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

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Abstract. 1. Soil is a diverse and heterogeneous environment where chemicals mediate numerous interactions between soil organisms and plants. To date, studies have extensively addressed volatile-mediated interactions between soil microorganisms and the effects of microbial volatiles on plant growth. Yet, to our knowledge, it remains to be explored whether volatiles from soil-borne fungi can influence plant interactions with root herbivores, facilitating or hampering performance of competitors that share the same host plant.

2. In the present study, we investigated the effects of volatiles emitted by the soil-borne 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.

3. Fungal volatiles slowed down the development of the root nematode cysts but increased their size, suggesting an enhanced egg load. In contrast, the performance of the insect root herbivore 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.

4. Together, our data show that volatiles from a soil-borne fungus can affect root interactions with root herbivores. Increased production of nematode eggs and plant growth promotion suggest a specific modulation of root-herbivore interactions by fungal volatiles.

Key words. *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 archaea. 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.

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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 *et al.* 2016; van Dam & Bouwmeester 2016; Kong *et al.* 2018). Therefore, soil microorganisms have evolved mechanisms to sense their neighbours (Johnson & 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 & 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.* 2017). Additionally, microbial volatiles can mediate inter-kingdom interactions with plants and animals (Wenke *et al.* 2010; Rasmann *et al.* 2012b; van Dam & Bouwmeester 2016; Schenkel *et al.* 2018). For example, volatiles emitted by saprophytic *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 & Sikora 1994). Also, volatiles from pathogenic and non-pathogenic soil microorganisms can promote plant growth and accelerate plant development (Casarrubia *et al.* 2016; Cordovez *et al.* 2017; Piechulla *et al.* 2017; Moisan *et al.* 2019). Reciprocally, plant volatiles can affect soil microorganisms. For example, root volatiles can attract beneficial bacteria to the rhizosphere (Rasmann & 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, microbial volatiles can indirectly mediate interactions between different organisms. For instance, microbial volatiles can enhance plant resistance to subsequent attackers, by providing chemical protection of the plant, hence preventing further microbial colonisation (Vorholt 2012; Junker & Tholl 2013), and by promoting plant growth and eliciting local and systemic resistance (Bitas *et al.* 2013; Sharifi & Ryu 2016; Cordovez *et al.* 2017). Recently, we showed that volatiles from different soil-borne fungi influence compensatory plant growth and resistance against a caterpillar and a root insect herbivore (Moisan *et al.* 2020). Together, these data indicate that microbial volatiles can indirectly mediate ecological interactions of plants with other organisms.

Yet, it remains largely unknown whether volatiles from a soil-borne fungus, directly and indirectly, impact plant interactions with root herbivores by facilitating or hampering the performance of the attacker. In this study, we investigated the effects of volatiles emitted by saprophytic *F. oxysporum* on the performance of the cyst nematode *Heterodera schachtii* and the insect root herbivore *Delia radicum* on *Brassica rapa* roots. We hypothesised that *F. oxysporum* volatiles would negatively impact the performance of the two root herbivores as they both represent competitors that share the same host plant.

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). 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 ϕ 9 cm Petri dishes for 7 days or in ϕ 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 volatiles from *F. oxysporum* using two-compartment pots (Fig. 1). Plants were grown in the top compartment, while the fungus was grown in the bottom compartment. 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, while preventing physical contact between the roots and the fungus. 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 filled with a sterile (γ-irradiated) soil mixture (1:1 v/v, ϕ 2 mm sieved Horticoop potting soil: sand). The bottom compartment contained either a Petri dish with *F. oxysporum* growing on 1/5th PDA medium or a control Petri dish with 1/5th PDA medium only. In the pots used for infestation with *D. radicum* larvae, both compartments were connected to each other by a connector ($h = 12.5$ cm, $\phi = 12.7$ cm), which allowed the weekly replacement of Petri dishes containing the fungus or control Petri dishes with fresh 7-day-old fungus or medium. Volatile exposure was initiated as soon as *B. rapa* seeds were sown, and was maintained in a greenhouse compartment (21 ± 2 °C; L16:D8; 70 ± 5% RH) throughout the experiment until the harvest of the plants. Volatile exposures (*F. oxysporum* and control) were replicated 14–15 times for each herbivore infestation.

Plant infestation with Heterodera schachtii *cyst nematodes*

Two-week-old *B. rapa* plants were inoculated with 1 ml of inoculum containing 550 ± 20 *H. schachtii* J2 juveniles, while roots were still exposed to fungal volatiles (see previous section; Fig. 1a). For this, cysts of *H. schachtii* were incubated in water at 25 °C, 4 days before inoculation. Newly hatched juveniles were sieved, and the concentration of the inoculum was adjusted by counting the juveniles under the microscope and making dilutions. To prevent flushing of the juveniles, no water was added to the plants during the first 2 days after inoculation. Aboveground parts of the plants were harvested 3 weeks after inoculation, and the cysts were given another 2 weeks to ripen. Roots were gently harvested and rinsed with water through two sieves ($\phi = 0.850$ mm and $\phi = 0.212$ 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 microscope: white to light yellow-coloured cysts were considered as unripe, and orange to dark orange-coloured cysts were considered as ripe (Gardner & Caswell-Chen 1997). To assess cyst size, cysts were photographed (Leica DFC450, Leica Microsystems B.V., Son, The Netherlands) under a microscope (Leica M205C),

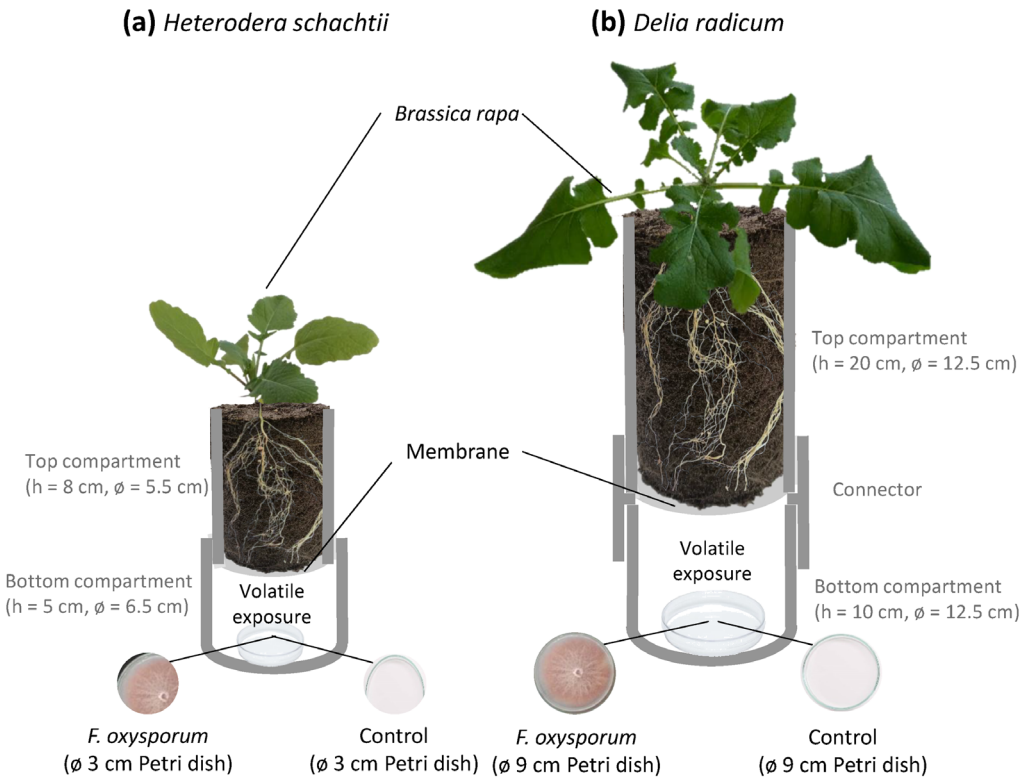

Fig. 1. Schematic representation of the two-compartment pot systems used in this study to expose roots of *Brassica rapa*, growing in soil in the top compartment, to volatiles from *Fusarium oxysporum* fungus growing in a Petri dish in the bottom compartment or to control Petri dish (medium only). Different pot sizes were used for the plant infestation with (a) *Heterodera schachtii* and (b) *Delia radicum*. [Colour figure can be viewed at wileyonlinelibrary.com].

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 per g of root dry weight was tested with a Student's *t*-test ($\alpha = 0.05$) while the total count of cysts was analysed with a generalised linear model with a quasi-Poisson distribution. 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 ($\alpha = 0.05$). 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's *t*-tests, and correlations with the number and size of cysts were assessed with Pearson correlation tests ($\alpha = 0.05$).

Plant infestation with Delia radicum *larvae*

Four-week-old *B. rapa* plants were infested with 10 newly hatched *D. radicum* larvae, while roots were still exposed to fungal volatiles (see section '*Plant exposure to* Fusarium oxysporum *volatiles*'; Fig. 1b). During the first 2 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*

The total count of cysts collected in fungal volatile-exposed plants did not differ with that of control plants (Fig. 1a; GLM; $P = 0.403$). However, fewer *H. schachtii* cysts were retrieved per milligram of dry roots from *B. rapa* plants exposed to *F. oxysporum* volatiles than from non-exposed control plants (Fig. 2a; $t = 2.5$; $P = 0.018$). 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. 2b; GLM; $P = 0.029$).

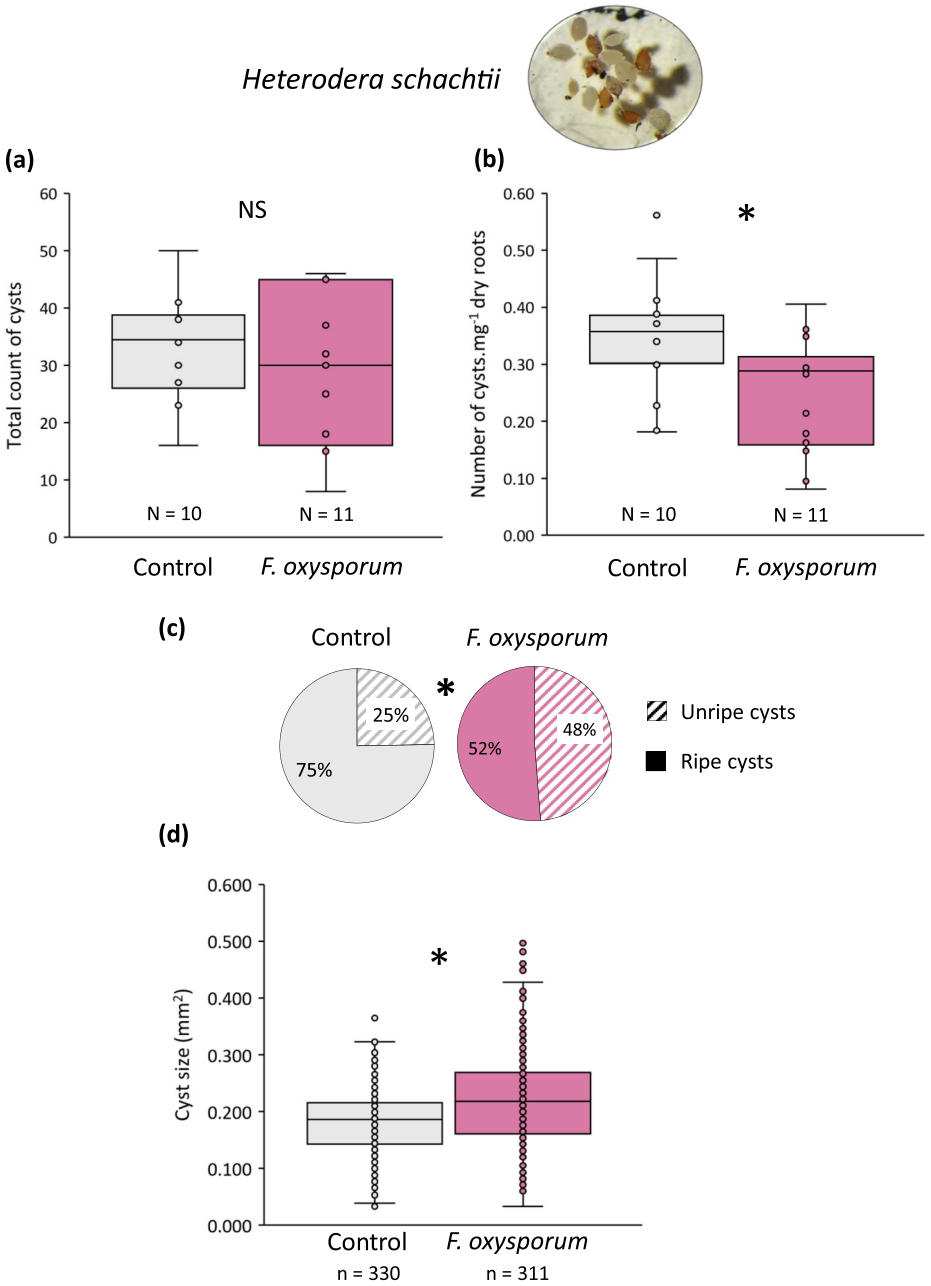

Fig. 2. (a) Number of *Heterodera schachtii* cysts collected in total and (b) per mg of dry weight of *Brassica rapa* roots, (c) mean percentage of unripe cysts (white to light yellow-coloured cysts) and ripe cysts (orange to dark orange-coloured cysts), and (d) size of the cysts collected on control 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 total number of collected cysts were tested with a generalised linear model with a quasi-binomial distribution, while the number of cysts per unit of dry roots was tested with a Student's *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 (*: $P < 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com].

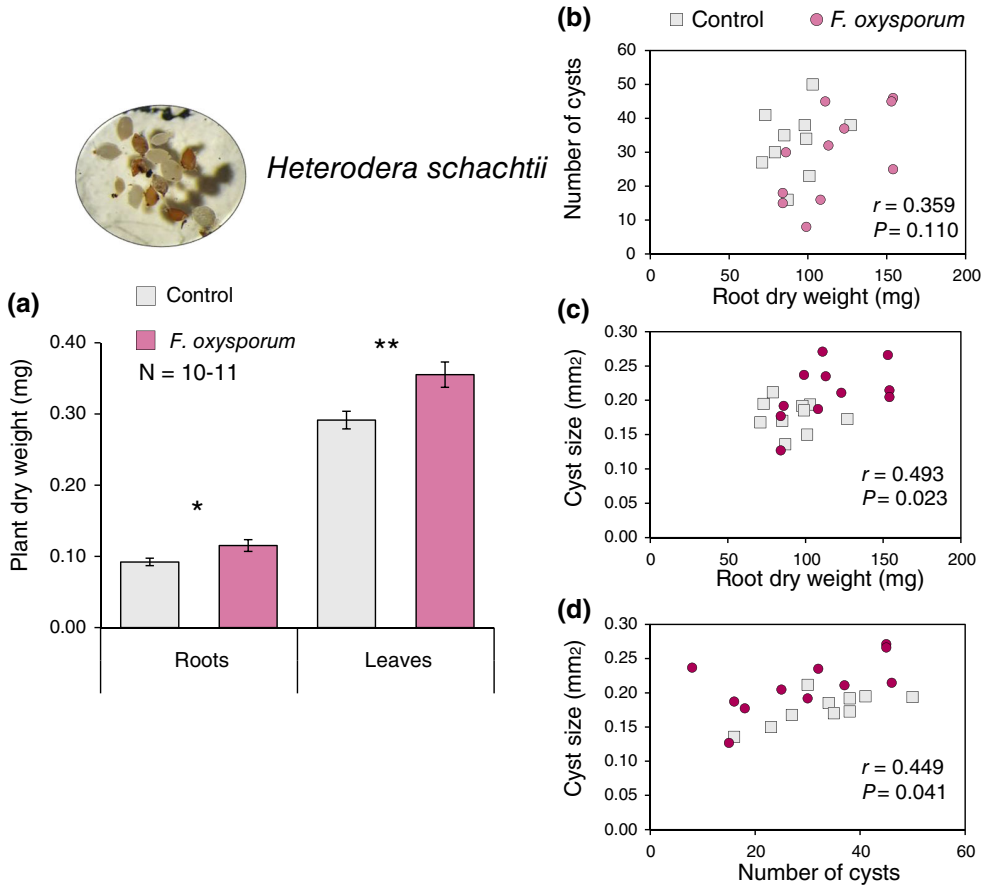

Fig. 3. (a) Root and leaf dry weight of *Heterodera schachtii*-infested *Brassica rapa* plants when exposed or not to *Fusarium oxysporum* volatiles. Pearson correlations between (b) the number of *H. schachtii* cysts collected per plant and the root dry weight, (c) the average size of *H. schachtii* 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's *t*-test (*: $P < 0.05$; **: $P < 0.01$). [Colour figure can be viewed at wileyonlinelibrary.com].

Moreover, cysts retrieved from plants exposed to the fungal volatiles were larger than those collected from control plants (Fig. 2c; $F = 5.8$; $P = 0.026$). Exposure to the fungal volatiles increased root and leaf weight of *H. schachtii*-infested plants (Fig. 3a; $t_{root} = -2.3$; $P_{root} = 0.034$; $t_{leaf} = -2.9$; $P_{leaf} = 0.009$). Overall, cyst size was positively correlated with root dry weight (Fig. 3b; $r = 0.493$; $P = 0.023$) and with the number of cysts retrieved (Fig. 3c; $r = 0.449$; $P = 0.041$).

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. 4a; GLM; $P = 0.731$). We found as many pupae and larvae from control plants and plants exposed to *F. oxysporum* volatiles (Fig. 4b; GLM; $P = 0.899$), and their weight did not differ (Fig. 4c; $F = 0.1$; $P = 0.468$). Exposure to the fungal volatiles did not impact plant growth of *D. radicum*-infested plants (Fig. 5a; $t_{root} = 0.2$; $P_{root} = 0.789$; $t_{leaf} = 0.0$; $P_{leaf} = 0.958$). Overall, *D. radicum* fresh weight was positively correlated with root dry weight (Fig. 5b; $r = 0.481$; $P = 0.015$) and with the number of individuals recollected (Fig. 5c; $r = 0.486$; $P = 0.014$).

Discussion

Our results show that interactions of *B. rapa* plants with root herbivores can be 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 the development and reproduction of the nematode *H. schachtii* were negatively and positively affected, 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. Together, our data show that fungal volatiles can specifically affect the performance of root herbivores and plant growth upon root herbivory.

Interaction between *B. rapa* roots and *H. schachtii* nematodes was hindered upon root exposure to *F. oxysporum* volatiles.

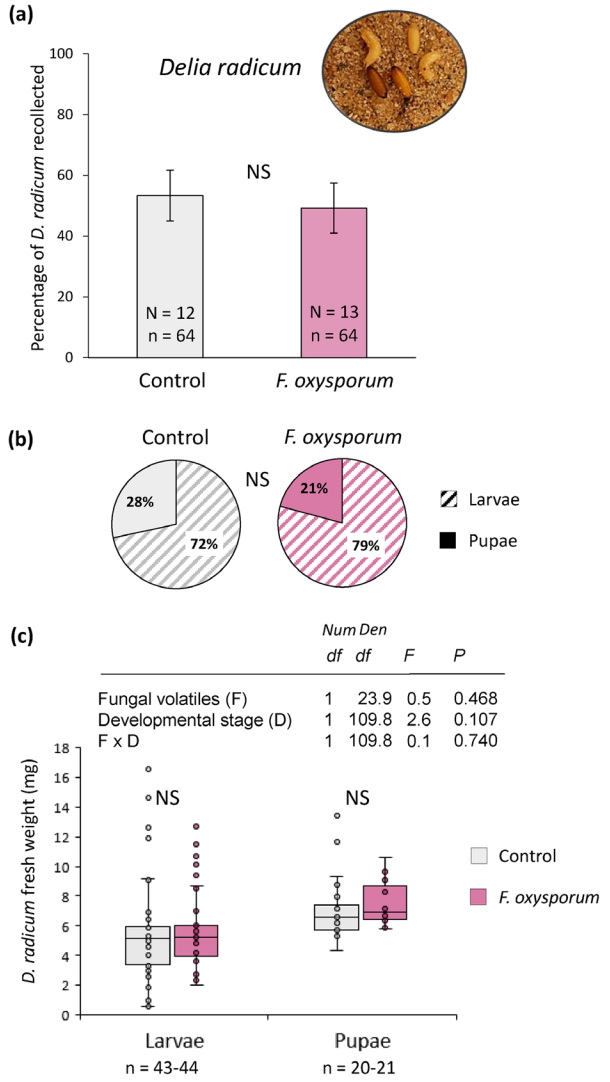

Fig. 4. (a) Percentage (mean ± SE) of *Delia radicum* recollected per *Brassica rapa* plant, (b) mean percentage of *D. radicum* larvae and pupae recollected per plant, and (c) fresh weight of *D. radicum* larvae and pupae recollected from control plants and from plants exposed to *Fusarium oxysporum* 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: $P > 0.05$). [Colour figure can be viewed at wileyonlinelibrary.com].

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 & Hallem 2016). Previous studies have shown that volatiles from soil or root-associated 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). Although we expected higher colonisation in fungal volatile-exposed plants that had more roots to colonise, we recorded similar number of cysts in control plants and in fungal volatile-exposed plants. This result suggests that volatiles from *F. oxysporum* did not impact root colonisation by the nematode.

Interestingly, *F. oxysporum* volatiles did slow down nematode development and potentially increased their reproduction rate. We hypothesise that these effects result from a modulation of plant defences by *F. oxysporum* volatiles. We previously demonstrated that pre-exposure of plant roots to volatiles from fungal pathogens, using same experimental set-ups, can alter plant growth and resistance to insect herbivores, despite the fact that the herbivores were not directly exposed to the fungal volatiles themselves (Cordovez *et al.* 2017; Moisan *et al.* 2019, 2020). Furthermore, we show that root pre-exposure to fungal volatiles can affect plant compensatory growth upon herbivory, suggesting an effect on plant primary metabolism (Moisan *et al.* 2020). Here, we postulate that upon root penetration and colonisation, juveniles encountered more structural (e.g. cuticle, wax layer) or chemical plant defences (e.g. reallocation of primary metabolites, accumulation of secondary metabolites), resulting in lower colonisation rate (Miroslaw *et al.* 2005). For instance, exposure of *Arabidopsis thaliana* plants to volatiles from *Bacillus amyloliquefaciens* resulted in the elevation of glucosinolates in leaves, which negatively affected the performance of an insect herbivore (Aziz *et al.* 2016). Survival and development of the nematodes depends on the establishment of the feeding site, that is the syncytium. For this, cyst nematodes manipulate plant machinery by inhibiting plant resistance responses (Gheysen & Mitchum 2011; Bohlmann & 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 can 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 may support higher numbers of eggs (Atkinson *et al.* 2001). Thus, plant growth promotion by fungal volatiles could have enabled higher food intake by female nematodes, thus increasing cyst size as a result of increased number of eggs. Further research, involving transcriptomic and metabolomic analyses of the plant responses to the fungal volatiles, will be needed to address the underlying mechanisms.

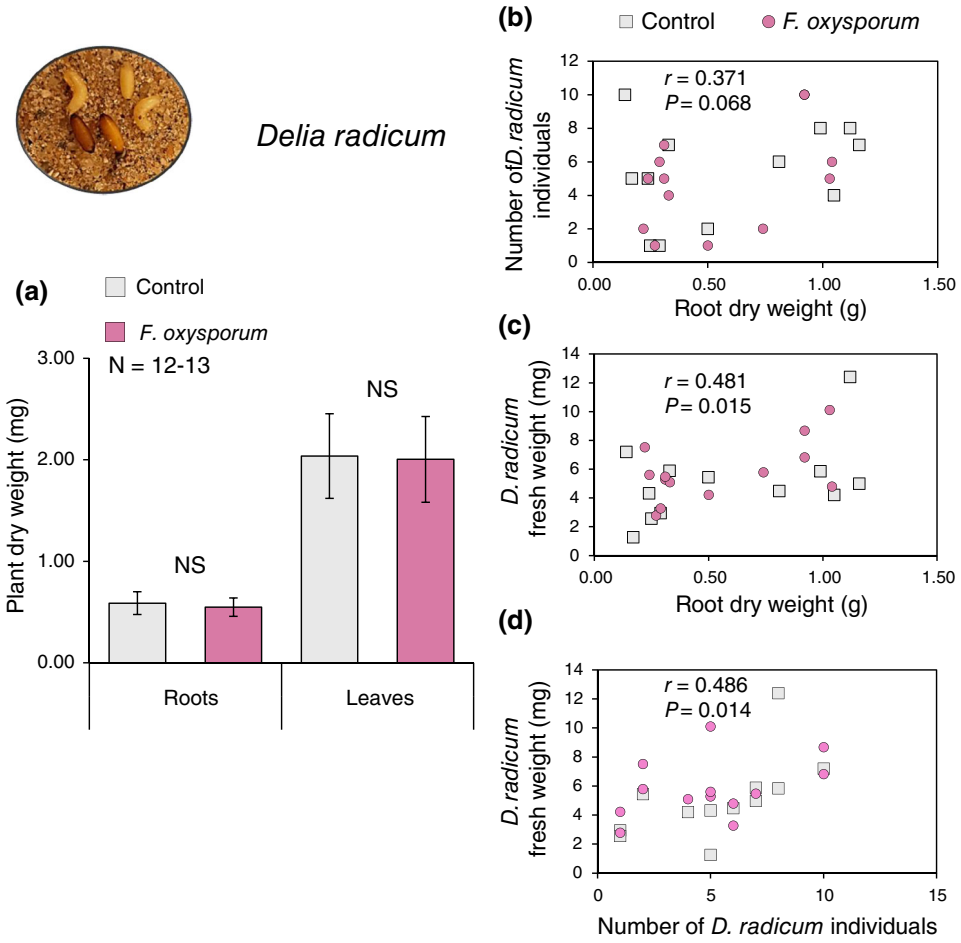

Fig. 5. (a) Root and leaf dry weight of *Delia radicum*-infested *Brassica rapa* plants when exposed or not to *Fusarium oxysporum* volatiles. Plant dry weight of control plants and plants exposed to *F. oxysporum* volatiles was compared with Student's *t*-test (NS: $P > 0.05$). Pearson correlations 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. [Colour figure can be viewed at wileyonlinelibrary.com].

Interestingly, unlike *H. schachtii* nematodes, larvae of *D. radicum* performed equally on control plants and plants exposed to fungal volatiles, and plant growth was unaffected. These differences may be explained by the size of the pots used. In the setup which is used for *D. radicum*, the top compartment was larger than that for *H. schachtii*, hence increasing the distance between the roots plus the insect herbivores and the source of the fungal volatiles. Thus, plant roots and insect larvae were less likely to be directly exposed to fungal volatiles. However, we recently showed, using the same large pots, that development of *D. radicum* can be slowed down in *B. rapa* roots when these plants are pre-exposed to *F. oxysporum* volatiles (Moisan *et al.* 2020). Thus, the setup used is effective. The difference in the results of the current study suggests that the direct effects (when the herbivore itself is also exposed to the volatiles, our current study) and the indirect effects (*via* plant-mediated changes when roots are exposed to the fungal volatiles prior to herbivore attack, Moisan *et al.* (2020)) of fungal volatiles on the performance of *D. radicum* can differ. Moreover, fungal volatiles may also elicit biochemical and physiological changes in the roots that can differentially affect plant resistance to specific attackers. For instance, colonisation of *A. 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 may expect differences in fungal volatile-induced plant responses as well. Taken together, our findings show that volatiles from a soil-borne fungus can modulate belowground interactions of plants with root attackers.

In conclusion, we show that fungal volatiles can diffuse through the soil matrix and induce plant phenotypic responses that, in turn, can affect the performance of root herbivores. The next step will be to investigate these interactions in more complex and natural ecosystems with highly competitive soil microbiomes and with more dynamic and complex blends of volatiles. Additionally, as *F. oxysporum* can parasite

nematode eggs and can colonise plant roots as an endophyte, it would be interesting to test more soil-borne fungi to determine if such effects on plant interactions with root herbivores can result from a manipulation of the fungus for its own benefit.

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Authors contribution

KM, MD, JMR and VC planned and designed the study. KM and ER performed the assay with the nematodes and KM performed the assay with the insect. KM processed the data. KM, MD, JMR and VC interpreted the data and wrote the manuscript.

Data availability statement

Data available on request from the authors.

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