatile analysis is based on headspace analysis followed by gas chromatography (GC) analysis (Farag, *et al.*, 2013). No single analytical method can comprehensively survey the entire set of volatile metabolites of a living organism i.e., plants or bacteria (Farag, *et al.*, 2013). The approach to the analysis of VOCs has radically changed during the last 15-20 years and the number of techniques available to extract VOCs is quite large. Examples include distillation techniques such as vacuum-, dry-, steam- or hydro-distillation (Li, *et al.*, 2014), highly effective solvent extraction techniques such as ultrasound (Alissandrakis, *et al.*, 2003), microwave-assisted extraction hydrodistillation (MA-HD) (Ferhat, *et al.*, 2006), or conventional extraction with organic solvents (liquid-liquid extraction, LLE) (Castro, *et al.*, 2004), pressurised solvent extraction (PSE), or supercritical fluid extraction (SFE) (Bicchi, *et al.*, 1999). However, most of these approaches are typically time-consuming and labour-intensive, use significant amounts of environmentally unfriendly solvents, and involve multi-step procedures, which can lead to analyte losses and a reduction of sensitivity (Mendes, *et al.*, 2012). In most cases, the search of adequate extraction techniques that minimise the use of harmful organic solvents or even use solvent-free procedures that could be easily implemented, has attracted the attention of many scientists in the last years. Nowadays, VOCs are usually extracted by headspace techniques which give a more realistic picture of the volatile profile emitted by a biological sample (Tholl, *et al.*, 2006). According to the definition of Kolb and Ettre, (Kolb & Ettre, 1997), headspace sampling is a solvent free technique aimed at sampling the gaseous of vapour phase in equilibrium (or not) with a solid or liquid matrix in order to characterise its composition. Traditionally, headspace sampling operates either in dynamic or static mode. Headspace techniques are based on either the static or dynamic accumulation of volatiles on polymers operating in absorption and/or adsorption modes, or on solvents (Bicchi, *et al.*, 1999).

Dynamic headspace sampling represents the most frequently used technique in plant volatile analysis (Tholl, *et al.*, 2006), and it consists of a continuous gas stream flowing

through the sample container as a carrier gas, which increases the headspace sample size. Dynamic headspace extraction can be carried out in a closed (i.e. closed-loop stripping) or open (i.e. purge and trap) system. VOCs are trapped and enriched on an adsorbing matrix and further eluted from the trap by organic solvents or the loaded trap is directly connected to the carrier gas stream of the gas chromatograph (GC), thus VOCs are desorbed onto the GC column by rapidly heating the trap to high temperatures (thermodesorption). Although dynamic headspace techniques are highly sensitive, impurities from the incoming air may lead to artifacts (Tholl*, et al.*, 2006).

In static headspace, samples are enclosed in a container (i.e. headspace vials) and VOCs are enriched on the adsorbing matrix without continuous gas stream as for dynamics techniques. After a period of time for equilibration, the headspace is sampled either by the withdrawal of a defined-volume aliquot from the headspace (i.e. by sample loop or gas-tight syringe) or the introduction of an adsorptive/absorptive material attached to the sampling device (Weingart*, et al.*, 2013). An important progress in static headspace technique was the development of solid phase microextraction (SPME).

In the following paragraphs two headspace sampling techniques used in the publications reported in this thesis will be described in more details. Solid phase microextraction, and on-line sampling.

3.2.1 Headspace solid phase microextraction (HS-SPME)

Most of the sample-preparation techniques currently available rely on trapping the analytes of interest from the sample (gas, liquid or solid) by an adsorbent material (Baltussen*, et al.*, 2002). The first headspace technique to appear was HS-SPME introduced by Zhang and Pawliszyn in 1993 (Zhang & Pawliszyn, 1993). They showed that analyte recovery from headspace by a fibre depends on two closely-related but distinct equilibria: the first is the matrix/headspace equilibrium responsible for the headspace composition, the second is the headspace/polymeric fibre coating equilibrium (Zhang & Pawliszyn, 1993). SPME is based on ad/absorption and desorption of volatiles from an inert fibre coated with different types of ad/absorbents (Tholl*, et al.*, 2006). The fibre is located inside the needle of a modified syringe and volatiles can be sampled by inserting the needle through a septum of a headspace collection container and pushing the plunger to expose the fibre (Figure 3).

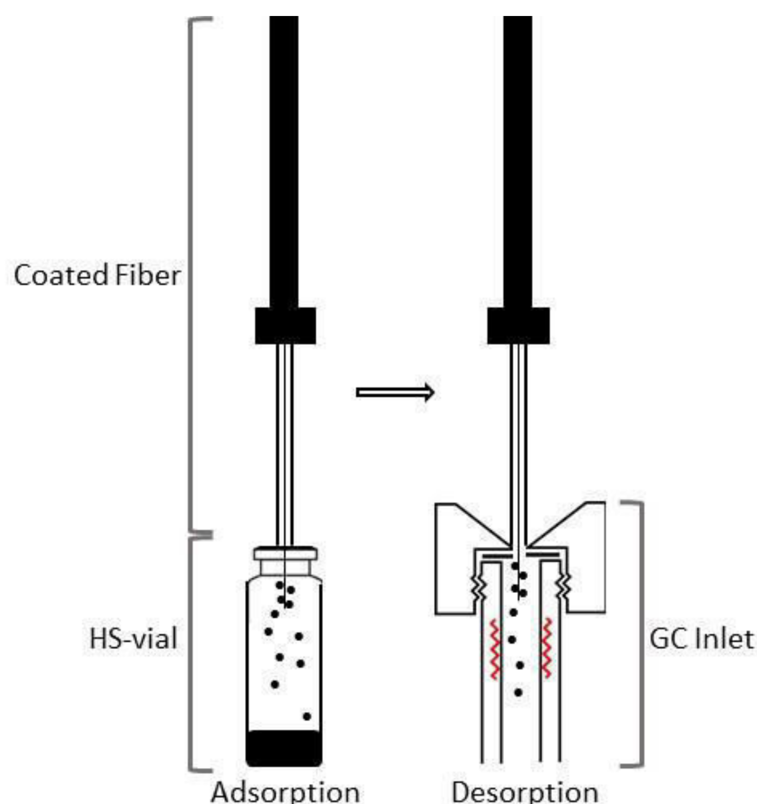


Figure 3. SPME sampling. The fibre is placed in the headspace above the sample during extraction. The amount of analyte extracted onto the fibre coating is at a maximum when the analyte concentration has reached the equilibrium between the sample matrix and the fibre coating. Next, the enriched analytes can be desorbed from the fibre to the chromatographic column through the GC inlet (modified from http://web.sonoma.edu).

Different coatings of the fibre are available, and they vary according to the chemical properties of the metabolites of interest. The coating is fixed on a fused silica core, and depending on the coating material, the analytes of interest are adsorbed or absorbed to the coating material (Table 1).

Table 1. Fibre coatings, thickness and polarity of different SPME fibres.

Coating	Thickness (µm)	Polarity
Polydimethyl-siloxane (PDMS)	7, 30, 100	Apolar
Polyacrilate (PA)	85	Polar
Polyethylene glycol (PEG)	60	Polar
Carbopack/Z PDMS	15	Bipolar
PDMS/Divinylbenzene (DVB)	65	Bipolar
Carboxen (CAR)/PDMS	85	Bipolar
DVB/CAR/PDMS	55/30	Bipolar

In SPME, the concentration of the analyte bound to the fibre is related to the concentration of the same analyte in the sample and it depends on the distribution constant (diffusion of the metabolites between the phases) which is temperature dependent. Ideally, the extraction of VOCs shall last until equilibrium between the metabolites on the fibre and the HS has been reached. Following equilibration between the fibre and the volatile sample (typically a few minutes to approximately one hour), the fibre is retracted into the needle and can be transferred to a gas chromatograph for direct thermal desorption (Tholl*, et al.*, 2006). Thermal desorption of VOCs from the fibre eliminates the need for solvents that may contain impurities which will interfere with sample analysis (Tholl*, et al.*, 2006). By carefully selecting the polarity and thickness of the fibre coating, VOCs of different polarity and volatility ranging from high-boiling or semi-volatile to volatile compounds can be sampled (Tholl*, et al.*, 2006). A further important factor that contributed greatly to the development of HS techniques is the increasing knowledge of sorption material. Such sorption materials (coatings) are homogeneous, non-porous materials in which the analytes can dissolve so that they do not undergo real bonding with the material, but are retained by dissolution (Baltussen*, et al.*, 2002). Several companies offer GC autosamplers with a SPME option for rapid successive processing of multiple samples (Tholl*, et al.*, 2006). Automated SPME-GC allows high-throughput analysis of volatile profiles emitted from plant foliage or bacteria, as presented in this thesis. Despite the enormous advantages of this technique, static headspace and in particular, SPME has some disadvantages. It is nearly impossible to quantify absolutely and precisely a large number of metabolites with diverse polarities and volatility at the same time with SPME. This problem is typical of complex biological samples, such as plant leaves or bacteria, which contain VOCs with various chemical and physical properties.

In order to reach the best results possible with the highest number of peaks, the equilibration time and temperature have been optimised before (Weingart, *et al.*, 2013) and extraction time and temperature used were 40 minutes and 60°C, respectively for the analysis of grapevine leaves (publication 2). Concerning the *Lysobacter* spp. VOCs (publication 3), 30 minutes at 25°C for both equilibration and extraction were used, and polydimethylsiloxane/divinylbenzene/carboxen (PDMS/DVB/CAR) fibre was used for both the publications.

3.2.2 On-line sampling

Automated VOC analysis systems with on-line capability have become indispensable for monitoring fast changes of volatile profiles from biological samples (Tholl*, et al.*, 2006). The need for real-time measurements has led to considerable interest in non-chromatographic methods, most often involving mass spectrometry [e.g. proton transfer reaction (PTR)-MS]. The major contribution made by PTR-MS is that it provides instruments by which traces of gases and volatiles in headspace above liquid and solid samples (e.g. plant leaves or bacterial cultures) can be measured qualitatively and quantitatively in real time, obviating sample collection into bags or onto traps. Thus, the sample and its analytes are not modified or disturbed, and, since many VOCs are fragile molecules, this is obviously advantageous (Smith & Španěl, 2011). A second innovation was the drift tube. Instead of employing a carrier gas to transport ions along the tube, the analyte/air sample is directly injected into the drift tube and analysed (Blake*, et al.*, 2009). In the following section, this technique is described in detail.

3.2.2.1 Proton-transfer reaction mass spectrometry (PTR-MS)

Proton-transfer reaction mass spectrometry (PTR-MS) was first developed by Professor Werner Lindinger and collaborators in the late 1990s (Lindinger*, et al.*, 1998). This instrument typically consists of four parts: discharge ion source, drift tube reaction chamber, mass analyser and ion detection unit (Figure 4).

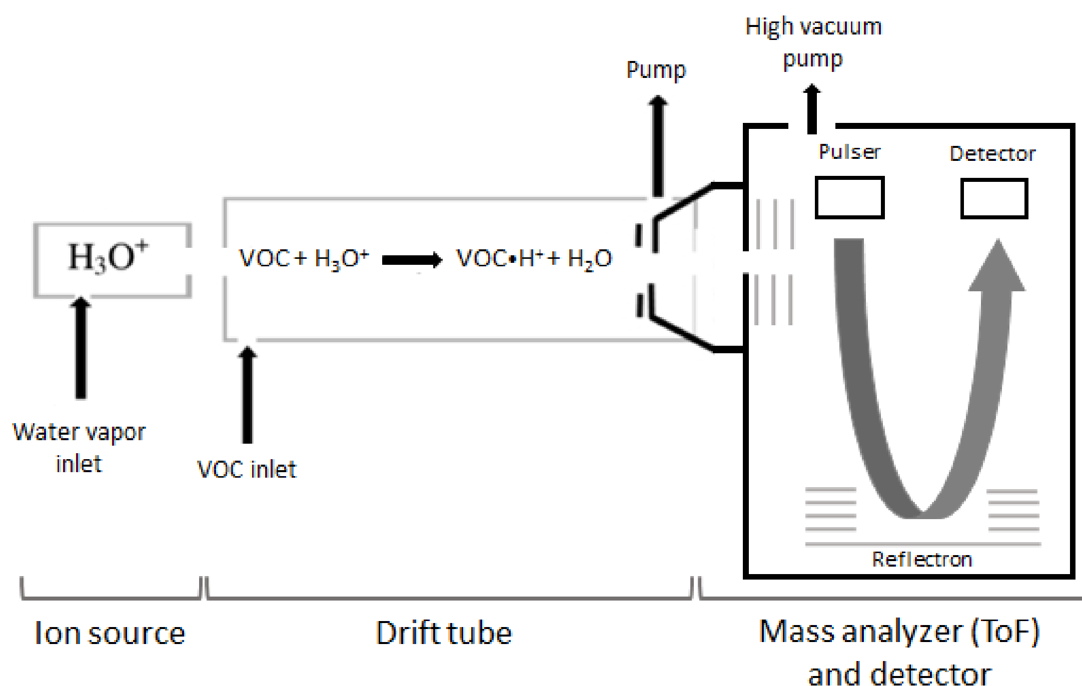
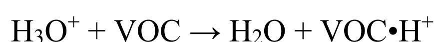


Figure 4. Proton transfer reaction-time of flight mass spectrometer (PTR-ToF-MS) scheme. The hydronium H_3O^+ are generated from water vapour and electrically forced to the drift tube where they collide with VOCs, transferring a proton to them. Protonated VOCs are guided to the ToF analyser where they are separated according to their *m/z* prior to detection (modified from https://alchetron.com).

In PTR-MS, the neutral VOC molecules are ionised via a proton transfer reaction, typically with the hydronium ion (H_3O^+). These ions are generated in the ion source by a hollow cathode discharge on the water vapour which is injected into the ion source region (Hansel, *et al.*, 1995). Ions produced in the ion source are allowed to enter into the PTR-MS drift tube. Here the gaseous samples are also injected. H_3O^+ ions undergo proton-transfer reaction with most organic species, while such reaction does not occur with air constituents, such as O_2, N_2, CO_2, or noble gases. This is because the proton affinity of water is smaller than most VOCs but larger than the constituents of clean air (Lindinger, *et al.*, 1998). Proton-transfer reactions take place in a buffer gas (usually air) which flows through the drift tube and protons are transferred from primary H_3O^+ ions to trace VOCs that have smaller proton affinity than water. The reaction is:



$$H_3O^+ + VOC \rightarrow H_2O + VOC{\bullet}H^+$$

Protonated VOCs are separated in a mass analyser according to the *m/z* ratio and finally detected by an ion detection unit. In this PhD thesis, I used a PTR-MS coupled with a time-of-flight (ToF) mass spectrometer. The instrument gives raw data in count per scan (cps) which have to be converted in absolute concentrations. For this reason, the instrument needs to be calibrated with a mixture of standard gases, provided by gas cylinders, with a known concentration (Materić*, et al.*, 2015). In the present PhD thesis, and in particular in publications 1 and 3, a PTR-ToF 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) was used. In the papers 1 and 3, the H_3O^+ primary ion mode was used and similar ion drift tube conditions were used, which means drift tube pressure was maintained to 2.3 mbar, the temperature of the drift tube was set at 110°C. Drift tube voltage was set to 550 V and 480 V for paper 1 and paper 3, respectively, while the *E/N* ratio (*E* represents the electric field strength and *N* represents the density of the drift tube gas molecules) was maintained at 140 Td for paper 1 and at 120 Td for paper 3 (1 Td = 1 Townsend = 10^{-17} Vcm^2).

3.3 Chromatographic separation of VOCs

The most widely used tool for qualitative and quantitative determination of VOCs is gas chromatography-mass spectrometry (GC-MS) (Blake*, et al.*, 2009). However, although it is a highly sensitive and reliable technique, absolute quantification needs strict sampling parameters and a detailed evaluation of matrix effects (Tholl*, et al.*, 2006). Furthermore, it is also necessary to pre-concentrate the samples for a few minutes, leading the GC-MS to be a relatively slow technique for separating and subsequently detecting VOCs (Blake*, et al.*, 2009, Materić*, et al.*, 2015). However, if speed is not important, than GC-MS is probably the most powerful technique available for measuring trace levels of VOCs (Blake*, et al.*, 2009). In the next section, GC will be described.

3.3.1 Gas chromatography (GC)

Plant and bacterial VOCs trapped on adsorbing matrices are routinely separated by the well-established technique of gas chromatography which is commonly used for separation of low boiling and low mass compounds, even in complex mixtures (Tholl*,*

et al., 2006, Materić*, et al.*, 2015). A gas chromatograph usually consists of a sample inlet, an oven with a chromatographic column, and a detector (Figure 5).

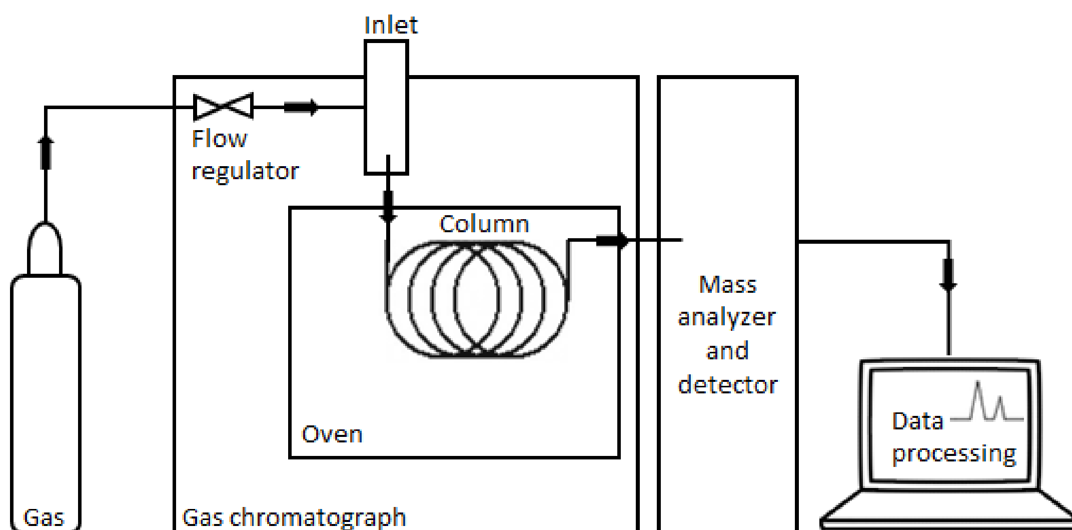


Figure 5. Gas chromatography-mass spectrometry instrument scheme. Samples are introduced via a heated inlet and then transported by the carrier gas (usually helium) through the column. Each of the VOCs interacts differently with the stationary phase of the column. Then, VOCs elute from the column at different times after injection (retention time) and reach the ion source, mass analyser and finally the detector (modified from https://www.netzsch-thermal-analysis.com).

In case of SPME, the enriched analytes from the sample must be desorbed from the coated fibre by placing it directly in a thermal desorption tube of the GC inlet, heated to 250-300°C. The thermically released volatiles can also be concentrated by a cold trap prior to their separation on the GC column (Tholl*, et al.*, 2006). Samples are introduced via a heated inlet and then transported by the mobile phase through the column (Materić*, et al.*, 2015). The core part of a gas chromatograph is the column, which is placed in the oven of the instrument. Generally, there are two types of columns: packed and capillary, however capillary columns are more commonly used in VOCs research (Materić*, et al.*, 2015). The stationary phase is a highly viscous film capable of absorbing and separating compounds according to their vapour pressure and their interaction with the stationary phase. Each of the VOCs interacts differently with the stationary phase of the column and is therefore differentially partitioned between the stationary phase and mobile phase which consists of a carrier gas flow. Generally, VOCs are separated on fused silica capillary columns coated with different stationary phases such as non-polar dimethyl

polysiloxanes (e.g. DB-5, HP-5) or more polar polyethylene glycol polymers such as Carbowax® 20M or DB-Wax) (Tholl*, et al.*, 2006). For our purposes, a DB-5MS non-polar column (5% diphenyl polysiloxane, 95% dimethyl-diphenyl polysiloxane; 30m of length, 0.25mm of internal diameter, 0.25µm of film thickness) has been used for publication 2, while a polar column HP-InnoWax [polyethylene glycol (PEG); 30m, 0.32mm, 0.5µm] has been used in publication 3. The GC instrument also contains a temperature-controlled oven, capable of being rapidly ramped up reproducibly from room temperature to over 300°C (Materić*, et al.*, 2015). An increase in temperature (temperature gradient) during separation leads to changes of the partition coefficient, thus VOCs widely differing in volatility elute from the column and reach the detector at different time points, resulting in a detection of substances with largely different boiling points within one run (Materić*, et al.*, 2015). Although GC is a very powerful tool for efficiently separating complex mixtures, it is not possible to achieve a complete separation of all analytes. This is because there are compounds which are very similar in terms of molecular structure that cannot be completely separated. For this reason, mass spectrometry (MS) detectors are needed to increase selectivity and detection in case of co-elution to the GC. In this PhD thesis and in particular in publications 2 and 3, two different CG instruments were used. In publication number 2, an Agilent 6890N (Agilent, Waldbronn, Germany) has been used, while in publication 3 an Auto System XL gas chromatograph (Perkin Elmer, Norwalk, CT, USA) has been used. One end of the GC column is connected to the inlet, and the other end is connected to the detector. Mass spectrometry detectors are the most popular type of detector for routine plant and bacterial volatile analysis (Tholl*, et al.*, 2006, Farag*, et al.*, 2013) and have also been used in this PhD thesis.

3.4 Detection of VOCs

The detection of VOCs requires analytical techniques that are sensitive enough in order to distinguish variations in the composition of the samples and their metabolism. Mass spectrometry is based on the principle that molecules are ionised and, depending on the ionisation technique, can be fragmented further into a number of structurally significant fragments which, together with the molecular ions, can be separated according to their mass-to-charge ratio by a magnetic or electromagnetic field (Knepil, 1999). A typical mass spectrometer consists of an ion source, a mass analyser, an ion

detector, a vacuum system and a computer. In the following sections, the types of ion source and mass analysers which were used in the presented thesis, will be explained in more detail.

3.4.1 Ion source

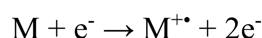
The analytes exiting the GC column are ionised in the ion source in a characteristic and reproducible way usually by either chemical ionisation (CI) or electron ionisation (EI).

3.4.1.1 Chemical ionisation (CI)

The CI is a "soft ionisation technique" and it forms intact ions called ion molecules which are nearly not fragmented. The charge of the ions formed is transferred to the sample molecules leading to protonation, deprotonation or ion-molecule adduct formation (Knepil, 1999). This process requires an ion-source pressure of about 0.1 to 1 mbar, while the pressure outside the ion source must be kept below 10^{-5} mbar to maintain a sufficient free path length without any collision with other molecules or ions. PTR used in publications 1 and 3 is based on chemical ionisation of the VOCs under investigation within a drift reactor tube (King*, et al.*, 2013).

3.4.1.2 Electron ionisation (EI)

EI is a "hard ionisation technique" and it was applied in this thesis (publications 2 and 3). It consists of the creation of the resulting positively charged molecule ions which are further fragmented due to their excess in internal energy. The ionisation process occurs within about 10^{-16} to 10^{-15} seconds and is several orders of magnitude faster than the oscillation period of a chemical bond. In the ion source, high-energy electrons are created from a resistively heated metal filament and accelerated across the source (typically using potential difference in the range 5–100 V) to the stream of neutral analyte molecules in the gas phase. The general equation is:



$$M + e^- \rightarrow M^{+\bullet} + 2e^-$$

As a consequence of the very rapid ionisation process, the geometry of the molecules does not change during ionisation (Knepil, 1999). The energy content of the electrons

employed for ionisation is usually expressed in electron volts (eV). The extent of fragmentation and thus the formed fragment ions depend on the energy used for ionisation. Typical ionisation energies of common organic analytes will range from 5 eV to 15 eV, however, having higher energy (usually set at 70 eV) than is required to affect ionisation, improves the efficiency and the repeatability of the ionisation process. Moreover, an energy of 70 eV does not alter the absolute or relative intensities of ions within the spectrum, which is of additional value when using literature libraries for analyte annotation/identification (Taylor, 2015).

3.4.2 Mass analyser

The most common gas chromatograph detectors are flame ionisation detectors (FID) and mass spectrometers (MS) (Materić*, et al.*, 2015). In the GC-MS analyses carried out in this thesis, I used a quadrupole mass analyser. Other common mass analysers that can be used in combination with GC are: ion trap (IT), triple quadrupole (QqQ) and time-of-flight (ToF), this latter has been used in publications 1 and 3. In all types of mass analysers, molecular ions and derived fragments are separated according to their mass-to-charge (*m*/*z*) ratio. The resulting mass spectra are plotted by the software of the GC instrument and the *m*/*z* values of the intact molecular ion and the corresponding fragments (x-axis) as well as the intensity (or abundance, y-axis) are shown (Figure 6). Each mass spectrum is characteristic for the substance measured and provides information about the structure of the analyte.

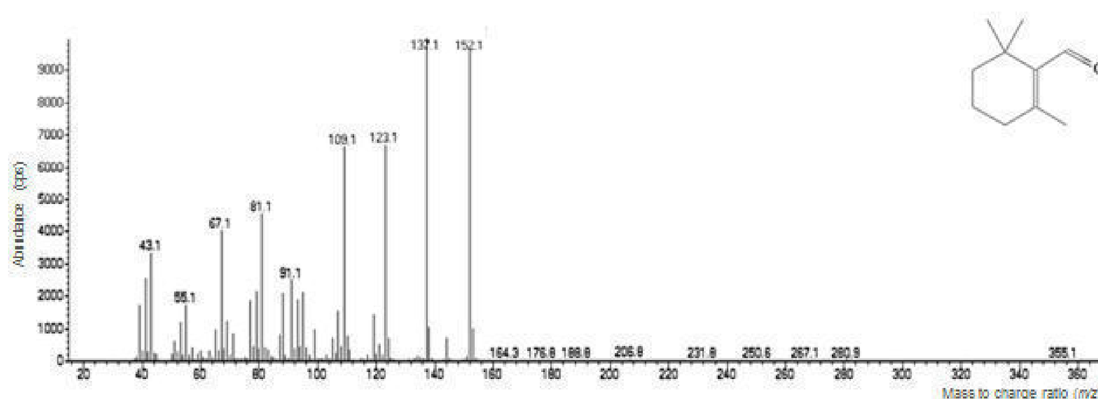


Figure 6. Mass spectrum of β-cyclocitral recorded for grapevine leaves of the hybrid Kober 5BB infected with *P. viticola* (publication 2).

3.4.2.1 *Quadrupole mass analyser*

Quadrupole mass analyser is the most popular device used in routine analysis (Herrmann, 2010). A typical quadrupole mass analyser consists of four rods of molybdenum alloys because of their inherent inertness (Figure 7). Because of the voltage oscillating at a radio frequency, each pair of oppositely located rods becomes successively positive, then negative and so forth in order to have always a pair of positive and negative rods. An ion travelling through the quadrupole will successively be attracted and then repelled from each rod, generating oscillating trajectories. In parallel to the oscillating voltage, direct current voltage is applied between the pairs of opposite rods, both of which can be ramped together with the alternating current voltage amplitude and direct current voltage value at a constant ratio. For each ion with a certain *m/z* ratio, there is a set of voltages which generates stable trajectories through the quadrupole, and only ions with this *m/z* can pass though the quadrupole and reach the detector. A typical transfer time through the quadrupole filter is between 25 and 250 ms. All other ions with different *m/*z leave the quadrupole or collide with the rods and thus do not reach the detector. In this thesis, a quadrupole was used in full scan mode and the recorded mass range ranged from 35 to 500 *m/z* and from 30 to 300 *m/z* for VOCs analysis of grapevine and *Lysobacter* VOCs (papers 2 and 3, respectively).

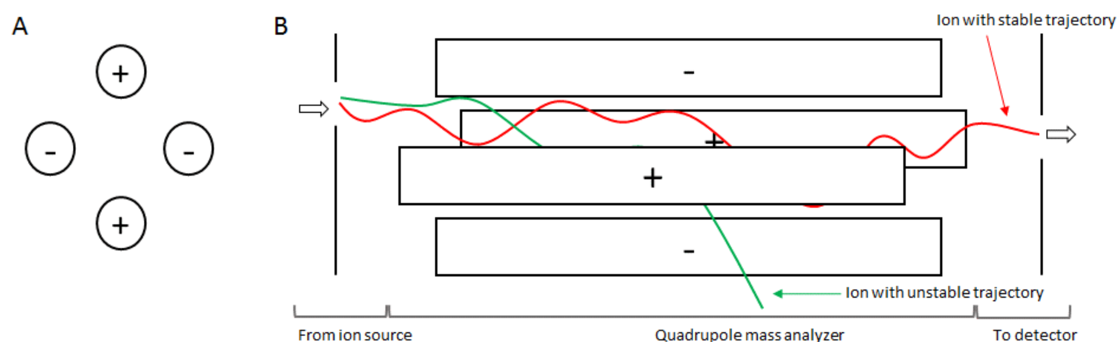


Figure 7. Scheme of a quadrupole mass analyser.

3.4.2.2 *Time of flight mass analyser*

Another mass analyser used in this PhD thesis is the time-of-flight (ToF) that was coupled with PTR (see also 3.2.2). The ions are accelerated by a defined voltage so that they have equal kinetic energy before entering the flight tube that is a field free drift region (Figure 8). This mass analyser works by deflecting a batch of ions into a field free flight tube and then separating them according to their flight times to a detector

(Blake, *et al.*, 2009). Since the kinetic energy gained by the ions through acceleration by the electronic field is equal for all ions and corresponds to $\frac{1}{2} mv^2$, where m is the mass of the ion and v is the ion velocity, the lower the ion's mass, the greater the velocity, and shorter its time of flight. The travel time through the flight tube to the detector can be transformed to the m/z value, and thus into a mass spectrum (Blake, *et al.*, 2009). The ability to acquire the whole mass spectrum at once at high mass resolution without sacrificing speed or sensitivity makes ToF-MS an excellent choice for qualitative analyses in the presence of complex matrices.

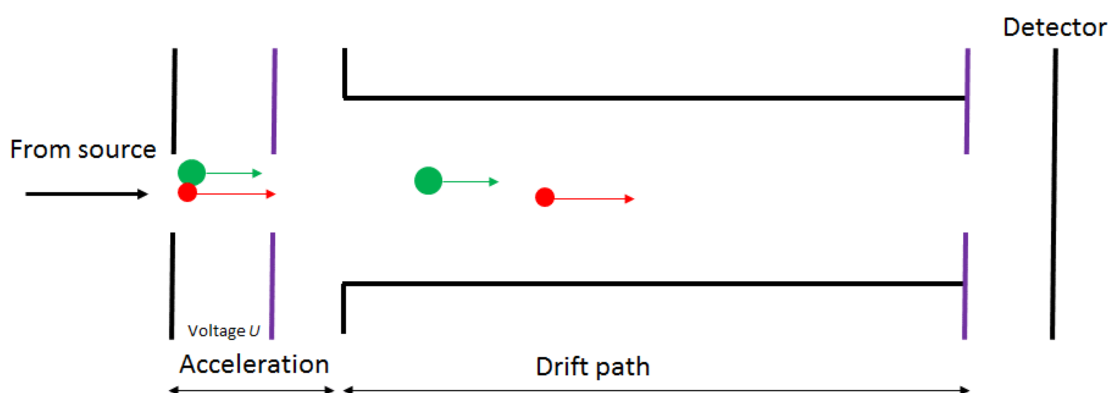


Figure 8. Scheme of a time of flight mass analyser.

3.4.3 Electron multiplier

The electron multiplier is used to detect the presence of ion signals emerging from the quadrupole mass analyser. The task of the electron multiplier is to detect every ion which has passed the mass filter. The operation of an electron multiplier is based on the secondary electron emission. Basically, a dynode is an electrode in high vacuum that emits electrons when an ion (or electron) with sufficient kinetic energy slams into it. When a charged particle (ion or electron) strikes a surface, it causes secondary electrons to be released from atoms in the surface layer. The ions are thus accelerated into the back of the dynode by a high negative potential (in case of positively charged ions). This process of emitting electrons is called secondary electron emission. The number of secondary electrons released depends on the type of incident primary particle, its energy and characteristic of the incident surface. Usually, two types of electron multiplier are used in mass spectrometry: the discrete-dynode electron multiplier and the continuous-dynode electron multiplier.

The discrete-dynode uses a series of dynodes that are chained together by resistors. A negative voltage is applied to the first dynode where the ions enter the multiplier device. The voltage difference between the front and the back of the discrete-dynode results in a gradual voltage drop down the direction of the electrons, thus an exponential increase in electron count occurs down the length of the chain. The continuous-dynode uses the same principle of the discrete-dynode, but it has an uninterrupted electrode that has a sufficient resistance to make the voltage gradually drop from the front to the back of the detector. In this way, the secondary electrons are accelerated towards the back of the detector by the potential drop. The output current is then converted to a voltage signal which finally can be translated to an intensity value.

3.5 Spectra analysis and compound identification/annotation

In order to achieve the aims of my PhD thesis, it was necessary to assign a biological role to the detected VOCs. For this purpose, it was necessary to at least annotate the metabolites and to compare relative concentrations between the samples. A key step in VOC profiling is compound annotation/identification. Compound identification in untargeted analyses with PTR-MS is challenging and usually requires further information to be pursuable (Cappellin*, et al.*, 2013). As reported in this PhD thesis, corroborating tentative identifications with compound annotation/identification by GC-MS, has been revealed as the most straightforward choice.

3.5.1 Spectra analysis in PTR-ToF-MS

Since no chromatographic separation of the biological VOCs is used in case of PTR-ToF-MS, the resulting mass spectra are highly complex and mass peak extraction procedures are mandatory in order to extract manageable datasets which can be used as inputs for data visualization or data mining procedures (Cappellin*, et al.*, 2013). Mass calibration in PTR-ToF-MS raw data is nowadays limited to external calibration which implies fixing a set of calibration coefficients employed during the entire data acquisition process. However, because of the fluctuations in instrumental parameters, such as fluctuations in ion tube length caused by temperature variations, such procedure does not guarantee high accuracy for a sufficient long time, hence there is also the need of internal calibration.

3.5.2 Annotation and identification in GC-MS

Identification of metabolites is essential to convert analytical data into meaningful biological knowledge (Creek*, et al.*, 2014). In 2007, the Metabolomics Standards Initiative (MSI) recommended minimum reporting standards of compound identification for chemical analysis in metabolomics These reporting standards were based on a four-level system ranging from level 1 (identified compound), levels 2 and 3 (putatively annotated compounds and compound classes) to level 4 (unidentified or unclassified metabolites which can be differentiated based on spectral data) (Sumner*, et al.*, 2007). Briefly, they include:

1. Identified compounds. A minimum of two independent and orthogonal properties relative to an authentic compound analysed under identical conditions are proposed as necessary to validate non-novel metabolite identifications (i.e. retention time/index and mass spectrum). The use of literature values reported for authentic reference standards by other laboratories are generally believed insufficient to validate a rigorous identification.

2. Putatively annotated compounds. Compounds without chemical reference standards, based on physiochemical properties and/or spectral similarity with public/commercial spectral libraries

3. Putatively characterised compound classes. Compounds based on physiochemical properties of a chemical compound class, or by spectral similarity to known compounds of a chemical class.

4. Unknown compounds. Although unidentified or unclassified, these metabolites can still be differentiated and quantified based on spectral data.

GC-MS offers two features, which permit the annotation/identification of sample compounds: mass spectrum and retention index.

Recently, Schymanski and collaborators (Schymanski*, et al.*, 2014) suggested a new level system to ease the identification step in high resolution-MS analysis (Figure 9). The differences include:

Level 1. Confirmed structure. This is the ideal situation and it includes the confirmation of the structure by measuring a reference standard.

Level 2. Probable structure. It considers two sub-levels: Level 2a: library, which involves matching literature or library spectrum data where the spectrum-structure

match is unambiguous. Level 2b: diagnostic, where no structures fit the experimental information, but no standard and literature information is available for confirmation.

Level 3. Tentative candidate. This level marks the evidence of a possible structure, however there are very few information about only one exact structure (i.e. positional isomers).

Level 4. Unequivocal formula. It gives information about the formula by using spectral information (i.e. adducts, isotopes, and fragments information), but there are no sufficient information about the possible structures.

Level 5. Exact mass (*m/z*). This level gives important information about the investigation, however no unequivocal information about the formula or structure exists. In this case it is possible to record a MS spectrum of a level 5 and save it as "unknown spectrum" in a database.

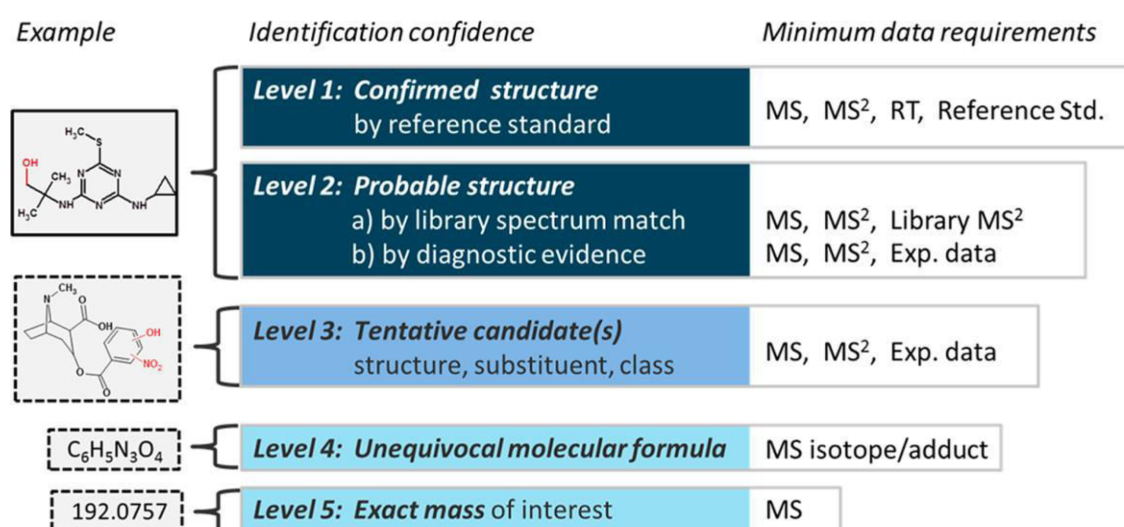


Figure 9. Proposed identification confidence levels in high resolution mass spectrometric analysis. Figure from Schymanski, *et al.* (2014).

3.5.2.1 Mass spectrum

In case of electron ionisation, the analytes reaching the mass spectrometer are bombarded with a stream of electrons carrying an energy of tens of electronvolts (eV), and some of this energy is transferred to the molecule to generate positive ions (molecular ion M^+). These ions are possessing an excess of internal energy and are therefore unstable and brake up into smaller fragments. Only charged fragments are accelerated, deflected and detected by the mass spectrometer and they produce a fragmentation patterns that can be displayed in form of mass spectra (MS). Ionisation

energy of most molecules are in the range of 7 to 15 eV. However, the majority of the MS instruments are set up with electron ionisation at a standardised ionisation energy (IE) of 70 eV. A reason to use this energy in the mass spectra of almost all libraries is that the ion signal is most intense at around 70 eV (Figure 10) and when the energy is decreased, the spectrum will be less fragment rich and the signal intensity decreases dramatically (www.shimadzu.com). Moreover, the plateau of the ionisation efficiency curve around 70 eV makes small variations in electron energy negligible (Hesse, 2004).

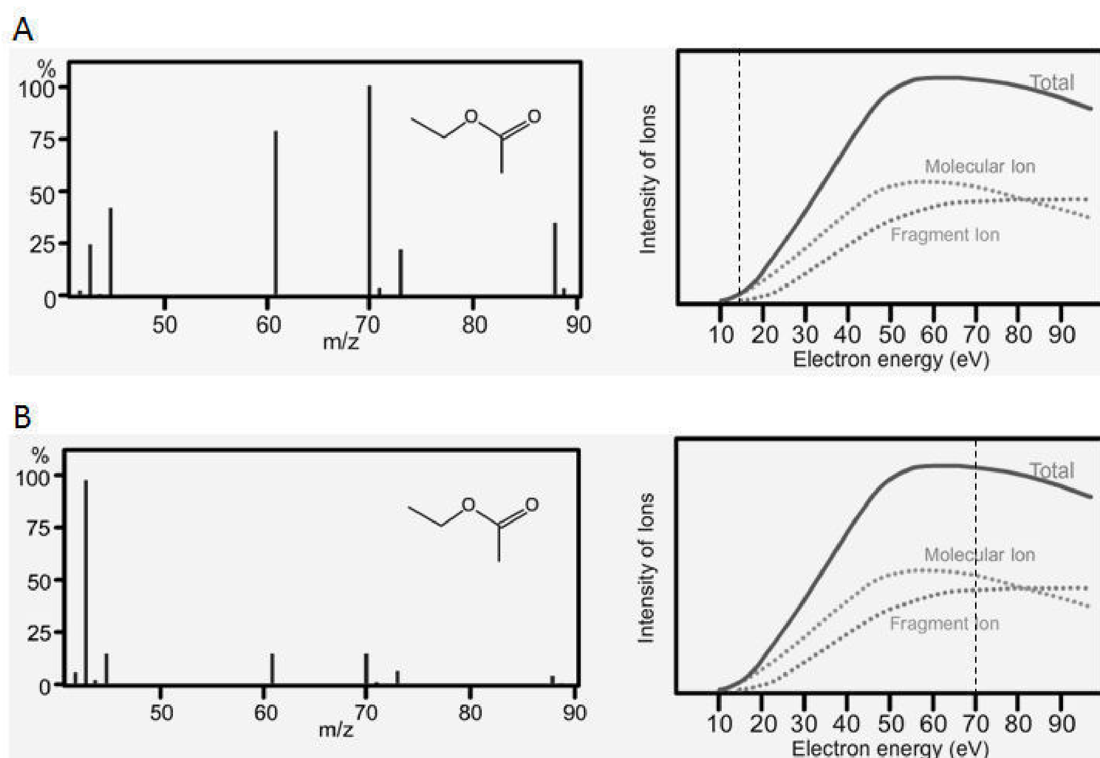


Figure 10. Spectrum of ethyl acetate obtained from an ionisation energy of 14 eV (A). Spectrum of ethyl acetate obtained from an ionisation energy of 70 eV (B) (figure from www.shimadzu.com).

This set up facilitates comparison of generated spectra with library spectra using manufacturer-supplied software or software developed by the National Institute of Standards (NIST). Spectral reference libraries are well established and extensively used. For example, in this PhD thesis, NIST (www.nist.gov) and in-house MS libraries have been used for annotation/identification of metabolites.

3.5.2.2 Retention index

The retention index of a compound is defined as a relationship between the retention of the analyte and two members of a homologous series enclosing it (i.e. alkane standards). In 1958, Kováts (Kováts, 1958) published a model to generate an isothermal retention index. Complex mixtures of VOCs are often preferably analysed using temperature programming regimes (Girard, 1996). For this reason, Van den Dool and Kratz (van Den Dool & Dec. Kratz, 1963) first produced an equation expanding the use of the retention index to linear temperature-programmed GC:

$$RI = 100(n + \frac{t_{Ri} - t_{Rn}}{t_{R(n+1)} - t_{Rn}})$$

t_{Ri}	Retention time of metabolite
t_{Rn}	Retention time of earlier eluting alkane
$t_{R(n+1)}$	Retention time of later eluting alkane
n	Number of C-atoms of earlier eluting alkane

The linear temperature-programmed retention index (LTPRI) is calculated based on the alkane standards, one eluting before and the other eluting after the analyte (Figure 11).

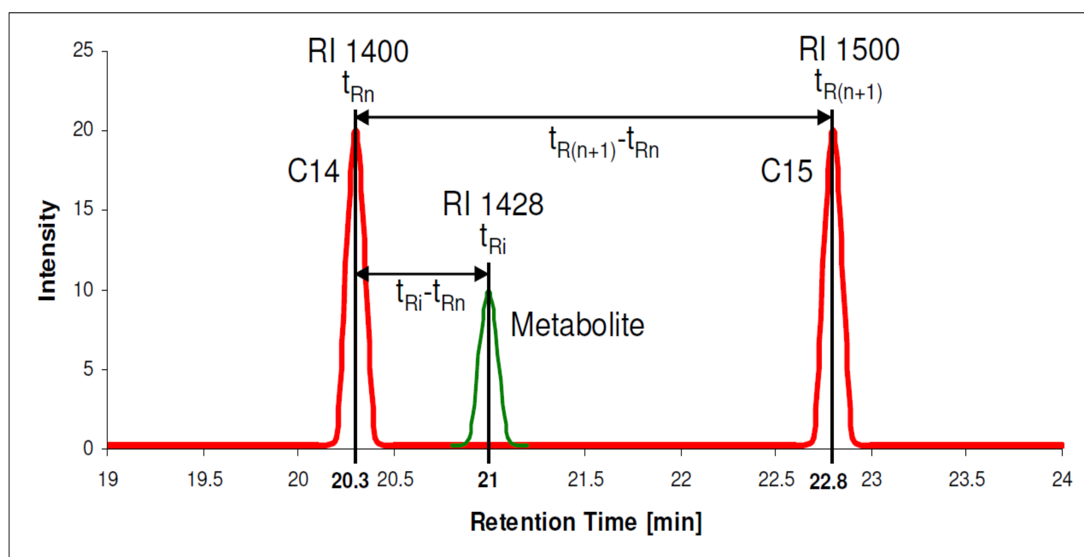


Figure 11. Calculation of the linear temperature-programmed retention index (LTPRI). C14: tetradecane, C15: pentadecane.

These features are provided by different software such as AMDIS software (Stein, 1999), Tagfinder (Luedemann*, et al.*, 2008), and MetaboliteDetector (Hiller*, et al.*, 2009) which has been used in this PhD thesis (publication 2). Starting from raw GC-MS data, MetaboliteDetector software detects and annotates potential metabolites.

Although the retention index is characteristic for each substance, it is not sufficient for an unambiguous annotation/identification when used as a single criterion. Therefore, the RI should be used in combination with mass spectra. In this thesis, RI were automatically calculated by TurboMass 5.4.0 software (Perkin Elmer, Norwalk, CT; USA) in publication 3 and by MetaboliteDetector software in publication 2. This software automatically determines appropriate quantification ions and performs an integration of single ion peaks. Finally, the analysis results can directly be converted to and illustrated as a data matrix making it accessible for further statistical analysis (Hiller*, et al.*, 2009).

3.5.2.3 Deconvolution

Frequently, compounds of highly complex metabolite mixtures (partly) co-elute (elute at similar time points) during GC-MS analysis. If the retention times of two or more components differ in only a few scans, they often tend to form a single, only slightly distorted peak in the total ion current (TIC) chromatogram. This means that it is impossible to extract pure mass spectra for these compounds. Although these components appear as a single compound within the TIC, the peaks of the single ion chromatograms can be separated on their apex. In order to detect these components as independent ones and to extract pure mass spectra, it is essential to perform a deconvolution step (Hiller*, et al.*, 2009). Deconvolution is the process of computationally separating partly co-eluting components and creating a pure spectrum for each component (Figure 12). Specifically, for each observed extracted ion current (EIC) chromatogram that results from two or more components, the contribution of each component to the EIC is calculated by the aid of a deconvolution algorithm (Du & Zeisel, 2013). One of the most popular algorithm is AMDIS (Automated Mass spectrometry Deconvolution and Identification System) (Stein, 1999). However, during this thesis I applied the MetaboliteDetector software to measure the spectra of publication 2.

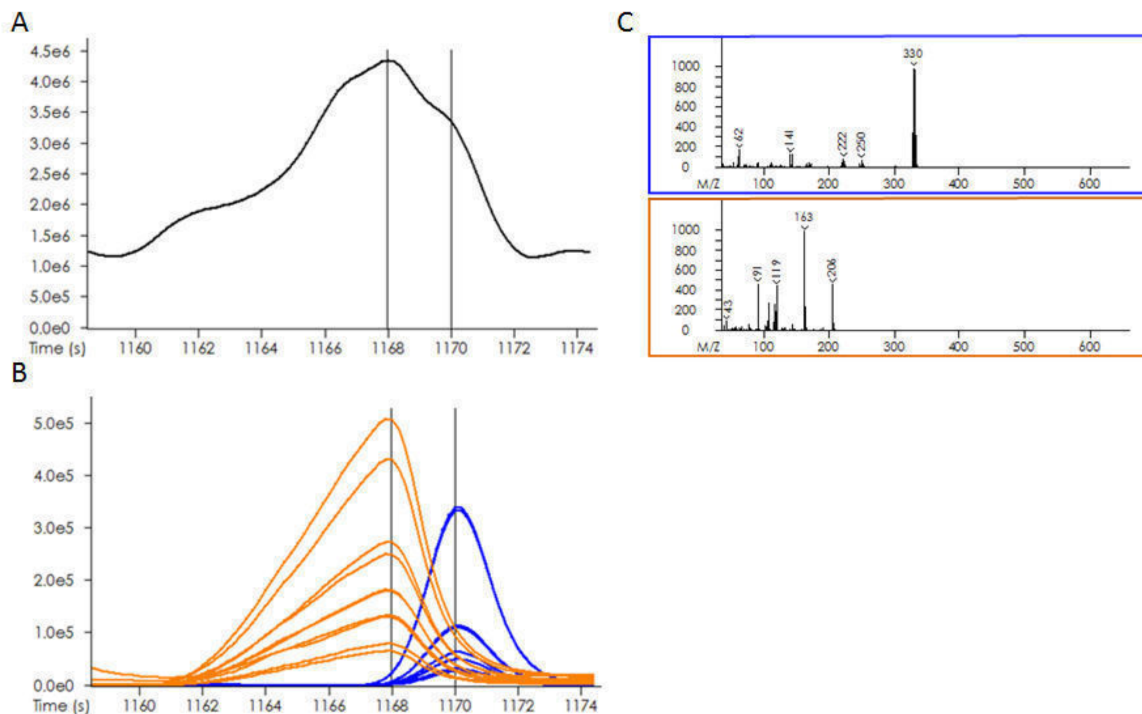


Figure 12. Total ion current (TIC) chromatogram of closely co-eluting compounds (A). Overlay of the single ion chromatograms depicted in orange and blue (B) are the result of a deconvolution step, giving their corresponding mass spectra (C) (www.leco.com).

The principle behind MetaboliteDetector software is that it detects chromatographic peaks from the beginning to the end by calculating the first derivative of the smoothing process obtained from the equation used by Savitzky-Golay (Savitzky & Golay, 1964) used to smooth both the spectral as well as the retention time (chromatographic) dimension of the raw data:

$$f(x) = \frac{-3(x_{-2} + x_{+2}) + 12(x_{-1} + x_{x+1}) + 17x}{35}$$

where $f(x)$ is the filtered value for the intensity x, while x-2, x-1, x+1, and x+2 are the intensities of the neighbouring mass peaks of the mass peak under investigation x. The resulting peak is valid if:

1) the peak consists of more than three values;
2) the height above the baseline in signal-to-noise units of the maximum peak value exceeds a predefined threshold;
3) the quality of the peak shape is in a predefined range.

The quality of the peak shape is named discrepancy index q_p which is the ratio of the nonideal to ideal slopes of the peak:

$$q_p = \frac{\text{sum of nonideal slopes}}{\text{sum of ideal slopes}} \times 100\%$$

where reasonable values of q_p should be between 0% and 10%. Finally, in order to determine the model peak shape for each perceived component, MetaboliteDetector software, sorts all single ion peaks of a compound having q_p values below 10% and the top 25% of the peaks in terms of the sharpness value are summed to form the model peak for that compound.

3.5.3 *Compound identification in PTR-ToF-MS*

As already mentioned above, compound identification and quantification in PTR-MS is challenging and usually requires two premises (Cappellin*, et al.*, 2013). The first one is that the chemical ionisation process gives a VOC•H^+ which do not undergo secondary ion-molecule reactions. The second premise is that the H_3O^+ ion signal is not depleted by reaction with organic analyte molecules (Goff & Klee, 2006). However, given the high mass resolution, PTR-ToF-MS strongly enhances compound identification, although isomers are not distinguishable. As no chromatographic separation step is used, for this reason, in this PhD work, the complementary combination between GC-MS and PTR-ToF-MS for identification/annotation of bacterial and plant VOCs has been used. PTR-ToF-MS is able to determine the compound sum formula, and recently, Cappellin and co-workers (Cappellin*, et al.*, 2011) proposed a method to automate the workflow analysis from PTR-ToF-MS raw spectra to data mining which starts from the dead time correction and internal calibration followed by baseline removal, noise reduction, peak detection and peak extraction. The output is a data matrix of peak intensities (Cappellin*, et al.*, 2011). Then, preliminary data visualisation methods or advanced data mining procedures may be applied.

3.5.3.1 *Dead time correction*

Commercial instruments are equipped with ToF systems whose linearity is affected by the detector dead time (Stephan*, et al.*, 1994). This means that ions reaching the ion

detecting device (usually a multi-channel plate) during dead time are irrevocably lost, causing distortion of intense mass signals. Analytical corrections of such effects are commonly based on Poisson statistics.

3.5.3.2 Internal calibration

Due to a lack of stability in instrumental parameters, external calibration in commercial PTR-ToF-MS instruments does usually not guarantee mass accuracy for sufficiently long time periods (Cappellin*, et al.*, 2011). A common solution to this problem is the use of an internal calibration based on the known exact mass of selected ions such as NO^+, O_2^+ and protonated acetone at nominal masses 30, 32 and 59, respectively which are permanently present in the analytical system (Cappellin*, et al.*, 2011).

3.5.3.3 Baseline removal and noise reduction

PTR-ToF-MS spectra are characterised by a baseline, which is subtracted by specific MATLAB functions that have been implemented for data pre-processing procedures (Cappellin*, et al.*, 2011). Furthermore, a PTR-ToF-MS spectrum can be composed of a high number of *m/z* values, each referring to an equally separated range of ion time of flights (Cappellin*, et al.*, 2011). Thus, if many spectra of the same sample are available, random noise is reduced and the quality of the signal is improved by simply averaging over all available spectra. This procedure is appropriate only if the spectra are properly aligned in terms of mass scale (Cappellin*, et al.*, 2011).

3.5.3.4 Peak detection

The peak position is determined by the *m/z* value of the protonated VOC, while the peak area represents the number of ions that reach the MS detector during the set acquisition time. Mass peaks referring to compounds of similar masses having close enough *m/z* values are superimposed within the spectrum, resulting in highly complex structures, approximately Gaussian especially in the top part, while asymmetries often appear as right-side (higher *m/z*) tails. In order to provide a satisfactory shape matching, Gaussian functions to fit mass spectrometric peaks are employed (Cappellin*, et al.*, 2011).

3.5.3.5 *Mass peak extraction*

Automatic peak extraction is performed on the spectra corresponding to each of the elements. For each nominal mass, a linear sum of Gaussian functions with fixed mean parameters is fitted and the peak heights are estimated as the maximum height of the corresponding Gaussian distribution. Peak widths are then determined from an average resolution estimated. At the end of the whole procedure, a data matrix of peak areas is constructed. Each row corresponds to different elements and each column corresponds to a peak with a defined *m/z* value. This matrix is the starting point for further data analysis and data mining (Cappellin*, et al.*, 2011).

3.6 Data analysis

Several data analysis software tools have been developed in order to process all the data collected by the instruments. TurboMass (Perkin Elmer, Norwalk, CT; USA) used in publication 3, MetaboliteDetector (Hiller*, et al.*, 2009) used in publication 2, represent useful software tools for GC-MS data analysis. However, the analysis of large amounts of data such as the volatilome of leaves or bacteria samples, needs robust statistical methods, as well. For this reason, software providing statistical tools can be used for further data treatment. In this thesis, R (www.r-project.org) used in publication 1, 2, and 3, and Statistica (Dell, Round Rock, TX, USA) used in publication 2 and 3, have been utilised for statistical data analysis.

3.7 GC-MS or PTR-MS for VOCs sampling?

The common techniques used in qualitative and quantitative analysis of VOCs are based on mass spectrometry instruments, with or without previous chromatographic separation (Materić*, et al.*, 2015). In this thesis, two different techniques have been used: gas chromatography-mass spectrometry (GC-MS), and proton-transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS). The first technique is based on a chromatographic separation of VOCs by capillary columns followed by a hard ionisation technique (electron ionisation EI), the second technique is based on a soft chemical ionisation (PTR) and it does not need any chromatographic separation, giving VOCs as their protonated molecular mass. The limited selectivity obtained from monitoring only the protonated parent mass leads the PTR-MS to measure only the total

concentration of volatile isomers and gives no information on the exact chemical composition of a sample constituent (Tani, *et al.*, 2003). For example, PTR-MS can only be used to measure the total concentration of monoterpene or sesquiterpene isomers and it gives no information on the terpene composition of a sample under investigation (Tani, *et al.*, 2003). For this reason, the combination of GC-MS and PTR-MS techniques is a powerful methodology for comprehensively measuring and identifying of VOCs (Farag, *et al.*, 2013), with PTR-MS having become a useful addition to GC analysis of plant and bacteria VOCs measurements. Indeed, in order to distinguish between isomers, combined experiments using GC (which provides high selectivity) together with the fast PTR-MS method are the most suitable to monitor and identify fast-changing concentration of VOCs at trace levels from different types of samples, such as plants or bacteria cultures. In this thesis, VOCs have been analysed combining the two mentioned techniques (exception for publication 1 where only PTR-ToF-MS has been used for a preliminary screening of VOCs emitted by three grapevine hybrids) in order to achieve the comprehensive measurement of VOCs from complex biological matrices. It shall be mentioned however that even in case of (one-dimensional) GC-MS, limitations exist for the analysis of the usual highly complex mixtures of VOCs with respect to chromatographic separation as well as compound identification.

In conclusion, although the PTR-MS method has found numerous applications and has greatly expanded the capability for relatively fast measurements of VOCs there remain significant weaknesses such as difficulties in the detection of particular VOC species (e.g. monoterpenes and sesquiterpenes). Furthermore, the lack of physical separation of the VOCs results in a limited selectivity obtained from monitoring only the protonated parent mass, which may lead to complications in the interpretation of ion signals measured (Jordan, *et al.*, 2009). On the other side, GC-MS is a powerful technique able to separate very similar chemical compounds (i.e. isomers such as α- and β-pinene) in case of at least partly chromatographic separation. However, GC-MS is not suitable for research where high time resolution is needed. Therefore, a complementary approach combining the two techniques is recommended in order benefit from the advantages of both GC-MS and PTR-MS.

4 VOCs and interactions in grapevine defence mechanisms and in biocontrol processes of beneficial bacteria

Damaged plants have been reported to release VOCs which can be used by nearby plants to recognise impending danger (Heil, 2014). Likewise, beneficial microorganisms are able to emit VOCs to inhibit the growth of phytopathogens (Weisskopf, 2013), promote plant growth (Ryu*, et al.*, 2003, Blom*, et al.*, 2011) or induce plant resistance (Ryu*, et al.*, 2004). In this chapter, two different interactions are outlined in order to better describe how plant and beneficial bacteria interplay with two important plant pathogens, respectively: *P. viticola* and *P. infestans*.

4.1 Interaction between grapevine and Plasmopara viticola

The biotrophic oomycete *P. viticola* (Berk. and Curt.) Berl. and de Toni is the causal agent of downy mildew, that is one of the most economically important grapevine diseases worldwide, particularly in warm and wet climates (Gessler*, et al.*, 2011). *P. viticola* is a heterotallic oomycete and it is an obligate biotrophic pathogen that overwinters as oospores in leaf litter and soils. *P. viticola* attacks all green parts of the grapevine where functional stomata are present, and it penetrates only these natural openings (Gessler*, et al.*, 2011). The first visible symptoms are yellowish lesions (called oil spots) on the adaxial leaf surface, while sporulation can be observed on the abaxial side of the leaf, on the surface of tendrils, inflorescences and young berries (Buonassisi*, et al.*, 2017). *P. viticola* overwinters as sexually oospores in fallen leaves and berries (Figure 13).

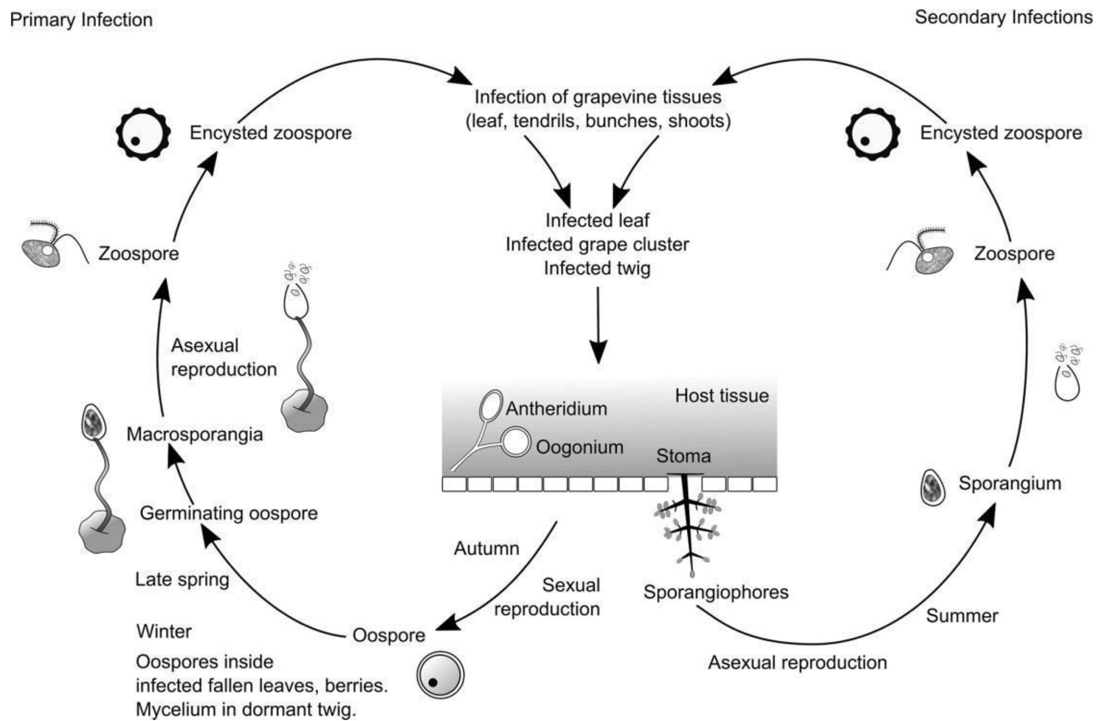


Figure 13. The life cycle of *P. viticola* (Buonassisi*, et al.*, 2017).

In spring, the oospores germinate giving macrosporangia which can be spread directly onto leaves by rain splash or have become airborne with turbulence (Gessler*, et al.*, 2011). It is assumed (Baldacci & Refatti, 1956) that the necessary conditions for primary infection are a minimum atmospheric temperature of 10°C, at least 10 mm of rain and a shoot length of at least 10 cm. Macrosporangia release zoospores which can swim in free water on the grapevine surface towards stomata, where they encyst. Zoospores produce a germinative tube which penetrate through the stomata and they form substomatal vesicles. After 48 hours, the substomatal vesicles develop and give rise to the intercellular mycelium. After seven-ten days of incubation time (Gessler*, et al.*, 2011), sporulation occurs on the abaxial surface of the host tissue and sporangiophores with sporangia emerge through stomata. *P. viticola* sporulation occurs with a minimum of 98% of relative humidity, 4 hours of darkness and an optimal temperature of 19°C (Gessler*, et al.*, 2011). After the sporulation, the sporangia are dispersed by wind and rain splash leading to the liberation of zoospores and marking the start of a new infection cycle (Buonassisi*, et al.*, 2017). Secondary infections can occur repeatedly during the summer season. At the end of grapevine growing season, *P. viticola* sexual production starts within the infected host tissue through the fertilization of oogonia by anteridia and the resulting oospore represents the

overwintering structure with a source of genetic variation and the new primary inoculum for the following season.

The wine industry relies predominantly on the European *V. vinifera* cultivars, which are highly susceptible to downy mildew (Gessler*, et al.*, 2011). Grapevine downy mildew is currently controlled with repeated applications of fungicides that lead to environmental pollution, development of resistant population of the pathogen and residual toxicity (Pimentel*, et al.*, 1992) which have sparked off growing interest in alternative approaches in the pest management, such as the application of resistance inducers on susceptible cultivars or the use of resistant *Vitis* spp. hybrids (Gessler*, et al.*, 2011). In particular, the application of substance known to induce resistance in susceptible *V. vinifera* cultivars has been largely characterised (Gessler*, et al.*, 2011). For example, treatments with chitosan (Aziz*, et al.*, 2006), plant extracts of *Solidago canadensis* (Harm*, et al.*, 2011) or the use of microorganisms such as *Trichoderma harzianum* T39 (Perazzolli*, et al.*, 2008) significantly increased grapevine resistance to downy mildew. On the other side, resistance traits have been identified in American wild grapevine species including *V. riparia, V. rupestris, V. amurensis and Muscadinia rotundifolia*, probably because of their co-evolution with the pathogen in the place of origin (Boso*, et al.*, 2014). Indeed, efforts to insert resistant traits into *V. vinifera* genotypes have yielded some commercially important resistant interspecific hybrids, such as BC4 (Kozma*, et al.*, 2009), Kober 5BB (Cadle-Davidson, 2008), SO4 (Boso*, et al.*, 2014) and Solaris (www.vivc.de) which efficiently halt hyphal growth. It is already known that grapevine resistance traits to *P. viticola* are quantitatively inherited (Moreira*, et al.*, 2011). These resistance traits are based on both physical (hairy and water repellent leaf surface) and chemical (phytoanticipins) constitutive factors (Kortekamp & Zyprian, 1999), and inducible defence mechanisms such as localised cell death, production of reactive oxygen species, synthesis of phytoalexins such as resveratrol and viniferins (Pezet*, et al.*, 2004, Chitarrini*, et al.*, 2017) and pathogenesis-related proteins (Kortekamp, 2006, Polesani*, et al.*, 2010, Malacarne*, et al.*, 2011). The involvement of grapevine VOCs in resistance mechanisms against *P. viticola* has been recently investigated. For example, the emission of (E,E)-α-farnesene in grapevine was recently associated with the resistance induced by a sulphated laminarin against downy mildew (Chalal*, et al.*, 2015), and, thanks to our contributions (publications 1 and 2), we demonstrated that grapevine VOCs of resistant genotypes can contribute to the plant defence against downy mildew inoculation. Particularly, downy mildew significantly

increased the production of defence-related VOCs in resistant, but not in susceptible grapevine genotypes (publication 1 and 2). Moreover, VOCs contribute to grapevine resistance against downy mildew by direct inhibition of downy mildew thanks to the accumulation of VOCs with direct inhibitory activities against *P. viticola* development (publication 2).

4.2 Interactions between Lysobacter *spp. and* Phytophthora infestans

Phytophthora infestans (from the Greek words "plant destroyer") causes potato late blight, a disease with major historical impact, although even nowadays *P. infestans* remains a major problem in agriculture and recalcitrant to disease suppression (Fry, 2008, Kamoun*, et al.*, 2015). *P. infestans* is a heterothallic oomycete, and it is a near-obligate hemibiotrophic pathogen under natural and agricultural conditions (Fry, 2008), which means that during infection, it has a biotrophic phase where it forms haustoria. This is then followed by a necrotrophic phase where hyphae kill the plant tissue for nutrient acquisition (Perfect & Green, 2001). Life cycle of *P. infestans* is divided in sexual and asexual stages. During the asexual phase, sporangia are produced on sporangiophores that grow from infected tissue (Figure 14) and are released from aerial dispersal during a drop in relative humidity or water splashes (Aylor*, et al.*, 2001).

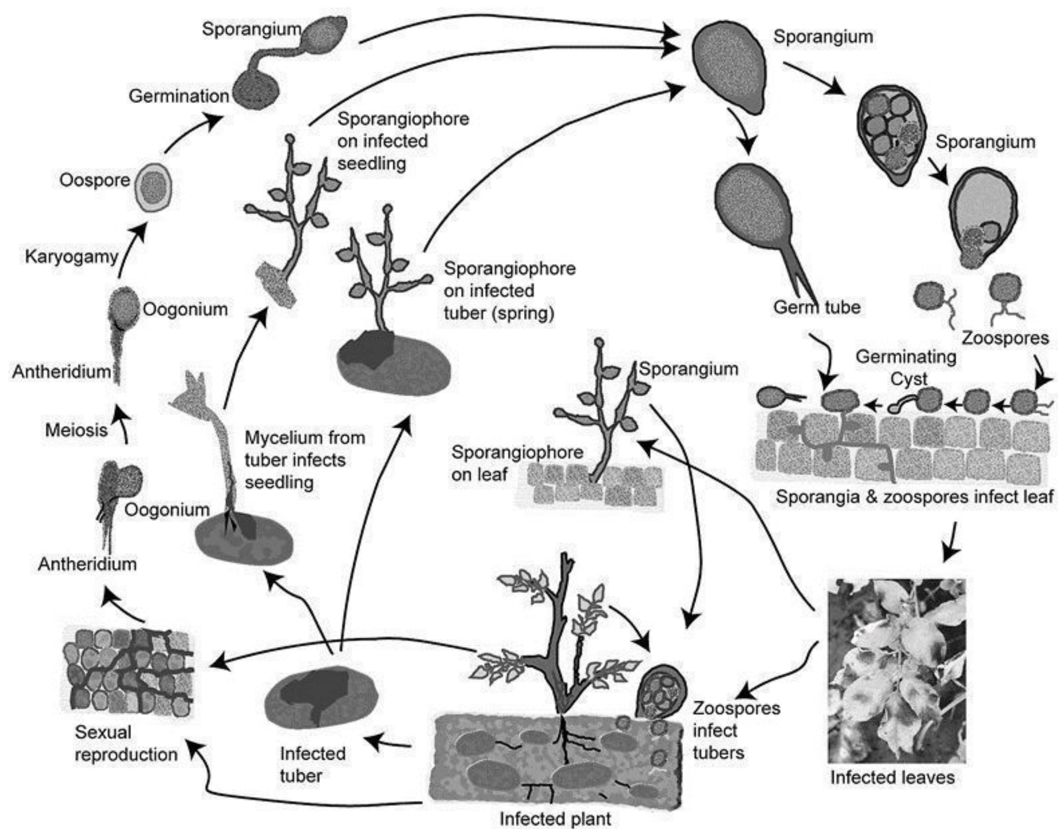


Figure 14. The life cycle of *P. infestans* (Vetukuri*, et al.*, 2012).

Sporangia in free water germinate either via germ tube at an optimal temperature between 20°C and 25°C, or release from 3 to 8 wall-less bi-flagellated motile zoospores when the optimal temperature is between 10°C and 15°C. *P. infestans* zoospores become encysted and germinate via a germ tube in order to penetrate plant tissue (Fry, 2008). Both sporangia and zoospores form germ tubes and appressoria prior to penetration (Tucker & Talbot, 2001). Symptoms are visible after two days from the infection and generally consist of small areas of necrosis. Then, the mycelium grows within and between plant cells, until the whole foliage is totally wilted one week after infection. Moreover, sporangia and zoospores can infect tubers which can be responsible for the inoculum during the following season. During the sexual phase, the sexual spore (called oospore) is formed by fertilisation between the anteridium and the oogonium, respectively the male and the female organ. The oospore represents a source of genetic variation is very robust and is able to survive in soil and at low temperatures

(Fay & Fry, 1997, Mayton*, et al.*, 2000) and it develop into a sporangium starting a new asexual cycle (Schumann & J., 2000).

The basic knowledge on *P. infestans* in the last decade is starting to have an impact on the management of the late blight disease. Indeed, to control late blight, farmers largely rely on agrochemicals, many with unknown modes of action and environmental effects (Kamoun*, et al.*, 2015). Moreover, resistance traits were selected in some pathogen populations, thus the efficacy of many fungicides declined noticeably (Fry, 2008). For these reasons, development of reliable, environmentally benign and economically feasible management tactics is of primary importance (Fry, 2008). Interactions between beneficial bacteria and plant pathogenic oomycetes occur in the rhizosphere and could lead the death of the phytopathogens or promote the growth of plants (Haas & Defago, 2005). In the last decade, increasing attention has been paid to the functional roles of bacterial VOCs in soil microbial interactions (Effmert*, et al.*, 2012), and bacteria belonging to the *Lysobacter* genus are frequently found in soil and showed a great potential for biological control of crop diseases (Hayward*, et al.*, 2010). The antagonistic effects of four type strains of *Lysobacter* spp. (*L. antibioticus, L. capsici, L. enzymogenes* and *L. gummosus*) are known for the production of lytic enzymes and antibiotics against numerous soil phytopathogens (Kobayashi & Yuen, 2007, Xie*, et al.*, 2012, Puopolo*, et al.*, 2016). However, little is known about the chemical composition of *Lysobacter* VOCs and their toxic activity against *P. infestans*. In this thesis, publication 3 answered to these question, demonstrating that the chemical profiles of the *Lysobacter* volatilome differed according to the growth medium and a protein-rich substrate maximised the toxic effect of the four *Lysobacter* type strains tested against *P. infestans*.

5 Conclusions and outlook

The interest in alternative approaches has become of primary importance in the management of plant pests because of the impact of synthetic fungicide overuse on human health and the environment, as well as the development of resistant populations of plant pathogens. The final aim of this PhD thesis was to identify possible novel alternative compounds of natural origin to control the two major crop pathogens *Plasmopara viticola* and *Phytophthora infestans* in order to deeply understand the role of VOCs in interactions between beneficial bacteria or grapevine, and plant pathogenic oomycetes. In order to reach this aim, my PhD studies were focused on VOCs and their role in the grapevine defence mechanisms against *P. viticola* and in the biocontrol processes of *Lysobacter* spp. against *P. infestans*. In particular, VOCs produced by four resistant *Vitis* spp. genotypes and four *Lysobacter* type strains were screened, identified and functionally characterised. In a first attempt, we found out that downy mildew significantly increased the emission of the compound classes of volatile monoterpenes and sesquiterpenes in the resistant grapevine genotypes Kober 5BB and SO4 (publication 1) but not in the susceptible *V. vinifera* cultivar Pinot noir *in vitro*. A more detailed analysis of grapevine VOCs produced by susceptible (Pinot noir) and resistant genotypes (BC4, Kober 5BB, SO4 and Solaris) by HS-SPME/CG-MS revealed the (putative) structures for a number of individual VOCs. Our results demonstrated that resistant genotypes produced monoterpenes, sesquiterpenes and other VOC classes upon *P. viticola* inoculation under greenhouse conditions, while Pinot noir did not. Furthermore, six identified VOCs were functionally tested by leaf disk assays and they were able to impair the development of downy mildew symptoms, suggesting that the production of VOCs from resistant grapevine genotypes acts as a post-infection mechanism with direct inhibitory activities against *P. viticola* (publication 2). Further metabolomics and transcriptomics studies are required in order to understand the role of grapevine VOCs in the activation of plant resistance mechanisms in more detail. This will also help to test and optimize their potential for future application in biocontrol with appropriate encapsulating formulation under field conditions. The second part of this thesis was focused on VOCs emitted by the beneficial microorganisms *Lysobacter* spp. in order to identify biocontrol VOCs with inhibitory activities against *P. infestans*. VOCs emitted by four type strains of *Lysobacter* spp. that were grown on two different growth media were analysed using HS-SPME/GC-MS and PTR-ToF-MS analyses

(publication 3). The *P. infestans* inhibiting effect of *Lysobacter* VOCs was demonstrated *in vitro* by dual-culture assays and the biocontrol activities and VOCs profiles were clearly dependent on the composition of the bacterial growth medium. These results highlight the importance of the nutrient source in order to induce the formation of volatile metabolites which results in biocontrol activity against *P. infestans*. Further validations are needed in order to understand the possible scenario of VOCs emitted by *Lysobacter* spp. under natural conditions in the soil. In conclusion, four grapevine (2-ethylfuran, 2-phenylethanol, β-cyclocitral or trans-2-pentenal) and four *Lysobacter* spp. VOCs (2,5-dimethyl pyrazine, 2-methoxy-3-methyl pyrazine, decanal and pyrrole) with strong inhibitory activity against *P. viticola* and *P. infestans* were identified respectively. Future studies will reveal the potential of these molecules from natural origin for being further developed into new bio-pesticides.

Part II

Overview of Part II

Downy mildew and late blight of potato and tomato are controlled by massive use of fungicides which cause environmental pollution and development of resistant pathogen populations (Chen*, et al.*, 2007, Fry, 2008). The main objectives of my PhD thesis were to better understand the role of plant and microbial VOCs in the communications among organisms and to identify alternative molecules from natural origin to control plant pathogens. The main tasks of this work were the studies of VOC involvement in the grapevine defence mechanisms against *P. viticola* and in the biocontrol processes of beneficial *Lysobacter* spp. against *P. infestans*. Plant defence is based on different mechanisms, and VOCs play an important role in response to insects and pathogens. Although resistance mechanisms and the production of secondary metabolites have been widely characterised in resistant grapevine genotypes, the emission of VOCs was not yet investigated following *P. viticola* inoculation. Our PTR-ToF-MS analysis revealed that resistant and susceptible grapevine genotypes clearly differed in terms of VOCs emission and downy mildew inoculation significantly increased the emission of terpenes in resistant genotypes. Thus, volatile terpenes could play an important role in grapevine defence against downy mildew (publication 1). In order to investigate the exact identity of grapevine VOCs, a HS-SPME/GC-MS analysis was then carried out. We confirmed that downy mildew significantly increased the emission of monoterpenes, sesquiterpenes and other VOC classes by resistant grapevine genotypes. Moreover, terpenoids, aldehydes, furans and alcohols were tested as pure VOCs against *P. viticola* by leaf disk assays and four of them impaired the development of downy mildew symptoms by treatments in air volume without direct contact with the leaf tissue (publication 2). The second task of this PhD project was focussed on the identification and functional characterization of VOCs emitted by *Lysobacter* spp. type stains. We found out that VOCs profiles and volatile-dependent biocontrol activity against *P. infestans* was affected by on the bacterial growth media. Volatile pyrazines, pyrrole and decanal significantly inhibited *P. infestans* and were mainly emitted by *Lysobacter* spp. grown on a protein-rich medium, demonstrating that the nutrient availability strongly affect the metabolic pathways and the biocontrol properties of *Lysobacter* spp. against plant pathogens (publication 3).

Publication 1 – Emission of volatile sesquiterpenes and monoterpenes in grapevine genotypes following *Plasmopara viticola* inoculation *in vitro*

Alberto Algarra Alarcon*, **Valentina Lazazzara***, Luca Cappellin, Pier Luigi Bianchedi, Rainer Schuhmacher, Georg Wohlfahrt, Ilaria Pertot, Franco Biasioli and Michele Perazzolli

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MOTIVATION OF THE STUDY

Grapevine resistance mechanisms and the production of non-volatile secondary metabolites upon *P. viticola* inoculation have been widely characterised in resistant genotypes. However, VOCs emission has not yet been investigated. This publication described a PTR-ToF-MS analysis for the detection of VOCs emitted by resistant and susceptible grapevine genotypes grown *in vitro* inoculated with *P. viticola*. The grapevine genotypes differed in terms of VOC emission and downy mildew inoculation significantly increased the emission of monoterpenes and sesquiterpenes in resistant but not in susceptible genotypes, indicating a possible role of volatile terpenes as toxic molecules against plant pathogens.

CONTRIBUTION OF THE PRESENTING AUTHOR

I contributed in the inoculation and in the measurement of the *in vitro* grapevine plants. I also performed the disease assessement, I was responsible for the biological interpretation of the data and I contributed to the writing of the manuscript.

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Emission of volatile sesquiterpenes and monoterpenes in grapevine genotypes following *Plasmopara viticola* inoculation *in vitro*

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The grapevine (*Vitis vinifera*) is one of the most widely cultivated fruit crops globally, and one of its most important diseases in terms of economic losses is downy mildew, caused by *Plasmopara viticola*. Several wild *Vitis* species have been found to be resistant to this pathogen and have been used in breeding programs to introduce resistance traits to susceptible cultivars. Plant defense is based on different mechanisms, and volatile organic compounds (VOCs) play a major role in the response to insects and pathogens. Although grapevine resistance mechanisms and the production of secondary metabolites have been widely characterized in resistant genotypes, the emission of VOCs has not yet been investigated following *P. viticola* inoculation. A Proton Transfer Reaction-Time of Flight-Mass Spectrometer (PTR-ToF-MS) was used to analyze the VOCs emitted by *in vitro*-grown plants of grapevine genotypes with different levels of resistance. Downy mildew inoculation significantly increased the emission of monoterpenes and sesquiterpenes by the resistant SO4 and Kober 5BB genotypes, but not by the susceptible *V. vinifera* Pinot noir. Volatile terpenes were implicated in plant defense responses against pathogens, suggesting that they could play a major role in the resistance against downy mildew by direct toxicity or by inducing grapevine resistance. The grapevine genotypes differed in terms of the VOC emission pattern of both inoculated and uninoculated plants, indicating that PTR-ToF-MS could be used to screen hybrids with different levels of downy mildew resistance. **Copyright © 2015 John Wiley & Sons, Ltd.**

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Keywords: PTR-ToF-MS; volatile organic compounds; grapevine; downy mildew resistance; *in vitro* plants

Introduction

The grapevine is one of the most widely grown fruit crops globally, both for fresh produce (table grapes) and processed products (wine) consumption. The grapevine industry relies predominantly on *Vitis vinifera*, which is susceptible to a large spectrum of pathogens. The biotrophic oomycete *Plasmopara viticola* is the causal agent of one of the most damaging diseases, namely, grapevine downy mildew.[1] Downy mildew is controlled by frequent applications of chemical fungicides, particularly in warm and wet climates,[1] but concerns about the environmental impact of pesticide overuse[2] and the development of resistant *P. viticola* populations[3] have sparked off growing interest in alternative approaches in the pest management, such as the use of resistant *Vitis* spp. hybrids.

Resistance traits have been identified in wild grapevine species, and the defense mechanisms to downy mildew have been characterized in resistant genotypes.[1] American grapevine species, such as *V. riparia*, *V. rupestris*, and *V. rotundifolia*, are resistant or tolerant to the disease, probably because of their long coevolution with the pathogen in the place of origin.[4] Soon after the introduction of the pathogen in Europe in the 19th century, breeding for grapevine resistance to downy mildew started by combining the quality traits of European cultivars (*V. vinifera*) with the downy mildew resistance traits of American hybrids.[1] Hybrids of *V. berlandieri* and *V. riparia*, such as SO4 and Kober 5BB (Kober), developed as resistant rootstocks to an important pest (*Daktulosphaira vitifoliae*), are also resistant to downy mildew.[4,5] Grapevine resistance to *P. viticola* is based on both constitutive factors (structural barriers, hairy and water repellent leaf surfaces, and phytoanticipins) and inducible defense mechanisms (localized cell death, production of reactive oxygen species, synthesis of phytoalexins and pathogenesis-related proteins).[6,7] Although the presence of non-volatile secondary metabolites, such as stilbenic phytoalexins and other antimicrobial compounds, have been largely characterized in resistant

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genotypes,[8–10] the emission of volatile organic compounds (VOCs) has not yet been investigated following downy mildew inoculation. Plants have evolved complex pathways to adapt to the environment, and plant VOCs play a crucial role in communications between plants and the other organisms.[11] Several studies have characterized the production of plant VOCs in response to herbivore attack and demonstrated the role of VOCs in the defense mechanisms, both by directly deterring herbivores and by attracting their predators.[11,12] VOCs are also emitted by plants in response to pathogen infection, and they typically consist of green leaf volatiles, terpenoids,[13,14] methyl jasmonate (MeJA),[15] methyl salycilate (MeSA),[16] aldehydes,[17,18] and terpenes.[19–22] However, little is known about the role of these VOCs in the defense mechanisms against pathogens.[20,23] Some pathogen-induced VOCs could have direct antimicrobial effects against pathogens,[24] such as 2-carene, β-caryophyllene, 2-hexenal, 2-nonenal, C9-aldehydes against *Botrytis cinerea*,[25] and trans-2-hexenal against *Monilinia laxa*.[26] Plant VOCs could also mediate the activation of defense reactions in distal parts of the same plant (within-plant signaling) or in neighboring plants (plant-plant communication).[20,27,28] For example, VOCs emitted by a resistant common bean were able to enhance the resistance to the fungus *Colletotrichum lindemuthianumin* in a susceptible cultivar,[20] indicating a major role for VOCs in plant-plant communication among resistant and susceptible genotypes.

The VOC emission by grapevine plants has been little investigated and has mainly been studied using gas chromatography-mass spectrometry (GC-MS) analysis, with different preparation methods, such as VOC trapping in activated charcoal[29] and direct VOC extraction.[30,31] A wide range of compounds have been identified, such as aldehydes, ketones, alcohols, esters, terpenoids, and aromatic compounds.[29–31] However, headspace analysis of VOCs using chromatographic techniques is a laborious and time-consuming approach. On the other hand, proton transfer reaction-mass spectrometry (PTR-MS)[32] is an ultra-high sensitivity technique for VOC detection[33] based on the principles of chemical ionization introduced by Munson and Field in the 1960s[34] and allows online analysis of air samples. The most innovative instruments couple a PTR source to a Time-of-Flight (ToF) mass analyzer[35] to achieve a mass resolution more than 5000 times greater than quadrupole-based instruments and time resolution of a full spectrum in a split second. The aim of this work was to analyze the VOC profile produced by different grapevine genotypes in response to *P. viticola* inoculation using PTR-ToF-MS analysis. *In vitro*-grown plants were used in order to ensure axenic conditions, meaning that only the grapevine species was cultivated in a sterile environment, to avoid the interference of possible contaminant microorganisms.[36] The axenic conditions also ensured constant high humidity to permit pathogen infection, symptom development, and sporulation. VOCs emitted by susceptible and resistant genotypes were screened at different time points after inoculation, and the VOC emission pattern was compared with the severity of the disease. The final goal was to further develop innovative methods for downy mildew control, based on a better understanding of the role of VOC emission in the grapevine defense mechanisms.

Materials and methods

Plant material

Plants of the susceptible *V. vinifera* cv Pinot noir ENTAV115 and the resistant grapevine hybrids (*V. berlandieri* × *V. riparia*) SO4[4] and Kober 5BB[5] were grown *in vitro* in 200 ml glass vessels for plant tissue culture (SigmaAldrich, St. Louis, MO, USA), covered with Magenta B-cap (SigmaAldrich) on 30 ml of half-strength Murashige–Skoog (MS) basal medium supplemented with 0.6 mg l^{-1} thiamine, 100 mg l^{-1} myo-inositol, 3% (w/v) sucrose and 0.6% (w/v) agar. Plants were grown for 2 months in a growth chamber at 23 ± 1 °C with a photoperiod of 16 h light. To measure the headspace air of the vessel, caps were replaced before VOC analysis with a sterile customized cap with four 5-mm holes, covered with 18 mm PTFE/silicone septa (Agilent Technologies, Santa Clara, CA, USA) and glued with melted silicone. Plants were then acclimated in a plant growth cabinet (Climacell CLC 707) at 23 ± 1 °C with a photoperiod of 16 h light for three days before VOC analysis.

Grapevine inoculation with *Plasmopara viticola*

A *P. viticola* population was collected from an untreated vineyard in northern Italy (Trentino region) in 2014 and maintained through subsequent inoculations onto Pinot noir plants under greenhouse conditions at 25 ± 1 °C, with a photoperiod of 16 h light and a relative humidity (RH) of 70 ± 10%.[37] To obtain a sterile inoculum suspension, infected leaves showing early symptoms of *P. viticola* (oil spots) were collected and washed in a 1% sodium hypochlorite solution for 10 min, with orbital shaking at 80 rpm.[38] These surface-sterilized leaves were washed twice for 5 min each in sterile distilled water, with orbital shaking at 80 rpm. Leaves were transferred with the abaxial side uppermost onto sterile moist filter paper (three foils) in autoclaved Petri dishes (9 cm diameter)[39] and incubated overnight in the dark at room temperature to permit downy mildew sporulation. Leaves bearing freshly sporulating lesions were transferred to 50-ml sterile tubes and gently washed with 4 ml of cold sterile distilled water. The inoculum suspension was filtered with a sterile fine net, and the concentration was adjusted to 4×10^4 sporangia ml^{-1} by counting with a hemocytometer.

For *P. viticola* inoculation, the abaxial surface of each leaf of *in vitro*-grown plants was inoculated with six to eight drops of 20 μl of the fresh sporangia suspension under sterile conditions (*P. viticola*-inoculated plants). As a control, the abaxial surface of each leaf of *in vitro*-grown plants was treated with six to eight drops of sterile distilled water under the same conditions (control plants). The plants were incubated overnight in the dark at 25 ± 1 °C to allow *P. viticola* infection, and on the following day each leaf of the *P. viticola*-inoculated and control plants was dried using sterile filter paper under sterile conditions. The plants were then incubated for 10 days in a plant growth cabinet (Climacell CLC 707) in the conditions described earlier.

Assessment of downy mildew severity and leaf weight

Development of downy mildew symptoms was visually assessed during incubation in the plant growth cabinet. At the end of VOC analysis, all the leaves of each plant were collected and washed in 1 ml of distilled water by gently vortexing to collect the sporangia. The sporangia suspension was centrifuged at 1600 × g for 5 min at 4 °C, the pellet was re-suspended in 50 μl of distilled water, and sporangia were counted with a hemocytometer under a light microscope. The leaves of each plant were dehydrated by incubating them at 80 °C for 48 h, and dry weight was measured (Table S1) to normalize the number of sporangia and the emission of VOCs per gram of leaf dry weight. We used leaf dry weight because it is less variable than fresh weight[40] and is positively correlated to the leaf area.[41]

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Headspace analysis of volatile organic compounds by PTR-ToF-MS

Analysis of the VOCs emitted by the grapevine plants was carried out by sampling the headspace air in the jars through the PTFE/silicone septa on the lid. A heated (110 °C) PEEK tube (diameter of 0.055 in.) was connected to one septum, and the headspace air was withdrawn using a PTR-TOF 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) at a rate of 100 standard cubic centimeters per minute for a total record of 180 s. A second PEEK tube was connected to another septum to permit the injection of humidified (80% of RH) zero air generated with a gas calibration unit equipped with a catalytic VOC scrubber (GCU, Ionicon Analytik GmbH, Innsbruck, Austria) to remove VOCs. After each measurement, both PEEK tubes were removed from the septa, and the jar was incubated in a plant growth cabinet for 3 days, to allow VOC accumulation in the headspace before subsequent measurement.

The PTR-TOF 8000 instrument was equipped for primary ion switching, and the H_3O^+ primary ion mode was used. The following conditions were set in the instrument drift tube: 110 °C drift tube temperature, 2.3 mbar drift pressure, 550 V drift voltage, leading to an *E*/*N* ratio (*E* represents the electric field strength and *N* represents the gas number density) of about 140 Td (1 Td = 10^{-17} Vcm^2). This *E*/*N* value was selected to limit the number of cluster ions present in the measured spectra and the excessive fragmentation of the product ions as described in the literature.[42] The ions exiting from the drift tube were detected using a ToF mass analyzer with the standard configuration (V mode). The sampling time per channel was 0.1 ns during ToF acquisition, accounting for about 350 000 channels for a mass spectrum ranging up to m/z = 400. Each individual spectrum was the sum of about 28 600 acquisitions lasting for 35 μs, resulting in a time resolution of 1 s. Because the analysis time for each sample was set to 180 s, 180 spectra were acquired for each jar during each measurement.

Two experiments were carried out independently. In the first experiment, five replicates (plants) of the Pinot noir and SO4 genotypes were analyzed for each condition (control and *P. viticola*-inoculated plants). Five untreated plants per genotype were uprooted, and jars containing only the substrate were used as negative controls for background correction of VOCs. In the second experiment, seven replicates (plants) of the Pinot noir, SO4, and Kober genotypes were analyzed for each condition. In this case, six plants per genotype were uprooted, and jars containing only the substrate were used as negative controls for background correction.

One day before *P. viticola inoculation* (T0) the headspace of each jar was cleaned by flushing with humidified zero air. One day later, plants of each genotype were inoculated with *P. viticola* or treated with water, and 1 day after inoculation, the headspace was cleaned by flushing with zero air. All samples were measured at 3-day intervals after the second headspace cleaning, corresponding to 4 (T4), 7 (T7), and 10 (T10) days after *P. viticola* inoculation. At each time point, the inoculated plants, control plants, and jars containing only the substrate were analyzed, using a randomized complete block design. Plants were kept in the plant growth cabinet between measurements.

Analysis of PTR-ToF-MS spectra

The ToF spectra produced by PTR-ToF-MS were processed according to the methodology reported by Cappellin *et al.*,[43] with slight modifications. In the first post-processing step, the spectra were corrected for count losses related to the detector dead time using a correction based on the Poisson statistics.[44] Because the external mass axis calibration provided by the acquisition software was not accurate, all spectra were subjected to internal mass axis calibration according to Cappellin *et al.*[43] This procedure led to a mass accuracy better than 0.001 Th and allowed the identification of the sum formula of the ions corresponding to the spectral peaks in most cases. To reduce the spectral noise, baseline removal and peak intensity extraction were carried out according to Cappellin *et al.*,[43] using modified Gaussian fits to the spectral peaks. The integrated signal over the 180 s of spectra acquisition was used for VOC concentration determination. Headspace VOC concentrations, expressed as ppbv (parts per billion by volume), were calculated from the peak intensities of both protonated parent molecules and known fragment ions, according to the formula described by

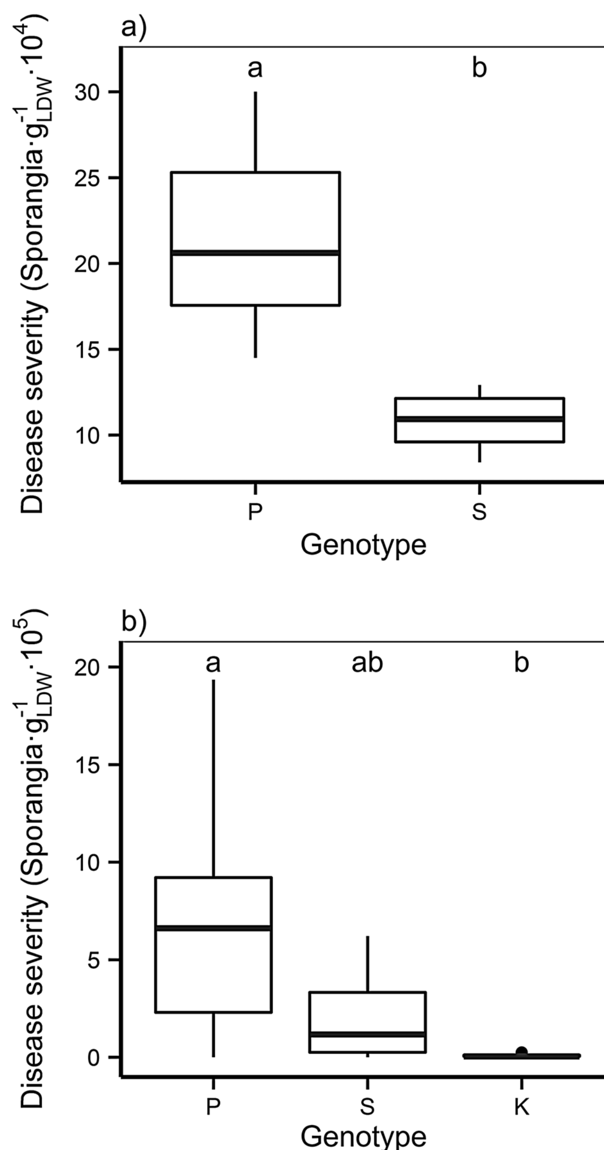

Figure 1. Downy mildew severity on the Pinot noir (P), SO4 (S), and Kober 5BB (K) genotypes in the (a) first and (b) second *in vitro* experiments. Disease severity was assessed 11 days after inoculation as the number of *Plasmopara viticola* sporangia per gram of leaf dry weight. Box plots of five and seven replicates (plants) are presented for each genotype in the first and second experiment, respectively. Different letters indicate significant differences among genotypes, according to the Kruskal–Wallis test ($p < 0.05$).

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Lindinger *et al.*,[32] considering H_3O^+ as primary ion and a constant reaction rate coefficient of $2 \times 10^{-9}\,cm^3\,s^{-1}$ in the calculations, and this introduced a systematic error of up to 30%.[45]

Identification of volatile organic compounds

Peak selection related to VOC emissions by the *in vitro*-grown plants was carried out as follows. For each spectrometric peak, the median of the VOC concentration (ppbv) was calculated for the jars containing only the substrate, control plants or *P. viticola* inoculated plants, for each genotype and time point. Values from the jars containing only the substrate were subtracted from the intensities of the respective peak of the control and *P. viticola* inoculated plants for each genotype and time point, in order to obtain the background-corrected headspace concentration. After background correction, the headspace concentration of each peak was normalized using the leaf dry weight of each plant and the number of days of emission and expressed as $ppbv\,g^{-1}\,d^{-1}$. In order to discard noise, a *t*-test analysis ($p = 0.05$) was carried out for each genotype and condition, and peaks with a background-corrected headspace concentration significantly greater than zero for at least two thirds of the time points were considered as significantly emitted by grapevine plants.

The molecular formula of significantly emitted VOCs was identified according to the mass/charge ratio (*m/z*), and the background-corrected headspace VOC emission was expressed as $\mu g\,g^{-1}\,d^{-1}$ for identified compounds. Significantly emitted VOCs were identified based on VOC reference standards (National Institute of Standards and Technology, NIST), our database of reference standard fragmentation patterns, and the available literature.

Statistical analysis

The background-corrected concentrations of the VOCs emitted by the grapevine genotypes were analyzed using an analysis of variance simultaneous component analysis (ASCA) to consider large

Table 1. Volatile organic compounds of *in vitro*-grown grapevines measured by Proton Transfer Reaction-Time of Flight-Mass Spectrometer analysis in the second experiment

Sample[a]	Volatile organic compound[b]: *m/z* = 42.0385 Alcohols/aldehydes/esters fragment isotope		*m/z* = 58.9833 Sulfur based compound [52,53]		*m/z* = 59.0491 + 60.0524 Acetone [50]		*m/z* = 81.0698 + 137.1324 + 138.1358 Monoterpenes [50,54]	*m/z* = 205.1950 + 206.1984 Sesquiterpenes [50,54]	
K_C_T0	n.d.		n.d.		n.d.		n.d.	n.d.	
P_C_T0	n.d.		0.005 ± 0.003	a	3.606 ± 3.115	a	n.d.	0.021 ± 0.034	a
S_C_T0	0.039 ± 0.033		0.002 ± 0.002	b	1.906 ± 1.653	a	n.d.	n.d.	
K_C_T4	n.d.		n.d.		n.d.		n.d.	n.d.	
K_I_T4	n.d.		n.d.		n.d.		n.d.	0.334 ± 0.247	a
P_C_T4	n.d.		0.003 ± 0.002	a	2.830 ± 1.590	a	n.d.	n.d.	
P_I_T4	n.d.		0.003 ± 0.001	a	3.241 ± 1.165	a	n.d.	0.025 ± 0.023	c
S_C_T4	0.022 ± 0.022	a	0.002 ± 0.001	a	1.685 ± 0.755	b	n.d.	n.d.	
S_I_T4	0.020 ± 0.020	a	n.d.		1.323 ± 1.544	b	0.235 ± 0.201	0.112 ± 0.086	b
K_C_T7	n.d.		n.d.		n.d.		n.d.	n.d.	
K_I_T7	n.d.		n.d.		n.d.		n.d.	0.545 ± 0.451	a
P_C_T7	n.d.		0.002 ± 0.001	a	3.363 ± 1.226	a	n.d.	n.d.	
P_I_T7	n.d.		0.002 ± 0.001	ab	4.044 ± 1.297	a	n.d.	0.073 ± 0.052	b
S_C_T7	0.015 ± 0.016		0.002 ± 0.001	ab	2.038 ± 0.944	b	n.d.	n.d.	
S_I_T7	n.d.		0.001 ± 0.001	b	1.891 ± 1.487	b	0.125 ± 0.148	0.394 ± 0.232	a
K_C_T10	n.d.		n.d.		n.d.		n.d.	n.d.	
K_I_T10	n.d.		n.d.		n.d.		n.d.	0.371 ± 0.444	a
P_C_T10	n.d.		0.003 ± 0.001	a	3.452 ± 1.153	a	n.d.	n.d.	
P_I_T10	n.d.		0.003 ± 0.002	a	4.456 ± 1.406	a	n.d.	0.056 ± 0.049	b
S_C_T10	0.017 ± 0.012	a	0.002 ± 0.001	a	2.343 ± 1.031	b	n.d.	0.010 ± 0.010	c
S_I_T10	0.019 ± 0.016	a	0.002 ± 0.001	a	2.414 ± 1.202	b	0.102 ± 0.074	0.411 ± 0.235	a

VOC, volatile organic compounds; PTR-ToF-MS, Proton Transfer Reaction-Time of Flight-Mass Spectrometer.
n.d. indicates not determined VOCs that showed a background-corrected headspace concentration not significantly greater than zero.
[a]Grapevine plants of the Pinot noir (P), Kober 5BB (K), and SO4 (S) genotype were inoculated (I) or not (C) with *Plasmopara viticola*, and VOCs were assessed at zero (T0), 4 (T4), 7 (T7), and 10 (T10) days after inoculation. Two independent experiments were carried out, and the results of the second experiment are reported in this table.
[b]The VOCs were identified by the mass/charge ratio (*m/z*) of the PTR-ToF-MS analysis and assigned to the class by looking up the *m/z* in the NIST database and the cited references. The background-corrected headspace concentrations are expressed as micrograms per gram of leaf dry weight per day ($\mu g\,g^{-1}\,d^{-1}$) and mean, and standard deviation of seven replicates (plants) are presented for each genotype and condition of the second experiment. For each time point, different letters indicate significant differences among genotypes and conditions according to the Kruskal–Wallis test ($p < 0.05$).

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metabolomics data sets with different time points.[46] The Kruskal–Wallis multiple comparison test ($p < 0.05$) was carried out on the background-corrected headspace concentrations of the significantly emitted peaks, in order to detect significant differences among genotypes and conditions at each time point. Statistical analysis and box plots were obtained with the open source program R,[47] using the MetStaT[48] and Agricolae[49] packages for ASCA and Kruskal–Wallis analysis, respectively. In the box plots presented, the box represents the range between the first and third quartiles; the line inside the box represents the median; the whiskers extend to the most extreme data within 1.5 times the interquartile range from the box, and the outliers are represented by separate dots.

Results and discussion

Two independent experiments were carried out, in order to characterize the VOC emission of different grapevine genotypes in response to *P. viticola* inoculation. The resistant SO4 and the susceptible Pinot noir genotypes were analyzed in the first experiment, and another resistant genotype called Kober was added in the second experiment.

Severity of downy mildew on grapevine genotypes

Downy mildew sporulation appeared on the abaxial leaf surface of the susceptible cultivar Pinot noir 7 days (T7) after inoculation. Pinot noir plants also showed sporulation on the upper leaf surface, petioles, and stems after 10 days (T10), as reported for susceptible grapevines grown *in vitro*.[50] SO4 showed only slight sporulation on the abaxial surface of the leaves and small necrotic spots at T10, in agreement with the resistance to downy mildew described in greenhouse and leaf disk tests.[4] Kober 5BB showed no sporulation and diffuse necrotic spots at T10, demonstrating the successful defense response. Necrotic spots were one of the earliest phenotypic differences that we noticed between susceptible and resistant genotypes, and they were attributed to a hypersensitivity reaction causing programmed cell death at the infection site and associated with reductions in pathogen performance and symptom development.[51]

Assessment of disease severity at T10 showed high production of sporangia on Pinot noir leaves (Fig. 1). The disease severity was lower in SO4 and Kober than in Pinot noir plants, in agreement with the resistance reported for these genotypes under greenhouse conditions.[4,5] The slight sporulation observed on the resistant hybrid SO4 could be due to the high humidity, which may have favored the development of downy mildew sporangia on *in vitro*-grown resistant grapevines.[36]

Table 2. Other volatile organic compounds of *in vitro*-grown grapevines measured by PTR-ToF-MS analysis in the second experiment

Sample[a]	Volatile organic compound[b]								
	$m/z = 77.0569$ ($C_3H_9O_2^+$)		$m/z = 84.0827$ ($C_5H_{10}N^+$)		$m/z = 95.0467$ ($C_6H_7O^+$)	$m/z = 113.0227$ ($C_5H_5O_3^+$)		$m/z = 149.1253$	
K_C_T0	n.d.		n.d.		0.080 ± 0.100	0.004 ± 0.006		n.d.	
P_C_T0	n.d.		n.d.		n.d.	n.d.		n.d.	
S_C_T0	n.d.		0.817 ± 0.545		n.d.	n.d.		n.d.	
K_C_T4	n.d.		n.d.		n.d.	n.d.		n.d.	
K_I_T4	n.d.		n.d.		0.065 ± 0.084	0.005 ± 0.005		0.028 ± 0.036	
P_C_T4	0.002 ± 0.002	a	n.d.		n.d.	n.d.		n.d.	
P_I_T4	0.002 ± 0.002	a	n.d.		n.d.	n.d.		n.d.	
S_C_T4	n.d		0.109 ± 0.094	b	n.d.	n.d.		n.d.	
S_I_T4	n.d		0.201 ± 0.128	a	n.d.	n.d.		n.d.	
K_C_T7	n.d.		n.d.		n.d.	0.003 ± 0.003	a	n.d.	
K_I_T7	n.d.		n.d.		n.d.	0.002 ± 0.002	b	0.043 ± 0.057	a
P_C_T7	0.003 ± 0.002	a	n.d.		n.d.	n.d.		n.d.	
P_I_T7	0.004 ± 0.002	a	n.d.		n.d.	n.d.		n.d.	
S_C_T7	n.d.		0.040 ± 0.040	b	n.d.	n.d.		n.d.	
S_I_T7	n.d.		0.179 ± 0.104	a	n.d.	n.d.		0.025 ± 0.033	ab
K_C_T10	n.d.		n.d.		n.d.	0.002 ± 0.001		n.d.	
K_I_T10	n.d.		n.d.		0.004 ± 0.005	n.d.		n.d.	
P_C_T10	0.004 ± 0.003	ab	n.d.		n.d.	n.d.		n.d.	
P_I_T10	0.005 ± 0.003	a	n.d.		n.d.	n.d.		n.d.	
S_C_T10	0.003 ± 0.002	ab	n.d.		n.d.	n.d.		n.d.	
S_I_T10	0.003 ± 0.002	b	0.143 ± 0.076		n.d.	n.d.		0.028 ± 0.031	

VOC, volatile organic compound; PTR-ToF-MS, Proton Transfer Reaction-Time of Flight-Mass Spectrometer.

n.d. indicates not determined VOCs that showed a background-corrected headspace concentration not significantly greater than zero.

[a]Grapevine plants of the Pinot noir (P), Kober 5BB (K), and SO4 (S) genotype were inoculated (I) or not (C) with *Plasmopara viticola*, and VOCs were assessed at zero (T0), 4 (T4), 7 (T7), and 10 (T10) days after inoculation. Two independent experiments were carried out, and the results of the second experiment are reported in this table.

[b]The VOCs were identified by the mass/charge ratio (m/z) of the PTR-ToF-MS analysis. The background-corrected headspace concentrations are expressed as micrograms per gram of leaf dry weight per day ($\mu g\, g^{-1}\, d^{-1}$), and the mean and standard deviation of seven replicates (plants) are presented for each genotype and condition of the second experiment. For each time point, different letters indicate significant differences among genotypes and conditions according to the Kruskal–Wallis' test ($p = 0.05$).

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Assessment of volatile organic compounds emitted by *in vitro*-grown grapevines

Although production of non-volatile secondary metabolites was widely largely characterized in grapevines following downy mildew inoculation,[8–10] this is the first time that the emission of VOCs was investigated in such conditions. The background-corrected headspace concentration of most of the significantly emitted VOCs was relatively low in both experiments (Tables 1, 2, and S2), demonstrating the potential of PTR-ToF-MS analysis to assess VOC emissions by *in vitro* plants. The low headspace concentrations of VOCs strongly limit the use of most traditional chromatographic techniques (e.g., GC-MS), while PTR-ToF-MS is a suitable method because of its high level of sensitivity, time resolution, and the lack of sample preparation or fiber incubation.

The ASCA analysis showed that the VOCs emitted by the *in vitro*-grown plants permits a clear distinction between the three grapevine genotypes in the absence of *P. viticola* infection (Fig. 2a). The first and the second principal components accounted for 86.9% and 13.1% of the variance, respectively, and the genotype factor accounted for the largest (39.3%) contribute to the sum of squares of the centered data. The time factor accounted for 16.2%; the interaction between time and genotype contributed for 10.8%, and the remaining 33.7% was due to residuals. After *P. viticola* inoculation, differences in the three genotypes were better highlighted by the significantly emitted VOCs, with the first and second principal components accounting for 90.7% and 9.3% of variance, respectively, and the genotype factor accounting for the largest percentage (60.1%) contribution to the sum of squares of the centered data (Fig. 2b). The sum of squares of the centered data for the time factor accounted for 6.3%; the interaction between time and genotype accounted for 2.4%, and the residuals for 31.2% in *P. viticola*-inoculated plants. Clustering of genotypes in separate regions of the ASCA analysis was better for inoculated plants (Fig. 2b) than for control plants (Fig. 2a), indicating that the resistance to downy mildew was mainly based on post-infection mechanisms, in agreement with gene expression analysis.[55]

The VOC emission by control plants differed significantly according to the plant genotype. For instance, the peaks found at $m/z = 58.9833$ and $m/z = 59.0491$, corresponding to sulfur-based compounds and acetone,[56] respectively, were emitted by Pinot noir and SO4 and not by Kober 5BB control plants at T0, T4, T7, and T10 in the second experiment (Table 1). The emission of sulfur-based compounds, acetone (Table 1), and five unidentified VOCs (Table 2 and S2) was not significantly affected by *P. viticola* inoculation, suggesting that it was mainly related to the grapevine genotype and was not stimulated in response to pathogen infection. However, the concentrations and profiles of sulfur-based compounds and acetone differed during the experimental repetition, possibly because of a diverse number of replicates or to a noisy background.

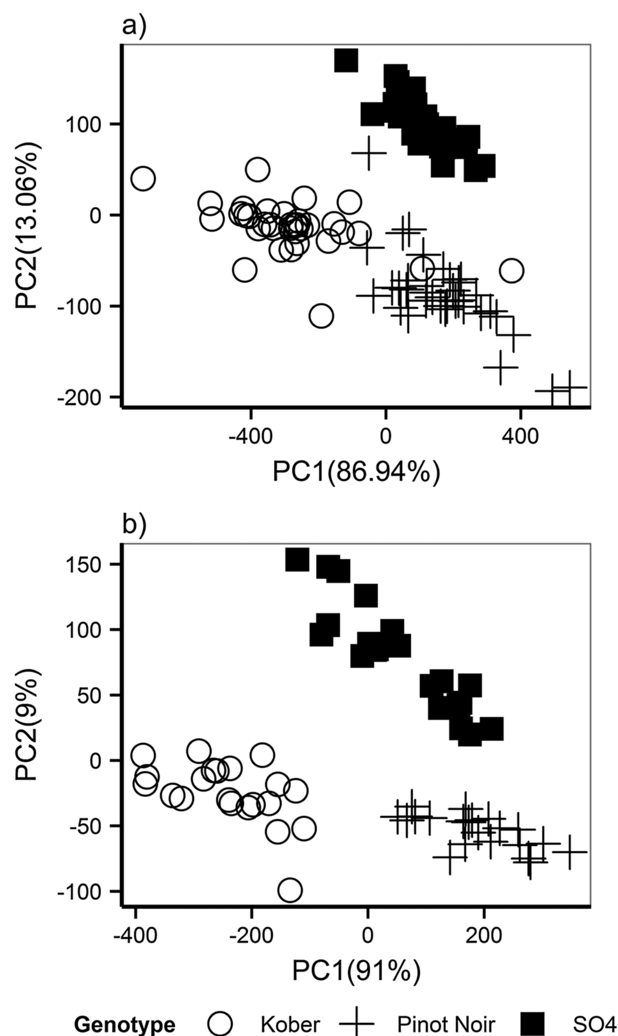

Figure 2. Analysis of variance simultaneous component analysis of volatile organic compounds emitted by (a) control (uninoculated) and (b) *Plasmopara viticola*-inoculated grapevines of the Pinot noir (crosses), Kober 5BB (open circles), and SO4 (solid squares) genotypes in the second *in vitro* experiment.

Emissions of sesquiterpenes

No emission of sesquiterpenes (*m/z* 205.1950 and 206.1984) by control plants was detected, except a slight emission by SO4 plants at T10 (Fig. 3a) and Pinot noir plants at T0 (Table 1). In particular, the SO4 genotype emitted sesquiterpenes at T4, T7, and T10 in the first experiment (Fig. 3a) and the second experiment (Fig. 3b). Kober 5BB plants emitted sesquiterpenes at T4, T7, and T10, while Pinot noir plants showed only slight emission after *P. viticola* inoculation. The emission of sesquiterpenes by *P. viticola*-inoculated plants was greater from resistant genotypes (SO4 and Kober 5BB) than from the susceptible genotype (Pinot noir). The level of sesquiterpene emission by *P. viticola*-inoculated SO4 and Kober 5BB plants was statistically comparable, except at T4 when it was greater from Kober 5BB than SO4.

Sesquiterpenes play a major role in resistance mechanisms against insect pests,[56,57] and their accumulation is also induced by inoculation with a pathogen or its metabolites, such as lettucenin A by *Bremia lactucae*, *Botrytis cinerea*, *and Pseudomonas syringae* pv. *phasealicola* in lettuce plants[58] and capsidiol by an elicitor from *Phytophthora megasperma* in tobacco cell cultures.[59] Lettucenin A and the sequiterpenes β-elemene exhibited antifungal activity against *B. cinerea*, *Br. lactucae*, *Ps. syringae* pv. *phasealicola*,[58] and *Magnaporthe oryzae*,[14] respectively, indicating the direct effect of sesquiterpenses against plant pathogens. Moreover, Himejima and colleagues[24] demonstrated that the sesquiterpene longifolene exhibited activity against *Bacillus subtilis* and *Brevibacterium ammoniagenes*. Likewise, the β-caryophyllene produced by tomato leaves was able to inhibit *B. cinerea*[25] and was implicated in

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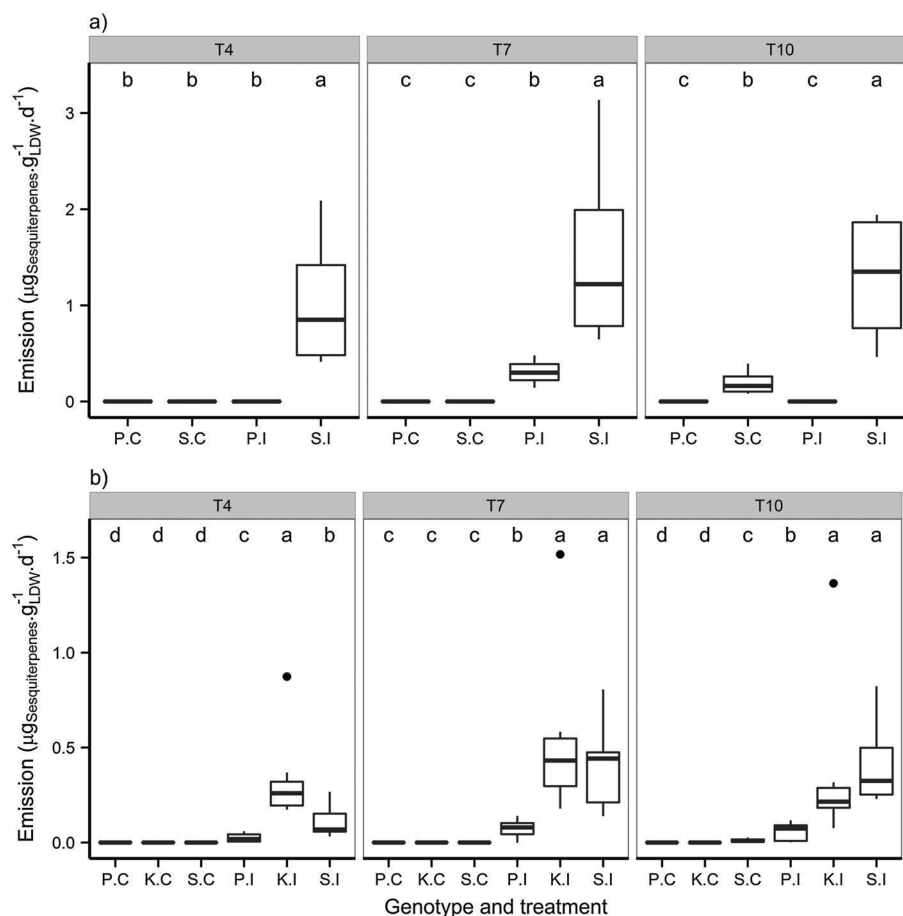

Figure 3. Emission of sesquiterpenes by grapevine plants inoculated with *Plasmopara viticola* in the (a) second experiment and (b) first *in vitro* experiment. Grapevine plants of the Pinot noir (P), Kober 5BB (K), and SO4 (S) genotypes were inoculated (I) or not (C) with *P. viticola*, and volatile organic compounds were assessed using Proton Transfer Reaction-Time of Flight-Mass Spectrometer analysis at 4 (T4), 7 (T7), and 10 (T10) days after inoculation. Box plots of background-corrected headspace concentrations from five and seven replicates (plants) are presented for each genotype and condition in the first and second experiments, respectively. For each time point, different letters indicate significant differences among genotypes and conditions, according to the Kruskal–Wallis test ($p < 0.05$).

the resistance of *Arabidopsis thaliana* to infections of *Ps. syringae* pv. *tomato* DC3000 on floral stigmas,[60] suggesting a possible role for sesquiterpenes as toxic molecules against pathogens.

Emissions of monoterpenes

Emission of monoterpenes (*m/z* 137.1325, 138.1358, and 81.0698) was only detected from *P. viticola*-inoculated plants of the SO4 genotype (Fig. 4). In particular, SO4 plants showed emission of monoterpenes at T4, T7, and T10 following *P. viticola* inoculation in both experiments. On the other hand, Pinot noir and Kober 5BB plants did not emit monoterpenes from either the control or *P. viticola*-inoculated plants. The control plants did not emit monoterpenes, except for the SO4 genotype at T4 in the first experiment, demonstrating that the production of volatile monoterpenes was activated in response to *P. viticola* inoculation. Although the volatile terpenes emitted by *P. viticola*-inoculated plants could be synthesized either by the grapevine or the pathogen, their emission was high in genotypes with low pathogen development, suggesting that they are mainly produced by plant cells.

Pathogen-dependent emission of monoterpenes was previously reported in rice plants after inoculation with *Magnaporthe grisea*.[21] However, the role of monoterpenes in plant defense against plant pathogens has been little investigated to date. The monoterpene linalool displayed antimicrobial activities against *Xanthomonas citri*, *Penicillium italicum*,[61] and *C. lindemuthianum*.[20] Likewise, the monoterpenes γ-terpinene, β-pinene, and α-pinene inhibit growth and germination of *Leptographium* spp.,[62] as well as citral, citronellal, and linalool inhibited spore germination and hyphal growth of the fungal pathogen *Alternaria alternata*.[52] Citral interfered with the membranes of *Aspergillus* spp. spores,[53] and α-pinene, β-pinene, 3-carene, limonene, and terpinolene showed antimicrobial activity against *Saccharomyces cerevisiae*,[24] suggesting the general antifungal role of monoterpenes against phytopathogens. Limonene was also able to inhibit the growth of the pathogens *Mucor mucedo*[24] and *C. lindemuthianum*.[20] Moreover, the monoterpene allo-ocimene, together with C6-aldehydes, was able to confer greater resistance against *B. cinerea* by inducing the expression of defense genes in *A. thaliana*,[54] also demonstrating the role of monoterpenes to induce plant resistance. To summarize, it seems that monoterpenes could have several roles in the defense mechanisms against pathogens, suggesting that the production of monoterpenes in SO4 plants may possibly be associated with the defense mechanisms activated in resistant genotypes to downy mildew.

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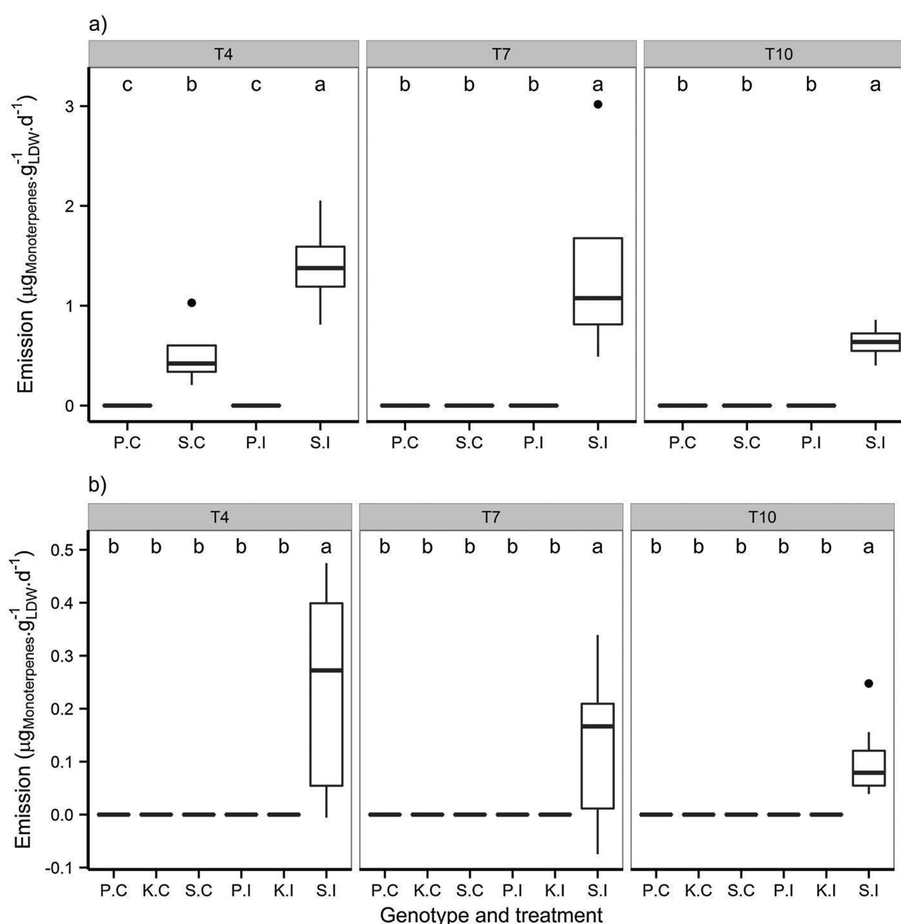

Figure 4. Emission of monoterpenes by grapevine plants inoculated with *Plasmopara viticola* in the (a) first experiment and (b) second *in vitro* experiment. Grapevine plants of the Pinot noir (P), Kober 5BB (K), and SO4 (S) genotypes were inoculated (I) or not (C) with *P. viticola*, and volatile organic compounds were assessed using Proton Transfer Reaction-Time of Flight-Mass Spectrometer analysis at 4 (T4), 7 (T7), and 10 (T10) days after inoculation. Box plots of background-corrected headspace concentrations from five and seven replicates (plants) are presented for each genotype and condition in the first and second experiments, respectively. For each time point, different letters indicate significant differences among genotypes and conditions, according to the Kruskal–Wallis test ($p < 0.05$).

Conclusions

There is no information available in the literature on the VOCs emitted by grapevine plants following inoculation with *P. viticola*. Here, we have shown that the PTR-ToF-MS is a powerful tool for analyzing VOC emission by *in vitro*-grown grapevines after downy mildew inoculation, which may be also extended to other pathosystems. The analysis of *in vitro*-grown plants allowed precise identification of compounds produced by control plants under axenic conditions and in response to *P. viticola* inoculation, without possible external factors and contaminants. Our method discriminated between the three tested genotypes based on VOC emissions and showed significant differences between the American hybrids and the *V. vinifera* genotype both in *P. viticola*-inoculated and control plants. The emission of monoterpenes by SO4 plants and the emission of sesquiterpenes by SO4 and Kober 5BB plants were significantly greater than in Pinot noir after inoculation. Because monoterpenes and sesquiterpenes are known to be involved in plant resistance to insects and pathogens, our results raise the question of the possible role of volatile terpenes in the defense reaction against *P. viticola* in resistant genotypes. Clarifying this role requires assessment of direct toxic activity against *P. viticola* and/or the validation of their role in the activation of plant resistance, which will be further investigated in the future.

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Publication 2 – Downy mildew symptoms can be reduced by volatile organic compounds of resistant grapevine genotypes

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MOTIVATION OF THE STUDY

Grapevine VOCs represent possible mediators of plant defence against phytopathogens such as *P. viticola* (publication 1). However, the contribution of VOCs in grapevine defence mechanisms against downy mildew has not yet been investigated. In this study, HS-SPME/GC-MS analysis was used to analyse VOC profiles of four resistant and one susceptible grapevine genotypes upon *P. viticola* inoculation under greenhouse conditions. Results revealed that downy mildew significantly increased the production of defence-related VOCs in resistant, but not in susceptible grapevine genotypes. Particularly, active VOCs against *P. viticola* were identified and they possibly contribute to grapevine resistance by direct inhibition of downy mildew in the emitting tissues and in systemic parts of locally attacked plants.

CONTRIBUTION OF THE PRESENTING AUTHOR

I performed all the experiments, analysed the grapevine samples and performed data analysis. Furthermore, I was responsible for writing the manuscript.

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Downy mildew symptoms on grapevines can be reduced by volatile organic compounds of resistant genotypes

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Running title: Volatile defence metabolites of downy mildew-resistant genotypes

Abstract

Volatile organic compounds (VOCs) play a crucial role in the communication of plants with other organisms and are possible mediators of plant defence against phytopathogens. Although the role of non-volatile secondary metabolites has been largely characterised in resistant genotypes, the contribution of VOCs to grapevine defence mechanisms against downy mildew (caused by *Plasmopara viticola*) has not yet been investigated. In this study, more than 50 VOCs from grapevine leaves were annotated/identified by headspace-solid-phase microextraction gas chromatography-mass spectrometry analysis. Following *P. viticola* inoculation, the abundance of most of these VOCs was higher in resistant (BC4, Kober 5BB, SO4 and Solaris) than in susceptible (Pinot noir) genotypes. The post-inoculation mechanism included the accumulation of 2-ethylfuran, 2-phenylethanol, β-caryophyllene, β-cyclocitral, β-selinene and trans-2-pentenal, which all demonstrated inhibitory activities against downy mildew infections in water suspensions. Moreover, the development of downy mildew symptoms was reduced on leaf disks of susceptible grapevines exposed to air treated with 2-ethylfuran, 2-phenylethanol, β-cyclocitral or trans-2-pentenal, indicating the efficacy of these VOCs against *P. viticola* in receiver plant tissues. Our data suggest that VOCs contribute to the defence mechanisms of resistant grapevines and that they may inhibit the development of downy mildew symptoms on both emitting and receiving tissues.

Introduction

Plants are constantly exposed to environmental stressors and have evolved complex ways to defend themselves against pathogens, herbivorous arthropods, parasitic plants and neighbouring plant competitors[1]. Plants can produce a wide variety of volatile organic compounds (VOCs), which play a crucial role in the interaction of plants with other organisms and in the regulation of plant responses against biotic stresses[1,2]. VOCs constitute approximately 1% of plant secondary metabolites[3] and are usually lipophilic molecules that can freely diffuse into the environment and pass biological membranes, thanks to their low molecular weight and high vapour pressure[4]. Based on their structure and biosynthetic pathways, plant VOCs can be divided into four main classes: terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives and those derived from non-aromatic amino acids[2,3]. Volatile terpenoids are synthesized by the cytosolic mevalonic acid and plastidial methylerythritol phosphate pathway, which leads to the formation of carotenoids, mono-, di-, hemi- and sesquiterpenes[2]. Phenylpropanoid/benzenoid compounds are the second largest class of plant VOCs and they originate from phenylalanine through the shikimate/phenylalanine biosynthetic pathway[2]. Volatile fatty acid derivatives mainly derive from linoleic and linolenic acids through the lipoxygenase pathway[2], while volatile amino acid derivatives contain nitrogen and sulphur and are synthesized from alanine, valine, leucine, isoleucine or methionine[3].

The production and roles of plant VOCs in response to mechanical wounding or herbivore insects have been extensively investigated, but little is known about their involvement in defence mechanisms against pathogens[5,6]. Pathogen-induced VOCs typically consist of methyl salicylate (MeSA)[7-9], mono- and sesquiterpenes[7,8,10-12], heterocyclic compounds[7], green leaf

volatiles (GLVs) and ketones[10,11]. Three possible modes of action against pathogens have been attributed to plant VOCs, namely direct inhibition of microbial growth, induced and associational resistance[6]. For example, GLVs[13] and β-caryophyllene[14] directly inhibited bacterial growth and trans-2-hexenal reduced the germination of *Monilinia laxa*[15] and *Botrytis cinerea* conidia[16]. Likewise, monoterpenes (limonene and β-linalool), nonanal and methyl jasmonate (MeJA) inhibited the germination of *Colletotrichum lindemuthianum*[6], and esters (methyl propanoate and methyl prop-2-enoate) reduced the development of *Fusarium culmorum* and *Cochliobolus sativus*[17]. As a result of induced and associational resistance, VOCs can contribute to disease reduction in systemic parts of a locally attacked plants or in neighbouring plant receivers[6]. For example, VOC blends emitted by resistant plants[6,18] induced defence-related processes in neighbouring plants, such as monoterpenes (α-pinene and β-pinene)[19], MeSA[20], MeJA and GLVs[21]. Finally, VOCs can be adsorbed to the cuticle of a receiver plant and these 'sticky' VOCs can persist on the leaf surface[22], thereby exerting inhibitory effects against fungal pathogens and establishing the associational resistance[6].

The involvement of plant VOCs in resistance mechanisms against pathogens is supported by specific VOC emission profiles in resistant and susceptible genotypes of maize to *Aspergillus flavus*[23], citrus plants to *Candidatus* liberibacter asiaticus[24] and grapevine plants to *Plasmopara viticola*[12]. In the latter, the emission of the sesquiterpene and monoterpene classes was found to be more pronounced in downy mildew-resistant than in susceptible grapevine genotypes[12], and the emission of a sesquiterpene [(E,E)-α-farnesene] was associated with the resistance induced by a sulphated laminarin against downy mildew[25]. Downy mildew, caused by the biotrophic oomycete *Plasmopara viticola*, is one of the most destructive diseases of the grapevine[26]. Resistance traits have been identified in wild grapevine species (*Vitis riparia*, *V.*

rupestris, *V. amurensis* and *Muscadinia rotundifolia*) and the defence mechanisms against downy mildew have been investigated in resistant genotypes[27]. For example, physical (hairy and water repellent leaf surface) and chemical (phytoanticipins) barriers represent constitutive factors against pathogen infection[28], while the accumulation of reactive oxygen species, pathogenesis-related proteins and non-volatile secondary metabolites (stilbenic phytoalexins and other antimicrobial phenolic compounds) has been shown to be a key post-inoculation mechanism involved in limiting *P. viticola* infection[29-31]. Although resistant genotypes produce some VOC classes after *P. viticola* inoculation[12], identification of the underlying compounds and their functional role in grapevine resistance mechanisms have not yet been investigated. The aim of this study was to annotate/identify VOCs produced by resistant and susceptible grapevine genotypes in response to *P. viticola* inoculation using headspace-solid-phase microextraction gas chromatography-mass spectrometry analysis (HS-SPME/GC-MS) and to test their effects against downy mildew. Due to the obligate biotrophic lifestyle of *P. viticola*, inhibitory effects of VOCs can be tested only in the presence of host tissues and the final goal was to better understand the contribution of grapevine VOCs to limit downy mildew development in susceptible leaves.

Results

Profiles of VOCs detected in grapevine leaves. The evaluation of resistance levels confirmed a lower degree of resistance for the susceptible *V. vinifera* cultivar Pinot noir ENTAV 115 in both greenhouse experiments, as compared with the four downy mildew-resistant genotypes: BC4 [*M. rotundifolia* × *V. vinifera*[32]], Kober 5BB [*V. berlandieri* × *V. riparia*[33]], SO4 [*V. berlandieri* × *V. riparia*[34]] and Solaris [Merzling (Seyve-villard 5276 × Freiburg 379-52) ×

Geisenheim 6493 (Severnyi × Muscat Ottonel); www.vivc.de] (Fig. 1). Specifically, leaves of susceptible Pinot noir plants showed dense sporulation of *P. viticola*, chlorotic spots and the absence of necrosis (mean OIV-452 score: 3), while those of BC4, Kober 5BB, SO4 and Solaris showed diffuse necrotic spots with sparse or absent sporangiophores (mean OIV-452 scores ranged from 7 to 9).

Leaf samples were collected immediately before inoculation (0 dpi) and six days post inoculation (6 dpi) with *P. viticola*, frozen in liquid nitrogen, ground to a fine powder and subjected to VOCs analysis according to the protocol optimized for grapevine leaves by Weingart, et al. [63] (Supplementary Fig. S1). A total of 56 and 52 VOCs were found in the five grapevine genotypes in the first and second experiment, respectively. In particular, 41 VOCs were properly annotated and 16 were found as unknown compounds according to the measured retention index (RI) of the HS-SPME/GC-MS analysis (Supplementary Tables S1, S2 and S3). Three pairwise comparisons were analysed to detect VOCs with significant changes in abundance (Kruskal-Wallis test $p \leq 0.05$ and a fold change > 1.5) between each resistant genotype and Pinot noir at 0 dpi (R *vs.* PN 0 dpi) and 6 dpi (R *vs.* PN 6 dpi) or between 6 and 0 dpi for each genotype (6 *vs*. 0 dpi). VOC profiles of the tested grapevine genotypes were mainly consistent in the two experiments, and they differed according to the grapevine genotypes and time points (Figure 2, Supplementary Tables S1 and S2). Slight differences in VOC abundance occurred in resistant genotypes and the susceptible Pinot noir at 0 dpi (constitutive differences). On the other hand, the abundance of most of the annotated VOCs was consistently higher in resistant genotypes than in Pinot noir at 6 dpi in both experiments (post-inoculation differences). This is also reflected by the observation that most of the VOCs

showed an increase in abundance at 6 dpi as compared with 0 dpi within each resistant genotype, but not in Pinot noir.

More specifically, VOCs were divided into six metabolite groups according to changes in abundance between resistant and susceptible genotypes consistently found in both experimental repetitions. The first metabolite group included two sesquiterpenes (γ-cadinene, δ-cadinene) and unknown compound 1, whose abundance was consistently higher in all resistant genotypes than in Pinot noir at 6 dpi in both experiments (Metabolite group 1). Moreover, γ-cadinene and δ-cadinene already showed higher constitutive levels in two (Kober 5BB and SO4) and three (BC4, Kober 5BB and SO4) resistant genotypes as compared with Pinot noir at 0 dpi in both experiments, respectively.

The metabolite group 2 summarises 12 compounds whose abundances were consistently higher in two or more resistant genotypes than in Pinot noir at 6 dpi in both experiments. β-caryophyllene, β-selinene and ledol and the two unknown compounds 2 and 3 were consistently more abundant in *P. viticola*-inoculated leaves of three resistant genotypes (BC4, Kober 5BB and Solaris) than in Pinot noir. The abundance of 2-ethylfuran and β-cyclocitral was higher in *P. viticola*-inoculated leaves of the resistant genotypes Kober 5BB and Solaris as compared with Pinot noir in both experiments. All the other unknown compounds (from 4 to 8) of this metabolite group showed higher levels in *P. viticola*-inoculated leaves of two of the four resistant genotypes as compared with Pinot noir at 6 dpi in both experiments.

The abundance of 17 VOCs was consistently higher in only one resistant genotype as compared with Pinot noir at 6 dpi in both experiments (Metabolite group 3). Specifically, α-caryophyllene, α-muurolene and epizonarene were consistently more abundant in *P. viticola*-

inoculated leaves of BC4 than in Pinot noir. The abundance of trans-2-pentenal and unknown compound 9 was higher in Kober 5BB than in Pinot noir. Together with γ− and δ−cadinene, trans-2-pentenal and unknown compound 9 belonged to a group of four VOCs that were not only found to be induced by the pathogen inoculation but were also constitutively more abundant in Kober 5BB than in Pinot noir before inoculation. Moreover, α-eudesmol, γ-selinene and β-linalool showed higher abundance in Kober 5BB as compared with Pinot noir after *P. viticola* inoculation. *P. viticola* inoculation increased the abundance of a diester (diisobutyl phthalate) and eight VOCs [2-penten-1-ol-(E), β-ionone, 2-phenylethanol, decanal, ethyl-benzaldehyde and unknown compounds 10, 11 and 12] in SO4 and Solaris as compared with Pinot noir at 6 dpi, respectively.

In contrast to the high number of *P. viticola*-induced VOCs, two alcohols (3-ethyl-4-methyl-1-pentanol and benzyl alcohol) and two aldehydes [2,4-heptadienal (E-E)- and benzenacetaldehyde] were consistently less abundant in at least one resistant genotype and time point as compared with Pinot noir (Metabolite group 4). While the results described for metabolite groups 1-4 were consistent across both experiments, the profiles of 15 VOCs differed in the two experiments (Metabolite group 5). Moreover, five VOCs (α-copaene, germacrene B, germacrene D, dihydroactinidiolide and (+)-aromadendrene) and one VOC (octanoic acid) were detected only in the first or second experiment, respectively (Metabolite group 6).

Effects of pure VOCs on downy mildew severity. Eight VOCs were selected according to their consistent changes in abundance between resistant and susceptible genotypes in both experiments and they were tested as single pure compounds against *P. viticola* at different

dosages in water suspension and air volume (Supplementary Fig. S1). More specifically, a mixture of ɣ- and δ-cadinene isomers was selected since they were consistently more abundant in all resistant genotypes at 6 dpi; three compounds (β-caryophyllene, β-selinene and ledol) and two compounds (2-ethylfuran and β-cyclocitral) were selected due to their consistently higher abundance in three (BC4, Kober 5BB and Solaris) and two resistant genotypes (Kober 5BB and Solaris) as compared with Pinot noir at 6 dpi respectively; trans-2-pentenal and 2-phenylethanol were selected as Kober 5BB (at 0 and 6 dpi) and Solaris (at 6 dpi) specific compounds, respectively. Leaf disks inoculated with the *P. viticola* sporangia suspension only (control) displayed severe sporulation at 6 dpi, while those inoculated with sporangia suspensions containing 10.0 g/L of each pure VOC had no disease symptoms (Fig. 3A). However, treatments with 10.0 g/L in water suspension of cadinene, ledol, trans-2-pentenal, 2-ethylfuran and β-cyclocitral caused phytotoxic effects on leaf tissues (diffuse chlorotic spots). Trans-2-pentenal, 2-ethylfuran and β-cyclocitral prevented downy mildew symptoms at the dosage of 1.0 g/L with no visible phytotoxic effects (Fig. 3B). At a VOC concentration of 0.1 g/L in water suspension, only trans-2-pentenal reduced downy mildew symptoms (Fig. 3C), with a disease reduction (efficacy) of 29.0 ± 9.2% (mean ± standard error, both expressed as a percentage), calculated according to the following formula: (disease severity of control disks - disease severity of VOC-treated disks)/(disease severity of control disks) × 100. No reduction in downy mildew severity was observed with pure VOCs at 0.01 g/L each (efficacy ranged from 0.0 ± 0.1% to 0.1 ± 0.0%) or with a blend of eight (2-phenylethanol, cadinene, β-caryophyllene, β-selinene, ledol, trans-2-pentenal, 2-ethylfuran, and β-cyclocitral) or three (trans-2-pentenal, 2-ethylfuran, and β-cyclocitral) pure VOCs at dosages of 0.1 (efficacy of 0.0

± 0.0% and 0.3 ± 0.1%, respectively) or 0.01 g/L in water suspension for each compound (efficacy of 0.0 ± 0.0% and 0.2 ± 0.1%, respectively; Kruskal-Wallis test, $p > 0.05$).

The eight pure VOCs were also tested against *P. viticola* at different dosages in air volume without direct contact with the leaf tissue. These tests showed that 2-phenylethanol, trans-2-pentenal, 2-ethylfuran and β-cyclocitral reduced downy mildew symptoms at a dosage of 20 mg/L in air volume, while cadinene, β-caryophyllene, β-selinene and ledol did not (Fig. 4A). Leaf disks exposed to β-caryophyllene at a concentration of 50 mg/L in air volume showed phytotoxic effects, while those exposed to cadinene (78.0 ± 8.9%), β-selinene (85.6 ± 4.8%) and ledol (94.0 ± 0.9%) showed a disease severity comparable to control disks (98.8 ± 0.8%, Kruskal-Wallis test $p > 0.05$) and therefore these VOCs were not further used in activity tests. By lowering the concentration to 5.0 and 0.5 mg/L in air volume, only trans-2-pentenal reduced downy mildew symptoms with an efficacy of 100.0 ± 0.1% and 46.7 ± 10.3%, respectively (Figs 4B and 4C). The dependence of efficacy on the concentration was tested in more detail for trans-2-pentenal, and at a concentration of 2.5 mg/L in air volume it was able to completely suppress downy mildew symptoms (efficacy 100.0 ± 0.1%) without any visible phytotoxic effects (Fig. 5).

The four active VOCs in air volume (2-ethylfuran, 2-phenylethanol, β-cyclocitral or trans-2-pentenal) were further characterised with microscopic analysis, using the lowest concentrations at which the highest efficacy without visible phytotoxicity was observed (i.e. optimised concentrations), namely 2.5 mg/L in air volume of trans-2-pentenal and 20 mg/L in air volume of 2-ethylfuran, 2-phenylethanol or β-cyclocitral. Aniline blue-staining revealed marked differences between control and VOC-treated leaf disks after *P. viticola* inoculation (Fig. 6). At 1 dpi, the pathogen had already penetrated the stomata of control leaf disks, and

encysted zoospores and substomatal vesicles were visible. The number of zoospores that had successfully entered stomata at 1 dpi was reduced in leaf disks treated with 2-phenylethanol, 2-ethylfuran or β-cyclocitral, while no infection structures were visible on trans-2-pentenal-treated disks. At 2 dpi, elongated and branched hyphae with haustoria were visible in control leaf disks, while primary haustoria and primary hyphae were occasionally visible in 2-phenylethanol-, 2-ethylfuran-, and β-cyclocitral-treated leaf disks. Again, no pathogen structures were visible in trans-2-pentenal-treated leaf disks at 2 dpi and sporulation was still not visible at 6 dpi. At 6 dpi, *P. viticola* mycelium had already spread to the parenchyma and produced sporangiophores in control leaf disks, while *P. viticola* sporulated areas were reduced in 2-phenylethanol-, 2-ethylfuran- and β-cyclocitral-treated samples.

Effects of pure VOCs on *Plasmopara viticola* sporangia. In order to assess the effects on *P. viticola* sporangia, the four active VOCs were tested at their respective optimised concentrations in air volume, as calculated from the experiments described above. Trans-2-pentenal and β-cyclocitral reduced sporangia length and width, while 2-phenylethanol and 2-ethylfuran did not (Figs 7A and 7B). However, sporangia vitality was not affected by VOC treatments and the disease severity of disks inoculated with 2-phenylethanol- (77.8 ± 4.5%), 2-ethylfuran- (80.4 ± 2.7%), β-cyclocitral- (76.2 ± 10.4%) and trans-2-pentenal-treated sporangia (77.3 ± 11.4%) was comparable to that of control sporangia (79.8 ± 1.7%; Kruskal-Wallis test $p > 0.05$).

Discussion

VOCs are known to play a crucial role in the communication between plants and other organisms[1,2] and three possible modes of action against plant pathogens have been hypothesised so far[6]. More specifically, it has been shown that VOCs can directly inhibit pathogen growth, induce plant resistance mechanisms in neighbouring plants and mediate associational resistance by adsorption to the cuticle of receiver tissues[6,35]. The emission of some VOC classes has been demonstrated in resistant grapevines after *P. viticola* inoculation[12,35], but annotation of their chemical structures and assessment of their functional roles in defence mechanisms have not yet been investigated. In agreement with previous literature on transcriptional regulation and accumulation of non-volatile metabolites[29,30], VOC profiles were mainly related to post-inoculation mechanisms and significant increases in abundances were detected for 20 annotated VOCs at 6 dpi as compared with 0 dpi in all the four resistant grapevine genotypes tested (BC4, Kober 5BB, SO4 and Solaris). The role of VOCs in grapevine defence mechanisms was supported by the higher abundance in resistant genotypes as compared with the susceptible *V. vinifera* cultivar (Pinot noir) after *P. viticola* inoculation. For example, 11 terpenoids (α- and β-caryophyllene, α-muurolene, α-eudesmol, β-linalool, γ- and δ-cadinene, β- and γ-selinene, epizonarene and ledol) showed higher abundance in at least one resistant genotype as compared with Pinot noir, in agreement with the increased emission of this VOC class by *P. viticola*-inoculated plants previously shown by proton-transfer-reaction time-of-flight mass spectrometry analysis[12]. Moreover, the abundance of trans-2-pentenal, γ- and δ-cadinene also differed in resistant and susceptible genotypes before *P. viticola* inoculation, suggesting their involvement in constitutive defence mechanisms of resistant genotypes as well. Moreover, the majority of the unknown compounds

(from 1 to 12) showed an increase in abundance in resistant genotypes as compared with Pinot noir at 6 dpi, but further studies are required to better identify the chemical structure and potential roles of these compounds.

Functional assays demonstrated that treatments with 2-ethylfuran, 2-phenylethanol, β-caryophyllene, β-cyclocitral, β-selinene and trans-2-pentenal in water suspensions reduced the development of downy mildew symptoms on Pinot noir leaf disks with no visible phytotoxic effects. Previous studies have indicated that VOCs are more abundant in the emitting leaf than in its surrounding gas space[6] and they possibly accumulate in correspondence with the stomata[36], suggesting that active VOCs can reach sufficiently high concentrations at stomatal infection sites to limit *P. viticola* infections. Thus, VOCs synthesized by resistant genotypes possibly contribute to resistance mechanisms by inhibiting downy mildew development in the plant emitter. Two active VOCs (β-caryophyllene and β-selinene) inhibited *P. viticola* only when applied in the water sporangia suspension, possibly due to the relatively low volatility of sesquiterpenes[37], and they may form protective envelopes on the leaf surfaces of the plant emitter, with scarce migration to neighbouring plants. Moreover, four active VOCs (2-ethylfuran, 2-phenylethanol, β-cyclocitral and trans-2-pentenal) also prevented downy mildew symptoms on the susceptible Pinot noir leaf disks when applied in the air volume without direct contact with leaf tissues, indicating possible migration of these VOCs from plant emitters to neighbouring plants receivers. Previous studies have suggested that resistance induction can be impaired in detached leaves[38] and this plant defence mechanism can show negligible effects on leaf disks[6], indicating that these four active VOCs may contribute to associational resistance and reduce the severity of downy mildew symptoms on receiver plant tissues. Indeed, it has been reported that some VOCs can be adsorbed to the cuticle of the receiver and persist on its

leaf surface[22] and display inhibitory activities against phytopathogens through associational resistance mechanisms[6]. However, inhibitory effects of VOCs against downy mildew can be tested only in the presence of host tissues, due to the obligate biotrophic lifestyle of *P. viticola*. Thus, possible indirect effects of VOCs on host tissues cannot be totally excluded, such as the induction of plant resistance or slight phytotoxic effects. Indeed, sporangia vitality was not affected by the exposure to air treated with the four active VOCs, and only trans-2-pentenal and β-cyclocitral slightly reduced sporangia diameter. Thus, active VOCs could act against *P. viticola* once zoospores are released from sporangia and/or they possibly need the presence of host tissues to display the inhibitory activities. However, more precise functional and molecular studies on zoospore motility and grapevine resistance induction after VOC treatments are required to better understand the VOC activities against *P. viticola*. Moreover, stereochemical analyses are also needed to identify the stereoisomeric configuration of grapevine VOCs and the specificity of stereoisomers against downy mildew.

In the conditions applied in our study, trans-2-pentenal strongly inhibited downy mildew symptoms and was found in Kober 5BB plants before and after *P. viticola* inoculation. Trans-2-pentenal belongs to α,β-unsaturated aldehydes, which are categorised as GLVs[39]. Due to their chemical structure, α,β-unsaturated aldehydes can react with nucleophiles (such as protein sulphydryl or amino groups)[40] and cause morphological deformations, collapse and deterioration of fungal structures[16], as in the case of trans-2-hexenal against *B. cinerea*[16] and *M. laxa*[15]. Therefore, the same mechanism of action can be hypothesised for trans-2-pentenal against *P. viticola* and can also explain the phytotoxic effects observed on grapevine leaf disks at dosages higher than 5 mg/L in air volume. Moreover, 2-phenylethanol, 2-ethylfuran and β-cyclocitral displayed moderate efficacy against downy mildew in air volume and they were

mainly produced by one (Solaris) and two (Kober 5BB and Solaris) resistant genotypes after *P. viticola* inoculation, respectively. 2-phenylethanol (also known as benzeneethanol or phenylethyl alcohol) is present in plant essential oils[41] and has previously been shown to have antimicrobial activity against *Escherichia coli* and *Rhizoctonia solanacearum*[41], *Penicillium digitatum* and *P. italicum*[42], *Candida albicans*, Gram-positive and negative bacteria[43]. Moreover, it has been reported that 2-ethylfuran accumulated during fatty acid oxidation in wild rocket[44] and olive oil[45], and it has nematicidal activity against *Meloidogyne incognita*[46]. Likewise, Ikawa, et al. [47] and Ozaki, et al. [48] respectively demonstrated that β-cyclocitral inhibited *Chlorella pyrenoidosa* and *Cyanobacterium microcystis*.

Sesquiterpenes (β-caryophyllene and β-selinene) reduced downy mildew symptoms in water suspensions and terpenes have already been classified as markers of genetic[12] and induced resistance[25] against grapevine downy mildew. Terpenes are generally recognised to contain antimicrobial metabolites[49] and can interfere with mitochondrial membranes causing microbial cell death[50]. Infections of *Magnaporte oryzae* and *Pseudomonas syringae* pv. *maculicola* increased the emission of β-caryophyllene in rice leaves[51] and tobacco plants[8], respectively. Our results are also in agreement with Huang, et al. [14], who demonstrated that β-caryophyllene inhibits *P. syringae* pv. *tomato* DC3000 in water suspensions and not in air volume (without direct contact). Likewise, β-selinene reduced downy mildew symptoms in water suspension and has previously been found in plant essential oils, with antimicrobial activities against *Staphylococcus aureus* and *C. albicans*[52], *Bacillus licheniformis* and *Trypanosoma brucei brucei*[53]. Moreover, our results indicate that resistant genotypes can produce at least a further six terpenes (α-caryophyllene, α-eudesmol, α-muurolene, β-linalool, γ-selinene and epizonarene), one isoprenoid (β-ionone), one alcohol [2-penten-1-ol-(E)] and

two aldehydes (decanal and ethyl-benzaldehyde), and some of these VOCs are known for their inhibitory activities against plant pathogens. For example, β-linalool inhibited *C. lindemuthianum*[6] and *P. aeruginosa*[54] and induced resistance against *Xanthomonas oryzae* in rice[55]. Likewise, β-ionone showed fungistatic activity against *C. musae*[56] and decanal inhibited *Phytophthora infestans*[57], indicating that several putative defence-related VOCs are synthesized by resistant genotypes in response to *P. viticola*.

In conclusion, *P. viticola* inoculation significantly increased the production of defence-related VOCs in resistant, but not in susceptible grapevine genotypes. Resistant grapevines accumulated six VOCs (2-ethylfuran, 2-phenylethanol, β-caryophyllene, β-cyclocitral, β-selinene and trans-2-pentenal) that reduced downy mildew symptoms on leaf disks and other putative defence-related VOCs (β-linalool, β-ionone and decanal) that possibly contribute to the inhibition of *P. viticola* infection. Moreover, downy mildew symptoms were impaired on leaf disks of susceptible grapevines exposed to air treated with 2-ethylfuran, 2-phenylethanol, β-cyclocitral or trans-2-pentenal, indicating that these four active VOCs possibly contribute to grapevine defence against downy mildew in systemic parts of a locally attacked plants or in neighbouring plants. Particularly, trans-2-pentenal was the most efficient VOC identified in this study and it represent a promising molecule from natural origin that could be further developed for downy mildew control of grapevine possibly with appropriate encapsulating formulations. Thus, VOCs could contribute to grapevine defence against downy mildew, but further metabolomic and transcriptomic analyses are required to investigate the possible VOC adsorption of the leaf cuticle and the possible indirect effects of VOCs on plant tissues, such as the activation of resistance mechanisms.

Methods

Inoculation of grapevine plants and assessment of disease severity. Grapevine rooted cuttings were grown under greenhouse conditions as described by Banani, et al. [58]. A *P. viticola* population was collected from an untreated vineyard in the Trentino region (northern Italy) and maintained by subsequent inoculations on *V. vinifera* Pinot Noir plants under greenhouse conditions[59]. Grapevines were inoculated with a suspension of *P. viticola* sporangia (2.5×10^5 sporangia/mL) as described by Perazzolli, et al. [59]. The degree of downy mildew resistance was assessed at 7 dpi according to the OIV-452 descriptor[60], and category scores from 1 (the most susceptible) to 9 (totally resistant) were assigned according to disease symptoms[61]. Ten replicates (plants) per genotype were assessed in a randomised complete block design. The experiment was carried out twice in two consecutive years (namely first and second experiment).

Sample collection and VOC analysis. Leaf samples were collected immediately at 0 dpi and 6 dpi with *P. viticola*, to maximise the accumulation of non-volatile stilbenic phytoalexins[29,62]. Each sample comprised three leaves (from the fourth-sixth node) immediately frozen in liquid nitrogen, with five replicates (plants) being collected for each genotype and time point.

Samples were processed according to the protocol optimized by Weingart, et al. [63] for grapevine leaves. Each frozen leaf sample was ground to a fine powder using a mixer-mill disruptor (MM301 Retsch) for 30 sec at 30 Hz, with pre-cooled 10 mL stainless steel beakers (Retsch) and a 9 mm stainless steel ball (Retsch). Leaf powder was transferred into 50 mL tubes and stored at -80°C. Each sample (100 mg) was weighed in a 20 mL headspace vial (HS vials; Gerstel, Mülheim a.d. Ruhr), which was immediately sealed with a screw cap, assembled

with a 1.3 mm silicone/PTFE septum (Supelco). As a quality control sample (QC sample), equal aliquots of each leaf sample were homogenised to determine technical variability[63]. Samples were measured in a randomised complete block design and a QC sample (100 mg) was analysed every eight grapevine samples.

VOCs were measured using HS-SPME/GC-MS analysis according to Weingart, et al. [63]. Briefly, each HS vial was placed in the auto-sampler at 15°C (MPS2XL, Gerstel), after 20 min at 60°C, a Divinylbenzene/Carboxen/Polydimethylsiloxane fibre (2 cm 50/30 µm; Supelco, Sigma-Aldrich) was inserted into the HS vial and the VOC extraction was carried out for 40 min at 60°C. Analytes were desorbed in splitless mode at 250°C for 2 min using an Agilent 6890 N gas chromatograph coupled to a quadrupole mass spectrometer 5975B Mass Selective Detector (MSD; Agilent Technologies). A non-polar DB-5MS column (Agilent Technologies) was operated at a constant 1 mL/min-flow of helium. The oven temperature was ramped from 35°C to 260°C with an increase of 5°C per minute and the transfer line was set at 270°C. Mixed alkane standard solutions for RI calibration were included in the sample list to ensure stable retention times and three SPME conditions were applied to obtain good peak shapes[63].

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 MetaboliteDetector software, version 3.020151231 Ra-Linux[64]. The mass spectrum deconvolution settings were: peak threshold of 4, minimum peak height of 4, deconvolution width (scans) of 5, 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, www.nist.gov) and with an in-house library of authentic reference standards. Compound annotation was achieved imposing a

relative deviation of RI value lower than 2%[63] and according to the highest mass spectrum similarity score, which was set to more than 70% after first successful annotation, in order to include low-abundance substances or substances where the deconvolution process did not lead to a complete elimination of interfering mass signals[63]. The in-house library was obtained with authentic reference standards in duplicate using the instrument and parameters reported above. VOCs with an average signal-to-noise ratio (S/N) lower than 10 (used as the limit of quantification[65]) were checked manually and only included in the data matrix if their abundance was significantly higher than 10 times S/N for at least one time point or genotype. To assess the technical precision of each experiment, the relative standard deviation of peak areas was calculated for every compound detected in the QC sample (RSD = 100*standard deviation / average of peak areas) and compounds with a RSD greater than 30% were discarded[66]. For each of the two experiments, five replicates (plants) were analysed per genotype and time point.

Standard solutions and pure VOCs. Alkane standard solutions from C_8 to C_{20} (40 mg/L each in hexane) and C_{21} to C_{40} (40 mg/L each in toluene) were purchased from Sigma-Aldrich. 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. [63].

Pure VOCs were selected according to the SPME/GC-MS results, such as Benzenethanol, β-caryophyllene, trans-2-pentenal, 2-ethylfuran and β-cyclocitral (Sigma-Aldrich); cadinene (a mixture of ɣ-cadinene and δ-cadinene; (BOC Sciences); β-selinene and ledol (Xiamen Freede Industry). Pure VOCs were used in functional assays and for identity confirmation with HS-SPME/GC-MS analysis (Supplementary Fig. S2)

Effects of pure VOCs against downy mildew. Leaves (from the fourth-sixth node) of Pinot noir plants were sterilised as described by Palmieri, et al. [67]. Leaf disks (18 mm diameter) were placed onto wet sterilised filter paper in Petri dishes, with the abaxial surface uppermost. Each pure VOC was diluted ten-fold in DMSO (Sigma-Aldrich) and serially diluted in distilled water to obtain the appropriate concentration for each treatment.

To assess the effects of pure VOCs against *P. viticola* in water suspension, each leaf disk was inoculated with five 5 µL-drops of a *P. viticola* suspension (2.5×10^5 sporangia/mL), mixed with 0 (control), 0.01, 0.1, 1.0 and 10.0 g/L of the respective pure VOC (VOC-treated), calculated assuming the complete VOC dissolution in the water suspension. Dishes were incubated in the dark at 24 ± 1° C overnight, then dried under a laminar hood and incubated for six days under greenhouse conditions as described by Palmieri, et al. [67].

To assess the effects of pure VOCs on *P. viticola* in air volume, the respective pure VOC (0, 0.05, 0.5, 2.0 and 5.0 mg) was applied to a filter paper disk on the dish lid (without physical contact with the leaf tissue) as previously described[6,68], corresponding to a concentration of 0 (control), 0.5, 5.0, 20 and 50 mg/L in air volume (VOC-treated) calculated assuming the complete VOC evaporation from the filter paper. Dishes were sealed with Parafilm (Beims) and incubated in the dark at 24 ± 1° C for 24 h. Each leaf disk was inoculated with five 5 µL-drops of a *P. viticola* suspension (2.5×10^5 sporangia/mL), the respective pure VOC was applied again to the filter paper disk in the appropriate concentration. Dishes were sealed with Parafilm and incubated in the dark at 24 ± 1° C overnight. Leaf disks were dried under a laminar hood and incubated for six days under greenhouse conditions.

Disease severity was assessed at 6 dpi as a percentage of the leaf disk surface covered by sporulation[69], calculated as the sum of the five inoculum drops. Each inoculum drop was scored as: 0%, no sporulation; 10%, scarce sporulation; 20%, dense sporulation. Five replicates (dishes) were assessed for each treatment and the experiments (i.e. in water suspension and air volume) were carried out twice.

Inoculated disks were collected at 1, 2 and 6 dpi and stained with aniline blue as reported by Lenzi, et al. [70] by incubation in 1 M KOH at 95°C for 15 min and staining with 0.05% aniline blue (Sigma-Aldrich) in 0.067 M K_2HPO_4 at pH 8 for 15 min Leaf disks (18 mm diameter) were observed under a LMD7000 microscope (Leica Microsystems) using an A4 filter (320–400 nm excitation, 400 nm dichroic mirror and 470 nm emission). Three leaf disks were analysed for each treatment and time point, and the experiment was carried out twice.

Effects of VOCs on *Plasmopara viticola* sporangia. Sporulated leaves of Pinot noir plants were collected, leaf disks (18 mm diameter) were cut out and placed onto wet sterilised filter paper in Petri dishes, with the abaxial surface uppermost. The respective pure VOC was applied to a filter paper disk placed on the dish lid (without physical contact with the leaf tissue) at a concentration of 0 (control), 2.5 and 20 mg/L in air volume (VOC-treated), dishes were sealed with Parafilm and incubated at 24 ± 1°C overnight. Sporangia were collected by washing five disks for each replicate in 2 mL of cold distilled water. Sporangia length and width were measured with a LMD7000 microscope (Leica Microsystems). One hundred sporangia were measured for each replicate (dish of five disks each), five replicates were assessed for each treatment and the experiment was carried out twice.

In order to assess sporangia vitality, each sporangia suspension (adjusted to 2.5×10^5 sporangia/mL) was used to inoculate Pinot noir leaf disks as described above. Nine replicates (dishes with five disks each) were assessed for each treatment and the experiment was carried out twice.

Statistical analysis. Each experiment was carried out twice and data on the degree of downy mildew resistance, disease severity and sporangia dimension were analysed using the Statistica 13.1 software (Dell). Each experimental repetition was analysed singularly 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 then used to detect significant differences among treatments ($p \leq 0.05$).

VOC abundance was processed using an in-house R-script (R version 3.1.0). Data were inspected for outliers using the Dean-Dixon outlier test[71]. The Kruskal-Wallis test ($p \leq 0.05$) and a fold change of VOC abundance greater than 1.5 were set to classify VOCs with significant changes in abundance in three pairwise comparisons: i) between each resistant genotype and Pinot noir before inoculation (R *vs.* PN 0 dpi) or ii) six days post inoculation with *P. viticola* (R *vs.* PN 6 dpi) and iii) between 6 and 0 dpi for each genotype (6 *vs*. 0 dpi).

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Author contributions

M.P. and R.S. conceived the study, coordinated the experiments and wrote the manuscript. V.L. performed the experiments, analysed the data and wrote the manuscript. C.B. wrote the R scripts and analysed the data. A.P. contributed to chemical analysis and authentic reference standard measurements. I.P. supervised the experiments and revised the manuscript. M.P. and R.S. contributed equally to the coordination of this work. All authors have read and approved the paper before submission.

Competing interests: All authors declare no competing interests.

Figures

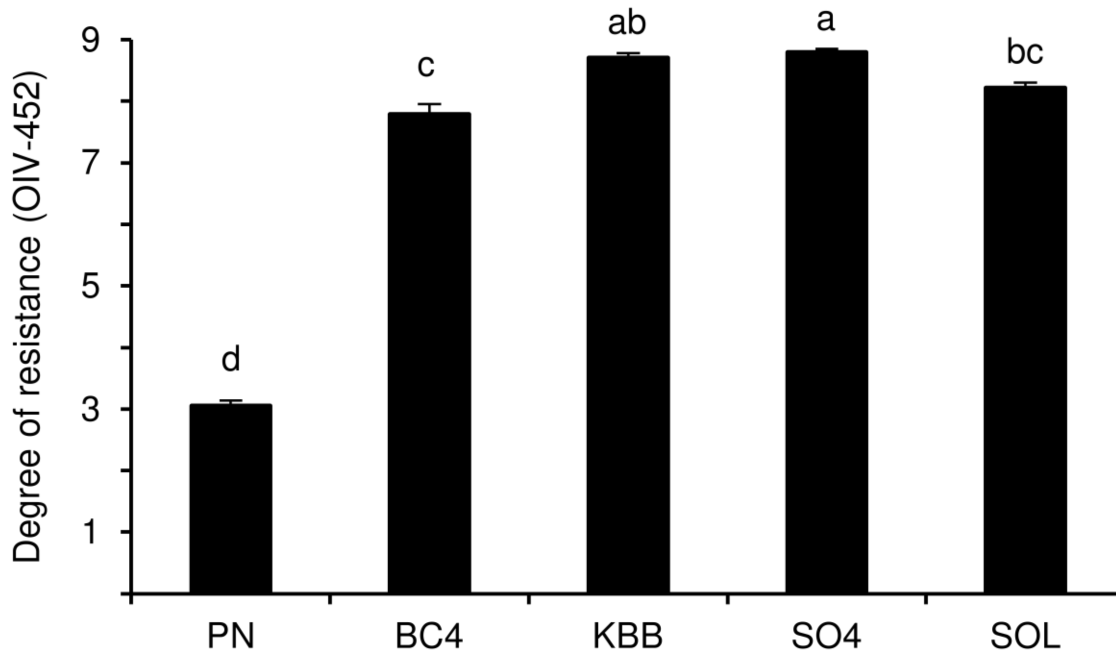


Figure 1. Degree of resistance of grapevine plants to downy mildew. Susceptible (Pinot noir; PN) and resistant grapevine plants [BC4, Kober 5BB (KBB), SO4, and Solaris (SOL)] were inoculated with *Plasmopara viticola* and the degree of resistance was assessed at seven days post inoculation according to the OIV-452 scores. Classes were assigned from the most susceptible (class 1) to the totally resistant (class 9) phenotype, according to the occurrence of sporangiophores and necrotic spots[60]. As Kruskal-Wallis test indicated no significant differences between two experiments ($p > 0.05$, n = 5 replicates per experiment), data from the two experiments were pooled. The pooled mean and standard error values of ten replicates (plants) are reported for each genotype. Different letters indicate significant differences among genotypes according to the Kruskal-Wallis test ($p \leq 0.05$).

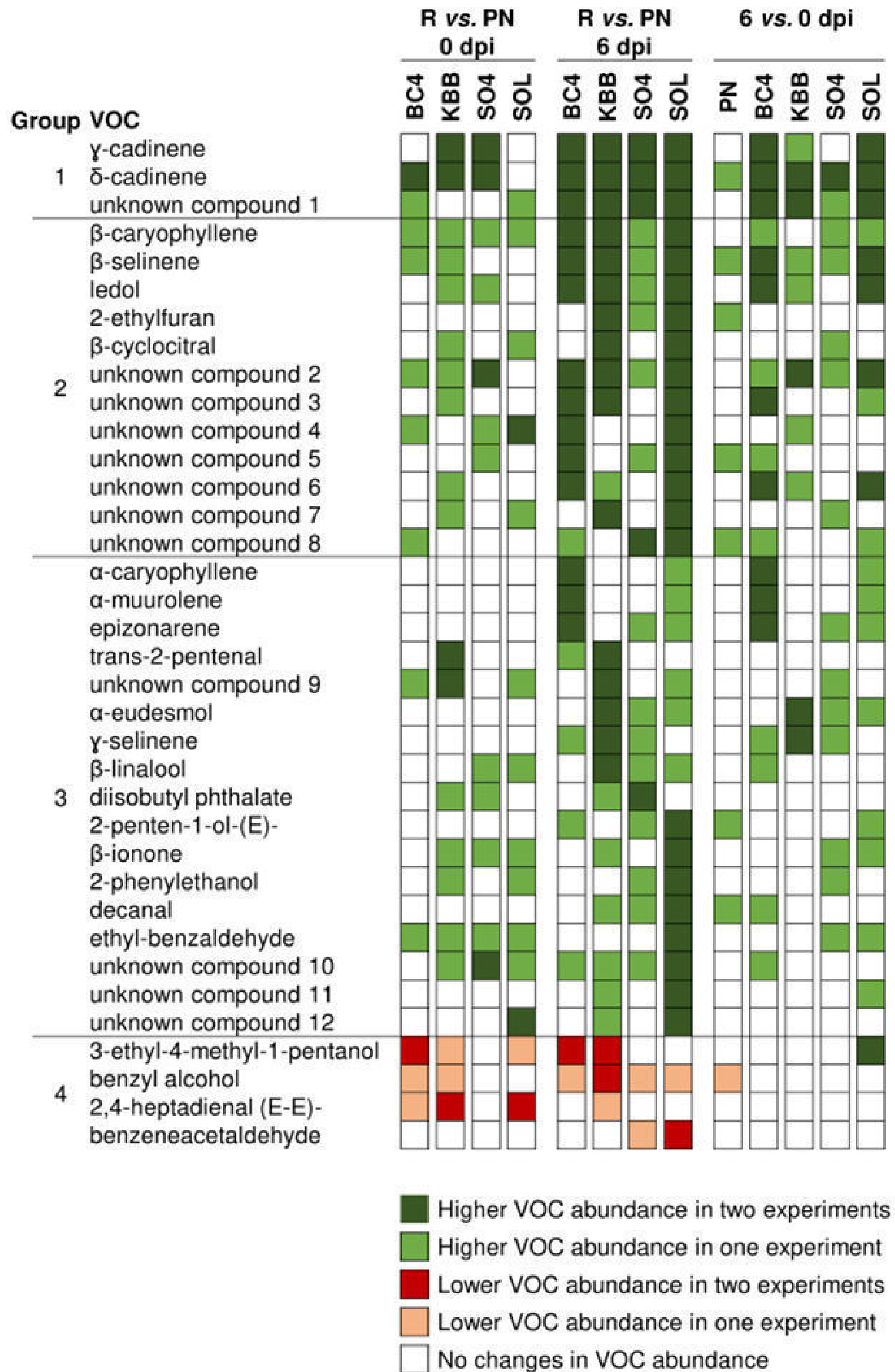


Figure 2. Profiles of volatile organic compounds (VOCs) of grapevine leaves. Susceptible (Pinot noir; PN) and resistant grapevine plants [BC4, Kober 5BB (KBB), SO4, and Solaris (SOL)] were inoculated with *Plasmopara viticola* and VOCs were detected before inoculation (0 dpi) and six days post inoculation (6 dpi) with *P. viticola* in two greenhouse experiments

(Supplementary Tables S1 and S2). Three pairwise comparisons were carried out between VOC abundance in each resistant genotype and Pinot noir at 0 dpi (R *vs.* PN 0 dpi) or at 6 dpi (R *vs.* PN 6 dpi) and between 6 and 0 dpi for each genotype (6 *vs.* 0 dpi). Green and red cells indicate significantly higher and lower VOC abundance (Kruskal-Wallis test $p \leq 0.05$ and fold change > 1.5) in two (dark colour) or one (light colour) experiment, respectively. Metabolite groups were identified according to the VOC profiles: higher abundance in all resistant genotypes in both experiments at 6 dpi (Group 1), higher abundance in two or more resistant genotypes in both experiments at 6 dpi (Group 2), VOCs with a higher abundance in only one resistant genotype in both experiments at 6 dpi (Group 3), VOCs with a lower abundance in at least one resistant genotype in both experiments (Group 4) as compared with Pinot noir.

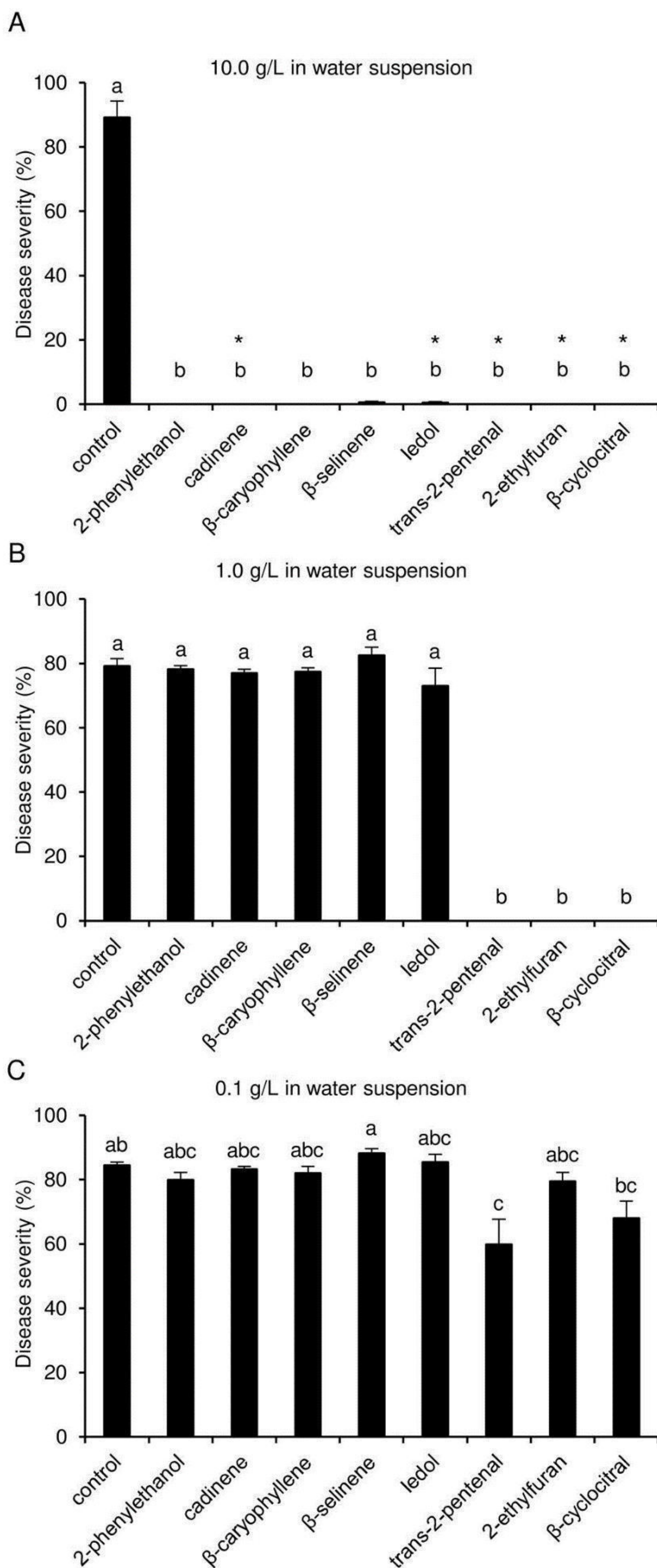


Figure 3. Effects of pure volatile organic compounds (VOCs) on downy mildew in water suspension. Leaf disks were inoculated with a *Plasmopara viticola* suspension without VOCs (control) or with 10.0 (**A**), 1.0 (**B**) and 0.1 (**C**) g/L of pure VOCs in water suspension (corresponding to 0.5, 0.05 and 5×10^{-3} mg/L in air volume, respectively). Five replicates (dishes with five disks each) were assessed for each treatment and the experiment was carried out twice. As the Kruskal-Wallis test indicated no significant differences between the two experiments ($p > 0.05$, n = 5 replicates per experiment), data from the two experiments were pooled. The pooled mean and standard error values of ten replicates are presented for each treatment. For each chart, different letters indicate significant differences among treatments according to the Kruskal-Wallis test ($p \leq 0.05$). Asterisks indicate phytotoxic effects on leaf disks.

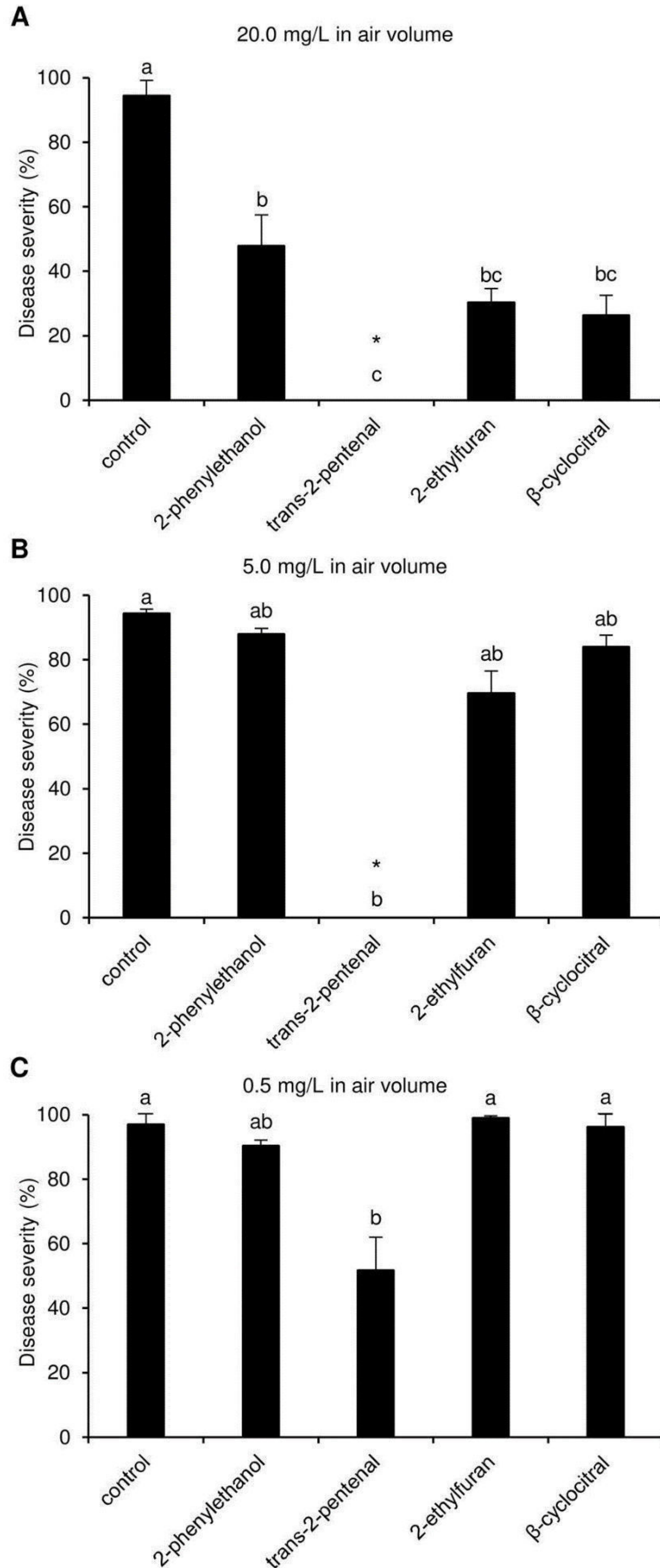


Figure 4. Effects of pure volatile organic compounds (VOCs) on downy mildew in air volume. Leaf disks were treated with water (control) or a pure VOC at concentrations of 20.0 mg/L (**A**), 5.0 (**B**) and 0.5 (black) mg/L in air volume, on a filter paper disk without contact with leaf tissues. Five replicates (dishes with five disks each) were assessed for each treatment and the experiment was carried out twice. As the Kruskal-Wallis test indicated no significant differences between the two experiments ($p > 0.05$, n = 5 replicates per experiment), data from the two experiments were pooled. The pooled mean and standard error values of ten replicates from the two experiments are presented for each treatment. For each chart, different letters indicate significant differences among treatments according to the Kruskal-Wallis test ($p \leq 0.05$). Cadinene, β-caryophyllene, β-selinene and ledol (20.0 mg/L in air volume) did not affect downy mildew severity as compared with the control disks (Kruskal-Wallis test $p > 0.05$) and severity data are therefore not shown here. Asterisks indicate phytotoxic effects on leaf disks.

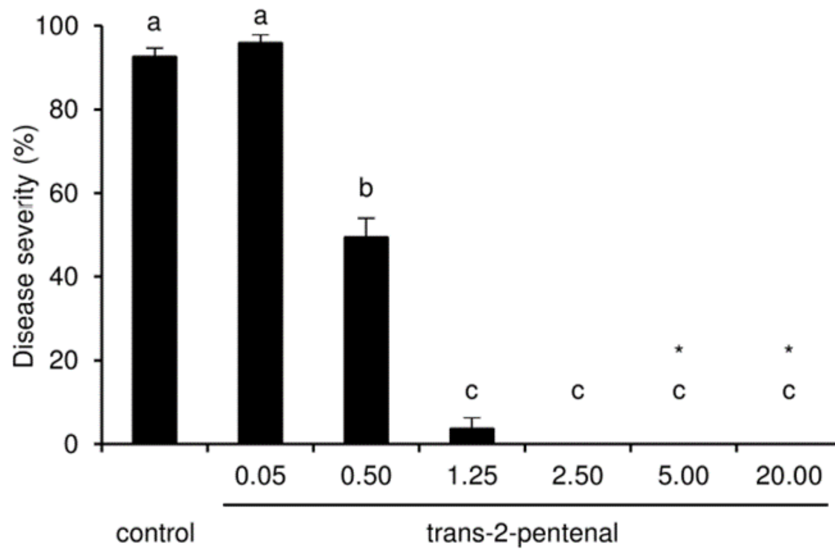


Figure 5. Effects of different concentrations of trans-2-pentenal on downy mildew in air volume. Leaf disks were treated with water (control) or trans-2-pentenal at different concentrations expressed in mg/L of air volume. Trans-2-pentenal was applied on a filter paper disk without contact with leaf tissues. Five replicates (dishes with five disks each) were assessed for each concentration and the experiment was carried out twice. As Kruskal-Wallis test indicated no significant differences between two experiments ($p > 0.05$, n = 5 replicates per experiment), data from the two experiments were pooled. The pooled mean and standard error values of ten replicates from the two experiments are presented for each treatment. Letters indicate significant differences among concentrations according to the Kruskal-Wallis test ($p \leq 0.05$). Asterisks indicate phytotoxic effects on leaf disks.

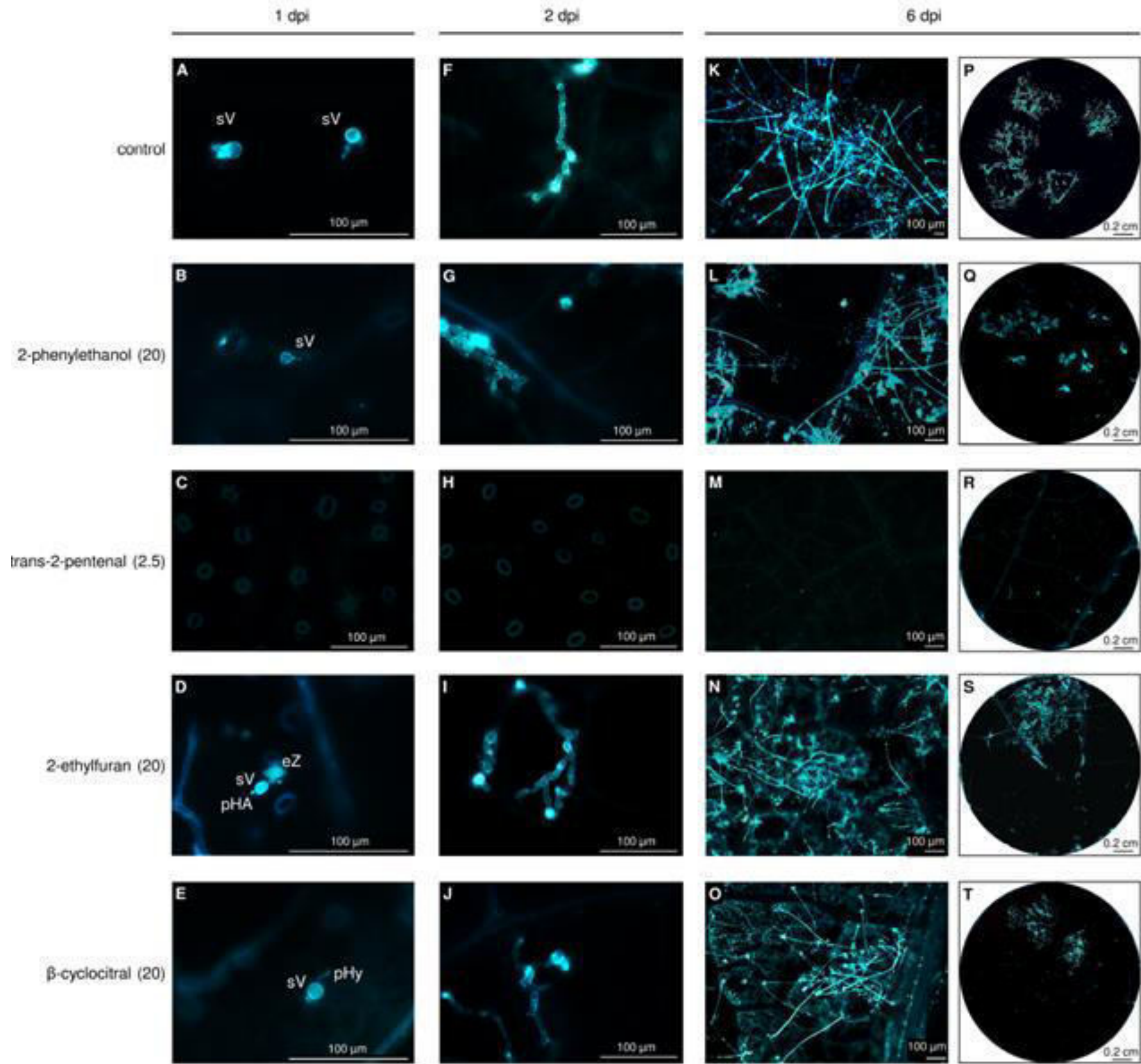


Figure 6. Effects of pure volatile organic compounds (VOCs) on downy mildew development. Leaf disks were treated with water (control), 2.5 mg/L (trans-2-pentenal) or 20 mg/L in air volume (2-phenylethanol, 2-ethylfuran or β-cyclocitral) on a filter paper disk without contact with leaf tissues. Disks were inoculated with *Plasmopara viticola* and the respective pure VOC was applied again to the filter paper disk. Pathogen development was monitored at one (**A-E**), two (**F-J**) and six (**K-T**) days post inoculation (dpi) using aniline blue staining. A representative leaf disk of ten is shown for each treatment and the experiment was carried out twice. Abbreviations: eZ, encysted zoospore; pHA, primary haustorium, pHy, primary hyphae; sV, substomatal vescicle. VOC concentrations, expressed as mg/L in air volume, are shown in brackets.

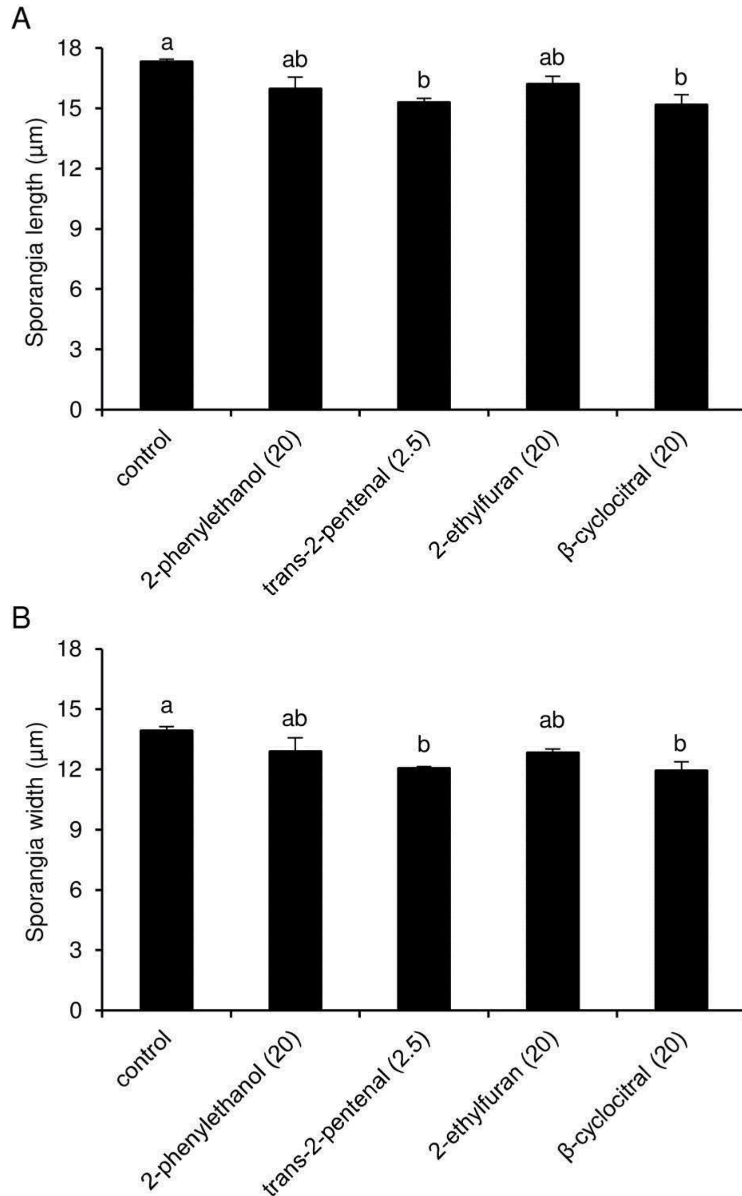


Figure 7. Effects of pure volatile organic compounds (VOCs) on *Plasmopara viticola* sporangia. Sporulated leaf disks were treated with water (control), 2.5 mg/L (trans-2-pentenal) or 20 mg/L in air volume (2-phenylethanol, 2-ethylfuran or β-cyclocitral) on a filter paper disk without contact with leaf tissues. Dishes were incubated overnight, after which *P. viticola* sporangia length (**A**) and width (**B**) were assessed. One hundred sporangia were measured for each replicate, five replicates (dishes) were assessed for each treatment and the experiment was carried out twice. As the Kruskal-Wallis test indicated no significant differences between the two experiments ($p > 0.05$), data from the two experiments were pooled. The pooled mean and standard error values of ten replicates are presented for each treatment. For each chart, different letters indicate significant differences according to the Kruskal-Wallis test ($p \leq 0.05$). VOC concentrations, expressed as mg/L in air volume, are shown in brackets.

Figure S1. Overview of the experimental design. Leaf samples of the susceptible *Vitis vinifera* cultivar Pinot noir and four resistant *Vitis* spp. hybrids (BC4, Kober 5BB, SO4 and Solaris) were collected immediately before inoculation (0 dpi) and six days post inoculation (6 dpi) with *Plasmopara viticola*. Ground leaves were subjected to headspace-solid-phase microextraction gas chromatography-mass spectrometry analysis (HS-SPME/GC-MS) and two independent experimental repetitions were analysed to annotate/identify volatile organic compounds (VOCs). VOCs were selected according to their different levels in resistant and susceptible genotypes after pathogen inoculation and they were tested as single pure compounds in the functional assays. Two protocols were tested to asses the effect of pure VOCs against *P. viticola* i) in water suspension and ii) in air volume without direct contact with the leaf tissue.

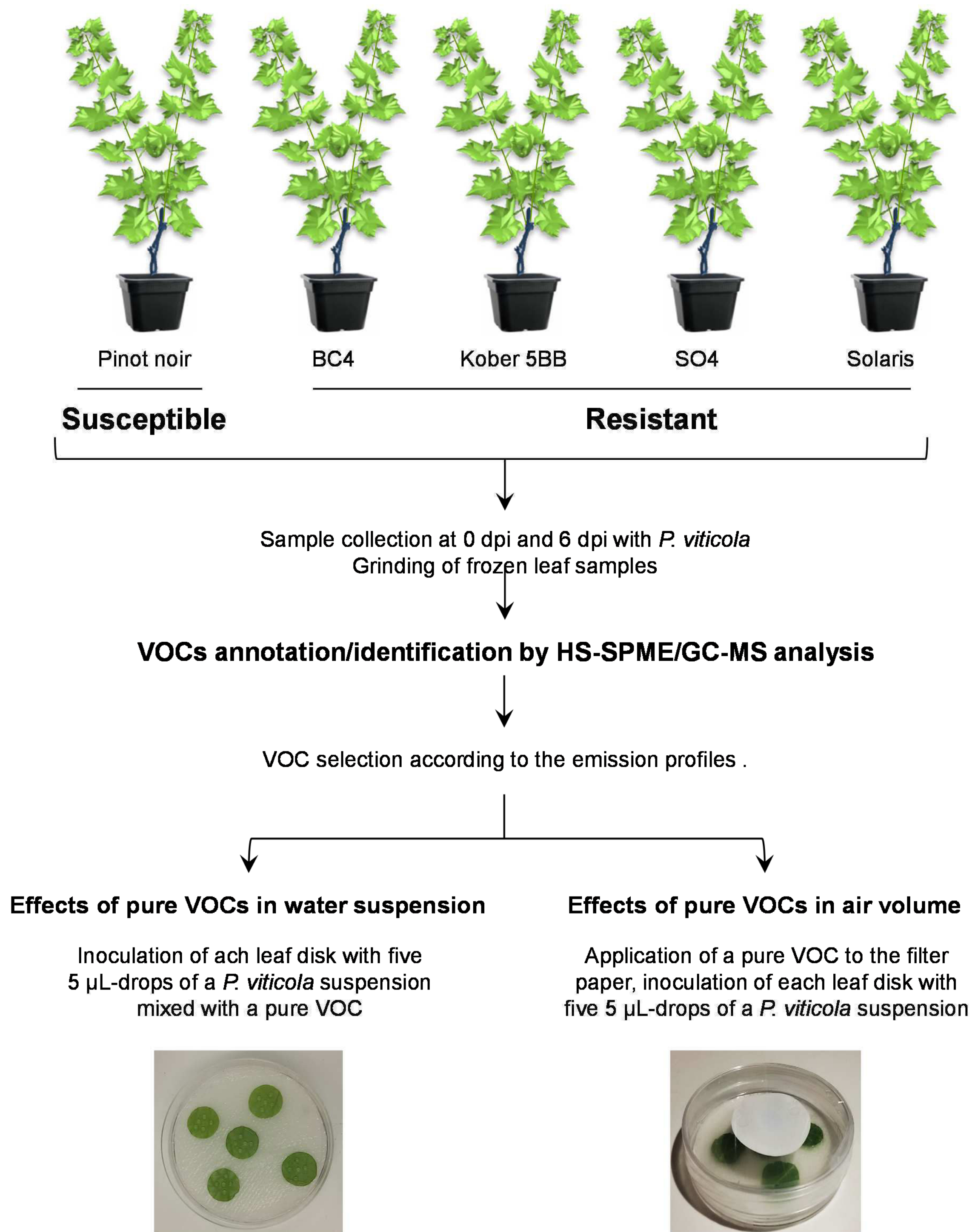
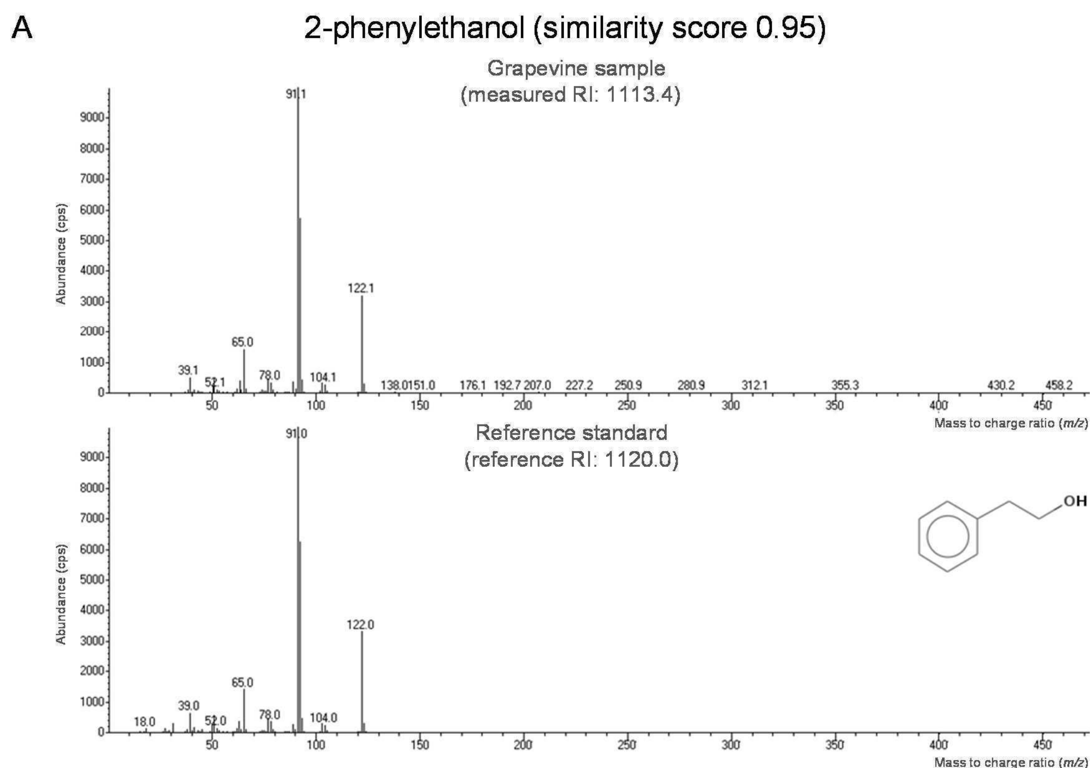
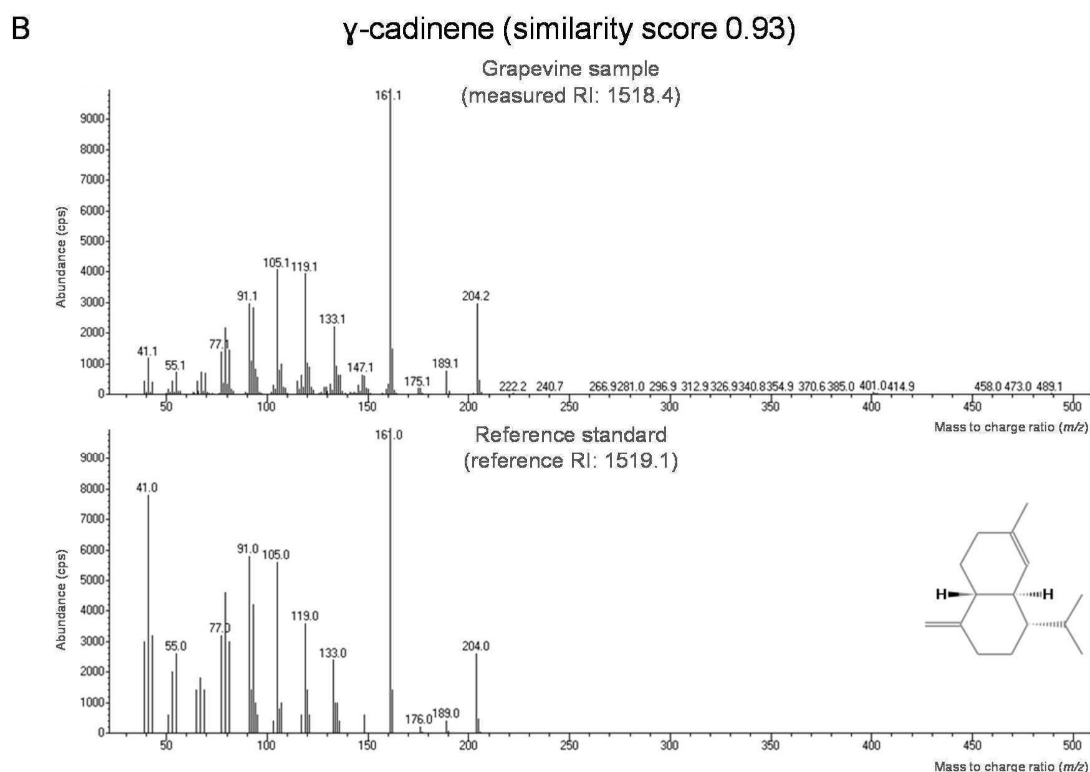
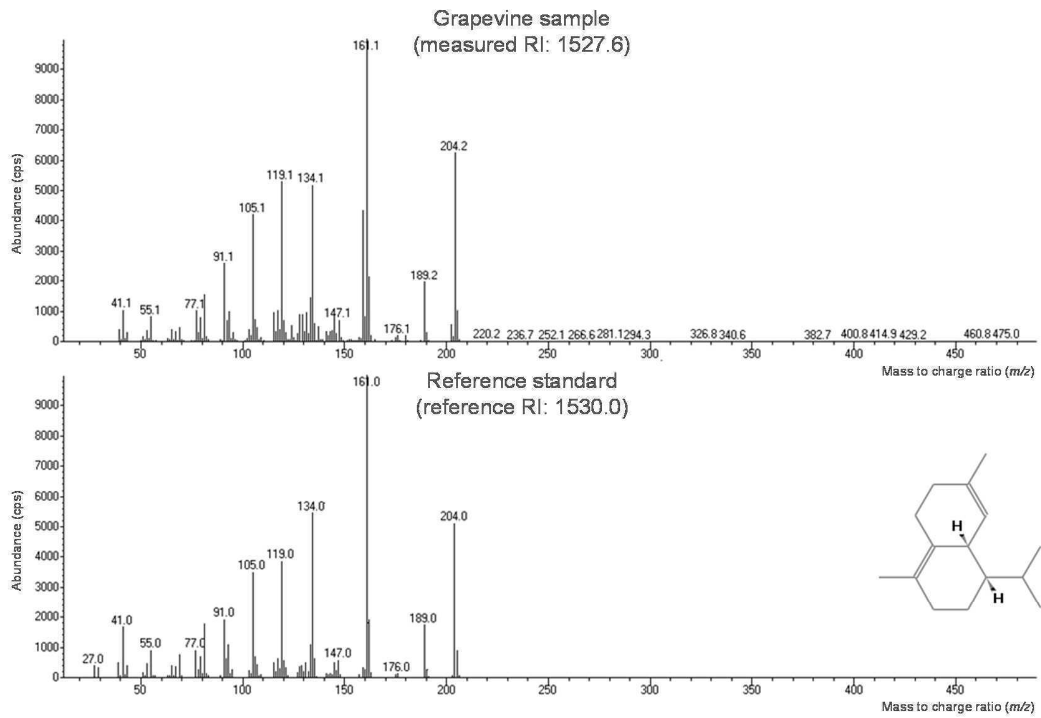


Figure S2. Comparison of the measured mass spectra of the volatile organic compounds (VOCs) in grapevine leaf samples with that of the corresponding pure VOC: 2-phenylethanol (A), ɣ-cadinene (B), δ-cadinene (C), β-caryophyllene (D), trans-2-pentenal (E), 2-ethylfuran (F), and β-cyclocitral (G). The mass spectrum similarity score and retention index values are reported for each VOC.

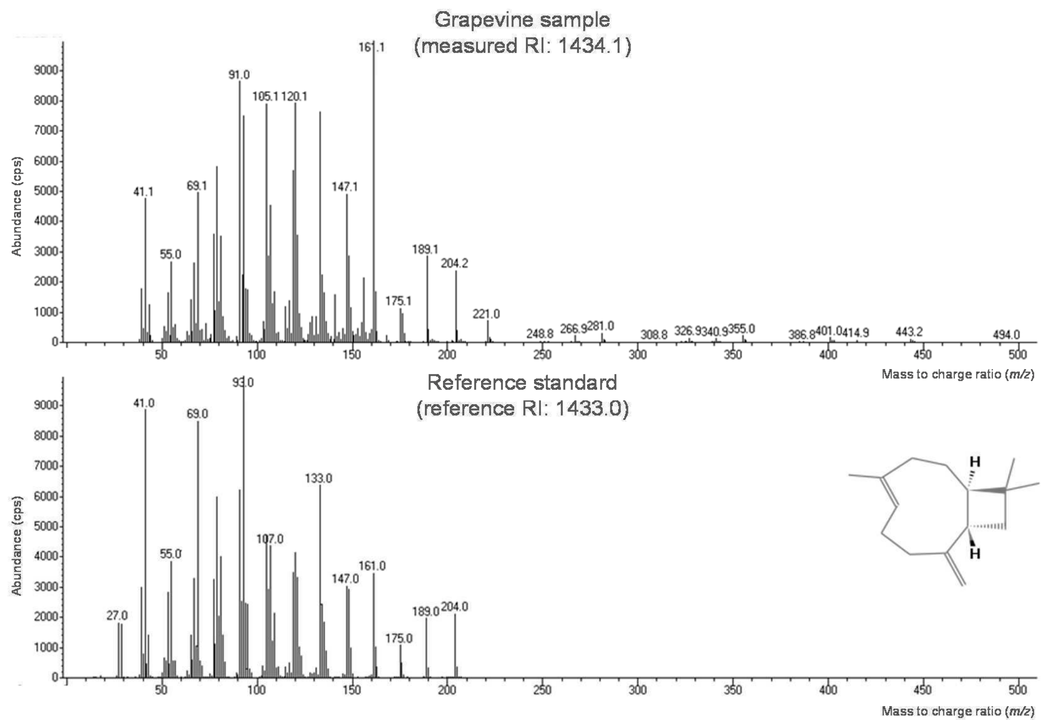




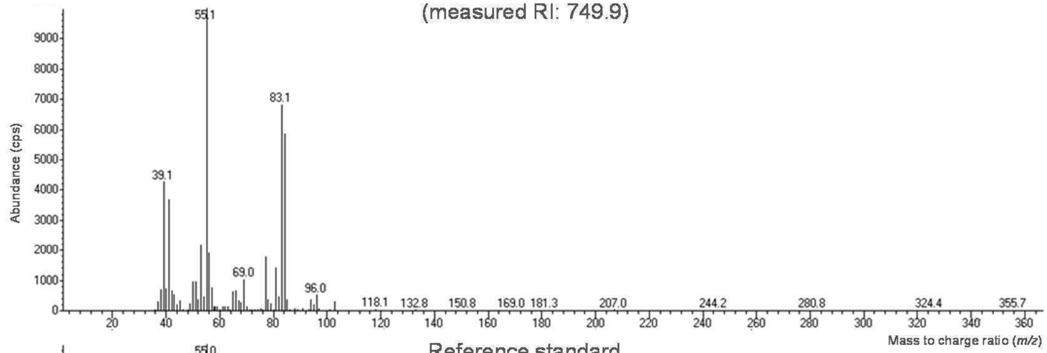
C δ-cadinene (similarity score 0.98)

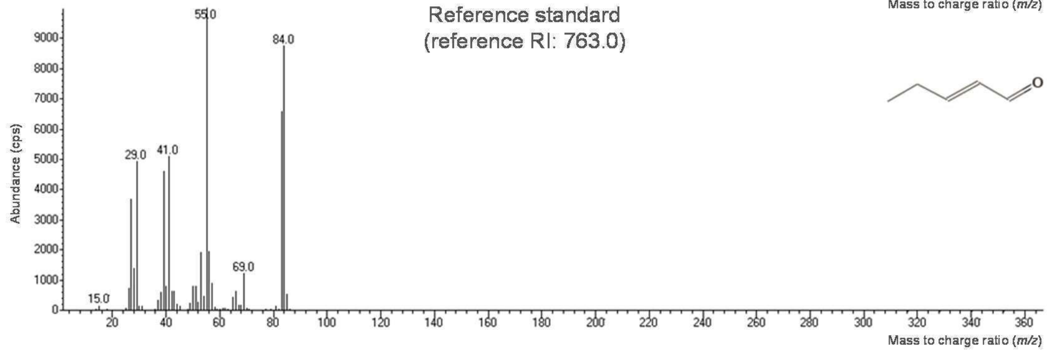


D β-caryophyllene (similarity score 0.94)

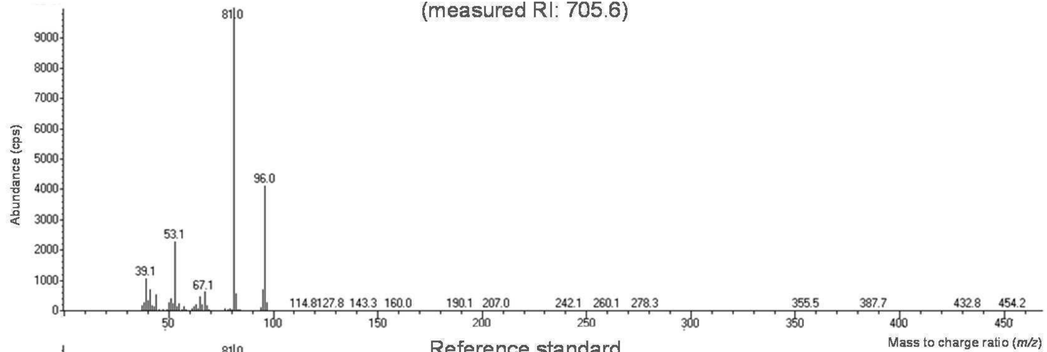


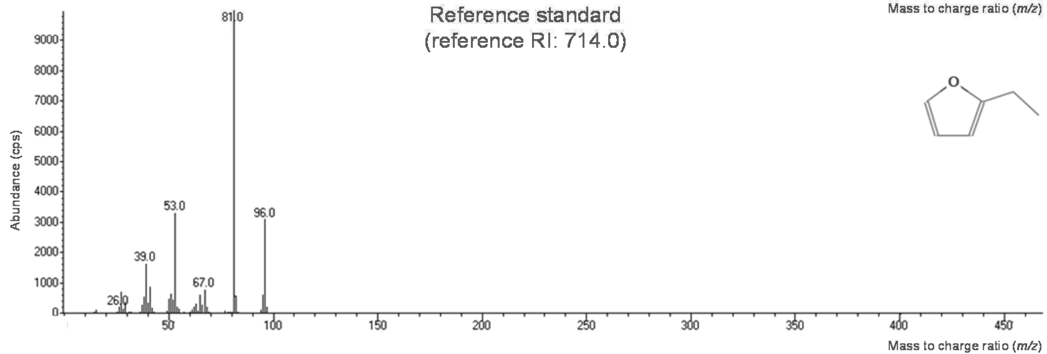
E trans-2-pentenal (similarity score 0.81)



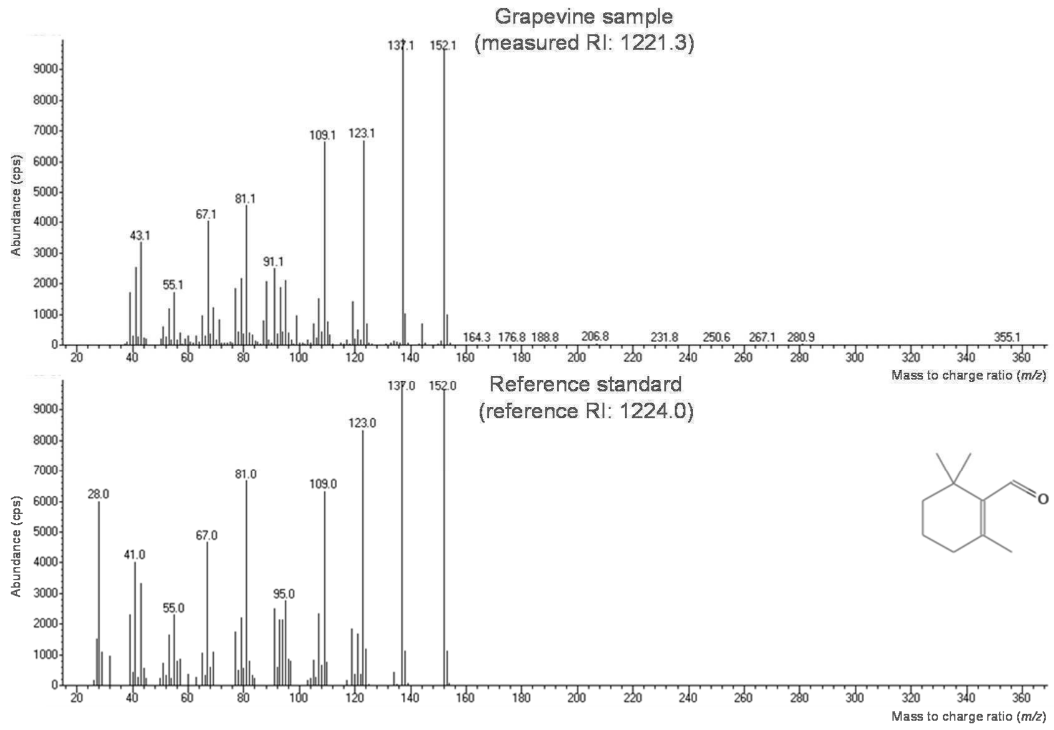


F 2-ethylfuran (similarity score 0.94)





G β-cyclocitral (similarity score 0.94)



Supplementary Tables and Table Legends

Table S1. Volatile organic compounds (VOCs) detected by headspace-solid phase microextraction-gas chromatography-mass spectrometry from five grapevine genotypes in the first experiment.

Table S2. Volatile organic compounds (VOCs) detected by headspace-solid phase microextraction-gas chromatography-mass spectrometry from five grapevine genotypes in the in the second experiment.

Leaf samples were collected from susceptible [Pinot noir (PN)] and resistant [BC4, Kober 5BB (KBB), SO4, Solaris (SOL)] grapevine genotypes before inoculation (0 dpi) and six days post inoculation (6 dpi) with *Plasmopara viticola* and volatile organic compounds (VOCs) were measured using a headspace-solid phase microextraction-gas chromatography-mass spectrometry analysis (HS-SPME-GC-MS). Two independent repetitions of the experiment were carried out (namely first and second experiment).

Column A. VOCs were grouped in six metabolite groups according to their profiles in: VOCs with a higher abundance in all resistant genotypes as compared with Pinot noir in both experiments in at least one time point (Group 1); VOCs with a higher abundance in two or more resistant genotypes as compared with Pinot noir in both experiments in at least one time point (Group 2), VOCs with a higher abundance in only one resistant genotype as compared with Pinot noir in both experiments in at least one time point (Group 3); VOCs with a lower abundance in at least one resistant genotype as compared with Pinot noir in both experiments in at least one time point (Group 4); VOCs with different abundance profiles in the two experiments (Group 5); VOCs only found in the first or in the second experiment (Group 6).

Column B. Names of VOCs found in grapevine leaves using a HS-SPME-GC-MS analysis. Green cells represent VOCs with increased abundance consistent in the two experiments. Orange cells represent VOCs with decreased abundance consistent in the two experiments. White cells represent VOCs with increased or decreased abundance in one of the two experiments.

Column C. CAS Registry Numbers. Source: http://webbook.nist.gov/chemistry/
Column D. Measured retention index (Measured RI).
Column E. Retention index measured from an in-house library of authentic reference standards (Reference RI).
Column F. Measured retention time (Measured RT).

Columns G, M, W, AG, AQ. Mean of absolute peak area (abundance) expressed as counts per seconds (cps) of five biological replicates (plants) at 0 dpi.
Columns H, N, X, AH, AR. Standard error of absolute peak area (abundance) expressed as cps of five biological replicates at 0 dpi.

Columns I, O, Y, AI, AS. Mean of absolute peak area (abundance) expressed as cps of five biological replicates at 6 dpi.
Columns J, P, Z, AJ, AT. Standard error of absolute peak area (abundance) expressed as cps of five biological replicates at 6 dpi.

Columns K, Q, AA, AK, AU. Fold change (FC) values between 0 and 6 dpi for each genotype. Values are reported for significant changes ($p \leq 0.05$ of Kruskal-Wallis test and FC fold change > 1.5). Coloured cells represent consistent statistical differences in the two experiments (green and orange for VOC with increased or decreased peak area, respectively).

Columns L, R, AB, AL, AV. Asterisks indicated significant differences between 0 and 6 dpi for each genotype according to a Kruskal-Wallis test ($p \leq 0.05$) with a fold change of VOC abundances greater than 1.5. Coloured cells represent consistent statistical differences in the two experiments (green and orange for VOC with increased or decreased peak area, respectively).

Columns S, AC, AM, AW. Fold change (FC) values between of each resistant genotype against Pinot noir at 0 dpi. Values are reported for significant changes ($p \leq 0.05$ of Kruskal-Wallis test and FC fold change > 1.5). Coloured cells represent consistent statistical differences in the two experiments (green and orange for VOC with increased or decreased peak area, respectively).

Columns T, AD, AN, AX. Asterisks indicated significant differences of each resistant genotype against Pinot noir at 0 dpi according to a Kruskal-Wallis test ($p \leq 0.05$) with a fold change of VOC abundances greater than 1.5. Coloured cells (green and orange for VOC with increased or decreased peak area, respectively) represent consistent statistical differences in the two experiments.

Columns U, AE, AO, AY. Fold change (FC) values between of each resistant genotype against Pinot noir at 6 dpi. Values are reported for significant changes ($p \leq 0.05$ of Kruskal-Wallis test and FC fold change > 1.5). Coloured cells represent consistent statistical differences in the two experiments (green and orange for VOC with increased or decreased peak area, respectively).

Columns V, AF, AP, AZ. Asterisks indicated significant differences of each resistant genotype against Pinot noir at 6 dpi according to a Kruskal-Wallis test ($p \leq 0.05$) with a fold change of VOC abundances greater than 1.5. Coloured cells represent consistent statistical differences in the two experiments (green and orange for VOC with increased or decreased peak area, respectively).

Supplementary Table S3. Deconvoluted mass spectra of unknown compounds. Deconvoluted spectra were automatically generated using MetaboliteDetector software and the 20 most abundant ions are reported for each compound. Exceptions are unknown compounds 6 and 13, which exhibited less than 20 ions in their deconvoluted spectra. The intensity of the base peak (the most intense peak of the mass spectrum) was set to 100% and the intensities of the other most abundant ions of each deconvoluted spectrum are expressed relative to the base peak ion.

Publication 3 – Growth media affect the volatilome and antimicrobial activity against *Phytophthora infestans* in four *Lysobacter* type strains

Valentina Lazazzara*, Michele Perazzolli*, Ilaria Pertot, Franco Biasioli, Gerardo Puopolo, Luca Cappellin

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*Valentina Lazazzara and Michele Perazzolli contributed equally to this work

MOTIVATION OF THE STUDY

Lysobacter spp. are key determinants of soil suppressiveness against phytopathogens and their production of non-volatile antimicrobial metabolites has been extensively demonstrated. However, the chemical composition and antagonistic properties of *Lysobacter* VOCs have only been poorly investigated until now. The VOC profiles of four *Lysobacter* type strains was studied by PTR-ToF-MS and HS-SPME/GC-MS. Our results revealed that growth media significantly affect the chemical profile and the functional properties of the four *Lysobacter* type strains against *P. infestans* growth. Particularly, four *Lysobacter* spp. VOCs (2,5-dimethyl pyrazine, 2-methoxy-3-methyl pyrazine, decanal and pyrrole) showed strong inhibitory activity against *P. infestans.*

CONTRIBUTION OF THE PRESENTING AUTHOR

I participated in preparing all the bacterial samples and I performed the dual-culture experiments. Moreover, I contributed to major parts of the manuscript.

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Growth media affect the volatilome and antimicrobial activity against *Phytophthora infestans* in four *Lysobacter* type strains

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ABSTRACT

Bacterial volatile organic compounds (VOCs) play important ecological roles in soil microbial interactions. *Lysobacter* spp. are key determinants of soil suppressiveness against phytopathogens and the production of non-volatile antimicrobial metabolites has been extensively characterised. However, the chemical composition and antagonistic properties of the *Lysobacter* volatilome have been poorly investigated. In this work, VOC emission profiles of four *Lysobacter* type strains grown on a sugar-rich and a protein-rich medium were analysed using solid-phase microextraction gas chromatography–mass spectrometry and proton transfer reaction-time of flight-mass spectrometry. *Lysobacter antibioticus*, *L. capsici*, *L. enzymogenes* and *L. gummosus* type strains were recognised according to their volatilome assessed using both headspace mass spectrometry methods Moreover, the chemical profiles and functional properties of the *Lysobacter* volatilome differed according to the growth medium, and a protein-rich substrate maximised the toxic effect of the four *Lysobacter* type strains against *Phytophthora infestans*. Antagonistic (pyrazines, pyrrole and decanal) and non-antagonistic (delta-hexalactone and ethanol) VOCs against *Ph. infestans* or putative plant growth stimulator compounds (acetoin and indole) were mainly emitted by *Lysobacter* type strains grown on protein- and sugar-rich media respectively. Thus nutrient availability under soil conditions could affect the aggressiveness of *Lysobacter* spp. and possibly optimise interactions of these bacterial species with the other soil inhabitants.

1. Introduction

Microorganisms produce a wide variety of secondary metabolites, including antibiotics, toxins, pigments and volatile organic compounds (VOCs). Volatile organic compounds are molecules of high vapour pressure and low molecular weight that readily diffuse through water- and gas-filled pores in soil environments (Schmidt et al., 2015). VOCs emitted by bacteria belong to different chemical classes (alcohols, aldehydes, alkenes, benzenoids, ethers, lactones, ketones, terpenoids and sulphur compounds) and are generated by complex metabolic pathways (Audrain et al., 2015; Schmidt et al., 2015; Schulz and Jeroen, 2007). Bacterial VOCs play essential ecological roles in communications with soil microorganisms, nematodes, insects and plants (Effmert et al., 2012; Kai et al., 2009). Notably, bacterial VOCs can inhibit spore germination and mycelial growth of several phytopathogens (De Vrieze et al., 2015; Kai et al., 2007; Weisskopf, 2013), promote plant growth (Blom et al., 2011; Ryu et al., 2003) and induce plant resistance (Lee et al., 2012; Ryu et al., 2004). The chemical composition of the bacterial volatilome is defined by genetic determinants and can be used as a chemotaxonomic marker in standardised conditions (Peñuelas et al., 2014). However, composition and functional properties of the bacterial bouquet are influenced by the nutrient source where bacteria are grown (Asari et al., 2016; Blom et al., 2011; Bruce et al., 2003; Fiddaman and Rossall, 1994; Garbeva et al., 2014; Weise et al., 2012), indicating metabolic changes in VOC production according to nutrient availability and growth conditions in the soil (Insam and Seewald, 2010).

Bacteria belonging to the *Lysobacter* genus are frequently found in

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soil and increased disease suppression of soil phytopathogens correlated significantly with increased populations of *L. antibioticus*, *L. capsici* and *L. gummosus* (Postma and Schilder, 2015; Postma et al., 2008). The *Lysobacter* genus (Christensen and Cook, 1978) includes species that are efficient antagonists of phytopathogens and potential candidates for biological control of crop diseases (Hayward et al., 2010; Kobayashi and Yuen, 2007). In particular, *L. antibioticus* DSM 2044^T (ATCC 29479), *L. enzymogenes* DSM 2043^T (ATCC 29487) and *L. gummosus* DSM 6980^T (ATCC 29489) were described as *Lysobacter* type strains by Christensen and Cook (1978), and the antagonistic mechanisms of these species have been extensively characterised (Folman et al., 2003, 2004; Ko et al., 2009; Qian et al., 2009; Yu et al., 2007). For example, *L. antibioticus* HS124 produced lytic enzymes and a toxic compound against *Phytophthora capsici* (Ko et al., 2009). Likewise, the production of lytic enzymes and antibiotics was shown for *L. enzymogenes* 3.1T8 (Folman et al., 2003, 2004), *L. enzymogenes* C3 (Yu et al., 2007) and *L. enzymogenes* OH11 (Qian et al., 2009) against *Fusarium graminearum*, *Pythium aphanidermatum*, *Py. ultimum*, *Ph. capsici, Rhizoctonia solani* and *Sclerotinia sclerotiorum*. The antagonistic properties of *L. gummosus* were associated with proteolytic degradation of biofilm (Gökçen et al., 2014) and biosynthesis of antifungal metabolites (Meyers et al., 1985). Furthermore, the type strains *L. capsici* DSM 19286^T (YC5194) (Park et al., 2008) and *L. capsici* AZ78 (Puopolo et al., 2014a,b, 2016) produced secondary metabolites that inhibit the growth of phytopathogenic fungi (*Botrytis cinerea*, *Colletotrichum gloeosporioides*, *F. oxysporum* and *R. solani*) and oomycetes (*Ph. infestans*, *Plasmopara viticola* and *Py. ultimum*) respectively.

Although the production of extracellular lytic enzymes (proteases, glucanases, chitinases and cellulases) and antimicrobial compounds (pyrazines, tetramic acid-containing macrolactams and other antifungal factors) has been widely characterised in *Lysobacter* spp. (Puopolo et al., 2014a; Xie et al., 2012), the possible contribution of VOCs in antagonistic processes has been poorly investigated. The limited studies available (Sang et al., 2011; Zou et al., 2007) suggest a significant potential for VOC-mediated antagonistic processes. In particular, the VOCs emitted by *L. gummosus* KCTC 12132 and *L. enzymogenes* ISE13 inhibited mycelial growth of nematicidal fungi (*Paecilomyces lilacinus* and *Pochonia chlamydosporia*) (Zou et al., 2007) and phytopathogenic microorganisms (*C. acutatum* and *Ph. capsici*) (Sang et al., 2011) respectively.

The aim of this study was to elucidate the antagonistic potential of *Lysobacter* spp., based on a better understanding of the emission profiles and functional properties of VOCs. Therefore, we used four *Lysobacter* type strains (*L. antibioticus* DSM 2044^T, *L. capsici* DSM 19286^T, *L. enzymogenes* DSM 2043^T and *L. gummosus* DSM 6980^T) as representative of the biocontrol *Lysobacter* spp. (Hayward et al., 2010; Kobayashi and Yuen, 2007; Postma and Schilder, 2015; Postma et al., 2008) and we assessed both volatilome composition and antagonistic effects against *Ph. infestans*, the causal agent of late blight of potato and tomato plants (Fry, 2008). The VOCs produced by the type strains on two growth media were analysed by solid-phase microextraction gas chromatography–mass spectrometry (SPME/GC–MS) and proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS) to precisely analyse the chemical composition and rapidly monitor the emission profiles, respectively (Jordan et al., 2009b).

2. Materials and methods

2.1. Propagation of the Lysobacter type strains and the plant pathogenic oomycete

The *Lysobacter* type strains *L. antibioticus* DSM 2044^T, *L. capsici* DSM 19286^T, *L. enzymogenes* DSM 2043^T and *L. gummosus* DSM 6980^T were grown on Luria-Bertani Agar (LBA, Sigma-Aldrich, St. Louis, MO, USA) for 72 h at 27 °C and cell suspensions of each strain were prepared by flooding LBA dishes with 5 ml of sterile isotonic solution (0.85% NaCl). Bacterial cells were scraped from the medium surface with a sterile spatula and collected in a sterile 15 ml-tube. The resulting cell suspensions were centrifuged (4300 × *g* for 15 min); pelleted cells were suspended in sterile isotonic solution to a final optical density of 0.1 at 600 nm (OD_{600}), corresponding to 1 × 10^8 cells/ml (Puopolo et al., 2016).

The *Ph. infestans* isolate (kindly provided by M. Finckh and A. Butz, University of Kassel, Germany) was grown on pea agar medium (PAM, 12.5% frozen peas and 1.2% agar in distilled water) at 17 °C, as described by Puopolo et al. (2015).

2.2. Bacterial growth conditions for headspace analysis of volatile organic compounds

For headspace VOC analysis, 5 ml of sterilised nutrient agar (NA, Oxoid, Basingstoke, United Kingdom) or potato dextrose agar (PDA, Oxoid) were poured into sterile 20 ml headspace vials (HS vials, Supelco, Sigma-Aldrich) and they were left open under a laminar flow for 2 h at room temperature to avoid condensation. Each HS vial was then inoculated with 20 µl of the cell suspension of a *Lysobacter* type strain (1 × 10^8 cells/ml) and left to dry under a laminar flow for 1 h at room temperature. Each HS vial was tightly sealed with a sterilised 18 mm screw metal cap assembled with silicone/PTFE septa of 1.3 mm (Supelco, Sigma-Aldrich). Additional HS vials containing non-inoculated NA or PDA (Uninoculated) were used as controls to exclude VOCs released from the culture medium in the absence of bacteria (Kluger et al., 2013). HS vials were incubated at 25 °C for ten days to accumulate VOCs before the headspace VOC assessment by SPME/GC–MS and PTR-ToF-MS analysis. This time point was selected because it showed the greatest antagonism of the *Lysobacter* type strains against *P. infestans*.

The number of *Lysobacter* cells developed in each inoculated HS vial was assessed one day after headspace VOC analysis (11 days after inoculation). Each HS vial was flooded with 4 ml of sterilised isotonic solution (0.85% NaCl) and bacterial cells were scraped from the medium surface by vigorous vortexing for 30 s. The cell concentration of the resulting suspension was assessed by converting the OD_{600} values, with OD_{600} = 0.1 corresponding to 1 × 10^8 cells/ml (Puopolo et al., 2016), and the quantity of *Lysobacter* cells was then calculated for each HS vial.

2.3. Headspace analysis of volatile organic compounds using solid-phase micro extraction gas chromatography–mass spectrometry (SPME/GC–MS) analysis

Headspace VOC analysis was carried out with SPME/GC–MS using an Auto System XL gas chromatograph coupled with a Turbo Mass Gold Mass spectrometer (Perkin Elmer, Norwalk, CT, USA). For measurement automatisation and standardisation, the instrument was coupled with a thermostated autosampler (CTC CombiPAL, CTC Analytics, Zwingen, Switzerland) and HS vials were kept at 25 °C. After equilibration for 30 min, VOCs were extracted and pre-concentrated with solid phase microextraction (SPME) using 2 cm PDMS/DVB/CAR fibre (Supelco, Bellafonte, PA, USA), according to Endrizzi et al. (2012). The fibre collected VOCs from the headspace for 30 min and desorbed them into the GC injector for 5 min at 250 °C. The chromatographic separation was performed via an HP-Innowax fused-silica capillary column (length 30 m, inner diameter 0.32 mm, film thickness 0.5 µm; Agilent Technologies, Palo Alto, CA, USA). The GC oven temperature programme was the following: 40 °C for 3 min, raised from 40 °C to 220 °C at 4 °C/min, 220 °C for 1 min, increased from 220 °C to 250 °C at 10 °C/min and 250 °C for 1 min. The carrier gas was helium with a constant column flow rate of 1.5 ml/min. The transfer line temperature was maintained constant at 220 °C. Upon exiting the column, compounds were ionised via electron impact at 70 eV and detected with a quadrupole mass spectrometer with a mass/charge ratio (*m/z*) ranging from 30 to 300

Thomson. Spectra analysis was carried out using TurboMass 5.4.0 software (Perkin Elmer, Norwalk, CT; USA). Mass measure parameters were: background subtraction with a polynomial order of 1 and a below curve of 33%, smooth mode with a peak width of 0.75 Da, minimum peak width at half height of 4. Compound annotation was achieved by comparing the spectra with the NIST-98/Wiley library (National Institute of Standards and Technology, www.nist.gov) using a mass spectrum similarity greater than 85%, and by matching retention indices (RI) of authentic reference standards computed under the same chromatographic conditions with the C7-C30 *n*-alkane series (Supelco, Sigma-Aldrich) using a maximum tolerance of 4% RI deviation. The VOC content of each sample was reported as the absolute peak area obtained with the TurboMass 5.4.0 software. Five replicates (HS vials) of *L. enzymogenes* and four replicates of *L. antibioticus*, *L. capsici* and *L. gummosus* were used for each media.

2.4. Headspace analysis of volatile organic compounds using proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS)

Rapid headspace VOC analysis was carried out using a commercial PTR-TOF 8000 instrument (Ionicon Analytik GmbH, Innsbruck, Austria) (Jordan et al., 2009a). The instrument was coupled with an adapted thermostated autosampler (MPS Multipurpose Sampler, Gerstel) and HS vials were kept at 25 °C. During VOC headspace measurement, 40 sccm of zero air were injected into the HS vial through a needle heated to 40 °C, and the outflow going through a second heated needle (40 °C) was delivered via Teflon fittings to the PTR-ToF-MS. Zero air was produced via a catalytic VOC scrubber (GCU unit, Ionicon Analytik, Innsbruck, Austria). HS vials were measured in random order and each measurement lasted for 3 min, with a waiting time of 5 min between samples to avoid memory effects. The PTR-ToF-MS was operated in H_3O^+ primary ion mode. The following conditions were set in the instrument drift tube: 2.3 mbar drift pressure, 480 V drift voltage, 110 °C drift tube temperature, leading to an E/N value (E corresponding to electric field strength and N to gas number density) of about 120 Townsend (Td; 1 Td = 10^{-17} Vcm^2). The primary and product ions exiting the drift tube region were detected using a time-of-flight (ToF) mass spectrometer operated with its standard configuration (V mode). Each acquisition consisted of 350,000 channels with a sampling time of 0.1 ns per channel of ToF acquisition, resulting in a mass spectrum ranging up to $m/z = 400$. Each individual spectrum was the sum of about 28,600 acquisitions lasting for 35 µs, resulting in a time resolution of 1 s. Because the analysis time for each sample was set to 3 min, 180 spectra were acquired for each vial during each measurement.

PTR-ToF-MS spectra were processed according to the methodology reported by Cappellin et al. (2011a), with slight modifications. As the first data processing step, signal distortions related to detector dead time were calculated using a correction approach based on Poisson statistics, according to Cappellin et al. (2011b). Because the external calibration provided by the acquisition software did not achieve sufficient mass accuracy, internal mass calibration was carried out according to Cappellin et al. (2011b) and a mass accuracy of greater than 0.001 Th was obtained. Subsequent data processing of noise reduction, baseline removal and peak intensity extraction were carried out according to Cappellin et al. (2011b) using modified Gaussians to fit spectral peaks. Headspace VOC concentrations, expressed as parts per billion by volume (ppbv), were estimated from the integrated signal over the 3 min of spectra acquisition using the formula described by Lindinger et al. (1998), considering hydronium H_3O^+ as primary ion and a constant reaction rate coefficient of 2×10^{-9} cm^3/s in the calculations. This approach introduces a systematic deviation of up to 30% that can be accounted for if the actual rate coefficient is known (Cappellin et al., 2012b). Four replicates (HS vials) of *L. enzymogenes* and five of *L. antibioticus*, *L. capsici* and *L. gummosus* were grown on PDA. Five replicates of *L. capsici* and four of *L. antibioticus*, *L. enzymogenes* and *L. gummosus* were grown on NA.

2.5. Functional analysis of bacterial volatile organic compounds against Phytophthora infestans

Split Petri dishes (92 mm of diameter) with two compartments and ventilation cams (Sarstedt, Nümbrecht, Germany) were used to analyse the effect of VOCs emitted by *Lysobacter* type strains on *Ph. infestans* growth. Sterilised NA or PDA (5 ml) were poured into one half of the split dish (*Lysobacter*-growth side) and 5 ml of sterilised PAM were poured into the other half (*Phytophthora*-growth side). Once dried, 50 µl of the cell suspension of the *Lysobacter* type strain (1×10^8 cells/ml) were spread onto the *Lysobacter*-growth side of the split dish containing NA or PDA using sterile spatulas. As a control, split dishes containing non-inoculated NA or PDA (Uninoculated) on the *Lysobacter*-growth side were used. Dishes were sealed with Parafilm tape (Beims, Neenah, WI, USA) and incubated at 25 °C in the dark for 72 h. Subsequently, *Ph. infestans* plugs (5 mm) were cut from the edge of ten-day-old colonies grown on PAM, as described by Puopolo et al. (2016), and a plug was placed at the centre of the *Phytophthora*-growth side of each split dish. Inoculated dishes were sealed with Parafilm tape and mycelial growth was evaluated by measuring the diameter (parallel to the edge of the dish) of the *Ph. infestans* colony after seven days of incubation in the dark at 20 °C, corresponding to ten days after *Lysobacter* spp. inoculation. Each *Ph. infestans* plug exposed to VOCs of *Lysobacter* type strains grown on NA or PDA was then transferred to fresh PAM dishes and the colony diameter was measured after seven days of incubation in the dark at 20 °C. Seven replicates (split dishes) were analysed for each *Lysobacter* type strain and each growth medium and the functional assay against *Ph. infestans* was carried out twice.

VOCs were selected according to their emission profiles; pure 2,5-dimethyl pyrazine, 2-methoxy-3-methyl pyrazine, decanal, delta-hexalactone, ethanol and pyrrole were purchased (Sigma-Aldrich) and tested against *Ph. infestans*. Sterilised PAM was poured into one half of a split dish (*Phytophthora*-growth side) and a pure VOC was applied to a filter paper disk placed into the other half (VOC side) at dosage of 20 mg per split dish corresponding to 190 mg/L (VOC-treated) of air volume, which is a dosage compatible for VOC-mediated functional assays (De Vrieze et al., 2015; Fernando et al., 2005). As control, distilled water was applied to a filter paper disk into the VOC side of control dishes. Each dish was sealed with Parafilm tape, incubated at 25 °C in the dark for 72 h and inoculated with a *Ph. infestans* plug (5 mm) into the *Phytophthora*-growth side. The diameter (parallel to the edge of the dish) of each *Ph. infestans* colony was measured after seven days of incubation in the dark at 20 °C and the inhibition of *Ph. infestans* growth (percentage) was calculated according to the following formula: (growth in control dishes — growth in VOC-treated dishes)/(growth in control dishes) × 100. Seven replicates (split dishes) were analysed for each treatment and the experiment was carried out twice.

2.6. Statistical analysis

To obtain background-corrected headspace VOC concentration, the background signal (the signal corresponding to the mean signal for empty HS vials) was subtracted from VOC emission values of both SPME/GC–MS and PTR-ToF-MS analysis. Emitted VOCs were identified as peaks with a background-corrected headspace concentration significantly greater than the corresponding signal for uninoculated HS vials for at least one strain and growth medium, according to the Kruskal-Wallis test with Bonferroni correction ($p \leq 0.05$).

Volatile emission data of SPME/GC–MS and PTR-ToF-MS analysis were analysed using in-house routines written in R (www.r-project.org), including the Agricolae package (https://cran.r-project.org/web/packages/agricolae/index.html) and Glmnet package (https://cran.r-project.org/web/packages/glmnet/index.html). Data exploration with principal component analysis (PCA) was carried out using in-house

routines written in R, on normalised variables that were obtained by subtracting the mean and dividing by the standard deviation, to obtain more homogeneous variables and prevent variance from being concentrated in few variables, affecting the results of PCA (Afifi et al., 2011).

Bacterial class prediction models based on VOC data were developed with both SPME/GC–MS and PTR-ToF-MS datasets, using the least absolute shrinkage and selection operator (LASSO) method described by Tibshirani (1996). Briefly, a linear model can be represented by the following equation: $Y = X \times B + E$, where Y is the matrix of the properties to be predicted (dependent variable), X is the matrix of the measurements to be employed in the prediction (independent variable), B is the matrix of regression coefficients to be estimated in the model optimisation procedure and E is the matrix of residuals. In the LASSO method, the model can be represented by the following equation: $Y = X \times B + E + \lambda \times |B|$, which includes a penalisation term ($\lambda \times |B|$) of the absolute values of coefficient B, multiplied by a factor λ, corresponding to the penalty coefficient to be optimised. During model optimisation, the size of the penalty coefficient λ needs to be optimised and cross-validation is used for this purpose (Tibshirani, 1996). Models for all possible λ values were calculated simultaneously as an ordinary linear regression, as reported by Hastie et al. (2009). The considered study was a multiclass problem (i.e. growth media and strain classes) and a LASSO model for each class was therefore developed to predict whether a sample belonged to the class (model value 1) or not (model value 0). Performance evaluation of the classification methods was carried out using a leave-one-out (LOO) procedure and confusion matrices (Cappellin et al., 2012a; Westerhuis et al., 2008).

VOC emission values, *Ph. infestans* colony diameters and *Lysobacter* cell numbers ($\log_{10}$-transformed) were analysed using Statistica 13.1 software (Dell, Round Rock, TX, USA) and a Kruskal-Wallis test with Bonferroni correction was applied to detect significant differences ($p \leq 0.05$) among *Lysobacter* type strains and growth media.

3. Results

3.1. Profiles of volatile organic compounds differed according to the Lysobacter type strains and growth media

VOC emission profiles measured by SPME/GC–MS analysis varied in *Lysobacter* type strains and growth media, with the first and sixth principal components (explaining 36.7 and 3.8% of variance respectively) of PCA analysis discriminating samples according to the growth medium (Fig. 1A). Moreover, marked similarities of VOC emission profiles occurred between HS vials belonging to the same *Lysobacter* type strain, independently of the growth medium (Fig. 1B). Specifically, the second and sixth principal components of PCA (explaining 18.6 and 3.8% of variance respectively) discriminated *Lysobacter* type strains, and HS vials of the same type strain grown on the two media clustered together.

A total of 77 VOCs were detected by the SPME/GC–MS analysis, and the emission profiles of 70 of them differed according to the growth medium and type strain (Fig. 2 and Table S1). No differences in bacterial growth were found between PDA and NA, except for *L. antibioticus* (Table 1), and VOC differences were mainly related to bacterial metabolism rather than to the growth rate. Specifically, the emission of 17 VOCs was higher for all *Lysobacter* type strains on PDA as compared with NA (PDA-specific VOCs, Cluster 1, Table S1), such as 3-methyl-2-buten-1-ol, 1-tridecanol, 1-tetradecanol, 1-pentadecanol, according to the Kruskal-Wallis test with Bonferroni correction ($p \leq 0.05$). PDA-specific profiles were also found for three ketones [delta-hexalactone (Fig. 3A), dihydro-5-pentyl-2(3H)-furanone and 2-hexadecanone], an organosulfur compound (methyl thiolacetate), a heterocyclic compound (indole; Fig. 3B) and five unknown compounds. Likewise, higher emission of methyl 2-methyl butanoate, 4-methyl-1-pentanol and (Z)-3-decen-1-ol was measured for all *Lysobacter* type strains grown on PDA as compared with NA. The emission profiles of PDA-specific VOCs differed for *Lysobacter* type strains, and the emission of 1-tridecanol, 1-tetradecanol, 1-pentadecanol, delta-hexalactone, 2-hexadecanone, methyl thiolacetate, five unknown compounds, 4-methyl-1-pentanol and (Z)-3-decen-1-ol by *L. enzymogenes* was higher as compared with the other type strains on PDA. Moreover, 3-methyl-2-buten-1-ol and indole were emitted mainly by *L. gummosus* and *L. capsici* respectively, and dihydro-5-pentyl-2(3H)-furanone was mainly

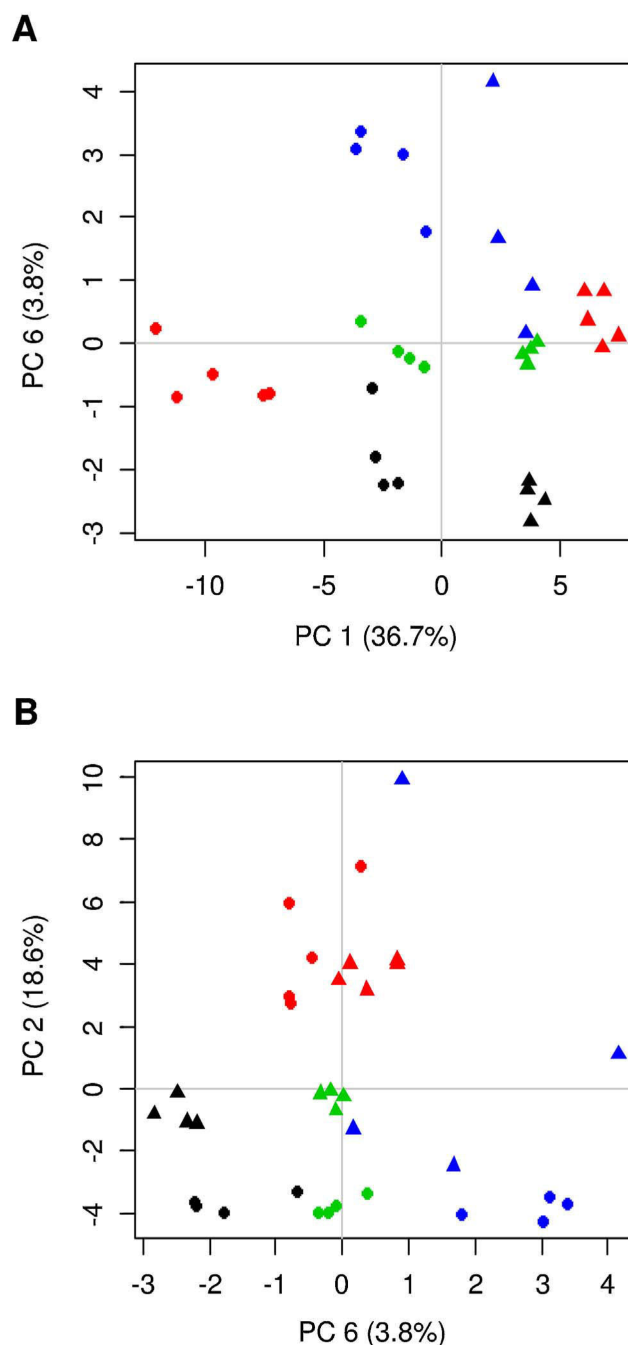

Fig. 1. Principal component analysis (PCA) of volatile organic compounds (VOCs) emitted by *Lysobacter* type strains. PCA was based on VOCs measured using solid-phase microextraction gas chromatography–mass spectrometry (SPME/GC–MS) for *Lysobacter antibioticus* DSM 2044^T (red), *L. capsici* DSM 19286^T (blue), *L. enzymogenes* DSM 2043^T (black) and *L. gummosus* DSM 6980^T (green) grown for ten days on nutrient agar (triangles) or potato dextrose agar (circles). The percentage of variance explained by the principal components (PC) is reported in brackets for PC1 and PC6 **(A)** or PC6 and PC2 **(B)**.

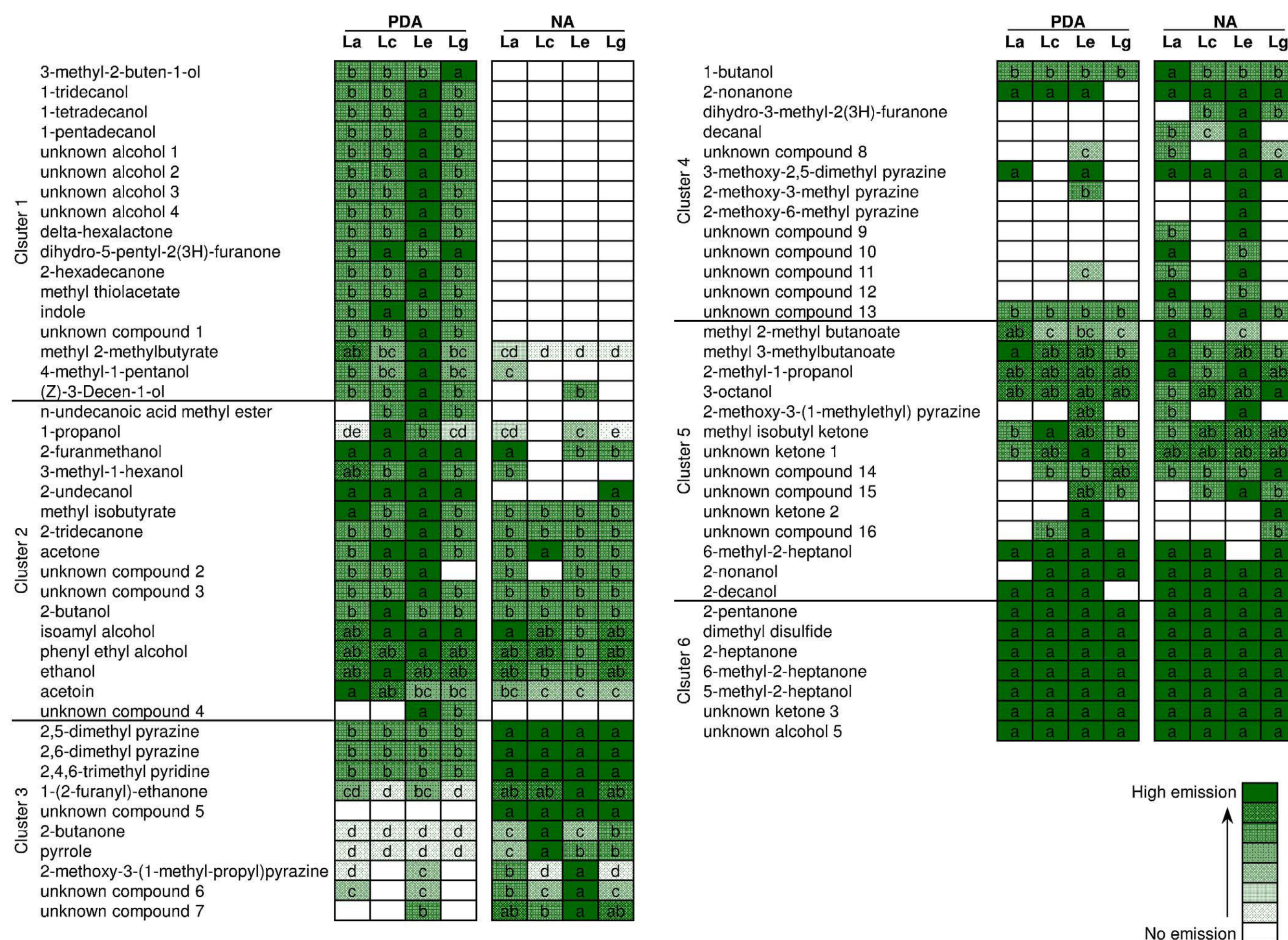

Fig. 2. Profiles of volatile organic compounds (VOCs) emitted by *Lysobacter* type strains. Headspace VOC analysis was carried out using solid-phase microextraction gas chromatography–mass spectrometry (SPME/GC–MS) for *Lysobacter antibioticus* DSM 2044^T (La), *L. capsici* DSM 19286^T (Lc), *L. enzymogenes* DSM 2043^T (Le) and *L. gummosus* DSM 6980^T (Lg) grown for ten days on nutrient agar (NA) or potato dextrose agar (PDA). For each compound, the intensity of the colour gradient and letters are based on a Kruskal-Wallis test with Bonferroni correction ($p \leq 0.05$) on VOC emission data (Table S1). Compounds were grouped based on their emission profiles into: VOCs with higher emission by all *Lysobacter* type strains on PDA as compared with NA (Cluster 1), VOCs with higher emission by some *Lysobacter* type strains on PDA as compared with NA (Cluster 2), VOCs with higher emission by all *Lysobacter* type strains on NA as compared with PDA (Cluster 3), VOCs with higher emission by some *Lysobacter* type strains on NA as compared with PDA (Cluster 4), VOCs with different (Cluster 5) or consistent (Cluster 6) emission by *Lysobacter* type strains on both growth media.

emitted by both *L. gummosus* and *L. capsici*.

The emission of 16 VOCs was higher for some *Lysobacter* type strains on PDA as compared with NA (Cluster 2, Table S1). For example, the emission of the *n*-undecanoic acid methyl ester was specific for three type strains grown on PDA (*L. capsici, L. enzymogenes* and *L. gummosus*) and it was not detected in other HS vials. These three type strains also showed higher emission of 1-propanol, 2-furanmethanol and 3-methyl-1-hexanol on PDA as compared with NA. The emission of 2-undecanol and methyl isobutyrate was higher on PDA as compared with NA for three type strains (*L. antibioticus*, *L. capsici* and *L. enzymogenes*) and two

Table 1
Number of *Lysobacter* type strain cells developed during volatile organic compound assessment.

VOC analysis[1]	Media[2]	*Lysobacter* type strain concentration[3]			
		L. antibioticus	*L. capsici*	*L. enzymogenes*	*L. gummosus*
SPME/GC–MS	PDA	$7.75 \pm 0.32 \times 10^9$ ab	$4.77 \pm 0.38 \times 10^9$ bc	$7.23 \pm 0.49 \times 10^9$ ab	$1.12 \pm 0.03 \times 10^{10}$ a
	NA	$7.72 \pm 0.42 \times 10^8$ c	$5.82 \pm 1.30 \times 10^9$ abc	$5.86 \pm 0.92 \times 10^9$ abc	$4.14 \pm 2.09 \times 10^9$ abc
PTR-ToF-MS	PDA	$6.80 \pm 0.30 \times 10^9$ ab	$4.87 \pm 0.66 \times 10^9$ bc	$6.79 \pm 0.18 \times 10^9$ ab	$1.06 \pm 0.08 \times 10^{10}$ a
	NA	$1.40 \pm 0.09 \times 10^9$ c	$5.02 \pm 0.74 \times 10^9$ abc	$5.09 \pm 0.24 \times 10^9$ abc	$4.01 \pm 1.45 \times 10^9$ abc

[1] Headspace analysis of the volatile organic compounds (VOCs) emitted by *Lysobacter antibioticus* DSM 2044^T, *L. capsici* DSM 19286^T, *L. enzymogenes* DSM 2043^T and *L. gummosus* DSM 6980^T was carried out using solid-phase microextraction gas chromatography–mass spectrometry (SPME/GC–MS) and proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS).

[2] The *Lysobacter* type strains were grown for ten days at 25 °C on potato dextrose agar (PDA) or nutrient agar (NA) in headspace vials before VOC analysis.

[3] Growth of the *Lysobacter* type strains was measured one day after VOC assessment (11 days after inoculation). Bacterial cells were collected from the headspace vials and the number of *Lysobacter* cells for each vial was calculated by measuring optical density at 600 nm [optical density of 0.1 corresponds to 1×10^8 cells/ml according to Puopolo et al. (2016)]. Mean and standard deviation values of *Lysobacter* cells from four to five replicates are presented for each type strain and growth media. For each headspace VOC analysis, different letters indicate significant differences according to a Kruskal-Wallis test with Bonferroni correction ($p \leq 0.05$).

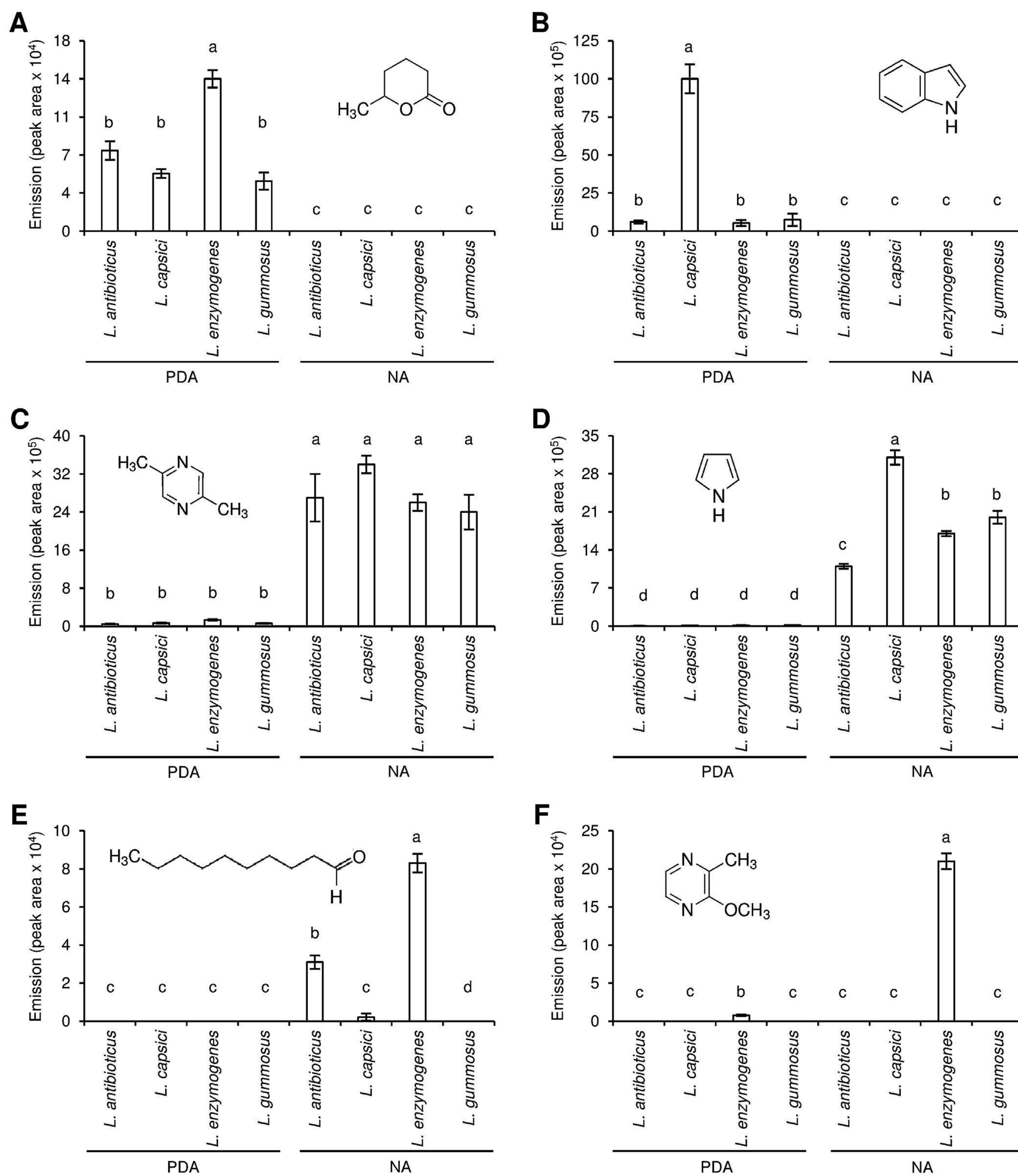

Fig. 3. Profiles of selected volatile organic compounds (VOCs) emitted by *Lysobacter* type strains. Emission of delta-hexalactone (**A**), indole (**B**), 2,5-dimethyl pyrazine (**C**), pyrrole (**D**), decanal (**E**) and 2-methoxy-3-methyl pyrazine (**F**) was measured using solid-phase microextraction gas chromatography–mass spectrometry (SPME/GC–MS) for *L. antibioticus* DSM 2044^T, *L. capsici* DSM 19286^T, *L. enzymogenes* DSM 2043^T and *L. gummosus* DSM 6980^T grown for ten days on nutrient agar (NA) or potato dextrose agar (PDA). For each compound, mean and standard error values of the absolute peak area from four to five replicates are reported for each *Lysobacter* type strain and growth medium. Different letters indicate significant differences according to the Kruskal-Wallis test with Bonferroni correction ($p \leq 0.05$). The structural formula is reported for each compound.

type strains (*L. antibioticus* and *L. enzymogenes*) respectively. On PDA, emission of 1-propanol by *L. capsici* and *L. enzymogenes* was higher as compared with *L. gummosus*, while that of methyl isobutyrate by *L. antibioticus* and *L. enzymogenes* was higher as compared with *L. capsici* and *L. gummosus*. Five compounds (2-tridecanone, acetone, 2-butanol and two unknown compounds) were emitted mainly by one PDA-grown *Lysobacter* type strain. Specifically, *L. enzymogenes* was characterised by the highest emission of 2-tridecanone and two unknown compounds,

while *L. capsici* was characterised by the most significant emission of 2-butanol as compared with the other PDA-grown strains. Moreover, higher emission of isoamyl alcohol and phenyl ethyl alcohol by *L. enzymogenes*, ethanol by *L. capsici* and acetoin by *L. antibioticus* and *L. capsici* was found on PDA as compared with NA.

All the *Lysobacter* type strains showed higher emission of ten VOCs on NA as compared with PDA (NA-specific VOCs, Cluster 3, Table S1), such as 2,5-dimethyl pyrazine (Fig. 3C), 2,6-dimethyl pyrazine, 2,4,6-trimethyl pyridine, 1-(2-furanyl)-ethanone and an unknown compound, with consistent emission profiles for all NA-grown *Lysobacter* type strains. NA-specific profiles were also found for 2-butanone, pyrrole (Fig. 3D), 2-methoxy-3-(1-methyl-propyl) pyrazine and two unknown compounds. Emission of these VOCs differed for NA-grown *Lysobacter* type strains: 2-butanone and pyrrole were mainly emitted by *L. capsici*, while 2-methoxy-3-(1-methyl-propyl) pyrazine and two unknown compounds were mainly emitted by *L. enzymogenes,* as compared with the other NA-grown type strains.

The emission of 15 VOCs was higher on NA as compared with PDA for some type strains (Cluster 4, Table S1), as in the case of 1-butanol and 2-nonanone emission by *L. antibioticus* and *L. gummosus* respectively. Dihydro-3-methyl-2(3H)-furanone, decanal (Fig. 3E) and an unknown compound were specifically emitted by more than two *Lysobacter* type strains grown on NA, with the highest emission by *L. enzymogenes*. Emission of 3-methoxy-2,5-dimethyl pyrazine by *L. capsici* and *L. gummosus* was higher on NA as compared with PDA, while its emission by *L. antibioticus* and *L. enzymogenes* was comparable on NA and PDA medium. Moreover, 2-methoxy-3-methyl pyrazine (Fig. 3F) and 2-methoxy-6-methyl pyrazine were emitted exclusively by *L. enzymogenes*, with lower and no emission by *L. enzymogenes* on PDA respectively. Likewise, four unknown compounds (named from 13 to 16) were mainly emitted by NA-grown *L. antibioticus* and *L. enzymogenes*.

The emission profiles differed for *Lysobacter* type strains on both media for 14 VOCs (Cluster 5, Table S1). For example, the emission of methyl 2-methyl butanoate and methyl 3-methyl butanoate was higher by *L. antibioticus* was higher as compared with the other type strains on both growth media. On NA, the emission of 2-methyl-1-propanol by *L. antibioticus* and *L. enzymogenes* was higher as compared with *L. capsici* and 3-octanol emission by *L. gummosus* was higher as compared with *L. antibioticus.* Moreover, 2-methoxy-3-(1-methylethyl) pyrazine was mainly emitted by *L. enzymogenes* on both media and to a lower extent by *L. antibioticus* on NA, while methyl isobutyl ketone was mainly emitted by *L. capsici* as compared with *L. antibioticus* and *L. gummosus* on PDA. Consistent emission of seven VOCs (2-pentanone, dimethyl disulfide, 2-heptanone, 6-methyl-2-heptanone, 5-methyl-2-heptanol, and two unknown compounds) was detected in *Lysobacter* type strains and growth media (Cluster 6, Table S1) and they were possibly produced by constitutive metabolic pathways.

3.2. Lysobacter type strains and their growth media can be recognised by modelling the profiles of volatile organic compounds

Differences in VOC emission among *Lysobacter* type strains grown on the two growth media were used to predict the medium on which the bacteria were grown. Optimisation of the LASSO model corresponded with a linear combination of the original variables (VOCs annotated by SPME/GC–MS analysis) with a coefficient of zero, except for the coefficient associated with the 2,4,6-trimethyl pyridine variable (Table S1). This result highlighted that a simple univariate model, built on the SPME/GC–MS emission data of 2,4,6-trimethyl pyridine alone, was sufficient to predict the growth substrate on the basis of only one bacterial VOC. The prediction performance was assessed with a LOO procedure and the success rate of growth media prediction using LASSO was 100%, meaning that the growth media could be predicted with high level of accuracy (Table 2).

PTR-ToF-MS data (Table S2) confirmed the marked differences in VOC emissions by *Lysobacter* type strains grown on PDA and NA. In agreement with the SPME/GC–MS analysis, the growth of *Lysobacter* type strains was comparable on PDA and NA (except for *L. antibioticus;* Table 1). LASSO modelling based on PTR-ToF-MS data resulted in prediction performance of the growth media comparable to that obtained with SPME/GC–MS data and cross-validation using a LOO procedure provided a prediction success rate of 100% (Table 2). The LASSO procedure was able to predict growth media on the basis of only two bacterial VOCs associated with peaks at *m/z* of 68.050 and 129.091 (Table S2). Although annotation of the compounds associated with PTR-ToF-MS spectral peaks is difficult (Cappellin et al., 2011a), the peak at *m/z* of 68.050 corresponds with the $C_4H_6N^+$ ion, which is consistent with a fragment ion of 2,4,6-trimethyl pyridine reaction with H_3O^+.

LASSO modelling was used for *Lysobacter* type strain prediction on the basis of VOC emission profiles. Since the medium prediction had a 100% success rate, the different growth media were modelled as separate classes and the success rate of strain prediction was 97 and 90% with LASSO modelling based on SPME/GC–MS and PTR-ToF-MS data respectively (Table 2). Specifically, HS vials belonging to *L. enzymogenes* and *L. antibioticus* grown on both PDA and NA were correctly classified by SPME/GC–MS analysis, as well as *L. capsici* and *L. gummosus* grown on PDA. HS vials of *L. gummosus* were confused only once with *L. capsici* when grown on NA. Likewise, all *Lysobacter* type strains grown on PDA were correctly classified according to their volatilome assessed with PTR-ToF-MS analysis. On NA, HS vials of *L. gummosus* were confused only once with *L. antibioticus* and those of *L. enzymogenes* were confused twice with *L. capsici.* The LASSO modelling based on SPME/GC–MS and PTR-ToF-MS data associated non-zero coefficients with 11 VOCs (Table S1) and 12 peaks (Table S2) to distinguish *Lysobacter* type strains respectively, indicating that a linear model built using only these compounds was sufficient to discriminate the bacterial strains tested. Specifically, the emission profiles of methyl 2-methyl butanoate and methyl 3-methyl butanoate were characteristic for *L. antibioticus* on PDA, while those of 2-butanol, methyl thiolacetate and 3-methyl-2-buten-1-ol specified the emission of *L. capsici*, *L. enzymogenes* and *L. gummosus* respectively. On NA, the volatilome of *L. antibioticus* was characterised by the emission profiles of 2-furanmethanol and unknown compound 14, and *L. capsici*, *L. enzymogenes* and *L. gummosus* were specified by acetone, pyrazine (2-methoxy-3-methyl pyrazine and 2-methoxy-6-methyl pyrazine) and 3-octanol emission respectively.

3.3. Volatile organic compounds emitted by Lysobacter type strains grown on nutrient agar and not on potato dextrose agar inhibit Phytophthora infestans growth

VOCs emitted by *L. antibioticus*, *L. capsici*, *L. enzymogenes* and *L. gummosus* grown on NA inhibited the mycelial growth of *Ph. infestans* (Fig. 4A and 4B). Conversely, VOCs produced by the four *Lysobacter* type strains grown on PDA did not affect *Ph. infestans* growth. When transferred to new PAM dishes, the growth of *Ph. infestans* plugs previously exposed to VOCs produced by NA-grown *Lysobacter* type strains was compromised as compared with *Ph. infestans* plugs exposed to uninoculated NA (Fig. 4C). Moreover, the growth of plugs exposed to VOCs produced by PDA-grown *Lysobacter* type strains was comparable (Kruskal-Wallis test, $p > 0.05$) with those exposed to uninoculated NA and PDA (data not shown).

Functional assays demonstrated that pure 2,5-dimethyl pyrazine, 2-methoxy-3-methyl pyrazine, decanal and pyrrole inhibited the *Ph. infestans* growth (Table 3) and they were mainly emitted by the NA-grown *Lysobacter* type strains. Conversely, pure delta-hexalactone and ethanol, that were mainly emitted by the PDA-grown *Lysobacter* type strains, did not significantly inhibit the *Ph. infestans* growth.

Table 2
Confusion matrix for bacteria strain prediction based on the least absolute shrinkage and selection operator method (LASSO) with a leave-one-out (LOO) procedure based on solid-phase microextraction gas chromatography–mass spectrometry (SPME/GC–MS) and proton transfer reaction-time of flight-mass spectrometry (PTR-ToF-MS) data.

			LASSO predicted class							
			PDA				NA			
			L. antibioticus	*L. capsici*	*L. enzymogenes*	*L. gummosus*	*L. antibioticus*	*L. capsici*	*L. enzymogenes*	*L. gummosus*
	SPME/GC–MS data									
Real class	PDA	*L. antibioticus*	**4**	0	0	0	0	0	0	0
		L. capsici	0	**4**	0	0	0	0	0	0
		L. enzymogenes	0	0	**5**	0	0	0	0	0
		L. gummosus	0	0	0	**4**	0	0	0	0
	NA	*L. antibioticus*	0	0	0	0	**4**	0	0	0
		L. capsici	0	0	0	0	0	**4**	0	0
		L. enzymogenes	0	0	0	0	0	0	**5**	0
		L. gummosus	0	0	0	0	0	**1**	0	**3**
	PTR-ToF-MS data									
	PDA	*L. antibioticus*	**5**	0	0	0	0	0	0	0
		L. capsici	0	**5**	0	0	0	0	0	0
		L. enzymogenes	0	0	**4**	0	0	0	0	0
		L. gummosus	0	0	0	**5**	0	0	0	0
	NA	*L. antibioticus*	0	0	0	0	**4**	0	0	0
		L. capsici	0	0	0	0	0	**4**	0	0
		L. enzymogenes	0	0	0	0	0	**2**	**2**	0
		L. gummosus	0	0	0	0	**1**	0	0	**4**

SPME/GC–MS and PTR-ToF-MS data were obtained from *Lysobacter antibioticus* DSM 2044^T, *L. capsici* DSM 19286^T, *L. enzymogenes* DSM 2043^T and *L. gummosus* DSM 6980^T grown on potato dextrose agar (PDA) or nutrient agar (NA).
Columns represent the class predicted with the LASSO method based on SPME/GC–MS or PTR-ToF-MS data and rows represent the real class. Diagonal entries of the matrix correspond to the number of samples correctly classified for each class and off-diagonal entries correspond to prediction errors.

4. Discussion

In the last decade, increasing attention has been paid to the functional roles of bacterial VOCs in soil microbial interactions (Effmert et al., 2012; Kai et al., 2009). Strains belonging to *L. antibioticus*, *L. capsici* and *L. gummosus* play a major role in the soil suppressiveness against *R. solani* (Postma and Schilder, 2015; Postma et al., 2008) and strains belonging to *L. enzymogenes* are involved in the biocontrol of several phytopathogens (Hayward et al., 2010; Kobayashi and Yuen, 2007). The incidence of *Lysobacter* spp. in soil is influenced by soil type, plant cover, seasonal factors and organic amendments (Hayward et al., 2010; Postma et al., 2008), but little is known about the ecological role of VOCs emitted by strains belonging to this genus. Although the synthesis of non-volatile antimicrobial metabolites against phytopathogens has been widely studied (Puopolo et al., 2014a; Xie et al., 2012), the possible contribution of *Lysobacter* VOCs to antagonistic processes has been poorly investigated (Sang et al., 2011; Zou et al., 2007). In this work we analysed the VOC profiles emitted by four *Lysobacter* type strains grown on PDA and NA using two types of headspace analysis. The growth media had a different nutrient composition: PDA (sugar-rich media) contained a high sugar content (20 g/l glucose), while NA (protein-rich media) mainly contained proteins (5 g/l peptone and 2 g/l yeast extract) with a low sugar content (Fiddaman and Rossall, 1994). VOC emission by the *Lysobacter* type strains changed radically according to the growth medium, and the comparable growth rate on both media indicated that volatilome differences were mainly related to metabolic changes instead of biomass formation. The composition and functional properties of the bacterial volatilome are known to be influenced by the growth substrate (Asari et al., 2016; Blom et al., 2011; Bruce et al., 2003; Garbeva et al., 2014; Weise et al., 2012), indicating metabolic adaptation of VOC production according to nutrient availability in the soil (Insam and Seewald, 2010). However, the composition of the bacterial bouquet is defined by genetic determinants (Peñuelas et al., 2014) and can be used to identify the four *Lysobacter* type strains grown on PDA with LASSO modelling. Specifically, butanoates (methyl 2-methyl butanoate, methyl 3-methyl butanoate), 2-butanol, methyl thiolacetate and 3-methyl-2-buten-1-ol specified the emission of *L. antibioticus*, *L. capsici*, *L. enzymogenes* and *L. gummosus* on PDA respectively. On NA, 2-furanmethanol and two pyrazines (2-methoxy-3-methyl pyrazine and 2-methoxy-6-methyl pyrazine) discriminated the emission of *L. antibioticus* and *L. enzymogenes* respectively. Interestingly, *Lysobacter* type strains emitted some strain-specific VOCs independently of the growth media. For example, *L. antibioticus* emitted higher amounts of two methyl esters (methyl 2-methyl butanoate and methyl 3-methyl butanoate) as compared with the other type on both media.

The functional properties of the *Lysobacter* volatilome changed radically according to the growth medium and the VOC-mediated biocontrol effects of the four *Lysobacter* type strains against *Ph. infestans* were enhanced on the protein-rich medium as compared with the sugar-rich medium. Likewise, a protein-rich medium (tryptone soya agar) increased the VOC-mediated antagonism of *Serratia* spp. against five sapstain fungi as compared with a sugar-rich medium (malt extract agar) (Bruce et al., 2003). In our experiments, higher emission of six pyrazines [2,5-dimethyl pyrazine, 2,6-dymethyl pyrazine, 2-methoxy-3-(1-methyl-propyl) pyrazine, 3-methoxy-2,5-dimethyl pyrazine, 2-methoxy-3-methyl pyrazine and 2-methoxy-6-methyl pyrazine] was found by NA-grown as compared with PDA-grown *Lysobacter* type strains and two pure pyrazines (2,5-dimethyl pyrazine, 2-methoxy-3-methyl pyrazine) inhibited the *Ph. infestans* growth. Pyrazines are synthesised by alanine, valine, leucine and isoleucine (Dickschat et al., 2005) and addition of amino acids to the growth medium increased the bacterial production of pyrazines (Beck et al., 2003; Bungert et al., 2001), in agreement with higher emission by the NA-grown as compared with the PDA-grown *Lysobacter* type strains. Pyrazines and related heterocyclic compounds were involved in antimicrobial activities (Baldwin et al., 2013; Beck et al., 2003). Specifically, 2,5-dimethyl pyrazine and 2-ethyl-3,5-dimethyl pyrazine were emitted by a biocontrol strain of *Bacillus pumilus* and pure 2,5-dimethyl pyrazine showed antagonistic activity against *Ph. infestans* (De Vrieze et al., 2015) and *Phaeomoniella chlamydospora* (Haidar et al., 2016). Likewise, the VOC-mediated antimicrobial activity of the antagonist *B. megaterium* BP17 (Munjal et al., 2016) and *Pseudomonas putida* BP25 (Sheoran et al., 2015) was attributed to the emission of pyrazines, and four pure molecules (2,5-

dimethyl pyrazine, 2-ethyl-3-methyl pyrazine, 2-ethyl pyrazine and 2-methyl pyrazine) showed inhibitory activities against *Ph. capsici*, *Ralstonia solanacearum* and *Magnaporthe oryzae* (Munjal et al., 2016). As shown for pyrazine emission by *Paenibacillus* spp. (Rybakova et al., 2016), three pyrazines [2-methoxy-3-(1-methyl-propyl) pyrazine, 2-methoxy-3-methyl pyrazine and 2-methoxy-6-methyl pyrazine] were species-specifically emitted, indicating the involvement of some species-specific biosynthetic pathways.

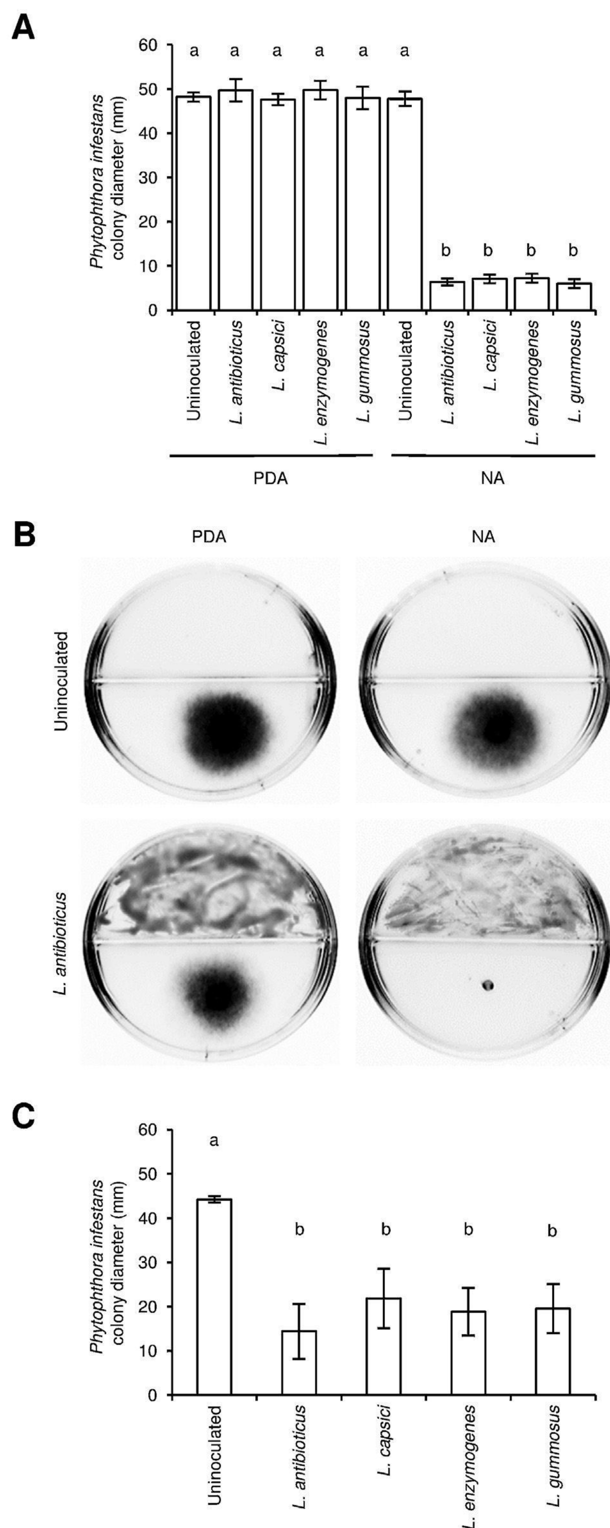

Fig. 4. Antagonistic activity of volatile organic compounds (VOCs) produced by *Lysobacter* type strains against *Phytophthora infestans*. *Lysobacter antibioticus* DSM 2044^T, *L. capsici* DSM 19286^T, *L. enzymogenes* DSM 2043^T and *L. gummosus* DSM 6980^T were grown for 72 h at 25 °C on potato dextrose agar (PDA) or nutrient agar (NA) in split dishes and uninoculated dishes were used as controls (Uninoculated). One plug of *Ph. infestans* was placed on the other side of each dish containing the pea agar medium (PAM) and colony diameter was measured seven days after incubation at 20 °C (**A**). Representative pictures of *Ph. infestans* growth (lower side of each dish) in split dishes with NA and PDA growth medium inoculated (*L. antibioticus*) or not (Uninoculated) with *L. antibioticus* (upper side of each dish) (**B**). Each *Ph. infestans* plug exposed to VOCs emitted by *Lysobacter* type strains grown on NA was then transferred to a fresh PAM dish and colony diameter was measured seven days after incubation at 20 °C (**C**). Seven replicates (dishes) were analysed for each treatment and the experiment was carried out twice. Mean and standard error values of mycelium diameters obtained from 14 replicates pooled from two experiments are presented for each bacterial strain and growth media. Different letters indicate significant differences according to the Kruskal-Wallis test with Bonferroni correction ($p \leq 0.05$).

Table 3
Antagonistic activity of pure volatile organic compounds (VOCs) on *Phytophthora infestans* growth.

Treatment	Inhibition (%) of *Phytophthora infestans* growth
Control	0.00 ± 4.86 a
2,5-dimethyl pyrazine	93.66 ± 1.44 bc
2-methoxy-3-methyl pyrazine	97.03 ± 1.08 c
Decanal	94.52 ± 0.82 bc
delta-hexalactone	8.55 ± 3.59 a
Ethanol	29.33 ± 6.99 ab
Pyrrole	96.58 ± 0.96 c

Each pure VOC was applied to a filter paper disk in split dishes at dosage of 20 mg per split dish corresponding to 190 mg/L of air volume (VOC-treated) and distilled water was applied in control dishes (control). One plug of *Ph. infestans* was placed on the other side of each dish containing the pea agar medium (PAM) and colony diameter was measured seven days after incubation at 20 °C. The inhibition of *Ph. infestans* growth (percentage) was calculated according to the following formula: (growth in control dishes — growth in VOC-treated dishes)/(growth in control dishes) × 100. Seven replicates (split dishes) were analysed for each treatment and the experiment was carried out twice. Mean and standard error values of 14 replicates pooled from two experiments are presented for each treatment. Different letters indicate significant differences according to the Kruskal-Wallis test with Bonferroni correction ($p \leq 0.05$).

All the *Lysobacter* type strains showed higher emission of 2,4,6-trimethyl pyridine when grown on NA as compared to PDA and this VOC was also produced by the antagonistic strains *Collimonas fungivorans* Ter331 and *C. pratensis* Ter91 (Garbeva et al., 2014), suggesting its contribution to bacterial biocontrol processes. Pyrrole was also emitted by NA-grown *Lysobacter* type strains and inhibited the *Ph. infestans* growth. Likewise, pyrrole derivatives exhibited antagonistic activity towards phytopathogens, such as pyrrolnitrin [3-chloro-4-(20-nitro-30-chlorophenyl) pyrrole], which is a broad spectrum antifungal metabolite produced by several bacterial species (Saraf et al., 2014). The emission of 1-butanol by *L. antibioticus* was higher on NA as compared with PDA and the pure compound inhibited mycelial growth of *F. oxysporum* and *Moniliophthora perniciosa* (Chaves-López et al., 2015). Likewise, 1-butanol derivatives (3-methyl-1-butanol, 2-methyl-1-butanol and 1-butanol, 3-methyl-acetate) inhibited the mycelial growth of *Py. ultimum*, *R. solani* and *S. sclerotiorum* (Fialho et al., 2011; Strobel et al., 2001), suggesting their contribution to the antagonistic activity against phytopathogens. Dihydro-3-methyl-2(3H)-furanone and decanal were mainly emitted by the NA-grown *L. enzymogenes* and the last one inhibited the *Ph. infestans* growth, indicating their potential biocontrol activities as already shown for 3-(1-Hexenyl)-5-methyl-2-(5H)-furanone (Paulitz et al., 2000) and decanal (Fernando et al., 2005). Nine VOCs specifically emitted by the NA-grown *Lysobacter* type strains were found as unknown substances and more sensitive chromatographic techniques are required to better characterise these compounds.

A blend of 17 VOCs was specifically emitted by all the *Lysobacter*

type strains grown on PDA and not on NA, such as three aliphatic alcohols (1-tridecanol, 1-tetradecanol and 1-pentadecanol). In particular, 1-tridecanol and 1-tetradecanol showed no antifungal activity against *Saccharomyces cerevisiae* (Kubo et al., 2003) and *S. sclerotiorum* (Giorgio et al., 2015) respectively, in agreement with the absence of *Ph. infestans* inhibition with VOCs emitted by the PDA-grown *Lysobacter* type strains. Delta-hexalactone and ethanol were mainly emitted by the PDA-grown *Lysobacter* type strains and they did not show antagonistic activity against *P. infestans*. Likewise, ethanol was produced by two *Serratia* spp. strains grown on the sugar-rich medium (malt extract agar) and not on the protein-rich medium (tryptone soya agar) (Bruce et al., 2004) and did not seem to be implicated in VOC-mediated biocontrol processes (Bruce et al., 2003). Indole emission was detected from the PDA-grown *Lysobacter* type strains and low dosages of this VOC promoted *Arabidopsis thaliana* growth (Blom et al., 2011). Likewise, acetoin, responsible for stimulation of *A. thaliana* growth (Ryu et al., 2003), was mainly emitted by *L. antibioticus* and *L. capsici* on PDA, in agreement with higher emission by *B. amyloliquefaciens* grown on a sugar-rich medium (M9 agar supplemented with glucose) as compared with protein-rich media (tryptic soy agar and Luria-Bertani agar) (Asari et al., 2016).

In conclusion, specific VOCs of *Lysobacter* spp. were identified and a tool for recognising four *Lysobacter* type strains *in vitro* was developed according to VOC emission profiles, assessed using SPME/GC–MS or PTR-ToF-MS analysis. The chemical profiles and functional properties of *Lysobacter* VOCs differed according to the growth medium, suggesting that appropriate nutrient sources should be preferred in dual culture assays in order to maximise biocontrol efficacy against phytopathogens. Bacterial VOC production in soil can differ according to community composition and nutrient availability (Insam and Seewald, 2010), suggesting a possible adaptation to the soil environment and inhabitants. Although our results were obtained from *in vitro*-grown bacteria, we hypothesized a possible scenario of *Lysobacter* VOCs that need further validation under soil conditions. Particularly, protein sources deriving from the lytic activities of phytopathogenic or saprophytic fungi may stimulate the production of antimicrobial VOCs by *Lysobacter* type strains (volatile pyrazines, pyrrole and decanal) to maximise antagonism to soil microbial inhabitants, such as *Ph. infestans*. Conversely, an increase in sugar availability due to root exudates in the rhizosphere (Jones et al., 2004) may change the volatilome of *Lysobacter* type strains, possibly to increase the production of plant growth stimulators (acetoin and indole) and non-antimicrobial compounds (1-tridecanol, 1-tetradecanol, delta-hexalactone and ethanol) to maximise beneficial interaction with the plant. However, further studies are required to investigate the volatilome shift and properties of *Lysobacter* spp. in soil conditions.

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Conflict of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.micres.2017.04.015.

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Part III

Curriculum vitae

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Education

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University of Natural Resources and Life Sciences (BOKU), Vienna
Doctoral studies of Natural Resources and Life Sciences;Diploma program: Food Chemistry and Biotechnology
Center for Analytical Chemistry, Department of Agrobiotechnology(IFA-Tulln), Austria
Plant pathology and applied Microbiology group, Department of Sustainable Agro-ecosystems and Bioresources (DASB), Foundation Edmund Mach, Italy

10/2009 – 10/1013
University of Bari "Aldo Moro", Italy
Master degree in Biotecnologie per la qualità e la sicurezza dell'alimentazione umana, passed with highest distinction
Master thesis: "Development of high-efficiency transformation for functional genomics of raspberry (*Rubus idaeus* L.)"

10/2006 – 12/2009
University of Bari "Aldo Moro", Italy
Bachelor degree in Biotecnologie per le produzioni agricole ed alimentari, passed with distinction
Bachelor thesis: "Comparative analysis of the regulatory regions of the gene *p1* coding for a transcription factor in different species of corn using a Genome Walking technique" (Italian)

Personal skills and competences

Social skills:	Good team spirit, ability to adapt to multicultural environments, good communication skills
Technical skills:	Plant pathology, molecular biology, microbiology, metabolomics, GC-MS, PTR-ToF-MS, data analysis
Artistic skills:	2005 - 5th year diploma in Piano conferred at the Conservatorio "N. Piccinni" of Bari.

List of scientific publications

All the publications are integral part of the presented thesis.

International reviewed publications (SCI ranked)

1. *Emission of volatile sesquiterpenes and monoterpenes in grapevine genotypes following* Plasmopara viticola *inoculation* in vitro.
Alberto Algarra Alarcon*, Valentina Lazazzara*, Luca Cappellin, Pier Luigi Bianchedi, Rainer Schuhmacher, Georg Wohlfahrt, Ilaria Pertot, Franco Biasioli and Michele Perazzolli
Journal of Mass Spectrometry, 50, 1013-1022. 2015.
DOI: 10.1002/jms.3615
*Alberto Algarra Alarcon and Valentina Lazazzara have contributed equally to this work

2. *Downy mildew symptoms can be reduced by volatile organic compounds of resistant grapevine genotypes*
Valentina Lazazzara, Christoph Bueschl, Alexandra Parich, Ilaria Pertot, Rainer Schuhmacher, Michele Perazzolli
Scientific Reports, in press (Manuscript SREP-17-39525). 2018.
DOI: https://doi.org/10.1038/s41598-018-19776-2

3. *Growth media affect the volatilome and antimicrobial activity against* Phytophthora infestans *in four* Lysobacter *type strains*
Valentina Lazazzara*, Michele Perazzolli*, Ilaria Pertot, Franco Biasioli, Gerardo Puopolo, Luca Cappellin
Microbiological Research, 201, 52-62. 2017.
DOI: http://dx.doi.org/10.1016/j.micres.2017.04.015
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Proceeding of conferences

Lazazzara V., Bueschl C., Parich A., Pertot I., Schuhmacher R., Perazzolli M. (2016). Identification of volatile organic compounds emitted by different grapevine genotypes in response to downy mildew infection. *Journal of Plant Pathology* 98 (Supplement): S19

Oral presentations

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Lazazzara, V., Bueschl, C., Parich, A., Pertot, I., Schuhmacher, R., Perazzolli, M. (2017). Identification and activity testing of volatile organic compounds (VOCs) found in different grapevine genotypes in response to downy mildew infection. 13th ASAC JunganalytikerInnen-Forum of Austrian Society for Analytical Chemistry. 12-13 May 2017, Vienna, Austria.

Lazazzara, V., Bueschl, C., Parich, A., Pertot, I., Schuhmacher, R., Perazzolli, M. (2017). Identification and functional characterization of grapevine volatile organic compounds for the sustainable control of downy mildew. Oral communication and Abstract, International Congress “FUTURE IPM 3.0: towards a sustainable agriculture”. 16-20 October, Riva del Garda, Italy.

Poster presentations

Lazazzara V., Algarra Alarcon A., Cappellin L., Bianchedi P.G., Wohlfahrt G., Pertot I., Biasioli F. and Perazzolli M. (2015). Profiling of volatile sesquiterpenes and monoterpenes in grapevine genotypes after *Plasmopara viticola* inoculation by PTR-ToF-MS analysis. Poster and Abstract, 11th JunganalytikerInnerforum of Austrian Society for Analytical Chemistry. 12-13 June, Innsbruck, Austria.

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