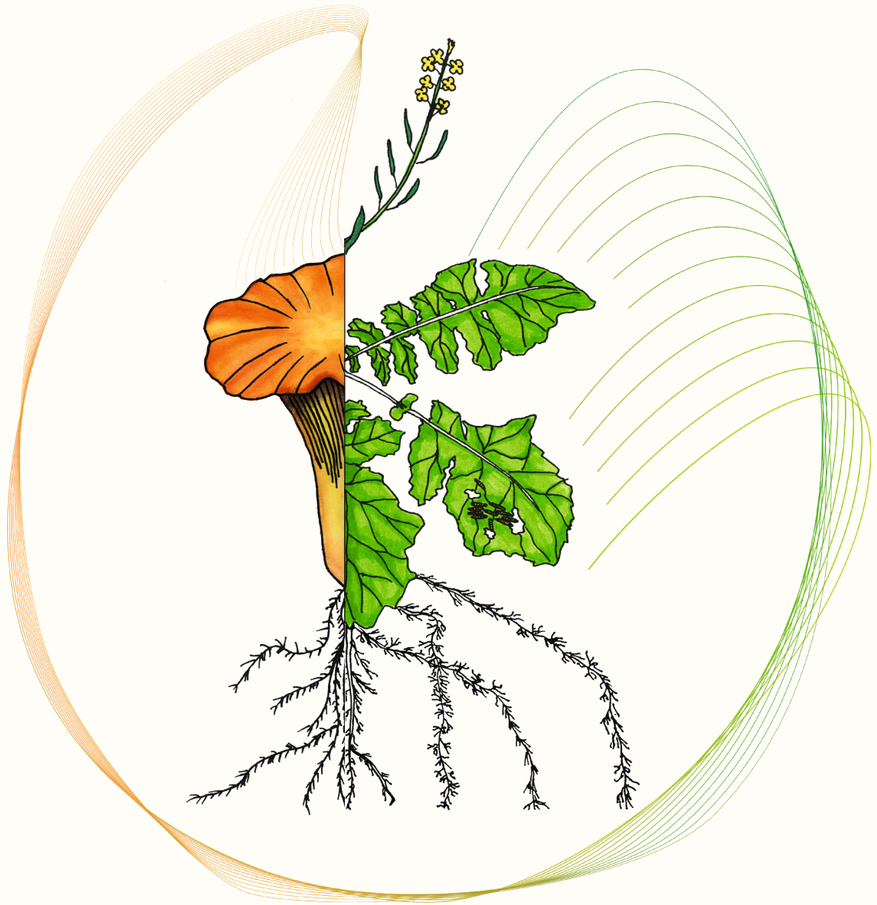


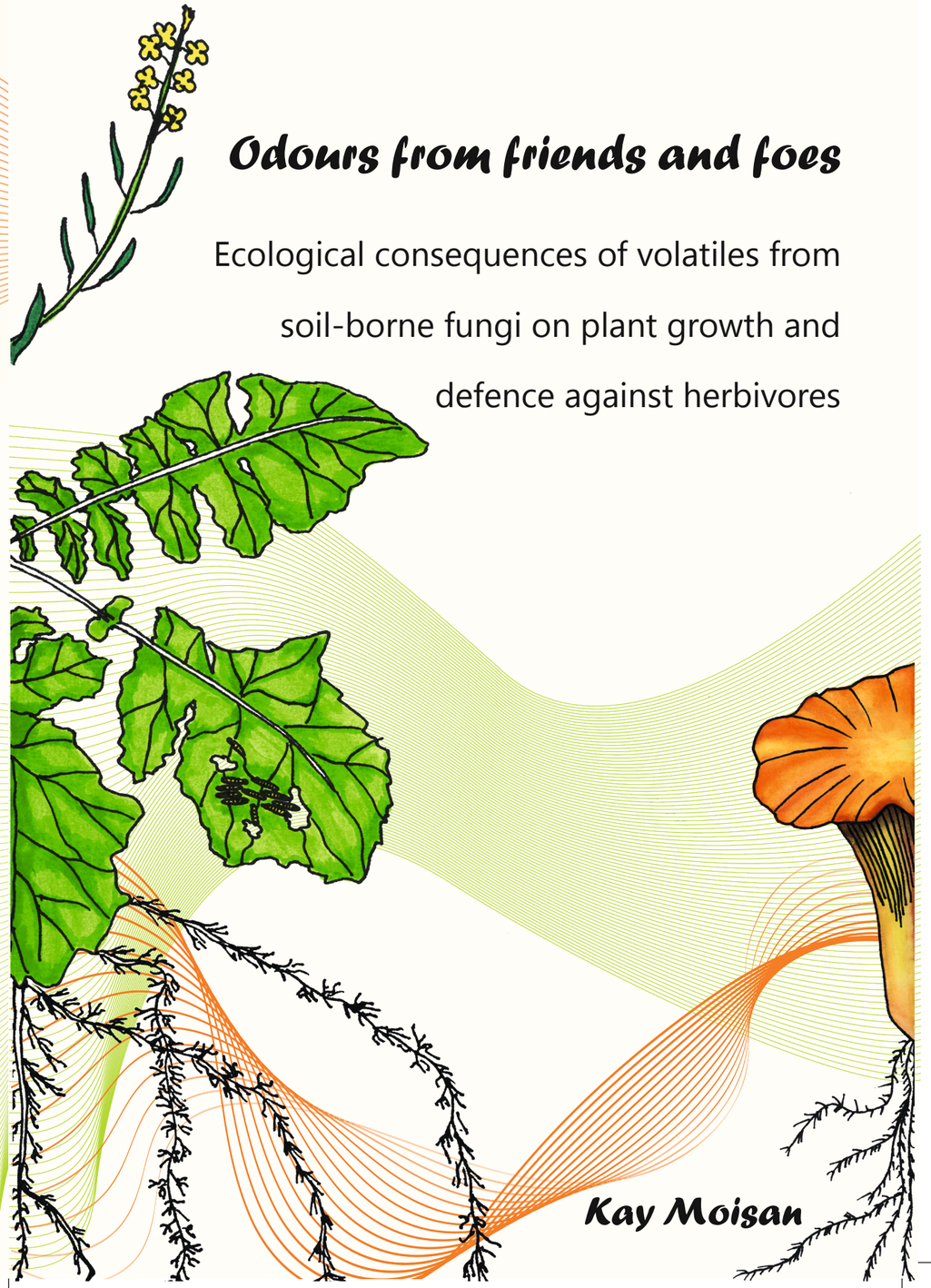
Odours from friends and foes

Kay Moisan

2020

Odours from friends and foes

Ecological consequences of volatiles from soil-borne fungi on plant growth and defence against herbivores



Kay Moisan

Invitation

It is my pleasure to invite you, digitally or physically, to the public defence of my PhD thesis entitled:

Odours from friends and foes: ecological consequences of volatiles from soil-borne fungi on plant growth and defence against herbivores

Friday October 16th 2020
at 11am in the Aula of
Wageningen University,
Generaal Foulkesweg 1a,
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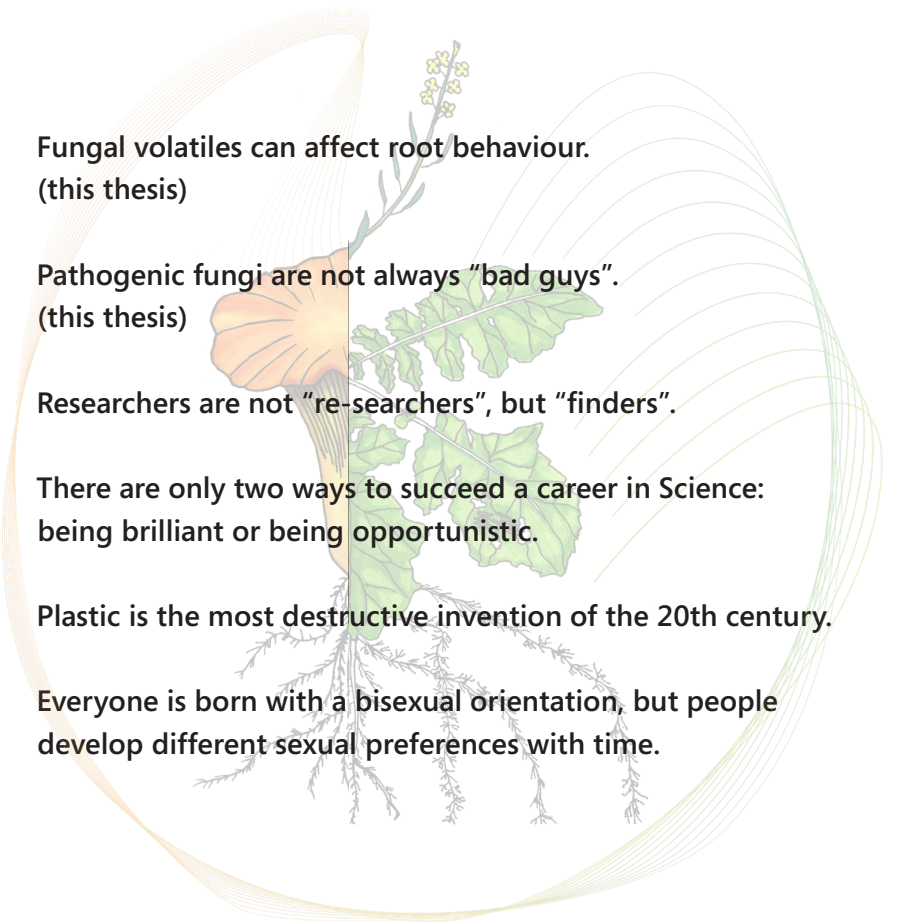
Propositions



1. Fungal volatiles can affect root behaviour.
   (this thesis)

2. Pathogenic fungi are not always "bad guys".
   (this thesis)

3. Researchers are not "re-searchers", but "finders".

4. There are only two ways to succeed a career in Science:
   being brilliant or being opportunistic.

5. Plastic is the most destructive invention of the 20th century.

6. Everyone is born with a bisexual orientation, but people
   develop different sexual preferences with time.

Propositions belonging to the thesis entitled:
"Odours from friends and foes: ecological consequences of volatiles from soil-borne fungi on plant growth and defence against herbivores"

Kay Moisan

Wageningen, October 16th 2020

Odours from friends and foes

Ecological consequences of volatiles from soil-borne fungi on plant growth and defence against herbivores

Kay Moisan

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This research was conducted under the auspices of the graduate school Experimental Plant Sciences.

Odours from friends and foes

Ecological consequences of volatiles from soil-borne fungi on plant growth and defence against herbivores

Kay Moisan

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 16 October 2020
at 11 a.m. in the Aula.

To my father,

Gant ar boan hag an amzer a-benn a bep tra e teuer.
(With effort and time, one can accomplish everything)

-Breton proverb-

Kay Moisan

Odours from friends and foes: ecological consequences of volatiles from soil-borne fungi on plant growth and defence against herbivores

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Chapter 1

General Introduction

General introduction

Plants are naturally exposed below- and aboveground to macro- and microorganisms, which form the phytobiome. Members of the phytobiome comprise deleterious organisms such as herbivores and pathogens, as well as mutualists such as natural enemies of herbivores and plant-growth promoting microbes. Together, these members form complex networks of positive and negative interactions and can directly or indirectly influence each other *via* chemical cues (Raaijmakers *et al.*, 2009; Stam *et al.*, 2014; Leach *et al.*, 2017). For instance, soil microorganisms can positively or negatively affect plant health upon root colonisation. By secreting molecules, soil-borne microbes may promote plant growth and enhance the plant's ability to respond to attack by various organisms and to environmental stresses (Pineda *et al.*, 2010; Berendsen *et al.*, 2012; Raaijmakers, 2015; Vandenkoornhuyse *et al.*, 2015; Dicke, 2016). Over the past two decades, a considerable number of studies has acknowledged the importance of volatiles in mediating interactions within the phytobiome (Heil, 2014; Piechulla and Degenhardt, 2014; Schmidt *et al.*, 2015; Dicke, 2016; Schulz-Bohm *et al.*, 2017b). Despite recent advances, only the tip of the iceberg that covers volatile-mediated interactions has been unveiled so far. Below, I describe the current knowledge of the ecological roles of microbial and plant volatiles for ecosystem functioning and plant health.

Biosynthesis and emission of volatiles

Biosynthetic pathways and emission of volatile organic compounds

Volatiles are synthesised by all living organisms including plants, animals, and microorganisms (Wenke *et al.*, 2010; Peñuelas *et al.*, 2014; Tumlinson, 2014) and are part of their primary and secondary metabolism. Volatiles are characterised by small molecules (< 20 carbon atoms) with a low molecular mass (< 300 Da), low boiling point (< 200 °C) and high vapour pressure (> 0.01 kPa at 20 °C) (Dudareva *et al.*, 2004; Maffei *et al.*, 2011; Morath *et al.*, 2012). Upon their release into the environment, volatiles can diffuse in the water and gas phases, and thus can be transported over "long" distances in the air and soil. These properties make them good candidates as infochemicals (Dicke and Sabelis, 1988) that mediate intra- and interspecific interactions. Volatiles comprise inorganic compounds such as CO_2 or N_2O (Bryant, 1979; Baggs, 2011), and organic compounds (VOCs). In plants, the main VOC biosynthetic pathways include the shikimic acid pathway, terpenoid pathways (mevalonic acid and methylerythritol phosphate pathways) and lipoxygenase pathway (Laothawornkitkul *et al.*, 2009; Dudareva *et al.*, 2013), whereas bacterial and fungal VOCs result from aerobic metabolism, heterotrophic carbon metabolism, fermentation, amino acid catabolism, terpenoid biosynthesis, fatty-acid degradation and sulphur reduction (Pichersky *et al.*, 2006; Maffei, 2010; Maffei *et al.*, 2011; Davis *et al.*, 2013; Peñuelas *et al.*, 2014). Currently, there is ample evidence that VOCs are induced in response to certain

stimuli or conditions, *i.e.* when microorganisms compete for resources in a nutrient-poor environment (Baldwin, 2010; Insam and Seewald, 2010; Audrain *et al.*, 2015; Saxena, 2016) or upon attack of plants by herbivores (Paré and Tumlinson, 1999; Baldwin, 2010; Maffei, 2010). VOCs can be either stored in the emitter's tissues or synthesised *de novo* before being emitted in the environment (Widhalm *et al.*, 2015; Tissier *et al.*, 2017). Several mechanisms, other than passive diffusion, were proposed to describe the transport of VOCs from their synthesis site to their release in the environment (Insam and Seewald, 2010; Widhalm *et al.*, 2015; Tissier *et al.*, 2017). The emission of VOCs is mostly determined by their physicochemical characteristics, such as solubility, volatility and diffusivity. For the rest of this chapter, I will focus solely the roles of plant and microbial VOCs in terrestrial ecosystems.

Composition and dynamics of VOC blends

More than 1700 plant volatiles have been described to be emitted from roots, leaves and flowers (Steeghs *et al.*, 2004; Knudsen *et al.*, 2006; Baldwin, 2010; Schenkel *et al.*, 2015), with sesquiterpenoids representing the most abundant group of compounds (Harrison *et al.*, 2013). With respect to microorganisms, such as fungi and bacteria, 1860 VOCs have been described and listed in the mVOC 2.0 database (Korpi *et al.*, 2009; Morath *et al.*, 2012; Schenkel *et al.*, 2015; Lemfack *et al.*, 2017). Typical fungal VOCs include 1-octen-3-ol and geosmin (Hung *et al.*, 2015; Dickschat, 2017). Fungal VOC blends are mostly dominated by alcohols and benzenoids, whereas bacterial VOC blends, are dominated by alkenes, alcohols, ketones and terpenes (Peñuelas *et al.*, 2014). VOCs are rarely emitted as single compounds, instead they are part of a blend that comprises up to a hundred VOCs, balanced in species-specific ratios (Stotzky *et al.*, 1976; Ueda *et al.*, 2012; Dudareva *et al.*, 2013). Whereas some VOCs are commonly emitted by bacteria, fungi and plants (Schenkel *et al.*, 2015; Quintana-Rodriguez *et al.*, 2018), other VOCs are emitted only by specific phylogenetic groups or species (Farag *et al.*, 2006; Splivallo *et al.*, 2007a; Hertel *et al.*, 2016), and can, therefore, be used for taxonomic characterisation (Spinelli *et al.*, 2012; Cordovez *et al.*, 2015; Oliveira *et al.*, 2015; Tóth *et al.*, 2016). A recent study suggested that fungal VOC profiles may be also used for chemotyping different fungal lifestyles, *i.e.* ectomycorrhizal, pathogenic and saprophytic species (Müller *et al.*, 2013). However, in that study only a few representatives were tested for each lifestyle and further investigations are needed to validate these findings. VOC emission is highly dynamic and may follow temporal dynamics, for instance the circadian clock in plants (Fenske and Imaizumi, 2016; Schuman *et al.*, 2016). Additionally, environmental conditions can affect VOC emission in plants and microorganisms, such as the composition and richness of the substrate, temperature or humidity (Wheatley *et al.*, 1997; Gouinguené and Turlings, 2002; Loreto *et al.*, 2014; Weikl *et al.*, 2016). Furthermore, VOC emission is also correlated with the age and phenology of the emitter (Splivallo *et al.*, 2007a; Dudareva *et al.*, 2013; Simon *et al.*, 2017). Therefore, VOC emission is context-dependent.

Occurrence and importance of VOCs for ecosystem functioning

Effects of plant VOCs above-and belowground

Plant VOCs mediate a myriad of intra- and inter-kingdom interactions (Heil and Karban, 2010; Maffei, 2010; Poelman *et al.*, 2012; Pierik *et al.*, 2014; Schiestl, 2015). Plant VOCs can mediate within-plant and between-plant interactions. For instance, VOCs emitted by one tissue can provide information about its status to a more distant tissue of the same plant, hence communicate in a faster way than *via* vascular tissues (Karban *et al.*, 2006; Heil and Silva Bueno, 2007). Additionally, VOCs emitted by one plant can also positively or negatively affect growth of con- or heterospecific neighbouring plants (Dicke *et al.*, 2003; Kegge and Pierik, 2010). Interestingly, upon attack by a herbivore or a pathogen, plants release a specific blend of VOCs referred to as Herbivore-Induced Plant Volatiles (HIPVs) and Pathogen-Induced Plant Volatiles (PIPVs), respectively. HIPVs and PIPVs can have a direct negative effect on the coloniser's performance (Scala *et al.*, 2013; Hammerbacher *et al.*, 2019). Furthermore, when released aboveground or belowground, these HIPVs and PIPVs can be exploited by others such as neighbouring plants (Baldwin *et al.*, 2002; Dicke *et al.*, 2003; Peng *et al.*, 2011) and natural enemies of that attacker, which, in turn, alleviate the herbivore and pathogen pressure on plants (Dicke and Baldwin, 2010; Rasmann *et al.*, 2012b; Schulz-Bohm *et al.*, 2018). Thus, the emission of HIPVs and PIPVs is part of indirect plant resistance mechanisms. Beside resistance, VOCs are also crucial for plant reproduction. Indeed, floral VOCs attract pollinators that ensure pollen transfer (Muhlemann *et al.*, 2014; Schiestl, 2015). Together, these examples illustrate the importance of plant VOCs for plant survival and reproduction *via* the modulation of plant interactions with mutualists and antagonists.

Effects of microbial VOCs belowground

Because of its porous structure, soil provides different sites filled with water or gases that harbour diverse communities of soil microorganisms (Ruamps *et al.*, 2011). In this dark environment, soil microorganisms emit blends of VOCs that mediate important ecological interactions with con- and heterospecific organisms in the soil (Hung *et al.*, 2015; van Dam and Bouwmeester, 2016; van Dam *et al.*, 2016; Werner *et al.*, 2016). First, microbial VOCs can act as defensive molecules that exert antagonistic activity against microbial competitors (Minerdi *et al.*, 2009; Schmidt *et al.*, 2015; Schulz-Bohm *et al.*, 2015; Werner *et al.*, 2016; Enespa and Chandra, 2017; Schulz-Bohm *et al.*, 2017a). For instance, bacterial VOCs can negatively affect the germination of fungal spores or inhibit growth of hyphae (Wheatley, 2002; Vespermann *et al.*, 2007; Kai *et al.*, 2009) as well as growth of certain bacteria (Schulz *et al.*, 2010; Dandurishvili *et al.*, 2011) and nematodes (Bui *et al.*, 2019). Similarly, some fungal VOCs may have antifungal, antibacterial, nematicidal or self-inhibiting properties (Strobel *et al.*, 2001; Bennett *et al.*, 2012; Werner *et al.*, 2016; Piechulla *et al.*, 2017). Interestingly, microbial VOCs can also influence the behaviour of soil organisms, *e.g.* repelling protists or nematodes (Werner *et al.*, 2016; Schulz-Bohm *et al.*, 2017a). In some cases, microbial VOCs

can stimulate growth of fungi and bacteria (Vespermann *et al.*, 2007; Garbeva *et al.*, 2014a; Werner *et al.*, 2016). Thus, microbial VOCs play an essential role in shaping soil fauna and soil microorganism communities. More recently, microbial VOCs have been described to impact plant growth and defence (Ryu *et al.*, 2003). To date, the effects of VOCs in plant-microbe interactions are still far less understood compared with the effects of VOCs in microbe-microbe interactions.

Microbial VOC-mediated interactions with plants

Plant perception of microbial VOCs

Plants may be exposed to VOCs emitted by plant-colonising microorganisms (endophytes and ectophytes) and by microorganisms growing near the plants. Microbial VOCs are released directly in the plant tissues or into the environment (Junker and Tholl, 2013; Peñuelas *et al.*, 2014; Sudipta and Debdulal, 2019). To date, however, the mechanisms of VOC perception by plants as well as the transduction pathways remain poorly understood. In leaves, it was hypothesised that exogenous VOCs enter the intercellular space through the stomata or diffuse through the cuticle, and dissolve into cell membranes as a function of their polarity (Seco *et al.*, 2007; Heil, 2014; Matsui, 2016). In roots, however, the mechanisms remain largely unknown. Wenke and Piechulla (2013) coined the term mVAMP (microbial volatile-associated molecular pattern), and it was hypothesised that mVAMPs function similarly as MAMPs (microbe-associated molecular patterns), DAMPs (damage-associated molecular pattern) and PAMPs (pathogen-associated molecular pattern), where specific VOCs are recognised by cell receptors and induce a signal-transduction cascade that leads to differential plant responses (Tyagi *et al.*, 2018). There is indeed growing evidence that microbial VOCs induce manifold changes at the plant transcriptomic, proteomic and metabolomic levels (Kwon *et al.*, 2010; Bailly and Weisskopf, 2012; Cordovez *et al.*, 2017; Ameztoy *et al.*, 2019; Tyagi *et al.*, 2019), which consequently alter plant chemical and physical traits.

From plant growth inhibition to growth promotion and faster flowering

Since the first report of Ryu *et al.* (2003), a growing body of studies has reported the effects of bacterial and fungal VOCs on plant growth and development (Farag *et al.*, 2006; Bitas *et al.*, 2013; Garnica-Vergara *et al.*, 2015; Kanchiswamy *et al.*, 2015b; Piechulla *et al.*, 2017; Fincheira and Quiroz, 2018). At the seed stage, microbial VOCs can accelerate or delay germination (Ogura *et al.*, 2000; Hung *et al.*, 2014; Cordovez *et al.*, 2017). The effects on plant growth ranged from plant death (Blom *et al.*, 2011; Weise *et al.*, 2013) up to ten-fold biomass increase (Paul and Park, 2013). Yet, plant growth was predominantly promoted upon exposure to microbial VOCs (Fincheira and Quiroz, 2018). Microbial VOC-exposed plants also exhibited larger leaves (Ryu *et al.*, 2003; Lee *et al.*, 2012; Bitas *et al.*, 2015), higher accumulation of starch in leaves (Ezquer *et al.*, 2010; Li *et al.*, 2011)

and increased height (Han *et al.*, 2014; Sánchez-López *et al.*, 2016). In addition to plant growth promotion, microbial VOCs may also accelerate plant development and induce earlier flowering (Sánchez-López *et al.*, 2016). Several scenarios were proposed to explain these plant phenotypic responses. ***(i)*** Microbial VOCs can enhance photosynthetic rate by increasing photosynthetic efficiency and chlorophyll content *via* translational modifications and *via* modulation of thiol reduction-oxidation of cysteins (Zhang *et al.*, 2008; Minerdi *et al.*, 2011; Hung *et al.*, 2013; Fincheira and Quiroz, 2018; Ameztoy *et al.*, 2019). ***(ii)*** Microbial VOCs can enhance plant nutrition and uptake of minerals (Kwon *et al.*, 2010; Fincheira and Quiroz, 2018). For example, dimethyl-disulfide produced by a *Bacillus* strain acts as a source of sulphur, and can be directly assimilated and incorporated into plant protein metabolism (Hofmann, 2013; Meldau *et al.*, 2013). Alternatively, microbial VOCs can change root architecture by increasing root surface area, number of lateral roots and hairs, which in turn facilitate nutrient uptake (Ortiz-Castro *et al.*, 2008; Splivallo *et al.*, 2009; Ditengou *et al.*, 2015; Garnica-Vergara *et al.*, 2015; Casarrubia *et al.*, 2016). ***(iii)*** Microbial VOCs can also affect plant hormone homeostasis (Bailly and Weisskopf, 2012; Bhattacharyya *et al.*, 2014), which orchestrates vital plant growth and development (Davies, 2010). These changes in plant hormone homeostasis may be directly linked to plant phenotypic responses to microbial VOCs such as growth promotion through cell expansion (Zhang *et al.*, 2007; Bitas *et al.*, 2015) and cell division (Zhang *et al.*, 2008; Sánchez-López *et al.*, 2016) or changes in root architecture (Splivallo *et al.*, 2009; Garnica-Vergara *et al.*, 2015). Therefore, modulation of the hormone-signalling machinery and hormone transport by microbial VOCs can affect plant growth and development (Bailly and Weisskopf, 2012; Bhattacharyya *et al.*, 2014; Tyagi *et al.*, 2018).

Helping plants to endure harsh environmental conditions

Plant growth can be challenged when plants face abiotic stresses, such as salinity, drought, exposure to heavy metals or sulphur and iron deficiency, and microbial VOCs can help plants to endure these stresses by sustaining plant growth (Zhang *et al.*, 2008; Han *et al.*, 2014; Liu and Zhang, 2015; Zamioudis *et al.*, 2015; Jalali *et al.*, 2017; Camarena-Pozos *et al.*, 2019). Coined as tolerance (Schafer, 1971), the capability of plants to endure stresses that damage plant tissues or limit plant development can be measured by the degree to which plant growth is affected by a given stress relative to its growth in the undamaged state (Strauss and Agrawal, 1999). Remarkably, microbial VOCs can influence the degree of plant tolerance to abiotic stresses (Bhattacharyya *et al.*, 2014; Liu and Zhang, 2015). For instance, VOCs emitted by *Bacillus subtilis* can alleviate the negative effects of high salinity or drought on *A. thaliana* growth by regulating sodium transporters (Zhang *et al.*, 2008) and by closing stomata to prevent water loss, respectively (Cho *et al.*, 2008). Here again, plant hormones play a crucial role in orchestrating such plant responses and microbial VOCs, as mentioned above, can influence hormone homeostasis.

Enhancing plant resistance to pathogens and herbivores

Microbial VOCs can enhance plant resistance to a range of attackers (Salas Marina *et al.*, 2011; Lee *et al.*, 2012; Farag *et al.*, 2013; Naznin *et al.*, 2014; Kottb *et al.*, 2015; Aziz *et al.*, 2016; Sharifi and Ryu, 2016; Cordovez *et al.*, 2017; Tahir *et al.*, 2017a). For instance, exposure of *A. thaliana* and tobacco plants to 2,3-butanediol leads to the reduction of disease symptoms of bacterial leaf pathogens, *e.g. Pseudomonas syringae* or *Erwinia carotovora,* and fungal pathogens, *e.g. Botrytis cinerea* (Ryu *et al.*, 2004; Han *et al.*, 2006; Kishimoto *et al.*, 2007). First, microbial VOCs can directly inhibit pathogen growth because of their antifungal and antibacterial properties (Schmidt *et al.*, 2015; Sharifi and Ryu, 2016; Ulloa-Benítez *et al.*, 2016; Werner *et al.*, 2016; Rojas-Solís *et al.*, 2018). Additionally, microbial VOCs can enhance plant resistance, for example by altering the regulation of the plant hormones jasmonic acid, salicylic acid and ethylene (Farag *et al.*, 2013; Sharifi and Ryu, 2016; Cordovez *et al.*, 2017; Lee *et al.*, 2019; Tyagi *et al.*, 2019) that orchestrate plant defensive responses (Pieterse *et al.*, 2012). Because different plant attackers induce specific hormonal signalling pathways in plants (Davies and Gilbert, 1985; Erb *et al.*, 2012; Checker *et al.*, 2018), hormone homeostasis changes caused by microbial VOCs may differentially affect the attacker. To date, far fewer studies have investigated the effects of microbial VOCs on plant resistance to insects compared with that of pathogens (Song and Ryu, 2013; D'Alessandro *et al.*, 2014; Aziz *et al.*, 2016; Cordovez *et al.*, 2017). *Spodoptera exigua* larvae performed worse on *A. thaliana* exposed to VOCs from *Bacillus amyloliquefaciens* than on control plants, whereas the opposite effect was observed for *Mamestra brassicae* caterpillars feeding on *A. thaliana* plants exposed to VOCs from *Rhizoctonia solani* (Aziz *et al.*, 2016; Cordovez *et al.*, 2017). Some microbial VOCs were shown to augment sulphur metabolism (Martínez-Medina *et al.*, 2017) and the accumulation of defensive sulphur-containing plant metabolites like glucosinolates (Aziz *et al.*, 2016). So far, a thorough understanding of how microbial VOCs affect the performance of insect herbivores is still lacking. Apart from effects on insect performance, microbial VOCs can also impact insect behaviour (Becher *et al.*, 2012; Davis *et al.*, 2013). For instance, 1-octen-3-ol emitted by phyllospheric fungi is attractive to numerous insect species for oviposition, and could therefore have direct effects on higher trophic levels as well (Davis *et al.*, 2013). For instance, VOCs emitted by wheat infested with *Aspergillus* and attacked by the weevil *Sitophilus granarius* were less attractive to the parasitoids *Lariophagus distinguendus* compared with pathogen-free plants (Steiner *et al.*, 2007). In an open field, application of 2,3-butanediol in the soil of maize plants that were infested with *Spodoptera littoralis* larvae attracted more *Cotesia marginiventris* parasitic wasps than non-exposed larvae-infested plants (D'Alessandro *et al.*, 2014). Similarly, cucumber plants drenched in a solution with 3-pentanol and 2-butanone recruited more ladybirds than control plants (Song and Ryu, 2013). Yet, it remains unknown whether microbial VOCs can affect plant VOC emission from a distance, for instance HIPV emission, thus influencing the attraction of natural enemies upon herbivory. Alongside with resistance, plants may also endure herbivory and the loss of consumed tissues *via* tolerance mechanisms (Stowe *et al.*, 2000; Leimu and Koricheva, 2006). Direct plant colonisation by

soil microorganisms has already been shown to enhance plant tolerance to herbivory (Hermosa *et al.*, 2013; Contreras-Cornejo *et al.*, 2016). However, it is unknown whether plant tolerance to herbivores is influenced microbial VOCs as well.

Current challenges and outstanding questions

The studies mentioned above demonstrate that microbial volatilomics has become an emerging topic in plant research in the last two decades. However, despite technological advances in the collection and analysis of VOCs (Steeghs *et al.*, 2004; van Dam *et al.*, 2016; Majchrzak *et al.*, 2020), research on microbial VOCs still faces challenges and technical issues that limit profound insight in the ecological implications of the findings (Li *et al.*, 2016). Most studies conducted so far have employed *in vitro* bio-assays to test the effects of microbial VOCs on plant growth (Kai *et al.*, 2016; Song *et al.*, 2019), by using in 55% of the cases the model plant *Arabidopsis thaliana* growing in a two-compartment Petri dish (Fincheira and Quiroz, 2018). This setup provides many advantages, such as a rapid screening of phenotypic plant responses and the isolation of the microbial strain to test, but also disadvantages. Some of the plant phenotypes observed in closed systems, like growth promotion, may result from experimental artefacts such as the accumulation of microbial CO_2 (Kai and Piechulla, 2009; Piechulla and Schnitzler, 2016). Moreover, growing plants in Petri dishes implies the exposure of both shoots and roots to the VOCs, which may not reflect the occurrence of such interactions in natural ecosystems. Therefore, the development of new methods to test microbial VOC-mediated interactions with plants is needed (Song *et al.*, 2019). To date, only a few studies addressed plant exposure *in planta* to the complete volatile blend emitted by the test microorganism (Park *et al.*, 2015; Cordovez *et al.*, 2017; Tahir *et al.*, 2017b; Tahir *et al.*, 2017c; Bui *et al.*, 2019). However for most studies, the challenge of identifying "bioactive" compounds lies in the complexity and dynamics of VOC blends (Ueda *et al.*, 2012) and the identity of the receiver. Thus, from an ecological perspective, it is crucial to address whole VOC blends with their specific concentration and ratios to fully understand plant responses to microbial VOCs.

In addition to these technical limitations, fundamental understanding of such ecological interactions is still lacking. To date, research has mainly focussed on screening VOCs from a large array of beneficial bacteria and fungi for their potential application in agriculture. Yet, in natural ecosystems, plants are likewise exposed to VOCs from deleterious microorganisms, and very little is known about plant responses to volatile cues from potential enemies. At the start of my project, only three studies had addressed plant responses to VOCs from pathogenic microorganisms (Ezquer *et al.*, 2010; Minerdi *et al.*, 2011; Fiers *et al.*, 2013). Since then, new studies have shown that pathogenic fungi may also impact plant growth (Bitas *et al.*, 2015; Casarrubia *et al.*, 2016; Sánchez-López *et al.*, 2016; Cordovez *et al.*, 2017; Fincheira and Quiroz, 2018; Li and Kang, 2018). However, it is currently unknown whether plants respond differentially to VOCs emitted by pathogenic and non-pathogenic

microorganisms. Furthermore, beyond plant growth, the ecological consequences of plant responses to microbial VOCs for other members of the phytobiome remain understudied. So far, studies focussed almost exclusively on microbial VOC-induced plant resistance to bacterial and fungal pathogens (Ryu *et al.*, 2004; Han *et al.*, 2006; Salas Marina *et al.*, 2011; Kottb *et al.*, 2015; Sharifi and Ryu, 2016; Tahir *et al.*, 2017c). However, plants are threatened by diverse herbivorous enemies aboveground and belowground, and employ different defence strategies, *i.e.* resistance and tolerance, to cope with these attackers. Therefore, more research is needed to unravel the ecological consequences of microbial VOCs on plant interactions with deleterious organisms, such as insect herbivores, as well as mutualists like natural enemies of insect herbivores.

General objective and research questions of this thesis

The overall aim of this PhD project was to assess the effects of fungal volatiles on plant health, in particular plant growth and defence to herbivores aboveground and belowground. To tackle these questions, I focussed on chemical and ecological aspects of fungal volatile-mediated interactions with plants and herbivores. By combining *in vitro* laboratory studies, greenhouse *in planta* bioassays and field experiments, I explored the ecological consequences of such volatile-mediated interactions for plant growth and defence.

The major research questions addressed in this thesis are (Fig. 1):

- Do pathogenic and non-pathogenic soil-borne fungi emit distinct VOC profiles and do plants discriminate between these VOC blends? (Chapters 2 and 6)
- Does root exposure to fungal VOCs affect plant direct and indirect resistance and tolerance to belowground and aboveground herbivory? (Chapters 3 and 4)
- Do fungal VOCs affect the performance of root herbivores? (Chapter 5)

Study system

To address the questions raised above, I used two different study systems. I chose two brassicaceous plants: the model plant *Arabidopsis thaliana* to build on existent knowledge (Fig. 2a) and the wild turnip *Brassica rapa* to address more relevant ecological questions (Fig. 2b), with their associated herbivores, and I selected a range of soil-borne fungi associated with Brassicaceae.

Arabidopsis thaliana, also known as thale cress, is a small rosette plant with a fast cycle of approximately six weeks from germination to mature seeds (www.arabidopsis.org). Although most *A. thaliana* accessions are winter annuals, they may be still exposed to herbivory (Mosleh Arany *et al.*, 2009). In my PhD project, I used *Mamestra brassicae* L. (Lepidoptera: Noctuidae), a generalist herbivorous insect species that feeds on Brassicaceae. This lepidopteran species, known as the cabbage moth, is an important pest on a broad range of crops including cabbage and turnip (Ahuja *et al.*, 2011). It takes about two months for the larvae to go through the five moults before turning into adults. The insect occurs generally with two generations per year.

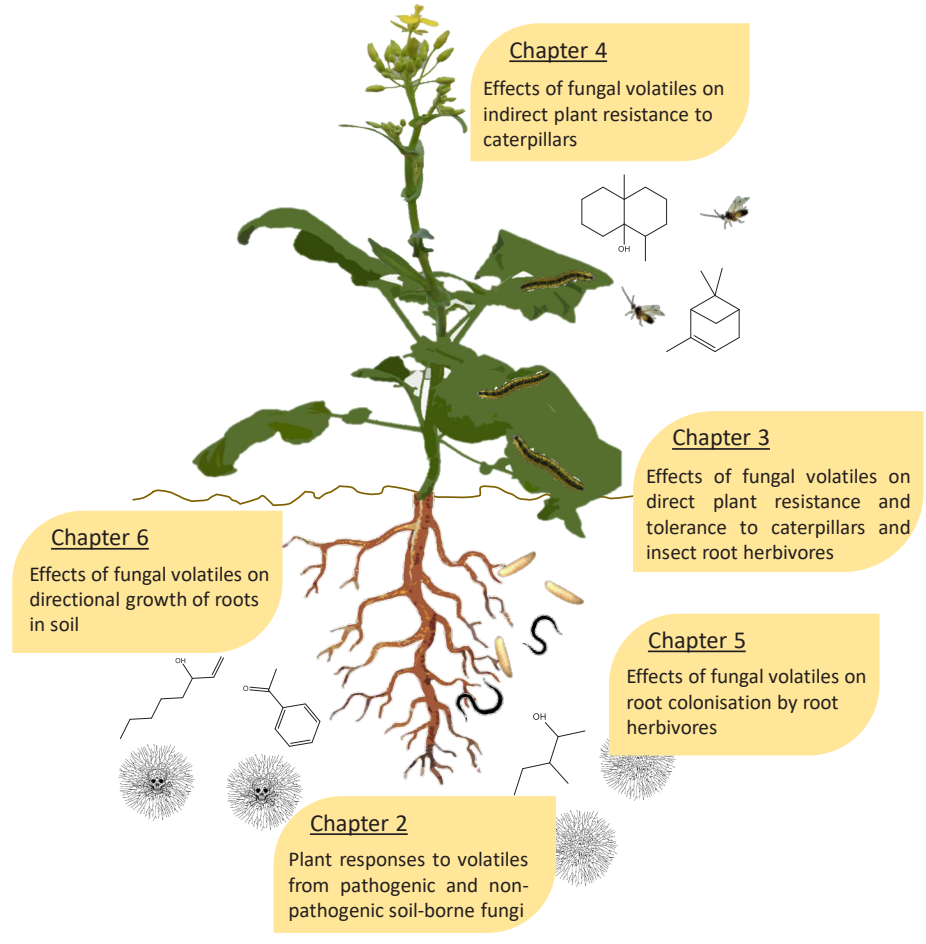


Figure 1. Schematic overview of the main objectives addressed in each chapter presented in this thesis.

Brassica rapa, also known as the wild turnip, is an annual flowering plant that occurs in ecosystems such as cropland, weedy fields or roadsides. This species also comprises domesticated subspecies and cultivars such as the cultivated turnip *Brassica rapa subsp. rapa,* which is distributed worldwide. Roots, leaves and flowers of *B. rapa* are subjected to attack by a large range of insect herbivores as well as pathogens (www.cabi.org). Here, I used the caterpillar *Pieris brassicae* (Lepidoptera: Pieridae) and its parasitoid *Cotesia glomerata* (Hymenoptera: Braconidae), the insect root herbivore *Delia radicum* (Diptera: Anthomyiidae) and the plant-parasitic nematode *Heterodera schachtii* (Tylenchida: Heteroderidae). Also known as Large Cabbage White butterfly, *P. brassicae* is a specialist insect herbivore, whose larvae feed on brassicaceous plants. First instar caterpillars of *P. brassicae* feed on leaves, whereas later instars move to the inflorescences to feed on buds and flowers (Lucas-Barbosa *et al.*, 2013). Natural enemies of this caterpillar comprise the parasitic wasp *C. glomerata,* a gregarious endoparasitoid that targets Pieridae larvae in the first or second instars (Sato and Ohsaki, 2004). Eggs of the Cabbage Root fly, *D. radicum,* are laid on the surface of the soil, next to the stem of brassicaceous species (Nottingham, 1988). Upon hatching, larvae crawl down to the roots and feed on the outer layer of primary roots, inflicting dramatic damage to roots. Nematodes of *H. schachtii* are commonly named as sugar beet nematodes after their primary host plants. Juveniles enter the roots and travel until main vascular tissues where they establish a feeding site. After fertilisation, female nematodes continue feeding as their eggs develop inside, forming a cyst (Smith *et al.*, 2004).

I selected eleven soil-borne fungi based upon the literature by following three criteria: 1) the fungi spend part of their life in soil as saprophytes; 2) the fungi co-occur in the regions where *A. thaliana* and *B. rapa* are naturally present; 3) the fungi were previously described as interacting with brassicaceous plants. Five of the eleven selected fungi are reported as economically important pathogens of brassicaceous plant species. By penetrating through lateral roots and root hairs, *Verticillium longisporum* and *V. dahliae* (Ascomycota) cause vascular wilt in more than 400 plant species, including brassicaceous species (Karapapa *et al.*, 1997; Fradin and Thomma, 2006; Zhou *et al.*, 2006). Similarly, pathogenic *Fusarium oxysporum* (Ascomycota) causes vascular wilt in a wide range of crops and the *forma specialis* used here, *raphani,* is particularly adapted to infecting radish plants and possibly other relatives (Bosland and Williams, 1987; Diener and Ausubel, 2005). *Sclerotinia sclerotiorum* (Ascomycota) causes stem rot and watery soft rot in more than 400 plant species. The fungus can enter the plant either in the leaves and flowers *via* arpogenic germination or in the roots *via* myceliogenic germination (Dickman and Mitra, 1992). *Rhizoctonia solani* (Basidiomycota) encompasses several anastomosis groups that infect different plant species. After entering plants through roots or lower stems, the fungus typically causes damping-off or bottom rot, and its large host range includes members of the Brassicaceae (Pannecoucque and Höfte, 2009). The six other fungi that I used in my study were reported as endophytes of Brassicaceae or saprophytes (Kumud *et al.*, 1986; Ishimoto *et al.*, 2000; Junker *et al.*, 2012; Keim *et al.*, 2014), and thus I categorised

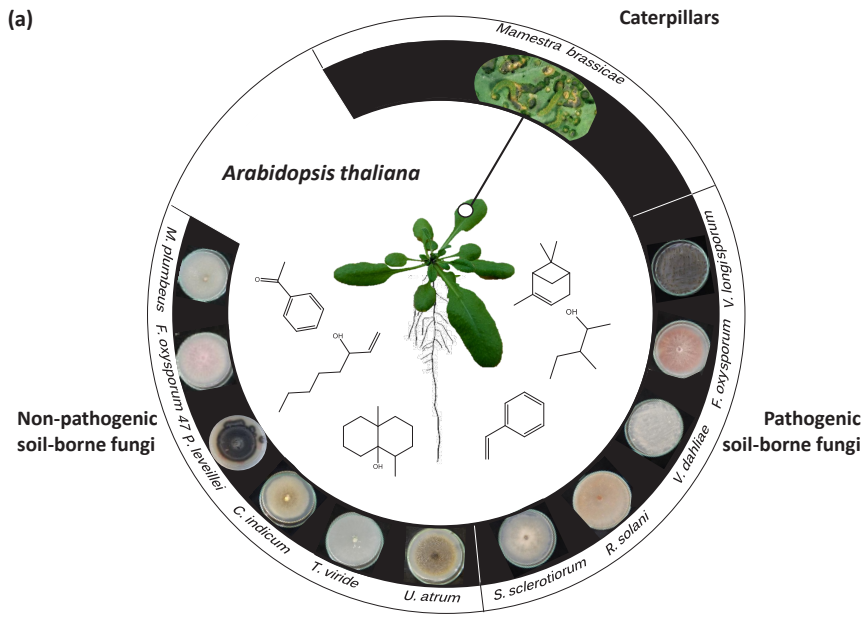


Figure 2. Schematic overview of the two study systems used **(a)** with *Arabidopsis thaliana* and **(b)** with *Brassica rapa*. Chemical structures in the middle depict examples of fungal volatile organic compounds.

them as non-pathogenic. *Trichoderma viride* (Ascomycota) is a fungus well-known for its plant growth promotion properties and its biocontrol activity by parasitising other fungi (Harman *et al.*, 2004). *Fusarium oxysporum* Fo47 (Ascomycota) is a non-pathogenic strain that contributes to disease suppression in soils (Alabouvette, 1999; Aimé *et al.*, 2013). For the other fungi, *Ulocladium atrum* (Ascomycota), *Chaetomium indicum* (Ascomycota), *Phoma leveillei* (Ascomycota) and *Mucor plumbeus* (Zygomycota), no particular effect on plant growth or defence has been reported.

Outline of the thesis

In **Chapter 2**, I screened responses of *Arabidopsis thaliana* seedlings when exposed *in vitro* to volatiles emitted by a range of soil-borne fungi, and I profiled VOCs emitted by these fungi, and quantified fungal CO_2 emission. In this chapter, I aimed at comparing the VOC blends emitted by pathogenic and non-pathogenic fungi and at unravelling whether plants could discriminate between these VOC blends and respond specifically in terms of growth, development and resistance. The effects of fungal volatiles on plant growth were assessed after one week *in vitro* volatile exposure, but also after plant transplantation in soil following one week *in vitro* volatile exposure. I also scored the flowering time of volatile-exposed *A. thaliana* seedlings and the performance of the caterpillar *Mamestra brassicae* feeding on these volatile-exposed plants.

In **Chapter 3**, I designed a two-compartment pot system in which only roots, growing in soil, were exposed *in planta* to fungal volatiles. Using this pot system, I investigated whether fungal volatiles influence plant defence against aboveground and belowground herbivory. I addressed in particular plant resistance and tolerance upon infestation of volatile-exposed plants by either the caterpillar *P. brassicae* or the root herbivore *D. radicum*. I expected that the effects of fungal volatiles would differ between roots and aerial plant tissues, thus differentially impacting the performance of the insect feeding on these tissues. To test this, I scored insect biomass and developmental stage, and I monitored different plant traits such as plant biomass, plant flowering time and number of buds and flowers.

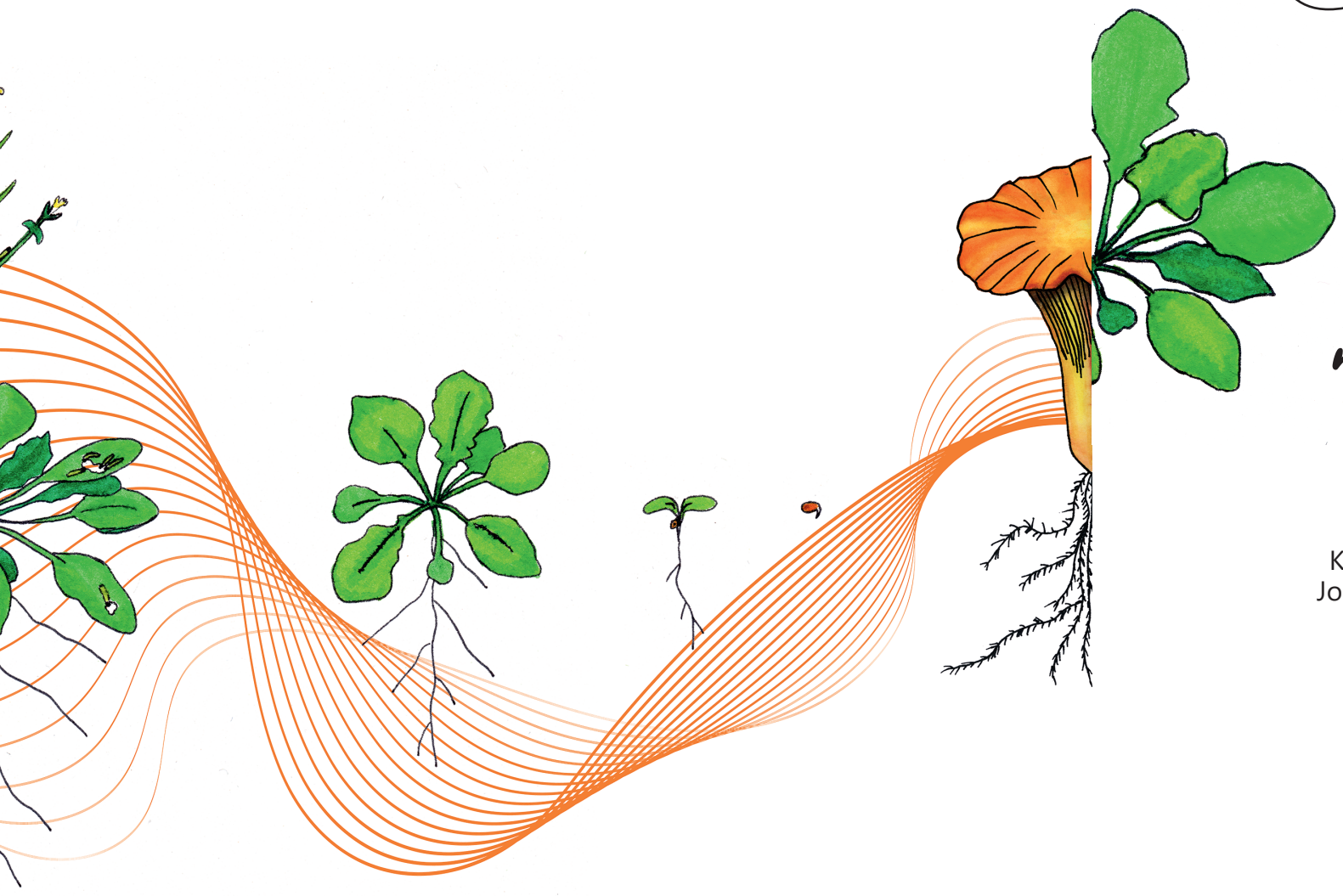
Interestingly, the perception of volatiles can affect volatile emission of the receiver. There is growing evidence that leaf volatiles from an attacked plant can increase volatile emission by a neighbouring plant, thus enhancing its indirect resistance. In this line, I speculated that fungal volatiles perceived by plant roots would influence plant volatile emission, in particular upon attack of the plant by an herbivore. In **Chapter 4**, I carried out a greenhouse assay and a common-garden experiment in which fungal volatile-exposed plants were infested with *P. brassicae* larvae. I investigated whether root exposure to fungal volatiles affects plant recruitment of natural enemies of the herbivore, which include predators and parasitoids, such as the parasitic wasp *C. glomerata*. Additionally, I profiled and compared the blends of herbivore-induced plant volatiles emitted by plants exposed to fungal volatiles.

Roots are expected to be more influenced by volatiles from soil-borne fungi than shoots because of their direct exposure. In addition, fungal volatiles emitted in the soil may directly affect organisms among the soil biota. In **Chapter 5**, I focussed on root-herbivore interactions upon continuous exposure of fungal volatiles. Using the root herbivore *D. radicum* and the cyst nematode *H. schachtii,* I proxied the effects of fungal volatiles on herbivore performance by scoring the number of individuals, their developmental stage as well as their size. To further understand how fungal volatiles can influence roots, I designed a root olfactometer to test whether root behaviour is affected by fungal volatiles (**Chapter 6**). By providing roots with the choice to grow either towards or away from volatiles emitted by a fungus, I investigated whether root directional growth is influenced by volatiles from soil-borne fungi. I hypothesised that root responses vary between the emitted VOC blends and that roots would be repelled by volatiles emitted by pathogenic fungi.

In **Chapter 7**, I summarise and discuss the main findings of my PhD project and integrate them in broader ecological and evolutionary perspectives of volatile-mediated interactions between soil-borne fungi and plants. Moreover, I present directions for future research to unveil the ecological roles of volatiles in ecosystem functioning.

Acknowledgements

I thank Viviane Cordovez, Dani Lucas-Barbosa, Jos Raaijmakers and Marcel Dicke for their helpful comments and advice on an earlier version of this chapter.



Chapter 2

Volatiles of pathogenic and non-pathogenic soil-borne fungi affect plant development and resistance to insects

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Abstract

Plants are ubiquitously exposed to a wide diversity of (micro)organisms, including mutualists and antagonists. Prior to direct contact, plants can perceive microbial organic and inorganic volatile compounds (hereafter: volatiles) from a distance that in turn may affect plant development and resistance. To date, however, the specificity of plant responses to volatiles emitted by pathogenic and non-pathogenic fungi and the ecological consequences of such responses remain largely elusive. We investigated whether *Arabidopsis thaliana* plants can differentiate between volatiles of pathogenic and non-pathogenic soil-borne fungi. We profiled volatile organic compounds (VOCs) and measured CO_2 emission of eleven fungi. We assessed the main effects of fungal volatiles on plant development and insect resistance. Despite distinct differences in VOC profiles between the pathogenic and non-pathogenic fungi, plants did not discriminate, based on plant phenotypic responses, between pathogenic and non-pathogenic fungi. Overall, plant growth was promoted and flowering was accelerated upon exposure to fungal volatiles, irrespectively of fungal CO_2 emission levels. Additionally, plants became significantly more susceptible to a generalist insect leaf-chewing herbivore upon exposure to the volatiles of some of the fungi, demonstrating that a prior fungal volatile exposure can negatively affect plant resistance. These data indicate that plant development and resistance can be modulated in response to exposure to fungal volatiles.

Keywords

Arabidopsis thaliana, fungal volatiles, plant development, plant pathogens, plant resistance

Introduction

Plants are exposed to diverse communities of insects and microorganisms, ranging from beneficial organisms, such as natural enemies of herbivores and plant-growth promoting rhizobacteria, to deleterious organisms such as pests and pathogens (Raaijmakers *et al.*, 2009; Bardgett and van der Putten, 2014; Stam *et al.*, 2014). To cope with antagonists and maximise mutualistic interactions, plants have evolved specific mechanisms to recognise elicitors from pathogens and insect herbivores, also referred to as microbe-associated molecular patterns and herbivore-associated molecular patterns, respectively.

Upon recognition of pathogenic microbes colonising the roots, local and systemic responses are induced in the plant which may affect plant development and resistance (Chagas *et al.*, 2018). Recognition of pathogens by plants usually leads to programmed cell death at the site of infection, known as the hypersensitive response, and accumulation of reactive oxygen species, preventing further infection of the plant tissues (Wojtaszek, 1997; Coll *et al.*, 2011). Upon infection, plants may also reallocate their resources, which in turn affects growth and accelerates reproductive development (Korves and Bergelson, 2003; Berger *et al.*, 2007). Also non-pathogenic microorganisms that colonise roots can alter plant development and induce resistance against a range of biotic stresses (Bent, 2006; Pineda *et al.*, 2010; Pieterse *et al.*, 2014) and abiotic stresses (van Wees *et al.*, 2008). Furthermore, some non-pathogenic microorganisms can promote plant growth through facilitation of nutrient uptake or hormone production, and *via* symbiotic interactions such as nitrogen fixation (van Loon, 2007; Bhattacharyya and Jha, 2012). Plants colonised by these beneficial soil microorganisms may also accelerate flowering, thus promoting their reproductive output (Koide and Dickie, 2002; Wolfe *et al.*, 2005). Hence, both pathogens and non-pathogenic microorganisms can affect plant fitness.

Remarkably, plants can also respond to microorganisms from a distance *via* the perception of microbial volatiles, encompassing volatile organic compounds (VOCs) and inorganic volatile compounds such as CO_2 and NO (Schmidt *et al.*, 2015). Already at the seed stage, microbial volatiles can affect plant development and delay seed germination (Ogura *et al.*, 2000; Hung *et al.*, 2014). At later growth stages, exposure to microbial volatiles may affect flowering time positively or negatively (Xie *et al.*, 2009; Sánchez-López *et al.*, 2016). In response to exposure to microbial volatiles, plant growth can be promoted (Naznin *et al.*, 2013; Kanchiswamy *et al.*, 2015a; Bailly and Weisskopf, 2017; Piechulla *et al.*, 2017) or inhibited (Wenke *et al.*, 2012; Lo Cantore *et al.*, 2015; Lee *et al.*, 2016). Interestingly, plant exposure to microbial volatiles can also enhance plant resistance to foliar pathogens (Farag *et al.*, 2013; Naznin *et al.*, 2014; Sharifi and Ryu, 2016). However, effects of microbial volatiles on plant resistance to herbivorous insects are overlooked and contrasting (Song and Ryu, 2013; D'Alessandro *et al.*, 2014; Aziz *et al.*, 2016; Cordovez *et al.*, 2017). Collectively these studies suggest a degree of specificity in plant responses to microbial volatiles.

Perception of distinct volatile profiles by plants may be one of the mechanisms underlying specificity of plant responses to different microorganisms. Fungal species with different lifestyles, such as ectomycorrhiza, pathogens and saprophytes are indeed known to emit unique VOCs which can be used as additional biomarkers for phylogenetic delineation (Müller *et al.*, 2013; Cordovez *et al.*, 2015; Oliveira *et al.*, 2015). Palma *et al.* (2018) recently proposed that profiling of the presence/absence of certain subsets of VOCs could be used to determine if a given microbial species is pathogenic to humans. Yet, it remains unclear whether plants can discriminate between volatiles of pathogenic and non-pathogenic fungi and respond accordingly (Bitas *et al.*, 2015; Casarrubia *et al.*, 2016; Sánchez-López *et al.*, 2016; Cordovez *et al.*, 2017; Tahir *et al.*, 2017c; Hernández-Calderón *et al.*, 2018; Li *et al.*, 2018). Here, we hypothesized that plants can distinguish between volatiles of pathogenic and non-pathogenic soil-borne fungi. Volatiles of pathogenic fungi may be perceived as a 'warning' of the presence of a potential antagonist, allowing plants to prepare for the attack, whereas volatiles of non-pathogenic fungi may be perceived as an information of a potential mutualist, prompting plants to facilitate direct contact. To test this hypothesis, we (1) selected multiple pathogenic and non-pathogenic soil-borne fungi, (2) analysed their VOC profiles and quantified their CO_2 emission, and (3) compared the phenotypic responses of *Arabidopsis thaliana* seedlings upon exposure to these fungal volatiles. More specifically, we investigated if exposure of seedlings to fungal volatiles affects plant development and resistance to a generalist herbivorous leaf-chewing insect.

Materials and Methods

Study system

The annual plant *Arabidopsis thaliana* L. (Brassicaceae) was used as model plant. Eleven soil-borne fungi (belonging to ten different species) were selected to investigate their volatile-mediated interactions with *A. thaliana*.

Fungi were selected on the basis of three criteria: 1) part of the fungal life cycle is saprophytic; 2) fungi were previously isolated from brassicaceous plants; and 3) fungi co-occur in regions where *A. thaliana* is naturally present (Table S1). We selected five fungi that are economically important pathogens of brassicaceous crops (Table S2, Fig. S1): *Verticillium longisporum* (Zhou *et al.*, 2006); *Verticillium dahliae* (Fradin and Thomma, 2006); *Sclerotinia sclerotiorum* (Dickman and Mitra, 1992)*; Fusarium oxysporum* f. sp. *raphani* (Leeman *et al.*, 1995) and *Rhizoctonia solani* (Pannecoucque and Höfte, 2009). The six other fungi are non-pathogenic to *A. thaliana*, being either rhizospheric fungi or endophytes: *Trichoderma viride* (Harman *et al.*, 2004)*; Ulocladium atrum* (Junker *et al.*, 2012)*; Chaetomium indicum* (Keim *et al.*, 2014)*; Fusarium oxysporum* Fo47 (Alabouvette, 1999); *Phoma leveillei* (Junker *et al.*, 2012) and *Mucor plumbeus* (Ishimoto *et al.*, 2000).

We selected the leaf chewer *Mamestra brassicae* L. (Lepidoptera: Noctuidae) as a generalist herbivorous insect species. This lepidopteran species, known as the cabbage moth, is an important pest on a broad range of crops including cabbage plants (Ahuja *et al.*, 2011).

Culture of fungi, plants and insects

Arabidopsis thaliana seeds (accession Columbia-0) were surface-sterilised by exposure to chlorine gas for 3 h in a desiccator (Cordovez *et al.*, 2017) and stratified in the dark for 3-4 days at 4 °C. Six seeds were sown per 9 cm ø Petri dish containing half-strength Murashige–Skoog medium (Duchefa, The Netherlands) with vitamins and supplemented with 5% sucrose. The medium pH was set at 5.8. Petri dishes were sealed with plastic wrap (Darco Pack B.V., The Netherlands) and kept vertically in a climate cabinet (21 ± 1 °C; 16:8 h, L:D; 70 ± 5% RH) for one week prior to the exposure to the fungal volatiles. One Petri dish was treated as one biological replicate.

All Petri dishes with fungi were initiated with a mycelial plug collected from a fungal culture, apart from *V. dahliae* and *V. longisporum*, for which the spores were used. For the collection of fungal volatiles, fungi were grown in 7 cm ø glass Petri dishes. For all bioassays in which plants were exposed to the fungal volatiles, fungi were grown in ø 3 cm plastic Petri dishes. All fungi were cultured on 1/5th strength Potato Dextrose Agar (1/5th PDA), prepared with 7.8 g of PDA (Oxoid) and 14 g of technical agar number 3 (Oxoid). The medium pH was set at 7. Petri dishes were incubated at 25 °C in the dark until fungi reached a diameter of 3 cm (Table S1). Thus, the fungi *T. viride* and *M. plumbeus* were incubated for 4 days; *V. longisporum, V. dahliae, R. solani, U. atrum, F. oxysporum 47* and *F. oxysporum* f.sp. *raphani* were incubated for 7 days; *C. indicum, S. sclerotiorum* and *P. leveillei* were incubated for 10 days at 25 °C. Fungi with different incubation times were grown with their respective controls, *i.e.* medium alone incubated for 4, 7 or 10 days.

Caterpillars of *M. brassicae* were reared on *Brassica oleracea* L. var. *gemmifera* cv. Cyrus (Brussels sprouts) plants. The adults were kept in glass containers and fed with a sucrose solution (10%) in a climate chamber (22 ± 2 °C; 16:8 h, L:D; 50 ± 5% RH). Freshly laid eggs were used in the experiments.

Collection and analysis of fungal volatile organic compounds

To profile VOCs emitted by pathogenic and non-pathogenic fungi, we collected VOCs from the headspace of the 11 fungi. As a control, we collected and determined VOCs emitted by the medium used to culture the different fungi (1/5th PDA). Fungal VOCs were collected for 2 h using a dynamic headspace set-up and each fungus was replicated six times. VOCs were collected from fungi previously grown in the dark at 25 °C for 4, 7 or 10 days (Table S1), in glass Petri dishes (7 cm ø) containing 1/5th PDA medium. Each Petri dish was placed individually inside a 0.5 L glass jar (previously autoclaved) with an inlet and an outlet. Synthetic air was flushed into the jar at a flow rate of 300 mL min^{-1} *via* a Teflon tube inserted through the inlet in the jar lid. Fungal VOCs were collected in a tube filled with 90 mg Tenax

TA 25/30 mesh (Grace-Alltech, Germany) connected directly to the outlet on the lid of the glass jar, and air was sucked out at a flow rate of 200 mL min^{-1} (224-PCMTX8, air-sampling pump Deluxe, Dorset, UK; equipped with an inlet protection filter) for 2 h in a greenhouse compartment (25 ± 2 °C, 16:8 h, L:D, 60 ± 5% RH). Fungal dry weight was determined and used to normalise the peak area of each compound. For this, mycelium-containing agar was cut into pieces and transferred to a glass beaker with demineralised water (Garbeva *et al.*, 2014b). The agar was then melted in a microwave oven and filtered over a tea strainer. The remaining hyphae were rinsed with hot water and excess water was removed by placing the tea strainer on filter paper. Fungal hyphae were stored in a micro-centrifuge tube at −80 °C until freeze drying. Hyphae were subjected to freeze-drying for 24 h at -50 ± 2 °C, and dry weight was measured.

Headspace samples were analysed by gas chromatography equipped with a thermo-desorption unit (Ultra 50:50, Markes, Llantrisant, UK) and coupled to a mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Fungal VOCs were desorbed from the Tenax in the thermo-desorption trap unit by heating from 25 °C to 250 °C (5 min hold) at a rate of 60 °C min^{-1} in splitless mode. Released compounds were focused at 0 °C in a cold trap (ID 1.80 mm) filled with Tenax and charcoal. By flash heating of the cold trap to 280 °C at 40 °C sec^{-1} (hold 10 min), VOCs were transferred to the analytical column (30 m × 0.25 mm ID, 1 µm film thickness, DB-5, Phenomenex, Torrence, CA, USA) for 4 min at a constant flow of 1 mL min^{-1}. The oven temperature programme started at 40 °C and immediately rose at a rate of 5 °C min^{-1} to 280 °C (hold 4 min). Column effluent was ionized by electron impact ionization at 70 eV. Mass scanning was carried out from *m/z* 35 to 300 at 4.70 scans sec^{-1}.

The detected VOCs were identified by comparison of the mass spectra with those of NIST (National Institute of Standards and Technology, USA), Wiley libraries and the Wageningen Mass Spectral Database of Natural Products, by comparing the experimentally calculated linear retention index with the literature values and by using the mVOC 2.0 database (Lemfack *et al.*, 2017). Only VOCs detected in the samples with a peak area 4-fold higher than that of control samples (VOCs from the fungal medium alone), and detected in at least 50% of the replicates of one of the fungal species were selected for further analysis. Qualitative comparisons of VOC profiles produced by pathogenic and non-pathogenic fungi were plotted in Venn diagrams. Total Ion Chromatograms (TIC) were used to generate values for peak area, and the VOC profiles of pathogenic and non-pathogenic fungi were analysed by multivariate analyses through a Projection to Latent Structures Discriminant Analysis (PLS-DA) (SIMCA 15 software, Umetrics AB, Umeå, Sweden). This model was evaluated using a 7-fold cross-validation test (N = 200; One-way ANOVA) and with R^2 and Q^2 estimates. Variable importance in projection (VIP) values were generated for each compound. Compounds with a VIP > 1.2 were listed as the most important for the model.

Short-term effects of exposure to fungal volatiles on plant growth

To investigate whether a continuous exposure to fungal volatiles affects plant growth, one-week-old *A. thaliana* seedlings were exposed *in vitro* to volatiles of the different fungi for two weeks, and their weight was compared with that of their respective control plants (*i.e.* exposed only to the medium incubated for 4, 7 or 10 days) (Table S1). For this, a three-compartment Petri dish was designed in which *A. thaliana* seedlings and the fungi were co-cultivated, but physically separated allowing volatile-mediated interactions only, as previously described (Cordovez *et al.*, 2017). Six *A. thaliana* seedlings were cultivated in a 9 cm ø Petri dish, whereas one of the 11 fungi was cultivated in a 3 cm ø Petri dish. The Petri dishes containing the seedlings and the fungi were enclosed inside a third 14.5 cm ø Petri dish that was sealed with plastic wrap (Darco Pack B.V., The Netherlands) and incubated vertically in a climate cabinet (21 ± 1 °C; 16:8 h, L:D; 70 ± 5% RH). Plants were harvested after two weeks of exposure, and root and leaf weight of volatile-exposed and control plants were dried overnight at 55 °C and weighed. Each fungal volatile-exposure was replicated six times. The increase in root and leaf dry weight, as well as the change in ratio root:leaf dry weight, of volatile-exposed plants relative to the control plants were expressed in percentages, *i.e.* an increase in plant weight of 0% corresponds to a similar weight as control plants. These data were statistically analysed using One Sample Student's *t*–test (H_0 = 0; α = 0.05). A Two Sample Student's *t*-test was additionally performed to statistically assess the differences of plant responses (root and shoot weight) to the pathogenicity of the volatile-emitting fungi (α = 0.05).

Long-term effects of exposure to fungal volatiles on plant development

To investigate whether plant responses to fungal volatiles are maintained after temporary exposure, one-week-old plants were exposed for one week to fungal volatiles *in vitro* as described in the previous section. Exposure was disrupted after one week and the plants alone were transplanted to soil. Four seedlings were randomly selected from each Petri dish and transplanted to a plastic pot (ø 10 cm, H = 7.8 cm) filled with a sterile mixture of sand and potting soil (1:1 v/v; Horticoop potting soil ø 2 mm sieved). Plants were grown in a greenhouse compartment until harvesting (21 ± 2 °C; 16:8 h, L:D; 70 ± 5% RH). Date of the first flower per pot was monitored daily and plants were harvested 17 days after transplantation. To assess plant growth, roots and shoots were dried overnight at 55 °C and weighed. Each fungal volatile-exposure was replicated eight times. The increase of root and shoot weight, as well as the change in ratio flower:leaf dry weight, of volatile-exposed plants relative to the control plants were expressed in percentages, *i.e.* an increase in plant weight of 0% corresponds to a similar weight as control plants. These data were statistically analysed using One Sample Student's *t*–test (H_0 = 0; α = 0.05). Change in flowering time relative to control plants was likewise tested for each volatile-exposed plants using One Sample Student's *t*–test (H_0 = 0; α = 0.05). A Two Sample Student's *t*-test was additionally performed to statistically assess the differences in plant responses (plant weight and flowering time) to the pathogenicity of the volatile-emitting fungi (α = 0.05). Correlation

between change in flower dry weight and change in flowering time of exposed plants relative to control plants was tested with Pearson correlation test.

Effects of exposure to fungal volatiles on plant resistance to a generalist insect herbivore

To test whether a temporary exposure of plants to fungal volatiles affects plant resistance to a generalist insect, one-week-old *A. thaliana* seedlings were first exposed to fungal volatiles *in vitro* for one week, and then exposure was disrupted. Four seedlings were randomly selected from each Petri dish and transplanted to pots filled with a sterile mixture of sand and potting soil. Three days following transplantation into soil, five fresh eggs of *M. brassicae* were transferred to each of the four plants per pot, hence 20 eggs per pot. When fewer than 50% of the larvae hatched per pot, neonate larvae were manually added to reach a minimal larval density of 10. Larval fresh weight was determined at 3 and 7 days post-hatching (dph). Larval density was reduced to 10 larvae at 3 dph to simulate natural dispersal and predation in nature (Johansen, 1997). During the period in which plants were infested with the insects, plants were covered with a plastic cylinder (Duchefa, Haarlem, the Netherlands; H = 14 cm, upper ø 11.5 cm, lower ø 9 cm) and that was closed with a mesh and sealed with rubber bands to prevent caterpillars from escaping. Each treatment was replicated 1-3 times per batch and in 7 consecutive batches. The change in fresh weight of larvae feeding on volatile-exposed plants relative to that of larvae feeding on their respective control plants was expressed in percentages, *i.e.* a change in larval weight of 0% corresponds to a similar weight as in control plants, and was statistically analysed using One Sample Student's t–test ($H_0 = 0$; $\alpha = 0.05$) at each of the two time points. Additionally, a Two-Sample Student's *t*-test was performed to statistically analyse the differences in larval fresh weight between larvae feeding on plants previously exposed to volatiles of pathogenic fungi and that of on plants exposed to volatiles of non-pathogenic fungi ($\alpha = 0.05$).

Collection and quantification of fungal CO_2

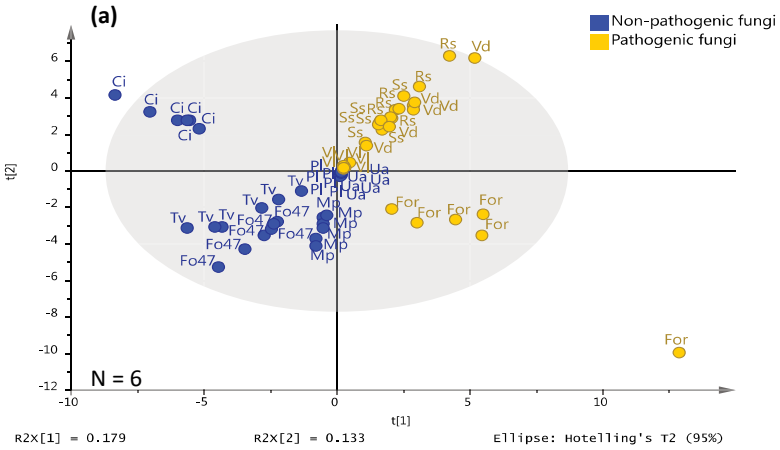
To determine the emission of fungal CO_2 and to unravel its potential role in promoting *A. thaliana* growth, we quantified CO_2 concentration for each fungus in the absence and presence of plants. After being pre-incubated for 4, 7 or 10 days in a 3 cm ø Petri dish containing 1/5th PDA (see *section Culture of fungi, plants and insects*), fungi were enclosed individually with or without plants, and 1/5th PDA was used as control. Plant exposure was performed as described above (see *section Short-term effects of exposure to fungal volatiles on plant growth*). To allow sampling of the headspace with a syringe, one 14 mm ø hole was made in each lid of the large Petri dishes (14.5 cm ø), and a butyl rubber stopper was inserted (Rubber BV, Den Haag, the Netherlands). Prior to use, these lids were sterilised by rinsing with ethanol and exposing to UV light for 15 min in a flow cabinet. A volume of 250 µL was sampled manually from the headspace of the large Petri dishes after 7 and 14 days of incubation through the lid septa, and directly injected into a Trace Ultra GC gas chromatograph (Interscience, The Netherlands). The GC was equipped with a methanizer (flame ionization detector in combination with a hydrogenation reactor which converts CO_2

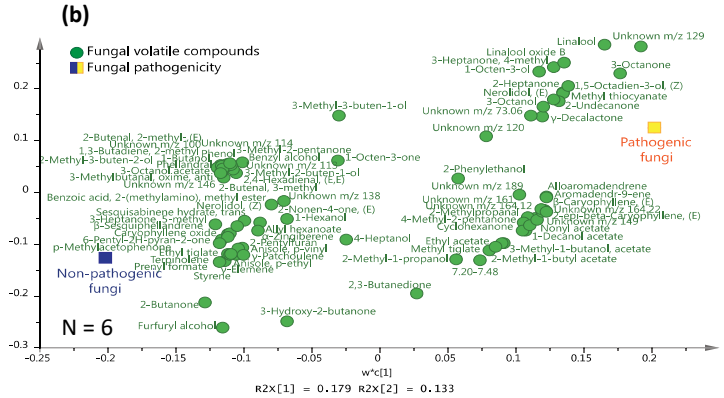
into CH_4) and at Rt-QBOND (30 m, 0.32 mm ID, cat# 19744) capillary column. Helium was used as a carrier gas with a flow of 5 ml min^{-1} and a split ratio of 1:4. The oven temperature was set at 50 °C. The data was acquired with Chromeleon 7.02 (Thermo Scientific, Germany). CO_2 concentration was calculated for all 11 fungi and their respective controls (medium alone pre-incubated for 4, 7 or 10 days) in the presence or absence of plants. Peak areas of CO_2 standards at 1200 ppm and 2000 ppm in synthetic air (Westfalen AG, Germany) were used for calibration. Main effects of the fungal species and sampling time on CO_2 concentration were tested using an ANOVA. Each fungus and control was replicated 5-8 times when enclosed alone and 2-7 times when co-cultivated with *A. thaliana* plants. At the end of the 14 days of co-cultivation, all plants were harvested, dried and weighed. Correlation between the average plant dry weight upon co-cultivation for 14 days with the fungi and the average CO_2 concentration measured after 14 days when the fungi were enclosed alone was tested with a Pearson correlation test.

Results

Profiling of fungal volatile organic compounds

Analysis of the VOC headspace of the 11 soil-borne fungi revealed a total of 82 discrete VOCs (Table S3). Approximately 15% of these compounds were detected for pathogenic as well as non-pathogenic fungi, whereas 38% and 47% of the compounds were unique to the pathogenic and non-pathogenic fungi, respectively (Fig. S2a). Among the pathogenic fungi, 58% of the compounds were unique to a fungal species, and 42% of the compounds were detected in the VOC profiles of at least two different species (Fig. S2b). There was no VOC that was common to all pathogenic fungi. Among the non-pathogenic fungi, 51% of the compounds were unique to a species, and 49% of the compounds were detected in at least two different fungi (Fig. S2c). Three co-eluted alcohols (3-methyl-1-butanol, 2-methyl-1-butanol and 1-pentanol) were detected in the VOC profiles of all non-pathogenic species but were also found in the VOC profiles of some pathogenic fungi (Fig. S2). Overall, the VOC profiles of the pathogenic fungi separated from the profiles of the non-pathogenic fungi: 18% and 13% of the total variance was explained by the first and second principal components, respectively (Fig. 1; PLS-DA; $R^2 = 0.6$; $Q^2 = 0.6$, $P_{CV\ ANOVA} < 0.001$). Eleven VOCs had a VIP > 1.2 and these contributed the most to the separation between VOC profiles of pathogenic and non-pathogenic fungi (Fig. 1c and Table S4). The vast majority of these VOCs was detected only in pathogenic fungi. Thus, the VOC profiles of pathogenic and non-pathogenic fungi can be clearly discriminated.





(c)

	Emitting fungi	VIP
Unknown m/z 129	Rs; Ss; Vd	1.73247
Linalool	Rs; Ss	1.58972
3-octanone	Rs; Vd	1.53772
Linalool oxide B	Ss	1.34451
3-heptanone, 4-methyl	Ss	1.28484
Furfuryl alcohol	Mp	1.28081
Methyl thiocyanate	Rs	1.25267
2-butanone	Ss; Ci; Mp	1.21264
1-octen-3-ol	Rs; Ss; Vd; Vl; Ci; Tv; Ua	1.20929
1,5-octadien-3-ol, (Z)	Rs	1.2069
2-heptanone	Vd	1.2023

Emitted by non-pathogenic fungi only
Emitted by pathogenic fungi only
Emitted by both fungal groups

N = 6

Figure 1. Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile organic compounds (VOCs) collected from the headspace of pathogenic and non-pathogenic fungi of *Arabidopsis thaliana*. **(a)** Grouping pattern of samples according to the first two principal components and the Hotelling's T2 ellipse confining the confidence region (95%) of the score plot. **(b)** Contribution of individual VOCs to the first two principal components is shown in the loading plot of the PLS-DA. **(c)** List of VOCs with values of variable importance in projection (VIP) > 1.2. Different letters indicate the distribution of the samples of the 11 different fungi: Ci = *Chaetomium indicum*, Fo47 = *Fusarium oxysporum* 47, For = *F. oxysporum* f.sp. *raphani*, Mp = *Mucor plumbeus*, Pl = *Phoma leveillei*, Rs = *Rhizoctonia solani*, Ss = *Sclerotinia sclerotiorum*, Tv = *Trichoderma viride*, Ua = *Ulocladium atrum*, Vd = *Verticillium dahliae*, Vl = *Verticillium longisporum*.

Short-term effects of continuous exposure to fungal volatiles on plant growth

To test whether fungal volatiles affect plant growth, one-week-old *A. thaliana* seedlings were exposed *in vitro* to volatiles of the different fungi for two weeks, and plant weight of volatile-exposed plants was compared with that of their respective control plants (Fig. 2 and Fig. S3b and S3c). Plant exposure to fungal volatiles had a positive main effect on both leaf and root weight (Fig. 2a and 2b; One Sample Student's *t*-test; $H_0 = 0$; $P_{leaf} < 0.001$; $P_{root} < 0.001$), and this increase was similar for leaves and roots (Fig. 2c; One Sample Student's *t*-test; $H_0 = 0$; $P = 0.805$). Only plants exposed to volatiles of *M. plumbeus* did not exhibit a significant increase in leaf nor root weight (Fig. 2a and 2b; One Sample Student's *t*-test; $H_0 = 0$; $P_{leaf} = 0.179$; $P_{root} = 0.194$). Plants exposed to volatiles of *T. viride* and of *S. sclerotiorum* had a reduced root:leaf ratio relative to control plants (Fig. 2c; One Sample Student's *t*-test; $H_0 = 0$; $P_{T.viride} = 0.001$; $P_{S.sclerotiorum} = 0.046$). In contrast, plants exposed to volatiles of *F. oxysporum* 47 had a higher root:leaf ratio relative to their respective control plants (Fig. 2c; One Sample Student's *t*-test; $H_0 = 0$; $P = 0.041$). The effects of fungal volatiles on plant growth were not associated with the pathogenicity of the fungus. Shoot and root dry weight, as well as the root:leaf ratio, did not differ between plants exposed to volatiles of pathogenic fungi and those of non-pathogenic fungi (Fig. 2a, 2b and 2c; Two Sample Student's *t*-test; $P_{leaf} = 0.632$; $P_{root} = 0.325$; $P_{ratio} = 0.949$).

Long-term effects of temporary exposure to fungal volatiles on plant development

To test whether plant responses to fungal volatiles can be sustained after temporary exposure, we assessed the weight of plants exposed *in vitro* to fungal volatiles for only one week after which they were transplanted to soil in the absence of the volatile-emitting fungus (Fig. 3 and Fig. S3b and S3c).Plant exposure to fungal volatiles had a positive main effect on shoot (Fig. 3a; One Sample Student's *t*-test; $H_0 = 0$; $P < 0.001$) and root dry weight (Fig. 3b; One Sample Student's *t*-test; $H_0 = 0$; $P < 0.001$). Particularly, plants exposed to volatiles of *T. viride* and *U. atrum* had an increase in both shoot (Fig. 3a; One Sample Student's *t*-test; $H_0 = 0$; $P_{T.viride} < 0.001$; $P_{U.atrum} = 0.010$) and root dry weight (Fig. 3b; One Sample Student's *t*-test; $H_0 = 0$; $P_{T.viride} = 0.004$; $P_{U.atrum} = 0.031$). In addition, plant exposure to fungal volatiles had a positive main effect on the flower:leaf ratio (Fig. 3c; One Sample Student's *t*-test; $H_0 = 0$; $P < 0.001$). Shoot and root dry weight as well as the flower:leaf ratio did not differ between plants exposed to volatiles of pathogenic fungi and that of non-pathogenic fungi (Fig. 3a, 3b and 3c; Two Sample Student's *t*-test; $P_{shoots} = 0.559$; $P_{roots} = 0.338$; $P_{ratio} = 0.193$). Overall, *A. thaliana* plants exposed to fungal volatiles produced flowers sooner than control plants (Fig. 4a; One Sample Student's *t*-test; $H_0 = 0$; $P < 0.001$). Only volatiles of *S. sclerotiorum* delayed flowering (Fig. 4a; One Sample Student's *t*-test; $H_0 = 0$; $P = 0.019$). No association with pathogenicity was found (Fig. 4a; Two Sample Student's *t*-test; $P = 0.520$). Flowering time negatively correlated with flower dry weight (Fig. 4b; Pearson correlation; $N = 77$; $r = -0.807$; $P = 0.003$).

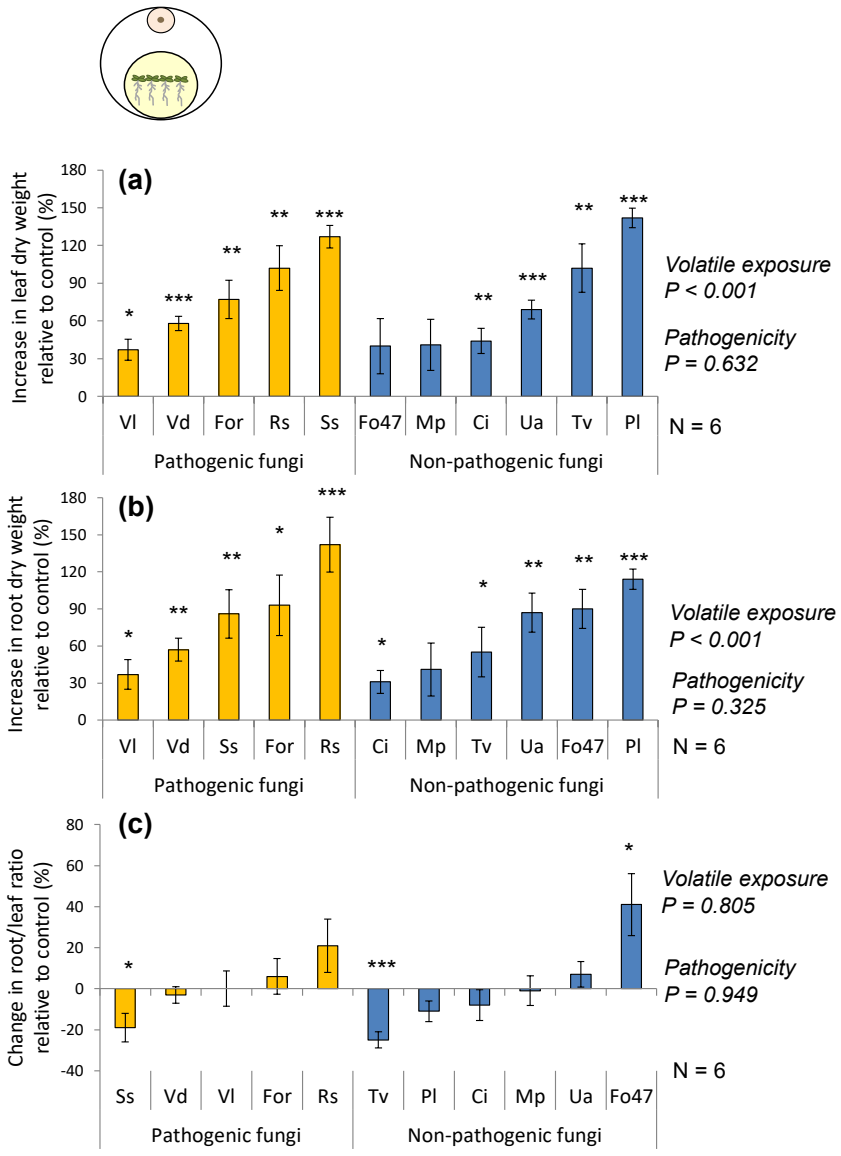


Figure 2. Increase in **(a)** leaf and **(b)** root dry weight (mean % ± SE), and **(c)** change in root:leaf ratio (mean % ± SE) of *Arabidopsis thaliana* after 2 weeks of *in vitro* exposure to fungal volatiles. Data are shown as relative to control plants; an increase of 0% in plant weight or ratio corresponds to a similar weight or ratio as in control plants. Ci = *Chaetomium indicum*, Fo47 = *Fusarium oxysporum* 47, For = *F. oxysporum f.sp. raphani*, Mp = *Mucor plumbeus*, Pl = *Phoma leveillei*, Rs = *Rhizoctonia solani*, Ss = *Sclerotinia sclerotiorum*, Tv = *Trichoderma viride*, Ua = *Ulocladium atrum*, Vd = *Verticillium dahliae*, Vl = *Verticillium longisporum*. Main effect of the volatile exposure was tested using One Sample Student's *t*-test (H_0 = 0), and difference of plant weight increase in response to volatiles of different fungal pathogenicity was tested using Two Sample Student's *t*-test at α = 0.05. Asterisks indicate statistical differences with the respective control plants (* P < 0.05; ** P < 0.01; *** P < 0.001) using One Sample Student's *t*-test (H_0 = 0).

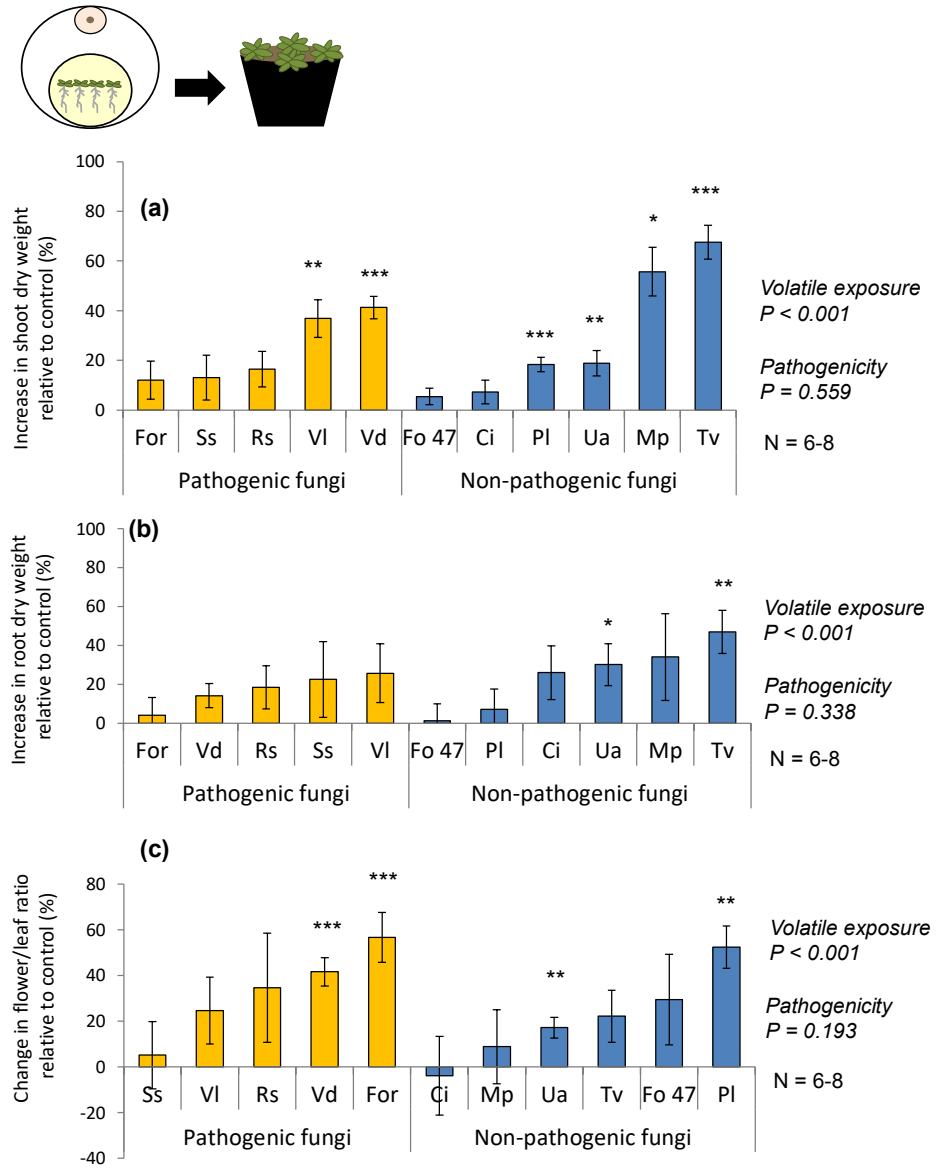


Figure 3. Increase in **(a)** shoot and **(b)** root dry weight (mean % ± SE), and **(c)** change in flower:leaf ratio (mean % ± SE) of *Arabidopsis thaliana* exposed temporary to fungal volatiles *in vitro,* and subsequently grown in soil for 2.5 weeks. Data are shown as relative to control plants; an increase of 0% in plant weight or ratio corresponds to the same weight or ratio as in control plants. Ci = *Chaetomium indicum*, Fo47 = *Fusarium oxysporum* 47, For = *F. oxysporum* f.sp. *raphani,* Mp = *Mucor plumbeus*, Pl = *Phoma leveillei*, Rs = *Rhizoctonia solani,* Ss = *Sclerotinia sclerotiorum*, Tv = *Trichoderma viride*, Ua = *Ulocladium atrum*, Vd = *Verticillium dahliae*, Vl = *Verticillium longisporum*. Main effect of the volatile exposure was tested using One Sample Student's *t*-test (H_0= 0), and difference of plant weight increase in response to volatiles of different fungal pathogenicity was tested using Two Sample Student's *t*-test at α = 0.05. Asterisks indicate statistical differences with the respective control plants (* P < 0.05; ** P < 0.01; *** P < 0.001) using a One Sample Student's *t*-test (H_0 = 0).

Effects of temporary exposure to fungal volatiles on plant resistance to a generalist insect herbivore

To test whether a temporary exposure of plants to fungal volatiles affects plant resistance to a generalist insect, we assessed the weight of larvae feeding on plants previously exposed to fungal volatiles and on control plants at two time points (Fig. 5 and Fig. S3b and S3d). Plant exposure to fungal volatiles had a positive main effect on larval weight at 3 days post hatching (dph) (Fig. 5a; One Sample Student's *t*-test $H_0 = 0$; $P = 0.013$) and at 7 dph (Fig. 5b; One Sample Student's *t*-test $H_0 = 0$; $P = 0.001$). In particular, larvae feeding on plants previously exposed to volatiles of *S. sclerotiorum* were larger at 3 dph than larvae fed on control plants (Fig. 5a; One Sample Student's *t*-test $H_0 = 0$; $P_{S.\ sclerotiorum} = 0.011$). Additionally, larvae feeding on plants previously exposed to volatiles of *S. sclerotiorum* and *R. solani* were also larger than those feeding on control plants at 7 dph (Fig. 5b; One Sample Student's *t*-test $H_0 = 0$; $P_{S.\ sclerotiorum} = 0.013$; $P_{R.\ solani} = 0.018$). Larval fresh weight, at 3 dph and 7 dph, did not differ between larvae feeding on plants exposed to volatiles of pathogenic fungi and that of plants exposed to volatiles of non-pathogenic fungi (Fig. 5a and 5b; Two Sample Student's *t*-test; $P_{3dph} = 0.112$; $P_{7dph} = 0.132$). Larval weight at 7 dph was correlated with shoot dry weight of infested plants previously exposed to fungal volatiles (Fig. S4a; Pearson correlation; $N = 68$; $r = 0.330$; $P = 0.006$) but not with the difference of shoot weight between uninfested and infested plants (Fig. S4b; Pearson correlation; $N = 62$; $r = 0.045$; $P = 0.726$).

Collection and quantification of fungal CO_2

To assess the potential contribution of fungal CO_2 to the plant growth-promoting effects observed *in vitro*, we quantified the CO_2 concentration for each fungus and control (*i.e.* medium alone) in the absence and presence of *A. thaliana* plants for 7 and 14 days (Fig. 6). CO_2 concentration differed between fungi when they were growing alone (Fig. 6a; ANOVA; $P < 0.001$) as well as in co-cultivation with *A. thaliana* (Fig. 6b; ANOVA; $P < 0.001$). Detailed output of the pairwise differences between the fungal volatile exposures is reported in the electronic supplemental material (Fig. S5). In both situations, *i.e.* when fungi were enclosed alone or co-cultivated with plants, CO_2 concentration overall decreased with time (Fig. 6a and Fig. 6b; ANOVA; $P < 0.001$). Plant dry weight after 14 days of co-cultivation with fungi did not correlate with CO_2 concentration measured after 14 days when the fungi were enclosed alone (Fig. 6c; Pearson correlation test; $r = 0.279$; $P = 0.355$) nor with CO_2 concentration measured after 14 days of co-cultivation (Fig. 6d; Pearson correlation test; $r = -0$.

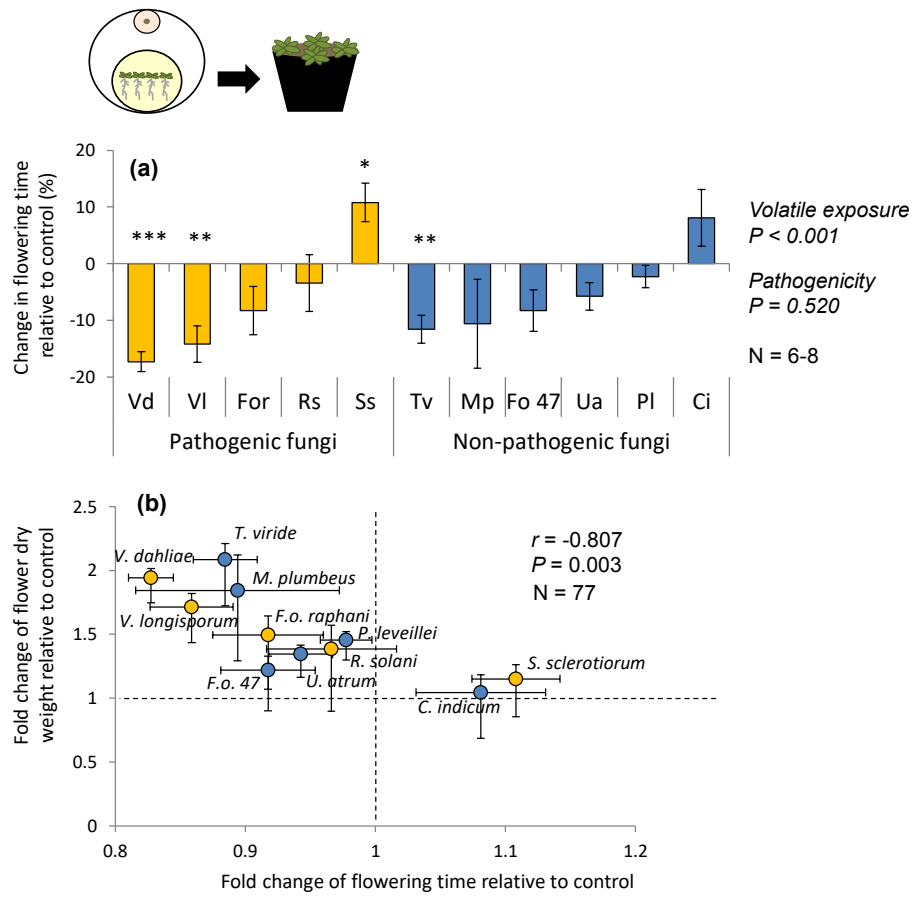


Figure 4. (a) Change in flowering time (mean % ± SE) of *Arabidopsis thaliana* exposed temporary to fungal volatiles *in vitro*, and subsequently grown in soil 2.5 weeks. **(b)** Pearson correlation between the fold change of flower dry weight (mean % ± SE) and flowering time (mean % ± SE) of *A. thaliana* exposed to the fungal volatiles relative to control. Ci = *Chaetomium indicum*, Fo47 = *Fusarium oxsyporum* 47, For = *F. oxysporum* f.sp. *raphani*, Mp = *Mucor plumbeus*, Pl = *Phoma leveillei*, Rs = *Rhizoctonia solani*, Ss = *Sclerotinia sclerotiorum*, Tv = *Trichoderma viride*, Ua = *Ulocladium atrum*, Vd = *Verticillium dahliae*, Vl = *Verticillium longisporum*. Main effect of the volatile exposure was tested using a One Sample Student's *t*-test ($H_0 = 0$), and difference of flowering time in response to volatiles of different fungal pathogenicity was tested using Two Sample Student's *t*-test at $\alpha = 0.05$. Aste-risks indicate statistical differences with the respective control plants (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) using a One Sample Student's *t*-test ($H_0 = 0$). For the Pearson correlation, dash lines represent the control plants.

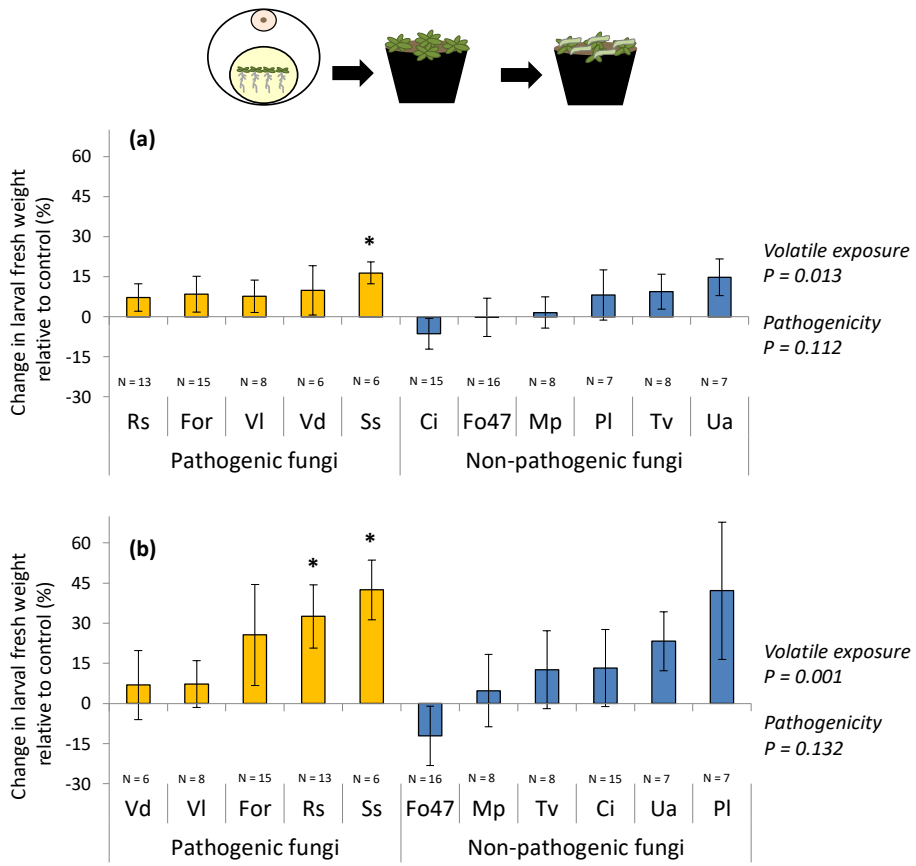


Figure 5. Change in larval fresh weight (mean ± SE) of *Mamestra brassicae* at **(a)** 3 days post-hatching and **(b)** 7 days post-hatching when feeding on *Arabidopsis thaliana* exposed temporary to fungal volatiles *in vitro,* and subsequently grown in soil. Ci = *Chaetomium indicum*, Fo47 = *Fusarium oxsyporum* 47, For = *F. oxysporum* f.sp. *raphani,* Mp = *Mucor plumbeus*, Pl = *Phoma leveillei*, Rs = *Rhizoctonia solani,* Ss = *Sclerotinia sclerotiorum*, Tv = *Trichoderma viride*, Ua = *Ulocladium atrum*, Vd = *Verticillium dahliae*, Vl = *Verticillium longisporum.* Main effect of the volatile exposure was tested using a One Sample Student's *t*-test (H_0 = 0), and difference of larval fresh weight between plants exposed to volatiles of different fungal pathogenicity was tested using Two Sample Student's *t*-test at α = 0.05. Asterisks indicate statistical differences with the respective control plants (* *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001) using One Sample Student's *t*-test (H_0 = 0). "N" indicates the number of pots that were infested with 20 larvae.

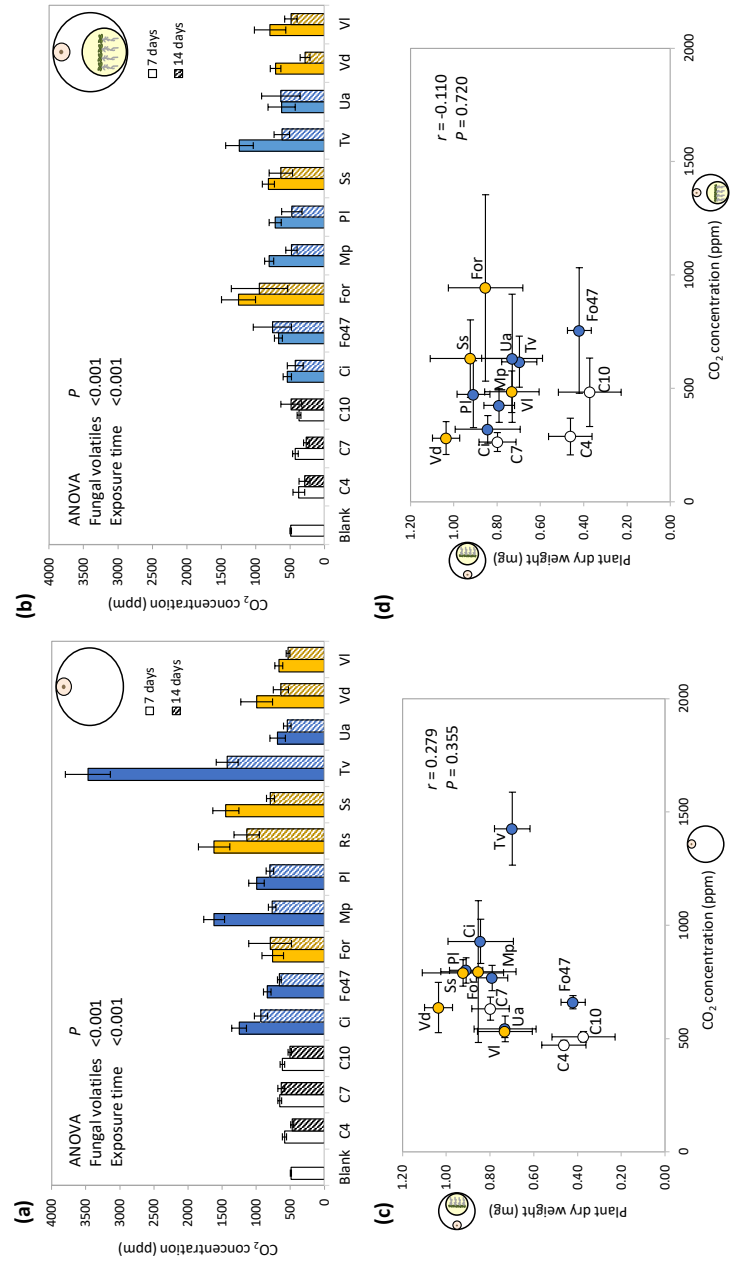


Figure 6. CO_2 concentration (mean ± SE) measured for the eleven fungi and their respective controls when **(a)** enclosed alone, and **(b)** co-cultivated with *Arabidopsis thaliana* seedlings for 7 and 14 days. Pearson correlation between the average plant dry weight (mean ± SE) upon co-cultivation (mean ± SE) measured after 14 days when the fungi were enclosed alone, and **(d)** the average CO_2 concentration (mean ± SE) measured after 14 days when the fungi were co-cultivated with plants. Blank = empty Petri dish, C4, C7 and C10 = medium alone pre-incubated for 4, 7 and 10 days, Ci = *Chaetomium indicum*, Fo47 = *Fusarium oxysporum* 47, For = *Fusarium oxysporum* f.sp. *raphani*, Mp = *Mucor plumbeus*, Pl = *Phoma leveillei*, Rs = *Rhizoctonia solani*, Ss = *Sclerotinia sclerotiorum*, Tv = *Trichoderma viride*, Ua = *Ulocladium atrum*, Vd = *Verticillium dahliae*, Vl = *Verticillium longisporum*. Upon co-cultivation with plants, each fungal volatile exposure was replicated 2-7 times, and when the fungus was incubated alone, each volatile exposure was replicated 5-8 times. Due to fungal overgrowth on plant compartment, exposure with *R. solani* volatiles was excluded from the analysis upon co-cultivation with *A. thaliana*. Main effects of the fungal volatiles and exposure time were tested using ANOVA. Detailed output of the pairwise differences between the fungal volatile exposures is reported in the electronic supplemental material (Fig. S5).

Discussion

We studied the effects of volatiles emitted by pathogenic and non-pathogenic fungi on plant development and resistance to insects and found that overall, fungal volatiles increased plant weight and accelerated plant development, irrespective of the pathogenicity of the fungus and of the fungal CO_2 emission. Based on these phenotypic changes, plants do not seem to discriminate between volatiles of pathogenic and non-pathogenic soil-borne fungi, despite distinct compositional differences in the VOC profiles of these two groups. Interestingly, volatiles of pathogenic and non-pathogenic fungi can both affect plant resistance and development. Overall, *A. thaliana* plants exposed to fungal volatiles flowered earlier, and plants exposed to volatiles of some fungi in particular became more susceptible to herbivory by generalist caterpillars. These results indicate that plant exposure to fungal volatiles can affect plant development and resistance.

The profiling of fungal VOCs showed that some compounds were species-specific, whereas a few compounds were present in the VOC profiles of both pathogenic and non-pathogenic fungi, such as the typical mushroom odour, 1-octen-3-ol. Previous studies showed that soil-borne fungi and bacteria emit unique VOCs, which can be used as microbial signatures (De Lucca *et al.*, 2010; Bos *et al.*, 2013; Müller *et al.*, 2013; Cordovez *et al.*, 2015). More specifically, fungal VOCs may allow the classification of different fungal lifestyles, including pathogenicity (Müller *et al.*, 2013). It is important to bear in mind, however, that the denomination of "pathogen" is specific to a plant host-fungus system and may differ in another system. In the present study, we did not detect VOCs that were common to all pathogenic fungi and that were absent in the VOC profiles of non-pathogenic fungi. Similarly, we did not detect VOCs that were common to all non-pathogenic fungi and that were absent in the VOC profiles of pathogenic fungi tested in this study. Together, these data indicate that a large diversity of VOCs is emitted by different soil-borne fungal species. Differences between the VOC profiles of pathogenic and non-pathogenic fungi were mostly explained by compounds exclusively emitted by the pathogenic fungi we tested. These findings highlight the importance of the presence/absence of these VOCs in discriminating the profiles of the pathogenic and non-pathogenic fungi in this system. The distinct VOC profiles observed in the conditions tested provide a solid empirical basis to further investigate if plants respond differently upon exposure to volatiles from pathogenic and non-pathogenic fungi.

Interactions between plants and fungi are among the oldest interactions on Earth, and microbial volatiles act as an ancient cue involved in plant–microbe interactions, which was shaped during evolutionary history and established before the development of higher plants (Sharifi and Ryu, 2018b). Therefore, it is plausible to expect plants to have evolved differential responses to volatile cues from pathogenic and non-pathogenic microorganisms surrounding them, in particular when volatile profiles are distinct, as plants could benefit from anticipating the arrival of pathogens and mutualists. Interestingly, although only pathogenic fungi represent an actual threat to plant fitness, plants did not differentially respond to fungal volatiles in the present study. Fungal VOCs were collected for 2 h in

the absence of plants and therefore, this snapshot does not include possible changes in fungal VOC emission in the presence of a plant. Potentially, fungi in the vicinity of plants may perceive the presence of their hosts (Hegedus and Rimmer, 2005; Chagas *et al.*, 2018), *e.g. via* root volatiles, and respond by changing their own VOC emission (Venturi and Keel, 2016). A change in fungal VOCs, *e.g.* emission of similar VOCs found in non-pathogenic microorganisms or in roots (Schenkel *et al.*, 2015), may render pathogens undetectable to plants that consequently cannot distinguish pathogenic from non-pathogenic microorganisms. This VOC "dialogue" between fungi and plants may be dynamic over time (Fincheira and Quiroz, 2018). Further real-time analysis of the fungal VOCs over a longer period of time in the absence and presence of plants may shed light on these two-way interactions (van Dam *et al.*, 2012).

In the present study, plant weight was overall enhanced and flowering was accelerated upon exposure to fungal volatiles, irrespective of the pathogenicity of the fungus and its CO_2 emission. These results indicate that plants respond to a wide range of volatiles produced by different fungal species, and are in line with results of recent studies that have shown a critical role of microbial volatiles in plant growth and health (Kanchiswamy *et al.*, 2015a; Piechulla *et al.*, 2017; Sharifi and Ryu, 2018b). In addition to fungal VOCs, fungal CO_2 may also accumulate in closed systems and lead to plant growth promotion (Kai and Piechulla, 2009; Piechulla and Schnitzler, 2016). Our data show that enclosure of fungi alone indeed increased the CO_2 concentration in our experimental set-up, and that CO_2 emission differs between the fungi. However, CO_2 concentration overall decreased with time, in the presence but also in the absence of plants. We speculate that this is most likely due to the fact that the plastic used for wrapping the Petri dishes was not fully airtight and allowed some CO_2 diffusion. Hence, the set-up used for the volatile exposure is, in fact, not completely closed. In the present study, plant dry weight did not correlate with CO_2 concentrations measured when the fungi were enclosed in the presence nor in the absence of plants. In fact, plants exposed to the highest CO_2-emitting fungi did not show the strongest growth-promotion. These results indicate that increased CO_2 in our experimental set-up was not the main driver for the observed plant growth-promoting effects. Additional evidence shows that in closed system, fungal CO_2 alone does not trigger growth promotion to the same extent as when plants are also exposed to VOCs (Sánchez-López *et al.*, 2016; Cordovez *et al.*, 2017). However, we cannot exclude that other inorganic volatile compounds may have contributed as well to the plant responses observed in our study (Sánchez-López *et al.*, 2016; García-Gómez *et al.*, 2019).

Our study presents evidence that plants can have long-term effects following volatile exposure, which affects plant developmental processes. A temporary plant exposure to the fungal volatiles was sufficient to observe enhanced plant growth and accelerated development. To date, only a few studies have correlated an increased plant weight with a change in developmental traits (Xie *et al.*, 2009; Hung *et al.*, 2013; Sánchez-López *et al.*, 2016). Interestingly, the greater the increase in flower weight, the earlier plants flowered, which implies faster development rather than merely growth promotion. Acceleration of

flowering is a common plant response to stress among short-lived plants, and therefore our findings suggest that plants may have perceived fungal volatiles as a 'warning'. Diverse biotic stresses have been shown to trigger faster flowering (Lucas-Barbosa *et al.*, 2013; Pashalidou *et al.*, 2013), and exposure of plants to microbial volatiles at the seedling stage may likewise affect the transition from vegetative to reproductive stages (Sánchez-López *et al.*, 2016). This reproductive escape strategy might be particularly important for annual plant species to prevent pathogens to colonise and infect the plant (Douglas, 2008). Early flowering might be costly for plants that rely on insect pollination because it may desynchronise the presence of pollinators and the flowering state of the plant (Kudo and Ida, 2013; Petanidou *et al.*, 2014). However, *A. thaliana* that we studied does not rely on pollinators for reproduction and, therefore, acceleration of development in response to a potential threat seems to be an advantageous strategy for this self-compatible short-lived species to ensure reproduction.

In the present study, direct resistance of *A. thaliana* seedlings against herbivory by *M. brassicae* was overall negatively affected by the volatile exposure, and led to a significantly better *M. brassicae* performance on plants exposed to volatiles of some fungi than on control plants. Increased plant weight could have provided the herbivore with more plant material that, in turn, can enhance its performance (Aziz *et al.*, 2016). In this study, larval weight was positively correlated with the shoot fresh weight of infested plants but not with the difference of shoot weight between uninfested and infested plants, suggesting that the larvae did not grow faster by consuming more plant material. Instead, these results indicate a change in the plant primary and secondary metabolites leading to increased plant susceptibility. Indeed, upon perception of microbial volatiles, plant chemistry can be altered, in particular secondary metabolites involved in resistance such as glucosinolates, rendering plants more or less resistant to a subsequent attacker (Aziz *et al.*, 2016). Alternatively, *A. thaliana* plants exposed to the volatiles emitted by *R. solani* and *S. sclerotiorum* could have also become more nutritious for the caterpillars, *e.g.* due to an alteration of the leaf carbon:nitrogen ratio or starch accumulation (Ezquer *et al.*, 2010), which in turn can enhance the conversion of plant material into larval body mass (Lincoln *et al.*, 1993). We hypothesise that these plant responses may result from the perception of linalool, *i.e.* the only VOC that was exclusively detected in the VOC profiles of *R. solani* and *S. sclerotiorum*. This monoterpene alcohol can positively and negatively affect insect attraction to plants (Aharoni *et al.*, 2003; Xiao *et al.*, 2012) but the effects on plant direct resistance against herbivorous insects remain to be elucidated (Ton *et al.*, 2007; Xiao *et al.*, 2012). Interestingly, linalool may also negatively affect *A. thaliana* growth (Aharoni *et al.*, 2003). Further metabolomic analyses of plants exposed to volatiles of *R. solani* and *S. sclerotiorum* and to linalool alone will shed light on the mechanisms underlying such increase of *M. brassicae* performance on these plants.

In conclusion, our results show that plants do not respond differentially to volatile cues from the tested pathogenic compared to non-pathogenic fungi, although these fungal groups emitted distinct VOC profiles. Plants respond with accelerating development, sometimes at

the cost of reduced resistance to insect herbivores. Despite the reduced resistance in some volatile-exposed plants, plants can potentially and ultimately benefit from accelerated development by sustaining fitness and by escaping from potential threats. Similarly to the initial stages of microbe-associated molecular pattern recognition, plants may not be able to discriminate pathogenic fungi from non-pathogenic fungi upon perception of volatiles (Wenke and Piechulla, 2013). Our findings provide new fundamental insight into plant-microbe interactions showing that despite distinct VOC profiles, volatiles of pathogenic and non-pathogenic fungi can both affect plant resistance and development. These findings have significant implications for the understanding of how plants respond to chemical cues from soil-borne fungi in terms of growth and resistance, and how these responses may be exploited to improve durable production of crops.

Acknowledgements

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Chapter 2

Supplemental Materials

Materials and Methods

Pathogenicity to *A. thaliana* Col-0 of *R. solani, S. sclerotioum* and *V. longisporum* strains which had not been previously described in the literature was tested (Table S1). Fungi were inoculated on 17 day-old *A. thaliana*, and plants were monitored for infection one and two weeks after inoculation. All plants were grown individually in plastic pots (7 x 7 x 8 cm) filled with 100% sterile sand supplemented with half-strength Hoagland solution (Hoagland and Arnon, 1950), and kept in a climate chamber (24 ± 1 °C; 16:8 h, L:D; 70 ± 5% RH) until plants had developed six fully expanded leaves. For *R. solani,* a mycelium plug (5 mm) was buried in the sand directly in contact with the roots. For *S. sclerotiorum,* a mycelium plug was inoculated on the 5th leaf of the *A. thaliana* rosette. For *V. longisporum,* plants were up-rooted, roots were incubated in a conidial suspension (10^6 conidia ml^{-1}) for 3 min and subsequently replanted. Control plants were inoculated either with a plug of agar only or by dipping roots into water. After the fungal inoculation, plants remained covered with a plastic lid for one week to ensure high humidity, favourable for the fungal infection. Each inoculation was replicated six times. Disease incidence was calculated as the percentage of diseased leaves over the total number of leaves when regarding *R. solani* and *S. sclerotiorum*, whereas for *V. longisporum,* shoot dry weight was measured. Fungus-inoculated plants and control plants were compared using the two-sample Student's *t*-test (α = 0.05).

Results

Rhizoctonia-inoculated plants displayed disease symptoms (yellow/white leaves) at 14 dai (Fig. S1a; two-sample Student's *t*-test; *P* = 0.016). Symptoms of *Sclerotinia*-inoculated *A. thaliana* were relatively more subtle, and were visible only at the spot of inoculation on the leaf at 8 dai (Fig. S1b; two-sample Student's *t*-test; *P* = 0.024). Plants inoculated with *V. longisporum* were smaller than control plants at 15 dai (Fig. S1c; two-sample Student's *t*-test; *P* = 0.003).

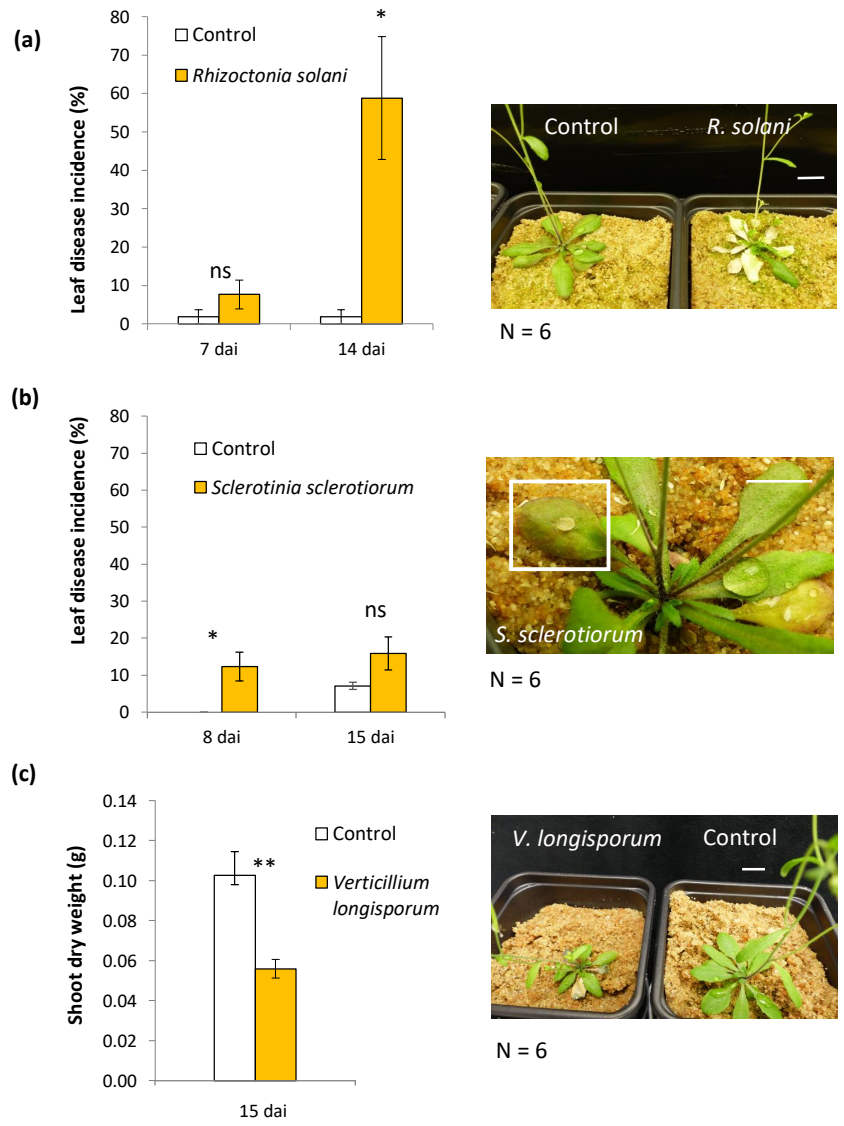


Figure S1. Disease incidence of *Arabidopsis thaliana* inoculated with the soil-borne fungal pathogens *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Verticillium longisporum.* Disease incidence was calculated as the percentage of leaves (± SE) with disease symptoms at 7 and 14 days after inoculation (dai) for plants inoculated with *R. solani* **(a),** and after 8 and 15 dai for plants inoculated with *S. sclerotiorum* **(b)**, whereas disease incidence in plants inoculated with *V. longisporum* **(c)** was determined by the shoot dry weight (± SE) at 15 dai. Asterisks indicate statistical differences (*: *P* < 0.05; **: *P* < 0.01) using two-sample Student *t*-test.

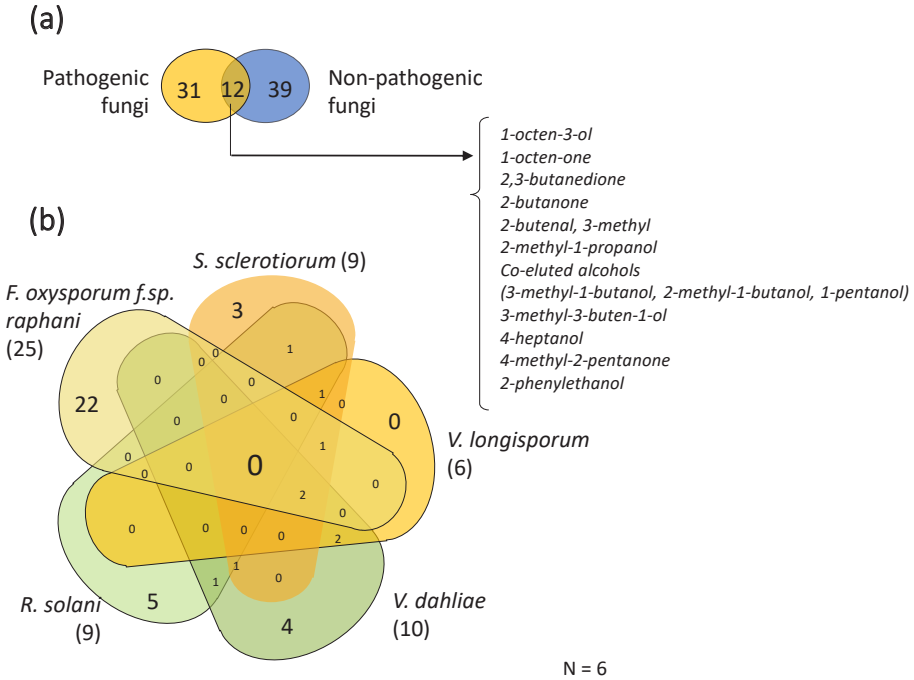


Figure S2. Venn diagrams comparing the qualitative differences of the headspace volatile organic compounds detected for pathogenic and non-pathogenic fungi. **(a)** Total number of unique and shared volatiles detected for pathogenic and non-pathogenic fungi, **(b)** within the pathogenic fungi, and **(c)** within the non-pathogenic fungi.

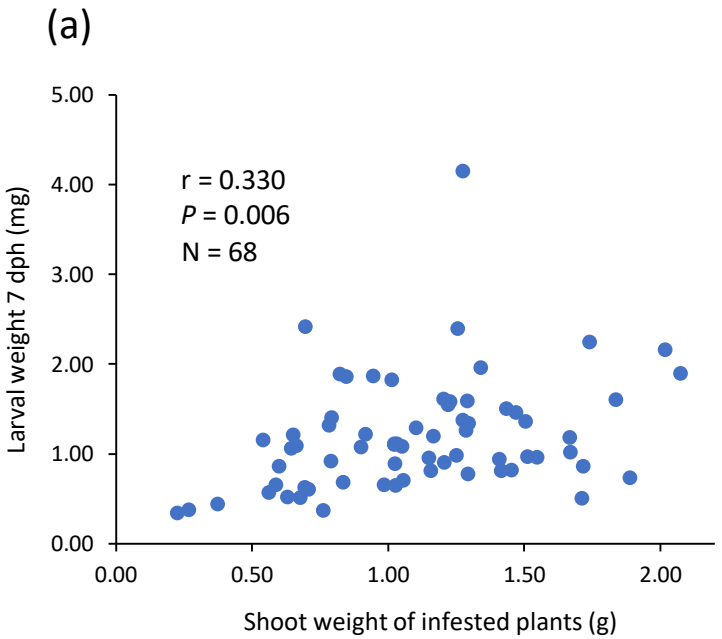
				t	df	P-values
(b)	Leaf	Volatile exposure		13.285	61	<0.001
		Pathogenicity		-0.481	60	0.632
	Root	Volatile exposure		12.331	61	<0.001
		Pathogenicity		-0.993	60	0.325
	Root: leaf	Volatile exposure		0.248	61	0.805
		Pathogenicity		-0.064	60	0.949
	Shoot	Volatile exposure		8.915	76	<0.001
		Pathogenicity		0.587	75	0.559
	Root	Volatile exposure		5.341	76	<0.001
		Pathogenicity		0.965	75	0.338
	Flower: leaf	Volatile exposure		5.894	76	<0.001
		Pathogenicity		-1.313	75	0.193
	Flowering time	Volatile exposure		-4.109	76	<0.001
		Pathogenicity		0.646	75	0.520
	3 dph	Volatile exposure		2.516	107	0.013
		Pathogenicity		-1.603	106	0.112
	7 dph	Volatile exposure		3.381	107	0.001
		Pathogenicity		-1.518	106	0.132

			t	df	P-values
(d)	3 dph	Cl	-1.109	14	0.286
		Fo47	-0.035	15	0.973
		For	1.269	14	0.225
		Mp	0.258	7	0.804
		Pl	0.867	6	0.419
		Rs	1.398	12	0.187
		Ss	3.926	5	0.011
		Tv	1.4	7	0.204
		Ua	2.339	6	0.058
		Vd	1.07	5	0.334
		Vl	1.185	6	0.281
	7 dph	Cl	0.932	14	0.367
		Fo47	-1.106	15	0.286
		For	1.352	14	0.198
		Mp	0.358	7	0.731
		Pl	1.635	6	0.153
		Rs	2.732	12	0.018
		Ss	3.772	5	0.013
		Tv	0.868	7	0.414
		Ua	2.122	6	0.078
		Vd	0.541	5	0.612
		Vl	0.764	6	0.474

			t	df	P-values
(c)	Leaf	Cl	4.394	5	0.007
		Fo47	1.808	5	0.130
		For	5.071	5	0.004
		Mp	2.035	2	0.179
		Pl	18.462	5	<0.001
		Rs	5.728	5	0.002
		Ss	14.001	5	<0.001
		Tv	5.296	5	0.003
		Ua	9.222	5	<0.001
		Vd	10.508	5	<0.001
		Vl	4.458	4	0.011
	Root	Cl	3.268	5	0.022
		Fo47	5.719	5	0.002
		For	3.792	5	0.013
		Mp	1.928	2	0.194
		Pl	13.830	5	<0.001
		Rs	6.415	5	0.001
		Ss	4.381	5	0.007
		Tv	2.734	5	0.041
		Ua	5.547	5	0.003
		Vd	6.103	5	0.002
		Vl	3.141	4	0.035
	Root: leaf	Cl	-1.067	5	0.335
		Fo47	2.728	5	0.041
		For	0.714	5	0.507
		Mp	-0.200	2	0.860
		Pl	-2.251	5	0.074
		Rs	1.584	5	0.174
		Ss	-2.643	5	0.046
		Tv	-6.462	5	0.001
		Ua	1.199	5	0.284
		Vd	-0.657	5	0.540
		Vl	-0.051	4	0.962
	Shoot	Cl	1.878	6	0.109
		Fo47	0.125	7	0.904
		For	0.448	7	0.667
		Mp	1.526	3	0.225
		Pl	0.673	5	0.531
		Rs	1.649	6	0.150
		Ss	1.152	6	0.293
		Tv	4.251	7	0.004
		Ua	2.795	6	0.031
		Vd	2.273	7	0.057
		Vl	1.701	6	0.140
	Root	Cl	-0.228	6	0.827
		Fo47	1.488	7	0.180
		For	5.179	7	0.001
		Mp	0.544	3	0.624
		Pl	5.674	5	0.002
		Rs	1.454	6	0.196
		Ss	0.347	6	0.740
		Tv	1.956	7	0.091
		Ua	3.740	6	0.010
		Vd	6.679	7	<0.001
		Vl	1.686	6	0.143
	Flower: leaf	Cl	1.623	6	0.156
		Fo47	-2.269	7	0.058
		For	-1.940	7	0.930
		Mp	-1.351	3	0.269
		Pl	-1.129	5	0.310
	Flowering time	Rs	-0.678	6	0.523
		Ss	3.179	6	0.019
		Tv	-4.686	7	0.002
		Ua	-2.342	6	0.058
		Vd	-10.009	7	<0.001
		Vl	-4.416	6	0.004

		t	df	P-values
(a)	7 dai	-1.398	10	0.203
	14 dai	-3.531	10	0.016
	8 dai	3.216	7	0.024
	15 dai	1.359	7	0.216
	15 dai	-4.534	7	0.003

Figure S3. Output of the Student two-samples *t*-tests and Student one-sample *t*-tests ($H_0 = 0$) performed **(a)** for the disease incidence, **(b)** for the effects of volatile exposure and pathogenicity on plant dry weight, flowering time and larvae fresh weight, **(c)** for the individual effects of the fungal species on plant dry weight and flowering time, and **(d)** for the individual effects of the fungal species on larvae fresh weight.



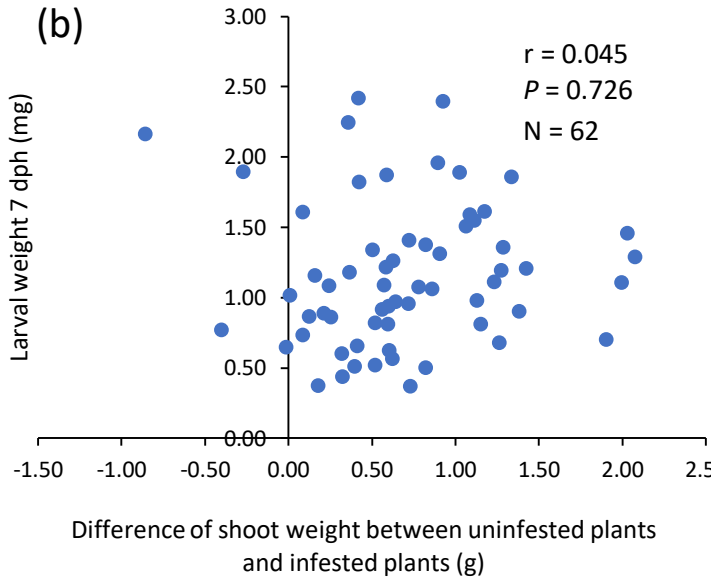


Figure S4. Pearson correlation between fresh weight of *Mamestra brassicae* larvae at 7 days post hatching and **(a)** shoot fresh weight of infested *Arabidopsis thaliana* previously exposed to volatiles of *Rhizoctonia solani, Chaetomium indicum, Fusarium oxysporum f.sp. raphani, Fusarium oxysporum* 47 and non-exposed plants, and **(b)** with the difference of shoot weight between non-infested and infested *A. thaliana* previously exposed to volatiles of *R. solani, C. indicum, F. oxysporum f.sp. raphani, F. oxysporum* 47 and non-exposed plants.

(b)

contrast	p.value	contrast	p.value	contrast	p.value
C10 - C4	1.000	C7 - Mp	0.463	For - Ss	0.533
C10 - C7	1.000	C7 - Pl	0.840	For - Tv	0.994
C10 - Ci	1.000	C7 - Ss	0.350	For - Ua	0.185
C10 - Fo47	0.979	C7 - Tv	0.001	For - Vd	0.020
C10 - For	0.014	C7 - Ua	0.787	For - Vl	0.151
C10 - Mp	0.976	C7 - Vd	0.998	Mp - Pl	1.000
C10 - Pl	0.998	C7 - Vl	0.657	Mp - Ss	1.000
C10 - Ss	0.894	Ci - Fo47	0.993	Mp - Tv	0.449
C10 - Tv	0.095	Ci - For	0.008	Mp - Ua	1.000
C10 - Ua	0.995	Ci - Mp	0.992	Mp - Vd	0.998
C10 - Vd	1.000	Ci - Pl	1.000	Mp - Vl	1.000
C10 - Vl	0.988	Ci - Ss	0.937	Pl - Ss	1.000
C4 - C7	1.000	Ci - Tv	0.064	Pl - Tv	0.416
C4 - Ci	0.999	Ci - Ua	0.999	Pl - Ua	1.000
C4 - Fo47	0.788	Ci - Vd	1.000	Pl - Vd	1.000
C4 - For	0.001	Ci - Vl	0.997	Pl - Vl	1.000
C4 - Mp	0.596	Fo47 - For	0.774	Ss - Tv	0.971
C4 - Pl	0.883	Fo47 - Mp	1.000	Ss - Ua	1.000
C4 - Ss	0.443	Fo47 - Pl	1.000	Ss - Vd	0.972
C4 - Tv	0.005	Fo47 - Ss	1.000	Ss - Vl	1.000
C4 - Ua	0.834	Fo47 - Tv	0.994	Tv - Ua	0.681
C4 - Vd	0.998	Fo47 - Ua	1.000	Tv - Vd	0.142
C4 - Vl	0.737	Fo47 - Vd	0.997	Tv - Vl	0.634
C7 - Ci	0.999	Fo47 - Vl	1.000	Ua - Vd	1.000
C7 - Fo47	0.760	For - Mp	0.078	Ua - Vl	1.000
C7 - For	0.000	For - Pl	0.077	Vd - Vl	0.999

(a)

contrast	p.value	contrast	p.value	contrast	p.value
C10 - C4	1.000	C7 - Ci	0.389	Fo47 - Ss	0.727
C10 - C7	1.000	C7 - Fo47	1.000	Fo47 - Tv	<.0001
C10 - Ci	0.199	C7 - For	1.000	Fo47 - Ua	1.000
C10 - Fo47	0.997	C7 - Mp	0.063	Fo47 - Vd	1.000
C10 - For	0.989	C7 - Pl	0.958	Fo47 - Vl	1.000
C10 - Mp	0.026	C7 - Rs	0.005	For - Mp	0.414
C10 - Pl	0.793	C7 - Ss	0.235	For - Pl	1.000
C10 - Rs	0.002	C7 - Tv	<.0001	For - Rs	0.056
C10 - Ss	0.109	C7 - Ua	1.000	For - Ss	0.773
C10 - Tv	<.0001	C7 - Vd	0.999	For - Tv	<.0001
C10 - Ua	1.000	C7 - Vl	1.000	For - Ua	1.000
C10 - Vd	0.972	Ci - Fo47	0.858	For - Vd	1.000
C10 - Vl	1.000	Ci - For	0.894	For - Vl	0.999
C4 - C7	1.000	Ci - Mp	1.000	Mp - Pl	0.884
C4 - Ci	0.196	Ci - Pl	0.998	Mp - Rs	0.999
C4 - Fo47	0.997	Ci - Rs	0.954	Mp - Ss	1.000
C4 - For	0.989	Ci - Ss	1.000	Mp - Tv	<.0001
C4 - Mp	0.026	Ci - Tv	<.0001	Mp - Ua	0.063
C4 - Pl	0.789	Ci - Ua	0.376	Mp - Vd	0.649
C4 - Rs	0.002	Ci - Vd	0.970	Mp - Vl	0.049
C4 - Ss	0.107	Ci - Vl	0.324	Pl - Rs	0.295
C4 - Tv	<.0001	Fo47 - For	1.000	Pl - Ss	0.991
C4 - Ua	1.000	Fo47 - Mp	0.380	Pl - Tv	<.0001
C4 - Vd	0.971	Fo47 - Pl	1.000	Pl - Ua	0.949
C4 - Vl	1.000	Fo47 - Rs	0.053	Pl - Vd	1.000

Figure S5. Statistical output of the pairwise comparisons between the fungal volatile exposures in the absence **(a)** and presence **(b)** of *Arabidopsis thaliana* seedlings, using Tukey post-hoc tests.

Table S1. List of fungi with information on accession number, abbreviations used in this study, source, pathogenicity and incubation time.

Fungus	Accession number	Abbreviations	Source	Pathogenicity	Incubation time
Chaetomium indicum	CBS 356.92	Ci	Westerdijk Fungal Biodiversity Institute (NL)	No	10 days
Fusarium oxysporum Fo47	Fo 47	Fo47	Agroécologie group - INRA Dijon (FR)	No	7 days
Fusarium oxysporum f.sp. raphani	WCS600	For	Microbe-Plant-Interactions group-Utrecht University (NL)	Yes	7 days
Mucor plumbeus R2	n/a	Mp	NIOO-KNAW collection (NL)	No	4 days
Phoma leveillei	CBS 373.69	Pl	Westerdijk Fungal Biodiversity Institute (NL)	No	10 days
Rhizoctonia solani AG-2-2 IIIB	n/a	Rs	Sugar Beet Research Institute (NL)	*Yes	7 days
Sclerotinia sclerotiorum	CBS 397.54	Ss	Westerdijk Fungal Biodiversity Institute (NL)	*Yes	10 days
Trichoderma viride	CBS 101227	Tv	Westerdijk Fungal Biodiversity Institute (NL)	No	4 days
Ulocladium atrum	CBS 193.67	Ua	Westerdijk Fungal Biodiversity Institute (NL)	No	7 days
Verticillium dahliae JR2	n/a	Vd	Phytopathology group Wageningen University (NL)	Yes	7 days
Verticillium longisporum	CBS 110219	Vl	Westerdijk Fungal Biodiversity Institute (NL)	*Yes	7 days

The pathogenicity reported for these fungi is based on the literature available for brassicaceous species. The strains for which the pathogenicity was not specifically confirmed in *Arabidopsis thaliana* Col-0 are marked with "*Yes" in the pathogenicity and were tested *via* a pathogenicity assay in this study.

Table S2. List of fungal pathogens used in this study and their corresponding host range specificity, disease symptoms and infection mechanisms.

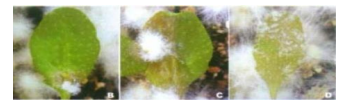
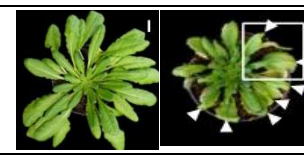
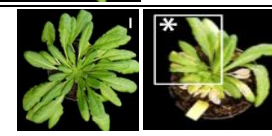
Fungal species	Host-range	Disease	Infection mechanism	Symptoms in Arabidopsis	References
***Fusarium oxysporum f. sp.* raphani**	Radish and Arabidopsis	Yellow/wilt	Through root tips, wounds in the roots, or at the formation point of lateral roots		Bosland and Williams, 1987; Diener and Ausubel, 2005
Rhizoctonia solani	Broad, including bean, lettuce, sugar beet, and cabbage	Bottom Rot/ Damping-off	Through direct penetration of the plant cuticle/epidermis or by means of natural openings in the plant		Sneh *et al.* ,1986; Perl-Treves *et al.* , 2004; Foley *et al.*, 2013
Sclerotia sclerotiorum	Broad (more than 400 species) including Arabidopsis	Stem rot and watery soft rot	Through flowers or by direct germination on leaves (arpogenic germination) or through roots, crowns and lower plant parts (myceliogenic germination)		Li *et al.* , 2003; Dixelius *et al.*, 2004
Verticillium dahliae	Broad, including eggplant, cabbage, and tomato	Wilt	Through lateral roots and root hairs		Tjamos *et al.* , 2005; Fradin and Thomma, 2006; Zhou *et al.* 2006
Verticillium longisporum	Brassica crops	Wilt	Through lateral roots and root hairs		Karapapa *et al.*, 1997; Zhou *et al.* 2006

Table S3. Volatile organic compounds (mean peak area 10^{-6} mg fungal biomass^{-1} ± SD) quantified in the headspace of the pathogenic fungi and non-pathogenic fungi using total ion chromatograms.

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
Amino acid and fatty acid derivates											
1,3-butadiene, 2-methyl	ND	ND	ND	ND	ND	951 ± 231	ND	ND	ND	ND	ND
1,5-octadien-3-ol, (Z)	ND	10 ± 4	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-butanol	ND	ND	ND	ND	ND	47 ± 15	ND	ND	ND	ND	ND
1-decanol acetate	12 ± 11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1-hexanol	ND	ND	ND	ND	ND	ND	207 ± 185	ND	ND	ND	ND
1-octen-3-ol	ND	405 ± 119	21 ± 15	BT (3.91)	16 ± 5	91 ± 50	ND	ND	ND	27 ± 14	5 ± 3
1-octen-3-one	ND	17 ± 7	ND	ND	ND	5 ± 1	ND	ND	ND	26 ± 32	ND
2,3-butanedione	230 ± 170	ND	ND	ND	ND	ND	BT (3.21)	199 ± 58	ND	ND	BT (2.15)
2,4-hexadienal, (E,E)	ND	ND	ND	ND	ND	8 ± 5	ND	ND	ND	ND	ND
2-butanone	ND	ND	56 ± 39	ND	ND	106 ± 59	ND	411 ± 67	ND	ND	ND

Ci: *Chaetomium indicum*, Fo47: *Fusarium oxysporum* 47, For*: F. oxysporum* f.sp. *raphani,* Mp: *Mucor plumbeus*, Pl: *Phoma leveillei*, Rs: *Rhizoctonia solani,* Ss*: Sclerotinia sclerotiorum*, Tv: *Trichoderma viride*, Ua: *Ulocladium atrum*, Vd: *Verticillium dahliae*, Vl: *Verticillium longisporum*. "ND" indicates that the compound was not detected in the samples or in less than 50% of the samples of the fungus; "BT" indicates that the compound was detected but the peak area was below the selected threshold of 4 (emission 4-fold higher than controls). Ratio of peak area measured in the sample over the controls is reported between brackets in the latter case.

Table S3. *Continued.*

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
Amino acid and fatty acid derivates *(continued)*											
2-butenal, 2-methyl-,(E)	ND	ND	ND	ND	ND	2 ± 1	ND	ND	ND	ND	ND
2-butenal, 3-methyl	21 ± 15	ND	ND	ND	ND	279 ± 58	ND	15 ± 5	ND	ND	ND
2-heptanone	BT (1.61)	ND	ND	4 ± 1	ND	ND	ND	ND	ND	ND	ND
2-methyl-1-butyl acetate	9 ± 14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2-methyl-1-propanol	2056 ± 885	ND	ND	ND	ND	ND	621 ± 644	449 ± 115	61 ± 8	111± 37	156 ± 75
2-methyl-3-buten-2-ol	ND	ND	ND	ND	ND	948 ± 136	ND	ND	ND	ND	ND
2-methyl propanal	81 ± 885	ND	ND	ND	ND	ND	BT (2.09)	ND	ND	ND	ND
2-nonen-4-one, (E)	ND	ND	ND	ND	ND	ND	ND	ND	ND	87 ± 85	ND
2-pentylfuran	ND	ND	ND	ND	ND	ND	ND	ND	ND	43 ± 12	ND
2-undecanone	ND	ND	ND	25 ± 11	ND	ND	ND	ND	ND	ND	ND
3-heptanone, 4-methyl	ND	ND	11 ± 2	ND	ND	ND	ND	ND	ND	ND	ND

Ci: *Chaetomium indicum*, Fo47: *Fusarium oxysporum* 47, For*: F. oxysporum* f.sp. *raphani,* Mp: *Mucor plumbeus*, Pl: *Phoma leveillei*, Rs: *Rhizoctonia solani,* Ss*: Sclerotinia sclerotiorum*, Tv: *Trichoderma viride*, Ua: *Ulocladium atrum*, Vd: *Verticillium dahliae*, Vl: *Verticillium longisporum*. "ND" indicates that the compound was not detected in the samples or in less than 50% of the samples of the fungus; "BT" indicates that the compound was detected but the peak area was below the selected threshold of 4 (emission 4-fold higher than controls). Ratio of peak area measured in the sample over the controls is reported between brackets in the latter case.

Table S3. *Continued.*

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
Amino acid and fatty acid derivates *(continued)*											
3-heptanone, 5-methyl	ND	ND	ND	ND	ND	6 ± 2	ND	ND	ND	46 ± 26	ND
3-hydroxy-2-butanone	195 ± 141	ND	ND	ND	ND	ND	ND	612 ± 246	ND	ND	8 ± 9
3-methyl-1-butanol and/or 2-methyl-1-butanol and/or 1-pentanol*	7434 ± 3342	ND	359 ± 138	1747 ± 1907	131 ± 66	85 ± 28	1102 ± 1336	4440 ± 522	103 ± 34	272 ± 140	200 ± 64
3-methyl-1-butanol, acetate	26 ± 33	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
3-methyl-2-buten-1-ol	ND	ND	ND	ND	ND	309 ± 163	ND	ND	ND	ND	ND
3-methyl-2-pentanone	ND	ND	ND	ND	ND	36 ± 25	ND	ND	ND	ND	ND
3-methyl-3-buten-1-ol	33 ± 18	ND	10± 4	24 ± 12	25 ± 11	113 ± 35	21± 23	ND	7± 4	ND	ND
3-methyl butanal, oxime, anti	ND	ND	ND	ND	ND	6 ± 1	ND	ND	ND	ND	ND

Ci: *Chaetomium indicum*, Fo47: *Fusarium oxysporum* 47, For*: F. oxysporum* f.sp. *raphani,* Mp: *Mucor plumbeus*, Pl: *Phoma leveillei*, Rs: *Rhizoctonia solani,* Ss*: Sclerotinia sclerotiorum*, Tv: *Trichoderma viride*, Ua: *Ulocladium atrum*, Vd: *Verticillium dahliae*, Vl: *Verticillium longisporum*. "ND" indicates that the compound was not detected in the samples or in less than 50% of the samples of the fungus; "BT" indicates that the compound was detected but the peak area was below the threshold of 4 (emission 4-fold higher than controls). "*" indicates the presence of 3-methyl-1-butanol and/or 2-methyl-1-butanol and/or 1-pentanol due to a high co-elution.

Table S3. *Continued.*

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
Amino acid and fatty acid derivates *(continued)*											
3-octanol	ND	ND	ND	26± 33	11 ± 6	ND	ND	ND	ND	ND	ND
3-octanol acetate	ND	ND	ND	ND	ND	6 ± 2	ND	ND	ND	ND	ND
3-octanone	ND	66 ± 23	ND	150 ± 80	ND	ND	ND	ND	ND	ND	ND
4-heptanol	1951 ± 1740	ND	1262 ± 2094	ND	411 ± 375	ND	1270 ± 1239	635 ± 730	963 ± 721	893 ± 830	777 ± 955
4-methyl-2-pentanone	68 ± 59	ND	ND	ND	ND	ND	BT (1.74)	ND	3± 2	ND	ND
6-pentyl-2H-pyran-2-one	ND	ND	ND	ND	ND	ND	ND	16 ± 17	ND	2023 ± 575	16 ± 12
allyl hexanoate	ND	ND	ND	ND	ND	ND	ND	ND	ND	13 ± 12	ND
cyclohexanone	17 ± 17	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ethyl acetate	190 ± 251	ND	ND	ND	ND	ND	BT (2.24)	ND	ND	ND	ND
ethyl tiglate	ND	ND	ND	ND	ND	ND	12 ± 7	ND	ND	ND	ND
furfuryl alcohol	ND	ND	ND	ND	ND	ND	ND	22± 6	ND	ND	ND

Ci: *Chaetomium indicum*, Fo47: *Fusarium oxysporum* 47, For*: F. oxysporum* f.sp. *raphani,* Mp: *Mucor plumbeus*, Pl: *Phoma leveillei*, Rs: *Rhizoctonia solani,* Ss*: Sclerotinia sclerotiorum*, Tv: *Trichoderma viride*, Ua: *Ulocladium atrum*, Vd: *Verticillium dahliae*, Vl: *Verticillium longisporum*. "ND" indicates that the compound was not detected in the samples or in less than 50% of the samples of the fungus; "BT" indicates that the compound was detected but the peak area was below the selected threshold of 4 (emission 4-fold higher than controls). Ratio of peak area measured in the sample over the controls is reported between brackets in the latter case.

Table S3. *Continued.*

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
Amino acid and fatty acid derivates *(continued)*											
γ-decalactone	ND	ND	ND	7 ± 4	ND	ND	ND	ND	ND	ND	ND
methyl tiglate	20± 29	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
nonyl acetate	14 ± 12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
prenyl formate	ND	ND	ND	ND	ND	ND	343 ± 160	ND	ND	ND	ND
Benzenoids											
2-phenylethanol	ND	ND	ND	196 ± 168	67± 19	ND	ND	172 ± 35	ND	ND	BT (1.40)
anisole, p-ethyl	ND	ND	ND	ND	ND	ND	123 ± 84	ND	ND	ND	ND
anisole, p-vinyl	ND	ND	ND	ND	ND	ND	22 ± 12	ND	ND	ND	ND
benzyl alcohol	ND	ND	ND	ND	ND	5 ± 2	ND	ND	ND	ND	ND
phenol	ND	ND	ND	ND	ND	18 ± 8	ND	ND	ND	BT (1.93)	ND
p-methyl acetophenone	ND	ND	ND	ND	ND	ND	94 ± 39	ND	ND	ND	ND

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Table S3. *Continued.*

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
Benzenoids *(continued)*											
styrene	ND	ND	ND	ND	ND	ND	798 ± 172	ND	ND	ND	ND
Monoterpenoids											
linalool	ND	13 ± 9	40 ± 20	ND	ND	ND	ND	ND	ND	ND	ND
linalool oxide B	ND	ND	16± 6	ND	ND	ND	ND	ND	ND	ND	ND
phellandral	ND	ND	ND	ND	ND	18 ± 5	ND	ND	ND	ND	ND
terpinolene	ND	ND	ND	ND	ND	ND	16 ± 8	ND	ND	ND	ND
Sesquiterpenes											
2-epi-β-caryophyllene, (E)	24 ± 20	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
alloaromadendrene	33 ± 21	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
α-zingiberene	ND	ND	ND	ND	ND	ND	ND	ND	ND	38 ± 15	ND

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Table S3. *Continued.*

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
Sesquiterpenes *(continued)*											
aromadendr-9-ene	215 ± 139	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
β-caryophyllene, (E)	8± 5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
β-sesquiphellandre	ND	ND	ND	ND	ND	ND	ND	ND	ND	60± 28	ND
caryophyllene oxide	ND	ND	ND	ND	ND	ND	ND	ND	ND	62 ± 26	ND
ɣ-elemene	ND	ND	ND	ND	ND	ND	13 ± 5	ND	ND	ND	ND
ɣ-patchoulene	ND	ND	ND	ND	ND	ND	145 ± 100	ND	ND	ND	ND
nerolidol, (E)	ND	5 ± 3	ND	ND	ND	ND	ND	ND	ND	ND	ND
nerolidol, (Z)	ND	ND	ND	ND	ND	ND	ND	ND	ND	31 ± 22	ND
sesquisabinene hydrate, (E)	ND	ND	ND	ND	ND	ND	ND	ND	ND	31 ± 18	ND

Ci: *Chaetomium indicum*, Fo47: *Fusarium oxysporum* 47, For*: F. oxysporum* f.sp. *raphani,* Mp: *Mucor plumbeus*, Pl: *Phoma leveillei*, Rs*: Rhizoctonia solani,* Ss*: Sclerotinia sclerotiorum*, Tv: *Trichoderma viride*, Ua: *Ulocladium atrum*, Vd: *Verticillium dahliae*, Vl: *Verticillium longisporum*. "ND" indicates that the compound was not detected in the samples or in less than 50% of the samples of the fungus; "BT" indicates that the compound was detected but the peak area was below the selected threshold of 4 (emission 4-fold higher than controls). Ratio of peak area measured in the sample over the controls is reported between brackets in the latter case.

Table S3. *Continued.*

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
N-containing compounds											
benzoic acid, 2-(methylamino), methyl ester	ND	ND	ND	ND	ND	ND	ND	ND	ND	77 ± 91	ND
S-containing compounds											
methyl thiocyanate	ND	27 ± 8	ND	ND	ND	ND	ND	ND	ND	ND	ND
Unknown compounds											
unknown m/z 100	ND	ND	ND	ND	ND	19 ± 5	ND	ND	ND	ND	ND
unknown m/z 113	ND	ND	ND	BT (1.40)	ND	6 ± 1	ND	ND	ND	ND	ND
unknown m/z 114	ND	ND	ND	ND	ND	4 ± 1	ND	ND	ND	ND	ND
unknown m/z 120	ND	ND	ND	59 ± 95	ND	ND	ND	ND	ND	ND	ND
unknown m/z 129	ND	22 ± 24	25 ± 26	36 ± 29	ND	ND	ND	ND	ND	ND	ND

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Table S3. *Continued.*

	Pathogenic fungi					Non-pathogenic fungi					
	For	*Rs*	*Ss*	*Vd*	*Vl*	*Ci*	*Fo 47*	*Mp*	*Pl*	*Tv*	*Ua*
Unknown compounds *(continued)*											
unknown m/z 138	ND	ND	ND	ND	ND	ND	BT (1.11)	ND	ND	34 ± 49	ND
unknown m/z 146	ND	ND	ND	ND	ND	19 ± 5	ND	ND	ND	ND	ND
unknown m/z 149	25 ± 20	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
unknown m/z 161	1003± 714	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
unknown m/z 164.12	110 ± 79	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
unknown m/z 164.22	23 ± 15	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
unknown m/z 189	13 ± 14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
unknown m/z 73	ND	4 ± 4	ND	ND	ND	ND	ND	ND	ND	ND	ND
TOTAL VOCs	**12848 ± 6110**	**572 ± 159**	**1380± 1886**	**2277 ± 2187**	**526 ± 334**	**3097 ± 609**	**4055 ± 2469**	**6977 ± 909**	**818 ± 782**	**3584 ± 1708**	**1027 ± 868**

Ci: *Chaetomium indicum*, Fo47: *Fusarium oxysporum* 47, For: *F. oxysporum* f.sp. *raphani*, Mp: *Mucor plumbeus*, Pl: *Phoma leveillei*, Rs: *Rhizoctonia solani*, Ss: *Sclerotinia sclerotiorum*, Tv: *Trichoderma viride*, Ua: *Ulocladium atrum*, Vd: *Verticillium dahliae*, Vl: *Verticillium longisporum*. "ND" indicates that the compound was not detected in the samples or in less than 50% of the samples of the fungus; "BT" indicates that the compound was detected but the peak area was below the selected threshold of 4 (emission 4-fold higher than controls). Ratio of peak area measured in the sample over the controls is reported between brackets in the latter case.

Table S4. List of the variable importance in projection (VIP) values, generated by the PLS-DA model, for each volatile organic compound of the fungal headspace.

Compound	VIP	Compound	VIP
unknown m/z 129	173.247	2-butenal, 3-methyl	0.980837
linalool	158.972	unknown m/z 73	0.976175
3-octanone	153.772	2-methylpropanal	0.972724
linalool oxide B	134.451	4-methyl-2-pentanone	0.967578
3-heptanone, 4-methyl	128.484	γ-elemene	0.961433
furfuryl alcohol	128.081	3-heptanone, 5-methyl	0.960106
methyl thiocyanate	125.267	3-methyl-1-butanol, acetate	0.955109
2-butanone	121.264	perilla ketone	0.950891
1-octen-3-ol	120.929	3-methyl-2-buten-1-ol	0.950432
1,5-octadien-3-ol, (*Z*)	120.690	ethyl acetate	0.945610
2-heptanone	120.230	2,3-butanedione	0.938122
2-undecanone	115.551	2-pentylfuran	0.935906
3-octanol	113.733	co-elution compounds	0.933692
3-hydroxy-2-butanone	108.861	methyl tiglate	0.931901
nerolidol, (*E*)	106.419	p-methylacetophenone	0.929315
unknown m/z 164.22	106.124	2,4-hexadienal, (*E,E*)	0.925762
2-methyl-3-buten-2-ol	105.348	3-methyl-2-pentanone	0.925220
1,3-butadiene, 2-methyl	104.689	prenyl formate	0.919730
β-caryophyllene, (*E*)	104.492	2-methyl-1-butyl acetate	0.918724
unknown m/z 100	103.726	terpinolene	0.912356
unknown m/z 161	103.495	caryophyllene oxide	0.896991
unknown m/z 149	103.401	α-zingiberene	0.895012
γ-decalactone	102.718	anisole, p-vinyl	0.890064
unknown m/z 164.12	102.491	β-sesquiphellandrene	0.881373
1-butanol	102.276	ethyl tiglate	0.863652
phellandral	102.154	γ-patchoulene	0.857320
unknown m/z 113	102.145	unknown m/z 189	0.848463
aromadendr-9-ene	102.043	anisole, p-ethyl	0.837471
3-octanol acetate	102.026	sesquisabinene hydrate, (*E*)	0.836242
2-epi-β-caryophyllene, (*E*)	101.620	2-methyl-1-propanol	0.814313
3-methylbutanal, oxime, anti	101.537	nerolidol, (*Z*)	0.790710
nonyl acetate	101.447	3-methyl-3-buten-1-ol	0.751673
unknown m/z 146	101.429	allyl hexanoate	0.723355
alloaromadendrene	101.412	2-nonen-4-one, (*E*)	0.701143
2-butenal, 2-methyl-,(*E*)	101.339	unknown m/z 120	0.695870
1-decanol acetate	101.139	benzoic acid, 2-methylamino	0.636713
benzyl alcohol	100.526	unknown m/z 138	0.563735
unknown m/z 114	0.998043	1-hexanol	0.544389
cyclohexanone	0.995420	2-phenylethanol	0.460180
styrene	0.993565	1-octen-3-one	0.413256
phenol	0.984344	4-heptanol	0.399529



Chapter 3

Fungal volatiles influence plant defence against aboveground and belowground herbivory

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Abstract

Plants have evolved resistance traits that negatively affect attackers, and tolerance traits that sustain plant growth despite herbivore damage. These mechanisms often co-occur in a mixed-defence strategy, balancing resistance and tolerance. These plant defences can be enhanced upon interaction with soil microorganisms. Here, we investigated the effects of volatiles emitted by soil-borne fungi on plant defence to insect herbivory, and on plant phenology. We exposed roots of *Brassica rapa* plants to volatiles emitted by four soil-borne fungi. As a proxy of plant resistance, we assessed the performance of *Pieris brassicae,* a caterpillar feeding on leaves and inflorescences, and of *Delia radicum,* an insect root herbivore. As a proxy of plant tolerance, we compared growth of volatile-exposed plants challenged with or without insects. Additionally, we assessed the effects on plant phenology by recording bolting time and by counting the number of buds and flowers. Plant exposure to fungal volatiles differentially affected plant resistance to above- and belowground herbivory. Performance of *P. brassicae* caterpillars differed between the fungal volatile-exposed plants but were variable between experiment batches. In contrast, the effects of fungal volatiles on *D. radicum* performance was predominantly negative, indicating an increased plant resistance. Despite root consumption by *D. radicum,* root dry weight remained unchanged in infested plants compared with uninfested ones, irrespectively of the volatile exposure, suggesting compensation for the tissue loss, sometimes at the cost of undamaged aboveground tissues. When *B. rapa* plants were attacked by *P. brassicae* caterpillars, only exposure to volatiles of some fungi led to compensation for the loss of aboveground tissues consumed by the caterpillars, which differed between leaves and inflorescences. Furthermore, bolting was accelerated in response to volatiles of some fungi, resulting in more buds and flowers, which suggests a potential enhancement of plant fitness. Our data show that fungal volatiles can influence the mixed-defence strategies of *B. rapa* plants, balancing plant resistance and tolerance to above- and belowground herbivory. These effects may be variable and were fungus-specific. Ultimately, plant fitness may be enhanced upon root exposure to fungal volatiles.

Keywords

Brassica rapa, compensatory growth, *Delia radicum*, *Pieris brassicae,* plant fitness, plant phenology, resistance, tolerance

Introduction

Plants are part of complex and dynamic communities, coined as the phytobiome (Leach *et al.*, 2017). Members of the phytobiome include plant-associated microorganisms that live inside, on the surface or adjacent to plant tissues. This plant microbiome can influence several plant phenotypic traits, altering plant interactions with their associated organisms and plant fitness (Junker and Tholl, 2013; Philippot *et al.*, 2013; Dicke, 2016; Roat and Saraf, 2017; Hassani *et al.*, 2018). In particular, soil and root-associated microorganisms can play a substantial role in modulating plant defence strategies to diverse biotic and abiotic stresses, with positive or negative consequences for plant fitness (Berendsen *et al.*, 2012; Choudhary *et al.*, 2016).

Plant defence strategies consist of resistance and tolerance mechanisms that co-occur in a mixed-defence strategy (Stowe *et al.*, 2000; Leimu and Koricheva, 2006), and plant colonisation by microorganisms can affect these two components concurrently (Hermosa *et al.*, 2013; Contreras-Cornejo *et al.*, 2016). Microbial colonisation can condition plants to respond faster and stronger to a subsequent stress (Martinez-Medina *et al.*, 2016; Mauch-Mani *et al.*, 2017) or can induce systemic plant resistance, *e.g.* by negatively affecting the preference or performance of a herbivore or pathogen *via* plant metabolomic changes (van de Mortel *et al.*, 2012; Pieterse *et al.*, 2014; Pangesti *et al.*, 2016; Etalo *et al.*, 2018). These plant responses occur locally at the site of colonisation, but also systemically affect chemical and physical plant traits. As a consequence, root colonisation by soil microorganisms can affect subsequent plant interactions aboveground and *vice versa* (Bezemer and van Dam, 2005; van Dam and Heil, 2010; Pineda *et al.*, 2017). Alongside resistance, tolerance, *i.e.* the capability of plants to endure stresses that damage plant tissues or limit plant development, can be modulated upon microbial colonisation as well. Tolerance can be measured by the degree to which plant growth is affected by a given stress relative to its growth in the undamaged state (Strauss and Agrawal, 1999), and plant colonisation by microorganisms can influence the degree of stress tolerance compared to uncolonised plants. For instance, microorganisms can alleviate the negative effects of high salinity on plant growth by boosting photosynthetic rate (Han and Lee, 2005; Yang *et al.*, 2009). These studies exemplify that plant colonisation by microorganisms can enhance plant defence to biotic and abiotic stresses, and maintain plant fitness.

Remarkably, without direct physical contact with plants, microorganisms, such as fungi and bacteria, can also affect plant growth and defence through the emission of volatile organic and inorganic compounds (Bitas *et al.*, 2013; Kanchiswamy *et al.*, 2015a; Piechulla *et al.*, 2017; Tyagi *et al.*, 2018; Moisan *et al.*, 2019; Chapter 2 of this thesis). Volatiles emitted by pathogenic and beneficial microorganisms can promote plant growth (Casarrubia *et al.*, 2016; Lee *et al.*, 2016; Cordovez *et al.*, 2018; Fincheira and Quiroz, 2018; Ameztoy *et al.*, 2019), and accelerate plant development (Sánchez-López *et al.*, 2016; Moisan *et al.*, 2019; Chapter 2 of this thesis), for instance by increasing nutrient uptake (Liu and Zhang, 2015) or by altering phytohormone homeostasis (Zhang *et al.*, 2007; Bailly and Weisskopf,

2012). Microbial volatiles can also enhance plant resistance to fungal, bacterial or oomycete pathogens (Farag *et al.*, 2013; Kottb *et al.*, 2015; Jain *et al.*, 2017) and to insect herbivores (Aziz *et al.*, 2016; Cordovez *et al.*, 2017; Moisan *et al.*, 2019; Chapter 2 of this thesis). They can do so, either directly by inhibiting the attacker's activity (Vespermann *et al.*, 2007; Bailly and Weisskopf, 2017) or indirectly by eliciting plant resistance (Ryu *et al.*, 2005; Sharifi and Ryu, 2016). Upon abiotic stresses, such as salinity or drought, microbial volatiles can improve plant tolerance and sustain plant growth (Han *et al.*, 2014; Liu and Zhang, 2015; Jalali *et al.*, 2017; Camarena-Pozos *et al.*, 2019). Yet, to our knowledge, it remains unknown whether microbial volatiles affect plant tolerance to insect herbivory and whether these responses are specific to the plant tissue(s) being attacked. Additionally, how plant resistance and tolerance to herbivory are concurrently modulated by microbial volatiles has not been addressed. Therefore, here, we investigated the effects of volatiles emitted by soil-borne fungi on plant tolerance and resistance to above- and belowground insect herbivory, and on plant phenology. For this, we selected the brassicaceous plant species *Brassica rapa* and its natural herbivores: the Cabbage Root fly, *Delia radicum* (Diptera: Anthomyiidae), whose larvae feed on *B. rapa* roots, and the Large Cabbage White butterfly, *Pieris brassicae* (Lepidoptera: Pieridae), whose caterpillars feed on leaves and inflorescences of *B. rapa* plants. Furthermore, we selected four soil-borne fungi (*Fusarium oxysporum*, *Rhizoctonia solani, Ulocladium atrum* and *Phoma leveillei*) that all co-occur and interact with brassicaceous plants, and have a saprophytic phase in their cycle.

Materials and Methods

Study system

Brassica rapa L. (Brassicaceae) is an annual plant, also known as the wild turnip. Occurring in ecosystems such as cropland, weedy fields or roadsides, *B. rapa* is subjected to attack by insect herbivores as well as pathogens. The *B. rapa* accession used in this study originated from a wild population in Maarssen (The Netherlands). All *B. rapa* seeds were first surface-sterilised before sowing by exposure to chlorine gas for 4 h in a desiccator and stratified at 4 °C in the dark for 3 to 4 days (Cordovez *et al.*, 2017).

Volatiles emitted by the four soil-borne, *F. oxysporum f. sp. raphani* (WCS600, provided by Utrecht University, The Netherlands), *R. solani* AG2-2 IIIb (provided by the Sugar Beet Research Institute, The Netherlands), *U. atrum* (CBS 193.67, from the Westerdijk Fungal Biodiversity Institute, The Netherlands) and *P. leveillei* (CBS 373.69, from the Westerdijk Fungal Biodiversity Institute, The Netherlands) differentially affect plant growth and resistance *in vitro* (Moisan *et al.*, 2019; Chapter 2 of this thesis). In the present study, all fungi were inoculated in ø 9 cm plastic Petri dishes containing 1/5th strength Potato Dextrose Agar (1/5th PDA). This medium was prepared with 7.8 g of PDA (Oxoid) and 14 g of Bacto^{TM}Agar (Becton Dickinson). The pH was set at 7. Fungi were incubated at 25 °C in the dark for 7 days before the start of the exposure.

First instar caterpillars of *P. brassicae* feed on leaves, whereas later instars move to the inflorescences to feed on buds and flowers (Lucas-Barbosa *et al.*, 2013). Caterpillars were reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv Cyrus) in a climate room (22 ± 2 °C; 16:8 h, L:D; 60 ± 10% RH). Larvae of *D. radicum* feed for most of their development on primary roots of Brassicaceae, which results in growth retardation or plant mortality (Ahuja *et al.*, 2011; Shuhang *et al.*, 2016). Larvae were reared on rutabaga (*Brassica napus* subsp. *napobrassica*) in a climate cabinet (20 ± 1 °C; 16:8 h, L:D).

Plant exposure to fungal volatiles

To expose *B. rapa* roots to fungal volatiles *in vivo*, we designed a two-compartment pot system (Fig. 1). One sterile *B. rapa* seed was sown in the top compartment (h = 20 cm, ø = 12.5 cm) filled with a sterile (*i.e.* autoclaved twice at 121 °C for 20 min with 24 hours interval in between) soil mixture (1:1 v/v, ø 4 mm sieved Horticoop potting soil:sand), whereas the test fungus (*F. oxysporum, R. solani, U. atrum* or *P. leveillei*) was grown in a ø 9 cm Petri dish enclosed in the bottom compartment (h = 10 cm, ø = 12.5 cm). Both compartments were connected to each other by a cylinder (h = 12.5 cm, ø = 12.7 cm), and separated by a nylon membrane of 1 µm mesh width (Plastok associates Ltd., Birkenhead Wirral, UK, ø= 14.5 cm) that allowed air exchange between the two compartments. Volatile exposure was initiated in a greenhouse compartment (21 ± 2 °C; 16:8 h, L:D; 70 ± 5% RH) with seven-day-old fungi as soon as *B. rapa* seeds were sown, and was maintained for four weeks, after which *B. rapa* plants had 6-8 fully developed leaves. Control plants were exposed to a Petri dish containing 1/5th PDA medium only. Petri dishes containing the fungi and control were replaced weekly with Petri dishes containing fresh seven-day-old fungi or fresh 1/5th PDA medium. A total of 30 plant replicates was prepared for each fungal volatile exposure and divided in two experimental batches, with one week interval.

Plant infestation with aboveground and belowground herbivores

After the four-week volatile exposure, all Petri dishes were removed permanently from the bottom compartments, and the *B. rapa* plants were either infested with one of the two insect herbivores or remained uninfested. For *P. brassicae,* twenty newly hatched caterpillars were placed on the third fully expanded leaf. For *D. radicum,* ten newly hatched larvae were placed close to the plant stem and watched until all larvae crawled down to the roots. Per experimental batch, the 15 plant replicates of each fungal volatile exposure were divided as follows: 5 replicates infested with *P. brassicae,* 5 replicates infested with *D. radicum* and 5 replicates remained uninfested.

Effects of fungal volatile exposure on growth of uninfested plants

To assess the effects of fungal volatile exposure on plant growth, we measured the dry weight of six-week-old uninfested *B. rapa* plants after the four weeks of exposure to fungal volatiles. Roots, leaves and inflorescences were harvested, dried at 105 °C for 16 h, and weighed. Effects of root exposure to fungal volatiles on the total plant dry weight as

well as on the dry weight of the different plant tissues were separately tested with linear models (PROC GLM in SAS v. 9.4). Fungal volatile exposure, batch, and their interactions were included in the model as fixed factors (scripts available on Online Supplemental Materials). Upon a significant main effect of the fungal volatile exposures, post-hoc tests were performed using the *t*-distribution.

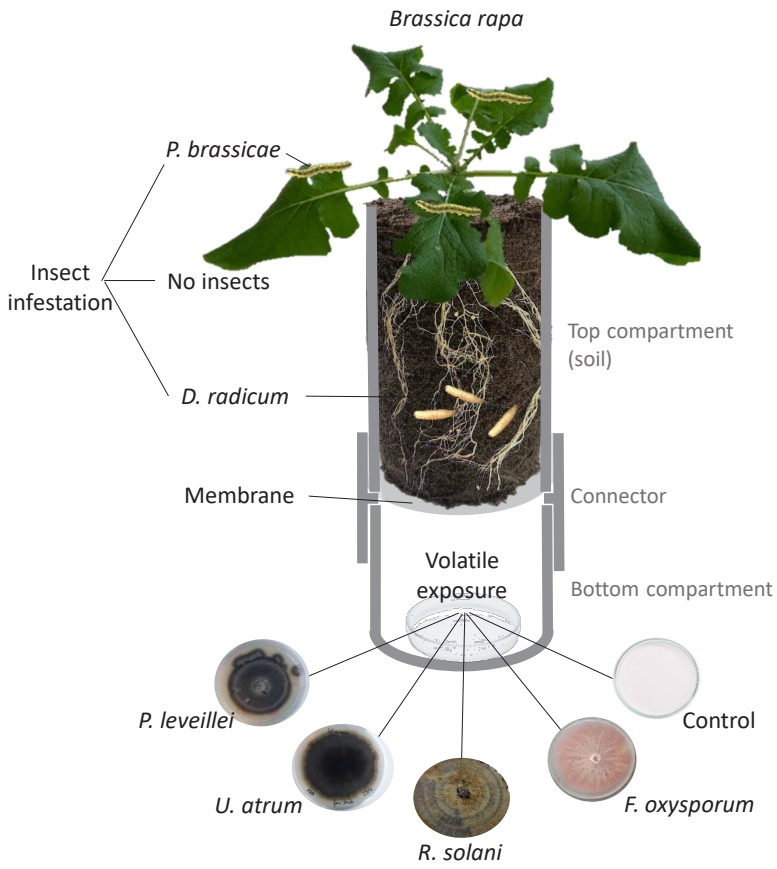


Figure 1. Schematic representation of the two-compartment pot system used in this study to expose roots of *Brassica rapa,* growing in soil in the top compartment, to volatiles emitted by one fungus growing in a Petri dish in the bottom compartment or by a control Petri dish. Plants were subsequently infested with either caterpillars of the aboveground herbivore, *Pieris brassicae,* or with larvae of the belowground herbivore, *Delia radicum;* or remained uninfested (no insects).

Effects of fungal volatile exposure on plant growth upon aboveground and belowground herbivory

To assess the effects of fungal volatile exposure on plant tolerance to aboveground and belowground herbivores, we measured the dry weight of *B. rapa* plants whose roots were previously exposed to fungal volatiles and then infested with either *P. brassicae* caterpillars or with *D. radicum* larvae, and compared it to that of volatile-exposed uninfested plants. Using a mixed model (PROC MIXED in SAS), we specified an unstructured covariance matrix that allows for correlations and inequality of variances among plant tissues of the same plant (Online Supplemental Materials). Fungal volatile exposure, plant tissue, herbivory and batch, and their interactions were included in the model as fixed factors. From this model, we generated differences of least squares means of plant tissue dry weights (1) between *B. rapa* infested with *P. brassicae* and uninfested *B. rapa* plants, and (2) between *B. rapa* infested with *D. radicum* and uninfested *B. rapa* plants, within each volatile exposure (Table S1). For each plant tissue and volatile exposure, the effect of herbivory was tested by comparing the above described differences to zero, using the *t* distribution ($\alpha = 0.05$). For the visualisation of the data, we plotted the Cohen's D effect sizes by dividing the differences of least square means by the pooled standard deviation of each plant tissue.

Effects of fungal volatile exposure on aboveground and belowground herbivore performance

To assess the effects of fungal volatile exposure on plant resistance to aboveground and belowground herbivores, we measured the performance of *P. brassicae* caterpillars and *D. radicum* larvae on plants whose roots were previously exposed to fungal volatiles. Individual fresh weight of *P. brassicae* caterpillars was assessed at 3 days post infestation (dpi) and at 7 dpi. At 3 dpi, all twenty *P. brassicae* caterpillars were recollected, and larval density was reduced by 50% to mimic natural field predation and dispersal (Lucas-Barbosa *et al.*, 2013). For this, ten larvae per plant were randomly selected, individually weighed and placed back on their respective plants until the second measurement at 7 dpi. Caterpillar fresh weight was ^{10}log-transformed. Separately for the two timepoints, we used a linear mixed model (LMM) in R (v.3.4.0) (R Development Core Team, 2011) with fungal volatile exposure, batch, and their interaction as fixed factors and plant replicate as a random factor (Online Supplemental Materials). Effects of fungal volatile exposure on the caterpillar fresh weight were compared using approximate *F*-tests with degrees of freedom calculated according to the method of Kenward and Roger (Kenward and Roger, 1997), followed by post-hoc tests using the *t*-distribution. At 14 dpi, we also scored the developmental stage (larvae or pupae) of recollected *D. radicum*, and individually weighed the recollected insects. A mixed model similar to that used for *P. brassicae* was used to analyse *D. radicum* fresh weight. Additionally, the number of recovered *D. radicum* (out of 10 infested insects) and the fraction of recovered *D. radicum* pupae were analysed using a beta-binomial (to handle binomial overdispersion) generalised linear model (GLM) and logit link function (Online Supplemental Materials). For this, we used the glmmTMB package in R (Brooks *et al.*, 2017). In this model, fungal volatiles, batch and their interaction were included as fixed

factors. Upon a significant main effect of the fungal volatile exposures, likelihood ratio post-hoc tests (LRT) were performed. Furthermore, to estimate whether the insect performance was correlated with food intake or food available, we calculated Pearson correlations (1) between the average *P. brassicae* fresh weight at 7 dpi and leaf and inflorescence dry weights, (2) between *D. radicum* fresh weight, the root dry weight and the number of *D. radicum* individuals recollected, and (3) between the average *P. brassicae* fresh weight at 7 dpi or the *D. radicum* fresh weight per fungal volatile exposure and the mean of tissue dry weight difference of uninfested and infested plants ($\alpha = 0.05$).

Effects of fungal volatile exposure on plant bolting upon herbivory

To assess the effects of fungal volatile exposure on phenology of *B. rapa* plants upon aboveground and belowground herbivory, we recorded bolting date, as well as total count of buds and flowers of six-week-old *B. rapa* plants. The cumulative percentage of bolting *B. rapa* plants whose roots were exposed to volatiles of different fungi and upon different herbivories was plotted against time. Time until bolting was statistically analysed in SAS using a Cox proportional hazard model (PROC PHREG; Lin and Wei, 1989), which can handle incomplete (censored) observations of plants that did not bolt within 42 days (Online Supplemental Materials). In the Cox model, bolting time was allowed to depend on fungal volatile exposure, herbivory, batch and their interactions. Total count of buds and flowers of *B. rapa* plants following root exposure to volatiles of different fungi and upon different herbivories was modelled with a GLM with a log-link and a negative binomial distribution (type 1; Hilbe, 2011). Upon a significant main effect of the fungal volatile exposures in one of the two models described above, LRT post-hoc tests were performed. To assess the relative investment of plants in bud development, the number of buds and flowers per unit of plant dry weight was calculated and analysed with an ordinary linear model. Fungal volatile exposure, herbivory and batch, and their two-way interactions were included in the models as fixed factors.

Results

Effects of fungal volatile exposure on growth of uninfested plants

Fungal volatiles affected total dry weight of uninfested *B. rapa* plants (Fig. 2; LM; $P = 0.039$). Growth of control plants did not differ from growth of volatile-exposed plants (Fig. 2; post-hoc tests; all $P > 0.050$), however plants exposed to *F. oxysporum* volatiles were overall smaller than plants exposed to *R. solani* volatiles (Fig. 2; post-hoc tests; $P = 0.032$) and to *U. atrum* volatiles (Fig. 2; post-hoc tests; $P = 0.003$). Fungal volatiles particularly affected leaf dry weight (Fig. 2; LM; $P = 0.044$) but not root or inflorescence dry weights (Fig. 2; LM; $P = 0.147$; $P = 0.175$, respectively). Plants exposed to volatiles from *U. atrum* had higher leaf dry weight than the control plants and the other fungal volatile-exposed plants (Fig. 2; post-hoc tests; all $P < 0.050$). Volatile exposure had a significant interaction with batch on

the total plant dry weight (Fig. 2; LM; $P = 0.022$) and leaf dry weight (Fig. 2; LM; $P = 0.036$), so the differential effects of fungal volatiles were not constant between the two batches.

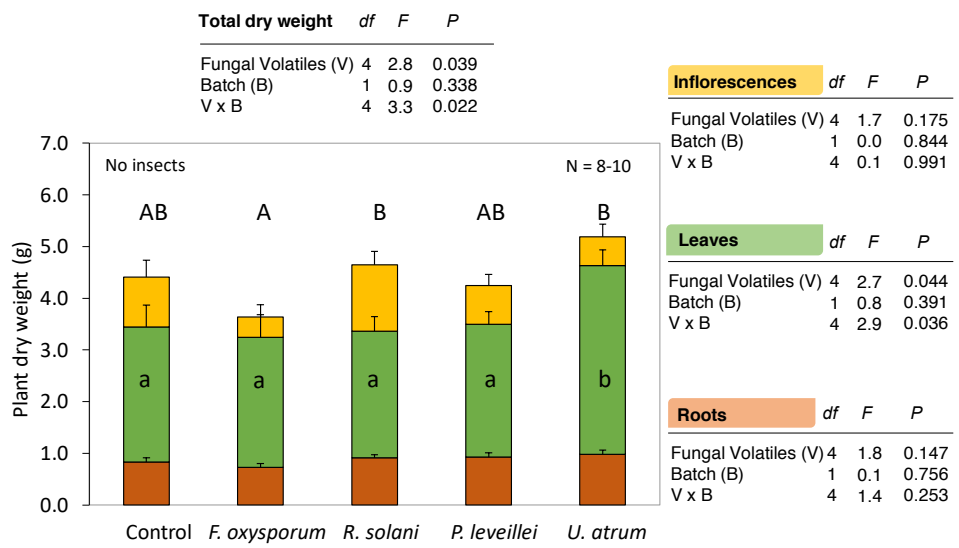


Figure 2. Root, leaf and inflorescence dry weight (mean + SE) of uninfested six-week-old *Brassica rapa* plants exposed for four weeks to volatiles of four different fungi (*F. oxysporum, R. solani, P. leveillei, U. atrum*). Main effects of the fungal volatile exposure, batch, and their interactions were tested using linear models. "N" indicates the number of plant replicates. Uppercase letters indicate pairwise differences of the total plant dry weight between the fungal volatile exposures, while lowercase letters indicate pairwise differences of the leaf dry weight between the fungal volatile exposures ($\alpha = 0.05$).

Effects of fungal volatile exposure on plant growth upon aboveground and belowground herbivory

Overall, herbivory by *P. brassicae* negatively affected weight of aboveground tissues. This reduction was expected as *P. brassicae* caterpillars feed on aboveground tissues. Yet, the effects differed between tissues. When infested with *P. brassicae,* inflorescences of control plants (*i.e.* plants not exposed to fungal volatiles) and plants whose roots were exposed to *R. solani* volatiles weighed less than inflorescences of uninfested plants (Fig. 3a; *t*-tests; $P_{(control)} = 0.038$; $P_{(R.solani)} = 0.005$). Also, *P. brassicae*-infested plants exposed to *U. atrum* volatiles had lower leaf weight than uninfested plants (Fig. 3a; *t*-test; $P = 0.020$). However, leaf and inflorescence weights of plants whose roots were exposed to *F. oxysporum* and *P. leveillei* volatiles did not significantly differ between *P. brassicae*-infested plants and uninfested plants (Fig. 3a and Table S1a; *t*-tests; all $P > 0.050$).

Belowground, despite an overall reduction, infestation by *D. radicum* did not significantly impact individual root weight, irrespectively of fungal volatiles they had been exposed to (Fig. 3b and Table S1b; *t*-tests; all $P > 0.050$). However, infestation by *D. radicum* resulted in

lower inflorescence weight upon exposure to *R. solani* volatiles (Fig. 3b; *t*-test; $P = 0.023$), and in lower leaf weight upon exposure to *U. atrum* volatiles (Fig. 3b; *t*-test; $P = 0.039$), compared with uninfested plants.

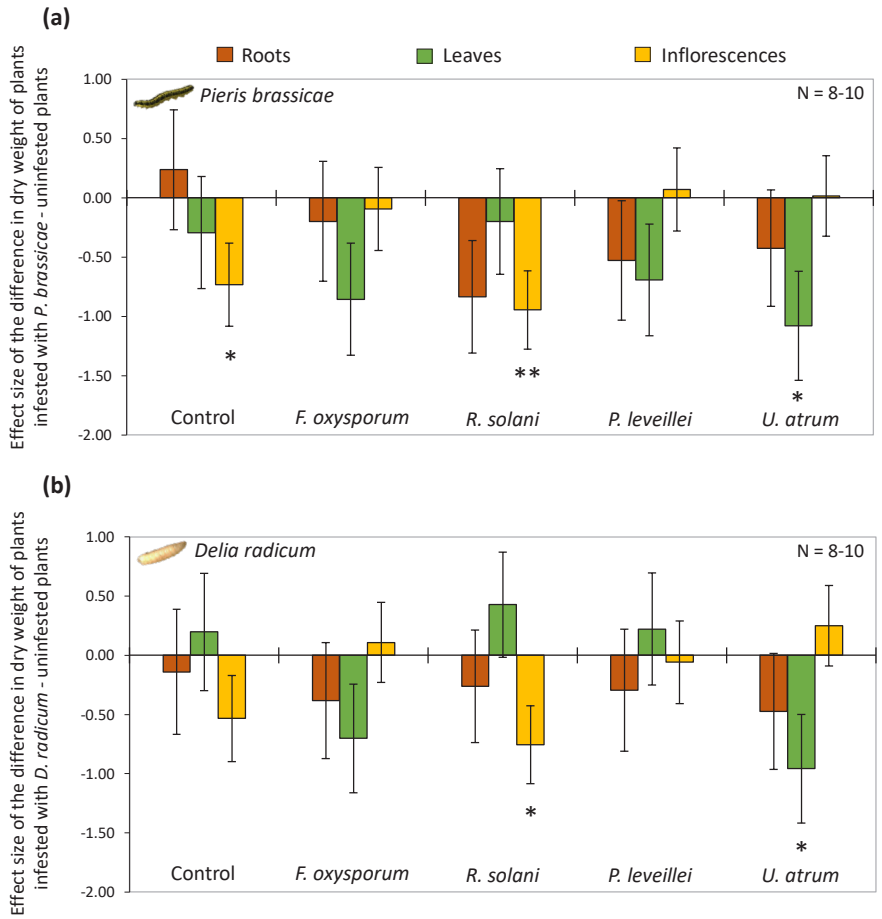
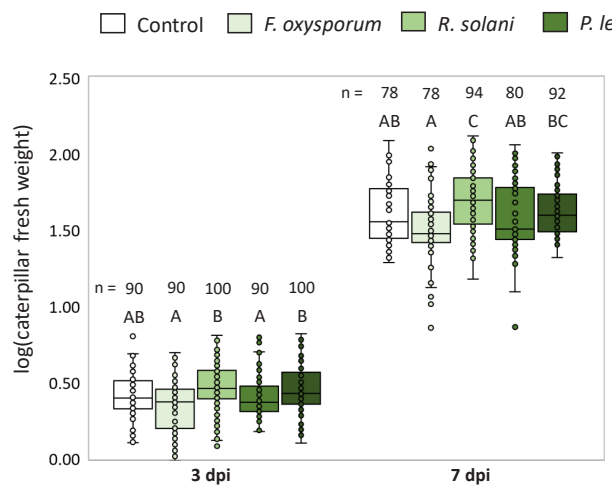


Figure 3. Cohen's D effect sizes [(differences of least squares means / pooled SD) ± (SE of the differences / pooled SD)] in root, leaf and inflorescence dry weight of **(a)** six-week-old *Brassica rapa* plants infested with *Pieris brassicae* and uninfested plants, and of **(b)** six-week-old *B. rapa* plants infested with *Delia radicum* and uninfested plants, when exposed to volatiles of four different fungi (*F. oxysporum, R. solani, P. leveillei, U. atrum*). Differences of least squares means were generated using a mixed model that allows for correlations and inequality of variances among plant tissues of the same plant, and were statistically tested per plant tissue and per fungal volatile exposure using the *t* distribution (*: $P < 0.050$; **: $P < 0.010$). "N" indicates the number of plant replicates. Detailed information of the least squares means can be found in Table S1.

Effects of fungal volatile exposure on aboveground and belowground herbivore performance

Plant exposure to fungal volatiles affected *P. brassicae* caterpillar weight at 3 and 7 dpi (Fig. 4; LMM; $P = 0.014$; $P = 0.006$, respectively). At 3 dpi, caterpillars feeding on plants whose

roots were exposed to volatiles from *R. solani* or *U. atrum* were larger than those feeding on plants exposed to volatiles from *F. oxysporum* or *P. leveillei* (Fig 4; post-hoc tests; $P < 0.050$). At 7 dpi, caterpillars feeding on plants exposed to *R. solani* volatiles were larger than those feeding on control plants and plants exposed to volatiles from *F. oxysporum* or *P. leveillei* (Fig 4; post-hoc tests; $P < 0.050$). Additionally, batch has a significant effect on caterpillar fresh weight at the two time points (Fig. 4; LMM; $P = 0.037$; $P < 0.001$, respectively), and interacts with fungal volatile exposure at 7 dpi (Fig. 4; LMM; $P = 0.002$). Caterpillar weight at 7 dpi was neither correlated with leaf nor inflorescence weight of *P. brassicae*-infested plants (Table S2b; Pearson correlation tests; all $P > 0.050$), nor with the difference of weight of these plant tissues between *P. brassicae*-infested and uninfested plants (Table S2c; Pearson correlation tests; all $P > 0.050$). Plant exposure to fungal volatiles did not affect the number of *D. radicum* we recollected (Table S3; GLM; $P = 0.058$). However, it did affect the insect developmental stage reached by the larvae (Fig. 5a; GLM; $P < 0.001$). In three out of the four fungal volatile exposures tested (*F. oxysporum, R. solani* and *P. leveillei*), we recollected fewer pupae than in control plants (Fig. 5b; LRT post-hoc; all $P < 0.050$), except for plants exposed to *U. atrum* volatiles (Fig. 5a; LRT post-hoc; $P = 0.723$). Fresh weight of *D. radicum* was not affected by fungal volatile exposure (Fig. 5b; GLM; $P = 0.081$) and was not correlated with the number of *D. radicum* recollected (Table S2a; Pearson correlation test; $P = 0.219$), nor with the difference in root weight between *D. radicum*-infested and uninfested *B. rapa* plants (Table S2c; Pearson correlation test; $P = 0.275$).



7 dpi		Num df	Den df	F	P
Fungal volatiles	(V)	4	37.0	4.3	0.006
Batch	(B)	1	37.0	35.7	<0.001
V x B		4	37.0	5.1	0.002

3 dpi		Num df	Den df	F	P
Fungal volatiles	(V)	4	37.0	3.6	0.014
Batch	(B)	1	37.0	4.7	0.037
V x B		4	37.0	2.5	0.057

Figure 4. Fresh weight (log10) of *Pieris brassicae* caterpillars at 3 and 7 days post-infestation (dpi) when feeding on leaves and inflorescences of *Brassica rapa* plants exposed to volatiles of four different fungi (*F. oxysporum, R. solani, P. leveillei, U. atrum*). Each box-and-whisker shows the distribution of the dataset into quartiles: the minimum, first quartile, median, third quartile, and maximum. Dots show the distribution of each measurement (individual larvae). Main effects of the volatile exposure, time point (dpi), batch, and their interactions were tested using a mixed model, with plant replicate as a random factor. Uppercase letters indicate overall effects of volatile exposure using post-hoc tests ($P < 0.05$). "n" indicates the number of caterpillars recollected. Each plant was infested with 10 *P. brassicae* neonates, and each volatile exposure was replicated 9-10 times.

However, the insect fresh weight and the number of individuals recollected were overall positively correlated with root dry weight of *D. radicum*–infested plants (Table 2a; Pearson correlation tests; P = 0.023; P = 0.031, respectively).

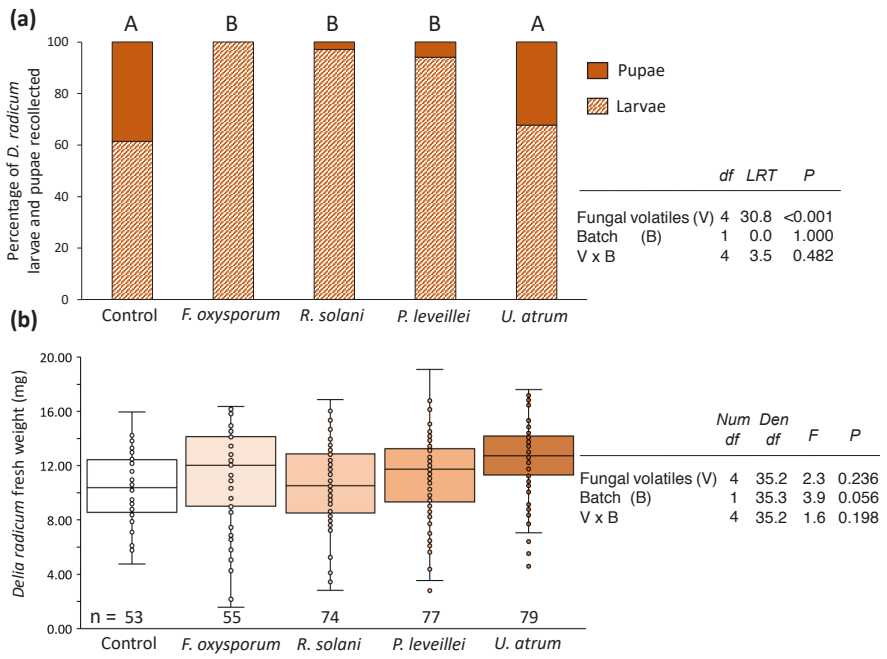


Figure 5. (a) Percentage of larvae and pupae of *Delia radicum* collected from roots of *Brassica rapa* exposed to volatiles of four different fungi (*F. oxysporum, R. solani, P. leveillei, U. atrum*) and **(b)** fresh weight of the individuals. "n" indicates the total number of individuals (pupae and larvae) recollected. Each box-and-whisker shows the distribution of the dataset into quartiles: the minimum, first quartile, median, third quartile, and maximum. Dots show the distribution of individual *D. radicum*. For the fraction of larvae and pupae, main effects of the volatile exposure, batch, and their interaction were tested using a generalised linear model with a beta-binomial distribution, and for the insect fresh weight we used a mixed model, with plant replicate as a random factor. Uppercase letters indicate pairwise differences in percentages of larvae and pupae between fungal volatile exposures using likelihood ratio post-hoc tests (LRT). Each plant was infested with 10 *D. radicum* neonates, and each volatile exposure was replicated 7-10 times.

Effects of fungal volatile exposure on plant bolting upon herbivory

Fungal volatiles affected bolting time of *B. rapa* plants (Fig. 6; Cox; P = 0.002). Overall, plants exposed to *P. leveillei* and *R. solani* volatiles bolted faster than control plants (Fig. 6; LRT post-hocs; $P_{P.leveillei}$ = 0.012; $P_{R.solani}$ = 0.018) and plants exposed to *U. atrum* volatiles (Fig. 6; LRT post-hocs; $P_{P.leveillei}$ = 0.005; $P_{R.solani}$ = 0.007) or *F. oxysporum* volatiles (Fig. 6; LRT post-hocs; $P_{P.leveillei}$ = 0.002; $P_{R.solani}$ = 0.004). The effects of fungal volatiles on bolting time of *B. rapa* plants was not influenced by herbivore infestation (Fig. 6; Cox; P = 0.177). In addition, fungal volatiles affected the total number of buds and flowers of *B. rapa* plants (Fig. 7; GLM;

P < 0.001) but not the number of buds and flowers per unit of plant dry weight (Fig. S1; LM; P = 0.491). On average, plants whose roots were exposed to *P. leveillei* and *R. solani* volatiles had more buds and flowers than control plants (Fig. 7; LRT post-hocs; $P_{P.leveillei}$ = 0.008; $P_{R.solani}$ = 0.022) and plants exposed to *F. oxysporum* or *U. atrum* volatiles (Fig. 7; LRT post-hocs; P < 0.050). Effects of the fungal volatiles were influenced by the experimental batch (Fig. 7; GLM; P = 0.005).

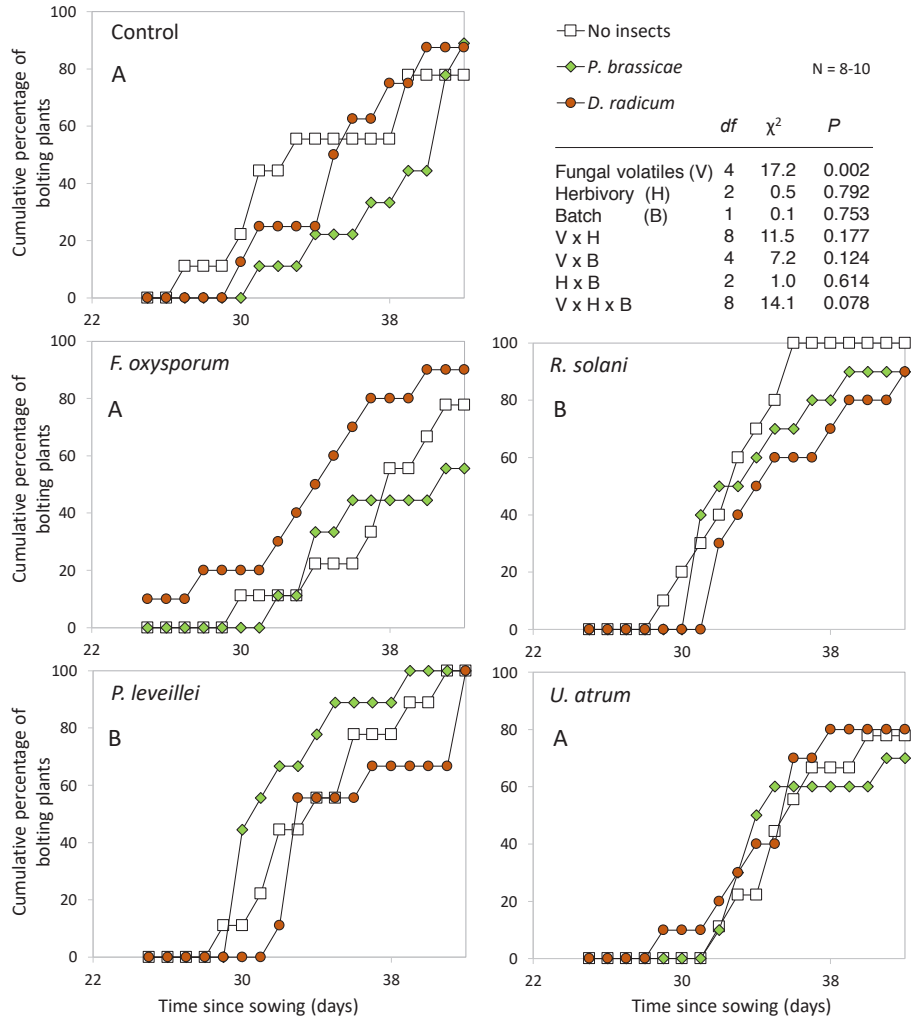
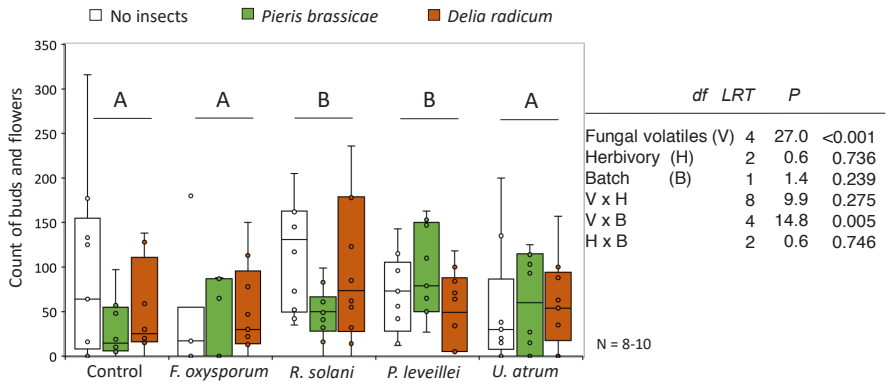


Figure 6. Cumulative percentage of bolting of *Brassica rapa* plants upon different herbivore infestations: no insects, *Pieris brassicae* and *Delia radicum* and when exposed to volatiles emitted by different fungi. Main effects of the volatile exposure, herbivory and batch, and their interactions were tested using a Cox proportional hazard model. Uppercase letters in the panels indicate pairwise differences between the fungal volatile exposures using likelihood ratio tests. "N" indicates the number of plant replicates.



	df	LRT	P
Fungal volatiles (V)	4	27.0	<0.001
Herbivory (H)	2	0.6	0.736
Batch (B)	1	1.4	0.239
V x H	8	9.9	0.275
V x B	4	14.8	0.005
H x B	2	0.6	0.746

Figure 7. Total count of buds and flowers of six-week-old *Brassica rapa* plants when exposed to volatiles of four different fungi (*F. oxysporum, R. solani, P. leveillei, U. atrum*) and upon different herbivore infestations. Each box-and-whisker shows the distribution of the dataset into quartiles: the minimum, first quartile, median, third quartile, and maximum. Dots show the distribution of each plant. Main effects of the volatile exposure, herbivory, batch, and their interactions were tested using a generalised linear model with a negative binomial distribution. Uppercase letters indicate pairwise differences between the fungal volatile exposures using likelihood ratio tests. "N" indicates the number of plant replicates.

Discussion

We found that volatiles emitted by four soil-borne fungi differentially affected *B. rapa* resistance and tolerance to herbivory by *P. brassicae* caterpillars and *D. radicum* larvae. Effects on *P. brassicae* performance varied between the different fungi and between the batches, whereas the effects on *D. radicum* performance was predominantly negative, indicating an increased plant resistance. Despite an overall reduction of root weight upon attack by the root herbivore *D. radicum, B. rapa* plants tolerated the damages caused by the herbivore and compensated for the loss of root tissues, albeit that other (undamaged) plant tissues were smaller in some volatile-exposed plants. In contrast, attack by *P. brassicae* caterpillars led to an overall reduction of aboveground tissues and compensation varied between the tissues and between the fungal volatile exposures. Root exposure to *R. solani* or *P. leveillei* volatiles accelerated plant phenology, which resulted in more buds and flowers overall. Altogether, our data show that fungal volatiles can influence plant mixed-defence strategy, balancing plant resistance and tolerance to aboveground and belowground herbivory. Yet, these effects are variable and differ between fungal species. Potentially, exposure of *B. rapa* roots to some fungal volatiles may ultimately enhance plant fitness.

Fungal volatiles influenced plant growth and tolerance upon herbivory

Exposure of *B. rapa* roots to fungal volatiles affected growth of uninfested plants, making plants either smaller or larger, and also impacted plant (re)growth upon herbivory, thus influencing plant tolerance. Although *D. radicum* larvae feed solely on roots, especially

primary roots (van Dam and Raaijmakers, 2006), we did not observe an effect on root weight compared to uninfested plants, suggesting that plants remained tolerant and compensated for the loss of root tissues (Mesmin *et al.*, 2019), regardless of the fungal volatile exposure. Interestingly, upon certain fungal volatile exposure, this compensatory growth occurred at the cost of undamaged aboveground tissues. These findings suggest that compensatory plant growth to root herbivory may result from a reallocation of resources from aboveground tissues to roots (Núñez-Farfán *et al.*, 2007; van Dam, 2009), and that plant exposure to fungal volatiles can specifically influence this reallocation, sometimes at the cost of undamaged tissues, thus negatively affecting plant tolerance to root herbivory.

In contrast, herbivory by *P. brassicae* caterpillars resulted in a reduction of *B. rapa* plant weight compared to uninfested plants. Nonetheless, these tissue losses differed between leaves and inflorescences and between fungal volatile exposures. These differences suggest that root exposure to fungal volatiles may have differentially influenced compensatory plant growth upon herbivory by *P. brassicae* caterpillars, for instance by investing resources to growth of either inflorescences or leaves. Microbial volatiles were indeed previously shown to induce starch and sugar accumulation in leaves (Ezquer *et al.*, 2010; Li *et al.*, 2011; Sánchez-López *et al.*, 2016). A thorough analysis of resource partitioning and allocation to storage and defence will provide a better understanding of plant tolerance to herbivory following plant exposure to fungal volatiles. In this study, we did not monitor the position and feeding of the caterpillars over time, but it is also plausible that, beside plant tolerance, fungal volatile exposure may have also influenced the tissues on which *P. brassicae* caterpillars fed, which would result from resistance mechanisms (Lucas-Barbosa *et al.* 2017). A detailed analysis of the feeding damage on the different tissues may shed light on this hypothesis.

Effects of fungal volatiles on plant resistance differ between insect herbivores

Most fungal volatile exposures, apart from exposure to *U. atrum* volatiles, resulted in a reduced *D. radicum* development rate, indicating increased direct plant resistance (antibiosis). Increased developmental time of the insect herbivore can be detrimental to the host plant due to prolonged food intake by the root herbivore, but could also be beneficial due to longer exposure time to natural enemies (Benrey and Denno, 1997). In this study, fresh weight of *D. radicum* did not differ between volatile-exposed plants nor the amount of roots consumed by *D. radium*. Therefore, slower development of the insect would be advantageous for the host plant in natural environments. Slower development of the insect may result from changes in plant chemistry and root architecture which can lead to chemically or structurally more resistant roots upon fungal volatile exposure. To our knowledge, this study is the first to address root herbivory upon root exposure to microbial volatiles. Differential increase of indole glucosinolates in the main roots can slow down larval development (van Dam and Raaijmakers, 2006). Additionally, root exposure to fungal volatiles may alter architecture of primary and lateral roots (Ditengou *et al.*, 2015; Garnica-Vergara *et al.*, 2015; Casarrubia *et al.*, 2016), which, in turn, negatively impacted insect

performance (Felkl *et al.*, 2005; Werner *et al.*, 2016). It would be additionally interesting to explore whether fungal volatiles affect behaviour and preference (antixenosis) of root herbivores (Werner *et al.*, 2016) for a comprehensive overview of plant resistance.

Aboveground, performance of the caterpillars was also affected by root exposure to fungal volatiles. *Pieris brassicae* caterpillars feeding on plants whose roots were exposed to *R. solani* volatiles were larger than those feeding on control plants and fungal volatile-exposed plants, indicating higher plant susceptibility. This finding corroborates with previous studies (Cordovez *et al.*, 2017; Moisan *et al.*, 2019; Chapter 2 of this thesis). As the average caterpillar fresh weight did not correlate with the difference in shoot weight of infested plants and uninfested plants, we conclude that caterpillar performance was not correlated with the amount of total aboveground tissues consumed. Instead, the increase of caterpillar fresh weight may be explained by nutritive and defensive traits of the specific plant tissue(s) upon which caterpillars were feeding (Smallegange *et al.*, 2007; Wetzel *et al.*, 2016; Lucas-Barbosa *et al.* 2017). Plant exposure to fungal volatiles may have altered metabolite levels in some plant tissues, making the tissues repellent/attractive or unpalatable to the insect herbivores, thus positively or negatively impacting insect performance (Schoonhoven *et al.*, 2005; Smallegange *et al.*, 2007). For instance, *Arabidopsis thaliana* seedlings exposed to bacterial VOCs had an accumulation of glucosinolates in leaves, which diminished the performance of the caterpillars *Spodoptera exigua* (Aziz *et al.*, 2016). We speculate that *P. brassicae* larvae feeding on *R. solani*-exposed plants fed on tissues of higher nutritional values or of lower concentration in defensive compounds. Yet, the effects of fungal volatiles on *P. brassicae* performance were also influenced by the experimental batches, which suggests that the effects may be variable. A thorough analysis of plant primary and secondary metabolites (including volatiles) in the different tissues and daily monitoring of the insect feeding sites will further improve the understanding of the modulation of plant resistance to herbivory by fungal volatiles.

Acceleration of bolting time by fungal volatiles suggests enhancement of plant fitness

Effects of fungal volatiles on plant phenology were fungus-species specific. Plant exposure to volatiles emitted by *R. solani* or *P. leveillei* accelerated overall plant bolting and enhanced production of buds and flowers. As discussed above, an acceleration of bolting could be disadvantageous for the plant as *P. brassicae* caterpillars prefer to feed on inflorescences, which enhances their performance (Smallegange *et al.*, 2007), but it can also result from an escape strategy to reproduce faster (Lucas-Barbosa *et al.*, 2013). Acceleration of flowering seems a common plant phenomenon in response to volatiles emitted by fungi of different lifestyles (Sánchez-López *et al.*, 2016; Cordovez *et al.*, 2017; Moisan *et al.*, 2019; Chapter 2 of this thesis). Furthermore, our data show that despite lower resistance (*e.g.* higher consumption of reproductive tissues by caterpillars) and lower tolerance (*e.g.* resource reallocation from inflorescences to roots upon herbivory by *D. radicum* larvae), *B. rapa* plants still benefit from the exposure to *R. solani* volatiles and display overall more buds and flowers than control plants. In contrast, plant exposure to *P. leveillei* volatiles did not affect growth

of uninfested *B. rapa* plants. Instead, we observed positive effects of the volatile exposure on plant resistance and tolerance compared with control plants, which spared plant reproductive tissues. By producing more buds and flowers and quicker, plants increase their chance of reproductive success. Yet, it remains to be tested whether pollinator attraction and seed set are ultimately influenced by root exposure to fungal volatiles. To conclude, fungal volatiles can affect plant growth and defence to herbivores specifically, and this balance can potentially enhance plant fitness.

Conclusions

Our results show that fungal volatiles can influence plant mixed-defence strategies, balancing plant resistance and tolerance to above- and belowground herbivory in a fungus-specific manner. Yet, it remains to be investigated how these results obtained in controlled conditions with single fungal isolates can be extrapolated to natural ecosystems where plant roots are exposed to volatiles emitted simultaneously or in sequence by diverse fungal and other microbial communities. In such future studies, one should also address how these responses affect subsequent plant interactions with mutualists, for instance pollinators that are essential for reproduction of obligate outcrossing plant species such as *B. rapa*.

Acknowledgements

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Chapter 3

Supplemental Materials

Table S1. Statistical output of the differences of least square mean estimates and their standard errors generated for exposure to volatiles from each fungus for the different plant tissue dry weights (a) between *B. rapa* infested with *P. brassicae* and uninfested *B. rapa* plants, and (b) between *B. rapa* infested with *D. radicum* and uninfested *B. rapa* plants. For each plant tissue and volatile exposure, the effect of herbivory was tested by comparing the above described differences to zero, using the *t* distribution (*P** < 0.05). Names of the fungi were abbreviated as follow: "Fo = *Fusairum oxysporum*", "Pl = *Phoma leveillei*", "Rs = *Rhizoctonia solani*", "Ua = *Ulocladium atrum*"

(a)

Contrast						Estimate	SE	*t*	*P*
Volatiles	**Herbivory**	**Tissue**	**Volatiles**	**Herbivory**	**Tissue**				
Control	*P. brassicae*	Flower	Control	No insect	Flower	-0.6144	0.293	-2.1	0.039
Control	*P. brassicae*	Leaf	Control	No insect	Leaf	-0.2796	0.451	-0.62	0.537
Control	*P. brassicae*	Root	Control	No insect	Root	0.054	0.115	0.47	0.639
Fo	*P. brassicae*	Flower	Fo	No insect	Flower	-0.0782	0.293	-0.27	0.790
Fo	*P. brassicae*	Leaf	Fo	No insect	Leaf	-0.817	0.451	-1.81	0.073
Fo	*P. brassicae*	Root	Fo	No insect	Root	-0.0452	0.115	-0.39	0.695
Pl	*P. brassicae*	Flower	Pl	No insect	Flower	0.0595	0.293	0.2	0.840
Pl	*P. brassicae*	Leaf	Pl	No insect	Leaf	-0.6607	0.451	-1.46	0.146
Pl	*P. brassicae*	Root	Pl	No insect	Root	-0.12	0.115	-1.04	0.299
Rs	*P. brassicae*	Flower	Rs	No insect	Flower	-0.7931	0.276	-2.87	0.005
Rs	*P. brassicae*	Leaf	Rs	No insect	Leaf	-0.1904	0.425	-0.45	0.655
Rs	*P. brassicae*	Root	Rs	No insect	Root	-0.1899	0.108	-1.75	0.082
Ua	*P. brassicae*	Flower	Ua	No insect	Flower	0.0137	0.285	0.05	0.962
Ua	*P. brassicae*	Leaf	Ua	No insect	Leaf	-10.301	0.438	-2.35	0.020
Ua	*P. brassicae*	Root	Ua	No insect	Root	-0.0967	0.112	-0.87	0.388

(b)

Contrast						Estimate	SE	t	*P*
Volatiles	**Herbivory**	**Tissue**	**Volatiles**	**Herbivory**	**Tissue**				
Control	*D. radicum*	Flower	Control	No insect	Flower	-0.448	0.3063	-1.46	0.1464
Control	*D. radicum*	Leaf	Control	No insect	Leaf	0.1881	0.4714	0.4	0.6907
Control	*D. radicum*	Root	Control	No insect	Root	-0.0318	0.1201	-0.26	0.7919
Fo	*D. radicum*	Flower	Fo	No insect	Flower	0.0916	0.2848	0.32	0.7485
Fo	*D. radicum*	Leaf	Fo	No insect	Leaf	-0.6696	0.4383	-1.53	0.1295
Fo	*D. radicum*	Root	Fo	No insect	Root	-0.0868	0.1116	-0.78	0.4384
Pl	*D. radicum*	Flower	Pl	No insect	Flower	-0.0492	0.2931	-0.17	0.8671
Pl	*D. radicum*	Leaf	Pl	No insect	Leaf	0.2121	0.451	0.47	0.6392
Pl	*D. radicum*	Root	Pl	No insect	Root	-0.0674	0.1173	-0.57	0.5666
Rs	*D. radicum*	Flower	Rs	No insect	Flower	-0.6345	0.2763	-2.3	0.0235
Rs	*D. radicum*	Leaf	Rs	No insect	Leaf	0.4082	0.4252	0.96	0.3392
Rs	*D. radicum*	Root	Rs	No insect	Root	-0.0598	0.1083	-0.55	0.5819
Ua	*D. radicum*	Flower	Ua	No insect	Flower	0.2087	0.2848	0.73	0.4654
Ua	*D. radicum*	Leaf	Ua	No insect	Leaf	-0.9152	0.4383	-2.09	0.0391
Ua	*D. radicum*	Root	Ua	No insect	Root	-0.1079	0.1116	-0.97	0.3358

Table S2. Correlations between **(a)** *Delia radicum* fresh weight, number of *D. radicum* recollected and root dry weight of infested plants upon different fungal volatile exposures, between **(b)** the average *Pieris brassicae* fresh weight per pot at 7 days post infestation (dpi) and the leaf and inflorescence dry weight of infested plants upon different fungal volatile exposures, and between **(c)** average of *P. brassicae* or *D. radicum* fresh weight per fungal volatile exposure and the mean of plant dry weight differences of uninfested and infested plants. "*r*" refers to Pearson coefficients and "*P*" to the *P*-values of the Pearson correlation tests and "N" to the number of plant replicates.

	Control	***F. oxysporum***	***R. solani***	***P. leveillei***	***U. atrum***	**All**
D. radicum fresh weight *vs*	*r* = 0.451	*r* = 0.627	*r* = -0.130	*r* = 0.721	*r* = 0.257	*r* = 0.339
root dry weight	*P* = 0.262	*P* = 0.071	*P* = 0.721	*P* = 0.044	*P* = 0.473	*P* = 0.023
D. radicum fresh weight *vs*	*r* = -0.705	*r* = 0.704	*r* = 0.186	*r* = 0.385	*r* = 0.038	*r* = 0.185
number *D. radicum* recollected	*P* = 0.051	*P* = 0.034	*P* = 0.608	*P* = 0.306	*P* = 0.918	*P* = 0.219
Number *D. radicum* recollected *vs*	*r* = 0.460	*r* = 0.605	*r* = -0.236	*r* = 0.024	*r* = -0.023	*r* = 0.321
root dry weight	*P* = 0.252	*P* = 0.084	*P* = 0.511	*P* = 0.948	*P* = 0.950	*P* = 0.031
N=	8	9	10	8	10	45

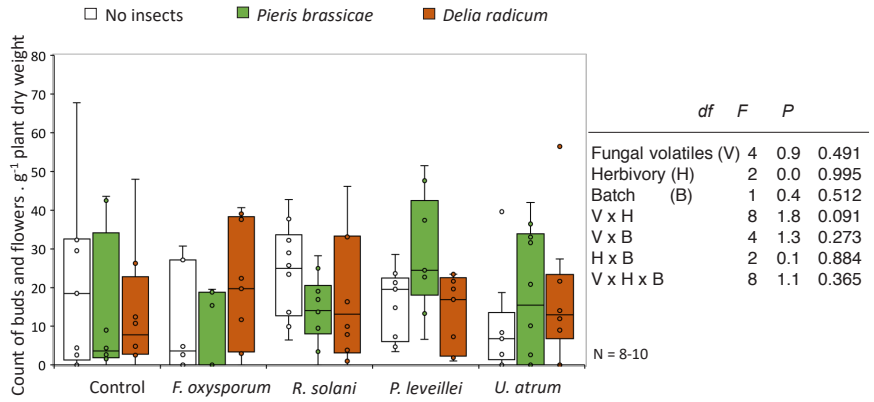
	Control	***F. oxysporum***	***R. solani***	***P. leveillei***	***U. atrum***	**All**
Fresh weight *P. brassicae vs*	*r* = 0.570	*r* = 0.064	*r* = 0.258	*r* = -0.083	*r* = 0.431	*r* = 0.251
flower dry weight	*P* = 0.109	*P* = 0.870	*P* = 0.473	*P* = 0.831	*P* = 0.213	*P* = 0.088
Fresh weight *P. brassicae vs* leaf	*r* = 0.102	*r* = 0.519	*r* = -0.642	*r* = -0.507	*r* = -0.594	*r* = -0.134
dry weight	*P* = 0.795	*P* = 0.152	*P* = 0.045	*P* = 0.163	*P* = 0.070	*P* = 0.369
Fresh weight *P. brassicae vs*	*r* = 0.302	*r* = 0.428	*r* = -0.535	*r* = -0.561	*r* = -0.583	*r* = -0.035
flower+leaf dry weight	*P* = 0.429	*P* = 0.251	*P* = 0.111	*P* = 0.116	*P* = 0.077	*P* = 0.815
N=	9	9	10	9	10	47

	Mean/volatile exposure
P. brassicae fresh weight *vs*	*r* = -0.671
flower dry weight difference	*P* = 0.215
P. brassicae fresh weight *vs*	*r* = 0.614
leaf dry weight difference	*P* = 0.271
D. radicum fresh weight *vs*	*r* = -0.610
root *dry* weight difference	*P* = 0.275
N=	5

Table S3. Total count of larvae and pupae of *Delia radicum* collected from roots of *Brassica rapa* plants exposed to volatiles of four different fungi (*Fusarium oxysporum, Rhizocotina solani, Phoma leveillei and Ulocladiu atrum*). Main effects of the volatile exposure, batch, and their interaction were tested using a generalised linear with a beta-binomial distribution. Each plant was infested with 10 *D. radicum* neonates.

	Number of *D. radicum* recollected	**Number of plants**
Control	53	7
F. oxysporum	55	9
R. solani	74	10
P. leveillei	77	9
U. atrum	79	10

	df	*LRT*	*P*
Fungal volatiles (V)	4	9.1	0.058
Batch (B)	1	2.6	0.108
V x B	4	8.0	0.090

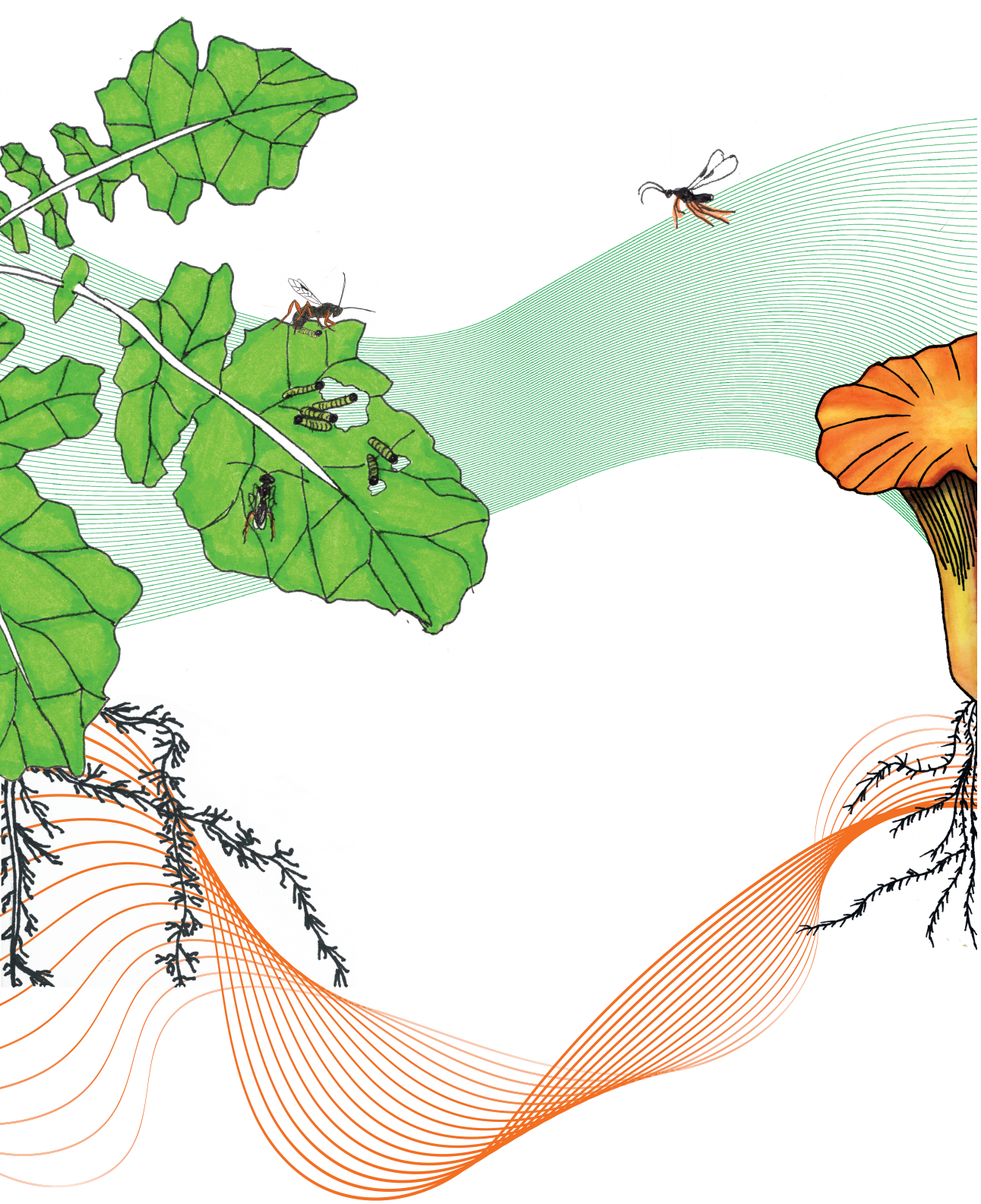


	df	*F*	*P*
Fungal volatiles (V)	4	0.9	0.491
Herbivory (H)	2	0.0	0.995
Batch (B)	1	0.4	0.512
V x H	8	1.8	0.091
V x B	4	1.3	0.273
H x B	2	0.1	0.884
V x H x B	8	1.1	0.365

Figure S1. Number of buds and flowers per g of dry weight of two-week-old *Brassica rapa* plants when exposed to volatiles of different fungal species and upon different herbivory infestations. Each box-and-whisker shows the distribution of the dataset into quartiles: the minimum, first quartile, median, third quartile, and maximum. Dots show the distribution of each plant. Main effects of the volatile exposure, herbivory and batch, as well as, their interactions were tested using a linear model. "N" indicates the number of plant replicates

Online Supplemental Materials

All R and SAS scripts used for the statistical analyses performed in the present study are available online: https://doi.org/10.5281/zenodo.3696621



Chapter 4



No evidence of modulation of indirect plant resistance by volatiles from soil-borne fungi

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Abstract

Upon attack by an herbivore, plants emit specific blends of volatile organic compounds (VOCs) that can attract natural enemies of the herbivore, thus serving as indirect plant resistance. Apart from insect herbivores, plant-colonising microorganisms can also affect plant VOC emission, including these Herbivore-Induced Plant Volatiles (HIPVs), which, in turn, can affect the behaviour of natural enemies of the herbivore. Interestingly, volatiles emitted by microorganisms can likewise modulate plant chemistry prior to colonisation, and enhance direct plant resistance. Yet, it remains elusive whether microbial volatiles influence HIPV emission and indirect plant resistance as well. In this study, we investigated whether volatiles emitted by soil-borne fungi can influence the emission of HIPVs and the recruitment of natural enemies of *Pieris brassicae* larvae attacking *Brassica rapa* plants. We found that exposure of *B. rapa* roots to volatiles from four soil-borne fungi did not affect the number of *P. brassicae* larvae recollected from these plants in a common garden experiment in the field, suggesting a neutral effect of the fungal volatiles on predation. Likewise in a greenhouse, similar numbers of larvae were parasitised by *Cotesia glomerata* wasps on control plants as on fungal volatile-exposed plants. Additionally, chemical analysis of HIPV profiles did not reveal differences between control plants and fungal volatile-exposed plants that were both infested by *Pieris brassicae* larvae. Together, our data indicate that root exposure to fungal volatiles did not affect indirect plant resistance to an insect herbivore. These findings provide new insight into influence of plant defence by fungal volatiles that are discussed in the context of other effects of fungal volatiles on plant performance.

Keywords

Common-garden experiment, fungal VOCs, HIPVs, parasitoids, predators, recruitment

Introduction

Upon attack by an herbivore, plants emit specific volatile organic compounds (VOCs) that can attract natural enemies of the herbivores, hence acting as an indirect plant resistance mechanism (Schoonhoven *et al.*, 2005). Green Leaf Volatiles (GLVs), typically six-carbon compounds, are usually among the first VOCs emitted upon attack. These so-called Herbivore-Induced Plant Volatiles (HIPVs) can be released locally at the site of attack but also systemically, and predators and parasitoids can exploit these HIPVs as cues to locate their prey or hosts on infested plants (Rasmann *et al.*, 2005; Mumm and Dicke, 2010; Hare, 2011). The composition and ratios of VOCs within an HIPV blend can greatly influence the behaviour of parasitoids or predators (Uefune *et al.*, 2013). Indeed, changes, even minor, in the VOC blends can influence the degree of attraction of natural enemies to the infested plant (Poelman *et al.*, 2009; Snoeren *et al.*, 2010; Bukovinszky *et al.*, 2012; Tamiru *et al.*, 2015; Douma *et al.*, 2019). In *Brassica* plants infested with *Pieris brassicae* eggs, recruitment of parasitoids is particularly important upon hatching of the larvae because *Cotesia* wasps parasitise only first and second instar larvae, which can lead to up to 40% of larval mortality (Lucas-Barbosa *et al.*, 2014). Predation of *P. brassicae* eggs or larvae by carnivorous arthropods such as ground beetles, spiders, social wasps or birds also contributes to the reduction of herbivores in the field (Schmaedick and Shelton, 2000). Together, recruitment of parasitoids and predators can lead to nearly 100% of *P. brassicae* mortality (Lucas-Barbosa *et al.*, 2014). Therefore, recruitment of natural enemies through HIPV emission is essential to alleviate the herbivore pressure on plants, and represents an important component of plant resistance.

Interestingly, plant-colonising microorganisms can modulate plant VOC emission (Junker and Tholl, 2013; Farré-Armengol *et al.*, 2016), which in turn affects indirect plant resistance (Pineda *et al.*, 2010). Upon plant colonisation, microorganisms induce systemic resistance and may affect regulation of VOC biosynthetic pathways, thus inhibiting or enhancing the emission of individual VOCs (Pieterse *et al.*, 2000; Pineda *et al.*, 2010; Pangesti *et al.*, 2015b; Sharifi *et al.*, 2018). For instance, *Arabidopsis thaliana* seedlings inoculated with the plant growth-promoting bacterium *Pseudomonas simiae* (formerly *P. fluorescens*) and infested with aphids had a higher VOC emission than non-colonised aphid-infested plants. These changes in HIPV emission rates rendered the plants less attractive to an aphid parasitoid (Pineda *et al.*, 2013). Because each parasitoid and predator species may exploit different plant VOCs for oviposition or foraging (McCormick *et al.*, 2012), effects of microorganism-induced HIPV changes on the behaviour of natural enemies of the herbivore can range from negative to positive (Pineda *et al.*, 2013; Pangesti *et al.*, 2015b; Ponzio *et al.*, 2016). Therefore, plant-colonising microorganisms can modulate plant VOC emission positively or negatively, and consequently affect indirect plant resistance (Dicke, 2016).

Interestingly, prior to plant colonisation, volatiles emitted by microorganisms can already affect plant growth and enhance plant resistance against attackers by altering plant chemistry. Plant responses to volatiles from fungi or bacteria may lead to changes in plant

transcriptome, proteome and metabolome (Kwon *et al.*, 2010; Aziz *et al.*, 2016), promoting growth (Bailly and Weisskopf, 2012; Piechulla *et al.*, 2017; Fincheira and Quiroz, 2018) and increasing plant resistance to leaf pathogens like *Pseudomonas syringae* or *Botrytis cinerea* (Salas Marina et al., 2011; Lee et al., 2012; Kottb et al., 2015; Tahir et al., 2017). Although less extensively studied, microbial volatiles can also affect plant resistance to insect herbivores (Moisan *et al.*, 2019; Chapter 2 of this thesis). For instance, *A. thaliana* seedlings exposed to VOCs from *Bacillus amyloliquefaciens* were more resistant to *Spodoptera exigua* larvae than non-exposed plants (Aziz *et al.*, 2016). These bacterial volatile-exposed plants displayed elevated glucosinolate levels, *i.e.* secondary metabolites present in brassicaceous plants that can be converted into an array of derivatives that are toxic to generalist herbivores. Interestingly, *A. thaliana* plants exposed to VOCs from *Rhizoctonia solani* emitted a different VOC blend and emitted in a lower amount than non-exposed plants (Cordovez *et al.*, 2017). These preliminary findings indicate that microbial VOCs can modulate from a distance plant VOC emission and plant chemical responses to herbivory, thus potentially also HIPVs. Interestingly, recent studies showed that direct application of individual microbial VOCs on plants or in the soil can enhance recruitment of natural enemies to insect-infested plants (Song and Ryu, 2013; D'Alessandro *et al.*, 2014). However, it remains unknown whether this enhanced recruitment of natural enemies is mediated *via* a change in plant VOC emission, and whether these plant responses are similar when exposed to microbial volatiles from a distance. In this chapter, we explored whether the emission of HIPVs by *P. brassicae*-infested *Brassica rapa* plants is influenced by the exposure of roots to volatiles emitted by four soil-borne fungi, and whether these changes affect the recruitment of parasitoids and predators of the herbivores.

Materials and Methods

Study system

The wild turnip, *B. rapa* L. (Brassicaceae), accession used in this study originated from a wild population in Maarssen (The Netherlands). All *B. rapa* seeds were surface-sterilised before sowing by exposure to chlorine gas for 4 h in a desiccator and stratified at 4 °C in the dark for 3 to 4 days (Cordovez *et al.*, 2017).

We selected four soil-borne fungi that co-occur and interact with brassicaceous plants. Volatiles emitted by these fungi differentially affect *Arabidopsis thaliana* growth and resistance to a leaf insect herbivore (Moisan *et al.*, 2019; Chapter 2 of this thesis). *Fusarium oxysporum f. sp. raphani* and *Rhizoctonia solani* AG2-2 IIIb are two saprophytic fungi, occurring worldwide, that can turn into necrotrophic pathogens of brassicaceous species (Leeman *et al.*, 1995; Pannecoucque and Höfte, 2009). *Chaetomium indicum* and *Trichoderma viride* are also commonly occurring saprophytic fungi that can colonise brassicaceous plants as non-pathogenic endophytes (Junker *et al.*, 2012). For the present

study, all fungi were inoculated in ø 9 cm plastic Petri dishes containing 1/5th strength Potato Dextrose Agar (1/5th PDA). This medium was prepared with 7.8 g of PDA (Oxoid) and 14 g of Bacto™Agar (Becton Dickinson). The pH was set at 7. Fungi were incubated at 25 °C in the dark for 7 days before the start of the experiment.

We selected the specialist insect herbivore *P. brassicae* L. (Lepidoptera: Pieridae), also known as the Large Cabbage White butterfly, since larvae commonly feed on *Brassica* species. Butterflies lay eggs on the leaves, and upon hatching, larvae feed on leaves and move later to the flowers. Larvae were reared on Brussels sprouts plants (*Brassica oleracea* L. var. *gemmifera* cv Cyrus) in a climate room (22 ± 2 °C; 16:8 h, L:D; 60 ± 10% RH). *Cotesia glomerata* (Hymenoptera: Braconidae) is an endoparasitoid that can parasitise several species within the Pieridae, although *P. brassicae* is its main host (Geervliet and Brodeur, 1992). Wasps exploit plant volatiles of infested plants to locate their hosts, and can complete their development when ovipositing in first or second instars larvae (Brodeur *et al.*, 1996). In a greenhouse compartment (25 ± 2 °C; 16:8 h, L:D; 60 ± 10% RH), wasps were reared in *P. brassicae* larvae feeding on *B. oleracea*, and were fed with droplets of honey.

Plant exposure to fungal volatiles

Brassica rapa roots were exposed to fungal volatiles using a two-compartment pot system (Chapter 3 of this thesis). In short, one sterile *B. rapa* seed was sown in the top compartment filled with a sterile soil mixture (1:1 v/v, potting soil:sand). A Petri dish containing the test fungus (*F. oxysporum, R. solani, C. indicum* or *T. viride*) growing on 1/5th PDA medium or a Petri dish with 1/5th PDA medium only (*i.e.* control) was enclosed in the bottom compartment. Both compartments were connected to each other by a cylinder, and separated by a nylon membrane of 1 µm mesh width (Plastok associates Ltd., Birkenhead Wirral, UK) that allowed air and volatile exchange between the two compartments. Exposure to fungal volatiles was initiated with seven-day-old fungi as soon as *B. rapa* seeds were sown, and was maintained for four weeks, after which *B. rapa* plants had 6-8 fully developed leaves. Plants were grown in a greenhouse compartment (21 ± 2 °C; 16:8 h, L:D; 70 ± 10% RH). Petri dishes containing the fungi and control were replaced weekly with Petri dishes containing fresh seven-day-old fungi or fresh 1/5th PDA medium. After four weeks of exposure to fungal volatiles, the bottom compartments with the Petri dishes were removed permanently, thus terminating plant exposure to fungal volatiles.

Recruitment of natural enemies by larvae-infested plants in the open field

Following four weeks of growth in the greenhouse while being exposed to fungal volatiles (see section 2.2), uninfested plants were transplanted to a common garden to test the effects of prior plant exposure to fungal volatiles (*F. oxysporum, R. solani* and *C. indicum*) on plant recruitment of natural enemies upon attack by an insect herbivore. Plants were planted in a randomised complete block design with a distance of 0.8 m between plants. Plants were initially covered with a mesh tent (90 x 30 x 30 cm, supported by wooden sticks) to prevent

plant infestation with herbivores naturally present in the environment while enabling plant acclimation to field conditions. After 5 days, the mesh tents were removed, and plants were infested with twenty *P. brassicae* neonates. One additional set of control plants (*i.e.* exposed to 1/5th PDA medium only) remained covered with the mesh tents (thereafter named 'protected controls') throughout the experiment to assess larval recollection rate upon exclusion of aboveground predation and parasitisation. Therefore, we assumed that larval recollection rate on these protected controls would reflect larval survival when taking into account natural mortality and soil predation, while on uncovered plants, larval recollection would reflect larval survival upon aboveground predation in addition. Each treatment (*i.e.* plants exposed to volatiles emitted by *F. oxysporum, R. solani, C. indicum,* or control and protected control plants) was replicated 8 times. Experiments were carried out at the experimental farm of Wageningen University (51°59'20.23"N, 5°39'56.34"E) in July 2018. Plants were harvested 48 h after the infestation, and *P. brassicae* larvae were recollected and dissected under a stereo microscope to assess parasitisation. Identification of the parasitic wasp was based on visual evaluation of the number of eggs found (*e.g.* solitary *versus* gregarious species). The percentage of recollected larvae was calculated and compared between fungal volatile-exposed plants using a generalised linear mixed model that follows a beta-binomial distribution (to correct for overdispersion). Pairwise comparisons between treatments were performed using Tukey post-hoc tests ($\alpha = 0.05$).

Recruitment of parasitic wasps by larvae-infested plants in greenhouse conditions

To investigate if prior plant exposure to fungal volatiles affects parasitisation of *P. brassicae* larvae, *C. glomerata* wasps were offered 24 h to parasitise larvae in a five-choice set-up. After four weeks of exposure to fungal volatiles (see section 2.2), *B. rapa* plants (including non-exposed control plants) were infested with 20 *P. brassicae* neonates, and five plants, one of each treatment, were placed together in a circle inside a mesh tent (70 x 73 x 105 cm). Each plant was placed at equidistance from the release point of the wasp, and positioning of the plants inside the tent was randomised in every tent. One naïve female wasp (4-6 day-old) was released per tent and allowed to parasitise the host larvae feeding on the five plants during 24 h. The wasp was released from the top of a wooden pedestal (h = 38 cm) placed in the centre of the circle, where two Petri dishes with water and honey were also placed. A total of 13 tents (*i.e.* replicates) was prepared and each female wasp was only tested once. If the wasp died or escaped before the end of the 24 h-period, the replicate was removed from the analysis. Tents were placed in a separate greenhouse compartment (23 ± 2 °C; 16:8 h, L:D; 60 ± 10% RH).

To record larval parasitisation, *P. brassicae* larvae were recollected from the plants and dissected under a stereo microscope to check for the presence of *C. glomerata* eggs. Additionally, we estimated the amount of leaf damage per plant by analysing photographs of the infested leaves with ImageJ software, and we measured fresh weight of plant aboveground tissues. Differences in the leaf fresh weight and the amount of leaf damage between the treatments were tested with one-way ANOVA ($\alpha = 0.05$). Additionally,

correlation between leaf damage and leaf fresh weight was analysed with Pearson correlation test ($\alpha = 0.05$). The percentage of parasitised larvae out of the recollected larvae per plant was calculated and compared between the different treatments using a generalised linear mixed model that follows the beta-binomial distribution (to correct for overdispersion). Fungal volatile exposure was included as a fixed factor and leaf damage as covariate, whereas tent number and position of the plants in the tent were included as random factors.

Collection and analysis of headspace VOCs from infested plants

After four weeks of exposure to fungal volatiles, plants were infested with twenty *P. brassicae* neonates on the third fully expanded leaf. Twenty-four hours after plant infestation, VOCs emitted by infested plants were collected *via* dynamic headspace collection. Prior to the collection, all plants were watered with 50 mL of tap water. Soil and pots were then covered with aluminium foil, and aboveground plant parts were enclosed in a customised oven bag (Toppits® Bratschlauch, Minden, Germany, polyester, 2 bags of 30 × 30 × 50 cm tapped together with PTFE strips). *Pieris brassicae* larvae were kept on the plants during the VOC collection. Charcoal-filtered synthetic air (nitrogen 80%, oxygen 20%; Linde) was flushed into the bag at a flow rate of 300 mL min^{-1} *via* a PTFE tube, and air was sucked out at a flow rate of 250 mL min^{-1} (air-sampling pump Deluxe, Dorset, UK; equipped with an inlet protection filter). VOCs were collected for 90 min in stainless steel Thermodesorption (TD) tubes filled with 200 mg Tenax TA (20/35 mesh; CAMSCO). Each treatment, *i.e.* previous plant root exposure to volatiles of one fungus (*F. oxysporum, R. solani, C. indicum* or *T. viride*) or to the control (*i.e.* medium only), was replicated 8-11 times. As background, we collected VOCs from a pot filled with the soil mixture and covered with aluminium foil in the absence of plants. VOC collection was carried out in a greenhouse compartment (25 ± 2 °C; 16:8 h, L:D; 60 ± 10% RH).

After VOC collection, TD tubes were stored at room temperature and were subjected to a dry-purge (~25 psi of helium, for 10 min at room temperature) until GC–MS analysis. Headspace samples were analysed using a gas chromatograph (hereafter GC; Trace GC Ultra; Thermo Electron) with a thermodesorption unit (hereafter TD; Ultra TD and Unity modules; Markes International) and coupled to a mass spectrometer (hereafter MS; EI–single quadrupole Trace DSQ; Thermo Electron). The (TD)–GC–MS was controlled *via* Thermal Desorption System Control Program (Markes International) and Thermo Xcalibur (Thermo Fisher Scientific) software. During the primary desorption, VOCs were desorbed from the tubes for 10 min at 250 °C, and re-adsorbed on an electrically cooled sorbent trap at 0 °C. Compounds were desorbed from this trap during the secondary desorption at a heating rate of 40 °C s^{-1} and kept at 280 °C for 10 min, and were transferred to the GC column in splitless mode. The temperature of the sample flow path was set to 185 °C. The chromatographic process was carried out at a flow rate of the carrier gas of 1 mL min^{-1}. The oven temperature was programmed from 40 °C (2 min hold time) to 280 °C (4 min hold time) at 6 °C min^{-1}. This resulted in a 46-min temperature programme. The MS transfer line

was set to 275 °C. The energy of the electron beam was set to 70 eV, and the temperature of the ion source was set to 250 °C. The mass spectrometer scanned m/z 35–400 at a rate of 4.7 scans s^{-1}. The GC column was a ZB–5MS Zebron (Phenomenex); 30 m × 0.25 mm × 1.00 µm, with 10 m Guardian End. The stationary phase of this column consists of 5% polysilarylene – 95% dimethylpolysiloxane. Helium gas was used for desorption and chromatographic processes.

A standard mixture of linear alkanes was also analysed by GC-MS for the determination of the arithmetic retention index (AI) values of the VOCs. For this, a working solution of linear alkanes was prepared by diluting a commercial mixture of the compounds (C7–C30; Supelco) with petroleum ether 40–60 (puriss. p.a.; Sigma–Aldrich). The concentration of each alkane in the working solution was approximately 10 µg mL^{-1}. An aliquot of 0.5 µL of this working solution was added to a TD tube using a 10 µL-glass syringe. Analysis of the alkane mixture was performed in similar conditions than those described above for the samples. AI values above 800 were determined for all VOCs of the samples based on their retention times and those of the linear alkanes, through the following formula from Adams (2001):

$$\mathrm{AI} = (100 \times \mathrm{C}z) + \frac{100 \times (\mathrm{RTy} - \mathrm{RTz})}{\mathrm{RT}z+1 - \mathrm{RTz}}$$

Cz is the number of C-atoms of the alkane (z) eluting just before the VOC of interest (y), RTy is the retention time of y, RTz is the retention time of z, and RTz+1 is the retention time of the alkane (z+1) eluting just after y, thus RTz < RTy < RTz+1.

GC–MS data were processed using the MetAlign–MSClust software pipeline. MetAlign was developed by Wageningen Food Safety Research (Wageningen University and Research, Wageningen, The Netherlands) and MSClust was developed by Tikunov *et al.* (2012). MetAlign and MSClust are freeware available online (https://www.wur.nl/nl/Onderzoek-Resultaten/Onderzoeksinstituten/food-safety-research/show-rikilt/MetAlign.htm). In brief, MetAlign corrects the baseline and eliminates the noise of each GC–MS output file. Subsequently, it aligns the individual mass peaks in all files. MSClust then clusters the aligned mass peaks so that mass spectra of putative compounds are constructed (Lommen, 2009). Only mass peaks with a retention time within 5–38 min and in the 55–400 m/z-range were further processed. VOCs were tentatively annotated by comparing their mass spectra with those of the reference databases NIST (National Institute of Standards and Technology, USA), Wiley libraries and the Wageningen Mass Spectral Database of Natural Products, by comparing the list of VOCs from *P. brassicae*-infested *B. rapa* reported by (Danner *et al.*, 2018), and by comparing the calculated AI with those in the literature. Relative peak intensity was determined for each VOC in all samples by selecting an individual mass peak with $<9.95 \times 10^7$ counts to prevent saturation of the sensor. Only VOCs from the samples whose peak intensity statistically differed from the background (Student *t*-test, $\alpha = 0.05$) were accounted as plant headspace VOCs and further processed. Selected VOCs were additionally normalised with the shoot fresh weight. Per treatment, average and standard error of peak intensity values were calculated. Differences of peak intensity between the different fungal volatile-exposed plants were statistically tested for each VOC using the non-parametric Kruskal-Wallis test ($\alpha = 0.05$). Additionally, a heatmap indicating the relative peak intensity of each plant VOC per plant treatment was plotted (MetaboAnalyst 4.0; https://www.metaboanalyst.ca; Chong *et al.*, 2019). Separation of the VOC blends emitted by the different fungal volatile-exposed plants was analysed using a Principal Component Analysis (SIMCA 15 software, Umetrics AB, Umeå, Sweden).

Results

Recruitment of natural enemies by larvae-infested plants in the open field

Recollection rate of *P. brassicae* larvae differed between the protected controls and the uncovered plants (Fig. 1; GLMM; $\chi^2 = 14.9$; $P = 0.005$). Approximatively 77% of the larvae were recollected from protected controls, whereas 54% were recollected from uncovered controls. On average, 45% of larvae were recollected from all uncovered plants. In particular, more larvae were retrieved from protected controls than from plants exposed to *R. solani* volatiles (Fig. 1; $t = 3.3$; $P = 0.020$) and *F. oxysporum* volatiles ($t = 3.4$; $P = 0.014$). However, among uncovered plants, similar numbers of larvae were recollected from fungal volatile-exposed plants as from control plants (Fig. 1; GLMM; $\chi^2 = 2.3$; $P = 0.510$). Out of the 268 larvae recollected in total from the uncovered plants, only five were parasitised, either by *C. glomerata* wasps or by *C. rubecula* wasps (Table S1). All parasitised larvae were found on plants exposed to *C. indicum* volatiles. Parasitisation of the larvae was too low to be statistically analysed, most likely due to abiotic stress during the field period (heat waves reaching 36 °C).

Recruitment of parasitic wasps by larvae-infested plants in greenhouse conditions

Fungal volatiles did not affect parasitisation rates of *P. brassicae* larvae when female wasps were offered host larvae feeding on five differently treated plants (*i.e.* control plants and plants exposed to volatiles of each of the four fungi). *Cotesia glomerata* wasps parasitised similar numbers of larvae on control plants as on plants previously exposed to any of the fungal volatiles (Fig. 2; GLMM; $\chi^2 = 1.0$; $P = 0.916$). Additionally, the amount of leaf damage did not affect parasitisation rate (Fig. 3; GLMM; $\chi^2 = 2.8$; $P = 0.093$). Plant exposure to fungal volatiles did neither affect leaf fresh weight (Fig. 3a. ANOVA; $F = 0.3$; $P = 0.858$) nor the amount of leaf damage by *P. brassicae* larvae (Fig. 3b; Kruskal-Wallist; $H = 5.0$; $P = 0.286$). Leaf damage was negatively correlated with leaf fresh weight (Fig. 3c; Pearson correlation; $r = -0.482$; $P < 0.001$).

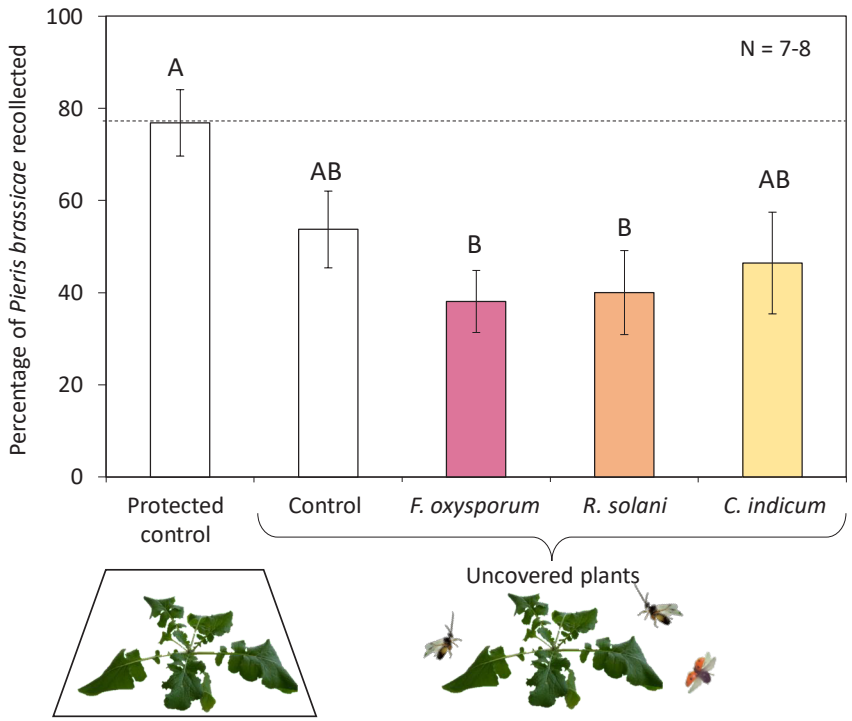

Figure 1. Percentage (mean ± SE) of *Pieris brassicae* larvae recollected in a common garden experiment from protected control *Brassica rapa* plants, uncovered control plants and uncovered plants previously exposed to volatiles emitted by different fungi (*Fusarium oxysporum, Rhizoctonia solani* and *Chaetomium indicum*) 48 hours after infesting each plant with 20 *P. brassicae* neonates. The dashed line represents the number of larvae recollected when predation is excluded. Differences in recollection rate were tested using a generalised linear model with a beta-binomial distribution. Uppercase letters indicate pairwise differences between the treatments using Tukey post-hoc tests (α = 0.05). "N" indicates the number of plant replicates.

Collection and analysis of headspace VOCs from infested plants

A total of 26 discrete VOCs were detected from the plant headspace, with terpenoids being the chemical class with the most numerous compounds (Table S2). Multivariate analysis of the VOC blends resulted in a model with three significant principal components (Fig. 4; PCA; R^2 = 0.694, Q^2 = 0.293). The PCA shows that samples of control plants did not separate from the samples of fungal volatile-exposed plants: 47.5% and 11.4% of the total variance was explained by the first and second principal components, respectively (Fig. 4). All 26 VOCs were detected in the blends of all treatments, *i.e.* of control plants as well as fungal volatile-exposed plants (Table S2). Additionally, peak intensity of individual compounds did not differ between the treatments for any of the VOCs (Table S1; Kruskal-Wallis tests; all P > 0.05). We also did not detect differences in blend composition between the treatments for each chemical class separately (Fig. S1). Although the processing of the peaks using single mass peak does not enable the calculation of total VOC emission, we observed that

the relative intensity of *C. indicum* VOC emission was higher than in the other treatments for most of the VOCs (Fig. S1).

	χ^2	P
Fungal volatiles	1.0	0.916
Leaf damage	2.8	0.093



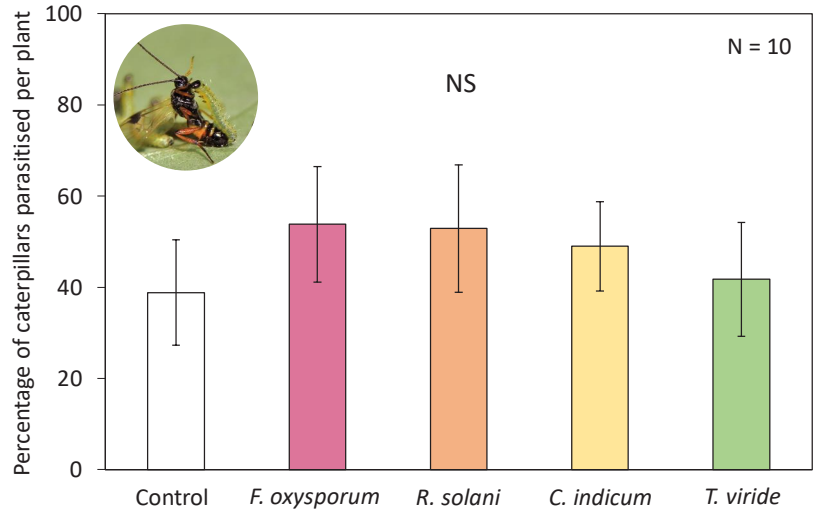

Figure 2. Percentage (mean ± SE) of *Pieris brassicae* larvae parasitised when one female *Cotesia glomerata* wasp was given 24 hours in a tent to parasitise larvae that had been feeding for 24 hours from five treated *Brassica rapa* plants (*i.e.* control plants and plants whose roots had been previously exposed to volatiles emitted by four different fungi: *Fusarium oxysporum, Rhizoctonia solani, Chaetomium indicum* and *Trichoderma viride*). Each tent contained one plant of each treatment, and each plant was infested with 20 *P. brassicae* neonates. Differences in parasitisation were analysed using a generalised linear mixed model with a beta-binomial distribution, with fungal volatiles as main factor, amount of leaf damage as covariate, and tent number and pot position as random factors. "N" indicates the number of tents prepared (*i.e.* replicates).

Discussion

We found that the number of *P. brassicae* larvae recollected from *B. rapa* plants whose roots had been exposed to volatiles from soil-borne fungi did not differ with that of control plants when subjected to natural predation in the field. Furthermore, in a greenhouse experiment, parasitisation of *P. brassicae* larvae by *C. glomerata* wasps was similar on fungal volatile-exposed plants and control plants. Interestingly, profiling of HIPV blends emitted upon attack by *P. brassicae* larvae reveals neither qualitative nor quantitative differences between control plants and plants exposed to fungal volatiles. Taken together, we did not

find evidence that root exposure to fungal volatiles affects indirect resistance of *B. rapa* plant to herbivory by *P. brassicae* larvae.

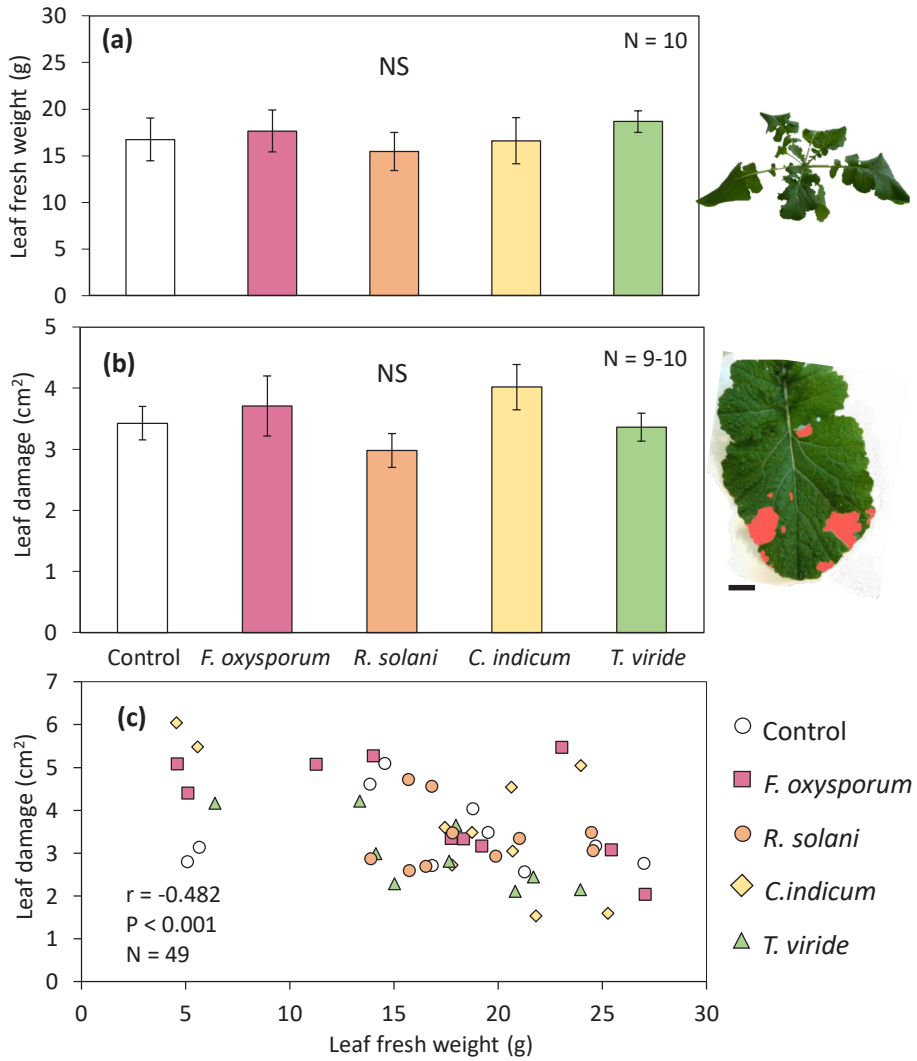

Figure 3. (a) Leaf fresh weight (mean ± SE), **(b)** amount of leaf damage (mean ± SE) upon feeding by *Pieris brassicae* larvae for 48 hours on four-week-old *Brassica rapa* plants whose roots had been previously exposed to volatiles emitted by different fungi (*Fusarium oxysporum, Rhizoctonia solani, Chaetomium indicum* and *Trichoderma viride*) or control plants. **(c)** Pearson correlation between leaf damage and leaf fresh weight. Damaged areas (in red on the picture) were estimated by analysing photographs of the infested leaves with ImageJ software. Differences in leaf fresh weight were analysed with a one-way ANOVA, and differences in amount of leaf damage were analysed with a Kruskal-Wallis test. "NS" refers to "non-significant" differences (α = 0.05) and "N" indicates the number of plant replicates.

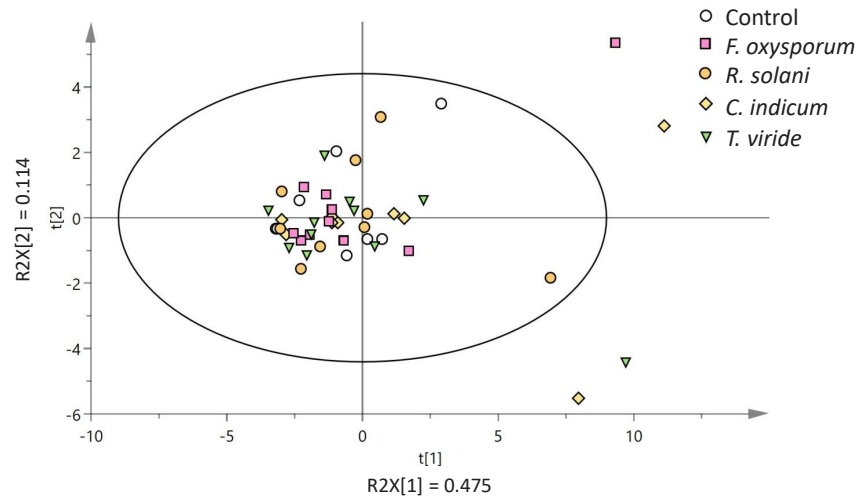

Figure 4. Principal Component Analysis (PCA) of the volatile organic compounds (VOCs) collected from the headspace of *Brassica rapa* plants infested with *Pieris brassicae* larvae and whose roots had been exposed to volatiles emitted by the soil-borne fungi: *Fusarium oxysporum f.sp. raphani, Rhizoctonia solani, Chaetomium indicum* and *Trichoderma viride*. Grouping pattern of samples according to the first two principal components and the Hotelling's T2 ellipse confining the confidence region (95%) of the score plot.

In the present study, we did not detect differences in HIPV emission between non-exposed plants and plants whose roots were exposed to fungal volatiles. To our knowledge, this is the first study that investigated whether belowground plant exposure to microbial volatiles modulates the emission of HIPVs aboveground. Plant VOC blends comprise typical HIPVs of Brassicaceae such as the GLVs 3-hexen-1-ol and 3-hexenyl acetate, as well as glucosinolate derivatives like 3-butenyl isothiocyanate (Danner *et al.*, 2018). We recorded neither qualitative nor quantitative differences between HIPV blends emitted upon herbivory by control *B. rapa* plants and those emitted by fungal volatile-exposed plants. Interestingly, soil inoculation with non-pathogenic soil-borne bacteria and fungi can affect HIPV emission (Bezemer and van Dam, 2005; Pineda *et al.*, 2013; Pangesti *et al.*, 2015b), and plant exposure to microbial volatiles may impact levels of secondary metabolites (Aziz *et al.*, 2016). Therefore, belowground plant stimulation by microorganisms can affect aboveground plant chemistry (Ohgushi *et al.*, 2018), including plant VOCs, although the contribution of microbial volatiles in these phenotypical changes remains unclear. Preliminary findings indicate that VOC emission from *A. thaliana* seedlings can be altered upon plant exposure to volatiles from the soil-borne fungus *R. solani* (Cordovez *et al.*, 2017). Interestingly, plant VOC emission by aboveground tissues can be altered in response to leaf exposure to volatiles from con- and heterospecific organisms. For instance, volatiles emitted by a herbivore-infested plant can prime the emission of HIPVs in neighbouring intact plants (Ruther and Kleier, 2005; Ninkovic *et al.*, 2019). Similarly, volatiles emitted by an insect herbivore can also affect HIPV emission (Helms *et al.*, 2014; Bittner *et al.*, 2019). In the present study, we did not find an effect of root exposure to fungal volatiles on HIPV emission. To date, knowledge about the

mechanisms of plant perception of volatiles and the signal-transduction cascade remains sparse. Plants may specifically respond to microbial volatiles released in their environment (Moisan *et al.*, 2019; Chapter 2 of this thesis), and these responses may be local at the site of perception, *i.e.* in this case roots, or systemic. The tissue that is exposed to the microbial volatiles may indeed differentially affect plant VOCs, and hence plant interactions mediated by plant VOCs. For instance, the application of the bacterial VOC 2,3-butanediol to the headspace of maize plants did not affect the attraction of the parasitoid upon herbivory, whereas inoculation of the VOC into the soil enhanced parasitoid attraction (D'Alessandro *et al.*, 2014), suggesting soil-mediated effects on HIPV emission (Pangesti *et al.*, 2015a). Further research is required to explore how soil characteristics and soil microbial community composition modulate plant responses to microbial volatiles below- and aboveground.

Root exposure to fungal volatiles did not affect recruitment of predators and parasitoids by plants whose leaves were under attack. In the field, based on the number of *P. brassicae* larvae recollected from the protected control plants that were not exposed to predation, we assume that approximately 20% of the *P. brassicae* larvae were missing across all treatments because of natural disease, desiccation or possibly starvation as a result of direct plant resistance. Yet, we previously showed that exposure of *B. rapa* roots to volatiles from *F. oxysporum* and *R. solani* in a greenhouse did not affect the mortality of *P. brassicae* larvae after three days of feeding, even though it affected their biomass (Chapter 3 of this thesis). Therefore, the percentage of missing larvae when predation is excluded is assumed to be similar between plant exposure to the different fungal volatiles. The rest of the larvae missing from uncovered plants, *i.e.* the difference between the percentage of larvae recollected from protected plants and the percentage of larvae recollected from uncovered plants, is assumed to result from predation. Predation rate of *P. brassicae* larvae in the open field did not differ between uncovered control plants and fungal volatile-exposed plants. Similarly, exposure of *B. rapa* roots to fungal volatiles did not affect parasitisation of *P. brassicae* larvae by *C. glomerata* wasps in the greenhouse. In contrast, other studies showed that direct application of microbial VOCs onto the plant can positively impact herbivore predation rates (Song and Ryu, 2013; D'Alessandro *et al.*, 2014). For instance, drenching of cucumber seedlings with the bacterial VOCs 3-pentanol and 2-butanone increased the number of ladybirds, *i.e.* general predators, in an open field and led to a reduction of an aphid population (Song and Ryu, 2013). Additionally, inoculation of the bacterial VOC 2,3-butanediol in the soil surrounding maize plants attacked by *S. littoralis* larvae can positively affect the attraction of *Cotesia marginiventris* parasitoids (D'Alessandro *et al.*, 2014). Plant VOCs, and in particular HIPVs, are known as cues used by parasitic wasps and predators to locate infested plants from a distance (Dicke and Sabelis, 1988; Mattiacci *et al.*, 1995), while other plant traits, such as cuticular traits or host-related traces, can be used after contact with an infested plant as cues to locate and select the larvae to parasitise or predate (Mattiacci and Dicke, 1995; Nufio and Papaj, 2001; Obonyo *et al.*, 2010). To date, no study has investigated whether plant exposure to microbial volatiles from a distance can influence HIPV and plant recruitment of natural enemies of herbivores. In the present study,

we did not detect any difference in the HIPVs emitted by infested *B. rapa* plants whose roots had been exposed to fungal volatiles compared with non-exposed plants. Thus, attraction of the natural enemies of *P. brassicae* larvae to infested plants is unlikely to be affected by root exposure to fungal volatiles. Interestingly, *Cotesia* wasps are also known to select the most suitable host species for parasitoid development and oviposit accordingly (Brodeur *et al.*, 1998). Thus, different larval biomass (Chapter 3 of this thesis) could have affected the number of parasitoid eggs laid inside the larvae feeding on fungal volatile-exposed plants (not measured here), which may impact encapsulation by the larvae. Ultimately, successful recruitment of natural enemies as indirect plant resistance depends on the number of larvae killed by the predators and parasitoids. Further research addressing plants and herbivores during their full life cycle may provide more insights into the ultimate effects of fungal volatiles on the alleviation of herbivore pressure on the exposed plants. Altogether, our data suggest that exposure of *B. rapa* roots to volatiles from soil-borne fungi does neither affect HIPV emission upon herbivory by *P. brassicae* larvae nor the predation and parasitisation of the larvae, thus sustaining indirect plant resistance. We have previously demonstrated that direct plant resistance as well as plant growth upon herbivory can be positively or negatively modulated by root exposure to fungal volatiles (Chapter 3 of this thesis). Therefore, volatiles emitted belowground by soil microorganisms may be important modulators of plant defence (Dicke, 2016).

Acknowledgements

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Chapter 4

Supplemental Materials

Table S1. Total number of *Pieris brassicae* larvae recollected from plants whose roots had been previously exposed to volatiles from *Chaetomium indicum* and number of parasitised larvae with the identity of the parasitic wasp (*Cotesia glomerata* or *C. rubecula*).

Replicate	Nr larvae recollected	Nr parasitised larvae	Identity of the wasp
Plant 1	1	0	-
Plant 2	16	0	-
Plant 4	14	2	*C. glomerata* (2x)
Plant 5	9	1	*C. rubecula* (1x)
Plant 6	10	2	*C. glomerata* (2x)
Plant 7	2	0	-
Plant 8	13	0	-

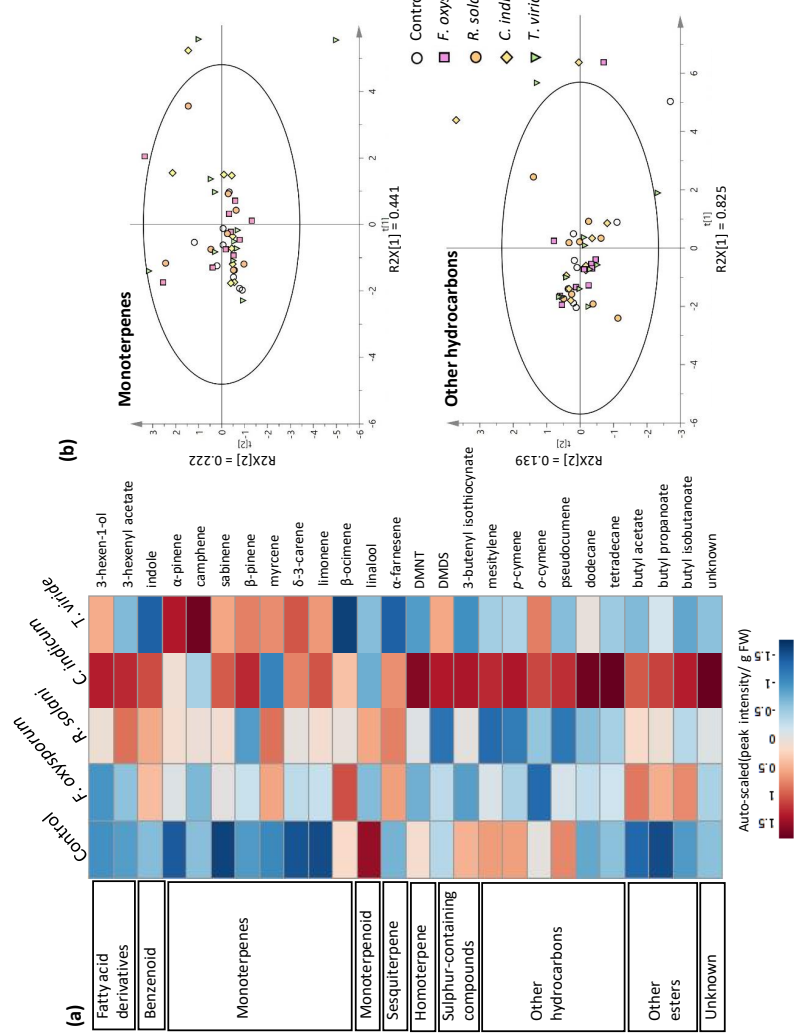


Figure S1. (a) Heatmap of the volatile organic compounds (VOCs) detected in the headspace of *Brassica rapa* plants infested with *Pieris brassicae* larvae and whose roots had been exposed to volatiles emitted by the soil-borne fungi: *Fusarium oxysporum f.sp. raphani*, *Rhizoctonia solani*, *Chaetomium indicum* and *Trichoderma viride*. VOCs were sorted per chemical class. To generate this heatmap, we used values subjected to auto-scaling (peak intensity / g shoot fresh weight). Plant VOCs were collected after 4 weeks of fungal volatile-exposure and 24 hours of herbivory. Each treatment was replicated 8-11 times. **(b)** Principal Component Analysis (PCA) of the monoterpenes and other hydrocarbons between the different plant treatments. "DMNT" stands for 4,8-dimethyl-1,3,7-nonatriene and "DMDS" for dimethyl-disulfide.

Table S2. Relative peak intensity [mean (SE)], CAS registry number and the calculated Arithmetic retention Index (AI) of volatile organic compounds (VOCs) collected in the headspace of *Brassica rapa* plants infested with *Pieris brassicae* larvae and whose roots had been exposed to VOCs emitted by *Chaetonium indicum*, *Fusarium oxysporum*, *Rhizoctonia solani* or *Trichoderma viride*, or non-exposed plants. Differences of individual peak intensity between fungal VOC-exposed plants were tested with Kruskal-Wallis tests (α = 0.05).

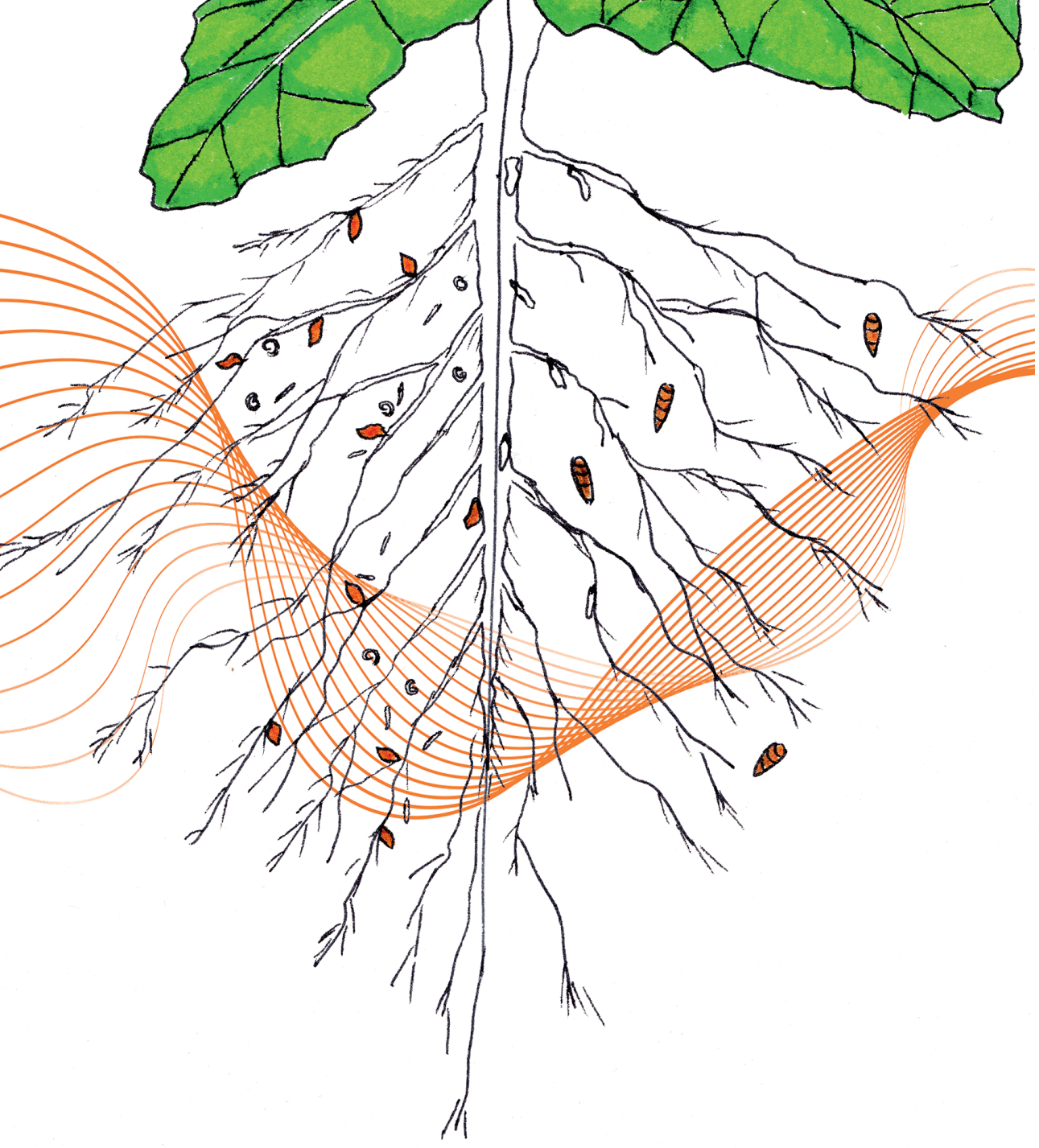
VOC code	Putative identity	CAS registry number	Calculated Arithmetic retention Index (AI)	Control (N = 8)	*C. indicum* (N = 9)	*F. oxysporum* (N = 10)	*R. solani* (N = 9)	*T. viride* (N = 11)	*P* values Kruskal-Wallis test
Fatty acid derivatives									
2064	(3E or 3Z)-hexen-1-ol ($C_6H_{12}O$)	928-97-2 or 928-96-1	856	18 (6)	65 (33)	18 (6)	39 (18)	49 (31)	0.631
5989	(3E or 3Z)-hexenyl acetate ($C_8H_{14}O_2$)	3681-82-1 or 3681-71-8	1004	11 (3)	17 (6)	12 (4)	16 (6)	12 (4)	0.708
Phenylpropanoid/benzenoid									
14833	indole (C_8H_7N)	120-72-9	1311	7 (3)	13 (4)	11 (5)	11 (5)	4 (1)	0.191
Monoterpenes ($C_{10}H_{16}$)									
4149	α-pinene	80-56-8	942	64 (9)	99 (22)	95 (12)	100 (14)	129 (44)	0.513
4592	camphene	79-92-5	961	10 (3)	13 (3)	9 (1)	20 (5)	45 (32)	0.676
5276	sabinene	3387-41-5	981	21 (4)	29 (6)	25 (3)	26 (4)	27 (5)	0.955
5490	β-pinene	127-91-3	989	10 (2)	13 (2)	11 (1)	10 (2)	13 (2)	0.724
5561	myrcene	123-35-3	991	15 (6)	14 (3)	45 (23)	50 (33)	48 (22)	0.631
6454	δ-3-carene	13466-78-9	1018	54 (11)	82 (14)	69 (8)	73 (11)	85 (14)	0.501
6959	limonene	138-86-3	1038	8 (1)	11 (2)	10 (1)	10 (1)	11 (2)	0.851
7368	(E or Z)-β-ocimene	3779-61-1 or 3338-55-4	1049	16 (4)	16 (3)	17 (6)	16 (3)	13 (3)	0.661
Monoterpenoid									
8936	linalool ($C_{10}H_{18}O$)	78-70-6	1102	158 (141)	13 (8)	20 (14)	98 (73)	20 (11)	0.407
Sesquiterpene ($C_{15}H_{24}$)									
20059	(E,E)-α-farnesene	502-61-4	1508	19 (11)	25 (9)	24 (11)	25 (11)	16 (8)	0.832
Homoterpene									
9393	(E)-DMNT ($C_{11}H_{18}$)	19945-61-0	1115	30 (12)	38 (10)	25 (11)	29 (10)	24 (6)	0.794

All values were divided per row by either 10^3, 10^4 or 10^5 and should be regarded as relative abundance of individual VOC between treatments using individual mass peaks. All values were normalised with the shoot fresh weight. VOC codes were generated by MSClust software as "centrotype (or cluster) index numbers"; they can be considered as arbitrary codes attributed to the VOCs. Classification of VOCs into chemical classes is not exclusive and may be rearranged differently. "N" indicates the number of plant samples in each treatment. "DMNT" stands for 4,8-dimethyl-1,3,7-nonatriene and "DMDS" for dimethyl-disulfide.

Table S2. *Continued.*

VOC code	Putative identity	CAS registry number	Calculated Arithmetic retention Index (AI)	Control (N = 8)	*C. indicum* (N = 9)	*F. oxysporum* (N = 10)	*R. solani* (N = 9)	*T. viride* (N = 11)	*P* values Kruskal-Wallis test
Other sulphur/nitrogen-containing compounds									
706	dimethyl disulfide ($C_2H_6S_2$)	624-92-0	< 800	7 (2)	8 (3)	7 (1)	6 (1)	7 (2)	0.977
5367	3-butenyl isothiocyanate (C_5H_7NS)	3386-97-8	985	36 (12)	47 (17)	20 (4)	30 (13)	18 (4)	0.437
Other hydrocarbons									
5934	mesitylene (C_9H_{12})	108-67-8	1002	9 (3)	10 (2)	9 (2)	7 (1)	8 (2)	0.977
6653	*p*-cymene ($C_{10}H_{14}$)	99-87-6	1027	9 (3)	10 (2)	8 (2)	7 (1)	8 (2)	0.912
6774	pseudocumene (C_9H_{12})	95-63-6	1031	29 (10)	31 (8)	26 (8)	22 (4)	24 (5)	0.832
6814	*o*-cymene ($C_{10}H_{14}$)	527-84-4	1033	8 (2)	9 (2)	7 (1)	8 (1)	9 (2)	0.912
11906	dodecane ($C_{12}H_{26}$)	112-40-3	1200	32 (3)	44 (10)	34 (5)	32 (6)	36 (7)	0.851
17147	tetradecane ($C_{14}H_{30}$)	629-59-4	1400	32 (3)	53 (14)	36 (7)	34 (9)	33 (7)	0.676
Other esters									
1547	butyl acetate ($C_6H_{12}O_2$)	123-86-4	812	5 (2)	15 (6)	14 (7)	11 (4)	8 (2)	0.363
3238	butyl propanoate ($C_7H_{14}O_2$)	590-01-2	906	19 (5)	44 (13)	38 (13)	34 (8)	32 (7)	0.372
4360	butyl isobutanoate or isobutyl butanoate ($C_8H_{16}O_2$)	97-87-0 or 539-90-2	952	6 (2)	9 (3)	8 (3)	7 (1)	7 (1)	0.977
Other									
826	unknown	unknown	< 800	35 (9)	155 (70)	44 (12)	62 (28)	35 (18)	0.155

All values were divided per row by either 10^3, 10^4 or 10^5 and should be regarded as relative abundance of individual VOC between treatments using individual mass peaks. All values were normalised with the shoot fresh weight. VOC codes were generated by MSClust software as "centrotype (or cluster) index numbers"; they can be considered as arbitrary codes attributed to the VOCs. Classification of VOCs into chemical classes is not exclusive and may be rearranged differently. "N" indicates the number of plant samples in each treatment. "DMNT" stands for 4,8-dimethyl-1,3,7-nonatriene and "DMDS" for dimethyl-disulfide.



Chapter 5



Volatiles from the fungus Fusarium oxysporum affect interactions of Brassica rapa plants with root herbivores

Kay Moisan, Marcel Dicke, Jos M. Raaijmakers, Elvira Rachmawati, Viviane Cordovez

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Abstract

Soil is a dark and crowded environment where chemicals mediate a tremendous amount of interactions between soil organisms that may compete or benefit from each other. To date, studies have extensively addressed interactions between rhizospheric microorganisms and the effects of microbial volatiles in microbe-microbe interactions. Yet, to our knowledge, it remains to be explored whether volatiles from a soil-borne fungus influence plant colonisation by root attackers, facilitating or hampering performance of competitors that share the same host plant. In the present study, we investigated the effects of volatiles emitted by the fungus *Fusarium oxysporum* on the performance of two root herbivores: the plant parasitic cyst nematode, *Heterodera schachtii,* and the insect root herbivore, *Delia radicum,* upon infestation of *Brassica rapa* roots. Our results show that *F. oxysporum* volatiles slowed down the development of *H. schachtii* cysts but increased their size and, hence, egg load. In contrast, the performance of *D. radicum* was unaffected by the exposure of roots to fungal volatiles. Additionally, fungal volatiles promoted the growth of plants infested with the root nematode, but not of those infested with the insect root herbivore. Together, our data show that volatiles from a soil-borne fungus differentially affect root colonisation by two herbivore species. Increased production of nematode eggs and plant growth promotion suggest a specific modulation of root-herbivore interactions by fungal volatiles.

Keywords

Delia radicum, Heterodera schachtii, nematodes, plant growth, root colonisation

Introduction

Soil is the most diverse ecosystem on Earth and harbours dense and complex communities of macro- and microorganisms, including earthworms, arthropods, protozoa, nematodes, fungi, oomycetes, bacteria and archea. These soil organisms interact directly or indirectly with each other and constitute dynamic soil food webs with several trophic levels (Susilo *et al.*, 2004; Morriën, 2016). In this crowded environment, soil organisms have to compete for resources and escape from predators and parasites, while still interacting with mutualists.

To survive in this dark environment, soil microorganisms rely on physical and chemical cues to assess their surroundings, and to interact with con- and heterospecific organisms (van Dam and Bouwmeester, 2016; van Dam *et al.*, 2016; Kong *et al.*, 2018). Therefore, soil microorganisms have evolved mechanisms to sense their neighbours (Johnson and Nielsen, 2012; Rasmann *et al.*, 2012a; Hiltpold *et al.*, 2013), and interact *via* the production of chemical compounds. Among these, volatile compounds (hereafter: volatiles) mediate important direct interactions between soil organisms (Raaijmakers *et al.*, 2009; Johnson and Rasmann, 2015; Schmidt *et al.*, 2015). In particular, in the rhizosphere, described as "a playground and battlefield for soil-borne pathogens and beneficial microorganisms" by Raaijmakers *et al.* (2009), microbial volatiles can inhibit the growth of microbial competitors (Schmidt *et al.*, 2015; Schulz-Bohm *et al.*, 2017b). Additionally, microbial volatiles can mediate inter-kingdom interactions (Wenke *et al.*, 2010; Rasmann *et al.*, 2012b; van Dam and Bouwmeester, 2016; Schenkel *et al.*, 2018). For example, volatiles emitted by *Fusarium oxysporum* strains can negatively affect egg hatching of the root-knot nematode *Meloidogyne incognita* (Terra *et al.*, 2018) or reduce nematode population size (Hallmann and Sikora, 1994). Volatiles from pathogenic and non-pathogenic soil microorganisms can promote plant growth and accelerated plant development (Casarrubia *et al.*, 2016; Cordovez *et al.*, 2017; Piechulla *et al.*, 2017; Moisan *et al.*, 2019; Chapter 2 of this thesis). Reciprocally, plant volatiles can affect soil microorganisms. For example, root volatiles can attract beneficial bacteria to the rhizosphere (Rasmann and Turlings, 2016; Schulz-Bohm *et al.*, 2018). Therefore, behaviour and performance of one soil organism can be directly affected by volatiles emitted by con- and heterospecific organisms in the soil.

Interestingly, volatiles can also mediate indirect interactions between soil organisms, *e.g.* by involving higher trophic levels or *via* a shared interacting organism. Similar to herbivore-induced plant volatiles aboveground, root volatiles may also be released in soil as a "cry for help", attracting natural enemies of the attacker (Ali *et al.*, 2013; Rasmann and Turlings, 2016; van Dam *et al.*, 2016; Massalha *et al.*, 2017). For instance, infection by fungal pathogens induced root volatiles (Fiers *et al.*, 2013), which attracted specific bacterial strains with anti-fungal activity (Schulz-Bohm *et al.*, 2018). Insect-damaged maize roots attracted entomopathogenic nematodes through the emission of herbivore-induced root volatiles (Rasmann *et al.*, 2005). Moreover, microbial volatiles can enhance plant resistance to subsequent attackers, by providing physical and chemical protection of the plant, hence preventing further microbial colonisation (Vorholt, 2012; Junker and Tholl, 2013), and by

eliciting plant resistance responses (Bitas *et al.*, 2013; Sharifi and Ryu, 2016; Cordovez *et al.*, 2017). Together, these data indicate that volatiles in soil can indirectly mediate ecological interactions between different soil organisms.

Yet, it remains largely unknown whether volatiles from a soil-borne fungus, directly and indirectly, impact root colonisation by further root attackers, facilitating or hampering the performance of competitors that share the same host plant. In this study, we investigated the effects of volatiles emitted by the asymptomatic endophyte *F. oxysporum* on the performance of the cyst nematode *Heterodera schachtii* and the insect root herbivore *Delia radicum* on *Brassica rapa* roots.

Materials and Methods

Plant, herbivore and fungal materials

Seeds of the wild turnip, *B. rapa* (accession Maarssen, The Netherlands), were sterilised as previously described (Moisan *et al.*, 2019; Chapter 2 of this thesis). Briefly, all *B. rapa* seeds were surface sterilised by exposure to chlorine gas for 4 h in a desiccator, and stratified at 4 °C in the dark for 3-4 days. Before the start of the experiments, *F. oxysporum f. sp. raphani* was cultured on 1/5th PDA medium, either in ø 9 cm Petri dishes for 7 days or in ø 3 cm Petri dishes for 3 days. The plant parasitic nematode, *H. schachtii,* was reared on roots of sugar beet plants (*Beta vulgaris* L.), and larvae of the cabbage root fly, *D. radicum,* were reared on rutabaga (*Brassica napus* subsp. *napobrassica*) roots.

Plant exposure to Fusarium oxysporum *volatiles*

Brassica rapa roots were exposed to *F. oxysporum* volatiles using two-compartment pots (Chapter 3 of this thesis). Size of the pots differed between the infestations of the two root herbivores as they naturally infest plants at different stages: *H. schachtii* nematodes perform better on plants in the pre-emergence seedling stage (Fedorko, 1962; Griffin, 1981), whereas larvae of *D. radicum* require sufficient development of the root system for feeding, thus adult flies preferably oviposit on larger plants (Ellis *et al.*, 1979; Dosdall *et al.*, 1996). In both experiments, one sterile *B. rapa* seed was sown in the top compartment (for *H. schachtii:* h = 8 cm, ø = 5.5 cm; for *D. radicum:* h = 20 cm, ø = 12.5 cm) filled with a sterile (γ-irradiated) soil mixture (1:1 v/v, ø 2 mm sieved Horticoop potting soil:sand). The bottom compartment (for *H. schachtii:* h = 5 cm, ø = 6.5 cm; for *D. radicum:* h = 10 cm, ø = 12.5 cm) contained either a Petri dish with *F. oxysporum* growing on 1/5th PDA medium or a control Petri dish with the medium only (for *H. schachtii:* ø 3 cm Petri dish; for *D. radicum:* ø 9 cm Petri dish). Both compartments were separated by a nylon membrane of 1 µm mesh width (Plastok associates Ltd., Birkenhead Wirral) that allowed air exchange between the two compartments. In the pots used for infestation with *D. radicum* larvae*,* both compartments were connected to each other by a cylinder (h = 12.5 cm, ø = 12.7 cm), which allowed the

weekly replacement of Petri dishes containing the fungus or control Petri dishes with fresh seven day-old fungus or medium. Volatile exposure was initiated as soon as *B. rapa* seeds were sown, and maintained throughout the experiments in a greenhouse compartment (21 ± 2 °C; 16:8 h, L:D; 70 ± 5% RH) until harvest. Volatile exposures (*F. oxysporum* and control) were replicated 14-15 times for each herbivore infestation.

Plant infestation with Heterodera schachtii *cyst nematodes*

Cysts of *H. schachtii* were incubated in water at 25 °C, four days before inoculation. Newly hatched juveniles were sieved, and the concentration of the inoculum was adjusted by counting the juveniles under the stereo microscope and making dilutions. After two weeks of root exposure to volatiles, each *B. rapa* plant received 1 mL of inoculum containing 550 ± 20 *H. schachtii* J2 juveniles with a pipette. To prevent flushing of the juveniles, no water was added to the plants during the first two days after inoculation. Aboveground parts of the plants were harvested three weeks after inoculation, and the cysts were given another two weeks to ripen. Roots were gently harvested and rinsed with water through two sieves (ø = 0.850 mm and ø = 0.212 mm). Sieves were rinsed with water, and cysts in suspension in this water were collected and counted under the microscope. Roots were also checked under the microscope for remaining cysts. Collected cysts were stored in Petri dishes at 4 °C until measurement. To assess the degree of cyst maturation, cyst colouration was scored based on visual evaluation under the stereo microscope: white to light yellow-coloured cysts were considered as unripe, and orange to dark orange-coloured cysts were considered as ripe (Gardner and Caswell-Chen, 1997). To assess cyst size, cysts were photographed (Leica DFC450, Leica Microsystems B.V., Son, The Netherlands) under a microscope (Leica M205C), and pictures (400 dpi resolution) were processed with ImageJ software to measure individual cyst size. In addition, roots and leaves were dried at 70 °C for 3 days and weighed. Effects of *F. oxysporum* volatiles on the number of collected cysts was tested with a Student *t*-test (α = 0.05). Differences of percentages of unripe and ripe cysts collected from control and volatile-exposed plants were tested using a quasi-binomial (to handle binomial overdispersion) generalised linear model with fungal volatile exposure as a fixed factor (α = 0.05). Differences in cyst size were analysed using a linear mixed model with plant replicate as a random factor. Differences of root and leaf dry weight were tested with Student *t*-tests, and correlations with the number and size of cysts were assessed with Pearson correlation tests (α = 0.05).

Plant infestation with Delia radicum *larvae*

After four weeks of root exposure to the fungal volatiles, *B. rapa* plants were infested with ten newly hatched *D. radicum* larvae. During the first two days after infestation, no water was added to the plant to prevent flushing of the larvae. Two weeks after infestation, plants were harvested and *D. radicum* individuals were recollected from the soil and roots. Larvae and pupae were counted and individually weighed. The fraction of recollected *D. radicum* (out of 10 larvae infested per plant) and the fraction of *D. radicum* pupae (out of the insects

recollected) were analysed with a quasi-binomial (to handle binomial overdispersion) generalised linear model and logit link function, using fungal volatiles as a fixed factor. Additionally, a linear mixed model, using plant replicate as a random factor, was used to analyse fresh weight of *D. radicum* larvae and pupae. Correlations between insect fresh weight, number of insects and root dry weight were assessed with Pearson correlation tests ($\alpha = 0.05$).

Results

Plant infestation with Heterodera schachtii *cyst nematodes*

Fewer *H. schachtii* cysts were retrieved per gram of roots from *B. rapa* plants exposed to *F. oxysporum* volatiles than from non-exposed control plants (Fig. 1a; $t = 2.5$; $P = 0.018$). On average, 25% of the cysts collected from control plants were unripe, whereas this number reached 48% in plants exposed to the fungal volatiles (Fig. 1b; GLM; $P = 0.029$). Moreover, cysts retrieved from plants exposed to the fungal volatiles were larger than those collected from control plants (Fig. 1c; $F = 5.8$; $P = 0.026$). Exposure to the fungal volatiles increased root and leaf weight of *H. schachtii*-infested plants (Fig. 2a; $t_{root} = -2.3$; $P_{root} = 0.034$; $t_{leaf} = -2.9$; $P_{leaf} = 0.009$). Overall, cyst size was positively correlated with root dry weight (Fig. 2b; $r = 0.493$; $P = 0.023$) and with the number of cysts retrieved (Fig. 2c; $r = 0.449$; $P = 0.041$).

Plant infestation with Delia radicum *larvae*

On average, 53% and 49% of the larvae that were infested on control plants and plants exposed to *F. oxysporum* volatiles respectively were recollected (Fig. 3a; GLM; $P = 0.731$). We found as many pupae and larvae from control plants and plants exposed to *F. oxysporum* volatiles (Fig. 3b; GLM; $P = 0.899$), and their weight did not differ (Fig. 3c; $F = 0.1$; $P = 0.468$). Exposure to the fungal volatiles did not impact plant growth of *D. radicum*-infested plants (Fig. 4a; $t_{root} = 0.2$; $P_{root} = 0.789$; $t_{leaf} = 0.0$; $P_{leaf} = 0.958$). Overall, *D. radicum* fresh weight was positively correlated with root dry weight (Fig. 4b; $r = 0.481$; $P = 0.015$) and with the number of individuals recollected (Fig. 4c; $r = 0.486$; $P = 0.014$)

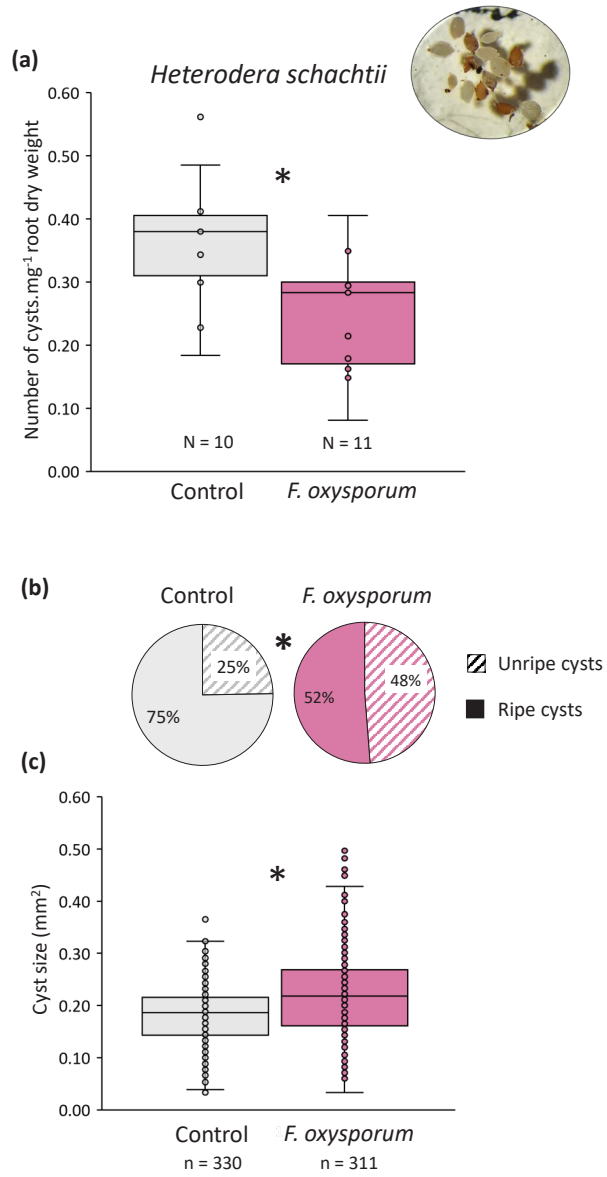


Figure 1. (a) Number of *Heterodera schachtii* cysts collected per mg of dry weight of *Brassica rapa* roots, **(b)** mean percentage of unripe cysts (white to light yellow-coloured cysts) and ripe cysts (orange to dark orange-coloured cysts), and **(c)** size of the cysts collected on control plants and on plants exposed to *Fusarium oxysporum* volatiles. Plants received 1 mL of inoculum containing 550 ± 20 *H. schachtii* juveniles. "N" indicates the number of plant replicates and "n" indicates the total number of cysts collected. Each box-plot shows the distribution of the dataset into quartiles: the minimum, first quartile, median, third quartile, and maximum. Dots show the distribution of each cyst measurement. Effects of *F. oxysporum* volatiles on the number of collected cysts was tested with a Student *t*-test. Differences of percentages of light and dark coloured-cysts collected from control and volatile-exposed plants were tested using a generalised linear model with a quasi-binomial distribution. Differences of cyst size were tested using a linear mixed model, with plant replicate as a random factor (*: $P < 0.05$).

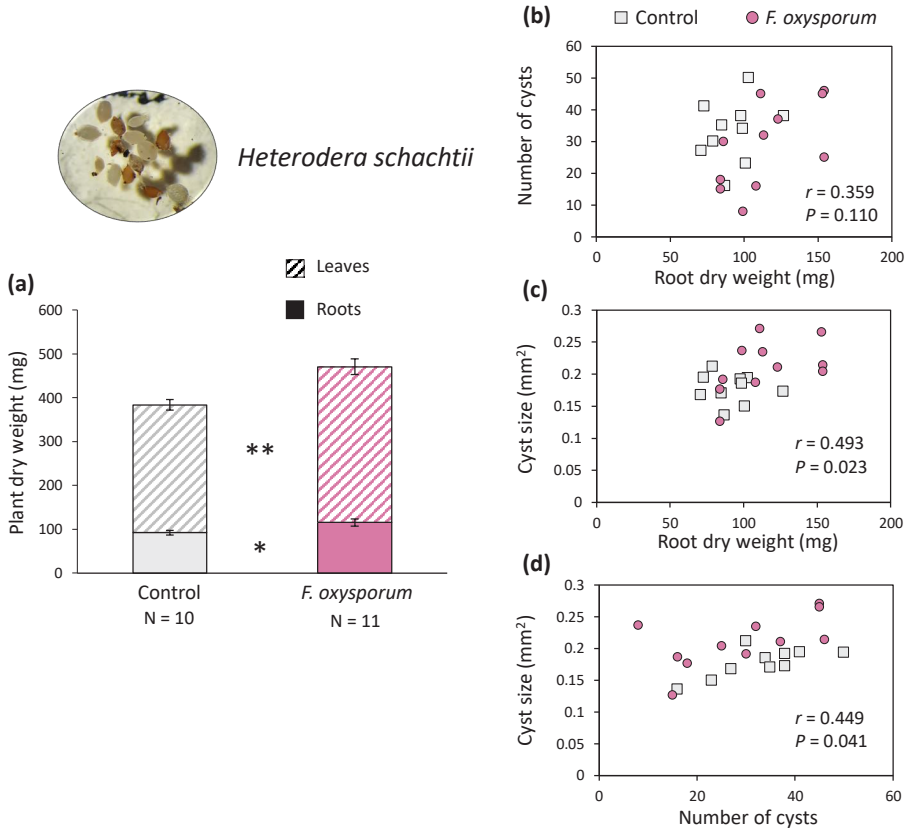


Figure 2. (a) Root and leaf dry weight of *Heterodera schachtii*-infested *Brassica rapa* plants when exposed or not to *Fusarium oxysporum* volatiles. Pearson correlations between **(b)** the number of *H. schachtii* cysts collected per plant and the root dry weight, **(c)** the average size of *H. schachtii* cysts per plant and the root dry weight, and **(d)** the average cyst size per plant and the number of collected cysts. "N" indicates the number of plant replicates. Plant dry weight of control and volatile-exposed plants was compared with Student *t*-test (*: $P < 0.05$; **: $P < 0.01$).

Discussion

Our results show that colonisation of *B. rapa* roots by either of two herbivore species is differentially affected by volatiles emitted by the soil-borne fungus *F. oxysporum*. We did not find an effect of fungal volatiles on the performance of the insect root herbivore *D. radicum,* whereas root colonisation and reproduction of the nematode *H. schachtii* was negatively and positively impacted, respectively. Besides potential direct effects of fungal volatiles on the attackers, plant growth promotion upon fungal volatile exposure suggests possible indirect effects mediated by the plant as well.

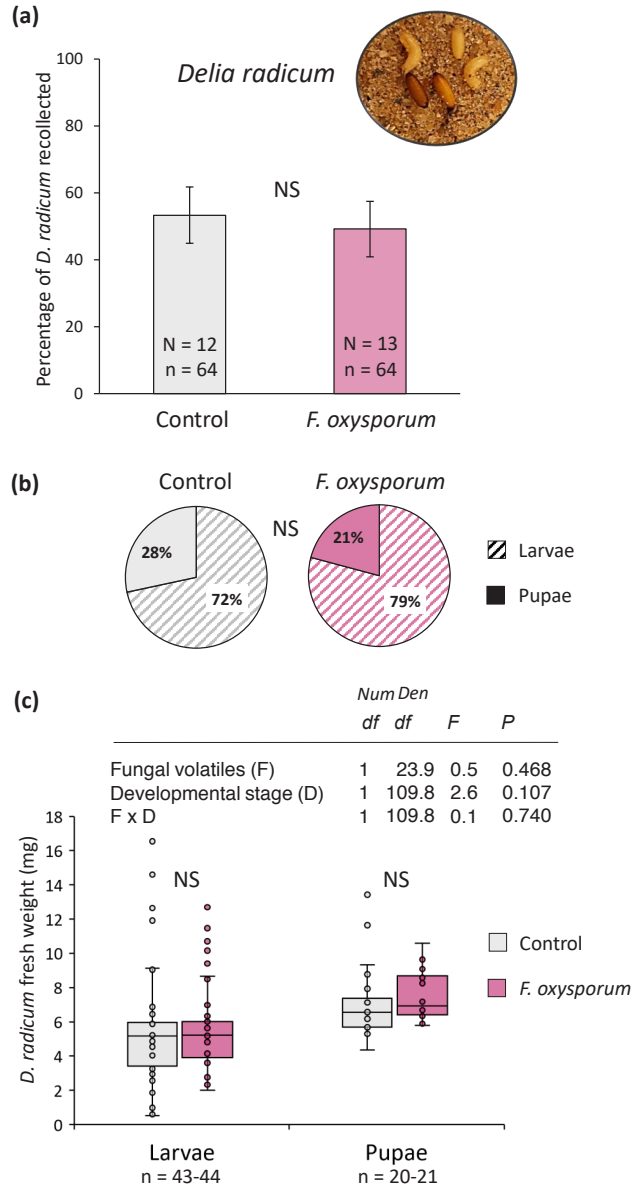


Figure 3. (a) Percentage (mean ± SE) of *Delia radicum* recollected per *Brassica rapa* plant, **(b)** mean percentage of *D. radicum* larvae and pupae recollected per plant, and **(c)** fresh weight of *D. radicum* larvae and pupae recollected from control plants and from plants exposed to *Fusarium oxysporum* volatiles. Each plant was infested with 10 neonates. "N" indicates the number of plant replicates and "n" indicates the total number of pupae and larvae recollected. Each box-plot shows the distribution of the dataset into quartiles: the minimum, first quartile, median, third quartile, and maximum. Dots show the distribution of each measurement (individual *D. radicum*). Effect of *F. oxysporum* volatiles on the percentages of recollection was tested using a generalised linear model with a quasi-binomial distribution. Differences in insect fresh weight between plants exposed to *F. oxysporum* volatiles and control plants were analysed used a linear mixed model, with plant replicate as a random factor (NS: $P > 0.05$).

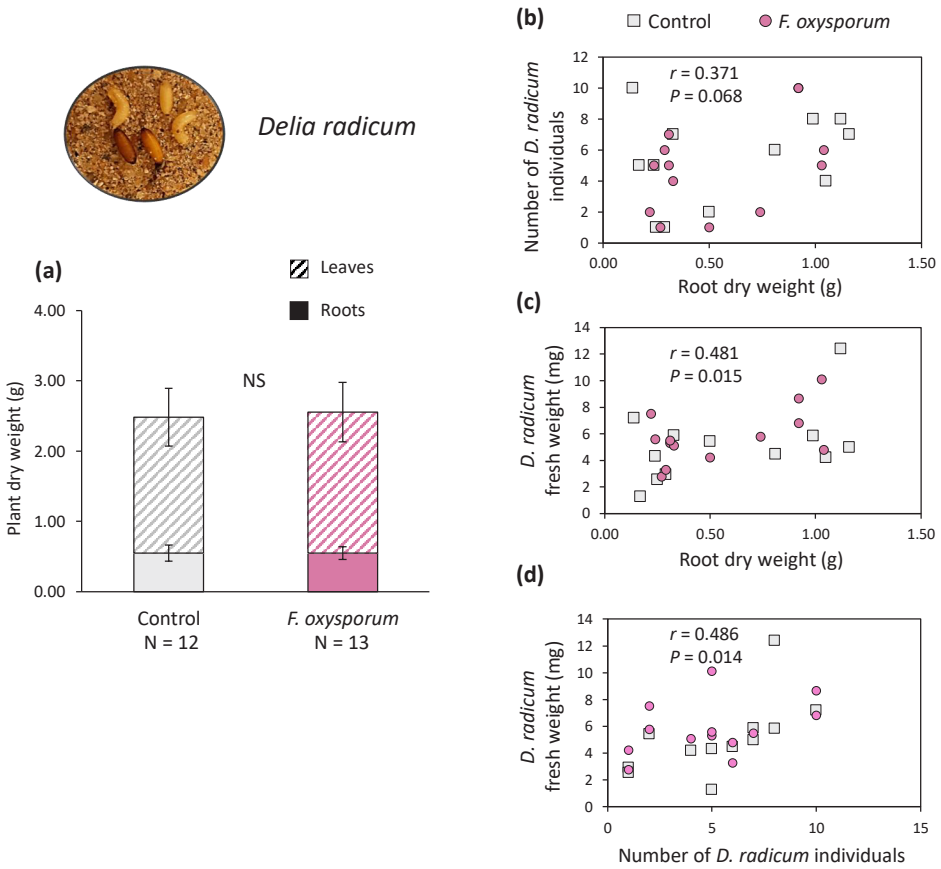


Figure 4. (a) Root and leaf dry weight of *Delia radicum*-infested *Brassica rapa* plants when exposed or not to *Fusarium oxysporum* volatiles. Plant dry weight of control plants and plants exposed to *F. oxysporum* volatiles was compared with Student *t*-test (NS: $P > 0.05$). Pearson correlations between **(b)** the number of *D. radicum* recollected per plant and the root dry weight, **(c)** the average fresh weight of *D. radicum* per plant and the root dry weight, and **(d)** the average fresh weight of *D. radicum* per plant and the number of individuals recollected. Number of individuals refers to the sum of larvae and pupae. "N" indicates the number of plant replicates.

Root exposure to *F. oxysporum* volatiles influenced the interaction between *B. rapa* roots and *H. schachtii* nematodes: upon continuous fungal volatile exposure, root colonisation was reduced and cyst development was slowed down, whereas egg load was increased. Growth of fungal volatile-exposed plants was also promoted. In contrast, fungal volatiles did neither affect performance of the cabbage root fly *D. radicum* nor plant growth. Together, our data show that fungal volatiles differentially affect the performance of two root herbivores and plant growth upon root herbivory. We hypothesise that specific enhancement of the nematode egg load and plant growth may result from the modulation by fungal volatiles for the fungus's own interest. Upon contact with the roots, *F. oxysporum* may indeed benefit

from colonising the roots as an asymptomatic endophyte and parasitising the nematodes (Roberts *et al.*, 1981; Nicolay and Sikofu, 1989; Gao *et al.*, 2008).

Interaction between *B. rapa* roots and *H. schachtii* nematodes was hindered upon root exposure to *F. oxysporum* volatiles. Fungal volatiles could have directly affected the nematodes themselves in the soil. Nematodes are indeed endowed with high sensitivity to volatiles for their navigation in soil (Rasmann *et al.*, 2012a; Rengarajan and Hallem, 2016). Previous studies have shown that volatiles from roots or soil microorganisms can affect nematode development and behaviour (Wuyts *et al.*, 2006; Turlings *et al.*, 2012; Cheng *et al.*, 2017; Sharma *et al.*, 2019; Wolfgang *et al.*, 2019). For example, volatiles emitted by some *F. oxysporum* strains negatively affect egg hatching of the root-knot nematode *Meloidogyne incognita* (Terra *et al.*, 2018). Additionally, some bacterial volatiles can act as nematicides and chemotactic agents to *M. incognita* (Cheng *et al.*, 2017). Thus, it is plausible that fungal volatiles negatively affected survival of *H. schachtii* juveniles in soil or disrupt their foraging behaviour, resulting in lower colonisation rate of plant roots. A lower colonisation may exert less herbivore pressure on the plant and allowed enhanced growth.

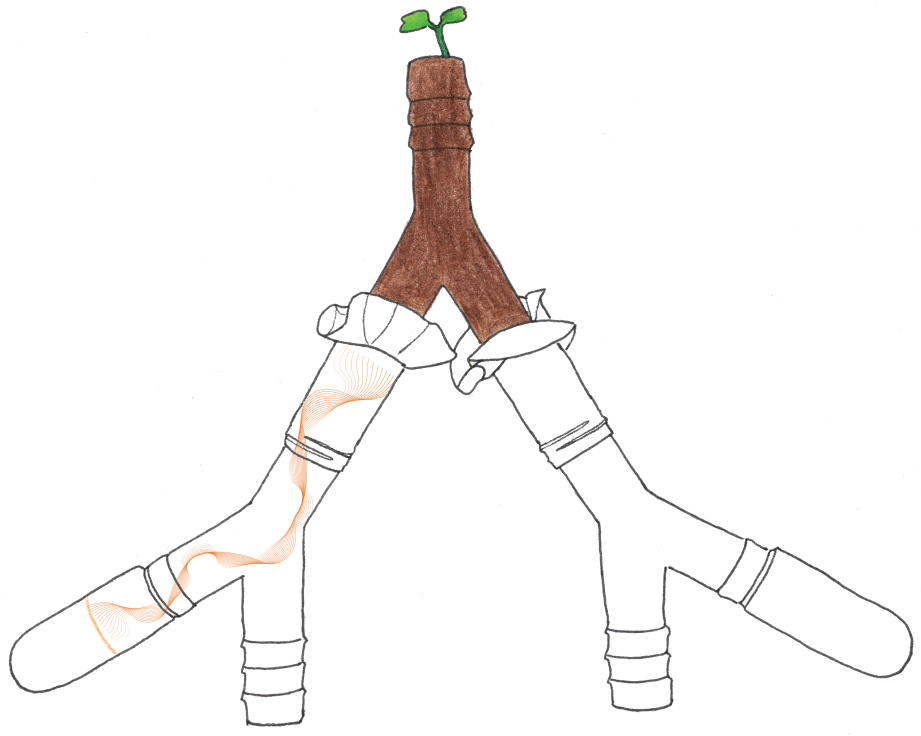
Additionally, *F. oxysporum* volatiles may also have indirectly affected the nematode performance *via* plant-mediated changes. We previously demonstrated that root exposure to volatiles from fungal pathogens can alter plant growth and resistance to caterpillars (Cordovez *et al.*, 2017; Moisan *et al.*, 2019; Chapter 2 of this thesis). Upon root penetration and colonisation, juveniles may have encountered more structural and chemical plant defences, resulting in lower colonisation rate (Miroslaw *et al.*, 2005). Furthermore, survival and development of the nematodes depend on the establishment of the feeding site, *i.e.* the syncytium. For this, cyst nematodes manipulate plant machinery by inhibiting plant resistance responses (Gheysen and Mitchum, 2011; Bohlmann and Sobczak, 2014). Root exposure to fungal volatiles may have modified plant resistance responses to cell damage caused by the nematodes, thus influencing establishment of the syncytium. Additionally, biochemical and physiological changes in the host plant affect the quantity and quality of nutrients available in the syncytium, thus influencing the cyst content (Betka *et al.*, 1991; Gaur *et al.*, 1995). Cysts, which are the dead body of the female, contain the new progeny in the form of eggs, and larger cysts support higher numbers of eggs. Thus, plant growth promotion by fungal volatiles could have enabled higher food intake by female nematodes, hence increasing the number of eggs. Yet, all these effects remain hypothetic and further research investigating these hypotheses will shed light on the underlying mechanisms.

Interestingly, effects of *F. oxysporum* volatiles differed between the two root herbivores tested. Unlike *H. schachtii* nematodes, larvae of *D. radicum* performed equally on control plants and plants exposed to fungal volatiles, and plant growth was unaffected. First of all, we hypothesise that these differences can be explained by the size of the pots used. In the set-up which is used for *D. radicum*, the top compartment was larger than for *H. schachtii*, hence making roots and the insect herbivore further away from the bottom compartment with the source of the fungal volatiles. Thus, plant roots and insect larvae were less likely

to be directly exposed to fungal volatiles. Yet, previous studies showed that behaviour of newly hatched *D. radicum* larvae is affected by root volatiles (Košťál, 1992; Johnson and Nielsen, 2012), but to our knowledge, little is known about effects of microbial volatiles on the insect root herbivore. Secondly, fungal volatiles may also elicit biochemical and physiological changes in the roots that can affect plant resistance to specific attackers. For instance, colonisation of *Arabidopsis thaliana* roots by the rhizobacteria *Pseudomonas simiae* led to reduced performance of the caterpillar *Mamestra brassicae* (Pangesti *et al.*, 2016), whereas it enhanced the performance of the aphid *Myzus persicae* (Pineda *et al.*, 2013). As *D. radicum* larvae and *H. schachtii* nematodes cause different types of root damage, one can expect differences in fungal volatile-induced plant responses as well. Lastly, we speculate that the fungus modulates plant interactions with other organisms for its own interest. Since *F. oxysporum* is a plant endophytic fungus, we hypothesise that fungal volatiles foster root colonisation by herbivores when they facilitate further root colonisation by the fungus. Root colonisation by nematodes increases root branching (Willig *et al.*, 2018) and increases number of possible entry points for the fungus (Bergeson, 1972; Back *et al.*, 2002). Also, plant responses to nematode feeding may decrease plant resistance to fungal colonisation, rendering roots suitable to fungal development (Back *et al.*, 2002). Additionally, *F. oxysporum* can also parasitise eggs of plant-parasitic nematodes (Roberts *et al.*, 1981; Nicolay and Sikofu, 1989; Gao *et al.*, 2008). Thus, slower cyst development and increase of nematode egg number may be beneficial to *F. oxysporum,* offering more eggs to parasitise as an alternative host. Actual inoculation of *F. oxysporum* on these fungal volatile-exposed *B. rapa* plants following root herbivory will confirm these hypotheses. Taken together, our findings show that volatiles from a soil-borne fungus can modulate interactions of plants with root attackers belowground.

Acknowledgements

We thank Unifarm of Wageningen University for growing the rutabaga plants used to rear the root insect herbivore. We also thank the Microbe-Plant-Interactions group of Utrecht University (NL) for providing us with the strain *F. oxysporum* f.sp. *raphani,* Nicole van Dam (iDiv, DE) for providing us with seeds of *B. rapa,* and the Sugar Beet Institute in Bergen op Zoom (NL) for providing us with the nematode cysts. A particular thanks to Elma Raaijmakers, Ellen van Oorschot, Roel Wagenaar and Gerard Korthals for their kind advices on the nematode assay. This research was partially funded by an NWO Spinoza award to MD.





Chapter 6

Volatiles from soil-borne fungi affect directionnal growth of roots

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Abstract

Volatiles play major roles in mediating ecological interactions between soil (micro)organisms and plants. For instance, it is well-established that microbial volatiles can increase root biomass and lateral root formation. To date, however, it is unknown whether microbial volatiles affect directional root growth. As proof-of-concept, we designed a root Y-tube olfactometer, and tested the effects of volatiles from four soil-borne fungi on directional growth of *Brassica rapa* roots in soil. Subsequently, we compared the fungal volatile organic compounds (VOCs) previously profiled with Gas Chromatography-Mass Spectrometry. We show that directional root growth was differentially affected by fungal volatiles. Roots grew more frequently towards volatiles from the root pathogen *Rhizoctonia solani,* whereas volatiles from the other three saprophytic fungi did not impact directional root growth. GC-MS profiling showed that seven VOCs were exclusively emitted by *R. solani*. Our results show that directional root growth in soil can be affected by volatiles from soil-borne fungi. Although the specific fungal VOCs that affect directional root growth and the underlying signal transduction cascade were not yet identified, these findings provide novel insight in root growth in soil and raised new questions about the intriguing chemical cross-talk between roots and soil-borne fungi.

Keywords

Chemotaxis, fungal volatiles, *Rhizoctonia solani,* root attraction, root olfactometer, root pathogen

Introduction

Roots are a vital plant organ that ensures anchoring in the soil and mechanical support, as well as water and nutrient acquisition (Erktan *et al.*, 2018). The rhizosphere, *i.e.* the narrow zone surrounding and influenced by roots, harbours dynamic communities of microorganisms that exploit root exudates as food source (Philippot *et al.*, 2013; Hassan *et al.*, 2019). Root exudates, including volatiles, can recruit beneficial microorganisms from a distance to enhance plant performance (Rasmann *et al.*, 2005; Haichar *et al.*, 2014; Schulz-Bohm *et al.*, 2018). However, plant pathogens, plant-parasitic plants and nematodes can also exploit root volatiles to locate their host plants (Rasmann *et al.*, 2012b; Ruyter-Spira *et al.*, 2013).

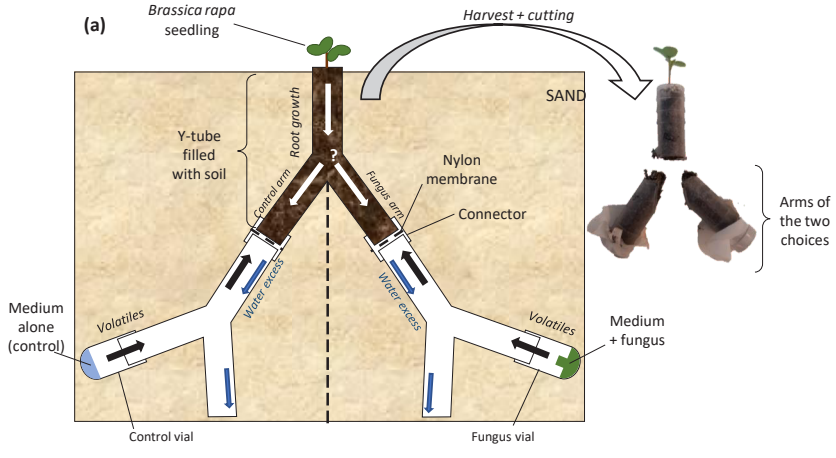
Soil microorganisms also produce a large array of volatiles that can promote plant growth and affect root architecture, *e.g.* shortening of the primary roots and enhancing lateral root formation (Garnica-Vergara *et al.*, 2015; Casarrubia *et al.*, 2016; Piechulla *et al.*, 2017; Moisan *et al.*, 2019; Chapter 2 of this thesis). To date, however, it remains unknown whether microbial volatiles can affect directional root growth. Considering that plant pathogenic and non-pathogenic soil-borne microorganisms emit distinct volatile blends (Müller *et al.*, 2013; Moisan *et al.*, 2019; Chapter 2 of this thesis), plants may be able to discriminate among volatiles from pathogenic and non-pathogenic beneficial microorganisms and respond differently. In particular, roots are expected to grow away from soil-borne pathogens. To investigate whether volatiles from soil-borne fungi affect root biomass and root directional growth in soil, we designed a root Y-tube olfactometer, providing roots of *Brassica rapa* with the choice to grow towards one volatile source: either towards volatiles emitted by the soil-borne fungus or towards volatiles from the agar medium used for fungal growth (*i.e.* the control).

Materials and Methods

Plant material and fungi

Brassica rapa L. (Brassicaceae), known as wild turnip, is an annual plant with a non-tuberous tap root. The accession used in this study originated from a wild *B. rapa* population in Maarssen, The Netherlands (Danner *et al.*, 2015). Seeds were surface-sterilised with chlorine gas as previously described by Cordovez *et al.* (2017). The four soil-borne fungi used in this study were *Fusarium oxysporum* f. sp. *raphani* (WCS600), *Rhizoctonia solani* AG2-2 IIIb, *Chaetomium indicum* (CBS 356.92) and *Trichoderma viride* (CBS 101227). We previously showed that volatiles emitted by these fungi differentially affected *Arabidopsis thaliana* growth *in vitro* (Moisan *et al.*, 2019; Chapter 2 of this thesis). Both *C. indicum* and *T. viride* can colonise the rhizosphere or endosphere of roots of Brassicaceae (Junker *et al.*, 2012), whereas *F. oxysporum* and *R. solani* can be pathogenic to brassicaceous species (Leeman

et al., 1995; Pannecoucque and Höfte, 2009). However, their pathogenicity was not known yet for *B. rapa*, and therefore tested in this study (Box S1). The results of these bioassays showed that *R. solani* was indeed pathogenic to *B. rapa* with the typical root lesions and spear-tip symptoms and a concomitant reduction in root biomass. For the other three fungi, no disease symptoms nor adverse effects on root or shoot biomass of *B. rapa* were observed (Fig. S1). All fungi were cultured on 1/5th strength Potato Dextrose Agar (1/5th PDA) at 25°C.



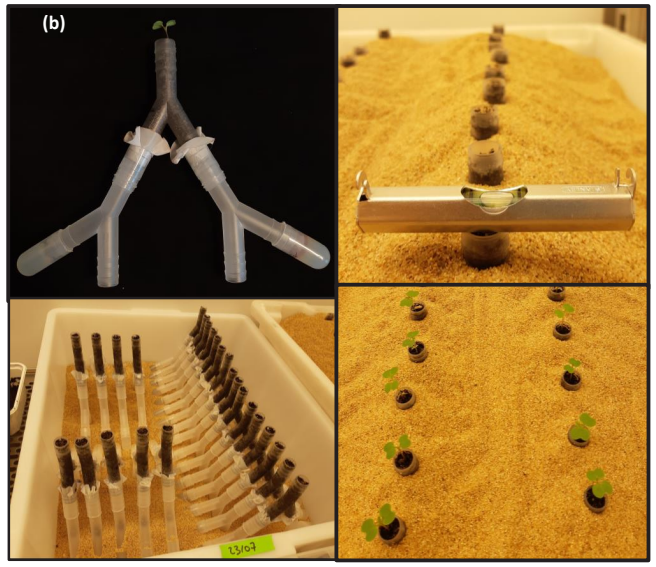


Figure 1. (a) Schematic representation of the root Y-tube olfactometer set-up. One *Brassica rapa* seed was sown on the top of the Y-tube containing soil, and primary root of the germinated seedling was "offered the choice" to grow into one of the arms of the Y-tube: either towards the volatiles emitted by the test fungus on agar or towards the volatiles from the agar medium only, *i.e.* the control. **(b)** Photographs of the root Y-tube olfactometers during the experiment. Each olfactometer was buried in a box filled with sand, and kept in a growth cabinet for seven days. At the harvest, the Y-tubes were cut into three parts**.** Photo credits: Kay Moisan.

Root Y-tube olfactometer assay

The Y-tube olfactometer consisted of a Y-shaped plastic tube (Bürkle GmbH, Bad Bellingen, Germany) filled with a mixture of gamma-irradiated potting soil and sand (1:1 v:v potting soil and sand, ø 2 mm sieved), with the two arms pointing downwards. Each arm was connected to an empty tube which was connected to a vial (bottom of 13 mL screw cap tube, Sarstedt AG & Co. KG, Nümbrecht, Germany) containing agar medium with or without one of the four fungi (Fig. 1a). Prior to inoculation, these vials were surface-sterilised with 70% ethanol in a flow cabinet, rinsed with ample sterile water, and dried by evaporation and kept under UV light for 45 min. In each vial, 3 mL of 1/5th PDA medium was poured, and the fungi were inoculated on the same day as the sowing of the seeds. Tubes connecting the vials to the arms of the Y-tube had an opening in the middle to allow excess water to flow out. Also, a nylon membrane (1 µm mesh size, Plastok associates Ltd., Birkenhead Wirral, UK) was placed between these tubes and the arms of the Y-tube to allow exchange of volatiles, while preventing physical contact between the roots and the fungus.

After assembling all tubes together, sterile *B. rapa* seeds were sown on the soil surface on top of the Y-tubes. Primary roots of the germinated seedlings could grow downwards into one of the two arms of the Y-tube: either towards the volatiles emitted by the test fungus or towards the volatiles from the agar medium only, *i.e.* the control. The four fungi were individually tested against the control, and as a negative control we also tested control against control. One seed was sown per olfactometer and each fungal species was replicated in 30 independent root olfactometers. To mimic natural darkness conditions for the fungi and roots growing in the soil, the assembled root olfactometers were buried in a box filled with sand and placed in a growth cabinet (21 °C; 16:8 h L:D; 70% RH). Positioning of the olfactometers followed a random complete block design, and the position of the fungus and control sides was alternated every two replicates. To avoid geotropism/gravity bias, each olfactometer was checked for horizontality with a level bubble measuring tool (Fig. 1b). Plants were harvested seven days after sowing to focus on the "choice" of the primary roots and to prevent growth of lateral roots in the two arms of the Y-tube. Y-tubes were cut with a hot blade to separate the two arms of the Y-tube (Fig. 1a). Roots were collected from both arms of the Y-tube and washed to remove soil particles. Length of the primary roots was measured, and all roots were dried at 70 °C for 5 days and weighed. Replicates with non-germinated seeds or replicates where the control agar medium developed microbial contamination were discarded. The "choice" of the primary roots (*i.e.* arm of the Y-tube in which the primary root grew) were analysed with binomial tests, and frequencies of root growth towards fungal volatiles were compared between the four fungi using a χ^2 test (H_0 = 0.50). Differences in root dry weights between control and fungal volatile-exposed plants were analysed with paired-sample Student *t*-tests, and differences in leaf dry weights upon choice of the primary root were analysed with Student *t*-tests (α = 0.05).

Analysis of fungal volatile organic compounds

To compare the volatile organic compound (VOC) profiles of the four fungi, we statistically analysed VOC blends that were previously profiled as part of a larger number of soil-borne fungi (Moisan *et al.*, 2019; Chapter 2 of this thesis). Total Ion Chromatograms were used to generate values for peak area, and the VOC profiles of the four selected fungi were analysed through a Projection to Latent Structures Discriminant Analysis (PLS-DA) (SIMCA 15, Umetrics AB, Umeå, Sweden). This model was evaluated using a 7-fold cross-validation test (N = 200; One-way ANOVA) and with R^2 and Q^2 estimates.

Results and discussion

Fungal volatiles differentially affected directional growth of *B. rapa* roots (Fig. 2a). Primary roots grew more frequently towards volatiles from *R. solani* than towards volatiles from the agar medium control (Fig. 2a; *Obs.Freq* = 0.72; *P* = 0.033). In contrast, root directional growth was not significantly affected by the other three fungi (Fig. 2a; *F. oxysporum*: *Obs. Freq* = 0.64; *P* = 0.076; *C. indicum: Obs.Freq* = 0.62; *P* = 0.097; *T. viride; Obs.Freq* = 0.56; *P* = 0.175). These results suggest that *B. rapa* roots perceive and respond to volatile blends emitted by specific soil-borne fungi. Interestingly, directional growth was significantly affected but length of the primary roots (Fig. 2b; Student *t*-tests; *P* > 0.05) and total root dry weight (Fig. 2c; Student *t*-tests; *P* > 0.05) were not impacted by the "choice" of the primary roots. Whereas fungal volatiles promoted root biomass *in vitro* (Casarrubia *et al.*, 2016; Cordovez *et al.*, 2017; Moisan *et al.*, 2019; Chapter 2 of this thesis), our results indicate that root growth rate of *B. rapa* seedlings was not increased in soil upon exposure to the fungal volatiles.

To begin to unravel which specific volatiles emitted by *R. solani* contribute to the attraction of *B. rapa* roots, we analysed the VOC profiles of all four fungi obtained from our previous study when fungi were cultured on the same media as used in the present study in the absence of plants (Moisan *et al.*, 2019; Chapter 2 of this thesis). Comparison of the four VOC profiles together shows that *C. indicum* and *F. oxysporum* profiles differ from each other and from the profiles of *R. solani* and *T. viride,* whereas VOC profiles of *R. solani* and *T. viride* were more similar compared to the other two fungi (Fig. 3a; PLS-DA; R^2 = 0.95; Q^2 = 0.90, $P_{CV\ ANOVA}$ < 0.001). Yet, VOC profiles of *T. viride* and *R. solani* were distinct (Fig. S2; PLS-DA; R^2 = 0.94; Q^2 = 0.90, $P_{CV\ ANOVA}$ < 0.001). Qualitative comparison reveals that only two VOCs, 1-octen-3-ol and 1-octen-3-one, were commonly emitted by *R. solani, T. viride* and *C. indicum,* while 7 and 13 VOCs were unique to *R. solani* and *T. viride,* respectively (Fig. 3b). The seven VOCs unique to *R. solani* include 3-octanone, diethyl-phtalate, linalool, methyl thiocyanate, nerolidol and two unknown VOCs (Fig. 3b and c). Surprisingly, none of these individual VOCs have, to our knowledge, been previously shown to promote root growth, but instead had adverse effects on seed germination and root growth (Table S1).

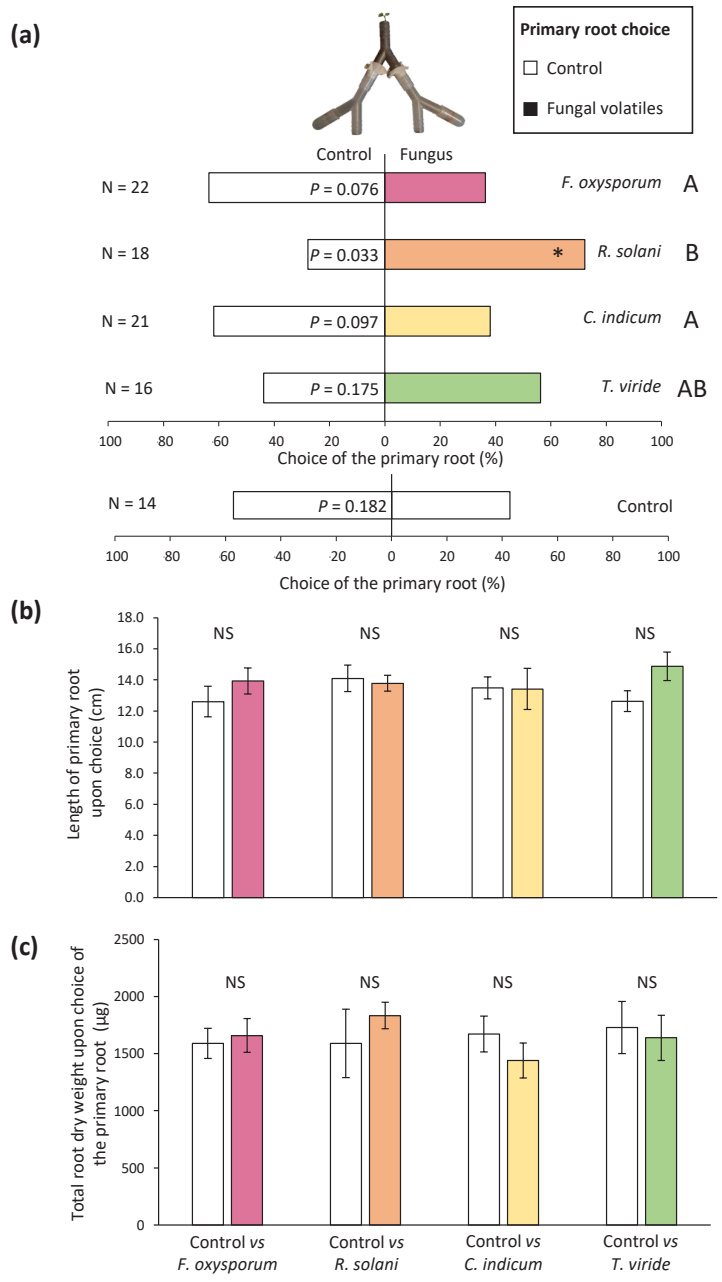
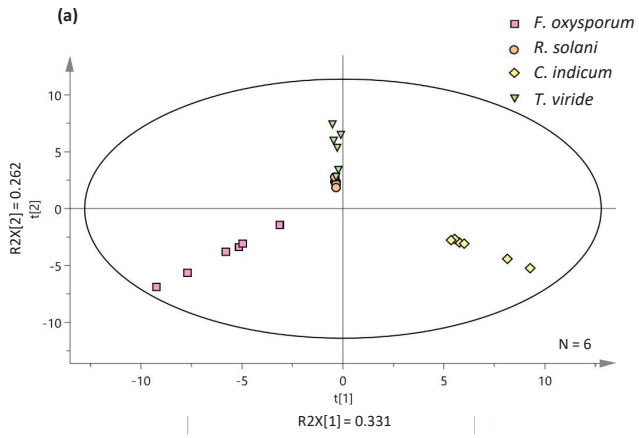


Figure 2. (a) Percentages of *Brassica rapa* primary roots growing into the control arm (*i.e.* towards volatiles of the agar medium) or into the fungus arm (*i.e.* towards fungal volatiles) seven days after sowing. Control *versus* control was used as a negative control. "N" indicates the number of root olfactometer replicates. Choices of primary roots were analysed with binomial tests (H_0 = 0.50), and frequencies of choices towards the fungal volatiles between the four different two-choices combinations were compared using χ^2 tests. Uppercase letters indicate pairwise differences between control-*versus*-fungus choices. **(b)** Length (mean ± SE) of the primary root and **(c)** total root dry weight (mean ± SE) upon choice of the primary root towards the control or the fungal volatiles. Differences in primary root length and root dry weight were analysed with Student *t*-tests (NS: *P* > 0.050).



(b)

Compound name	F.oxysporum	R. solani	C. indicum	T. viride
1,3-butadiene, 2-methyl			■	
1-butanol			■	
1-decanol acetate	■			
1-octen-3-ol		■	■	■
1-octen-3-one		■	■	■
1-pentanol			■	
2,3-butanedione	■			
2,4-hexadienal, *(E,E)*			■	
2-butanone			■	
2-butenal, 2-methyl			■	
2-butenal, 3-methyl	■		■	
2-epi-beta-caryophyllene, *(E)*	■			
2-methyl-1-butanol				■
2-methyl-1-butyl acetate	■			
2-methyl-1-propanol	■			■
2-methyl-3-buten-2-ol			■	
2-methylcyclopentanone			■	
2-methylpropanal	■			
2-nonen-4-one, *(E)*				■
2-pentylfuran				■
3-heptanone, 5-methyl			■	■
3-hydroxy-2-butanone	■			
3-methyl-1-butanol	■		■	

Compound name	F.oxysporum	R. solani	C. indicum	T. viride
3-methyl-1-butanol acetate	■			
3-methyl-2-buten-1-ol			■	
3-methyl-2-pentanone			■	
3-methyl-3-buten-1-ol	■		■	
3-methylbutanal	■			
3-methylbutanal, oxime, anti			■	
3-octanol acetate			■	
3-octanone		■		
4-heptanol	■			
4-methyl-2-pentanone	■			
6-pentyl- 2H-pyran-2-one				■
alloaromadendrene	■			
allyl hexanoate				■
alpha-zingiberene				■
aromadendr-9-ene	■			
ascaridole	■			
benzoic acid, 2-methylamino				■
benzyl alcohol			■	
beta-sesquiphellandrene				■
caryophyllene oxide				■
cyclohexanone	■			
diethyl-phtalate		■		
ethyl acetate	■			

Compound name	F.oxysporum	R. solani	C. indicum	T. viride
gamma-humulene	■			
linalool		■		
methyl thiocyanate		■		
methyl tiglate	■			
nerolidol, *(E)*		■		
nerolidol, *(Z)*				■
nonyl acetate	■			
phellandral			■	
phenol			■	■
phenol, 3-methyl				■
sesquisabinene hydrate, trans				■
unknown *m/z* 73		■		
unknown *m/z* 100			■	
unknown *m/z* 113			■	
unknown *m/z* 114			■	
unknown *m/z* 129		■		
unknown *m/z* 138				■
unknown *m/z* 146			■	
unknown *m/z* 149	■			
unknown *m/z* 161	■			
unknown *m/z* 164 (27.92)	■			
unknown *m/z* 164 (28.71)	■			
unknown *m/z* 189	■			

Figure 3. (a) Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile organic compounds (VOCs) collected from the headspace of the soil-borne fungi: *Fusarium oxysporum f.sp. raphani, Rhizoctonia solani, Chaetomium indicum* and *Trichoderma viride.* Grouping pattern of samples according to the first two principal components and the Hotelling's T2 ellipse confining the confidence region (95%) of the score plot. **(b)** List of compounds detected in the fungal VOC headspace of the four fungi.

Plant responses may depend on the concentration of the specific VOCs or on a combination of several of these VOCs (Ueda *et al.*, 2012). Also the timing and duration of VOCs exposure may determine the phenotypic outcome. Thus, it remains unknown which active compounds or groups of compounds are responsible for the attraction of *B. rapa* roots. Further research exploring the production and dynamics of these fungal VOCs *in situ, i.e.* in the presence of roots, will shed more light on these interactions and will help to pinpoint the specific VOCs that mediate these root phenotypic responses.

To date, only limited information is available about the mechanisms of root perception to chemicals in soil and the signal-transduction cascades (Chen *et al.*, 2012; Delory *et al.*, 2016; Sharifi and Ryu, 2018b; Tyagi *et al.*, 2018). Yet, it is known that roots do not grow randomly through the soil matrix, but can grow towards nutrient patches (Hutchings and de Kroon, 1994; de Kroon and Mommer, 2006). Different physical and chemical stimuli may influence directional root growth such as a gradient in soil moisture (hydrotropism), light (phototropism) or gravity (gravitropism) (Eapen *et al.*, 2005; Mochizuki *et al.*, 2005; Band *et al.*, 2012). Thus, directional root growth may likely be influenced by volatiles in soil as well (Wenke *et al.*, 2010; Yokawa *et al.*, 2014). However, the mechanisms underlying the change in root directional growth are still unknown. The emergence of new technologies that allow non-invasive root imaging, such as X-ray or MRI (Nagel *et al.*, 2012; Rogers *et al.*, 2016; Atkinson *et al.*, 2019) can provide new insight in how root direction is affected by microbial volatiles *in situ*.

Intriguingly, *B. rapa* primary roots were only attracted to volatiles from *R. solani,* which was the only fungus that infected roots of the *B. rapa* accession used (Fig. S1). This result was counter-intuitive as we hypothesised that roots would predominantly grow towards beneficial fungi, while avoiding pathogens. The attraction of roots towards volatiles from a pathogenic fungus would provide clear advantages to the fungus, hence our findings suggest a manipulation of the plant growth by the fungus. A much larger set of pathogenic and non-pathogenic fungi need to be tested to support this assumption. Yet, the actual costs and benefits of the volatile emitter and receiver in soil ecosystems remain to be elucidated. Here, we showed that leaf dry weight was not affected upon choice of the primary roots towards the fungal volatiles (Fig S3**;** Student *t*-tests; $P > 0.05$). In conclusion, our study provides a first step towards deciphering root growth responses to volatiles emitted by soil-borne fungi. It raises fundamental questions about the evolutionary ecology of such interactions between roots and fungi, and in particular the concept of "fatal attraction".

Acknowledgements

We thank the Microbe-Plant-Interactions group of Utrecht University (Utrecht, NL) for providing us with the strain *F. oxysporum f.sp. raphani*, the Sugar Beet Research Institute (Bergen op Zoom, NL) for providing us with the strain *R. solani* AG2-2 IIIb, and thank Nicole van Dam (iDiv, Leipzig, Germany) for providing the seeds of *B. rapa*. We also thank Elvira Rachmawati for her assistance in the pilot assays of this setup. This research was partially funded by an NWO Spinoza award to MD.

Chapter 6

Supplemental Materials

Box S1. Protocol used to test the pathogenicity of *Rhizoctonia solani, Fusarium oxysporum, Chaetomium indicum* and *Tricherderma viride* on *Brassica rapa.*

To determine the pathogenicity of the four fungal strains on *B. rapa*, 1-week-old *B. rapa* seedlings were inoculated with the fungi, and disease incidence was recorded after two weeks. For this, surface-sterilised seeds were sown in plastic pots (6 x 6 x 7 cm) filled with autoclaved sand supplemented with Hyponex nutrient solution, and kept in a growth cabinet (25 ± 1 °C; 16:8 h, L:D; 70 ± 5% RH). For *R. solani*, an agar plug with mycelium was buried in the sand directly in contact with the roots. For the other three fungi, *B. rapa* plants were up-rooted, and roots were incubated for 3 min in a solution of conidia suspended in sterile demi-water (10^6 conidia mL^{-1}). Seedlings were subsequently replanted in sand. Control plants were inoculated either with a plug of sterile agar or by dipping roots into sterile demi-water. After the fungal inoculation, pots were kept inside trays with transparent lids for one week to ensure high humidity, favourable to the fungal infection. Each inoculation was replicated six to seven times. Two weeks after the inoculation, plants were harvested and disease symptoms (*e.g.* lesions, discoloration vascular tissue, wilting) were recorded in the aerial tissues and in the roots, the latter by using a binocular (Leica Microsystems B.V., Son, The Netherlands). Additionally, dry weights of roots and leaves were measured by drying plant tissues at 70 °C for 3 days. Root and leaf dry weight of fungus-inoculated plants and control plants (non-inoculated) were compared using one-way ANOVA and two-samples Student's *t*-tests (α = 0.05).

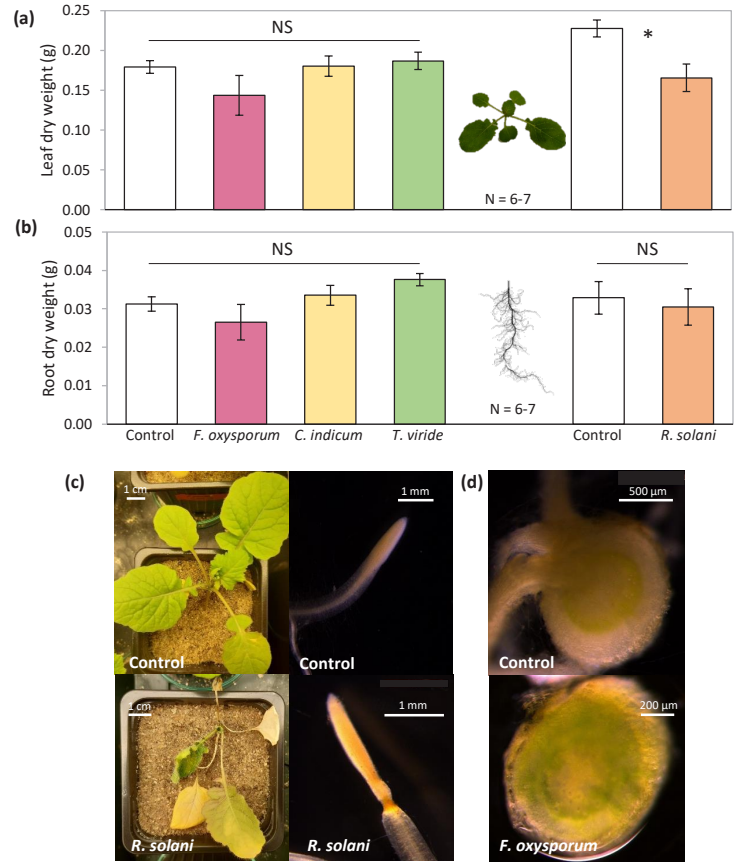


Figure S1. (a) Leaf and **(b)** root dry weight (mean ± SE) of two week-old *Brassica rapa* plants inoculated either with a conidia suspension of *Fusarium oxysporum*, *Chaetomium indicum* or *Trichoderma viride*, or inoculated with mycelium of *Rhizoctonia solani*, and their respective controls. **(c)** Photographs of the aboveground tissues and root tips of control plants and plants inoculated with *R. solani*. **(d)** Photographs of root cross sections of control plants and plants inoculated with *F. oxysporum*. Controls were either inoculated with sterile demi-water (used to suspend the fungal conidia) or with a plug of sterile agar (used to culture *R. solani* mycelium). Differences of dry weight were tested with one-way ANOVA and Student *t*-tests, and asterisks indicate statistical differences ($P < 0.05$; NS: $P > 0.05$).

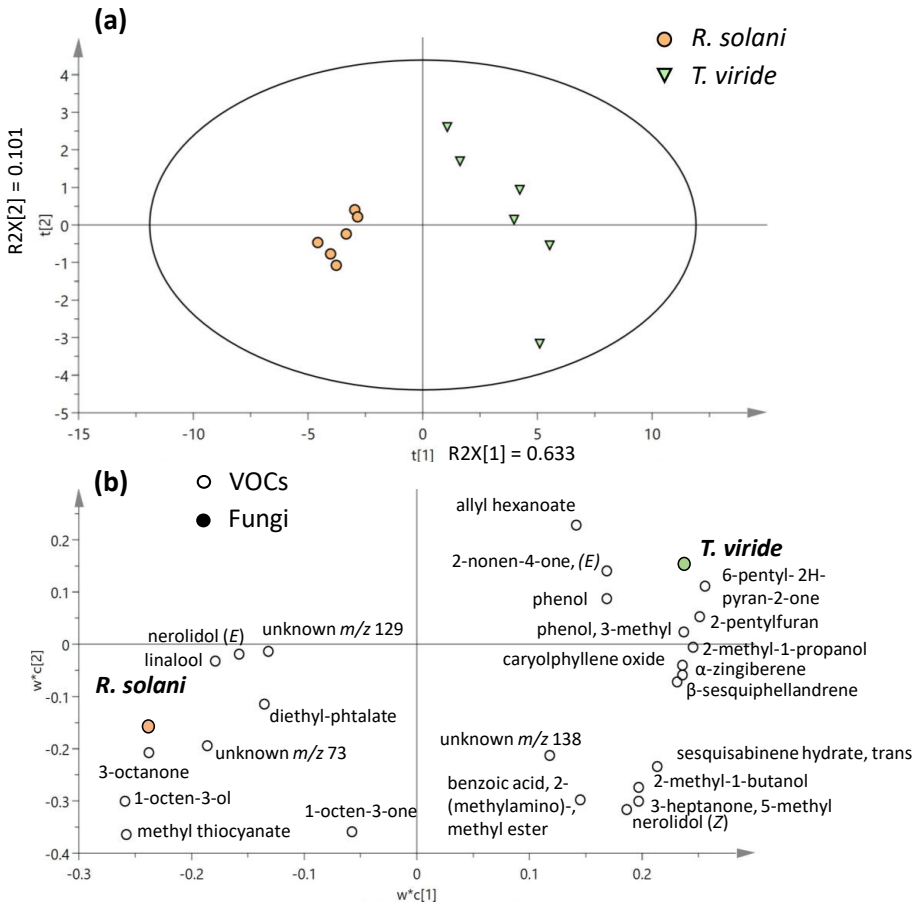


Figure S2. Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile organic compounds (VOCs) collected from the headspace of *Rhizoctonia solani* and *Trichoderma viride*. **(a)** Grouping pattern of samples according to the first two principal components and the Hotelling's T2 ellipse confining the confidence region (95%) of the score plot. **(b)** Loading plot showing the contribution of individual VOCs to the first two principal components.

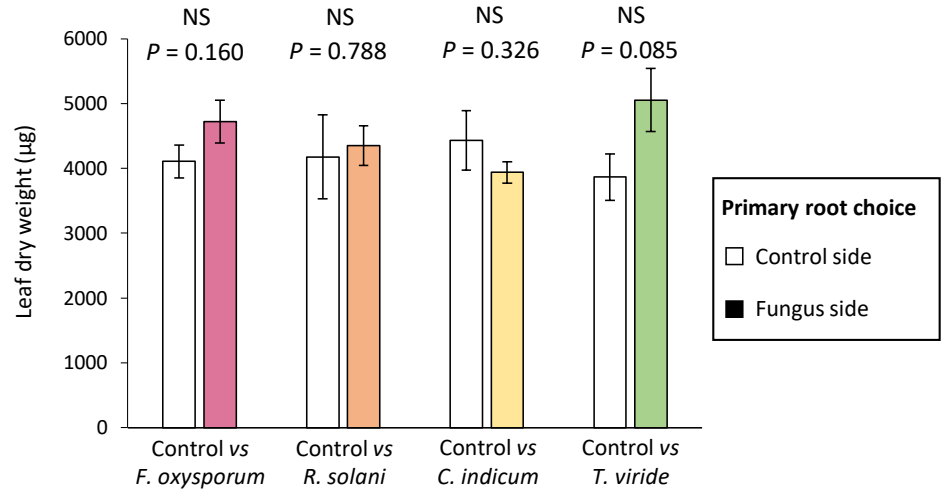
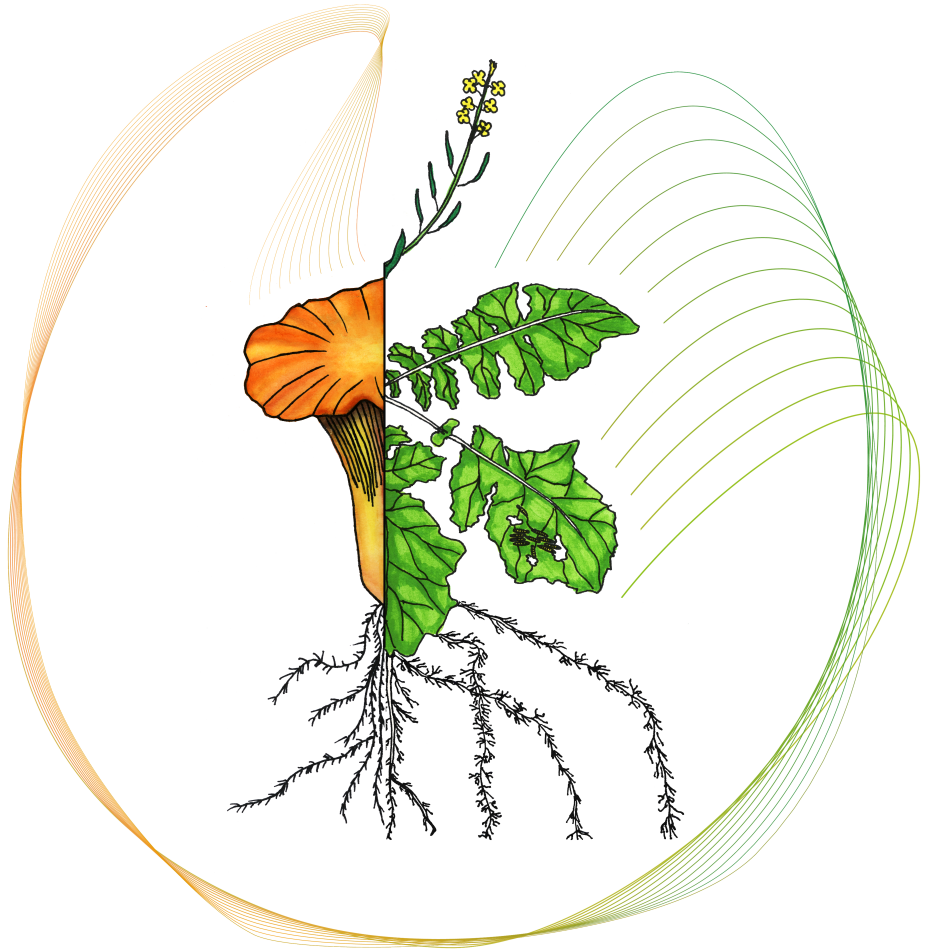


Figure S3. Leaf dry weight (mean ± SE) of seven day-old *Brassica rapa* seedings upon choice of the primary root towards the control or fungal volatiles. The fungi tested were *Fusarium oxysporum f.sp. raphani, Rhizoctonia solani, Chaetomium indicum* and *Trichoderma viride.* Differences of leaf dry weights and root:leaf ratios were analysed with Student *t*-tests (NS: $P > 0.05$).

Table S1. List of unique volatile organic compounds (VOCs) identified in the headspace of *Rhizoctonia solani,* and their reported effects on plants and soil organisms

VOC	Receiver	Effects	References
3-octanone	*Arabidopsis thaliana*	Leaf bleaching at high concentrations	Spivallo *et al.* (2007b)
		Reduction ofroot length	
		No effect on plant growth at low concentrations	Cordovez *et al.* (2017)
		Phytotoxic effects if high concentrations	
		Inhibition of root germination and growth	Lee *et al.* (2014)
	Gossypium hirsutum (cotton)	Inhibition of root elongation	Bradow (1993)
diethyl-phtalate	Several crops (lettuce, rice...)	Inhibition of germination	Xuan *et al.* (2006)
	Spinacia oleracea (spinach) and *Pisum sativum* (pea)	Inhibition of germination and growth Reduction of root length	Herring and Bering (1988)
	Raphanus sativus (radish)	Retardation of growth	Saarma *et al.* (2003)
	Spirodela polyrhiza (duckweed)	Oxidative stress	Cheng *et al.* (2012)
methyl-thiocyanate	*Lactuca sativa* (lettuce)	Possible inhibition of germination	Brown and Morra (1996)
	Rhizoctonia solani	Inhibition of growth	Ossowicki *et al.* (2017)
linalool	*Steinernema* and *Heterorhabditis* nematodes	Possitive or negative effects on chemotaxis	Laznik and Trdan (2013)
	Hordeum vulgare (barley)	Increase of root elongation	Wardle and Short (1982)
	Lactuca sativa (lettuce)	Inhibition of seed germination at high concentrations	
nerolidol	*Hordeum vulgare* (barley)	Reduction of root elongation at high concentrations	Wardle and Short (1982)
	Lactuca sativa (lettuce) and *Lepidium sativum* (cress)	Inhibition of seed germination	



Chapter 7

General Discussion

General discussion

Volatiles can mediate a myriad of ecological interactions within and between plants, animals and microorganisms, both above- and belowground (Heil and Karban, 2010; Heil, 2014; Piechulla and Degenhardt, 2014; Schmidt *et al.*, 2015; Dicke, 2016; Schulz-Bohm *et al.*, 2017b). In particular, volatiles from plant-associated microorganisms may modulate plant phenotypic plasticity to a larger extent than previously thought (Vandenkoornhuyse *et al.*, 2015; Dicke, 2016; Hassani *et al.*, 2018). For instance, volatiles emitted by bacteria and fungi were shown to tremendously impact plant growth and resistance (Bailly and Weisskopf, 2012; Piechulla *et al.*, 2017; Sharifi and Ryu, 2018a). Yet, little is known about plant responses to volatiles emitted by microorganisms with different lifestyles and the ecological consequences of such responses for subsequent plant interactions with herbivores. In my PhD project, I investigated whether plants perceive and discriminate between volatiles from pathogenic and non-pathogenic soil-borne fungi. I explored the effects of fungal volatiles on plant growth as well as plant defence to herbivores above- and belowground. Here, I summarise the main findings of my PhD project and discuss them in a broader ecological and evolutionary context.

Plant responses to fungal volatiles, from *in vitro* setups to *in vivo* exposure

Fungal volatile organic compounds as main drivers of plant growth promotion in vitro

Since the first discovery by Ryu *et al.* (2003), a considerable literature has emerged that further investigates plant growth promotion mediated by microbial volatiles (Farag *et al.*, 2006; Bitas *et al.*, 2013; Fincheira and Quiroz, 2018). The use of *in vitro* setups, such as bi- and tripartite Petri dishes and plastic containers, enabled the isolation of individual microbial strains to test and the separation of the microorganism from the plant while still allowing volatile exchange (Kai *et al.*, 2016). These methods allow a fast screening of plant responses to volatiles from a large array of microbial species under controlled conditions (**chapter 2**). Thanks to this approach, it is well established today that microbial volatiles may negatively or positively affect plant growth (Kottb *et al.*, 2015; Piechulla *et al.*, 2017; Fincheira and Quiroz, 2018). Specific volatile organic compounds (VOCs) such as 2,3-butanediol, dimethyl-disulfide (DMDS), 2-pentylfuran, acetoin, indole or β-caryophyllene have been identified as "bioactive" VOCs that promote plant growth individually (Chung *et al.*, 2016; Werner *et al.*, 2016; Fincheira and Quiroz, 2018). Besides VOCs, microorganisms also produce inorganic volatiles, such as CO_2, which could likewise positively affect plant growth. Thus, for studies using closed experimental systems, such as sealed Petri dishes, ascribing the observed plant growth promotion to microbial VOCs has often been questioned in the light of the accumulation of microbial CO_2 (Kai and Piechulla, 2009; Piechulla and Schnitzler, 2016).

In **chapter 2**, I assessed the growth of *Arabidopsis thaliana* seedlings when exposed *in vitro* to volatiles emitted by different soil-borne fungi. The effects of fungal volatiles ranged from no effect to biomass increase up to 150%. The increase in plant biomass was not correlated with the CO_2 concentrations measured in the presence or absence of the plants. Thus, these findings corroborate other studies that showed that microbial CO_2 is not the main driver of plant growth promotion but likely contributes to the effects induced by microbial VOCs (Sánchez-López *et al.*, 2016; Cordovez *et al.*, 2017; García-Gómez *et al.*, 2019). Therefore, *in vitro* setups, even in closed systems, represent a good starting point to assess and compare plant responses to microbial VOCs between different species or between microorganisms with different lifestyles (Casarrubia *et al.*, 2016; Sánchez-López *et al.*, 2016; **chapter 2**).

Fungal VOC-mediated plant interactions in soil

Recently, new setups have been developed to move one step closer towards more natural conditions by exposing plants *in vivo* to microbial VOCs (Fig. 1; Park *et al.*, 2015; Kai *et al.*, 2016; Tahir *et al.*, 2017c; Song *et al.*, 2019). In **chapter 3**, I designed a two-compartment pot system where plants were grown in soil, while still being physically separated from the fungus. In this system, VOCs emitted by the test fungus, which was kept in the bottom compartment, could disperse through the soil matrix to the top compartment where the plant was growing. In soil, microbial VOCs diffuse in gaseous and liquid phases, adsorb to soil particles or are subjected more rapidly to physicochemical degradation than in Petri dish assays (Insam and Seewald, 2010; Peñuelas *et al.*, 2014; Delory *et al.*, 2016). Therefore, it is expected that the further the VOC's receiver is located from the emitter, the lower the VOC concentration will be and the smaller the effects on the emitter. Currently, however, little is known on how persistent and active VOC blends are with increasing distance from their release point (Braasch and Kaplan, 2012). It was shown that the entomopathogenic nematode *Heterorhabditis megidis* was capable of locating its hosts (*i.e.* an insect root herbivore) 50 cm away from infested plants by tracing back the root VOCs in soil (Rasmann *et al.*, 2005; Turlings *et al.*, 2012). Similarly, VOCs emitted by *Carex arenaria* roots infested with the fungal pathogen *Fusarium culmorum* attracted beneficial bacteria from a distance of 12 cm (Schulz-Bohm *et al.*, 2018). Using the new pot system with *in vivo* root exposure, I showed in **chapters 3, 4 and 5** that plants still respond to fungal VOCs when growing in 20-cm-long pots, and these responses include faster development (**chapter 3**) and altered plant resistance to herbivory (**chapters 4 and 5**). Moreover, fungal VOCs can be attractive to roots growing in soil (**chapter 6**). Together, these findings show that fungal VOCs can disperse through the soil matrix and trigger plant responses from a distance.

Spatial and temporal scale of plant responses to fungal VOCs

Other advantages of the two-compartment pot system include the possibility of testing various plant species during their entire development, *i.e.* until the reproductive stage, and of exposing only plant roots to VOCs from soil-borne microorganisms. These allowed us

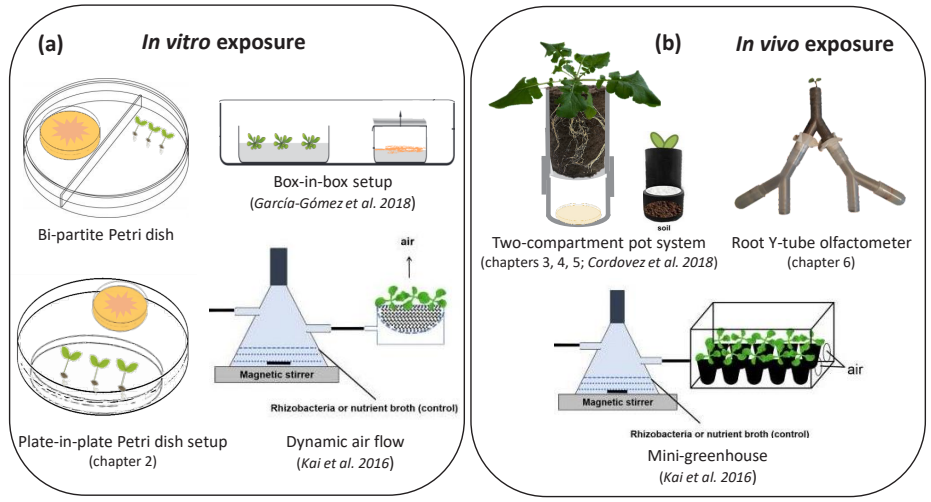


Figure 1. Experimental setups employed for *in vitro* or *in vivo* exposure of plants (roots and/or shoots) to microbial volatiles

to address spatial and temporal effects of plant responses to fungal VOCs. Interestingly, plant responses to fungal VOCs are not limited to the plant tissues that are exposed to the VOCs, but may affect below- and aboveground plant compartments systemically. For instance, exposure of *Brassica rapa* roots to VOCs from *Rhizoctonia solani* or *Phoma leveillei* affected bolting time and the number of buds and flowers of the exposed plants (**chapter 3**). Additionally, in **chapters 3 and 5**, I showed that not only plant resistance to root attackers can be affected by root exposure to fungal VOCs, but plant resistance to a caterpillar feeding on aerial plant tissues can also be influenced, even though these aerial plant tissues were not directly exposed to the fungal VOCs. Other studies that addressed plant responses upon root exposure to microbial VOCs *in planta* also reported enhanced shoot growth (Park *et al.*, 2015; Tahir *et al.*, 2017c; Cordovez *et al.*, 2018), and effects of microbial volatiles on plant growth depended on which tissues were exposed (Cordovez *et al.*, 2018). In addition, exposure of *A. thaliana* plants to VOCs from *Antirrhinum majus* flowers negatively affect growth of *A. thaliana* roots (Horiuchi *et al.*, 2007). Therefore, it seems a common phenomenon that VOC perception in plants leads to local as well as systemic responses. Yet, the signal-transduction cascade underlying systemic plant responses to VOCs remains poorly understood to date. Moreover, the effects of fungal VOCs on plants depend on the traits investigated. In **chapter 3**, I showed that exposure of *B. rapa* roots to fungal VOCs did not affect root biomass, whereas in **chapter 6**, directional growth of the primary root of *B. rapa* was influenced by fungal VOCs upon seed germination. These results also suggest that plant responses are dependent on the plant developmental stage as well as the duration of VOC exposure. Nevertheless, the effects of plant exposure to fungal VOCs in an early developmental stage, such as germinating seeds or seedlings, may

influence later plant developmental processes like flowering (Sánchez-López *et al.*, 2016; **chapter 3**), even after termination of the VOC exposure (Xie *et al.*, 2009; Cordovez *et al.*, 2017; **chapter 2**).

Modulation of plant defensive responses by volatiles from soil-borne fungi

Fungal VOCs influence plant growth upon herbivory

In response to stresses that adversely affect plant growth, one plant defence strategy consists of enduring the loss of biomass *via* compensatory growth (Strauss and Agrawal, 1999). This mechanism, coined tolerance (Schafer, 1971), can be enhanced by plant-associated microorganisms (Yang *et al.*, 2009; Pineda *et al.*, 2010). Recently, it was demonstrated that microbial VOCs can also help plants to endure abiotic stresses through the stimulation of plant growth (Bhattacharyya *et al.*, 2014; Liu and Zhang, 2015; Jalali *et al.*, 2017; Camarena-Pozos *et al.*, 2019). In **chapter 3**, I showed that exposure of *B. rapa* plants to fungal VOCs also affects biomass of different plant tissues upon attack by a caterpillar or a root insect herbivore, making plants less or more tolerant to herbivory aboveground and belowground. I report for the first time that prior plant exposure to fungal volatiles influences plant growth upon herbivore attack. I hypothesised that fungal VOCs could have stimulated growth of specific plant tissues. By reallocating resources from intact tissues to damaged ones, fungal VOCs may render a plant more tolerant to herbivory. Further research into resource partitioning will shed more light on the relative importance of the presumed mechanisms.

Influence of fungal VOCs on plant interactions with members of the phytobiome

Fungal VOCs can modulate plant resistance by affecting, directly or indirectly, the performance and behaviour of plant attackers and of mutualists from higher trophic levels. First, VOCs from soil-borne fungi can directly inhibit growth of other soil organisms, including plant pathogens, hence preventing root colonisation (Strobel *et al.*, 2001; Schmidt *et al.*, 2015; Werner *et al.*, 2016; Enespa and Chandra, 2017). Microbial VOCs can also mediate important inter-kingdom interactions belowground and affect the composition of soil communities (Wenke *et al.*, 2010; Rasmann *et al.*, 2012b; van Dam and Bouwmeester, 2016; van Dam *et al.*, 2016; Schulz-Bohm *et al.*, 2017b). In **chapters 3 and 5**, I showed that fungal VOCs can negatively affect root colonisation by root herbivores such as insects and nematodes. Aboveground, plant-associated organisms are less likely to be directly exposed to VOCs from soil-borne microorganisms, yet they may still be affected indirectly *via* plant chemical and phenotypic changes induced by fungal VOCs. Previous studies have shown that plant exposure to microbial VOCs reduced disease severity of leaf pathogens (Salas Marina *et al.*, 2011; Lee *et al.*, 2012; Farag *et al.*, 2013; Naznin *et al.*, 2014; Tahir *et al.*, 2017a). The induction of different plant-defence-related signal-transduction pathways was

suggested as the mechanism underlying enhanced resistance (Kishimoto *et al.*, 2007; Sharifi and Ryu, 2016; Cordovez *et al.*, 2017; Liu *et al.*, 2020). Up- or down-regulation of these signalling pathways may lead to changes in plant primary and secondary metabolism, *e.g.* accumulation of glucosinolates in *A. thaliana* leaves upon exposure to bacterial VOCs (Aziz *et al.*, 2016), which in turn may positively or negatively impact subsequent plant ecological interactions. So far, studies addressing the consequences of plant exposure to microbial VOCs on plant-insect interactions have been sparse and contrasting (Song and Ryu, 2013; D'Alessandro *et al.*, 2014; Aziz *et al.*, 2016; Cordovez *et al.*, 2017). For example, *Mamestra brassicae* caterpillars feeding on *A. thaliana* plants previously exposed to *R. solani* VOCs performed better than those on non-exposed control plants (Cordovez *et al.*, 2017), whereas *Spodoptera exigua* caterpillars performed worse on plants exposed to *Bacillus amyloliquefaciens* VOCs (Aziz *et al.*, 2016). Similarly, in **chapters 2 and 3,** I demonstrated that direct resistance of *A. thaliana* and *B. rapa* plants to caterpillars and root herbivores can be positively or negatively affected by VOCs from different fungal species. Furthermore, as a mechanism of plant indirect resistance, plants may attract natural enemies of the herbivores upon the release of herbivore-induced plant volatiles (HIPVs) (Dicke and Baldwin, 2010; Turlings *et al.*, 2012). In **chapter 4**, I did not find evidence that emission of HIPVs was affected by plant exposure to fungal VOCs, and recruitment of natural enemies of the insect herbivore was not jeopardised. In contrast, it was previously shown that direct application of bacterial VOCs in the soil or on insect-infested maize and cucumber plants attracted more parasitic wasps and predators than non-exposed plants (Song and Ryu, 2013; D'Alessandro *et al.*, 2014). Collectively, these findings highlight the ecological effects of VOCs from soil-borne fungi on mediating below- and aboveground interactions with members of the phytobiome. Moreover, plant responses to fungal VOCs are specific to the fungal species they are exposed to.

Public information or secret messages: do plants decipher fungal volatiles?

Diversity of fungal VOCs and potential use for chemotyping fungal lifestyles

VOCs are ubiquitous in all ecosystems and mediate numerous intra- and interkingdom interactions between plants, microorganisms and animals (Baldwin, 2010; Schmidt *et al.*, 2015; van Dam *et al.*, 2016; Schulz-Bohm *et al.*, 2017b). To date, approximately 1900 VOCs have been reported from fungi and bacteria (Korpi *et al.*, 2009; Morath *et al.*, 2012; Schenkel *et al.*, 2015; Lemfack *et al.*, 2017). Contrary to $CO_{2,}$ produced by all aerobic organisms, some VOCs can be emitted only by certain species and under certain growth conditions, which may make them more reliable cues to be exploited by the interacting organisms (Turlings *et al.*, 2012). Microbial VOC emission is highly dynamic and is influenced by different environmental conditions, such as substrate composition, the age of the microbe, temperature and humidity, as well as the presence of competitors (Wheatley *et al.*, 1997;

Spraker *et al.*, 2014; Weikl *et al.*, 2016; Guo *et al.*, 2019). Therefore, VOC profiles are context-dependent. Additionally, emission of microbial VOC blends is also species specific (Fischer *et al.*, 1999; Cordovez *et al.*, 2015; Guo *et al.*, 2019). While some individual compounds, such as 1-octen-3-ol, are commonly emitted by fungi, partial or total VOC blends can function as microbial VOC signatures, and therefore be used for taxonomic characterisation (Spinelli *et al.*, 2012; Cordovez *et al.*, 2015; Oliveira *et al.*, 2015; Tóth *et al.*, 2016). In **chapter 2,** I show that different soil-borne fungi emit a large diversity of VOCs and share only a few VOCs. Interestingly, VOC emission was recently suggested to be also specific to fungal lifestyle, hence VOC profiles may be used to discriminate ectomycorrhizal and pathogenic fungi from saprophytic ones (Müller *et al.*, 2013). Remarkably, in **chapter 2**, I also demonstrated that a range of plant pathogenic and non-pathogenic soil-borne fungi emitted distinct VOC blends in the absence of plants. Yet, VOC emission is dynamic and may change over time and in the presence of a host plant. Additionally, labelling of the fungi is difficult before contact with the host as fungi can switch their lifestyle from saprophytic to neutral endophytic or pathogenic (biotrophic or necrotrophic) upon certain biological and environmental conditions. However, it is unclear when this switch takes place (Hegedus and Rimmer, 2005; Minerdi *et al.*, 2011; Zeilinger *et al.*, 2015; Lofgren *et al.*, 2018). Thus, it remains unknown whether plants are able to perceive distinct VOC blends or specific individual VOCs (Ueda *et al.*, 2012), and translate these into information about the fungal lifestyle.

Plants smelling their enemies, a myth?

While plants are exposed to multiple attackers, such as herbivores and pathogens, that threaten their growth and reproductive success, plants may benefit from the help of mutualists, *e.g.* natural enemies of the herbivores or bacteria with antifungal activity. To anticipate enemies, *i.e.* getting ready for the attack, and to accommodate mutualists, *i.e.* facilitating beneficial interactions, plants are expected to discriminate between their friends and enemies as early as possible, to maximise fitness. However, the discrimination between mutualists and antagonists is not always a clear cut-off. For instance, upon colonisation of plant tissues, both pathogenic and beneficial microorganisms are initially recognised as alien by plants (Box 1). Moreover, the release of virulence factors can be delayed in some pathogenic microorganisms. Thus, these microorganisms cannot be immediately recognised by plants as pathogens. Apart from the complexity of recognition, it would be also advantageous for plants to anticipate such interactions with deleterious organisms even before physical contact. Recently, it was shown that plants could exploit VOCs from insect herbivores to anticipate further attack. Indeed, defences of *Pinus sylvestris* and *Solidago altissima* plants were primed upon perception of VOCs emitted by *Diprion pini* and *Eurosta solidaginis*, respectively (Helms *et al.*, 2014; Yip *et al.*, 2017; Bittner *et al.*, 2019). However, to date, it remains unknown whether plants can anticipate a future infection upon perception of VOCs from pathogens. The rhizosphere, *i.e.* the narrow zone of soil around the roots, harbours complex and dynamic microbial communities that comprise deleterious and beneficial microorganisms (Raaijmakers *et al.*, 2009). Plants were shown to actively and selectively recruit beneficial microorganisms in the rhizosphere *via* root

Box 1: Plant recognition of pathogenic and beneficial microorganisms upon colonisation

To successfully colonise their host, plant-associated microorganisms aim at manipulating or evading plant resistance responses. In this regard, plant survival relies on its ability to discriminate, as early as possible, beneficial microorganisms from pathogenic microorganisms that impair plant growth and cause disease, and to respond accordingly. To resist microbial invasion, plants have evolved immune responses upon recognition of non-self structures. However, in this process, the distinction between pathogens and beneficial microorganisms can be arduous since they share many of the same attributes (Preston Gail, 2004; Zeilinger *et al.*, 2015). Plant primary immune responses to microorganisms involve the recognition of common microbial features (Microbial-Associated Molecular Patterns, MAMPs), such as flagellin, which could therefore target non-pathogenic microorganisms as well. Upon colonisation by pathogenic microorganisms, these plant primary responses may lead to the abortion of microbial growth (Jones and Dangl, 2006). However, to overcome plant primary responses, successful pathogens release specific effectors that suppress plant immunity. Upon recognition of these virulence factors by surveillance proteins (so-called R proteins), resistant plants trigger another array of immune responses which cause, among other responses, hypersensitive cell death response at the infection site (Chisholm *et al.*, 2006; Jones and Dangl, 2006). In this infinite arm race, plants and pathogens co-evolve, with on one hand pathogens diversifying the production of effectors to counter plant immune responses, and on the other hand plants tailoring R proteins to recognise these new effectors (Keen, 1990; Chisholm *et al.*, 2006; Jones and Dangl, 2006). The "winner" of this race in time determines the susceptibility or resistance of the plant against the pathogen. In the case of plant colonisation by beneficial microorganisms, recognition of MAMPs results in a mild activation of plant immune responses (van Wees *et al.*, 2008). To successfully colonise the roots further, mutualists require hijacking of the plant immune system (Zamioudis and Pieterse, 2012). For instance, mycorrhizal fungi or rhizobia (*i.e.* nitrogen-fixing bacteria) secrete specific effectors that suppress primary plant responses and release Myc/Nod factors, respectively, to trigger symbiotic signalling and plant reprogramming (Lopez-Gomez *et al.*, 2011; Zamioudis and Pieterse, 2012). Other non-symbiotic mutualists, such as plant growth-promoting rhizobacteria and fungi, may undergo phenotypic changes as well to evade plant immune responses (Zamioudis and Pieterse, 2012). Interestingly, some of these interactions are not compatible, for instance, interactions between brassicaceous plants and mycorrhizal fungi, which is indicative of a high degree of specificity between plant-microbe systems. Therefore, the discrimination of friends and foes upon colonisation is a challenging task for plants and only a high degree of coordination and specific molecular dialogues between the plant and the microorganism will determine the outcome of the interaction (Preston Gail, 2004; Zeilinger *et al.*, 2015).

exudates (Berendsen *et al.*, 2018; Hu *et al.*, 2018), including VOCs (Schulz-Bohm *et al.*, 2018; Liu and Brettell, 2019). According to the root-brain hypothesis, plants "possess a sensory-based cognition which leads to behaviour and decisions", and roots are regarded as a plant "brain-like" organ (Baluška *et al.*, 2009). In line with this hypothesis, roots are also expected to exploit chemical cues in the soil such as microbial VOCs and to specifically respond to cues from microbial mutualists and antagonists. Since pathogenic and non-pathogenic fungi emit distinct VOC blends (**chapter 2**), I expected plants to discriminate between them. However, throughout my thesis, I showed that plants do not differentially respond to VOCs from pathogenic and non-pathogenic fungi. Instead, some plant responses were common to VOCs from both pathogenic and non-pathogenic fungi, while other responses were specific to the fungal strain emitting the VOCs. For instance, *in vitro* exposure of *A. thaliana* seedlings to VOCs from the pathogenic fungi *Verticillium dahliae and V. longisporum* and from the non-pathogenic fungus *Trichoderma viride* significantly accelerated plant flowering, whereas VOCs from the pathogenic fungus *Sclerotinia sclerotiorum* delayed it (**chapter 2**). Additionally, developmental time of an insect root herbivore feeding on *B. rapa* roots previously exposed *in vivo* to VOCs from the pathogenic fungus *R. solani* and from the non-pathogenic fungus *Fusarium oxysporum* was increased, whereas it was unaffected by VOCs from the non-pathogenic fungus *Ulocladium atrum* (**chapter 3**). These findings corroborate other studies that reported plant growth promoting effects or acceleration of plant development by VOCs from pathogenic microorganisms (Sánchez-López *et al.*, 2016; Hernández-Calderón *et al.*, 2018). Potentially, some fungal VOCs may be perceived as a common stress signal that accelerates plant development and reproduction. However, further investigations are needed to confirm whether this represents an escape strategy by plants. Together, these results suggest that plants do not discriminate between VOCs from pathogenic and non-pathogenic fungi.

Fungal VOCs: a manipulative weapon from opportunistic fungi?

Interestingly, in the plant responses to fungal VOCs reported above, the physical separation between plant roots and the test fungus was artificially maintained throughout the experiments. However, in the soil, VOCs can affect taxis of the receiving organisms such as bacteria (Schulz-Bohm *et al.*, 2018), nematodes (Rasmann *et al.*, 2005; Turlings *et al.*, 2012), protists (Schulz-Bohm *et al.*, 2017a) and likely also plant roots (Yokawa *et al.*, 2014). So far, it had remained unexplored whether root directional growth is affected by microbial VOCs in soil. In **chapter 6**, I developed a Y-tube root olfactometer to assess plant directional growth in response to VOCs from soil-borne fungi. Unexpectedly, roots of *B. rapa* were attracted to VOCs from *R. solani, i.e.* the only of the four fungal strain tested that caused disease symptoms in *B. rapa*. Additionally, fungal VOCs enhanced biomass of intact roots and of roots upon herbivory (**chapters 3 and 5**), and affected root architecture, *e.g.* shortening of the primary roots and enhancing lateral root formation (Garnica-Vergara *et al.*, 2015; Casarrubia *et al.*, 2016; Cordovez *et al.*, 2017). Although plants would only benefit from growing towards a beneficial fungus, an increase of the root system and attraction of roots would give clear advantages to the fungus. Motility of filamentous fungi is indeed limited

to the growth and branching of hyphae and the production of spores (Brand and Gow, 2009). In the vicinity of a host plant, taxis of fungal hyphae can be positively affected by chemical cues from the host plant, such as root exudates, although the reported responses were limited to only a few millimetres (Jansson *et al.*, 1988). Therefore, as suggested by Werner *et al.* (2016), VOCs may represent good infochemical candidates for opportunist fungi to perceive and manipulate a host plant from a distance. For instance, fungal VOCs can attract the host and stimulate it to increase the root surface, facilitating the colonisation by the fungus. Yet, to my knowledge, it has never been tested whether fungal VOC emission is modified upon the perception of VOCs from the roots of a host plant. Interestingly, *A. thaliana* seedlings first exposed to VOCs from the pathogenic fungus *R. solani* and then inoculated by this pathogen displayed more diseased leaves than non-exposed plants (Cordovez *et al.*, 2017). These findings suggest that VOCs from this pathogenic fungus reduced plant defence before contact to facilitate its future infection. However, more pathogenic fungi have to be investigated to confirm this hypothesis and further test the possible "fatal attraction" of roots towards VOCs from pathogenic fungi.

Concluding remarks and future perspectives

Using the two-compartment pot system, I showed that perception of volatiles from soil-borne fungi by roots in soil can influence growth and defence of *A. thaliana* and *B. rapa* plants against herbivores, and potentially enhance plant fitness. These effects were specific to the fungal species and to the VOC blends that roots were exposed to. Yet, fungi produce a large diversity of VOCs, and it is still unclear today whether plants respond to the full fungal VOC blend or selectively to a few bioactive compounds (Ueda *et al.*, 2012). Whereas biosynthesis and emission of VOCs have been well documented, it is surprising that mechanisms of VOC perception and signal-transduction cascades in the receiving organisms remain elusive (Heil, 2014; Tyagi *et al.*, 2018). Additionally, it remains unknown whether these interactions reflect a dynamic VOC dialogue between plants and fungi where both partners perceive and respond to each other, or whether it is a one-way interaction of one influencing the other or one eavesdropping on VOCs from the other. VOC dialogues have been already described in interactions between bacteria and fungi (Spraker *et al.*, 2014; Guo *et al.*, 2019), and preliminary data indicate that plants may also adjust their VOC emission in response to perception of fungal VOCs (Cordovez *et al.*, 2017). For a comprehensive understanding of VOC-mediated interactions, one would need to monitor real-time VOC emission in a defined system (Majchrzak *et al.*, 2020), and to mark the VOCs to identify the emitter, which raises several biological and technical challenges.

Furthermore, in my PhD thesis, I addressed plant responses to fungal VOCs in one-to-one interactions. However, in nature, soil harbours dense communities of soil organisms (1 g of soil = 10^9 bacteria, 10^6 fungi, 10^4 protozoa, 10^2 nematodes and other 10^4 invertebrates) that potentially all secrete VOCs. Taken together, soil may resemble a mosaic of odorous

hotspots where VOCs can disperse in soil or be taken up or degraded as soon as they are released. Aboveground, VOCs have been shown to disperse a few hundred meters away in the air, and could, therefore, be used as long-range infochemicals that mediate interactions between the emitter and receiver (Kessler *et al.*, 2008; Braasch and Kaplan, 2012). Yet, volatiles can be blended with others in the air, and the signalling molecules may be lost in this complex landscape (Aartsma *et al.*, 2017). Therefore, VOC-mediated interactions in soil are likely complex and dynamic as well.

Box 2: Outstanding questions

- ❖ How do plants perceive fungal VOCs? Do they respond to the full VOC blends or selectively to a few bioactive compounds? What is the signalling transduction cascade?
- ❖ Do plants and soil-borne fungi exchange VOCs in a dynamic dialogue?
- ❖ Do fungal VOCs affect microbial community composition and activity in soil?
- ❖ How do plants respond to simultaneous VOCs emitted by microbial communities that include beneficial as well as pathogenic microorganisms and in their natural substrates?
- ❖ Does prior root exposure to VOCs from pathogenic fungi facilitate plant infection upon root colonisation?
- ❖ Do fungal VOCs alter plant interactions with pollinators?

From my data, I conclude that plants did not discriminate between VOCs from pathogenic fungi and non-pathogenic fungi when exposed separately. To my knowledge, there is no evidence yet that plants possess specific receptors that recognise microbial VOCs similarly to MAMP recognition. As long as physical interaction is prevented, plants may respond specifically to VOCs from a distance, regardless of the potential pathogenicity of the fungus that is undefined before contact. To further explore the evolutionary history of such VOC-mediated interactions, it would be particularly interesting, though challenging, to assess the effects on the plant and fungus's fitness. To thrive and propagate, plants rely, among others, on the attraction of mutualists, such as natural enemies of herbivores, plant growth-promoting microorganisms and pollinators. In particular, recruitment of beneficial microorganisms in the soil strongly influences plant phenotypic plasticity, and VOCs can modulate plant interactions with mutualists (Dicke, 2016; Liu and Brettell, 2019). I show in my thesis that attraction of natural enemies of herbivores was not influenced by plant

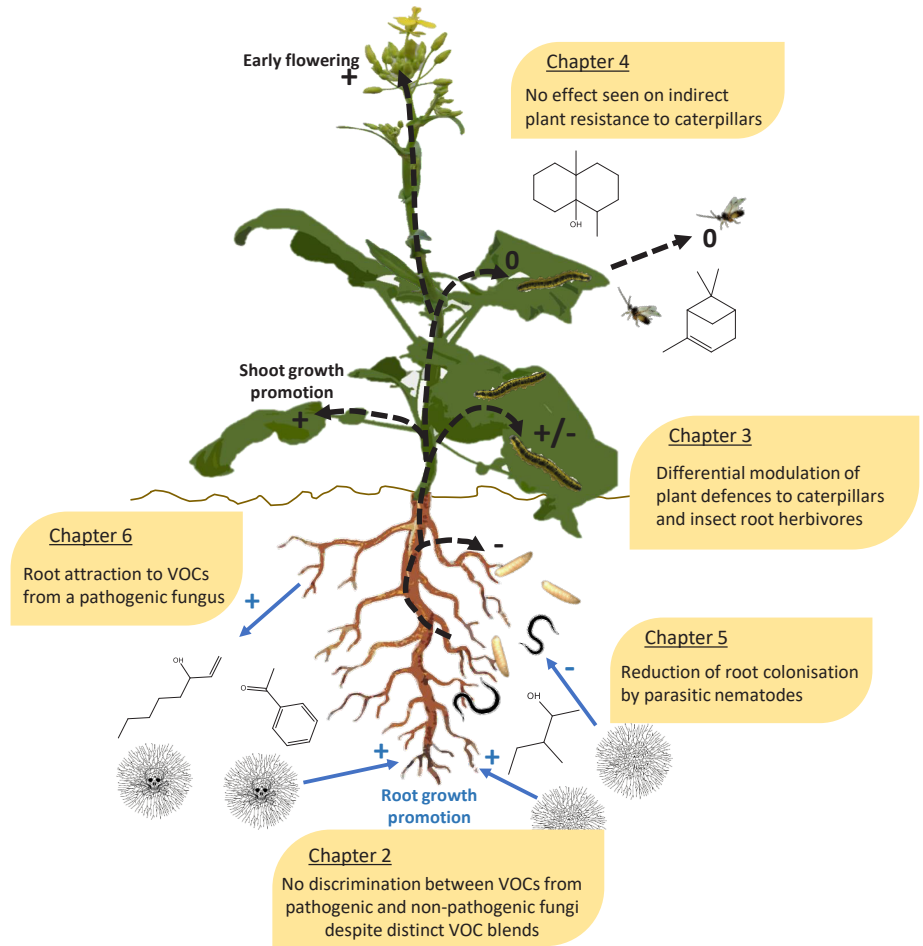


Figure 2. Schematic overview of the major findings from each chapter presented in this thesis. Solid blue lines indicate direct effects of the fungal volatile organic compounds (VOCs), whereas dashed black lines indicate potential effects mediated by the plants. "+" indicate a positive effect, "-" a negative effect and "0" a neutral effect.

exposure to fungal VOCs. Yet, we still lack knowledge on how other plant mutualists can be directly (belowground) or indirectly (aboveground) affected by fungal VOCs and fungal VOC-mediated plant changes. As a result, it is unknown whether plant fitness is ultimately impacted by plant exposure to fungal VOCs. On the other hand, if the separation of the fungus and roots is not artificially maintained, fungal pathogens may actually benefit from such VOC-mediated interactions with plants. Enhancement of plant growth and attraction of roots may indeed facilitate further root colonisation and infection. Yet, production of VOCs may be costly. A thorough assessment of biosynthetic and ecological costs and benefits for plant and fungi under realistic field conditions within the context of the full phytobiome will shed light on the evolution of such interactions. In fig. 2, I summarise the main findings of my PhD thesis as well as the outstanding questions (Box 2).

Together, the findings of my thesis make an important step in the exploration of VOC-mediated interactions between plants and soil-borne fungi and unveil several ecological consequences for plants in interactions with attackers and the enemies of their attackers.

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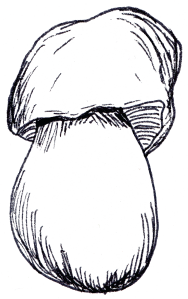

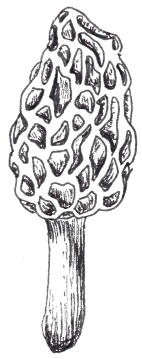

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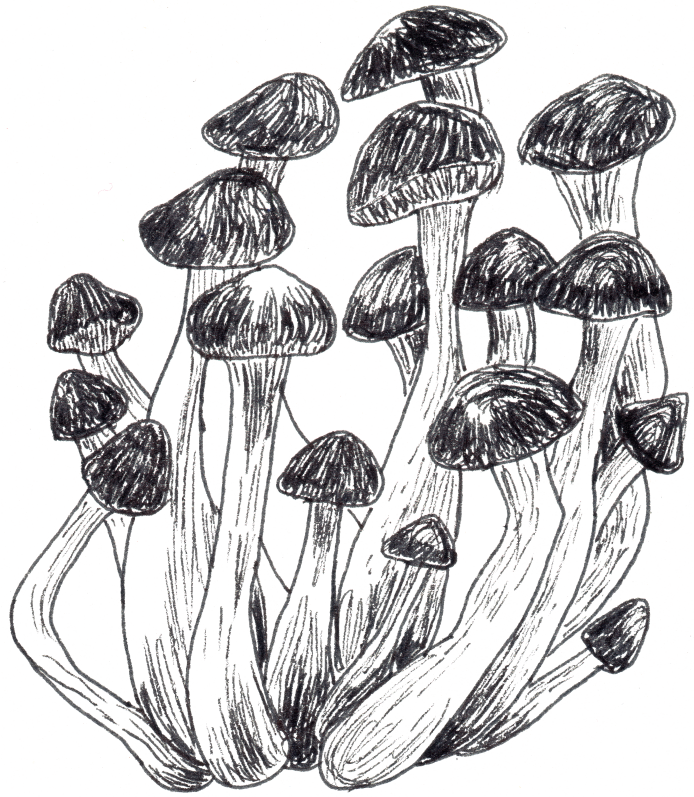

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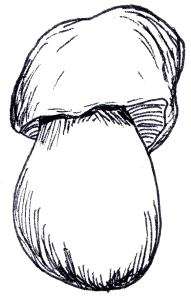
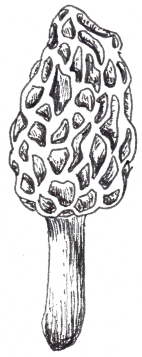




Summaries

English, French and German



Summary

Over the past two decades, a considerable number of studies has acknowledged the importance of volatiles in mediating ecological interactions within and between plants, animals and microorganisms, both above- and belowground. In particular, plant exposure to microbial volatile organic compounds (mVOCs) were shown to alter plant physiology, such as promoting growth of shoots and roots or accelerating plant development, which can affect subsequent ecological plant interactions. For instance, plants exposed to specific mVOCs are more resistant to leaf pathogens than non-exposed plants. Most of these studies have focussed on phenotypic plant responses to chemically diverse VOCs from a large number of beneficial bacterial and fungal genera. However, in natural ecosystems, plants are also exposed to VOCs from deleterious microorganisms. Yet, little is known to date about plant responses to VOCs from plant pathogenic microorganisms. Therefore, the **overall aim of my PhD project** was to investigate the effects of VOCs from soil-borne fungi on plant growth and defence to herbivores aboveground and belowground, and to assess whether plants distinguish between VOCs from pathogenic and non-pathogenic fungi.

After the selection of five soil-borne fungi pathogenic to *Arabidopsis thaliana* and six non-pathogenic soil-borne fungi, chemical profiling of these eleven soil-borne fungi revealed distinct VOC blends between the pathogenic and the non-pathogenic fungi (**chapter 2**). Subsequent *in vitro* bioassays with *A. thaliana* seedlings indicated that plant exposure to VOC blends from each of these fungi can positively affect plant growth, accelerate flowering and reduce resistance to an herbivorous insect. Yet, these effects were fungal species-specific and not related to the fungal pathogenicity, suggesting that plants did not discriminate between VOCs from their foes and friends.

To move towards more natural conditions where only plant roots are exposed to the VOCs from soil-borne fungi, I designed a two-compartment pot system (**chapter 3**). Plants were growing in the top soil compartment, while being physically separated from the fungus that was placed in the bottom compartment. Using this experimental design, the effects of fungal VOCs on growth and defence of *Brassica rapa* plants against aboveground and belowground insect herbivory were studied, as well as the effects on plant bolting. The results show that fungal VOCs can modulate the mixed-defence strategies of *B. rapa* plants, balancing direct plant resistance and tolerance to above- and belowground herbivory. These effects were variable and fungal species-specific. Plant exposure to certain VOC blends also lead to an earlier flowering and an increased number of buds and flowers, suggesting that plant fitness can be enhanced upon root exposure to fungal VOCs.

Besides direct resistance, plants may also recruit natural enemies of the herbivores through the emission of Herbivore-Induced Plant Volatiles (HIPVs) as an indirect resistance mechanism. By conducting common-garden and greenhouse experiments (**chapter 4**), I showed that *B. rapa* plants upon root exposure to fungal VOCs recruited as many parasitoids

of *Pieris brassicae* larvae as the non-exposed plants. Additionally, the larval survival rate in the field suggested a neutral effect of the fungal VOCs on larval predation. Furthermore, no differences were found between the HIPV blends emitted by non-exposed plants and those emitted by plants whose roots had been exposed to fungal VOCs. Collectively, these data suggest that VOCs from the fungi tested did not influence indirect plant resistance.

Belowground, plant roots and root herbivores are expected to be more influenced by VOCs from soil-borne fungi than plant shoots. By investigating the colonisation of roots by the insect herbivore *Delia radicum* and the cyst nematode *Heterodera schachtii* (**chapter 5**), I showed that performance of the two herbivores as well as plant growth were differentially affected by continuous exposure to VOCs from the soil-borne fungus *Fusarium oxysporum*. Besides the effects on plant growth and resistance, I also explored for the first time whether VOCs from soil-borne fungi can influence the direction of root growth. To test this, I designed a root olfactometer and provided roots of *B. rapa* seedlings with the choice to grow either towards or away from VOCs emitted by soil-borne fungi (**chapter 6**). The results show that directional root growth was differentially affected by VOCs from four different soil-borne fungi. *Brassica rapa* roots were attracted to VOCs from the root pathogen *Rhizoctonia solani,* while no significant effects on root taxis were observed for the other three fungi. Profiling of the VOCs exclusively emitted by *R. solani* did not reveal compounds with known positive effects on root growth. Remarkably, roots were attracted only to VOCs from the pathogenic fungus tested. How common this response is and what the mechanisms are that determine directional root growth towards or away from fungal VOCs remain to be investigated.

In conclusion, my thesis addressed a number of technical challenges to study VOC-mediated interactions between soil-borne fungi and plant roots both *in vitro* and in more ecologically relevant conditions (**chapter 7**). The results of my thesis revealed that VOCs from soil-borne fungi can positively affect plant performance from a distance, and can directly or indirectly influence plant interactions with other organisms, both belowground and aboveground. These findings highlight the importance of fungal VOCs for plant fitness and ecosystem functioning. Whereas plants did not seem to discriminate between VOCs from pathogenic fungi and VOCs from non-pathogenic fungi, a detailed mechanistic understanding of plant perception of VOCS from friends and foes will be needed to better understand the functional and evolutionary roles of fungal VOCs in natural and agro-ecosystems.

Résumé

Durant les deux dernières décennies, un nombre croissant d'études ont démontré l'importance des volatiles en tant que médiateurs d'interactions écologiques en surface et sous terre entre les plants, les animaux et les microorganismes. En particulier, l'exposition des plantes aux volatiles microbiens peut altérer la physiologie de la plante, comme par exemple stimuler sa croissance ou accélérer son développement, ce qui peut affecter les futures interactions écologiques de la plante. Certaines plantes exposées à des volatiles microbiens spécifiques deviennent, par exemple, plus résistantes à des pathogènes foliaires que des plantes non exposées. Jusqu'à aujourd'hui, la recherche s'est principalement focalisée sur les volatiles émis par les bactéries et champignons bénéfiques à la plante. Cependant, dans les écosystèmes naturels, les plantes peuvent également être exposées à des volatiles émis par des microorganismes néfastes. Aujourd'hui, nous ne savons que très peu sur comment les plantes réagissent aux volatiles émis par des pathogènes potentiels. C'est pourquoi, **l'objectif principal de ce projet de thèse** est d'évaluer les effets écologiques des volatiles émis par des champignons vivant dans le sol sur la croissance et sur la défense des plantes face aux herbivores, et de déterminer si la plante arrive à distinguer les odeurs (volatiles) de champignons pathogènes des champignons non-pathogènes.

J'ai commencé ce projet de thèse par tester un large panel de champignons du sol et évaluer *in vitro* les réponses des plantes *Arabidopsis thaliana* exposées à ces volatiles (**chapitre 2**). Après avoir sélectionné cinq champignons pathogènes à *A. thaliana* et six champignons non pathogènes et analysé leurs émission de composés organiques volatils (COVs), nous avons conclu que les champignons pathogènes et non pathogènes produisent des mélanges de COVs distincts. A travers des expériences *in vitro*, j'ai démontré que l'exposition d'*A. thaliana* aux COV fongiques peut affecter positivement la croissance de la plante, accélérer la floraison et réduire la résistance de la plante contre une chenille. Cependant, ces effets étaient spécifiques à l'espèce de champignons et n'étaient pas corrélés à l'émission de CO_2 ni au caractère pathogène du champignon. Ces résultats suggère que la plante n'a pas distingué les COVs émis par les pathogènes de ceux émis par les champignons non pathogènes malgré leurs COVs distincts.

Pour me rapprocher de conditions un peu plus naturelles où seulement les racines des plantes sont exposées aux COV de champignons du sol et où ces COV fongiques peuvent circuler dans le sol, j'ai développé dans le **chapitre 3** un système de pots à deux compartiments. Les plantes poussaient dans le sol dans le compartiment supérieur, tout en étant physiquement séparées du champignon qui était placé dans le compartiment inférieur. Ce système de pot m'a permis d'étudier *in vivo* les réponses des plantes *Brassica rapa* face aux COV fongiques jusqu'à la floraison ainsi que les effets sur les interactions avec des herbivores des feuilles et des racines. Mes données montrent qu'en effet, les COV fongiques peuvent influencer les stratégies mixtes de défense des plantes en influençant de part et d'autre la résistance et la tolérance de la plante face aux herbivores se nourrissant de différents tissues (racines, feuilles, fleurs). Ces effets étaient toutefois variables et spécifiques

aux espèces de champignons. Par ailleurs, certains COV fongiques ont également induit une accélération de la floraison et l'augmentation du nombre de bourgeons et de fleurs, ce qui suggère que la fitness des plantes peut être améliorée dans certains cas par l'exposition des plantes à ces COVs.

En plus de la résistance directe, les plantes peuvent aussi recruter les ennemies naturels des herbivores par le biais de Volatiles de Plantes Induits par l'Herbivore (VPIH) en tant que mécanisme de résistance indirecte. A travers mes expériences en champs et en serre, j'ai démontré dans le **chapitre 4** que les plantes *B. rapa* dont les racines ont été exposées aux COV fongiques et pour lesquelles les feuilles ont été infestées par les chenilles *Pieris brassicae* recrutaient autant de parasitoïdes et de prédateurs que les plantes non exposées. De même, je n'ai pas détecté de différences entre les VPIHs émis par les plantes exposées aux COV fongiques et ceux émis par les plantes contrôle. Ensemble, mes données indiquent que les COV fongiques (pour les champignons que j'ai testés) n'influencent pas la résistance indirecte des plantes *B. rapa*.

Sous terre, les racines et les herbivores sont supposément plus susceptibles d'être influencés par les COVs de champignons du sol car ils sont directement exposés à ces odeurs. Dans le **chapitre 5**, j'ai étudié la colonisation des racines des plantes *B. rapa* par l'insecte herbivore *Delia radicum* et par les nématodes *Heterodera schachtii* lors d'une exposition continue aux COVs émis par le champignon *Fusarium oxysporum*. J'ai démontré que la performance des deux herbivores, ainsi que la croissance de la plante, sont différemment affectés by les COV fongiques. Outre les effets sur la croissance et résistance des plantes, j'ai également étudié si les COV fongiques peuvent influencer la croissance directionnelle des racines. Afin de tester cela, j'ai développé un olfactomètre pour racines en forme de Y inversé où les racines devaient choisir entre pousser vers ou contre les COVs émis par un champignon du sol (**chapitre 6**). Mes résultats montrent que la croissance directionnelle des racines est en effet différemment influencée par les COVs de quatre espèces de champignons. Les racines de *B. rapa* étaient en particulier attirées par les COVs émis par *Rhizoctonia solani*, un champignon pathogène des racines, tandis que les COVs des trois autres champignons n'ont pas impacté significativement la croissance directionnelle des racines. L'analyse des COVs émis seulement par *R. solani* n'a pas permis d'identifier des composés ayant des propriétés de stimulation de croissance des racines. C'est pourquoi les mécanismes de modulation de la croissance des racines par les COV fongiques demeurent encore inconnus. Toutefois, il est surprenant de noter que les racines de *B. rapa* ont été seulement attirées par les COVs émis par l'unique champignon pathogène testé ici. Il serait intéressant d'élargir cette question à d'autres microorganismes pour déterminer si ce phénomène est commun ou unique à *R. solani*.

Dans la discussion générale de cette thèse (**chapitre 7**), je résume et discute les résultats principaux de ma thèse. Dans un premier temps, je souligne d'abord les challenges techniques associés à l'étude des interactions médiées par les COVs entre les champignons et les plantes dans un contexte semi-naturel. Ensuite, je discute les résultats de ma thèse.

Mes résultats révèlent que les COV fongiques du sol peuvent affecter positivement la performance de la plante et ce, à distance. Ils peuvent également influencer, directement ou indirectement, les interactions des plantes avec d'autres organismes sous terre et au-dessus du sol. Ces résultats souligne l'importance des COV fongiques pour la fitness de la plante et le fonctionnement des écosystèmes. Alors que les plantes n'ont pas discriminé entre les COVs émis par les champignons pathogènes de ceux émis par les champignons non-pathogènes, une étude approfondie des mécanismes de perception de COVs par les plantes serait nécessaire afin d'élucider le fonctionnement et les rôles d'évolution des COVs fongiques dans des écosystèmes naturels et des agroécosystèmes.

Zusammenfassung

Im Laufe der letzten zwei Jahrzehnte hat eine beachtliche Anzahl von Studien die Bedeutung von Gerüchen (flüchtigen Stoffen) in ökologischen Wechselbeziehungen zwischen Pflanzen, Tieren und Mikroorganismen bestätigt, sowohl über als auch unter der Erde. Insbesondere wurde gezeigt, dass sich die Physiologie von Pflanzen verändert, wenn diese sogenannten mikrobiellen flüchtigen organischen Verbindungen (englisch Microbial Volatile Organic Compounds, mVOCs) ausgesetzt sind. Dies kann, etwa durch gefördertes Pflanzenwachstum oder beschleunigte Pflanzenentwicklung, nachfolgende ökologische Wechselbeziehungen von Pflanzen beeinflussen. So sind zum Beispiel Pflanzen, die bestimmten mVOCs ausgesetzt waren, resistenter gegen Blattpathogene als solche, die diesen Stoffen nicht ausgesetzt waren. Die meisten der bisherigen Studien haben sich auf phänotypische Reaktionen von Pflanzen auf VOCs nützlicher Bakterien und Pilze konzentriert. In natürlichen Ökosystemen sind Pflanzen allerdings auch VOCs schädlicher Mikroorganismen ausgesetzt. Daher war **das Ziel meiner Doktorarbeit**, die Auswirkungen von VOCs bodenlebend Pilze auf das Wachstum von Pflanzen und die Abwehr gegen Pflanzenfresser zu erforschen und zu bestimmen, inwieweit Pflanzen zwischen VOCs pathogener und nicht-pathogener Pilze unterscheiden können.

Zu Beginn meiner Doktorarbeit untersuchte ich die Reaktion von *Arabidopsis thaliana* auf flüchtige Stoffe einer großen Anzahl bodenlebender Pilze *in vitro* (**Kapitel 2**). Aus diesen Pilzen habe ich fünf mit einer pathogenen Wirkung auf *Arabidopsis thaliana*, sowie sechs nicht-pathogene Pilze ausgewählt. Die Analyse der VOCs dieser Pilze hat gezeigt, dass die pathogenen und nicht-pathogegen Pilze unterschiedliche VOCgemische abgeben. Die anschließenden *in vitro* Versuche mit Pflanzen haben gezeigt, dass VOCs von Pilzen einen positiven Effekt auf das Pflanzenwachstum haben, die Blütenbildung beschleunigen und zu einer verminderten Resistenz gegen ein pflanzenfressendes Insekt führen können. Diese Effekte waren von der Pilzart abhängig und wurden nicht von dem durch die Pilze freigesetzten CO_2 ausgelöst. Diese Ergebnisse legen nahe, dass Pflanzen nicht zwischen den VOCs unterschieden, die von ihren Freunden und Feinden abgegeben werden.

Um unter natürlicheren Bedingungen zu arbeiten, bei denen nur die Pflanzenwurzeln den VOCs von bodenlebenden Pilzen ausgesetzt sind, habe ich ein zweigeteiltes Topfsystem entwickelt (**Kapitel 3**). Die Pflanzen wuchsen in dem oberen, erdgefüllten Teil und waren physikalisch vom Pilz getrennt, der in den unteren Teil des Topfes gesetzt wurde. Mithilfe dieses experimentellen Designs wurden die Effekte von den VOCs der Pilze auf das Wachstum von *Brassica rapa* Pflanzen, die Bildung von Blütenständen und die Verteidigung gegen oberirdische und unterirdische Pflanzenfresser untersucht. Die Ergebnisse zeigen, dass Pilz VOCs die Verteidigungsstrategien von Pflanzen beeinflussen und die direkte Pflanzenresistenz und -toleranz gegenüber unter- und oberirdischer Herbivorie regulieren. Diese Effekte waren variabel und hingen von der Art des Pilzes ab. Pflanzen, die den VOCgemischen bestimmter Pilzarten ausgesetzt waren, zeigten außerdem eine frühere Blüte und eine erhöhte Anzahl an Knospen und Blüten, was vermuten lässt, dass der

Wurzelkontakt mit Pilz VOCsn die Fitness der Pflanzen erhöhen kann.

Neben der direkten Resistenz können Pflanzen sich auch indirekt verteidigen , wobei sie die natürlichen Feinde der Herbivoren durch die Abgabe von Herbivoren-induzierten Pflanzen VOCs (englisch Herbivore-Induced Plant Volatiles, HIPVs) anlocken. Mithilfe von Freiland- (‘common garden’-Experimente) und Gewächshausexperimenten (**Kapitel 4**), konnte ich zeigen, dass *B. rapa* Pflanzen, deren Wurzeln Pilz VOCs ausgesetzt waren und deren Blätter von *Pieris brassicae* Raupen befallen waren, genauso viele Parasitoiden und Fressfeindeder Raupen anlocken wie die nicht-exponierten Kontrollpflanzen. Die Überlebensrate der Raupen im Freiland lässt zudem einen neutralen Effekt der Pilz VOCs auf die Prädationsrate vermuten. Darüber hinaus wurden keine Unterschiede in HIPV-Gemischen zwischen nicht-exponierten Pflanzen und Pflanzen, deren Wurzeln den Pilz VOCs ausgesetzt waren, gefunden. Zusammengefasst lassen diese Ergebnisse vermuten, dass die getesteten Pilz VOCs die indirekte Resistenz der Pflanzen nicht beeinflussen.

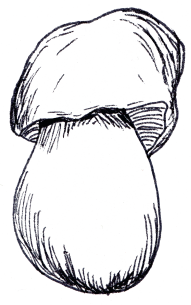
Man kann davon ausgehen, dass Wurzeln und Wurzelherbivoren stärker von den VOCs der bodenlebenden Pilze beeinflusst zu werden, da sie diesen direkt ausgesetzt sind. In **Kapitel 5**, habe ich den Befall von Wurzeln durch Larven des Insekts *Delia radium* und den Zystenfadenwurm *Heterodera schachtii* an *B. rapa* Pflanzen beobachtet, während diese kontinuierlich den VOCs des Pilzes *Fusarium oxysporum* ausgesetzt waren. Ich konnte beweisen, dass das Gewicht dieser zwei Pflanzenfresser sowie das Wachstum der Pflanze auf verschieden Weise von den Pilz VOCs beeinflusst werden. Neben den Effekten auf das Wachstum und die Resistenz der Pflanzen, habe ich ebenfalls untersucht, ob das zielgerichtete Wachstum der Wurzeln durch Pilz VOCs beeinflusst werden kann. Hierzu habe ich ein Olfaktometer für Wurzeln in der Form eines umgekehrten Y entwickelt, in dem die Wurzeln einer Pflanze entweder in Richtung der VOCs eines Bodenpilzes oder in die Gegenrichtung wachsen konnten (**Kapitel 6**). Meine Ergebnisse zeigen, dass das Richtungswachstum von Wurzeln tatsächlich auf unterschiedliche Weise von den VOCs vier verschiedener Pilze beeinflusst wird. Die Wurzeln von *B. rapa* wurden insbesondere von den VOCs des Wurzelpathogens *Rhizoctonia solani* angezogen, während die VOCs der drei anderen Pilze das Wurzelrichtungswachstum weitaus weniger beeinflusst haben. Trotz einer Analyse der f**ür** *R. solani* spezifischen VOCs gelang es nicht, die Verbindungen zu identifizieren, welche das Wachstum der Wurzeln stimulieren. Die Mechanismen durch welche VOCs von Pilzen das Wurzelwachstum regulieren müssen daher noch erforscht werden. Nichtsdestoweniger ist es erstaunlich, dass die Wurzeln von *B. rapa* ausschließlich von den VOCs des einzigen hier getesteten pathogenen Pilzes angezogen wurden. Es wäre interessant, Folgeexperimente auf weitere Mikroorganismen auszubreiten, um zu beobachten, ob dieses Phänomen allgemeingültig ist oder nur für *R. solani* gilt.

In der allgemeinen Diskussion dieser Doktorarbeit (**Kapitel 7**) findet sich eine Zusammenfassung und eine Diskussion zu den wichtigsten Ergebnissen meiner Arbeit. Zunächst hebe ich die technischen Herausforderungen hervor, welche mit der Erforschung der durch VOCs vermittelten Interaktionen zwischen Pilzen und Pflanzen in einer halb-

natürlichen Umgebung einhergehen. Anschließend diskutiere ich die Ergebnisse meiner Arbeit. Meine Forschungsergebnisse beweisen, dass VOCs von Bodenpilzen Pflanzen selbst aus der Entfernung positiv beeinflussen können. Darüber hinaus können sie, direkt oder indirekt, die Interaktionen von Pflanzen mit anderen Organismen unter oder über der Erde beeinflussen. Diese Ergebnisse unterstreichen die Wichtigkeit von Pilz-VOCs für das Wohlergehen von Pflanze sowie für das Funktionieren von Ökosystemen . Da Pflanzen nicht zwischen den VOCs pathogenener und nicht-pathogener Pilze unterscheiden, ist die Erforschung der Mechanismen mit welchen VOCs durch Pflanzen wahrgenommen werden notwendig, um die Funktionsweise und die Evolution von pilzlichen VOCs in Ökosystemen und Agroökosystem zu verstehen.









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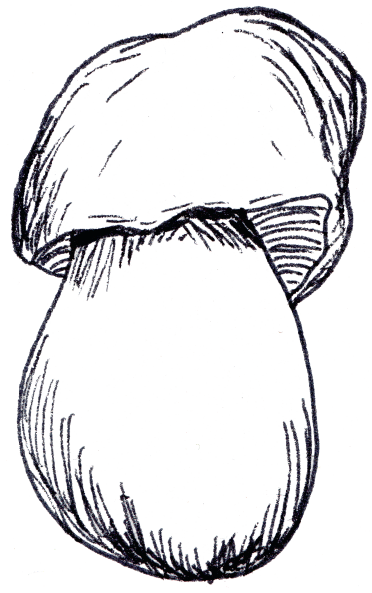


Acknowledgements

This story, my story in Wageningen, started with a button that said “Submit”. Back then in 2012, I was sitting in my bedroom in France, and I was about to submit my application to start my MSc program at Wageningen University. I was a bit nervous and obviously I had no idea that clicking on this button would completely change my life. I was “meant to” be an agronomist and finished my 5 years studies at the Ecole Supérieure d’Agriculture in Angers, but instead I decided to quit the program after 3 years. At that time, the only person who fully supported me in my application to Wageningen University was my dad, **Jean-Paul**. Dad, I remember you helped me so much with writing my CV and cover letter in English. Somehow, it feels like you already knew at that time about everything great that would happen to me here. I will always remember that sparkle in your eyes when we clicked together on that button. Sadly, I did not get the chance to share with you the great news of my enrolment at Wageningen University a few months later, but I am very grateful for all the support and everything you have done for me to get here. I have kept this life motto of yours in mind “There is only one way to go: forward”, so I did. You have been a great mentor, who taught me and inspired me very much. You have been yourself a great researcher and professor, I hope my work today makes you proud as much as I am of you.

Although I did not intend first to do a PhD when arriving in Wageningen, I quickly changed my mind after carrying out my two MSc theses, which turned out to be great experiences that yielded to my PhD project. For this reason, I would like to thank first Dani Lucas-Barbosa. **Dani**, I remember very well meeting you the first time after Marcel connected us. My thesis with you was my very first research project I had ever performed, and I was super excited about working with pollinators in the field. Thank you for introducing me to Entomology, I felt rapidly like part of this big family. Later, you also helped me a lot when writing my PhD proposal and setting up the whole project. Without your help, I would not be standing here today for my defence. I am very thankful for everything you taught me since we have met, and for all the energy and passion you shared. Your critical and demanding mind has always pushed me to do my very best. Besides work, I definitely miss our Swing dance classes together in Utrecht. Many thanks as well for introducing me to Viviane Cordovez**. Vivi,** I cannot believe how much has happened since we know each other. We both grew a lot! As a MSc student, I was so fascinated about your PhD project, I was so happy to take part in your PhD journey and to follow up with my own PhD project. Thank you so much for teaching me, with patience and passion, about the fascinating world of microorganisms and the lab work. In general, many thanks for all the good fun, the support and help you gave me since the beginning, and for all the inspiring talks we had about my project. I really appreciated to have you along my PhD as a friend, a supervisor, a co-promotor. I hope that to some extent, I managed to convince you that insects can be also fun and tasty bugs too ;-).

Besides my two co-promotors, this work would also not have been possible without the help of my two promotors, Jos Raaijmakers and Marcel Dicke**. Jos,** I also remember the first time

we met while I was conducting a mini-research project about soil suppressiveness for the EABI course at the Laboratory of Phytopathology. I immediately saw you as a very brilliant and friendly person. I think this first impression was completely right. Your expertise has been very valuable to my project during our brainstorming. I also particularly appreciated your contribution in making my manuscripts/ chapters more concise. Somehow, you know exactly how to sharpen ideas and messages, I learnt a lot from you (although admittedly, this acknowledgment section is not the best example of conciseness). Thank you for allowing me to be part of your team, I enjoyed it a lot to work at the NIOO with wonderful colleagues. Discussing with true microbiologists helped me to think with a different angle. Many thanks for your enthusiasm and positive energy that always contributed to the pleasant atmosphere of the meetings, I enjoyed a lot working with you. Lastly, **Marcel**. I actually don't know where to start, I am so grateful for everything: thank you for allowing me to write my PhD proposal at Entomology, thank you for all your support and help since the start, thank you for all the freedom and trust you gave me throughout my project, thank you for just being there whenever needed (I still wonder how you manage to find time). I feel very privileged that I could work in your team as well. You have been a true mentor for me in these last years as a scientist and team leader. Thanks for all the nice chats and walks. I think in general I had a very privileged position for working at both NIOO and Entomology for my PhD, and for having such great supervisors. All your contributions to and support of this project made my PhD journey a fantastic life experience, merci! I think we made altogether a great team and somehow very well balanced in terms of expertise, gender and nationality :-).

To my dear paranymphs, Katherine and Cristina**,** first of all thank you very much first for helping me with organising my PhD defence and celebrations afterwards. I am sure you will do a great job. **Kathe**, our friendship started already some years ago when we both were doing our MSc thesis at Entomology with Dani. I am so happy you came back to Ento to start your own PhD project. You are such a hard worker, and I am sure you will go through your PhD project successfully. Thank you for all the love (and hugs), fun and trust. No doubt that the Barragan blood and Colombian sun are totally part of you. I feel very lucky to have you as a friend, as a sister. **Cristina**, I am also very happy that the "Wageningse black hole" brought you back to Wageningen after your thesis with Vivi. You are such an AMAZING friend too, always cheerful and with amazing musical taste :-). Thanks so much for the great fun we had during lunches, coffee breaks and for all activities (drinks, dinners, ice creams, concerts…) we had outside NIOO. Have fun playing the bass in the band :-)

There are so many more people I would like to thank for their help and friendship. Let me start with Entomology first. **Lucille,** my Frenchie friend. First of all, thank you for introducing me to the field of Entomology. I have never met someone more passionate about insects than you. You taught me a lot about bugs, but also about snails, spiders and any other "funny" animals that people normally dislike but you always see the beauty in each. I have always admired your broad knowledge of biology and your eagerness to always learn more. From the moment I met you, it has always been obvious to me that you will be among the top researchers in Entomology, your work and dedication are truly inspiring to me. Thanks as well for your support, good mood and friendship during my PhD. **Sandeep,** my dear friend, you are (together with Je-seung) among the sweetest persons I have ever met. Thank you for your limitless kindness and generosity. Thanks also for inviting me to your wedding in India. This was a life experience that I will never forget. I cannot wait now to meet your daughter. **Antonio,** thank you for being an amazing friend and office mate. Together with Kathe, you were my family here in Wageningen, and I know I could always count on you. Thanks for all the fun, support and talks we have had. Many thanks as well for all the delicious Italian food you cooked for us. I look forward to visiting you in Italy as soon as it is possible. **Maite,** a PhD without you would have been boring. Thanks a lot for all your positive Spanish energy and support. It was great to have you as flat mate for a little while before the covid-19 crisis. Many thanks as well for keeping me in shape by making me run so much while playing squash or jogging in the forest. I already miss you here but I know we will keep in touch :-). **Alexandre**, thank you for your help with the VOC analyses and for your patience and zen attitude in general, I learnt a lot from you. I enjoyed a lot the (philosophical) chats and coffee/chocolate we had together at Forum during breaks. Thank you as well for the tennis matches, it was great to play together. **Jeroen,** thanks for your crazy nerdiness, for sharing the Netflix account (it saved me during the lockdown) and for the fun videogames on Nintendo. **Daan,** thanks for always being there for everyone and for being simply yourself, you are an amazing friend. I admire a lot your knowledge and ease in statistics and how well you can explain them to others. **Quint,** thank you for being a playful office mate and colleague of the flower team. It was great to work side-by-side and I am very happy with your new position in Zürich! To all other entomologists **Bram, Alessia, Thibault, Karol, Peter, Davy, Els, Mitchel, Max, Tessa, Karen, Stijn, Marieke, Julia, Alexander, Steve, Shaphan, Julien, Pieter, Ilka, Rob, Patrick, Maximilien, Yidong, Rody, Julian, Janneke, Marilyn, Margaret, Filippo, Gabriela, Chantal, Hanneke, Helen, Jeanine, Helena, Kyra, Luuk, Parth, Yavanna, Martine** and to all staff members and BSc/ MSc students, many thanks for all the great atmosphere at Entomology.

There are also a few groups within Entomology that I would like to specifically thank. To the current and former members of the Party Committee of Entomology: **Davy, Max, Katherine, Els, Yidong, Pieter, Bram** and **Maite.** Thank you for the great fun with organising Ento events, it was super cool. I hope these social activities can start soon again. Many thanks as well to my Viking fellows for the three editions of Viking Race in 2018: **Tim, Yidong, Peter, Rieta, Maite, Hans, Sandeep, Tessa, Jitte, Thibault** and **Chantal.** It was SO much fun to pass obstacles, run, swim, and crawl in the mud/snow with you. I can't stop laughing whenever watching the videos again. Also, thanks to **Quint, Luuk, Tessa, Bram** and **Daan** for the good fun with playing music together in the Brassiska band. You guys rock! Thank you to Max and Ilka for the great help with translating the summary of this PhD thesis in German. To all the fungal girls I supervised during my PhD: **Marcela, Silvia, Els, Giuditta, Liana** and **Elvira**, I want to say thank you. You all did a great job, and it was a pleasure to work with you. In fact, I also learned a lot from each of you. Thank you for your contribution

to this thesis. I am very happy that many of you pursue today a PhD project as well. Last but not least, thanks to all people that indirectly helped for my experiments: thanks to **Léon, Frans, André, Pieter, Seppe** and **Kimmy** for helping with the insect cultures; thanks to the Unifarm people, **Sean, Alex, Wim, Herman** and **Julia** that provided plants for my cultures and experiments and helped with the field work. A big thank you as well to **Gerrit** for the great collaboration and help with the statistics. I learnt so much from you, many many thanks for your patience to teach me R. Finally, I would like to thank all people working at the reception and the ones working on the maintenance and cleaning of the building for providing such pleasant working conditions in Radix.

Next to Entomology, I also met wonderful people/friends at the NIOO. Thanks first to my dear friends **Ohana, Késia, Ruth, Afnan, Je-seung, Nurmi, Cristina, Desalegn, Raul, Ben, Vivi, Roos, Adam, Lara, Ana, Marta** and **Alex** for all the support and your positive energy at work. This was so stimulating! Thanks as well for all chocolates, cookies and ice creams we shared, my belly will be forever grateful. To the Brazilian girls in particular, **Ohana, Késia, Afnan** and **Vivi,** thank you for always being there and for all the cute desk decorations/ presents/cards you organised for people's birthdays. Thanks for all the good memories we have together at dinners (especially at Sa Lolla and Watami haha) and for our endless discussions about Netflix series during lunch**. Je-seung**, thank you so much for your infinite kindness. I cannot remember how many times you saved my experiments by watering my plants in weekends or during holidays, you are my HERO! I am extremely grateful for everything, and I am very happy to count you among my closest friends. We witnessed each other start of the PhD, and I am super proud today that we both graduate from our PhDs. **Nurmi,** we know each other from Entomology and I am very glad that we could continue our friendship at the NIOO. Thank you for your kind support and advice throughout my PhD. **Alex,** thanks for sharing Russian chocolates and your evil 1000 pieces puzzle. My addiction to this puzzle could have almost delayed the submission of my thesis haha. **Roos,** many thanks for all the nice chats we had about kitesurfing, tropical holidays or biology in general. Thank you as well for giving me the opportunity to help a tiny bit with the setting up of your guest house. I was happy to put my IKEA furniture building skills at use. Special thanks also my office mates **Matthias, Fleur, Nori,** and **Marcio.** It was fun to be surrounded by bio-informaticians working with four screens. Many thanks for helping me whenever I struggled with running my R codes (which means a lot).

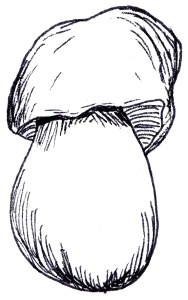
I would like also to thank **Jos** and all members of his team: **Desalegn, Ben, Natalia, Raul, Vivi, Victor, Irene, Chunxu, Sang-Yoon, Staline, Je-seung, Ruth, Juan, Roos, Xu, Yiying, Dominika, Mariana, Francisco, Mahdere**, **Lilian,** and all students. It was great to be part of the same team. Thank you for your feedback on my work and for the nice discussions/ brainstorming during the meetings and coffee breaks. To the current and former Party Planners **Kristin, Marta, Jelmer, Adam, German, Fleur, Purva, Lara, Ana, Lukas,** thank you as well for the good fun with organising borrels, Christmas dinners and BBQs at the NIOO. More generally, I thank the whole **Microbial Ecology department**. Before starting my MSc thesis, I didn't know much about microbiology and molecular techniques. I learnt a lot from your presentations, posters and seminars. A specific thank to **Saskia, Marianne, Roos, Femke, Roel, Paulien, Gregor, Hans** and **Marion** for their help in maintaining the labs, ordering chemicals and materials and helping whenever needed**.** Sorry for all glassware I broke during my PhD and for starting a fire in the flow cabinet of the lab, oupsi... **Roel,** many thanks in particular for your help with taking my soil to the gamma irradiation and your precious guidance in the nematode assay. Nematodes are quite fascinating organisms and I am very happy today to work with nematodes in my new position. At last, I would like to thank as well all ladies working at the reception for always being so kind and willing to help. Altogether, you all contributed to the great atmosphere of NIOO too.

Outside work, many people indirectly helped me to make my PhD a fantastic life experience. I would like first to thank my corridor mates of the student housing in Haarweg: **Sasja, Jori, Merel,** and **Marcela,** for everything we shared including the dinners, movie nights and board game evenings. Thanks to my dear music fellows, **Daniel, Tessa, Max,** and **Tim,** for all the great fun of playing together. Thanks to the student associations that organise super nice events for internationals. In particular, I enjoyed a lot the international dinners of **IxESN** and I was delighted to join the Elderly walk team. It was a true pleasure to help and it certainly improved my Dutch to talk with locals. Thanks as well to **Esther** and all volunteers of the vineyard in Wageningen to give me the opportunity to help with the grape harvest. The **city of Wageningen** is such a dynamic place filled with amazing people. Thanks to all, I can say today that Wageningen became home. I feel I will be forever bound to this place, and a piece of my heart will forever remain here. My mother is used to call Wageningen the "hobbit village" (the Shire in the Lord of the Rings book), not at all because of the short size of people or hairy feet but simply because of the cosiness of the place. I could not agree more with her and I am proud to have being a "hobbit" for 7.5 years.

Despite the geographical distance, my family has also been very important to me. Thanks to my mother **Brigitte,** my twin sister **Tania,** my sister **Armelle,** my brother **Patrick** with his wife **Stéphanie** and their two beautiful kids **Ewen** and **Nolan** for the immeasurable support through their visits, pictures and Skype calls. I don't say it frequently enough but I love you and I miss you a lot! I would not have been able to pull this off without you. Thank you to **Tania** in particular for helping me with the edition of this book and my mother for helping with translating in German parts of the summary. I am very happy and proud that you both contributed to this work too. Thank you also to my colleague at Koppert, **Lisa**, for the help with the illustrations. A special thank to my beloved rabbit. **Missa**, you have been such a great companion. Thank you for all the love you gave me for seven years. Rest in peace in the most beautiful place of Wageningen. Last but not least, **Julie**, thank you so much for all your support. You have been always here in the good and in the difficult moments. We know each other for more than 10 years now, and your love makes me the happiest woman for the last 5.5 years. I can't wait to move to our apartment in The Hague and to start this new life together. Merci mon amour.



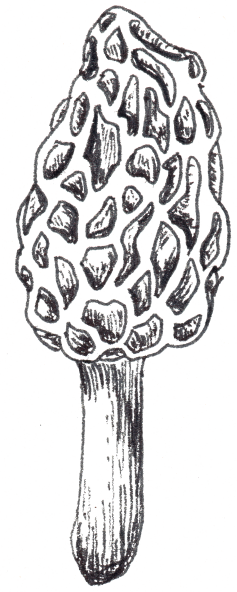






About the author

Curriculum Vitae, List of publications, Education Statement



Curriculum Vitae



Kay Moisan was born on January 11th, 1991 in Nantes (France) together with her twin sister, Tania. Since she masters walking, Kay has always enjoyed being outside. As a kid, her parents could not stop her from climbing trees or playing in the garden with her cats. Thanks to the guidance of her dad, Kay discovered the joy of growing vegetables in the garden: sowing the seeds and taking care of the plants until the harvest and the consumption of these fresh veggies. This opened up Kay's vocation: she loves plants and she wants to protect them. For her Bachelor degree, Kay studied at the Ecole Supérieure d'Agriculture in Angers (France). There, she was trained as an agronomist and learnt the applied aspects of crop production. For her Master degree, she moved to Wageningen (The Netherlands) and studied the MSc program in Plant Sciences with a specialisation in Entomology and Plant Pathology at Wageningen University. She performed her major MSc thesis at the Laboratory of Entomology (WUR), and studied insect-plant interactions in field and greenhouse conditions. For her minor MSc thesis, she worked at the Department of Microbial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW), and she explored the interactions of microbial volatiles with plants. At the end of her MSc program, Kay enrolled to the EPS Graduate Program that allowed her to write her own PhD proposal. As she could not choose between microbes and insects, her two new loves, she wrote a PhD proposal that combines microbe-plant-insect interactions and addressed the effects of fungal volatiles on plant growth and resistance to insect herbivores. After graduating from her MSc program with *cum laude* in 2015, Kay started this very exciting PhD project. Just like for her two MSc theses, her PhD project consisted of a collaboration between the Laboratory of Entomology (WUR) and the Department of Microbial Ecology at NIOO and was supervised by the same supervisors: Prof. dr Marcel Dicke, Prof. dr Jos Raaijmakers, dr Dani Lucas-Barbosa and dr Viviane Cordovez. The findings of this project are presented in this thesis.

List of publications

Published papers

Cordovez V., Mommer L., **Moisan K.,** Lucas-Barbosa D., Pierik R., Mumm R., Carrion V.J. & Raaijmakers J. M. (2017). Plant phenotypic and transcriptional changes induced by volatiles from the fungal root pathogen *Rhizoctonia solani*. *Frontiers in Plant Sciences*, 8, 1262.

Moisan K., Cordovez V., van de Zande E.M., Raaijmakers J.M., Dicke M. & Lucas-Barbosa D. (2019). Volatiles of pathogenic and non-pathogenic soil-borne fungi affect plant development and resistance to insects (**Chapter 2** of this thesis). *Oecologia*, 190, 589-604.

Moisan K., Lucas-Barbosa D., Villela A., Greenberg L., Cordovez V., Raaijmakers J.M. & Dicke M. (2020). No evidence of modulation of indirect plant resistance of *Brassica rapa* by volatiles from soil-borne fungi (**Chapter 4** of this thesis). *Ecological Entomology,* 45(5), 1200-1211.

Moisan K., Aragón M., Gort G., Dicke M., Cordovez V., Raaijmakers J.M. & Lucas-Barbosa D. (2020). Fungal volatiles influence plant defence against aboveground and belowground herbivory (**Chapter 3** of this thesis). *Functional Ecology, in press*, https://doi.org/10.1111/1365-2435.13633

Moisan K., Raaijmakers, J. M., Dicke M., Lucas-Barbosa D. & Cordovez V. (2020). Volatiles from soil-borne fungi affect directional growth of roots (**Chapter 6** of this thesis). *Plant, Cell and Environment, in press*, https://doi.org/10.1111/pce.13890

Submitted papers

Moisan K., Dicke M., Raaijmakers J.M., Rachmawati E. & Cordovez V. (2020). Volatiles from the fungus *Fusarium oxysporum* affect interactions of *Brassica rapa* plants with root herbivores (**Chapter 5** of this thesis). Submitted for publication to *Ecological Entomology* (under review).

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Kay Moisan
Date: 16 October 2020
Group: Laboratory of Entomology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
VOC-mediated interactions between plants and soil-borne fungi: effects on plant growth and plant resistance	18 Jan 2016	1.5
► **Writing or rewriting a project proposal**		
► **Writing a review or book chapter**		
► **MSc courses**		
Subtotal Start-Up Phase		1.5

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
Annual EPS PhD student days - Soest, NL	9-10 Feb 2017	0.6
Annual EPS PhD student days - Soest, NL	15-16 Feb 2018	0.6
► **EPS theme symposia**		
EPS theme 2 'Interactions between Plants and Biotic Agents' - Leiden, NL	22 Jan 2016	0.3
EPS theme 2 'Interactions between Plants and Biotic Agents' - Wageningen, NL	23 Jan 2017	0.3
EPS theme 2 'Interactions between Plants and Biotic Agents' - Amsterdam, NL	24 Jan 2018	0.3
EPS theme 2 'Interactions between Plants and Biotic Agents'- Wageningen, NL	1 Feb 2019	0.3
► **Lunteren Days and other national platforms**		
28th Entomologendag - Ede, NL	16 Dec 2016	0.3
29th Entomologendag - Ede, NL	15 Dec 2017	0.3
30th Entomologendag - Ede, NL	14 Dec 2018	0.3
Netherlands Annual Ecology Meeting - Lunteren, NL	14-15 Feb 2017	0.6
► **Seminars (series), workshops and symposia**		
Insect-Plant Interaction workshops - Amsterdam, NL	19 Nov 2015	0.3
Insect-Plant Interaction workshops - Leiden, NL	22 Nov 2016	0.3
Insect-Plant Interaction workshops - Wageningen, NL	7 Nov 2017	0.3
Insect-Plant Interaction workshops - Leiden, NL	9 Oct 2018	0.3
Wageningen PhD symposium - Wageningen, NL	26 Apr 2016	0.3
Wageningen PhD symposium - Wageningen, NL	3 May 2017	0.3
Yearly Entomology Research Exchange Meeting (YELREM) - Bennekom, NL	1 Jun 2016	0.3
Yearly Entomology Research Exchange Meeting (YELREM) - Bennekom, NL	24 May 2018	0.3
Yearly Entomology Research Exchange Meeting (YELREM) - Bennekom, NL	4 Jun 2019	0.3
NIOO Science Days - Heeze, NL	11-12 Nov 2015	0.6
NIOO Research Days - Soesterberg, NL	8-9 Nov 2017	0.6
NIOO Research Day - Driebergen-Zeist, NL	7 Nov 2019	0.3
Workshop "Metabolomics in Chemical Ecology" - Wageningen, NL	31 Oct - 1 Nov 2016	0.5
Mini-symposium "Chemical communication" - Wageningen, NL	8 May 2018	0.3
► **Seminar plus**		
► **International symposia and congresses**		
Jean-Marie Delwart Foundation Symposium 2015 "Plant communication: signal production, reception, transduction and propagation" - Brussels, BE	30-31 Oct 2015	0.5
International workshop "Plant-soil-microbe interactions for crop and pest management" - Wageningen, NL	30 Jun 2016	0.2
DE	14-16 Sep 2016	0.8
16th International Symposium on Insect-Plant Relationships - Tours, FR	2-6 Jul 2017	1.3
Gordon Research Conference and seminar on Plant-Volatiles - Lucca, IT	3-9 Feb 2018	1.7
International Plant-Soil Feedback Symposium - Wageningen, NL	7 Mar 2019	0.3
miCROPe 2019 Symposium "Microbe-assisted crop production – opportunities, challenges and needs"- Vienna, AU	2-5 Dec 2019	1.2
► **Presentations**		
Poster "Proposal presentation: Warning or blessing: do plants differentiate between volatiles from pathogenic and non-pathogenic soil-borne fungi?" COST Action Worshop FA1405, Leipzig, DE	14-16 Sep 2016	1.0
Talk "Warning or blessing? Volatiles emitted by pathogenic and non-pathogenic fungi affect plant development and resistance to insects" at the International Symposium on Insect-Plant Relationships, Tours, FR	5 Jul 2017	1.0
Poster "The volatile cascade: effects on insect behaviour" at the 29th Entomologendag (poster), Ede, NL	15 Dec 2017	1.0
Talk "Warning or blessing? Volatiles emitted by pathogenic and non-pathogenic fungi affect plant development and resistance to insects" at the Gordon Research Conference and Seminar, Lucca, IT	3-9 Feb 2018	1.0
Poster "Warning or blessing: volatiles emitted by pathogenic and non-pathogenic fungi affect plant growth and resistance to insects" at the Gordon Research Conference and Seminar - Plant Volatiles, Lucca, IT	3-9 Feb 2018	1.0
Talk "The power of smell: do fungal volatiles affect plant resistance to insects?" at the Insect-Plant Interaction workshop 2018, Leiden, NL	9 Oct 2018	1.0
Talk "Fungal volatiles induce plant growth and resistance responses aboveground and belowground" at the EPS Theme 2 symposium 2019, Wageningen, NL	1 Feb 2019	1.0
Poster + poster pitch "Can soil microbes enhance plant health without direct contact?" at miCROPe 2019, Vienna, AU	2 Dec 2019	1.0
► **IAB interview**		
► **Excursions**		
Subtotal Scientific Exposure		22.9

3) In-Depth Studies	date	cp
► **Advanced scientific courses & workshops**		
Advanced Statistics course Design of Experiments (PE&RC & WIAS)	19-21 Dec 2016	0.8
Introduction to R for statistical analysis (PE&RC & SENSE)	23-24 Oct 2017	0.6
Generalized Linear Models (PE&RC & SENSE)	14-15 Jun 2018	0.6
Mixed Linear Models (PE&RC & SENSE)	20-21 Jun 2018	0.6
► **Journal club**		
Insect-Plant Interaction meeting at Entomology (every 2 weeks)	2015-2019	0.8
Microbial Ecology Department literature meeting (once per week)	2015-2019	1.0
► **Individual research training**		
Subtotal In-Depth Studies		4.4

4) Personal Development	date	cp
► **General skill training courses**		
Techniques for Writing and Presenting a Scientific Paper	10-21 Mar 2017	1.2
Reviewing a Scientific Paper	7 Jun 2018	0.1
Career Perspectives	12 Sep - 10 Oct 2019	1.6
Writing propositions for your PhD	4 Sep 2019	0.0
Last Stretch of the PhD Programme	4 Sep 2019	0.0
► **Organisation of meetings, PhD courses or outreach activities**		
Convening parallel session at Netherlands Annual Ecology Meeting	14 Feb 2017	0.3
Chairing one session at Wageningen PhD symposium	03 May 2017	0.1
Organiser of NIOO-KNAW PhD discussion group (1 per month)	2015-2017	0.6
► **Membership of EPS PhD Council**		
Subtotal Personal Development		3.9

TOTAL NUMBER OF CREDIT POINTS*	32.7

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.

** A credit represents a normative study load of 28 hours of study.*

The research presented in this thesis was performed at the Laboratory of Entomology of Wageningen University (WUR) and at the Department of Microbial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW).

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