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von

Pingyin Guan

aus

Shanxi, China

Dekan: Prof. Dr. Reinhard Fischer

Referent: Prof. Dr. Peter Nick

Korreferent: Prof. Dr. Holger Puchta

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Erklärung

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Contents

Abbreviations

PAMPs: pathogen-associated molecular patterns

PRRs: pattern recognition receptors (PRRs)

PTI: PAMP-triggered immunity

ETS: effector-triggered susceptibility

ETI: effector-triggered immunity

LRR: site-leucine-rich repeat

NLRs: nucleotide-binding site-leucine-rich repeat receptors

VmE02: *Valsa mali* E02

tetranin1: Tet1

tetranin2: Tet2

FLS2: flagellin-sensing 2

MAPK: mitogen-activated protein kinase

T3SS: type III protein secretion system

SA: salicylic acid

JA: jasmonic acid

CaM: calmodulin

CMLs: CaM-like proteins

CBLs: calcineurin B-like proteins

CDPKs or CPKs: Ca^{2+}-dependent protein kinases

CCaMK: calcium/CaM-dependent protein kinase

CAMTA: CaM-binding transcription activators

BcPG1: endopolygalacturonase1 from *B. cinerea*

ROS: reactive oxygen species

O_2^-: superoxide

H_2O_2: hydrogen peroxide

•OH: hydroxyl radicals

RboHs: respiratory burst oxidase homologues

O_2: oxygen

HR: hypersensitive response

PCD: programmed cell death

SNE1: *suppressor of necrosis 1*

PsCRN63: *P. sojae* crinkling- and necrosis-inducing proteins 63

PsojNIP: *P. sojae* necrosis-inducing protein (PsojNIP)

CaMV: Cauliflower mosaic virus

MAPs: microtubule-associated proteins

F-actin: filaments actin

ABPs: actin-binding proteins

ADFs: ACTIN DEPOLYMERIZING FACTORS

MAPKKK or MEKK: MAPK kinase kinase

MAPKK or MKK: MAPK kinase

MPK: MAPK

StSy: stilbene synthase

ER: endoplasmic reticulum

PAL: phenylalanine ammonia lyase

RS: resveratrol synthase

MC2: metacaspase 2

MC5: metacaspase 5

JAR1: jasmonate-resistant 1

JAZ1: the jasmonate ZIM/tify-domain protein 1

GTDs: Grapevine Trunk Diseases

EPSs: Exopolysaccharides

4-HBAL: 4-hydroxy-benzaldehyde

HPLC: high-performance liquid chromatography

MS: mass spectrometry

BAF: biotin-aneurin-folic

PDA: Potato Dextrose Agar

4-HBA: 4-hydroxybenzyl alcohol

DPI: Diphenylene-iodonium chloride

DMSO: dimethylsulfoxide

IBWF: Institut für Biotechnologie und Wirkstoff-Forschung gGmbH

Semi-qPCR: Semi-quantitative PCR

qRT-PCR: Quantitative real-time PCR

EF1: elongation factor 1α

ΔpH: pH change

SE: standard error

Zusammenfassung

Esca (engl. Grapevine Trunk diseases (GTDs)) wirkt sich im zunehmenden Maße auf die Produktivität und Lebensdauer von Weinbergen aus. Im Gegensatz zu klassischen Krankheiten prägt sich Esca nicht gemäß Kochs Postulat aus und kann nicht durch die Gegenwart oder Abwesenheit eines bestimmten Mikroorganismus erklärt werden, weshalb ein neues Konzept zum Verständnis und zur Bekämpfung dieser Krankheit benötigt wird.

Im Verlauf der Esca-Krankheit entstehen Blattsymptome meist durch spezifische Pilzmetaboliten, die nicht dauerhaft produziert werden, sondern wahrscheinlich vom physiologischen Status des Wirts abhängen. Die vorliegende Arbeit geht von einem Modell aus, in dem der Ausbruch von Esca durch die Aktivierung des ruhenden metabolischen Potentials des mikrobiellen Endophyten ausgelöst wird, so dass Toxine vom induzierten Stamm über den Transpirationsstrom in die Blätter gelangen. Um Zugang zu den Immunsystem-modulierenden Substanzen, die in Abhängigkeit von den Kulturbedingungen hergestellt wurden, zu bekommen, haben wir zuerst Extrakte von Pilzen, die mit Esca assoziiert sind, auf ihr Potential hin untersucht, basale Abwehrreaktionen in Zellkulturen von Weinreben auslösen zu können. In der verwendeten Zellkultur waren Mikrotubuli mit GFP markiert, um Zellskelettreaktion als frühe Abwehrreaktion auslesen zu können. Wir konnten zwei unterschiedliche Extrakte des gleichen Organismus (*Eutypa lata*) identifizieren, die in Abhängigkeit vom Kulturmedium, unterschiedliche Aktivität in der Induktion von Mikrotubuli-Antworten und der Expression von Abwehrgenen aufwiesen.

Durch aktivitätsgeleitete Fraktionierungsverfahren dieser beiden Extrakte wurde O-Methylmellein als Kandidat für eine immunmodulierende Substanz ermittelt und im Folgenden im Hinblick auf ihre Wirkungsweise analysiert. Wir zeigen, dass O-Methylmellein an sich keine Immunantwort auslösen kann (d.h. es wirkt nicht als Elicitor), aber einige Abwehrantworten, die durch den Elicitor flg22 ausgelöst werden, wie zum Beispiel die Expression von Abwehrgenen und die Reaktion von

Aktinfilament, verstärkt. Interessanterweise wurde eine der frühesten bekannten flg22-induzierten Immunantworten, Kalziumaufnahme (beobachtet durch Alkanisierung des Apoplasten), durch O-Methylmellein nicht ausgelöst.

Darüber hinaus wurden Eutypinol und Siccayne auch in Kulturextrakten von *Eutypa lata* identifiziert. Zusammen mit zwei weiteren in der Literatur beschriebenen Metaboliten, die von *Eutypa lata* produziert werden, Eutypine und Eulatinol, haben wir in dieser Studie ihre Effekte auf die Abwehr von Weinreben untersucht. Obwohl diese Metaboliten ähnliche Strukturen haben, lösten sie unterschiedliche Immunreaktionen in Weinreben aus. Wir fanden heraus, dass Eutypine Kalziumaufnahme und Expression von Abwehrgenen induzieren konnte, während Eutypinol nur den Abbau kortikaler Mikrotubuli verursachte. Siccayne und Eulatinol haben die Abwehr von Weinreben leicht aktiviert. Folglich könnte Eutypine ein Elicitor der basalen Immunantwort sein, während die anderen drei Metaboliten wenig Auswirkung auf die Immunantwort haben. Da diese Metaboliten ähnliche chemische Strukturen aber unterschiedliche biologische Aktivitäten aufweisen, wurde angenommen, dass die Unterschiede auf unterschiedlicher Affinität für den jeweiligen Rezeptor auf der Plasmamembran beruhen könnten.

Um die Affinitäten der Metaboliten für bestimmte Rezeptor der Weinrebe zu verifizieren, haben wir vier entsprechende chemische Homologe von Eutypine, Eutypinol, Eulatinol und Siccayne (die sich nur durch das Fehlen der Alkingruppe unterscheiden) namens 4-Hydroxybenzaldehyde (4-HBAL), 4-Hydroxybezyl-Alkohol (4-HBA), 4-Methoxyphenol und Hydroquinon. Im Vergleich zu den jeweiligen *Eutypa lata* Metaboliten haben diese chemischen Homologe ähnliche, jedoch schwache, Immunantworten in *Vitis rupestris* Zellen ausgelöst. Es wurde geschlussfolgert, dass es einen spezifischen Rezeptor auf der Zellmembran von *V. rupestris* gibt, welcher eine hohe Affinität für die Seitengruppen des phenolischen Rings von Eutypine, nicht aber der anderen Metaboliten Eutypinol, Eulatinol und Siccayne, aufweist.

Diese Entdeckungen zeigen, dass *Eutypa lata*, ein Pilz der mit Esca in Verbindung steht, Substanzen absondern kann, die als Elicitor oder Verstärker der basalen

Immunantwort wirken. Die spezifische Erkennung des Elicitors Eutypine durch einen Rezeptor der Weinrebe könnte über die Aldehyd- und Alkinseitengruppen des Phenolrings stattfinden. Folglich wird neben Elicitoren, die die basale Immunantwort auslösen und Effektoren, die die basale antibakterielle Immunität herunterregulieren, durch *Eutypa lata,* einem Endophyten des Esca-Syndroms, ein dritter Typ von chemischen Signalen sekretiert, der die basale Immunität verstärkt. Das könnte im Zusammenhang mit der Konsortienbildung von gegenseitig konkurrierenden Mikroorganismen eine Rolle spielen.

Abstract

Grapevine Trunk Diseases (GTDs) are progressively affecting vineyard longevity and productivity worldwide. In contrast to classical diseases, GTDs do not follow the Koch postulates, and cannot be explained by mere presence or absence of a particular microbial organism, requiring a different concept to understand and combat these diseases.

Foliar symptoms of GTDs are mostly due to specific fungal metabolites which are not produced constitutively but probably depend on the physiological status of the host. The current work was motivated by a model, where the outbreak of GTD results from the activation of the silent metabolic potencies in the microbial endophyte, such that toxins are transferred from the infected trunk to leaves via the transpiration stream. To get access to immunity-modulating compounds that were regulated depending on the conditions of cultivation, we first screened extracts from fungi associated with GTDs for their association with basal defence responses in suspension cells of grapevine, where microtubules were tagged by GFP to monitor the response of the cytoskeleton as an early readout of defence. We identified two extracts from the same organism (*Eutypa lata*) that, depending on the cultivation medium, differed in their activity to induce a microtubular response and the expression of defence genes.

By activity-guided fractionation of these two extracts, O-methylmellein was identified as a candidate for a modulator of immunity, and subsequently analysed with respect to its mode of action. We demonstrate that O-methylmellein cannot induce immune responses by itself (i.e. does not act as elicitor), but can amplify some of the defence responses triggered by the bacterial elicitor flg22, such as the induction level of defence genes and actin filament responses. Interestingly, the earliest known event of flg22 triggered immunity, calcium influx (monitored by apoplastic alkalinisation), was not deployed by O-methylmellein.

Furthermore, eutypinol and siccayne were also identified from *Eutypa lata* culture

extracts. Along with two other reported metabolites which are produced by *Eutypa lata*, eutypine and eulatinol, we measured their effects on grapevine defence in this study. Although these metabolites have similar structures, they induced different grapevine immune responses. We found that eutypine could activate the calcium influx and expression of defence genes while eutypinol only caused degradation of the cortical microtubules. Siccayne and eulatinol slightly activated grapevine defence responses. Thus, eutypine might be an elicitor of basal immunity while the other three metabolites have few effects on immune responses. Since these metabolites have similar chemical structures but different biological activities, it was assumed that the differences might be the result of the differential affinity for the respective receptor at the plasma membrane.

To verify the affinity levels of metabolites and specific grapevine receptors, we have tested four corresponding chemical homologues (just differing in the lack of the alkyne moiety) of eutypine, eutypinol, eulatinol and siccayne such as 4-hydroxybenz aldehyde (4-HBAL), 4-hydroxybenzyl alcohol (4-HBA), 4-methoxyphenol and hydroquinone. Compared with the respective *Eutypa lata* metabolite, the chemical homologues induced similar but weak immune responses in *V. rupestris* cells. It was concluded that there is a specific receptor on the cell membrane of *V. rupestris* that has a high affinity with the side groups of the phenolic ring of eutypine, rather than other metabolites eutypinol, eulatinol and siccayne.

These findings show that *Eutypa lata*, as fungus linked with GTD, can secrete compounds that act as elicitors or amplifiers of basal immunity. The specific recognition of the elicitor eutypine by the grapevine receptor might be associated with the aldehyde and alkyne moieties of the phenolic ring. Thus, in addition to elicitors that can trigger basal immunity, and effectors that down-modulate antibacterial basal immunity, once it had been activated, *Eutypa lata* as GTD associated endophyte seems to secrete a third type of chemical signal that amplifies basal immunity and might play a role in the context of consortia of mutually competing microorganisms.

1 Introduction

1.1 Plant innate immunity

In contrast to most animals, plants are sessile organisms, which make immunity even more crucial to ward off intruders. However, plants lack a somatic adaptive immune system. Instead, they have evolved innate immunity composed of several layers. The perception of pathogen-associated molecular patterns (PAMPs) by cell-surface pattern recognition receptors (PRRs) leads to an effective resistance response known as PAMP-triggered immunity (PTI) (**Fig. 1**) (Boller and Felix, 2009; Boutrot and Zipfel, 2017; Couto and Zipfel, 2016; Jones and Dangl, 2006). In the second round of evolutionary warfare, pathogens can adapt to the immunity of their host by secreting specific molecules, termed effectors, into plant cells, to evade detection or suppress PTI, leading to effector-triggered susceptibility (ETS) of the host (**Fig. 1**) (Cook et al., 2015). Subsequently, in consequence of prolonged co-evolution with the pathogen, the host species can acquire means to detect these effectors and re-install a second layer of immunity, called effector-triggered immunity (ETI). In this context, intracellular nucleotide-binding site-leucine-rich repeat (LRR) receptors (NLRs) which genetically have been discovered as *Resistance (R)* loci play an important role (**Fig. 1**) (Jones et al., 2016; Pieterse et al., 2009).

1.1.1 PAMPs that mainly trigger basal immunity

PAMPs are often highly conserved molecules, harbouring signatures characteristic of a whole class of microbes, such as oomycete glucans, fungal chitin or bacterial flagellin, lipochitooligosaccharides, peptidoglycan, or lipopolysaccharides, which are essential for the normal life cycle of microorganisms, such that there is stringent selection against loss of these molecules (Boutrot and Zipfel, 2017; Macho and Zipfel, 2014; Shinya et al., 2015). Furthermore, the medium-chain 3-hydroxy fatty acid is confirmed as the active ingredient of bacterial lipopolysaccharide to trigger immunity in *Arabidopsis* plants (Kutschera et al., 2019).

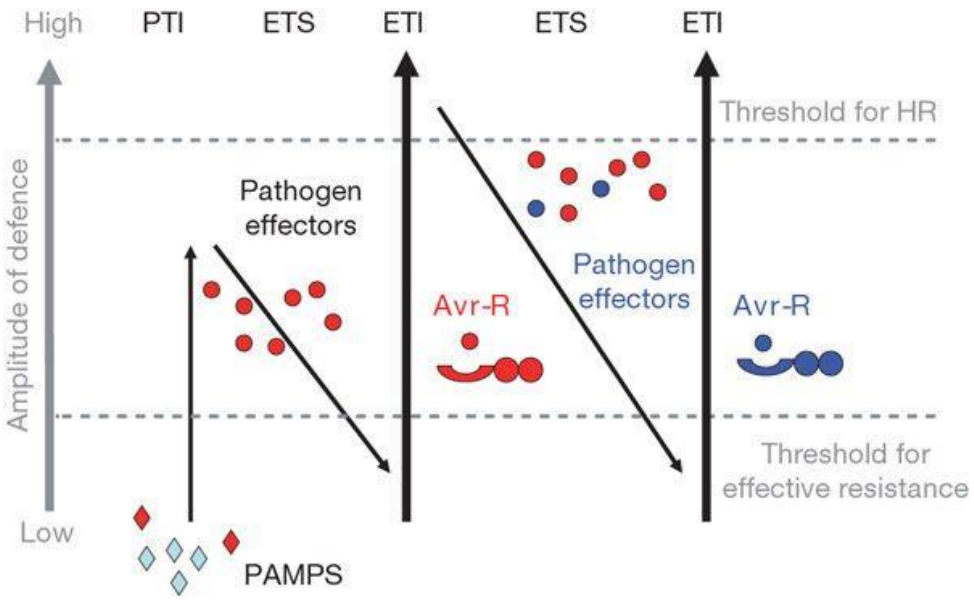


Figure 1 A zigzag model illustrates the quantitative output of the plant immune system (Jones and Dangl, 2006). PAMPs, pathogen-associated molecular patterns; PTI, PAMP-triggered immunity; ETS, effector-triggered susceptibility; ETI, effector-triggered immunity.

It is hypothesised that plants easily recognise the low-complexity metabolite of bacteria to activate immune responses (Kutschera et al., 2019). More recently, a small cysteine-rich protein has been identified as novel PAMP from the necrotrophic fungus *Valsa mali*, *V.* mali E02 (VmE02), which is widely spread in oomycetes and fungi (Nie et al., 2019). In addition to the well-known salivary elicitors of classical chewing herbivores (Maffei et al., 2012), two elicitors of the sucking herbivore two-spotted spider mite *Tetranychus urticae*, tetranin1 (Tet1) and tetranin2 (Tet2), have been characterised (Iida et al., 2019).

The most comprehensive understanding of plant responses to PAMPs has been achieved for the PTI triggered by bacterial flagellin (Buscaill et al., 2019; Gómez-Gómez and Boller, 2002). This building block of bacterial flagella harbours a conserved peptide motif, flg22, which is recognised by the flagellin-sensing 2 (FLS2) receptor on the plasma membrane. Binding of the ligand leads to a conformational change of this transmembrane receptor, which activates a calcium influx channel, an apoplastic oxidative burst, rapid responses of the cytoskeleton, activation of mitogen-activated protein kinase (MAPK) signalling, and activation of a

transcriptional cascade through the transcription factors WRKY22/29 and WRKY25/33, accompanied by biosynthesis of stress-related hormones such as ethylene, or jasmonic acid (Boller and Felix, 2009; Chang and Nick, 2012; Chang et al., 2017; Chinchilla et al., 2006; Chinchilla et al., 2007; Guan et al., 2015; Zipfel et al., 2004).

1.1.2 Modulators that directly regulate innate immunity (normal effectors)

These elicitor-triggered defence responses can be manipulated by effectors secreted by the pathogen. For example, the phytopathogenic bacterium *Pseudomonas syringae* can use its type III protein secretion system (T3SS) to inject effectors that can inhibit the flg22-triggered immunity (Popov et al., 2016). For the oomycete, *Plasmopara viticola*, the causative agent of Grapevine Downy Mildew, RxLR effectors can suppress basal immunity in grapevine (for review see Xiang et al., 2016). In addition to these examples for effector proteins, also non-proteinaceous molecules, which are fungal secondary metabolites, can act as effectors. A famous example is the *P. syringae* toxin coronatine which mimics, with hyperbolic efficiency, the plant hormone jasmonic acid (Weiler et al., 1994).

1.1.3 Modulators that indirectly manipulate innate immunity (special effectors)

Pathogen effectors can support infection success also indirectly by modulating targets outside of innate immunity (Pelgrom and Van den Ackerveken, 2001). For instance, there exist examples of effectors altering the antagonistic balance between salicylic acid (SA) and jasmonic acid (JA) signalling (El Oirdi et al., 2011; Üstün and Börnke, 2015), activating transcriptional reprogramming of the cell (McLellan et al., 2013; Song et al., 2015), affecting host vesicular transport (Dagdas et al., 2016; Du et al., 2015; Guo et al., 2016), or increasing the partitioning of host assimilates towards microbial nutrition (Chen et al., 2010).

1.1.4 Three modes of action of pathogen secretions

Thus, the outcome of a pathogen-host encounter depends on chemical signals that are secreted by the invader and that are subject to evolutionary pressure. Formally, there exist three modes of action for such chemical signals: (i) elicitors (such as PAMPs) can activate host defence, (ii) effectors can silence host defence, after it had been activated by elicitors, (iii) the third type of signal might be “amplifiers” that promote host defence, once it had been activated.

1.2 Cellular signal signatures in plant defence

The perception of pathogen-derived biomolecules or pathogen attack will induce a series of plant cellular responses. Distinct molecular and biochemical modules mediate differential cellular responses and integrate plant innate immunity to handle a broad spectrum of pathogens (Yu et al., 2017).

1.2.1 Calcium signals

The calcium ion, a ubiquitous intracellular second messenger, plays a crucial role in numerous plant signalling pathways, conveying a diverse range of internal and external stimuli to appropriate physiological and gene expression responses (Demidchik and Shabala, 2018; Dodd et al., 2010; Edel et al., 2017). The stimulus-induced changes of calcium cytosolic concentration are termed as Ca^{2+} signals, or ‘calcium signatures’, including oscillations, elevations, standing waves and, more rarely, standing gradients (Feijó and Wudick, 2018; McAinsh and Pittman, 2009). The calcium signatures are essential for a plant decoding the different stimuli and eliciting the appropriate response, such as the recognition of plant pathogenic microbes (Zipfel and Oldroyd, 2017), or expression of stress genes in plants (Lenzoni et al., 2018; Whalley and Knight, 2013). However, the mode of action of calcium signature is not yet known. The specific information carried by the calcium signature is thought to be decoded via the calcium-binding proteins, like calmodulin (CaM) and CaM-like proteins (CMLs), calcineurin B-like proteins (CBLs), Ca^{2+}-dependent protein kinases (CDPKs or CPKs), and calcium/CaM-dependent protein kinase (CCaMK) (Hashimoto and Kudla, 2011; Yuan et al., 2017). The accumulation of calcium-binding

proteins accompanied by the accretion of the calcium signatures are demonstrated to be necessary for plant immunity (Seybold et al., 2014; Tsuda and Somssich, 2015). For example, the different calcium signatures are decoded by CaM-binding transcription activators (CAMTA) to regulate the expression of different genes in *Arabidopsis thaliana* (Liu et al., 2015; Whalley and Knight, 2013).

The activation of a rapid influx of Ca^{2+} and H^{+} is one of the earliest cellular immune responses (Nürnberger, 1999). In case of grapevine defence, the bacterial elicitors harpin, flagellin and rhamnolipids, or the fungal elicitors chitin and endopolygalacturonase1 from *B. cinerea* (BcPG1), are proved to trigger calcium influx (Aziz et al., 2006; Chang and Nick, 2012; Qiao et al., 2010; Trdá et al., 2014; Vandelle et al., 2006; Varnier et al., 2009). Interestingly, the protein hydrolysates from soybean and casein also trigger calcium influx and other grapevine immune responses (Lachhab et al., 2014). The flg22-triggered influx of calcium can be blocked by $GdCl_3$, a specific inhibitor of mechanosensitive calcium channels (Ding and Pickard, 1993). However, $GdCl_3$ failed to block the calcium influx induced by harpin (Chang and Nick, 2012). It is hypothesised that the Gd-sensitive calcium channels may be unnecessary for the ion fluxes triggered by Harpin. Therefore, the perception of elicitors can trigger different calcium signatures, which may be decoded by grapevine to produce different defence responses. Besides, the roles of calcium signatures in ETI are less investigated.

1.2.2 Apoplastic Respiratory Burst

The transient generation of apoplastic reactive oxygen species (ROS), such as superoxide (O_2^{-}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (•OH), is one of the earliest cellular events during pathogen invasion or after treatment with PAMPs (Jabs et al., 1997; Torres et al., 2006; Wojtaszek, 1997). They play an essential role in mediating different plant adaptive responses (Hazman et al., 2015; Mittler et al., 2011; Scheler et al., 2013; Torres, 2010). In plants, some specific NADPH oxidases (respiratory burst oxidase homologues, RboHs) have been identified to be associated with the generation of superoxide anion by transferring electrons to oxygen (O_2).

Subsequently, the superoxide anion converts to H_2O_2 which acts as a further signal used for transduction (Marino et al., 2012).

In case of the grapevine defence, flg22 and harpin both induced a rapid production of ROS but at different kinetic patterns. Previously, it has been found that the oxidative burst triggered by flg22 is later than alkalinisation and even the activation of defence related transcripts. So, generation of ROS may act as the downstream signals in the flg22-induced defence pathway of grapevine (Chang and Nick, 2012). However, apoplastic ROS are necessary for the induction of defence genes triggered by harpin (Chang et al., 2011). The role of ROS burst may be variable according to the different PAMPs in grapevine defence.

1.2.3 Hypersensitive response (HR)

Plant hypersensitive response (HR), a form of programmed cell death (PCD), is thought to be triggered by the perception of the pathogen at the attempted infection sites (Lam et al., 2001; Morel and Dangl, 1997). This cellular response blocks the pathogen infection by rapidly sacrificing tissue as early as possible, mainly against (hemi)biotrophic microorganisms (Feechan et al., 2015). (Hemi)Biotrophic pathogens have evolved the corresponding mechanism to suppress HR by secreting effectors, like the SNE1 (*suppressor of necrosis 1*) effector from the hemibiotrophic oomycete *Phytophthora infestans* (Kelley et al., 2010), or type III effectors from *Pseudomonas syringae* pathovar *tomato* DC3000 (Guo et al., 2009). Interestingly, two effectors produced by the oomycete *Phytophthora sojae* have distinct effects on HR, *P. sojae* crinkling- and necrosis-inducing proteins 63 (PsCRN63) effector induces HR while the PsCRN115 suppress the cell death induced by *P. sojae* necrosis-inducing protein (PsojNIP) or PsCRN63. However, both of them are needed for full pathogenesis (Liu et al., 2011).

In contrast, necrotrophic pathogens take nutrients from dead or dying cells. They kill or weaken the cells by producing phytotoxins and cell wall degrading enzymes (Coll et al., 2011). Therefore, the plant HR machinery may be utilised by some necrotrophs

to promote their virulence, such as the fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum* (Govrin and Levine, 2000). Also, several PAMPs induce HR, for example, the oomycete elicitins cryptogein and capsicein from *Phytophthora cryptogea* and *Phytophthora capsici* induce leaf necrosis (Ricci et al., 1989). The elicitor harpin also induces a significant HR in resistant *Vitis rupestris* cells, but flg22 failed to do so (Chang and Nick, 2012). In some cases, HR has fewer connections with plant resistance (Coll et al., 2011).

Furthermore, the different types of immunity require a mechanism that during early signalling "decides" which pathway is activated. As discussed in the following section, there is evidence that this role is played by the cytoskeleton, microtubules and actin filaments.

1.2.4 Microtubules

Plant microtubules are long and hollow cylinders made up of highly conserved linear polymers constructed from heterodimers of α- and β-tubulins (Nakamura et al., 2004; Nogales, 2000; Weisenberg, 1972). The characteristic of nonlinear dynamic assembly and catastrophic disassembly of plant microtubules, makes them always stay in one of two consistent phases, growth (polymerisation) and rapid shrinkage (depolymerisation) (Mitchison and Kirschner, 1984; Nakamura et al., 2004). The dynamic equilibrium of the microtubule growth and rapid shrinkage is essential for the acquisition of differentiated cell shape (Paradez et al., 2006). Despite their important roles in plant cell growth and differentiation, microtubules are also necessary for dealing with biotic and abiotic stress (Hardham, 2013; Li and Staiger, 2018; Nick, 2013; Park et al., 2018). In the last years, the microtubule network has been found closely associated with host defence responses and can be affected by either the attack of symbiotic and pathogenic microbes (Hardham, 2013; Li and Staiger, 2018). The reorganisation of host cell microtubules occurs during the Cauliflower mosaic virus (CaMV) attack (Martinière et al., 2009), the inoculation with symbiotic bacterium *Mesorhizobium loti* or purified *M. loti* lipochitin oligosaccharide signal molecules (Nod factors) (Vassileva et al., 2005), the formation of plant-parasitic

nematode feeding sites (Caillaud et al., 2008; de Almeida Engler and Favery, 2011), or the infection by filamentous oomycetes and fungi (Hardham et al., 2008; Hoefle et al., 2011; Takemoto et al., 2003). Moreover, the chemical and genetic disruption of microtubule network also increases the plant susceptibility to pathogens (Cheong et al., 2014; Guo et al., 2016; Hardham, 2013; Quentin et al., 2016). Hence microtubules may promote plant immunity against pathogens.

The interactions between pathogen secretions and microtubule remodelling are less investigated. More recently, it has been found that only several elicitors related to the hypersensitive response, such as cryptogein (Binet et al., 2001) or harpin (Qiao et al., 2010), can disrupt host cell microtubules. And the microtubules or microtubule-associated proteins (MAPs) are identified as targets of specific pathogen effectors (Park et al., 2018; Yoshida et al., 2006). One of these effectors, HopZ1a, is found in *Pseudomonas syringae* pathovar tomato (Pst), strain DC3000 and increases its DC3000 virulence and flagellin-triggered callose by targeting tubulin and disrupting the microtubule network (Lee et al., 2012). To conclude, microtubules may play a role in the initiation of ETI, but not PTI (Li and Staiger, 2018).

1.2.5 Actin cytoskeleton

The plant actin cytoskeleton, collection of actin filaments with their accessory and regulatory proteins, belongs to the most abundant protein in most eukaryotic cells (Svitkina, 2018). It extensively participates in more protein-protein interactions than any other known proteins (Dominguez and Holmes, 2011). The actin cytoskeleton network is composed of filaments (F-actin) actin and filament bundles or cables which are generated by the monomers (G-actin). The transition of the F-actin and G-actin can be triggered by nucleotide hydrolysis, ions, and a large number of actin-binding proteins (ABPs) (Day et al., 2011; Dominguez and Holmes, 2011). These properties and abilities allow actin cytoskeleton participating in a plethora of essential plant cellular functions, such as cell division (Kimata et al., 2016) and elongation (Takatsuka et al., 2018), cell development (Deeks et al., 2007), pollen germination and tube growth (Zhu et al., 2017), as well as response to biotic

(Henty-Ridilla et al., 2013; Kang et al., 2014; Li and Staiger, 2018; Qiao et al., 2010) and abiotic stresses (Kadota et al., 2009; Malerba et al., 2010).

In most plant-microbe interactions, the primary role of actin cytoskeleton has been identified as the track for transporting antimicrobial components to the plasma membrane and cell wall (Hardham et al., 2007; Li and Staiger, 2018). Remodelling of the actin cytoskeleton has been observed when the host is infected by pathogens (Li et al., 2015a; Porter and Day, 2016). Also, mechanical stress which is probed with nanoindentation technique or a needle applied to the cell wall induces rapid actin reorganisation (Branco et al., 2017; Hardham et al., 2008). In the sites where fungi or oomycetes tried to penetrate plant cells, which causes similar mechanical stress, radial actin bundles were often observed (Kobayashi et al., 1997b; Opalski et al., 2005; Takemoto et al., 2003). Besides, the actin cytoskeleton also accumulates around the nuclear, which is hypothesised to be needed for repositioning the nucleus to the infection site to trigger quick immune responses (Eichmann et al., 2004). Whenever plant actin cytoskeleton was disrupted genetically or pharmacologically, the penetration frequency of fungi and oomycetes has been increased (Kobayashi and Hakuno, 2003; Kobayashi et al., 1997a; Kobayashi et al., 1997b; Yang et al., 2014). During infection of biotrophic bacteria, which colonise the surface of leaves or proliferate in intracellular spaces between mesophyll cells, the density of host cell actin filaments was increased and later decreased, and bundling of actin filaments was observed (Henty-Ridilla et al., 2013).

Several PAMPs and effectors are found to disrupt the actin cytoskeleton to inhibit plant defence (Park et al., 2018), such as, HopW1, an effector secreted by *Pseudomonas syringae*, which targets F-actin to disrupt actin filaments in *vitro* and the actin cytoskeleton in *planta* (Kang et al., 2014). Knockout of the accompanying receptors (FLS2, EFR, and LYK1) of these PAMPs (flg22, elf26, and chitin, respectively), results in failure to disrupt the actin organisation (Henty-Ridilla et al., 2014; Henty-Ridilla et al., 2013; Li et al., 2015b). The actin organisation and generation of higher order structures, filament bundles or cables, are controlled both by interactions of actin with more than 100 ABPs and by ABPs sensing the cellular

environment (Pollard et al., 2000; Pollard and Cooper, 2009). Mutations of the key ABPs, such as ACTIN DEPOLYMERIZING FACTORS (ADFs) (Henty-Ridilla et al., 2014), CP (Li et al., 2015b), or myosin XI (Cai et al., 2014; Yang et al., 2014), will disrupt the actin cytoskeleton and are more easily infected by bacteria and fungi. More and more experiments indicated that the actin reorganisation may be a conserved hallmark of the plant immune response.

Furthermore, the signal output processed by the cytoskeleton has to be conveyed to the nucleus to modulate the expression of defence genes. The last decade has been increasingly rapid advances in the field of the MAPK cascade, which has been verified as the central element of this signal transport from the membrane to the nucleus.

1.2.6 MAPK cascades

MAPK cascades, highly conserved signalling modules, play pivotal roles in intracellular signal transduction processes of eukaryotes (Dóczi et al., 2012). MAPK cascade commonly comprises three sequentially activated kinases of a MAPK kinase kinase (MAPKKK or MEKK), a MAPK kinase (MAPKK or MKK), and a MAPK (MPK) and links upstream receptors to downstream targets (Meng and Zhang, 2013). Thus, MAPK cascade signalling is necessary for the plant to establish resistance against pathogens, such as phytoalexin biosynthesis, or transcription of defence genes (Meng and Zhang, 2013; Pitzschke et al., 2009). The perception of PAMPs by several PRRs can activate the MAP kinases and induce defence responses. Well-studied PRRs include the bacterial flagellin receptor FLS2 (Gómez-Gómez and Boller, 2000), the bacterial elongation factor EF-Tu receptor EFR (Zipfel et al., 2006), and the chitin receptor CERK1 from *Arabidopsis* (Miya et al., 2007; Wan et al., 2008). Besides, several effectors have been found to inhibit MAP kinase cascades (Bi and Zhou, 2017). For instance, flg22 triggered rapid and transient activation of MPK3, MPK4 and MPK6 (Droillard et al., 2004). However, the type III secretion system effector HopAI1 of *Pseudomonas syringae* directly inactivated MPK3 and MPK6 by dephosphorylation and suppressed the flg22-triggered immune responses in tomato plants (Zhang et al.,

2007). And the harpin protein activated the MPK4 and MPK6 in *Arabidopsis* (Desikan et al., 2001).

MAPK cascades are also necessary for mediating most signals transduction of grapevine defence responses. When they were suppressed by PD98059, an inhibitor of MAPK kinase, the transcriptions of defence genes triggered by flg22 and harpin were repressed (Chang and Nick, 2012). Moreover, the activation of grapevine *MYB14*, a specific transcription factor controlling stilbene biosynthesis (Höll et al., 2013), was reported to be mediated by the MAPK cascades in grapevine (Duan et al., 2016). The overexpression of the *VqMAPKKK38*, a grapevine MAPKKK gene, positively regulated the accumulation of SA-induced stilbene and induction of the gene of stilbene biosynthesis (*StSy*) and *MYB14* in grapevine leaves (Jiao et al., 2017). Thus, in the grapevine-pathogen interactions, MAPK cascades might amplify and transduce the extracellular signals into cellular immune responses.

1.2.7 Defence gene expression

Pathogen invasion generally elicits a rapid and dynamic transcription of regulatory and resistance metabolites and phytoalexin-synthesis genes, which protect the plant from the pathogen (Kushalappa et al., 2016; Tsuda and Somssich, 2015). As a major feature of plant immunity, transcriptional reprogramming can be modulated by several plant signals or pathogen secretions in different patterns. The transcription factors and co-regulatory proteins associated with discrete transcriptional complexes, which are the important and well-studied regulators, have been identified to dominate the pathogen-responsive gene expression (Moore et al., 2011). The expression of specific transcription factors and associated co-factors can be activated by the recognition of PAMPs or effectors, like directly regulated by immune receptors, phosphorylated by MAPKs or acting downstream of Ca^{2+} signalling, leading to various transcriptional changes (Li et al., 2016; Yu et al., 2017). The induction of a large number of defence genes has been found in response to the treatment with different PAMPs, such as flg22, elf18, bacterial peptidoglycan, or fungal chitin (Gust et al.,

2007; Wan et al., 2008; Zipfel et al., 2006). The transcriptional reprogramming in response to PTI and that induced by ETI is largely overlap (Li et al., 2016).

Pathogens recruit virulence effector molecules to manipulate cellular transcriptional reprogramming by modifying chromatin structure, affecting epigenetic regulation or altering transcription. These effectors target the nucleus and named as 'nucleomodulin' (Bierne and Cossart, 2012). For example, *P. infestans* RXLR effector Pi03192 enhances the host susceptibility through preventing the translocation of the potato-membrane-bound NAC transcription factors from the endoplasmic reticulum (ER) to the host nucleus (McLellan et al., 2013).

In grapevine, the signals triggered by flg22 or harpin lead to the significant transcription of defence related genes such as phenylalanine ammonia lyase (PAL), stilbene synthase (StSy) and resveratrol synthase (RS) (Chang and Nick, 2012). The accumulation of resveratrol also activates the jasmonate defence pathway (Tassoni et al., 2005). The synthesis of phytoalexins represents a central element of grapevine basal immunity. The expression of genes associated with PAMP-triggered immunity can be used as a hallmark of immune response to identify the novel elicitor or effectors, such as the finding of the conserved nematode signalling molecule ascarosides (Manosalva et al., 2015).

1.3 Grapevine Trunk Diseases (GTDs)

Grapevine Trunk Diseases (GTDs), a major problem in viticulture and wine industry, because they diminish vineyard longevity and productivity and cause tremendous economic losses worldwide (Agustí-Brisach et al., 2011; Bertsch et al., 2013; Gramaje et al., 2018; Mondello et al., 2018; Munkvold et al., 1994). The annual financial cost of replacement of worldwide grapevine (*Vitis vinifera*) affected by GTDs has been evaluated to be equivalent to 1.132 billion euros per year (Hofstetter et al., 2012).

1.3.1 The microbes involved in GTDs

The complexity of GTDs results from a diverse range of phytopathogens, the

uncertain asymptomatic period of latency within the vine, the yearly erratic manifestation of the foliar symptom, lack of effective control strategies (Reis et al., 2019). In most cases, the fungal spores infect the grapevine through pruning wounds, and also the mechanical and frost wounds. Several taxonomically unrelated groups of ascomycetous fungi have been isolated from the infected trunks, and are discussed as causative agents for a couple of syndromes, such as *Botryosphaeria* dieback, Esca, *Eutypa* dieback, Petri disease, Black Foot, and *Phomopsis* dieback (**Fig. 2**) (Bertsch et al., 2013; Cobos et al., 2015; Rolshausen et al., 2010). Species in *Botryosphaeria* dieback include *Botryosphaeria dothidea, Lasiodiplodia theobromae, Diplodia seriata, D. mutila, D. corticola, Dothiorella iberica, D. viticola, Neofusicoccum parvum, N. australe, N. luteum, N. vitifusiforme,* and *N. viticlavatum* (Crous et al., 2006; Úrbez-Torres and Gubler, 2009; Úrbez-Torres et al., 2009; van Niekerk et al., 2004). *Phaeomoniella chlamydospora, Togninia minima* and many species of *Phaeoacremonium* are also isolated from the esca or black measles and Petri disease grapevine (Crous and Gams, 2000; Crous et al., 1996; Gramaje et al., 2009).

Figure 2 Typical symptoms of eutypa dieback, esca and botryosphaeria dieback in leaves and wood of Chardonnay grapevines (Bertsch et al., 2013). (a-c) Eutypa dieback; (a, b) Eutypa lata; (c) wood cross-section of discoloured tissue; (d-f) Esca; (d) typical tiger-like necrosis and chlorosis; (e) the dead shoots and leaves; (f) white rot trunk. (g-k) Botryosphaeria dieback; (g) yellowish-orange spots on leaves; (k) leaf desiccation; (j) infected fruits; (h) brown streaking under bark; (i) grey rotted sector. All pictures were taken from Sauvignon grapevine except for h, from Cabernet-Sauvignon grapevine.

Fungi *Eutypa lata* has been reported to be involved in the development of *Eutypa* dieback (Moller and Kasimatis, 1978). In addition, some Basidiomycetes, for instance, *Fomitoporia mediterranea*, are also found in the affected, often discoloured, woody tissues (Cloete et al., 2015; Fischer, 2002; Gramaje et al., 2018). To date, as many as 133 fungal species belonging to 34 genera, have been reported to participate in GTDs (Gramaje et al., 2018). In addition to these fungi, also bacteria inhabit the wood tissues of both, asymptomatic and GTD-diseased grapevine, and might interact with the fungal colonisers in a complex and unknown manner (Bruez et al., 2015).

1.3.2 The epidemiology of GTDs

The epidemiology and aetiology of GTDs remain unclear and poorly understood (Bertsch et al., 2009). Surprisingly, the same fungi can also be isolated from healthy grapevine trunks and in similar frequencies with the diseased grapevine, without causing disease symptoms, such, that they are viewed as latent pathogens (González and Tello, 2011; Hofstetter et al., 2012; Verhoeff, 1974). Whether this microbial endoflora will lead to disease or remain latent, depends on the stress status of the host. Especially heat and drought stress, but also the host genotype are relevant in this context (Graniti et al., 2000; Surico et al., 2006). The devastating damage caused by this type of disease, is also caused by the fact that the symptoms can progress slowly, often over many years, and then break out suddenly, leading to a so-called apoplectic breakdown, where the grape dies within a few days, often at the time, when its productivity is maximal. This type of disease obviously differs from the classical case of infectious diseases following Koch's postulates (actually first published by his disciple Löffler, 1884). Especially, the first postulate (the parasitic organism has to be found in all cases, where the disease is observed, and there is no case, where the parasitic organism is absent, when the disease is observed), is not met.

1.3.3 Fungal secondary metabolites in GTDs

Because the GTD related pathogens colonise the trunk of grapevine and have never been isolated from leaves, the extracellular compounds that are produced by these pathogens are hypothesised to be linked to the foliar symptoms associated with the

GTDs (Bruno and Sparapano, 2006; Fontaine et al., 2016). Therefore, a variety of fungal secondary metabolites have been isolated and characterised chemically (Masi et al., 2018). Their toxicity has also been tested on the protoplasts, calli and leaves of various *Vitis* species and *V. vinifera* cultivars (Andolfi et al., 2011).

Eutypa lata, one of the common fungi related to GTDs, produces an array of structurally related secondary metabolites, mainly acetylenic phenols and heterocyclic analogues (Jiménez-Teja et al., 2006). Several of these metabolites have been isolated and characterised from the culture filtrate of an unspecified strain of *Eutypa lata*, such as eutypine (Renaud et al., 1989; Tsoupras et al., 1988), which was first considered as the principal phytotoxin in causing foliar symptoms (Tey-Rulh et al., 1991). However, eutypine is not found in several pathogenic strains of *Eutypa lata* (Molyneux et al., 2002; Smith et al., 2003). Therefore, the foliar symptoms are believed to be induced by more than one metabolite (Mahoney et al., 2003; Octave et al., 2006). The corresponding alcohol and presumptive detoxification product of eutypine, eutypinol, has been identified as an essential metabolite in many strains but are not toxic to grapevine (Molyneux et al., 2002). Further experiments implemented as a leaf disk bioassays indicated that benzofuran, eulatinol, and eulatacromene also have toxic effects similar to eutypine on the leaves of “Cabernet Sauvignon” (Mahoney et al., 2003). Which one plays the key role in causing the dieback is still not clear.

The esca complex is composed of a wood rot and a vascular disease, mainly caused by *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* (Mugnai et al., 1999). Various metabolites, including naphthalenone pentaketides, have been purified from the culture extracts of *Phaeomoniella chlamydospora* and *Phaeoacremonium aleophilum* (Evidente et al., 2000; Tabacchi et al., 2000). The biological activity of these metabolites was measured on grapevine leaves, calli and living protoplasts (Andolfi et al., 2011). The filtrates of *Phaeomoniella chlamydospora* and the solutions of metabolite scytalone, isosclerone or pullulan, caused similar symptoms on grapevine leaves and berries (Bruno et al., 2007). Exopolysaccharides (EPSs) and polypeptides secreted by *Phaeomoniella chlamydospora* and

Phaeoacremonium aleophilum were also able to induce esca-like foliar symptoms (Luini et al., 2010; Sparapano et al., 2000). The metabolites of the basidiomycetous fungus *Fomitiporia mediterrane,* such as 4-hydroxy-benzaldehyde (4-HBAL), dihydroactinolide and 6-formyl-2,2-dimethyl-4-chromanone are also identified but less analysed (Tabacchi et al., 2000). The mode of action of these metabolites, essentially naphthoquinones, involved in the esca complex needs to be further tested.

Many Botryosphaeriaceae species have been identified to be associated with GTDs in recent years. These fungi produce a large number of metabolites: mainly naphthalenone pentaketides, melleins and polyphenols (Andolfi et al., 2011). The high molecular weight hydrophilic compounds, produced by *Botryosphaeria dothidea*, *Diplodia seriata*, *Dothiorella viticola*, *Neofusicoccum luteum* and *N. parvum*, have shown toxic properties on grapevine leaves (Martos et al., 2008). And the metabolites produced by *N. parvum*, (3R,4R)-(-)-4-hydroxy-mellein, (3R,4S)-(-)-4-hydroxy-mellein, isosclerone, and tyrosol, have shown phytotoxic activity on tomato plants (Evidente et al., 2010). These toxins may directly or indirectly cause foliar symptoms.

These metabolites belong to different classes of natural compounds and have been well identified. Sometimes, different fungi can secret the same metabolite with toxic properties. The common metabolites, like naphthalenone pentaketides and polyphenols, which are produced by *Pm. aleophilum, Pa. chlamydospora* and Botryosphaeriaceae fungi, might be involved in causing foliar symptoms. In GTDs, there are also some metabolites that may be secreted by individual fungi. These metabolites can be used to distinguish particular fungi from others. For example, the melleins and their derivatives are considered as specific metabolites of the botryosphaeriaceous fungi; the anthraquinones, found only in *Pa. chlamydospora*; the acetylenic phenols and dihydro-γ-pyrones, both specific to *E. lata* (Andolfi et al., 2011; Cabras et al., 2006; Djoukeng et al., 2009).

1.3.4 Fungal secondary metabolites and plant defence

Although the toxic substances produced by pathogens related to GTDs *in vitro* and probably also *in vivo* are capable of reproducing similar symptoms that are found in the field, the mechanism of action of these substances remains blurred. Studies to investigate the direct impact of fungal metabolites in the virulence of the respective producing pathogens are rarely reported. Recently, the total extracellular compounds produced by *Diplodia seriata* and *Neofusicoccum parvum*, which are associated with Botryosphaeria dieback, both induced significant expression of defence genes in *Vitis vinifera* cv. Chardonnay cells. But the transcriptional levels of defence genes showed a different pattern (Ramírez-Suero et al., 2014). The secondary metabolite, mellein, a characteristic phytotoxin of Botryosphaeriaceae and produced by both *Diplodia seriata* and *Neofusicoccum parvum*, only triggered a lower-level expression of defence genes (Ramírez-Suero et al., 2014). Besides, the extracellular proteins produced by the two fungi also induced significant transcription of defence genes (Bénard-Gellon et al., 2015). The metabolite produced by the fungus *Eutypa lata*, eutypine, was also hypothesised to be an important virulence factor in causing the symptoms of Eutypa dieback (Deswarte et al., 1996a). Eutypine affects the growth and development of grapevine might through disrupting the normal biological functions of cell mitochondria (Deswarte et al., 1996b). However, the role of eutypine in grapevine defence is unclear.

1.3.5 The classical methods to control GTDs

The classical approach of plant protection is to kill the potential pathogen by toxic chemicals. In the case of Grapevine Trunk Disease, a toxic product sodium arsenite was employed and killed most of these fungi through the xylem. However, because of its toxicity for humans including the wine growers as first targets, this product has been banned in Europe on good grounds (European Commission 2009). This plant-protection strategy not only causes a negative ecological footprint, it is also completely inappropriate in case of a pathogen that does not meet Koch's postulates. Other effective fungicides like flusilazole and carbendazime were formulated in 1996 and retired from the market in 2010. Therefore, there is no marketised fungicide

which is permitted by the authorities to control these diseases (INT, 2016).

Since conventional approaches fail to control this type of diseases (Wagschal et al., 2008), we need to search for new strategies. The disease is not caused by the mere presence of a particular microbial organism, but by a change of its behaviour. In other words: there must be signals, which are exchanged between pathogen and host, and decide on the outbreak of GTDs symptoms.

1.4 Strategies for identification of fungal PAMPs/modulators

Depending on fractionation and purification of culture extracts by ion-exchange chromatography, a wide range of PAMPs have been characterised from bacteria, fungi, or oomycetes (Boutrot and Zipfel, 2017). This technology can also be used for purification of PAMPs/modulators from the culture filtrates of GTDs related fungus. Whether these purified compounds can be recognised by PRRs and trigger PTI-responses in grapevine or act as effectors or "amplifiers" that can manipulate PTI-responses, needs to be investigated further.

1.4.1 Purification of PAMPs/elicitors from culture extracts

Many PAMPs have been isolated and identified from the culture filtrates by using the ion exchange chromatography and high-performance liquid chromatography (HPLC) approaches, which can physically separate substrate from the mixture. These analytical tools are usually combined with mass spectrometry (MS) analyses to elucidate the chemical structure of organic compounds or the sequencing of oligonucleotides (Boutrot and Zipfel, 2017). Bacterial flagellin, one of the best-studied elicitors, was purified by anion exchange chromatography from liquid culture extracts and identified by N-terminal protein sequencing (Felix et al., 1999). Due to the tremendous advantages of MS in revealing the nature of unknown organic biomolecules, MS is considered as the favoured tool to identify PAMPs in recent years. And several novel PAMPs have been identified by using this technology, such as the glycoside hydrolase from *Phytophthora sojae* (Ma et al., 2015), the glycolipidic ascarosides from the nematode *Meloidogyne sp.* (Manosalva et al., 2015), the

protein PeBL1 from the bacterium *Brevibacillus laterosporus* (Wang et al., 2015), and secondary metabolite chrysophanol from the fungus *Trichoderma harzianum* (Liu et al., 2016; Liu et al., 2007).

Very little is currently known about the identification of PAMPs/elicitors produced by fungi related to GTDs. Although some culture filtrates produced by *Eutypa lata*, *Trichoderma atroviride* or different fungi associated with Botryosphaeria dieback are reported to induce obvious grapevine immune responses, only a limited number of elicitors have been identified (Bénard-Gellon et al., 2015; Mutawila et al., 2017; Ramírez-Suero et al., 2014).

1.4.2 Identification of modulators based on flg22-elicited PTI

PAMPs or elicitors are acting primarily, i.e. they can trigger a defence response by themselves. However, there might be compounds that are acting secondarily. These would not induce any response by themselves, but they would modulate a defence response triggered by a PAMP or elicitor. In fact, this is the operational definition of an effector. Different from the traditional effector that is known to suppress the basal immunity activated by PAMPs or elicitors, the special effector “amplifiers” seems counter-intuitive at first glance. What selective advantage should a pathogen draw from even further amplifying host defence? However, in the natural context, a pathogen is seldom alone but has to compete with other pathogens for the host resources. Since attacked host cells will activate chemical warfare to ward off the intruders, a pathogen can acquire mechanism to evade or degrade such host compounds and, thus, outcompete its rival without the need of direct attack, representing a kind of plant version of a famous Chinese war trick (借刀杀人 *jiè dāo shā rén*, kill somebody with a borrowed sword, Sun, 1993). We will in the following use the term amplifiers for this type of immunity-modulating signals.

While plant cells cultures have been classical systems to study the effect of elicitors (Hahlbrock et al., 1995), effectors have been exclusively identified from experiments, where plants were inoculated with pathogens, often by making use of mutagenesis,

or transgenic strategies. To be able to monitor the effect of immunity “modulators” (effectors or amplifiers) in cell culture, would require that first, basal immunity is activated. As one of the best-studied elicitor, flg22 was used to trigger PTI as a platform to test the modulators.

1.5 Scope of this study

Due to the mass destruction, long duration of the latency period, and the lack of effective control methods, GTDs are of rapidly growing concern in all wine producing countries. Foliar symptoms of GTDs are mostly caused by the extracellular metabolites (signals) produced by the fungi hidden in the trunk, which are not constitutive but may depend on the physiological status of the host. Although plenty of metabolites secreted by these fungi have been isolated and identified, the mode of action of these compounds in grapevine physiological status or defence is less investigated.

Therefore, we addressed the following questions:

1. What are the signals exchanged between pathogen and host?
2. Which fungal metabolite could be the elicitor or modulator candidate?
3. What are the differences in the immune responses triggered by elicitors and their chemical homologues?

In order to answer these questions, we used two grapevine suspension lines expressing fluorescent markers for microtubules and actin, as early readouts for defence responses activated by fungal metabolites. Furthermore, the flg22 was used to trigger PTI as a platform to follow the activity of fungal "modulators". By screening cultural filtrates from fungi involved in GTDs, we have used a bioactivity-guided fractionation strategy to identify compounds secreted by *Eutypa lata*. The effect of culture filtrates and fractionations from candidate filtrates on the expression of defence genes are measured in this study. Based on the HPLC-MS analysis, promising fractionations have been identified from candidate culture filtrates.

Subsequent analysis of the mode of action of one fractionation, O-methylmellein, in the context of plant defence shows that this fungal secondary metabolite acts as an amplifier for flg22-triggered PTI. In addition to the secondary metabolite O-methylmellein, we also checked the mode of action of other identification products and their analogues, like eutypine, eutypinol, eulatinol, siccayne. Furthermore, the respective chemical analogues of those *Eutypa lata* metabolites in grapevine defence, like 4-HBAL (the analogue of eutypine), 4-hydroxybenzyl alcohol (4-HBA, the analogue of eutypine), 4-methoxyphenol (the analogue of eulatinol), hydroquinone (the analogue of siccayne), were also analysed in this study.

2 Materials and methods

2.1 Cell cultures

Suspension cell cultures of *V. rupestris* expressing the fluorescent tubulin marker *GFP-AtTUB6* (Guan et al., 2015), and *V. vinifera* L. cv. 'Chardonnay' expressing the actin marker *FABD2–GFP* (Akaberi et al., 2018) were used in this experiment. The cells were cultivated in liquid medium containing 4.3 g-L^{-1} Murashige and Skoog salts (Duchefa, Haarlem, The Netherlands), 30 g-L^{-1} sucrose, 200 mg-L^{-1} K_2HPO_4 100 mg-L^{-1} inositol, 1 mg-L^{-1} thiamine and 0.2 mg-L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8, supplemented with either 30 mg-L^{-1} hygromycin (tubulin marker line), or kanamycin (actin marker line), respectively. Cells were subcultured weekly by transferring 5 ml (*V. rupestris*) or 10 ml (*V. vinifera*) cells into 30 ml fresh medium in 100 ml Erlenmeyer flasks. The suspension was incubated at 27 °C in the dark on a horizontal shaker (KS250 basic, IKA Labortechnik, Staufen, Germany) at 150 rpm. If not stated otherwise, all these treatments were conducted with cells at the onset of the expansion phase (day 4 after subcultivation).

2.2 Fungal culture extracts

Culture filtrate extracts of *Phaeomoniella chlamydospora* (HMG), *Phaeoacremonium minimum* (HMG), *Eutypa lata* (HMG), *Fomitiporia mediterranea* (HMG), *Botrytis cinerea* (HMG), *Roesleria subterranea* (HMG), *Guignardia bidwellii* (HMG), *Eutypa lata* IBWF E16012 (BAF), *Eutypa lata IBWF* E16012 (PDA), *Eutypa lata* 5.1 (BAF), *Eutypa lata* 5.1 (PDA), *Eutypa lata* 5 (BAF), *Eutypa lata* 5 (PDA), *Eutypa lata* HKM2 (BAF), *Eutypa lata* HKM2 (PDA), all of which associated with trunk diseases of grapevine, were kindly provided by the Institut für Biotechnologie und Wirkstoff-Forschung gGmbH (IBWF). The culture extracts were dissolved in 100% methanol to 10 mg/ml and used in the 25 µg/ml.

Phaeomoniella chlamydospora CBS 229.95 (*Pch*) and *Phaeoacremonium minimum* (*Togninia minima*) CBS 100398 (*Pmi*) were purchased from the CBS-KNAW culture

collection (CBS-KNAW Fungal Biodiversity Centre, The Netherlands) (Markwart et al., 2019). The *Botrytis cinerea* strain was maintained as previously published (Schüffler et al., 2009). *Guignardia bidwellii* was obtained from the Centraalbureau voor Schimmelcultures (CBS, Fungal Biodiversity Centre, The Netherlands) as described previously (Buckel et al., 2017). The *Eutypa lata* strains (HKM2, IBWF E16121; HKM5, IBWF E16122 and HKM5.1, IBWF E16123) were kindly provided by apl. Prof. Dr. Hanns-Heinz Kassemeyer (Staatliches Weinbauinstitut Versuchs- und Forschungsanstalt für Weinbau und Weinbehandlung). The *Eutypa lata* strain IBWF E16012 is part of the IBWF culture collection and was isolated by Linda Muskat in a Hessian vineyard. The *Roesleria subterranea* strain 1303-K is also part of the IBWF culture collection and was provided by Isabell Büttel. For maintenance the strains were cultured on Yeast Malt Glucose (HMG) agar (4 g/L yeast extract, 10 g/L malt extract, 10 g/L glucose, 2 % agar, pH 6.5), biotin-aneurin-folic (BAF) agar (0.1 % yeast extract; 1 % glucose; 2 % maltose; 0.2 % peptone; 0.05 % KH_2PO_4; 0.04 % $MgSO_4 \bullet 7H_2O$; 0.007 % $CaCl_2 \bullet 2H_2O$; 0.001 % $FeCl_3 \bullet 6H_2O$; 0.0002 % $ZnSO_4$) or Potato Dextrose Agar (PDA) (dehydrated mashed potatoes 2% w/v, glucose 2% w/v, pH 5.5) and were transferred to new agar plates every two to four weeks. The fungi were cultivated in 500 mL HMG in 1 L Erlenmeyer flasks and at an orbital shaker (120 rpm, 22±1 °C) (Kramer et al., 2009). The availability of free glucose within the medium was monitored by Diabur test 5000 strips (Roche, Germany). Once the free glucose in the medium was depleted the fermentation was stopped by separating culture broth from the mycelium by filtration.

2.3 Chemicals

The peptide flg22 was synthesised by a commercial provider (GenScript) and diluted in sterile H_2O. Diphenylene-iodonium chloride (DPI, Sigma-Aldrich, Deisenhofen, Germany), an inhibitor of NADPH oxidase (Bolwell and Wojtaszek, 1997), was prepared in dimethylsulfoxide (DMSO) to a stock solution of 10 mM and used in a final concentration of 10 µM. $GdCl_3$, an inhibitor of calcium influx (Ding and Pickard, 1993), was prepared in distilled water to a stock of 10 mM and used in a final concentration of 100 µM.

Fungal metabolites O-methylmellein, eutypine, eutypinol, eulatinol, and siccayne were kindly provided by the IBWF. The fungal metabolites were dissolved in 100% methanol to a stock of 10 mM and mostly used in 10 µM.

The chemical homologues of fungal metabolites, 4-HBAL (Sigma-Aldrich, Deisenhofen, Germany), 4-HBA (Sigma-Aldrich, Deisenhofen, Germany), 4-methoxyphenol (Sigma-Aldrich, Deisenhofen, Germany), hydroquinone (Sigma-Aldrich, Deisenhofen, Germany) were prepared in 2% methanol to 10 mM and mostly used in 50 µM.

To test for potential effects of solvents, controls without inhibitors, metabolites, or chemicals, but the same concentration of solvent was used in a parallel set of experiments.

2.4 Extracellular alkalinisation

Extracellular alkalinisation was measured by combining a pH meter (Schott handy lab, pH 12) with a pH electrode (Mettler Toledo, LoT 403-M8-S7/120), and recorded by a paperless readout (VR06; MF Instruments GmbH, Albstadt-Truchtelfingen, Germany). Before the addition of chemicals, cells were equilibrated on an orbital shaker for at least one hour.

Fungal culture extracts treatments;

To determine the effect of exogenous fungal culture extracts on extracellular alkalinisation, cells of the tubulin marker line (*V. rupestris* TuB6) were treated by 25 µg/ml of the different extracts or methanol as solvent control for one hour after the equilibration.

O-methylmellein and flg22 treatments;

To follow the dose-response for O-methylmellein, the cells were inoculated with 10-100 µM of O-methylmellein, along with solvent controls with 0.1-1% methanol. A different experiment tested the effect of flg22 (1 µM) with 25 µM O-methylmellein, or the 0.25% methanol as solvent control.

Eutypine and its analogues treatments;

To measure the dose-response of calcium influx induced by fungal metabolites, the cells were inoculated with eutypine, eutypinol, eulatinol, siccayne either in a concentration of 10 µM or 25 µM, 0.1% or 0.25% methanol as the solvent control respectively.

4-HBAL and other homologues treatments;

To evaluate the effect of the homologues of fungal metabolites on the cellular calcium influx, the cells were treated with 50 µM 4-HBAL, 50 µM 4-HBA, 50 µM 4-methoxyphenol, or 50 µM hydroquinone, the 0.01% methanol as the solvent control, respectively.

To test the impact of $GdCl_3$ on 4-HBAL- or 4-HBA-dependent extracellular alkalinisation, the cells were co-incubated with 50 µM 4-HBAL, 50 µM 4-HBA, either with or without 100 µM $GdCl_3$, the 0.01% methanol as the solvent control, respectively.

The pH changes (ΔpH) were recorded over time, and values for ΔpH were calculated as differentials of treatment versus mock control using the peak values to estimate ΔpH_{max}. The experiments were repeated at least five times.

2.5 RNA extraction and cDNA synthesis

Following the various treatments, total RNA was purified using the Universal RNA Purification Kit (ROBOKLON, Berlin, Germany), according to the protocol of the producer. The genomic DNA contamination from the sample was removed by using the DNase (Invitrogen, Karlsruhe, Germany). The RNA quantity and quality were measured spectrophotometrically (NanoDrop, Radnor, USA), and, in parallel, by electrophoresis on a 0.8% agarose gel. The mRNA was transcribed into cDNA using the M-MuLV cDNA Synthesis Kit (New England Biolabs; Frankfurt am Main, Germany) according to the instructions of the manufacturer. The amount of RNA template was 1 µg.

2.6 Semi-quantitative PCR

Semi-quantitative PCR (Semi-qPCR) was performed as described previously (Duan et al., 2015). The elongation factor 1α (*EF1*) was used as an internal standard to quantify the transcript levels of *PAL*, *RS*, *StSy*, and *JAZ1*. These genes were amplified by semi-qPCR using the primers and conditions given in Supplementary **Table S1** of the Supplementary material. The PCR was performed using *Taq* polymerase from New England Biolabs (NEB, Frankfurt, Germany). Each experiment was repeated with three biological replicates, each in three technical replicates.

To screen the effect of HPLC fractions obtained from the *Eutypa lata IBWF E16012* (BAF), and the *Eutypa lata IBWF E16012* (PDA) extracts on the transcription of defence-related genes, cells were treated for one hour with 20 µl solution of fractions or the concentration of methanol as a mock control.

Semi-qPCR results were quantified by quantitative image analysis using the freeware ImageJ (https://imagej.nih.gov/ij/) from the digital images recorded for the electrophoretically separated amplicons. For each band, the integrated density was measured along a probing line transecting the band, and the results integrated into an Excel spreadsheet. The methanol solvent control was used as the internal standard for relative quantification of the other bands on the gel. For visualisation of the complex and extensive data sets, relative changes of induction levels were calculated as the relative surplus of expression for the fraction from the BAF supernatant over the corresponding fraction from the PDA supernatant. These were encoded in a colour code from dark blue (inhibited response) till dark red (enhanced response) and plotted along with the elution profile (**Fig. 5**).

2.7 Real-time PCR analysis

Quantitative real-time PCR (qRT-PCR) was conducted using a CFX96^{TM} real-time PCR cycler (Bio-RAD, USA) as described previously (Wang et al., 2019). Values for relative transcript abundance were calculated using elongation factor 1α (*EF1*) as an internal

standard. Data analysis were performed using the $2^{-\Delta\Delta ct}$ method (Livak and Schmittgen, 2001).

Fungal culture extracts treatments;

To evaluate the effect of fungal extracts on the transcription of defence-related genes, cells were treated for one hour with 25 µg/ml of the corresponding extract or the equivalent concentration of methanol as a mock control.

O-methylmellein and flg22 treatments;

To test the effect of O-methylmellein on the induction of defence genes, cells were treated for one hour with 25 µM O-methylmellein or 0.25% methanol as solvent control for one hour.

To measure the modulating effect of O-methylmellein on the flg22-triggered expression of defence genes, the cells were inoculated with a combined flg22 (1 µM) with 25 µM O-methylmellein or 0.25% methanol as solvent control for indicated time points (0 h, 0.5 h, 1 h or 2 h).

To map the role of different events for the signalling triggered by O-methylmellein, cells were incubated by a combination of flg22 (1 µM) and O-methylmellein (25 µM) along with the calcium influx inhibitor $GdCl_3$ (100 µM), or the NADPH oxidase inhibitor DPI (10 µM), or DMSO as solvent control for DPI, for one hour.

Eutypine and its analogues treatments;

To determine the transcript levels of phytoalexin-synthesis genes activated by fungal metabolites, the cells were respectively treated by 10 µM eutypine, 10 µM eutypinol, 10 µM eulatinol, 10 µM siccayne, or the 0.1% methanol as the solvent control for one hour.

4-HBAL and 4-HBA treatments;

To assess the relationship between the chemical structure and biological activity in the induction of defence genes, the cells were treated with 50 µM 4-HABL, 50 µM

4-HAB, the combinations of 50 µM 4-HABL and different concentrations (10 µM, 50 µM, 100 µM) of 4-HBA, or the 0.01% methanol as the solvent control for indicated time points (0 h, 1 h, 3 h or 6 h).

Transcripts of the genes *PAL*, *RS*, *StSy*, *JAR1*, *JAZ1*, *MC2*, and *MC5* were amplified by qRT-PCR using the primers and conditions given in **Table S1** of the Supplementary material. Each experiment was repeated with three biological replicates, each in three technical replicates.

2.8 Live-cell visualisation of the cytoskeleton

Making use of the GFP tag linked to either the tubulin marker *At*TuB6, or to the actin marker FABD2, the cytoskeleton could be monitored in living cells of grapevine.

Fungal culture extracts treatments;

The responses of cortical microtubules to *Eutypa lata* IBWF E16012 BAF (25 µg/ml) and IBWF E16012 PDA (25µg/ml) extracts were monitored over time in individual cells by spinning-disc confocal microscopy.

O-methylmellein and flg22 treatments;

Likewise, the effect of 25 µM O-methylmellein on microtubules and actin filaments was assessed 30 or 60 min after addition to the compound, in parallel to experiments testing the effect of, 0.25% methanol as solvent control, as well as 1 µM of flg22 either without or with 25 µM of O-methylmellein.

Eutypine and its analogues treatments;

The responses of cortical and central microtubules to 10 µM eutypinol, 10 µM eutypine, and 10 µM siccayne were assessed 30 or 60 min after addition to the compound, 0.1% methanol as solvent control.

The effect of 10 µM eutypinol on cortical and central microtubules was also monitored 8 hours after the addition to the compound, 0.1% methanol as the

solvent control.

Confocal z-stacks were recorded with an AxioObserver Z1 (Zeiss, Jena, Germany) using a 63 × LCI-Neofluar Imm Corr DIC objective (NA 1.3), the 488 nm emission line of an Ar-Kr laser, and a spinning-disc device (YOKOGAWA CSU-X1 5000). The images were operated via the ZEN 2012 (Bule edition) software platform to generate orthogonal projections from the recorded stacks and to export in TIFF format.

To quantify the degree of actin aggregation a strategy modified from Schwarzerová et al. (2002) was used. Intensity profiles were collected in the cell periphery over time-lapse series of the same cell, avoiding the nuclear region using a line width of 10 pixels and the spline averaging option (ImageJ, https://imagej.nih.gov/ij/). The profile shows peaks to microtubules separated by troughs. Depletion of microtubules will render these peaks thinner and discontinuous, while the troughs will become flattered. This phenomenon can be quantified by calculating the standard error over the profile. This standard error can, therefore, be used as a readout for the degree of actin aggregation. The initial value (5 min after addition of the respective extract) was used as an internal standard to record the relative change of microtubule thickness relative to this initial value.

2.9 Determination of mortality

To determine mortality, the Evans Blue dye exclusion test was used (Gaff and Okong'o-Ogola 1971).

O-methylmellein and flg22 treatments;

A time course of the response to 25 µM O-methylmellein, 1 µM flg22, or a combination of both compounds along with 0.25% methanol as the solvent control or 9 µg/ml harpin as the positive control were recorded, using time intervals of 6 h, 12 h, 24 h, 48 h, and 72 h, respectively.

Eutypine and its analogues treatments;

To measure the effect of *Eutypa lata* metabolites on mortality, the cells were treated with 10 µM eutypine, 10 µM eutypinol, or 10 µM siccayne, 0.1% methanol as the solvent control for 24 h, 48 h, and 72 h.

4-HBAL and 4-HBA treatments;

To evaluate the effect of 4-HBAL and 4-HBA on mortality, the cells were treated with 50 µM 4-HBAL, 50 µM 4-HBA, or a combination of both compounds, 0.01% methanol as the solvent control for 24 h, 48 h, and 72 h.

For each sample, aliquots of 200 µL cells were transferred into custom-made staining chambers, to remove the medium, incubated for 3 min in 2.5% (w/v) Evans Blue, and then washed with distilled water several times. Aliquots of 40 µL stained cells were microscopically observed using an AxioImager Z.1 microscope (Zeiss, Jena) equipped with an ApoTome microscope slider through the filter sets 38 HE. Evans Blue penetrates dead cells resulting in blue staining of the cell. At least 1000 cells were scored per sample. Data represent a population of 3000 cells scored over three independent experiments.

3 Results

3.1 The response to fungal culture filtrates is associated with the expression of basal immunity

3.1.1 Fungal culture filtrates induce calcium influx

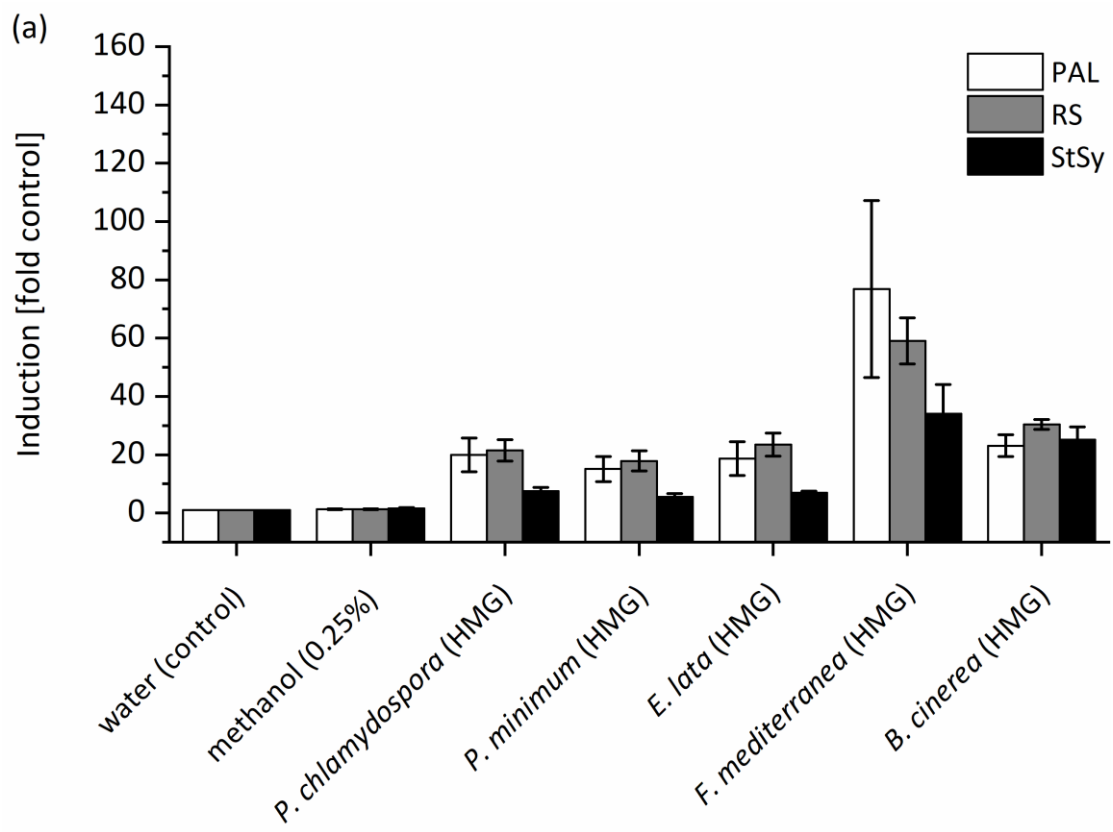
Fungal extracts contain a wide range of secondary metabolites, and some of them may modulate plant defence. Among those, elicitors can activate defence primarily, while effectors and amplifiers do not induce a defence response by themselves, but secondarily modulate a response that had been activated by an elicitor. Fungal cultural filtrates are expected to harbour significant amounts of elicitors, since products of remodelling or breakdown of the fungal cell wall, such as chitins should act as PAMPs and deploy a basal immune response. Under these conditions, the operational criteria for an amplifier are the same as those for an elicitor: a cultural filtrate that is able to induce markers of a defence response would qualify as a candidate. To find out such candidate "pools" of defence modulators, we tested the effects of fungal filtrates by measuring extracellular alkalinisation (indicative of calcium influx through proton coimport) as a rapid readout for a defence response. The cells were incubated with fifteen cultural extracts (the respective media are given in brackets) from fungi associated grapevine diseases, with a focus on GTDs, particularly *Eutypa lata* (**Tab. 1**): *Phaeomoniella chlamydospora* (HMG), *Phaeoacremonium minimum* (HMG), *Eutypa lata* (HMG), *Fomitiporia mediterranea* (HMG), *Botrytis cinerea* (HMG), *Roesleria subterranea* (HMG), *Guignardia bidwellii* (HMG), *Eutypa lata* IBWF E16012 (BAF), *Eutypa lata* IBWF E16012 (PDA), *Eutypa lata* 5.1 (BAF), *Eutypa lata* 5.1 (PDA), *Eutypa lata* 5 (BAF), *Eutypa lata* 5 (PDA), *Eutypa lata* HKM2 (BAF), *Eutypa lata* HKM2 (PDA). In most treatments, the pH increased rapidly after the addition of these extracts. The most significant ΔpH was triggered by *P. chlamydospora* (HMG) extract, up to 0.6. The response to *Eutypa lata* ranged from less than 0.05 and more than 0.5 depending on the tested strain indicative of considerable intra-specific variability (**Tab. 1**).

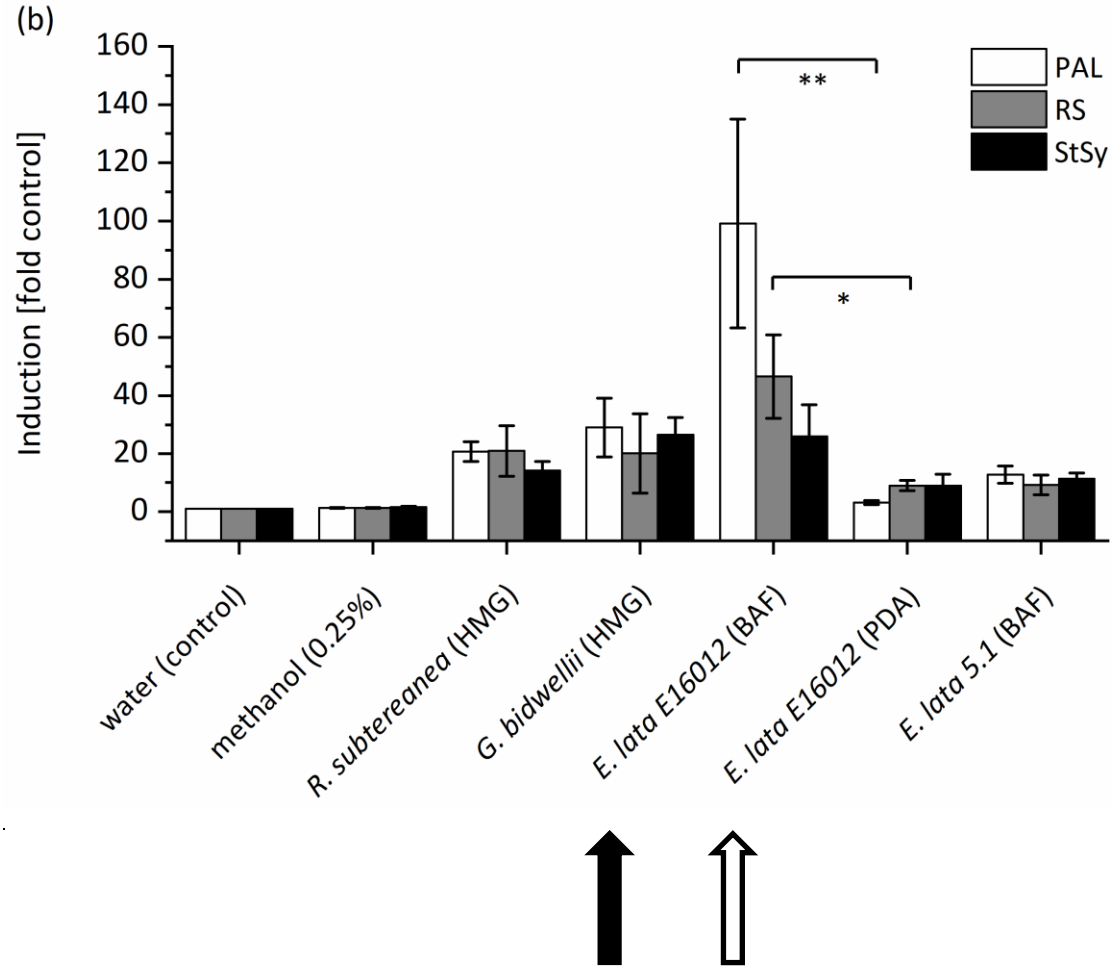
Table 1 Extracellular alkalinisation of *V. rupestris* cells in the presence of fungal culture filtrates. The ΔpH was recorded after 20 min of suspension cells incubated with 25 µg/ml culture filtrates, 0.25% methanol as the solvent control, water as the control. The experiments were repeated at least five times.

Medium	Organismus	ΔpH (after 20 min)
water	--	0
methanol	--	-0.0055
HMG	*Phaeomoniella chlamydospora*	0.60
HMG	*Phaeoacremonium aleophilum*	0.54
HMG	*Eutypa lata*	0.55
HMG	*Fomitiporia mediterranea*	0.21
HMG	*Botrytis cinerea*	0.24
HMG	*Roesleria subterranea*	0.13
MEM	*Guignardia bidwellii*	0.24
BAF	*Eutypa lata* E16012	0.40
PDA	*Eutypa lata* E16012	0.39
BAF	*Eutypa lata* 5.1	0.22
PDA	*Eutypa lata* 5.1	0.24
BAF	*Eutypa lata* 5	0.01
PDA	*Eutypa lata* 5	0.05
BAF	*Eutypa lata* HKM2	0.14
PDA	*Eutypa lata* HKM2	0.22

3.1.2 Fungal culture filtrates induce expression of defence genes

As a second readout for defence, the activation of phytoalexin-synthesis genes was monitored. All filtrates activated a strong (up to 77-fold in case of *F. mediterranea*) increase in steady-state transcript levels for *PAL*, *RS*, and *StSy* (**Fig. 3**). In some cases (in almost all accessions from *E. lata*), the expression of *StSy* was significantly lower than that seen for *PAL*, or *RS*. Among those samples, a particular pair was interesting, because, here, the expression of phytoalexin genes was strongly modulated by differences in the media: for *Eutypa lata* IBWF E16012, these transcripts were strongly induced during cultivation with BAF medium, while the same strain produced a much lower transcript level (**Fig. 3b**, black and white arrows), when cultivated on PDA. Interestingly, the extracellular alkalinisation induced by these two extracts was similar (**Tab. 1**). Nevertheless, the response of phytoalexin genes differed, whereby the response to culture filtrate obtained from BAF-cultivated fungi was much stronger than that seen for PDA. The amplitude of this difference was dependent on the investigated transcript, though: For *PAL* transcripts, the difference between the media was almost one order of magnitude, for *RS* still around a factor of 5, and even for *StSy* around 3. We, therefore, decided to use this contrasting pair of culture filtrates for activity-guided fractionation of immunity modulators, because here the same fungal strain obviously produced quite different immunity modulator activities, depending on the conditions.


(a)
Induction [fold control]
160
140
120
100
80
60
40
20
0
PAL
RS
StSy
water (control)
methanol (0.25%)
P. chlamydospora (HMG)
P. minimum (HMG)
E. lata (HMG)
F. mediterranea (HMG)
B. cinerea (HMG)


(b)
Induction [fold control]
160
140
120
100
80
60
40
20
0
**
*
PAL
RS
StSy
water (control)
methanol (0.25%)
R. subtereanea (HMG)
G. bidwellii (HMG)
E. lata E16012 (BAF)
E. lata E16012 (PDA)
E. lata 5.1 (BAF)

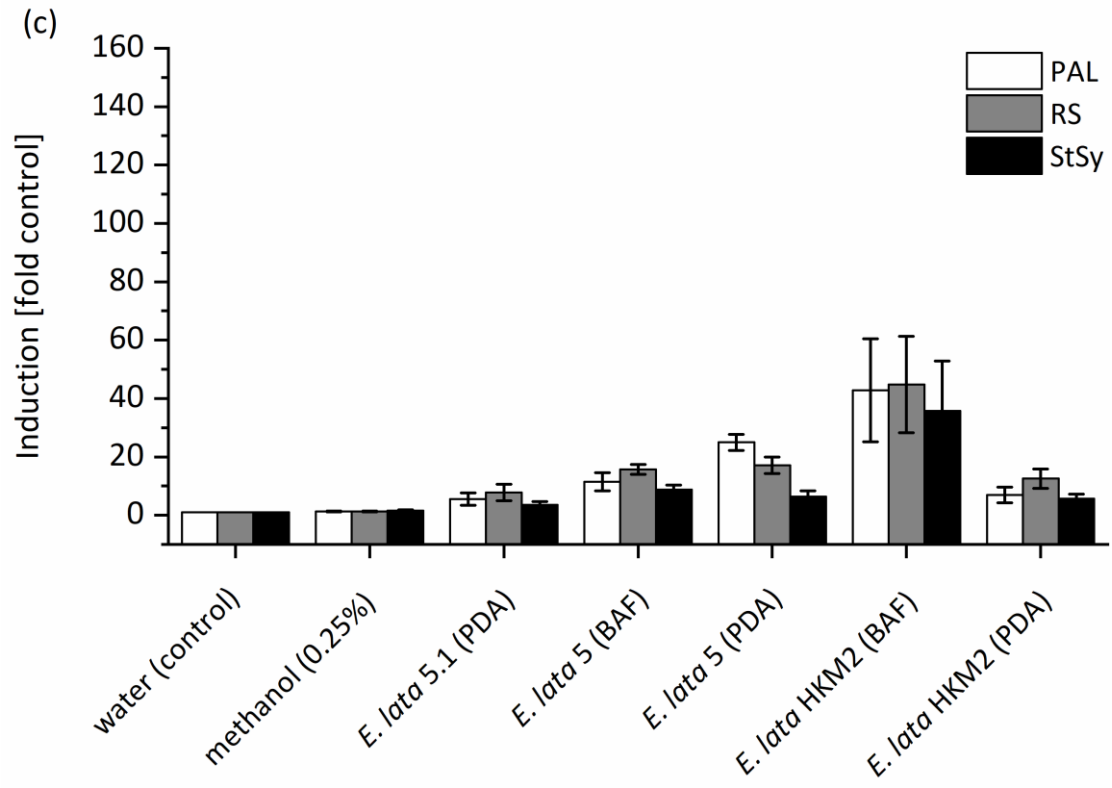


Figure 3 Phytoalexin-synthesis genes expression of different cultural extract treatments in *V. rupestris* cells. The treatment concentration of fungal cultural filtrates was 25 µg/ml cultural filtrates, 0.25% methanol and water acted as the solvent control and the control, respectively. Error bars stand ± standard error (SE) of the mean; *, P< 0.05; **, P< 0.01 (Student's t-test), n=3.

3.1.3 Culture filtrate *Eutypa lata* E16012 (BAF) induces microtubule depolymerisation

The reorganisation of host cell microtubules has been reported as rapid response to infection by oomycetes or fungi (Hardham et al., 2008; Hoefle et al., 2011; Takemoto et al., 2003). To test, whether secondary metabolites secreted by *Eutypa lata* IBWF E16012 can evoke a microtubular response, we followed microtubules after addition of the two culture filtrates along with solvent control (0.25% methanol). For this solvent control, microtubules became thinner and less continuous, but maintained their overall integrity, even if viewed 35 min after addition of the solvent (**Fig. 4a**). The same was observed, if extracts obtained from cultures raised in PDA medium were applied (**Fig. 4c**). In contrast, microtubules were significantly disintegrated and strong aggregations of fluorescent signals appeared in the cell centre in response to extracts obtained from cultures raised in BAF medium (**Fig. 4b**). Quantification of the microtubule response over time (**Fig. 4d**) showed around 10% reduction in average microtubule diameter (caused by the gaps of microtubule continuity) for the treatment with solvent or PDA extract, while for BAF extract, the score dropped to around half of the initial value. Thus, the extract from BAF-cultivated *E. lata* was not only active with respect to phytoalexin-related transcripts, but also with respect to microtubule remodelling.

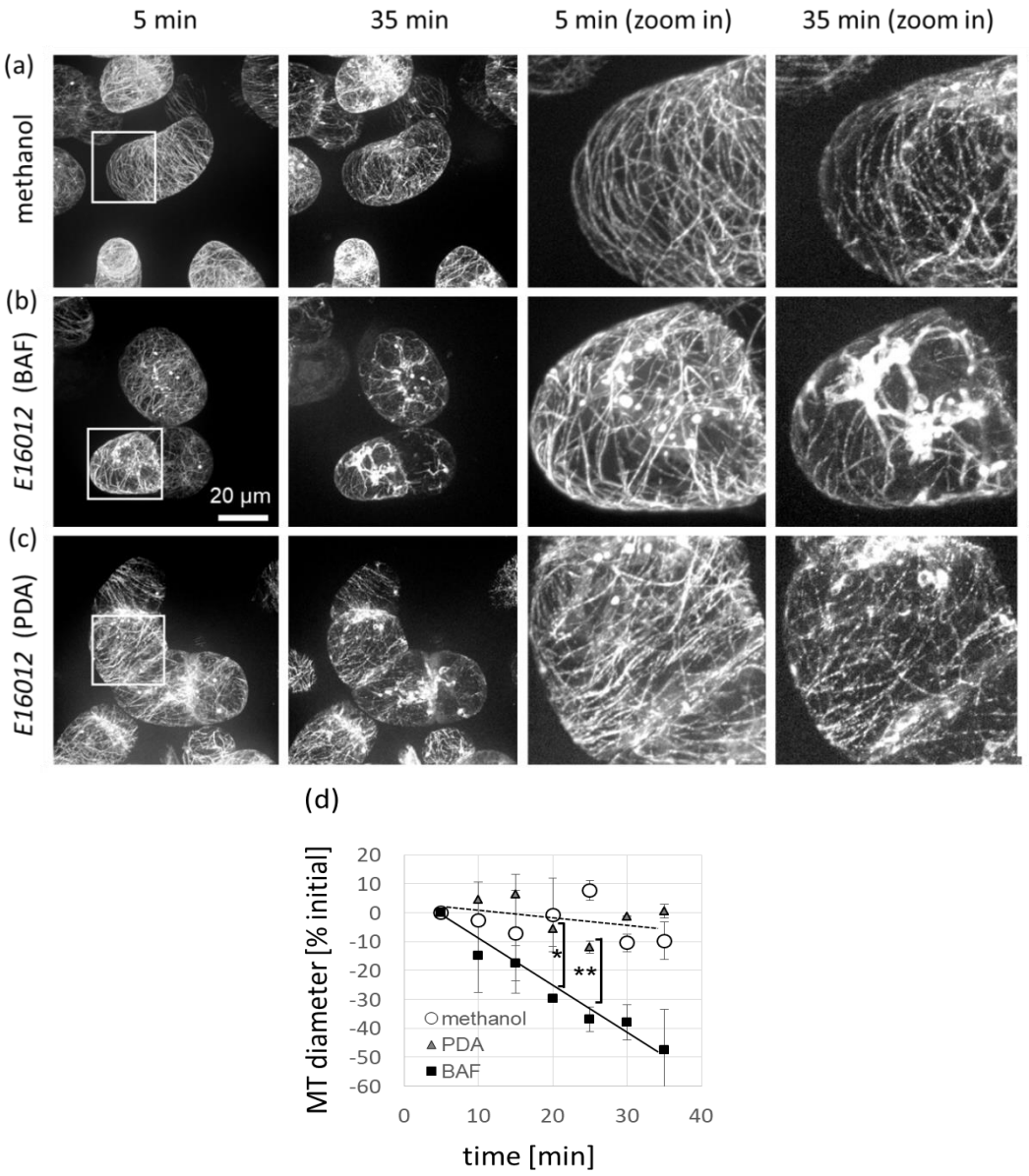


Figure 4 Microtubular responses to culture filtrates of *Eutypa lata* IBWF E16012 in the cells of *V. rupestris* expressing the fluorescent tubulin marker GFP-*At*TUB6. Microtubules in the cells of *V. rupestris with* 0.25% methanol solvent control (a), 25 µg/ml E16012 (BAF) filtrate (b) and 25 µg/ml E16012 (PDA) filtrate (c) for 5 min and 35 min. For each treatment, a representative confocal section from a z-stack along with the time course (5, 10, 15, 20, 25, 30, 35 min) of the same cell, microtubules fusion with GFP for visualisation, and zoom-in of microtubule were shown. (d) The alteration of microtubule thickness in relative units along with the time course of the treatments. Error bars stand ± standard error (SE) of the mean; *, P< 0.05; **, P< 0.01 (Student's t-test), n=3. Observations are representative of at least four independent experimental series with a population of 50 individual cells for each treatment. Bars: 20 µm.

3.2 Activity-guided fractionation of *Eutypa lata* E16012 filtrates leads to the isolation of secondary metabolites

3.2.1 Activity-guided fractionation of IBWF E16012 culture filtrates

Since the strain *Eutypa lata* IBWF E16012, if cultivated in BAF medium, secreted compounds that efficiently activate phytoalexins transcripts along with a microtubular response, while the same strain was not producing this bioactivity, if cultivated in PDA medium, we used these contrasting conditions of the same biological material for a bioactivity-guided fractionation strategy.

We, therefore, fractionated the two filtrates IBWF E16012 (BAF) and IBWF E16012 (PDA) by preparative HPLC and collected the fractions in 96-well plates. The bioactivity of these fractions was then assessed by measuring steady-state transcript levels of genes involved in phytoalexins synthesis (*PAL*, *RS*, *StSy*) along with *JAZ1* as readout for the activity of basal immunity (Chang et al., 2017) in *V. rupestris* cells as reporter (**Suppl. Fig. S1, S2**). To identify those fractions that differed in bioactivity between the two culture filtrates, the gene expression-obtained for the fraction generated from the more active BAF-derived extract was calibrated against the value observed for the same fraction in the less active PDA-derived extract. When these changes in bioactivity were projected upon the elution profiles (**Fig. 5a**), two major hotspots of bioactivity emerged: (1) The fractions eluting earlier than 6.5 min produced stronger activation of *StSy*, if these fractions originated from the BAF culture filtrate. (2) The fractions eluting between 9 and 13 min produced a stronger activation for *PAL* (to a lesser extent also of *StSy*), if coming from the BAF culture filtrate. Since *PAL* was the gene activity, where the bioactivity of the two cultures contrasted most (**Fig. 3b**), the second hotspot was then subjected to closer scrutiny, searching for molecular components that might correlate with bioactivity.

In several cases, the identification of molecular candidates correlating with gene activation was achieved (**Fig. 5**) by subsequent mass spectrometry. Of special interest was a peak eluting at 9.05 min, because this peak was significantly larger in case of

the BAF elution profile (1250 mAU) as in case of the PDA profile (950 mAU). The lead compound identified in this peak, was O-methylmellein (**Fig. 5a, b,** 1). The subsequent peak, eluting between 9.6 and 9.8 min was instead more similar between the two culture filtrates and contained siccayne and eutypinol (**Fig. 5a, b**, 2). Additional molecular candidates, such as FS E16012-4, FS E16123-1, FS E16123-3, or FS E16123-5 (**Fig. 5a, b,** 3) were isolated, but could not be structurally elucidated (**Suppl. Tab. S2, S3**),.

Since the peak harbouring O-methylmellein differed significantly in abundance between the corresponding fractions from the two culture filtrates, and since this difference was correlated with a differential activation of *PAL*, as first committed step of the phenylpropanoid pathway giving rise to lignin as well as to stilbenes, we wondered, whether O-methylmellein acted as elicitor, or whether it might act, in concert with other compounds present in the fraction, as amplifier.

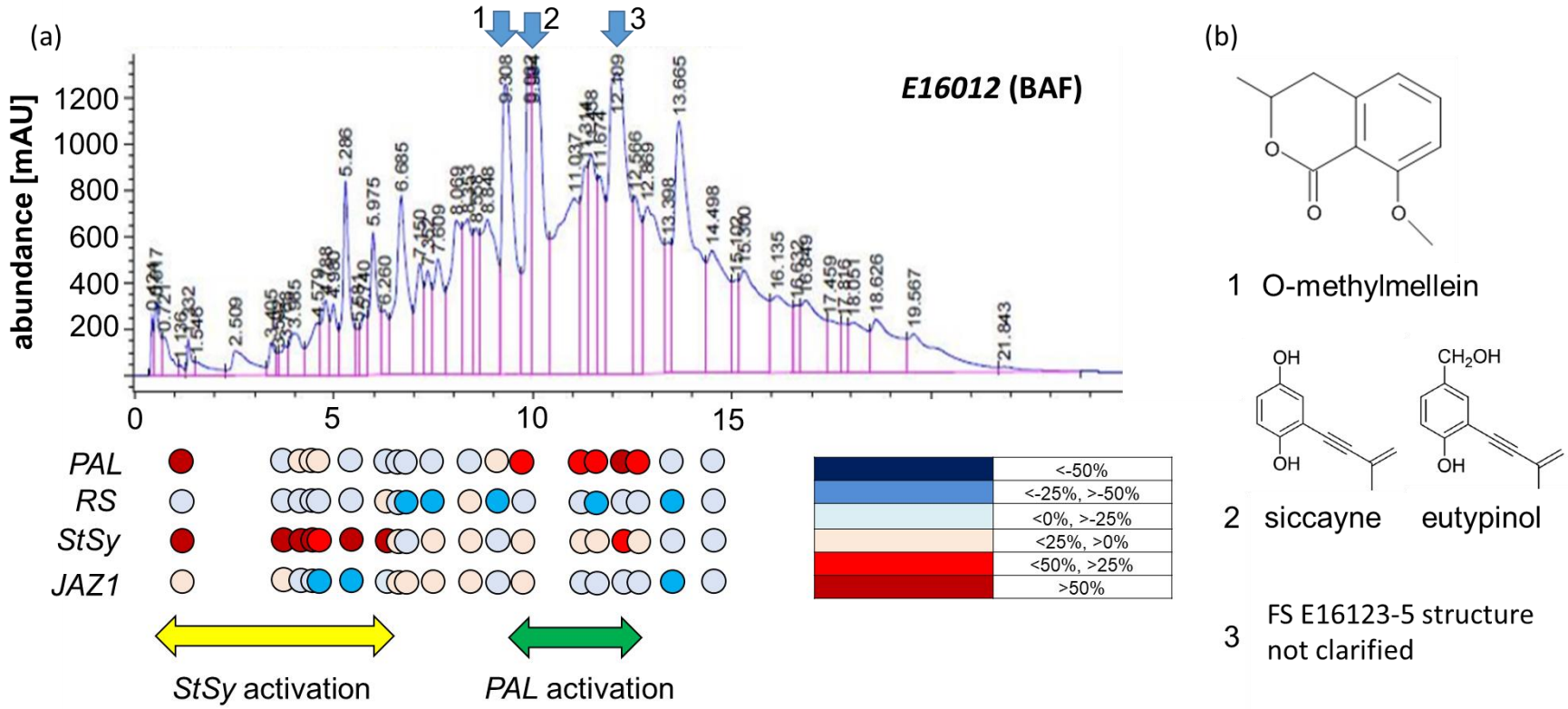


Figure 5 Fractionation of the *Eutypa lata* IBWF E16012 (BAF) culture filtrate and the effect of fractions on the expression of phytoalexins genes.

(a) The composition of E16012 (BAF) culture filtrate was fractionated by the HPLC along with time. Elta (BAF-PDA)/PDA in % showing the stimulation due to medium change, elution time interpolated from the two runs. (b) Secondary metabolites identified from *Eutypa lata* IBWF E16012 extracts: (1) O-methylmelleim; (2) siccayne and eutypinol; (3) FS E123-5; The relative induction levels were expressed by using different colours: dark blue: <-50%; blue: <-25%, >-50%; light blue: <0%, >-25%; light red: <25%, >0; red: <50%, >25%; dark red: >50%.

3.2.2 The candidate modulator O-methylmellein and grapevine defence

3.2.2.1 O-methylmellein is not acting as an elicitor

If O-methylmellein was active as an elicitor, it should, when administered as a pure compound, induce hallmarks of basal immunity, such as activation of calcium influx, or the activation of phytoalexin synthesis genes as compared to other defence-related genes.

Firstly, we measured the effect of different concentrations of O-methylmellein and corresponding concentrations of the solvent methanol on extracellular alkalinisation in *V. rupestris* cells as a readout for a potential activation of calcium influx (**Suppl. Fig. S3**). O-methylmellein could not induce any extracellular alkalinisation that significantly exceeded the (relatively weak) response triggered by the solvent methanol alone, even for a concentration as high as 100 µM (**Suppl. Fig. S3**). In order to validate this finding, the bacterial elicitor flg22 (1 µM) was used as a positive control along with 0.25% methanol and 25 µM O-methylmellein to trigger extracellular pH change (Chang and Nick, 2012). As expected, the flg22 triggered a rapid and significant extracellular alkalinisation, up to 1.0 in 25 min. However, the O-methylmellein only induced a much weaker pH change, around 0.05 (**Fig. 6a**).

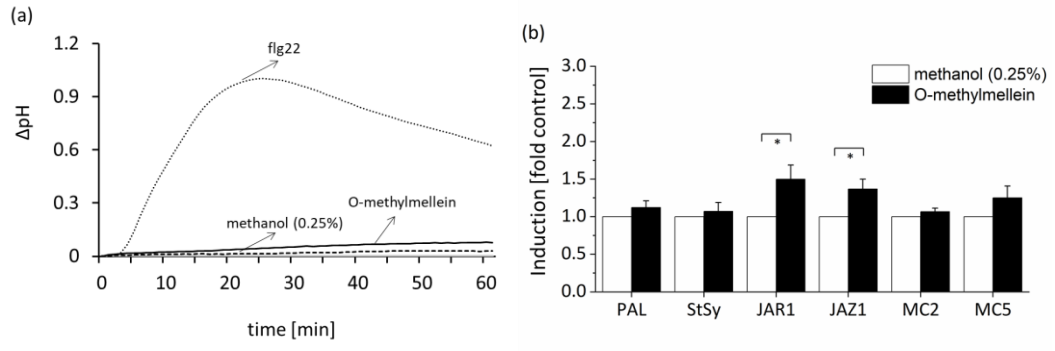


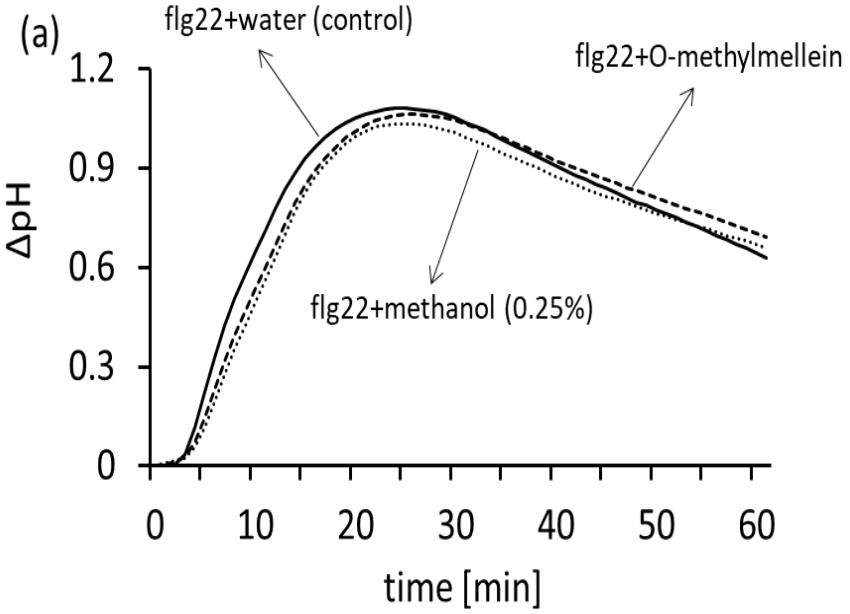
Figure 6 Effect of O-methylmellein on extracellular alkalinisation and induction of defence genes in transgenic *V. rupestris* cell line. (a) Cells were challenged with 25 µM O-methylmellein, 1 µM flg22 as the positive control and 0.25% methanol as the solvent control, respectively. Cellular pH changes (ΔpH) were recorded by the pH meter. (b) Cells were elicited by 25 µM O-methylmellein for 1 hour, 0.25% methanol as solvent control. The expression of defence genes (*PAL, StSy, JAR1, JAZ1, MC2* and *MC5*) was measured by qPCR. Error bars stand ± standard error (SE) of the mean, n=3.

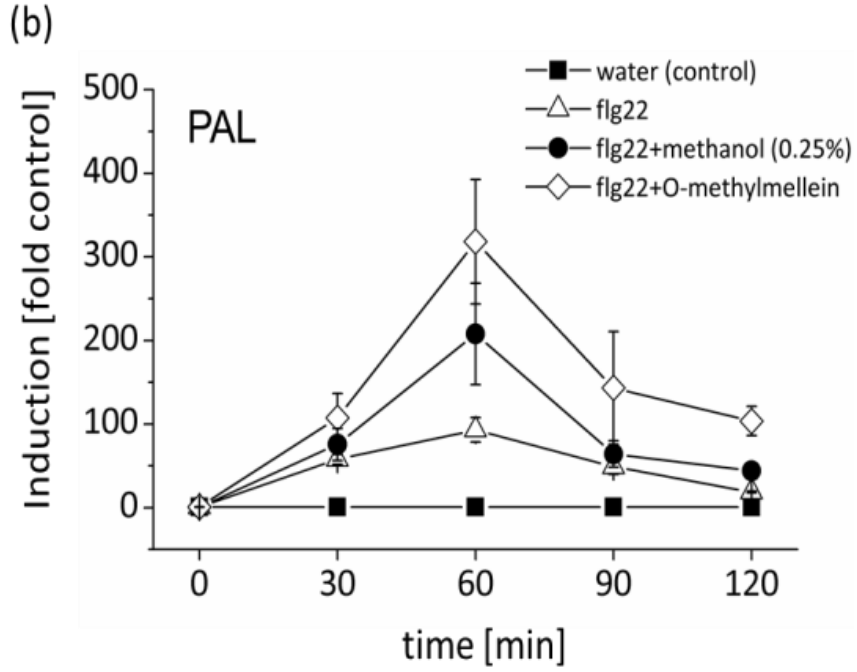
To test, whether O-methylmellein would induce defence-related genes, we measured steady-state transcript levels by real time qPCR for *PAL* (as readout for the activation of the phenylpropanoid pathway), *StSy* (as readout for the activation of the phytoalexin-branch of this pathway), *JAZ1* (as readout for the jasmonate signalling monitoring basal immunity), *JAR1* (as readout for synthesis of active jasmonate), and the metacaspases *MC2*, and *MC5* (as readouts for cell-death related defence) in response to 25 µM O-methylmellein and 0.25% methanol as solvent control, respectively. There was no significant induction for any of these genes, except a slight elevation for *JAZ1* and *JAR1* indicative of a mild activation of basal immunity (**Fig. 6b**). This was in sharp contrast with the strong activation of *PAL* seen for the supernatant from the BAF culture (**Fig. 3b**), and the still considerable induction of this gene observed by the respective HPLC fraction (induction factor over solvent control 4.6±0.534, n=3).

Thus, O-methylmellein neither can induce calcium influx, nor has any significant response of defence-related genes, and therefore does not qualify as elicitor.

3.2.2.2 O-methylmellein amplifies flg22-triggered induction of phytoalexins-synthesis genes, but not extracellular alkalinisation

Some compounds secreted by the pathogen might affect plant immune response indirectly, rather than activating the first level of plant immunity. To test, whether O-methylmellein is able to regulate the basal immune responses stimulated by flg22, we used the same strategy as described above and examined extracellular alkalinisation along with transcript levels of phytoalexins synthesis genes (**Fig. 7**), but administered O-methylmellein (25 µM) in combination with flg22. Control experiments tested the effect of flg22 alone, or in combination with 0.25% methanol (as a solvent for O-methylmellein), and water (as solvent control for flg22). As to be expected, flg22 induced a significant pH response, up to 1.06 in 25 min (**Fig. 7a**), no matter, whether it was combined with O-methylmellein or just with the equivalent concentration of the solvent methanol (0.25%). Therefore, O-methylmellein does not modulate flg22-triggered extracellular alkalinisation in cells of *V. rupestris*.


(a)
flg22+water (control)
flg22+O-methylmellein
flg22+methanol (0.25%)
ΔpH
1.2
0.9
0.6
0.3
0
0
10
20
30
40
50
60
time [min]


(b)
PAL
water (control)
flg22
flg22+methanol (0.25%)
flg22+O-methylmellein
Induction [fold control]
500
400
300
200
100
0
0
30
60
90
120
time [min]

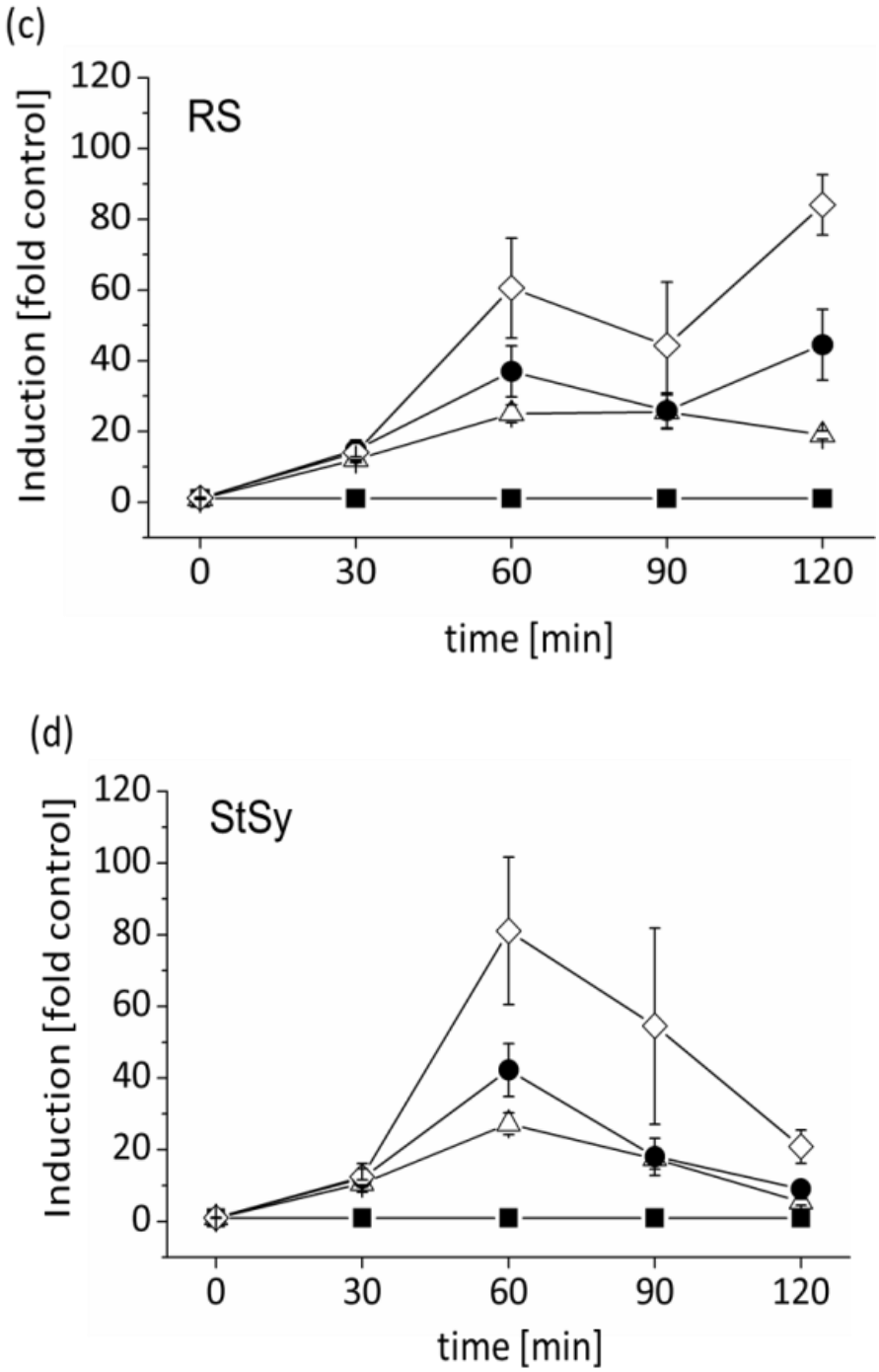


Figure 7 Modulate effect of O-methylmellein on flg22-triggered extracellular alkalinisation and defence genes transcription in transgenic *V. rupestris* cell line. (a) The extracellular pH responses to 1 µM flg22 combined with either water control, 0.25% methanol solvent control or 25 µM O-methylmellein was recorded over time. (b) (c) (d) Cells were treated with 1 µM flg22 alone (white triangle) or combined with either 0.25% methanol solvent control (black round) or 25 µM O-methylmellein (white rhombus) for 0-120 min, water as control (black square). The transcription of genes *PAL* (b), *RS* (c), and *StSy* (d) was measured by qPCR. Error bars stand ± standard error (SE) of the mean, n=3.

Nevertheless, O-methylmellein might, by activation of a pathway that is independent of calcium, modulate the expression of phytoalexin-synthesis genes. Using the same experimental design, the expression of *PAL*, *RS*, and *StSy* was followed over time (**Fig. 7b-d**). As expected, flg22 induced the transcripts for all three genes, detectable already at 30 min after addition of this elicitor, and reaching a peak at 60 min and slowly dissipated for longer timer intervals (**Fig. 7b, c, d**). The induction of *PAL* transcripts (**Fig. 7b**) was more pronounced (around 100-fold) as compared to *RS* (**Fig. 7c**) and *StSy* (**Fig. 7d**) that were both induced around 25-fold. In combination with 0.25% methanol (the solvent control for O-methylmellein), this induction was slightly, but significantly enhanced (for *PAL* by a factor of 2, for *RS* and *StSy* by around a third). Interestingly, O-methylmellein that by itself failed to cause any induction of *PAL* (**Fig. 6b**), was strongly enhancing the response of this transcript to flg22 (**Fig. 7b**): O-methylmellein in combination with flg22 induced *PAL* transcripts 318 fold, much stronger than the 208-fold induction seen for methanol with flg22, or the 93-fold induction obtained for flg22 alone (**Fig. 7b**). Similar enhancements were observed for the other genes, albeit with a different temporal pattern for RS: Here, the decrease of transcript levels after 60 min was replaced by further increase when flg22 was accompanied by O-methylmellein (**Fig. 7c**). For *StSy*, the combined treatment of O-methylmellein with flg22 reached an induction of 81-fold at 60 min, as compared to 42 fold for flg22 with methanol, 27 fold for flg22 alone (**Fig. 7d**). Irrespective of the tested gene and the amplitude of the response to flg22 alone, O-methylmellein enhanced the amplitude of the response to flg22 by a factor of 3. Thus, O-methylmellein interacts with flg22 in a multiplicative manner.

3.2.2.3 The amplifier activity of O-methylmellein is independent of calcium influx, but dependent on RboH

To further explore the amplification of flg22 triggered defence signalling by O-methylmellein, we either blocked calcium influx by 100 µM $GdCl_3$, or inhibited the NADPH oxidase RboH by 10 µM DPI prior to the co-treatment of O-methylmellein and flg22. Again, the induction of phytoalexin-synthesis genes *PAL*, *RS*, and *StSy* was monitored by qPCR. Since DPI has to be dissolved in DMSO, a respective solvent control was included into this experiment. When the Ca^{2+} channels were blocked by

$GdCl_3$, the three genes were only slightly (by about 10-15%) and with the exception of *PAL*, not significantly, suppressed (**Fig. 8**). In contrast, the transcription level of *PAL* and *RS* were strongly inhibited by DPI, down to 0.5 and 0.7, respectively (**Fig. 8**). Interestingly, this inhibition was not observed in the case of *StSy*. Thus, on the level of phytoalexins-gene expression, the amplification of the flg22 response by O-methylmellein is mostly independent of calcium influx, for all three genes. Instead, the expression of *PAL* and *RS* is strongly dependent on RboH activity (which is not the case for *StSy*).

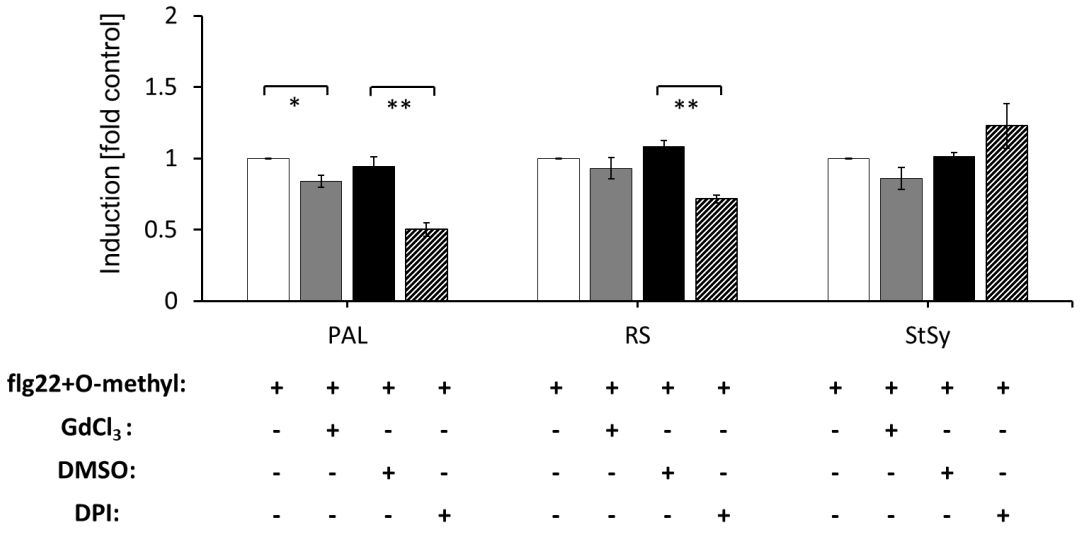


Figure 8 Roles of Gd-sensitive calcium channels and ROS on the O-methylmellein (O-methyl) modulated defence genes transcription triggered by flg22 in transgenic *V. rupestris* cell line.
Cells were challenged by the combination of 1 µM flg22 and 25 µM O-methylmellein alone, or with 100 µM $GdCl_3$ (the calcium channel blocker), 10 µM DPI (the NADPH oxidase inhibitor), 0.1% DMSO as solvent control of DPI. The induction of the genes PAL, RS, StSy was measured by qPCR. Error bars indicate ± standard error (SE) of the mean; *, P< 0.05; **, P< 0.01 (Student's t-test), n=3.

3.2.2.4 O-methylmellein amplifies flg22-triggered actin disassembly, but not microtubules

Motivated by the previous result that the *Eutypa lata* E16012 (BAF) extract, but not the *Eutypa lata* E16012 (PDA) extract induced microtubule depolymerisation (**Fig. 4**),

we wondered, whether O-methylmellein would act on microtubules. The microtubule responses were observed by spinning-disc confocal microscopy after treating the *V. rupestris* TuB6 cells either with flg22, or with O-methylmellein, or combinations thereof for 30 or 60 min. As solvent control, 0.25% methanol was used in a parallel experiment. We found that microtubules were partially depolymerised as compared to the solvent control (**Fig. 9a**), when the cells were incubated with flg22 for 30 or 60 min (**Fig. 9b**). However, O-methylmellein had no effects on microtubules (**Fig. 9c**), and even the combined treatment did not enhance the slight microtubule depolymerisation seen for flg22 alone (**Fig. 9d**). Therefore, O-methylmellein did not mimic the microtubule-depolymerisation activity of the BAF culture filtrate (**Fig. 4**).

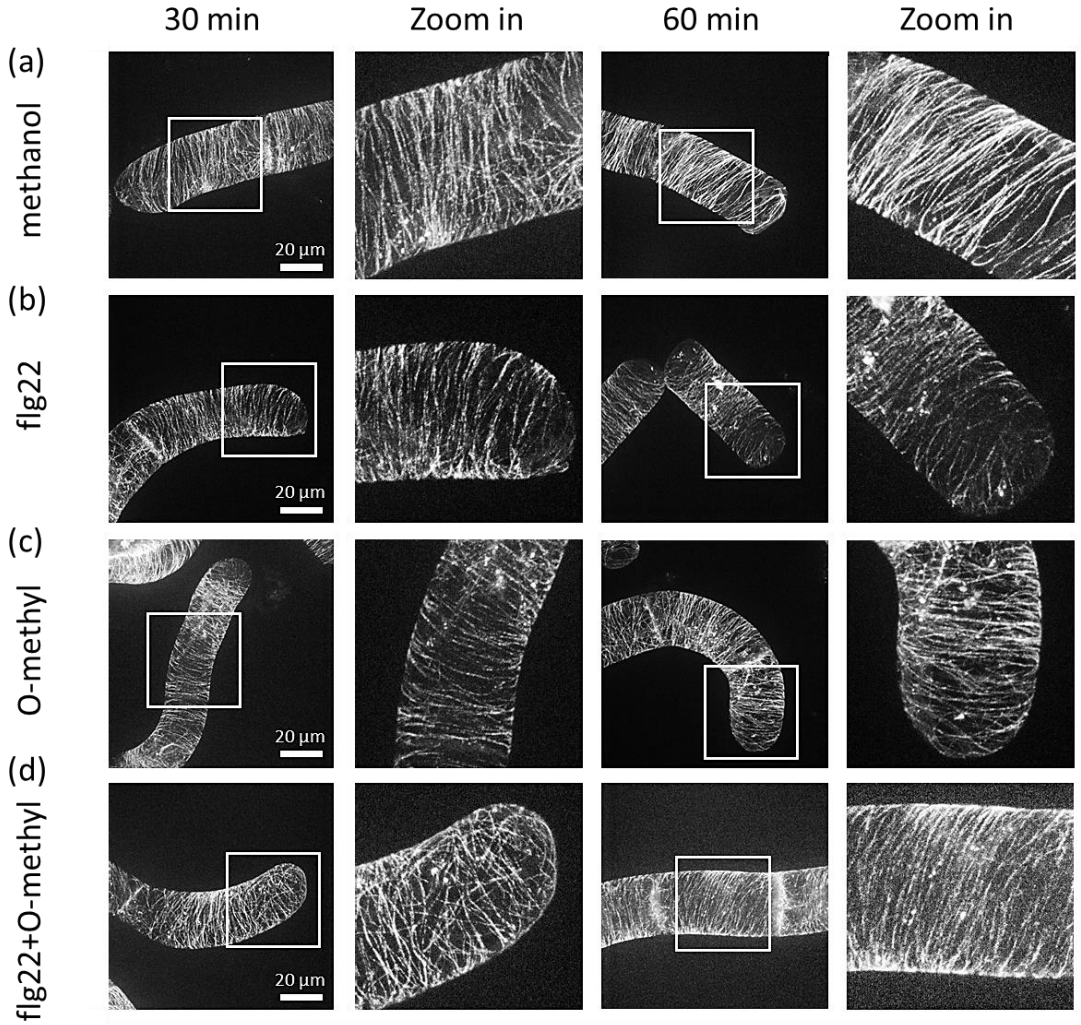


Figure 9 Microtubular responses to flg22 and O-methylmellein (O-methyl) in the cells of *V. rupestris* expressing the fluorescent tubulin marker GFP-*At*TUB6. The response of Vitis cells to the 0.25% methanol solvent control (a), 1 µM flg22 (b), 25 µM O-methylmellein (c) or combined flg22 and O-methylmellein (d) for 30 min and 60 min was observed under the spinning-disc confocal microscopy. For each treatment, a representative confocal section from a z-stack along with different time points (30 min and 60 min), visualisation of GFP fused with microtubule, and zoom-in of microtubule were shown. Observations were representative of at least four independent experimental series with a population of 50 individual cells for each treatment. Bars: 20 µm.

At sites of attempted penetration of fungi and oomycetes, often radial actin bundles were observed (Kobayashi et al., 1997b; Opalski et al., 2005; Takemoto et al., 2003). Moreover, several PAMPs and effectors were found to disrupt the actin cytoskeleton associated with the inhibition of plant defence (Park et al., 2018). We, therefore, repeated the experiment in the same design, but following this time actin filaments by spinning-disc confocal microscopy, making use of a cell line from *V. vinifera* L. cv. 'Chardonnay' expressing the actin marker FABD2–GFP. While in the methanol control, a rich meshwork of fine, cortical actin filaments was observed (**Fig. 10a**), we found that both, flg22 (**Fig. 10b**), and O-methylmellein (**Fig. 10c**) induced a depletion of these cortical strands, while longitudinal actin cables appeared (**Fig. 10b, c**). This response was already detectable at 30 min after induction, and was more prominent after 60 min. This bundling was more pronounced for O-methylmellein as compared to flg22. For O-methylmellein, the bundled cables also contracted upon the nucleus (**Fig. 10c**). This actin reorganisation was strongly enhanced for the combined treatment (**Fig. 10d**). Here, already after 30 min, the cortical meshwork had disappeared almost completely, and the thick cables of actin had already strongly contracted to the nucleus. At 60 min, patches of actin appeared, as they are characteristic for actin-nucleation sites becoming manifest when actin is disassembled by actin-eliminating compounds (Maisch et al. 2009).

Thus, while O-methylmellein is neither acting on microtubules, nor amplifying the (mild) effect of flg22 on microtubules, there is a clear and strong amplification of the flg22 effect upon actin filaments.

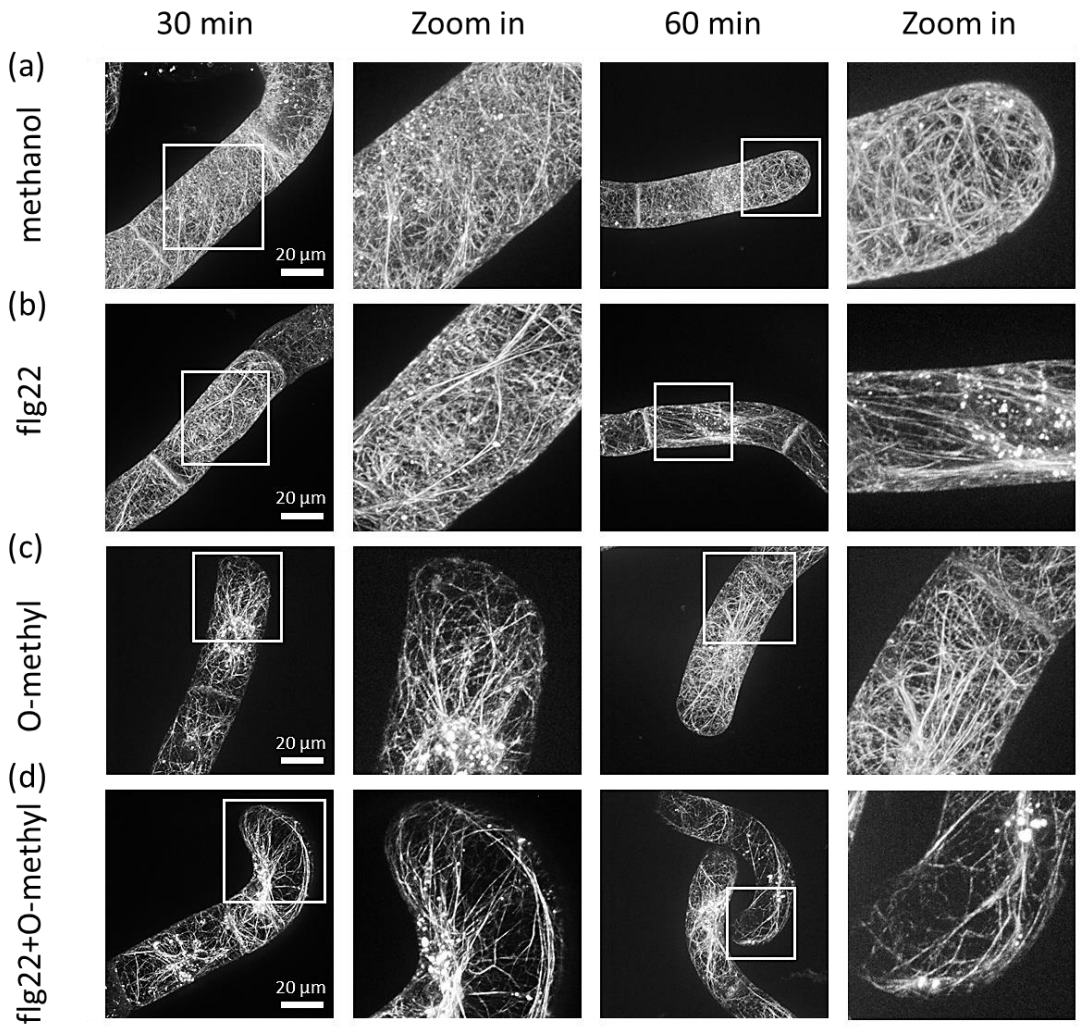


Figure 10 Responses of actin filaments to flg22 and O-methylmellein (O-methyl) in the cells of *V. vinifera* L. cv. 'Chardonnay' expressing the actin marker FABD2–GFP. The *V. vinifera* cells were treated either with the 0.25% methanol solvent control (a), 1 µM flg22 (b), 25 µM O-methylmellein (c) alone or combined (d) for 30 min and 60 min. The zoom-in of actin filaments in different time points (30 min and 60 min) was shown. Observations were representative of at least four independent experimental series with a population of 50 individual cells for each treatment. Bars: 20 µm.

3.2.2.5 O-methylmellein is not fungal phytotoxic for grapevine cell

The outbreak of GTD symptoms is already discussed to be caused by the secretion of fungal phytotoxins. Mellein and its derivatives had been reported to induce leaves necrosis in *Vitis vinifera* (Djoukeng et al., 2009). We also probed for potential cell mortality induced by O-methylmellein. The *V. rupestris* TuB6 cells were treated with 1 µM flg22, 25 µM O-methylmellein, or combined for 6 h, 12 h, 24 h, 48 h, and 72 h. 0.25% methanol as solvent control. The cell mortality was measured by using the Evans Blue assay. Compared with solvent control, there were no significant differences in cell mortality in the treatment of O-methylmellein or O-methylmellein combined with flg22 over time **(Fig. 11)**. For example, after 48 h, the cell mortality was around 5.3% in methanol treatment, and 8.6%, 7.6%, 7.2% in the treatments of flg22, O-methylmellein or combined flg22 with O-methylmellein, respectively **(Fig. 11)**. After 72 h, the cell mortality induced by flg22 was yet downregulated from 10% to 6.6% **(Fig. 11)**. The results showed that O-methylmellein has no toxic effect on grapevine cells in 25 µM.

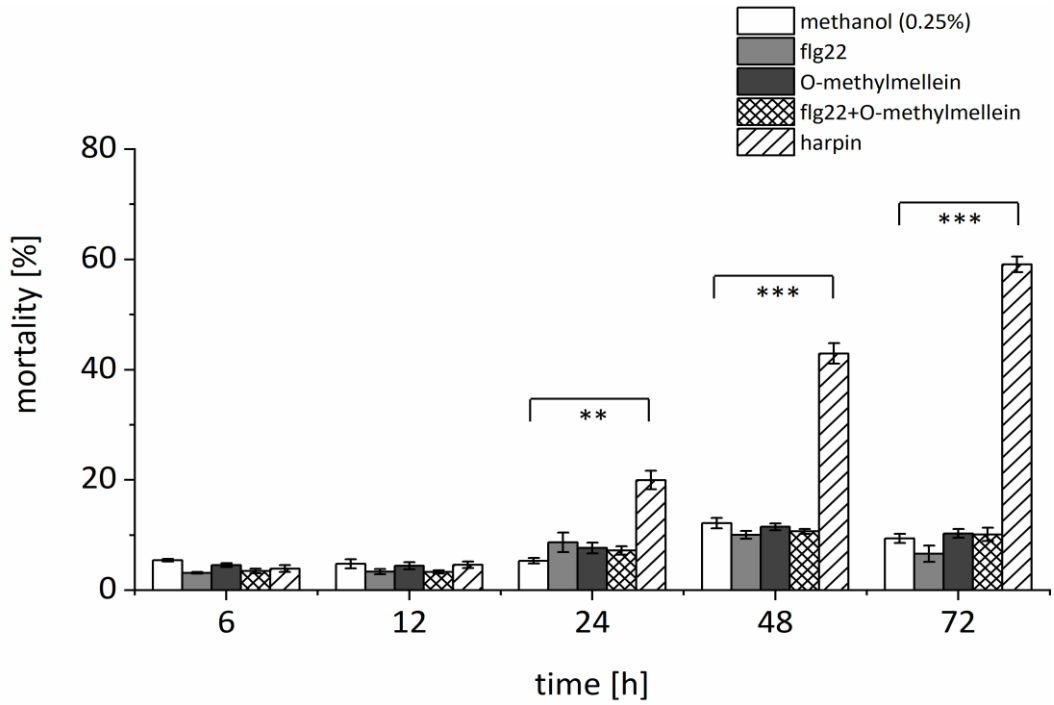
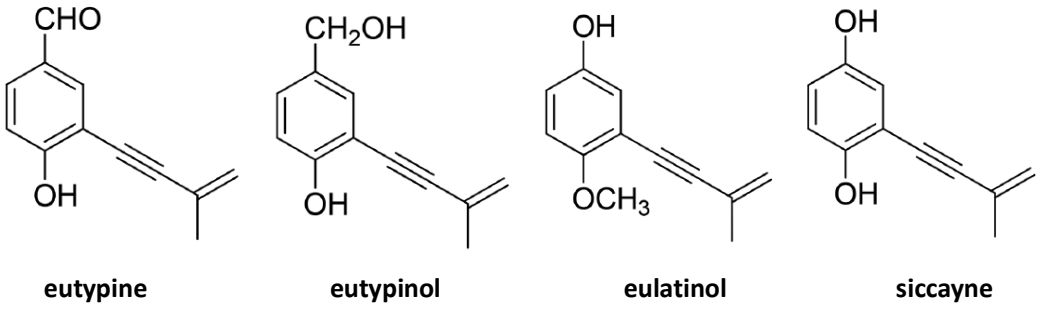


Figure 11 Effect of flg22 and O-methylmellein on cell mortality in transgenic *V. rupestris* cell line. Cells were incubated with 1 µM flg22, 25 µM O-methylmellein, combined flg22 and O-methylmellein using 0.25% methanol as solvent control or 9 µg/ml harpin as the positive control along with the time (6 h-72 h). Error bars indicate ± standard error (SE) of the mean; *, P< 0.05; **, P< 0.01; *** P< 0.001 (Student's t-test), n=3.

3.3 Eutypinol and its analogues produced by *Eutypa lata* induce grapevine defence

Since eutypinol and siccayne have also been identified from *Eutypa lata* IBWF E16012 culture extracts, we further analysed their effects on grapevine defence (**Suppl. Tab. S2**, **S3**; **Fig. 5**). Because of the similar chemical structure of eutypinol and siccayne, it was assumed that they may have similar biological activity and can be recognised by the same cell-surface receptor. To identify this hypothesis, we further employed the other two *Eutypa lata* metabolites that have a similar chemical structure with eutypinol: eutypine and eulatinol (**Fig. 12**). These metabolites are usually found in secretions of *Eutypa lata* fungi (Masi et al., 2018). However, their effects on grapevine cellular signal signatures are less investigated.



eutypine **eutypinol** **eulatinol** **siccayne**

Figure 12 Structure of metabolites produced by *Eutypa lata* fungi involved in GTDs (Masi et al., 2018).

3.3.1 Eutypine elicits calcium influx, but its analogue eutypinol cannot

To evaluate the effect of those metabolites on calcium influx, we treated *V. rupestris* cells with 10 µM and 25 µM eutypine, eutypinol, eulatinol and siccayne, 0.1% and 0.25% methanol as the solvent control and measured the extracellular pH changes by pH meter. Only eutypine induced an obvious and quick pH change rather than other metabolites, around 0.16 (10 µM) and 0.66 (25 µM) after 20 min (**Tab. 2**). Neither eutypinol, eulatinol nor siccayne could activate extracellular pH changes (**Tab. 2**). We concluded that eutypine activates calcium influx.

Table 2 Extracellular alkalinisation of *V. rupestris* cells to different fungal metabolites. Cells were treated with eutypine, eutypinol, eulatinol and siccayne in two concentrations (10 µM and 25 µM), 0.1% and 0.25% methanol as the solvent control, respectively. After 20 min, the cellular ΔpH was recorded. Data were from at least five repetitions.

Metabolites	Concentrations	ΔpH (after 20 min)
methanol	0.1%	0.01
	0.25%	0.04
eutypine	10 µM	**0.16**
	25 µM	**0.66**
eutypinol	10 µM	-0.02
	25 µM	0.08
eulatinol	10 µM	-0.01
	25 µM	0.01
siccayne	10 µM	-0.03
	25 µM	-0.04

3.3.2 Eutypine induces the expression of defence genes

To identify whether the calcium influx induced by *Eutypa lata* metabolites was related to the expression of defence genes, we treated Vitis cells with 10 µM eutypine, 10 µM eutypinol, 10 µM eulatinol and 10 µM siccayne for 1 hour, 0.1% methanol as the solvent control and the transcription of defence genes *PAL*, *RS*, *StSy* and *JAZ1* was measured by qRT-PCR. We found that eutypine induced significant transcription of the genes *PAL* and *RS*, up to 12 fold (**Fig. 13**). The induction of the gene *StSy* was slightly induced but also activated to 8 fold (**Fig. 13**). Eutypinol could not induce the transcription of those defence genes and even suppressed the induction level of gene *JAZ1*, to 0.7 (**Fig. 13**). Eulatinol and siccayne have fewer effects on expression of these genes (**Fig. 13**). Only siccayne slightly induced the transcription of RS, around 1.8 fold (**Fig. 13**). Although those metabolites produced by *Eutypa lata* fungi have very similar chemical structure, their effects on the expression of basal immunity were greatly different. Those results indicated that the

side groups of the phenolic ring of eutypinol and its analogues might play a role in activating grapevine basal immune responses.

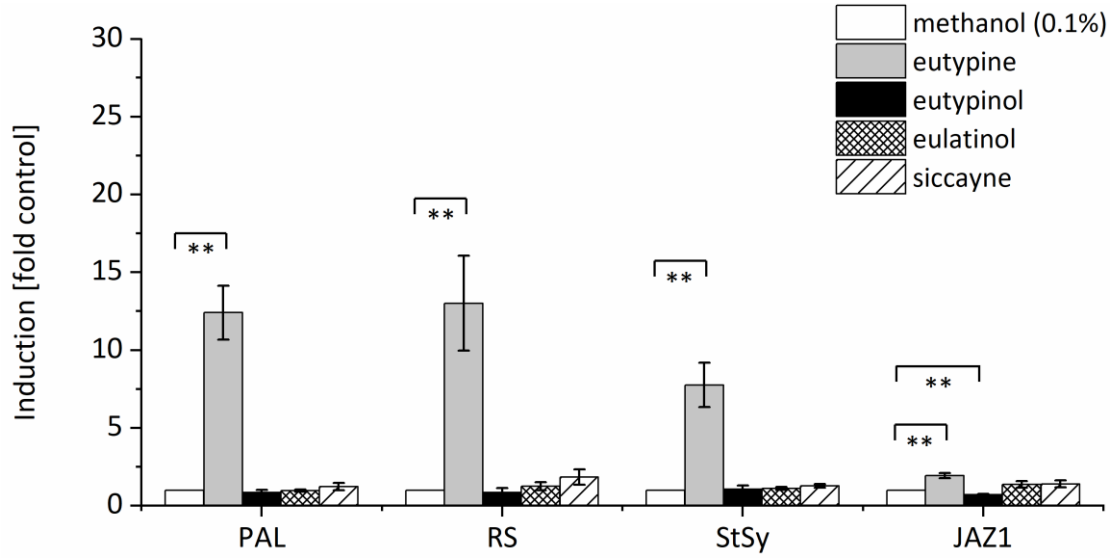


Figure 13 Expression of defence genes induced by secondary metabolites produced by *Eutypa lata* fungi in transgenic *V. rupestris* cell line. Cells were treated with 10 µM eutypine, 10 µM eutypinol, 10 µM eulatinol, 10 µM siccayne using 0.1% methanol as solvent control for 1 hour. The induction of defence genes (*PAL*, *RS*, *StSy*, *JAZ1*) was measured by qPCR. Error bars indicate ± standard error (SE) of the mean; *, P< 0.05; **, P< 0.01 (Student's t-test), n=3.

3.3.3 Eutypinol, eutypine and siccayne induce obvious cell mortality

Due to the obvious toxic effect of eutypine on leaves found in the previous research, eutypine was reported as one of the important toxins for causing grapevine foliar symptoms of GTDs (Tey-Rulh et al., 1991). However, a recent study has identified that eutypine was not the product of some pathogenic *Eutypa lata* (Mahoney et al., 2005). In contrast, the eutypinol was verified not to be a phytotoxin and found in almost all the pathogenic *Eutypa lata* fungi (Lardner et al., 2006; Mahoney et al., 2005). Besides, siccayne and eulatinol were also reported not to be toxic for grapevine leaves (Mahoney et al., 2003). In addition, the toxicity of fungal metabolites might be related to grapevine immunity induced by PAMPs or effectors, like the hypersensitive response. Therefore, we tested their toxicities on grapevine cells by using the same concentration of 10 µM for 24 hours, 48 hours, and 72 hours.

We found that eutypine could induce significant cell mortality after 24 hours, around 7% (**Fig. 14**). And the cell death peaked after 48 hours of treatment, up to 16% (**Fig. 14**). To our surprise, eutypinol and siccayne also induced cell mortality significantly. For example, after 48 hours, the cell mortality induced by eutypinol was around 13% (**Fig. 14**), in the treatment with siccayne, it was 11% (**Fig. 14**). But eulatinol had a less toxic effect on grapevine cells at the concentration of 10 µM. These results showed that eutypine, eutypinol and siccayne all induced mortality in grapevine cells, and they might play a role in the formation of foliar symptoms caused by *Eutypa lata* fungi involved in GTDs.

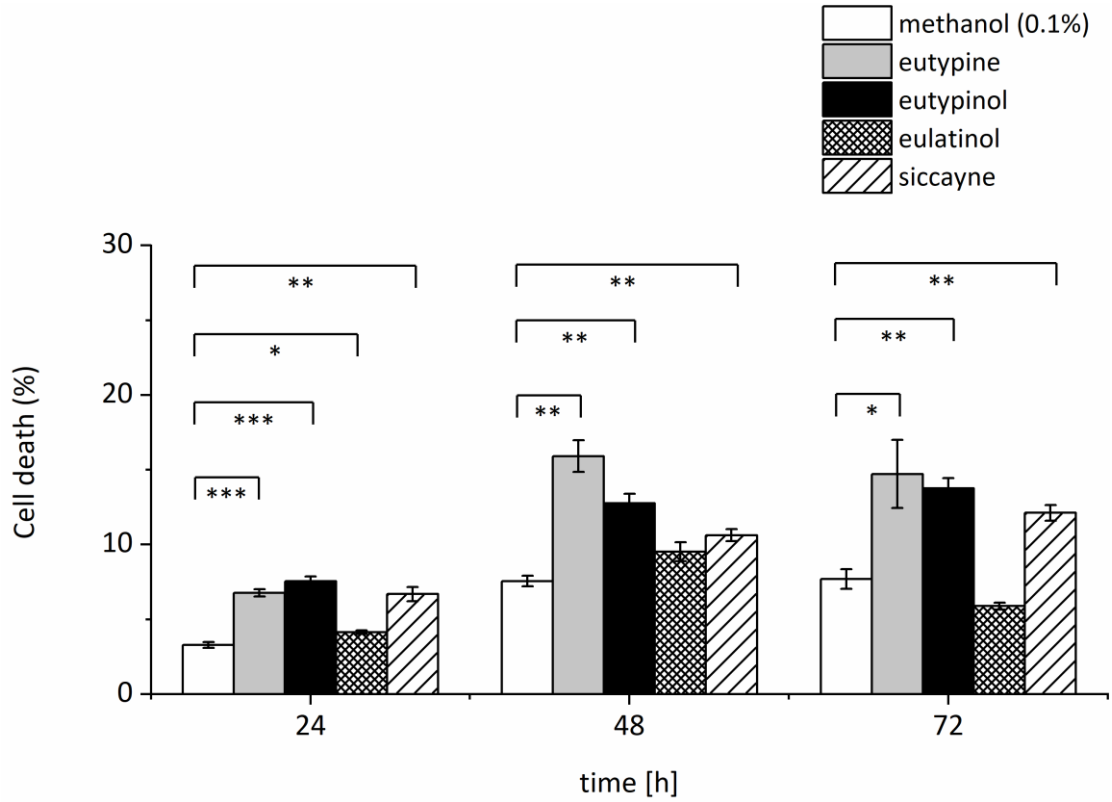


Figure 14 Effect of secondary metabolites produced by *Eutypa lata* fungi on cell death in transgenic *V. rupestris* cell line. Cells were incubated with 10 µM eutypine, 10 µM eutypinol, 10 µM eulatinol, 10 µM siccayne and 0.1% methanol as solvent control for 24 hours, 48 hours and 72 hours. The cell mortality was measured by using the Evans blue staining assay. Error bars indicate ± standard error (SE) of the mean; *, P< 0.05; **, P< 0.01; ***, P< 0.001 (Student's t-test), n=3.

3.3.4 Eutypinol degrades cortical microtubules

The reorganisation of the microtubule network was reported to be associated with cell-death elicitor (like bacterial elicitor harpin) treatments or ETI. So, the microtubule response was used as a quick readout of cellular signals induced by PAMPs or modulators. Since previous results showed that eutypine, eutypinol and siccayne induced clear cell death (**Fig. 14**), we assumed that some of them might be cell-death elicitors or effectors. For example, eutypine, which could induce grapevine basal immunity and cell death (**Fig. 13, 14**), might be an elicitor candidate, or eutypinol, which inhibited the induction of gene *JAZ1* and induced cell mortality (**Fig. 13, 14**), might be an effector. Thus, we incubated *Vitis* cells of *V. rupestris* expressing the fluorescent tubulin marker GFP-*At*TUB6 with 10 µM eutypine, 10 µM eutypinol and 10 µM siccayne for 30 min and 60 min, 0.1% methanol was used as the solvent control. The microtubule network was observed under confocal spinning-disc microscopy.

Cortical microtubules were slightly depolymerised after 30 min of eutypinol treatment (**Fig. 15c**). In 60 min, almost all the cortical microtubules disappeared and central microtubules bundled (**Fig. 15c**). In the treatment of 10 µM eutypine, we also observed that cortical microtubules disrupted but less depolymerisation occurred in two time points (**Fig. 15b**). However, siccayne did not affect the microtubule network (**Fig. 15d**). These results indicated that eutypine and eutypinol could disrupt the cortical microtubules but they showed different effectiveness.

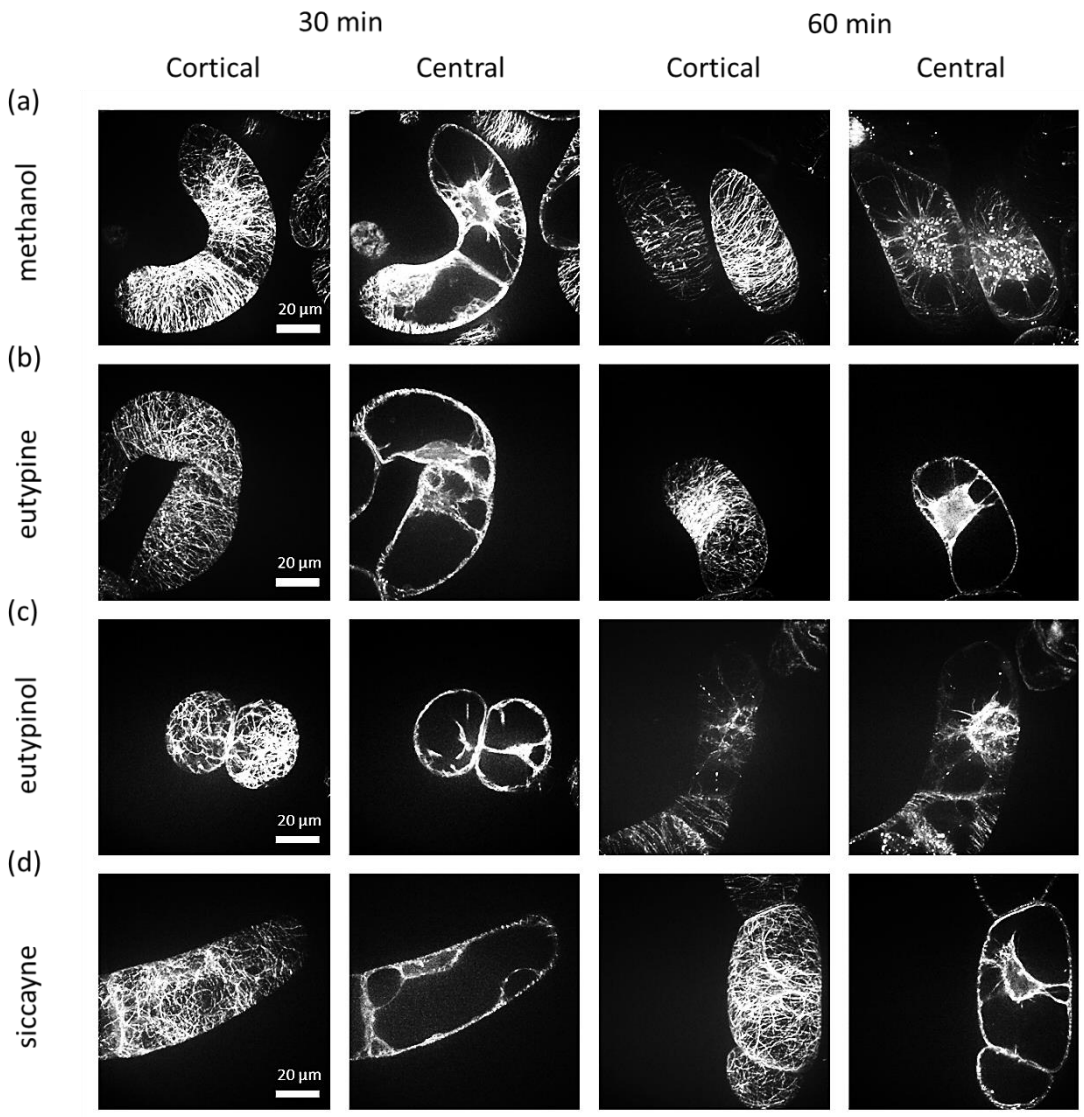


Figure 15 Microtubular responses to secondary metabolites produced by *Eutypa lata* fungi in cells of *V. rupestris* expressing the fluorescent tubulin marker GFP-AtTUB6. Cells were treated with either 0.1% methanol solvent control (a), 10 µM eutypine (b), 10 µM eutypinol (c), 10 µM eulatinol or 10 µM siccayne (d) for 30 min and 60 min. For each treatment, a representative confocal section from a z-stack along with two time points: 30 min and 60 min, visualization of GFP fused with microtubule, cortical and central microtubules were shown. Observations are representative of at least four independent experimental series with a population of 50 individual cells for each treatment. Bars: 20 µm.

3.3.5 The microtubule depolymerisation induced by eutypinol can be restored

Because of the significant microtubule depolymerisation was observed in the treatment with eutypinol 30 min and 60 min (**Fig. 15c**), we further treated the cells with eutypinol for 8 hours to check if the microtubules can be restored. Interestingly, the disappeared microtubules induced by eutypinol in 60 min and return back in 8 hours (**Fig. 16b**). There were fewer differences in cortical microtubules between eutypinol treatment and methanol control. Compared with solvent control, eutypinol induced more microtubule polymerizations. The rehabilitation of the cortical microtubule network indicated that microtubule might act as a signal in defence responses induced by eutypinol.

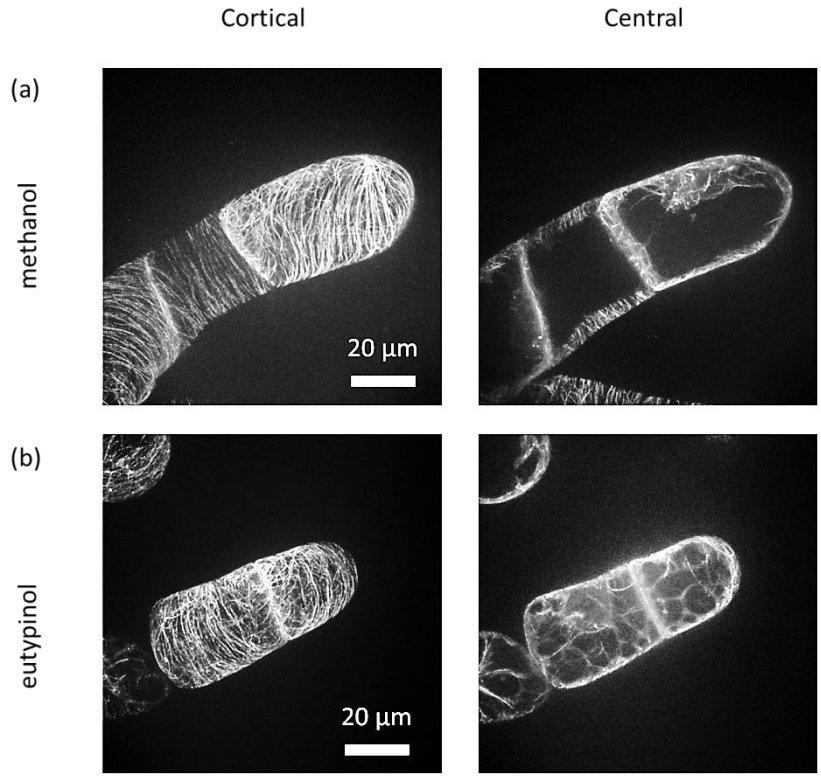


Figure 16 Microtubular responses to eutypinol in cells of *V. rupestris* expressing the fluorescent tubulin marker GFP-*At*TUB6. Cells were challenged 10 µM eutypinol (b) for 8 hours. 0.1% methanol was chosen as the solvent control (a). Two treatments were all emerged in cortical and central microtubules, respectively. Observations were based on at least four independent experimental series with a population of 50 individual cells for each treatment. Bars: 20 µm.

3.4 Chemical analogues of *Eutypa lata* metabolites induced grapevine defence

Since eutypinol and its analogues have a similar chemical structure but showed significantly different effects on the grapevine defence, we assumed that the differences might be due to a differential affinity for the respective receptor at the plasma membrane. Specifically, the functional difference was structurally exclusively related to the side groups of the phenolic ring, while the alkyne moiety was the same. We, therefore, selected the respective analogues that were commercially available and just differed in the lack of the (biologically apparently irrelevant) alkyne moiety. The effect of these chemical analogues of *Eutypa lata* metabolites on grapevine defence was then measured in this study (**Fig. 17**).

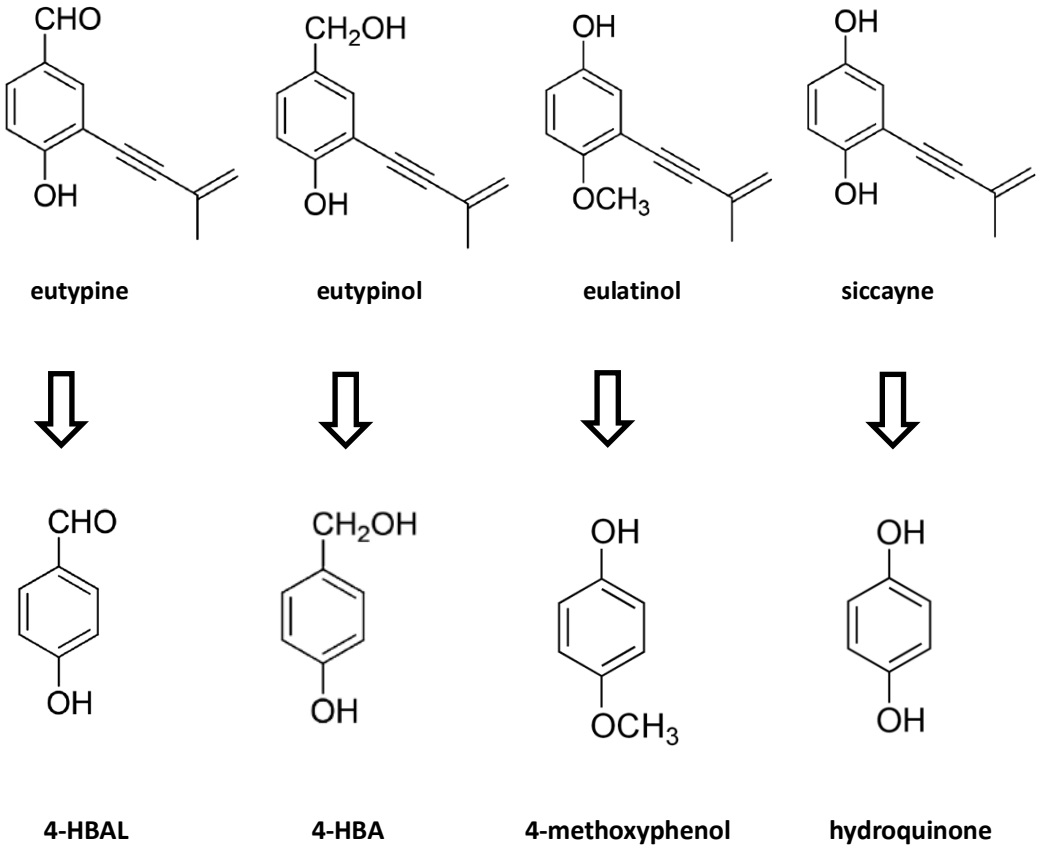


Figure 17 Structure of chemical analogues of *Eutypa lata* metabolites (Masi et al., 2018).

3.4.1 Analogues of eutypine and eutypinol, 4-HBAL and 4-HBA, activate calcium influx

Compared with the methanol control, 4-HBAL and 4-HBA induced much stronger pH responses, which indicated the calcium influx was activated, about 0.53 and 0.46 after 20 min, respectively. But the other two compounds, 4-methoxyphenol hydroquinone, had no effects on the extracellular alkalinisation (**Tab. 3**). Since the calcium influx could be evaluated by measuring the extracellular alkalinisation and was also considered as one of the earliest signals of grapevine immunity, we might conclude that 4-HBAL and 4-HBA could activate basal immune responses.

Table 3 Extracellular alkalinisation of *V. rupestris* cells to chemical analogues of *Eutypa lata* metabolites. Extracellular pH responses to 50 µM 4-HBAL, 50 µM 4-HBA, 50 µM 4-methoxyphenol, 50 µM hydroquinone were recorded by pH meter over time. The ΔpH (after 20 min) was shown. The experiments were repeated at least five times.

Chemical analogues of *Eutypa Lata* metabolites	ΔpH (after 20 min)
methanol (0.01%)	0.01
4-HBAL (50 µM)	**0.46**
4-HBA (50 µM)	**0.49**
4-methoxyphenol (50 µM)	-0.01
hydroquinone (50 µM)	0.01

3.4.2 The activation of calcium influx induced by 4-HBAL and 4-HBA is related to Gd-calcium channels

To identify the calcium influx induced by 4-HBAL and 4-HBA is related to Gd-calcium channels or not, we used 100 µM $GdCl_3$ combined with 50 µM 4-HBAL or 4-HBA to treat *Vitis* cells. Extracellular pH responses to 4-HBAL and 4-HBA were separately inhibited by $GdCl_3$ (**Fig. 18**). The peak of pH change induced by 4-HBAL was downregulated by $GdCl_3$ from 0.47 to 0.18 at 18 min (**Fig. 18**). The pH response activated by 4-HBA was also inhibited from 0.58 to 0.11 in 26 min (**Fig. 18**). Although the calcium influx could be activated by both 4-HBAL and 4-HBA, the incubation time

to the peak point was different. We concluded that the calcium influx induced by two compounds was associated with Gadolinium-blocked calcium channel and can be strongly inhibited by $GdCl_3$.

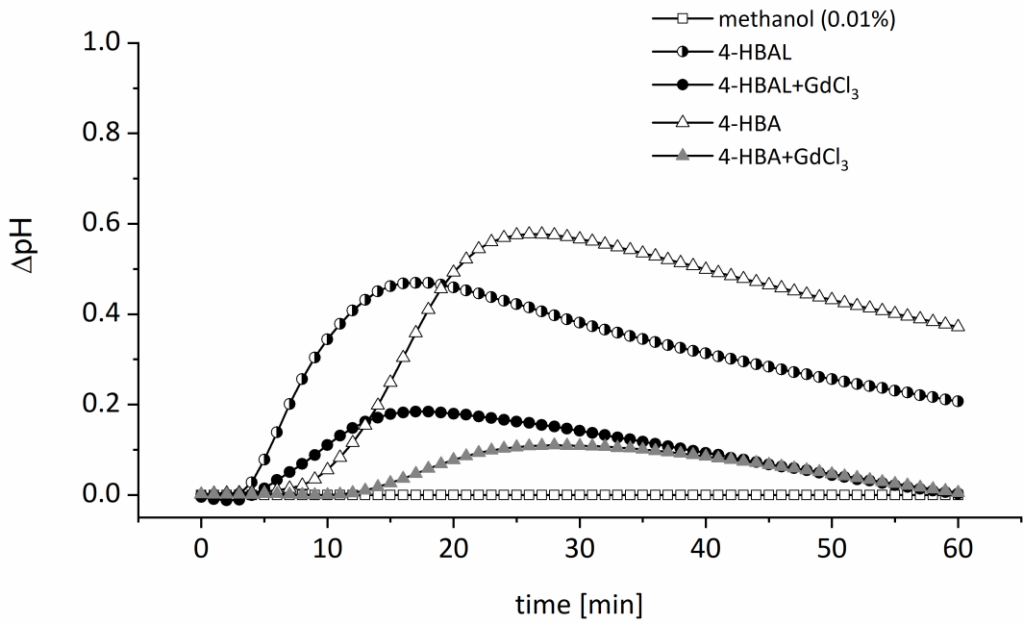


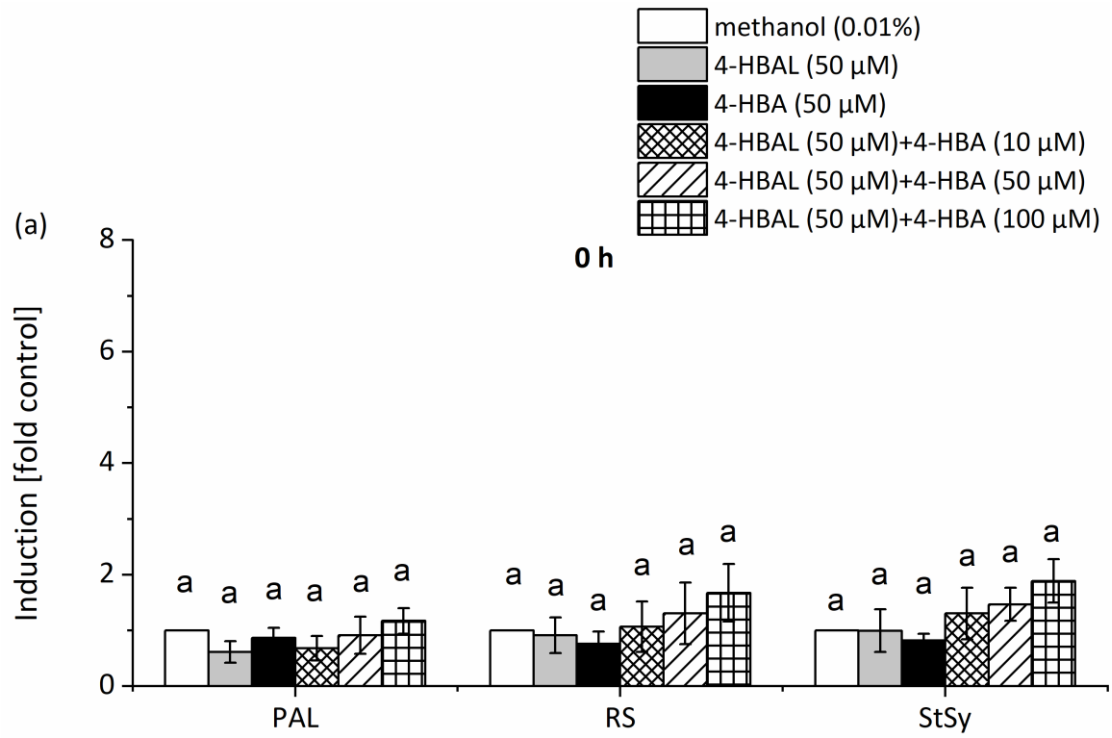
Figure 18 Effect of $GdCl_3$ on apoplastic alkalinisation induced by 4-HBAL and 4-HBA. Extracellular pH was evaluated in response to 50 µM 4-HBAL, 50 µM 4-HBA alone or separately combined with 100 µM $GdCl_3$. 0.01% methanol as solvent control. The experiments were repeated at least five times.

3.4.3 4-HBAL and 4-HBA induce weak transcription of defence genes

Because of the 4-HBAL and 4-HBA both activate the calcium influx, one of the earliest defence signals in plant defence, we assumed that two compounds of similar structure could be recognised by the same receptor. And this receptor may also recognise eutypine and eutypinol, the candidate cell-death elicitor and the candidate effector. To verify this hypothesis, we treated cells with 4-HBAL and 4-HBA alone or combined them in a different set of concentrations: the 10 µM, 50 µM, 100 µM 4-HBA combined with 50 µM 4-HBAL, respectively, for 0 hour, 1 hour, 3 hours, 6 hours. The transcription of defence gens *PAL*, *RS* and *StSy* were measured by qPCR.

Both 4-HBAL and 4-HBA induced the expression of the genes *PAL*, *RS*, and *StSy* after 1 hour (**Fig. 19b**). However, this induction level was decreased by 3 hours and 6 hours

after the start of the experiment (**Fig. 19c, d**). In combined treatments, we found that the induction level of defence genes was slightly increased, but had no significant differences with separate treatments except the treatment of 50 µM 4-HBAL with 100 µM 4-HBA (**Fig. 19**). Besides, the expression levels induced by the two compounds were much lower than eutypine in 1 hour (**Fig. 13, 19b**). For example, the induction of the gene *PAL* induced by 50 µM concentrations of the two compounds was around 4 fold while it was 12 fold induced by 10 µM eutypine (**Fig. 13, 19b**). These results indicated that the recognition of eutypine was much more stable and effective than the recognition of the two compounds by the same hypothesised effector. And this may be related to the additional ligand (the alkyne moiety) of eutypine that was different with 4-HBAL.



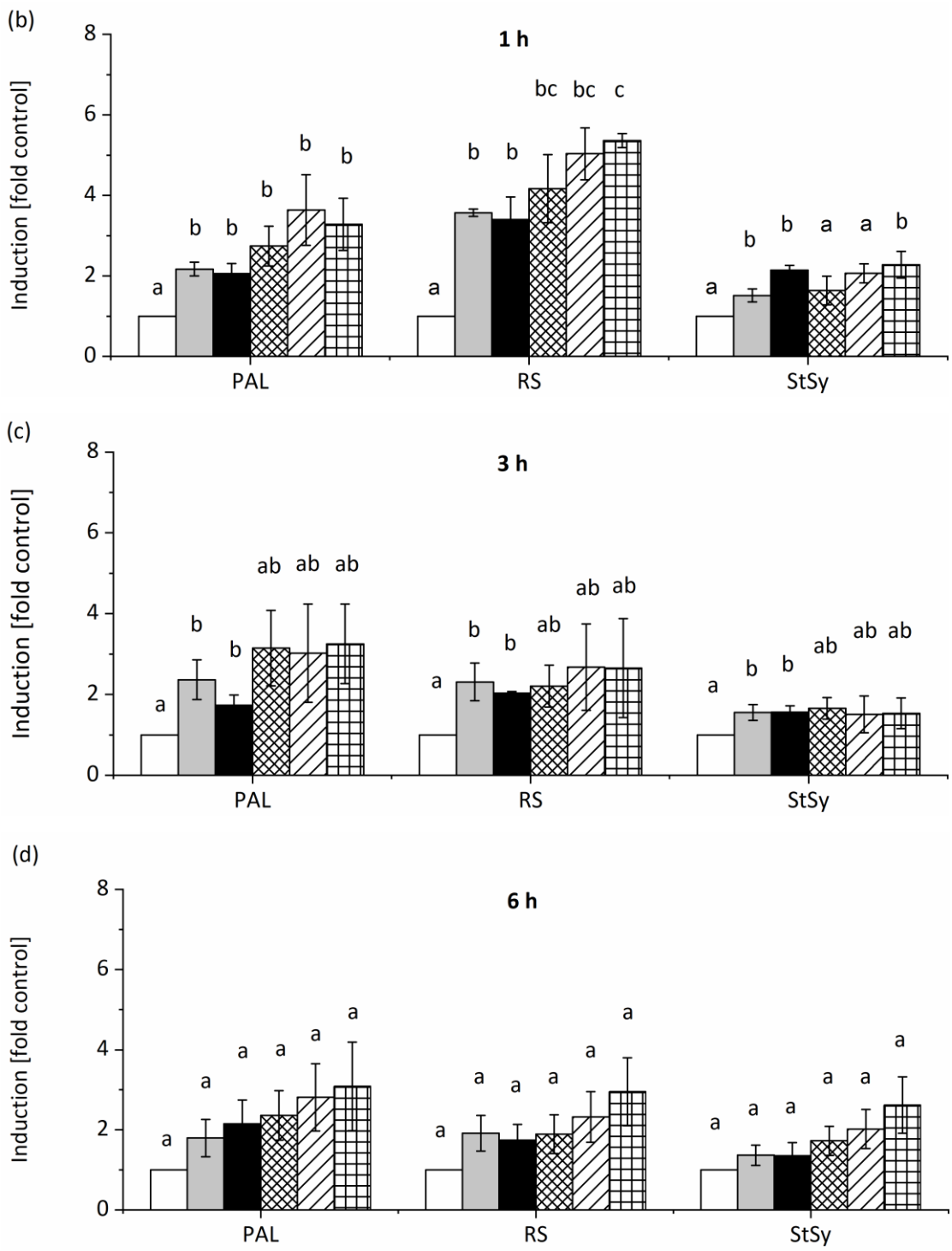


Figure 19 Induction of phytoalexin-synthesis genes by 4-HBAL, 4-HBA or different combinations. Cells were treated with 50 µM 4-HBAL, 50 µM 4-HBA or 50 µM 4-HBAL crossed with three concentrations of 4-HBA: 10 µM, 50 µM, 100 µM, for 0 to 6 hours with sequential equivalent-time sampling. The transcription of phytoalexin-synthesis (*PAL*, *RS*, *StSy*) was recruited via qPCR. Error bars indicate ± standard error (SE) of the mean; different *lowercase letters* indicate the significance at P=5 % (Student's t-test), n=3.

3.4.4 4-HBAL and 4-HBA induce slight cell death

Both eutypine and eutypinol were found to be effective inducers of cell death (**Fig. 14**), thus, their chemical analogues might have similar biological functions. The *Vitis* cells were incubated with 50 µM 4-HBAL, 50 µM 4-HBA alone, or a combination of both in the same concentration, 0.01% methanol served as the solvent control. After 24 hours, there was no significant cell mortality in all treatments (**Fig. 20**). We have found a slight increase in cell death at 50 µM 4-HBAL after 48 hours, around 18%, but it was not statistically significant. Therefore, we concluded that 4-HBAL and 4-HBA only could slightly induce cell mortality.

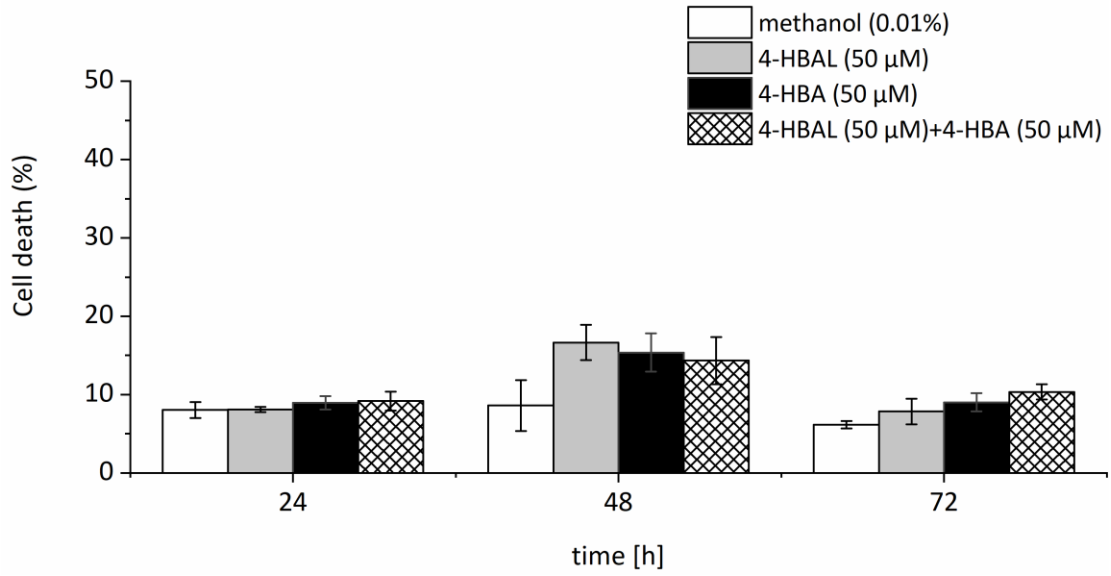


Figure 20 Effect of 4-HBAL and 4-HBA on cell death in transgenic *V.rupestris* cell line. Cell mortality was measured response to 50 µM 4-HBAL, 50 µM 4-HBA or their combination for 24 hours, 48 hours and 72 hours. Error bars indicate ± standard error (SE) of the mean, n=3.

3.5 Summary of results

In the current study, an easy and fast assay which combined the measurement of cellular signatures and HPLC-MS analysis was introduced to screen and identify signals exchanged between pathogenic fungi and grapevine. Firstly, the biological activities of fifteen fungal culture extracts were evaluated by measuring their effects on cellular calcium influx and defence genes expression. About 60 fractions of the two candidate extracts, *Eutypa lata* IBWF E16012 (BAF) and *Eutypa lata* IBWF E16012 (BAF) which have shown similar effects on calcium influx but significantly different in the transcription of defence genes, were isolated by HPLC. According to their effects on the induction of defence genes, 40 fractionations have been analysed and identified by HPLC-MS.

One of those fractionations was identified as O-methylmellein which was firstly isolated from *Eutypa lata* fungi. O-methylmellein acts as an "amplifier" which can enhance the flg22-triggered induction of defences genes and actin filaments bundling. Two fractionations were identified as eutypinol and siccayne that have similar chemical structures. In order to understand the relationship between the chemical structures and biological activities, eutypine and eulatinol which are two analogues of eutypinol and siccayne, were employed and tested. Only eutypine induced significant grapevine immune responses. To further reduce the scope of the effective chemical ligands of those metabolites, the effect of four homologues of *Eutypa* metabolites (4-HBAL, 4-HBA, 4-methoxyphenol, hydroquinone) were analysed. 4-HBAL and 4-HBA activated similar levels of immune responses, such as similar calcium influx and gene expression.

Taken all together, the metabolites produced by fungi involved in GTD could modulate or activate grapevine defence. And their mode of action was closely related to their specific chemical structures.

4 Discussion

GTDs are considered as a major destructive threat to viticulture worldwide, since many pathogens are involved, the latency period is long, and our understanding of their epidemiology is poor (Bertsch et al., 2009; Gramaje et al., 2018). Different to most plant diseases, GTDs do not follow Koch's postulate and cannot be efficiently controlled by means of fungicides. Meanwhile, as a consequence of global warming, the incidence of GTDs is also increasing further. Our rationale for this novel type of disease is to focus on the signals exchanged between grapevine and pathogens. These signals are most likely secondary metabolites produced by fungi, and probably arise in response to other chemical signals, may it be from competing or cooperating fungi, may it be from the host itself. This chemical communication culminates in the formation of the toxins that cause the foliar symptoms as a terminal manifestation of GTDs (Fontaine et al., 2016). As worked out in the introduction, these fungal metabolites may act either primarily (as elicitors), or secondarily as effectors, or even amplifiers that can enhance the grapevine defence. Therefore, understanding the mode of action of these signals might help us to control the GTDs and reduce economic losses.

In order to detect those signals and verify their roles in GTDs, the current study pursued a bioactivity-guided fractionation strategy from a pair of culture filtrates that originated from the same fungal strain of *Eutypa lata*, but differed in biological activity combining the measurement of cellular defence signatures with preparative HPLC and subsequent structural analysis. Several metabolites have been identified by this approach, such as O-methylmellein, eutypinol, siccayne, and their effects on the grapevine defence are also investigated.

4.1 The defence modulator O-methylmellein

The metabolite O-methylmellein produced by *Eutypa lata* E16012 has been identified as a defence-modulating compound in this study. When the mode of action for this

compound was investigated, we found that O-methylmellein acts as an amplifier of flg22-triggered basal immunity. This amplification was multiplicative, and correlated with a synergistic effect on actin filaments, without any significant effect upon microtubules.

These findings lead to the following questions that will be discussed in the following:

1. By which pathway can O-methylmellein stimulate flg22-triggered PTI?
2. What role does the cytoskeleton play in this pathway?
3. What might be the biological function of a fungal amplifier?

4.1.1 Functional modularity of compounds in fungal culture extracts

The starting material for the activity-guided fractionation strategy pursued in the current study was a panel of culture filtrates from fungi involved in GTDs. As expected, these extracts stimulated typical hallmarks of grapevine immunity, such as the calcium influx and induction of phytoalexin-synthesis genes (**Tab. 1; Fig. 3**). However, the biological activities of those culture extracts varied depending on the strains and growth media. The finding that even the same fungal isolate can produce different types of metabolites is not unusual and has been studied intensively for mycotoxins produced in food-contaminating fungi (Betina, 1989; Kokkonen et al., 2005). Also for *Eutypa lata*, metabolomic variability has been demonstrated (Mahoney et al., 2005). In general, different substrates and environmental conditions have been proposed as essential factors (Kokkonen et al., 2005; Lardner et al., 2006). To date, the factors which control these metabolic responses have remained elusive. This is also true for the current study – why *E. lata* strain E16012 generates significantly higher immunity activation in response to BAF medium as compared to PDA medium is not known. However, for the purpose of the current study this question, as interesting as it may be, may remain open. It was sufficient to find a situation, where extracts collected from the same fungal strain activated different immune responses, because this means that different extracts might contain different metabolites. As to be expected, the functional difference extends beyond a

single compound and is multi-faceted, as will be explained in the following:

The transcription of defence genes which encode enzymes of the biosynthesis of stilbenes was induced by almost all the culture extracts (**Fig. 3**). Some stilbenes, such as resveratrol and oxidised stilbene oligomers, such as δ-viniferin, play a vital role in protecting grapevine from pathogen attacks and can be induced by several PAMPs (Chang and Nick, 2012; Pezet et al., 2004). Grapevine antifungal proteins and specific stilbenes have been found to accumulate in the infected xylem of GTDs indicative of activated defence (Spagnolo et al., 2014; Yadeta and Thomma, 2013). Since these extracts primarily (i.e. without the need to add other compounds) induced transcripts of phytoalexins-synthesis genes, it is safe to conclude that some compounds present in those extracts act as PAMPs able to activate basal grapevine immunity.

While the presence of putative PAMPs seems to be a general feature of all extracts, there clear differences with respect to the amplitude of the triggered responses. We were able to identify E16012 (BAF) and E16012 (PDA) extracts as a contrasting pair with significant differences of bioactivity despite originating from the same fungal strain (**Fig. 3b**). Those differences might be the result of differences in the abundance of elicitors, but they might as well come from differences in the abundance of effectors (that would inhibit induction levels caused by the elicitor), or in the abundance of “amplifiers” (that would promote induction levels caused by the elicitor) in E16012 (BAF). Of course, nature will not do us the favour to keep these three possibilities apart for the sake of more convenient scientific analysis. Instead, combinations, and complex interactions (both antagonistic or synergistic) of different immunity modulators are to be expected from the fractionation of complex fungal culture filtrate. To trim down complexity, we have, therefore, focussed on the first step of phytoalexins synthesis, PAL, because this bioactivity was strongly amplified in the filtrate collected from *E. lata* strain E16012 upon cultivation in BAF over that seen for cultivation in PDA, such that there would be a realistic chance to arrive at a candidate molecule behind this differential activity. In fact, we succeeded to separate in the HPLC profile two regions that differentially activated *StSy* and *PAL* (**Fig. 5**), and

to identify O-methylmellein as *bona fide* candidate correlated with the higher bioactivity (with respect to induction of *PAL*) in the *E. lata* E16012 (BAF) culture filtrate.

The same conclusion has to be drawn for the reorganisation of the microtubule network which was differentially triggered by the two culture filtrates (**Fig. 4**). This microtubule response correlated with the activation of phytoalexins-synthesis genes, and can be used as an early readout for a defence response in grapevine (Qiao et al., 2010). The functional context of this microtubule response is not clear. While the microtubules have been proposed as tracks for the delivery of cell-wall components and also other defence agents to the fungal infection sites (Schmidt and Panstruga, 2011), this concept ignores that exocytosis in plants runs mostly through actin filaments (Boevink et al., 2002), and that depolymerisation of cortical microtubules would impede, rather than promote, transport of such vesicles, because transport in cortical microtubule arrays requires sustained treadmilling (Shaw et al., 2003). While the functional context of microtubules in defence is far from understood, there exist several reports that pathogen effectors can specially target microtubules or MAPs to disrupt the microtubule network correlated with a suppression of host defence (Park et al., 2018). While our data (**Fig. 4**) show that the two extracts differ in their activity for microtubule disruption, and that this correlates with their differential induction of phytoalexin-synthesis genes (**Fig. 3b**), it is also clear that O-methylmellein is not responsible for this effect (**Fig. 9**).

In summary, while we have isolated O-methylmellein as candidate compound correlated with elevated induction of *PAL* transcripts, we find that O-methylmellein is not the elicitor primarily triggering induction of *PAL*, nor is O-methylmellein the compound responsible for the microtubule disruption triggered by the *E. lata* E16012 (BAF) culture filtrate. Does this mean that O-methylmellein was “the wrong horse”, i.e. a compound without any relation to plant immunity? Our mode-of-action study led to a different conclusion: O-methylmellein is not the elicitor, but it is definitely active with respect to immunity, namely, as an amplifier. Before we will discuss the potential pathway, how O-methylmellein boosts basal immunity, once it had been

triggered by a different (still unknown) elicitor, we can already draw a general conclusion: the response to the culture filtrate is composed of different elements that can be separated: a general elicitation of basal immunity including activation of a calcium channel, followed by activation of phytoalexins-synthesis genes (triggered by a yet unidentified compound), disruption of microtubules (triggered by a yet unidentified compound), amplification of phytoalexin-gene expression (mediated by O-methylmellein), and amplification of defence-related actin remodelling (mediated by O-methylmellein). In other words: the fungus employs a panel of different compounds to steer plant immunity in a modular manner.

4.1.2 O-methylmellein as an amplifier: what can we conclude on the pathway

O-methylmellein is a derivative of mellein, a compound which had first been isolated from the saprobiotic *Aspergillus melleus* (Nishikawa, 1933). A potential role as virulence factor for phytopathogenic fungi had been proposed by work on the necrotrophic wheat pathogen *Parastagonospora nodorum* (Chooi et al., 2015). Here, a polyketidsynthase had been found to be upregulated during infection, and was later shown to generate mellein. Mellein impaired germination of wheat and alfalfa, indicative of a potential phytotoxic effect. However, the concentrations required for this effect, were high, which would not be expected if mellein was a specific regulator. More importantly, when the responsible polyketide synthase was knocked down in the fungus, this did not impair infection success, such that mellein does not qualify as a virulence factor. Thus, the mode of action of mellein and its derivative O-methylmellein are far from understood. Also in our hands, O-methylmellein did not show any significant cytotoxic activity (**Fig. 11**). Therefore, it does not qualify as a molecular candidate for the toxin that causes the foliar symptoms in GTDs. For the same reason, O-methylmellein cannot be considered as a potential trigger for programmed cell death. The strategy to elicit untimely programmed cell death could be conceived and is, in fact, used by some pathogens, such as *Liberibacter asiaticus* causing the Huanglongbing disease of *Citrus* (Orbović et al., 2015).

O-methylmellein positively regulated the induction of defence genes triggered by

elicitor flg22, but had no effects on flg22-triggered calcium influx (**Fig. 7**). This amplification effect was always 3-fold, no matter, to what extent the respective gene was induced by flg22. Thus, the interaction of flg22 and O-methylmellein is multiplicative, and not additive, which means that the signalling triggered by O-methylmellein and that triggered by flg22 must be shared to a certain extent. From the measurement of extracellular pH, we know that the merging point must be downstream of the calcium channel.

As the implication of this conclusion, the enhancement effect of O-methylmellein on the flg22-triggered expression of defence genes would be predicted to be insensitive to $GdCl_3$, a blocker of calcium influx that can suppress flg22-triggered alkalinisation (Qiao et al., 2010). This implication was tested experimentally and confirmed (**Fig. 8**). Instead, a significant (albeit not complete) inhibition was observed, when the other important stress input, the membrane located NADPH oxidase Respiratory burst oxidase Homologue (RboH) was blocked by diphenylene iodonium (**Fig. 8**). In grapevine cells, this input has been found to initiate cell-death related defence in response to the bacterial elicitor harpin (Chang and Nick, 2012) or the product of plant oxylipin metabolism, 3-cis-hexenal (Akaberi et al., 2018), and was also found to be necessary for the resulting gene activation (Chang et al., 2011). Thus, O-methylmellein as amplifier shares a specific signature (dependence on RboH, independence of calcium influx) with cell-death related defence. However, it is not culminating in cell death. Thus, there must be a point, where O-methylmellein signalling diverges from the signalling leading to programmed cell death.

There is a further, very specific, hallmark of defence-related cell death: massive and rapid remodelling of actin filaments that have been found for both, harpin (Chang et al., 2015) as well as for 3-cis-hexenal (Akaberi et al., 2018). We checked this phenomenon, and found that O-methylmellein induced actin remodelling, and, more importantly, boosted actin remodelling in response to flg22 in a synergistic manner (**Fig. 10**). This effect was specific, because it was not seen for the microtubule network (**Fig. 9**).

Thus, supported by (non-intuitive) implications that have been experimentally confirmed, the following model emerges for the signalling events triggered by O-methylmellein: activation of RboH, leading to an apoplastic oxidative burst, penetration of reactive oxygen species, probably through aquaporins into the cytoplasm (Eggenberger et al., 2017), remodelling of actin (Chang et al., 2015), and activation of phytoalexins genes. The divergence from the signalling leading to defence-related cell death seems to be close to actin remodelling. The convergence with the signalling deployed by flg22 is downstream of calcium influx, but upstream of gene expression. A straightforward hypothesis would locate this convergence at MAPK signalling. A testable implication of this hypothesis would be that treatment with the specific inhibitor PD98059 should disrupt the amplifier activity of O-methylmellein (Chang and Nick, 2012).

While the individual events of this pathway have been demonstrated, the model sketched down above does not answer the question, why O-methylmellein can deploy this signalling only in the presence of flg22, and not by itself. In other words, what is the reason that this compound cannot act as an elicitor, but only as (multiplicative) amplifier? In the following, we will discuss this as a possible consequence of the cytoskeletal activity.

4.1.3 Does actin play a role for elicitor sensitivity?

The remodelling of actin filaments is clearly detectable already 30 min after addition of O-methylmellein (**Fig. 10**) and, thus, clearly precedes the amplification of flg22-induced transcripts (**Fig. 7**). How might actin remodelling interfere with defence? Most works on this topic have focussed on so-called penetration resistance, where actin filaments reorganise around a site of attempted penetration and are involved in the deposition of callose, as well as in the accumulation of vesicles with phytoalexins or defence proteins (reviewed in Day et al., 2011). A further phenomenon that has attracted attention, is the movement of the nucleus towards the penetration site (Gus-Mayer et al., 1998). The functional relevance of this phenomenon is not clear (for review see Griffis et al., 2014), it might be rather a side phenomenon and caused

by the rapid accumulation of actin filaments around the nucleus, because the perinuclear actin cage tethers the nucleus in concert with the plant-specific class-XIV kinesin KCH1 (Frey et al., 2010; Durst et al., 2014). Although the function of actin filaments for defence is far from understood, the importance of actin remodelling is supported by the fact that silencing of *TaADF4*- is necessary to render wheat resistant against *P. striiformis f. sp.tritici* strain (Tang et al., 2016). In the case of the effector HopG1 from the *P. syringae* remodelling of actin filament organisation was shown to correlate with increased susceptibility and symptom development (Shimono et al., 2016).

Although these reports show the importance of actin, they do not really help to understand, how the remodelling of actin cytoskeleton should lead to an amplifying effect of flg22-triggered gene expression. The multiplicative interaction of O-methylmellein with flg22-triggered signalling (**Fig. 7b-d**) occurs without increasing the amplitude of calcium influx (**Fig. 7a**). Through interaction with its co-receptor BAK1, flf22 can deploy different signal pathways (including phosphorylation cascades and release of reactive oxygen species) that run in parallel to calcium influx (reviewed in Robatzek and Wirthmüller, 2013), and would be candidates for the target of amplification by O-methylmellein. The binding of the ligand, flg22, to its cognate receptor FLS2, located at the plasma membrane, not only activates signalling, but subsequently, endocytotic uptake of this receptor (Robatzek et al., 2006). This process is dependent on actin.

The amplifying effect of O-methylmellein could, therefore, be explained by an actin dependent self-amplification: the rapid actin remodelling induced by O-methylmellein (**Fig. 10c**), would suppress endocytotic recycling of FLS2, such that FLS2-triggered signalling would not be shut off. Among other targets, the reactive oxygen species triggered by FLS2 signalling would accumulate to higher levels, which would accentuate actin remodelling (manifest as synergistic activity of flg22 with O-methylmellein, **Fig. 10d**), and thus would amplify the activation of phytoalexin genes by flg22. Why this amplification is not seen with calcium influx (**Fig. 7a**) might be due to the fact that this response is already saturated – if flg22 can open all

available channels, it is hard to see a stimulation by O-methylmellein. This could be tested in the future by conducting dose-response studies with suboptimal concentrations of flg22. The molecular mechanism underlying actin remodelling is certainly complex, because actin is complex, but a prime candidate is the actin-capping proteins that are responsive to ROS and have been shown to modulate actin filaments in response to various PAMPs including chitin, flg22, and elf26 (Li et al., 2017; Li et al., 2015b). Additional players may be actin-depolymerisation factors (Eggenberger et al., 2017).

Thus, the amplifier effect of O-methylmellein can be explained by a working model, where ROS-dependent actin remodelling increases the sensitivity towards flg22, by prolonging the lifetime of the flagellin receptor at the membrane. Sensitivity is used here in the strict sense (Galland, 1991), meaning that the dose-response curve is shifted to lower concentrations of ligand (an implication that will be tested in future work by conducting detailed dose-response studies for flg22 and O-methylmellein, with the option of quantitative modelling).

4.2 The defence elicitor eutypine and its analogues

The *Eutypa lata* metabolite eutypine was found to trigger grapevine defence while our identification product eutypinol only induce cortical microtubule depolymerisation. Although most *Eutypa* lata metabolites are identified as acetylenic phenols and heterocyclic analogues that have similar structures (Jiménez-Teja et al., 2006), they activated significant grapevine cellular events. This might be related to the different affinities with the receptor on the grapevine cell-surface. Besides, the fungal metabolites might have other special targets to weaken grapevine cell, for example, eutypinol might affect the dynamic balance of cortical microtubule network. Through the comparative study of *Eutypa lata* metabolites and their chemical homologues, we assumed that the aldehyde and alkyne moieties of the phenolic ring of eutypine maybe the combining site and "stabiliser" in combination with grapevine receptor, respectively.

Based on the experimental results, the following questions will be discussed:

1. Why *Eutypa lata* fungi produce so many phenolic metabolites with similar structures?
2. How the aldehyde and alkyne moieties of eutypine affect the eutypine induced immunity levels?

4.2.1 Most phenolic metabolites produced by *Eutypa lata* are elicitors or toxins

Eutypinol and siccayne had been identified and isolated from *Eutypa lata* IBWF E16012 culture extracts (**Suppl. Tab. S2, S3**; **Fig. 5**). They have similar chemical structures with eutypine and eulatinol (**Fig. 12**). The later experiments have shown that the eutypine may act as a novel PAMP of activating immune responses in *Vitis* cells (**Tab. 2; Fig. 13**). But other three *Eutypa lata* metabolites failed to induce basal immunity (**Tab. 2; Fig. 13**). Eutypine elicited strong calcium influx that is reported as the hallmark of grapevine basal immunity (**Tab. 2**) (Chang and Nick, 2012). The calcium influx can be evaluated by measuring the apoplastic alkalinisation as readout (Felix et al., 1993). Besides, the defence genes are only activated by eutypine (**Fig.**

15). The perception of PAMPs by the cell-surface receptor usually elicits a series of cellular signals (Couto and Zipfel, 2016). Those cellular events evoked by eutypine support that eutypine is a novel PAMP candidate. This leads to a simple model that only eutypine is recognised by the cell-surface receptor and activate PTI responses, like calcium influx and induction of defence genes. Although eutypine and its analogues have many similar chemical structures, the potential receptor on the cell membrane may have a high affinity with eutypine rather than other three analogues. In conclusion, the side groups of the phenolic ring of eutypine might play an essential role in the highly affinitive recognition between eutypine and grapevine receptor.

Significant cell death has been observed in all the time points of the eutypine, eutypinol and siccayne treatments (**Fig. 14**). In the plant-pathogen interaction, the cell death can be induced or modulated by pathogen toxins (Coll et al., 2011), elicitors (Chang and Nick, 2012; Ricci et al., 1989), and effectors (Liu et al., 2011). Based on existing experimental results, it is difficult to tell which group they belong to. Since eutypine was only detected in the crude sap and inflorescences of the infected grapevine but not in healthy plants, posing questions about its role in the virulence of *Eutypa lata* (Tey-Rulh et al., 1991). Previously it was shown that eutypine was toxic to Cabernet-Sauvignon microcutting leaves and caused leaf necrosis in a dose-independent manner (Tey-Rulh et al., 1991). And, the toxicity of eutypine was also verified in *V. vinifera* cv. Gamay cell cultures (Afifi et al., 2003) and *V. vinifera* cell-suspension cultures (Colrat et al., 1999). Therefore, eutypine is considered as an important virulence factor that transported through the sap to shoots or leaves of the grapevine and involved in the development of the foliar symptoms.

However, eutypine has not been isolated from some pathogenic *Eutypa lata* strains (Mahoney et al., 2005; Molyneux et al., 2002; Smith et al., 2003). There might be other phytotoxins produced by *Eutypa lata* can cause foliar symptoms. This kind of search has never stopped. And some new phytotoxins have been identified, such as eulatachromene and 2-isopropenyl-5- formyl benzofuran (Smith et al., 2003). The two identifications, eutypinol and siccayne, has been identified as phytotoxins in this

study, which were opposite with other's leaf experiments (eutypinol and siccayne were considered as non-toxic products for a long time) (Mahoney et al., 2003; Smith et al., 2003). Maybe, the cells are more sensitive to phytotoxins than grapevine leaves. Besides, the obvious cell death induced by the E16012 (PDA) extract may be related to eutypinol and siccayne (data not shown). The eutypinol is reported as one of the most abundant products and produced by 97% *Eutypa lata* isolates and may act as a chemical marker of *Eutypa lata* involved diseases (Lardner et al., 2006; Mahoney et al., 2005). Since the eulatinol is almost exclusively isolated from the artificial culture filtrate of *Eutypa* fungi and may have fewer effects on grapevine defence (Mahoney et al., 2003). Although eutypine induced significant cell death and immune responses, it is difficult to link its toxic with the perception of the receptor. In addition to eutypine, eutypinol and siccayne might be important phytotoxins in inducing the expression of disease symptoms.

Cortical microtubule depolymerisation, readout of grapevine defence, was only induced by eutypinol (**Fig. 15**). And the remodel of the microtubule network was able to be recovered after treating with eutypinol for 8 hours (**Fig. 16**). The restored microtubules indicated that the microtubule network might act as a signal in the eutypinol activated signalling pathway. There are no significant differences in microtubule network between other *Eutypa lata* metabolites treatments and solvent control (**Fig. 15**). Since the eutypinol had been identified from both E16012 (BAF) and E16012 (PDA) extracts (**Suppl. Tab. S2, S3**), we might conclude that eutypinol contribute to the microtubule depolymerisation induced by E16012 (BAF) extracts (**Fig. 4**). Although the reorganisation of the microtubule network could be induced by several hypersensitive response related elicitors, it is widely deemed as the cellular signal of ETI (Li and Staiger, 2018). This is because of the microtubules or MAPs are sometimes as specific targets of several pathogen effectors (Park et al., 2018). Besides, the eutypinol even suppressed the induction of gene *JAZ1*; we might view eutypinol as a candidate effector.

To conclude, grapevine might have a specific receptor that can only recognise eutypine rather than other similar metabolites. The perception of eutypine might

activate a series of grapevine immune responses, like the calcium influx, induction of defence genes, but not the microtubule depolymerisation. Eutypine, eutypinol, and siccayne might be the important phytotoxins contributing to the expression of the symptoms of *Eutypa lata* involved GTDs. Eutypinol esspecially activates a transitory cortical microtubule depolymerisation and might be a candidate effector. The specific product, eulatinol, which was only found in fungal culture filtrate, has fewer effects on the *Eutypa lata* pathogenicity.

Thus, all three phenolic metabolites (eutypine, eutypinol, and siccayne) produced by *Eutypa lata* fungi in the trunk act as toxins to weaken grapevine and might induce foliar symptoms. But the grapevine only can recognise eutypine, not its corresponding alcohol eutypinol or another metabolite siccayne. Interestingly, eutypinol and siccayne have been identified from E16012 (BAF) and E16012 (PDA) culture extracts with a large amount.

We might assume that fungi probably use a very efficient survival strategy:

a. Screening stage;
   Eutypa lata fungi produce many toxins that have a similar structure (to save energy) to test the grapevine recognition system;
b. Directional production stage;
   After screening, fungi prefer to produce toxins that cannot be detected by the grapevine, like eutypinol and siccayne, or metabolites that can activate grapevine immunity, like eutypine, or effectors to inhibit host immuty, or amplifiers to promot host immunity to kill competitors.

4.2.2 Is the aldehyde ligand of eutypine and 4-HBAL play a crucial role in triggering grapevine defence?

The calcium influx activated by 4-HBAL and 4-HBA, chemical homologues of eutypine and eutypinol, could be blocked by $GdCl_3$ (**Fig. 17, 18**). Another two homologues of eulatinol and siccayne, 4-methoxyphenol and hydroquinone, had no effects on the

extracellular calcium influx (**Fig. 17**; **Tab. 3**). It had been reported that calcium influx was one of the earliest defence signals in grapevine immunity system (Chang and Nick, 2012). Thus, 4-HBAL and 4-HBA might stimulate basal grapevine immunity.

4-HBAL and 4-HBA also weakly induced the transcription of defence genes (**Fig. 19b, 19c**). In co-treatment of 4-HBAL and different concentrations of 4-HBA, we found the induction levels of those genes slightly increased but not too much (**Fig. 19**). Both 4-HBAL and 4-HBA have shown slight toxic to grapevine cells. In the co-treatment experiment, cell mortality even down regulated (**Fig. 20**).

The previous experiments have shown only eutypine, the homologue of 4-HBAL, could induce grapevine immunity. Because of 4-HBAL and eutypine have similar chemical structures, only lack the alkyne moiety, we assumed that they might be recognised by the same receptor and initiated calcium influx. Furthermore, 4-HBAL is a natural phytotoxin produced by several fungal classes involved in GTD (Andolfi et al., 2011; Tabacchi et al., 2000). From an evolutionary perspective, 4-HBAL is not only the chemical homologue but also a candidate precursor of eutypine. However, the induction level of immune responses induced by eutypine and 4-HBAL had a great difference. The hypothesis was formed that eutypine has a higher affinity with the grapevine receptor than 4-HBAL. And the difference may be a result of the specific ligand (alkyne moiety) between them.

Eutypinol failed to activate grapevine immunity while its homologue 4-HBA induced defence related cellular events (**Tab. 2**; **Fig. 13, 18, 19**). Maybe, the particular receptor on the grapevine cell-surface only recognises 4-HBA, not eutypinol. 4-HBA, one of active ingredients of *Gastridua elata* Blume (Orchidaceae) (Tian ma in Chinese), a typical Chinese medicinal herb, has been widely used for the treatment of central and peripheral nervous disorders (Chen and Sheen, 2011). Our study has shown that 4-HBA can also induce grapevine immunity, providing a novel role of 4-HBA in the plant-pathogen interaction. However, the mode of action of 4-HBA was unclear so far.

4-HBAL and 4-HBA have very similar chemical structures and similar effects on grapevine defence. The most important thing is that there are no significant differences between the combined and single treatments of 4-HBAL or 4-HBA (**Fig. 19**). They might share a similar receptor in the grapevine.

Taken all together, the chemical homologues of eutypine and eutypinol, 4-HBAL and 4-HBA, could evoke similar grapevine immune responses. They may be recognised by a similar receptor, which also has a high affinity with eutypine but not eutypinol. In the comparison of the chemical structures, eutypine has an aldehyde ligand in the aromatic ring while eutypinol does not (**Fig. 19**). We might conclude that the aldehyde ligand of eutypine may be specially recognised by grapevine receptors. Compared with eutypine, 4-HBAL lacks an alkyne moiety ligand and has activated weaker immune responses. It was assumed that the alkyne moiety increases eutypine's affinity with grapevine receptors. The alkyne moiety ligand might play a stabiliser role for grapevine receptor to recognise eutypine.

4.3 Conclusion

After multiple screening and identification of the metabolites secreted by fungi related to GTDs, three compounds (O-methylmellein, eutypinol, siccayne) have been selected from the *Eutypa lata* IBWF E16012 culture extracts. The mode of action of these compounds along with other reported fungal metabolites and chemical analogues have been investigated in grapevine. The conclusion was the following:

1. Some metabolites of the fungal culture extracts act as PAMPs that can induce basal grapevine immunity.

2. O-methylmellein acts as a multiplicative amplifier of flg22-triggered basal immunity might be conducted by raising the longevity of the flagellin receptor at the membrane through the ROS-dependent actin remodelling.

3. Eutypine is an elicitor that can trigger both basal grapevine immunity and cell mortality. Other analogues eutypinol and siccayne cannot activate grapevine defence but induce clear cell mortality. Eulatinol has no effects on grapevine defence.

4. As the homologue of eutypine, fungal metabolite 4-HBAL is also an elicitor that can trigger immune responses. The eutypinol homologue 4-HBA, which is the active compound of Traditional Chinese medicine Tianma, activates similar defence responses with 4-HBA. 4-HBAL and 4-HBA might be recognised by the same grapevine receptor.

5. The perception of eutypine by grapevine receptor might be related with the “binding site” aldehyde ligand and “stabiliser” alkyne moiety ligand in the aromatic ring.

4.4 Outlook: Hunting the function of the modifier

The concept of a fungal signal that boosts the defence response of the host, appears counterintuitive, at first sight. However, a second look makes clear that amplifiers can confer a selective advantage under the conditions of microbial consortia colonising grapevine trunks, and including both, fungi and bacteria (Bruez et al., 2015; Gramaje et al., 2018). In the world of microbes, competition for limited resources is commonly decided by chemical warfare, for instance, by secretion of anti-microbial compounds that will weaken the competitor, but leave the donor unharmed, because it harbours a mechanism to degrade the compound.

In the case of O-methylmellein, the competition might run indirectly through a “rail shot” by leaving the job of killing the competitor to the host. This would allow *Eutypa lata* to outcompete bacteria. On the other hand, O-methylmellein has been found to exert high fungitoxic activity against the GTD-associated fungus *Botryosphaeria obtuse* as a potential competitor, and *Botrytis cinerea*, a necrotrophic generalist (Glauser et al., 2009). If O-methylmellein acted as anti-microbial compound, it will be necessary to understand, how *E. lata* itself can escape the action of its own toxin. If O-methylmellein acted as “borrowed knife” (Sun, 1993), it will be necessary to understand, how *E. lata* itself can escape the immune response of the host. In this research, O-methylmellein might weaken its competitors (mainly bacteria) by enhancing grapevine defence. A third possibility should be kept in mind, however. The dominant gene activation seen in response to the amplifier effect of O-methylmellein concerns *PAL*, the first committed step of the phenylpropanoid pathway. This pathway generates not only phytoalexins of the host, but also monolignols, i.e. the building blocks for the main food source of the fungus. The fact that the chemical factors leading to stimulation of *PAL* can be separated from those responsible for the stimulation of *StSy* (**Fig. 5a**), would fit to a scenario, where the fungus reprogrammes host metabolism towards lignin production, while simultaneously suppressing (by different signals) the phytoalexins-generating side branch of this pathway.

No matter, which of these three scenarios is used to describe the interaction with host and competitors, all three possibilities would ask for metabolic reprogramming of *E. lata*, which on the molecular level, should become visible as activation of otherwise silent genes, such as the members of the extensive polyketide synthase clusters. The (circumstantial) discovery that these silent potencies can be stimulated by cultivation in BAF medium indicates that the metabolic reprogramming is regulated by external factors. These might be signals from the host plant itself, or signals from competing microorganisms. To hunt down the real biological function of O-methylmellein will require to hunt down the context under which O-methylmellein is generated. We have, therefore, launched a project, where we will co-cultivate different fungal signal "donors" with grapevine cells as signal "receptors" in a manner that they can chemically interact, but remain physically separate.

Besides, among all the phenolic metabolites produced by *E. lata*, only eutypine acts as PAMP that might be recognised by the grapevine receptor, specifically. That is, eutypinol and siccayne escape from the grapevine's monitoring system. For *E. lata* fungi, those metabolites which fail to activate host defence but have toxic effects are ideal products. This might be the explanation of the previous finding that eutypinol has been isolated from almost all the *E. lata* strains. Eutypinol and siccayne might play a role in causing grapevine trunk or foliar symptoms.

Furthermore, is the eutypinol just a simple toxin? Even the *E. lata* reduce the synthesis and secretion of eutypine selectively, there might be other fungal PAMPs that activate grapevine defence, like chitin. Thus, *E. lata* fungi cannot avoid activating all levels of grapevine defence. Another more advanced strategy used by fungi might be producing effectors to inhibit grapevine defence. Eutypinol is assumed as an effector candidate since it can downregulate the induction of *JAZ1* and degrade the cortical microtubule (**Fig 13, 15**). Therefore, the effect of eutypinol on flg22 or eutypine triggered PTI need to be tested in the grapevine cell line.

Besides, more comparative experiments need to be conducted with different phenolic compounds to verify the role of the aldehyde and alkyne moiety ligand in

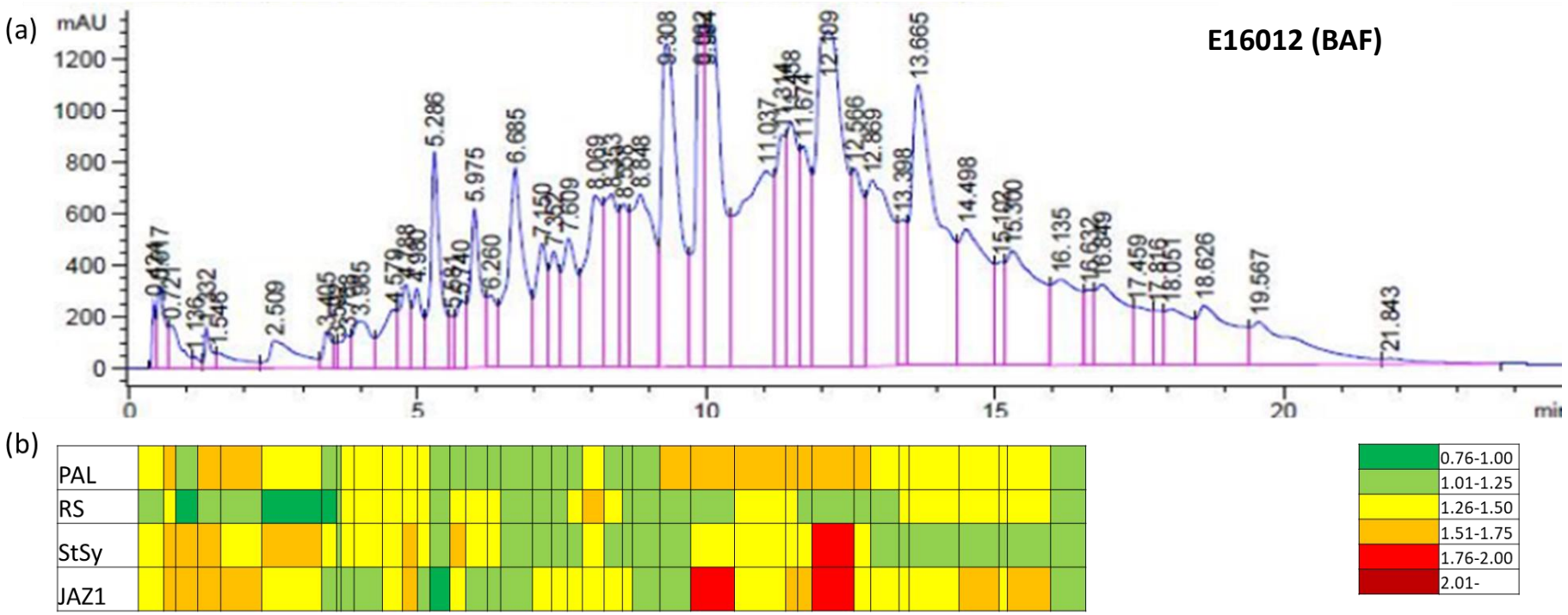
inducing grapevine defence. Since we partly understand the mode of action of the main metabolites produced by *E. lata* fungi, the next project will focus on the grapevine side, such as, how to detect fungal signals? And we will also screen the specific grapevine metabolites which related to GTDs. Maybe we can identify several compounds produced by fungi or grapevine which can act as the alarm signals of the outbreak of GTDs and provide suggestions to grape growers.

Appendix

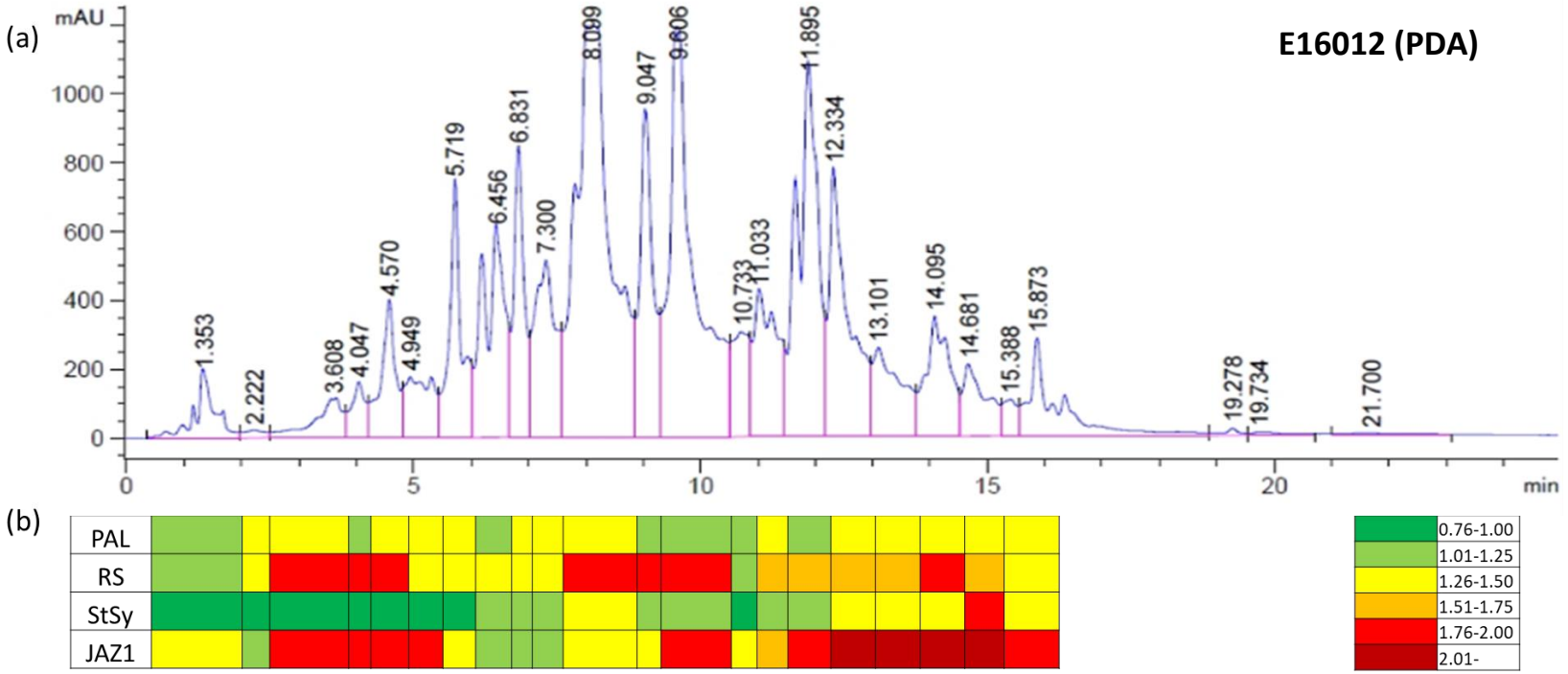
Table S1 List of oligonucleotide primers used for expression analysis by semi-quantitative and quantitative PCR.

Name	GenBank accession no.	Primer sequence 5'-3'	Reference
EF1α	EC959059	Sense:5'-GAACTGGGTGCTTGATAGGC-3' Antisense:5'-AACCAAAATATCCGGAGTAAAAGA-3'	Reid *et al*. (2006)
PAL	X75967	Sense:5'-TGCTGACTGGTGAAAAGGTG-3' Antisense: 5'-CGTTCCAAGCACTGAGACAA-3'	Belhadj *et al*. (2008)
RS	AF274281	Sense:5'-TGGAAGCAACTAGGCATGTG-3' Antisense: 5'-GTGGCTTTTTCCCCCTTTAG-3'	Duan *et al*. (2015)
StSy	X76892	Sense:5'-CCCAATGTGCCCACTTTAAT-3' Antisense: 5'-CTGGGTGAGCAATCCAAAAT-3'	Duan *et al*. (2015)
VvMC2	KC494645	Sense: 5'-TGGGGAGGTCATTTCCTTTAG-3' Antisense: 5'-GGTTGATCGCATTGAATTTAGC-3'	Gong *et al*. (2019)
VvMC5	KC494648	Sense: 5'-GAGGGTTGCCGCATTACGA-3' Antisense: 5'-GCACCTTGCACGGTTTGGT-3'	Gong *et al*. (2019)
JAR1	XM_002280702.2	Sense:5'-GAGAATTGCGGATGGTGATA-3' Antisense: 5'-CTAAAGGCGAAAGAGGTT-3'	Figueiredo *et al*. (2015)
JAZ1	JF900329	Sense: 5'-TGCAGTCTGTTGAGCCAATACATA-3' Antisense: 5'-CACGTTTCCGGACTTCTTTACAC-3'	Ismail A *et al*. (2012)

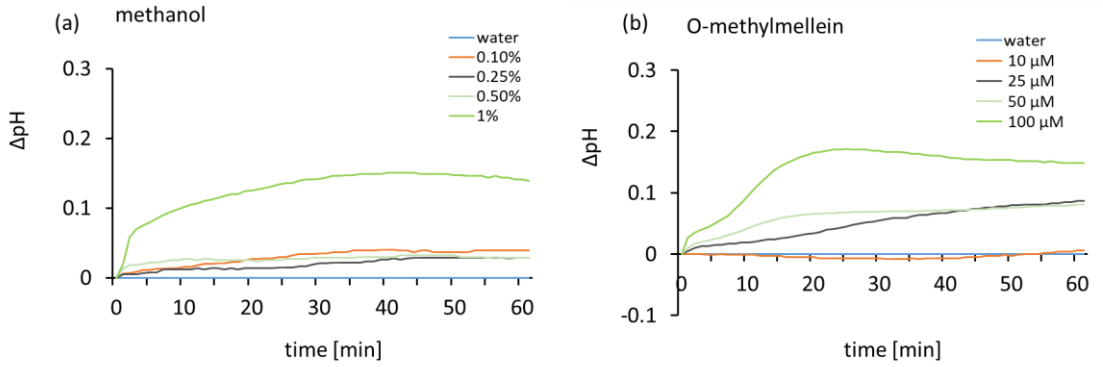
(EF1α, elongation factor 1α; PAL, phenylalanine ammonia lyase; RS, resveratrol synthase; StSy, stilbene synthase; MC2, metacaspase 2；MC5, metacaspase 5；JAR1, jasmonate-resistant 1; JAZ1, the jasmonate ZIM/tify-domain protein 1, a transcriptional repressor)



Supplement figure 1 Fractionation of the *Eutypa lata* IBWF E16012 (BAF) culture filtrate (a) and the effect of fractions on the expression of phytoalexins genes (b). (a) The composition of E16012 (BAF) culture filtrate was fractionated by the HPLC with time. (b) The induction of phytoalexins genes to 39 fractions was measured by the Semi-qPCR. The integrated density of bands of Semi-qPCR results was measured by using the Line Width of ImageJ. These values were calculated and formed by Excel. The methanol solvent control was considered as the ground level (1.00). The relative induction levels were expressed by using different colours: green : 0.76-1.00; light green 1.01-1.25; yellow 1.26-1.50; orange 1.51-1.75; red 1.76-2.00; dark red >2.0



Supplement figure 2 Fractionation of the *Eutypa lata* IBWF E16012 (PDA) culture filtrate (a) and the effect of fractions on the expression of phytoalexins genes (b). (a) The composition of E16012 (PDA) culture filtrate was fractionated by the HPLC with time. (b) The expression of phytoalexins genes induced by 21 fractions was measured by the Semi-qPCR. The Semi-qPCR results was analysed by using the Line Width of ImageJ. These values were calculated and formed by Excel. The methanol solvent control was considered as the ground level (1.00). The relative induction levels were expressed by using different colours: green : 0.76-1.00; light green 1.01-1.25; yellow 1.26-1.50; orange 1.51-1.75; red 1.76-2.00; dark red >2.01.



Supplement figure 3 Dose response of apoplastic alkalinisation to O-methylmelleim over time in transgenic *V.rupestris* cell line. Extracellular pH was measured in response to 0.1% - 1% methanol (a) and 10 µM – 100 µM O-methylmellein (b) along with time for 60 min. Water as the solvent control. The experiments were repeated at least five times.

Table S2 HPLC-MS analysis result of compositions of

***Eutypa lata* IBWF E16012 (BAF) filtrate (Provided by IBWF).**

BAF medium		
Well-position	**Compound**	**Notes**
A3 (0.51 min)	Unidentified	-
A6 (1.33 min)	Unidentified	UV spectra similar to B1
A10 (2.51 min)	Unidentified	-
B9 (3.77 min)	Unidentified	-
B5 (4.79 min)	Unidentified	UV spectra similar to B3 and C3
B3 (5.29 min)	Unidentified	UV spectra similar to B5 and C5
B1 (5.98 min)	Unidentified	UV spectra similar to A6
C2 (6.26 min)	Unidentified	-
C3 (6.69 min)	Unidentified	UV spectra similar to B3 and C5
C7 (7.61 min)	Unidentified	-
C6 (7.35 min)	Unidentified	-
C9 (8.07 min)	**FS E16012-4**	**UV spectra and mass match**

BAF medium		
Well-position	**Compound**	**Notes**
C12 (8.85 min)	Unidentified	-
D11 (9.31 min)	**O-methylmellein**	**UV spectra and mass match**
D9 (9.92 min)	**Siccayne/ Eutypinol**	**UV spectra match; mass not available**
D3 (11.31 min)	Unidentified	-
D2 (11.68 min)	Unidentified	-
E1 (12.11 min)	Unidentified	-
E10 (14.50 min)	Unidentified	-
E3 (12.57 min)	Unidentified	-
E6 (13.40 min)	Unidentified	UV spectra similar to E7, F11 and F12
E7 (13.67 min)	Unidentified	UV spectra similar to E6, F11 and F12
F12 (15.10 min)	Unidentified	UV spectra similar to E6, E7 and F11
F11 (15.30 min)	Unidentified	UV spectra similar to E6, E7 and F12

Table S3 HPLC-MS analysis result of compositions of

***Eutypa lata* IBWF E16012 (PDA) filtrate (Provided by IBWF).**

PDA medium		
Well-position	**Compounds**	**Notes**
A6 (1.35 min)	Unidentified	-
B10 (3.61 min)	Unidentified	-
B8 (4.05 min)	Unidentified	-
B6 (4.57 min)	Unidentified	-
B5 (4.95 min)	Unidentified	-
B1 (5.72 min)	Unidentified	-
C1 (6.20 min)	Unidentified	-
C2 (6.46 min)	Unidentified	-
C4 (6.83 min)	Unidentified	-
C6 (7.30 min)	Unidentified	-

PDA medium		
Well-position	**Compounds**	**Notes**
C9 (8.10 min)	**FS E16123-1**	**UV spectra and mass match**
D12 (9.05 min)	**O-methylmellein**	**UV spectra and mass match**
D10 (9.61 min)	**Siccayne/ Eutypinol**	**UV spectra match; mass not available**
D6 (10.73 min)	Unidentified	-
D4 (11.03 min)	Unidentified	-
D2 (11.60 min)	Unidentified	-
D1 (11.90 min)	**FS E16123-5**	**UV spectra and mass match**
E2 (12.33 min)	**FS E16123-3**	**UV spectra and mass match**
E5 (13.10 min)	Unidentified	-
E9 (14.10 min)	Unidentified	-

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