No evidence of modulation of indirect plant resistance of *Brassica rapa* plants by volatiles from soil-borne fungi

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Abstract. 1. Upon herbivory, plants emit specific herbivore-induced plant volatiles (HIPVs) that can attract natural enemies of the herbivore thus serving as indirect plant resistance. Not only insect herbivores, but microorganisms may also affect HIPV emission before or after plant colonisation, which in turn can affect behaviour of natural enemies of the herbivore. Yet, it remains elusive whether volatiles from microorganisms influence HIPV emission and indirect plant resistance.

2. In this study, we investigated whether exposure of *Brassica rapa* roots to volatiles from soil-borne fungi influence HIPV emission and the recruitment of natural enemies of *Pieris brassicae* larvae.

3. Using a two-compartment pot system, we performed greenhouse and common-garden experiments, and we profiled plant HIPV emission.

4. We found that exposure of plant roots to fungal volatiles did not affect the number of *P. brassicae* larvae recollected from the plants, suggesting a neutral effect of the fungal volatiles on natural 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 revealed no qualitative and quantitative differences between control plants and fungal volatile-exposed plants that were both infested with *P. brassicae* larvae.

5. 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 the influence of indirect plant resistance by fungal volatiles that are discussed together with the effects of fungal volatiles on direct plant resistance.

Key words. 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 throughout the plant, and predators and parasitoids can exploit these HIPVs as cues to locate their prey or hosts on infested plants (Rasmann et al., 2005; Mumm & 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 & 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 & 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 emitted VOCs in larger quantities 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). Each parasitoid and predator species may exploit different plant VOCs for oviposition or foraging (McCormick et al., 2012). Therefore, effects of microorganism-induced HIPV changes on the behaviour of natural enemies of the herbivore can range from negative (i.e. repellence) to positive (i.e. attraction) (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).

Prior to plant colonisation, volatiles emitted by microorganisms can affect plant growth and enhance plant resistance against attackers by altering plant chemistry (Ortíz-Castro et al., 2009; Chung et al., 2016). 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 & Weisskopf, 2012; Piechulla et al., 2017; Fincheira & 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). Although less extensively studied, microbial volatiles can also affect plant resistance to insect herbivores (Moisan et al., 2019). 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 nonexposed 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. A few 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 & 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 from a distance to volatile blends from soil-borne microorganisms. In the present study, 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 the consequences for the recruitment of parasitoids. For three of these fungi, we also addressed whether these changes affect the recruitment of predators in the field.

Materials and methods

Study system

The wild turnip, B. rapa L. (Brassicaceae), accession used in this study originated from a wild population in Maarsen (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 affected Arabidopsis thaliana growth and resistance to a leaf insect herbivore (Moisan et al., 2019). Fusarium oxysporum f. sp. raphani and Rhizoctonia solani AG2-2 IIIb are two saprophytic fungi, occurring worldwide that may turn into necrotrrophic pathogens of brassicaceous species (Leeman et al., 1995; Pannecoque & Höfte, 2009). Chaetomium indicum and Trichoderma viride are also commonly occurring saprophytic fungi that can colonise brassicaceous plants as nonpathogenic endophytes (Junker et al., 2012). For the present study, all fungi were inoculated in 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. Larvae were reared on Brussels sprouts plants (Brassica oleracea L. var. gemmifera cv Cyprus) in a climate room (22 ± 2°C; L16:D8; 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 & Brodeur, 1992). Wasps exploit plant volatiles of infested plants to locate their hosts (Brodeur et al., 1996). In a greenhouse compartment (25 ± 2°C; L16:D8; 60 ± 10% RH), wasps were reared.
in *Brassica rapa* 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 (Fig. 1). One sterile *B. rapa* seed was sown in the top compartment filled with a sterile soil mixture (1:1 vol/vol, 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, while preventing physical contact between the roots and the fungus. Exposure to fungal volatiles was initiated with seven-day-old fungi as soon as *B. rapa* seeds were sown, and was maintained for 4 weeks, after which *B. rapa* plants had 6–8 fully developed leaves. Plants were grown in a greenhouse compartment (21 ± 2°C; L16:D8; 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 4 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 4 weeks of growth in the greenhouse while being exposed to fungal volatiles (see previous section), uninfested plants were transplanted (without the fungi) to a common garden to test the effects of prior root 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 20 *B. rapa* 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 disease, desiccation or possibly starvation, 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 the 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 (α = 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 setup. After 4 weeks of exposure to fungal volatiles (see Section 2.2), *B. rapa* plants (including nonexposed 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 as food for the wasps. 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; L16:D8; 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 (α = 0.05). Additionally, correlation between leaf damage and leaf fresh weight was analysed with Pearson correlation test (α = 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 4 weeks of exposure to fungal volatiles, plants were infested with 20 *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 taped 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. To identify background VOCs, 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; L16:D8; 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) before 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 mz 35–400 at a rate of 4.7 scans s⁻¹. The GC column was a ZB–5MS Zebtron (Phenomenex); 30 m × 0.25 mm i. d. × 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):

\[
AI = (100 \times C_\zeta) + \frac{100 \times (RT_y - RT_\zeta)}{RT_\zeta + 1 - RT_\zeta}
\]

C_\zeta is the number of C-atoms of the alkane (z) eluting just before the VOC of interest (y), RT_y is the retention time of y, RT_\zeta is the retention time of \zeta, and RT_\zeta + 1 is the retention time of the alkane (z + 1) eluting just after y thus RT_\zeta < RT_y < RT_\zeta + 1.

GC–MS data were processed using the MetAlign–MSClust software pipeline (Lommen, 2009; Tikunov et al., 2012). 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. MScClust then clusters the aligned mass peaks so that mass spectra of putative compounds are constructed. Only mass peaks with a retention time within 5–38 min and in the 55–400 mz-range were further processed. VOCs were tentatively annotated by comparing their mass
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 h 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. [Colour figure can be viewed at wileyonlinelibrary.com].

An individual mass peak was selected for each putatively identified VOC, with its intensity across samples relating to the abundance of the compound. Per VOC, only aligned mass peaks displaying peak height values <9.95 × 10^7 counts across all samples were used. Only VOCs from the samples whose peak intensity statistically differed from the background (Student t-test, α = 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. Per VOC, values were divided by 10^3, 10^4, or 10^5, and are reported in Table S1. Differences of peak intensity between the different fungal volatile-exposed plants were statistically tested for each VOC using the nonparametric Kruskal-Wallis test (α = 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).

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. 2; GLMM; χ^2 = 14.9; P = 0.005). Approximately 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. 2; 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. 2; GLMM; χ^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*.
Fungal volatiles and indirect plant resistance

Fig. 3. Percentage (mean ± SE) of *Pieris brassicae* larvae parasitised when one female *Cotesia glomerata* wasp was given 24 h in a tent to parasitise larvae that had been feeding for 24 h 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). [Colour figure can be viewed at wileyonlinelibrary.com].

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. 3; 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. 4c; Pearson correlation; $r = -0.482; P < 0.001$).

Collection and analysis of headspace VOCs from infested plants

A total of 26 discrete VOCs was detected from the plant headspace and relatively quantified, 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. 5; 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. 5). 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 test; $P > 0.05$). We also did not

| Fungal volatiles | 1.0 | 0.916 |
|------------------|-----|------|
| Leaf damage      | 2.8 | 0.093|
Fig. 4. (a) Leaf fresh weight (mean ± SE), (b) amount of leaf damage (mean ± SE) upon feeding by *Pieris brassicae* larvae for 48 h on 4-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 “nonsignificant” differences ($\alpha = 0.05$) and “N” indicates the number of plant replicates. [Colour figure can be viewed at wileyonlinelibrary.com].

detect differences in blend composition between the treatments within the monoterpenes and other hydrocarbons (Figure S1b). We observed that the relative intensities of *C. indicum* VOC emission were higher than in the other treatments for most of the VOCs (Figure S1a).

**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.

In the present study, we did not detect differences in HIPV emission between nonexposed plants and plants whose roots were exposed to fungal volatiles. To our knowledge, this is the first study that investigated whether exposure of roots from a distance to a whole microbial volatile blend modulates the emission...
Fungal volatiles and indirect plant resistance

Fig. 5. 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. (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 volatiles to the principal components is shown in the loading plot of the PCA. “DMNT” stands for “4,8–dimethyl–1,3,7-nonatriene”. [Colour figure can be viewed at wileyonlinelibrary.com].

of HIPVs aboveground. All 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). Interestingly, direct soil inoculation with non-pathogenic soil-borne bacteria and fungi can affect HIPV emission (Bezemer & 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 Arabidopsis seedlings can be altered upon plant exposure to volatiles from the soil-borne fungus R. solani (Cordovez et al., 2016). 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 & 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 remain sparse. Plants may specifically respond to microbial volatiles released in their environment (Moisan et al., 2019), 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 wasps Cotesia marginiventris to the headspace volatiles and 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 wasps Cotesia marginiventris to the headspace volatiles. The rest of the larvae missing from uncovered plants, are 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 volatile from F. oxysporum and R. solani in a greenhouse did not affect the mortality of P. brassicae larvae after 3 days of feeding, even though it affected their biomass (unpublished data). Therefore, the percentage of missing larvae when predation is excluded is assumed to be similar between plant exposures 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, are 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 & 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 & Ryu, 2013). Additionally, inoculation of the bacterial VOC 2,3-butanediol in the soil surrounding maize plants attacked by S. littoralis larvae positively affected 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 (Dike & 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 & Dike, 1995; Nuñez & 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 nonexposed 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 exploit HIPVs as indicators of the host quality to select the most suitable host species for parasitoid development and oviposit accordingly (Brodeur et al., 1998; Fatoouros et al., 2005). Furthermore, other (nonvolatile) secondary plant metabolites may have been altered by fungal volatiles and may directly affect the larval performance (Aziz et al., 2016). Different larval biomass 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 (i.e. immune response involving the isolation of the parasitoid eggs into a capsule which kills the eggs). 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 affects neither HIPV emission upon herbivory by P. brassicae larvae nor the predation and parasitisation of the larvae, thus sustaining indirect plant resistance. We have previously reported that direct plant resistance as well as plant growth upon herbivory can be positively or negatively modulated by root exposure to fungal volatiles (Moisan et al., 2019). Therefore, volatiles emitted belowground by soil microorganisms may be important modulators of plant resistance (Dike, 2016).

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**Contribution of authors**

KM, DLB, VC, JMR and MD planned and designed the study. KM performed the volatile collection and the parasitisation assay in the greenhouse, and KM and LOG carried out the common-garden experiment. AV processed the volatile data, and KM performed the volatile collection and the parasitisation rate. This research was partially funded by an NWO Spinoza award to MD. The authors declare that there is no conflict of interest.

**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Supporting Information**

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

**Box S1.** Supplemental information of the Materials & Methods of the VOC analysis

**Table S1.** Total number of *Pieris brassicae* larvae recollected from fungal volatile-exposed *Brassica rapa* plants with the number of parasitised larvae

**Fig. S1.** Analysis per chemical/biosynthetic class of volatile organic compounds collected from the headspace of fungal volatile-exposed *Brassica rapa* plants infested with *Pieris brassicae* larvae

**Table S2.** Relative peak intensity of volatile organic compounds collected in the headspace of fungal volatile-exposed *Brassica rapa* plants infested with *Pieris brassicae* larvae

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