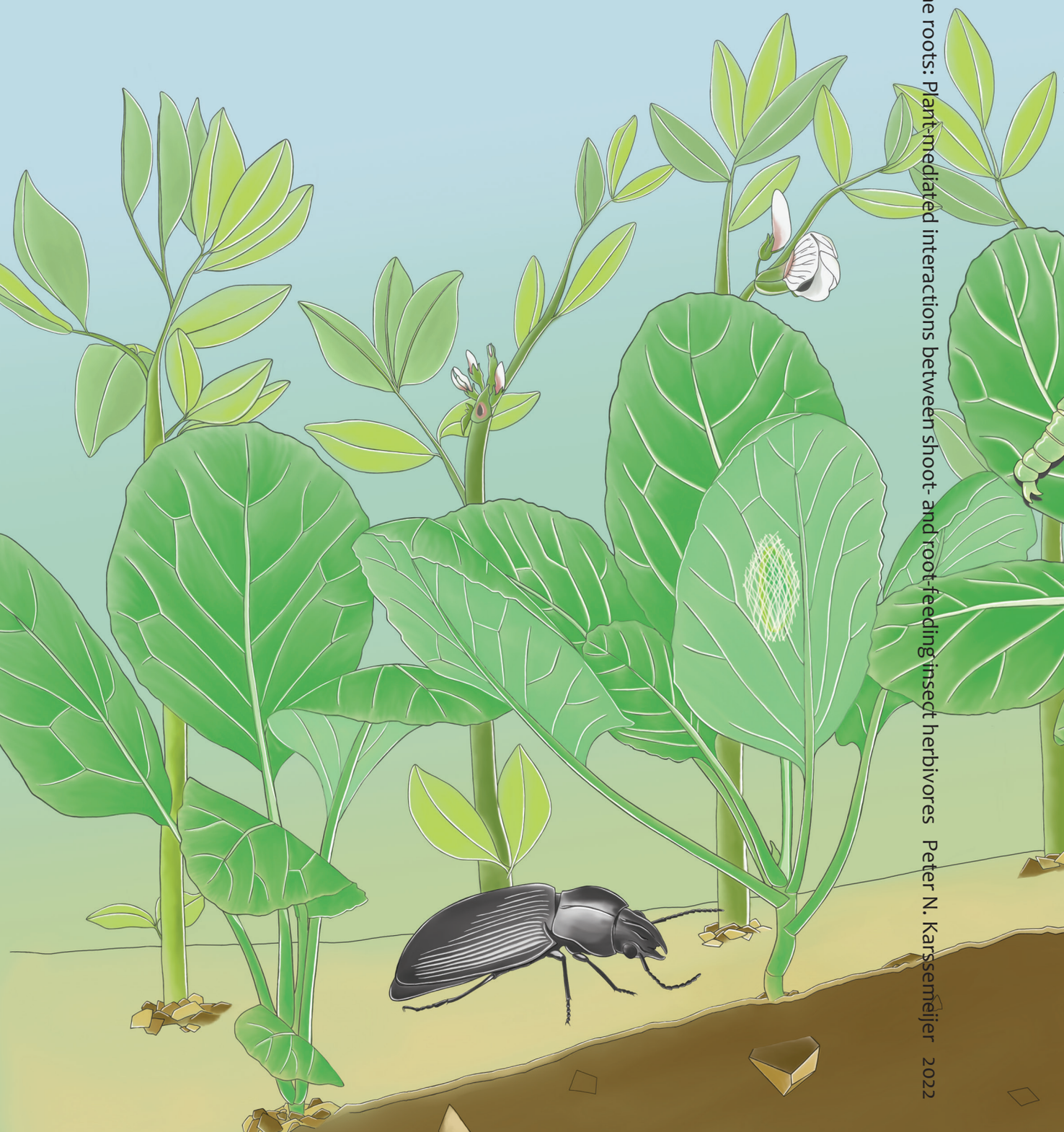
Down to the roots:
Plant-mediated interactions between shoot- and root-feeding insect herbivores



Down to the roots: Plant-mediated interactions between shoot- and root-feeding insect herbivores Peter N. Karssemeijer 2022

Peter N. Karssemeijer

Propositions

1. *Delia radicum* larvae manipulate plant defence to reduce aliphatic glucosinolate biosynthesis.
(this thesis)

2. *Brassica oleracea* plants integrate rhizosphere microbes in defence responses against insect herbivores.
(this thesis)

3. Theoretical intelligence is overvalued.

4. Exploiting biodiversity is essential for sustainable agriculture.

5. Greenwashing delays much-needed climate action.

6. Classification of people into being “higher” and “lower” educated fuels elitism.

7. Male feminists are needed to crack the glass ceiling from above.

Propositions accompanying the thesis, entitled

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Peter N. Karssemeijer
Wageningen, 8 April 2022

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This research was conducted under the auspices of the Graduate School Experimental Plant Sciences

Down to the roots:

Plant-mediated interactions between shoot- and root-feeding insect herbivores

Peter N. Karssemeijer

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 8 April 2022
at 4 p.m. in the Aula.

Peter N. Karssemeijer
Down to the roots: Plant-mediated interactions between shoot- and root-feeding insect herbivores
230 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2022)
With references, with summary in English

ISBN: 978-94-6447-128-1
DOI: https://doi.org/10.18174/565517

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Chapter 1

General introduction

Plants and the tree of life

The first plants grew on terrestrial Earth over 450 Million year ago (Mya) (Kenrick & Crane, 1997). Early fossils show simple plants, much like liverworts that exist today. In the Devonian (420-360 Mya), plants had developed many of the structures we still see today. Vascular tissue, leaves, roots, and seeds evolved to cope with the difficulties of life on land (Kenrick & Crane, 1997). Since then, land plants have been the dominant primary producers in nearly every terrestrial ecosystem. Nowadays, over 350,000 species of plants are named and described (Royal Botanic Gardens Kew, 2016), ranging from towering trees to the smallest weed and from expansive grasses to the crops we eat. The downside of being at the basis of virtually all life on land is that everyone wants to eat you.

Indeed, plants are on the menu for terrestrial life forms throughout the tree of life. Plant attackers comprise insects, arthropods, nematodes, mites, mammals, fungi, bacteria, viruses, oomycetes, unicellular eukaryotes, and even other plants. In natural ecosystems, these attackers help safeguard biodiversity by reducing dominant species (Carson & Root, 2000). However, in agricultural settings, a substantial amount of food is lost every year to pests and diseases (Oerke, 2006; Deutsch *et al.*, 2018). To avoid economic losses, farmers commonly deploy chemical pesticides to protect their crops, causing great environmental costs and public health risks (Schulz & Liess, 1999; Goldman, 2014; Hallmann *et al.*, 2014; Kessler *et al.*, 2015; Stykel *et al.*, 2018; Calvo-Agudo *et al.*, 2019). These environmental and public health concerns led countries to ban or restrict the use of a number of insecticidal chemicals, including DDT and several neonicotinoids (Carvalho, 2017; European Commission, 2018a; European Commission, 2018b; European Commission, 2018c), driving the need for alternatives. In recent years, there is a consumer-driven trend towards sustainability, and circular and sustainable agriculture have become buzz-words for policymakers. To reach such a transition to a more sustainable food production system, it is vital to deepen our understanding of plants and their attackers.

Insect herbivores

Insect herbivores are one of the largest groups of plant attackers. They are subdivided into feeding guilds, of which the main groups are chewing, piercing/sucking, and mining. The earliest evidence of arthropod herbivory dates back nearly to the rise of land plants, around 420 Mya, and by the Triassic (250 Mya) all major insect feeding guilds are represented in the fossil record (Labandeira *et al.*, 2014; Pinheiro *et al.*, 2016). By recent estimates, there are currently 5.5 million species of insects, of which around one million are named (Stork, 2018). Roughly half of all these insect species is thought to be herbivorous (Schoonhoven *et*

al., 2005). The majority of these species are specialist herbivores, feeding only on a narrow range of host plants, while a much smaller group of herbivorous insects are generalists, feeding on a wide range of host plants from multiple plant families (Ali & Agrawal, 2012). Only a fraction of insect herbivore species reach the level of agricultural pest (Schoonhoven *et al.*, 2005), yet those that do leave a strong mark. Throughout written history we find examples of devastating pest outbreaks. For instance, one of the biblical plagues of Egypt describes swarms of locusts devouring the fields (Exodus 10:3–6), much like the outbreaks of the desert locust *Schistocerca gregaria* Forskåll (Orthoptera: Acrididae) seen throughout Africa in recent years.

Herbivorous insects differ in their preferred feeding site within the host plant. The most commonly studied species feed on leaves, but there are many others that predominantly feed on flowers or roots. Root herbivores can be particularly devastating agricultral pests (Johnson *et al.*, 2016a). For instance, the western corn root worm *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is widespread throughout the US and Europe, and costs of damage and control are estimated to be many billions of US$ annually (Gray *et al.*, 2008). Reasons that root herbivores are so damaging include their persistence, many root herbivores have long larval cycles leading to prolonged damage, and the cryptic nature of their attack, which means that they often go unnoticed until severe aboveground damage is observed (Johnson *et al.*, 2016a; Johnson *et al.*, 2016b). This cryptic nature is perhaps best described by an example; cicadas in floodplain forests in Illinois can reach densities of 600,000 larvae per hectare, yet they are only visible when they emerge as adults once every 17 years (Dybas & Davis, 1962).

Johnson *et al.* (2016b) outlined how insect root herbivores differ from aboveground feeders. Firstly, root herbivores are generally less mobile, as movement through the soil matrix is more challenging than through air. Second, root feeders are often long-lived, larval stages that last more than a year are no exception. Third, root feeders are less diverse than shoot herbivores, and the majority belong to the chewing feeding guild. That said, other feeding guilds are also represented belowground including xylem-feeding cicadas, phloem-feeding root aphids, and root-mining fly larvae (Brown & Gange, 1990). Fourth, root herbivores have to cope with a wide range of abiotic factors such as moisture content, temperature and soil texture, and their low mobility means they cannot easily escape these microclimates (Barnett & Johnson, 2013; Johnson *et al.*, 2016b). Finally, and arguably most importantly, there are many other organisms that inhabit the soil directly around plant roots; the rhizosphere (Johnson & Rasmann, 2015). The rhizosphere is a microbial hotspot due to extensive root exudation of plant nutrients. Research into functions of this “second genome” of plants has taken off in recent years, revealing that plants strongly influence the microbes surrounding

their roots (Berendsen *et al.*, 2012). Furthermore, the soil is inhabited by an abudance of nematodes (Neher, 2010), including entomopathogenic nematodes that present formidable natural enemies for soil-dwelling insects (Georgis *et al.*, 2006; Jones *et al.*, 2013). All in all, the environment inhabited by insect root herbivores differs in many ways from the aboveground.

A peek inside the plant arsenal

Look around you in most places on Earth, and you will see lush greenery, a strong indication that plants have some means to defend themselves. Indeed, plants have evolved defence mechanisms in order to repel, deter, tolerate or hamper their attackers. While some of these defences are obvious to the eye, such as sharp spines and thick bark, the most important aspects of plant defence involve a highly diverse arsenal of specialised toxic chemicals and proteins which are usually invisible (Fig. **1**).

Coevolution has shaped plant defence against insects, leading to an immense diversity of plant defence mechanisms as well as coping strategies by insect herbivores. To get an idea of the extent of coevolution between plants and insects, let us consider two factors. First, defence mechanisms were already present in the earliest land plants, over 400 Mya, illustrated both by the fossil record and by the presence of overlapping defence mechanisms in all plants including the ancient liverwort linneage (Labandeira *et al.*, 2014). Second, both plant and insect species are capable of rapid evolution. When released from herbivore pressure, plants are able to rapidly evolve changes in defence mechanisms (Agrawal *et al.*, 2012). Moreover, in modern times, plant breeders have imposed strong selection pressures on insect herbivores by developing resistant crop varieties, and there are ample examples where insect pests have overcome a novel resistance mechanism within a limited timeframe (Thrall *et al.*, 2011; Douglas, 2018). Combine the knowledge of this long coexistence and the potential for rapid evolutionary changes, and you can imagine the extent of coevolution in plant-insect interactions.

Plant defence traits can be categorised by their mode of action (direct versus indirect) and their inducibility (Erb & Reymond, 2019). Direct defences are those traits that target phytophagous insects directly, such as spines, thick cuticles, chemical defences, or proteinase inhibitors that interfere with digestion by the insect (Erb & Reymond, 2019). Indirect defences, on the other hand, are traits that influence the interactions between herbivores and their natural enemies. These traits include extrafloral nectaries to provide a food source for natural enemies, and plant volatiles that help predators and parasitoids to find their hosts or prey (Dicke & Baldwin, 2010). Defence traits can be expressed constitutively,

but can also be induced upon perception of a herbivore (Erb & Reymond, 2019). Induced defences are less costly in terms of energy, as they are only expressed upon attack (Kessler, 2015). Furthermore, inducible defences provide flexibility when plants need to respond to various attackers (Kessler, 2015; Mertens *et al.*, 2021b). The levels of defence, as well as the balance between constitutive and induced traits, differs between plant species and even between tissues within the same plant (Tsunoda *et al.*, 2017).

Plant defence traits differ between leaves and roots. Whereas in aerial parts of plants, spines and glandular trichomes may be effective in hampering insect herbivores, one can imagine that these structures have little effect in the soil matrix. Plants strengthen their roots using organic polymers such as lignin and suberin, and this provides resistance to herbivory (Johnson *et al.*, 2010). Furthermore, like leaves, roots are equiped with chemical defences, but differences in compounds or concentrations between root and shoot tissues have been described in many species (Rasmann & Agrawal, 2008; van Dam, 2009). For instance, cabbage roots contain high concentrations of the aromatic glucosinolate gluconasturtiin, whereas this compound is hardly produced in leaves (van Dam *et al.*, 2009). Moreover, some secondary metabolites are produced only in roots and are transported to defend other tissues. These include various alkaloids in the Asteraceae and Solanaceae families, including nicotine in tobacco plants (Dawson, 1941; Erb *et al.*, 2009b). Indirect defence can also occur in roots; for instance, maize roots attacked by western corn rootworm larvae emit high concentrations of the volatile compound (*E*)-β-caryophyllene which attracts entomopathogenic nematodes from the surrounding soil (Rasmann *et al.*, 2005).

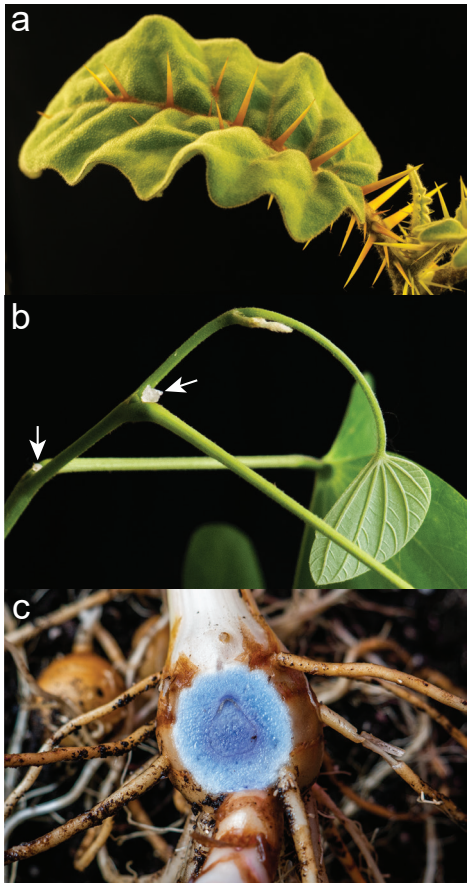

Figure 1. Examples of plant defence traits. Spines on *Solanum pyracanthum* (**a**), extrafloral nectaries that provide sugar to natural enemies in leaf axils of *Bauhinia variegata* (**b**), blue colouring by secondary metabolites of *Curcuma aeroginosa* rhizomes (**c**). Plants grown and photos taken by the author.

Plant roots may also make use of soil microbes when defending against root herbivores. The soil directly around the roots is a microbial hotspot, powered by extensive exudation of nutrients from plant roots (Mendes *et al.*, 2013). Upon herbivory the composition of root exudates changes, resulting in changes in the microbial community composition (Hu *et al.*, 2018; Friman *et al.*, 2021). Rhizosphere microbes include strains that can stimulate plant defence (Pineda *et al.*, 2010; Pieterse *et al.*, 2014). Furthermore, among the microbial rhizosphere inhabitants are entomopathogenic fungi and bacteria, which may provide indirect defence against insect herbivores (Johnson & Rasmann, 2015; Rasmann & Turlings, 2016). Whether plants have evolved to specifically attract and nurture entomopathogenic microbes has not been directly studied to date. If indeed they do, this would make root exudation in some way analogous to the provision of extrafloral nectar in shoot tissues.

The inner workings of plant defence

In the previous section I have established that plants evolved a vast arsenal of defences against insect herbivores, and that these defences can be induced upon attack. In order to regulate induced defence, plants have evolved an intricate network of molecular regulation. Starting from the perception of an attacker, signaling pathways are activated and fine-tuned to respond with adequate inducible defence traits. Much of the knowledge on these molecular mechanisms comes from research on the model plant *Arabidopsis thaliana*, and its interactions with various pathogens (Jones & Dangl, 2006). However, there are many similarities between the molecular aspects of plant responses to pathogens and insect herbivores (Kessler & Baldwin, 2002; Erb & Reymond, 2019).

Perception of an attacker is the first step to defence. Plants are equipped with pattern recognition receptors, proteins that have an extracellular part that can recognise specific molecular patterns and relay this information inside the cell (Acevedo *et al.*, 2015). Many of these receptors are known, and they can respond to molecular patterns associated with mechanical damage, microbes, or herbivores. Molecular patterns can be cues for plant tissue damage, such as plant protein fragments, or proteins present in insect saliva (Huffaker *et al.*, 2013; Acevedo *et al.*, 2015). As there is much variation in the composition of insect saliva, plants are able to respond differently to specific insects. Perception is rapidly followed, within seconds in some cases, by depolarization of the plasmamembrane and rise in cytosolic Ca^{2+} ions (Maffei *et al.*, 2004). Some insect herbivores are able to counter recognition. For instance, Colorado potato beetles harbor bacteria in their saliva that trigger a pathogen response in the plant, thereby suppressing plant defence that would be effective against the insect (Chung *et al.*, 2013).

Following perception of an attacker, the defence response is initiated through a network of signalling pathways (Pieterse *et al.*, 2009). The phytohormone jasmonic acid (JA) lies at the core of regulation of plant responses to insect herbivores (Erb & Reymond, 2019). Jasmonates are a class of oxylipid signaling molecules, which are derived from the precursor α-linolenic acid, a membrane component of chloroplasts (Wasternack & Hause, 2013). Activation of defence responses regulated by JA is achieved through degradation of JAZ proteins that act as repressors of transcription factors. The most important of these transcription factors is MYC2, which is regarded as a master regulator of JA responses (Kazan & Manners, 2013). Phytohormonal signaling pathways are interconnected, and signaling in response to insect herbivores is further modulated by salicylic acid (SA), ethylene (ET), and abscisic acid (ABA). The general consensus is that SA regulates responses against biotrophic pathogens and piercing-sucking insect herbivores, ET together with JA against necrotrophic pathogens, ABA together with JA against chewing insect herbivores, and ABA against abiotic stresses (Pieterse *et al.*, 2012). Together, these four hormonal pathways, through a network of positive and negative feedback loops, fine-tune plant responses to biotic and abiotic stresses (Pieterse *et al.*, 2012).

While most studies on plant defence have focused on the leaves, regulation of defence in plant roots has gained popularity in recent years (Johnson & Rasmann, 2015; Johnson *et al.*, 2016b). As in shoot tissues, JA plays a central role in regulating defence against root herbivores (Johnson *et al.*, 2016b). Despite differences in plastids that are the source of the jasmonate precursor α-linolenic acid (root plastids develop into storage organs rather than chloroplasts), roots are capable of synthesizing jasmonic acid (Grebner *et al.*, 2013). However, herbivore-induced biosynthesis of jasmonates is weaker in roots compared to shoots (Erb *et al.*, 2012a). This could be caused by organ-specific enzymes that may differ slightly in their activity; in *A. thaliana*, wound-induced JA biosynthesis is mediated by the lipoxygenase LOX2 in leaves and by LOX6 in roots (Grebner *et al.*, 2013). Moreover, there are differences in signaling within the JA pathway between shoot and root tissues (Acosta *et al.*, 2013). Indeed, even though induction of JA is weaker in roots, it can cause a strong response in downstream processes such as production of secondary metabolites (Erb *et al.*, 2012a). Interestingly, the phytohormonal network of JA, SA, ET and ABA, which is important for fine-tuning the response in shoot tissues, appears to be different in roots (Johnson *et al.*, 2016b). This may be due to ancillary functions of these hormones in root-specific processes, for instance lateral root formation (Saini *et al.*, 2013). Despite recent advances, our understanding of defence regulation in plant roots is limited.

When discussing molecular regulation of plant defence in roots, it is worth mentioning the interactions between plants and plant-parasitic nematodes. These microscopic worms feed exclusively on roots and engage in intimate molecular interactions with their host plants.

Due to the economic importance of some plant-parasitic nematodes, especially those that feed in specialised galls or cysts, much effort has gone into understanding the molecular mechanisms underlying their interactions with the host (Jones *et al.*, 2013; Goverse & Smant, 2014). The phytohormones JA, SA, and ET are involved in regulation of plant defence against plant-parasitic nematodes and some nematodes are able to suppress these pathways during successful infection (Nahar *et al.*, 2011; Kyndt *et al.*, 2012; Kammerhofer *et al.*, 2015; Martínez-Medina *et al.*, 2021). Arguably more importantly than phytohormonal signaling, plant-parasitic nematodes make use of effectors to suppress plant defences, which plants tackle with highly specific resistance genes (Kandoth & Mitchum, 2013). Although the types of interactions with the host plants differ substantially between plant-parasitic nematodes and insect root herbivores, they show that plant roots are capable of engaging in specialised molecular defence responses.

Induced plant defence responses are not only triggered locally, but systemically throughout the plant. This way, herbivory on the leaves of plants can trigger a defence response in distal leaves and even in roots, and *vice versa* (Soler *et al.*, 2013). For instance, when cotyledons of *Arabidopsis* seedlings are wounded, induction of JA in the roots quickly follows (Acosta *et al.*, 2013). Similarly, when tobacco leaves are wounded and treated with oral secretions of *Manduca sexta* Linneaus (Lepidoptera: Sphingidae) caterpillars, concentrations of JA increase in stems, systemic leaves, and roots within two hours (Machado *et al.*, 2018). Systemic induction may play a role in the accumulation and transport of secondary metabolites that are only produced in certain tissues, like nicotine which is only produced in roots. Further, systemic induction can aid plants in regulating their defences in complex ecological environments in which attackers are rarely alone.

Plant-mediated species interactions

Insect herbivores do not wait for their turn to attack, and as such, plants often face multiple stresses simultaneously. Through systemic induction of plant defence, plant attackers can affect one another even when they are spatially or temporally separated; a concept termed plant-mediated species interaction. The first examples of interactions between organisms via plant traits were recorded by phytopathologists, although they did not call them plant-mediated species interactions but systemic acquired resistance (Durrant & Dong, 2004). Virologist Frank Ross, in 1961, found that tobacco plants treated with tobacco mosaic virus on one leaf were better defended against the same virus in distal leaves (Ross, 1961). Further studies indicated that systemic acquired resistance was effective against a broad range of pathogens, indicating that multiple species of pathogens interacted via induction of plant defence (Ryals *et al.*, 1996; Durrant & Dong, 2004). Later, researchers discovered that certain microbes in the rhizosphere

could aid in defence against leaf pathogens and insect herbivores, a process termed induced systemic resistance (van Peer *et al.*, 1991; Pieterse *et al.*, 2014). These interactions also occur between different insect herbivores feeding on the same plant, and entomologists refer to them as plant-mediated species interactions (Masters & Brown, 1992; Stam *et al.*, 2014).

Plant-mediated species interactions can result in antagonism or facilitation between insect herbivores sharing a host plant (Kaplan & Denno, 2007; Stam *et al.*, 2014). For instance, *Pieris brassicae* L. (Lepidoptera: Pieridae) caterpillars perform better on plants previously colonised by *Brevicoryne brassicae* L. (Hemiptera: Aphididae) aphids (Soler *et al.*, 2012). Over the past decades, a myriad of studies have described interactions between numerous combinations of insect herbivores on numerous host-plant species (Kaplan & Denno, 2007). Factors such as feeding guild, feeding site, and sequence of arrival determine whether facilitation or antagonism occurs (Johnson *et al.*, 2012; Stam *et al.*, 2014; Huang *et al.*, 2017). Systemic induction of plant defence mediates these interactions, and as such, they also occur from root to shoot tissues and *vice versa* (Soler *et al.*, 2013; Papadopoulou *et al.*, 2018). Indeed, in maize plants, root feeding by *D. v. virgifera* larvae leads to increased plant resistance to *Spodoptera litoralis* Boisduval (Lepidoptera: Noctuidae) caterpillars on the leaves (Erb *et al.*, 2009a). In the opposite direction, *P. brassicae* caterpillars induced resistance against root-feeding *Delia radicum* L. (Diptera: Anthomyiidae) larvae in *Brassica nigra* plants (Soler *et al.*, 2007). Antagonism between above- and belowground chewing herbivores was confirmed in a meta-analysis of studies investigating plant-mediated interactions between shoots and roots (Johnson *et al.*, 2012), but the underlying mechanisms are far from understood, especially for interactions from shoot to root.

Aboveground herbivores may affect defence in roots in several ways. A recent study showed that simulated leaf chewing facilitates the performance of plant parasitic nematodes on roots, and that a functional JA pathway is required for this plant-mediated interaction (Machado *et al.*, 2018). On the other hand, aboveground sap-feeding whiteflies induced resistance against *Agrobacterium* in roots via the SA pathway (Song *et al.*, 2015). Thus, both JA- and SA-mediated signaling occur in both shoots and roots. More downstream in the defence response, aboveground chewing herbivory can lead to increased levels of secondary metabolites in roots, such as glucosinolates and tannins, potentially affecting root herbivore performance (Soler *et al.*, 2013; Huang *et al.*, 2014). Aboveground sap-feeding herbivores also induce changes in root primary and secondary metabolites (Johnson *et al.*, 2009; Kutyniok & Müller, 2012). Moreover, whiteflies and aphids feeding on leaves can alter root exudation and recruitment of rhizosphere microbes (Kim *et al.*, 2016; Kong *et al.*, 2016). Furthermore, aboveground herbivory can alter volatile production in roots, leading to differences in attractiveness to root herbivore larvae and their natural enemies (Rasmann & Turlings, 2007; Huang *et al.*, 2017). Finally, the plant

response to root herbivores may be altered when an aboveground herbivore was previously present. For instance, aboveground herbivores may cause priming of defence in plant roots, leading to a faster or stronger response upon root herbivory. In conclusion, defence against insect root herbivores can be altered by prior aboveground herbivory. Learning more about these fascinating aboveground-belowground interactions aids our understanding of nature, and may lead to novel strategies for sustainable crop production.

Thesis objective

I hope this introduction sparked in you the same enthusiasm and passion that I feel about the complex interactions between plants and insect herbivores above- and belowground. It is from this passion that I wrote a research proposal five years ago which eventually led to this thesis.

The main objective of my research was **to identify and understand the effects of shoot-feeding insect herbivores, *via* plant-mediated interactions, on root-feeding insect herbivores**. I chose to study effects of both chewing and sap-feeding aboveground herbivores, as these feeding guilds are known to induce different plant defence responses (Fig. **2**). Furthermore,

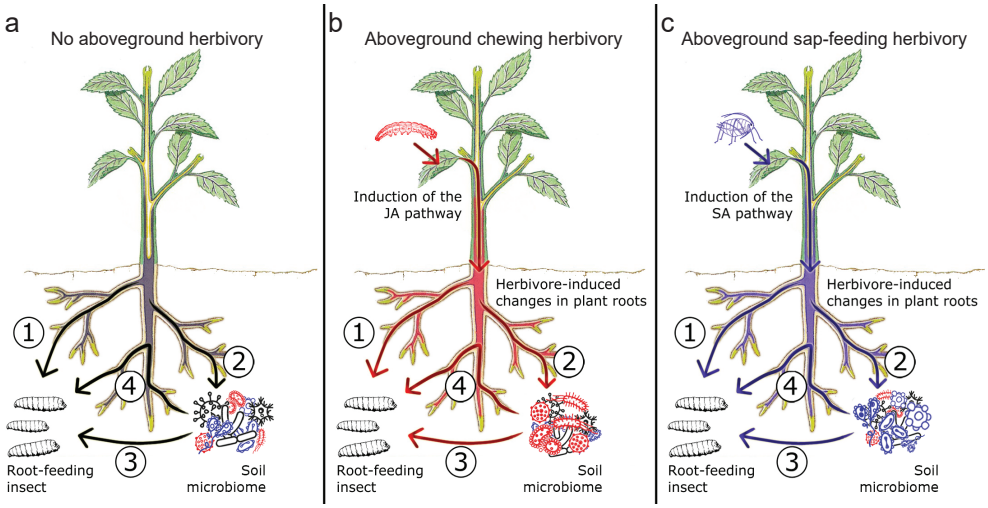

Figure 2. Schematic representation of aboveground-to-belowground plant-mediated species interactions in scenarios when no herbivores (**a**), chewing herbivores (**b**) or sap-feeding herbivores (**c**) are present on leaves. Plant roots may affect root-feeding insects directly (**1**), e.g. by secondary metabolites, and indirectly via the soil microbiome (**2**). The soil microbiome can, in turn, affect root-feeding insects directly (**3**), e.g. by entomopathogenic fungi, or indirectly by inducing the plant immune system (**4**), e.g. by beneficial soil bacteria. Aboveground herbivory induces changes in plant traits, starting with the induction of the jasmonic acid (JA) or salicylic acid (SA) pathways by chewing and sap-feeding insect herbivores, respectively. These pathways lead to changes in root traits, which, in turn, may change both direct and indirect (via the soil microbiome) effects on root-feeding insects. This figure was made by the author in 2016 as the central figure of the research proposal that led to this thesis.

very few studies had investigated the effects of aboveground sap-feeders on root herbivores (Johnson *et al.*, 2009; Soler *et al.*, 2013). Aboveground herbivores may affect root herbivores via differences in plant defence, or indirectly via changes in the root-associated microbiome. During the four years of my PhD project, I studied many aspects of plant-mediated species interactions between aboveground and belowground herbivores. To bridge the molecular and ecological fields that draw my interest, I combined experiments with a molecular focus, greenhouse bioassays, and a field study.

Study system

The experiments that compose this thesis involve interactions between *Brassica oleracea* plants and three of its major insect herbivore pests: the cabbage root fly *D. radicum*, the diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae), and the cabbage aphid *B. brassicae*. All three species of insect herbivores are considered specialists of the Brassicaceae family and are commonly found in the field.

The plant

Brassica oleracea is a biennial plant species in the Brassicaceae family. Plants in this family have evolved the ability to produce glucosinolates, a class of secondary metabolites that are used in defence against insects and pathogens (Hopkins *et al.*, 2009; Textor & Gershenzon, 2009). The chemical structure of glucosinolates is composed of a core and a variable side chain. Over 120 different glucosinolates have been described, most of which can be assigned to three groups based on the amino acid precursor of the side chain. Indole glucosinolates are derived from tryptophan, aliphatic glucosinolates from methionine, and aromatic glucosinolates from phenylalanine or tyrosine (Hopkins *et al.*, 2009). Glucosinolates are not toxic until they are cleaved by a plant enzyme called myrosinase, leading to toxic breakdown products like isothiocyanates and nitriles. By storing glucosinolates and myrosinases in separate plant cells, the toxic products are only formed upon tissue damage. The model species *A. thaliana* is also a member of the Brassicaceae family, and a vast knowledge base is available for this species. For instance, the genes involved in biosynthesis and regulation of glucosinolates have been extensively characterised in this species (Sønderby *et al.*, 2010). The vast knowledge on the molecular plant biology of this model species can, with care, be used to gain better understanding of brassicaceous crop species. In this thesis, I mostly used Brussels sprouts plants, *Brassica oleracea* var. *gemmifera* cv. “Cyrus”.

The root herbivore

The star insect herbivore in this thesis is the cabbage root fly, *D. radicum*. The adults feed on floral resources, and the larvae are specialist herbivores of plants in the Brassicaceae family. They predominantly feed by burrowing through the tap root, causing stunted growth, drought symptoms, and even plant death. *Delia radicum* is a serious pest on various crops in temperate regions (Finch, 1989). Current control measures in conventional farms include neonicotinoid seed coatings, which are banned in the European Union.

Cabbage root flies have been studied extensively over the past century. In particular, host-plant selection behaviour of the female flies is well characterised. Female flies are attracted to volatile breakdown products of glucosinolates (Hawkes & Coaker, 1979), and assess the leaf colour and shape when they approach a potential host plant (Roessingh & Städler, 1990). Upon landing on a leaf, the fly tastes the chemical profile using receptors on her tarsi, where glucosinolates act as oviposition stimulants (Roessingh *et al.*, 1992). If all is well, she walks around the leaf and petiole to the stem, where she circles around the stem base, possibly to assess the diameter as a proxy for root size and eventually deposits multiple eggs in the soil (Zohren, 1968; Schoonhoven *et al.*, 2005). After several days the eggs hatch and the larvae disperse through the soil searching for the cabbage tap root, where they rapidly disappear into the plant tissue. Around two weeks later, the larvae have grown sufficiently to pupate, which they do in the soil just outside the root. Being specialists on brassicaceous plants, larvae are likely to have a strategy for coping with glucosinolate plant defences. Indeed, enzymes that can detoxify isothiocyanates derived from aromatic glucosinolates were discovered in the *D. radicum* larval gut microbiome (Welte *et al.*, 2016). Other detoxification strategies or means of manipulating host-plant defences have not been described. Moreover, the transcriptional regulation of plant defence responses against *D. radicum* larvae have not been characterised.

The foliar herbivores

To study the plant-mediated effects of aboveground herbivores on *D. radicum*, I included two species of foliar herbivores in most of the experiments in this thesis: leaf-chewing *P. xylostella* (Lepidoptera: Plutellidae) caterpillars and sap-feeding *B. brassicae* (Hemiptera: Aphididae) aphids. These insect herbivores are commonly found on *Brassica* hosts in fields in the Netherlands, indicating that the interactions studied in this thesis are realistic and ecologically relevant. Indeed, in a three-year-long field trial in the Netherlands, over 50 percent of plants studied were colonised by *P. xylostella* and *B. brassicae* (Mertens *et al.*, 2021b)2021b. In one of the chapters of this thesis, other foliar chewing herbivores are included besides *P. xylostella*;

these are *P. brassicae*, *Athalia rosae* L. (Hymenoptera: Tenthredinidae), *Phaedon cochleariae* Fabricius (Coleoptera: Chrysomelidae), *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and *Autographa gamma* L. (Lepidoptera: Noctuidae).

Thesis outline

In **chapter 2**, I studied the plant-mediated effects of *P. xylostella* and *B. brassicae* on the performance of *D. radicum*. To unravel the molecular mechanisms underlying these interactions, I included measurements of phytohormones and gene expression within these phytohormonal pathways. I discuss the plant responses to *D. radicum* in roots, and how aboveground herbivores may modulate them. This work was a stepping stone for **chapter 3**, in which I used RNA-sequencing to investigate how the plant root transcriptome changes in response to herbivory above- and/or belowground. The transcriptomic analysis led to novel hypotheses on plant responses to root herbivory and plant-mediated effects. I tested two of these hypotheses in follow-up experiments. First, I used mutant *B. oleracea* plants to study whether aliphatic glucosinolates confer resistance to *D. radicum*, and second, I studied whether *P. xylostella* primes plant roots to respond faster to *D. radicum*.

Female *D. radicum* flies integrate cues from leaves in their oviposition choice behaviour as described above. As such, plant-mediated effects of aboveground herbivores may have additional effects on *D. radicum* through their host-searching behaviour. Furthermore, while plant-mediated effects of aboveground chewers on root chewers have been targeted in many studies, they usually include only a single species combination. In **chapter 4**, I studied the plant-mediated effects of six different foliage-chewing herbivores on *D. radicum* preference and performance and induction of plant defence. The species of foliar herbivores spanned three insect orders and included both specialist and generalist herbivores. The combination of measurements and inclusion of multiple inducing herbivores allowed me to address two hypotheses in the field of insect-plant interactions: that “mother knows best”, i.e. that oviposition preference is linked to higher larval performance, and that generalist and specialist herbivores induce distinct plant responses.

Plants influence the rhizosphere microbiome through root exudation, and this is altered upon herbivory. This way, plant-mediated species interactions may be mediated by changes in the soil microbiome. In **chapter 5**, I performed a plant-soil feedback experiment. In this experiment, I analysed the rhizosphere microbiome after treating plants with above- and belowground herbivores or beneficial microbes. Furthermore, the soil conditioned by these treated plants was used to grow a new set of plants on which plant defence against *D. radicum* was tested.

Plant-mediated effects found in greenhouse studies are not necessarily translatable to a field setting. Therefore, in **chapter 6**, I studied *D. radicum* oviposition and abundance in the field. To connect the field data to results from the previous greenhouse experiments, I assessed the aboveground herbivore community prior to measurements of *D. radicum* oviposition and abundance. Moreover, when searching the roots and surrounding soil for *D. radicum* larvae and pupae, other belowground macrofauna was also recorded. Plants assessed for these experiments were part of a large intercropping trial, where different cropping systems were compared. In this chapter, I discuss how different cropping systems affect *D. radicum* oviposition and abundance, and how these measurements were connected to the abundance of above- and belowground macrofauna.

Finally, **chapter 7** offers a general discussion of the entire thesis, with a focus on our current understanding of plant defence against *D. radicum* and the mechanisms underlying plant-mediated species interactions.

Acknowledgements

I want to thank my promotors**, Joop van Loon** and **Marcel Dicke**, for their feedback on this chapter.

Photo by Hans Smid

Chapter 2

Foliar herbivory by caterpillars and aphids differentially affects phytohormonal signalling in roots and plant defence to a root herbivore

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Plant, Cell & Environment, 2020, 43 (3), 775-786

DOI: https://doi.org/10.1111/pce.13707

Abstract

Plant-mediated interactions are an important force in insect ecology. Through such interactions, herbivores feeding on leaves can affect root feeders. However, the mechanisms regulating the effects of aboveground herbivory on belowground herbivores are poorly understood. Here, we investigated the performance of cabbage root fly larvae (*Delia radicum*) on cabbage plants (*Brassica oleracea*) previously exposed to aboveground herbivores belonging to two feeding guilds: leaf chewing diamondback moth caterpillars (*Plutella xylostella*) or phloem-feeding cabbage aphids (*Brevicoryne brassicae*). Our study focusses on root-herbivore performance and defence signalling in primary roots by quantifying phytohormones and gene expression. We show that leaf herbivory by caterpillars, but not by aphids, strongly attenuates root herbivore performance. Aboveground herbivory causes changes in primary roots in terms of gene transcripts and metabolites involved in plant defence. Feeding by belowground herbivores strongly induces the jasmonate pathway in primary roots. Caterpillars feeding on leaves cause a slight induction of the primary root jasmonate pathway, and interact with plant defence signalling in response to root herbivores. In conclusion, feeding by a leaf chewer and a phloem-feeder differentially affect root-herbivore performance, root-herbivore induced phytohormonal signalling and secondary metabolites.

Introduction

Most research on insect-plant interactions focusses on what is visible aboveground (Kaplan & Denno, 2007; Poelman *et al.*, 2008; Poelman *et al.*, 2010; Stam *et al.*, 2014; Papadopoulou & van Dam, 2017), yet there is a hidden world beneath our feet, with its own organisms, ecological interactions, food webs, and abiotic environment (Rasmann *et al.*, 2005; Erb *et al.*, 2011b; Johnson *et al.*, 2012; Johnson & Rasmann, 2015). What happens belowground often has major impacts on what we see aboveground. For instance, some of the worst agricultural pests are soil-dwelling, and they drastically affect plant health (Brown & Gange, 1990; Johnson *et al.*, 2016b).

However, plants are by no means defenceless. When attacked by insects, plants respond in terms of gene expression, signal transduction *via* phytohormonal pathways, and eventually responses such as the biosynthesis of secondary metabolites (Pieterse *et al.*, 2009; Erb & Reymond, 2019). In leaves, chewing herbivores commonly induce a defence response mediated by the jasmonic acid (JA) pathway, whereas phloem feeders usually induce the salicylic acid (SA) pathway (Pieterse *et al.*, 2012). Root herbivores induce the JA pathway, although the regulation is different from the aboveground-induced JA pathway, while they seem not to induce the SA pathway (Erb *et al.*, 2012a; Acosta *et al.*, 2013; Johnson *et al.*, 2016b). Defence responses occur not only locally, but throughout the plant. While most studies on systemic responses focus on aboveground tissues, in response to induction in either another leaf or in the roots (Soler *et al.*, 2012; Papadopoulou & van Dam, 2017), there is an increasing body of literature showing that roots respond to leaf herbivory as well (Machado *et al.*, 2013; Soler *et al.*, 2013; Gulati *et al.*, 2014; Huang *et al.*, 2014; Kim *et al.*, 2016; Kong *et al.*, 2016; Machado *et al.*, 2018).

Organisms that are spatially separated can interact via such systemic responses, and in this way the aboveground and belowground communities are linked (Stam *et al.*, 2014). An example of this is induced systemic resistance, in which non-pathogenic rhizosphere microbes enhance defence against aboveground attackers (Berendsen *et al.*, 2012; Pieterse *et al.*, 2014; Pineda *et al.*, 2017). Insect herbivores also affect each other through such plant-mediated interactions (Stam *et al.*, 2014). Herbivores feeding on aboveground plant parts can have a strong impact on root herbivores (Johnson *et al.*, 2012; Soler *et al.*, 2013), but there are large gaps in our understanding of the underlying mechanisms.

The type of defence response, and thus the plant-mediated effect on subsequent herbivores, that is initiated by a feeding herbivore depends largely on the feeding guild (e.g. chewing or phloem-feeding) of the inducing insect (Stam *et al.*, 2014). Chewing herbivores on

leaves generally negatively impact root-feeding insects (Hunt-Joshi & Blossey, 2005; Erb *et al.*, 2011c; Johnson *et al.*, 2012), and this has been correlated to changes in secondary metabolites such as tannins or glucosinolates (Soler *et al.*, 2013; Huang *et al.*, 2014). A recent study showed that simulated leaf chewing facilitates the performance of plant parasitic nematodes on roots, and that a functional JA pathway is required for this plant-mediated interaction (Machado *et al.*, 2018). Furthermore, not only direct defence but also the feeding preference of root herbivores (Erb *et al.*, 2015), and attraction of their natural enemies (Rasmann & Turlings, 2007; Soler *et al.*, 2007), can be affected by aboveground induction. Sap-feeding herbivores have been shown to induce changes in primary metabolites (Johnson *et al.*, 2009), secondary metabolites (Kutyniok & Müller, 2012), root exudation and recruitment of rhizosphere microbes (Kim *et al.*, 2016). However, the effect of these changes on root herbivores are not consistent; root chewing beetle larvae grew larger on barley plants induced by aphids on leaves (Johnson *et al.*, 2009), but not on Chinese tallow trees (Huang *et al.*, 2014). Conversely, aphids induce resistance against root-feeding aphids on *Cardamine pratensis* and against root-feeding nematodes on *Arabidopsis* (Salt *et al.*, 1996; Kutyniok & Müller, 2012). The latter was correlated with slight differences in root glucosinolates (Kutyniok & Müller, 2012). Furthermore, aboveground feeding by whiteflies induced resistance against *Agrobacterium* in roots in an SA-dependent manner (Song *et al.*, 2015). Thus, the feeding guild of the aboveground inducer appears to matter for the plant-mediated effects on root herbivores.

Here, we study how aboveground insect herbivores with different feeding modes affect the performance of root herbivores, and the potential underlying mechanisms. As a study system, we used *Brassica oleracea* plants and their interaction with several specialist insect herbivores. This system has been previously used to study interactions between folivorous insects (Kroes *et al.*, 2015), and transcriptomic responses to various insects on leaves (Kroes *et al.*, 2017; Sarde, 2019). Furthermore, in a closely related plant species, *Brassica nigra*, *Pieris brassicae* caterpillars were found to negatively affect the root-chewing herbivore *Delia radicum*, the cabbage root fly (Soler *et al.*, 2007). Here, we studied how the chewing herbivore *Plutella xylostella*, the diamondback moth, and the phloem feeder *Brevicoryne brassicae*, the cabbage aphid, affect *D. radicum* in roots. All three species are specialist herbivores of the Brassicaceae family. To shed light on the underlying mechanisms, we examined defence signalling in *B. oleracea* roots. We studied how plants respond to *D. radicum* feeding on the roots, as well as to *P. xylostella* or *B. brassicae* on the leaves. Furthermore, we investigated whether aboveground herbivory modulates the plant response to root herbivory.

Materials and methods

Study system

Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv "Cyrus") were used for all experiments. Plants were grown in a glasshouse compartment in potting soil (Lentse potgrond, Lent, the Netherlands) at 22±2 °C, 50-70% RH, with a 16:8 L:D cycle.

Brevicoryne brassicae L. (Hemiptera: Aphididae) aphids and *Plutella xylostella* L. (Lepidoptera: Plutellidae) caterpillars were reared on Brussels sprouts plants at 22±2 °C, 50-70% RH, with a 16:8 L:D cycle. *Delia radicum* L. (Diptera: Anthomyiidae) was collected near Zeewolde, the Netherlands, in 2013 and was reared on swede (*Brassica napobrassica*) at 20±1 °C, 50-70% RH, 16:8 L:D cycle.

Root herbivore performance

Three-week-old Brussels sprouts plants were infested with 10 *P. xylostella* L1 caterpillars or 10 *B. brassicae* apterous adults. Insects were constrained to the youngest fully expanded leaf ('induced leaf' hereafter) by placing cotton wool around the petiole, this was also done for control plants. In this way, inducing herbivores always started feeding on the same leaf, and most remained on that leaf for the duration of the experiment. Aboveground inducers were allowed to feed on the leaf for a total of six days, after which they were carefully removed with a fine brush. Plants which were cross-infested or on which removal of aboveground insects was unsuccessful were removed from the analysis. After two days of aboveground herbivory, 10 *D. radicum* neonate larvae were placed directly on the main root of all plants, just below the soil surface. Plants were distributed over a single greenhouse compartment in blocks to be able to test and correct for spatial differences. All plants received 50 mL of Hyponex (Unifarm, Wageningen, the Netherlands) twice weekly. Plants were watered three times each week. The amount of water given was varied depending on the estimated weight of the pots, as water uptake differs largely depending on the severity of root-herbivore damage. Twenty days after *D. radicum* induction, plants were individually bagged with mesh nets. From this moment on, plants were checked daily for emerged adults, which were collected and immediately frozen at -18 °C. Root fly survival to adulthood was scored, as well as their body weight (Sartorius CP2P micro balance, Germany) and hind tibia length (Dino-Lite Edge digital microscope, Taiwan).

Gene expression analysis

Induction of plants was carried out as above. Plants were harvested 6 h and 24 h after the start of infestation (hpi) with *D. radicum*. Main roots were cut off using scissors and disks of the induced leaf were collected using a 1 cm diameter cork borer; these tissues were immediately frozen in liquid nitrogen and stored at -80 °C. Each sample consisted of three pooled plants.

RNA was extracted using the Bioline Isolate II plant RNA kit (GCbiotech, the Netherlands) according to the manufacturer's instructions. After RNA extraction, cDNA libraries were prepared (SensiFAST™, Bioline). To quantify gene expression, qPCR was performed using SYBR Green (SensiFAST™, Bioline) and primers designed specifically for *B. oleracea* (Table S1). For each tissue type, 10 random samples were analysed for six reference genes (*Act-2*, *Btub*, *EF1a*, *GAPDH*, *PER4*, *SAR1a*) to calculate the best combination of reference genes using GeNorm; these were *Btub* and *GAPDH* for leaves, and *Act-2* and *Btub* for roots (Vandesompele *et al.*, 2002). In leaves, the expression of *LOX2* and *PR1* was assessed. In roots, the transcript levels of *LOX6*, *AOS*, *VSP2*, *MYC2*, *PAL*, *ACS*, *ABA2*, *ORA59*, *PDF1.2* and *PR1* were quantified. Relative expression, normalised to the selected reference genes and the 6 h control sample and taking into account primer efficiency, was calculated using the CNRQ (Calibrated Normalized Relative Quantity) method in qBase+ version 3.1 (Biogazelle, Zwijnaarde, Belgium).

Phytohormone analysis

From the same samples that were used for gene expression, a portion was lyophilised (Snijders type 2040 lyophylizer, Tilburg, the Netherlands). Phytohormone analysis was performed as in Vadassery *et al.* (2012) on an Agilent 1200 series HPLC system (Agilent Technologies) with the modification that a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany) was used. Since it was observed that both the D6-labeled JA and D6-labeled JA-Ile standards (HPC Standards GmbH, Cunnersdorf, Germany) contained 40% of the corresponding D5-labeled compounds, the sum of the peak areas of D5- and D6-compounds was used for quantification. Concentration of cis-OPDA and OH-JA were determined relative to the quantity of the internal standard D6-JA applying a response factor (RF) of 1.0. OH-JA-Ile and COOH-JA-Ile were quantified relative to D6-JA-Ile: RF 1.0. Sulfo-JA was determined relative to the quantity of the internal standard D6-JA: RF 6.0.

Statistics

Differences in gene expression levels and metabolite concentrations between the samples were explored through a multivariate approach, using Partial Least Squares Discriminant

Analysis (PLS-DA) in SIMCA-P version 15 (Umetrics, Umeå, Sweden). Initial models with all measured variables were used to assess variable importance in projection (VIP) values. Final models were generated by removing the least important variables (VIP < 0.75).

All other statistical analyses were carried out in R (R Core Development Team, 2017) using the packages lme4, fitdistrplus, lmtest, and lsmeans (Zeileis & Hothorn, 2002; Bates *et al.*, 2015; Delignette-Muller & Dutang, 2015; Lenth, 2016). Distributions were assessed by checking QQ-plots, histograms, and using the functions shapiro.test and descdist. Survival of *D. radicum* was analysed using a GLM with a Poisson distribution. *Delia radicum* development time, weight and hind tibia length, gene expression levels, and metabolite concentrations were analysed by (G)L(M)M (Generalized Linear Mixed Model) using either Gaussian or gamma distributions, with block (position in the greenhouse) and as a random factor where relevant. As multiple flies emerged from most plants, for *D. radicum* development time, weight, and hind tibia length, plant was included as a random factor to avoid pseudoreplication. Model selection was done by comparing Akaike Information Criterion (AIC) values.

Results

Plant-mediated effects of aboveground herbivores on *D. radicum*

To investigate whether aboveground herbivory affects *D. radicum* performance on *B. oleracea* roots, a no-choice experiment was performed (Fig. **1**). Leaf chewing by *P. xylostella* negatively affected survival to adulthood of *D. radicum* (GLM: χ^2 = 8.55, df = 2, p = 0.014), causing a reduction of *ca.* 43% in survival compared to the control. Survival of root flies following phloem feeding by *B. brassicae* infestation on the leaves was intermediate and not different from survival on either control or *P. xylostella* treated plants. Other performance parameters of the flies were unaffected (Fig. **S1** Development time: GLM: χ^2 = 0.18, df = 2, p = 0.92, Weight: LMM: χ^2 = 2.16, df = 2, p = 0.34, Tibia length: GLMM: χ^2 = 0.05, df = 2, p = 0.98). This experiment was repeated in a slightly different setup with similar results (Fig. **S2**).

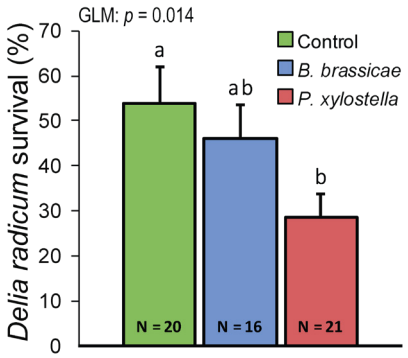

Figure 1. Survival of *Delia radicum* to adulthood on *Brassica oleracea* var. *gemmifera* plants. Two days prior to *D. radicum* infestation, plants were induced by either *Plutella xylostella* or *Brevicoryne brassicae* on the leaves. Error bars indicate standard errors of the mean. Means having no letters in common differ significantly (Tukey's LSD, p < 0.05).

Plant responses to above- and belowground herbivory

Effects of the treatments on gene transcription and metabolite concentrations were assessed through multivariate analyses (Fig. **2**). In total, transcript levels of ten genes and concentrations of nine metabolites involved in plant defence were measured in the primary roots. For all samples together, the first principal component (PC, R^2 = 0.552), clearly separates samples with and without *D. radicum* (Fig. **2a,b**; NC = 4, Q^2 = 0.74, $p_{CV\text{-}ANOVA} < 0.001$), indicating that *D. radicum* has a strong effect on the set of genes transcribed and metabolite concentrations. *Delia radicum* feeding induced the expression of genes and biosynthesis of metabolites in the jasmonate pathway such as *LOX6*, JA-Ile, *MYC2* and *ORA59* (Fig. **2b**). Furthermore, the second PC (R^2 = 0.191) separates samples taken at 6 hpi from samples at 24 hpi. To further investigate the effects of the aboveground treatments, a separate model was built using only samples from the 24 h time point without root herbivory (Fig. **2c,d**; NC = 4, Q^2 = 0.92, $p_{CV\text{-}ANOVA}$ = 0.0017). This model shows a separation of the *P. xylostella* induced root samples from the other two treatments on the first PC (R^2 = 0.463). Breakdown products of JA, such as OH-JA-Ile, COOH-JA and Sulfo-JA, appear to be important for this separation (Fig. **2d**). The second PC (R^2 = 0.199) separates roots of plants induced by *B. brassicae* from control roots. Similar results were obtained when this model was repeated for the 6 h time point (Fig. **S3**; NC = 3, Q^2 = 0.8, $p_{CV\text{-}ANOVA}$ = 0.0034). Finally, a model was made to explore differences between the *D. radicum* induced roots. Here, the first PC separates samples of roots from plants fed upon by *P. xylostella* plus *D. radicum* (R^2 = 0.291) from the other two treatments (Fig. **2e,f**; NC = 2, Q^2 = 0.58, $p_{CV\text{-}ANOVA}$ = 0.044). JA-Ile and *ABA2* are associated with roots of plants that were only infested with *D. radicum*, while the dual infested plants by *P. xylostella* and *D. radicum* is associated with OH-JA, *ACS*, and ABA. The second PC (R^2 = 0.201) separates root samples of plants induced by *D. radicum* only from samples induced by both *D. radicum* on roots and *B. brassicae* on leaves. For the 6 h samples, no separation was seen between *D. radicum* induced roots (Fig. **S3**; NC = 2, Q^2 = 0.43, $p_{CV\text{-}ANOVA}$ = 0.32).

Induction of plant defence by *Delia radicum*

Primary roots of plants exhibit a jasmonate response when damaged by *D. radicum* larvae. Jasmonate biosynthesis genes *AOS* and *LOX6* were upregulated by *D. radicum* feeding (Fig. **3a**, Fig. **S4**). The bioactive jasmonates JA and JA-Ile were strongly induced following *D. radicum* herbivory (Fig. **3f**). Compared to control, JA increased 10-fold and 20-fold, while JA-Ile increased 25-fold and 42-fold, after 6 h and 24 h, respectively. Further downstream, the JA-related transcription factors *MYC2* and *ORA59* were induced by *Delia* herbivory (Fig. **3i,k**), and after 24 h of feeding, *VSP2* and *PDF1.2*, two genes encoding defence proteins, were activated (Fig. **3j,l**).

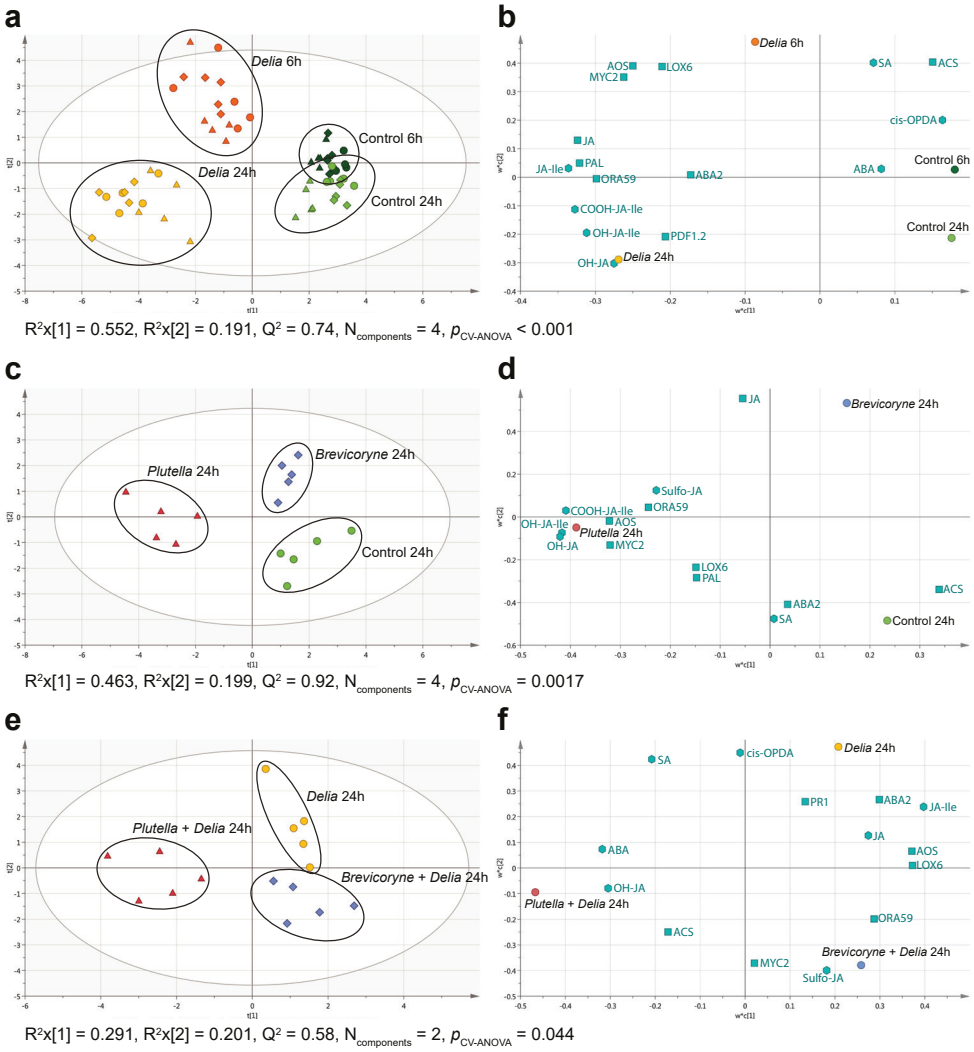

Figure 2. PLS-DA analyses illustrating the defence response of *Brassica oleracea* primary roots to *Delia radicum* and two aboveground herbivores in terms of defence-related genes and metabolites. Score plots (**a,c,e**) show separation of samples based on the PLS-DA model, loading plots (**b,d,f**) show the contribution of each gene/metabolite included in the model. The first model (**a,b**) shows contrasts between plants infested by *D. radicum* for 6 h and 24 h and plants without root herbivory. The second model (**c,d**) shows differences between the response of primary roots to different aboveground herbivores in the absence of root herbivory. The third model (**e,f**) shows how primary roots respond to *D. radicum* in the presence of aboveground herbivores. Final models were generated by discarding the least important genes/metabolites from full models (VIP < 0.75). Both the second and third models only show the 24 h time point. Aboveground treatments were: no aboveground herbivores, indicated by circles; *Plutella xylostella* larval feeding, indicated by triangles; *Brevicoryne brassicae* infestation, indicated by diamonds. Grey ellipses in score plots indicate Hotelling's T2 (95%). Black circles delineate treatment groups, they have no statistical value. In loading plots, squares show genes and hexagons show metabolites.

In addition to the jasmonate pathway, other hormonal pathways also changed in response to *D. radicum* feeding. The phenylpropanoid pathway marker *PAL* was activated in plants infested by *D. radicum* (Fig. **3d**), but SA levels were unchanged (Fig. **S3**). After 24 h of *Delia* feeding, primary roots had decreased *ACS* transcription, indicating lower ET biosynthesis

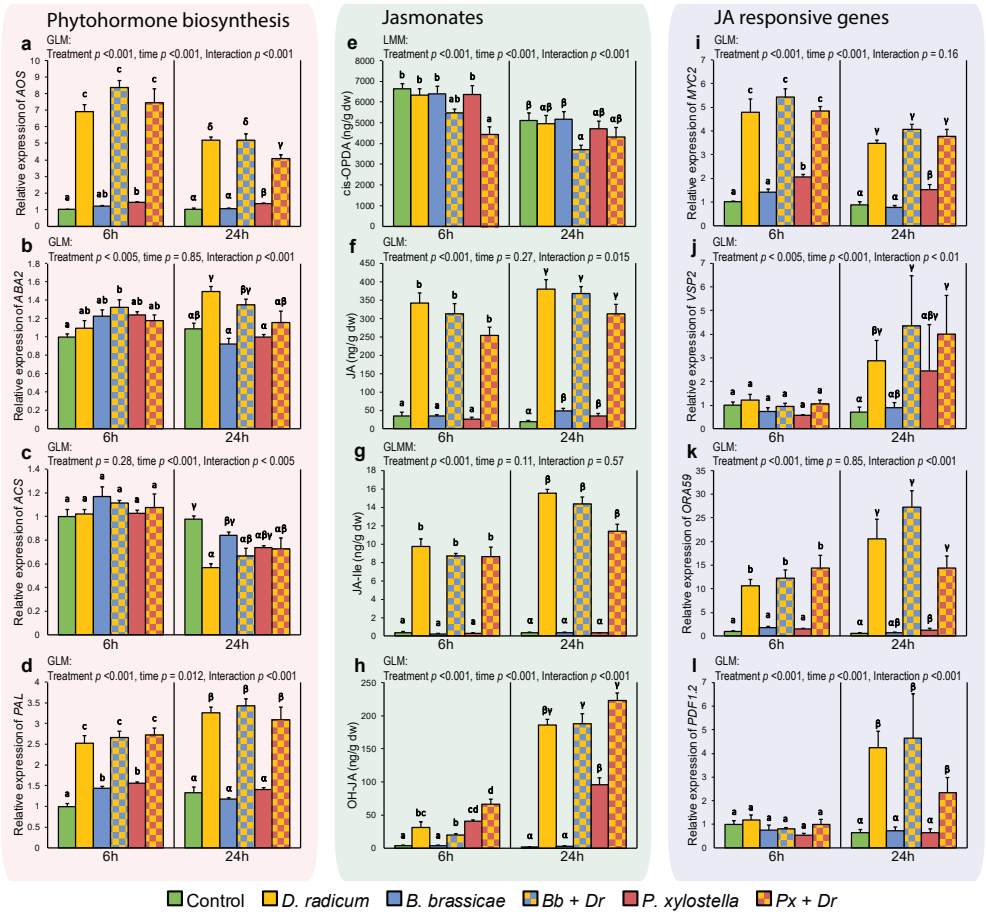

Figure 3. Expression of genes and concentrations of metabolites related to defence signalling in primary roots of *Brassica oleracea* var. *gemmifera* plants induced by aboveground (*Brevicoryne brassicae* (*Bb*) and *Plutella xylostella* (*Px*)) and belowground (*Delia radicum* (*Dr*)) insect herbivores. The red panel shows genes related to biosynthesis of defence-related phytohormones, namely AOS (**a**), ABA2 (**b**), ACS (**c**), and PAL (**d**) as markers for biosynthesis of jasmonic acid, abscisic acid, ethylene, and salicylic acid, respectively. The green panel shows concentrations of jasmonate precursor cis-OPDA (**e**), bioactive jasmonates JA (**f**) and JA-Ile (**g**), and JA catabolite OH-JA (**h**). The blue panel shows genes regulated by JA, transcription fators MYC2 (**i**) and ORA59 (**k**), and downstream genes VSP2 (**j**) and PDF1.2 (**l**). Time points indicate time since *D. radicum* induction, plants were infested with aboveground herbivores 48 h prior to this. Error bars indicate standard errors of the mean, N = 5, each sample represents 3 pooled plants. Different letters indicate statistically significant differences between treatments within a time point (Tukey's LSD, $p < 0.05$).

(Fig. **3c**), while *ABA2*, an ABA biosynthesis gene, was upregulated (Fig. **3b**). Conversely, there was a trend for lower ABA hormone levels 24 h after root herbivore induction compared to control roots (Fig. **S3**; Tukey's LSD; z = 2.79, $p = 0.058$)

Effects of *Plutella xylostella* on primary root defence signalling

Folivory by *P. xylostella* systemically enhanced defence responses in the primary roots. Transcription of *AOS*, involved in biosynthesis of JA, was slightly upregulated relative to control plants in response to caterpillar feeding on leaves (Fig. **3a**). Indeed, JA levels were slightly increased at the 24 h time point (72 h after *P. xylostella* induction) compared to control samples (Fig. **3f**). The jasmonate-regulated transcription factors *MYC2* and *ORA59* were also expressed at higher levels in *Plutella*-induced roots compared to control (Fig. **3i,k**). However, compared to control plants none of the active components of the JA pathway were increased as much by *P. xylostella* as they were by local induction by *D. radicum* (Fig. **3**). Interestingly, inactive jasmonates (OH-JA, OH-JA-Ile, COOH-JA-Ile) accumulated in the primary roots of *Plutella*-infested plants (Fig. **3h**, Fig. **S4**).

Effects of *Brevicoryne brassicae* on primary root defence signalling

Aboveground feeding by aphids had little effect on the jasmonate pathway in the primary roots. Aside from a slight increase in JA levels at the 24 h time point (72 h after aphid infestation) relative to control roots, no other markers were changed in response to *B. brassicae* infestation on the leaves (Fig. **3**). However, at the 6 h time point (54 h after the start of aphid induction), *PAL* expression was upregulated following *B. brassicae* treatment compared to control roots, and a *PR1* response was seen (Fig. **3d**, Fig. **S4**). Root SA concentrations were not altered by aboveground *B. brassicae* feeding (Fig. **S4**). Interestingly, the SA pathway marker gene *PR1* was unaffected in local tissues where aphids fed (Fig. **S5**), even though several colonies had formed on each induced leaf by the time of harvest.

Interactive effects between above- and belowground inducers on root defence signalling

The plant response to *D. radicum* was altered when plants had previously been infested with aboveground herbivores. When both *D. radicum* and *P. xylostella* were present, *AOS* and *LOX6* were downregulated after 24 h compared to plants that were only induced by *D. radicum*, implying lower jasmonate biosynthesis rates in these roots (Fig. **3a**, Fig. **S4**). Levels of cis-OPDA in the root were lower in plants exposed to dual herbivory, while single herbivore treatments did not affect the concentration of

this jasmonate precursor (Fig. **3e**). Expression levels of downstream genes in the JA cascade, *MYC2*, *ORA59*, *PDF1.2*, and *VSP2*, did not differ between plants induced by *D. radicum* only and dual-infested plants (Fig. **3i,j,k,l**). Interestingly, the upregulation of *ABA2* 24 h after *Delia* induction was not found when *P. xylostella* was present on the plants (Fig. **3b**). Finally, while SA hormone concentrations were not affected by aphids alone, the combination of *B. brassicae* and *D. radicum* caused a decrease in this signalling compound relative to control roots (Fig. **S4**).

Discussion

Our data show that leaf herbivory has a strong effect on root herbivores, and that this effect is dependent on the feeding guild of the aboveground attacker (Fig. **4**). We show that leaf herbivory causes changes in transcript levels and signalling compounds in primary roots. In particular, we show that the jasmonate pathway is induced by root herbivores, and that aboveground herbivores induce changes in this pathway in the roots, which may underlie

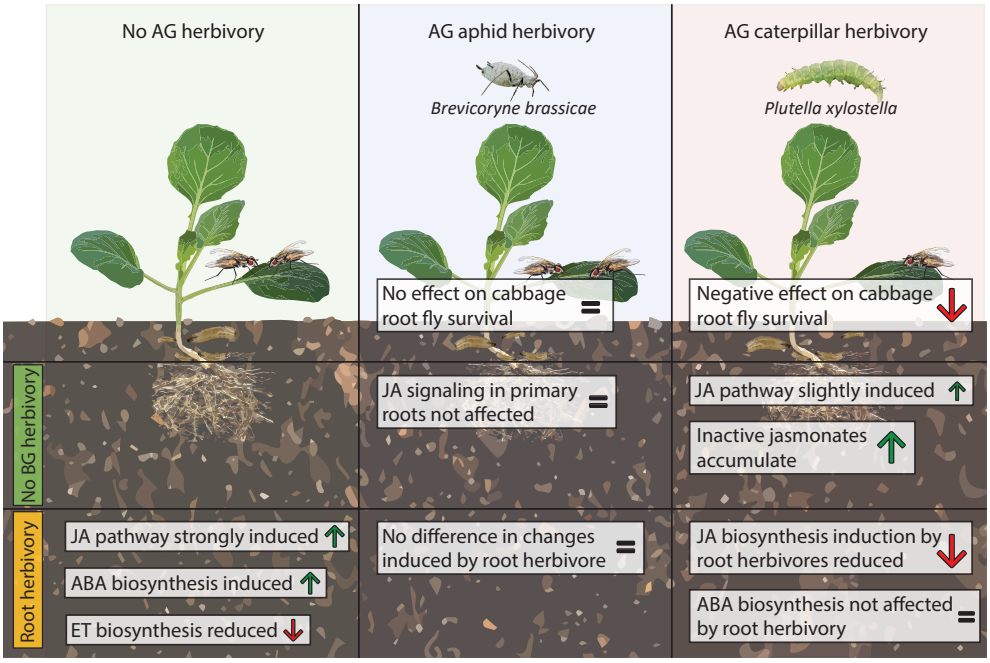

Figure 4. Overview of the effects of aboveground herbivory by aphids (*Brevicoryne brassicae*) or caterpillars (*Plutella xylostella*) on root herbivore (*Delia radicum*) survival and primary root defense signalling. A distinction is made between defence signalling induced by aboveground herbivores alone, and how aboveground herbivores affect the plant response to root herbivores. Abbreviations: AG; aboveground, BG; belowground, JA; jasmonic acid, ABA; abscisic acid, ET; ethylene.

the plant-mediated interaction described here. Furthermore, aboveground herbivores interact with defence induction by root herbivores, leading to a different signal signature in the primary root.

Responses of plants to *D. radicum* involve a strong activation of jasmonates. The JA pathway is well known for regulating defence against chewing herbivores, both in leaves and roots; in rice, mutants lacking a functional JA response were more susceptible to root herbivores (Lu *et al.*, 2015; Erb & Reymond, 2019). Furthermore, both root herbivory and jasmonate treatment triggers maize roots to produce of a volatile compound that attracts entomopathogenic nematodes (Rasmann *et al.*, 2005; Erb *et al.*, 2011a). The magnitude of jasmonate induction by *D. radicum* is quite surprising, as Erb *et al.* (2012a) reported that many plant species lack a strong jasmonate burst in their roots, instead relying on a more subtle increase compared to leaves. In leaves of the same cultivar as we use here, the magnitude of JA induction after 24h of feeding by several caterpillar species was shown to lie between 4-fold and 11-fold (Bruinsma *et al.*, 2009; Bruinsma *et al.*, 2010), much less compared to the 20-fold increase we find in primary roots responding to root-feeding maggots. Possibly, this is due to our focus on primary roots, whereas to the best of our knowledge previous studies did not distinguish between root tissues in terms of jasmonate concentrations. Root tissues that have a higher value in terms of plant fitness have higher levels of chemical defences in *Brassica* species and maize, in line with the optimal defence theory (Robert *et al.*, 2012b; Tsunoda *et al.*, 2017). Possibly this is also true for the high inducibility of jasmonates in primary roots found here.

In addition to the JA pathway, *D. radicum* induced changes in the expression of ABA and ET biosynthesis genes after 24 h, suggesting that these hormones play a role in later stages of the defence response. Since the symptoms of root herbivory by *Delia* resemble those of drought, involvement of ABA is not surprising, as it is the main regulator of abiotic stress resistance (Finkelstein *et al.*, 2002). Furthermore, ABA was shown to play a role in the response of maize to the root herbivore *Diabrotica virgifera virgifera* (Erb *et al.*, 2009a). Moreover, from studies on leaf defence signalling, we know that ABA and ET are important in fine-tuning JA responses (Pieterse *et al.*, 2009). In aboveground tissues, MeJA induction regulates over 3500 transcripts in *Arabidopsis thaliana* (Hickman *et al.*, 2017), and onion thrips (*Thrips tabaci*) feeding influences the transcription of about 10% of all *B. oleracea* genes (Sarde, 2019). Not all of these are involved in defence, as JA regulates many other processes, such as the regulation of root growth, formation of root hairs, lateral roots, and adventitious roots (Wasternack & Feussner, 2018). Indeed, activation of the jasmonate cascade does not always lead to enhanced defence. Exogenous jasmonate treatment of the root caused a decline in *Delia* pupation in broccoli plants, *B. oleracea*, but had the opposite

effect in turnip, *B. rapa* (Pierre *et al.*, 2012b). Possibly, ABA and ET in roots fine-tune the JA response to a specific sub-set of genes.

There is ample evidence that chewers feeding on leaves negatively affect chewers feeding on roots, which is in line with our findings (Masters & Brown, 1992; Soler *et al.*, 2007; Erb *et al.*, 2011b; Johnson *et al.*, 2012). While survival of root chewers is usually reduced in these interactions, growth is often increased, which may lead to some level of compensation (Johnson *et al.*, 2012). Here, however, other performance parameters of root chewers were unchanged by aboveground herbivory, so the surviving *D. radicum* individuals did not benefit from reduced competition. Several mechanisms have been proposed to explain these plant-mediated interactions. Primary metabolism seems a likely candidate for mediating interactions between above- and belowground herbivores, as tolerance is thought to be achieved by plants that allocate their resources in roots upon leaf attack (Schwachtje & Baldwin, 2008). Indeed, leaf herbivory has been found to increase allocation of resources to roots (Holland *et al.*, 1996; Schwachtje *et al.*, 2006). On the other hand, carbohydrate storage decreases in roots following leaf herbivory (Machado *et al.*, 2013). Others have pointed to increased secondary metabolites as the main mediators of antagonism between above and belowground chewers (Soler *et al.*, 2013). A well-documented example of this is found in Chinese tallow trees (*Triadica sebifera*), on which leaf chewers negatively affected flea beetle larvae in the roots, but conspecific adult beetles feeding on the leaves did not. In this system, root tannin concentrations in the different treatments correlated with the performance of the root herbivores (Huang *et al.*, 2014). In *B. oleracea*, an increase in indole glucosinolates was recorded in roots of plants challenged by *P. brassicae* caterpillars aboveground, which was suggested to play a role in a negative effect on *D. radicum* (Soler *et al.*, 2007). However, whether these toxins provide defence against the specialist *D. radicum* is debatable, because glucosinolates did not correlate with *D. radicum* performance in several studies (Pierre *et al.*, 2012b; van Geem *et al.*, 2015). Furthermore, *D. radicum* harnesses gut microbes that can disarm toxic isothiocyanates resulting from the breakdown of gluconasturtiin, an aromatic glucosinolate (Welte *et al.*, 2016), and it may well possess methods to detoxify aliphatic and indolic glucosinolates as well. Research on *A. thaliana* has shown that flavonoids rather than glucosinolates are involved in defence against specialist insects (Onkokesung *et al.*, 2014; Onkokesung *et al.*, 2019). To understand the mechanism underlying the interaction between *P. xylostella* and *D. radicum*, more components of root defence (e.g. secondary metabolites and defensive proteins) should be investigated, and manipulative approaches should be used.

Aboveground infestation by *P. xylostella* causes changes in root defence signalling. In maize, aboveground caterpillar feeding failed to induce jasmonate levels in roots (Erb *et al.*, 2009a),

while in tobacco, an increase in root jasmonates is recorded two hours after the application of leaf damage plus caterpillar oral secretion (Machado *et al.*, 2018). Indeed, a functional jasmonate pathway was needed to allow plant-mediated facilitation of aboveground simulated herbivory on nematodes in tobacco roots (Machado *et al.*, 2018). Here, we report a slight increase in JA levels in roots following leaf herbivory. A small increase may, however, have a large impact in roots (Erb *et al.*, 2012a). Furthermore, we find an increase in genes encoding enzymes catalysing JA biosynthesis and downstream transcription factors. Among the differences, roots of plants infested with *P. xylostella* on the leaves harboured much higher levels of jasmonate derivatives that are mostly inactive in signal transduction (Wasternack & Hause, 2013). Accumulation of jasmonate derivatives indicates that a jasmonate response occurred before our measurements started. This earlier jasmonate response could have led to more defensive metabolites, or could have primed plant defence in the roots, enabling the plant to respond more rapidly to *D. radicum*. Interestingly, some JA derivatives may retain partial activity, for instance, OH-JA treatment leads to slight induction of JA-related marker genes in *Arabidopsis* and a faster induction by JA-Ile treatment when applied together (Smirnova *et al.*, 2017). Furthermore, recently the inactive OH-JA-Ile was synthetically reactivated by modifications that can theoretically occur in nature, and these reactivated compounds can activate defence against *Manduca sexta* caterpillars (Jimenez-Aleman *et al.*, 2015; Jimenez-Aleman *et al.*, 2017).

In addition to altering the basal levels of defence in systemic tissues, plant-mediated interactions can involve defence priming, in which the induced response is altered because of a previous event (Erb *et al.*, 2008). Indeed, plants previously infested by *P. xylostella* responded differently to *D. radicum*. Transcripts of JA biosynthesis genes were less abundant in co-infested plants compared to plants only infested by *D. radicum*. This reduction could be the result of negative feedback in the jasmonate pathway, suggesting an earlier plant response to *D. radicum* when *P. xylostella* was already present (Chini *et al.*, 2007; Liu *et al.*, 2019). Alternatively, *D. radicum* may have fed less, or died early on roots of *P. xylostella* induced plants. In maize plants, root herbivores were shown to avoid plants that were previously induced by caterpillars (Erb *et al.*, 2015). However, because *D. radicum* tunnels through the tap root of cabbage plants, their feeding behaviour is hard to observe, especially in the early stages of infestation. Interestingly, while *D. radicum* infested roots contained higher levels of *ABA2* transcripts at 24 h, this was attenuated when *P. xylostella* was present. It is tempting to suggest that fine-tuning differences within the JA pathway, or differences in other ABA-regulated genes, may play a role in the plant-mediated interaction between *P. xylostella* and *D. radicum*. To investigate this further, a transcriptomic approach with more time points is required.

Plant-mediated interactions between different feeding guilds are rarely studied, in particular the effects of aboveground phloem-feeding insects on belowground chewers. In *T. sebifera*, aphids had no effect on root-feeding flea beetle larvae (Huang *et al.*, 2014). In barley, aphids did not affect survival of root-feeding wireworm larvae, but positively influenced their growth (Johnson *et al.*, 2009). In line with these two studies, the effect of aphids on *D. radicum* in our study was weak. Interestingly, other belowground feeders are more strongly affected by aboveground induction. For instance, *B. brassicae* negatively affected plant-parasitic nematode performance in roots of *A. thaliana* (Kutyniok & Müller, 2012), and on *Cardamine pratensis*, leaf feeding aphids negatively affected root feeding aphids (Salt *et al.*, 1996). In aboveground tissues, phloem feeding insects and chewers have been shown to facilitate one another (Soler *et al.*, 2012). The finding that this does not occur between foliar aphids and root-feeding insects may indicate that mechanisms underlying these interactions do not travel into the roots.

Although the plant-mediated effects of aphids on belowground chewers may be weak or absent, this does not necessarily indicate a lack of induction of belowground defence. Systemic effects of aphids from leaves to roots have been reported in terms of primary metabolites (Masters & Brown, 1992; Johnson *et al.*, 2009), secondary metabolites (Kutyniok & Müller, 2012), and root exudates (Kim *et al.*, 2016). Another phloem-feeding hemipteran, *Bemisia tabaci*, induces genes involved in biosynthesis of jasmonates and anthocyanins of maize roots (Park *et al.*, 2015). In our study, however, aboveground infestation of aphids had little effect on the measured root defence markers. It is quite possible that we missed changes induced by the aphids, because we focussed mainly on markers in the jasmonate defence pathway. On the other hand, SA levels and *PR1* transcripts in roots did not exhibit a strong aphid response either. Aphid-induced effects can be highly density dependent (Kroes *et al.*, 2015), perhaps a higher initial number of aphids would yield a different result. The differences induced by aphids that we observed, such as a slight increase in *PAL* transcripts, as well as changes we may have missed, did not change plant defence against *D. radicum* at the aphid density we studied.

Delia radicum appears to elicit a suboptimal defence response in their host plants, because induction by *P. xylostella* leads to much more effective defence. Herbivorous insects are known to be able to manipulate their host's immune system by using effectors in their saliva (Consales *et al.*, 2012; Acevedo *et al.*, 2015) or even by symbiosis with microorganisms (Ziebell *et al.*, 2011; Chung *et al.*, 2013; Kazan & Lyons, 2014), leading to induced susceptibility. For instance, Colorado potato beetle (*Leptinotarsa decemlineata*) larvae use bacteria in their saliva to trick their host plant into an SA-based defence response (Chung *et al.*, 2013). Root herbivores can also cause induced susceptibility, *e.g. D. v. virgifera* aggregate on maize

roots, and facilitate each other in a plant-mediated manner (Robert *et al.*, 2012a). It is unknown whether *D. radicum* possesses a similar mechanism, although it seems feasible. Especially because *D. radicum* shows aggregated distributions in cabbage fields (Mukerji & Harcourt, 1970), prefers to oviposit on conspecific-damaged plants (Baur *et al.*, 1996b), and also performs better on plants previously damaged by conspecifics (Pierre *et al.*, 2012b). A targeted search for host-manipulation mechanisms by *D. radicum* is likely to provide insights into the evolutionary arms race between brassicaceous plants and these specialist root-feeding herbivores.

Conclusion

The current study shows that aboveground herbivores, depending on the species, can influence root herbivores. We show that aboveground herbivory influences not only the basal defence, but also root-herbivore induced defence in primary roots. Research on interactions between aboveground and belowground herbivory improves the understanding of plants as a whole organism. This can help not only in breeding for better crops, but also to better understand ecological processes in nature, where plants are always dealing with multiple stressors in multiple organs.

Acknowledgements

We thank **Andre Gidding**, **Frans van Aggelen**, and **Pieter Rouweler** for rearing the insects, and **Sean Geurts** for growing the experimental plants. We thank **Pieter Rouweler** and **Julia Friman** for help during the experiments. Finally, we thank **Matthias Erb** and **two anonymous reviewers** for helpful comments on an earlier version of the manuscript.

Supporting information

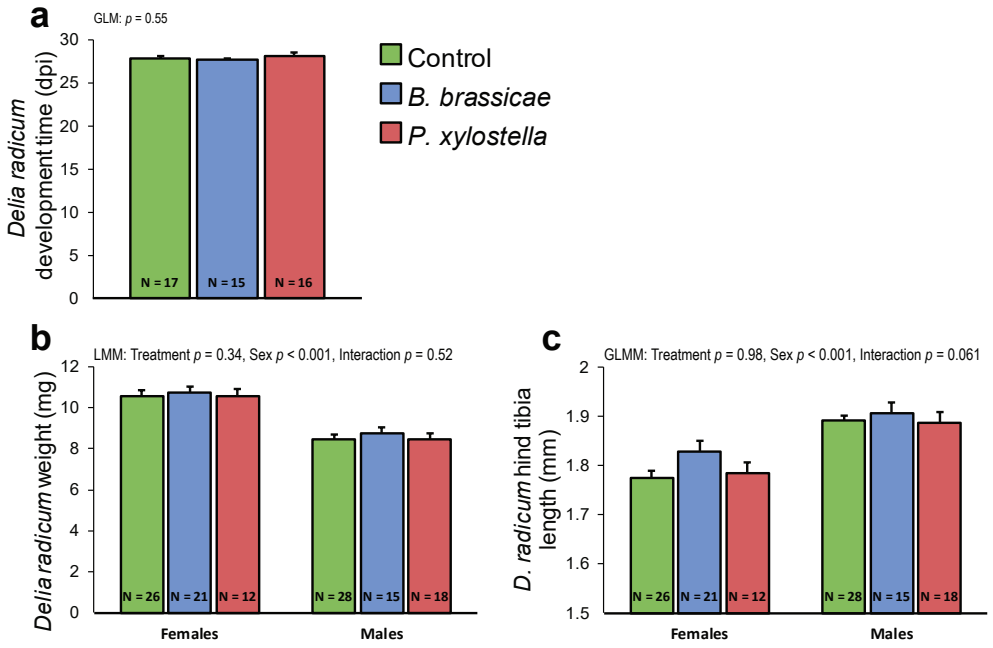

Figure S1. Development time from neonate to adult (**a**), adult weight (**b**) and adult hind tibia length (**c**) of *Delia radicum* on *Brassica oleracea* var. *gemmifera* plants. Two days prior to *D. radicum* infestation, plants were induced by either *Plutella xylostella* or *Brevicoryne brassicae* on the leaves. Error bars indicate standard errors of the mean.

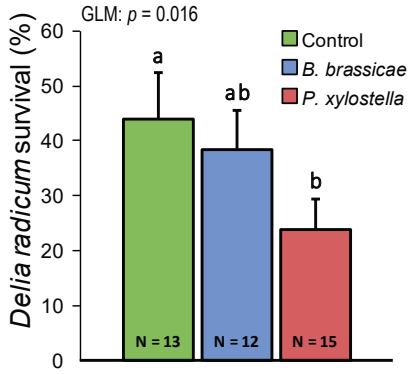

Figure S2. Survival of Delia radicum flies to adulthood on *Brassica oleracea* var. *gemmifera* plants. Prior to *D. radicum* infestation, plants were induced by either *Plutella xylostella* or *Brevicoryne brassicae*. Methods similar as described in material and methods, with two exceptions: aboveground inducers were placed on the plant 7 days before *D. radicum* and left for 6 days, and 10 *D. radicum* larvae were used. Error bars indicate standard errors of the mean. Different letters indicate statistically significant differences (Tukey's LSD, $p < 0.05$).

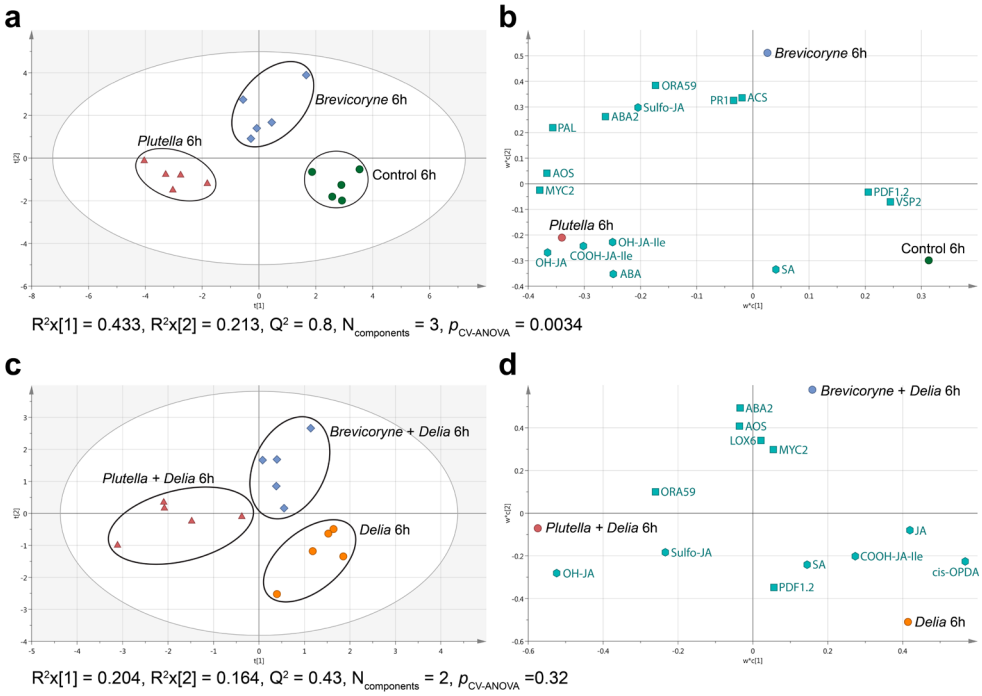

Figure S3. PLS-DA analyses illustrating the defence response of *Brassica oleracea* primary roots to *Delia radicum* and two aboveground herbivores in terms of defence related genes and metabolites. Score plots (**a,c**) show separation of samples based on the PLS-DA model, loading plots (**b,d**) show the contribution of each gene/metabolite included in the model. The first model (**a,b**) shows differences between the response of primary roots to different aboveground herbivores in the absence of root herbivory. The second model (**c,d**) shows how primary roots respond to *D. radicum* in the presence of aboveground herbivores. Final models were generated by discarding the least important genes/metabolites from full models (VIP < 0.75). Both models were made using only one time point, 6 h after *D. radicum* infestation. Aboveground treatments are indicated by shapes, circles: no aboveground herbivores, triangles: *Plutella xylostella*, diamonds: *Brevicoryne brassicae*. Grey ellipses in score plots indicate Hotelling's T2 (95%). Black circles delineate treatment groups, they have no statistical value. In loading plots, squares show genes and hexagons show metabolites.

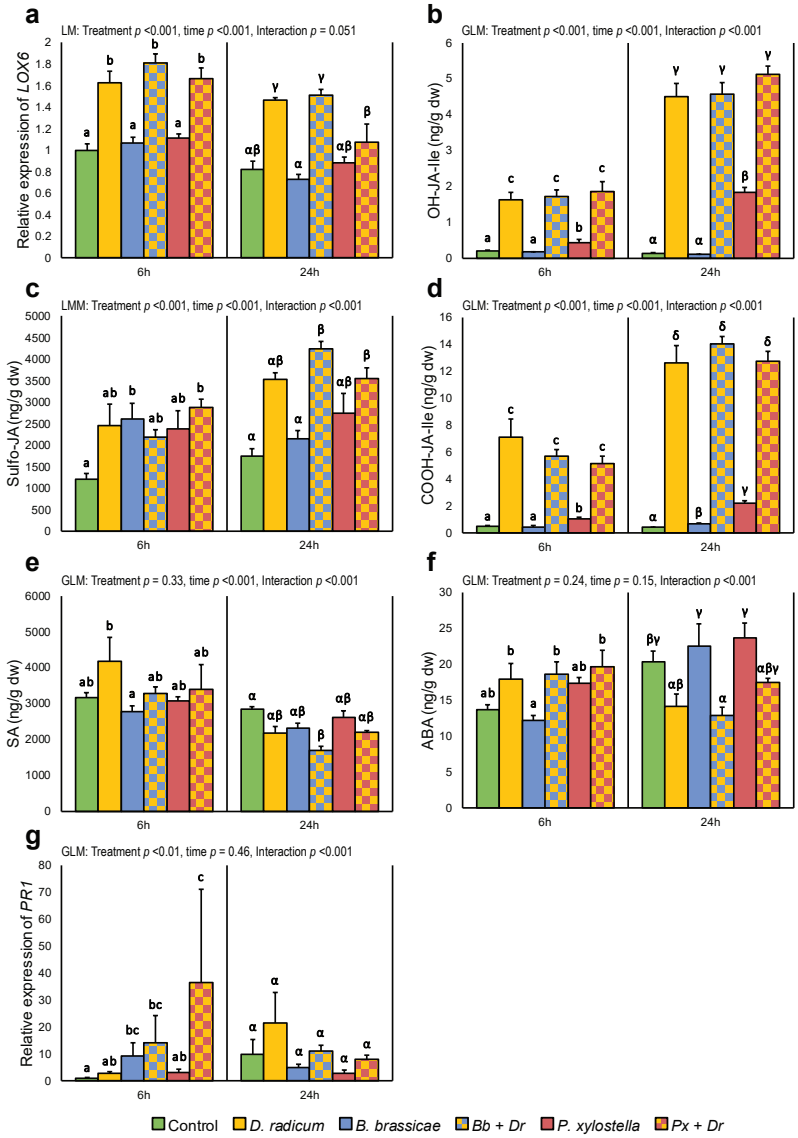

Figure S4. Expression of genes (**a, g**) and concentrations of metabolites (**b-f**) related to defence signalling in primary roots of *Brassica oleracea* var. *gemmifera* plants induced by aboveground (*Brevicoryne brassicae* (*Bb*) and *Plutella xylostella* (*Px*)) and belowground (*Delia radicum* (*Dr*)) insect herbivores. Time points indicate time since *D. radicum* induction, plants were infested with aboveground herbivores 48 h prior to this. Error bars indicate standard errors of the mean, N = 5, each sample represents 3 pooled plants. Different letters indicate statistically significant differences between treatments within a time point (Tukey's LSD, $p < 0.05$).

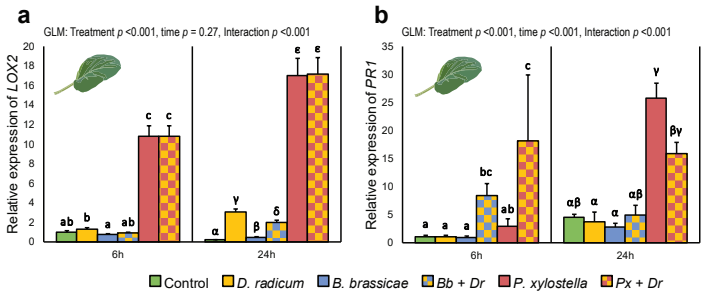

Figure S5. Expression of *LOX2* (**a**) and *PR1* (**b**) in leaves of *Brassica oleracea* var. *gemmifera* plants induced by aboveground (*Brevicoryne brassicae* (*Bb*) and *Plutella xylostella* (*Px*)) and belowground (*Delia radicum* (*Dr*)) insect herbivores. Samples were taken at the site of leaf damage. Time points indicate time since *D. radicum* induction, plants were infested with aboveground herbivores 48 h prior to this. Error bars indicate standard errors of the mean, N = 5, each sample represents 3 pooled plants. Different letters indicate statistically significant differences between treatments within a time point (Tukey's LSD, $p < 0.05$).

Table S1. Primers used for qPCR analysis of *Brassica oleracea* roots and leaves.

qPCR primers used for *B. oleracea* roots

Gene acronym	Gene amplified	Sequence Fw	Sequence Rv
PR1	Bo3g088360	GTCAACGAGAAGGCTAACTATAACTACG	TTACACCTTGCTTTGCCACATCC
MYC2	Bo5g086990	GGCTGGACCTACGCTATATTCTGG	AGAAAAACCACTCCGTATCCGT
ACS	Bo9g091320	ACTACGGTTGGCTGAAAGAC	GAGAAACGTTCAGCTTCACC
PAL	Bo6g067250	TCGCTATGGCTTCTTACTGCTCTG	GAGGTCTTACGAGATGAGATGAGTCC
VSP2	Bo2g159220	GACTATCTCACTTCCCCACAG	CGGGTCTAT CTTCTCTGTCC
LOX6	Bo6g098790 + Bo2g056010	AGGAGCTGCCAATTCGAAGC	CGCCTGTTCCAAAGTCATTCCA
AOS	Bo2g116210	ACCGCTTGCGACTAGGGATC	CAAAGTCCTTACCGGCGCAC
ABA2	Bo6g028000	GCATCGCTCGTCTGTTCCAC	CGGCGAAGTCAACAGCGTTA
ORA59	Bo5g007280 + Bo8g114710	AGGAAAGGGATAAGAGTGTGGCT	TCAAAGCTATCACCGGAGACTC
PDF1.2	Bo2g086460	CTCTCGAAGCACCAACAATG	CCATGTCGTGCTTTCTCAAGG

qPCR primers used for *B. oleracea* leaves

Gene acronym	Gene amplified	Sequence Fw	Sequence Rv
LOX2	5 *LOX2* orthologs	GCCATTGAGTTGACTCGTCC	GGATGCATGGCACTTAGTTGT
PR1	Bo3g088360	GTCAACGAGAAGGCTAACTATAACTACG	TTACACCTTGCTTTGCCACATCC

qPCR primers used for *B. oleracea* reference genes

Gene acronym	Gene amplified	Sequence Fw	Sequence Rv
GADPH	Bo5g017500	GCTACGCAGAAGACAGTTGATGG	TGGGCACACGGAAGGACATAC
Act-2	Bo5g117040	ACATTGTGCTCAGTGGTGGA	TCTGCTGGAATGTGCTGAGG
Btub	Bo2g124350, Bo7g067360, Bo9g059850	GTCAAGTCCAGCGTCTGTGA	TCACACGCCTGAACATCTCC
EF1a	Bo9g142520	GGTACCTCCCAGGCTGATTG	TCAGGTAKGAAGACACCTCCTTG
PER4	Bo7g095750	TATCCTCTGCAGCCTCCTCA	ACACACAGACTGAAGCGTCC
SAR1a	Bo3g052780	ATCTCTAGCCACCGTTCCCT	TTCCTGACGATGCTGCACAT

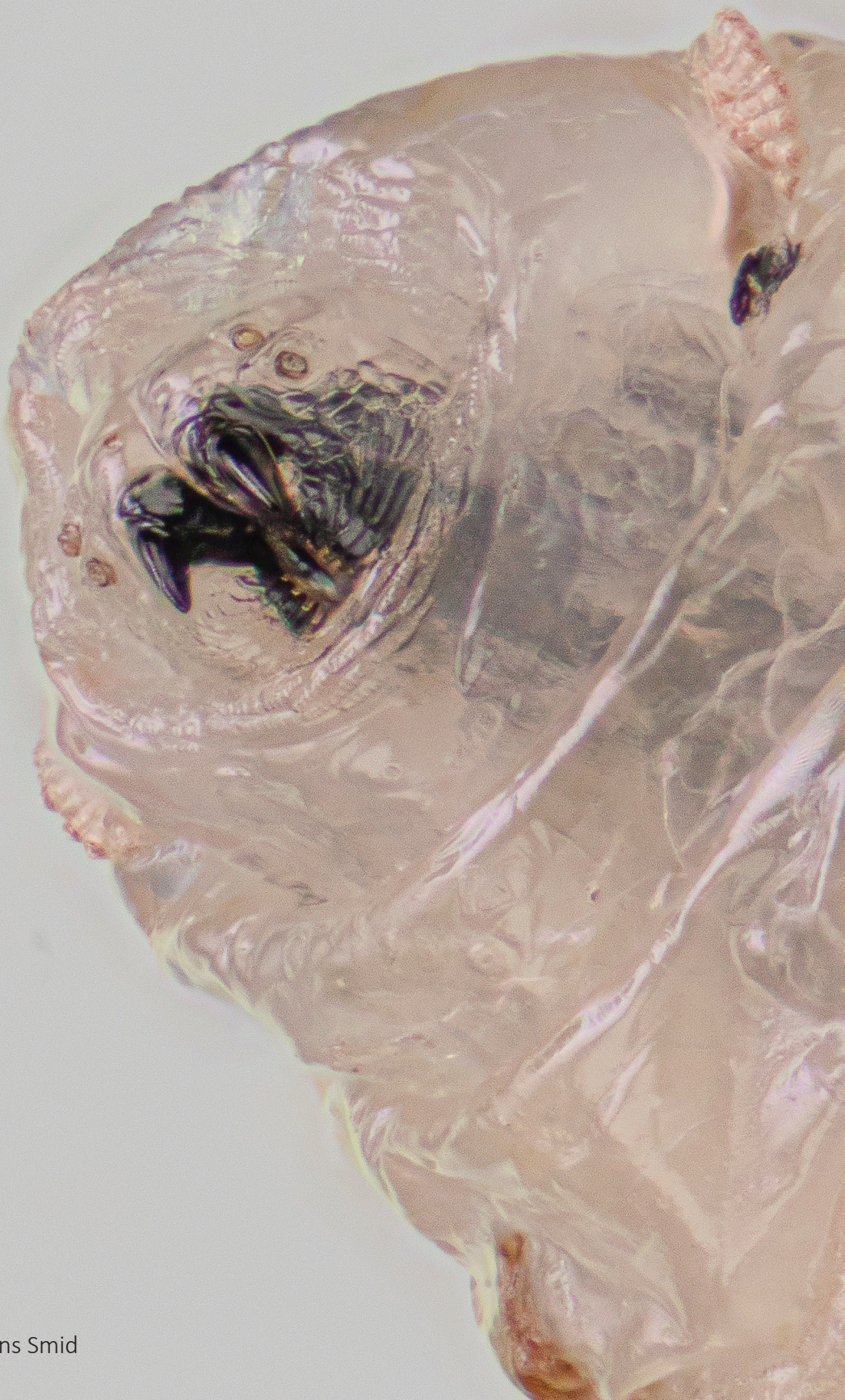

Photo by Hans Smid

Chapter 3

Specialist root herbivore modulates plant transcriptome and downregulates defensive secondary metabolites in a brassicaceous plant

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Under review

Abstract

Plants face attackers above and belowground. Insect root herbivores can lead to severe crop losses, yet the underlying transcriptomic responses have rarely been studied. We studied the dynamics of the transcriptomic response of Brussels sprouts (*Brassica oleracea* var. *gemmifera*) primary roots to feeding damage by cabbage root fly larvae (*Delia radicum*), alone or in combination with aboveground herbivory by cabbage aphids (*Brevicoryne brassicae*) or diamondback moth caterpillars (*Plutella xylostella*). This was supplemented with analyses of phytohormones and the main classes of secondary metabolites; aromatic, indole and aliphatic glucosinolates. Root herbivory leads to major transcriptomic rearrangement that is modulated by aboveground feeding caterpillars, but not aphids, through priming soon after root feeding starts. The root herbivore downregulates aliphatic glucosinolates. Knocking out aliphatic glucosinolate biosynthesis with CRISPR-Cas9 results in enhanced performance of the specialist root herbivore, indicating that the herbivore downregulates an effective defence. This study advances our understanding of how plants cope with root herbivory and highlights several novel aspects of insect plant interactions for future research. Further, our findings may help breeders develop a sustainable solution to a devastating root pest.

Introduction

Crop losses due to insect herbivory are a drain on food resources and finding sustainable solutions for crop protection is imperative to reach the UN's zero hunger goal by 2030. To this end, insight in the molecular interactions between plants and insect herbivores is important, as plant breeders can exploit this when selecting future-proof crops.

Plants are engaged in an evolutionary arms race with insect herbivores that feed on their leaf and root tissues. When under attack, plants attempt to defend themselves by producing toxic secondary metabolites or anti-nutritional proteins such as proteinase inhibitors (Erb & Reymond, 2019). These defence responses are orchestrated by an intricate network of phytohormones. Jasmonic acid (JA) plays a central role in plant defence against insects, together with salicylic acid (SA), abscisic acid (ABA) and ethylene (ET) (Erb *et al.*, 2012b; Pieterse *et al.*, 2012; Erb & Reymond, 2019). Plant defences can hamper herbivore growth and development, or lead to behavioural avoidance by the herbivores. However, insect herbivores might overcome plant defence by detoxifying plant toxins (Welte *et al.*, 2016), or by tricking plants into inducing a suboptimal defence response (Chung *et al.*, 2013). Most studies on plant defence focus on leaves, but roots are also threatened by insect herbivores and are receiving more attention in recent years (Johnson & Rasmann, 2015; Johnson *et al.*, 2016b). It is established that, despite many similarities, defence in plants roots differs from that in leaves. For instance, profiles of glucosinolates (GSLs), the main group of secondary plant metabolites in brassicaceous plants, differ substantially between roots and shoots (Tsunoda *et al.*, 2017). In terms of defence signalling, JA plays a central role in the defence against root herbivores like in foliar tissues, but the functions of SA, ABA and ET are less clear.

In the past decade, sequencing technology has broadened our understanding of defence signalling in plants. This led to extensive studies on how *Arabidopsis thaliana* (*Arabidopsis* hereafter) plants respond to exogenous application of JA (Hickman *et al.*, 2017; Zander *et al.*, 2020), SA (Hickman *et al.*, 2019), or combinations of these hormones (Hickman *et al.*, 2019). Coolen *et al.* (2016) studied how the *Arabidopsis* leaf transcriptome changes after stress by drought, infection by the necrotrophic pathogen *Botrytis cinerea*, chewing insect herbivory by *Pieris rapae*, or combinations of these three stresses. This study revealed that the last stress that plants were exposed to dominated the transcriptomic response, but also that earlier stresses left a legacy, which had consequences for the effectiveness of the defence response (Coolen *et al.*, 2016). Plant responses to insect root herbivores have rarely been the subject of transcriptome analyses (Barr *et al.*, 2010), presenting a sizeable knowledge gap.

Plants are seldomly attacked by a single herbivore in natural settings; rather, they must cope with different insect herbivores throughout the growing season (Stam *et al.*, 2014). By activating plant defence, herbivores feeding on the same plant can affect each other. Such plant-mediated interactions can have long-lasting effects on the insect community associated with plants under field conditions (Poelman *et al.*, 2008)2008. The feeding mode, degree of specialisation, and sequence of arrival of herbivores can be determining factors in the outcome of plant-mediated interactions (Erb *et al.*, 2011b; Johnson *et al.*, 2012; Stam *et al.*, 2014). Additionally, plants appear to be more adapted to respond to commonly occurring combinations of insect herbivores (Mertens *et al.*, 2021a). Plant-mediated interactions between insect herbivores can occur across plant compartments, even though the herbivores are not in direct contact (Johnson *et al.*, 2012; Biere & Goverse, 2016; Papadopoulou & van Dam, 2017). In most published studies, foliar herbivory by chewing herbivores negatively affects belowground chewers, and *vice versa* (Johnson *et al.*, 2012; Papadopoulou & van Dam, 2017). On the other hand, feeding by leaf chewers can induce susceptibility to root feeding nematodes (Biere & Goverse, 2016). Such interactions suggest that induction of plant defence occurs not only locally, but also in distal systemic tissues.

Indeed, defence signalling can cross the root-shoot interface (Ankala *et al.*, 2013; Gulati *et al.*, 2014; Wang, G *et al.*, 2019). For instance, in tobacco plants, herbivory on leaves triggers a systemic signal in roots to produce nicotine, a secondary metabolite that is only produced in root tissue and which is transported to foliar tissues for defence (Gulati *et al.*, 2014). Experiments with mutant tomato plants revealed that intact JA biosynthesis is more important in shoots than roots when defending against root knot nematodes. In this case, following infestation of the roots, an electrical signal moves up the stem to trigger JA biosynthesis in leaves, which then activates defence in roots (Wang, G *et al.*, 2019). Another well-studied example of plant defence signalling that crosses the root-shoot interface occurs when beneficial microbes in the rhizosphere trigger induced systemic resistance in the leaves (Berendsen *et al.*, 2012). This is an example of defence priming, in which the plant is prepared for future attack, leading to a faster and/or stronger response when that attack occurs (Conrath *et al.*, 2015; Hilker *et al.*, 2016). Priming is not limited to microbes; for instance, insect eggs may induce defence priming against caterpillars that hatch from those eggs (Hilker & Fatouros, 2015; Valsamakis *et al.*, 2020). Defence priming is a potential mechanism underlying interactions between different herbivores feeding on the same plant (Hilker *et al.*, 2016).

Here, we investigate transcriptomics in primary roots of a plant that is exposed to root herbivory, alone or in combination with aphids or caterpillars that were placed on the leaves two days earlier. The study system consists of Brussels sprouts (*Brassica oleracea*

var. *gemmifera*) and three of its pest species, cabbage aphids (*Brevicoryne brassicae*), diamondback moth caterpillars (*Plutella xylostella*) and cabbage root fly larvae (*Delia radicum*). These three insect species are important pests of cabbage and often occur together. In this study system, we previously discovered that diamondback moth caterpillars negatively affect root-feeding cabbage root fly larvae, but cabbage aphids do not (Karssemeijer *et al.*, 2020 - **chapter 2**). Based on the transcriptome analysis in the present study, two hypothesis-driven follow-up experiments were carried out. In the first, we tested whether aliphatic GSLs confer defence against *D. radicum* using *myb28* knockout *B. oleracea* plants. In the second, we investigated whether *P. xylostella* primes a faster plant defence response against *D. radicum*.

Material and methods

Study system

Brassica oleracea L. plants were used throughout the experiments. Plants were grown in a glasshouse compartment at 22±2 °C, 50-70% RH, with a 16:8 L:D cycle.

Delia radicum L. (Diptera: Anthomyiidae) was kept in a climate cabinet at 20±1 °C, with 50-70% RH and a 16:8 L:D cycle, larvae were reared on rutabaga (*Brassica napus* L. var. *napobrassica*) and adult flies were fed honey and a mixture of yeast, sugar and milk powder (1:1:1). *Brevicoryne brassicae* L. (Hemiptera: Aphididae) and *Plutella xylostella* L. (Lepidoptera: Plutellidae) were reared on Brussels sprouts (*Brassica oleracea* var. *gemmifera* cv. Cyrus) plants at 22±2 °C, 50-70% RH, with a 16:8 L:D cycle.

Transcriptomics of the herbivore-induced primary root

Plant treatments

Three-week-old Brussels sprouts plants were induced by placing either ten *B. brassicae* apterous adults or ten *P. xylostella* L1-L2 larvae on a leaf. The induced leaf was always the same, i.e. the third leaf counted from the bottom. Two days later, half of the plants received ten *Delia radicum* neonate larvae added to the base of the plant's stem. This resulted in six treatments: Control (C), *B. brassicae* (*Bb*), *P. xylostella* (*Px*), *D. radicum* (*Dr*), *B. brassicae* followed by *D. radicum* (*Bb* + *Dr*), and *P. xylostella* followed by *D. radicum* (*Px* + *Dr*). Plants were harvested just before adding *D. radicum* larvae (0 h), and 3, 6, 9, 24, and 48 hours after adding the *D. radicum* larvae. Per treatment at each time point, six biological replicates were harvested, of which four were selected for sequencing and chemical analysis. When

harvesting, plants were uprooted, the secondary roots were cut off using scissors, and the primary roots were separated by cutting the stem at the position where the soil surface had been. Primary root samples were immediately frozen in liquid nitrogen. All equipment was cleaned using RNaseZap (Thermofisher Scientific) before harvesting of each sample.

RNA-seq and read processing

Total RNA was extracted using Maxwell 16 LEV Plant RNA kit (Promega), subjected to poly-A isolation, digestion, and cDNA synthesis, followed by end repair and ligation of a universal adapter. Sequencing was performed to an average depth of 39 M reads, 150-bp paired end, per sample (Illumina HiSeq X, Genewiz). Quality of reads was assessed using fastQC (Andrews, 2010) and multiQC (Ewels *et al.*, 2016). Reads were processed with Trimmomatic (Bolger *et al.*, 2014)2014 and aligned to the cabbage TO1000 genome (Parkin *et al.*, 2014), using STAR (Dobin *et al.*, 2013)2013. On average, 92.7% of the reads were aligned to the genome (Table **S9**). Alignment was lowered in *D. radicum*-infested samples, suggesting that insect RNA is present in the raw data (Fig. **S1**). When we assembled the unmapped reads using Trinity, many reads indeed mapped to dipteran species, in particular the Australian sheep blowfly *Lucilia cuprina*, which is one of the most closely related species to *D. radicum* in the NCBI database. Raw sequencing data is available from the European Nucleotide Archive (https://www.ebi.ac.uk/ena/), under study accession PRJEB49273.

Differential gene expression

Read counts were processed in R using the DESeq2 package (Love *et al.*, 2014). Genes with less than ten counts on average across all samples were omitted, resulting in a total of 30,908 genes. To calculate differentially expressed genes (DEGs), a model with a combined factor for the different time points and treatments was run. The model was releveled to the control treatment for each time point, and genes were classified as DEG if they were different from the control of the relevant time point with a false discovery rate (FDR) lower than 0.0001 and log2 fold change (LFC) higher than 2 using the apeGLM shrinkage estimator (Zhu *et al.*, 2018). A separate analysis was performed in DESeq2 to calculate DEGs in multiple herbivore treatments relative to *D. radicum* alone; here, an FDR lower than 0.05 was used as threshold.

PCA analysis

Principal component analysis (PCA) was performed with PCAexplorer (Marini & Binder, 2019). Variance stabilised counts were used as input, and the top 10,000 most variant genes

were included. Association between the covariates and the first two principal component axes were assessed using Kruskal-Wallis tests. Genes in the top and bottom loadings of the first PC were extracted and individually functionally characterised. The closest *Arabidopsis* homolog was identified using PLAZA 4.5 (Van Bel *et al.*, 2017), and the function of these genes was manually assigned based on descriptions in the TAIR (Berardini *et al.*, 2015) and UniProt (The UniProt Consortium, 2021) databases.

Clustering

Normalised counts of the differentially expressed genes were clustered in R using the dynamictreecut function in WGCNA (Langfelder & Horvath, 2008; Langfelder *et al.*, 2008). Clusters were subjected to GO enrichment analysis, relative to all 30,908 expressed genes in the dataset, using PLAZA 4.5 (Van Bel *et al.*, 2017).

Analysis of defence pathways

Arabidopsis genes involved in plant defence pathways were selected based on recent literature on JA (Wasternack & Feussner, 2018), SA (Rekhter *et al.*, 2019; Zhang & Li, 2019), ABA (Cutler *et al.*, 2010; Yoshida *et al.*, 2015; Hauser *et al.*, 2017), ET (Chang *et al.*, 2013; Pattyn *et al.*, 2021), and glucosinolate biosynthesis (Gigolashvili *et al.*, 2009; Sønderby *et al.*, 2010; Pfalz *et al.*, 2016), catabolism (Barth & Jander, 2006; Sugiyama & Hirai, 2019), and transport (Jørgensen *et al.*, 2017). Many *Arabidopsis* genes have multiple homologs in cabbage due to a whole genome duplication event. Therefore, we used the PLAZA integrative orthology viewer as a method by which multiple homologs can be extracted per gene based on four evidence types: synteny, BLAST, orthologous gene family, and/or hierarchical trees (Van Bel *et al.*, 2017). Cabbage homologs with at least 2 evidence types were selected. In some cases, multiple *Arabidopsis* genes matched a cabbage homolog with equal evidence types; if the other *Arabidopsis* gene was also in the query, the cabbage gene was retained and the name adjusted (e.g. *LOX3/4*), if not, the gene was discarded. Finally, the *MAM* and *AOP* genes were selected based on earlier studies on *B. oleracea* (Yi *et al.*, 2015; Abrahams *et al.*, 2020).

Effect of aliphatic glucosinolates on *D. radicum*

To assess the effects of aliphatic glucosinolates on *D. radicum* performance, we used a *myb28* cabbage mutant in which aliphatic GSL biosynthesis is strongly knocked down. In the *myb28* line, two copies of the *MYB28* gene (Bo2g161590 and Bo9g014610) were knocked out using CRISPR-Cas9 technology (Neequaye *et al.*, 2021). The genetic background of these

plants is *B. oleracea* DH1012, a doubled haploid line obtained from a crossing of *B. oleracea* var. *alboglabra* and *B. oleracea* var. *italica*.

Seeds were sown in seedling soil and seedlings were transplanted after 8 days into regular potting soil. Starting 12 days after transplanting, plants were fertilised thrice weekly. One cotyledon was harvested from each plant 26 days after transplanting for genotyping (Methods **S1**). Five weeks after transplanting, 10 *D. radicum* neonate larvae were placed on the primary root just below the soil surface of each induced plant. Control plants remained uninfested. To assess GSL contents during the larval feeding stage, a subset of plants was harvested 5 days post infestation (dpi). All other plants were harvested 18 dpi, after *D. radicum* pupated in the soil. Primary root samples were collected as above, ground while frozen in liquid nitrogen, lyophilised (Christ Alpha 1-4 LD Plus), and subjected to chemical analysis. After harvesting, pots were covered in mesh nets to catch flies emerging from their pupae. Nets were checked daily for emergence, flies were caught and stored in glass vials. Flies were dried and weighed to the nearest 0.001 mg (Sartorius CP2P, Germany).

Priming of plant defence against *D. radicum* by *P. xylostella*

Plant treatments

Early transcriptional responses of *B. oleracea* plants to *D. radicum* were studied to assess whether defence was primed by *P. xylostella*. Two experiments were performed, in the first, three-week-old Brussels sprouts plants were induced by 10 *P. xylostella* L1-L2 larvae on a leaf as described above. Two days later, half of the plants received 10 *D. radicum* neonate larvae directly on the primary root, resulting in four treatments: control (C), *P. xylostella* (*Px*), *D. radicum* (*Dr*) and *P. xylostella* followed by *D. radicum* (*Px* + *Dr*). After 15, 30, 60 and 120 minutes of *D. radicum* feeding, primary roots were sampled as described above.

In a second experiment, we studied whether priming by *P. xylostella* would occur if there was a period without infestation between the two herbivores. To this end, three-week-old Brussels sprouts plants were induced by 10 *P. xylostella* L1-L2 larvae on a leaf for two days, after which they were removed. After a period without infestation of 24 hours or 7 days, 10 *D. radicum* neonate larvae were introduced directly on the primary root and samples were taken 30 and 60 min later. In this experiment, plants were divided over three treatments: control (C), *D. radicum* (*Dr*) and *P. xylostella* followed by *D. radicum* (*Px* + *Dr*).

Gene expression analysis

Primary root samples were ground while frozen in liquid nitrogen with a mortar and pestle, followed by RNA extraction (Isolate II Plant RNA kit, GCBiotech) and cDNA synthesis (SensiFAST, Bioline). Gene expression was quantified by quantitative polymerase chain reaction (qPCR) analysis (SensiFAST SYBR, Bioline; CFX96™ Real-Time System, Bio-rad). The optimal combination of reference genes was determined using GeNorm (Vandesompele *et al.*, 2002) in qbase+ (Biogazelle, Belgium), these were *Btub* and *SAR1a* for the first experiment and *PER4* and *SAR1a* for the second. We measured transcript levels of *AOS*, *MYC2*, *CYP81F4*, and *MYB28* (Table S7). Relative expression was calculated in qbase+, taking into account primer efficiency and interrun calibration where appropriate.

Chemical analyses

For phytohormone analysis, 14-96 mg of fresh frozen leaf samples and 8-102 mg of fresh frozen root samples were extracted with 1 ml of 80% methanol (v:v) containing 40 ng D4-SA, 40 ng D6-ABA (Santa Cruz Biotechnology, USA), 40 ng D6-JA, and 8 ng D6-JA-Ile (HPC Standards GmbH, Germany). Phytohormone analysis was performed as in Vadassery *et al.* (2012) on an Agilent 1200 series HPLC system (Agilent Technologies) with the modification that a tandem mass spectrometer QTRAP 6500 (SCIEX, Darmstadt, Germany) was used in multiple reaction monitoring (MRM) mode with parameters listed in Supplementary Table 8. Concentration of cis-OPDA, and OH-JA were determined relative to the quantity of the internal standard D6-JA applying a response factor (RF) of 1.0. OH-JA-Ile and COOH-JA-Ile were quantified relative to D6-JA-Ile: RF 1.0.

Glucosinolates (GSL) were analysed by HPLC-UV as described in Burow *et al.* (2006) in two sets of samples. The first set consisted of 14-96 mg of fresh frozen leaf samples and 8-102 mg of fresh frozen root, and the second of 6-25 mg of lyophilised root material. In short, tissue was extracted with 1mL 80% methanol solution (v:v) containing 50nmol of 4-hydroxybenzyl GSL. A 600µL aliquot of the raw extract was loaded onto DEAE Sephadex A 25 columns and treated with arylsulfatase for desulfation (Sigma-Aldrich) (Brown *et al.*, 2003). The eluted desulfoglucosinolates were separated using high performance liquid chromatography (Agilent 1100 HPLC system, Agilent Technologies) on a reversed phase C-18 column (Nucleodur Sphinx RP, 250 x 4.6 mm, 5µm, Machrey-Nagel, Düren, Germany) with a water (A)-acetonitrile (B) gradient (0-1 min, 1.5% B; 1-6 min, 1.5-5% B; 6-8 min, 5-7% B; 8-18 min, 7-21% B; 18-23 min, 21-29% B; 23-23.1 min, 29-100% B; 23.1-24min 100% B and 24.1-28 min 1.5% B; flow 1.0 mL min-1). Detection was performed with a photodiode array detector and peaks were integrated at 229 nm. Desulfated GSL were identified by

comparison of retention time and UV spectra to those of purified standards previously extracted from *Arabidopsis* (Brown *et al.*, 2003) or by analysis of the desulfoglucosinolate extracts on an LC-ESI-Ion-Trap-mass spectrometer (Esquire6000, Bruker Daltonics). We used the following molar response factors for quantification of individual GSL relative to the internal standard, 4-hydroxybenzyl GSL: aliphatic GSL 2.0, indole GSL 0.5 (83), 2-phenylethyl GSL 2.0. The following GSLs were detected in the samples: 2-propenyl GSL (sinigrin), 3-butenyl GSL (gluconapin), 3-methylsulfinylpropyl GSL (glucoiberin), 4-methylsulfinylbutyl GSL (glucoraphanin), 3-methylthiopropyl GSL (glucoiberverin), 4-hydroxy-indol-3-ylmethyl GSL (hydroxyglucobrassicin), 4-methylthiobutyl GSL (glucoerucin), indol-3-ylmethyl GSL (glucobrassicin), 4-methoxy-indol-3-ylmethyl GSL (methoxyglucobrassicin), 2-phenylethyl GSL (gluconasturtiin), and 1-methoxy-indol-3-ylmethyl GSL (neoglucobrassicin).

Statistical analysis

All statistical analyses were performed in R version 3.6.3 (R Core Development Team, 2017). For statistical analysis of phytohormones and glucosinolates, a small fraction (1.23×10^{-7}) was added to all data to circumvent measurements below the detection threshold. Nonetheless, several individual jasmonate derivatives could not be statistically analysed because too many measurements fell below the detection threshold. We instead analysed JA, JA-Ile, and the sum of jasmonates. We used (generalized) linear models ((G)LM) for data analysis using the lme4 package, with a Gamma (log or inverse link) or Gaussian distribution. Based on AIC, we selected the best model that included all fixed factors. *Delia radicum* emergence was analysed by generalized linear mixed model (GLMM) with a binomial distribution and an identifier of the plant as a random factor to avoid pseudoreplication. Significance of fixed factors was assessed with likelihood ratio tests using the lmtest package. Posthoc analysis by Tukey's HSD pairwise comparisons were analysed where appropriate using the emmeans package.

Results

Delia radicum feeding causes a major transcriptomic shift in the primary roots

Multivariate analysis revealed distinct patterns in the transcriptome of the primary root following root herbivory. The first principal component (PC) clearly separates the root transcriptomes based on the presence or absence of *D. radicum* (Fig. **1a**). The second PC separates samples by the different time points at which the roots were sampled following root infestation (Fig. **1b**). There is a distinction between infested root tissue sampled very early (3 h), early (6 and 9 h) and later (24 and 48 h). In roots that are not infested with *D. radicum*, the transcriptome of the primary root was affected by *P. xylostella* infestation

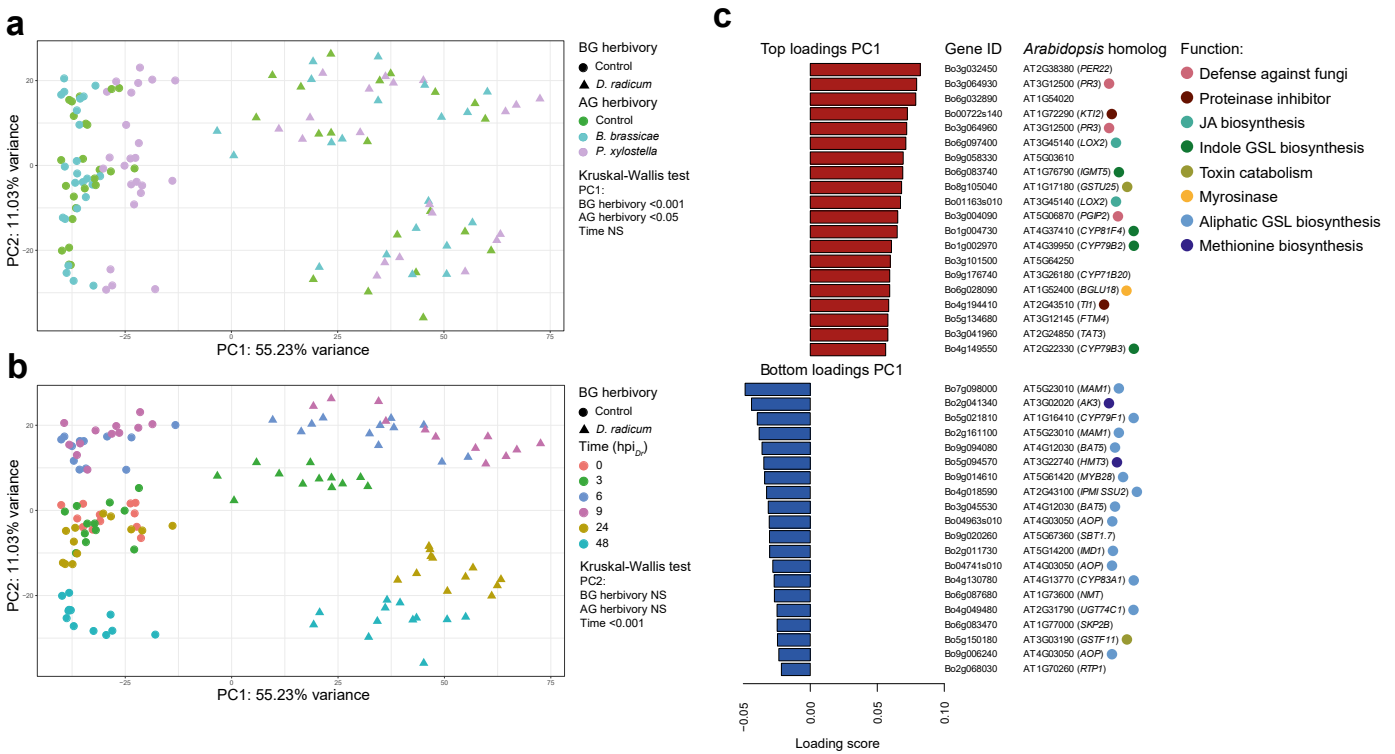

Figure 1. Principal component analysis of *Brassica oleracea* transcriptomes of the primary root when subjected to aboveground (AG) herbivory by *Brevicoryne brassicae* or *Plutella xylostella* and belowground (BG) herbivory by *Delia radicum.* Aboveground herbivores were introduced 48 h prior to infestation by *D. radicum.* Samples are coloured by herbivore treatments (**a**) or time points (**b**). Top and bottom loadings of the first principal component and the function of their *Arabidopsis thaliana* homologs (PC1; **c**). hpi$_{Dr}$: hours post infestation by *D. radicum.*

compared to uninfested control plants or plants with an aboveground infestation by *B. brassicae* aphids. In addition, we ran a separate PCA for each time point (Fig. **S2**), showing that after 3 and 48 hours of feeding by *D. radicum*, plants pretreated with *P. xylostella* exhibited a transcriptomic profile separate from the other samples with root-infestation by *D. radicum*. This effect was not evident at other time points.

We functionally characterised genes that contributed most to the separation on the first PC (Fig. **1c**, Table **S1**). Top loadings of PC1 (i.e. genes associated with the positive values on PC1, corresponding with *D. radicum*-infested roots) include genes involved in well-known defence processes, such as JA biosynthesis, proteinase inhibitors, indole GSL biosynthesis, GSL catabolism, and also a peroxidase gene (*PER22*) which strongly responded to root infestation. Bottom loadings of PC1 (i.e. genes associated with negative values on PC1, corresponding with uninfested roots) consist almost exclusively of genes involved in the biosynthesis of aliphatic GSLs and their amino-acid precursor methionine.

Transcriptome of the primary root in response to *D. radicum*

In total, 8469 genes were differentially expressed between control and any of the treatments over the course of the experiment, 4702 were up- and 3868 were downregulated (Fig. **2a**); this corresponds to roughly 14% of the genome. Most of these genes responded to infestation by the root herbivore *D. radicum*. As soon as 3 h after infestation by the root herbivore, over a thousand genes were upregulated in the roots. Feeding by shoot herbivores alone did not lead to many differentially expressed genes in the roots; their largest effect was seen at the latest time point, 96 h after shoot herbivory had started. When specifically filtering for genes differentially regulated by *B. brassicae* across multiple time points, we found that a cabbage homolog of the sugar transporter *SWEET11* (Bo3g111200) was induced in roots of aphid-infested plants. At several time points, more genes were differentially expressed when plants were facing multiple herbivory: for instance, after 3, 6, 9 and 48 h, more genes were downregulated in plants infested with both *P. xylostella* and *D. radicum* compared to plants only infested by *D. radicum*. To further assess the effects of shoot herbivores on the root transcriptomic response to *D. radicum*, we analysed DEGs of dual-infested plants relative to plants only infested with *D. radicum* (Fig. **S3**). This analysis revealed that *P. xylostella* mainly caused changes at the first and last time points. Infestation by *B. brassicae* had little effect compared to infestation with *D. radicum* only.

To gain more insight in the functions of the differentially expressed genes, a clustering analysis was executed. This resulted in seven clusters of upregulated genes and nine clusters of downregulated genes in response to *D. radicum* feeding (Fig. **2b**). For each cluster, we

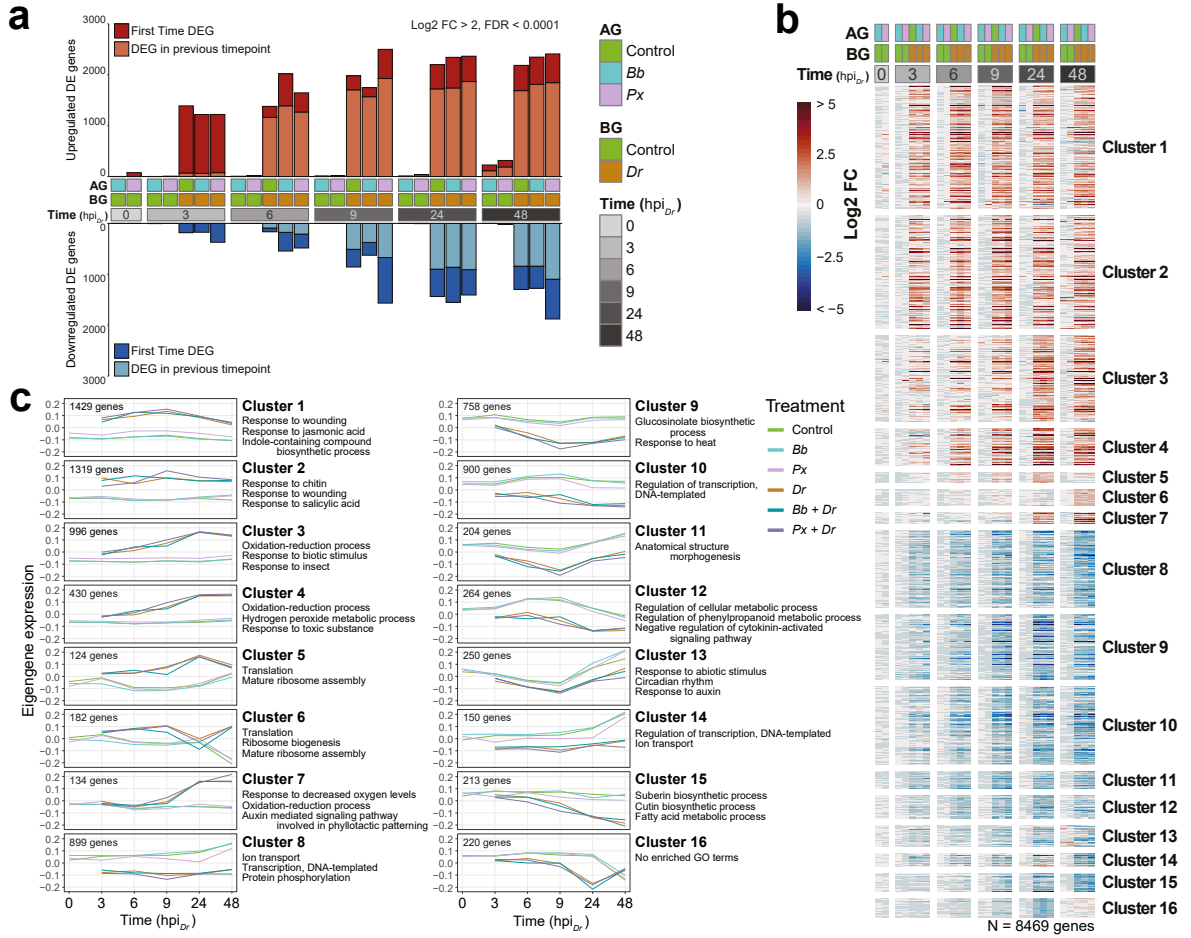

Figure 2. (**a**) Differentially expressed genes (DEGs) in *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding AG arrived two days prior to infestation by *D. radicum*. (**b**) Cluster analysis of DEGs. (**c**) Eigengene expression and enriched GO terms of each cluster. FC: fold change relative to control for each time point. FDR: false discovery rate. hpi$_{Dr}$: hours post infestation by *D. radicum*.

performed gene ontology (GO) enrichment analysis (Fig. **2c,** Table **S2**). Clusters 1 and 2 include genes that are upregulated rapidly upon infestation by *D. radicum*, and are involved in responses to wounding, chitin, jasmonic acid, salicylic acid, and in the biosynthesis of indole GSLs. Clusters 3, 4 and 7 encompass genes that respond to root herbivory at later time points, and include genes involved in oxidation-reduction processes. Further, we found that processes involved in the production of proteins, i.e. translation, ribosome biogenesis, were upregulated by *D. radicum*, peaking at 9 (cluster 4) and 24 h (cluster 5) after infestation. In clusters of downregulated genes, we found genes involved in GSL biosynthesis (cluster 9). Other processes downregulated upon infestation by *D. radicum* include ion transport and protein phosphorylation (cluster 8), regulation of circadian rhythm and responses to auxin (cluster 13), and biosynthesis of cutin and suberin (cluster 15). None of the clusters specifically correspond with changes in the roots induced by *B. brassicae* or *P. xylostella* feeding on aboveground tissues.

Jasmonic acid and ethylene are involved in the plant response to *D. radicum*

We studied the expression of genes involved in biosynthesis and signalling of phytohormones involved in plant defence against insect herbivory (Fig. **3**). Jasmonic acid signalling appears to play a central role in the plant response to *D. radicum*, as biosynthesis, regulation and signalling in this pathway are rapidly upregulated upon infestation (Fig. **3a**). This upregulation of genes is reflected in jasmonate concentrations (Fig. **3b**, Table **S3**). Inactivated jasmonates, such as hydroxy-JA, were found in higher concentrations in roots of plants exposed to *P. xylostella* feeding on the leaves compared to control plants (Fig. **S4**, Table **S3**). Genes involved in biosynthesis of ethylene were induced upon *D. radicum* infestation, especially *ACS2*, *ACO2* and *ACO4* were upregulated strongly and in early stages of the defence response (Fig. **3c**). Conversely, *ACS6* (Bo9g091320), was downregulated by *D. radicum* after 24 and 48 h. Further, expression of transcription factors *ERF1* and *ERF2* and ethylene response gene *PR3* were strongly upregulated by *D. radicum* (Fig. **3c**). There seems to be no clear role of ABA in the defence response against *D. radicum*. Some genes, such as *NCED9* and *RAB18*, are upregulated, whereas others, such as *ABA1*, *ABF3* and *RD29B*, are slightly downregulated (Fig. **3d**). Furthermore, none of the treatments affected ABA hormone levels in primary roots (Fig. **S4**, Table **S1**). Infestation by *D. radicum* did not have a uniform effect in SA biosynthesis and signalling, *ICS* genes are downregulated while other biosynthesis genes are upregulated (Fig. **3e**). Concentrations of SA were not affected by infestation (Fig. **S4**, Table **S1**). In conclusion, jasmonates, together with ethylene, appear to be involved in the plant response against *D. radicum*.

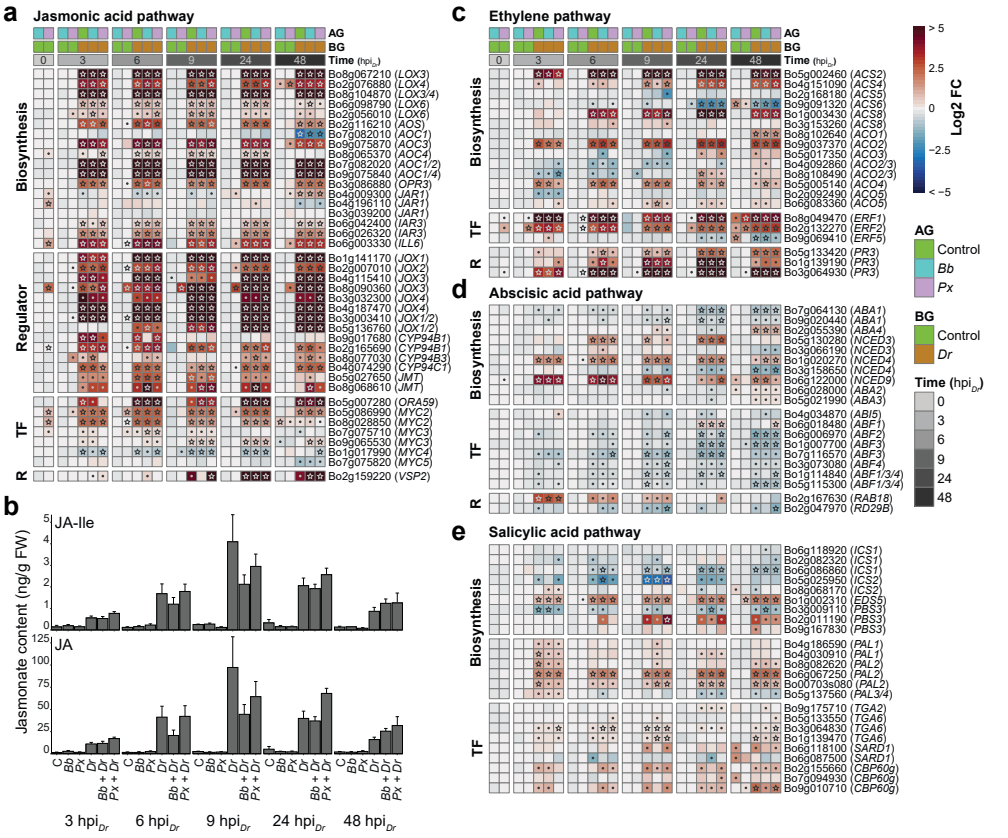

Figure 3. Phytohormonal response of *Brassica oleracea* primary roots to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding AG were introduced two days prior to root infestation by *D. radicum*. Fold changes of genes involved in the jasmonic acid pathway relative to control (**a**), concentrations of JA-Ile and JA; error bars represent standard error of the mean (N = 3 - 6) (**b**), fold changes of genes involved in ethylene (**c**), abscisic acid (**d**) and salicylic acid (**e**) signalling relative to control. Genes differentially expressed in the uninfested control of that time point are indicated by stars (FDR <0.0001) and dots (FDR <0.05). Statistical analyses of jasmonate concentrations are presented in Table **S3**. TF: transcription factor, hpi$_{Dr}$: hours post infestation by *D. radicum*. FC: fold change relative to control for each time point. C: control. N = 4 for each treatment x timepoint combination, N = 4 - 6 for chemical analyses.

Infestation by *D. radicum* leads to contrasting responses in glucosinolate biosynthesis

PCA and cluster analyses revealed that the plant response to feeding by *D. radicum* involves regulation of GSL biosynthesis. We analysed the expression of genes in the indole and aliphatic GSL pathways and the concentrations of these secondary metabolites (Fig. **4**). Nine GSLs were detected in the primary root samples, four indole GSLs, four aliphatic GSLs and

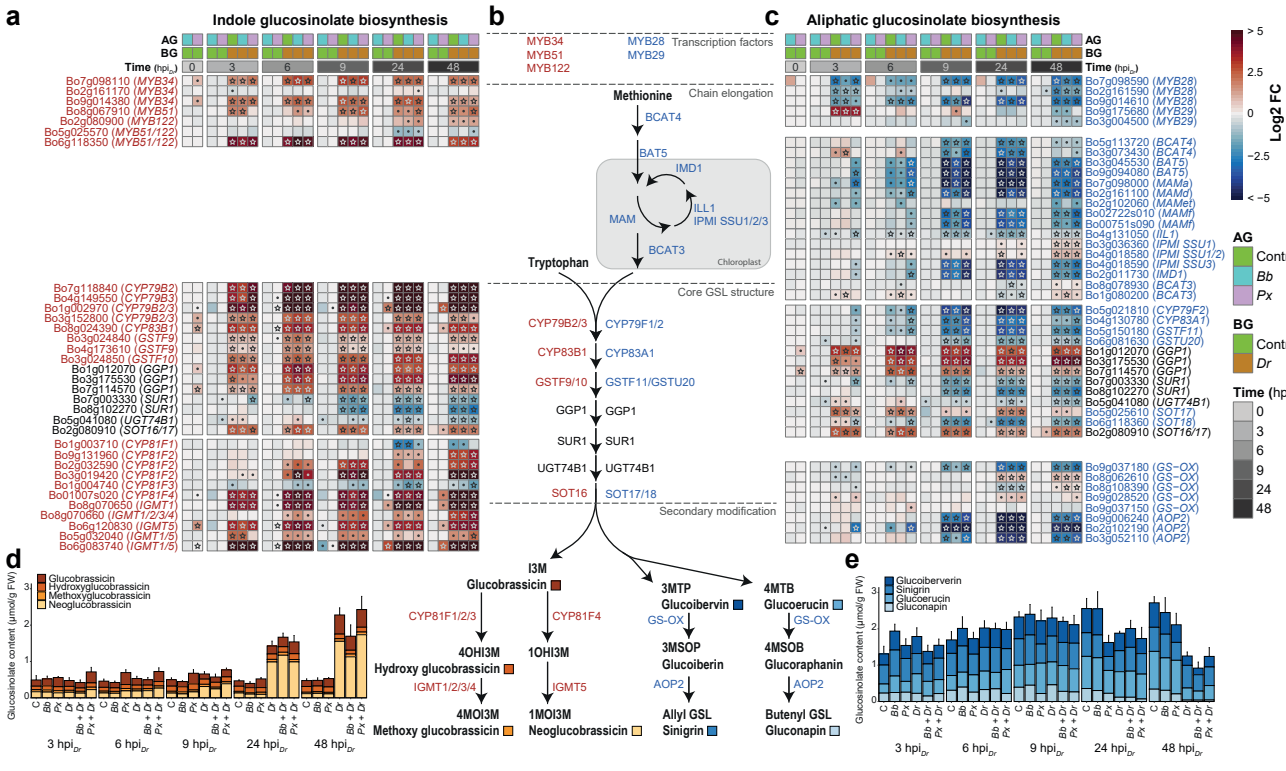

Figure 4. Fold changes relative to control for genes involved in indole (**a**) and aliphatic (**c**) glucosinolate (GSL) biosynthetic pathways in *B. oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding aboveground were introduced on plants two days prior to root infestation by *D. radicum*. Overview of GSL biosynthesis pathways derived from the amino acids tryptophan (indole) and methionine (aliphatic) (**b**). Concentrations of indole (**d**) and aliphatic (**e**) GSLs, means are plotted per compound and error bars represent the standard error of the total (N = 3 - 6). Genes presented in red are involved in indole GSL biosynthesis, those presented in blue are involved in aliphatic GSL biosynthesis, and genes presented in black are shared between the two pathways. Gene expression levels different from the uninfested control of that time point are indicated by stars (FDR <0.0001) and dots (FDR <0.05). For analyses of GSL concentrations, statistical information can be found in Table **S4**. hpi_{Dr}: hours post infestation by *D. radicum*. FC: fold change relative to control for each time point. C: control. FW: fresh weight. hpi_{Dr}: hours post infestation by *D. radicum*.

the aromatic GSL gluconasturtiin (Fig. **S5**). Indole GSL biosynthesis was rapidly upregulated upon *D. radicum* infestation (Fig. **4a**). Four transcription factors (Bo7g098110, Bo9g014380, Bo8g067910 and Bo6g118350) were already upregulated 3 h after infestation. In the core GSL biosynthesis pathways, genes encoding enzymes specific for indole GSL are upregulated under the influence of *D. radicum*. Several genes are involved in biosynthesis of the core structure for both aliphatic and indole GSL. Of these genes, the *B. oleracea* homolog of *GGP1* is upregulated by *D. radicum* whereas *SUR1* and *UGT74B1* are downregulated. In the indole GSL secondary modification steps, *CYP81F4* and *IGMT5* are most strongly upregulated by *D. radicum*, and indeed, the GSLs glucobrassicin and neoglucobrassicin are especially abundant in response to *D. radicum* feeding (Fig. **4d**). Notably, in plants experiencing dual herbivory by *P. xylostella* and *D. radicum*, these two compounds are produced faster compared to plants only exposed to *D. radicum* (Fig. **4d**, Table **S4**). Several genes in the indole GSL pathway (e.g. *MYB34*, *CYP79B2/3*, *CYP81F4*) are also upregulated in *P. xylostella* induced plants (Fig. **4a**).

Conversely, aliphatic GSL biosynthesis was downregulated in plants induced by *D. radicum* (Fig. **4c**). The three cabbage homologs of *MYB28*, in particular, were downregulated early on in the plant response to *D. radicum*. Interestingly, *MYB29* (Bo9g15680) was upregulated 3 h after *D. radicum* infestation but downregulated at later time points. Genes encoding proteins involved in chain elongation and core aliphatic GSL biosynthesis, such as *BAT5* and *CYP79F2*, were downregulated in plants infested by both *P. xylostella* on the leaves and *D. radicum* on the roots after 3 h, whereas this downregulation is seen after 6 h in plants only infested with *D. radicum*. Concentrations of aliphatic GSLs in primary roots showed a reduction of glucoiberverin from 24 h and gluconapin at 48 h after *D. radicum* infestation (Fig. **4e**). In plants dually infested with *B. brassicae* plus *D. radicum*, glucoerucin was also reduced 48 h after *D. radicum* started feeding, compared to control plants (Fig. **4e**).

We assessed the expression of genes involved in GSL catabolism and transport. Expression of genes encoding several myrosinases, enzymes that catabolise GSL into toxic breakdown products, was affected by *D. radicum* infestation (Fig. **S5**). While transcription of genes encoding classic myrosinase homologs (*TGG1-6* in *Arabidopsis*) was not strongly affected by *D. radicum* infestation, we found upregulation of genes encoding several of the more recently discovered atypical myrosinases (*BGLU18-33* in *Arabidopsis*). In particular, mRNA levels of cabbage homologs of *BGLU18*, *PYK10*, *BGLU25* and *BGLU31/32* were strongly increased upon *D. radicum* herbivory. Moreover, we assessed the expression of five *B. oleracea GTR* homologs (Fig. **S5**), which encode proteins responsible for GSL transport throughout the plant (Andersen *et al.*, 2013). Differences in expression upon *D. radicum* infestation occurred mainly for two of these genes: a *GTR1/2* homolog (Bo3137030) was slightly upregulated, and a *GTR2/3* homolog (Bo5025960) was downregulated. The functions of these genes have not been studied for *B. oleracea*; thus, assumptions on how

the changes in *GTR* gene expression affect GSL transport are premature. Nevertheless, the concentrations of GSLs in roots and leaves did not show clear evidence for GSL transport (Fig. **S5**). For instance, the reduction of aliphatic GSLs in *D. radicum* infested roots did not lead to higher aliphatic GSL concentrations in leaves. Likewise, the increase in indole GSLs in roots did not coincide with a reduction of leaf indole GSL concentrations (Fig. **S5**).

Aliphatic glucosinolates provide defence against *D. radicum*

Aliphatic GSL biosynthesis, regulated by the transcription factor MYB28, was downregulated upon *D. radicum* root herbivory (Fig. **4**). We hypothesised that this downregulation would reduce plant defence and favour *D. radicum* performance. To address this hypothesis, we studied *D. radicum* performance using a *myb28* knockout cabbage line (Neequaye *et al.*, 2021). Successful development of *D. radicum*, quantified as adult fly emergence, increased from 60% on control plants to 82% on *myb28* mutants (Fig. **5a**), while adult fly weight was

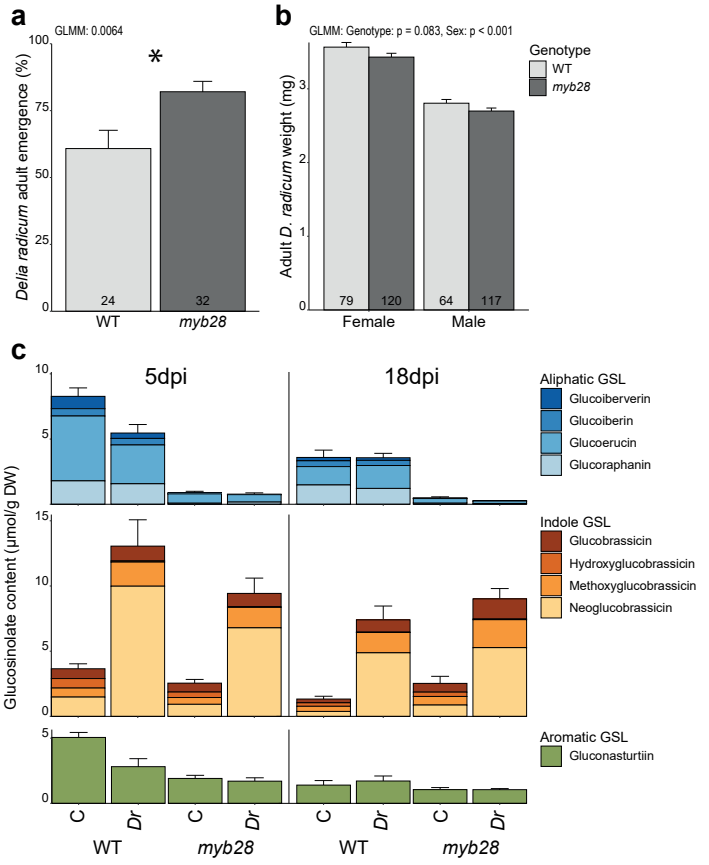

Figure 5. Emergence (**a**) and adult weight (**b**) of *Delia radicum* feeding on wild type (WT) or *myb28* knockout *B. oleracea* plants. Numbers at the bottom of bars represent the number of plants or flies. (**c**) Glucosinolate (GSL) concentrations in primary roots at 5- and 18-days-post-infestation by *D. radicum* (dpi). Means are plotted per compound and error bars represent the standard error of the total; N = 10 plants for all groups, excect for *D. radicum* infested plants at 18 dpi, in which case all plants used for *D. radicum* survival assay were measured (N = 24 - 32). Scaling of the Y-axis is identical for the three classes of GSLs. For analyses of GSL concentrations, statistical information can be found in Table **S5**. GLMM: generalized linear mixed model. DW; dry weight. C: Control. *Dr*: *D. radicum*.

not affected (Fig. **5b**). Glucosinolate content in primary roots of these plants was measured at 5 (during larval feeding) and 18 (after pupation) days post infestation, and revealed that indeed, aliphatic GSL production is effectively knocked down in *myb28* plants (Fig. **5c**, Table **S5**). Further, in accordance with our prior results (Fig. **4**), in both wild-type and *myb28* plants, indole GSLs are present in higher concentrations in plants induced by *D. radicum* (Fig. **5c**, Table **S5**).

Plutella xylostella primes the defence response against *D. radicum*

Transcriptome analysis revealed that *P. xylostella* induces changes in the early (3 hpi) plant response to *D. radicum* (Fig. **S2a**, Fig. **S3**). We therefore hypothesised that the plant response to *D. radicum* may be primed by prior shoot feeding by *P. xylostella*, leading to a faster or stronger response. To study this, we sampled primary roots at very early time points after induction by *D. radicum* on plants with or without prior feeding by *P. xylostella* and quantified transcripts of genes involved in JA, indole GSLs, and aliphatic GSLs (Fig. **6a**).

Expression levels of *AOS*, *MYC2*, and *CYP81F4* were higher 15 min after the start of *D. radicum* feeding when there had been a prior infestation by *P. xylostella* compared to the other treatments, whereas plants exposed to *D. radicum* alone responded after 30 or 60 min (Fig. **6b-d**, Table **S6**). Expression of *MYB28* was downregulated after 60 min of *D. radicum* feeding in plants induced with the root herbivore alone or in combination with *P. xylostella*, but the downregulation was stronger in dual infested plants (Fig. **6e**). After two hours, expression levels of all four genes were the same in plants induced by *D. radicum* alone or in combination with *P. xylostella* (Fig. **6b-e**).

We then studied whether priming of defence against *D. radicum* by *P. xylostella* would be retained over time. To this end, we introduced a period without infestation of 24 hours or 7 days between removal of *P. xylostella* and exposure to *D. radicum*. When such a period without infestation was introduced, a faster response in terms of gene expression was no longer observed in terms of expression of *AOS, MYC2* and *MYB28* (Fig. **6f-k, m**, Table **S6**). Expression of *CYP81F4* was higher in plants that had been exposed to *P. xylostella* prior to *D. radicum* with a period without infestation of 7 days, but not with a period without infestation of 24 hours (Fig. **6h, l**). Thus, prior infestation by *P. xylostella* leads to a faster response to *D. radicum*, but the effect of *P. xylostella* infestation diminishes after 24 or more hours since their feeding had stopped.

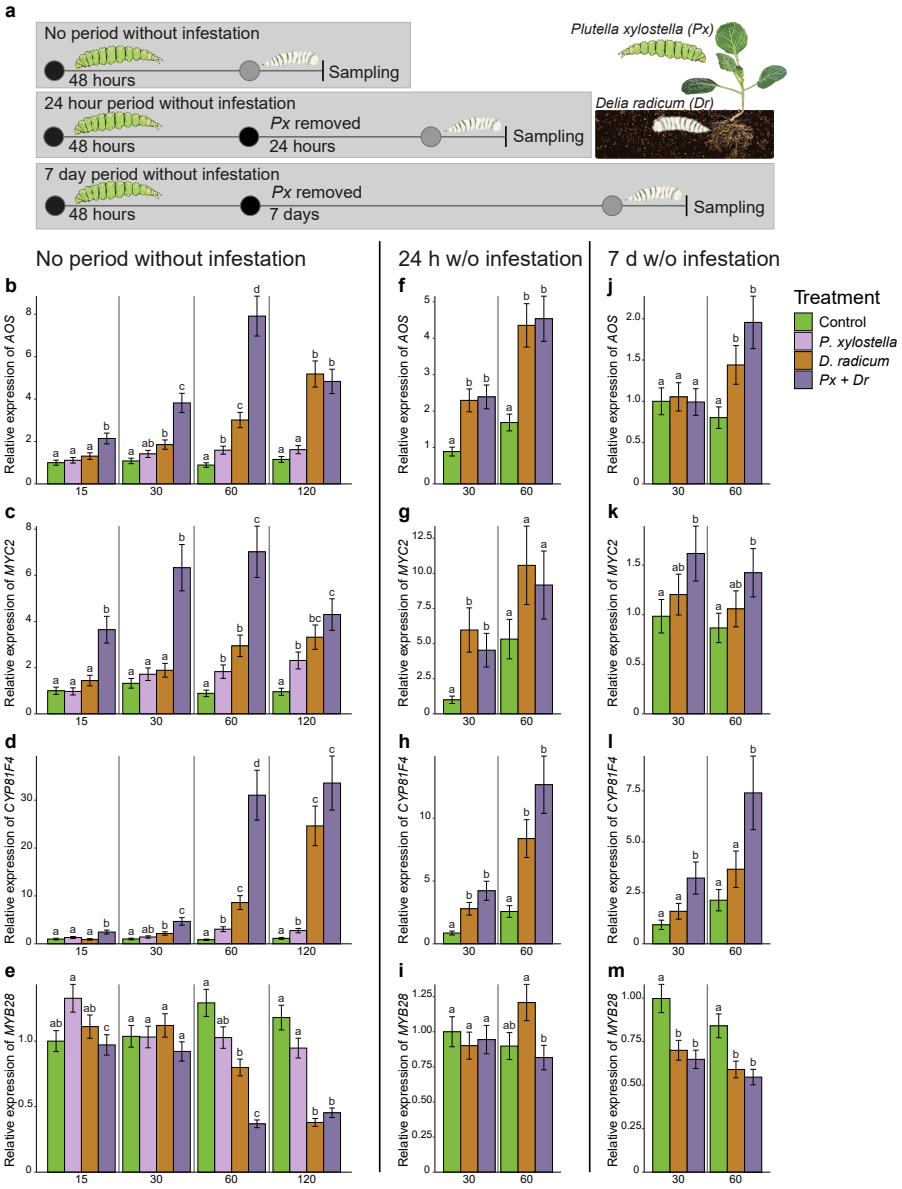

Figure 6. (**a**) Experimental setup to study the effects of *Plutella xylostella* (*Px*) leaf herbivory on the gene expression response of *B. oleracea* primary roots to the root herbivore *Delia radicum* (*Dr*). *Plutella xylostella* fed for 2 days prior to *D. radicum* arrival directly afterwards or after a period without infestation of 24 hours or 7 days. Relative expression of *AOS* (**b**, **f**, **j**), *MYC2* (**c**, **g**, **k**), *CYP81F4* (**d**, **h**, **l**), and *MYB28* (**e**, **i**, **m**). Statistical information of main effects can be found in Table S6. Different letters indicate differences (Tukey's HSD: $P < 0.05$) in pairwise comparisons between treatments within sampling times. w/o = without. N = four biological replicates consisting of three plants.

Discussion

Plant transcriptomic responses to root herbivory are rarely studied. Our in-depth transcriptomic analyses of plant responses to insect root herbivores show that plants invest heavily in responding to root herbivory, as demonstrated by major transcriptome reconfiguration in primary root tissue. Many of these genes are involved in processes commonly observed in response to herbivore attack, such as responses to wounding and jasmonic acid. Other processes clearly involved in the plant response to *D. radicum* are oxidation-reduction, responses to chitin, and translation. We found contrasting regulation of indole and aliphatic GSL in the response to cabbage root fly, and we provide evidence indicating that aliphatic GSL are toxic to this specialist herbivore. Prior herbivory by *B. brassicae* aphids or *P. xylostella* caterpillars did not lead to a large shift in the plant response to *D. radicum*. However, we found that prior caterpillar attack on the leaves primes for an earlier defence response in the roots.

Transcription of genes involved in oxidation-reduction and response to peroxide was increased upon *D. radicum* feeding. Reactive oxygen species such as hydrogen peroxide are produced upon wounding and occur during the early onset of plant defence (Erb & Reymond, 2019). The gene associated most strongly with *D. radicum* herbivory in our multivariate analysis was a homolog of the *Arabidopsis PER22* gene, a class III peroxidase that is secreted into the apoplastic space and confers resistance to cold stress in *Arabidopsis* (Kim *et al.*, 2012). Interestingly, Class III peroxidases and hydrogen peroxide production are involved in resistant wheat genotypes against the dipteran pest *Mayetiola destructor* (Liu *et al.*, 2009). Larvae of this pest feed from within induced galls in wheat stems, and while *D. radicum* does not induce galls, resistance may be achieved in a similar manner as young larvae feed from within the primary root.

Primary roots facing *D. radicum* herbivory have more transcripts of genes involved in responses to chitin. This finding is strengthened by our multivariate analysis, where we found that two homologs of the *Arabidopsis PR3* gene respond strongly to root herbivory, which encodes a JA/ET inducible chitinase (van Loon *et al.*, 2006). Notably, maize roots infested with *Diabrotica virgifera virgifera* also exhibited increased expression of chitinases (Barr *et al.*, 2010). Chitinases have been studied extensively for their antifungal activity (van Loon *et al.*, 2006; Grover, 2012). Since the rhizosphere is a microbial hotspot, it makes sense for plants to prepare for defence against opportunistic pathogens upon root herbivory (Johnson *et al.*, 2016b). However, recent research shows that maize chitinases play a role in defence to insects by disrupting the peritrophic matrix in the midgut of *Spodoptera exigua* caterpillars, thereby enhancing pathogen infection (Mason *et al.*, 2019; Han *et al.*, 2021).

Thus, responses to chitin may be targeted directly at *D. radicum*, or at secondary infection by fungal pathogens.

Upon root herbivory, the primary roots of cabbage plants show a change in many transcripts encoding proteins involved in translation and ribosome biogenesis. Ribosomes are large complexes comprised of rRNA and ribosomal proteins. By changing the composition of these ribosomal proteins, of which over 250 are known in *Arabidopsis*, plants may be able to regulate translation under stress (Martinez-Seidel *et al.*, 2020). Indeed, in roots of *Arabidopsis* plants, deficiency in phosphate and iron leads to changes in ribosomal composition (Wang *et al.*, 2013). Moreover, using TRAP-seq, a novel sequencing technique which specifically targets mRNA bound to ribosomes, Kimberlin *et al.* (2021) found that wounding leads to changes in transcripts associated to ribosomes. This level of regulation in plant defence against herbivorous insects presents an exciting avenue for future research.

The phytohormones JA and ET are involved in the plant defence response against *D. radicum*. Previous research established that JA and ET coordinate responses to necrotrophic pathogens, whereas JA and ABA regulate responses to chewing herbivores (Pieterse *et al.*, 2012). We previously reported that *D. radicum* caused downregulation of ET biosynthesis in primary roots based on expression of the marker gene *ACS6* (Karssemeijer *et al.*, 2020 - **chapter 2**). While our current data support that *ACS6* is downregulated, other *ACS* genes and ET response genes are strongly induced upon *D. radicum* infestation, underlining that conclusions based on marker gene expression should be made with care. Biosynthesis and signalling in the ABA pathway were not upregulated by root herbivory. Thus, while JA regulates responses to insect herbivores in both leaves and roots, finetuning of the response by ET and ABA appears to be different in the root response to *D. radicum*. There are multiple possible explanations for activation of JA and ET rather than ABA in response to root herbivory. Firstly, each of these three hormones have ancillary functions in root tissue, for instance in regulation of root development (Saini *et al.*, 2013; Johnson *et al.*, 2016b). This may cause differences in defence regulation between shoots and roots, as disrupting hormone homeostasis could affect normal root growth. Secondly, upregulating responses to necrotrophic pathogens may be beneficial when responding to root herbivores, as their feeding sites can be used for infection. Finally, many herbivores can manipulate their host-plant defences (Acevedo *et al.*, 2015; Favery *et al.*, 2020). For instance, Colorado potato beetles carry bacteria in their saliva that induce SA instead of JA when administered to tomato plants (Chung *et al.*, 2013). A similar mechanism is not yet known for *D. radicum*.

Root herbivory by *D. radicum* leads to a strong induction of indole GSLs, whereas aliphatic GSLs are downregulated. By transcriptomic and chemical analysis of the same samples, we clearly show the close connection between GSL biosynthesis gene expression and the accumulation of different GSLs. Comparison of GSL concentrations in leaves and roots, as well as expression patterns of GSL transporter (*GTR*) genes, suggests that local production drives root GSL accumulation. In *B. rapa*, *GTR* genes were strongly induced by *D. radicum*, but this did not coincide with changes in GSL concentrations in distal tissues, suggesting that *GTR* genes may be involved in GSL retention rather than distal transport (Touw *et al.*, 2020). Previous studies found similar GSL regulation in response to *D. radicum* and other herbivores, where indole GSLs are highly inducible while aliphatic GSLs are mostly non-responsive or downregulated (van Dam & Raaijmakers, 2006; Textor & Gershenzon, 2009; Pierre *et al.*, 2012b; Touw *et al.*, 2020). This is somewhat counterintuitive, as breakdown products of aliphatic GSL are more toxic to herbivorous insects (Jeschke *et al.*, 2016)2016. Using mutant *Arabidopsis* plants, Müller *et al.* (2010) found that generalist herbivores were negatively affected by both indole and aliphatic GSL, but specialist herbivores were not affected. Many specialist herbivores of brassicaceous plants have evolved mechanisms to cope with GSLs (Textor & Gershenzon, 2009; Jeschke *et al.*, 2016), and some species are even capable of sequestering specific GSLs for their own defence (Yang *et al.*, 2021). *Delia radicum* harbours gut microbes that can detoxify breakdown products of the aromatic GSL gluconasturtiin (Welte *et al.*, 2016). Several studies did not find a link between GSL contents and *D. radicum* performance in wild or cultivated *B. oleracea*, but the relationship was not directly studied (Pierre *et al.*, 2012b; van Geem *et al.*, 2015). Here, we made use of transgenic *B. oleracea* plants to show that *D. radicum* survival increases when feeding on plants with knocked down aliphatic GSL concentrations. This finding implies that downregulation of aliphatic GSL biosynthesis is adaptive for the root herbivore, and plants that do not respond in this manner are expected to be more resistant to *D. radicum*.

A logical next step would be to investigate why plants are equipped with this seemingly maladapted response to herbivory. Plants may respond more strongly to pathogens infecting wounds caused by *D. radicum*, and indole GSLs are indeed effective against pathogens (Bednarek *et al.*, 2011). On the other hand, similar differential regulation of indole and aliphatic GSLs in response to herbivory has been found in leaf tissue as well (Gols *et al.*, 2008), where the risk of secondary pathogen infection is likely lower. Possibly physiological constraints play a role, because the two biosynthesis pathways share several enzymatic steps (Sønderby *et al.*, 2010). Further, both indole and aliphatic GSL biosynthesis transcription factors are part of MYB subfamily 12, which form a complex with MYC2/3/4 through a shared motif (Millard *et al.*, 2019). Competition between different MYBs and their MYC interaction partners may occur (Millard *et al.*, 2019), and could potentially underlie

the observed differential regulation of aliphatic and indole GSLs. Studying how these two pathways are so differently regulated upon herbivory despite molecular commonalities may provide novel insights in regulation of plant defence.

Hydrolysis of GSLs is a crucial step in the production of toxic breakdown products, such as nitriles, isothiocyanates and glucobrassinin, a process catalysed by myrosinases. Recent advances show that brassicaceous plants are equipped with two types of myrosinase enzymes, differing in amino acids at the active site (Sugiyama & Hirai, 2019). In response to *D. radicum*, expression of classic myrosinases did not change much while atypical myrosinases were upregulated. Whereas classic myrosinases localise in specific cells throughout the plant and hydrolyse both indole and aliphatic GSLs, atypical myrosinases accumulate in ER bodies and show activity towards indole GSLs (Kissen *et al.*, 2009; Zhao *et al.*, 2015; Nakano *et al.*, 2017). Interestingly, ER bodies are only constitutively expressed in roots of *Arabidopsis*, while they may be produced upon wounding of leaves, presenting yet another difference between shoot and root defences (Ogasawara *et al.*, 2009; Nakano *et al.*, 2014). Different myrosinases may yield different hydrolysis products, with consequences for defensive activity (Zhao *et al.*, 2015). Moreover, there are many myrosinase-associated proteins, which alter the breakdown products into their final form, providing much potential for specific regulation of toxins (Textor & Gershenzon, 2009). Finally, like many components of plant defence pathways, atypical myrosinases have other functions besides hydrolysing indole GSLs. BGLU18 and BGLU33 can hydrolyse biologically inactive ABA-*O*-glucoside, resulting in bioactive ABA (Han *et al.*, 2019), and PYK10 can hydrolyse scopolin into scopoletin *in vitro* (Ahn *et al.*, 2009; Nakano *et al.*, 2014). Scopoletin, in turn, plays an important role in communication between roots and the rhizosphere microbiome as well as iron uptake (Ahn *et al.*, 2009; Nakano *et al.*, 2014; Stringlis *et al.*, 2019). As a follow-up study, it would be interesting to elucidate which biologically active compounds are produced from the accumulated neoglucobrassicin in *D. radicum* infested roots.

Our transcriptome data show that *P. xylostella* changed the plant response to *D. radicum* at the earliest time point studied. When we studied responses in the first hour following root herbivory, a clearly faster response was seen in *P. xylostella* induced plants, and thus priming by the caterpillar infestation, which may be responsible for the plant-mediated antagonism we previously recorded (Karssemeijer *et al.*, 2020 - **chapter 2**). It would be interesting to study the speed of induction in similar systems, as antagonism between AG and BG chewers is commonly found (Johnson *et al.*, 2012). Interestingly, after two hours, the genes we studied were expressed at the same level regardless of priming, indicating that especially the onset of the response was altered. Another element of priming is its retention over time, or 'memory' (Hilker *et al.*, 2016), which can even be transferred through seeds to the next

generation (Rasmann *et al.*, 2012). When we introduced a non-infested time interval, we no longer observed a faster response to root herbivory, indicating that continuous feeding by *P. xylostella* is required for priming to be sustained.

Aboveground herbivory by *B. brassicae* aphids or *P. xylostella* caterpillars did not lead to major differences in the overall transcriptomic response to *D. radicum*, demonstrated by similar patterns in our cluster analysis. This corresponds with earlier findings that the latest stressor has a dominant effect over earlier induction (Coolen *et al.*, 2016). Shoot infestation by the caterpillars had more effect than shoot infestation by the aphids, and we found most differences between single and dual infested plants in the first and last time point studied. Our data further show that, in the absence of root herbivory, foliar herbivores induce most primary root genes at the latest time point studied, i.e. 96 h after they had been placed on the leaf. Perhaps later in the plant response to *D. radicum*, foliar herbivores impact the pattern of induction more strongly than in the time points we studied.

In the current study, we present an extensive analysis of primary root responses to a specialist root herbivore. We provide clear evidence that aliphatic GSL can interfere with the performance of specialist insect herbivores. Our study opens new avenues of research in insect plant interactions. Using 'omics' approaches teaches us that there is much more to learn about interactions between plants and their surroundings. While much remains to be discovered to fully grasp the nature and evolution of these interactions, this study advances our understanding of how plants cope with root herbivores.

Acknowledgements

We are grateful to **Julia Friman**, **Thibault Costaz**, **Davy Meijer**, **Toya Nath Joshi**, **Janneke Bloem**, **Patrick Verbaarschot**, **Sandeep Sarde** and **Ina van de Kamp** for their valuable help during the experiments, and to **Yidong Wang** for the insect illustrations. The authors thank **Teun van Duffelen** for his work on assembly and mapping of unmapped reads. We thank **Pieter Rouweler**, **André Gidding** and **Frans van Aggelen** for insect rearing, and **Sean Geurts** and **Gaby Jaramillo** for growing plants. We thank **Syngenta Seeds NL** for performing the RNA-seq as an in-kind contribution to this project, and in particular **Jan Bruin** and **Bill Briggs**.

Supporting information

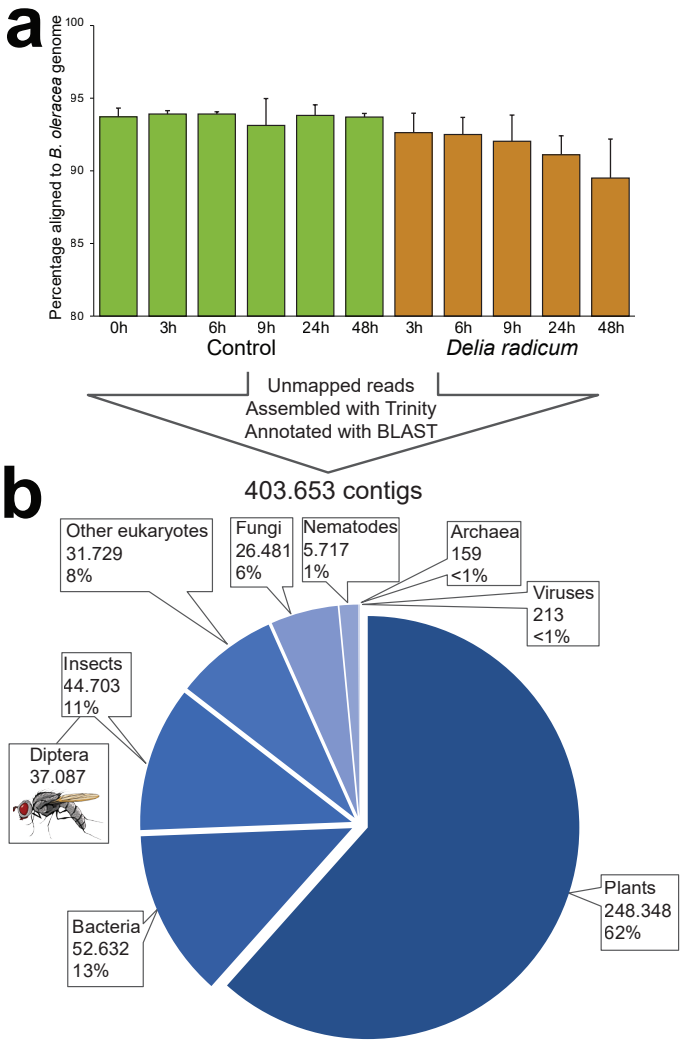

Figure S1. Percentage of RNAseq reads mapped to the *Brassica oleracea* TO1000 genome, grouped by time and whether or not plants were infested with *D. radicum* (**a**). Bars represent mean plus standard error, N = 12. Reads that did not align to the cabbage genome were assembled using Trinity into 403,653 contigs, which were annotated with BLAST. Number of contigs per group of organisms (**b**), Eukaryotes were subdivided into Phyla or Classes. Within the Insects, most contigs were annotated to Diptera, and within the Diptera, most contigs annotated to *Lucilia cuprina* (20,767).

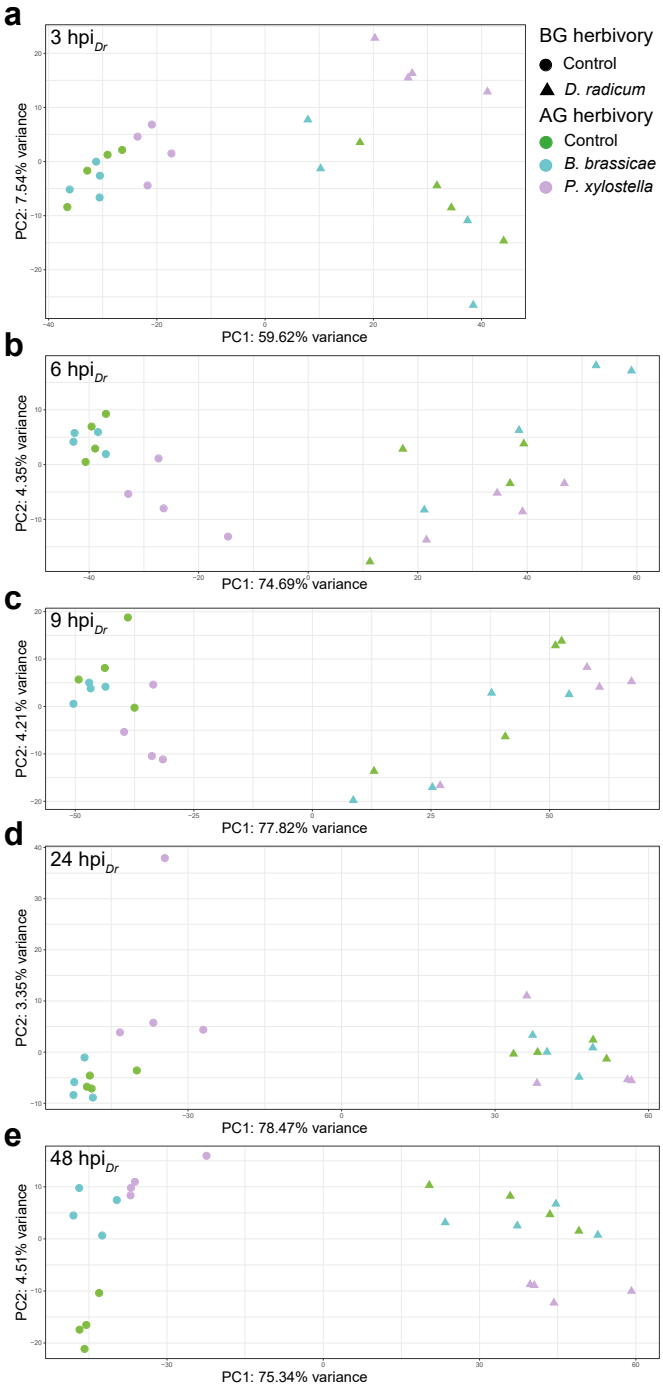

Figure S2. Principal component analysis (PCA) of *Brassica oleracea* transcriptomes of the primary root under the influence of aboveground (AG) herbivory by *Brevicoryne brassicae* or *Plutella xylostella* and belowground (BG) herbivory by *Delia radicum* for 3 (**a**), 6 (**b**), 9 (**c**), 24 (**d**), 48 (**e**) hours. Aboveground herbivores arrived 48 hours prior to infestation by *D. radicum*. hpi$_{Dr}$: hours post infestation by *D. radicum*.

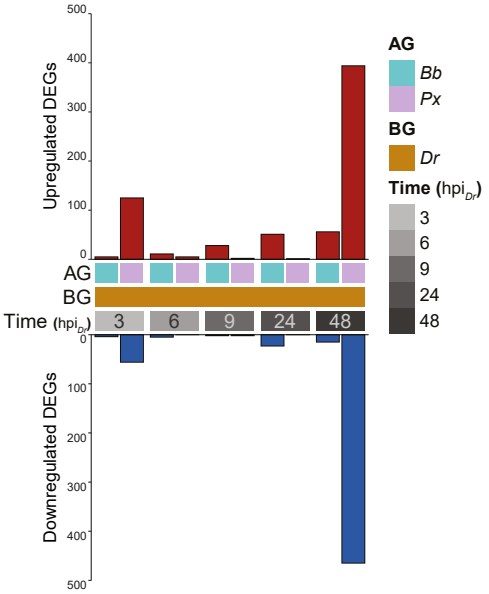

Figure S3. Significantly differentially expressed genes (DEGs; FDR <0.05) relative to root-herbivore (*Delia radicum* (*Dr*)) induced samples in *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*). Herbivores feeding AG were introduced two days prior to infestation by *D. radicum*. FDR: false discovery rate. hpi$_{Dr}$: hours post infestation by *D. radicum*.

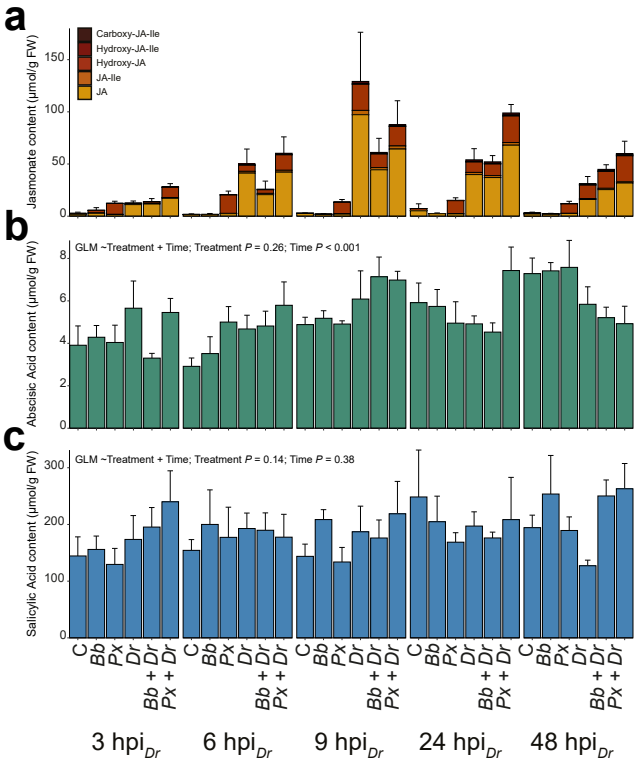

Figure S4. Concentrations of jasmonates (**a**) abscisic acid (**b**) and salicylic acid (**c**) in *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding AG arrived two days prior to root infestation by *D. radicum*. Corresponding statistical information in Table S3. For jasmonates, means are plotted per compound and error bars represent the standard error of the total (N = 3-6). FW: fresh weight. C: control. hpi$_{Dr}$: hours post infestation by *D. radicum*.

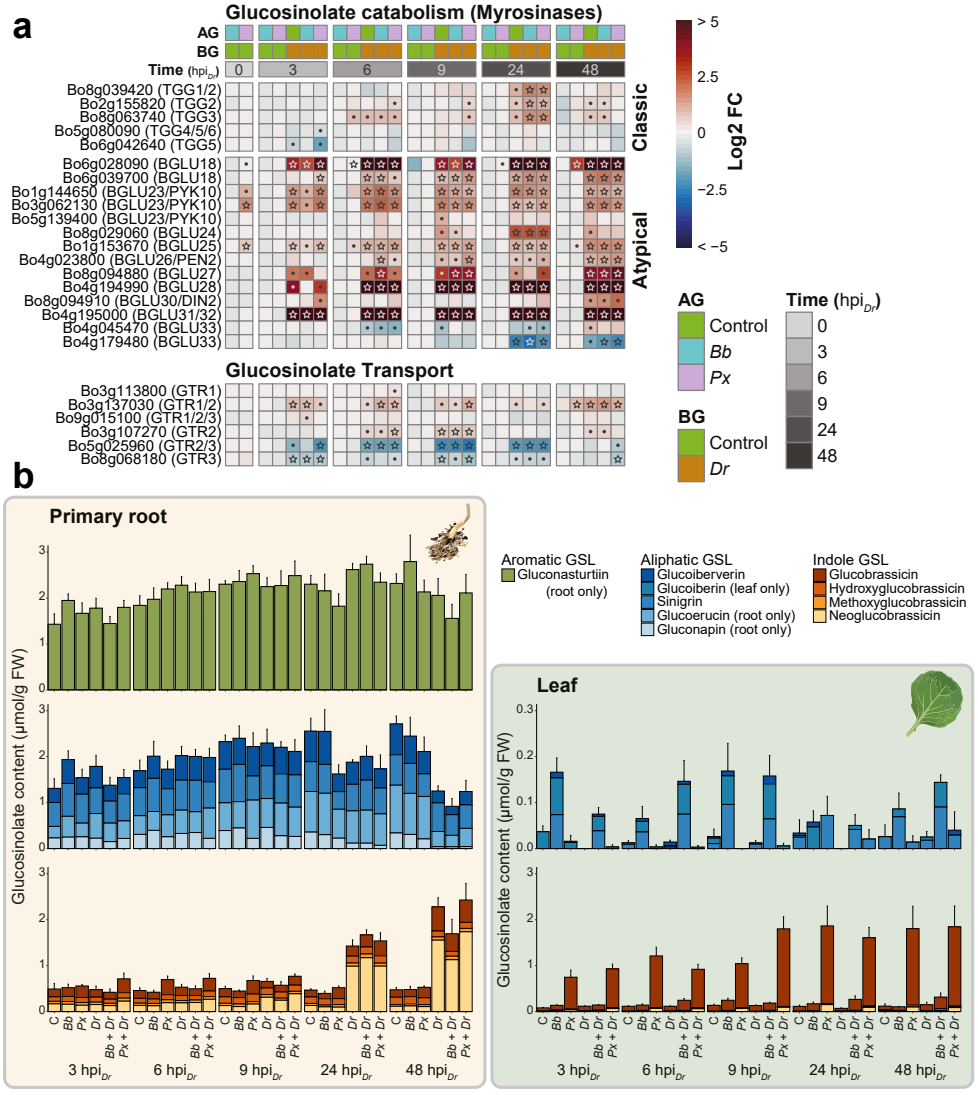

Figure S5. Fold changes relative to control for genes involved in glucosinolate catabolism and transport (**a**) in *B. oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*) and belowground (BG) herbivory by *Delia radicum* (*Dr*). Herbivores feeding aboveground were introduced on plants two days prior to root infestation by *D. radicum*. Concentrations of glucosinolates in *B. oleracea* primary roots and leaves (**b**) of plants from the same experiment used for RNAseq analysis (N = 3 - 6). For analyses of GSL concentrations, statistical information can be found in Table S4. Scaling of the y-axis is identical for the three classes of GSLs, with exception of leaf aliphatic GSL. Means are plotted per compound and error bars represent the standard error of the total amount. FW: fresh weight. C: control. hpi_{Dr}: hours post infestation by *D. radicum*.

Methods S1: Genotyping of *myb28* and WT plants.

In the experiments performed to test the effects of aliphatic glucosinolates (GSL) on *Delia radicum*, two cabbage genotypes were used: a wildtype (WT) DH1012 line, and a *myb28* knockout line in the same DH1012 background. In the latter line, two of the three copies of *MYB28* (Bo9175680 and Bo2g161590) have been knocked out using CRISPR-Cas9 (Neequaye *et al.*, 2021)2021. Seeds were kindly provided by Mikhaela Neequaye and Lars Østergaard, who created this mutant line.

Seeds were sown in seedling soil and seedlings were transplanted after 8 days into regular potting soil (Lentse potgrond nr4). We genotyped all *myb28* plants used for the experiments and a subset of WT plants. To this end, a cotyledon was harvested from each plant 26 days after transplanting using a sharp razor to limit wounding. Rapid DNA extraction was performed using the MyTaq™ Extract-PCR Kit (Bioline). A small piece of cotyledon was directly submerged into lysis buffer and crushed using tweezers. Three plants were pooled for each sample. The razor and tweezers were cleaned between samples using a 10% bleach solution, followed by 70% ethanol and finally MQ water. This mixture was incubated at 75 °C for 5 min followed by 95 °C for 10 min. Samples were briefly spun down to reduce debris, and liquid was diluted 10 times prior to PCR. An initial PCR for the Act-2 reference gene was performed to confirm the extraction technique (not shown).

For genotyping, we tested the deletion in the *MYB28* ortholog on chromosome 9 (Bo9175680), as this can easily be visualised on using gel electrophoresis. Mutation in the chromosome 2 ortholog (Bo2g161590) was assumed, as the seeds were harvested from homozygous plants for both mutations. PCR was performed using MyTaq HS Red Mix (Bioline) and primers spanning the deletion, with the following protocol: Initial denaturation 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 15 sec, annealing at 60 °C for 30 sec and elongation at 72 °C for 1 min, and final elongation at 72 °C for 5 min. The primers amplify a 1534bp product of the Bo9g175680 *MYB28* gene in WT plants, and a 1008bp product in *myb28* plants (Forward primer: AGAGTTCTCATCAACCGATCT, Reverse primer: ACCTTTCTGCTTAGGCACGA, designed by Mikhaela Neequaye). Samples were loaded on a 1 % agarose gel with Midori green. By gel electrophoresis, we confirmed the mutant *myb28* allele in all samples (Figure **M1**).

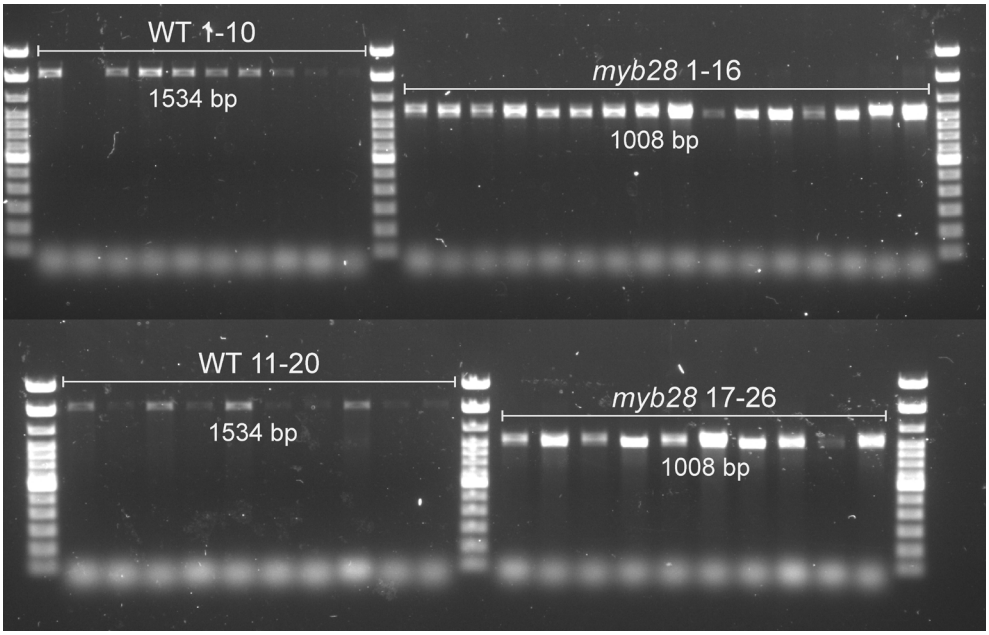

Figure M1. Gel electrophoresis of *MYB28* (Bo9g175680) PCR products in cotyledons of wild type (WT) and *myb28* mutant *Brassica oleracea* plants. Each reaction contained cotyledons of three plants. bp = base pairs

Supporting tables

Table S1. *Brassica oleracea* genes identified as top and bottom loadings of PC1 (Fig. **1**) and the functional characterisation of their *Arabidopsis thaliana* homologs based on TAIR (www.arabidopsis.org) and Uniprot (www.uniprot.org) descriptions. N/A indicates that functions are not clear from these descriptions. PC1 corresponds with *Delia radicum* herbivory (Fig. **1**). Hence, top loadings are genes associated with *D. radicum*-infested primary roots, and bottom loadings are genes associated with uninfested primary roots.

Top loadings

Gene ID	*Arabidopsis* homolog	Function	Acronym	Name	Orthology evidence*
Bo3g032450	AT2G38380 (**PER22**)	Oxidation-reduction	PER22	PEROXIDASE 22	2,4
Bo3g064930	AT3G12500 (**PR3**)	Chitinase, Defence against fungi	PR3	PATHOGENESIS-RELATED 3	1,2,3,4
Bo6g032890	AT1G54020	Lipid metabolism			2,4
Bo00722s140	AT1G72290 (**KTI2**)	Proteinase inhibitor	KTI2	KUNITZ TRYPSIN INHIBITOR 2	4
Bo3g064960	AT3G12500 (**PR3**)	Chitinase, Defence against fungi	PR3	PATHOGENESIS-RELATED 3	1,2,4
Bo6g097400	AT3G45140 (**LOX2**)	JA biosynthesis	LOX2	LIPOXYGENASE 2	2,4
Bo9g058330	AT5G03610	Lipid metabolism			1,2,4
Bo6g083740	AT1G76790 (**IGMT5**)	Indole GSL	IGMT5	INDOLE GLUCOSINOLATE O-METHYLTRANSFERASE 5	2,3,4
Bo8g105040	AT1G17180 (**GSTU25**)	Toxin catabolism	GSTU25	GLUTATHIONE S-TRANSFERASE TAU 25	2,4
Bo01163s010	AT3G45140 (**LOX2**)	JA biosynthesis	LOX2	LIPOXYGENASE 2	2,4
Bo3g004090	AT5G06870 (**PGIP2**)	Pectinase inhibitor, Defence against fungi	PGIP2	POLYGALACTURONASE INHIBITING PROTEIN 2	1,2,4
Bo1g004730	AT4G37410 (**CYP81F4**)	Indole GSL	CYP81F4	CYTOCHROME P450, FAMILY 81, SUBFAMILY F, POLYPEPTIDE 4	2,4
Bo1g002970	AT4G39950 (**CYP79B2**)	Indole GSL	CYP79B2	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 2	2,3,4
Bo3g101500	AT5G64250	N/A			1,2,3,4
Bo9g176740	AT3G26180 (**CYP71B20**)	N/A	CYP71B20	CYTOCHROME P450, FAMILY 71, SUBFAMILY B, POLYPEPTIDE 20	1
Bo6g028090	AT1G52400 (**BGLU18**)	Myrosinase/ABA catabolism	BGLU18	BETA GLUCOSIDASE 18	2,3,4
Bo4g194410	AT2G43510 (**TI1**)	Proteinase inhibitor	TI1	TRYPSIN INHIBITOR PROTEIN 1	2,4
Bo5g134680	AT3G12145 (**FTM4**)	Transition to flowering	FLOR1	FLORAL TRANSITION AT THE MERISTEM4	1,2,4
Bo3g041960	AT2G24850 (**TAT3**)	N/A	TAT3	TYROSINE AMINOTRANSFERASE 3	1,2,4
Bo4g149550	AT2G22330 (**CYP79B3**)	Indole GSL	CYP79B3	CYTOCHROME P450, FAMILY 79, SUBFAMILY B, POLYPEPTIDE 3	2,3,4

Table S1 continued

Bottom loadings

Gene ID	*Arabidopsis* homolog	Function	Acronym	Name	Orthology evidence*
Bo7g098000	AT5G23010 (**MAM1**)	Aliphatic GSL	MAM1	METHYLTHIOALKYLMALATE SYNTHASE 1	1,2,3,4
Bo2g041340	AT3G02020 (**AK3**)	Methionine biosynthesis	AK3	ASPARTATE KINASE 3	1,2,4
Bo5g021810	AT1G16410 (**CYP79F1**)	Aliphatic GSL	CYP79F1	CYTOCHROME P450 79F1	2,4
Bo2g161100	AT5G23010 (**MAM1**)	Aliphatic GSL	MAM1	METHYLTHIOALKYLMALATE SYNTHASE 1	1,2,4
Bo9g094080	AT4G12030 (**BAT5**)	Aliphatic GSL	BAT5	BILE ACID TRANSPORTER 5	1,2,3,4
Bo5g094570	AT3G22740 (**HMT3**)	Methionine biosynthesis	HMT3	HOMOCYSTEINE S-METHYLTRANSFERASE 3	1,2,3,4
Bo9g014610	AT5G61420 (**MYB28**)	Aliphatic GSL	MYB28	MYB DOMAIN PROTEIN 28	2,4
Bo4g018590	AT2G43100 (**IPMI2**)	Aliphatic GSL	IPMI SSU2	ISOPROPYLMALATE ISOMERASE SMALL SUB-UNIT 2	1,2,4
Bo3g045530	AT4G12030 (**BAT5**)	Aliphatic GSL	BAT5	BILE ACID TRANSPORTER 5	1,2,3,4
Bo04963s010	AT4G03050 (**AOP**)	Aliphatic GSL	AOP3	2-OXOGLUTARATE-DEPENDENT DIOXYGENASE	BLAST
Bo9g020260	AT5G67360 (**ARA12**)	Seed coating	SBT1.7	SUBTILISIN-LIKE SERINE PROTEASE 1.7	2,4
Bo2g011730	AT5G14200 (**IMD1**)	Aliphatic GSL	IMD1	ISOPROPYLMALATE DEHYDROGENASE 1	1,2,3,4
Bo04741s010	AT4G03050 (**AOP**)	Aliphatic GSL	AOP3	2-OXOGLUTARATE-DEPENDENT DIOXYGENASE	2,4
Bo4g130780	AT4G13770 (**CYP83A1**)	Aliphatic GSL	CYP83A1	CYTOCHROME P450, FAMILY 83, SUBFAMILY A, POLYPEPTIDE 1	2,3,4
Bo6g087680	AT1G73600 (**NMT**)	Phosphocholine biosynthesis	NMT	PHOSPHOETHANOLAMINE METHYLTRANSFERASE3	1,2,3,4
Bo4g049480	AT2G31790 (**UGT74C1**)	Aliphatic GSL	UGT74C1	UDP-GLYCOSYLTRANSFERASE 74C1	2,4
Bo6g083470	AT1G77000 (**SKP2B**)	Transition to photomorphogenesis	SKP2B	F-BOX PROTEIN SKP2B	1,2,3,4
Bo5g150180	AT3G03190 (**GSTF11**)	Toxin catabolism	GSTF11	GLUTATHIONE S-TRANSFERASE F11	1,2,3,4
Bo9g006240	AT4G03050 (**AOP**)	Aliphatic GSL	AOP3	2-OXOGLUTARATE-DEPENDENT DIOXYGENASE	2,4
Bo2g068030	AT1G70260 (**RTP1**)	Defence against biotrophic pathogens	RTP1	RESISTANCE TO PHYTOPHTHORA PARASITICA 1	2,3,4

* Based on PLAZA: 1 = Tree-based ortholog, 2 = Orthologous gene family, 3 = Anchor point (synteny), 4 = Best hit family (BLAST)

Table S2. Gene Ontology (GO) enrichment of gene clusters (Fig. **2b, c**). GO terms in bold are shown in the main text figure.

Cluster	GO Identifier	Log2 Fold Enrichment	P-Value	Subset Ratio	Description
Cluster 1	GO:0044710	0.67	2.96E-17	27.70%	single-organism metabolic process
Cluster 1	**GO:0009611**	**1.83**	**8.90E-15**	**5.03%**	**response to wounding**
Cluster 1	GO:0009073	3.1	1.26E-13	1.98%	aromatic amino acid family biosynthetic process
Cluster 1	GO:0010446	4.16	4.19E-10	0.91%	response to alkaline pH
Cluster 1	GO:0071467	4.16	4.19E-10	0.91%	cellular response to pH
Cluster 1	GO:0071469	4.16	4.19E-10	0.91%	cellular response to alkaline pH
Cluster 1	GO:1900067	4.16	4.19E-10	0.91%	regulation of cellular response to alkaline pH
Cluster 1	**GO:0009753**	**1.57**	**1.32E-09**	**4.45%**	**response to jasmonic acid**
Cluster 1	GO:0006568	3.25	7.20E-08	1.15%	tryptophan metabolic process
Cluster 1	GO:0006586	3.25	7.20E-08	1.15%	indolalkylamine metabolic process
Cluster 1	**GO:0042435**	**2.57**	**1.28E-07**	**1.65%**	**indole-containing compound biosynthetic process**
Cluster 1	GO:0055114	0.8	3.41E-07	10.72%	oxidation-reduction process
Cluster 1	GO:0000162	3.4	4.22E-07	0.99%	tryptophan biosynthetic process
Cluster 1	GO:0046219	3.4	4.22E-07	0.99%	indolalkylamine biosynthetic process
Cluster 1	GO:0006790	1.26	1.61E-06	4.70%	sulfur compound metabolic process
Cluster 1	GO:0009867	1.87	4.29E-06	2.31%	jasmonic acid mediated signaling pathway
Cluster 1	GO:1901657	1.41	7.03E-05	3.13%	glycosyl compound metabolic process
Cluster 1	GO:0044272	1.51	6.49E-04	2.39%	sulfur compound biosynthetic process
Cluster 1	GO:0010439	3.11	7.06E-04	0.74%	regulation of glucosinolate biosynthetic process
Cluster 1	GO:0007623	1.41	7.72E-04	2.64%	circadian rhythm
Cluster 1	GO:0048511	1.41	7.72E-04	2.64%	rhythmic process
Cluster 1	GO:0044712	1.05	8.59E-04	4.29%	single-organism catabolic process
Cluster 1	GO:0010438	3.31	8.86E-04	0.66%	cellular response to sulfur starvation
Cluster 1	GO:0006952	0.63	2.99E-03	8.99%	defence response
Cluster 1	GO:0055085	0.75	5.01E-03	6.43%	transmembrane transport
Cluster 1	GO:0009607	0.61	6.49E-03	8.99%	response to biotic stimulus
Cluster 1	GO:0008272	2.87	1.34E-02	0.66%	sulfate transport
Cluster 1	GO:0010035	0.55	2.10E-02	9.40%	response to inorganic substance
Cluster 1	GO:0006970	0.64	2.74E-02	7.09%	response to osmotic stress
Cluster 1	GO:0009651	0.67	3.00E-02	6.51%	response to salt stress
Cluster 2	GO:0042221	0.7	7.44E-17	26.68%	response to chemical
Cluster 2	GO:0010243	2.13	3.05E-16	4.48%	response to organonitrogen compound
Cluster 2	**GO:0010200**	**2.35**	**3.89E-16**	**3.79%**	**response to chitin**
Cluster 2	**GO:0009611**	**1.77**	**1.52E-12**	**4.82%**	**response to wounding**
Cluster 2	GO:0001101	0.82	6.12E-10	13.94%	response to acid chemical
Cluster 2	**GO:0009751**	**1.57**	**4.18E-08**	**4.04%**	**response to salicylic acid**
Cluster 2	GO:0006952	0.87	6.21E-08	10.59%	defence response
Cluster 2	GO:0043207	0.87	7.05E-08	10.59%	response to external biotic stimulus
Cluster 2	GO:0051707	0.87	7.05E-08	10.59%	response to other organism
Cluster 2	GO:0098542	0.94	8.86E-07	8.26%	defence response to other organism
Cluster 2	GO:0080167	1.53	1.59E-06	3.53%	response to karrikin
Cluster 2	GO:0002376	1.17	2.91E-06	5.34%	immune system process

Table S2 continued

Cluster	GO Identifier	Log2 Fold Enrichment	P-Value	Subset Ratio	Description
Cluster 2	GO:0009620	1.22	3.70E-06	4.91%	response to fungus
Cluster 2	GO:0006979	1.07	1.00E-05	5.85%	response to oxidative stress
Cluster 2	GO:0009617	1	1.15E-05	6.45%	response to bacterium
Cluster 2	GO:0045087	1.2	1.18E-05	4.73%	innate immune response
Cluster 2	GO:0050832	1.27	6.54E-05	3.87%	defence response to fungus
Cluster 2	GO:0009737	0.85	1.14E-04	7.31%	response to abscisic acid
Cluster 2	GO:0080134	1.1	1.36E-04	4.73%	regulation of response to stress
Cluster 2	GO:0031349	1.68	2.44E-04	2.24%	positive regulation of defence response
Cluster 2	GO:0006796	0.55	6.85E-04	13.17%	phosphate-containing compound metabolic process
Cluster 2	GO:0045088	1.56	7.66E-04	2.32%	regulation of innate immune response
Cluster 2	GO:0009414	0.92	3.46E-03	4.99%	response to water deprivation
Cluster 2	GO:0071446	1.89	5.05E-03	1.46%	cellular response to salicylic acid stimulus
Cluster 2	GO:0044710	0.36	5.32E-03	22.20%	single-organism metabolic process
Cluster 2	GO:0045089	1.81	5.78E-03	1.55%	positive regulation of innate immune response
Cluster 2	GO:0009863	1.92	7.37E-03	1.38%	salicylic acid mediated signaling pathway
Cluster 2	GO:0016310	0.59	9.30E-03	9.55%	phosphorylation
Cluster 2	GO:0015893	1.91	1.64E-02	1.29%	drug transport
Cluster 2	GO:0071456	2.46	1.70E-02	0.86%	cellular response to hypoxia
Cluster 2	GO:0019748	0.97	4.19E-02	3.53%	secondary metabolic process
Cluster 2	GO:0009753	1.05	4.32E-02	3.10%	response to jasmonic acid
Cluster 3	GO:0006855	2.53	2.23E-05	1.95%	drug transmembrane transport
Cluster 3	**GO:0055114**	**0.84**	**6.39E-05**	**10.96%**	**oxidation-reduction process**
Cluster 3	GO:0042744	2.34	3.81E-04	1.83%	hydrogen peroxide catabolic process
Cluster 3	GO:0019748	1.32	4.53E-04	4.51%	secondary metabolic process
Cluster 3	GO:1901136	2.29	1.49E-03	1.71%	carbohydrate derivative catabolic process
Cluster 3	**GO:0009607**	**0.72**	**6.36E-03**	**9.74%**	**response to biotic stimulus**
Cluster 3	**GO:0009625**	**2.12**	**1.36E-02**	**1.58%**	**response to insect**
Cluster 3	GO:0006026	3.07	2.37E-02	0.85%	aminoglycan catabolic process
Cluster 3	GO:0006030	3.07	2.37E-02	0.85%	chitin metabolic process
Cluster 3	GO:0006032	3.07	2.37E-02	0.85%	chitin catabolic process
Cluster 3	GO:0016998	3.07	2.37E-02	0.85%	cell wall macromolecule catabolic process
Cluster 3	GO:0046348	3.07	2.37E-02	0.85%	amino sugar catabolic process
Cluster 3	GO:1901072	3.07	2.37E-02	0.85%	glucosamine-containing compound catabolic process
Cluster 4	**GO:0055114**	**1.07**	**5.65E-04**	**12.93%**	**oxidation-reduction process**
Cluster 4	**GO:0042743**	**2.6**	**2.56E-03**	**2.90%**	**hydrogen peroxide metabolic process**
Cluster 4	GO:0042744	2.72	1.13E-02	2.37%	hydrogen peroxide catabolic process
Cluster 4	**GO:0009636**	**2.49**	**1.48E-02**	**2.64%**	**response to toxic substance**
Cluster 4	GO:0098754	2.64	1.73E-02	2.37%	detoxification
Cluster 4	GO:0071555	1.55	2.02E-02	5.28%	cell wall organization
Cluster 4	GO:0006562	5.34	4.34E-02	0.79%	proline catabolic process
Cluster 5	**GO:0006412**	**4**	**2.71E-55**	**56.73%**	**translation**
Cluster 5	**GO:0042256**	**4.76**	**8.08E-14**	**13.46%**	**mature ribosome assembly**

Table S2 continued

Cluster	GO Identifier	Log2 Fold Enrichment	P-Value	Subset Ratio	Description
Cluster 6	**GO:0006412**	**3.83**	**8.18E-72**	**50.31%**	**translation**
Cluster 6	**GO:0042254**	**3.23**	**1.27E-10**	**11.66%**	**ribosome biogenesis**
Cluster 6	**GO:0042256**	**4**	**1.15E-09**	**7.98%**	**mature ribosome assembly**
Cluster 6	GO:0006414	4.23	4.95E-04	3.68%	translational elongation
Cluster 7	**GO:0036293**	**3.63**	**1.15E-03**	**6.31%**	**response to decreased oxygen levels**
Cluster 7	**GO:0055114**	**1.62**	**1.95E-03**	**18.92%**	**oxidation-reduction process**
Cluster 7	**GO:0060774**	**7.26**	**4.17E-02**	**1.80%**	**auxin mediated signaling pathway involved in phyllotactic patterning**
Cluster 8	**GO:0006811**	**1.16**	**1.33E-04**	**7.23%**	**ion transport**
Cluster 8	**GO:0006351**	**0.64**	**1.16E-03**	**16.04%**	**transcription, DNA-templated**
Cluster 8	GO:0097659	0.64	1.16E-03	16.04%	nucleic acid-templated transcription
Cluster 8	GO:0006355	0.65	1.88E-03	15.03%	regulation of transcription, DNA-templated
Cluster 8	GO:1903506	0.65	1.88E-03	15.03%	regulation of nucleic acid-templated transcription
Cluster 8	**GO:0006468**	**0.83**	**2.39E-03**	**9.97%**	**protein phosphorylation**
Cluster 8	GO:0030001	1.44	1.89E-02	3.32%	metal ion transport
Cluster 9	GO:0016144	3.11	9.21E-07	2.20%	S-glycoside biosynthetic process
Cluster 9	GO:0019758	3.11	9.21E-07	2.20%	glycosinolate biosynthetic process
Cluster 9	**GO:0019761**	**3.11**	**9.21E-07**	**2.20%**	**glucosinolate biosynthetic process**
Cluster 9	**GO:0009408**	**1.69**	**2.45E-04**	**4.09%**	**response to heat**
Cluster 9	GO:0009644	2.25	2.13E-03	2.20%	response to high light intensity
Cluster 10	**GO:0006355**	**0.68**	**5.50E-04**	**15.32%**	**regulation of transcription, DNA-templated**
Cluster 10	GO:1903506	0.68	5.50E-04	15.32%	regulation of nucleic acid-templated transcription
Cluster 11	**GO:0009653**	**1.36**	**1.48E-02**	**14.37%**	**anatomical structure morphogenesis**
Cluster 12	**GO:0031323**	**0.9**	**8.37E-03**	**23.30%**	**regulation of cellular metabolic process**
Cluster 12	**GO:2000762**	**4.78**	**1.29E-02**	**1.94%**	**regulation of phenylpropanoid metabolic process**
Cluster 12	**GO:0080037**	**4.63**	**2.01E-02**	**1.94%**	**negative regulation of cytokinin-activated signaling pathway**
Cluster 13	**GO:0009628**	**0.95**	**1.29E-03**	**24.64%**	**response to abiotic stimulus**
Cluster 13	GO:0006355	1.05	3.42E-03	19.81%	regulation of transcription, DNA-templated
Cluster 13	GO:1903506	1.05	3.42E-03	19.81%	regulation of nucleic acid-templated transcription
Cluster 13	**GO:0007623**	**2.42**	**6.72E-03**	**5.31%**	**circadian rhythm**
Cluster 13	GO:0048511	2.42	6.72E-03	5.31%	rhythmic process
Cluster 13	GO:0009725	0.97	1.63E-02	19.32%	response to hormone
Cluster 13	**GO:0009733**	**1.69**	**1.90E-02**	**8.21%**	**response to auxin**
Cluster 13	GO:0010228	1.93	3.34E-02	6.28%	vegetative to reproductive phase transition of meristem
Cluster 14	**GO:0006355**	**1.34**	**1.02E-03**	**24.19%**	**regulation of transcription, DNA-templated**
Cluster 14	GO:1903506	1.34	1.02E-03	24.19%	regulation of nucleic acid-templated transcription
Cluster 14	**GO:0006811**	**1.8**	**3.76E-02**	**11.29%**	**ion transport**
Cluster 15	**GO:0010345**	**5.56**	**2.25E-13**	**6.08%**	**suberin biosynthetic process**
Cluster 15	**GO:0010143**	**5.61**	**8.32E-11**	**4.97%**	**cutin biosynthetic process**
Cluster 15	**GO:0006631**	**2.52**	**2.78E-03**	**6.08%**	**fatty acid metabolic process**
Cluster 15	GO:0055114	1.28	9.01E-03	14.92%	oxidation-reduction process
Cluster 15	GO:1901957	5.73	1.53E-02	1.66%	regulation of cutin biosynthetic process
Cluster 15	GO:1901959	5.73	1.53E-02	1.66%	positive regulation of cutin biosynthetic process

Table S3. Statistical information regarding phytohormone analyses (Fig. **3b**) of *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*). Herbivores feeding AG were introduced two days prior to infestation by *D. radicum* (Dr). Posthoc analyses were performed within each timepoint using Tukey's HSD corrections. GLM: Generalized linear model. Gamma distribution with log link used unless otherwise specified.

Main effects

Variable	Model type	Model	Factor	Chisq	*P*-value
JA	GLM	~Treatment+Time	Treatment	79.939	**<0.0001**
	Gamma (inverse link)		Time	12.75	**0.013**
JA-Ile	GLM	~Treatment+Time	Treatment	87.772	**<0.0001**
	Gamma (inverse link)		Time	17.751	**0.0014**
Sum-JA	GLM	~Treatment+Time	Treatment	140.77	**<0.0001**
	Gamma (inverse link)		Time	19.996	**<0.001**
SA	GLM	~Treatment+Time	Treatment	8.1889	0.1461
	Gamma		Time	4.1364	0.3879
ABA	GLM	~Treatment+Time	Treatment	6.4549	0.2644
	Gamma		Time	22.402	**<0.001**

Pairwise comparisons, values show *P*-values of Tukey's HSD between treatments within each timepoint.

		3h					6h					9h					24h					48h				
		Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr
JA	C	0.99	1.00	**<.001**	**<.001**	**<.001**	0.99	1.00	**<.001**	**<.001**	**<.001**	0.99	1.00	**<.001**	**<.001**	**<.001**	0.99	1.00	**<.001**	**<.001**	**<.001**	0.99	1.00	**<.001**	**<.001**	**<.001**
	Bb		1.00	**<.001**	**<.001**	**<.001**		1.00	**<.001**	**<.001**	**<.001**		1.00	**<.001**	**<.001**	**<.001**		1.00	**<.001**	**<.001**	**<.001**		1.00	**<.001**	**<.001**	**<.001**
	Px			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**
	Dr				0.58	1.00				0.58	1.00				0.58	1.00				0.58	1.00				0.58	1.00
	BbDr					0.38					0.38					0.38					0.38					0.38
JA-Ile	C	1.00	0.89	**<.001**	**<.001**	**<.001**	1.00	0.89	**<.001**	**<.001**	**<.001**	1.00	0.89	**<.001**	**<.001**	**<.001**	1.00	0.89	**<.001**	**<.001**	**<.001**	1.00	0.89	**<.001**	**<.001**	**<.001**
	Bb		0.87	**<.001**	**<.001**	**<.001**		0.87	**<.001**	**<.001**	**<.001**		0.87	**<.001**	**<.001**	**<.001**		0.87	**<.001**	**<.001**	**<.001**		0.87	**<.001**	**<.001**	**<.001**
	Px			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**
	Dr				0.74	1.00				0.74	1.00				0.74	1.00				0.74	1.00				0.74	1.00
	BbDr					0.68					0.68					0.68					0.68					0.68
Sum of jasmonates	C	1.00	**<.001**	**<.001**	**<.001**	**<.001**	1.00	**<.001**	**<.001**	**<.001**	**<.001**	1.00	**<.001**	**<.001**	**<.001**	**<.001**	1.00	**<.001**	**<.001**	**<.001**	**<.001**	1.00	**<.001**	**<.001**	**<.001**	**<.001**
	Bb		**<.001**	**<.001**	**<.001**	**<.001**		**<.001**	**<.001**	**<.001**	**<.001**		**<.001**	**<.001**	**<.001**	**<.001**		**<.001**	**<.001**	**<.001**	**<.001**		**<.001**	**<.001**	**<.001**	**<.001**
	Px			**<0.05**	**0.03**	**<.001**			**<0.05**	**0.03**	**<.001**			**<0.05**	**0.03**	**<.001**			**<0.05**	**0.03**	**<.001**			**<0.05**	**0.03**	**<.001**
	Dr				0.78	0.97				0.78	0.97				0.78	0.97				0.78	0.97				0.78	0.97
	BbDr					0.37					0.37					0.37					0.37					0.37

Table S4. Statistical information regarding glucosinolate (GSL) analyses (Fig. **4d,e**) of *Brassica oleracea* primary roots in response to aboveground (AG) herbivory by *Brevicoryne brassicae* (*Bb*) or *Plutella xylostella* (*Px*). Herbivores feeding AG were introduced two days prior to infestation by *D. radicum* (Dr). Posthoc analyses were performed within each timepoint using Tukey's HSD corrections. (G)LM: (Generalized) linear model. Gamma distribution with log link used unless otherwise specified.

Main effects

Variable	Model type	Model	Factor	Chisq	*P*-value
Total GSL	LM	~Treatment+Time	Treatment	6.058	0.30
	Gaussian		Time	44.80	**<0.0001**
Total Indole	GLM	~Treatment*Time	Treatment	58.96	**<0.0001**
	Gamma		Time	65.26	**<0.0001**
			Interaction	117.79	**<0.0001**
Total Aliphatic	LM	~Treatment*Time	Treatment	13.25	**0.021**
	Gaussian		Time	22.097	**0.00019**
			Interaction	50.22	**0.00021**
Glucobrassicin	GLM	~Treatment*Time	Treatment	43.33	**<0.0001**
	Gamma		Time	23.97	**<0.0001**
			Interaction	67.68	**<0.0001**
Hydroxyglucobrassicin	GLM	~Treatment+Time	Treatment	5.26	0.39
	Gamma		Time	55.18	**<0.0001**
Methoxyglucobrasicin	GLM	~Treatment*Time	Treatment	13.23	**0.021**
	Gamma		Time	2.048	0.73
			Interaction	36.89	**0.012**
Neoglucobrassicin	GLM	~Treatment*Time	Treatment	98.59	**<0.0001**
	Gamma		Time	85.63	**<0.0001**
			Interaction	152.3	**<0.0001**
Glucoiberverin	GLM	~Treatment*Time	Treatment	9.99	0.075
	Gamma		Time	18.88	**0.00083**
			Interaction	67.73	**<0.0001**
Sinigrin	GLM	~Treatment+Time	Treatment	13.40	**0.020**
	Gamma		Time	8.63	**0.071**
Glucoerucin	GLM	~Treatment*Time	Treatment	2.20	0.82
	Gamma		Time	23.58	**<0.0001**
			Interaction	35.95	**0.016**
Gluconapin	GLM	~Treatment*Time	Treatment	18.50	**0.0024**
	Gamma		Time	23.77	**<0.0001**
			Interaction	88.53	**<0.0001**

Table S4 continued: Pairwise comparisons, values show *P*-values of Tukey's HSD between treatments within each timepoint.

		3h					6h					9h					24h					48h				
		Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr
Total Aliphatic	C	0.26	0.97	0.57	1.00	0.97	0.93	1.00	0.87	0.90	0.92	1.00	1.00	1.00	1.00	0.99	1.00	0.09	0.40	0.64	0.19	0.98	0.54	**<.005**	**<.005**	**<.005**
	Bb		0.76	1.00	0.39	0.76		0.95	1.00	1.00	1.00		1.00	1.00	0.99	0.97		0.10	0.41	0.65	0.20		0.94	**0.01**	**<.005**	**0.01**
	Px			0.96	0.99	1.00			0.92	0.93	0.95			1.00	1.00	1.00			0.98	0.89	1.00			0.15	**0.01**	0.14
	Dr				0.72	0.96				1.00	1.00				1.00	1.00				1.00	1.00				0.94	1.00
	BbDr					0.99					1.00					1.00					0.97					0.95
Total Indole	C	1.00	0.98	1.00	0.95	0.27	1.00	0.16	0.96	1.00	0.09	1.00	0.71	0.79	0.99	0.33	0.97	1.00	**<.001**	**<.001**	**<.001**	1.00	1.00	**<.001**	**<.001**	**<.001**
	Bb		1.00	1.00	0.82	0.47		0.11	0.84	0.96	0.06		0.38	0.47	0.86	0.11		0.79	**<.001**	**<.001**	**<.001**		1.00	**<.001**	**<.001**	**<.001**
	Px			0.94	0.58	0.72			0.63	0.39	1.00			1.00	0.97	0.99			**<.001**	**<.001**	**<.001**			**<.001**	**<.001**	**<.001**
	Dr				0.98	0.19				1.00	0.48				0.99	0.98				0.98	1.00				0.73	1.00
	BbDr					0.03					0.26					0.74					1.00					0.54
Glucobrassicin	C	0.99	0.34	1.00	0.98	**0.04**	0.98	**0.01**	1.00	0.97	0.13	0.94	0.27	0.93	0.96	1.00	0.89	0.35	0.58	0.16	**0.01**	1.00	0.70	**<.005**	**<.005**	**<.005**
	Bb		0.73	0.94	0.77	0.18		**<.005**	0.93	1.00	**0.04**		**0.03**	1.00	1.00	0.80		**0.03**	0.07	**0.01**	**<.005**		0.57	**<.005**	**<.005**	**<.005**
	Px			0.19	0.07	0.93			**0.02**	**<.005**	0.94			**0.02**	**0.04**	0.48			1.00	1.00	0.78			**0.01**	0.22	**0.02**
	Dr				1.00	**0.01**				0.90	0.24				1.00	0.78				0.98	0.56				0.78	1.00
	BbDr					**<.005**					**0.01**					0.84					0.94					0.92
Methoxy-glucobrassicin	C	1.00	1.00	1.00	0.99	0.90	1.00	0.63	1.00	1.00	0.91	0.99	1.00	0.94	1.00	1.00	0.99	0.83	0.25	0.04	0.42	0.99	1.00	0.05	**0.02**	**0.03**
	Bb		1.00	0.99	0.90	0.99		0.84	1.00	1.00	0.98		1.00	1.00	0.95	1.00		0.46	0.06	0.01	0.13		1.00	0.26	0.12	0.16
	Px			0.98	0.89	0.99			0.81	0.90	0.99			0.98	1.00	1.00			0.93	0.54	0.99			0.14	0.06	0.08
	Dr				1.00	0.82				1.00	0.98				0.84	0.99				0.98	1.00				1.00	1.00
	BbDr					0.58					1.00					0.99					0.91					1.00
Neoglucobrassicin	C	1.00	0.96	1.00	0.89	0.81	1.00	0.66	0.70	0.58	**0.05**	0.99	0.97	**0.04**	0.25	**<.005**	0.95	0.87	<.001	<.001	<.001	1.00	1.00	**<.005**	**<.005**	**<.005**
	Bb		0.99	1.00	0.96	0.68		0.60	0.64	0.54	0.06		0.73	**<.005**	0.06	**<.005**		1.00	<.001	<.001	<.001		1.00	**<.005**	**<.005**	**<.005**
	Px			1.00	1.00	0.31			1.00	1.00	0.75			0.24	0.73	**0.04**			<.001	<.001	<.001			**<.005**	**<.005**	**<.005**
	Dr				0.98	0.58				1.00	0.71				0.97	0.98				0.99	1.00				0.89	1.00
	BbDr					0.18					0.81					0.64					0.99					0.69

Table S4 continued: Pairwise comparisons, values show *P*-values of Tukey's HSD between treatments within each timepoint.

		3h					6h					9h					24h					48h				
		Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr	Bb	Px	Dr	BbDr	PxDr
Glucoiberverin	C	**0.04**	0.96	0.18	0.99	0.96	0.78	0.97	0.34	0.43	0.37	1.00	1.00	0.96	1.00	0.98	1.00	0.13	0.69	0.86	0.26	0.99	0.67	**<.005**	**<.005**	**<.005**
	Bb		0.31	0.99	0.21	0.28		0.99	1.00	1.00	1.00		1.00	0.99	1.00	1.00		0.05	0.46	0.67	0.13		0.94	**<.005**	**<.005**	**0.01**
	Px			0.67	1.00	1.00			0.82	0.89	0.84			0.87	1.00	0.91			0.91	0.77	1.00			**0.05**	**<.005**	0.18
	Dr				0.53	0.64				1.00	1.00				0.98	1.00				1.00	0.98				0.62	0.99
	BbDr					1.00					1.00					0.99					0.92					0.28
Sinigrin	C	0.58	0.34	1.00	0.93	0.97	0.58	0.34	1.00	0.93	0.97	0.58	0.34	1.00	0.93	0.97	0.58	0.34	1.00	0.93	0.97	0.58	0.34	1.00	0.93	0.97
	Bb		**<.005**	0.55	0.11	0.16		**<.005**	0.55	0.11	0.16		**<.005**	0.55	0.11	0.16		**<.005**	0.55	0.11	0.16		**<.005**	0.55	0.11	0.16
	Px			0.38	0.90	0.84			0.38	0.90	0.84			0.38	0.90	0.84			0.38	0.90	0.84			0.38	0.90	0.84
	Dr				0.95	0.98				0.95	0.98				0.95	0.98				0.95	0.98				0.95	0.98
	BbDr					1.00					1.00					1.00					1.00					1.00
Glucoerucin	C	0.49	1.00	0.20	0.72	0.81	1.00	1.00	0.99	1.00	0.71	1.00	0.97	1.00	1.00	1.00	1.00	0.96	0.99	1.00	0.99	1.00	1.00	0.06	**0.01**	0.18
	Bb		0.82	1.00	1.00	1.00		1.00	0.99	1.00	0.81		0.94	1.00	1.00	1.00		0.95	0.99	0.99	0.99		1.00	0.16	**0.02**	0.36
	Px			0.49	0.95	0.98			1.00	1.00	0.93			0.98	1.00	0.99			1.00	1.00	1.00			0.13	**0.02**	0.32
	Dr				0.96	0.91				1.00	0.97				1.00	1.00				1.00	1.00				0.98	1.00
	BbDr					1.00					0.87					1.00					1.00					0.86
Gluconapin	C	1.00	1.00	1.00	0.61	1.00	0.96	0.98	1.00	1.00	0.83	1.00	0.52	1.00	0.91	0.84	1.00	0.73	**0.01**	**0.01**	**<.005**	1.00	0.70	**<.005**	**<.005**	**<.005**
	Bb		1.00	1.00	0.44	1.00		0.71	0.99	1.00	0.40		0.28	1.00	0.71	0.61		0.95	0.05	**0.05**	**<.005**		0.86	**<.005**	**<.005**	**<.005**
	Px			1.00	0.38	0.99			0.94	0.87	0.99			0.24	0.98	0.99			0.37	0.36	**0.01**			**<.005**	**<.005**	**<.005**
	Dr				0.69	1.00				1.00	0.70				0.67	0.56				1.00	0.63				1.00	1.00
	BbDr					0.74					0.55					1.00					0.64					1.00

Table S5. Statistical information regarding the *Brassica oleracea myb28* mutant experiment, including *Delia radicum* performance measures and primary root glucosinolate (GSL) analyses at 5 and 18 days post infestation (dpi; Fig. **5**). GL(M)M: Generalized linear (mixed) model. Gamma distribution with log link used unless otherwise specified.

***Delia radicum* performance measurements**

Variable	Model type	Model	Factor	Chisq	*P*-value
Emergence	GLMM	~ Genotype + Sex + (1|Plant)	Genotype	3.0076	**0.083**
	binomial		Sex	252.76	**<0.0001**
Weight	GLMM	~ Genotype + Sex + (1|Plant)	Genotype	3.0076	**0.083**
	Gamma		Sex	252.76	**<0.0001**
Development time	GLMM	~ Genotype + Sex + (1|Plant)	Genotype	0.03	0.86
(days post infestation)	Poisson		Sex	4.24	**0.040**

GSL measurements 5dpi

Variable	Model type	Model	Factor	Chisq	*P*-value
Total GSL	GLM	~ Genotype * Treatment	Genotype	24.39	**<0.0001**
	Gamma		Treatment	5.015	**0.025**
			Interaction	6.87	**0.0087**
Total Aliphatic GSL	GLM	~ Genotype * Treatment	Genotype	83.99	**<0.0001**
	Gamma		Treatment	1.58	0.21
			Interaction	1.41	0.23
Total Indole GSL	GLM	~ Genotype + Treatment	Genotype	2.23	0.13
	Gamma		Treatment	51.60	**<0.0001**
Glucoiberverin	GLM	~ Genotype * Treatment	Genotype	22.43	**<0.0001**
	Gamma (inverse link)		Treatment	2.97	0.085
			Interaction	2.017	0.16
Glucoiberin	GLM	~ Genotype + Treatment	Genotype	247.34	**<0.0001**
	Gamma (inverse link)		Treatment	0.016	0.90
Glucoerucin	GLM	~ Genotype + Treatment	Genotype	66.67	**<0.0001**
	Gamma		Treatment	2.72	0.099
Glucoraphanin	GLM	~ Genotype * Treatment	Genotype	91.82	**<0.0001**
	Gamma		Treatment	0.057	0.81
			Interaction	7.65	**0.0057**
Glucobrassicin	GLM	~ Genotype + Treatment	Genotype	0.80	0.37
	Gamma		Treatment	15.40	**<0.0001**
Hydroxyglucobrassicin	GLM	~ Genotype + Treatment	Genotype	2.73	0.098
	Gamma		Treatment	54.50	**<0.0001**
Methoxyglucobrassicin	GLM	~ Genotype + Treatment	Genotype	1.25	0.26
	Gamma		Treatment	62.50	**<0.0001**
Neoglucobrassicin	GLM	~ Genotype + Treatment	Genotype	1.69	0.19
	Gamma		Treatment	61.67	**<0.0001**
Gluconasturtiin	GLM	~ Genotype * Treatment	Genotype	18.57	**<0.0001**
	Gamma		Treatment	5.066	**0.024**
			Interaction	2.48	0.12

Table S5 continued

GSL measurements 18dpi

Variable	Model type	Model	Factor	Chisq	*P*-value
Total GSL	GLM	~ Genotype + Treatment	Genotype	2.16	0.14
	Gamma		Treatment	23.27	**<0.0001**
Total Aliphatic GSL	GLM	~ Genotype + Treatment	Genotype	74.37	**<0.0001**
	Gamma		Treatment	0.27	0.60
Total Indole GSL	GLM	~ Genotype * Treatment	Genotype	2.46	0.12
	Gamma		Treatment	54.19	**<0.0001**
			Interaction	2.48	0.12
Glucoiberverin	GLM	~ Genotype * Treatment	Genotype	14.23	**0.00016**
	Gamma (inverse link)		Treatment	0.65	0.42
			Interaction	8.045	**0.0046**
Glucoiberin	GLM	~ Genotype + Treatment	Genotype	545.27	**<0.0001**
	Gamma (inverse link)		Treatment	0.11	0.74
Glucoerucin	GLM	~ Genotype + Treatment	Genotype	39.26	**<0.0001**
	Gamma		Treatment	0	1.00
Glucoraphanin	GLM	~ Genotype + Treatment	Genotype	53.19	**<0.0001**
	Gamma		Treatment	0.56	0.46
Glucobrassicin	GLM	~ Genotype + Treatment	Genotype	14.0	**<0.0001**
	Gamma		Treatment	30.13	**<0.0001**
Hydroxyglucobrassicin	GLM	~ Genotype + Treatment	Genotype	0.20	0.66
	Gamma (inverse link)		Treatment	19.062	**<0.0001**
Methoxyglucobrassicin	GLM	~ Genotype + Treatment	Genotype	7.84	**0.0051**
	Gamma		Treatment	83.98	**<0.0001**
Neoglucobrassicin	GLM	~ Genotype * Treatment	Genotype	0.48	0.49
	Gamma		Treatment	54.07	**<0.0001**
			Interaction	3.55	**0.060**
Gluconasturtiin	GLM	~ Genotype * Treatment	Genotype	8.26	**0.0041**
	Gamma		Treatment	0.25	0.61

Table S6. Statistical information regarding gene expression analyses of *AOS*, *MYC2*, *CYP81F4*, and *MYB28* in primary roots of *Brassica oleracea* following root herbivory by *Delia radicum* with or without preinfestation by *Plutella xylostella* (Fig. **6**). Between infestation by *P. xylostella* and *D. radicum*, there was either no period without infestation, a 24 hour period without infestation, or a 7 day period without infestation. GLM: Generalized linear model. Gamma distribution with log link used unless otherwise specified.

No period without infestation

Variable	Period without infestation	Model type	Model	Factor	Chisq	*P*-value
AOS	0h	GLM	~ Treatment * Time	Treatment	74.56	**<0.0001**
		Gamma		Time	18.68	**0.00032**
				Interaction	61.23	**<0.0001**

Table S6 continued

No period without infestation

Variable	Period without infestation	Model type	Model	Factor	Chisq	*P*-value
MYC2	0h	GLM	~ Treatment * Time	Treatment	83.59	**<0.0001**
		Gamma		Time	6.50	0.090
				Interaction	28.005	**0.00095**
CYP81F4	0h	GLM	~ Treatment * Time	Treatment	69.90	**<0.0001**
		Gamma		Time	49.67	**<0.0001**
				Interaction	102.33	**<0.0001**
MYB28	0h	GLM	~ Treatment * Time	Treatment	20.014	**0.00017**
		Gamma		Time	10.88	**0.012**
				Interaction	86.97	**<0.0001**

24 hours period without infestation

Variable	Period without infestation	Model type	Model	Factor	Chisq	*P*-value
AOS	24h	GLM	~ Treatment + SamplingTime	Treatment	18.054	**<0.0001**
		Gamma		SamplingTime	9.23	**0.0024**
MYC2	24h	GLM	~ Treatment * SamplingTime	Treatment	6.87	**0.032**
		Gamma		SamplingTime	6.47	**0.011**
				Interaction	5.41	0.067
CYP81F4	24h	GLM	~ Treatment + SamplingTime	Treatment	18.77	**<0.0001**
		Gamma		SamplingTime	13.42	**0.00025**
MYB28	24h	GLM	~ Treatment * SamplingTime	Treatment	2.61	0.27
		Gamma		SamplingTime	0.080	0.78
				Interaction	5.49	0.064

7 days period without infestation

Variable	Period without infestation	Model type	Model	Factor	Chisq	*P*-value
AOS	7d	GLM	~ Treatment * SamplingTime	Treatment	6.70	**0.035**
		Gamma		SamplingTime	4.030	**0.045**
				Interaction	8.64	**0.013**
MYC2	7d	GLM	~ Treatment + SamplingTime	Treatment	5.30	0.071
		Gamma		SamplingTime	0.42	0.51
CYP81F4	7d	GLM	~ Treatment + SamplingTime	Treatment	11.74	**0.0028**
		Gamma		SamplingTime	7.065	**0.0079**
MYB28	7d	GLM	~ Treatment + SamplingTime	Treatment	15.47	**0.00044**
		Gamma		SamplingTime	2.43	0.12

Table S7. Primers used for qPCR of *Brassica oleracea* primary root samples.

Gene acronym	Gene amplified	Sequence Fw	Sequence Rv
GADPH	Bo5g017500	GCTACGCAGAAGACAGTTGATGG	TGGGCACACGGAAGGACATAC
Act-2	Bo5g117040	ACATTGTGCTCAGTGGTGGA	TCTGCTGGAATGTGCTGAGG
Btub	Bo2g124350, Bo7g067360, Bo9g059850	GTCAAGTCCAGCGTCTGTGA	TCACACGCCTGAACATCTCC
EF1α	Bo9g142520	GGTACCTCCCAGGCTGATTG	TCAGGTAKGAAGACACCTCCTTG
PER4	Bo7g095750	TATCCTCTGCAGCCTCCTCA	ACACACAGACTGAAGCGTCC
SAR1α	Bo3g052780	ATCTCTAGCCACCGTTCCCT	TTCCTGACGATGCTGCACAT
MYC2	Bo5g086990	GGCTGGACCTACGCTATATTCTGG	AGAAAAACCACTCCGTATCCGT
AOS	Bo2g116210	ACCGCTTGCGACTAGGGATC	CAAAGTCCTTACCGGCGCAC
CYP81F4	Bo1g004730	TGTGTCAGAAACGTTCAGGCT	ATGGCACGTCGTATCCTCCG
MYB28	Bo2g161590	CGGGAGAGATGAGCACAATACG	CAGCCCTCGAAGTTTCCTATCA

Table S8. Details of the analysis of phytohormones by LC-MS/MS using an Agilent HPLC 1260/QTRAP6500 instrument in negative ionisation mode. Abbreviations are: Q1, selected m/z of the first quadrupole; Q3, selected m/z of the third quadrupole; RT, retention time; DP, declustering potential (V); and CE, collision energy (V).

Compound	Internal standard	Q1	Q3	RT (min)	DP	CE
SA	D4-SA	136.93	93	3.3	-20	-24
JA	D6-JA	209.07	59	3.6	-20	-24
ABA	D6-ABA	263	153.2	3.4	-20	-22
JA-Ile	D6-JA-Ile	322.19	130.1	3.9	-20	-30
OPDA	D6-JA	290.9	165.1	4.6	-20	-24
OH-JA	D6-JA	225.1	59	2.6	-20	-24
OH-JA-Ile	D6-JA-Ile	338.1	130.1	3.0	-20	-30
COOH-JA-Ile	D6-JA-Ile	352.1	130.1	3.0	-20	-30
D4-SA		140.93	97	3.3	-20	-24
D6-JA		215	59	3.6	-20	-24
D5-JA		214	59	3.6	-20	-24
D6-ABA		269	159.2	3.4	-20	-22
D6-JA-Ile		328.19	130.1	3.9	-20	-30
D5-JA-Ile		327.19	130.1	3.9	-20	-30

Table S9. RNA-seq sample overview and processing. Numbers of reads shown in millions.

ID	Technology	Cycles	Run Type	Sample	Treatment	Time	Reads untrimmed	Reads trimmed	GC content	Alignment Percentage
LIY3053A1	ILLUMINA	150	Paired-End	C01	Control	0h	42.1	35.7	46%	92.13%
LIY3053A2	ILLUMINA	150	Paired-End	C02	Control	0h	44	39.3	46%	93.56%
LIY3053A3	ILLUMINA	150	Paired-End	C03	Control	0h	30.6	27.5	46%	93.72%
LIY3053A4	ILLUMINA	150	Paired-End	C04	Control	0h	41.4	37.4	46%	94.39%
LIY3053A5	ILLUMINA	150	Paired-End	B01	B_brassicae	0h	48.9	44.7	46%	93.99%
LIY3053A6	ILLUMINA	150	Paired-End	B02	B_brassicae	0h	37.9	33.7	46%	94.11%

Table S9 continued

ID	Technology	Cycles	Run Type	Sample	Treatment	Time	Reads untrimmed	Reads trimmed	GC content	Alignment Percentage
LIY3053A7	ILLUMINA	150	Paired-End	B03	B_brassicae	0h	51	45.8	46%	94.08%
LIY3053A8	ILLUMINA	150	Paired-End	B04	B_brassicae	0h	39.6	35.2	47%	94.09%
LIY3053A9	ILLUMINA	150	Paired-End	P01	P_xylostella	0h	37.1	31	46%	93.63%
LIY3053A10	ILLUMINA	150	Paired-End	P02	P_xylostella	0h	34	30.3	46%	94.12%
LIY3053A11	ILLUMINA	150	Paired-End	P03	P_xylostella	0h	27.4	24.2	46%	93.31%
LIY3053A12	ILLUMINA	150	Paired-End	P04	P_xylostella	0h	38	34.6	46%	93.36%
LIY3053A13	ILLUMINA	150	Paired-End	C07	Control	3h	40.9	37.7	47%	94.29%
LIY3053A14	ILLUMINA	150	Paired-End	C08	Control	3h	34.3	30.9	46%	94%
LIY3053A15	ILLUMINA	150	Paired-End	C09	Control	3h	49.4	44.2	46%	94.07%
LIY3053A16	ILLUMINA	150	Paired-End	C10	Control	3h	39	33.4	46%	93.77%
LIY3053A17	ILLUMINA	150	Paired-End	B07	B_brassicae	3h	37.9	32.1	46%	93.8%
LIY3053A18	ILLUMINA	150	Paired-End	B08	B_brassicae	3h	32.6	29.4	46%	93.8%
LIY3053A19	ILLUMINA	150	Paired-End	B09	B_brassicae	3h	36.3	32.8	46%	94.06%
LIY3053A20	ILLUMINA	150	Paired-End	B10	B_brassicae	3h	38.5	35.3	46%	93.89%
LIY3053A21	ILLUMINA	150	Paired-End	P07	P_xylostella	3h	42.8	39.5	46%	94.06%
LIY3053A22	ILLUMINA	150	Paired-End	P08	P_xylostella	3h	37.6	33.9	46%	93.34%
LIY3053A23	ILLUMINA	150	Paired-End	P09	P_xylostella	3h	45.6	41.4	46%	93.9%
LIY3053A24	ILLUMINA	150	Paired-End	P10	P_xylostella	3h	39.6	35.1	46%	93.84%
LIY3053A25	ILLUMINA	150	Paired-End	D07	D_radicum	3h	41.6	35.4	46%	91.77%
LIY3053A26	ILLUMINA	150	Paired-End	D08	D_radicum	3h	31.6	28.6	46%	93.38%
LIY3053A27	ILLUMINA	150	Paired-End	D09	D_radicum	3h	36.6	32.8	46%	93.12%
LIY3053A28	ILLUMINA	150	Paired-End	D10	D_radicum	3h	30.9	28.3	46%	92.76%
LIY3053A29	ILLUMINA	150	Paired-End	BD07	Brevi_Delia	3h	39.7	36.5	46%	93.62%
LIY3053A30	ILLUMINA	150	Paired-End	BD08	Brevi_Delia	3h	36.2	32.5	46%	93.67%
LIY3053A31	ILLUMINA	150	Paired-End	BD09	Brevi_Delia	3h	47.5	43	46%	90.23%
LIY3053A32	ILLUMINA	150	Paired-End	BD10	Brevi_Delia	3h	31.2	28	47%	93.15%
LIY3053A33	ILLUMINA	150	Paired-End	PD07	Plute_Delia	3h	42.5	37.8	46%	94.18%
LIY3053A34	ILLUMINA	150	Paired-End	PD08	Plute_Delia	3h	30.8	27.8	46%	89.95%
LIY3053A35	ILLUMINA	150	Paired-End	PD09	Plute_Delia	3h	35.1	31.5	46%	92.18%
LIY3053A36	ILLUMINA	150	Paired-End	PD10	Plute_Delia	3h	39.4	36.2	46%	93.4%
LIY3053A37	ILLUMINA	150	Paired-End	C13	Control	6h	44.7	41.1	46%	94.06%
LIY3053A38	ILLUMINA	150	Paired-End	C14	Control	6h	37.1	33.5	46%	93.81%
LIY3053A39	ILLUMINA	150	Paired-End	C15	Control	6h	45.9	40.3	46%	93.98%
LIY3053A40	ILLUMINA	150	Paired-End	C16	Control	6h	36.8	33.3	47%	93.97%
LIY3053A41	ILLUMINA	150	Paired-End	B13	B_brassicae	6h	41.9	37.9	46%	93.69%
LIY3053A42	ILLUMINA	150	Paired-End	B14	B_brassicae	6h	46	41.5	46%	94.01%
LIY3053A43	ILLUMINA	150	Paired-End	B15	B_brassicae	6h	35.8	32.3	46%	93.98%
LIY3053A44	ILLUMINA	150	Paired-End	B16	B_brassicae	6h	44	40.4	46%	94.08%
LIY3053A45	ILLUMINA	150	Paired-End	P13	P_xylostella	6h	41.9	38.2	46%	93.78%
LIY3053A46	ILLUMINA	150	Paired-End	P14	P_xylostella	6h	38.9	35.1	46%	94.09%
LIY3053A47	ILLUMINA	150	Paired-End	P15	P_xylostella	6h	38.2	33.8	46%	93.73%
LIY3053A48	ILLUMINA	150	Paired-End	P16	P_xylostella	6h	39	35.4	47%	93.69%
LIY3053A49	ILLUMINA	150	Paired-End	D13	D_radicum	6h	42.6	38.3	47%	92.12%

Table S9 continued

ID	Technology	Cycles	Run Type	Sample	Treatment	Time	Reads untrimmed	Reads trimmed	GC content	Alignment Percentage
LIY3053A50	ILLUMINA	150	Paired-End	D14	D_radicum	6h	21.5	19.8	46%	90.66%
LIY3053A51	ILLUMINA	150	Paired-End	D15	D_radicum	6h	40	36.1	46%	94%
LIY3053A52	ILLUMINA	150	Paired-End	D16	D_radicum	6h	37.8	34.7	46%	93.59%
LIY3053A53	ILLUMINA	150	Paired-End	BD13	Brevi_Delia	6h	17.9	16.6	46%	91.89%
LIY3053A54	ILLUMINA	150	Paired-End	BD14	Brevi_Delia	6h	34.6	31.4	46%	90.52%
LIY3053A55	ILLUMINA	150	Paired-End	BD15	Brevi_Delia	6h	39.5	35.4	46%	91.71%
LIY3053A56	ILLUMINA	150	Paired-End	BD16	Brevi_Delia	6h	38.7	35.4	46%	93.59%
LIY3053A57	ILLUMINA	150	Paired-End	PD13	Plute_Delia	6h	37.7	33.8	46%	93.13%
LIY3053A58	ILLUMINA	150	Paired-End	PD14	Plute_Delia	6h	51.1	45.8	46%	93.15%
LIY3053A59	ILLUMINA	150	Paired-End	PD15	Plute_Delia	6h	40.1	35.3	47%	93.6%
LIY3053A60	ILLUMINA	150	Paired-End	PD16	Plute_Delia	6h	47.2	43.1	46%	92.06%
LIY3053A61	ILLUMINA	150	Paired-End	C19	Control	9h	57	52.5	47%	94.04%
LIY3053A62	ILLUMINA	150	Paired-End	C20	Control	9h	39.7	36	46%	94.23%
LIY3053A63	ILLUMINA	150	Paired-End	C21	Control	9h	58.5	52.2	46%	94.36%
LIY3053A64	ILLUMINA	150	Paired-End	C22	Control	9h	55.4	50.7	47%	93.8%
LIY3053A65	ILLUMINA	150	Paired-End	B19	B_brassicae	9h	43.2	40	47%	93.56%
LIY3053A66	ILLUMINA	150	Paired-End	B20	B_brassicae	9h	38.3	34.5	46%	93.94%
LIY3053A67	ILLUMINA	150	Paired-End	B22	B_brassicae	9h	32.4	28.2	46%	93.95%
LIY3053A68	ILLUMINA	150	Paired-End	B23	B_brassicae	9h	43.7	40.1	46%	93.88%
LIY3053A69	ILLUMINA	150	Paired-End	P19	P_xylostella	9h	35.7	31.8	47%	88.62%
LIY3053A70	ILLUMINA	150	Paired-End	P20	P_xylostella	9h	28.4	24.4	46%	93.02%
LIY3053A71	ILLUMINA	150	Paired-End	P21	P_xylostella	9h	30.3	26.9	46%	89.98%
LIY3053A72	ILLUMINA	150	Paired-End	P22	P_xylostella	9h	41.4	38.2	46%	94.16%
LIY3053A73	ILLUMINA	150	Paired-End	D20	D_radicum	9h	46	42.5	46%	92.34%
LIY3053A74	ILLUMINA	150	Paired-End	D21	D_radicum	9h	41.8	37.5	46%	93.01%
LIY3053A75	ILLUMINA	150	Paired-End	D23	D_radicum	9h	31.4	27.4	46%	92.87%
LIY3053A76	ILLUMINA	150	Paired-End	D24	D_radicum	9h	45.4	41.7	46%	94.26%
LIY3053A77	ILLUMINA	150	Paired-End	BD19	Brevi_Delia	9h	30.3	26.7	46%	93.77%
LIY3053A78	ILLUMINA	150	Paired-End	BD20	Brevi_Delia	9h	29.7	25.7	46%	91.77%
LIY3053A79	ILLUMINA	150	Paired-End	BD21	Brevi_Delia	9h	38.8	34.3	46%	92.61%
LIY3053A80	ILLUMINA	150	Paired-End	BD22	Brevi_Delia	9h	20.2	17.9	47%	91.36%
LIY3053A81	ILLUMINA	150	Paired-End	PD19	Plute_Delia	9h	44.9	40.3	46%	89.01%
LIY3053A82	ILLUMINA	150	Paired-End	PD20	Plute_Delia	9h	33	29.3	46%	88.82%
LIY3053A83	ILLUMINA	150	Paired-End	PD21	Plute_Delia	9h	31.1	27.3	46%	90.75%
LIY3053A84	ILLUMINA	150	Paired-End	PD22	Plute_Delia	9h	46.5	42.4	46%	93.9%
LIY3053A85	ILLUMINA	150	Paired-End	C25	Control	24h	31.2	27.5	46%	94.33%
LIY3053A86	ILLUMINA	150	Paired-End	C27	Control	24h	27.6	24	46%	94.27%
LIY3053A87	ILLUMINA	150	Paired-End	C28	Control	24h	41.1	36.5	46%	94.24%
LIY3053A88	ILLUMINA	150	Paired-End	C29	Control	24h	23	20	46%	93.96%
LIY3053A89	ILLUMINA	150	Paired-End	B26	B_brassicae	24h	57.8	50.3	46%	91.6%
LIY3053A90	ILLUMINA	150	Paired-End	B27	B_brassicae	24h	42.1	36.7	46%	93.72%
LIY3053A91	ILLUMINA	150	Paired-End	B28	B_brassicae	24h	37.2	32	46%	93.78%
LIY3053A92	ILLUMINA	150	Paired-End	B29	B_brassicae	24h	49.8	45.7	46%	93.64%

Table S9 continued

ID	Technology	Cycles	Run Type	Sample	Treatment	Time	Reads untrimmed	Reads trimmed	GC content	Alignment Percentage
LIY3053A93	ILLUMINA	150	Paired-End	P25	P_xylostella	24h	31.8	28	46%	94.17%
LIY3053A94	ILLUMINA	150	Paired-End	P26	P_xylostella	24h	55	47.1	46%	93.78%
LIY3053A95	ILLUMINA	150	Paired-End	P28	P_xylostella	24h	40.2	35	46%	94.32%
LIY3053A96	ILLUMINA	150	Paired-End	P29	P_xylostella	24h	24.3	21.4	47%	93.88%
LIY3053A97	ILLUMINA	150	Paired-End	D25	D_radicum	24h	43.5	38.8	46%	89.78%
LIY3053A98	ILLUMINA	150	Paired-End	D27	D_radicum	24h	36.2	32.5	46%	91.46%
LIY3053A99	ILLUMINA	150	Paired-End	D28	D_radicum	24h	33.3	29.5	46%	90.93%
LIY3053A100	ILLUMINA	150	Paired-End	D29	D_radicum	24h	39.5	35.3	46%	93.54%
LIY3053A101	ILLUMINA	150	Paired-End	BD26	Brevi_Delia	24h	44.2	39.9	46%	90.49%
LIY3053A102	ILLUMINA	150	Paired-End	BD27	Brevi_Delia	24h	35.9	32.3	46%	92.08%
LIY3053A103	ILLUMINA	150	Paired-End	BD28	Brevi_Delia	24h	40.2	35.6	46%	90.16%
LIY3053A104	ILLUMINA	150	Paired-End	BD30	Brevi_Delia	24h	50.7	45.3	45%	89.48%
LIY3053A105	ILLUMINA	150	Paired-End	PD25	Plute_Delia	24h	34.8	30.9	47%	92.2%
LIY3053A106	ILLUMINA	150	Paired-End	PD26	Plute_Delia	24h	28.3	25.3	46%	89.4%
LIY3053A107	ILLUMINA	150	Paired-End	PD28	Plute_Delia	24h	30	26	46%	92.33%
LIY3053A108	ILLUMINA	150	Paired-End	PD30	Plute_Delia	24h	34.1	30.4	46%	91.43%
LIY3053A109	ILLUMINA	150	Paired-End	C31	Control	48h	32.9	29.5	46%	93.6%
LIY3053A110	ILLUMINA	150	Paired-End	C32	Control	48h	38.8	35.1	46%	93.73%
LIY3053A111	ILLUMINA	150	Paired-End	C33	Control	48h	35.3	31.1	46%	93.63%
LIY3053A112	ILLUMINA	150	Paired-End	C35	Control	48h	38.7	34.8	46%	94.05%
LIY3053A113	ILLUMINA	150	Paired-End	B31	B_brassicae	48h	33.6	29.9	46%	93.4%
LIY3053A114	ILLUMINA	150	Paired-End	B32	B_brassicae	48h	24.2	21.8	46%	93.97%
LIY3053A115	ILLUMINA	150	Paired-End	B33	B_brassicae	48h	34.5	30.2	46%	93.86%
LIY3053A116	ILLUMINA	150	Paired-End	B34	B_brassicae	48h	44.6	40	46%	94.04%
LIY3053A117	ILLUMINA	150	Paired-End	P31	P_xylostella	48h	39.3	35.4	46%	93.65%
LIY3053A118	ILLUMINA	150	Paired-End	P32	P_xylostella	48h	45.3	40.9	46%	93.22%
LIY3053A119	ILLUMINA	150	Paired-End	P33	P_xylostella	48h	44.7	39.9	46%	93.52%
LIY3053A120	ILLUMINA	150	Paired-End	P34	P_xylostella	48h	36.1	32.8	46%	93.67%
LIY3053A121	ILLUMINA	150	Paired-End	D31	D_radicum	48h	46.4	41.1	46%	87.62%
LIY3053A122	ILLUMINA	150	Paired-End	D32	D_radicum	48h	34.5	31.4	47%	91.82%
LIY3053A123	ILLUMINA	150	Paired-End	D33	D_radicum	48h	42.2	37.2	46%	86.47%
LIY3053A124	ILLUMINA	150	Paired-End	D34	D_radicum	48h	51.3	46.1	46%	91.02%
LIY3053A125	ILLUMINA	150	Paired-End	BD31	Brevi_Delia	48h	49.5	44.5	46%	91.57%
LIY3053A126	ILLUMINA	150	Paired-End	BD32	Brevi_Delia	48h	41.8	37.7	46%	89.44%
LIY3053A127	ILLUMINA	150	Paired-End	BD33	Brevi_Delia	48h	41.5	36.6	46%	89.13%
LIY3053A128	ILLUMINA	150	Paired-End	BD34	Brevi_Delia	48h	49.7	45.1	47%	92.5%
LIY3053A129	ILLUMINA	150	Paired-End	PD31	Plute_Delia	48h	49.4	42.8	45%	83.63%
LIY3053A130	ILLUMINA	150	Paired-End	PD32	Plute_Delia	48h	29	25.1	46%	91.83%
LIY3053A131	ILLUMINA	150	Paired-End	PD33	Plute_Delia	48h	42	35.5	46%	91.2%
LIY3053A132	ILLUMINA	150	Paired-End	PD34	Plute_Delia	48h	49.8	43	45%	87.8%

Photo by Wim van Egmond

Chapter 4

Leaf-chewing herbivores affect preference and performance of a specialist root herbivore

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Oecologia, 2022, in press

DOI: https://doi.org/10.1007/s00442-022-05132-9

Abstract

Plants interact with a diversity of phytophagous insects above- and belowground. By inducing plant defence, one insect herbivore can antagonise or facilitate other herbivores feeding on the same plant, even when they are separated in space and time. Through systemic plant-mediated interactions, leaf-chewing herbivores may affect the preference and performance of root-feeding herbivores. We studied how six species of leaf-chewing herbivores of *Brassica oleracea* plants affected the preference and performance of the root-feeding specialist *Delia radicum*. We also assessed how the different leaf-chewing herbivores affected defence-related gene expression in leaves and primary roots of *B. oleracea*, both before and after infestation with the root herbivore. Four out of six leaf-chewing herbivores tested negatively affected the performance of *D. radicum*. Surprisingly, we found that adult *D. radicum* females show a strong preference for plants infested with a leaf-chewing herbivore. Defence-related genes in primary roots of *B. oleracea* plants were affected by the leaf-chewing herbivores, but these changes were largely overruled upon local induction by *D. radicum*. Infestation by leaf herbivores makes plants more attractive for oviposition by *D. radicum* females, while decreasing larval performance. Therefore, our findings challenge the preference-performance hypothesis, which states that insects should choose the best feeding site for their offspring, when plant-mediated interactions are considered.

Introduction

Plants are members of complex and diverse ecological communities, and in natural and agricultural settings alike, they are under attack by insect herbivores above and belowground. In natural ecosystems, severe pest outbreaks are rare, and herbivory may even safeguard plant biodiversity by decimating dominant species (Carson & Root, 2000; Koerner *et al.*, 2018). In agriculture, however, farmers suffer significant crop losses from insect herbivory (Oerke, 2006; Deutsch *et al.*, 2018). Understanding how plants defend themselves and interact with different stressors may provide new information for plant breeders to select crop varieties that are better able to resist herbivory.

To cope with insect herbivores above and belowground, plants possess an intricate defence system. The first line of defence is composed of traits that are constitutively expressed, such as thick cuticles or basal concentrations of toxic secondary metabolites (Howe & Jander, 2008). Upon recognition of attack by an insect herbivore, induced defences are activated. These induced responses are regulated by a network of phytohormones, in which jasmonic acid (JA) is a central player (Pieterse *et al.*, 2009; Erb & Reymond, 2019). Induced defence leads to the activation of mechanisms such as the production of proteins that interfere with digestion in the insect gut, toxic secondary metabolites, or volatiles to attract natural enemies (Howe & Jander, 2008). Cues that trigger plant defence can be general, such as mechanical wounding of a leaf or root, or more specific, such as the recognition of insect saliva at the wounding site (Howe & Jander, 2008). Through recognition, plants are thought to be able to fine-tune their response to a variety of phytophagous insects. Specialist insect herbivores, which only feed on a single plant species or family, often evolved strategies to detoxify plant toxins or even use them for their own benefit (Müller *et al.*, 2001; Ratzka *et al.*, 2002; Abdalsamee & Müller, 2012). On the other hand, generalist herbivores, which feed on plants from many phytochemically unrelated families, rely on broad-spectrum detoxification enzymes or behavioural adaptations such as feeding on older leaves. Because generalists and specialists differ in their strategies for overcoming host defence, it has been suggested that induction of plant defence may also be different, although evidence for this is limited (reviewed by Ali and Agrawal (2012)).

In natural settings, plants are often attacked by multiple species of insect herbivores (Stam *et al.*, 2014). By defending against one herbivore, defence against a second herbivore may be altered. Hence, insect herbivores can interact with each other *via* induced plant defence, even when they are separated in time and space. These plant-mediated interactions are important ecological drivers. Insect herbivory early in the season can affect the community of insects surrounding that plant even after the initial

attacker is gone (Poelman *et al.*, 2008)2008. The outcome of plant-mediated interactions depends on the identity of the inducer. Factors such as feeding site and feeding mode are important in determining whether facilitation or antagonism between herbivores occurs (Stam *et al.*, 2014). Chewing herbivores generally mutually antagonise other chewing herbivores. On the other hand, caterpillars and aphids often mutually facilitate each other, as they induce different plant defence pathways. Plant defence is not only triggered locally, but systemically throughout the plant. For instance, leaf herbivory in maize triggers defence signalling in roots of maize plants (Ankala *et al.*, 2013). Since defence induction occurs systemically, plant-mediated interactions can also cross the shoot-root barrier. Indeed, leaf-chewing herbivores negatively affect the performance of root chewing herbivores (Johnson *et al.*, 2012).

A long-standing hypothesis in the field of entomology states that the oviposition preference of insects should be linked with the performance of their offspring; the so-called "mother knows best" or preference-performance hypothesis (Jaenike, 1978; Johnson *et al.*, 2006). To maximise fitness, female insects are expected to lay their eggs on the most suitable host plant for their larvae. A meta-analysis confirmed the hypothesis in situations without natural enemies or potential competitors (Gripenberg *et al.*, 2010). However, most studies on the preference-performance hypothesis focus on insects with an aboveground lifecycle, and whether this hypothesis also holds for root herbivores has received much less attention (Johnson *et al.*, 2006; Clark *et al.*, 2011; Menacer *et al.*, 2021). Furthermore, oviposition preference may be altered by the presence of another insect, either directly or via induced plant defence. Indeed, *Pieris brassicae* butterflies prefer to lay eggs on uninfested leaves rather than leaves already infested with other caterpillars (Blaakmeer *et al.*, 1994).

Oviposition choice of insects is governed by sensory stimuli, most importantly olfactory and gustatory. For many agricultural pests, oviposition selection behaviour has been studied in detail, in particular for the cabbage root fly *Delia radicum*; a specialist root herbivore of brassicaceous plants (Schoonhoven *et al.*, 2005). Brassicaceous plants produce glucosinolates (GSLs), which, upon attack, are hydrolysed by a separately stored myrosinase enzyme to form highly toxic breakdown products such as isothiocyanates (Hopkins *et al.*, 2009). Female *D. radicum* flies are attracted to volatile cues from cabbage plants, such as volatile breakdown products of GSLs (Hawkes & Coaker, 1979). When approaching a potential host plant, the flies assess leaf colour and shape (Roessingh & Städler, 1990). When the female lands on a leaf she inspects the chemical profile with taste receptors on her tarsi (Roessingh *et al.*, 1992; Roessingh *et al.*, 1997). She then walks around the leaf and petiole in a geotactic run and finally reaches the bottom of

the stem, where she circles around, perhaps to estimate the diameter of the stem as an indicator of root size (Schoonhoven *et al.*, 2005). Finally, she lays her eggs in the soil just next to the plant stem. The visual, volatile, and non-volatile chemical cues used in this behaviour can be altered by prior herbivory, allowing female flies to integrate information about other herbivores to make oviposition choices.

In this study, we test whether preference and performance of *Delia radicum* is affected by six different species of leaf-chewing herbivores. We selected both generalist and specialist species from several insect orders. We tested how feeding by these leaf herbivores affects the performance of *D. radicum* larvae on the primary roots of *Brassica oleracea* (Brussels sprouts) plants, and oviposition choice by female *D. radicum* flies. For two of the species of leaf herbivores tested, *Plutella xylostella* and *Pieris brassicae*, plant mediated antagonism towards root feeding *D. radicum* larvae was previously described (Soler *et al.*, 2007; Karssemeijer *et al.*, 2020 - **chapter 2**). To assess potential mechanisms underlying these interactions, we studied how leaf herbivores affected expression of three genes involved in JA, indole GSL and aliphatic GSL biosynthesis, in leaves and roots of plants both before and after infestation by cabbage root fly larvae. We hypothesise that leaf herbivores have a negative effect on the performance of *D. radicum*. We expect female *D. radicum* flies to lay eggs on uninfested control plants rather than plants infested with leaf herbivores, in accordance with the preference-performance hypothesis. We expect differences between leaf herbivores in terms of defence induction, as well as plant-mediated effects on *D. radicum* preference and performance, possibly corresponding with their diet breadth.

Materials and Methods

Study system

Three-week-old Brussels sprouts plants (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus) were used in all experiments. Seeds were sown in trays and transplanted after seven days into 8x8 cm pots. Experiments were performed in a greenhouse compartment (L16:D8 photoperiod, 22 ± 2 °C, 50-70 % relative humidity).

We used six species of leaf-chewing herbivores in the experiments. Two species of generalist herbivores, the cabbage moth (*Mamestra brassicae* L. (Lepidoptera: Noctuidae)) and silver Y (*Autographa gamma* L. (Lepidoptera: Noctuidae)), and two species of specialist herbivores, the large cabbage white (*Pieris brassicae* L. (Lepidoptera: Pieridae)) and diamondback moth (*Plutella xylostella* L. (Lepidoptera: Plutellidae)), were

reared on Brussels sprouts plants. Two more species of specialist herbivores, the turnip sawfly (*Athalia rosae* L. (Hymenoptera: Tenthredinidae)) and mustard beetle (*Phaedon cochleariae* Fabricius (Coleoptera: Chrysomelidae)), were reared on radish plants (*Raphanus sativus*). In the experiments, we used neonate larvae of *M. brassicae* and *P. brassicae*, and L1-L2 larvae of the other four species.

The cabbage root fly (*Delia radicum* L. (Diptera: Anthomyiidae)), a specialist herbivore, whose larvae feed on primary roots of brassicaceous plants, was reared on rutabaga (*B. napus* L. var. *napobrassica*). Flies were kept in cages (65x65x65 cm, Bugdorm, Taiwan), where they were provided with water, honey, and a 1:1:1 mixture of nutritional yeast, sugar, and milk powder. Eggs were collected by placing a slice of rutabaga in a Petri dish in the cage for several hours before taking it out and sealing it with Parafilm. After four days, neonate larvae hatched to be used for the experiments.

Plant treatments

In each of the experiments, three-week-old plants were treated by placing leaf-chewing herbivore larvae on the youngest fully expanded leaf. The petiole of this leaf was wrapped in cotton wool to prevent larvae from immediately moving to other leaves. We used ten larvae of *A. gamma*, *M. brassicae*, *P. cochleariae*, and *P. xylostella*, four *A. rosae* larvae or five *P. brassicae* larvae, to obtain roughly similar amounts of feeding damage (Fig. 1a). Control plants were not treated with any leaf herbivores but did receive a piece of cotton wool around the petiole. During the experiments, plants were placed on saucers and watered from the bottom as needed, and provided 50 mL of Hyponex (Unifarm, Wageningen) fertiliser twice weekly.

Delia radicum performance

To test the effect of feeding by different leaf herbivores on root herbivore performance, 24 plants per treatment were prepared as explained above. In addition to the cotton wool, small mesh bags were secured around the induced leaf to prevent larvae from escaping. As this experiment lasted more than a month, the soil was covered with a layer of sand to reduce attractiveness to fungus gnats (Diptera: Sciaridae). Two days after leaf infestation, 10 neonate *D. radicum* larvae were placed directly on the hypocotyl of all plants, including control plants. Three days post infestation (dpi) with *D. radicum* larvae, the mesh bags and leaf herbivores were removed. At 20 dpi, mesh bags were placed around each plant and sealed with a rubber band around the top of the pot. Bags were held up by two wooden sticks in the soil to give plants space to grow.

The first flies were observed at 24 dpi, but some may have emerged one or two days earlier in the weekend. After the first flies had emerged, every plant was checked daily for emergence. Flies were collected using an aspirator and stored in a freezer (-18 °C) after recording their sex and day of emergence. The experiment was terminated at 38 dpi. As an estimation of body size, the hind tibia length was measured using a digital microscope (Dino Lite Edge, Taiwan) (Soler *et al.*, 2007). After the experiment, we harvested the aboveground plant parts, dried them in an oven for seven days at 70 °C, and assessed the shoot dry weight (Sartorius CPC2 balance, Germany).

During this experiment, an infestation with thrips occurred in the greenhouse. To minimise the effects of thrips on our experiment, we excluded badly thrips-damaged plants from the analyses. Further, in some cases, plants were damaged by chewers that were missed during removal, these plants were also discarded.

Delia radicum oviposition preference

To study whether *D. radicum* oviposition preference is affected by leaf herbivory, we performed a two-choice experiment. Due to greenhouse space limitations, this experiment was performed in three rounds in subsequent weeks. For *A. gamma* and *M. brassicae*, data was collected in two rounds due to difficulties in synchronizing the rearing. Six weeks prior to each round, a separate *D. radicum* rearing cage was started. In short, eggs were collected as above and placed on halved rutabagas at a density of one egg per gram of root (Dr. Anne-Marie Cortesero, pers. com.), which were placed on a layer of sand in a plastic tray. Four weeks later, pupae were collected from the sand by sieving, and placed in cages (65x65x65 cm, Bugdorm, Taiwan) in a separate climate cabinet to minimise rutabaga scent exposure of emerging flies. Female flies used in the experiment were one to two weeks old, giving them enough time since pupation to mate and develop eggs.

Three-week-old Brussels sprouts plants were treated as above. As an oviposition substrate from which the eggs could later be extracted, we covered the top layer of soil with roughly 3 cm of white sand. Two days after leaf treatment, we prepared two-choice arenas in foldable cages (60x40x40 cm). In each tent, an untreated control plant was placed on one side, and a treated plant on the other, roughly 40 cm apart. Control cages had only untreated plants. In each cage, food (1:1:1 mixture nutritional yeast, sugar, and milk powder) and water was provided. Using Microsoft Excel, we randomized positions of cages in the greenhouse compartment and positions of control and treated plants within each cage. To start the oviposition trial, five naive gravid *D. radicum*

female flies were collected using an aspirator in a 50 mL tube, which was placed in the centre of each cage and opened. After 24 h, plants were removed from the cages, and the layer of white sand with the eggs was collected in plastic boxes by holding the pot sideways and gently tapping. Eggs stuck to the stems of plants were collected using a brush. Boxes were stored at 7 °C until further processing. Eggs were separated from sand by flotation using a Fenwick can, an instrument commonly used to collect cyst nematodes from soil (Fenwick, 1940). After collecting the eggs on a 500 µm sieve, they were immediately counted.

Induced gene expression

We tested how leaf and root herbivores affected transcript levels of genes related to plant defence. To this end, we treated plants as above with six species of leaf herbivores that were prevented from moving to other leaves by securing small mesh bags around the petioles. Plants were divided into two subsets. The first subset was harvested two days after induction by leaf herbivores. The second subset of plants was infested with ten *D. radicum* larvae after two days of leaf feeding and harvested 24 h later, an additional control treatment that did not receive root herbivores was included in this subset.

At the time of harvesting, plants were uprooted and the primary root was cut off using clean scissors. Samples were immediately wrapped in aluminium foil and frozen in liquid nitrogen. At the same time, systemic leaf tissue was collected from one leaf higher than the induced leaf using a 10 mm cork borer. Three leaf disks were harvested from the same leaf, placed in an Eppendorf tube, and frozen in liquid nitrogen. Tissue from three plants was pooled for each replicate. Harvesting took less than one minute per sample.

Root and shoot tissue was ground using mortar and pestle or plastic Eppendorf pestles, respectively, whilst keeping the sample cold in liquid nitrogen. From this ground tissue, RNA was extracted using the Isolate II Plant RNA kit (Bioline) and converted to cDNA using the SensiFAST cDNA synthesis kit (Bioline). We then performed qPCR using SensiFAST SYBR (Bioline), targeting defence related transcripts *AOS*, *MYB28*, and *CYP81F4* (Table S1). Optimal reference genes were selected based on GeNorm analysis, in which a random subset of 16 root or leaf samples were tested for six reference genes (Vandesompele *et al.*, 2002). For root tissue, *Act-2* and *PER4* were used as reference genes, and for leaf tissue *Act-2* and *SAR1a* were used. Three mixes of samples were included on each qPCR plate as interrun calibrators. Relative expression was calculated in qBase+ (Biogazelle, Belgium), taking into account primer efficiency and interrun calibration.

Statistics

Data was analysed using R version 3.6.3 (R Core Development Team, 2017) with packages lme4 (Bates *et al.*, 2015), emmeans (Lenth *et al.*, 2018), lmtest (Zeileis & Hothorn, 2002), and multcomp (Hothorn *et al.*, 2008). Depending on the data, (Generalized) Linear (Mixed) Models ((G)L(M)Ms) were used for data analysis with normal, gamma, Poisson, negative binomial or binomial distributions. In the analysis of *D. radicum* survival, tibia length and development time, each fly was considered a replicate and plant ID was included as a random factor to avoid pseudoreplication. For oviposition choice, round was included as a random factor.

We analysed each variable with two separate models. First, we used a model in which we tested whether leaf herbivore specialisation affected the variable, followed by pairwise comparisons. Secondly, we tested the effect of the individual treatments and compared each leaf herbivore to the control treatment.

Results

Leaf herbivory affects *D. radicum* emergence

Delia radicum adult emergence, as a proxy for survival, was affected by leaf herbivory depending on the specialisation of the herbivores (Fig. **1b**; Table **S2**). Specialist leaf herbivores negatively affected root fly emergence compared to uninfested plants, whereas emergence on plants treated with generalist herbivores did not differ from either control or specialist herbivore treatments. While the effect of individual treatments on the emergence of *D. radicum* was weak (GLMM; $X^2 = 10.54$ $P = 0.10$), comparison of the different treatments to the control treatment revealed that the generalist *M. brassicae* reduced *D. radicum* emergence, and there was a trend that *P. brassicae*, *P. cochleariae*, and *P. xylostella* slightly reduced root fly emergence. Hind tibia length of emerged flies was not affected by leaf herbivore treatments or specialisation (Fig. **1c**; Table **S2**). Males had longer hind tibia than females. Development time of cabbage root flies was not affected by leaf herbivores (Fig. **1d**; Table **S2**). Males emerged earlier than females. Shoot dry weight of the plants on which flies developed was not affected by leaf herbivory (Fig. **1e**; Table **S2**).

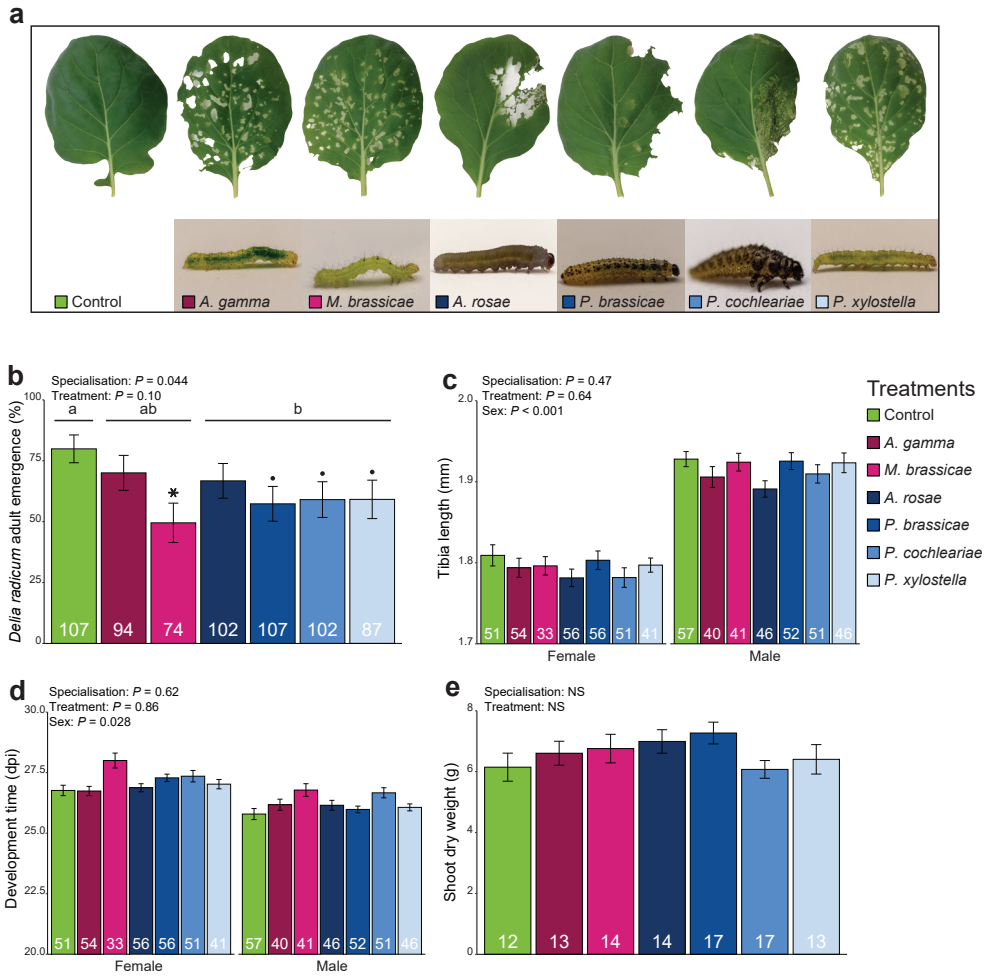

Figure 1. (**a**) Exemplary photographs of leaf damage by six leaf-chewing herbivores after 5 days of feeding on Brussels sprouts plants (*Brassica oleracea*). (**b**) *Delia radicum* adult emergence; (**c**) *Delia radicum* adult tibia length; (**d**) *Delia radicum* development time from egg until adult when feeding on plants previously subjected to leaf herbivory by one of six different leaf-chewing herbivores. (**e**) *Brassica oleracea* shoot dry weight at the end of the experiment. Leaf herbivores were placed on plants two days prior to *D. radicum* infestation and removed after they fed for 5 days. Two separate statistical analyses were performed, first to test the effect of leaf herbivore specialisation, and second to test effects of individual treatments. Magenta colours indicate generalist herbivores, whereas blue colours indicate specialist leaf herbivores of Brassicaceae. Stars ($P < 0.05$) and dots ($P < 0.10$) indicate differences between individual treatments and control, corrected for multiple testing using the false discovery rate method. Results of pairwise comparisons between herbivore specialisation groups are indicated with letters; groups having no letters in common differ significantly ($P < 0.05$). Bars represent mean and error bars represent the standard error of the mean. Leaf pictures by Laura Winzen, larvae pictures by Peter Karssemeijer.

Female *D. radicum* flies prefer to lay eggs on plants damaged by leaf herbivores

We studied the effects of leaf herbivory on oviposition preference of *D. radicum* using two-choice assays. Female *D. radicum* flies strongly preferred to lay eggs at the base of plants infested by leaf herbivores, both specialists and generalists (Fig. **2a**; Table **S2**). There was an effect of leaf herbivore treatments on *D. radicum* oviposition, and indeed, except for *M. brassicae*, feeding by each of the leaf herbivore species led to more *D. radicum* eggs compared to the control. The effect was less strong for *M. brassicae*, this could be due to a smaller sample size for this treatment. We also analysed the sum of eggs per cage (control and induced plants combined) and found that many more eggs were laid in cages that contained a plant infested with leaf herbivores compared to control cages (Fig. **2b**; Table **S2**).

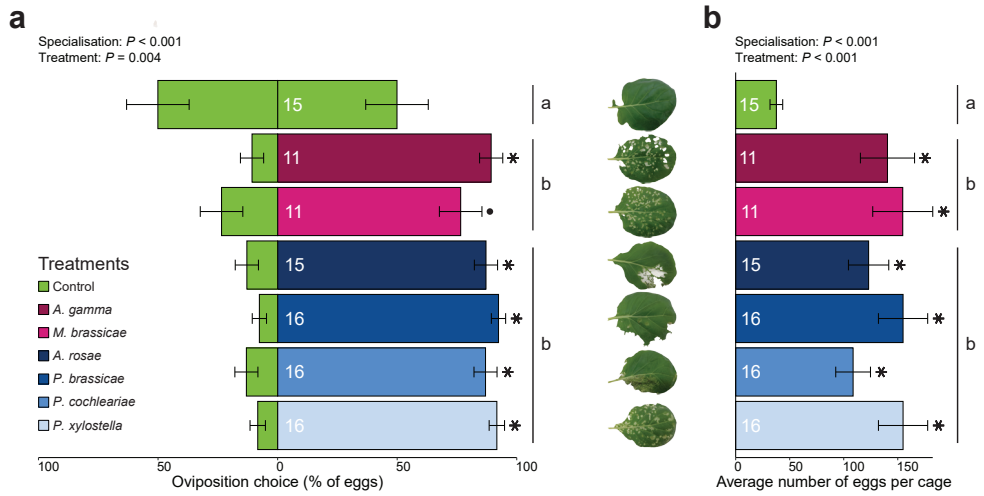

Figure 2. (**a**) *Delia radicum* oviposition choice when choosing between a plant previously subjected to leaf herbivory by six different leaf-chewing herbivores or an uninfested control plant and (**b**) the average total number of eggs per cage. Leaf herbivores were placed on plants two days prior to the start of the two-choice assay. Five *D. radicum* females were released in each cage and taken out 24 h later. Two separate statistical analyses were performed, first to test the effect of leaf herbivore specialisation, and second to test effects of individual treatments. Magenta colours indicate generalist herbivores, whereas blue colours indicate specialist leaf herbivores of Brassicaceae. Stars ($P < 0.05$) and dots ($P < 0.10$) indicate differences between individual treatments and control, corrected for multiple testing using the false discovery rate method. Results of pairwise comparisons between herbivore specialisation groups are indicated with letters; groups having no letters in common differ significantly ($P < 0.05$). Bars represent mean and error bars represent the standard error of the mean.

Effects of leaf and root herbivory on plant defence signalling

We studied the effects of leaf and root herbivory on systemic leaf (one leaf higher than the infested leaf) and primary root defence gene expression (Fig. **3**). Tissues were sampled at

two time points, at 0 hpi *i.e.* after leaf herbivores had fed for two days, which corresponds with the time of infestation for the *D. radicum* performance experiments (Fig. **1**) and the time where plants were presented to female *D. radicum* flies for the oviposition choice experiment (Fig. **2**). A second time point, after 24 h of *D. radicum* feeding, was sampled to study the effects of leaf herbivores on induction of plant defence by the root herbivore. Three genes were studied, *AOS* as a marker for JA biosynthesis, *CYP81F4* as a marker for indole GSL biosynthesis, and *MYB28* as a marker for aliphatic GSL biosynthesis. The latter two genes were previously found to be among the most strongly affected genes in the primary root response to *D. radicum* (**Chapter 3**).

Effects of leaf herbivores on the expression of plant defence genes in systemic leaves and in roots in the absence of *D. radicum*

In systemic leaves, transcript levels of *AOS, CYP81F4* and *MYB28* were affected by leaf herbivory at 0 hpi (Fig. **3a-c**, Table **S2**). All species of leaf herbivores induced expression of *AOS*, leading to a 4- to 8.5-fold increase. Generalists had a stronger effect on *AOS* transcript levels than specialists. Expression of *CYP81F4* was induced by *M. brassicae*, *P. brassicae*, *P. cochleariae*, and *P. xylostella*, but not by *A. gamma* and *A. rosae*. Transcript levels of *MYB28* were higher in systemic leaves of plants induced by specialists compared to generalists. When analysing at the species level, this effect is mainly caused by a strong induction of the gene by *P. xylostella* and a slight downregulation by *M. brassicae* compared to uninfested control plants.

In primary roots at 0 hpi, all three genes (*AOS, CYP81F4* and *MYB28*) were affected by leaf herbivory (Fig. **3d-f**, Table **S2**). Both generalist and specialist herbivores upregulated the expression of *AOS* as well as *CYP81F4*. Except for *P. xylostella*, each of the leaf herbivore species upregulated *AOS* expression relative to control. *CYP81F4* mRNA levels were induced in the roots by all six leaf herbivores. Expression of *MYB28* in the primary roots at 0 hpi was induced by specialists but not by generalist herbivores, compared to control roots. Interestingly, at the species level, the generalist chewers *A. gamma* and *M. brassicae* downregulated *MYB28* expression, whereas *P. brassicae*, *P. cochleariae* and *P. xylostella* caused upregulation of this gene.

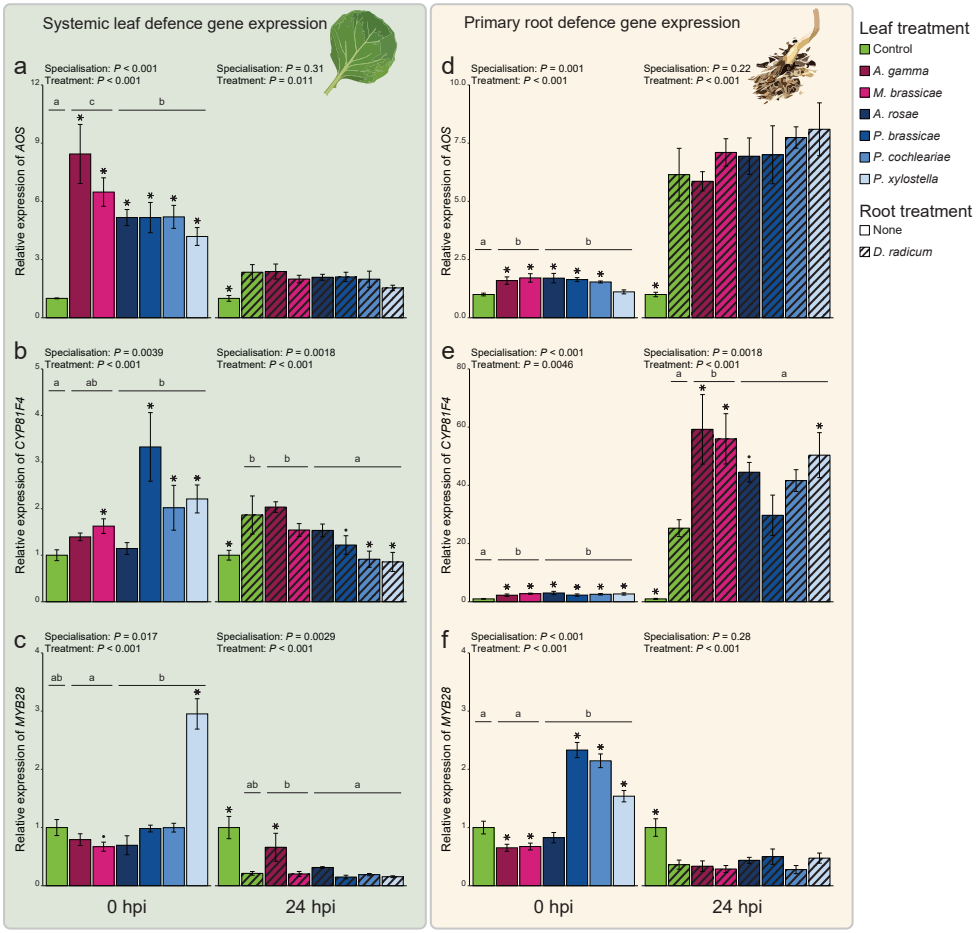

Figure 3. Defence gene expression in systemic leaves (left panel, **a-c**) and primary roots (right panel, **d-f**) of *Brassica oleracea* plants infested with six species of leaf-chewing herbivores either alone or in combination with the root herbivore *Delia radicum*. Systemic leaves were one leaf higher than the leaf infested with leaf herbivores. Leaf herbivores were placed on plants two days prior to *D. radicum* infestation. Two separate statistical analyses were performed, first to test the effect of leaf herbivore specialisation, and second to test effects of individual treatments. Magenta colours indicate generalist herbivores, whereas blue colours indicate specialists of the Brassicaceae family. Stars ($P < 0.05$) and dots ($P < 0.1$) indicate differences between individual treatments and control plants (0 hpi) or plants only infested with *D. radicum* (24 hpi), corrected for multiple testing using the false discovery rate method. Note that symbols above uninfested control plants at 24 hpi indicate a difference compared to *D. radicum* infested plants. Results of pairwise comparisons between herbivore specialisation groups are indicated with letters; groups having no letters in common differ significantly ($P < 0.05$). Hpi: hours post infestation by *D. radicum*. Bars represent mean and error bars represent the standard error of the mean. N = 4 replicates each consisting of three plants.

Effects of leaf herbivores on plant defence genes at 24 hpi after infestation with *D. radicum*

In systemic leaves at 24 hpi, expression of *AOS* was induced by *D. radicum* infestation. This effect was not altered when plants were treated with *D. radicum* in combination with generalist or specialist herbivores. Expression of *CYP81F4* was induced by *D. radicum*. Specialist herbivores in combination with *D. radicum* led to a lower expression of *CYP81F4* compared to plants treated only with *D. radicum*. Further, we measured lower *CYP81F4* expression levels in systemic leaves induced by *P. cochleariae* and *P. xylostella* in combination with *D. radicum* compared to plants treated by *D. radicum* alone. *Delia radicum* reduced the expression of *MYB28* in systemic leaves. Plants co-infested with *M. brassicae* and *D. radicum* had higher expression levels of *MYB28* compared to systemic leaves of plants treated with *D. radicum* alone.

In primary roots, 24 h after *D. radicum* infestation, a clear effect of the root herbivore was measured for all three genes (Fig. **3d-f**, Table **S2**). Primary root expression of *AOS* at 24 hpi was induced 6-fold by *D. radicum* compared to control, but did not differ between plants infested with *D. radicum* alone or in combination with leaf herbivores. Expression of *CYP81F4* was strongly induced by 24h of feeding by *D. radicum*. Furthermore, generalist herbivores had a synergistic effect with root herbivore induction, leading to higher expression of *CYP81F4* when compared to plants only infested with *D. radicum*. On the species level, higher levels of *CYP81F4* were measured in primary roots of plants treated with both *D. radicum* and *A. gamma*, *M. brassicae* or *P. xylostella* compared to plants only treated with the root herbivore. Primary roots that were infested with *D. radicum* for 24 h contained lower levels of *MYB28* mRNA compared to uninfested control. Expression of *MYB28* was not different between plants infested with *D. radicum* alone or in combination with leaf herbivores.

Discussion

We studied plant-mediated effects of leaf herbivores on preference and performance of the root herbivore *D. radicum* in the context of two hypotheses in the field of insect-plant interactions, *i.e.* that oviposition preference is linked to higher larval performance and that generalists and specialists induce distinct plant responses. Regardless of specialisation, most leaf herbivores negatively impacted *D. radicum* performance, albeit with marginal significance. This is in line with earlier findings that leaf chewers generally negatively affect root chewers (Johnson *et al.*, 2012). Female *D. radicum* flies strongly preferred to lay eggs on plants exposed to leaf herbivory, even though larval performance was reduced on those plants.

Leaf-chewing herbivores affect *Delia radicum* performance

Our results show that four out of six species of chewing folivores tested had a negative effect on *D. radicum* emergence or body size. Previously, negative plant-mediated effects of *P. brassicae* and *P. xylostella* on cabbage root fly emergence have been reported (Soler *et al.*, 2007; Karssemeijer *et al.*, 2020 - **chapter 2**). Shoot dry weight was not affected by the leaf herbivores, suggesting that lower performance was not caused by reduced plant growth but rather by differences in induced plant defence. We found that leaf herbivores induced JA biosynthesis marker gene *AOS* in both systemic leaves and primary roots. Treatment with JA can be sufficient to reduce *D. radicum* performance, although contrasting results have been reported (Pierre *et al.*, 2012a). Induction of JA biosynthesis was also found in the primary roots of plants induced by *A. gamma*, while performance was not affected by this treatment. In primary roots, leaf herbivores caused an increase in expression of indole GSL biosynthesis gene *CYP81F4*, which was strengthened further 24 h after *D. radicum* started feeding. Transcription related to aliphatic GSL biosynthesis was found to be both up- and downregulated depending on the species of leaf-chewing inducer prior to onset of root herbivory. *Delia radicum* performs better in the absence of aliphatic GSL (**chapter 3**), making this a potential mechanism for reduced performance on plants induced by *P. brassicae*, *P. cochleariae*, and *P. xylostella*. However, after *D. radicum* started feeding, this effect was reversed, and all plants showed a decrease in *MYB28* transcription similar to roots only treated with *D. radicum*. Root herbivore-induced expression of indole GSL biosynthesis gene *CYP81F4* was synergistically increased by several leaf herbivores. The toxicity of indole GSLs to *D. radicum* larvae has not been studied, however, most likely it is low.

Our results show no clear evidence of differences between generalist and specialist leaf herbivores in the induction of systemic plant defence or plant-mediated interaction with *D. radicum*. In terms of *D. radicum* preference and performance, effects of leaf herbivores were unidirectional, and differences appear between individual species. For instance, *M. brassicae* and *A. gamma*, the two generalist species we used and also the closest genetic relatives in our study, caused the strongest and weakest effect on *D. radicum* emergence, respectively. Similar species-specific rather than specialisation-driven plant-mediated interactions were reported between four species of leaf herbivores in wild radish (Agrawal, 2000). Generalist and specialist herbivores may induce distinct defence responses (Ali & Agrawal, 2012; Rowen & Kaplan, 2016). In systemic leaf gene expression, we indeed observe stronger induction of *AOS* by generalist herbivores compared to specialists. On the other hand, our gene expression analyses point much more towards species-specific responses; for instance, in systemic leaves, *MYB28* expression was strongly induced only by *P. xylostella*, and *CYP81F4* expression was induced by all herbivores except for *A. rosae*

and *A. gamma*. This corresponds with earlier studies that failed to find distinct patterns of induction by generalists and specialists (Reymond *et al.*, 2004; Bidart-Bouzat & Kliebenstein, 2011; Ali & Agrawal, 2012). For instance, microarray analysis of the *Arabidopsis thaliana* response to four chewing herbivores, two generalists and two specialists, revealed no effect of specialisation whereas the specialists *P. rapae* and *P. xylostella* elicited a very different response (Bidart-Bouzat & Kliebenstein, 2011). Upon feeding, chewing herbivores release saliva and regurgitant that may include effector proteins to interact with plant defence (Acevedo *et al.*, 2015), which could potentially explain species-specific responses. Further, some differences in effects of generalist versus specialist herbivores may be caused by the leaf age preferred for feeding. For instance, the specialist *P. xylostella* prefers to feed on the youngest developing leaves, whereas the generalist *M. brassicae* is found more often on older leaves. We excluded this effect from our study by constraining each herbivore to a single leaf, which may have masked differences between generalists and specialists.

Leaf herbivory affects oviposition of *Delia radicum*

Female *D. radicum* flies strongly preferred to lay eggs on plants induced by leaf herbivores. The oviposition behaviour of *D. radicum* has been recorded in much detail (Zohren, 1968; Schoonhoven *et al.*, 2005). *Delia radicum* aggregates in the field (Mukerji & Harcourt, 1970), and oviposition is stimulated on plants damaged by conspecifics, even when stems were cut and shoots were removed prior to testing (Baur *et al.*, 1996a). Plants infested with *Brevicoryne brassicae* or *Myzus persicae* aphids received fewer eggs by *D. radicum* females, possibly due to physical contact between searching flies and aphids (Finch & Jones, 1989), as the opposite effect was observed when *B. brassicae* aphids were removed prior to the test (Finch & Jones, 1987) although induction of plant defence by the aphids may also play a role. *Delia radicum* females were deterred from plants with *P. xylostella* eggs, but attracted to plants on which 2nd or 3rd instar larvae were feeding, in line with our findings (Finch & Jones, 1987). Finally, spraying plants with extracts of frass of *Evergestis forficalis* caterpillars works as an oviposition deterrent for cabbage root flies (Jones *et al.*, 1988). Caterpillar frass can induce a plant defence response, which could be a potential mechanism (Ray *et al.*, 2016). Specialist insect herbivores of Brassicaceae often use GSLs as oviposition stimulants (Textor & Gershenzon, 2009). Indeed, indole as well as aliphatic GSLs are oviposition stimulants for *D. radicum* upon contact (Roessingh *et al.*, 1992), and isothiocyanates that are produced upon GSL hydrolysis are volatile attractants for gravid females (Hawkes & Coaker, 1979). We found upregulation of GSL biosynthesis gene expression upon leaf herbivory by most species, and it is expected that constitutive GSLs stored in leaves are converted to isothiocyanates upon leaf damage (Textor & Gershenzon, 2009). However, GSLs do not trigger the entire response; in fact, a yet undescribed chemical compound found in cabbage, the so-called

cabbage identification factor (CIF), induces a much stronger oviposition response than GSLs (Roessingh *et al.*, 1997). To the best of our knowledge, whether concentrations of CIF differ upon herbivory has not been studied yet. In natural settings, there are many ways a plant can be mechanically damaged, such as by wind, heavy rain, or a rodent brushing past. Therefore, finding a completely undamaged plant like the control plants in our experiments is unrealistic in natural settings. Female *D. radicum* may not be adapted to recognise such plants as potential hosts, especially when stronger-smelling damaged plants are nearby.

Our finding that plants damaged by chewing folivores are more attractive for oviposition by *D. radicum* despite lower larval performance challenges the preference-performance hypothesis when plant-mediated interactions are considered (Jaenike, 1978; Johnson *et al.*, 2006). While many studies have confirmed the validity of this hypothesis (Gripenberg *et al.*, 2010), there are exceptions. Menacer *et al.* (2021) recently found support for the preference-performance hypothesis in *D. radicum* when comparing between cultivars of *B. rapa*, but not between cultivars of *S. alba*. Furthermore, *Otiorhynchus sulcatus* vine weevils laid more eggs on raspberry plants previously damaged by conspecific larvae and on raspberry plants with lower root mass, even though both these factors negatively impacted larval mass (Clark *et al.*, 2011). Our experiment investigated plant-herbivore interactions without the inclusion of natural enemies. *Trybliographa rapae* Westwood parasitoid wasps foraging for *D. radicum* larvae use volatile cues to locate hosts (Neveu *et al.*, 2002). The presence of other herbivores may change the volatile blend, thereby reducing parasitism (Rasmann & Turlings, 2007; Pierre *et al.*, 2011). Indeed, leaf herbivory by *P. brassicae* leads to lower parasitism of *D. radicum* in the roots in both laboratory and field conditions (Pierre *et al.*, 2011). Through lower parasitism, choosing for leaf-induced plants may yet be a beneficial strategy for *D. radicum* survival. To complement our greenhouse studies, preference and performance of *D. radicum* in the context of leaf herbivory should be studied in the field before firm conclusions can be made.

We observed that cages without induced plants received fewer eggs compared to cages with only uninfested control plants. Volatile and non-volatile cues from the induced plants, such as GSLs and their breakdown products, act as oviposition stimulants for female cabbage root flies, which could cause them to lay more eggs (Hawkes & Coaker, 1979; Roessingh *et al.*, 1992; Roessingh *et al.*, 1997). However, control plants should contain similar stimulants, albeit in lower concentrations. Because all cages were placed in a single greenhouse compartment, it is likely that volatile cues from induced plants were perceived by flies in cages that only contained control plants. Perhaps female flies in control cages laid fewer eggs because they perceived a more preferred host nearby, saving some of their egg load to deposit on those plants instead. It would be interesting to study this in detail, for instance by

testing egg deposition on control plants in a setup with or without herbivore-induced plants placed next to the cage.

Conclusion

Plants in natural or agricultural settings are often attacked by both above and belowground insect herbivores, linking the two communities. Our results show that leaf herbivores strongly affect the oviposition preference of *D. radicum* females, while slightly decreasing larval performance. Leaf herbivores induced defence-related gene expression in both systemic leaf and root tissue, which was largely overruled after the induction of the root herbivore. As a next step, a field study would be timely in which natural occurrences of both leaf and root herbivores would be studied in combination with their natural enemy community.

Acknowledgements

The authors are grateful to **Julia Friman** and **Julian Haller** for help during the experiments. This work could not have been completed without the insect and plants provided by **Pieter Rouweler**, **Sean Geurts**, **Arnout Berendsen**, **Andre Gidding**, **Frans van Aggelen**, **Alessia Vitiello** and **Daan Mertens**.

Supporting information

Table S1. Primers used for qPCR of *B. oleracea* leaves and primary roots.

Gene acronym	Gene amplified	Forward primer	Reverse primer
AOS	Bo2g116210	ACCGCTTGCGACTAGGGATC	CAAAGTCCTTACCGGCGCAC
CYP81F1	Bo1g004730	TGTGTCAGAAACGTTCAGGCT	ATGGCACGTCGTATCCTCCG
MYB28	Bo2g161590	CGGGAGAGATGAGCACAATACG	CAGCCCTCGAAGTTTCCTATCA
SAR1a	Bo3g052780	ATCTCTAGCCACCGTTCCCT	TTCCTGACGATGCTGCACAT
Btub	Bo2g124350, Bo7g067360, Bo9g059850	GTCAAGTCCAGCGTCTGTGA	TCACACGCCTGAACATCTCC
Act-2	Bo5g117040	ACATTGTGCTCAGTGGTGGA	TCTGCTGGAATGTGCTGAGG
PER4	Bo7g095750	TATCCTCTGCAGCCTCCTCA	ACACACAGACTGAAGCGTCC
GADPH	Bo5g017500	GCTACGCAGAAGACAGTTGATGG	TGGGCACACGGAAGGACATAC
EF1a	Bo9g142520	GGTACCTCCCAGGCTGATTG	TCAGGTAKGAAGACACCTCCTTG

Table S2. Overview of statistical tests and main effects regarding data presented in this chapter. (G)L(M)M: (Generalized) Linear (Mixed) Model.

***Delia radicum* performance experiment (Fig. 1)**

Variable	Model type	Model	Factor	Chisq	P-value
Emergence	GLMM (Bernoulli)	~ Specialisation + (1|Plant)	Specialisation	6.23	**0.044**
Emergence	GLMM (Bernoulli)	~ Treatment + (1|Plant)	Treatment	10.54	0.10
Tibia length	LMM	~ Specialisation + Sex + (1|Plant)	Specialisation	1.52	0.47
			Sex	337.21	**<0.0001**
Tibia length	LMM	~ Treatment + Sex + (1|Plant)	Treatment	4.25	0.64
			Sex	337.21	**<0.0001**
Development time	GLMM (Poisson)	~ Specialisation + Sex + (1|Plant)	Specialisation	0.96	0.62
			Sex	5.038	**0.025**
Development time	GLMM (Poisson)	~ Treatment + Sex + (1|Plant)	Treatment	2.57	0.86
			Sex	5.038	**0.025**
Shoot dry weight	LM	~ Specialisation	Specialisation	1.34	0.51
Shoot dry weight	LM	~ Treatment	Treatment	7.97	0.24

***D. radicum* oviposition experiment (Fig. 2)**

Variable	Model type	Model	Factor	Chisq	P-value
Oviposition choice	GLMM (binomial ~cbind(control, induced))	~ Specialisation + (1|Round) + (1|Cage)	Specialisation	15.12	**0.00052**
Oviposition choice	GLMM (binomial ~cbind(control, induced))	~ Treatment + (1|Round) + (1|Cage)	Treatment	19.086	**0.0040**
Total eggs	GLMM (negative binomial)	~ Specialisation + (1|Round)	Specialisation	38.61	**<0.0001**
Total eggs	GLMM (negative binomial)	~ Treatment + (1|Round)	Treatment	43.52	**<0.0001**

Table S2 continued

Gene expression experiment (Fig. 3)

Variable	Model type	Model	Factor	Chisq	P-value
Systemic leaf					
AOS 0h	GLM (Gamma)	~Treatment	Treatment	59.53	59.53
AOS 24h	LM	~Treatment	Treatment	18.27	**0.011**
CYP81F4 0h	GLM (Gamma)	~Treatment	Treatment	32.65	**<0.0001**
CYP81F4 24h	LM	~Treatment	Treatment	27.29	**0.00029**
MYB28 0h	GLM (Gamma)	~Treatment	Treatment	53.56	**<0.0001**
MYB28 24h	GLM (Gamma)	~Treatment	Treatment	56.42	**<0.0001**
Primary root					
AOS 0h	LM	~Treatment	Treatment	24.97	**0.00035**
AOS 24h	GLM (Gamma)	~Treatment	Treatment	60.33	**<0.0001**
CYP81F4 0h	LM	~Treatment	Treatment	18.74	**0.0046**
CYP81F4 24h	LM	~Treatment	Treatment	39.12	**<0.0001**
MYB28 0h	GLM (Gamma)	~Treatment	Treatment	71.40	**<0.0001**
MYB28 24h	GLM (Gamma)	~Treatment	Treatment	25.40	**0.00065**
Variable	**Model type**	**Model**	**Factor**	**Chisq**	**P-value**
Systemic leaf					
AOS 0h	GLM (Gamma)	~Specialisation*	Specialisation	53.71	**<0.0001**
AOS 24h	LM	~Specialisation*	Specialisation	2.33	0.31
CYP81F4 0h	GLM (Gamma)	~Specialisation*	Specialisation	11.09	**0.0039**
CYP81F4 24h	LM	~Specialisation*	Specialisation	12.70	**0.0018**
MYB28 0h	GLM (Gamma)	~Specialisation*	Specialisation	8.20	**0.017**
MYB28 24h	GLM (Gamma)	~Specialisation*	Specialisation	11.70	**0.0029**
Primary root					
AOS 0h	LM	~Specialisation*	Specialisation	13.81	**0.0010**
AOS 24h	GLM (Gamma)	~Specialisation*	Specialisation	3.007	0.22
CYP81F4 0h	LM	~Specialisation*	Specialisation	22.34	**<0.0001**
CYP81F4 24h	LM	~Specialisation*	Specialisation	12.60	**0.0018**
MYB28 0h	GLM (Gamma)	~Specialisation*	Specialisation	26.49	**<0.0001**
MYB28 24h	GLM (Gamma)	~Specialisation*	Specialisation	2.58	0.27

* Uninfested control samples were omitted for this analysis, as the relevant control is the *Delia radicum*-only infested treatment.

Photo by Hans Smid

Chapter 5

Shoot and root insect herbivory change the plant rhizosphere microbiome and affect cabbage-insect interactions through plant-soil feedback

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New Phytologist, 2021, 232 (6), 2475-2490

DOI: https://doi.org/10.1111/nph.17746

Abstract

Plant-soil feedback (PSF) may play a role in plant-insect interactions. Although plant defence differs between shoot and root tissues, few studies have examined root-feeding insect herbivores in a PSF context. We examined here how plant growth and resistance against root-feeding *Delia radicum* larvae was influenced by PSF. We conditioned soil with cabbage plants that were infested with herbivores that affect *D. radicum* through plant-mediated effects: leaf-feeding *Plutella xylostella* caterpillars and *Brevicoryne brassicae* aphids, root-feeding *D. radicum* larvae, and/or added rhizobacterium *Pseudomonas simiae* WCS417r. We analysed the rhizosphere microbial community, and in a second set of conspecific plants exposed to conditioned soil, we assessed growth, expression of defence-related genes, and *D. radicum* performance. The rhizosphere microbiome differed mainly between shoot- and root herbivory treatments. *Addition* of *P. simiae* did not influence rhizosphere microbiome composition. Plant shoot biomass, gene expression, and plant resistance against *D. radicum* larvae was affected by PSF in a treatment-specific manner. Soil conditioning overall reduced plant shoot biomass, *P. simiae*-amended soil causing the largest growth reduction. In conclusion, shoot- and root insect herbivores alter the rhizosphere microbiome differently, with consequences for growth and resistance of plants subsequently exposed to conditioned soil.

Introduction

Plants are members of complex communities, in which they interact with a plethora of other organisms such as insects and microbes (van der Heijden *et al.*, 2008; Berendsen *et al.*, 2012; Stam *et al.*, 2014). Plant responses to the biotic or abiotic environment can affect many of these interactions and can shape the roots and their associated microbiome (Sasse *et al.*, 2018; Stringlis *et al.*, 2019; Wang *et al.*, 2019; Delory *et al.*, 2020; Kostenko & Bezemer, 2020). Shaping of the root-associated microbial community may impact future plants growing in the same soil. The net effect of all biotic and abiotic properties of soil conditioned by plants that previously grew in it on plants subsequently growing in the same soil is called plant-soil feedback (PSF) (van der Putten *et al.*, 2013; Kaplan *et al.*, 2018; Bennett & Klironomos, 2019). Plant-soil feedback can affect the performance of plants positively (Kulmatiski *et al.*, 2017) or negatively (Ma *et al.*, 2017; Lekberg *et al.*, 2018). Although an increasing number of studies focuses on the effects of PSF on plant growth, its effect on plant resistance is less explored, in particular plant defence against belowground insect herbivores (Hu *et al.*, 2018).

Plants possess interconnected hormonal signaling pathways that respond to insect herbivory in both shoot and root tissue. Plant defences to insect herbivores are mainly regulated by the phytohormones jasmonic acid (JA) and salicylic acid (SA), but also other plant hormones such as abscisic acid (ABA) and ethylene (ET) are involved (Erb *et al.*, 2012b; Verma *et al.*, 2016). Plants respond to herbivory by upregulating primarily JA- or SA-associated signaling depending on the attacking insect species. Chewing insects generally induce JA production, whereas phloem-feeding insects induce SA biosynthesis (Erb *et al.*, 2012b; Stam *et al.*, 2014).

There are differences in plant defence and phytohormone regulation between plant shoot- and root tissues (Johnson *et al.*, 2016b). For instance, levels of the defensive glucosinolates in brassicaceous plants differ substantially between shoots and roots (Tsunoda *et al.*, 2017). In terms of phytohormonal signaling, JA is thought to be less inducible in roots compared to shoots (Erb *et al.*, 2012a; Tytgat *et al.*, 2013), but increased levels do occur after herbivore attack (Erb *et al.*, 2009a; Lu *et al.*, 2015; Karssemeijer *et al.*, 2020 - **chapter 2**), and SA may serve different functions in root and shoot tissues (Erb *et al.*, 2012a; Lu *et al.*, 2015).

Plant hormones do not only govern plant defence, they also influence root exudates and therefore consequently the microbiome around the plant root (Carvalhais *et al.*, 2015; Eichmann *et al.*, 2021). Therefore it is not surprising that feeding by shoot- and root

herbivores induces microbiome alterations, through altered plant root exudation (Dawson *et al.*, 2004; Kostenko *et al.*, 2012; Kim *et al.*, 2016; Kong *et al.*, 2016; Ourry *et al.*, 2018; Friman *et al.*, 2021). Herbivores can also influence the soil microbiome directly, for instance through caterpillar frass or aphid honeydew that mixes with soil (Frost & Hunter, 2004). The resulting changes in microbiome and soil properties can affect the chemical composition of subsequently growing plants (Meiners *et al.*, 2017) which in turn can affect herbivorous insects (Kostenko *et al.*, 2012). In this manner, phytohormone-mediated signaling pathways and by extension plant defence relying on types and levels of secondary metabolites, can be modified by PSF (Ma *et al.*, 2017; Hu *et al.*, 2018; Zhu *et al.*, 2018; Bennett & Klironomos, 2019). For instance, caterpillars of the cabbage moth *Mamestra brassicae* showed decreased performance when feeding on plants grown in soil conditioned by plants infested by root-feeding wireworms *Agriotes lineatus*, compared to caterpillars feeding on plants grown in soil conditioned by caterpillar-infested plants (Kostenko *et al.*, 2012). Thus, herbivores can affect plant defence through PSF, and the identity of the herbivore species in the conditioning phase may be an important factor. Because plants respond differently to insect herbivores depending on their feeding guild and feeding site, it is plausible that different types of insects cause different changes to the plant-associated microbe community. Whether the underlying microbial community changes are comparable between insect feeding guilds and feeding location has received little attention so far.

Some root-associated bacteria are known to boost plant growth, and consequently have been coined plant-growth-promoting rhizobacteria (PGPR). A number of these PGPR can induce systemic resistance (ISR) in the plant, a mechanism that enhances resistance against a range of plant attackers (Pineda *et al.*, 2010; Pieterse *et al.*, 2014; Friman *et al.*, 2021). These ISR-inducing bacteria can mediate PSF. *Arabidopsis thaliana* recruited an assemblage of ISR-inducing microorganisms after infection with downy mildew, *Hyaloperonospora arabidopsidis*, which subsequently increased plant resistance of plants grown in the same soil against the same pathogen (Berendsen *et al.*, 2018). Although plant-growth-promoting microbes are known to modulate plant resistance against insects (Pineda *et al.*, 2010), it remains to be investigated how these rhizobacteria affect plant defence against insects in plant conspecifics growing in the same soil.

Here, we studied how shoot- and root-feeding insect herbivores and beneficial rhizobacteria affect the rhizosphere microbiome, and how these differences through PSF affect plant growth and defence against a root herbivore in plants subsequently growing in the same soil. We conditioned soil by growing *B. oleracea* plants induced by either root-chewing *Delia radicum*, leaf-chewing *Plutella xylostella,* phloem-feeding *Brevicoryne brassicae*, or by adding growth-promoting and ISR-inducing PGPR *Pseudomonas simiae* WCS417r

to the soil. These inducers have previously been tested for their influence on *D. radicum* performance through plant-mediated effects, where *P. xylostella* negatively influenced *D. radicum* performance, *B. brassicae* had no effect (Karssemeijer *et al.*, 2020 - **chapter 2**), and *P. simiae* positively affected the insect (Friman *et al.*, 2020). After removal of the conditioning plants and insects, we used a mixture of sterilised and conditioned soil to grow a consecutive set of *B. oleracea* plants, for which we assessed growth, defence-related gene expression, and resistance against the root herbivore *D. radicum*. We aimed to elucidate the effect of the inducers on the rhizosphere microbial community, and how these changes may moderate plant-mediated interactions between biotic inducers. We hypothesised that the induction by leaf-chewing, root-chewing, and phloem-feeding insect herbivores would have distinct effects on the rhizosphere microbiome due to their respective induction of different phytohormones, and that plants grown in these soils would differ in resistance against *D. radicum*. We expected that *P. simiae* would increase plant growth in the feedback phase, and increase *D. radicum* performance.

Material and Methods

Plant growth conditions

Our study system consisted of *Brassica oleracea*, a globally important cultivated crop plant. *Brassica oleracea* var. *gemmifera* cv. "Cyrus" seeds (Syngenta Seeds, the Netherlands) were germinated in a seeding tray with seedling soil in a greenhouse with 21 ± 3 °C and 16 ± 3 °C day and night temperatures respectively. Natural daylight was supplemented with 400 Watt metal halide lamps (200 µmol m^{-2} s^{-1} photosynthetically active radiation) when photosynthetic active radiation (PAR) dropped below 400 µmol m^{-2} s^{-1}, in a 16:8 L:D cycle. After three days, plants were transplanted to 1 l pots containing potting soil and grown in greenhouse conditions for three weeks with identical settings as above at 60 ± 10 % RH. Plants were watered three times per week from the bottom until the soil was moist. Plants were additionally fertilised twice per week with 50 ml of Hyponex solution (NPK = 7:6:19, EC = 1.6). As the staring soil can be important in PSF experiments (French *et al.*, 2021), we used the same batch of soil throughout the experiment. Seedling and potting soil from the conditioning phase was bagged and stored at 4 °C for use in the feedback phase (Fig. **1**).

Insect rearing

Worldwide, the most important belowground feeding insect on *B. oleracea* is the specialist chewer cabbage root fly *Delia radicum* L. (Diptera: Anthomyiidae). The female flies deposit

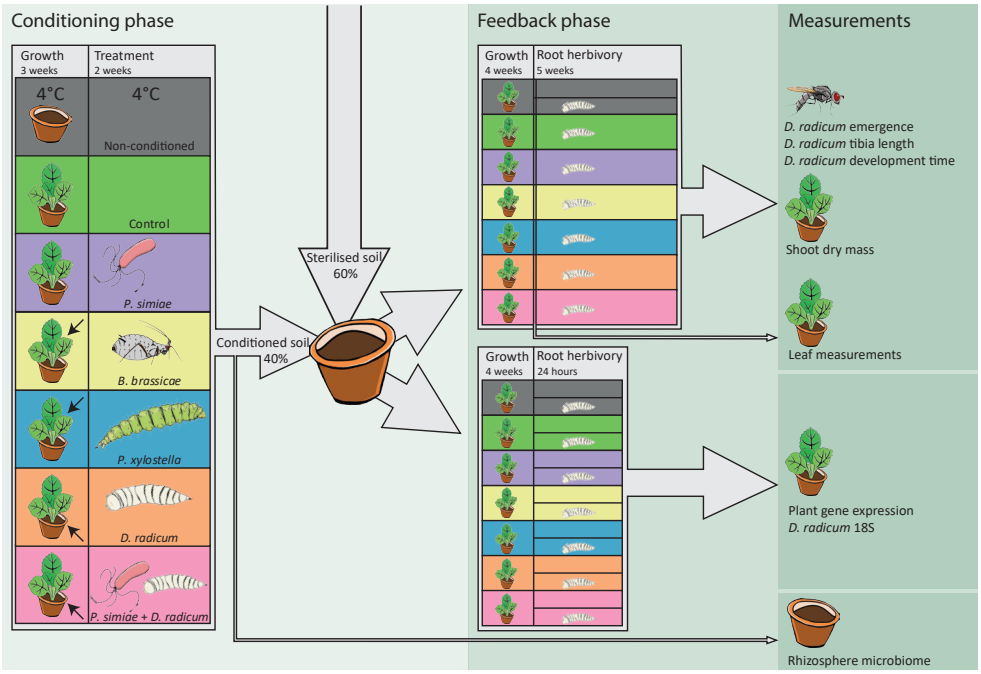

Figure 1. Overview of the experimental design. Soil was conditioned by *Brassica oleracea* plants that, after three weeks of growth, were induced by six treatments represented by coloured boxes in the conditioning phase. The treatments were uninfested plants (no herbivores, green), *Brevicoryne brassicae* (yellow), *Plutella xylostella* (blue), *Delia radicum* (orange), *Pseudomonas simiae* WCS417r (purple), *D. radicum* and *P. simiae* WCS417r (pink). Arrows in the leftmost panel indicate herbivore feeding locations (shoot or root). Additionally, soil was stored at 4 °C to be used as non-conditioned treatment in the feedback phase (gray). After two weeks of induction, plants and insects were removed and rhizosphere microbiome samples were taken. The remaining soil of each treatment was mixed with sterilized soil (40:60 v/v). These soil mixes were used to grow two new sets of *B. oleracea* plants, one set was used for gene expression assessment (24 h post infestation) and the other set for plant and insect assessment (five weeks post infestation). In the feedback phase, plants were exposed to *D. radicum* root herbivory, and the performance of the root herbivore was assessed, as well as plant performance and plant defense-related gene expression.

a cluster of eggs in the soil near the plant stem base. After hatching, the larvae feed in the primary root. The larvae leave the root to pupate in the soil and emerge later as adult flies. Experimental *D. radicum* larvae were reared on rutabaga roots (*B. napus* var. *napobrassica*) at 22 ± 1 °C, 70 % RH and a 16:8 L:D cycle. The flies were caught in Zeewolde in the Netherlands in 2013 and reared in the lab since. Adult flies were fed honey and a 1:1:1 mix of milk powder, sugar and yeast flakes. *Plutella xylostella* L. (Lepidoptera: Plutellidae) were reared on *B. oleracea* var. *gemmifera*. Second instar larvae were used in this experiment. *Brevicoryne brassicae* L. (Hemiptera: Aphididae) were reared on *B. oleracea* var. *gemmifera*, and wingless adults were used as inducers in the experiment. These insects were reared at 22 ± 2 °C, 70% RH and a 16:8 L:D cycle.

Pseudomonas simiae WCS417r growing conditions and solution preparation

The *Pseudomonas simiae* WCS417r (formerly *P. fluorescens*; (Berendsen *et al.*, 2015) bacterial inoculum was prepared by incubating bacteria on King's B (KB) medium agar plates supplemented with rifampicin (25 µg ml^{-1}) for 48 h at 28 °C. Cells were collected and suspended in sterilised 10 mM $MgSO_4$ solution. The suspension's optical density was adjusted to 1 x 10^9 CFU ml^{-1} (OD660 = 1.0).

Conditioning phase: induction with insects and rhizobacteria

After three weeks of growth, plants were infested with insects and/or exposed to *P. simiae* inoculum. Each treatment had 24 replicates divided over four trays with six plants placed in individual pots on saucers, to prevent sharing water between plants. Treatments were *D. radicum*, *D. radicum* plus *P. simiae* WCS417r, *P. xylostella*, *B. brassicae*, *P. simiae* WCS417r alone and control plants (Fig. **1**). Control plants were non-infested and non-inoculated. For infestation with *P. xylostella* (L2) or *B. brassicae* (apterous adults), 10 individuals were carefully transferred to the fourth leaf counted along the stem from the stem base to their respective treatment. To prevent insect contamination between the treatments, the petiole of the infested leaf was wrapped in cotton wool, bagged in a net and fixed with a piece of metal wire. The fourth leaves of the control plants were also wrapped in a similar manner. *Delia radicum* neonates were brushed on the carefully exposed stem base, just below soil level. For treatments that received *P. simiae* WCS417r, bacterial suspension was applied next to the stem with a syringe. Each pot received 20 ml solution, which equals 2×10^{10} CFU, and 8×10^7 CFU g^{-1} of soil. Control plants received 20 ml of sterilised 10 mM $MgSO_4$, applied in a similar manner as treatment plants.

Conditioning phase: soil and microbiome collection

Plants were exposed to insects and rhizobacterial inoculation for two weeks. Aboveground plant parts and primary roots were then removed from the soil. For soil microbiome analysis, around 3 g of secondary roots and root-attached soil were pooled from the six plants in each tray. Thus, the six plants in each tray were considered one biological replicate. Pooled roots were collected in 50 ml tubes containing 25 ml of sterilised buffer solution (6.33 g l^{-1} NaH_2PO_4 and 10.96 g l^{-1} NaH_2PO_4 x $2H_2O$). Tubes were vigorously shaken for 30 s, and centrifuged for 7 min at 3700 g. Supernatant was removed, as well as large chunks of root with sterilised tweezers. The soil slurry was transferred with a sterilised spoon into 1.5 ml tubes, and centrifuged for 5 min at 11,000 g. Supernatant was removed and samples were then stored at −80 °C. After taking microbiome samples, soils of all plants from the same

treatment were homogenised by mixing by hand, using clean gloves for each treatment. For soils conditioned with plants infested with *D. radicum*, special care was taken to remove any larvae from the soil.

Feedback phase: setup and measurements

Soil from the conditioning phase was mixed with γ-irradiated soil (>25 KGray, Steris, Ede, the Netherlands) in a ratio of 40 % conditioned soil: 60 % sterilised soil (v/v). The soil mixture was divided over 1 l pots, into 30 replicates per feedback treatment. We are aware of the discussion between mixed soil sampling strategy and independent soil sampling strategy in PSF experiments (Reinhart & Rinella, 2016; Cahill *et al.*, 2017; Gundale *et al.*, 2019). Since our experiment was performed in pots with similar starting soil, we believe the discussion is less applicable to our study.

A soil treatment was added consisting of pots containing a 40:60 mix of sterilised soil together with the original potting soil that was used in the conditioning phase (stored for 6 weeks at 4 °C), to include a treatment consisting of soil with a microbiome similar to that of the soil used as starting material in the conditioning phase; this treatment is hereafter referred to as 'non-conditioned'. *Brassica oleracea* seeds were sown on seedling soil, that had been stored at 4 °C from the start of the experiment, to expose the seeds to a similar microbiome as the first set of plants. After three days, the seedlings were transplanted to the feedback phase pots. Plants were grown for 25 days under the same greenhouse settings as during the conditioning phase. After one week of plant growth the pots were provided with sticks to later support insect nets. Plants were divided into two sets, one for gene expression analysis after 24 h of exposure to *D. radicum* larvae and the other for assessing plant and *D. radicum* performance.

Feedback phase: plant and root herbivore performance

After four weeks of growth, plants were infested with 10 neonate *D. radicum* larvae. Half of the plants grown on non-conditioned soil were infested with larvae, to assess effects of *D. radicum* on plant performance. The larval infestation was performed as described above. For insect performance measurements, all plants were individually covered with a mesh bag 10 days after infestation. Plants were inspected daily for emerged *D. radicum* adults, which were then collected, frozen, and stored at −20 °C. *Delia radicum* size was determined by measuring hind tibia length with a digital microscope (Dino-Lite Edge digital microscope, Taiwan) as a proxy for fly body size (Soler *et al.*, 2007; Karssemeijer *et al.*, 2020 - **chapter 2**). Developmental time was recorded as the time between larval infestation and adult emergence.

Plant performance in the feedback phase was assessed as leaf area of the second leaf after three weeks of plant growth as a proxy for plant size. Since measuring the leaf area might damage the leaf, we measured only leaf width and length in experimental plants. We then calculated the leaf area from the leaf measurements using the following formula: length x width x leaf area coefficient = leaf area. The coefficient was calculated by measuring width, length, and leaf area of ten *B. oleracea* non-experimental plants' leaves of similar size using LeafByte (Getman-Pickering *et al.*, 2020). Five weeks after infestation, the plant shoot was harvested and its biomass determined. Dry shoot biomass was recorded to the nearest 0.01 g (DK-6200-C-M, Allscales, USA) after drying at 105 °C for 24 h.

Plant defence-related gene expression analysis

After four weeks of growth on conditioned soil, half of the plants were infested with 10 neonate *D. radicum* larvae (Fig. **1**), to assess plant defence gene expression under plant-soil feedback conditions. After 24 h of infestation, primary roots were harvested by uprooting the plants, cutting off secondary roots, and freezing the primary root directly in liquid nitrogen. One leaf disk from three leaves per plant was collected with a 1 cm diameter metal puncher. Samples were pooled for three plants, and immediately frozen in liquid nitrogen to form one replicate. Samples were stored at −80 °C.

Frozen samples were ground in liquid nitrogen, with a mortar and pestle for roots, or with a small pestle directly in the collection tube for leaves. Plant RNA was extracted with Isolate II Plant RNA kit (GCbiotech, the Netherlands) following the manufacturer's instructions, and converted to cDNA (SensiFAST, Bioline). Quantitative polymerase chain reaction (qPCR) analysis was performed to test transcript levels of genes of interest (CFX96™ Real-Time System, Bio-rad, Hercules, CA, USA). The primer efficiency was calculated with qPCR by determining a standard curve with a dilution series. Reference genes *SAR1a*, *Btub*, *Act-2*, *PER4*, *GADHP* and *EF1a* were tested on 10 randomly selected samples from both roots and leaves to determine the optimal combination of reference genes using GeNorm (Vandesompele *et al.*, 2002) in qbase+ v.3.1 (Biogazelle, Zwijnaarde, Belgium). For roots, *Act-2* and *SAR1a* were used as reference genes, while for leaves *Btub* and *SAR1a* were used. We analysed transcript levels in roots for *LOX6*, *MYB28*, *CYP81F1*, *MYB72* and *PDR9*, and in leaves for *LOX2* and *MYB28* (Table **S1**). For *MYB72* and *PDR9*, two genes studied in *Arabidopsis* (At1g56160 and At3g53480, respectively), orthologous genes in *B. oleracea* were identified using the integrative orthology finder in PLAZA (Van Bel *et al.*, 2017).

Delia radicum biomass assessment

One of the main challenges when working with *D. radicum* is the difficulty of assessing larval performance. The larvae are small and colourless, and during the first days of feeding they dig into the root, making it difficult to find them back. To overcome this obstacle, we developed species-specific primers (see Supporting Information Methods **S1**, Table **S2**, Fig. **S1**). These primers specifically target the 18S region of *D. radicum*, without amplifying non-targets such as those found in fungus gnats and nematodes which may occur in the experimental soil. We used these primers in the root samples collected for gene expression analysis (**Fig. 1**) as a proxy of larval performance and normalised the quantity relative to the plant reference genes *Act-2* and *SAR1a*.

Soil microbiome analysis

Total genomic DNA (gDNA) from 0.25 ± 0.01 g of pooled rhizosphere soil was extracted using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The nucleic acid concentration and purity of samples were quantified with a spectrophotometer (DeNovix, Delaware, United States). For bacteria, the V4 region of the *16S* gene was amplified using the 515F/806R primers (Caporaso *et al.*, 2011) (Roche FastStart High Fi, 58 °C, 26 cycles). For fungi, the ITS2 region was amplified using the fITS9/ITS4R primers (Ihrmark *et al.*, 2012) (Qiagen HotStarTaq, 52 °C, 33 cycles). Microbial DNA was sequenced by Illumina MiSeq, 250bp paired-end, to a depth of 79,138 to 166,482 reads per sample. Amplification, library preparation and sequencing were performed by Génome Québec (Montreal, Canada). Raw sequencing data is available from the European Nucleotide Archive (https://www.ebi.ac.uk/ena/), under study accession (PRJEB47452).

Raw fastq files were processed using cutadapt (Martin, 2011) and the DADA2 pipeline (Callahan *et al.*, 2016). The code used for sample processing is available in the Supporting Information of the online publication of this chapter. After processing, 62,735 to 97,854 bacterial reads and 47,339 to 98,457 fungal reads remained per sample. Taxonomy was assigned using the SILVA v138 database (Quast *et al.*, 2013) for bacteria and the UNITE v8.2 database (Nilsson *et al.*, 2018) for fungi. We filtered ASVs (Amplicon Sequence Variants) with too few occurrences using the effective sample approach in metagenomeSeq (Paulson *et al.*, 2013).

Statistical analysis

Statistical analysis was performed in R, version 4.0.0 (R Core Development Team, 2017), with R studio version 1.2.5042. For microbiome analysis, counts were normalised using metagenomeSeq (Paulson *et al.*, 2013). Principle coordinate analysis (PCoA) was performed using Bray-Curtis dissimilarity in phyloseq (McMurdie & Holmes, 2013). PERMANOVA was done with 99,999 permutations using Bray-Curtis dissimilarity with the adonis function (Oksanen *et al.*, 2007), post hoc analysis was performed using the RVAideMemoire package (Hervé, 2020). We tested whether differences in variance could have caused significant differences using permutest, which were non-significant for both bacterial and fungal analyses, indicating that the PERMANOVA results are valid. Differential ASVs were calculated using DESeq2 (Love *et al.*, 2014), by comparing each treatment to the non-infested and non-inoculated group with a false discovery rate of 0.05.

We used the packages tidyverse, lme4, emmeans, lmtest, lattice and fitdistrplus for plant and insect data (Zeileis & Hothorn, 2002; Sarkar, 2008; Bates *et al.*, 2015; Delignette-Muller & Dutang, 2015; Lenth *et al.*, 2018; Wickham *et al.*, 2019). The distribution of each dataset was explored with QQ-plots, histograms, Shapiro-Wilk test and the function descdist with 2000 bootstrapped values. Analysis of leaf length, plant shoot dry biomass and gene expression levels was performed with generalized linear models either using Gamma or Gaussian distributions. Development time, fly emergence and hind tibia length of *D. radicum* were analysed by using generalized linear mixed models with Poisson, binomial and gamma distributions, respectively. Plant ID was used as a random factor to avoid pseudoreplication. Models were compared and chosen based on Akaike Information Criterion (AIC) values. In the case of multiple fixed factors, the best model that included both factors ('soil treatment' and 'sex' or 'time') was chosen. Significance of fixed factors was assessed using the lrtest function.

Results

Insect herbivore-induced alterations in the plant rhizosphere microbiome

Rhizospheres from plants in the conditioning phase were extracted and analysed for bacterial and fungal communities. We found 1311 bacterial and 187 fungal Amplicon Sequence Variants (ASVs), the majority of which belong to the phyla Proteobacteria and Ascomycota, respectively (Fig. **S2**).

Multivariate analysis revealed that microbial communities clustered by the presence and feeding location of inducing herbivores (Fig. **2**, Table **1**). The bacterial communities in rhizospheres of plants induced by root-feeding *D. radicum* clustered separately from those of plants induced by the shoot-feeding insects *B. brassicae* and *P. xylostella* and no herbivory (hereafter root herbivory, shoot herbivory, and no herbivory). These differences were confirmed by PERMANOVA (Table **1**), which showed that these three groups indeed differ in their bacterial communities (no herbivory – shoot herbivory: $F = 2.77$, $p < 0.001$, no herbivory – root herbivory: $F = 2.03$, $p < 0.001$, shoot herbivory – root herbivory: $F = 3.20$, $p < 0.001$). Within these three groups, treatments did not differ from each other (Control – *P. simiae*: $F = 1.17$, $p = 0.33$, *B. brassicae* – *P. xylostella*: $F = 0.84$, $p = 0.89$; *D. radicum* – *P. simiae* + *D. radicum*: $F = 1.04$, $p = 0.37$). Fungi were also affected by the treatments, rhizosphere fungal communities from plants treated with root herbivory separated from the other samples on the first principal component (Fig. **2**, Table **1**). Rhizosphere fungal communities were strongly affected by root herbivory, and only slightly by shoot herbivory (no herbivory – shoot herbivory: $F = 1.47$, $p = 0.01$; no herbivory – root herbivory: $F = 2.34$, $p < 0.001$; shoot herbivory – root herbivory: $F = 2.48$, $p < 0.001$). No differences were observed within the groups of shoot herbivory, root herbivory, or no herbivory (Control – *P. simiae*: $F = 0.99$, $p = 0.64$; *B. brassicae* – *P. xylostella*: $F = 0.86$, $p = 0.77$; *D. radicum* – *P. simiae* + *D. radicum*: $F = 1.42$, $p = 0.09$). Thus, feeding on either shoot or root tissue by herbivores appears to be an important factor in shaping the rhizosphere microbial community.

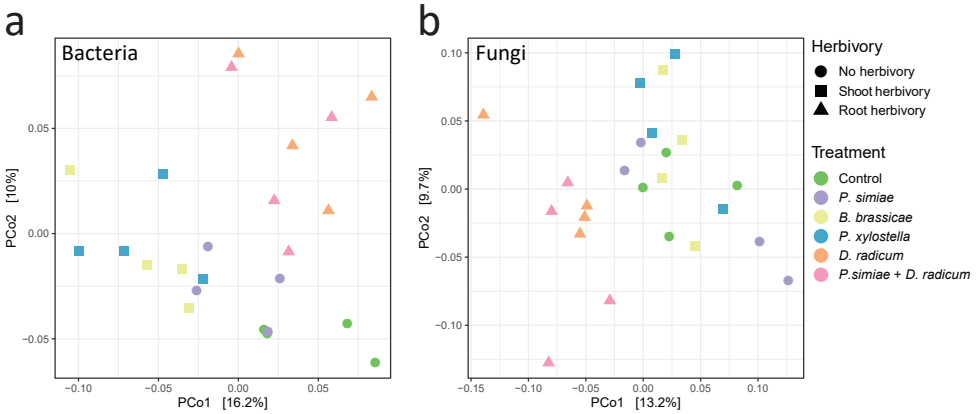

Figure 2. Principal Coordinate Analysis (PCoA) of bacterial (**a**) and fungal (**b**) rhizosphere communities. *Brassica oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. Control plants were non-infested and non-inoculated. After two weeks, rhizosphere samples were collected and pooled from six plants. Bacterial 16S and fungal ITS2 regions were sequenced. Colors distinguish no herbivory, shoot or root herbivory; treatments are represented by shapes.

Table 1. Effects of treatment and herbivory on bacterial and fungal communities, where herbivory consisted of six treatments; *Brassica oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella*, *Delia radicum*, inoculated with *Pseudomonas simiae* WCS417r, infested with *D. radicum* and inoculated with *P. simiae*, or non-infested/non-inoculated plants used as control. These treatments were grouped into shoot-, root-, or no herbivory to form the herbivory factor.

Variable	Model type	Model	*F*	R^2	*P*-value
Bacterial community	PERMANOVA	~Treatment	1.68	0.32	< 0.001
		~Herbivory	2.65	0.20	< 0.001
Fungal community	PERMANOVA	~Treatment	1.49	0.29	< 0.001
		~Herbivory	2.09	0.17	< 0.001

To identify specific changes caused by our treatments, we analysed differentially abundant ASVs (Fig. **3**). Based on visual representation of the Euclidean distance hierarchical tree, for both bacteria and fungi, rhizospheres of plants treated with root herbivory were separated from the shoot herbivory and no herbivory groups. Rhizospheres of plants treated with shoot herbivores also clustered in terms of bacteria, but not for fungal ASVs. For bacteria, most ASVs were differentially abundant between rhizospheres of plants treated with *B. brassicae* and *P. xylostella* and control plants. For fungi, the largest numbers of ASVs were found for plants infested by *D. radicum* and *P. simiae* + *D. radicum*.

A cluster of five bacterial ASVs is present in rhizospheres of plants treated with root herbivory, while being absent in the control treatment; these include two members of the family Enterobacteriaceae, a *Klebsiella*, a *Pseudomonas*, and *Verruccomicrobiom spinosum*. Among the fungal ASVs, *Candida tropicalis* has the most striking difference between treatments, and was strongly associated with rhizospheres of plants treated with *D. radicum*. Several differentially abundant ASVs, both bacteria and fungi, were negatively affected by infestation of the plants by *D. radicum* (without *P. simiae*); these ASVs are members of the bacterial families Nocardiaceae and Chitinophagaceae, genera *Bryobacter*, *Chryseobacterium* and *Roseiarcus*, and fungal order Helotiales, class Microbotryomycetes, and species *Candida palmioleophila* and *Coniochaeta fasciculata*.

Further, a group of highly abundant bacterial ASVs were quantitatively affected in the rhizospheres of *P. xylostella* and *B. brassicae*-treated plants compared to control plants. For instance, a member of the genus *Rhodanobacter* was the most abundant ASV in the overall bacterial community, and it was reduced from an average of 3700 normalised counts (4.8% relative abundance) in the rhizosphere of control plants, to 2600 (3.9% relative abundance) and 2500 (3.8% relative abundance) in rhizospheres of *P. xylostella* and *B. brassicae*-treated plants, respectively. Interestingly, several bacterial ASVs were depleted specifically in

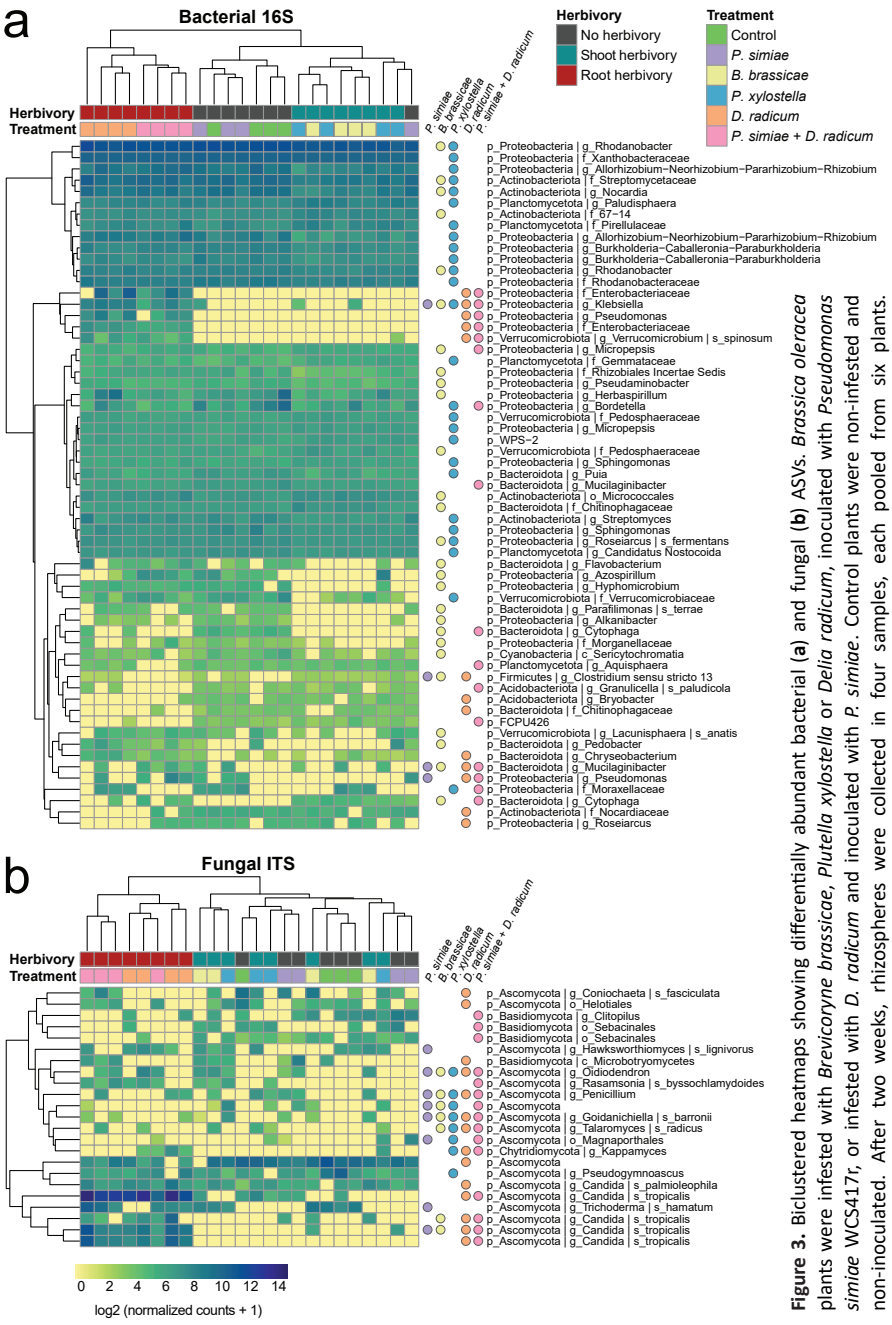

Figure 3. Biclustered heatmaps showing differentially abundant bacterial (**a**) and fungal (**b**) ASVs. *Brassica oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. Control plants were non-infested and non-inoculated. After two weeks, rhizospheres were collected in four samples, each pooled from six plants. Bacterial 16S and fungal ITS2 regions were sequenced. Differentially abundant ASVs were selected by DESeq2, with a threshold of FDR < 0.05 difference between treatment and control. Colored circles right of the heatmaps show whether the abundance of the ASV is significantly different between that treatment and control. Clustering by shoot and root herbivory and treatment is based on Euclidean distance. Colors show $\log_2$ (normalized count + 1).

Table 2. Effects of the factors soil treatment, root herbivory and sex on *Delia radicum* performance variables, and effects on plant performance and gene expression of *Brassica oleracea.*

Variable	Model type	Model	Factor	χ^2	df	*P*-value
D. radicum emergence	GLMM Binomial	~ Soil treatment + PlantID*	Soil treatment	25.62	6	< 0.001
D. radicum tibia length	LMM Normal	~ Soil treatment + Sex + PlantID*	Soil treatment	14.18	6	0.028
			Sex	68.87	1	< 0.001
D. radicum 18S	GLM Gamma	~ Soil treatment	Soil treatment	15.56	6	0.016
Leaf area	GLM Gamma	~ Soil treatment	Soil treatment	383.57	6	< 0.001
Plant dry mass	GLM Gamma	~ Soil treatment	Soil treatment	336.44	7	< 0.001
Root *LOX6*	GLM Gamma	~ Soil treatment + Root herbivory	Soil treatment	6.13	6	0.408
			Root herbivory	33.27	1	< 0.001
Root *MYB28*	GLM Gamma	~ Soil treatment × Root herbivory	Soil treatment	1.82	6	0.935
			Root herbivory	125.31	1	< 0.001
			Interaction	27.84	6	< 0.001
Root *CYP81F4*	GLM Gamma	~ Soil treatment × Root herbivory	Soil treatment	1.33	6	0.97
			Root herbivory	105.76	1	< 0.001
			Interaction	15.09	6	0.02
Root *MYB72*	GLM Gamma	~ Soil treatment + Root herbivory	Soil treatment	20.27	6	0.002
			Root herbivory	0.57	1	0.451
Root *PDR9*	GLM Gamma	~ Soil treatment × Root herbivory	Soil treatment	31.83	6	< 0.001
			Root herbivory	20.91	1	< 0.001
			Interaction	23.71	6	< 0.001
Leaf *LOX2*	LM Normal	~ Soil treatment × Root herbivory	Soil treatment	6.75	6	0.344
			Root herbivory	37.86	1	< 0.001
			Interaction	14.91	6	0.021
Leaf *MYB28*	GLM Gamma	~ Soil treatment × Root herbivory	Soil treatment	30.26	6	< 0.001
			Root herbivory	3.46	1	0.063
			Interaction	8.98	6	0.175

(G)L(M)M: (Generalized) Linear (Mixed) Model.

*PlantID was included in the models as a random factor to avoid pseudoreplication as multiple flies emerged from each plant.

rhizospheres of *B. brassicae*-treated plants compared to rhizosphere of control plants, including members of the genera *Flavobacterium*, *Azospirillum*, *Hyphomicrobium*, *Alkanibacter*, *Cytophaga*, and the species *Parafilimonas terrae*. Rhizospheres of plants inoculated with *P. simiae* only differed from those of non-infested/non-inoculated plants in four bacterial ASVs, while eight fungal ASVs were affected. Of those four bacterial ASVs in rhizospheres of *P. simiae*-inoculated plants, one is a *Pseudomonas* fully matching *P. simiae* WCS417r through a BLAST search. However, the sequenced 16S fragments are

identical to many strains in the related group Pseudomonads. Therefore we cannot verify that these fragments are explicitly from the strain used in the experiment; without specific bacterial testing, we cannot be certain of the origin of our recovered ASV. Two fungal ASVs, *Hawksworthiomyces lignovirorous* and *Trichoderma hamatum*, are specifically depleted in rhizospheres of plants inoculated with *P. simiae*.

Plant-soil feedback effects on plant performance

To assess whether rhizosphere microbiome alterations affected consecutively growing plants and their resistance to insect herbivores, *B. oleracea* plants were grown in the same soil previously conditioned by conspecific plants exposed to different treatments. The surface area of the second leaf was affected by soil conditioning (Fig. **4a**, Table **2**): plants grown on conditioned soil had smaller leaves. Plant shoot dry mass was also affected by soil conditioning (Fig. **4b**, Table **2**), where dry shoot biomass of plants grown on conditioned soil was lower compared to plants grown on non-conditioned soils. Plants grown on soil conditioned by plants inoculated with *P. simiae* were smaller compared to plants grown on soil conditioned by non-infested/non-inoculated plants. Plants grown on soil conditioned by plants treated with *P. xylostella* were larger, both in terms of leaf size and biomass.

Plant-soil feedback effects on *Delia radicum* performance

To examine belowground plant resistance in a plant-soil feedback context, we infested *B. oleracea* plants grown in conditioned soils with *D. radicum* larvae. Overall, *D. radicum* adult emergence was low in the experiment, on average 11.4% (N_{total} = 1970) of larvae developed into adults. In addition to these performance measurements, in the plants used for gene expression analysis, we examined larval performance through analysis of *D. radicum* 18S ribosomal RNA.

Emergence of *D. radicum* was affected by soil conditioning in a treatment-specific way (Fig. **5a**, Table **2**). Fewer flies emerged from plants grown on soil conditioned by plants infested by *D. radicum* compared to plants grown on soils conditioned by plants treated with *B. brassicae, P. simiae* or *D. radicum* together with *P. simiae*. Tibia length of adult flies was affected by soil conditioning (Fig. **5b**, Table **2**). Flies with smaller tibia length emerged from plants grown on soil conditioned by plants infested with *P. xylostella* compared to flies that emerged from plants grown on non-conditioned soil. Fly development time was similar for all treatments (data not shown).

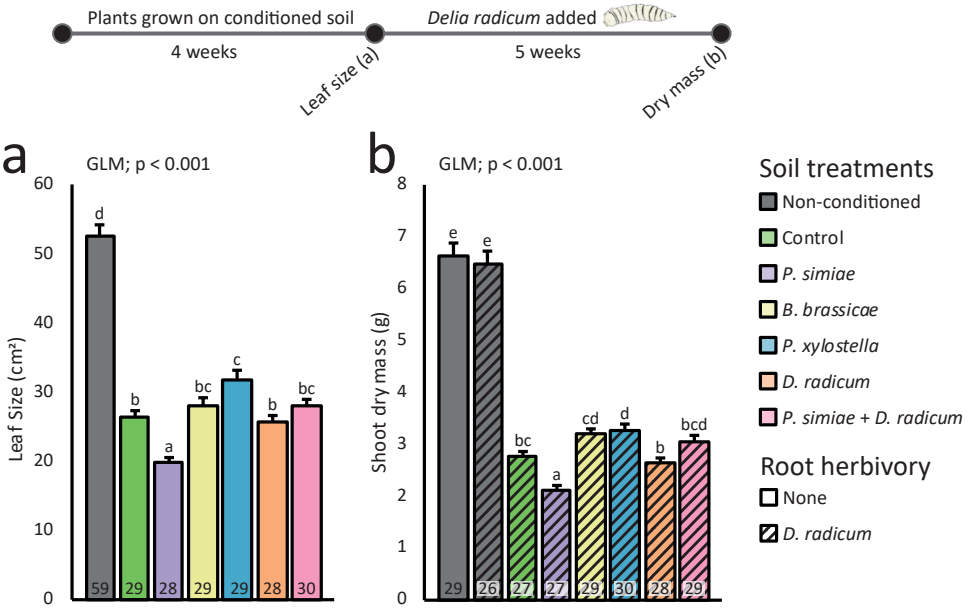

Figure 4. Size of leaf number 2 counted from the bottom on the stem (**a**) and dry shoot biomass after *Delia radicum* infestation (**b**) of *Brassica oleracea* plants grown in soil conditioned by conspecific plants exposed to herbivory, rhizobacterial inoculation or a combination. In the conditioning phase, *B. oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, or inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. Control plants were non-infested and non-inoculated. Plants were removed and the same soil was used to grow new *B. oleracea* plants. After three weeks of growth, leaf size of these new plants was quantified before infestation with insect herbivores (**a**). After five weeks of infestation, the plants were harvested, and dry shoot biomass was measured (**b**). All plants in the feedback phase, and a subset of plants on non-conditioned soil, were induced with 10 *D. radicum* larvae after three weeks of growth. Numbers in bars represent the number of included plants, bars with different letters within a panel are significantly different from one another (Tukey's HSD, $\alpha = 0.05$), and bars show mean + SE. GLM: Generalized Linear Model.

In the set of plants used for gene expression analysis 24 h post infestation, we quantified *D. radicum* 18S ribosomal RNA relative to plant reference genes as a proxy of *D. radicum* performance (Fig. **5c**, Table **2**). Relative quantities of *D. radicum* 18S were affected by soil-conditioning treatments. This analysis supports the observation that *D. radicum* performance was reduced in plants grown on soil conditioned by *D. radicum* compared to plants grown on non-conditioned soil or soil conditioned by control plants. Taken together, the results show that *D. radicum* was negatively affected when feeding on plants that had been growing in soil conditioned by plants also exposed to feeding by conspecific larvae.

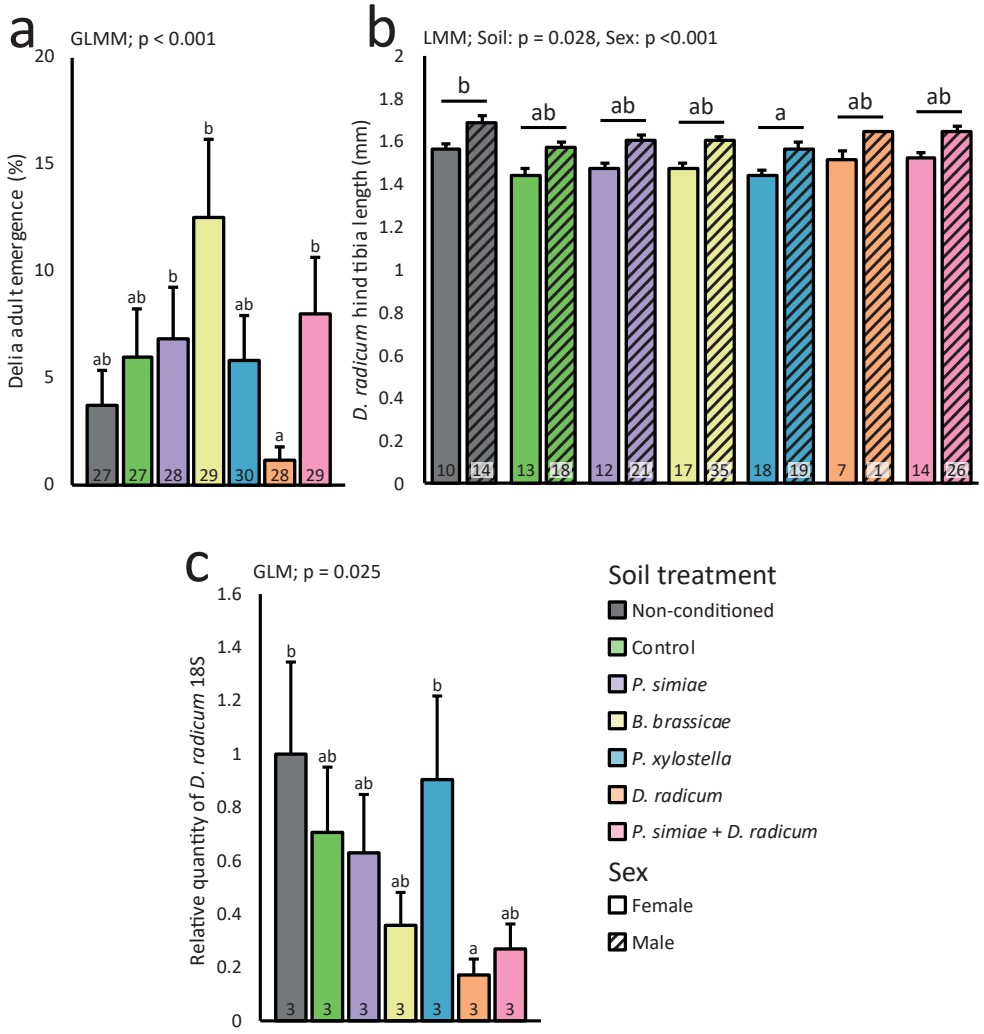

Figure 5. *Delia radicum* adult emergence (**a**), hind tibia length (**b**), and relative quantity of *D. radicum* 18S rRNA (**c**) in the primary roots of *Brassica oleracea* plants grown in soil conditioned by conspecific plants exposed to herbivory, rhizobacterial inoculation or a combination. In the conditioning phase, *B. oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *D. radicum*, or inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. Control plants were non-infested and non-inoculated. Plants were removed and the same soil was used to grow new *B. oleracea* plants. After three weeks of growth, these new plants were infested with *D. radicum* larvae, emerging flies counted and their hind tibia length measured, and in separate experimental plants the amount of *D. radicum* 18S was assessed 24 h after infestation. Numbers in bars represent the number of plants (**a**) flies (**b**), or pools of four plants (**c**), bars with different letters are significantly different from each other (Tukey's HSD, α = 0.05), and bars show mean + SE. Due to low sample size, no SE could be calculated for males in the *D. radicum* treatment (orange striped bar). Soil: Soil conditioning treatment, (G)LMM: (Generalized) Linear Mixed Model.

Gene expression in response to *Delia radicum* infestation and plant-soil feedback treatments

We assessed primary root defence responses to herbivory by *D. radicum* in plants grown on conditioned and non-conditioned soil, measured after 24 h of *D. radicum* infestation of the primary root. Expression in the roots of *LOX6*, a gene involved in JA biosynthesis, was induced by *D. radicum* regardless of soil conditioning (Fig. **6a**, Table **2**). Root transcript levels of *MYB28*, involved in the biosynthesis of aliphatic glucosinolates, were downregulated by *D. radicum* infestation (Fig. **6b**, Table **2**). The soil conditioning treatments did not affect root *MYB28* expression, but there was a significant interaction effect between *D. radicum* infestation and soil conditioning. When infested with *D. radicum*, transcript levels of *MYB28* were lower in plants grown on conditioned soils compared to non-conditioned. In contrast to *MYB28* downregulation by *D. radicum* infestation, mRNA levels of *CYP81F4*, encoding an enzyme involved in indole glucosinolates biosynthesis, were strongly upregulated by infestation. Type of soil conditioning did not influence *CYP81F4* transcript levels, but there was an interaction between *D. radicum* and soil conditioning (Fig. **6c**, Table **2**).

Expression of root *MYB72*, a transcription factor involved in induced systemic resistance and iron acquisition (van der Ent *et al.*, 2008; Palmer *et al.*, 2013), was affected by soil conditioning in a treatment-specific way, but not by *D. radicum* infestation (Fig. **6d**, Table **2**). Transcript levels of *PDR9*, a gene encoding a transporter involved in root exudation of coumarins, were affected by both soil treatment and *D. radicum* infestation, and there was an interaction between soil treatment and *D. radicum* infestation (Fig. **6e**, Table **2**). When no *D. radicum* was present, expression of *PDR9* was upregulated in primary roots of plants subjected to all soil conditioning treatments compared to plants grown on non-conditioned soil, especially when soil was conditioned by plants infested with *D. radicum*. This effect was attenuated upon *D. radicum* infestation, in which case transcript levels of *PDR9* did not differ between soil conditioning treatments.

Leaf transcript levels of *LOX2*, a marker gene for JA biosynthesis expressed in the shoot, were increased by root herbivory but not by soil conditioning; there was a significant interaction effect between soil conditioning and root herbivory (Fig. **S3a**, Table **2**). *MYB28* transcript levels in leaves were affected by soil conditioning treatments (Fig. **S3b**, Table **2**), but not by *D. radicum* infestation.

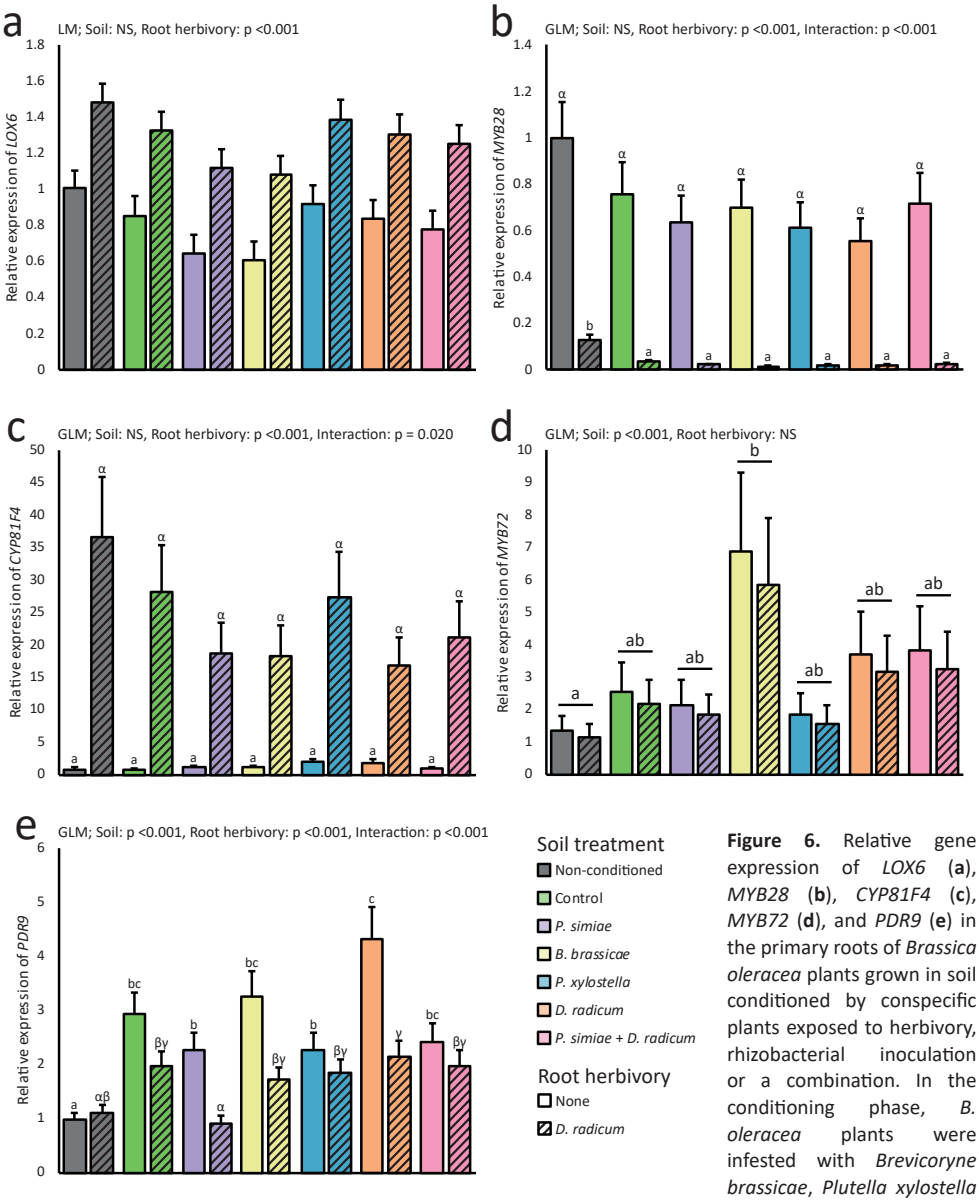

Figure 6. Relative gene expression of *LOX6* (**a**), *MYB28* (**b**), *CYP81F4* (**c**), *MYB72* (**d**), and *PDR9* (**e**) in the primary roots of *Brassica oleracea* plants grown in soil conditioned by conspecific plants exposed to herbivory, rhizobacterial inoculation or a combination. In the conditioning phase, *B. oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *D. radicum*, or inoculated with *Pseudomonas simiae* WCS417r, or infested with *D. radicum* and inoculated with *P. simiae*. Control plants were non-infested and non-inoculated. Plants were removed and the same soil was used to grow new *B. oleracea* plants. After three weeks of growth, half of these plants were infested with *D. radicum* (striped bars). All bars are set relative to the gene expression levels in primary roots of plants of non-infested plants grown in non-conditioned soil (grey bar). Bars with different letters are significantly different from one another, within *D. radicum* infested plants (Greek alphabet) or plants that did not receive root herbivores (Roman alphabet; Tukey's HSD, $\alpha = 0.05$), and bars show mean + SE. Soil: Soil conditioning treatment, NS: Not significant, (G)LM: (Generalized) Linear Model. N = 3 or 4 replicates of 3 pooled plants.

Discussion

Our study shows that the plant root microbiome is affected by insect attack to the plant and that plant growth and insect resistance are influenced via PSF mechanisms. Our results demonstrate that the bacterial rhizosphere community is differentially affected by shoot and root herbivory, whereas the fungal rhizosphere community is mostly affected by root herbivory. Although previous research shows that plant defence against shoot-feeding insects can be altered through PSF (Kostenko *et al.*, 2012; Bezemer *et al.*, 2013; Kos *et al.*, 2015a; Kos *et al.*, 2015b; Hu *et al.*, 2018; Pineda *et al.*, 2020), we here show novel evidence that the root-feeding insect *D. radicum* is negatively affected by conspecific feeding through PSF. While our data do not allow an unambiguous link to be established between the rhizosphere microbiome in the conditioning phase and the results in the feedback phase, it is most plausible that microbial changes underlie the reported PSF effects on plant growth and insect resistance.

Rhizosphere microbiome composition is differentially affected by shoot and root herbivory

We observed that herbivores feeding on the root or the shoot influenced the rhizosphere microbial community. Multivariate analysis revealed that bacterial rhizosphere communities were separated into three groups: (1) plants exposed to shoot herbivory, (2) plants exposed to root herbivory and (3) non-infested plants. We further observed that the fungal rhizosphere community was similar between plants fed on by shoot-feeding insects and non-infested plants, but was different from the fungal community of plants with root-feeding *D. radicum*. Thus, our results show that root herbivory has more impact on the plant rhizosphere community than the addition of *P. simiae* WCS417r. A previous study showed that *D. radicum* herbivory led to only minor changes in the fungal community, but caused major changes in both endo- and rhizosphere bacterial communities of oilseed rape, *B. napus* (Ourry *et al.*, 2018). Interestingly, our results show that *D. radicum* herbivory strongly increased the abundance of the soil yeast *Candida tropicalis*, a species containing known plant growth promoting strains (Amprayn *et al.*, 2012). None of the fungal ASVs that were different between the treatments are known to have entomopathogenic properties, although this was not directly studied for most of these species. Rhizospheres of *D. radicum*-infested plants showed an accumulation of several bacterial taxa (Enterobacteriaceae, *Klebsiella*, and *Pseudomonas*) that were previously found to be associated with the *D. radicum* gut microbiome (Lukwinski *et al.*, 2006; van den Bosch & Welte, 2020). The gut microbiome of another much-studied root herbivore, western corn rootworm, is thought to consist mostly of microbes selected from the surrounding soil (Dematheis *et al.*, 2012;

Ludwick *et al.*, 2019). Our findings hint at the interesting possibility of direct interactions between the microbiomes of the plant rhizosphere and the root herbivore gut. Perhaps, by selecting specific microbes from the soil and excreting them, root herbivores can influence the rhizosphere microbiome.

Herbivory by shoot-feeding insects was previously shown to affect the rhizosphere community, in line with our results (Yang *et al.*, 2011; Lee *et al.*, 2012; Bezemer *et al.*, 2013; Kong *et al.*, 2016; Zytynska *et al.*, 2020; Malacrinò *et al.*, 2021). On the other hand, some studies report similar rhizosphere microbiomes between shoot-herbivore-infested and non-infested plants (O'Brien *et al.*, 2018; Malacrinò *et al.*, 2021). The variation seen in the literature regarding rhizosphere microbiome responses to shoot herbivory could be explained by factors such as plant- and insect-specific responses, or different bulk soil bacterial communities in the starting soil.

Plant-soil feedback by differently treated conspecifics has adverse effects on plant growth

In the feedback phase of our experiment, we observed treatment-dependent responses in plant growth when grown on conditioned soils. Regardless of the treatment, plant growth was inhibited on conditioned soil compared to non-conditioned soil. Generally, such unfavorable legacy from plant conspecifics is termed negative PSF. In our experiment, shoot herbivory by *P. xylostella* on plants during the conditioning phase led to increased growth of plants in the feedback phase, compared to plants grown in soil conditioned by plants without herbivores. Hence, herbivory can affect not only the attacked plant, but also the growth of future plants growing in the same soil, via soil-mediated effects.

It is challenging to directly link changes in the rhizosphere microbiome of plants in the conditioning phase of our experiment with findings in the feedback phase. One potential discrepancy is that we sampled rhizosphere soil for microbiome analysis but transferred all soil in the pot to the feedback phase. The soil in the pots was completely colonised by roots at the end of the conditioning phase, therefore we believe that the overall bacterial community we transferred is representative of the rhizosphere community. Several PSF mechanisms other than the transfer of microbes could have contributed to our results. Fresh litter, such as fine roots, can stimulate the microbial activity (Fontaine et al. 2003), but can also negatively affect plant growth through the release of phytotoxic (allelopathic) and autotoxic compounds when decomposing (Bonanomi *et al.*, 2006). Extracellular self-DNA (eDNA) is also released from decomposing tissue, and can exert plant growth inhibition on grasses, forbs and *A. thaliana in vitro* (Mazzoleni *et al.*, 2015). These PSF mechanisms are

likely to have contributed to our results to some extent, as root fragments were present in the soil we transferred.

Surprisingly, the performance of *B. oleracea* was drastically decreased when grown in soil on which previously growing plants had been inoculated with *P. simiae*, compared to the other soil conditioning treatments. Root herbivory by *D. radicum* together with *P. simiae* inoculation of the plants during the conditioning phase restored plant biomass to a certain degree in the feedback phase. Although this PGPR strain is usually considered a beneficial rhizobacterium when applied to plants, including *B*. *oleracea* (Friman *et al.*, 2020), our results suggest that this beneficial effect may not persist through PSF. Notably, there are reports of rhizobacteria causing effects varying from plant-growth promotion to inhibition, depending on *e.g.* phosphate availability or rhizobacterial population density (Ciccillo *et al.*, 2002; Morcillo *et al.*, 2020). Although plant growth may have been boosted in two weeks of the conditioning phase, we regard this period as too short to leave lasting nutrient deficiencies in the soil, and therefore unlikely to have influenced our results. Further, we assume that the nutrient availability was sufficient for the experimental plants due to regular fertilization in our experiments and hypothesise that changes in the microbiome underlie the reduction in growth.

In contrast to our hypothesis, we found that inoculation with the rhizobacterium *P. simiae* did not affect overall microbial communities in the rhizosphere. Although there are studies that find an altered root community after addition of individual rhizobacterial species, others report no such effects (Herschkovitz *et al.*, 2005; Gadhave *et al.*, 2018; Wang *et al.*, 2018; Zytynska *et al.*, 2020). Even though the microbial community composition was not affected by the addition of *P. simiae* WCS417, the abundance of several distinct species was changed. It has been demonstrated that only a set of three bacterial soil species are sufficient to increase resistance in *A. thaliana* against a foliar fungal pathogen (Berendsen *et al.*, 2018). For example, *Trichoderma hamatum* was absent in rhizospheres of *P. simiae*-induced plants while it was present in the other treatments. This species is a known growth-promoting fungal species in *e.g.* pepper (Mao *et al.*, 2020). In this way, the addition of *P. simiae* may have suppressed other beneficial microbes in the rhizosphere, leading to a net negative effect on plant growth in our study.

Root herbivores can be affected via plant-soil feedback

Root herbivory by *D. radicum* during the conditioning phase led to lower performance of *D. radicum* in the feedback phase, in line with previous studies that recorded an alteration of plant resistance against insects through PSF (Kostenko *et al.*, 2012; Bezemer *et al.*, 2013; Kos

et al., 2015a; Kos *et al.*, 2015b; Hu *et al.*, 2018; Pineda *et al.*, 2020). Overall *D. radicum* adult emergence in our experiment was low compared to other studies using similar methods (Soler *et al.*, 2007; van Geem *et al.*, 2015; Karssemeijer *et al.*, 2020 - **chapter 2**). As a root miner, the insect is difficult to quantify in the early stages of its lifecycle. Therefore, we developed primers to supplement the emergence data with the quantification of *D. radicum* 18S ribosomal RNA after 24 h of feeding. This is a novel method to quantify root fly larval performance *in planta*; yet, similar methods are used to quantify plant parasitic nematode abundance in roots (Zijlstra & van Hoof, 2006; Braun-Kiewnick *et al.*, 2016). The *D. radicum* 18S ribosomal RNA method confirmed a lower performance of *D. radicum* on plants in the feedback phase growing in soil conditioned with *D. radicum*-infested plants. Notably, this technique can be further fine-tuned, for instance by dilution or selecting the optimal time-point for harvesting, and the results here should be interpreted in conjunction with the emergence data. Differences between the emergence data and 18S measurements may be due to different life stages targeted, as one measures performance of neonates while the other measures survival to adulthood.

The performance of *D. radicum* may have been affected by a change in plant defence, or by a direct influence of the soil microbiome. Lachaise *et al.* (2017) reported that differences in the soil microbiome affected *D. radicum* performance. *Delia radicum* infestation was previously shown to increase the abundance of *Bacillus* and *Paenibacillus* in the rhizosphere, which could have entomopathogenic properties (Ourry *et al.*, 2018). These bacterial species were not differentially affected in our study, perhaps due to different plant growth substrates. Without isolating specific rhizosphere microbes and testing their effects on the plant and the root herbivore larvae, we can only speculate about the underlying mechanisms.

In roots, most defence markers we studied were not affected by soil conditioning treatments, and thus they do not explain the difference in insect performance. However, we cannot rule out that soil microbes may have primed defence against *D. radicum*, leading to a faster defensive response. Indeed, two genes involved in ISR, *MYB72* and *PDR9*, were affected by soil conditioning treatments. The role of these genes in ISR has been especially studied in *A. thaliana*. Here, we found that soil conditioning changed the expression of their orthologues in *B. oleracea*. The transcription factor MYB72 has been identified as a key regulation node in *A. thaliana* roots in iron uptake and communication with the beneficial rhizobacterium *P. simiae* WCS417r (Verhagen *et al.*, 2004) and was later verified to play a central role in rhizobacterial ISR (van der Ent *et al.*, 2008). This transcription factor regulates the expression of genes involved in the shikimate, phenylpropanoid and nicotianamine biosynthesis pathways, including genes leading to the production and exudation of coumarins (Zamioudis *et al.*, 2014). These coumarins, in particular scopoletin, are secreted by the roots by the

transporter *PDR9*, where they play a dual role in both the plant response to iron deficiency and influencing the rhizosphere microbiome (Stringlis *et al.*, 2018; Stringlis *et al.*, 2019). This could be an indication that ISR plays a role in PSF. Interestingly, transcript levels of *LOX2* and *MYB28* in leaves were affected by soil conditioning treatments, a result which is in line with previous studies that found a link between shoot defence and plant-soil feedback in maize plants (Hu *et al.*, 2018). Our gene expression results underline that defence signaling in shoot and root is fundamentally different (Johnson *et al.*, 2016b).

Conclusion

In conclusion, our study demonstrates that shoot and root herbivory lead to distinct plant rhizosphere microbial communities, whereas inoculation of *P. simiae* to the soil has limited effects on the rhizosphere microbial community. Through PSF, plant performance and defence is altered in a treatment-dependent way for *B. oleracea* plants growing in soil conditioned by conspecific plants. The results presented here suggest that changes in the abundance of specific microbes, rather than the overall microbiome, may be more important for plant performance and defence.

Acknowledgments

The authors would like to thank **Laura Winzen**, **Camilo Rivera Arrivillaga**, **Viktoria Hartan** and **Giulia Berni** for experimental assistance, and **Pedro Beschoren da Costa** for discussions on data analysis. We would like to thank the **Laboratory of Entomology insect rearing team**, and **Kay Moisan** and **Max Wantulla** for help in rearing cabbage root flies, and **Sean Geurts** for growing plants. We thank **Yidong Wang** for drawing the insects and plants in our illustrations. Insightful comments by **Ian Kaplan** and **two anonymous reviewers** on an earlier version have been helpful to improve the manuscript.

Supporting information

Table S1. Primers for target and reference genes in *Brassica oleracea.*

Gene acronym	Gene amplified	Tissue used	Forward primer	Reverse primer
SAR1a	Bo3g052780	Leaf/Root	ATCTCTAGCCACCGTTCCCT	TTCCTGACGATGCTGCACAT
Btub	Bo2g124350, Bo7g067360, Bo9g059850	Leaf/Root	GTCAAGTCCAGCGTCTGTGA	TCACACGCCTGAACATCTCC
Act-2	Bo5g117040	Leaf/Root	ACATTGTGCTCAGTGGTGGA	TCTGCTGGAATGTGCTGAGG
PER4	Bo7g095750	Leaf/Root	TATCCTCTGCAGCCTCCTCA	ACACACAGACTGAAGCGTCC
GADPH	Bo5g017500	Leaf/Root	GCTACGCAGAAGACAGTTGATGG	TGGGCACACGGAAGGACATAC
EF1a	Bo9g142520	Leaf/Root	GGTACCTCCCAGGCTGATTG	TCAGGTAKGAAGACACCTCCTTG
LOX2	5 *LOX2* orthologs	Leaf	GCCATTGAGTTGACTCGTCC	GGATGCATGGCACTTAGTTGT
LOX6	Bo6g098790 + Bo2g056010	Root	AGGAGCTGCCAATTCGAAGC	CGCCTGTTCCAAAGTCATTCCA
CYP81F1	Bo1g004730	Root	TGTGTCAGAAACGTTCAGGCT	ATGGCACGTCGTATCCTCCG
MYB28	Bo2g161590	Leaf/Root	CGGGAGAGATGAGCACAATACG	CAGCCCTCGAAGTTTCCTATCA
MYB72	Bo3g185830	Root	AAACAAGTGGTCAAAGATCGCG	AGTCGTTTCTTGAGATGAGTGT
PDR9	Bo6g067490	Root	ATTCCACCACCTTCTATGCCG	ACTTGGTTGTATCTGGCTCC

Methods S1 *Delia radicum* biomass assessment.

To assess performance of *D. radicum* while the larvae are still within the primary root, we developed species-specific primers. As the goal was to be able to measure low quantities of *D. radicum* within cabbage roots, RNA of the 18S and 28S ribosomal subunits was targeted. During *in silica* primer development, specificity of *D. radicum* primers was optimised by testing specifically for BLAST hits on *Sciaridae*, Nematoda, Fungi and *B. oleracea*; as these are hypothesised to be the most common non-target organisms in our samples (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Four primer pairs were further tested, of which one was selected (in bold) for the experiment (Table **S2**).

Table S2. *Delia radicum* specific primer pairs.

Gene acronym	Forward primer	Reverse primer
***D. radicum* 18S**	**GCAAGATCGTTATTATGGTTGAACTCT**	**GAACCCTGATTCCCCGTTACC**
D. radicum 18S	CCGGTGGAGTTCTTATATGTATTAGGT	ACCAATGAAAGTAGAACAGAGGTCTTAT
D. radicum 28S	GATAATGGTGCTTCTGTGCTATTGTC	TTGAGAGATGTACCGCCCCA
D. radicum 28S	GATAATGGTGCTTCTGTGCTATTGTC	CCTTGAATTGGATCATACCGGAGTA

Stability of primers across different life stages of *D. radicum* was confirmed by testing primers on cDNA extracted from neonate larvae, 2- and 3-week-old larvae and pupae (Fig. **S3**). *Delia radicum* RNA was extracted with Isolate II Plant RNA kit (GCbiotech, the Netherlands) following the manufacturer's instructions. RNA was converted to cDNA using cDNA Synthesis Kit (SensiFAST, Bioline). Quantitative polymerase chain reaction (qPCR) analysis was performed to test transcript levels of genes of interest (CFX96™ Real-Time System, Bio-rad, Hercules, CA, USA). Expression data was processed using qBase and data analysed in R. Gel electrophoresis and melt curves indicated no non-target products of different lengths. Expression was stable across life-stages for each primer.

To assess the ability of this novel technique to discriminate between different larval densities in planta, 3-week-old *B. oleracea* plants were induced with 2, 4, 6, 8, 10 neonate *D. radicum* larvae. After 24h, primary roots were harvested by uprooting the plants, cutting off secondary roots, and freezing the samples directly in liquid nitrogen. Samples were pooled for three plants. Analysis was performed as described above.

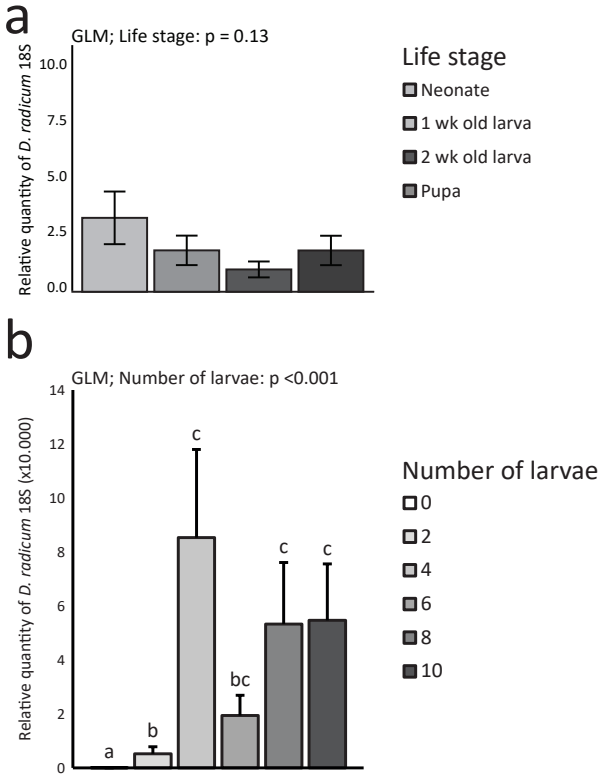

Figure S1. (**a**) Relative gene expression of *D. radicum* 18S rRNA in four life stages and (**b**) the relative quantity of *D. radicum* 18S rRNA in plants infested with different numbers of neonate larvae. Bars show mean ± SE; different letters are significantly different from one another. GLM: Generalized Linear Model. N = 4 insect individuals (**a**), or 3-5 pools of 3 plants (**b**).

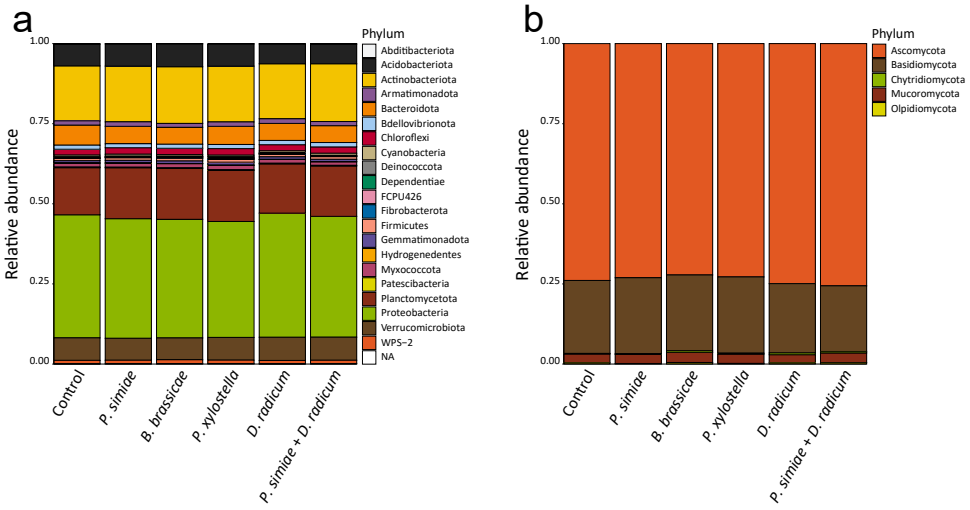

Figure S2. Relative abundance of bacterial (**a**) and fungal (**b**) phyla in rhizospheres of *Brassica oleracea* plants exposed to herbivory, rhizobacterial inoculation or a combination. NA = not available.

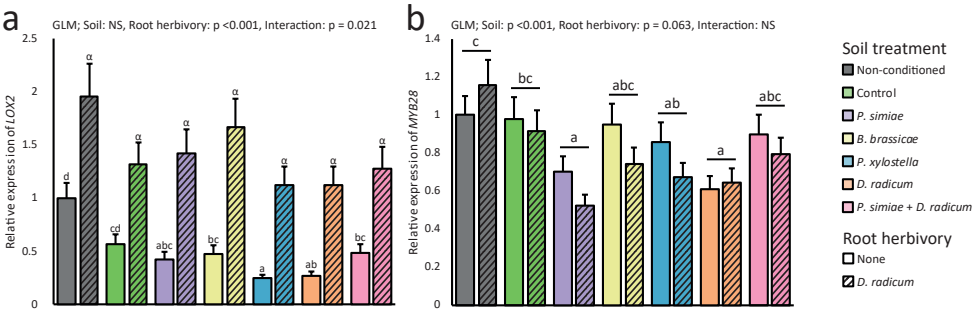

Figure S3. Relative gene expression of *LOX2* (**a**) and *MYB28* (**b**) in leaves of *Brassica oleracea* plants grown in soil conditioned by conspecific plants exposed to herbivory, rhizobacterial inoculation or a combination. In the conditioning phase, *B. oleracea* plants were infested with *Brevicoryne brassicae*, *Plutella xylostella* or *Delia radicum*, inoculated with *Pseudomonas simiae* WCS417, or infested with *D. radicum* and inoculated with *P. simiae*. Plants were removed and the same soil was used to grow new *B. oleracea* plants. After three weeks of growth, half of these plants were infested with *D. radicum* (x-axis). All bars are set relative to the gene expression levels in leaves from plants grown in non-conditioned soil and were non-infested (white bar). Bars show mean + SE; different letters are significantly different from one another, within control (Roman alphabet) or *D. radicum* infested (Greek alphabet) samples (Tukey's HSD, α = 0.05). Soil: soil conditioning treatment; NS: Not significant, GLM: Generalized Linear Model. N = 3 or 4 replicates of pools of three plants.

Photo by Hans Smid

Chapter 6

The effect of crop diversity and associated macrofauna on various life stages of the cabbage root fly

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Under review

Abstract

Root herbivores pose a major threat to agricultural crops. Their damage often goes unnoticed until the larvae reach their most devastating late instar stages and they are difficult to control. Crop diversification can reduce pest pressure without compromising yield. We studied how different diversified cropping systems affected the oviposition and abundance of the specialist cabbage root fly *Delia radicum*, the most important root herbivore in *Brassica* crops. The cropping systems incuded a monoculture, pixel cropping, and four variations of strip cropping with varying intra- and interspecific crop diversity, fertilization, spatial configuration, and presence of an (extra)floral nectar source. Furthermore, we assessed whether there was a link between *D. radicum* and other macrofauna present on the same plants. Cabbage root fly oviposition was much higher in strip cropping designs compared to the monoculture, and was highest in the most diversified strip cropping treatment. Despite the high number of eggs, there were no differences in the number of larvae and pupae between the cropping systems, indicating high mortality of *D. radicum* eggs and early instars especially in the strip cropping designs. We found no correlations between the presence of aboveground insect herbivores and the number of *D. radicum* on the plant. Within the belowground realm, we found positive correlations between the number of *D. radicum* and soil-dwelling predators and detritivores, and a negative correlation with other belowground herbivores. Our findings highlight that root herbivore presence is determined by a complex interplay of many factors, including nectar availability, spatial configuration of host plants, and other organisms residing near the roots.

Introduction

Insect root herbivores are major agricultural pests, causing damage leading to yield reduction in many crops, including maize, onion, and cabbage (Finch, 1989; Brown & Gange, 1990; Johnson *et al.*, 2016a). Due to their belowground life stages, root herbivores often go unnoticed and can be difficult to control. This is especially true for pest species with larvae that feed within the roots, such as the larvae of the cabbage root fly *Delia radicum* and the onion fly *D. antiqua* (Finch, 1989). Root herbivores are often controlled by preventive applications of insecticides (Johnson *et al.*, 2016a). These insecticides have detrimental effects on many species in the agro-ecosystem, including pollinators, parasitoid wasps, aquatic macroinvertebrates in farmland streams, and insectivorous birds (Schulz & Liess, 1999; Hallmann *et al.*, 2014; Kessler *et al.*, 2015; Calvo-Agudo *et al.*, 2019). Moreover, insecticides hamper human health (Goldman, 2014). To move towards sustainable agriculture, the use of insecticides should be reduced and alternatives are needed. One of these alternatives is to increase diversity in the agroecosystem to enhance ecology-based pest suppression.

Increasing diversity in agroecosystems has great potential to reduce pest outbreaks without compromising yield (Parker *et al.*, 2016; Tajmiri *et al.*, 2017; Mansion-Vaquié *et al.*, 2020; Juventia *et al.*, 2021). Cropping systems can be optimised to maximise benefits by increasing genetic, temporal, and spatial diversity (Ditzler *et al.*, 2021a). Intercropping, i.e. growing multiple crops in close proximity, is a promising practise to reach higher crop diversity. Crop diversification can enhance yield, as plants benefit from factors such as niche differentiation and more efficient resource use (Yu *et al.*, 2015; Li *et al.*, 2020). A recent meta-analysis showed that intercropping increases yield by 30 % compared to monocultures (Beillouin *et al.*, 2019). Intercropping may also reduce pest outbreaks through interference with host searching behaviour of the pest (Finch & Collier, 2000; Mansion-Vaquié *et al.*, 2020), or by enhancing the control activity of natural enemies (Trenbath, 1993; Khan *et al.*, 1997; Nilsson *et al.*, 2016; Tajmiri *et al.*, 2017). Inclusion of a nectar source, for instance, can boost the number and longevity of parasitoid wasps that parasitise on insect herbivores (Nilsson *et al.*, 2016). Further, crop diversification can lead to increased abundance and diversity of ground-dwelling arthropods, which may prey on root herbivore eggs and larvae (Finch, 1989; Booij *et al.*, 1997).

Various forms of intercropping are distinguished based on the spatial configuration of plants on the field, such as strip cropping and pixel cropping. In strip cropping, crops are cultivated in relatively narrow (3-6 m), alternating strips. In pixel cropping, crops are planted in a patchwork of squares. Strip cropping of just two crops can already reduce pest pressure

and enhance biodiversity (Ma *et al.*, 2007; Tajmiri *et al.*, 2017; Li *et al.*, 2019). Further diversification by including more crops in the strip cropping scheme (Parker *et al.*, 2016), mixing of cultivars (Koricheva & Hayes, 2018; Wetzel *et al.*, 2018), or reducing fertiliser inputs via additional nitrogen fixing plants (Ditzler *et al.*, 2021b) could benefit the agro-ecosystem even more.

We studied the interactions between white cabbage, *Brassica oleracea* L. var. *capitata*, and its most prominent root herbivore, the cabbage root fly *D. radicum* L. (Diptera: Anthomyiidae) in an agricultural setting. Cabbage root flies are specialised on the Brassicaceae family, and are an important pest of cabbage in temperate regions (Finch, 1989). Females carefully select their host plants and integrate cues on plant quality gathered from aboveground tissues in their host selection behaviour (Zohren, 1968; Schoonhoven *et al.*, 2005). Eggs are laid in the soil just next to the plant stem, and upon hatching the larvae make their way into the tap root to feed from within. After a three to four weeks, larvae pupate in the soil surrounding the plant, and emerge as adults. A variety of insect natural enemies feed on *D. radicum.* The parasitoid wasp *Trybliographa rapae* parasitises the larvae and emerges after pupation, and two species of staphylinid beetles, *Aleochara bipustulata* and *A. bilineata*, parasitise the pupae (Finch, 1989). Furthermore, eggs may be consumed by several ground beetle species, although their importance in pest control is subject of debate (Finch & Collier, 2007).

Here, we assessed how a range of increasingly diverse intercropping practices affected the oviposition and infestation by *D. radicum*. To improve management of *D. radicum* infestation, various intercropping studies have been carried out during the past decades (Tukahirwa & Coaker, 1982; Langer, 1996; Finch & Collier, 2000; Björkman *et al.*, 2010; Nilsson *et al.*, 2012; Meyling *et al.*, 2013; Nilsson *et al.*, 2016; Lamy *et al.*, 2017a). Most studies have focused on a cropping system with cabbage and an undercrop of clover, a nitrogen-fixing plant, which provides some level of control against *D. radicum* through interference with egg-laying behaviour (Finch & Collier, 2000). Here, we used different cropping systems, in which we tested how *D. radicum* was affected by increasing crop diversity, enhancing intraspecific variation via cultivar mixing, alteration of the fertilization regime by employing nitrogen-fixing secondary crops, and the addition of nectar sources. We expected to find fewer *D. radicum* eggs, larvae and pupae in more diverse cropping systems. Additionally, we assessed the community of natural enemies of *D. radicum* by rearing collected pupae from the field and by deploying pitfall traps. We expected the abundance of natural enemies to be higher in more diverse cropping systems, and this to translate into a lower abundance of *D. radicum* larvae and pupae. Finally, we monitored the above- and belowground macrofauna communities to establish whether they are correlated with *D. radicum* oviposition or infestation in a field setting. Previous greenhouse studies indicated that leaf-chewing

herbivores negatively affect *D. radicum* performance, whereas oviposition was stimulated on induced plants (Soler *et al.*, 2007; Karssemeijer *et al.*, 2020 - **chapter 2**; **chapter 4**). Therefore, we expect that the abundance of leaf-chewing herbivores is positively correlated with *D. radicum* oviposition, and negatively correlated with the abundance of *D. radicum* larvae and pupae.

Materials and Methods

Field setup

This study was carried out in the summer of 2020 as part of a large long term strip-cropping trial at Droevendaal Experimental Farm in Wageningen, the Netherlands (Juventia *et al.*, 2021). At the field site, white cabbage (*Brassica oleracea* var. *capitata*), wheat (*Triticum aestivum* L.), pumpkin (*Cucurbita maxima* L.), potato (*Solanum tuberosum* L.), barley (*Hordeum vulgare* L.), and grass (*Lolium multiforum* L.) were grown (Fig. **1a**). All data presented in this study relate to the white cabbage crop (*B. oleracea* var. *capitata* cv. Rivera). The experimental design consisted of six treatments (Fig. **1a**): (1) Reference, i.e. cabbage monoculture of 51 by 45 m, (2) Strip, i.e. alternating strips of cabbage and wheat (variety Lennox), (3) Strip_ cultivar; alternating strips of two cultivars of cabbage and two varieties of wheat (varieties Lennox and Lavett), (4) Strip_additive, i.e. alternating strips of cabbage (alone) and wheat together with broad bean (*Vicia faba* L., cv. Pyramid) to add floral and extrafloral nectar to the system for natural enemies of herbivores, (5) Strip_diversity; composite of strips of all six crops with two varieties per crop and a nectar source in the Poaceae crops (wheat with broad bean, barley with pea, grass with clover), (6) Pixel, adjacent 50 x 50 cm plots with each containing one of the six crops with two varieties per crop and a nectar source in the grass crops, in a random design. The same crops and additional nectar sources were planted in the Strip_diversity and Pixel treatments, but with a different spatial configuration.

The setup was an incomplete block design on four adjacent fields. On one field, the reference monoculture was planted on a 51 x 45 m plot, and a replicate of the Strip treatment was planted next to it. The other three fields contained replicates of all strip treatments (Strip, Strip_additive, Strip_cultivar, Strip_diversity). The rotation scheme of the long term field trial ensured that each treatment was planted on a plot that had a similar treatment the year prior. Every replication of the Strip, Strip_additive and Strip_cultivar treatments consisted of three strips per crop, of which the central two strips of cabbage where included in the measurements to reduce interference between treatments. In the Strip_diversity treatment, a single strip of each crop was planted per replication. In all treatments, each strip was 3 m wide and 42 or 54 m in length. Two replicates of the Pixel treatment were

planted in the fields, these were 12 by 7.5 m consisting of 360 pixels. Planting distance of cabbage was 38 cm within rows and 75 cm between rows, resulting in four rows per strip. In the Strip_cultivar and Strip_diversity treatments, a second cultivar of cabbage (*B. oleracea* var. *capitata* cv. Christmas Drumhead) was planted in the center two rows of each strip, as every fourth plant (Fig. **1a**). The Christmas Drumhead cultivar was used as it is known to be more attractive to shoot herbivores and parasitoids (Poelman *et al.*, 2009a; Poelman *et al.*, 2009b), and as such serves as a trap crop and to attract natural enemies into the strip. This second cultivar was also included in the Pixel treatment.

Fertilization was carried out two weeks prior to planting and varied between the treatments. One of the overarching goals of the field trial in which this study was carried out is to study different cropping systems, including differences in fertilization strategies. The Reference, Strip, and Strip_cultivar treatments received farm yard manure (35 t/ha), the Strip_additive and Strip_diversity treatments were fertilised using organic plant fertiliser (11-0-5 NPK) in a concentration matched to the manure in terms of nitrogen dosage. The Pixel treatment should have received a similar fertilization as the Strip_additive and Strip_diversity treatments, however due to unforeseen circumstances this was withheld. Moreover, there were differences in the precrop between the treatments, most notably the precrop for the Strip_additive, Strip_diversity and Pixel treatments included red clover (*Trifolium pratense* L.), a nitrogen-fixer. Cabbage plants were planted on the 25th of May, and harvested from the 15th of October until the 26th of November. The wheat intercrop was sown in the second half of March and harvested on July 31st, meaning that these rows were empty for two weeks before the last measurements were taken.

Data collection

We used three strategies to study *D. radicum* dynamics in the field. First, we assessed cabbage root fly oviposition and monitored aboveground herbivore communities to determine whether these predict oviposition by root flies. Second, we uprooted cabbage plants and their surrounding soil to quantify the number of *D. radicum* larvae and pupae and other above- and belowground macrofauna as well as the plant size. Third, we assessed potential (egg) predator abundance using pitfall traps.

Oviposition

We assessed the oviposition by cabbage root flies in each treatment using felt traps, a commonly used method (Bligaard *et al.*, 1999; Lamy *et al.*, 2020). Strips (4 x 100 cm) of black felt were wrapped around the stem of cabbage plants and secured using a safety

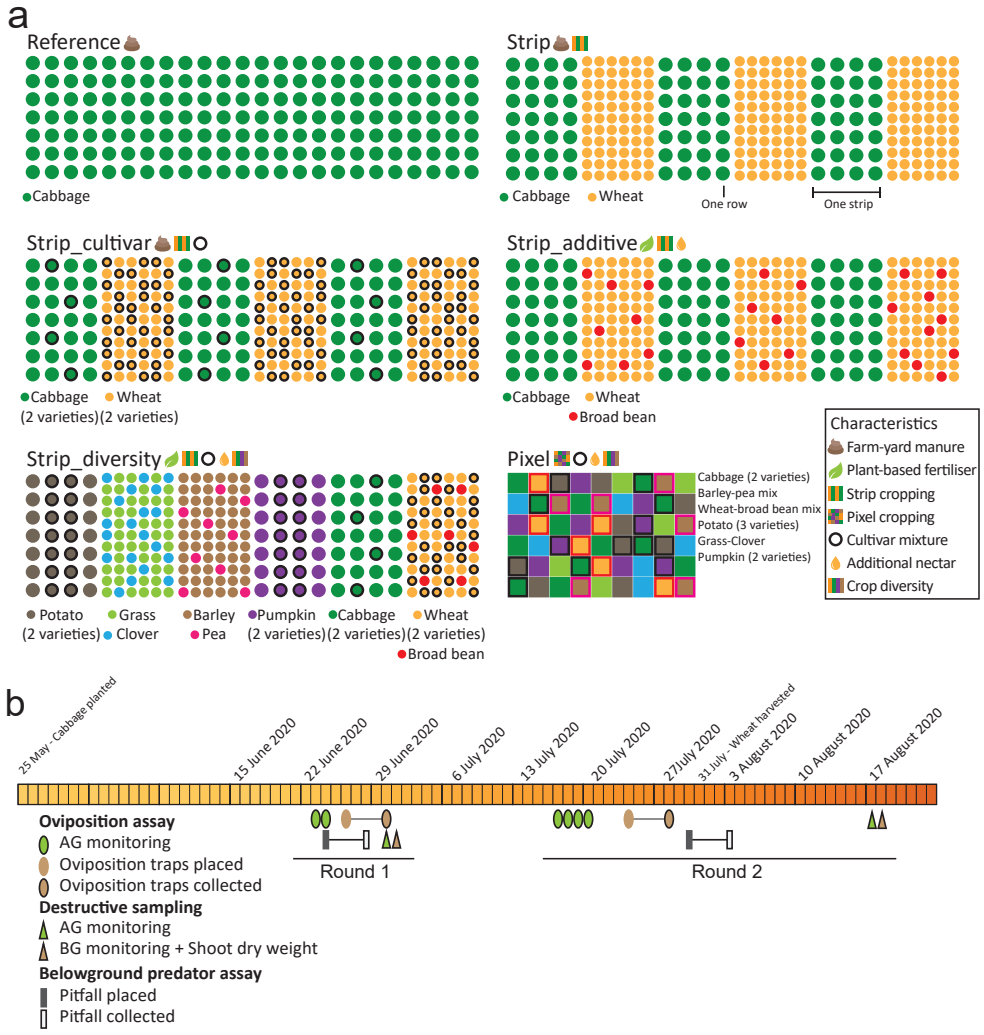


Figure 1. Experimental setup. (**a**) Illustrations of the different cropping systems (treatments). Each panel represents one replicate in the field, in which measurements were taken in the central cabbage strips. Circles represent a plant, black outlines represent a second crop cultivar. Icons behind treatment names indicate cropping system characteristics: type of fertilization, strip cropping, cultivar mixture, addition of a nectar source (broad bean), increased crop diversity (i.e. six crops), pixel cropping. (**b**) Timeline of the experiments throughout the field season of 2020. AG: aboveground, BG: belowground.

pin. Felt traps were collected five days after placing them and the number of eggs in each trap was counted. Three to seven days prior to placing the felt traps, the community of aboveground herbivores on each plant was assessed. Herbivores present on the plant were directly identified to species or family level. We measured the maximum plant radius

(distance between furthest leaf tips) as a non-destructive assay of plant size. Per treatment, 16 cabbage plants were assessed in each treatment replicate. The first and last 10 meters of each strip were not sampled, to account for field edge effects. For the Strip, Strip_additive, Strip_cultivar and Reference, 2 strips with each 8 plants and in the Strip_diversity 1 strip with 16 plants were sampled. The sampled plants were equally spread throughout the strip, and the order of the rows to be sampled was randomised. In the Pixel treatment, 16 random cabbage plants were chosen among the cabbages that were not on the edges of the Pixel field.

Destructive sampling

To quantify the abundance of *D. radicum* larvae and pupae, destructive sampling was performed two times during the field season (Fig. **1b**). As this was a time consuming sampling effort, timing was important. Therefore, throughout the season, we monitored a small number of cabbage plants in the fields every other week to confirm the presence of *D. radicum* larvae and pupae (Table S1). This pilot data, together with the oviposition data, provided the basis for timing the destructive sampling. One day before destructive sampling, the aboveground community of herbivores was assessed on each plant. When taking soil samples, shoots were first cut off and collected in paper bags. Then, soil samples were taken using an augur (20 cm diameter) to a depth of 20 cm with the cabbage tap-root at the center. To quantify plant dry biomass, shoot tissue was dried at 70 °C for 2 days and weighed. Soil samples were placed in plastic bags, secured using a tie-wrap, and stored at 7 °C until further analysis. The Pixel treatment was not included in this monitoring, as destructive sampling would interfere too much with other measurements in this treatment.

Within one week after collection and storage, soil samples were thoroughly searched for macrofauna. The cabbage taproots were carefully opened to find *D. radicum* larvae feeding within. When found, these cabbage root fly larvae were placed in small containers containing a small piece of rutabaga (*Brassica napus* var. *napobrassica*) to rear them to pupation. The collected pupae were stored in glass vials in a climate cabinet (20±1 °C) until eclosion to assess parasitism. Empty *D. radicum* puparia collected from the soil samples were also scored. During each round, eight plants were harvested per strip, two plants were randomly selected in each row, resulting in 232 samples per collection round.

Pitfall traps

Two times during the season, pitfall traps were placed to quantify the abundance of (egg) predators (Fig. **1b**). Pitfall traps consisted of a plastic cup (8.5 cm diameter) filled with 3

centimeters of water with odourless dish soap placed in the soil up to the rim, covered with a plastic roof (12.5 cm diameter) approximately 2 cm above the soil surface. One pitfall trap was placed in a predetermined random position in the one of the central rows of each strip, and traps were left in the field for 5 days. Macroinvertebrates captured in the pitfall traps were preserved in 70 % ethanol and identified. Carabid beetles were identified to the species level. Only the abundance of staphylinid and carabid beetles were statistically analysed.

Statistical analysis

Data was analysed using R (R Core Development Team, 2017), with packages vegan (Oksanen *et al.*, 2007), lme4 (Bates *et al.*, 2015), emmeans (Lenth *et al.*, 2018), ggeffects (Lüdecke, 2018). Individual plants or pitfall traps were the statistical units. Most of the data were counts, which we analysed using Generalized Linear Mixed Models (GLMM) with a negative binomial distribution. Plant quality measurements, shoot dry biomass and maximum radius, were analysed with a (G)LMM with a gamma or normal distribution. Field (a factor indicating the four fields on which the experiment was replicated) was included as a random factor. In the case of continuous explanatory variables, we used the ggpredict function to generate conditional predictions of the correlation between the response variable and the explanatory variables whilst keeping other variables and the random factor constant.

We performed a Principal Component Analysis (PCA) on the community of aboveground herbivores (oviposition data) and total macrofauna community (destructive sampling data) separately for each round. Using Redundancy Analysis (RDA) in which we added the number of cabbage root fly eggs or larvae and pupae as an explanatory variable, we further established whether cabbage root fly oviposition or abundance were correlated with other members of the plant associated macrofauna community. Finally, we tested correlations between cabbage root fly oviposition and abundance and other macrofauna using GLMM. For this analysis, species were grouped into explanatory variables based on feeding site and guild into aboveground chewers: aboveground phloem-feeders, belowground detritivores, belowground predators, and belowground herbivores.

Results

Cabbage root fly oviposition

We collected 1636 (average 4.08 per plant) and 6249 (average 18.61 per plant) cabbage root fly eggs in the two rounds of 304 sampled plants (Fig. **2a**). We did not identify these eggs to

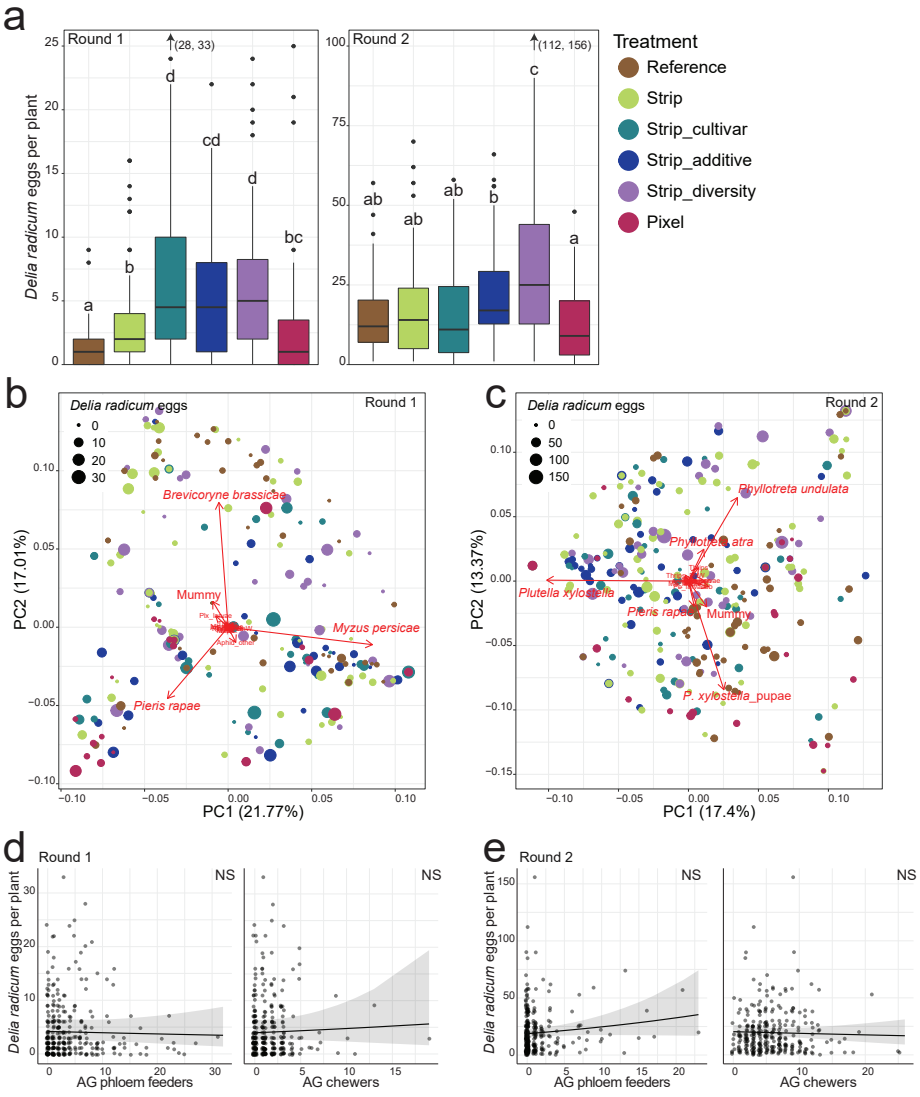


Figure 2. Oviposition of cabbage root fly in a strip cropping farm trial. (**a**) Effects of strip cropping treatments on the number of *D. radicum* eggs per plant, note that the Y-axis scale differs between the two rounds. Results of pairwise comparisons between treatments are indicated with letters; treatments having no letters in common differ significantly ($P < 0.05$). Arrows indicate outliers outside the plot area, their values are added between brackets. (**b, c**) Principal Component Analyses (PCA) of aboveground herbivore communities prior to placement of felt traps in round 1 and 2. Size of the data points reflects the number of eggs found on felt traps the week after the aboveground herbivore community was assessed. Note, the numbers of eggs were not included in the PCA analysis. (**d, e**) Conditional predictions of the relationship between aboveground phloem feeders or chewers and the abundance of *D. radicum* eggs in round 1 and 2; dots are the original data points, the line indicates the predicted values and the grey area depicts 95% confidence interval. AG: aboveground.

the species level, but based species identiy on pupal emergence (see below). We assume the vast majority of eggs were laid by *D. radicum* and a small fraction by the turnip fly *D. floralis*. More eggs were collected in round 2 (29 July 2020) compared to round 1 (30 June 2020; GLMM: $\chi^2 = 245.95$, $P < 0.001$). Oviposition by cabbage root flies differed among treatments (GLMM: $\chi^2 = 33.70$, $P < 0.001$), and there was an interaction effect between treatment and round (GLMM: $\chi^2 = 26.84$, $P < 0.001$). In the first round, cabbage root fly females deposited the lowest number of eggs per trap on plants in the monoculture (Reference). Compared to the monoculture (Reference), traps placed on plants in a pixel cropping design (Pixel) contained slightly more eggs, traps in a strip cropping design with wheat (Strip) contained twice as many eggs, and traps placed on cabbage plants in the three other strip cropping designs (Strip_cultivar, Strip_additive, Strip_diversity) contained roughly four times as many eggs. In the second round, differences were less pronounced than in the first round. Compared to the reference monoculture, traps placed on cabbage plants in strips of six crops (Strip_diversity) contained roughly twice as many eggs. None of the other treatments differed from the reference monoculture.

Multivariate analysis did not reveal clear patterns between the aboveground herbivore communities on cabbage plants prior to placing the felt traps and the number of *D. radicum* eggs collected (Fig. **2b, c**). The PCs were mostly explained by the aphids *Brevicoryne brassicae* and *Myzus persicae* and the caterpillar *Pieris rapae*, in the first collection round, and by *Plutella xylostella* caterpillars and *Phyllotreta undulata* flea beetles in the second round. The lack of a clear link between aboveground community and the number of eggs per felt trap was confirmed with redundancy analysis (Fig. **S1**), in which less that 0.5 % of variance was explained, and with univariate analysis (Fig. **2d, e**). The abundance of aboveground phloem feeders or chewers did not explain the number of eggs found on felt traps placed on the same plants in either round (GLMM, Round 1: Phloem_feeders; $\chi^2 = 0.16$, $P = 0.69$, Chewers; $\chi^2 = 0.30$, $P = 0.58$, Round 2: Phloem_feeders; $\chi^2 = 2.92$, $P = 0.087$, Chewers; $\chi^2 = 0.25$, $P = 0.62$).

For each plant, we measured the distance between the furthest leaf tips, i.e. radius, as a non-destructive measure of plant size and tested whether there was a correlation with the number of cabbage root fly eggs (Fig. **S2**). In both rounds, there were differences in radius between treatments (GLMM, Round 1: $\chi^2 = 75.44$, $P < 0.001$, Round 2: $\chi^2 = 146.08$, $P < 0.001$). Plants in the reference monoculture grew larger compared to the other treatments. In the first round, plants grown in strips next to wheat, either with or without a second cultivar (Strip, Strip_cultivar), grew larger compared to plants grown in rotated strips with six other crops (Strip_diversity) or strips with broad beans interspaced between wheat (Strip_additive). The latter two treatments received plant-based fertiliser instead of farm-yard

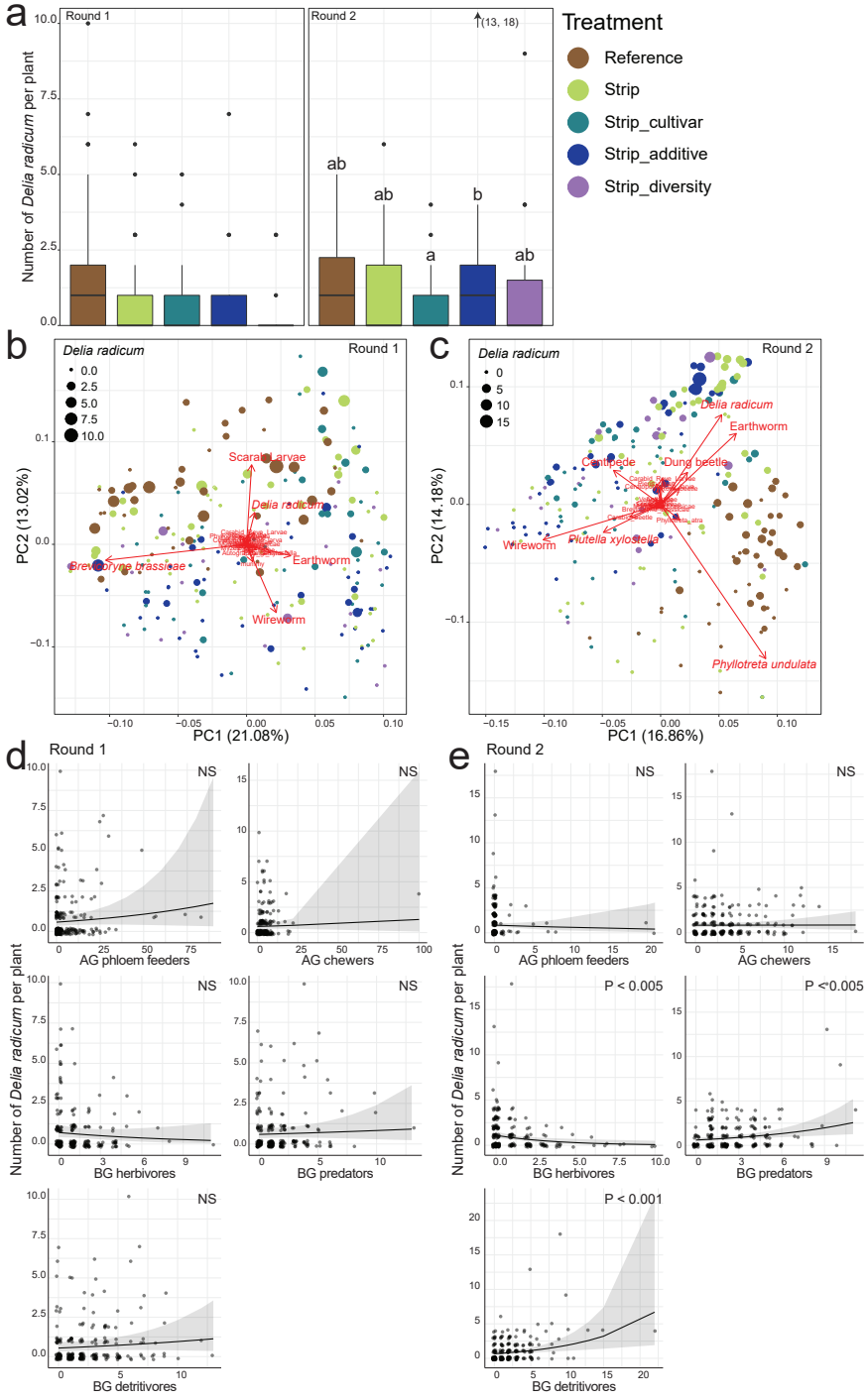
manure prior to planting. In the second round, plants grown in the pixel cropping system, in which fertiliser was not provided, grew smaller than any other treatment. A correlation between the number of cabbage root fly eggs and plant size was found in the first round but not in the second (Fig. **S2b**, GLMM; Round 1: $\chi^2 = 5.02$, $P = 0.025$, Round 2: $\chi^2 = 0.35$, $P = 0.55$). In the first round, cabbage root fly females oviposited more on larger plants.

Destructive sampling

For a different dataset obtained with destructive sampling we collected a total of 110 *D. radicum* larvae and 323 pupae from 464 plants in two rounds (Fig. **3**). While we did not identify larvae and pupae to the species level, adult emergence (see below) indicates that the vast majority was *D. radicum*, and a small fraction was *D. floralis*. More *D. radicum* were collected in the second round (Fig. **3a**, GLMM: $\chi^2 = 5.24$, $P = 0.022$), on average 0.96 per plant compared to 0.60 per plant in the first round. Strip cropping treatments affected the number of *D. radicum* with marginal significance (GLMM: $\chi^2 = 9.85$, $P = 0.043$) and there was an interaction effect between treatments and rounds (GLMM: $\chi^2 = 12.93$, $P = 0.012$). In the first round, there were no differences between treatments (Fig. **3a**). In the second round, we found no differences between any of the treatments and the reference monoculture. However, we did find more *D. radicum* on plants grown in strips with wheat combined with broad beans (Strip_additive) compared to plants grown in strips with multiple cultivars of cabbage and wheat (Strip_cultivar).

Multivariate analysis of above and belowground macrofauna in the two rounds revealed clustering of plants based on *D. radicum* larvae and pupae found on them (Fig. **3b, c**). This is in contrast to the analyses of the aboveground herbivore community in relation to *D. radicum* oviposition (Fig. **2b, c**). Redundancy analysis (Fig. **S3**) indicates that other macrofauna explains very little of the variance in *D. radicum* abundance, 0.78 % in round 1 and 1.98 % in round 2. Inspection of the strongest contributing factors suggests that scarab larvae and earthworms positively correlate with the number of *D. radicum* larvae and pupae in rounds

Figure 3. Abundance of *D. radicum* larvae and pupae in a strip cropping field trial. Plants were uprooted and the soil macrofauna was assessed as well as the number of *D. radicum* larvae and pupae in and around the cabbage taproot in a soil core of 20 cm diameter. The day before harvesting, aboveground herbivore communities were assessed. (**a**) Effects of strip cropping treatments on the number of *D. radicum* larvae and pupae. Results of pairwise comparisons between treatments are indicated with letters; treatments having no letters in common differ significantly ($P < 0.05$). Arrows indicate outliers outside the plot area, their values are added between brackets. (**b, c**) Principal Component Analyses (PCA) of above and belowground macrofauna in round 1 and 2. Size of the data points reflects the number of *D. radicum*. (**d, e**) Conditional predictions of the correlation between functional groups of the above and belowground macrofauna and the abundance of *D. radicum* in round 1 and 2; dots are the original data points, the line indicates the predicted values and the grey area depicts 95% confidence interval. Total *D. radicum* indicates the sum of larvae and pupae. AG: abovegound, BG: belowground. (**Figure 3** on next page).


a
Round 1
Round 2
(13, 18)
Number of Delia radicum per plant
ab
ab
a
b
ab
Treatment
Reference
Strip
Strip_cultivar
Strip_additive
Strip_diversity
b
Delia radicum
Round 1
PC2 (13.02%)
PC1 (21.08%)
Scarab Larvae
Delia radicum
Earthworm
Brevicoryne brassicae
Wireworm
c
Delia radicum
Round 2
PC2 (14.18%)
PC1 (16.86%)
Delia radicum
Earthworm
Centipede
Dung beetle
Wireworm
Plutella xylostella
Phyllotreta undulata
d
Round 1
e
Round 2
NS
P < 0.005
P < 0.001
AG phloem feeders
AG chewers
BG herbivores
BG predators
BG detritivores
Number of Delia radicum per plant

1 and 2, respectively. Moreover, wireworms (larvae of click beetles, family Elateridae) appear negatively correlated with *D. radicum* in both rounds. We further explored these associations by analysing the correlation between functional groups of above- and belowground macrofauna and the number of *D. radicum* (Fig. **3d, e**). In the first round, no correlation was found between any functional group and *D. radicum*. However, in the second round, *D. radicum* showed a negative correlation with belowground herbivores (GLMM: $\chi^2 = 6.80$, $P < 0.01$). This indicates that plants with more wireworms, by far the most abundant belowground herbivore other than *D. radicum* (Fig. **S4**), harbored fewer *D. radicum*. Furthermore, there was a positive correlation between the number of *D. radicum* and belowground predators and detritivores (GLMM: BG_Predators; $\chi^2 = 11.38$, $P < 0.001$, BG_Detritivores; $\chi^2 = 11.035$, $P < 0.001$). There was no correlation between the number of aboveground herbivores, regardless of feeding guild, and *D. radicum* in either round.

We assessed the shoot dry weight of all plants to assess whether this was correlated with the abundance of cabbage root fly larvae and pupae. In both rounds, strip cropping treatments affected cabbage shoot dry biomass (Round 1: GLMM; $\chi^2 = 31.34$, $P < 0.0001$, Round 2: LMM; $\chi^2 = 67.92$, $P < 0.0001$). Plants grown in the reference monoculture were heavier compared to plants in all other treatments (Fig. S5). In the first round, there was a strong correlation between plant size and the number of *D. radicum* (Fig. S5b, GLMM; $\chi^2 = 25.86$, $P < 0.0001$), larger plants harbored more cabbage root flies. This correlation was not found in the second round (GLMM; $\chi^2 = 0.21$, $P = 0.65$).

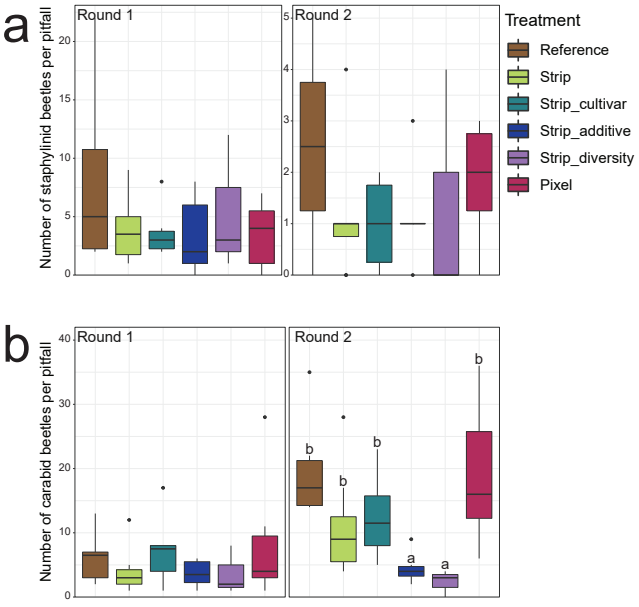


Figure 4. Staphylinid (**a**) and carabid (**b**) beetles collected in different cropping systems. Results of pairwise comparisons between treatments are indicated with letters; treatments having no letters in common differ significantly ($P < 0.05$).

Pitfall traps

To assess cabbage root fly natural enemies, we collected 209 staphylinid beetles and 627 carabid beetles in two rounds of pitfall trapping. Numbers of beetles per trap were highly variable. Carabid beetles were identified to the species level; the most abundant species were *Pterostichus melanarius* and *Harpalus rufipes* (Fig. **S6**). The number of staphylinid beetles did not differ between the treatments (GLMM: $\chi^2 = 3.70$, $P = 0.59$); more were found in the first than in the second collection round (GLMM: $\chi^2 = 22.52$, $P < 0.001$; Fig. **4a**). Carabid beetles were collected more in the second round (GLMM: $\chi^2 = 15.09$, $P < 0.001$), and were affected by the treatments (GLMM: $\chi^2 = 24.79$, $P < 0.001$; Fig. **4b**). In the second round, we found fewer carabids in the strip design with wheat combined with broad beans (Strip_additive) and the strip design with six crops (Strip_diversity) compared to the other four treatments (Reference, Strip, Strip_cultivar and Pixel).

We reared *D. radicum* pupae and larvae that had been collected in the destructive sampling experiment until eclosion to assess parasitism. In total, 126 adults emerged from the collected fly larvae and pupae: 92 cabbage root flies, 22 *A. bipustulata* staphylinid beetles, 3 *A. bilineata* staphylinid beetles, and 9 *T. rapae* parasitoid wasps. The majority of flies were *D. radicum*, however, a small proportion may have been *D. floralis*. Most *A. bipustulata* (18) emerged from pupae collected in the first round, whereas most *T. rapae* (8) emerged from pupae collected in the second round. Due to these low numbers, we did not perform statistical analysis of parasitism.

Discussion

We found that cabbage root fly oviposition was higher in strip cropping designs but that this did not result in higher infestation of cabbage with larvae and pupae. The highest numbers of cabbage root fly eggs were collected in the most diverse strip treatment, which included six crops (Strip_diversity). Interestingly, the numbers of larvae and pupae did not differ between treatments, suggesting that survival was lower in the treatments with plants grown in strips, in which we collected more eggs. This discrepancy between cabbage root fly oviposition and infestation may have several causes, including abiotic factors, natural enemies, or even plant-mediated interactions with other herbivores. Pitfall catches of carabid and staphylinid beetles were inconclusive for explaining the effects of natural enemies on cabbage root flies in our field. Moreover, the number of collected pupae was too low to make definitive statements on parasitism. Contrary to our expectations based on plant-mediated interactions between above and belowground herbivores (Soler *et al.*, 2007; Karssemeijer *et al.*, 2020 - **chapter 2**), we found no correlation between the

number of aboveground phloem feeders or leaf-chewers and cabbage root fly eggs, larvae or pupae. Other belowground macrofauna collected in our sampling effort did not explain much of the variation between plants in terms of root fly larvae. Although the effects were relatively weak, we did find that cabbage root fly larvae and pupae were negatively correlated with the abundance of other root herbivores, and positively with belowground detritivores and predators.

The resource concentration hypothesis states that insects would find their host plant more easily in a larger patch (Root, 1973). A potential mechanism is that host-searching behaviour is interupted when insects land on a non-host plant, which was demonstrated by reduced oviposition of *D. radicum* on cabbage plants with a clover undercrop (Finch & Collier, 2000). Our results seem contradictory to this hypothesis, as we found fewest eggs on plants in the reference monoculture. Potentially, if cabbage root fly females were equally attracted to monoculture and strip treatments from a long distance, the numbers of eggs may be explained by dilution as the density of cabbage plants was highest in the monoculture setting. Interestingly, cabbage root flies laid most eggs on plants grown in strips with six crops (Strip_diversity), which was the most diverse of the strip cropping treatments. On the other hand, plants grown in the pixel cropping system received few eggs, even though the composition of plants was similar to the Strip_diversity treatment. Therefore, it appears that the spatial configuration of plants within a field affects cabbage root fly oviposition. We speculate that, from the perspective of a female cabbage root fly, a strip of cabbage plants in an intercropped field may not be so different from a monoculture, since all cabbage plants still neighbour other cabbage plants. Indeed, a previous study showed that the distance between host cabbage plants and non-host clover plants should be less than 50 cm to result in a reduction of *D. radicum* oviposition (Tukahirwa & Coaker, 1982). As cabbage plants in the pixel cropping system neighboured mainly non-brassicaceous plants, this may have interfered with cabbage root fly host-searching behaviour in a similar manner as was demonstred for a clover undercrop (Tukahirwa & Coaker, 1982; Finch & Collier, 2000). These results suggest that interference with host-searching behaviour from neighbouring non-host plants is likely low in a strip intercropping system.

We found more cabbage root fly eggs on cabbage plants grown in a cropping systems that included broad beans as an additional (extra)floral nectar and nitrogen source (Strip_additive and Strip_diversity). The *Delia* genus is part of the dipteran family Anthomyiidae, derived from the ancient Greek words for flower (ἄνθος, anthos) and fly (μυῖα, myia), and the adults feed on floral resources. Thus, host-searching behaviour and foraging for food coincide in a cropping system that includes nectar, which may have stimulated oviposition on plants in close proximity of the nectar producing pants. Indeed, this has

been recorded in many herbivorous insect species of which the adults feed on floral resources (Wäckers *et al.*, 2007). However, in a previous study *D. radicum* oviposition was not affected by intercropping cabbage with dill and buckwheat as floral resources (Nilsson *et al.*, 2012). Perhaps broad beans, the main source of (extra)floral nectar in our setup, provide a more suitable food source for cabbage root flies. Differences in plant nutrition could provide an alternative explanation for the higher number of cabbage root fly eggs found in treatments with an additional nectar source. The strip treatments that included a legume species, e.g. broad bean, to provide nectar for natural enemies (Strip_additive and Strip_diversity), also received a plant-based fertiliser instead of farm-yard manure and a precrop with a grass-clover mixture instead of only grass. Thus, plants grown in these strips may differ in nutrient status due to the nitrogen-fixing properties of legumes. Oviposition of cabbage root flies may be affected indirectly by soil nutrients, for instance increased sulphur content makes oilseed rape plants (*B. rapa*) more attractive (Marazzi & Städler, 2005).

We also found a positive correlation between the abudance of all stages of the cabbage root fly and plant size in the first sampling round of our experiments. Laboratory choice experiments have previously shown that *D. radicum* indeed prefers to oviposit on larger plants when given the choice (Kostal & Finch, 1994). Interestingly, we did not find this correlation in the later sampling round. Flies earlier in the season may be more sensitive to differences in plant size, or there may be a threshold of plant size that flies use in their oviposition behaviour which most plants had surpassed in the second round. Alternatively, the contrast of plant sized might be more readily distinguishable early in the season, when soil cover is still relatively low.

There was a striking difference between the numbers of cabbage root fly eggs collected in the felt traps and larvae and pupae collected with the destructive sampling effort. The observed differences in *D. radicum* oviposition across treatments were not reflected in the collected larvae and pupae. Oviposition assays are used by farmers to assess whether insecticides should be applied, with an action threshold of seven eggs per trap per week (Bligaard *et al.*, 1999; Lamy *et al.*, 2017b). Our results show that this may not be a good proxy for cabbage root fly infestation, especially in diversified cropping systems. Indeed, cabbage yield in the same field trial did not seem to suffer from the high *D. radicum* egg densities: the highest yield was recorded in the Strip_diversity treatment, in which most cabbage root fly eggs were found (Lenora Ditzler, personal communication). The discrepancy between collected eggs and later life stages indicates a high mortality of eggs and early instar larvae, especially in those treatments in which most eggs were collected.

Many factors could have contributed to mortality of eggs and early instar larvae, including abiotic factors, plant defence and quality, and natural enemies. Eggs and early instars of *D. radicum* can be eaten by carabid and staphylinid beetles (Finch, 1989). However, it is difficult to estimate their importance based on the data we collected in this study. Carabid beetles of the genus *Bembidion* are considered important predators of *Delia* species (Finch & Elliott, 1992; Ferry *et al.*, 2007; Björkman *et al.*, 2010), but we only collected a very small number of these beetles. The carabid species with the highest activity density in our field, *P. melanarius*, is predominantly carnivorous (Turin, 2000). However, how much *P. melanarius* contributes to the control of *D. radicum* eggs and larvae is questionable. In a laboratory study, *P. melanarius* did not eat any eggs at all (Finch & Elliott, 1992; Finch, 1996), although this was contradicted in another experiment (Andersen *et al.*, 1983). While the second most abundant carabid species, *H. rufipes*, does feed on *D. radicum* eggs *in vitro*, it is considered to be predominantly a seed predator (Andersen *et al.*, 1983; Finch, 1996; Turin, 2000). We did find a positive correlation between the number of *D. radicum* and soil-dwelling predators in our destructive sampling effort. This may mean that predators were attracted to infested plants in search of cabbage root fly larvae and pupae to eat, or that similar abiotic conditions were preferred by both groups. Assays using sentinel *D. radicum* eggs or pupae glued to cards would be a valuable next step to assess predation and parasitism under field conditions (McHugh *et al.*, 2020), although care should be taken to make these sentinels realistic by covering them with soil (Finch & Collier, 2007). Furthermore, future studies should use DNA metabarcoding of carabid beetle gut contents (Roubinet *et al.*, 2018), as this would provide reliable estimates of which species feed on *D. radicum* in the field.

Through plant-mediated interactions, we expected to find a negative correlation between aboveground chewers and *D. radicum* oviposition and infestation. However, we did not record such an effect. This may be an indication that plant-mediated negative effects of aboveground chewers on *D. radicum* performance are magnified in greenhouse studies (Soler *et al.*, 2007; Karssemeijer *et al.*, 2020 - **chapter 2**). A meta-analysis of plant-mediated interactions between above- and belowground herbivores confirms that the effects in fields are much less pronounced than what is observed in greenhouse trials (Johnson *et al.*, 2012). This does not mean these interactions do not occur outside, as induction soon after planting in a field experment can affect the herbivore community throughout the growing season (Poelman *et al.*, 2008)2008. Since we monitored the aboveground herbivore community only around the same time as our *D. radicum* measurements, we may have missed important earlier inducers of systemic plant defence. We did find a negative correlation between *D. radicum* and other belowground herbivores, mainly consisting of wireworms (Elateridae) larvae. In milkweed plants, an asymmetrical interaction was found between specialist root-feeding *Tetraopes tetraophthalmus* larvae and wireworms. Mass of *T. tetraophthalmus*

increased when wireworms were present, while the latter decreased in the presence of the other (Erwin *et al.*, 2013). To the best of our knowledge, no studies have specifically investigated interactions between *D. radicum* and other root herbivores when they feed on the same plants.

Conclusion

The search for sustainable solutions to control *D. radicum* has spanned decades. Researchers have investigated many potential strategies to tackle this pest, from attempts with biological control agents (Finch, 1989; Chen *et al.*, 2003; Hartfield & Finch, 2003; Kapranas *et al.*, 2020), searching for natural plant resistance, and using cover crops (Finch & Collier, 2000; Meyling *et al.*, 2013), to the development of a push-pull system (Lamy *et al.*, 2017a; Lamy *et al.*, 2020). With this study, we contribute novel data regarding the effect of crop diversification on this devastating insect pest. While we did not find a beneficial effect of strip cropping on cabbage root fly oviposition from a farmer's perspective, our data does suggest increased natural biological control, leading to a similar pest pressure in various setups. Our findings highlight the complexity of plant-insect interactions in the field, in which many factors such as nectar availability and spatial configuration of plants coincide to determine pest dynamics. Because a silver bullet approach will most likely never be found to control this pest, an integrated approach should be considered to reduce damage in a sustainable manner. Such an approach would combine multiple strategies, including crop diversification, carefully selected crop varieties, measures to increase natural enemy populations, and novel pest control techniques.

Acknowledgements

This work could not have been completed without the help of many colleagues and students, who went out of their way to help us in the herculean effort of sifting through hundreds of soil samples in the destructive sampling effort during the Covid pandemic; for this we thank **Gabriel Joachim**, **Julia Friman**, **Camilo Rivera Arrivillaga**, **Robin Hendriks**, **Katherine Barragán-Fonseca**, **Janneke Bloem**, **Max Wantulla**, **Reinier Valstar**, **Evangelos Kontos**, **Nina Oskam**, **Misty Hu** and **Els van de Zande**. We are grateful to the strip cropping team for guidance and inspiration during the many meetings. Finally, we thank **Andries Siepel**, **Titouan le Noc**, and **Olivia Elsenpeter** for maintaining the field.

Supplementary figures

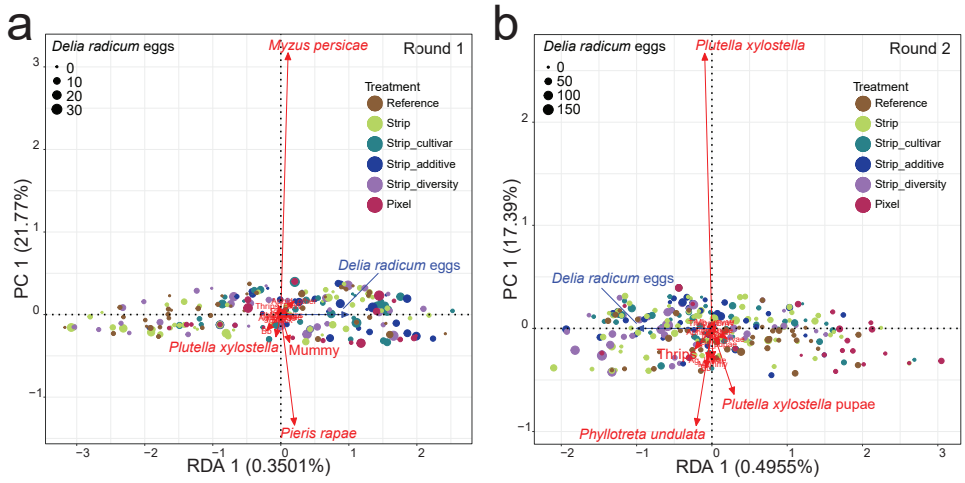


Figure S1. Redundancy analysis (RDA) of the aboveground herbivore community on cabbage plants grown in different cropping designs (treatments) in rounds 1 (**a**) and 2 (**b**). Constrained by the number of *Delia radicum* eggs collected on these plants. Size of the data points reflects the number of eggs found on felt traps the week after the aboveground herbivore community was assessed.

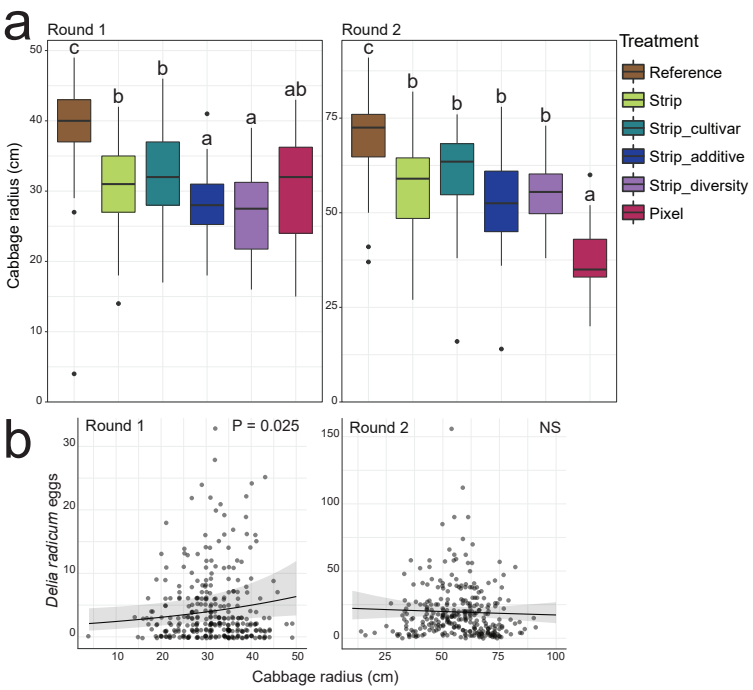


Figure S2. (**a**) Radius of cabbage plants in different cropping setups, measured as the distance between the furthest leaf tips. Results of pairwise comparisons between treatments are indicated with letters; treatments having no letters in common differ significantly ($P < 0.05$). (**b**) Correlation between cabbage radius and the number of *D. radicum* collected from those plants in round 1 and 2. NS: Not significant.

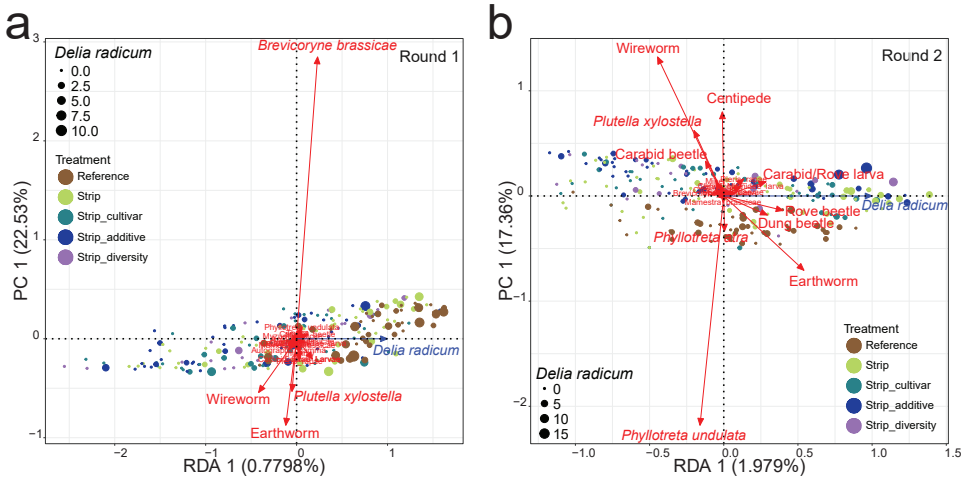


Figure S3. Redundancy analysis (RDA) of the above- and belowground macrofauna community on cabbage plants grown in different cropping designs (treatments) in rounds 1 (**a**) and 2 (**b**). Constrained by the number of *Delia radicum* larvae and pupae collected on these plants. Size of the data points reflects the number of *Delia radicum* on each plant.

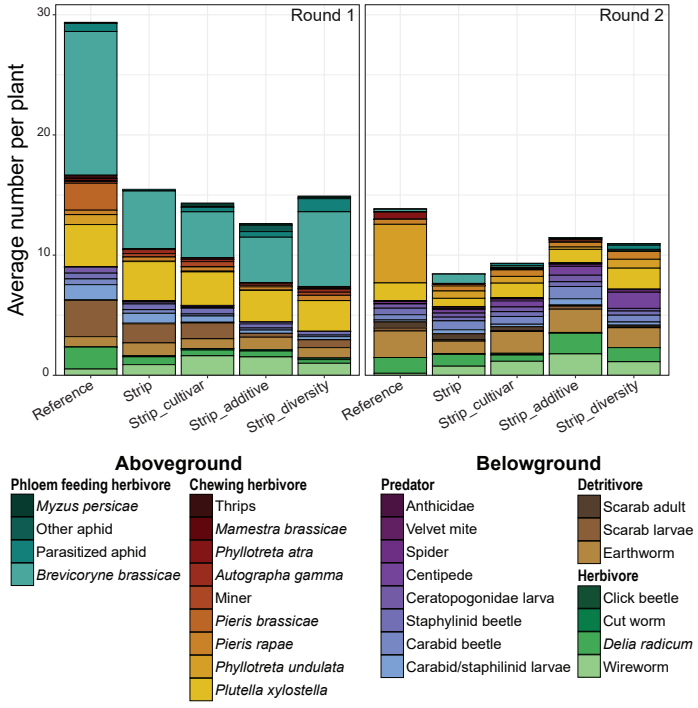


Figure S4. Above- and belowground macrofauna on and around cabbage plants in different cropping designs. Belowground macrofauna was assessed in a soil core (20 cm diameter, 20 cm depth) around cabbage plants. Aboveground herbivores on the plants were assessed one day prior to taking of soil cores. Different colour schemes indicate different functional groups.

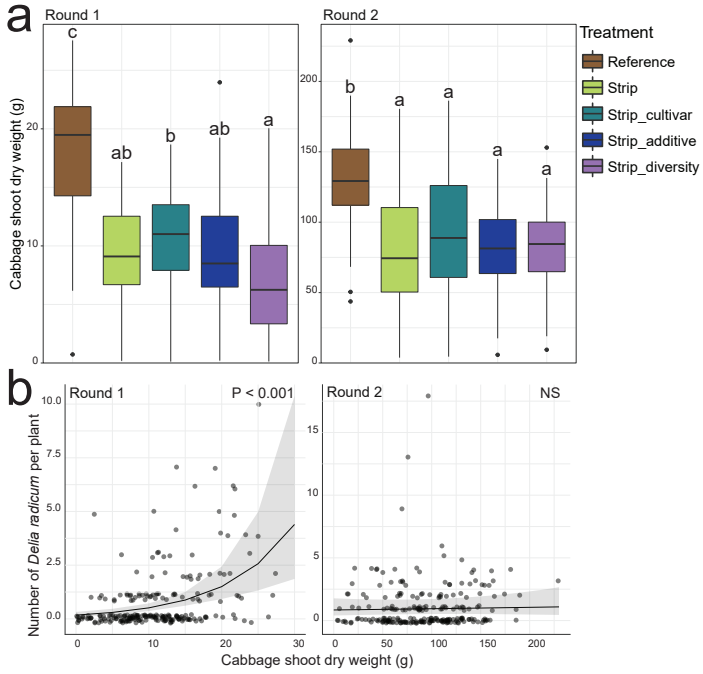


Figure S5. (**a**) Shoot dry weight of cabbage plants in different cropping setups. Results of pairwise comparisons between treatments are indicated with letters; treatments having no letters in common differ significantly ($P < 0.05$). (**b**) Correlation between cabbage shoot dry weight and the number of *D. radicum* collected from those plants in round 1 and 2. NS = Not significant.

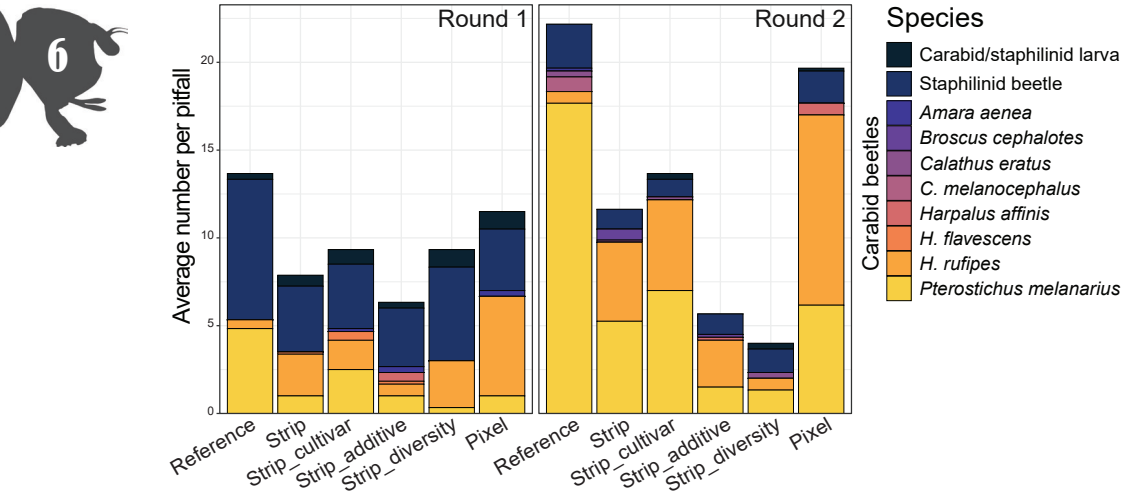


Figure S6. Pitfall catches in different cropping designs. Carabid beetles were identified to the species level.

Table S1. Number of cabbage root fly larvae and pupae (mean ± standard error) found in a biweekly monitoring effort throughout the field season. Every other week 24 plants were sampled, equally distributed over the four fields. Plants were taken from buffer strips, cabbage strips in between two treatments which are not used for any other measurement, to minimise interference with the field trial. Plants were collected with an augur (20 cm diameter). The roots and soil were carefully assessed for cabbage root fly larvae and pupae.

Date	N	Larvae	Pupae	Total
15 June 2021	23	1.17±0.46	0.57±0.19	1.74±0.49
29 June 2021	24	0.83±0.31	1.29±0.27	2.13±0.50
13 July 2021	24	0.50±0.16	0.50±0.21	1.00±0.31
27 July 2021	24	0.96±0.33	0.63±0.20	1.58±0.45
10 August 2021	24	0.38±0.19	0.83±0.30	1.21±0.42
24 August 2021	24	0.04±0.04	0.54±0.23	0.58±0.22
7 September 2021	24	0.00±0.00	0.25±0.14	0.25±0.14
21 September 2021	24	0.00±0.00	1.25±0.45	1.25±0.45
5 October 2021	24	0.00±0.00	0.13±0.07	0.13±0.07

Photo by Hans Smid

Chapter 7

General discussion

General discussion

Plants are at the mercy of the location in which they grow. To survive, they must be able to adapt to an immense diversity of stresses, which can occur alone or together in numerous combinations. Understanding how plants cope with their complex environment is a central challenge in biology. While ecologists and agronomists take a broad approach to understand general trends in (agro)ecosystems, most plant scientists do their best to narrow down to a single interaction or aspect of plant biology, to elucidate specific mechanisms or functions. In this thesis, I combined these two approaches by studying the interactions between above- and belowground herbivores in the laboratory, the greenhouse, and the field. The central objective of this thesis was to identify and understand the plant-mediated effects of aboveground herbivores on root-feeding herbivores.

To address this objective, I have extensively studied the interactions between cabbage plants and the cabbage root fly *Delia radicum* L. (Diptera: Anthomyiidae). Before addressing how aboveground herbivores affect this specialist cabbage root herbivore, it is important to understand how plant roots are defended. Several papers have reviewed plant defence in roots (van Dam, 2009; Erb, 2012; Johnson *et al.*, 2012), but knowledge on the underlying transcriptomics and signalling is scarce. Hence, before diving into plant-mediated interactions, I will discuss the current knowledge on plant defence signalling against root herbivores with a special focus on *D. radicum* (Fig. **1**).

The root response to *Delia radicum*

After hatching near a cabbage plant, *D. radicum* larvae quickly move towards the primary root. The larvae enter the roots, often via small crevices in the epidermis or hypocotyl, and disappear under the root epidermis where they start feeding on cortex tissue. The first days, larvae are hidden under the epidermis, and create feeding tunnels in the cortex tissue (for a timelapse video of larval feeding, see vimeo.com/508475837). While not much is known about the initial perception of cabbage root maggots by the plant roots, there is evidence for specificity compared to the root response to mechanical damage. For instance, the parasitoid wasp *Trybliographa rapae* Westwood (Hymenoptera: Figitidae) is attracted to volatiles emitted from roots and leaves of *D. radicum*-infested plants, but not from artificially damaged plants (Neveu *et al.*, 2002), even when only the leaves were presented and no direct volatiles from the larvae could be detected. Such specificity is most likely a result of recognition of elicitors by plant pattern recognition receptors, that may respond to components of the insect frass, exuviae, or saliva (Acevedo *et al.*, 2015; Erb & Reymond, 2019).

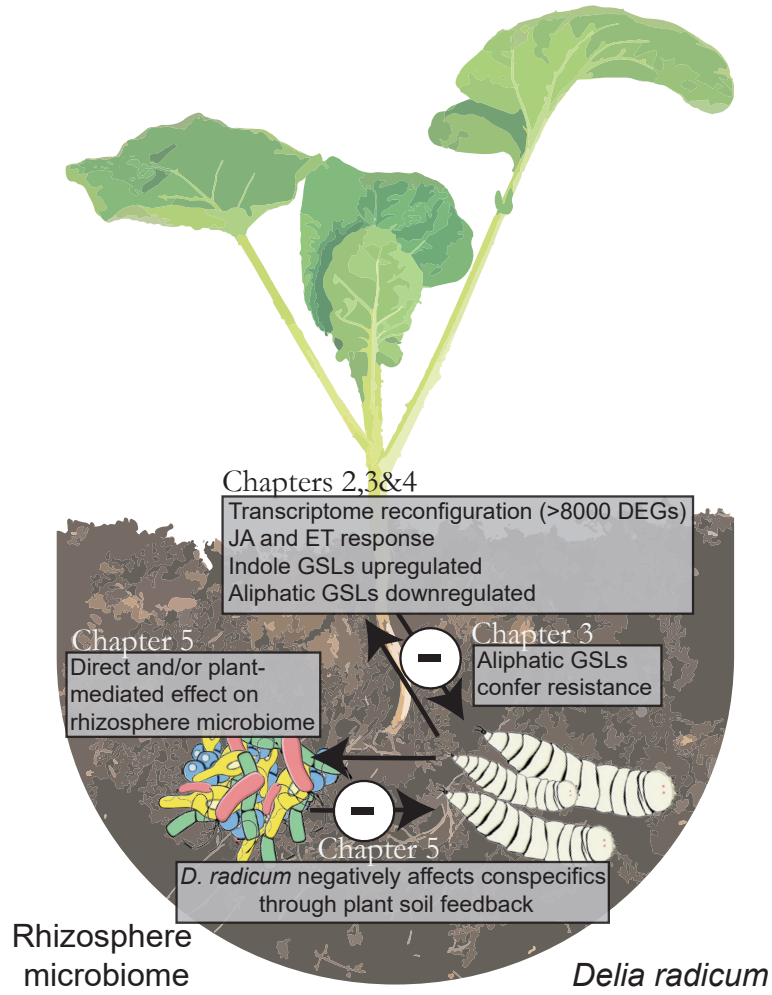


Figure 1. Overview of interactions between *Delia radicum* larvae, cabbage plants and the rhizosphere microbiome, with a focus on results of this thesis. Antagonistic effects are shown by a minus sign. Chapters in which effects are described are indicated. DEG: differentially expressed gene, JA: jasmonic acid, ET: ethylene, GSLs: glucosinolates. Insect and microbe drawings by Yidong Wang.

As soon as 30 minutes after placing larvae on a plant, a response can be measured in terms of gene expression within the primary root (**chapter 3**). Over the next two days, the cabbage primary root transcriptome is strongly reconfigured, leading to differential expression of thousands of genes (**chapter 3**). Genes encoding proteins involved in jasmonic acid (JA) and ethylene (ET) signalling are induced in the primary roots following *D. radicum* feeding (**chapters 2, 3, 4, 5**). While in previous studies the jasmonate burst upon root herbivory was

much milder compared to the response in leaves elicited by leaf-feeding herbivores (Erb *et al.*, 2012a), this does not seem to apply to cabbage primary roots in response to *D. radicum* (**chapters 2, 3**). Indeed, cabbage primary roots show a strong increase in jasmonates following *D. radicum* infestation. The *D. radicum*-induced root transcriptome further revealed exciting avenues for future research, such as the involvement of peroxidases, chitinases and ribosome reconfiguration in the primary root defence response.

One of the most obvious patterns emerging from the transcriptome analysis was an opposing regulation of indole and aliphatic glucosinolates (GSLs), secondary metabolites that are specific to the Brassicales order. Genes involved in the biosynthesis of indole GSLs were strongly upregulated after *D. radicum* infestation, while genes involved in biosynthesis of aliphatic GSLs were downregulated (**chapter 3**). Indeed, upon root herbivory, primary cabbage roots accumulate indole GSLs, in particular neoglucobrassicin, whereas concentrations of aliphatic GSLs are reduced or unchanged(van Dam & Raaijmakers, 2006; Touw *et al.*, 2020; **chapter 3**). In recent years, GSL transporters were shown to play a role in root-shoot allocation of GSLs (Andersen *et al.*, 2013; Jørgensen *et al.*, 2017). Expression of genes encoding these GSL transporters are upregulated by *D. radicum* in *B. rapa* roots (Touw *et al.*, 2020), while this effect is smaller in *B. oleracea* (**chapter 3**). Measurements of GSLs in leaves and roots of both species suggest that local production rather than distal transport is responsible for *D. radicum*-induced GSL patterns in primary roots (Touw *et al.*, 2020; **chapter 3**). Interestingly, when feeding on mutant plants that are impaired in aliphatic glucosinolate biosynthesis, *D. radicum* performance is increased, suggesting that these compounds confer resistance (**chapter 3**). As *D. radicum* feeding suppresses the biosynthesis of aliphatic GSLs, which appears to be beneficial to the insect rather than the plant, host-plant manipulation may be at play.

Aside from glucosinolates, plants in the *Brassica* genus produce many phenolic compounds such as lignin, anthocyanins, coumarins and flavonols (Cartea *et al.*, 2011; Park *et al.*, 2012; Zhuang *et al.*, 2019; Poveda *et al.*, 2021). These compounds are products of the phenylpropanoid biosynthetic pathway, which can also produce the phytohormone salicylic acid (Dixon *et al.*, 2002). Lignin, anthocyanins and flavonols can be involved in defence responses to pathogens and insect herbivores (Bernards & Båstrup-Spohr, 2008; Onkokesung *et al.*, 2014). Upon *D. radicum* herbivory, cabbage primary roots upregulate *PAL* gene expression (**chapters 2, 3**), which encodes a protein that is at the basis of the phenylpropanoid pathway in plants (Dixon *et al.*, 2002). Further investigation of genes involved in the phenylpropanoid pathway revealed that primary roots under attack most likely do not produce salicylic acid or flavonols (Mol, 2021; **chapter 5**), but may rather channel this pathway to producing lignin, anthocyanins or coumarins. An untargeted metabolomics screening would be timely to test which potential phytotoxins aside from GSLs are induced by *D. radicum*.

The soil directly around plant roots is a microbial hotspot, including many opportunistic pathogenic microbes (Johnson *et al.*, 2016b). Therefore, when plants respond to root herbivory it is not immediately clear who the primary target of the response is, the insect larvae or the microbes. Indeed, the plant response to *D. radicum* has similarities with responses to pathogens. Firstly, the primary root response to *D. radicum* appears mainly regulated by the JA and ET, a combination of phytohormones that was previously associated mostly with responses to necrotrophic pathogens (Pieterse *et al.*, 2012). Secondly, chitinases and indole GSLs are strongly upregulated upon *D. radicum* infestation (**chapter 3**), and these may be more effective against pathogens rather than insects (Bednarek *et al.*, 2011; Grover, 2012). In leaves of tomato plants, effective defence responses against the Colorado potato beetle are disrupted due to the presence of bacteria in the beetle's saliva (Chung *et al.*, 2013), suggesting that plants are poorly adapted to integrate defence responses against both insects and microbes. However, roots are always surrounded by a high density of microbes, so root defence responses may have evolved to better integrate responses to pathogens and insect herbivores.

To make matters more complicated, rhizosphere inhabitants themselves may also be involved in defence against root herbivores, either directly by infecting insect herbivores or indirectly by priming plant defence (Pieterse *et al.*, 2014; Johnson & Rasmann, 2015; Lachaise *et al.*, 2017). Upon herbivory, plants change the composition of their root exudates, which may lead to attraction of entomopathogenic nematodes (Rasmann *et al.*, 2005) and changes in the composition of rhizosphere microbes. Experiments with *Arabidopsis* mutants revealed a clear role of the JA pathway in shaping the root exudate composition, with consequences for the rhizosphere microbiome (Carvalhais *et al.*, 2015). The rhizosphere microbiome of plants infested with *D. radicum* is different from uninfested plants (Ourry *et al.*, 2018; **chapter 5**). Differences in microbiomes can, in turn, lead to differences in plant defence against insect herbivores (Lachaise *et al.*, 2017; Hu *et al.*, 2018). In **chapter 5**, I found that when new plants are grown on soils conditioned by *D. radicum*-infested plants, and these new plants are again infested with *D. radicum*, insect mortality is very high (**chapter 5**). This suggests that plants recruit rhizosphere microbes that may aid in defence against root herbivores.

Plant-mediated effects from shoot to root

In the first experiments included in this thesis, I showed that leaf chewing by *Plutella xylostella* L. (Lepidoptera: Plutellidae) caterpillars negatively affected the performance of root-feeding *Delia radicum* larvae, whereas *Brevicoryne brassicae* L. (Hemiptera: Aphididae) aphids had no such effect (**chapter 2**). Previous studies have shown that plant-mediated antagonism of foliar chewers on root herbivores is a common occurrence in many study

systems (Johnson *et al.*, 2012; Soler *et al.*, 2013). There are, of course, exceptions: in Chinese tallow trees, root-feeding *Bikasha collaris* Baly (Coleoptera: Chrysomelidae) larvae were facilitated by leaf-chewing conspecific adults, whereas three other species of leaf-chewing herbivores caused antagonistic effects (Huang *et al.*, 2014). Furthermore, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) caterpillars did not affect *Agriotes lineatus* L. (Coleoptera: Elateridae) or *Tecio solanivora* Povolný (Lepidoptera: Gelechiidae) larvae feeding on roots of cotton or potato plants, respectively (Bezemer *et al.*, 2003; Kumar *et al.*, 2016). Moreover, in maize, root feeding *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) larvae were negatively affected by foliar herbivory by *S. frugiperda* Smith (Lepidoptera: Noctuidae) caterpillars, but only when the caterpillars arrived first (Erb *et al.*, 2011b; Huang *et al.*, 2017). In **chapter 4**, I confirmed that the negative effect of leaf chewers on *D. radicum* is a general pattern, as four out of six foliar chewing herbivores included in the experiment negatively affected *D. radicum* performance. Testing the plant-mediated effects of sap-feeding aphids on chewing root herbivores was a novelty of **chapter 2**, but the absence of an effect may also indicate a publication bias, i.e. previous experiments testing these interactions may not have been published due to a lack of significant results. The few examples of studies that did include effects of sap-feeding foliar herbivores on root chewers showed either slight plant-mediated facilitation (Johnson *et al.*, 2009), or no effects (Huang *et al.*, 2014).

Plant-mediated interactions between shoot and root herbivores were mostly studied in short-term greenhouse experiments, and there is a need to validate the ecological relevance of these findings in field trials (Johnson *et al.*, 2012). To address this issue, in **chapter 6** I used an ecological approach to assess whether there were correlations between natural infestations of foliar herbivores and *D. radicum* abundance. Based on the results of **chapters 2 and 4**, I expected that foliar chewing herbivores would affect the abundance of *D. radicum*. However, contrary to my expectations, there was no correlation between the abundance of *D. radicum* and the numbers of chewing or sap-feeding foliar herbivores. A potential reason for the lack of a correlation is that there are very few completely unscathed "control" plants in a field setup, which may obscure subtle interactions.

Aside from plant-mediated effects on the performance of root herbivores, female insects may be affected in their host-searching behaviour when another herbivore is present. In **chapter 4**, I used a two-choice setup to test whether oviposition by *D. radicum* flies was affected by the presence of foliar herbivores. All six species of foliar chewing herbivores included in this experiment resulted in strong attraction of *D. radicum* females, despite lower larval survival of their offspring on these plants. Previous research indicated that *D. radicum* flies lay more eggs on plants infested by conspecifics in the roots or *P. xylostella* caterpillars on the leaves (Finch & Jones, 1987; Baur *et al.*, 1996a), whereas oviposition was lower on plants with *P. xylostella*

eggs or aphid infestation on the leaf (Finch & Jones, 1987; Finch & Jones, 1989). Together, these experiments show that plant-mediated species interactions can affect insect herbivores even before they start feeding. In the field, however, I found no correlation between the number of *D. radicum* eggs and the abundance of aboveground chewing or sap-feeding herbivores (**chapter 6**). This suggests that other factors, such as nectar availability as a food source for female flies, may have overruled the effects of foliar herbivores on *D. radicum* oviposition that were observed in the greenhouse trials.

When we broaden our scope to include other belowground attackers, i.e. nematodes and pathogens, we see that plant-mediated effects from above- to belowground tissues are a common occurrence (Biere & Goverse, 2016). Leaf-chewing herbivores facilitated root-parasitic *Meloidodyne incognita* nematodes on tobacco plants (Kaplan *et al.*, 2008; Machado *et al.*, 2018). On the other hand, foliar induction of tomato plants by *Manduca sexta* L. (Lepidoptera: Sphingidae) caterpillars induced resistance against *M. incognita* (Martínez-Medina *et al.*, 2021). Leaf-feeding aphids can increase resistance to root parasitic *Heterodera schachtii* nematodes on *Arabidopsis thaliana* (Kutyniok & Müller, 2012), and root exudates of aphid-induced potato plants reduce hatching of *Globodera pallida* nematodes (Hoysted *et al.*, 2018). In a field study, Kaplan *et al.* (2009) found that natural plant-parasitic nematode infestation was increased on plants with leaf-chewing herbivores, but reduced on plants infested with aphids. Insect herbivores feeding on leaves can also affect root resistance to microbial pathogens. For instance, aboveground feeding by *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae) whiteflies enhanced resistance against the soil-borne pathogens *Agrobacterium tumefaciens* and *Ralstonia solanacearum* in tobacco and pepper plants, respectively (Yang *et al.*, 2011; Song *et al.*, 2015). The many examples of plant-mediated species interactions from above- to belowground further highlight that shoot and root defences are interconnected.

Mechanisms underlying shoot to root interactions

Whilst identifying plant-mediated effects of foliar herbivores on the preference and performance of root herbivores is relatively straightforward, the real difficulty arises when attempting to understand how these interactions occur. Various mechanisms have been suggested, which are not mutually exclusive; they include changes in root-shoot resource allocation (Kaplan *et al.*, 2008; Johnson *et al.*, 2009), systemic induction or priming of plant defence (Erb *et al.*, 2008; Machado *et al.*, 2018), increased levels of secondary metabolites (Soler *et al.*, 2007; Huang *et al.*, 2014), interference with host manipulation (Martínez-Medina *et al.*, 2021), changes in root exudates (Hoysted *et al.*, 2018) or root emitted volatiles (Huang *et al.*, 2017), natural enemy attraction (Rasmann & Turlings, 2007), and shifts in the rhizosphere microbiome (Yang *et al.*, 2011). In this section, I will discuss

these potential mechanisms and whether they might apply to interactions between foliar herbivores and *D. radicum* in cabbage (Fig. **2**).

Primary metabolism

When plants are under attack in foliar tissues, allocating valuable primary resources to distal tissues may be a strategy to tolerate attack, as these resources can be used to regrow

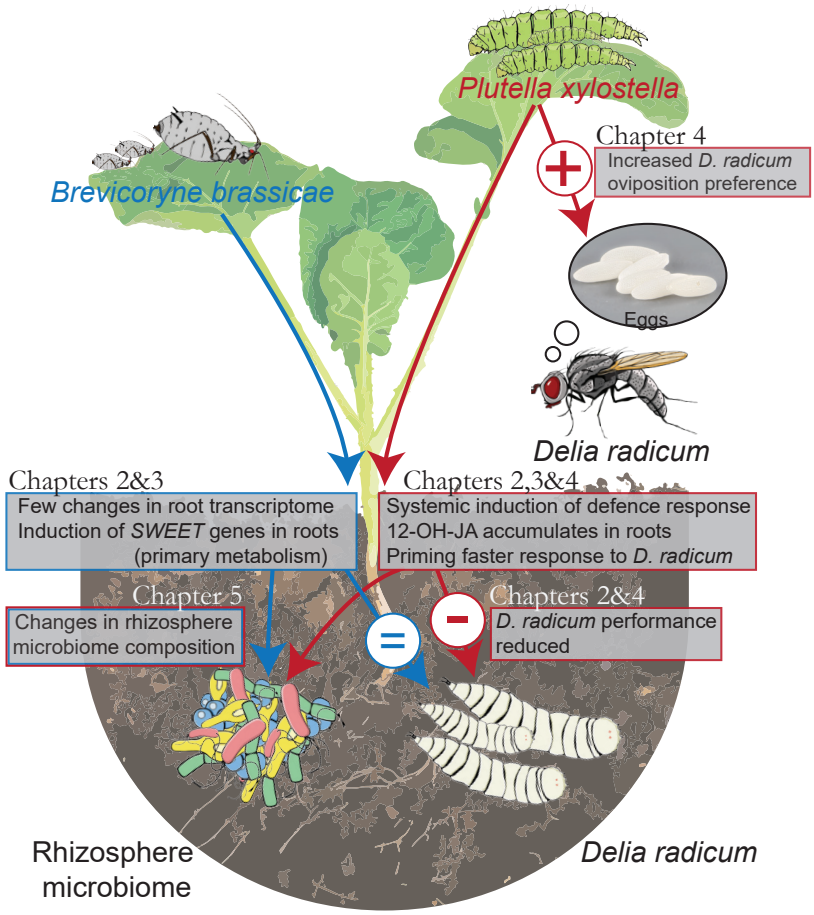


Figure 2. Overview of plant-mediated effects of *Brevicoryne brassicae* and *Plutella xylostella* on *Delia radicum*, with a focus on the proposed mechanisms studied in this thesis. Blue arrows and outlines indicate effects of *B. brassicae* leaf infestation, red arrows and outlines indicate effects of *P. xylostella* folivory. Chapters in which effects were found are indicated. Arrows indicate effects that are either positive (+), negative (-) or neutral (=). 12-OH-JA: 12-hydroxy-jasmonic acid. Insect and microbe drawings by Yidong Wang. Photo inlay of eggs by Hans Smid.

(Schwachtje *et al.*, 2006). This presents a potential mechanism by which foliar and root herbivores can affect one another. Changes in primary metabolites were suggested to play a role in plant-mediated facilitation of foliar aphids on root feeding wireworms (Johnson *et al.*, 2009), and foliar chewers on root-feeding nematodes (Kaplan *et al.*, 2008). While this mechanism may be involved in plant-mediated facilitation, the effects of foliage-chewing herbivores on root chewers such as *D. radicum* is generally negative and would not be easily explained by an increase in root nutrition. Indeed, leaf feeding by *P. brassicae* was detrimental for *D. radicum* on *B. nigra* roots, and no changes in root biomass or nitrogen content were measured (Soler *et al.*, 2007). In the RNAseq analysis of **chapter 3**, I found only a single gene that was consistently differentially regulated in roots infested aboveground with *B. brassicae* aphids. This gene (Bo3g111200) is a homolog of the *Arabidopsis SWEET11* gene, which encodes a sugar transporter involved in phloem loading of carbon (Durand *et al.*, 2016). Thus, changes in primary metabolites may have occurred in aphid-induced plants, but they did not affect resistance to *D. radicum* (**chapter 2**).

Plant defence signalling

Twenty years ago, van der Putten *et al.* (2001) argued that plant defence should be included in studies addressing above- and belowground interactions. Since then, many studies have established systemic induction of defences from shoot to root (Erb, 2012; Soler *et al.*, 2013; Biere & Goverse, 2016; Papadopoulou *et al.*, 2018). In many plant species, leaf-chewing herbivores can trigger a JA response in the roots, although there are exceptions to this rule (Erb *et al.*, 2009a; Soler *et al.*, 2013). In *Arabidopsis* seedlings, wounding of the cotyledons triggers transport of the JA precursor OPDA to the roots, triggering a systemic JA response (Schulze *et al.*, 2019). In **chapters 2, 3 and 4**, I found that six different leaf-chewing herbivore species trigger a JA-mediated defence response in roots, although this response is weak compared to local induction by *D. radicum*. OPDA levels in primary roots did not change in response to *P. xylostella*, which may be due to the timing of my measurements (starting 48 hours after the start of caterpillar feeding). Interestingly, the hydroxylated inactive form of JA, 12-OH-JA, accumulates in roots following *P. xylostella* herbivory (**chapters 2 and 3**). Whether this compound is transported from the leaves or produced directly in the roots, and thus indicative of an earlier accumulation of JA in roots, is unclear.

Even without a strong systemic defence response, foliar herbivores may affect root feeders by priming defences (Erb *et al.*, 2008). When defence is primed, the response to a local attacker can be stronger or faster, potentially leading to increased resistance (Hilker *et al.*, 2016). The primary root defence response to *D. radicum* leads to measurable changes in gene expression 30 minutes after induction. When plants are induced by *P. xylostella*

prior to root herbivory, this rate of induction is enhanced, and gene expression changes in the JA and indole GSL biosynthesis pathways can be measured 15 minutes after induction by root herbivores (**chapter 3**). Interestingly, the accumulated 12-OH-JA in *P. xylostella* induced plants might be responsible for this faster response. In *Arabidopsis* seedlings, gene expression responses triggered by the biologically active JA-Isoleucine conjugate (JA-Ile) were accelerated when 12-OH-JA was supplied together with JA-Ile (Smirnova *et al.*, 2017). The biological relevance of a faster response to *D. radicum*, as well as the potential mechanisms involving 12-OH-JA, should be studied in more detail.

Many herbivores have evolved mechanisms to manipulate their host plants. Such manipulation is often the result of effectors that interfere with the defence signalling cascade in plants (Consales *et al.*, 2012; Chung *et al.*, 2013; Acevedo *et al.*, 2015). Host-plant manipulation can be involved in plant-mediated interactions between herbivore species. For instance, a virulent population of *Nasonovia ribisnigri* Mosley (Hemiptera: Aphididae) aphids that is able to break a resistance gene in lettuce can facilitate conspecific aphids from an avirulent population that feed nearby, most likely through local suppression of plant defence (ten Broeke *et al.*, 2017). Herbivores can also interfere with host-manipulation of another herbivore feeding on the same plant, which is a potential mechanism for plant-mediated antagonism. Indeed, foliar feeding by *M. sexta* caterpillars attenuated repression of JA responses by *M. incognita* nematodes in roots, leading to reduced nematode performance (Martínez-Medina *et al.*, 2021). While I previously suggested that *D. radicum* may manipulate the host plant by suppressing toxic aliphatic GSL biosynthesis (**chapter 3**), this has not been directly studied yet. The recent publication of the *D. radicum* genome should facilitate studies targeting host-manipulation by screening for potential effectors (Sontowski *et al.*, 2021).

Plant defence chemistry

Defence responses lead to the production of defensive compounds and proteins, which in turn can confer resistance to herbivores. Systemic changes in toxic secondary metabolites have often been suggested as the mechanism underlying plant-mediated interactions between above- and belowground herbivores (Bezemer & van Dam, 2005; Soler *et al.*, 2007; Erb *et al.*, 2011b; Soler *et al.*, 2013; Erb *et al.*, 2015). For instance, effects of *P. brassicae* leaf feeding on *D. radicum* were correlated with changes in root glucosinolates (Soler *et al.*, 2007). Such changes could be caused by a systemically induced defence response or by translocation of secondary metabolites. Foliar herbivores trigger systemic production of nicotine in roots of tobacco plants, which is then transported to leaves for defence (Gulati *et al.*, 2014). In brassicaceous plants, GSLs can be transported throughout the plant

by glucosinolate transporters (Andersen *et al.*, 2013; Nambiar *et al.*, 2021), but I did not find evidence for GSL transport based on leaf and root concentrations following shoot and root herbivory (**chapter 3**). Moreover, I did not find differences in the primary root GSL profile induced by *P. xylostella* or *B. brassicae* (**chapter 3**). However, the priming effect by *P. xylostella* described above was also observed in terms of induction of indole GSLs, which were produced faster in dual-infested plants (**chapter 3**). Although different species of foliar herbivores either enhanced or reduced the expression of *MYB28* in roots of cabbage plants, these effects were overruled by local *D. radicum* induction (**chapter 4**). Naturally, other secondary metabolites aside from GSLs may have mediated the antagonism towards *D. radicum* caused by foliar herbivores.

Foliar herbivores also affect water-soluble or volatile secondary plant metabolites in both roots and shoots, potentially affecting belowground herbivores before they start feeding. For instance, *Myzus persicae* Sulzer (Hemiptera: Aphididae) aphids caused changes in root exudates of potato plants that inhibited hatching of *Globodera pallida* plant-parasitic nematodes (Hoysted *et al.*, 2018). Moreover, feeding on maize leaves by *S. littoralis* Boisduval (Lepidoptera: Noctuidae) or *S. frugiperda* caterpillars led to changes in root soluble phenolic acids and volatile emissions, respectively, both of which had deterrent effects on root-feeding *D. v. virgifera* larvae (Robert *et al.*, 2012a; Erb *et al.*, 2015; Huang *et al.*, 2017). Similar direct effects of root volatiles or exudates on *D. radicum* larvae have not been studied do date. However, behavioural effects of volatile secondary metabolites on adult cabbage root flies have been studied extensively. For instance, female flies are repelled from plants that emit high concentrations of dimethyldisulfide (DMDS), a compound that is strongly induced by larval feeding on the roots (Ferry *et al.*, 2009; Crespo *et al.*, 2012). While foliar herbivory by *Pieris brassicae* L. (Lepidoptera: Pieridae) caterpillars alone did not change the emission of this compound, the leaves of plants infested with both *P. brassicae* and *D. radicum* emitted increased amounts of this compound (Danner *et al.*, 2015). Furthermore, the headspace of *B. rapa* plants infested with foliar herbivores contained increased levels of volatile GSL hydrolysis products (Danner *et al.*, 2018), which are attractive to *D. radicum* females (Hawkes & Coaker, 1979). Herbivore induced changes in volatiles most likely mediated the oviposition-stimulating effects of foliar herbivory on *D. radicum* females found in **chapter 4**.

Involving a third party

Changes in volatile secondary metabolites emitted from shoots or roots can also influence natural enemies. If foliar herbivores interfere with indirect defence responses against root herbivores, they may result in an enemy-free space for the latter herbivore, thereby shifting the balance from plant-mediated antagonism to facilitation. This aspect of plant-mediated

interactions may be especially relevant in the field, as it can cause changes in population dynamics between herbivores and their natural enemies. When *S. littoralis* caterpillars feed on leaves of maize plants, they interrupt the attraction of entomopathogenic nematodes upon *D. v. virgifera* infestation (Rasmann & Turlings, 2007). The reduced attraction of entomopathogenic nematodes was linked to lower emissions of the volatile compound *(E)*-β-caryophyllene. Likewise, turnip plants infested with both *P. brassicae* caterpillars on leaves and *D. radicum* on roots were much less attractive to *T. rapae* compared to plants only infested in the roots, and this effect caused reduced parasitism rates in a field setup (Pierre *et al.*, 2011). In **chapter 6**, I assessed parasitism of *D. radicum* in a field trial, but the numbers of parasitoid individuals collected were too small to analyse whether their presence may have correlated with presence or absence of foliar herbivores.

Finally, foliar herbivores can affect the rhizosphere microbiome, which can have direct and indirect consequences for root herbivores (Friman *et al.*, 2021). Plants alter the composition of root exudates upon foliar herbivory (Marti *et al.*, 2013; Kim *et al.*, 2016). Additionally, foliar herbivores can influence soil microbes directly by dropping their frass, honeydew or exuviae onto the soil (Frost & Hunter, 2004). Indeed, it is well-established that foliar herbivores cause changes in the rhizosphere microbiome composition (Kostenko *et al.*, 2012; Kim *et al.*, 2016; Kong *et al.*, 2016; **chapter 5**). Differences in the rhizosphere microbiome can, in turn, influence root herbivores (Lachaise *et al.*, 2017). Thus, plant-mediated interactions between shoot- and root-feeding insect herbivores may be mediated by changes in the rhizosphere microbiome. With plant-soil feedback experiments, in which soil is conditioned by herbivore-infested plants, it is possible to isolate the effects of a changed rhizosphere microbiome to a certain extent. Plant-soil feedback experiments revealed that resistance against foliar herbivores can be altered when plants are grown on soils conditioned by herbivore-induced plants (Kostenko *et al.*, 2012). In **chapter 5**, resistance against *D. radicum* was not affected in plants grown in soil conditioned by plants that had been infested with the foliar herbivores *P. xylostella* or *B. brassicae*.

Interconnectivity

To conclude the previous section, there are many possible plant-mediated mechanisms by which foliar herbivores may affect root herbivores. Molecular plant scientists have taken great care to isolate mechanisms in the past decades, for instance by using mutant plants. In the natural world, however, these mechanisms do not act independently. As such, the outcome of plant-mediated interactions, whether facilitation or antagonism occurs, likely depends on a combination of many interconnected factors.

Many aspects of plant biology have a high degree of interconnectivity, which further increases the complexity of plant-insect interactions. Plant secondary metabolites often have functions other than being defence compounds (Erb & Kliebenstein, 2020). Glucosinolates, for instance, are involved in regulation of root growth and can be used by plants to store sulfur (Katz *et al.*, 2015; Zhang *et al.*, 2020). Moreover, some secondary metabolites, such as benzoxazinoids and coumarins, play a role in iron uptake. As such, the effectiveness of benzoxazinoids as defence compounds depends on the nutrition status of the soil the plants grow in. Hu *et al.* (2021) discovered that when iron is scarce, plants use benzoxazinoids to gather resources, but when iron is abundant, the same molecule is invested in defence. Coumarins, in addition to their role in defence and iron uptake, are recently emerging as important factors in shaping the root microbiome and regulating ISR in *Arabidopsis* (Stringlis *et al.*, 2018; Stassen *et al.*, 2021). Two genes involved in this process, *PDR9* and *MYB72*, also responded to soil conditioning treatments in cabbage, suggesting a similar role (**chapter 5**). These blurred lines between plant defence and other biological processes increase context-dependency of assumed plant-defence traits.

Another degree of interconnectivity emerges when we consider multiple plants grown in proximity. Plants downregulate their defences when perceiving competition for light by a neighbouring plant (Ballaré, 2014; Fernández-Milmanda *et al.*, 2020). A recent study revealed that genes involved in defence are also affected in roots of plants grown under shaded conditions (Rosado *et al.*, 2022), suggesting that root defences may be modulated when plants perceive a neighbouring competitor. Furthermore, plants can respond to herbivory on neighbouring plants. Volatiles emitted from damaged plants can activate defence responses in neighbouring plants, which may prepare them for future attack (Frost *et al.*, 2008; Hu, 2021). Similar communication among plants can occur through shared networks of mycorrhizal fungi associated with the roots, even between heterospecific plant species (Song *et al.*, 2010; Song *et al.*, 2015). Plant-plant communication can even be hijacked by insect herbivores, for instance, *B. tabaci* whiteflies induce plant volatiles that reduce defence in neighbouring plants (Zhang *et al.*, 2019). Through induction or priming of defence via plant-plant communication, insect herbivores might be able to interact even when they are feeding on neighbouring plants.

Zooming out to the ecosystem or agroecosystem scale reveals even more mechanisms that affect interactions between plants and insect herbivores feeding on them. Landscape elements such as forest edges, for instance, can affect foraging by natural enemies (Aartsma *et al.*, 2017). Within an agricultural field, transitioning from monoculture to a more diversified cropping system can influence insects on crop plants (Finch & Collier, 2000; Tajmiri *et al.*, 2017; Mansion-Vaquié *et al.*, 2020). The results of **chapter 6** show that *D. radicum* oviposition

can be strongly affected by changes in cropping systems (especially spatial configuration and nectar availability), whereas *D. radicum* infestation of the roots was not affected. The differences in results between eggs and later stages of *D. radicum* indicate high mortality of eggs and early instar larvae, yet I found no evidence for plant-mediated effects of shoot-feeding herbivores on *D. radicum* based on correlations. There were, however, correlations between the number of *D. radicum* larvae and pupae retrieved in the soil and the numbers of belowground predators, detritivores, and wireworms. These results suggest that other factors, such as nectar availability, spatial configuration of plants, and the presence of other belowground organisms, may overrule the effects of plant-mediated interactions between shoot- and root-feeding insect herbivores in the field.

Conclusion and Future directions

With this thesis, I contribute to understanding how plants interact with a complex and dynamic environment. My research and the examples included in this final chapter show that shoots and roots have different, but highly intertwined defence responses. In many cases, responses occurring in both compartments are important for an effective defence response (Biere & Goverse, 2016). This idea is further strengthened by the growing body of evidence that rhizosphere microbes are recruited by the plant to modulate defence responses. As such, the above- and belowground communities of plant-associated organisms are linked through the plant. Because plants interact intimately with such a highly diverse environment, they must integrate cues from different sources. Recent evidence suggests that plants are adapted to combinations of herbivores that occur often in a natural setting (Mertens *et al.*, 2021a; Mertens *et al.*, 2021b). Looking back at the progress made by the scientific community in the past decades, it is exciting to imagine what the next decades will reveal.

As the cabbage root fly is the main character of this thesis, I will use these final words to reflect on potential future directions of research on *D. radicum*. Extending the research using GSL mutants which I started in **chapter 3** would certainly yield interesting results, especially if aromatic and indole GSLs could be knocked out or overexpressed. Investigation of the role of peroxidases and chitinases in plant defence against *D. radicum* would also be justified based on my transcriptome analysis (**chapter 3**). In terms of the plant-mediated antagonism of *P. xylostella* on *D. radicum*, it would be interesting to further study defence priming of early responses. For instance, experiments with supplemented 12-OH-JA could substantiate whether this compound is involved in the priming I observed. Studying these very early plant defence responses in more detail may indicate how important the first minutes after infestation are in determining whether plant resistance can be reached. Rhizosphere microbes present many other intriguing avenues for future research. The results of **chapter 5** suggest that plants may actively recruit rhizosphere

microbes to aid in defence against *D. radicum*, but more experiments are needed to prove this. Such experiments could focus on the mechanisms underlying *D. radicum*-induced changes in the rhizosphere microbiome, for instance by studying root exudates or mutagenesis of genes responsible for root exudation, or on pinpointing the specific microbial strains responsible for the observed antagonistic effects. Studies involving rhizosphere microbiomes are currently popular (Berendsen *et al.*, 2012; Brunel *et al.*, 2020; Stassen *et al.*, 2021) and I expect many thrilling publications in the coming years.

The recent publication of a *D. radicum* genome paves the way for exciting discoveries (Sontowski *et al.*, 2021). With novel gene-editing technology, functional characterisation of *D. radicum* genes should be possible. Such experiments could reveal whether *D. radicum* possesses any means of detoxifying plant secondary metabolites aside from gut microbes, and whether the larvae produce effectors to interfere with plant defence signalling. Moreover, the *Delia* genus harbours closely related species with very different host ranges; *D. radicum* and *D. floralis* Fallén (Diptera: Anthomyiidae) specialise on brassicaceous plants, *D. antiqua* Meigen (Diptera: Anthomyiidae) specialises predominantly on the *Allium* genus, whereas *D. platura* Meigen (Diptera: Anthomyiidae) is a polyphagous pest on crops including Fabaceae and cereals (Gouinguené & Städler, 2006). Comparative genomics within the *Delia* genus could therefore lead to a deeper understanding of host-plant specialisation.

The above two paragraphs sketch mostly fundamental avenues for future research. Advancements in fundamental research are essential for developing novel applications in agriculture, to safeguard crops against insect herbivores and to support the necessary move towards sustainable agriculture. This will be essential in the coming decades, as the use of pesticides is increasingly restricted (Carvalho, 2017; European Commission, 2018a; European Commission, 2018b; European Commission, 2018c) and there is a growing societal need for sustainable crop production. These past years of studying insect-plant interactions made me greatly appreciate the complexity of a biological system that most people would not even think twice about, an ordinary cabbage plant and a handful of pesky insects. I strongly believe that introducing the wider public to fundamental research on insect-plant interactions is equally important as performing it; as it will hopefully inspire more people to admire plants, insects, and the natural world at large.

Acknowledgements

I greatly appreciate comments on an earlier version of this chapter by **Katherine Barragán-Fonseca**, **Joop J. A. van Loon** and **Marcel Dicke**.

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Summary

Plants are attacked by a diverse group of insect herbivores that feed on their roots and shoots. Over the course of millions of years, plants have evolved an intricate immune system. When a plant perceives an attacker, responses are rapidly triggered, leading to the activation of defence mechanisms aimed at deterring or resisting the attacker. Activation of plant defence does not only occur locally but throughout the plant; in part to prepare for attack in systemic tissues, and in part because plant secondary metabolites may be produced in systemic tissues and transported to the site where they are needed. Defence responses triggered by one insect herbivore can affect another insect herbivore feeding on the same plant, even if they are separated in space and time. Such plant-mediated interactions between insects are common in natural settings, as plants are rarely visited by only a single herbivore. The outcome of these interactions between insect herbivores can range from facilitation to antagonism, depending on many factors such as the identity and feeding site of the herbivores involved. Most studies on plant defence and plant-mediated species interactions have focussed on aboveground tissues. As a result, there is a gap in our understanding of regulation of plant defence in the roots, and how these defence responses may be modulated in plant-mediated interactions.

The **aim** of this thesis was **to identify and understand the effects of shoot herbivory, *via* plant-mediated interactions, on root herbivory**. My study system consisted of cabbage plants and three of the most common pest species in the field; shoot-feeding *Brevicoryne brassicae* aphids piercing-sucking on phloem and leaf-chewing *Plutella xylostella* caterpillars, and root-feeding *Delia radicum* maggots. I focussed on the effects of induction by the shoot feeders on defence in the roots and consequences for *D. radicum*. To understand how plant-mediated interactions may occur, I extensively studied plant defence in the primary roots of cabbage plants against *D. radicum* alone or in combination with the leaf herbivores.

In **chapter 2**, I tested whether *B. brassicae* and *P. xylostella* affected the performance of root-feeding *D. radicum* larvae. Plant-mediated antagonism of *D. radicum* occurred when *P. xylostella* was present on the plant, leading to a reduction in cabbage root fly survival by roughly 40 per cent. Leaf feeding by aphids did not affect cabbage root fly performance. Size and development time of the cabbage root flies was not affected by shoot herbivory.

To elucidate potential mechanisms of these plant-mediated interactions, I focussed on plant defence against the root herbivore in **chapters 2 and 3**. Infestation by *D. radicum* lead to thousands of differentially expressed genes in primary roots of cabbage plants in the first two days. Prior infestation by *B. brassicae* or *P. xylostella*, contrary to our expectations, did not have a large impact on the overall plant transcriptional response to *D. radicum*. Infestation by *D. radicum* appeared to overrule the systemic effects of leaf infestation.

However, we did find evidence for root-defence priming by *P. xylostella* caterpillars feeding on the leaves. Plants that were infested with *P. xylostella* responded faster to *D. radicum* compared to uninfested plants in terms of expression of several defence-related genes. The phytohormones jasmonic acid (JA) and ethylene (ET) play a central role in the root defence response. Following feeding by *P. xylostella* on the leaves, inactivated jasmonates accumulate in the primary root. The relevance of these compounds is yet unclear, but they may be involved in the aforementioned defence priming. When under attack by *D. radicum* larvae, primary roots rapidly started producing indole glucosinolates whereas the production of aliphatic glucosinolates was attenuated. Glucosinolates are defensive metabolites specific to the Brassicales order to which cabbage belongs. Using mutants that lack the ability to produce aliphatic glucosinolates altogether, I revealed that these metabolites confer resistance to *D. radicum*.

In **chapter 4**, I tested whether plant-mediated antagonism on *D. radicum* by *P. xylostella* is a general pattern of leaf-chewing herbivores. To this end, I tested the plant-mediated effects of six species of leaf-chewing herbivores on *D. radicum* performance and oviposition preference. The leaf herbivore species included both specialists that predominantly feed on plants in the Brassicaceae family, and generalists with a wide host range. Four out of six species negatively affected *D. radicum* performance, although the effects were relatively weak. Moreover, all six species had a strong impact on oviposition preference of female *D. radicum* flies. Infested plants were the preferred oviposition site, even though this may lead to reduced larval performance. In the discussion of **chapter 4**, I reflect on these results in the context of differential defence induction by specialist and generalist herbivores, and in the context of the mother-knows-best hypothesis which states that female insects should prefer to oviposit on plants that support higher larval performance.

Aside from interference or systemic induction of plant defence, another route by which insects may affect one another is through changes in the plant-associated rhizosphere microbiome. In **chapter 5**, I performed a plant-soil feedback experiment, in which soils were conditioned by plants infested with insect herbivores (*B. brassicae*, *P. xylostella* or *D. radicum*). The infested plants were removed, and the conditioned soils were used to grow a new set of cabbage plants on which plant defence against *D. radicum* was assessed. I assessed the fungal and bacterial communities in the rhizospheres of the removed plants directly after collecting the soils. All three herbivore species left their mark on the rhizosphere bacterial and fungal community, in which the feeding site (leaf or root) was an important factor. Performance of *D. radicum* larvae was reduced when they were feeding on plants grown on soils conditioned by plants infested with conspecific larvae. Root herbivore performance was not affected on soils conditioned by plants infested with leaf feeding *B.*

brassicae aphids or *P. xylostella* caterpillars. The results of this chapter suggest that plants recruit microbes to assist them in the defence against root herbivores.

In the final experimental chapter of this thesis, **chapter 6**, I investigated naturally occurring *D. radicum* oviposition and abundance in a field setting. We found no correlations between the oviposition or abundance of cabbage root flies and shoot herbivores, suggesting that the plant-mediated species interactions described in the previous chapters may be masked by the many other factors that play a role in a field setting. Another objective of this field experiment was to investigate how diversified cropping systems may affect *D. radicum*. I discovered that strip intercropping increased the number of *D. radicum* eggs but did not lead to higher infestation in terms of larvae and pupae. These results indicate high mortality of *D. radicum* eggs and young larvae in diversified cropping systems, potentially by increased activity of natural enemies. Furthermore, these results show that using *D. radicum* oviposition is a poor predictor for pest density.

In **chapter 7**, I discuss the findings of this research project and place them in the framework of plant defence in roots and plant-mediated interactions. This general discussion provides an extensive outline of plant defence against *D. radicum*, and the potential mechanisms that can cause plant-mediated species interactions. The results of this thesis advance the knowledge on plant defence against root-feeding insects and provide new insights on how plants interact with their complex environment.

Acknowledgements - Dankwoord

The work included in this thesis is a team effort and could not have been completed without the help of many people. In this section, I'd like to thank all those that have been involved in the making of this book on a professional and personal level.

Allereerst wil ik mijn promotoren bedanken. **Marcel**, toen ik in 2014 naar Wageningen kwam voor een minor gaf jij me met het vak *Insect Ecology* een prachtige introductie in de wereld van de Entomologie. Hierdoor was voor mij vrij snel duidelijk dat ik in Wageningen op de juiste plek was, en dat ik meer wilde leren over interacties tussen planten en insecten. Ik had de smaak te pakken en schreef me in voor een master programma Plantenwetenschappen met daarin zo veel mogelijk vakken over entomologie en het afweersysteem van planten. Tijdens deze master kwamen we elkaar vaak tegen, en toen ik een onderwerp zocht voor mijn scriptie was het denk ik geen verassing dat ik bij jou aanklopte. Ik volgde twee thesis projecten bij Entomologie, en jij begeleidde me bij het schrijven van het proposal dat uiteindelijk leidde tot dit proefschrift. Ik wil je vooral bedanken voor het vertrouwen dat je mij gegeven hebt, hierdoor kon ik het meeste van mijn studie maken en zonder dat vertrouwen was dit PhD project er niet geweest. **Joop**, jouw eeuwige geduld en vriendelijkheid maken je een geweldige begeleider, met jou erbij wordt elke meeting gezellig. Daarnaast bezit je een ongeloofelijke berg aan kennis, je lijkt soms een soort entomologische encylopedie. Bedankt voor al je goede advies en dat je altijd de állerkleinste foutjes in mijn drafts wist op te sporen. Marcel en Joop, jullie hebben beiden geweldige eigenschappen als begeleiders, en samen vullen jullie elkaar aan als in een koor – ik had me geen betere promotoren kunnen voorstellen. Jullie gaven mij de vrijheid om mijn project vorm te geven maar stonden altijd klaar als ik onzeker was over vervolgstappen. En tijdens de laatste fase hebben jullie telkens weer tijd gevonden om mijn drafts snel te bekijken zodat ik nooit op jullie heb hoeven wachten.

During the past years, I've been extremely lucky to receive help from many talented and motivated students. **Karthick**, thank you for your motivation and your hard work, it was truly a pleasure to connect with you on both a professional and personal level. I look forward to seeing the bright future that you will no doubt find for yourself! **Kris**, jij had mij eigenlijk niet nodig en hebt jezelf begeleid, en dat was maar goed ook want ik kon vaak niet aanwezig zijn op het lab. Het enige dat ik hoefde te doen was je afremmen omdat je ambities altijd groter waren dan je agenda. Ik vind het fantastisch dat jij op de laatste dag van mijn contract begonnen bent met je eigen PhD project, en symbolisch is het een mooie afsluiting dat jij "mijn" bureau de komende jaren gaat gebruiken. Ik kijk er naar uit om over vier jaar jouw boekje in handen te hebben. **Julian** and **Laura**, you showed me that there are many ways to reach excellence and it was fantastic to see you two develop and help each other during your thesis projects. Julian, your chaotic enthusiasm for science is an inspiration,

and I'm very happy to stay updated about all your adventures. Laura, your meticulous way of working is something we should all aim for. And then there were many others, **Bart**, **Teun**, **Toya**, and all the students that performed experiments in my project during courses I was involved in. You all poured your enthusiasm into this project, and taught me the joy of teaching and mentoring.

Frans, **André**, **Arnout** en **Pieter**, zonder alle insecten die jullie voor mij gekweekt hebben was dit werk niet mogelijk geweest! **Unifarm** personeel, in het bijzonder **Sean**, bedankt voor alle spruitkoolplantjes die jullie voor mij hebben opgekweekt. Jullie harde werk heeft veel geholpen om mijn experimenten soepel te laten verlopen. **Michael** and **Jonathan**, even though we have never met in person I'd like to thank you for your collaborations in the past years; your unbelievably rapid processing of countless samples for glucosinolate and phytohormone analyses greatly improved this thesis.

My dear Ento Family, it was wonderful to be part of such an incredible group of colleagues. The combination of a passion for entomology and your diverse backgrounds and personalities make the lab a truly magnificent place. I am proud to have been a member of the *Ento vikings* for very muddy strong obstacle runs and the *Tiger beetles* for the campus run!

Yidong, you were the first ento colleague whom I met. We had a lot of fun being lab-partners and started having dinners together. You showed me that I do not have to travel far from home to learn about faraway cultures, which brought me the joy of traveling even without leaving home! Thank you for inviting me into your life and for all the dinners you cooked for me, I am so happy to count you among my close friends. I hope some day we will succeed in making sticky rice sweets without having most of it stick to our hands. It's wonderful that you will stay in the Netherlands for the time being so we can continue to see each other. On a professional level, thank you for infusing my thesis with your artistic talent. Not only did you draw illustrations which I used in various chapters, but you also agreed to take care of the layout and cover drawing of this book, you have no idea how much stress that alleviated from me!

Julia, I feel very lucky that I happened to end up at the desk next to you. You have become one of my best friends. Thank you for lending me a hand in more experiments than I can count; working together with you was always smooth and you have secured a firm place in the acknowledgement section of all of my experimental chapters – aside from the one we authored together. I greatly appreciate all the discussions we had together, about teaching, science, life, the universe, and everything.. Our many phone calls during the pandemic made up for not being able to see our colleagues, and I'm glad we continue this in a weekly

boardgame night tradition together with Daniel. Our “study trip” to Rennes is a highlight of my PhD, and I’m happy that Juul and I were able to visit you in Sweden to see the area you grew up in and to meet your family. Thank you for being my paranymf!

Max, thank you for introducing me to Kölsch! I appreciate your humor and your critical way of thinking. I greatly respect that you always consider all possibilities and are not afraid to ask questions when in doubt, which I’m very confident will lead to a stunning thesis. Thank you for letting me “borrow” your eyes to see if my colorful figures were clear. And most of all, thank you for agreeing to being my paranymf, having you on the stage will surely keep the nerves at bay!

Kathe, it has been such a joy to sit across the desk from you the past years, you always made sure my vitamin C levels were on par by tossing mandarins at me. Thank you for all the good conversations, I’m glad we got the chance to work together and have many long talks during the preparations for Yidong’s defence. I hope one day I can come visit your family farm in Colombia. **Thibault**, thank you for all the nice walks around the campus and for showing me how to have lunch the French way, with a glass of good red wine. **Janneke**, het was fantastisch om met jou filmpjes en liedjes voor Julia’s verdediging te maken en jou tegelijkertijd beter te leren kennen. Ik heb Daði Freyr nog steeds in mijn hoofd. **Luuk**, ik vond het super leuk om voor mijn laatste hoofdstuk samen met jou kolen te slopen! Het was leuk om op deze manier een introductie te krijgen in veldecologie en het identificeren van allerlei bodemdiertjes. **Pieter**, bedankt voor alle goede gesprekken, en vooral voor al het slappe geouwehoer. **Rieta**, ik ben ontzettend blij dat jij betrokken raakte bij de laatste experimenten van mijn project. Het waren niet de makkelijkste proeven; de eerste werd opgeschud door de start van de pandemie en het veldseizoen was een enorme kluif. Jouw ervaring en kalmerende aanwezigheid hielpen om deze experimenten soepel te laten verlopen. Ik vond het super om met je te werken, en ben blij dat je als co-auteur op twee van mijn hoofdstukken staat. **Quint**, met jou valt haast geen serieus gesprek te voeren, bedankt daarvoor. Je was een geweldige scriptiebegeleider en het was fijn om je daarna nog even collega te mogen noemen. Ik zou het wel waarderen als je geen brandblussers meer naast mijn bed zet.. **Daan**, een proefschrift uit onze groep is niet compleet zonder een bedankje voor de Ento Statistiekguru; mijn kennis over statistiek heb ik grotendeels aan jou te danken, en als R weer een of andere irritante error gaf maakte jij altijd tijd om er even naar te kijken. Bedankt dat je altijd open staat voor een goed gesprek. **Tessa**, bedankt voor alle heerlijke taarten en voor de online versie van “Heel Ento bakt”. Ik hoop je nog vaak tegen te komen op Elfia. **Patrick**, bedankt voor je geduldige uitleg van moleculaire technieken en discussies over bioinformatica, ik heb veel van je geleerd! **Filippo**, thank you for your humour and your horrible taste in music; before meeting you I didn’t know that people actually listened to

experimental jazz by choice. **Alessia** and **Sandeep**, thank you for discussing bioinformatics with me, it gave me the confidence to continue learning. **Karen**, bedankt voor alle goede gesprekken over van alles en nog wat op het lab. Jouw enthousiasme voor de wetenschap is inspirerend en aanstekelijk. **Hans**, bedankt voor alle mooie foto's, en dat ik ze mocht gebruiken in dit boekje! **Angelique**, bedankt dat je zo'n luxe auto had geregeld voor het ritje naar Rennes van Julia en mij, reed als een zonnetje! **Shaphan**, thank you for all the cheerful greetings. **Kay**, **Lucille**, **Dani**, **Alexandre**, **Davy**, **Maite**, **Marieke**, **Parth**, **Marcela, Bram, Qi, Mitchel**, **Alex**; thank you for your presence at Ento and for all the nice conversations we shared. **Rocky**, thank you for staying in touch after leaving Wageningen, it's always a pleasure to chat with you.

Lieve vrienden, bedankt dat jullie er altijd zijn. Jullie interesse in mijn onderzoek hielp me om jargon van normale taal te scheiden, en door tijd met jullie door te brengen herinnerde ik me steeds dat er meer is dan alleen werk. **Michiel S.**, bedankt voor alle carpoolritjes naar Wageningen, en dat ik altijd op jou kan rekenen. **Joachim**, jij kan me als geen ander overhalen om alle remmen los te laten, bedankt voor alle katers. **Michiel U.**, bedankt voor je enthousiasme in alles dat je doet, zoals toen je me hielp om een filmpje over een uitkomende sluipwesp te monteren. **Lukas**, jij toont altijd oprechte interesse in alles dat ik doe, het perfecte luisterende oor. **Gulliver**, bedankt dat je altijd langs komt, ook al is het aan de andere kant van de wereld. **Maaike**, bij jou voel ik me altijd welkom en thuis. **Lex**, ons gezamelijke moestuintje op de Archi wakkerde mijn interesse in planten aan. De manier waarop jij met een open geest zoekt naar waar je gelukkig van wordt inspireert me.

Tot slot wil ik mijn familie bedanken. **Nico**, **Ina**, **Leendertjan**, **Rose**, **Roos**, **Johannes**; bedankt voor jullie onvoorwaardelijke liefde, het is een zegen om te weten dat jullie achter me staan, welke keuzes ik ook maak in mijn leven. **Sebastian**, meeting you was the highlight of my year. **Nico**, van jou heb ik mijn onderzoekende geest, waarmee ik vroeger als klein jongetje de wereld ontdekte, en waarmee ik nu nog steeds de wereld ontdek. **Ina**, van jou heb ik mijn optimisme, bedankt dat jij overal het mooie in ziet. Het was geweldig om de afgelopen jaren met jou te moestuinieren – door al jouw onkruidwieden kon ik me richten op datgeen waar ik het meeste genoegen uit haal; het zaaien en opkweken van alle gekke groentes die de revue gepasseerd hebben. **Hans** en **Anne**, het is fijn om jullie in mijn leven te hebben; door jullie wijsheid heb ik veel over mezelf geleerd. **Noor**, bedankt voor alle sokken. Superleuk dat jij "mijn" studie bent gaan doen in Utrecht! **Rik** en **Lenneke**, moge jullie plantjes spintvrij blijven.

Mijn lieve **Juul**, dit boek is slechts een hoofdstuk in de reis die wij samen maken, en ik kijk uit naar alle bladzijden die nog gaan komen. Met jouw veerkracht en authenticiteit ben jij een groot voorbeeld voor mij. Bedankt dat je me in alle fasen van mijn PhD ondersteund hebt, van het enthousiaste experimenteren tot het eenzame schrijven tijdens de pandemie. Jij kent mij als geen ander, je helpt me mijn gedachten op een rijtje te zetten, stelt me gerust als ik het overzicht even kwijt ben en schud me door elkaar als ik in een sleur dreig te belanden. Jouw liefde geeft mijn leven kleur.

Publications

Rusman Q, Karssemeijer PN, Lucas-Barbosa D, Poelman EH. 2019. Settling on leaves or flowers: herbivore feeding site determines the outcome of indirect interactions between herbivores and pollinators. Oecologia **191**(4): 887-896. https://doi.org/10.1007/s00442-019-04539-1

Karssemeijer PN, Reichelt M, Gershenzon J, van Loon JJA, Dicke M. 2020. Foliar herbivory by caterpillars and aphids differentially affects phytohormonal signalling in roots and plant defence to a root herbivore. Plant, Cell and Environment **43**(3): 775-786. https://doi.org/10.1111/pce.13707 (**chapter 2 of this thesis**).

Friman J*, Karssemeijer PN*, Haller J*, de Kreek K, van Loon JJA, Dicke M. 2021. Shoot and root insect herbivory change the plant rhizosphere microbiome and affects cabbage–insect interactions through plant–soil feedback. New Phytologist **232**(6): 2475-2490. https://doi.org/10.1111/nph.17746 (**chapter 5 of this thesis**).

Karssemeijer PN, Winzen L, van Loon JJA, Dicke M. 2022, in press. Leaf-chewing herbivores affect preference and performance of a specialist root herbivore. Oecologia. https://doi.org/10.1007/s00442-022-05132-9 (**chapter 4 of this thesis**).

Submitted

Karssemeijer PN, de Kreek KA, Gols R, Neequaye M, Reichelt M, Gershenzon J, van Loon JJA, Dicke M. 2021. Specialist root herbivore modulates plant transcriptome and downregulates defensive secondary metabolites in a brassicaceous plant. (**chapter 3 of this thesis**).

Karssemeijer PN*, Croijmans L*, Gajendiran K, Gols R, van Apeldoorn D, van Loon JJA, Dicke M, Poelman EH. The effect of crop diversity and associated macrofauna on various life stages of the cabbage root fly. (**chapter 6 of this thesis**).

*Shared first authorship

About the author

Written by Juul Oude Egberink

Peter was born on the 30th of March 1990, in a small but green place called Beek-Ubbergen. Even though there was not a lot to do around these parts, Peter had a blast growing up here. The fact that he lived in a pink miniature castle right on the edge of the forest helped immensely. The forest and all its inhabitants sparked a keen interest in nature in little Peter, and it has not left him since.

After high school, Peter travelled the world while thinking about the perfect study for him. To nobody's surprise, except maybe his own, Peter decided to study Biology at the Utrecht University. Here, he got introduced to the natural world he loved and knew so well on a deeper level. At some point during his BSc, Peter decided to do an evening workshop on insects which profoundly sparked his interest. After discovering his fondness for insects, Peter went on to do a minor on Entomology and Nematology at Wageningen University, he was sold. Wageningen proved to be an excellent place for Peter, and he flourished. Once again Peter discovered a whole new world when he started his BSc thesis at the Plant-Microbe Interactions group at Utrecht University. Under Dr. Silvia Proietti's amiable and watchful eye, Peter studied the molecular regulation of plant defense.

Peter decided to continue his studies in Wageningen, where he obtained his *cum laude* MSc degree in Plant Sciences with a specialization in Plant Pathology and Entomology. Herein, he focused on interactions between plants and their environment, with a special attention to plant antagonists. He followed quite some courses taught by Prof. Marcel Dicke. Inspired by his expertise, Peter approached Marcel for a Master thesis in Entomology. During this thesis, Peter worked in the lab of Prof. Carlos Ballaré in Buenos Aires, Argentina. He studied how competition for light affects plant defense while enjoying an excessive amount of empanadas and the some of most beautiful sunsets he would ever see.

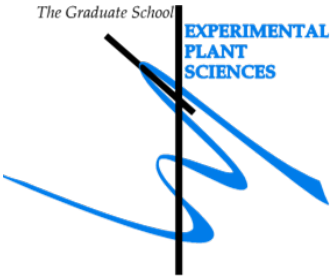
For his second thesis, under supervision of the enthusiastic Dr. Quint Rusman, he examined how herbivory by aphids affects pollinators. This thesis was awarded the East-

West Seeds graduation prize for Plant Sciences by the Royal Holland Society of Sciences and Humanities. While winning prizes, Peter was also working on an honors program. In this program he wrote a grant proposal to study plant-mediated interactions between above- and belowground interactions.

This proposal ultimately led to his PhD project at the Laboratory of Entomology, where he felt right at home. During his PhD, he investigated how caterpillars and aphids affect the root herbivore *Delia radicum*. He approached his subject from different angles: both targeted and untargeted analyses of genes and secondary metabolites, as well as behavioral and ecological approaches.

Peter is an avid researcher, and his quest for knowledge is not over yet. In the future, he would like to learn more about the ways plants interact with their surroundings. He hopes to keep studying the mysterious world of plants and insects.

Education Statement of the Graduate School Experimental Plant Sciences



Issued to: **Peter N. Karssemeijer**
Date: **08 April 2022**
Group: **Laboratory of Entomology**
University: **Wageningen University & Research**

1) Start-Up Phase	*date*	*cp*
► **First presentation of your project**		
Insect-Plant Interaction Lunch Meeting, Wageningen	27 Feb 2018	1,5
► **Writing or rewriting a project proposal**		
► **MSc courses**		
Subtotal Start-Up Phase		1,5

2) Scientific Exposure	*date*	*cp*
► **EPS PhD student days**		
Get2Gether Annual Experimental Plant Science PhD days, Soest	15-16 Feb 2018	0,6
Get2Gether Annual Experimental Plant Science PhD days, Soest	11-12 Feb 2019	0,6
► **EPS theme symposia**		
Theme 2 Symposium & Willie Commelin Scholten Day, Amsterdam	24 Jan 2018	0,3
Theme 2 Symposium & Willie Commelin Scholten Day, Wageningen	1 Feb 2019	0,3
Theme 2 Symposium & Willie Commelin Scholten Day, Online	9 Feb 2021	0,3
► **Lunteren Days and other national platforms**		
Annual Meeting Experimental Plant Sciences, Lunteren	9-10 Apr 2018	0,6
Annual Meeting Experimental Plant Sciences, Lunteren	8-9 Apr 2019	0,6
29th Annual Meeting of the Netherlands Entomological Society, Ede	15 Dec 2017	0,3
30th Annual Meeting of the Netherlands Entomological Society, Ede	14 Dec 2018	0,3
31st Annual Meeting of the Netherlands Entomological Society, Ede	13 Dec 2019	0,3
Yearly Entomology Lab Research and Exchange Meeting, Bennekom	24 May 2018	0,3
Yearly Entomology Lab Research and Exchange Meeting, Bennekom	6 Jun 2019	0,3
► **Seminars (series), workshops and symposia**		
12th Plant-Insect Interaction Workshop, Wageningen	7 Nov 2017	0,3
14th Plant-Insect Interaction Workshop, Amsterdam	14 Nov 2019	0,3
15th Plant-Insect Interaction Workshop, Wageningen	21 Oct 2021	0,3
EPS Flying Seminar: Urs Wyss	2 Oct 2017	0,1
Ento Seminar: Hein Sprong	28 Jan 2019	0,1

	Ento Seminar: Tobias Zust	8 Feb 2019	0,1
	Ento Seminar: Eveline Verhulst	6 May 2019	0,1
	Ento Seminar: Gorben Pijlman	27 May 2019	0,1
	Ento Seminar: Yoshihiro Yamada	7 Oct 2019	0,1
	Ento Seminar: Sanja Selakovic	20 Jan 2020	0,1
	Ento Seminar: Kamiel Spoelstra	30 Mar 2020	0,1
	Seminar: Jean-Francois Arrighi	18 Oct 2017	0,1
	Seminar: Giles Oldroyd	19 Oct 2017	0,1
	Seminar: Martin Heil	15 Oct 2019	0,1
	Seminar: Ted Turlings	9 Mar 2020	0,1
	Wageningen Evolution and Ecology Seminars (WEES): Jaboury Ghazoul	1 Mar 2018	0,1
	Wageningen Evolution and Ecology Seminars (WEES): Israel Págan	5 Apr 2018	0,1
	Wageningen Evolution and Ecology Seminars (WEES): Jeff Ollerton	31 May 2018	0,1
	Wageningen Evolution and Ecology Seminars (WEES): Britt Koskella	16 May 2019	0,1
	Wageningen Evolution and Ecology Seminars (WEES): Detmer Sipkema	19 Sep 2019	0,1
	Wageningen Evolution and Ecology Seminars (WEES): Enric Frago	17 Jun 2021	0,1
►	**Seminar plus**		
►	**International symposia and congresses**		
	10th European Plant Science Retreat, Utrecht	3-6 Jul 2018	1,0
	Plants, People, Planet Symposium, London, UK	4-5 Sep 2019	0,6
	17th Symposium on Insect-Plant Interactions, Leiden	25-30 Jul 2021	1,5
►	**Presentations**		
	10th European Plant Science Retreat, Utrecht (poster)	5 Jul 2018	1,0
	East-West Seed company visit, Suphan Buri, Thailand (oral)	5 Nov 2018	1,0
	Institut National de la Recherche Agronomique (INRA) Study Trip, Rennes, France (oral)	2 Jul 2019	1,0
	Plants, People, Planet Symposium, London, UK (poster)	4-5 Sep 2019	1,0
	Theme 2 Symposium & Willie Commelin Scholten Day, Online (oral)	9 Feb 2021	1,0
	17th Symposium on Insect-Plant Interactions, Leiden (poster)	25-30 Jul 2021	1,0
►	**3rd year interview**		
►	**Excursions**		
	East-West Seed company visit, Suphan Buri, Thailand	5-7 Nov 2018	0,9
	Study trip to lab of Prof. Anne-Marie Cortesero, Institut National de la Recherche Agronomique, Rennes, France	1-2 Jul 2019	0,6
Subtotal Scientific Exposure			18,1

3) In-Depth Studies		*date*	*cp*
►	**Advanced scientific courses & workshops**		
	9th Utrecht International Summer school on Environmental Signaling, Utrecht	28-30 Aug 2017	0,9

	The Power of RNA-seq, Wageningen	11-13 Jun 2018	0,9
	The Carpentries Workshop on Genomics Data, Wageningen	5-6 Feb 2019	0,6
	Functional Plant Bioinformatics (PLAZA) workshop, Ghent, Belgium	21-22 Oct 2019	0,6
	Chemical communication, Ede	2-6 Feb 2020	1,5
►	**Journal club**		
►	**Individual research training**		
Subtotal In-Depth Studies			4,5

4) Personal Development		*date*	*cp*
►	**General skill training courses**		
	Brain training, Wageningen	8 Nov 2017	0,3
	Intensive Writing Week, In'to Languages, Online	12-16 Jul 2021	1,0
►	**Organisation of meetings, PhD courses or outreach activities**		
	Organisation of 15th Plant-Insect Interaction Workshop (postponed), Wageningen	2020	0,5
	Organisation of 15th Plant-Insect Interaction Workshop, Wageningen	21 Oct 2021	1,2
►	**Membership of EPS PhD Council**		
Subtotal Personal Development			3,0

5) Teaching & Supervision Duties		*date*	*cp*
►	**Courses**		
	Ecophysiology	2017/2018/ 2019/2020	
	Insect-Plant Interactions	2018/2019	3,0
	Ecology 1	2018/2019	
	Molecular Aspects of Biointeractions	2019	
►	**Supervision of BSc/MSc students**		
	Toya Nath Joshi	2018	
	Laura Winzen	2019	
	Julian Haller	2019	
	Teun van Duffelen	2019	3,0
	Kris de Kreek	2020	
	Karthick Gajendiran	2020	
	Bart Mol	2020	
Subtotal Teaching & Supervision Duties			6,0

Total number of credit points*	**33,1**

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 & Research (WUR).

Cover design and thesis layout by Yidong Wang
Printed by Proefschriften.nl, Deventer, the Netherlands