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Faculty of Science

Plant defence system in multiple parallel biotic stresses

Doctoral dissertation

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Prague 2024

Abstract

Dual and multiple plant interactions with fungi and insect pests frequently occur in nature. Plants respond to such biotic stresses by activating distinct defence mechanisms; however, little is known about how plants cope with multiple stresses, and how symbiotic associations affect the subsequent fungal infections. The goal of such phytopathological studies is to better understand plant defence strategies and unravel sustainable and eco-friendly alternatives to chemical pesticides. Another way is usage of plant elicitors which can boost plant defence and make plants resistant to subsequent attackers. Firstly, I developed a choice test system to study the preference of insect pests to different leaf treatments, as described in Chapter 5.1.

In Chapter 5.2, I hypothesised that the combined interaction of fungal infection caused by *Leptosphaeria maculans* could alter oilseed rape palatability to *Plutella xylostella* caterpillars or *Brevicoryne brassice* aphids. Feeding preference tests were complemented by analyses of defence gene transcription, and levels of glucosinolates (GLSs) and volatile organic compounds (VOCs) in inoculated and non-inoculated leaves to determine the possible cause of insect choice. I confirmed that caterpillars and aphids prefer true leaves of oilseed rape to cotyledons; therefore true leaves were used for all further experiments involving oilseed rape. Leaves inoculated with *L. maculans* were more palatable to *P. xylostella* caterpillars in the early stage of disease development (3 days post inoculation, dpi), whereas the preference disappeared in the later stage (7 dpi). On the other hand, there was no preference of *B. brassicae* aphids at 3 dpi; however, *L. maculans* leaves were less palatable to aphids at 7 dpi. These differences in insect preference could be caused by the underlying mechanisms leading to changes in metabolic composition.

In Chapter 5.3, I hypothesized that specific resistance inducers can alter oilseed rape palatability to *P. xylostella* caterpillars. Indeed, leaves directly treated with benzothiadiazole (BTH) and ulvan were less palatable to caterpillars. Further studies need to be done to unravel this cause of larval choice.

In Chapter 5.4, I focused on the activation of salicylic acid (SA) and jasmonic acid (JA) signaling in *Arabidopsis* leaves during *B. brassicae* infestation in high spatio-temporal resolution. Despite the increasing understanding of transcriptomic changes upon infestation,

discrepancies in results frequently arise due to variations in sampling methodologies. To address this issue, I employed genetically encoded fluorescent biosensors, histochemistry, and quantitative reverse transcription PCR (qRT-PCR) techniques to accurately trace the activation patterns of distinct branches of phytohormonal signaling. I observed a rapid induction of SA and JA signaling in cells surrounding stylet puncture, co-localizing with callose deposition. For both *PR1* and *JAZ10* activation was detected at 24 hpi, increasing and spreading along the veins until 72 hpi and, to a lesser extent, within the epidermal pavement cells. The SA signaling wave appeared in parallel with JA-associated, and continued to increase in time. These results first show a local activation of SA- and JA-related responses after stylet penetration of *Arabidopsis* leaves and bring a detailed insight into the spatio-temporal complexity of plant defence activation during specialist aphid attack.

In Chapter 5.5, I hypothesized that root association with a plant-beneficial fungus *Metarhizium robertsii* will prime the plant for defence against subsequent *L. maculans* infection. However, I observed that symbiotic association by *M. robertsii* supported the infection compared to control group; additionally, plants were susceptible when treated with 0.05 % Triton X compared to water treatment. This might be due to observed changes in phytohormonal signaling responses, as *M. robertsii* and 0.05 % Triton X activated JA signaling and suppressed the activation of ET and oxidative stress responses.

Keywords: elicitors, glucosinolates, herbivores, pathogens, phytohormones, plant interactions, symbionts, volatiles

ACKNOWLEDGEMENTS

First and foremost, I would like to express my heartfelt gratitude to my mentor, Lenka. Without her unwavering support and guidance, this PhD journey and the completion of this thesis would not have been possible. Lenka provided me with the opportunity to undertake an internship during and after my Master's studies in the Laboratory of Pathological Plant Physiology at IEB, which ultimately led me to pursue a PhD. Thank you, Lenka, for your invaluable mentorship and warm collaboration.

I am also deeply grateful to my supervisor from ČZU, Prof. Ryšanek. His encouragement and willingness to assist with any issues I encountered were crucial to this journey's success.

My warmest thanks go to my Danish supervisor, Thure, who provided guidance and encouragement both before and during my stay in Copenhagen. Thure's mentorship extended beyond scientific advice, helping me maintain my mental well-being when challenges arose.

Special thanks are due to Meike, who became my supervisor after Thure's retirement. I highly appreciate your constructive feedback on my PhD thesis.

I am immensely grateful to my colleagues who supported me throughout this journey. I share the credit of my work with Bara, Tania, Morgane, Romča, and others whose assistance with lab work and collaboration led to some wonderful publications. Additionally, I extend my gratitude to all my colleagues from SOBI at the Faculty of Science in Copenhagen, who made my stay there so enjoyable. I will always cherish those memories.

My deepest gratitude goes to my parents, my sister, and my entire family and friends, whose emotional support sustained me throughout these years. Their understanding, especially when I couldn't come home for holidays due to my experiments or the need to care for my insects, was invaluable. Without their support, this achievement would not have been possible.

Lastly, I want to thank my partner for his unwavering support during the writing process, especially as our baby eagerly kicks from within, perhaps cheering for her mama.

This has been a wonderful journey, and I am excited about what the future holds.

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“Each step that we make in the more intimate knowledge of nature leads us to the entrance of new labyrinths.”

− Alexander von Humbolt, 1845;

quoted in Merlin Sheldrake’s *Entangled Life: How Fungi Make Our Worlds, Change Our Minds, and Shape Our Futures*

1 General introduction

The intricate interplay between plants and their biotic associates, ranging from antagonists such as insect pests and fungal pathogens and mutualists such as beneficial microbes, plays a fundamental role in shaping plant health and productivity and ecosystem functioning. Antagonists pose significant threats to plant health, often leading to reduced yields and economic losses. Conversely, mutualistic associations confer numerous benefits to plants, such as enhanced nutrient uptake, disease resistance, and reproductive success. The profound understanding of these relationships is central to the development of effective strategies for pest and disease management, as well as for harnessing the potential of beneficial organisms to promote plant health and productivity. By deciphering the mechanisms underlying plant interactions with both antagonists and mutualists, novel targets for sustainable agricultural practices can be identified, minimizing the reliance on chemical inputs and maximizing ecosystem services provided by beneficial organisms. In addition to mutualistic microbes, there exists a spectrum of compounds capable of inducing resistance in plants, potentially serving as effective alternatives to chemical pesticides. A comprehensive investigation into the mechanisms underlying the action of these compounds is imperative for their integration into agricultural strategies aimed at enhancing plant resilience and minimizing environmental impact.

Plant interactions with other organisms activate diverse components within the plant immune system, including phytohormonal signaling responses, the production of secondary metabolites, and volatile organic compounds. Despite the prevalence of multiple parallel biotic stresses in the plant kingdom, our understanding of these interactions remains limited. Therefore, there is a pressing need to expand our knowledge base, particularly regarding the relationships between different types of pathogens (biotrophs, necrotrophs, hemibiotrophs) and specific categories of insect herbivores (chewing, sucking), and plant mutualists. It is crucial to elucidate how infection by fungal pathogens, for instance, may alter plant susceptibility to insect pests, and *vice versa*. Moreover, there is a need to delve deeper into the interactions between plant pathogens and plant mutualists. Such insights are essential for devising tailored strategies for pest and disease management in agriculture.

To gain a comprehensive understanding of tripartite interactions involving plants, fungal pathogens and insect herbivores, my research focuses on investigating how infection by

the hemibiotrophic pathogen *Leptosphaeria maculans* influences the palatability of oilseed rape plants to the chewing insect herbivore *Plutella xylostella*. Specifically, I examine the alterations in phytohormonal signaling responses, conduct analyses of glucosinolate content, and investigate volatile profiles. In order to facilitate the thorough exploration of this study, the methodology for conducting insect choice test experiments was established in advance. Subsequently, the investigation delves into elucidating the impact of specific resistance inducers on the palatability of oilseed rape plants to *P. xylostella*. Moreover, the research continues on spatial analyses aimed at investigating phytohormonal signaling responses elicited during attacks by piercing-sucking insect herbivores. The specific focus is on the dynamics of salicylic and jasmonic acid signaling during *Brevicoryne brassicae* feeding in *Arabidopsis thaliana*. This pioneering investigation offers unprecedented insights into plant responses at the sites of aphid feeding, shedding light on previously unexplored aspects of plant-aphid interactions. Advancing my research, I delve deeper into tripartite interactions, particularly examining the intricate relationship between plant roots and beneficial fungi, and its impact on plant susceptibility to *L. maculans* infection. This focused investigation aims to illuminate the dynamics at play within this complex biological system.

2 Literature review

In the realm of ecological dynamics, plants engage in intricate interactions with pathogens, herbivores, and other organisms, giving rise to complex tripartite relationships. Central to these interactions is the activation of plant immunity mechanisms and associated signaling pathways. When faced with pathogens or herbivores, plants initiate a series of molecular responses aimed at defending against invasion. However, the outcome of these interactions is multifaceted, as the activation of plant immunity can have dual effects, either enhancing resistance to subsequent attackers or potentially increasing susceptibility. The intricacies of these interactions are influenced by various factors, including the specific nature of the assailant, the timing of the immune response, and the overall health and resilience of the plant. Understanding the dynamics of plant immunity and its impact on susceptibility to subsequent attackers is crucial for elucidating the broader ecological dynamics of tripartite interactions and devising strategies to enhance plant defences in natural and agricultural settings.

2.1 Plant immunity

The intricate interactions between plants and a diverse array of organisms, encompassing both antagonistic and mutualistic relationships, trigger remarkable alterations on physiological and molecular scales. As a first-layer protection against pathogens and pests, plants have formed defence structures such as trichomes and waxes, as well as production of toxic chemicals such as alkaloids, terpenoids and phenols that are generally effective against non-adapted attackers. However, to cope with aggressors that adapted to plant mechanical and chemical barriers, plants had to develop more complicated strategies, jointly termed as innate immunity. Plant cells recognize pathogen/microbe and herbivore signals, or microbe/herbivore damage signals, *i.e.* pathogen-/microbe-, herbivore- or damage-associated molecular patterns (PAMPs/MAMPs, HAMPs or DAMPs) such as microbial components (*e.g.* flagellin, chitin) or plant cell wall components, through pattern recognition receptors (PRRs) such as plasma-membrane bound proteins (*e.g.* receptor-like kinases, RLKs), and start the activation of defence reactions. These reactions are known as PAMP-/MAMP-triggered immunity (PTI/MTI), HAMP-triggered immunity (HTI) or DAMP-triggered immunity (DTI), collectively termed as

pattern-triggered immunity (PTI). After perceiving elicitors from a biotic stressor, an arms race between a plant and attacker is triggered, since attackers have also evolved to fight host defence responses (Delaunois et al., 2014; Jones and Dangl, 2006). Successful pathogens or insect herbivores, as well as mutualistic microbes can suppress or minimise PTI via effector proteins, which have forced host plants to develop resistance genes (*R* genes) coding for R proteins, *e.g.* nucleotide-binding leucine-rich repeat (NB-LRR) proteins (Tsuda et al., 2013), that are able to detect effectors and therefore activate effector-triggered immunity (ETI). Depending on the type of the attacker (*e.g.* pathogen, insect herbivore, beneficial microbe), as well as the lifestyle of the pathogen or the feeding guild of an insect, distinct elicitors and effectors are secreted and therefore different plant immunity responses are induced, such as cell wall and cuticle enhancement, and the activation of defence pathways.

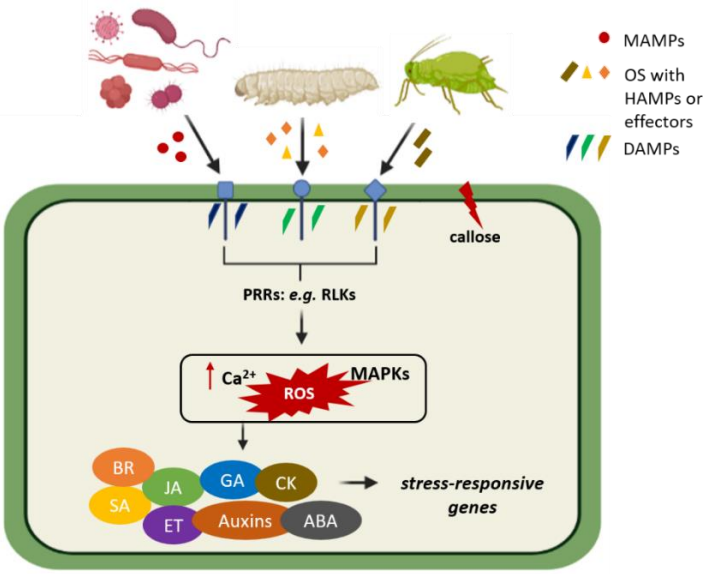

Figure 1. Activation of plant defence mechanisms after exposure to various biotic stresses. Plant perceives attacker-emitted molecules (MAMPs, HAMPs, DAMPs) by multiple receptors, such as PRR proteins and activates a cascade of responses in cytosol (rise of Ca^{2+}, ROS outburst, MAPK synthesis). That further triggers the phytohormonal biosynthesis, which leads to activation of downstream stress-responsive genes. Abbreviations: MAMPs = microbe-associated molecular patterns; OS = oral secretions; HAMPs = herbivore-associated molecular patterns; DAMPs = damage-associated molecular patterns; PRRs = pattern recognition receptors; ROS = reactive oxygen species; MAPKs = mitogen-activated protein kinases; BR = brassinosteroid; SA = salicylic acid; JA = jasmonic acid; ET = ethylene; GA = gibberellin; CK = cytokinin; ABA = abscisic acid.

2.1.1 PAMP-/MAMP-triggered immunity (PTI/MTI) and effector-triggered immunity (ETI)

When plants are attacked by pathogens or microbes, they get in contact with PAMPs or MAMPs, conserved microbial elicitors such as flagellin from bacterial flagella, or chitin present in fungal cell walls. Many PAMPs and MAMPs have already been described, and they generally belong to a group of proteins, carbohydrates or lipids (Newman et al., 2013). Moreover, as a response to tissue injury, plants are alarmed by so-called "danger" signals or DAMPs. The components of the plant cell wall, such as oligogalacturonides and cutin, or peptides present in the cytoplasm may act as such endogenous elicitors of plant defence (Lotze et al., 2007). The plant perceives MAMPs and DAMPs by PRRs: *e.g.* chitin elicitor receptor kinase 1 (CERK1) recognizes fungal chitin and bacterial peptidoglycan (Miya et al., 2007; Willmann et al., 2011), and initiates early plant defence signaling events. Firstly, outflow of plasma membrane K^+ and rise in cytosolic Ca^{2+} occurs, followed by reactive oxygen species (ROS) production, *i.e.* "the oxidative burst" that involves production of hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^{\cdot}$) and superoxide anion radical ($O_2^{\cdot-}$) (Gadjev et al., 2006; Verma et al., 2016). Moreover, Ca^{2+}-dependent protein kinases (CDPKs) and mitogen-activated protein kinases (MAPKs) are being accumulated. These events trigger a major transcriptional reprogramming leading to defence gene activation and accumulation of different proteins, such as pathogenesis-related (PR) proteins. Some of them have enzyme activities, *e.g.* chitinases which degrade microbial cell walls. Further, protein construction of physical barriers in the form of cell wall polymers is triggered, such as β-glucan callose, at the site of pathogen penetration into the plant tissue (Delaunois et al., 2014; Ferreira et al., 2007). This defence machinery is orchestrated by specific signaling pathways in which phytohormones play a central role (Fig. 1).

To suppress plant immune responses, or PTI/MTI, and promote their virulence, pathogens deliver an arsenal of effector proteins into plant cells. This ability to conquer plant resistance distinguishes harmful from non-harmful microbes. However, plants recognize delivered effectors by intracellular immune receptors encoded by many resistance (*R*) genes. If a plant carries *R* gene encoding a receptor to the perceived effector, this will result in incompatible (avirulent) interaction and induction of ETI. The perceived effector is then termed as avirulent (*Avr*) gene, or avirulent (Avr) protein (Jones and Dangl, 2006). Further activated

mechanisms are similar to those of PTI/MTI described above; in addition, to restrict the pathogen growth, ETI is followed by a hypersensitive response (HR) leading to a cell death localised at the infection site (Dodds and Rathjen, 2010; Jones and Dangl, 2006). On the other hand, if a pathogen does not contain the *Avr* gene, the interaction between the pathogen and the host will then be compatible (virulent); therefore, pathogen virulence is generally controlled by mutations or loss of *Avr* genes, which results in disease development (Petit-Houdenot et al., 2019). Nonetheless, for the infection to get established, efficient secretion of microbial effectors inside the plant is essential. For instance, pathogenic fungi have developed different lifestyles and therefore formed distinct effector systems. Such lifestyles can be biotrophic, *i.e.* pathogens thriving on the living host plant; necrotrophic, *i.e.* pathogens killing the host and obtaining nutrients from dead cells; and hemibiotrophic, *i.e.* pathogens infecting living plant cells during the initial part of their lifecycle and later switching to the necrotrophic stage. Biotrophs and hemibiotrophs evolved to penetrate cytoplasm and deliver effectors via infection structures, such as haustoria (Selin et al., 2016). Less is known about effectors secreted by necrotrophs and hemibiotrophs turning to necrotrophic phase during pathogenesis. However, it is suggested that both necrotrophic and hemibiotrophic pathogens secrete effectors to manipulate plant HR and cell death and use these events for their own benefit, which is then rightly termed as effector-triggered susceptibility (ETS; Shao et al., 2021). These necrotrophic/hemibiotrophic-secreted effectors are usually small proteins or secondary metabolites (Tan et al., 2010).

2.1.2 HAMP-triggered immunity (HTI) and effector-triggered immunity (ETI)

Upon feeding, insect herbivores elicit HAMPs, a multitude of cues present in their oral secretions (OS), cuticle, oviposition secretions, frass or honeydew secreted by some insects. HAMPs generally refer to insect-derived compounds which operate as elicitors, causing the activation of plant defence mechanisms, whereas similarly to pathogens, insects also produce effectors, the other group of compounds able to disrupt plant signaling responses (Jones et al., 2021). However, some compounds can act as both elicitors and effectors, which is dependent on plant species and a type of insect herbivore. Elicitor and effector delivery systems vary between insect herbivores and depend mainly on the insect feeding guild, since both groups of compounds are primarily secreted through insect OS (Erb and Reymond, 2019; Jones et al., 2021; Rodriguez and Bos, 2013). Two main insect feeding guilds are leaf chewers (*e.g.*

caterpillars of various Lepidoptera species) and piercing-sucking/phloem sap feeders (*e.g.* aphids in the order Homoptera).

2.1.3 Phytohormonal signaling

Phytohormones are essential for the regulation of plant growth, development, reproduction, as well as in biotic and abiotic stresses. Salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) take the main part of the plant defence chain. Other hormones, such as abscisic acid (ABA), auxins, gibberellic acid (GA), cytokinins (CKs), brassinosteroids (BRs),peptide hormones and strigolactones are considered as hormones that fine-tune plant defence responses (Pieterse et al., 2012; Torres-Vera et al., 2014). As shown in Fig. 1, when a plant is exposed to biotic stresses, various defence reactions are initially activated that trigger the phytohormonal biosynthesis.

2.1.3.1 Salicylic acid

Salicylic acid (SA) is one of the main plant hormones involved in both local and systemic acquired resistance (LAR and SAR) to plant pathogens and insect pests (Ding and Ding, 2020; Durrant and Dong, 2004; Vlot et al., 2009). SA is also involved in control of biochemical and physiological processes in plants (Rivas-San Vicente and Plasencia, 2011). The biosynthesis of SA as a phenolic compound can occur via two distinct enzymatic branches: isochorismate synthase (ICS) and phenylalanine ammonia lyase (PAL). These two pathways are both originating from chorismic acid (chorismate), the end product of metabolic shikimic acid (shikimate) pathway (Fig. 2A). SA biosynthesis has been well-studied in a model plant *A. thaliana* (Dempsey et al., 2011; Janda and Ruelland, 2015; Lefevere et al., 2020), and ICS pathway has been found to be the main one in plant response to pathogens (Wildermuth et al., 2001). Two *ICS* genes, *ICS1* and *ICS2* are encoded in the genome of *A. thaliana*, and *ICS1* is the main contributor to SA biosynthesis during biotic stress (Macaulay et al., 2017; Wildermuth et al., 2001). Wildermuth et al. (2001) confirmed that *ICS1* and ICS1-synthetized SA operate upstream of NON-EXPRESSOR PATHOGENESIS-RELATED PROTEIN 1 (NPR1) which interacts with transcription factors to alter the genes involved in pathogen attack, such as *pathogenesis-related 1* (*PR1*). However, the PAL pathway, comprised of four genes (*PAL1* -

PAL4) also contributes to SA biosynthesis and may be important during pathogen attack in *A. thaliana* (Huang et al., 2010), and may operate in harmonious manner with the ICS pathway regarding defence in other plant species (Shine et al., 2016). Both the ICS and PAL pathways are closely linked to biosynthetic pathways of aromatic amino acid (AA), such as phenylalanine, tyrosine and tryptophan, as chorismate is a precursor for production of such AAs and a key connector of the primary and secondary plant metabolism (Ding and Ding, 2020). the PAL pathway is a precursor for production of secondary metabolites in *Arabidopsis*, such as flavonoids which can be important in plant defence against pathogens and pests (Huang et al., 2010).

SA can be chemically converted into functional or inactive derivative compounds through methylation, glycosylation, AA conjugation or sulfonation, and most of the SA in plants is glycosylated or methylated (Rivas-San Vicente and Plasencia, 2011).

2.1.3.2 Jasmonic acid

Jasmonic acid (JA) and its derivatives known as jasmonates (JAs), such as jasmonyl isoleucine (JA-Ile), methyl jasmonate (MeJA) and 12-hydroxyjasmonic acid sulfate (12-OH-JA), are lipid-derived molecules involved in plant growth and stress response (Ghorbel et al., 2021; Wasternack and Hause, 2013). For instance, wounding increases the endogenous level of MeJA, but also the metabolic precursor *cis*-12-oxophytodienoic acid (OPDA; Fig. 2B) and activates defence responses (Wang et al., 2016).

JA biosynthesis is initiated in chloroplasts by release of α-linolenic acid (α-LeA); then, OPDA is formed in chloroplasts and exported to peroxisomes by JASSY protein, as it was shown that the loss of JASSY in *A. thaliana* abolishes the increase in expression of JA-regulated defence genes, such as *plant defensin 1.2* (*PDF1.2*) after wounding or pathogen attack (Guan et al., 2019). JA is finally formed in peroxisomes and further released to cytosol (Fig. 2B; Li et al., 2019; Wasternack and Hause, 2019). Then, JA is transformed into JA-Ile, MeJA or 12-OH-JA in the cytoplasm (Ruan et al., 2019). The main regulators involved in the JA signaling as activators or repressors are CORONATINE INSENSITIVE 1 (COI1), JASMONATE ZIM-DOMAIN (JAZ) and MYC proteins, which interact with each other in different ways in response to stresses (Boter et al., 2004; Chini et al., 2007; Xie et al., 1998). For instance, JA-Ile binding to COI1 targets JAZ proteins for degradation; therefore, MYC2 is activated

triggering the JA-regulated gene expression in *A. thaliana.* However, the lack of JA leads to JAZ and MYC2 interaction and suppression of JA-regulated defence genes (Chini et al., 2007).

2.1.3.3 Ethylene

Ethylene (ET) is a gaseous phytohormone, produced by a two-step enzymatic pathway, which begins with amino acid methionine, and it is also known as the "Yang cycle" (Fig. 2C; Li et al., 2019; Miyazaki and Yang, 1987; Pattyn et al., 2021). Even though ET biosynthesis looks simple, it is undoubtedly more complex. Nonetheless, its regulation is less well understood compared to other described phytohormonal biosynthetic pathways, namely SA and JA. For a considerable time, ET has been recognized as a hormone regulating plant growth and developmental processes (Li et al., 2019).

Based on the recent studies on *A. thaliana* ET-response mutants, Zhao et al. (2021) suggested a model of the ET signaling in *Arabidopsis* plants. Briefly, ET is perceived by ET receptors, *i.e.* ETHYLENE RESPONSE 1 and 2 (ETR1 and ETR2), ETHYLENE RESPONSE SENSOR 1 and 2 (ERS1 and ERS2) and EIN4, at the membrane of endoplasmic reticulum (ER) and converted through the Raf-like Ser/Thr kinase CTR1 and domain-containing protein EIN2. Then, EIN3/EIN3-LIKE (EIL) transcription factors get activated and trigger the expression of ET-responsive genes.

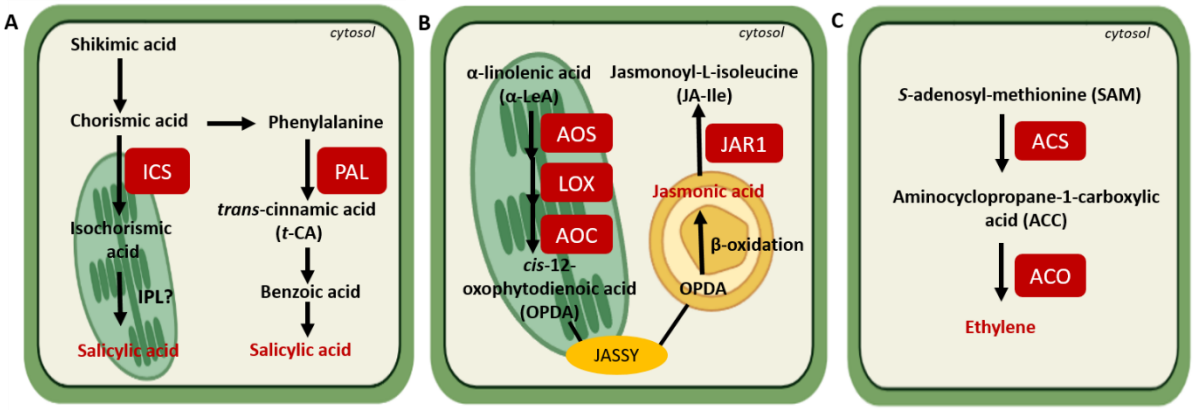

Figure 2. Biosynthesis of three main plant hormones: salicylic acid (SA), jasmonic acid (JA)/ jasmonoyl-L-isoleucine (JA-Ile) and ethylene (ET). **A:** SA biosynthesis via isochorismate (ICS) branch occurs in chloroplasts, where isochorismate synthase 1 and 2 (ICS1, ICS2) are stored, and their enzymatic activity converts chorismic acid to isochorismic acid. It is then suggested that SA is

synthesised by another enzyme, isochorismate pyruvate lyase (IPL). The enzyme phenylalanine ammonia lyase (PAL) operates in cytosol by converting phenylalanine to *trans*-cinnamic acid (*t*-CA). *t*-CA is a precursor of biosynthesis of secondary metabolites phenols, such as flavonoids and lignins. Further reaction converts *t*-CA to benzoic acid that leads to synthesis of SA. **B:** JA biosynthesis begins with the oxygenation of α-linolenic acid (α-LeA) in chloroplasts, which is then catalysed by 13-lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) to *cis*-12-oxophytodienoic acid (OPDA). OPDA produced in chloroplast is then transported by JASSY protein to peroxisomes, which eventually leads to JA formation. JA is further conjugated to JA-Ile by JA-amino synthetase (JAR1) in cytosol. **C:** ET synthesis starts with conversion of *S*-adenosyl-methionine (SAM) to 1-aminocyclopropane-1-carboxylic acid (ACC) by an enzyme ACC-synthase (ACS). ACC is then catalysed by ACC-oxidase (ACO), resulting in ET production.

2.1.3.4 Phytohormonal crosstalk

Studies on plant immunity underpin the pivotal role of cross-communicating hormones in the regulation of plant defence signaling network (Verhage et al., 2010). Cell wall composition and the activation of the cell wall defence responses against pathogenic and herbivorous attacks are regulated by phytohormonal crosstalk (Nafisi et al., 2015). Plant hormones communicate in a harmonious manner and create antagonistic or synergistic relationships as a response to environmental stimuli. There are many studies focusing on the antagonistic effect between specific signaling pathways, especially SA-mediated signaling towards JA/ET signaling (Li et al., 2006, 2019; Spoel et al., 2007). For instance, SA-induced gene *WRKY70* was reported to suppress the expression of JA-responsive gene *PDF1.2* in *A. thaliana* (Li et al., 2006, 2004). Since NPR1 protein is the main regulator of SA downstream signaling and the one required for the expression of *WRKY70* gene, it can therefore be suggested that NPR1 is essential for crosstalk between SA-JA/ET, and that the WRKY70 transcription factor can act as a connection point between JA and SA signaling (Li et al., 2019; Yang et al., 2019). SA and JA can also be synergistic, as it was found when tomato was infected with *Pseudomonas syringae* pv. *tomato* DC3000 (O'Donnell et al., 2003). Another example of synergism between hormones is SA, JA, ET and ABA signaling pathways being involved in regulation of ROS responses, such as H_2O_2 accumulation (Torres et al., 2006). Phytohormonal crosstalk is essential for plant survival, since it contributes to energy saving by maintaining the balance between the plant growth and response to abiotic and biotic stresses. All that would not

be possible without the interplay of many different genes and gene families finely orchestrated in a signaling network.

Phytohormonal activation against pathogens highly depends on a pathogen lifestyle: SA-mediated defence responses are commonly activated by biotrophic pathogens, whereas necrotrophs are generally affected by JA- and ET-mediated defences (Glazebrook, 2005; Verhage et al., 2010). However, in some pathosystems, SA plays a crucial signaling role in defence against necrotrophs, which is evident in *B. napus* and its resistance to *Sclerotinia sclerotiorum* (Nováková et al., 2014). Hemibiotrophs are generally inducing SA-related defence responses (Han and Kahmann, 2019). In addition, Šašek et al. (2012) found that SA- and ET-related genes were significantly upregulated in *B. napus* plants infected with *L. maculans*. Nonetheless, due to their complicated lifestyle, the understanding of plant defence against hemibiotrophs is still limited.

The way an insect pest feeds on the plant affects the activation of phytohormonal pathways in a distinct manner. Several studies revealed that leaf chewers generally induce only JA signaling pathway (Heil, 2008; Pangesti et al., 2016) and its derivatives MeJA and JA-Ile (Kroes et al., 2017a), while piercing-sucking herbivores tend to induce SA-mediated defence pathways; however, the involvement of JA and ET has also been reported during the aphid infestation (Kroes et al., 2017a; Moran et al., 2002). Even though SA and other phytohormones can be induced by aphids, the induction does not occur in all aphid–plant interactions and does not always enhance resistance to other insect pests (Hodge et al., 2019; Moran and Thompson, 2001).

2.1.4 Secondary plant metabolites

Apart from hormones, secondary plant metabolites such as glucosides, GLSs, alkaloids, terpenes and phenolics also play a key role in plant defence machinery. Secondary metabolites are classified into different groups based on their chemical structures. The chemistry and distribution of these compounds between plant species and plant organs is extremely diverse, and GLSs are one of the most studied ones.

2.1.4.1 Glucosinolates (GLSs)

Glucosinolates (GLSs) are non-volatile *S*-glycosylated thiohydroximate sulfate ester compounds characteristic for species from the Brassicales order, which include the model plant *A. thaliana* and a globally important crop *B. napus* (oilseed rape) and *Brassica oleracea* vegetables (*e.g.* cauliflower, broccoli, cabbage). Based on the amino acid from which are derived, GLS compounds are grouped in indole (tryptophan precursor), aliphatic (methionine, alanine, isoleucine, leucine, or valine precursor) and benzenic (phenylalanine or tyrosine precursor; Blažević et al., 2020). Aliphatic GLSs are the most diverse compared to other two classes, whereas benzenic are the least studied ones. Some of the most abundant indole and aliphatic compounds and a single benzenic compound in Brassicaceae are shown in Fig. 3 (Chhajed et al., 2020; Crocoll et al., 2016; Klopsch et al., 2018; Zhang et al., 2015). Moreover, GLS profile and the content of individual compounds differ depending on the plant species and organs within a plant. For instance, a total content of GLS compounds is higher in seeds and roots than in leaves of *B. napus* cultivars; however, aliphatic GLSs are dominating in both leaves and seeds, benzenic GLSs are prevailing in roots, and indole are more abundant in leaves compared to other organs (Kittipol et al., 2019; Velasco et al., 2008).

Intact GLSs are known to play a role in plant defence, and they can be both palatable and repugnant for pathogens and insect pests. Perceiving GLSs and their products by pathogens and insect herbivores highly depends on two factors: pathogen/herbivore feeding range and feeding mode. For instance, GLSs are highly responsible for plant palatability for specialist insect herbivores, and the preference is generally influenced by a GLS profile, not the total GLS content (Cole, 1997; Hopkins et al., 2009; Robin et al., 2017; Sun et al., 2009). Hence, females of *P. xylostella* prefer to oviposit on *Brassica* plants with a high level of indole GLSs, and moths are possibly sensing them through tarsal chemoreceptors when in contact with the leaf surface (Baur et al., 1998; Sun et al., 2009). Alternatively, GLSs can have lethal effects on generalist insect herbivores: Li et al. (2000) found that the aliphatic GLS sinigrin was toxic to *Spodoptera eridania* neonate, but not to the specialist *P. xylostella* caterpillars.

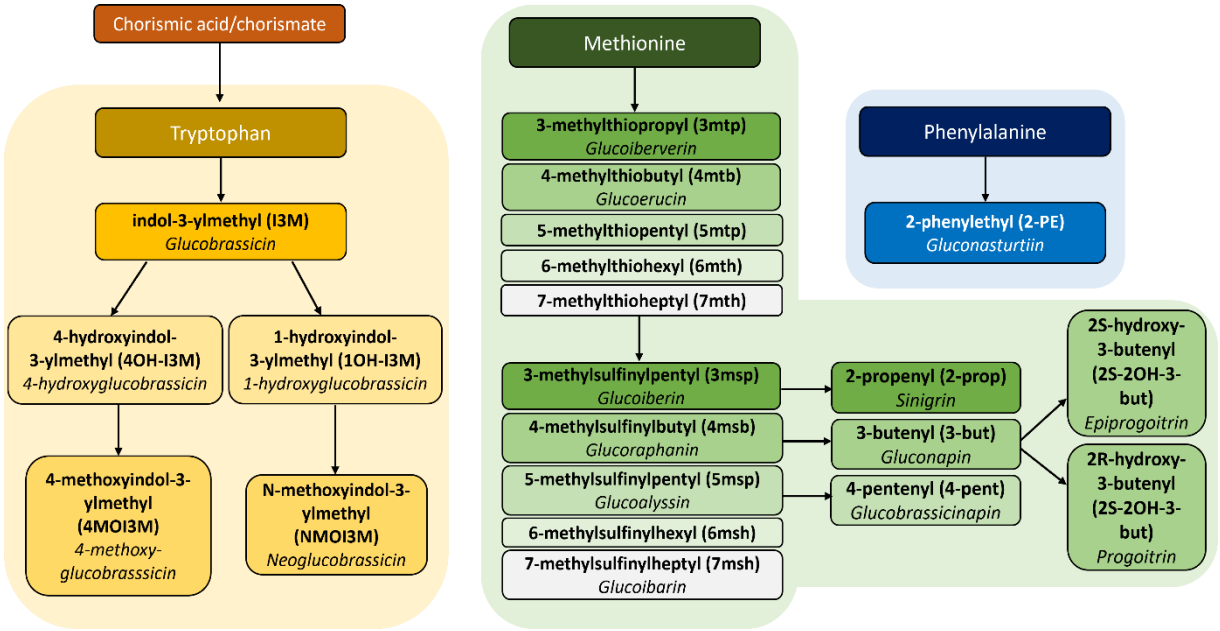

Figure 3. Simplified scheme of biosynthetic relations of some of the most abundant tryptophan-derived (indole), methionine-derived (aliphatic) and phenylalanine-derived (benzenic) GLS compounds in Brassicaceae. Compounds are known by their chemical names (bold letters) and common names (italic letters).

Piercing-sucking herbivores, such as aphids, show differential responses to different GLS profiles based on their host feeding range. For instance, reproduction rate of specialist *B. brassicae* was negatively affected by the strong induction of indole GLSs in Landsberg *erecta* accession of *A. thaliana*; however, generalist aphid *M. persicae* was not affected by this increase (Kuśnierczyk et al., 2007).

Fungal pathogens differing in their lifestyle and a range of hosts trigger the production of compounds belonging to different GLS groups. Hiruma et al. (2013) reported that growth of both specialist and generalist hemibiotrophic pathogens is negatively affected by synthesised indole compounds in *A. thaliana*. Furthermore, Buxdorf et al. (2013) studied two isolates of a generalist necrotrophic pathogen *Botrytis cinerea* and one isolate of *Alternaria brassicicola*, a specialised necrotroph on *Brassica* species. Using mutants of *A. thaliana* differing in the level of GLS compounds, they found that the response of *B. cinerea* is isolate-dependent, with the specialist being more strongly affected by aliphatic GLSs than by indole ones.

2.1.4.2 Glucosinolate-myrosinase system

For GLSs to be activated they must get in contact with enzymes called thioglucosidases, also known as myrosinases. Upon leaf tissue damage by herbivores, the velocity of GLS activation depends on the distance between laticifer-like S-cells, *i.e.* sulfur-rich cells where GLSs are stored, and myrosin cells, both located adjacent to the phloem (Kissen et al., 2009; Koroleva et al., 2000). Myrosin cells can easily be distinguished from S-cells because they have a lower content of lipids, higher content of endoplasmic reticulum and carry smoother protein bodies known as myrosin grains where the enzyme myrosinase is stored (Andréasson et al., 2001; Kumar, 2017). Once the integrity of S-cells and myrosin cells is compromised, *i.e.* in the case of tissue damage, their components get in contact, resulting in the formation of various hydrolysed products such as thiocyanates, isothiocyanates, and nitriles: the reaction known as a "mustard-oil bomb". These compounds can act as deterrents or attractants to a wide range of insect pests, and alarm insect predators and parasitoids (Hopkins et al., 2009). Generalist insects and pathogens are commonly negatively affected by the glucosinolate-myrosinase system, while specialists tend to use it in their own favour. For instance, herbivores specialised to feed on Brassicaceae may use herbivore-induced plant volatiles (HIPVs) as attractants for oviposition, as it was shown in studies with diamondback moth (*P. xylostella*): female moths were highly attracted to isothiocyanate compounds (ITCs), specifically aliphatic-derived iberin, iberverin, sulforaphane and 4-pentenyl ITC and benzenic-derived phenylethyl ITC, and therefore chose plants with high level of those compounds for oviposition (Hussain et al., 2020; Liu et al., 2020; Renwick et al., 2006; Sun et al., 2009).

However, specialist insects developed methods to overcome GLS-myrosinase system, primarily by using their own enyzmes. For instance, *P. xylostella* larvae use an enzyme sulfatase stored in their gut which eliminates sulfur from GLSs and converts them to desulfo-GLSs which are unable to get catalysed by plant myrosinase and therefore form toxic hydrolysis products, allowing larvae to feed on *Brassica* plants (Chen et al., 2020; Ratzka et al., 2002). Moreover, aphids specialised to feed on crucifers are able to accumulate GLSs in the hemolymph after ingestion or degrade them by their own myrosinase system and therefore use them for their own benefit (*e.g.* as a defence against predators; Winde and Wittstock, 2011). The cabbage aphid (*B. brassicae*) has its own myrosinase stored in thorax and head muscles and degrades the GLSs sinalbin and progoitrin to isothiocyanate compounds that lack the hydroxy group, which is not

the case with the plant myrosinase (Francis et al., 2002). The GLSs that have been accumulated in the hemolymph will be degraded by the aphid myrosinase after *B. brassicae* has been crushed by a predator, and that will further lead to release of volatiles as an alarm to other aphids in the colony; moreover, the predator will not enjoy the taste of the aphid and will probably leave the plant (Kumar, 2017). Due to this, cabbage aphid is also called "the walking mustard-oil bomb". Moreover, aphids can bend and manoeuvre stylet in between cells, avoiding the disruption of S-cells and myrosin cells and therefore suppressing the GLS-myrosinase reaction and production of toxic compounds (Francis et al., 2002; Winde and Wittstock, 2011).

Contrary to specialists, generalists do not seem to have the ability to sequester GLSs; for instance, generalist aphids tend to secrete high amounts of GLSs with their honeydew, which composition resembles that in the phloem sap. Kim and Jander (2007) observed that aliphatic GLSs pass through the gut of green peach aphid (*M. persicae*) intact when feeding on *A. thaliana*; however, indole GLSs tend to break down, either due to an unknown enzymatic activity in the phloem or simply because of the low stability of these compounds in the gut of aphids. Moreover, green peach aphid seems to induce an increased concentration of the indole compound 4-methoxyindol-3-ylmethyl (4-MOI3M) on the feeding sites in *Arabidopsis*, forming more resistant plants towards this generalist (Kim and Jander, 2007). Kuśnierczyk et al. (2007) found that both *B. brassicae* and *M. persicae* significantly elevated the transcription of genes involved in the synthesis of indole GLSs while feeding on *Arabidopsis* plants; however, they also observed a downregulation of genes coding for myrosinases.

2.1.4.3 Regulation of glucosinolate synthesis by phytohormones

One of the central players in GLS biosynthesis are phytohormones, which are regulating the GLS accumulation in plants in different ways (Mitreiter and Gigolashvili, 2021). From all the hormones, JA is a major and well-described hormone involved in the positive regulation of GLS production, especially the tryptophan-derived ones (Guo et al., 2013). Both ABA and ET are also involved in the positive regulation of GLS synthesis when applied alone (Mitreiter and Gigolashvili, 2021). On the other hand, BR was found to be a dominant inhibitor of GLS production: BR signaling decreased the levels of both aliphatic and indole compounds in transgenic *A. thaliana* plants overexpressing a gene involved in BR biosynthesis (Guo et al., 2013). This is not surprising since BR antagonises JA signaling (Mitreiter and Gigolashvili,

2021). Moreover, Guo et al. (2013) found that SA concentration plays a significant role in the regulation of aliphatic and indole GLS synthesis: higher concentrations of applied SA significantly reduced the GLS content in *A. thaliana*, and *vice versa*.

2.1.5 Induced resistance and "priming"

Plants evolved to perceive numerous environmental signals, such as stimuli from pathogens and herbivores, beneficial microbes, or chemicals that can lead to an induced state of resistance in plants. Induced resistance protects non-damaged plant parts against subsequent attacks. Such prepared plant mode to defend against stressors is known as "priming" and it involves quicker and stronger activation of plant defence responses during future attacks (Fig. 4). Molecular mechanisms that lead to a priming effect are not completely understood; however, two theories have been suggested: i) the role of MAPKs (Beckers et al., 2009; Conrath et al., 2015); ii) changes in DNA methylation and histone modifications may carry stress memory and initiate defence responses during the next attack (Chen and Tian, 2007; López Sánchez et al., 2016). Nonetheless, keeping in mind the complexity of the plant immune system, these theories should be calibrated based on the interaction between the plant and a type of the stimuli.

2.1.5.1 Organismal stimulants

PTI/MTI, HTI and ETI can trigger the activation of induced resistance signals in plant tissues distant from the infection and infestation sites, which may be detrimental to some pathogens and insect herbivores (Pieterse et al., 2014). A form of pathogen-induced resistance is generally known as systemic acquired resistance (SAR), and it is characterised by SA accumulation and activation of *pathogenesis-related* (*PR*) genes involved in SA signaling (*e.g. PR1*). On the other hand, herbivores causing wounding in plants trigger the activation of JA biosynthesis and JA-responsive gene expression in distant plant sites, known as herbivore-induced resistance (HIR; Pieterse et al., 2014). There is a considerable number of studies focused on relationships between plants and pathogens or plants and insect pests, and much less information is available about their interrelation, although plants are prevalently exposed to this complex of biotic stressors. When a plant is attacked by pathogens and pests simultaneously or sequentially, the impact of such combined stress is not just additive but triggers a whole network

of interactions in which these attackers can stimulate each other or create antagonistic relationships (Hauser et al., 2013). If the relationship between attackers will be synergistic or antagonistic, it primarily depends on the insect herbivore feeding guild and pathogen infection strategies and therefore on the activation of plant defence mechanisms. For instance, it is suggested that pathogens and pests can be mutually synergistic when a biotrophic pathogen attack or a sucking insect infestation and subsequent necrotrophic pathogen attack or a chewing insect infestation occurs. The reason for this is because these two groups (biotrophic pathogens/piercing-sucking insect pests and necrotrophic pathogens/chewing insect herbivores) of attackers tend to induce different phytohormonal defence signals that are in some cases mutually antagonistic: SA signaling and JA signaling, respectively. Thus, the plant resistance against a subsequent pathogen or insect pest will be repressed if antagonism between SA and JA signaling occurs (El Oirdi et al., 2011; Glazebrook, 2005).

A distinct type of organismal-acquired resistance is achieved by mutualistic relationships between plants and non-pathogenic microbes, which initiate different processes in the plant and generally do not include the activation of SA signaling. For the sake of distinction, the term SAR is used when induced resistance is triggered by a pathogenic microbe and is commonly SA-dependent, while the term induced systemic resistance (ISR) refers to a presence of beneficial microbe in the rhizosphere and is therefore SA-independent (Conrath et al., 2015; Pieterse et al., 2014). Hence, beneficial microbes tend to be associated with elevated expression of JA/ET-regulated genes, but only during the subsequent attack, as it was confirmed by an extensive study of the ISR transcriptome of *Arabidopsis* during the root colonisation by ISR-inducing rhizobacteria *Pseudomonas fluorescens* WCS417r and subsequent leaf infection by bacterial pathogen *P. syringae* pv. *tomato* DC3000 (Verhagen et al., 2004). Plant priming can also be achieved in terms of GLSs and myrosinases and insect-pathogenic fungi associating with plants. As observed in the study by Qing et al. (2023) and Cachapa et al. (2021), insect-pathogenic fungi *Metarhizium robertsii* and *Metarhizium brunneum*, which successfully associated with cauliflower roots, rendered plants more resistant to herbivory by *P. xylostella*, due to the faster and stronger increase in GLS content and myrosinase activity in fungus-inoculated plants than in fungus-free plants after herbivory.

2.1.5.2 Elicitors and chemical stimulants

Apart from living plant-associating organisms, induced resistance and priming can also be achieved by elicitors as components of various microorganisms (PAMPs: *e.g.* flagellin, chitin, ulvans), that are able to elicit plant defence by being recognized by plant PRRs which leads to activation of defence cascades and accumulation of defence related molecules and enzymes priming the plant for subsequent attack (Conrath, 2011). The second group of elicitors is comprised of chemical inducers that usually mimic signaling molecules in plant defence pathways, such as β-amino-butyric acid (BABA) and acibenzolar-S-methyl (ASM), also known as benzothiadiazole (BTH; Burketova et al., 2015).

Ulvans, sulphated polysaccharides isolated from the cell walls of green algae (*Ulva* spp.) can induce plant resistance against various pathogens and pests in different plant species (Agarwal et al., 2021; Burketova et al., 2015; Siah et al., 2018). Transcriptomics revealed that ulvan, primarily isolated from *U. armoricana*, activates the JA signaling pathway in *Medicago truncatula* (Jaulneau et al., 2010). Additionally, ulvan induces the expression of *pathogenesis-related* genes *PR2* and *PR3*, which are involved in ROS metabolism and the JA signaling pathway in wheat, thereby protecting the plant against the hemibiotrophic pathogen *Zymoseptoria tritici* (De Borba et al., 2021). Similarly, Přerovská et al. (2022) observed that low ulvan concentration triggers the upregulation of genes involved in JA (*VSP*), JA/ET (*βCHI*) and oxidative stress (*rbohF*) responses and downregulates the genes involved in ABA (*RD26*, *NCED3*) signaling; however, only higher concentrations (1 mg ml^{-1} and 10 mg ml^{-1}) of ulvan solution significantly decreased the lesion development by *L. maculans* in cotyledons of *B. napus* compared to control.

BTH, a chemical compound that mimics the SA signaling by triggering the upregulation of the *PR1* gene, contributes to plant resistance (Lawton et al., 1996). After spraying of *B. napus* with BTH, *PR1* gene was highly elevated 24 h after treatment and plants were more resistant to a specialist hemibiotroph *L. maculans, i.e.* the lesions were significantly smaller compared to non-treated plants (Kim et al., 2013; Šašek et al., 2012b). Beckers et al. (2009) observed that MAPK 3 and 6 accumulate during SAR by BTH and remain dormant until the subsequent attack, when they get activated and then trigger the production of other defence compounds.

2.1.5.3 Plant association with beneficial organisms in the rhizosphere

To survive in a hostile world, plants evolved to form mutualistic relationships with various microorganisms, from which they benefit in terms of growth and defence against non-beneficial organisms. Plant-beneficial microbes are in turn provided with shelter and nutrients. Beneficial microbes generally establish the relationship with their hosts as endophytes or endosymbionts, *i.e.* within the plant and not causing any visible symptoms of disease; however, they can also behave as ectophytes, *i.e.* reside on the root surface. For instance, plants from Fabaceae family create symbiotic relationships with *Rhizobium* species, endosymbiotic bacteria residing in root nodules, which are able to convert atmospheric nitrogen to ammonia which plants can utilise, while they in turn get provided with photosynthates (Taylor et al., 2020). Furthermore, bacteria *Pseudomonas fluorescens* and *P. putida* were found to stimulate growth of *A. thaliana* when interacting with roots, probably due to production of compounds with auxin-like activity, such as cyclodipeptides (CDPs; Ortiz-Castro et al., 2020).

When a plant gets in contact with a mutualistic microbe, *e.g.* nitrogen-fixing rhizobial bacteria, mycorrhizal fungus, or another root-associating microbe, its defence system might perceive it as a pathogenic association and therefore start the activation of defence signaling cascades. The initial stage of mutualistic colonisation is generally similar to infection by biotrophic pathogens and therefore activated defence responses are comparable between these two types of interactions (Nishad et al., 2020; Pieterse et al., 2012). Similarly to pathogens, plant-symbiotic organisms evolved an effector system which helps them overcome plant-activated reactions and establish a successful interaction with their host. Some effectors have been described and characterised, such as ectomycorrhizal secreted protein 7 (SP7) and arbuscular mycorrhiza-induced small secreted protein 7 (MiSSP7; Yamazaki and Hayashi, 2015). SP7 is known to suppress ET-mediated defence responses, whereas MiSSP7 promotes the expression of auxin-related genes and therefore controls the plant growth (Pieterse et al., 2012). Moreover, arbuscular mycorrhizal fungi generally suppress SA-mediated responses and activate JA signaling, which plays an important role in mycorrhiza-plant association (Nishad et al., 2020). This change in phytohormonal signaling renders plants more resistant towards necrotrophic pathogens and chewing insect herbivores. Apart from mycorrhiza, a lot of attention has been devoted to studies of other beneficial plant-associated microorganisms, such as entomopathogenic fungi.

2.1.5.4 Plant-associated entomopathogenic fungi

Entomopathogenic or insect-pathogenic fungi are generally studied as insect pathogens, as they can infect insects and possibly kill them. Due to their ability to penetrate insect exoskeleton and infect them (*e.g.* caterpillars, aphids, thrips), insect-pathogenic fungi are used as an alternative to chemical insecticides; additionally, these fungi are usually host-specific and do not represent a risk for plant-beneficial organisms (Bamisile et al., 2018; Vega et al., 2012). Recent advance in research of these microorganisms has uncovered their additional role as non-pathogenic plant-associated microbes. Insect-pathogenic fungi can establish a symbiotic relationship with its host plant: after infecting and killing insects in the soil, they provide the plant with insect-derived nitrogen via fungal mycelia, while plants in turn provide fungi with photosynthates (Behie et al., 2017, 2012). This symbiosis can lead to increased plant growth and boosting of plant defence mechanisms. A large number of entomopathogenic fungal species have been confirmed to act as endophytes: firstly, they have been isolated from various plant species as natural endophytes and secondly, they have been inoculated artificially and hence established their endophytic activity (Vega, 2008). Many insect-pathogenic fungi rendered plants more resistant towards different insect herbivores when associating as endophytes (Gange et al., 2019). Entomopathogenic fungi are dominated by the order Hypocreales, phylum Ascomycota, and show a wide diversity in plant hosts and host tissue (Vega, 2018). Among plant-associating insect-pathogenic fungi, *Beauveria* and *Metarhizium* species are the most studied ones. Since they reside in the rhizosphere, they firstly associate with roots of various plant species. However, *Beauveria bassiana* seems to be able to colonise all plant parts, whereas *Metarhizium* spp. generally stays only in/on the roots and in some cases within the stem and leaves, which is mostly dependent on plant species or cultivars and fungal species or strains (Behie et al., 2015; Rasool et al., 2021b). *Beauveria* and *Metarhizium* spp. successfully infect insect pests; however, more attention as plant endophytes was given to *Beauveria* than to *Metarhizium*. For instance, *B. bassiana* was found to reduce the growth and reproduction rate of many insect pests feeding on various plant species, such as caterpillars of *Ostrinia nubilalis* in corn and sorghum plants, or *Rhopalosiphum padi* aphids in wheat and bean (Bamisile et al., 2018; Rasool et al., 2021b). In a meta-analysis by Gange et al. (2019), greater negative effects of entomopathogenic fungal endophytes were observed in piercing-sucking insects than in

chewing ones after seed inoculation. However, positive or neutral effects on some insect pests, such as aphids, have also been reported (Jensen et al., 2019; Rasool et al., 2021b).

2.1.5.4.1 *Metarhizium* spp.

Insect-pathogenic fungi *Metarhizium* spp. (Ascomycota: Hypocreales: Clavicipitaceae) have been extensively used as insect biocontrol agents in the field; however, in some cases, fungi did not perform very well, which might be due to their neglected phylogenetic history as plant-associated microbes (Barelli et al., 2018). A comparative genomic analysis by Gao et al. (2011) has shown that *Metarhizium* spp. evolved from plant-symbiotic fungal species and have acquired the ability to infect insects as a later adaptation. Hence, there is a growing number of studies regarding the interaction between plants and entomopathogenic fungi such as *Metarhizium* spp. It was suggested that *Metarhizium* may associate only with plant roots (Barelli et al., 2016), or endophytically in belowground or aboveground plant tissues (Batta, 2013). Some studies suggest that while residing in the rhizosphere, *Metarhizium* fungi may trigger defence responses in the aboveground level of plants, such as GLS synthesis and phytohormonal signaling (Cachapa et al., 2021; Hu and Bidochka, 2021a). However, as with most microbe-induced resistance responses, results are inconsistent and often highly context-dependent. Qing et al. (2023) found that the production of indole and aliphatic GLSs increased in cauliflower leaves after root treatment with *M. robertsii* and subsequent leaf consumption by *P. xylostella* caterpillars was significantly reduced compared to non-inoculated plants. Similar results were obtained in the study with fungus *M. brunneum* by Cachapa et al. (2021): the fungus associated with roots of cauliflower when fungal-treated rice grains were introduced to the soil with cauliflower plants; subsequently, the myrosinase activity was higher in *M. brunneum*-inoculated plants after herbivory by *P. xylostella* than in *Metarhizium*-free plants after herbivory. In addition, *Metarhizium*-inoculated plants were less consumed by caterpillars compared to control plants. It can therefore be suggested that *M. robertsii* and *M. brunneum* "prime" the plant for defence against chewing herbivores. Moreover, Batta (2013) found that *M. anisopliae* increased the mortality of *P. xylostella* larvae on inoculated *B. napus*. Another study reported a negative effect of *M. robertsii* on insect performance: fungal inoculation of wheat seeds significantly reduced the population growth of the aphid *Rhopalosiphum padi* in wheat leaves when compared to non-inoculated plants; however, *M. brunneum* showed an

opposite effect and significantly increased the aphid population in wheat (Rasool et al., 2021b). Hu and Bidochka (2021b) found that *M. robertsii* can also play an important role in reduction of growth of plant pathogenic fungi, such as *Fusarium solani* during bean colonisation. Moreover, several studies observed a positive effect of *Metarhizium* on plant growth (Liao et al., 2017; Lugtenberg et al., 2016), whereas some have seen no effect (Cachapa et al., 2021). As there exist various *Metarhizium* species and strains that behave differently when introduced to the host plant, their role regarding plant defence is still poorly understood and needs further investigation. Nonetheless, current findings support that *Metarhizium* spp. can interact or interfere with the host plant physiology and defence signaling.

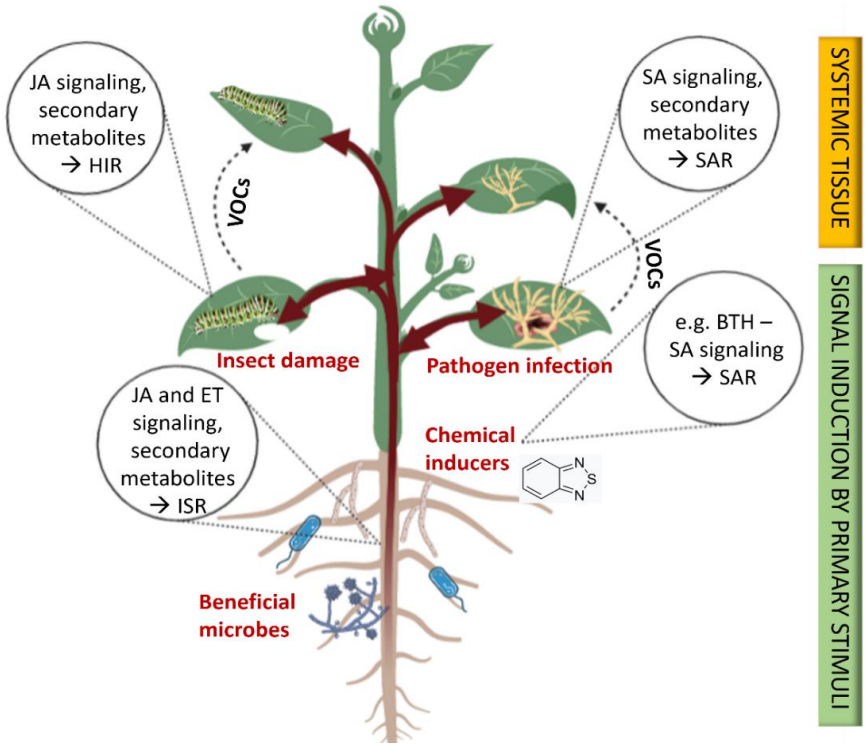

Figure 4. Induced resistance and priming effect in plants exposed to different biotic stresses. Interactions with beneficial microbes (*e.g.* root-associating insect pathogenic fungi) in the rhizosphere may trigger the induction of phytohormonal responses and secondary metabolites in the aboveground level. Chemical inducers applied in the soil or on the leaf surface can lead to induction of different defence mechanisms, *e.g.* benzothiadiazole (BTH) triggers the induction of SA signaling. Pathogen infection and insect herbivore infestation may trigger the induced resistance in systemic parts of the plant. Red arrows represent the induction of signaling by specific stimuli inside the plant, and dashed black arrows emitted volatile compounds (VOCs). Abbreviations: ISR = induced systemic resistance; HIR = herbivore-induced resistance; SAR = systemic acquired resistance.

2.2 Hemibiotrophic fungal pathogen *Leptosphaeria maculans*

2.2.1 Taxonomy of *Leptosphaeria maculans* and its life cycle

Leptosphaeria maculans, also known as *Plenodomus lingam* (de Gruyter et al., 2013) belongs to the phylum Ascomycota, subphylum Pezizomycotina, class Dothideomycetes, order Pleosporales and family Leptosphaeriaceae (Schoch et al., 2020). Characteristically for Ascomycota, *L. maculans* can reproduce asexually by mycelium and conidia called pycnidiospores, *i.e.* asexual spores released from fruiting bodies conidiophores known as pycnidia; however, it mainly reproduces sexually by ascospores formed inside sacs known as asci. In *L. maculans*, asci are produced within fruiting bodies ascocarps called pseudothecia (Agrios, 2005). Ascospores of *L. maculans* are transparent with vertical septa and are used to distinguish *L. maculans* from another species (Kaczmarek and Jędryczka, 2012). The sexual stage is generally called teleomorph, whereas the asexual stage is known as anamorph. *L. maculans* is initially saprophytic as it can survive for several years on host plant residues where sexual fruiting bodies pseudothecia and/or asexual pycnidia form spores which eventually get in contact with young *Brassica* crops. Ascospores formed in pseudothecia are dispersed by wind on long distances and represent the primary source of inoculum (Bousset et al., 2018; Fig. 5). *L. maculans* becomes biotrophic when ascospores fall on cotyledons or young leaves and start to germinate to produce germ tubes, which then penetrate the leaf apoplast through stomata and wounds. Then, *L. maculans* shortly switches to the necrotrophic stage and forms necrotic leaf lesions within which the asexual reproduction takes place, *i.e.* black pycnidiospores are produced in pycnidia inside the dead plant tissue and are dispersed by rain in the near distance representing a secondary inoculum source (Howlett et al., 2001; Rouxel and Balesdent, 2005; Fig. 5). The fungus then again switches to biotrophy when hyphae start spreading from the leaf spots endophytically, *i.e.* to the vascular system down the petiole mainly through xylem vessels with the goal to reach the stem while not causing any visible symptoms on the host plant (Chen and Howlett, 1996; Hammond and Lewis, 1987). However, when *L. maculans* finally reaches the crown of the stem, the juncture between the base of the stem and hypocotyl, it turns necrotrophic again and causes blackening known as stem canker or “blackleg” that eventually leads to premature ripening and yield loss of infected *Brassica* crops (Borhan et al., 2022).

As stated by Sheldrake (2021), mycelium is the means by which fungi obtain food; their hyphae are lengthy and divided, and they ingest all that is within their reach. One feature that distinguishes fungi from animals is that fungi incorporate their bodies into food, whereas animals incorporate food into their bodies.

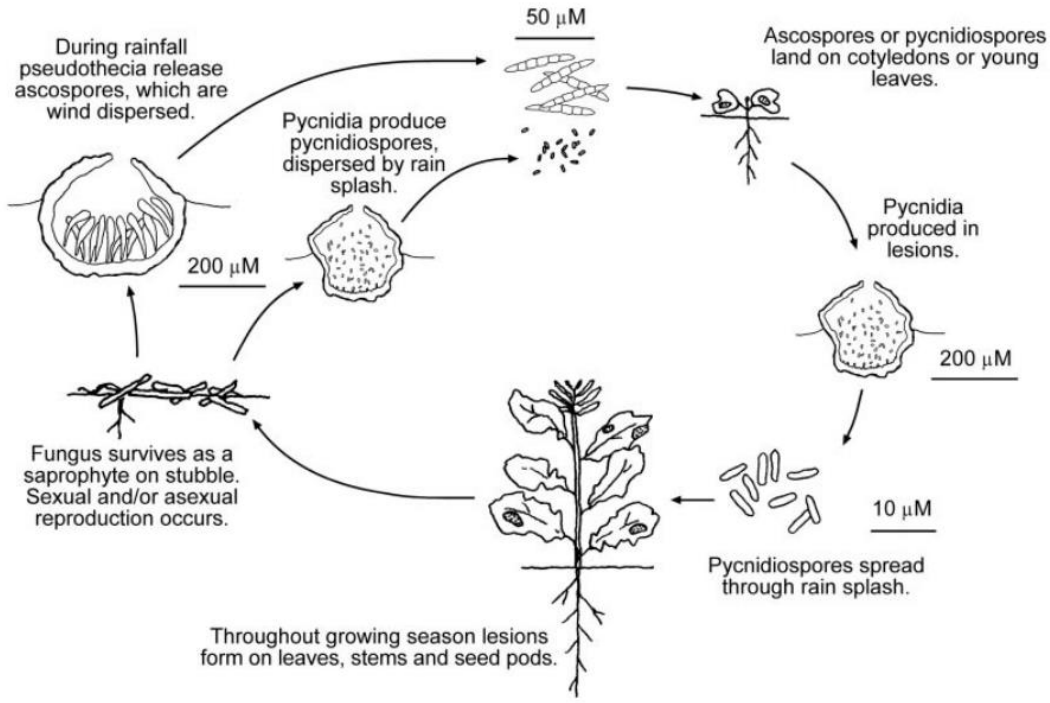

Figure 5. Life cycle of *Leptosphaeria maculans* on *Brassica* crops (Howlett et al., 2001).

2.2.2 *Leptosphaeria maculans* interaction with *Brassica napus*

An interesting example of incompatible and compatible interaction has been investigated on *L. maculans* and its host *B. napus*. Balesdent et al. (2002) found that a type of interaction and the infection outcome (resistant or susceptible) between different isolates of *L. maculans* and *B. napus* is mediated by the *Avr* gene of the pathogen, *e.g. AvrLm1* and a corresponding *R* gene, *i.e. Rlm1* in the host plant. For instance, JN3 isolate of *L. maculans* carrying *AvrLm1* gene causes small lesions resembling HR, whereas virulent JN2, an isolate of *L. maculans* lacking *AvrLm1* gene, causes significantly larger lesions on a Columbus cultivar carrying *Rlm1* (Šašek et al., 2012b). Therefore, *R*-mediated resistance (RMR) or qualitative resistance, *i.e.* gene-for-gene resistance is considered to be dependent on the combination of

the *AvrLm* gene in the pathogen and the corresponding *Rlm* gene in the plant, and is usually connected with cell death or HR (Borhan et al., 2022). The JN3 isolate seems to cause a stronger production of H_2O_2 in the Columbus cultivar than the virulent JN2 isolate, which confirms that oxidative burst is followed by HR (Mur et al., 2008; Šašek et al., 2012b). Above all, both JN2 and JN3 isolates trigger an intensive callose formation in Columbus plants; however, a higher amount of callose plugs, albeit at a lower intensity, was detected in JN2-infected cotyledons (Šašek et al., 2012). This difference was not detected in *B. napus* cv. Westar, an *Rlm1*-deficient cultivar, and therefore both fungal isolates produced severe damage (Šašek et al., 2012). Several other AvrLm proteins have been identified in *L. maculans*, along with Rlm proteins in *B. napus* (Fig. 6). Fungal effectors trigger the activation of MAPKs, which are linked to phytohormonal signaling pathways; for instance, MAPK3, 4 and 6 regulate the SA, JA, ET and ABA pathways (Jagodzik et al., 2018). Furthermore, the AvrLm4-7 effector has been reported to attenuate hormonal and ROS signaling in *B. napus* plants (Nováková et al., 2016). In addition, *L. maculans* also produces phytohormones by which could manipulate host defence responses (Leontovyčová et al., 2020; Trdá et al., 2017)

Along with qualitative resistance, *B. napus* can also form quantitative resistance (QR) to *L. maculans*, which remains poorly understood in comparison. QR is isolate-nonspecific resistance, generally influenced by multiple loci and by environmental factors. RMR is known as seedling resistance, whereas QR as adult plant resistance (Borhan et al., 2022). The combination of RMR and QR *via* development of resistant plant cultivars represents an efficient way of controlling *L. maculans* infection in the field (Petit-Houdenot et al., 2019).

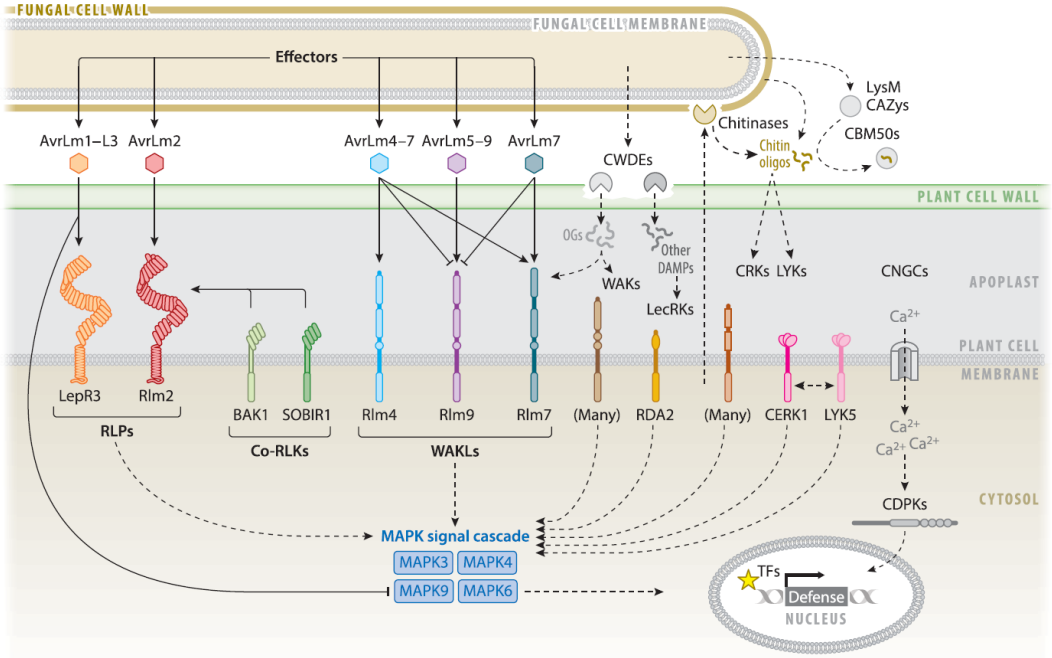

Figure 6. Molecular interactions in the *Brassica napus* – *Leptosphaeria maculans* pathosystem. Abbreviations: CAZys = carbohydrate-active enzymes; CDPKs = calcium-dependent protein kinases; CNGCs = cyclic nucleotide-gated channels; CRKs = cysteine-rich receptor-like kinases; CWDEs = cell wall-degrading enzymes; DAMPs = danger-associated molecular patterns; LecRKs = lectin receptor kinases; LYKs =lysin motif receptor-like kinases; MAPK = mitogen-activated protein kinase; OGs = oligogalacturonides; RLKs = receptor-like kinases; RLPs = receptor-like proteins; TFs = transcription factors; WAKs = wall-associated kinases; WAKL = wall-associated kinase-like (Borhan et al., 2022).

2.3 Chewing insect herbivores

When chewing caterpillars initiate the feeding process, they deliver specific elicitor compounds through saliva and regurgitant into the plant tissue, such as fatty-acid amino-acid conjugates (*e.g.* volicitin) and *β*-glucosidase (Jones et al., 2021), which induce early defence signaling responses in host plants. Depolarization of the plasma membrane occurs, followed by the rise in cytosolic Ca^{2+}, production of ROS and activity of MAPKs and CDPKs (Erb and Reymond, 2019; Fig. 1). Many lepidopterans evolved to produce effectors; one example is secretion of glucose oxidase (GOX), an enzyme involved in the production of H_2O_2 that some caterpillars may use to remove excessive sugars and increase the food quality, or to regulate plant defence mechanisms, as H_2O_2 is considered to be an upstream signal that triggers biosynthesis of plant hormones during caterpillar feeding (Babic et al., 2008; Jones et al., 2021; Merkx-Jacques and Bede, 2005). Nonetheless, GOX activity is highly dependent on both the caterpillar species and the plant host.

2.3.1 *Plutella xylostella* and its life cycle

One of the globally most destructive insect specialists on *Brassica* crops is *P. xylostella* or diamondback moth (Zalucki et al., 2012). The life cycle of *P. xylostella* (Fig. 7) begins as an adult moth, around 9 mm long and greyish brown in colour with a recognizable whitish diamond-shaped band along its back. After mating, female moths lay eggs on the host plant. As reported on Chinese kale (*Brassica oleracea* var. *alboglabra*), adults prefer to lay eggs on plant parts with less wax, such as cotyledons (Zhu et al., 2022). Volatiles, secondary metabolites, temperature and trichomes also play a role in oviposition stimulation (Silva and Furlong, 2012; Talekar and Shelton, 1993). However, when ovipositing on older plants of *B. oleracea* and *B. napus* without cotyledons, most eggs were laid on the abaxial side of the leaf, whereas in the case of *B. rapa*, the biggest number of eggs was detected on the adaxial leaf side (Silva and Furlong, 2012). The eggs are small, approximately 0.5 mm long and yellowish in colour, and can be clearly seen through binocular or hand lens (Fig. 7). After egg hatching and larval emergence, the feeding process is initiated on leaves. The larval stage is the primary cause of damage as caterpillars cause defoliation by chewing which results in major yield losses (Akandeh et al., 2016; Nouri-Ganbalani et al., 2018). In general, caterpillars do not find

cotyledons palatable and suitable for feeding, so when eggs hatch on cotyledons, caterpillars form silk nets which help them move to true leaves and feed there (Zhu et al., 2022). This process of net crawling and feeding changes the leaf structure and interestingly, lures females to oviposit on such damaged leaves (Silva and Furlong, 2012; Zhu et al., 2022). Caterpillars pass through four instars, each differing morphologically and in their method of damaging leaves (Fig. 7). First instar is around 1 mm long and pale white with dark head, and later stages are brownish to green with several short hairs, reaching the length of around 11 mm (Genç, 2021; Philips et al., 2014). Female and male caterpillars are easily distinguishable during the fourth instar, as only males have a pale mark on the eight abdominal segment (Genç, 2021). First instar creates so called "window-like" damage by mining only mesophyll cells, and later instars consume surface of leaves including the waxy layer (Sarfraz et al., 2005). When larvae are fully grown, they start to form silk cocoons on the host plant where they pupate (Fig. 7). The time to complete one life cycle varies based on temperature levels: it took 27 days at 18°C to complete one generation on cabbage (*B. oleracea* var. *capitata*) plants (Liu et al., 2002).

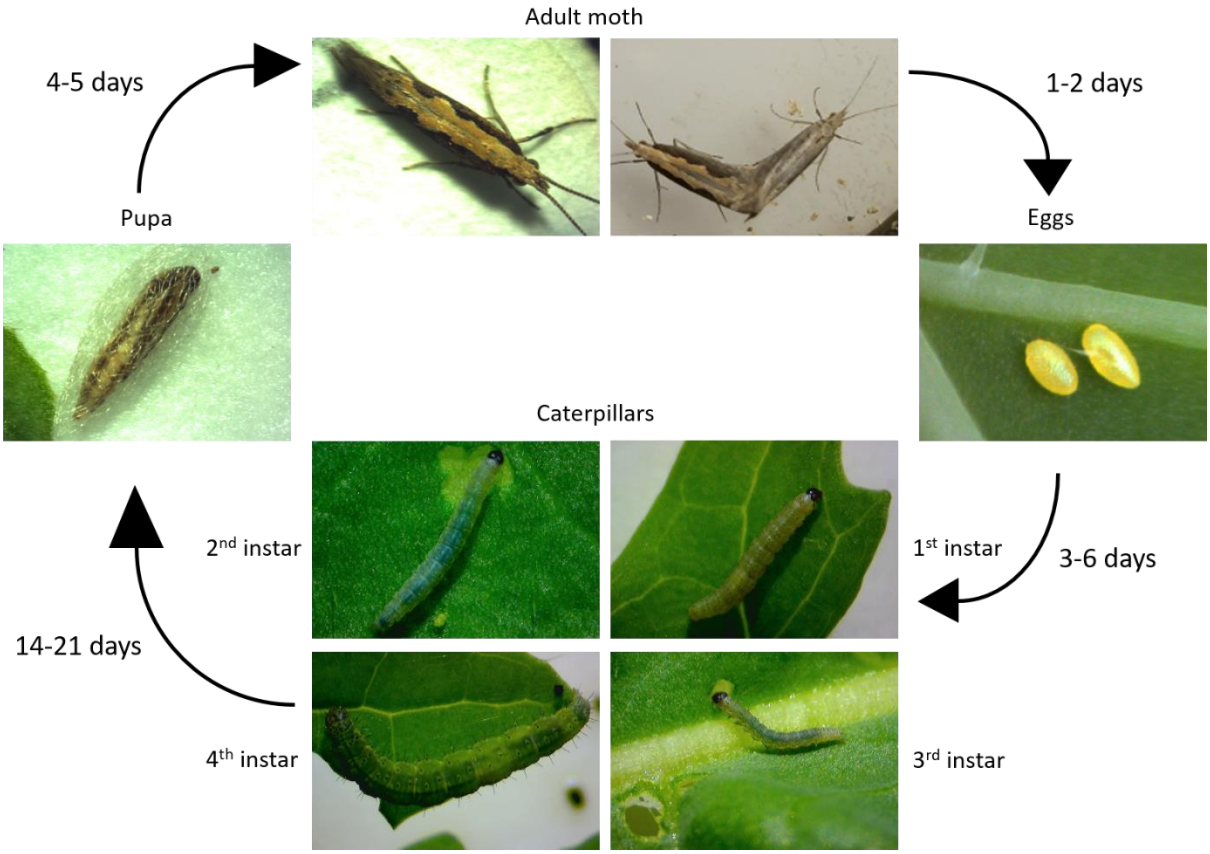

Figure 7. Life cycle of *Plutella xylostella.*

2.3.2 *Plutella xylostella* interaction with *Brassica* crops

As a chewing insect herbivore, *P. xyslostella* can cause defoliation of *Brassica* plants in the field. Plant secondary metabolites of Brassicaceae, *i.e.* glucosinolates (GLSs) serve as important cues for *P. xylostella* preference and performance as caterpillars are biochemically adapted to feed and adult moths are lured to oviposit on GLSs-containing plants (Chen et al., 2020; Ratzka et al., 2002; Robin et al., 2017; Sun et al., 2009). Along with GLSs, volatile organic compounds (VOCs) play a major role in moth choice for the acceptable plant to oviposit as they strongly respond to olfactory cues (Hussain et al., 2020; Liu et al., 2020; Renwick et al., 2006). For the larval stage, gustatory stimuli, *i.e.* GLSs are more important (Robin et al., 2017), and larvae are also able to choose food and move from one spot to another (Zhu et al., 2022). Exogenously applied plant hormones or plant defence responses induced by previous attackers may also play a role in *P. xylostella* behaviour. Nouri-Ganbalani et al. (2018) found that *B. napus* plants previously treated with synthetic JA or pre-infested by a specialist aphid *B. brassicae* significantly affected the activity of GOX by *P. xylostella* caterpillars: higher activity was detected on pre-treated compared to non-treated plants in both scenarios.

2.4 Piercing-sucking insect herbivores

Aphids, among the most important phloem-sap feeders, can achieve high population densities, transmit viral pathogens and cause severe damage on various crops worldwide (Singh and Singh, 2021). They evolved a characteristic elongated mouthpart, termed stylet by which they probe inside the leaf tissue and secrete salivary molecules on the way to phloem. Stylets tend to avoid the activation of plant defence reactions by moving intercellularly and excreting gelling saliva that serves as a stylet sheath; however, many mesophyll cells are punctured along the way and filled with watery saliva (Miles, 2007, 1959; Tjallingii, 2006). The massive secretion of watery salivary molecules occurs when stylet finally probes the sieve elements and initiates the feeding process. These molecules, or elicitor and effector proteins, trigger and manipulate plant defence responses during probing and feeding periods. At the same time, aphids tend to excrete honeydew on the leaf surface, a sugary liquid which also contains elicitor or effector molecules (Schwartzberg and Tumlinson, 2014). Some aphid-delivered effectors have been described, such as Mp10 and Mp42 in *Myzus persicae* (Bos et al., 2010). Nonetheless, when plant cells are punctured by the stylet, Ca^{2+} increases and triggers protein and callose synthesis, with the goal to clog sieve tubes and prevent the loss of phloem sap (Knoblauch and van Bel, 1998; Silva-Sanzana et al., 2020). Callose, a β-glucan polymer synthesized at the plasma membrane of plant cells, plays an important role in plant defence responses to aphid infestation (Silva-Sanzana et al., 2020). In response to these plant reactions, aphids start secreting effectors, such as Ca^{2+}-binding proteins, with the goal to suppress the blockage of nutrients and feed freely (Tjallingii, 2006; Will et al., 2009, 2007). However, the host plant may recognize these molecules, resulting in a cascade of defence signaling responses (Fig. 1). Apart from directly damaging the plant by sucking the nutrients, aphids can also harm the plant by transmitting viral phytopathogens and by excreting honeydew, that can attract ants or sooty mold which further decrease plant photosynthetic activity (Singh and Singh, 2021).

2.4.1 *Brevicoryne brassicae* and its life cycle

Cabbage aphid or *B. brassicae* is a specialist insect pest on *Brassica* crops native to Europe but is currently distributed across the world. The name "*Brevicoryne*" roughly translates from Latin as small ("brevi") pipes ("coryne"), as *B. brassicae* has two characteristic short

black cornicles or siphunculi on the abdomen, which can be seen through hand lens or stereomicroscope (Fig. 8A). The short cornicles and the greyish waxy coating are morphological features which distinguish *B. brassicae* from other aphids. Generally, aphids are ectothermic organisms, hence their biology largely depends on temperature. Soh et al. (2018) reported that *B. brassicae* successfully developed at temperatures ranging from 10°C to 30°C, and its development was inhibited at 35°C. Depending on the climate, life cycle of *B. brassicae* can be holocyclic (sexual reproduction) and anholocyclic (asexual reproduction; Fig. 8B). When winters are cold, non-winged/apterous females mate with winged/alatae males and lay eggs that overwinter on the same host plant. When the weather conditions are milder, apterous females are viviparous and reproduce parthenogenetically, *i.e.* give birth to young nymphs without mating. The nymphs go through four instars and turn into apterous females; however, if the host plant becomes overcrowded and the food quality declines, the fourth instar nymphs moult into migratory alatae females, which then fly to the new host plant and continue to reproduce parthenogenetically. Mainly such ability of aphid clonal reproduction brings deleterious outcome for the host plant.

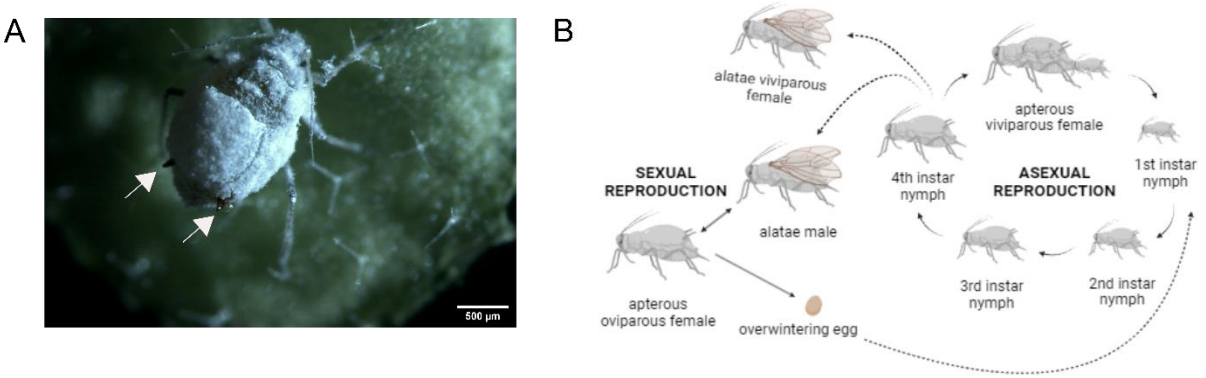

Figure 8. **A:** *Brevicoryne brassicae* adult feeding on *Arabidopsis thaliana*. White arrows point to the characteristic short cornicles. **B:** Life cycle of *B. brassicae.*

2.4.2 *Brevicoryne brassicae* interaction with *Brassica* crops

As other insect pests, winged *B. brassicae* aphid uses a set of visual and olfactory cues to locate the host plant (Döring, 2014). Then, for the host acceptance, aphids primarily rely on gustatory stimuli, and leaf structure and secondary metabolites play an important role in this

stage. For aphids to initiate the feeding process, stylet must first successfully penetrate the outer leaf layer (epidermis) of *Brassica* spp., then middle leaf tissues (mesophyll) composed of palisade and spongy parenchyma (Hao et al., 2020), after which it finally reaches the sieve elements and ingests the sap. However, there are many barriers that aphids need to overcome to start the feeding process, and they vary between plant species and cultivars (Gabryś and Pawluk, 1999; Hao et al., 2020). Electropenetrography (EPG) recordings of stylet probing periods through plant cells help to understand aphid resistance, and numerous stylet insertions and removals indicate that there might be certain factors present which hinder aphid feeding (Tjallingii, 2006). Firstly, the structure of the outer epidermal layer, *e.g.* thickness, trichome density, as well as the epidermis-released volatile and non-volatile chemical substances play an important role in host acceptance: Hao et al. (2020) observed that *B. brassicae* took longer time to start the first penetration on cultivars of *B. napus* with the thick epidermal layer and dense and long trichomes. It is also speculated that mesophyll cells of different *B. napus* cultivars might possess certain chemical stimuli which contribute to less or longer probing periods (Hao et al., 2020). When stylet reaches the phloem, aphids initiate the salivation in order to manipulate plant defence responses, *e.g.* sieve tube occlusion, and longer salivation periods indicate non-host or resistant plant species or cultivars (Tjallingii, 2006; Will et al., 2009, 2007). Nutritional quality of the phloem sap and secondary metabolites, such as GLSs play an important role in *B. brassicae* feeding as this specialist developed a biochemical adaptation to the GLS-myrosinase system (Cole, 1997; Francis et al., 2002). Mezgebe and Azerefegne (2021) found that drought-stressed *Brassica carinata* had a lower GLS content than well-watered plants; later, when infested by *B. brassicae*, the total GLS content increased significantly following the increased number of aphids.

Plant hormones also play a role in *B. brassicae* feeding: the upregulation of JA signaling, plant treatment with SA or ABA can have a negative effect on aphid reproduction (Dehghan et al., 2023; Khoshfarman-Borji et al., 2020; Kuśnierczyk et al., 2011).

It should also be noted that different cultivars may affect the aphid reproduction and longevity in different ways; hence the right choice of cultivar could control the aphid infestation (Jahan et al., 2013).

3 Scientific hypothesis and objectives

***Scientific hypothesis*:**

Plants under pathogen or insect pest attack, root association with beneficial fungi or treatment with resistance inducers activate a plethora of defence responses that may alter their palatability or attractiveness to subsequent attackers. The mechanisms underlying this altered susceptibility/resistance to subsequent infestation/infection were defined at the molecular level on the basis of transcriptome and metabolome analyses. The basis of the altered palatability can be revealed by changes in the plant secondary metabolites glucosinolates, and the basis of altered attractiveness can be revealed by changes in the plant-emitted volatile spectrum.

***Scientific objectives*:**

1. To study changes in phytohormonal signaling, glucosinolate and volatile level in diseased and infested plants and plants associated with beneficial fungi in different pathosystems.
2. To assess the impact of different resistance inducers on the palatability of plants to insect herbivores.

4 Materials and methods

4.1 Plant material

Oilseed rape (*Brassica napus* cv. Columbus), cauliflower (*Brassica oleracea* convar. *botrytis*, cv. Opaal) and *Arabidopsis* plants (*Arabidopsis thaliana*, ecotype Columbia-0; Col-0) were used for experiments. Oilseed rape is an important crop, and cauliflower is widely used vegetable in human nutrition. *Arabidopsis* is a well-described model plant, with completely sequenced genome and various mutants available, which are being used for basic physiology studies as well as to address the role of signaling pathways and phytohormones as plant responses to biotic stressors.

Oilseed rape was cultivated individually in seedling trays, hydroponically in perlite in Steiner cultivation medium (Steiner, 1984) placed in a controlled environment room (photoperiod 14 h light/10 h dark, 24/22°C, photon flux density 150 μmol m^{-2} s^{-1}). Second and third detached true leaves of three-week old plants (BBCH growth stage 12-13; Lancashire et al., 1991) were used in all experiments. Leaves were cut and chosen randomly from approximately 40 plants for experiments on insect feeding choice and for transcript (defence gene transcription by qRT-PCR) and metabolic profiling (GLSs and VOCs) analyses.

Cauliflower plants were cultivated in potting soil at 15 ± 5ºC under 16 h light/8 h dark cycle. Inoculation was performed by adding the spore suspension of the beneficial insect-pathogenic fungi in the soil around the germinating seeds. Untreated seedlings and seedlings treated with 0.05 % Triton X were used as a control treatment. Cotyledons or two first true leaves were further inoculated by spores of a pathogenic fungus and used for testing the fungal development and for the analyses of defence gene transcription (qRT-PCR) and GLSs. When cotyledons were used in the study, sprouting true leaves were removed. Roots were harvested to examine the colonies formed by the plant-beneficial fungus.

Arabidopsis plants were cultivated in the potting soil at 22ºC under 10 h light/14 h dark cycle and 65-70 % humidity. Wild type (Col-0) and transgenic lines of *A. thaliana* in the Col-0 background used for microscopic observation were *PR1::GUS*, *pPR1::NLS3xVenus* (Poncini et al., 2017), *pJAZ10::NLS3xVenus*, *pAOS::NLS3xVenus*, *pACS6::NLS3xVenus*, *pPR4/HEL::NLS3xVenus* (Marhavý et al., 2019). Four- to five-week-old plants were used for

preference and performance tests with the piercing-sucking insect herbivore, and for further analyses of plant defence responses.

4.2 Fungal inoculants

Leptosphaeria maculans JN2 and JN3 isolates, also referred to as v23.1.2 and v23.1.3 (Balesdent et al., 2002), and *L. maculans* JN3 isolate transformed with a pCAMBgfp construct (Sesma and Osbourn, 2004) carrying the *sGFP* gene (Šašek et al., 2012a) were cultivated on V8 solidified medium at 26°C in the dark. Sporulation cultures and conidial suspension were prepared according to Ansan-Melayah et al. (1995). Spores were washed once with autoclaved tap water after harvesting, diluted to 10^8 spore ml^{-1}, and stored at -20°C for a maximum period of 6 months.

After cutting, oilseed rape leaves were inoculated with spore suspension of the JN2 isolate (10^5 spore ml^{-1}) using vacuum infiltration. Briefly, leaves were immersed into conidial suspension in a glass beaker, and the beaker was placed in a desiccator for several minutes until the infiltration was successful. Detached leaves infiltrated with distilled water were used as controls. Subsequently, leaf petioles were submerged in distilled water and fixed with 0.3 cm glass beads.

True leaves of cauliflower plants were inoculated by making a puncture into the attached true leaf using a sterile needle and subsequently pipetting 10 µl drop of spore suspension (10^7 spore ml^{-1}) of the JN3 or JN3-GFP isolate. Cotyledons of cauliflower plants were inoculated with spore suspension (10^5 spore ml^{-1}) of the JN3-GFP isolate using syringe infiltration.

Metarhizium robertsii KVL 12-35, isolated from the Danish agricultural soil, was stored at -80ºC. The strain was then transferred to a quarter-strength dilution of SDAY: Sabouraud Dextrose Agar (SDA; Merck KGaA, Darmstadt, Germany) with Yeast extract (Merck KGaA, Darmstadt, Germany), and grown for three weeks at 25ºC. After three weeks, 10 ml of sterile 0.05 % Triton X was added to the plates and conidia were harvested from sporulating cultures by rubbing the surface with a spatula. The conidial suspension was then transferred to a 50 ml centrifuge tube, washed twice by centrifugation, and the pellet resuspended in sterile 0.05 % Triton X. After preparing a serial dilution, conidial concentration was estimated by adding 20 µl of spore suspension in a 0.2 mm hemocytometer (Fuchs-Rosenthal, Germany) and counting

the spores under microscope. Working suspensions were adjusted to 10^8 conidia ml^{-1} and stored at 4ºC for maximum 24 h. Germination test was then prepared on Sabouraud Dextrose Agar (SDA; Merck KGaA, Darmstadt, Germany) plate by transferring 100 µl of dilution used for spore counting. Plates were further stored at 25ºC and spores were counted under microscope 24 h after plating by cutting three small pieces of agar with a sterile knife. Germination rate was then calculated as the average of germinated spores from three agar pieces. For spores to be considered highly effective, germination rate should exceed 95 %. Fresh conidial suspension was prepared for each experimental repetition.

4.3 Insects

Caterpillars of diamondback moth (*Plutella xylostella*) were reared on broccoli (*Brassica oleracea* var. *italica*) and cabbage (*Brassica oleracea* var. *capitata*) in an enclosed cage (45 x 45 x 45 cm) placed at 18ºC under 16 h light/8 h dark cycle. After pupation and emergence of adult moths, a small cotton-wool wick soaked in saccharose solution was provided as a source of carbohydrates for adults. Third-instar caterpillars were used for experiments.

Cabbage aphids (*Brevicoryne brassicae*) were reared on broccoli plants in an enclosed cage (45 x 45 x 45 cm) and kept in a climate chamber at 23ºC under 16 h light/8 h dark cycle (Kroes et al., 2017b). The wingless (apterae) adults were used for experiments.

4.4 Plant elicitors

Benzothiadiazole (BTH; 1,2,3-benzothiadiazole-7-carbothioic acid S-methylester; commercial product BION 50 WG, Syngenta), was used for infiltration of oilseed rape cotyledons and true leaves (32 µM); 24 h after infiltration, plants were used for qRT-PCR analysis and choice tests with *P. xylostella* caterpillars. Plants infiltrated with distilled water were used as a control.

Ulvan extraction was performed by the method described previously (Přerovská et al., 2022). Then, the concentration of 1 mg ml^{-1} was prepared and used for spraying three-week old plants of oilseed rape; 48 h after spraying, plants were used for choice test experiments with *P. xylostella* caterpillars. Plants sprayed with distilled water were used as a control.

4.5 RNA isolation and gene transcription analysis of plant defence and signaling

Plant response to infection or infestation was mainly being studied on transcriptomic level. Briefly, leaf discs, around 150 mg in total weight per one sample, were cut and immediately frozen in liquid nitrogen.

Leaf tissues of oilseed rape and *Arabidopsis*, and cotyledons of cauliflower were homogenised in tubes with 1 g of 1.3 mm silica beads using a FastPrep-24 instrument (MP Biomedicals, USA). Total RNA was then isolated using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, USA) and treated with a DNA-free kit (Ambion, USA). The quantity of extracted RNA was further measured using NanoDrop. Subsequently, 1 μg of RNA was then converted to cDNA with M-MLV RNase H− Point Mutant reverse transcriptase (Promega Corp., USA) and an anchored oligo dT21 primer (Metabion, Germany).

Leaf tissues of cauliflower were homogenised using a Tissue Lyzer II (Qiagen, USA). Total RNA was then extracted using RNeasy Plant Mini Kit (Qiagen, USA) and treated with a DNase I, Amp Grade (Invitrogen, USA). The quantity of RNA was further measured using mySPEC (VWR, USA). Subsequently, 1 μg of RNA was then converted to cDNA using iScript cDNA Synthesis Kit (BIO-RAD, USA).

Gene transcription was then quantified by quantitative reverse transcription PCR (qRT-PCR) using a LightCycler 480 SYBR Green I Master kit and LightCycler 480 (Roche, Switzerland). The activation of plant defence signaling pathways was evaluated by transcription of marker genes associated with SA (*isochorismate synthase 1*, '*ICS1*'; *isochorismate synthase 2*, '*ICS2*'; *phenylalanine ammonia lyase 1*, '*PAL1*'; *pathogenesis related 1*, '*PR1*'; *pathogenesis related 2*, '*PR2*'; *pathogenesis related 5*, '*PR5*'; *WRKY70 transcription factor*, '*WRKY70*'), JA (*allene oxide synthase*, '*AOS*'; *lipoxygenase 2*, '*LOX2*'; *lipoxygenase 3*, '*LOX3*'; *vegetative storage protein*, '*VSP*'; *jasmonate ZIM-domain 8*, '*JAZ8*'; *jasmonate ZIM-domain 10*, '*JAZ10*'), ET (*ACC synthase 2*, '*ACS2*'; *ACC synthase 6*, '*ACS6*') and ET/JA (*β-chitinase*, '*βCHI*'; *hevein-like/pathogenesis related 4*, '*HEL/PR4*'; *plant defensin 1.2*, '*PDF1.2*'; *plant defensin 3*, '*PDF3*') pathways, and genes involved in oxidative stress (*respiratory burst oxidase homolog C*, '*rbohC*'; *respiratory burst oxidase homolog F*, '*rbohF*'; Tab. 1). Fungal mass was determined in cotyledons and true leaves of cauliflower using constitutively expressed *internal transcribed spacer 1* for *L. maculans*, '*LmITS1*'(Persson et al., 2009; Tab. 1). The relative transcription was calculated with an efficiency correction and

normalisation to the reference gene *ACT* (for oilseed rape; Tab. 1), *ACT8* and *SAND* (for *Arabidopsis*; Tab. 1), and *EF1α* and *SAND* (for cauliflower; Tab. 1). The data were finally compared to the open transcriptomic databases, *i.e.* Genevestigator (Hruz et al., 2008).

Table 1. Primer pairs used for qRT-PCR.

Plant	Gene	Accession No.	Primer	Sequence (5'->3')
Oilseed rape *(Brassica napus)*	***ACT***	AF111812	FP	CTGGAATTGCTGACCGTATGAG
			RP	TGTTGGAAAGTGCTGAGGGA
	PR1	BNU21849	FP	CATCCCTCGAAAGCTCAAGAC
			RP	CCACTGCACGGGACCTAC
	ICS1	EV225528	FP	CAAACTCATCATCTTCCCTC
			RP	AGCGTGACTTACTAACCAG
	PAL1	DQ341308.1	FP	GACTAATCTCATCTCGCAAG
			RP	ATTCTCCTCCAAGTGTCTTAG
	LOX3	EV113862	FP	GAAGTTTATGGCGGTGGT
			RP	CCTGTTTCTACGGTTAGGA
	VSP	CN726858	FP	CCTCTCACTTTCACTTCTCTTGC
			RP	GTTCGGCTTCGTCCTCAATG
	βCHI	X61488	FP	TGCTACATAGAAGAAATAAACGG
			RP	TTCCATGATAGTTGAATCGG
	PR4/HEL	FG577475	FP	GGAACACAAGGACTAATGC
			RP	TTTCGATAGCCATCACCA
	ACS2	HM450312	FP	TATAGAAGGAGCAACGCA
			RP	ACCGAGTCGTTGTAAGAATA
Arabidopsis thaliana	***ACT8***	At1g49240	FP	TTCATCGGCCGTTGCATTTC
			RP	AATGTCATCAGCATCGGCCA

	SAND	At2g28390	FP	CTGTCTTCTCATCTCTTGTC
			RP	TCTTGCAATATGGTTCCTG
	PR1	At2g14610	FP	AGTTGTTTGGAGAAAGTCAG
			RP	GTTCACATAATTCCCACGA
	PR2	At3g57260	FP	TATAGCCACTGACACCAC
			RP	GCCAAGAAACCTATCACTG
	PR5	At1g75040	FP	TCTCCAGTATTCACATTCTCTTCC
			RP	CAATTCAAATCCTCCATCGCC
	ICS1	At1g74710	FP	GCAAGAATCATGTTCCTACC
			RP	AATTATCCTGCTGTTACGAG
	ICS2	At1g18870	FP	TGTCTTCAAAGTCTCCTCTG
			RP	CTTCCTCCAAACTCATCAAAC
	AOS	At5g42650	FP	GAACCGCCTTTAATTTCTTG
			RP	GAGAGTAATGGATGGAGATTG
	LOX2	At3g45140	FP	ATCCCACCTCACTCATTACT
			RP	ATCCAACACGAACAATCTCT
	JAZ8	At1g30135	FP	TGTGACTTGGAACTTCGT
			RP	GATTCTTCATTTGGTTGTGG
	JAZ10	At5g13220	FP	GGTCGCTAATGAAGCAGCATC
			RP	TCTGTCTCCATCGACGACTCG
	ACS2	At1g01480	FP	GTTAAGCTCAATGTGTCTCC
			RP	AAGCAAATCCTAAACCATCC
	ACS6	At4g11280	FP	TTAGCTAATCCCGGCGATGG
			RP	ACAAGATTCACTCCGGTTCTCCA
	PDF1.2	At5g44420	FP	CTGTTACGTCCCATGTTAAA

			RP	TTACTCATAGAGTGACAGAGAC
	PR4/HEL	At3g04720	FP	CAAGTGTTTAAGGGTGAAGA
			RP	CATTGCTACATCCAAATCCA
Cauliflower *(Brassica oleracea)*	***EF1α***	XM_013730661	FP	ACAGGCGTTCTGGTAAG
			RP	GCAACAGTCTGCCTCAT
	SAND	XM_013775105	FP	GATCAATCGCTGAAGGTG
			RP	GAAATGCCAAAGTCCAAA
			RP	GACATGTCACCACTGCTCCA
	ICS1	XM_013732875	FP	GGCCAGGCCTAATCTGAATG
			RP	CTGCTTCTGATGAGAGAACACT
	WRKY70	XM_013744419	FP	GCAAGCTTGAGGATTCCGGT
			RP	TCTACAGTCCACGTCTCCGA
	AOS	XM_013765565	FP	TAACCCGTCCGATCAAAGCC
			RP	AGACCGTAGCTTCCAGGGAT
			RP	TCGAACCCGTTGGCTTGAAT
	LOX3	XM_013749276	FP	TCTGACCTCCAAAAGACCCT
			RP	ATCTTCACTGATGGCCGCAA
	ACS2	XM_013781510	FP	CTACCAAAATGCCCAAGCCT
			RP	AAGTCTGTCCAACGGGGATG
			RP	GGCGACAAAAGCGGAAAGTT
	PDF3	XM_013736136	FP	TGCAACAATGTGTTCCCTGC
			RP	TTGGTGTACTCGGTCTTCGG
	PR4/HEL	XM_013770974	FP	CTGGACAGCTTTTTGCGGTC
			RP	TCACAGTAGCTTGTGCTCCG
	rbohC	XM_013775186	FP	ATCGATGGTCCATACGGTGC

			RP	TCGCTTGTTCCATGCTCCAT
	rbohF	XM_013756987	FP	CTTGGCATTGGTGCAACTCC
			RP	GTTGCCACTGTTGCTTCCTG
	LmITS1	FJ172239	FP	GGTGTTGGGTGTTTGTTCCAC
			RP	GGCTGCCAATTGTTTCAAGG

4.6 Glucosinolate (GLS) analysis

Freeze-dried true leaves and cotyledons of oilseed rape, and true leaves of cauliflower were first ground in a pre-cooled mortar and 20 – 80 mg leaf material was then used for extraction with 300 µl of 85 % methanol (v/v) for 5 min at room temperature before 10 µM p-OH-benzyl glucosinolate was added as an internal standard. Then, 96-well filter plates (Millipore, cat. no. MAHVN 4550) with 45 µl Sephadex A-25 were loaded using the Millipore multiscreen column loader (Millipore, cat. no. MACL 09645), and 300 µl of water was added and equilibrated. After 2 – 6 h, the water was removed from 96-well filter plates using vacuum for 2 – 4 s. Then, 150 µl of supernatant was added into each well in the filter plates and vacuum applied for 2 – 4 s. The column was then washed two times with 100 µl of 70 % methanol and two times with 100 µl of water, and vacuum applied between each washing step. After washing, 20 µl of sulfatase solution was added, and the plate was incubated at room temperature. The next day, 100 µl of Milli-Q water was added to each well in the 96-well filter plates. Filter plates were then placed on top of 96-well plates (Millipore, cat. no. MDCPN2M50) and aligned. After a short centrifugation, samples were diluted to 1:10 with Milli-Q water for UHPLC/TQ-MS analysis (Burow et al., 2006; Crocoll et al., 2016).

4.7 Volatile organic compound (VOC) analysis

Analysis of VOCs was performed only on detached true leaves of oilseed rape inoculated with *L. maculans*. VOCs trapping from control and *L. maculans*-inoculated leaves was performed on day 0, and at 3 and 7 dpi in parallel arrangement: control leaves in one chamber vs. inoculated leaves in the second chamber during a single trapping event (Fig. 9). All corresponding trapping events were done at the same time of the day for each dpi and at precise

time intervals after removal from the cultivation chamber, with all time delays being the same. Four detached leaves were placed in a glass beaker containing glass balls and closed in a glass gas-tight trapping apparatus (4 l in volume). Synthetic air Zero Plus (purity 6.0, Air Products, Czech Republic) was supplied to the apparatus through an active charcoal Hydrocarbon trap (Restek Corporation, Bellefonte, PA, USA, Fig. S2). After 1 min air wash at flow 5 l min^{-1} and 10 min equilibration (system gas-tight, no air flow), a 20 min trapping period using Tenax TA sorbent tubes (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) was performed at 50 ml min^{-1} (1 l of gas phase) using programmable suction pumps (Gilian GilAir Plus, Sensidyne, LP, St. Petersburg, FL, USA).

LECO Pegasus 4D GC×GC-TOFMS system (Leco Corporation, St. Joseph, MI, USA) containing Agilent 7890 gas chromatograph (Agilent technologies, Santa Clara, CF, USA) equipped with a LECO quad-jet dual stage thermal modulator, Gerstel MultiPurpose Sampler (MPS), Gerstel Thermal Desorption Unit (TDU) and temperature programmed CIS4 inlet (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) was used for analysis. The Gas chromatograph was equipped with a precolumn (Phenomenex ZB 624plus, 1.3 m × 0.25 mm I.D. × 1.4 μm film, Phenomenex, Torrance, CA, USA), primary and modulatory column (SGE Analytical Science BPX-5, 29.3 + 0.1 m × 0.25 mm I.D. × 0.5 μm film, Trajan Scientific and Medical, Ringwood, Victoria, Australia), and secondary column and transfer line (SGE Analytical Science BPX-50, 1.9 + 0.21 m × 0.1 mm I.D. × 0.1 μm film). Helium with BIP (Built In Purifier) technology (purity 5.7) was used as a carrier gas (Air Products, Czech Republic). Tentative identification of compounds was based on comparison of their spectra with those of mass libraries (NIST, LECO/Fiehn Metabolomics Library, own user libraries).

ChromaTOF software (Leco Corporation, St. Joseph, MI, USA) served for initial processing. Due to the high complexity of data, where roughly 1500 peaks per chromatogram were detected in each sample (data processing at signal/noise = 10), the sensitivity was decreased (data processing at signal/noise = 100) leading to approximately 250 VOCs per chromatogram. Peak alignment, comparison of chromatograms and basic statistics was performed using the ChromaTOF Tile software (Leco Corporation, St. Joseph, MI, USA). A group of 55 VOCs, demonstrating the highest variability in their content between treatments, was then identified and final processing was performed in ChromaTOF software using the References module.

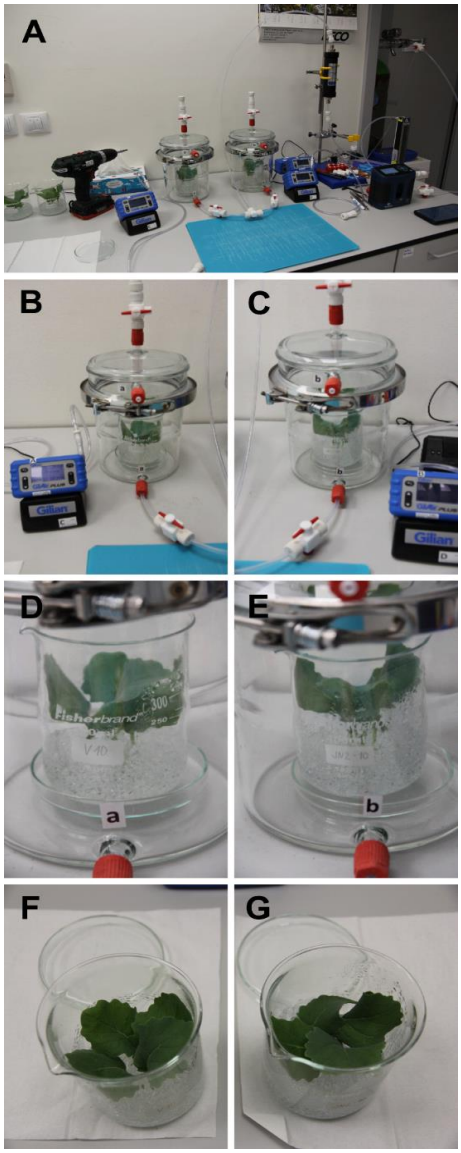

Figure 9. Volatile organic compound (VOC) trapping apparatus (**A**): the parallel VOC trapping in two chambers during a single trapping event; non-inoculated (control) leaves in chamber "a" (**B, D**); *Leptosphaeria maculans*-inoculated leaves in chamber "b" (**C, E**). Visual comparison of non-inoculated (control) leaves (**F**) and *L. maculans*-inoculated leaves (**G**) immediately after the trapping event.

4.8 Imaging of defence gene activation and fungal mycelium formation

To study the activation of expression in *A. thaliana* transgenic lines *pPR1::NLS3xVenus*, *pJAZ10::NLS3xVenus*, *pAOS::NLS3xVenus*, *pACS6::NLS3xVenus* and *pPR4/HEL::NLS3xVenus*, leaf discs from infested plants were used, with non-infested plants as a control. Venus fluorescence was detected by fluorescence microscope Zeiss AxioImager

ApoTome2 with EC Plan-Neofluar 5x/0.16 M27 objective and fluorescence cube FS09/GFP. The number of induced cells per leaf disc (6 mm-diameter) was quantified manually; at least 10 leaf discs from three independent plants were sampled. Illustrative images of *pPR1::NLS3xVenus* and *pJAZ10::NLS3xVenus* activation during live aphid feeding were captured using Stereomicroscope Leica M205FA, objective Plan-Apochromat 2.0x, Filter set ET GFP Ex470/40x Em525/50. Cellular-resolution images of *pPR1::NLS3xVenus* and *pJAZ10::NLS3xVenus* activation were captured using Zeiss LSM 880 inverted confocal laser scanning microscope (Carl Zeiss AG, Germany), Plan-Apochromat 20x/0.8 DIC M27 objective, Ex = 488 nm, detection 499-522 nm.

Fluorescence of mycelium of *L. maculans* JN3-GFP in cotyledons of *B. oleracea* was detected by fluorescence microscope Zeiss AxioImager ApoTome2 with EC Plan-Neofluar 5x/0.16 M27 objective and fluorescence cube FS09/GFP.

4.9 Histochemical staining for GUS activity and callose deposition

The histochemical GUS assay (Jefferson et al., 1987) was performed on four- to five-weeks old *PR1::GUS* transgenic lines of *A. thaliana*. Leaves were soaked in X-GLUC buffer for 16 h at 37°C as described previously (Krčková et al., 2018). Next, leaves were fixed and discoloured in ethanol:glacial acetic acid (3:1 v/v) and further rehydrated in different concentrations of ethanol, each applied for at least one h in successive order: 70 % ethanol, 50 % ethanol and 30 % ethanol. Fully discoloured leaves were then soaked in distilled water and left overnight in the dark. Later, leaves were incubated for at least 4 h in 150 mM K2HPO4 (pH 9.5) containing 0.01 % aniline blue (Sigma-Aldrich; #415049; Kalachova et al., 2020). Leaves were finally scanned on Epson Perfection V700 Photo (Suwa, Japan) and then analysed for callose deposition using Zeiss AxioImager ApoTome2 microscope, EC Plan-Neofluar 10x/0.3 M27 objective.

4.10 Root colonization test on selective media

Roots of cauliflower plants were harvested and washed with tap water to remove the soil. Different parts of roots were sampled by cutting 5 pieces of 2 cm roots that were subsequently submerged in 5 ml 0.05 % Triton X. Then, roots were drilled until only small pieces of roots

were left, vortexed and 100 µl of suspension was then placed on a plate with selective media containing peptone, glucose, agar, and antibiotics dodine (0.1 g ml^{-1}), streptomycin (0.6 g ml^{-1}), tetracycline (0.05 g ml^{-1}) and cyclohexamide (0.05 g ml^{-1}). Plates were further stored at 25°C for 14 days and the number of fungal colonies was then counted.

4.11 Statistical analyses

Data were predominantly analysed in R (R Development Core Team 2020) with a significance threshold of 5 %.

Regarding the evaluation of the feeding choice tests with cotyledons and true leaves of *B. napus* within vials by *P. xylostella* caterpillars, data were analysed using the mean consumption of leaf area in pixels, and the feeding choice tests with *P. xylostella* and *L. maculans* on true leaves within vials, using the mean consumption of leaf area in %. The effect of the experimental factors was tested using the linear model ('*lme4*' package, Bates et al., 2015) without transformation. As for the evaluation of the choice tests by *B. brassicae* aphids within Petri dishes, the effect of the experimental factors was tested using the generalized linear model (glm) and Poisson distribution without transformation. For details on significance tests, see univariate analyses below.

Univariate analyses were performed on feeding tests, gene transcription, GLS and VOC data. Regarding the VOC dataset, filtering was performed by removing the compounds if more than 50 % of all samples had a value no higher than 0.1 for that compound. Individual gene transcription, GLSs and VOCs, as well as the total content of aliphatic (AG), indole (IG) and all groups of GLSs were analysed against the treatment, the time and their interaction, using a linear model ('*lme4*' package, Bates et al., 2015), after transforming the data to the fourth root. After checking the normality and homoscedasticity of the model residues using the Shapiro-Wilk normality test, the significance of each term of the model was tested by the F-test as a type II analysis of variance ('*Anova*' function, '*car*' package, Fox and Weisberg, 2011). For significant factors, pairwise comparisons were tested by estimated marginal means in the R package '*emmeans*' (Lenth et al., 2019), using P-values corrected with the False Discovery Rate. The '*cld*' function from the '*multcomp*' package (Hothorn et al., 2008) was used to display pairwise comparisons.

Multivariate redundancy analyses (RDA) were performed on GLS and VOC data, using individual compounds, the fourth root transformation and the '*rda*' function from the '*vegan*' package (Oksanen et al., 2020). The '*RVAideMemoire*' package (Hervé, 2015) was used to test the significance of each term (same as the above univariate analyses) in the model with a type II permutation F-test for constrained multivariate analyses ('*MVA.anova*' function); to perform pairwise comparisons when a factor was significant ('*pairwise.factorfit*' function) and to plot the data for RDA ('*MVA.plot*' function). The '*ggplot*' package (Wickham, 2009) was used to generate the rest of the plots.

Data collected from the experiments with *B. brassicae* and *A. thaliana* were analysed and graphs were generated in GraphPad Prism 8 software (https://www.graphpad.com/scientific-software/prism/). Data distribution normality was checked using the Shapiro-Wilk test. Control and *B. brassicae*-infested leaf samples were compared within each time point with unpaired *t*-test for unequal variances. The maximum intensity projections obtained from 40 μm z-stacks were created using Zeiss ZEN Black software. Fiji software (https://fiji.sc/) was used for microscopy image analysis.

5 Individual studies and their results

5.1 Development of a feeding choice test method for studying leaf palatability for *Plutella xylostella* caterpillars

5.1.1 Development of a T-tube connected vial system

A feeding choice test for *P. xylostella* caterpillars was developed comprising two 100 ml plastic vials connected with a plastic T-shaped connector (Fig. 10). One detached leaf was placed in each vial, fixed with glass balls soaked in distilled water and covered with filter paper used to prevent caterpillars from drowning. Three third-instar caterpillars were added in the T-tube with a fine paintbrush and were given a choice to feed on two types of leaves for 48 h. Then, leaves were scanned, and the percentage of the damage was evaluated using BioLeaf (a professional mobile application for foliar analysis; Machado et al., 2016).

Figure 10. Vial system developed for performing larval feeding choice experiments.

5.1.2 Testing the reliability of the vial system using natural insecticides

The reliability of the vial system was confirmed using known natural insecticides: escin and neem oil. Escin, an active compound of *Aesculus hippocastanum* (horse chestnut) is playing a role in defence against pathogens or herbivores, and neem oil is generally used as an effective insecticide for spraying plants. Therefore, these two compounds were chosen as suitable positive controls.

Neem oil (1.5 ml l^{-1}) and escin (100 mM) were infiltrated into the true leaves of oilseed rape by vacuum. True leaves infiltrated with water were used as control. Leaves were left on

the room temperature to evaporate excess water, present in intercellular spaces of the leaf after infiltration, while being monitored to prevent the leaf from drying out. After fixing the leaves in the vials, three third-instar caterpillars were added in the T-tube.

The results indicate that *P. xylostella* caterpillars significantly prefer leaves without escin ($F_{1,18}$ = 39.33, P < 0.001; Fig. 11) and neem oil treatment ($F_{1,20}$ = 40.01, P < 0.001; Fig. 12).

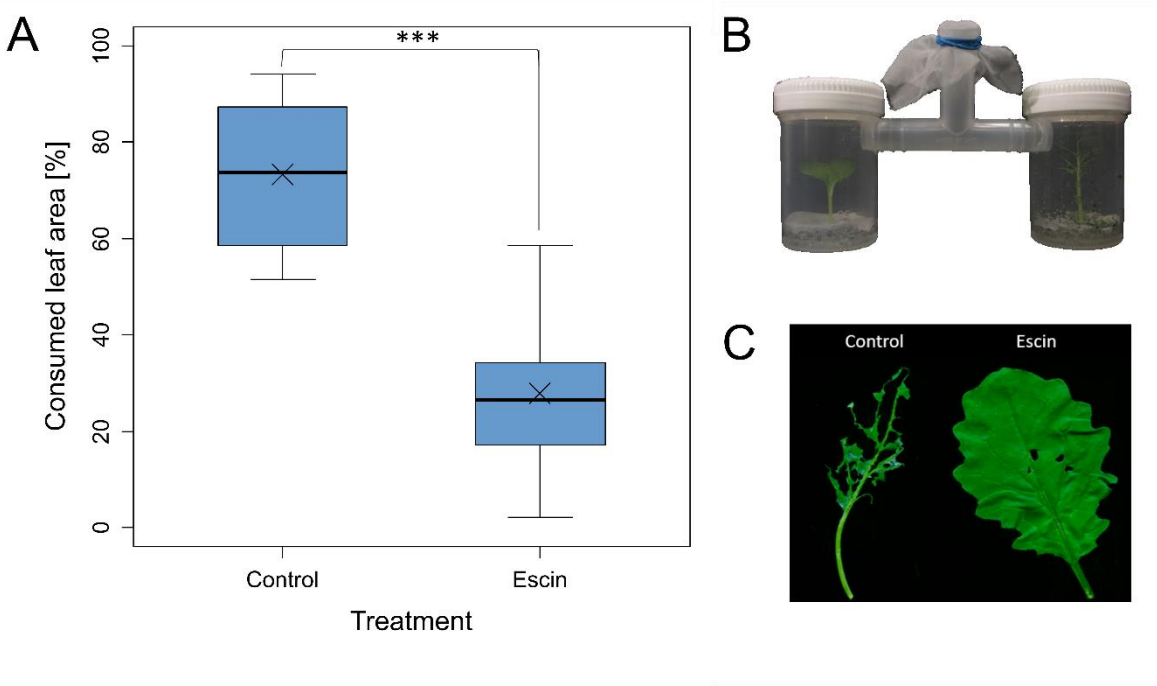

Figure 11. Feeding preference of *Plutella xylostella* caterpillars to control leaves of oilseed rape and leaves treated with escin. **A:** consumed leaf area (in %) caused by third-instar caterpillars after 48 h of feeding. Boxplot represents mean (´x´), median, 25th and 75th percentiles, and asterisks significant differences (P < 0.001); **B:** vial system developed for performing larval feeding choice experiments with one control leaf inside the left vial and one escin-treated leaf inside the right vial. Three caterpillars were placed in the T-tube and were given a choice to feed on two types of leaves; **C:** scanned leaves after 48 h of caterpillar feeding.

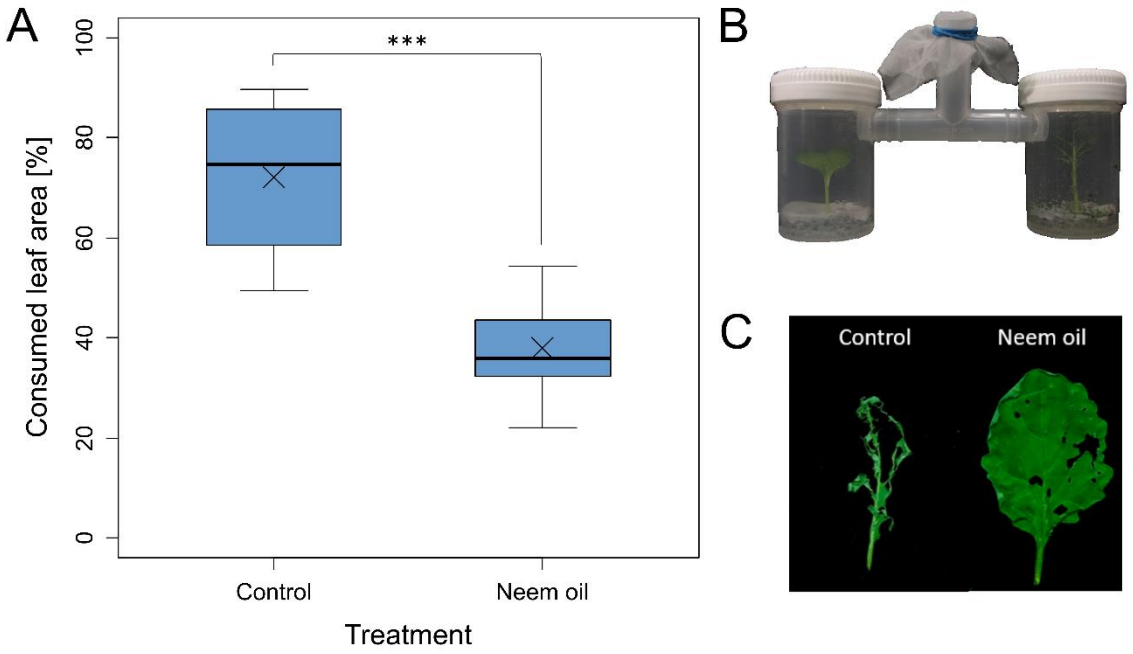

Figure 12. Feeding preference of *Plutella xylostella* caterpillars to control leaves of oilseed rape and leaves treated with neem oil. **A:** consumed leaf area (in %) caused by third-instar caterpillars after 48 h of feeding. Boxplot represents mean (′x′), median, 25th and 75th percentiles, and asterisks significant differences ($P < 0.001$); **B:** vial system developed for performing larval feeding choice experiments with one control leaf inside the left vial and one neem oil-treated leaf inside the right vial. Three caterpillars were placed in the T-tube and were given a choice to feed on two types of leaves; **C:** scanned leaves after 48 h of caterpillar feeding.

5.2 Can *Leptosphaeria maculans* infection alter palatability of oilseed rape for *Plutella xylostella* caterpillars and *Brevicoryne brassicae* aphids?

5.2.1 Introduction

Multiple and simultaneous attacks by pathogens and insect pests frequently occur in nature; however, little is known about how plants cope with such stresses. In particular, there is a scarcity of studies investigating the palatability of infected plants for subsequent herbivory. In this study, I specifically target oilseed rape due to its global economic significance as a crop. Despite its widespread cultivation, there remains a notable gap in our understanding of the combined biotic interactions affecting its management. Addressing this gap, the current study centers on the combined interaction of fungal infection caused by *L. maculans* and arthropod infestation by *P. xylostella* or *B. brassicae* in oilseed rape. The underlying hypothesis suggests that infection by the fungal pathogen *L. maculans* could significantly alter the palatability of oilseed rape for *P. xylostella*-chewing caterpillars or *B. brassicae*-sucking aphids. To test this hypothesis, feeding preference assays were conducted alongside analyses of defence gene transcription, as well as assessments of glucosinolate (GLS) and volatile organic compound (VOC) levels in *L. maculans*-inoculated and non-inoculated (control) leaves. These integrated approaches were employed to elucidate potential mechanisms underpinning larval choice and shed light on the intricate dynamics of plant-fungi-herbivore interactions.

5.2.2 Experimental design

Plutella xylostella

In order to determine the preference of *P. xylostella* caterpillars for different leaf stage/treatments, feeding choice test was performed on detached leaves of oilseed rape with one leaf placed in each vial. Three third-instar caterpillars were starving for 3 h in a separate plastic container before being transferred to the T-tube using a fine paintbrush. T-tube connected vials were randomly placed in a controlled environment under the same conditions as plants used for experiments were growing. During the next 48 h, caterpillars could choose the vial and feed on the leaf within, and switch from one vial to another.

Feeding choice tests were initially performed to determine the preference of *P. xylostella* caterpillars for true leaves or cotyledons of oilseed rape. After 48 h of exposure to herbivory, leaves were scanned and the percentage of the leaf area consumed by larvae was evaluated using BioLeaf (Machado et al., 2016) and the differences in leaf size between cotyledons and true leaves were corrected to leaf area in pixels using APS Assess 2.0 image analysis software (The American Phytopathological Society, St. Paul, MN, U.S.A.). Four separate experiments, with approximately ten replicates per treatment (cotyledon vs. true leaf) within each biological repetition were performed.

To test the effect of fungal infection on caterpillar preference, *L. maculans*-inoculated leaves were placed individually in each vial and the T-tube-connected vial served as a control (water-infiltrated/non-inoculated leaf). Then, 3 and 7 days post *L. maculans* inoculation (dpi), three third-instar caterpillars were added into the T-connector and could feed for 48 h. After leaf scanning, BioLeaf (Machado et al., 2016) was used for the leaf damage evaluation and given data were further analysed as a mean consumption. Four separate experimental repetitions for 3 dpi and two independent experimental repetitions for 7 dpi were performed, each repetition containing ten replicates per treatment (control vs. *L. maculans*-inoculated leaf).

For the GLS and gene transcription analyses one leaf was placed in the individual non-connected plastic vial, whereas for VOC analysis each glass beaker contained four leaves.

Brevicoryne brassicae

To test *B. brassicae* aphid preference for different leaf stages/treatments, feeding choice test was performed in Petri dishes.

Initially, cotyledons and true leaves were cut with a cork borer and placed on a wet filter paper inside a Petri dish (Fig. 14B). Then, three wingless adults were put in the middle and the total number of adults and nymphs on each leaf disc was counted 24 and 48 h after the infestation. Three separate experiments, with approximately ten replicates per treatment (cotyledon vs. true leaf) within each biological repetition were performed.

To determine the preference of *B. brassicae* aphids for *L. maculans*-inoculated and non-inoculated leaves, whole detached leaves (fungal-inoculated and non-inoculated) were placed opposite one another in a Petri dish (Fig. 17B). Then, 3 and 7 days post *L. maculans* inoculation

(dpi), ten wingless adults were put in the middle with a fine paintbrush and the total number of adults and nymphs on each leaf was counted 24 h after the infestation. Four separate experimental repetitions for 3 and 7 dpi were performed, each repetition containing ten replicates per treatment (control vs. *L. maculans*-inoculated leaf).

5.2.3 Results

5.2.3.1 *Plutella xylostella* caterpillars and *Brevicoryne brassicae* aphids prefer true leaves to cotyledons of oilseed rape

A clear preference of *P. xylostella* caterpillars for true leaves was observed (Fig. 13): significantly ($F_{1,72} = 55.56$, $P < 0.001$) larger leaf area was consumed from true leaves of oilseed rape than cotyledons. From all the cotyledons used in experiments, 54 % remained untouched after 48 h of herbivory. From these tests, true leaves were selected to examine the response of caterpillars in further experiments.

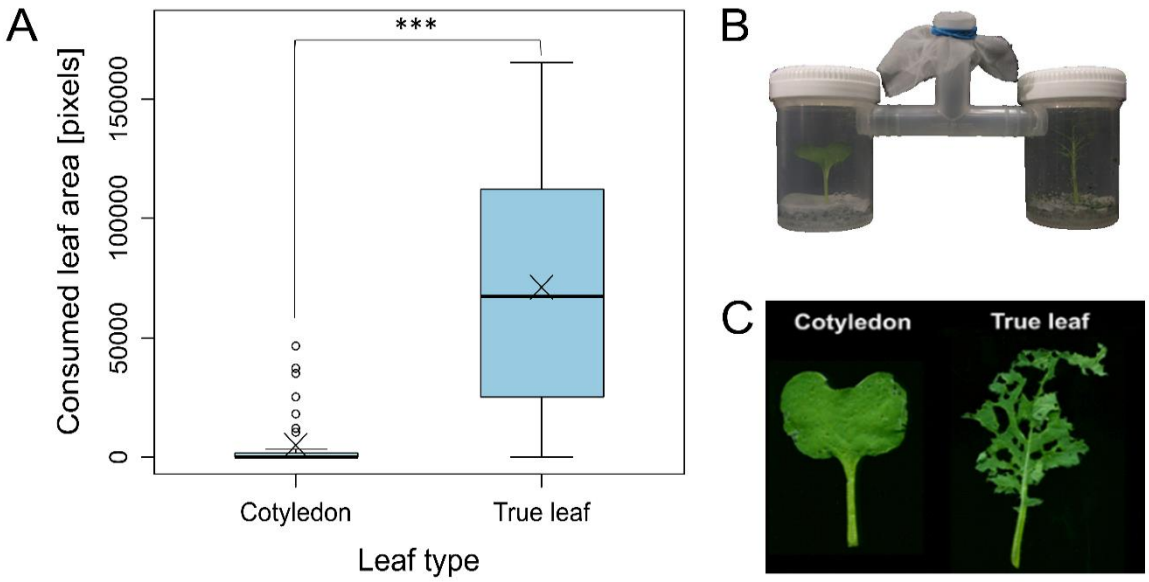

Figure 13. Feeding preference of *Plutella xylostella* caterpillars to detached cotyledons and true leaves of oilseed rape. **A:** consumed leaf area (in pixels) caused by third-instar caterpillars after 48 h of feeding. Boxplot represents mean (´x´), median, 25th and 75th percentiles, and asterisk a significant difference ($P < 0.001$); **B:** vial system developed for performing larval feeding choice experiments with one cotyledon

inside the left vial and one true leaf inside the right vial. Three caterpillars were placed in the T-tube and were given a choice to feed on two types of leaves; **C:** scanned leaves after 48 h of caterpillar feeding.

A significant (Chisq = 386.08, df = 1, P < 0.001) preference of aphids for true leaves was observed at both time points (Fig. 14). True leaves were therefore used in subsequent experiments with *L. maculans*-inoculated leaves.

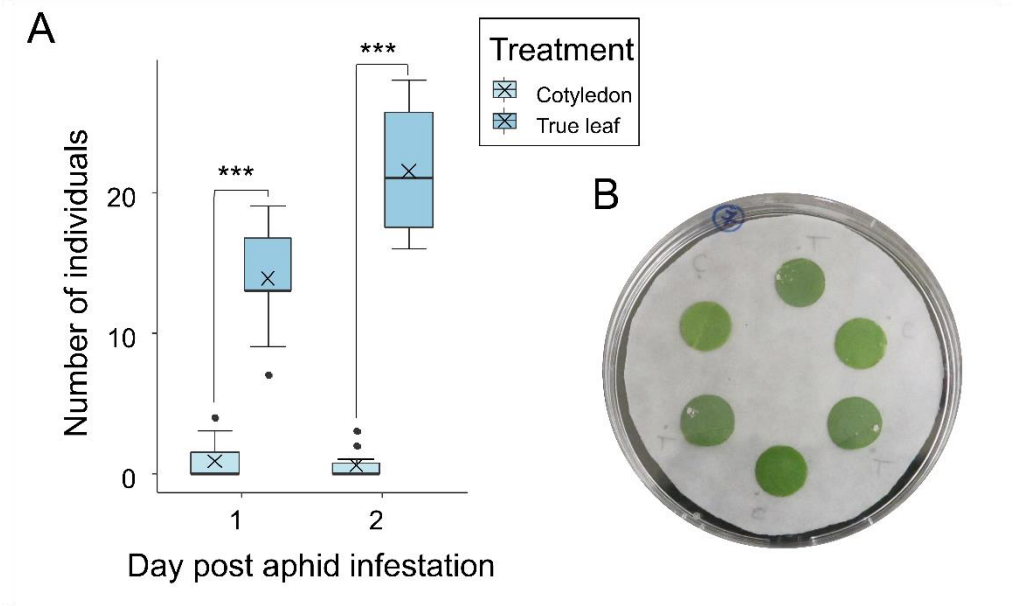

Figure 14. Feeding preference of *Brevicoryne brassice* aphids to cotyledon and true leaf discs of oilseed rape. **A:** count of number of individuals (wingless adults and nymphs) 1 and 2 days post aphid infestation. Boxplot represents mean (´x´), median, 25th and 75th percentiles, and asterisks significant differences (P < 0.001); **B:** choice test method in a Petri dish.

A significantly higher total content of aliphatic GLS, specifically glucoalyssin and glucoibarin, was detected in the true leaves of oilseed rape compared to the cotyledons (Fig. 15). One indole GLS, 4-methoxyglucobrassicin was also significantly higher in true leaves compared to cotyledons (Fig. 15).

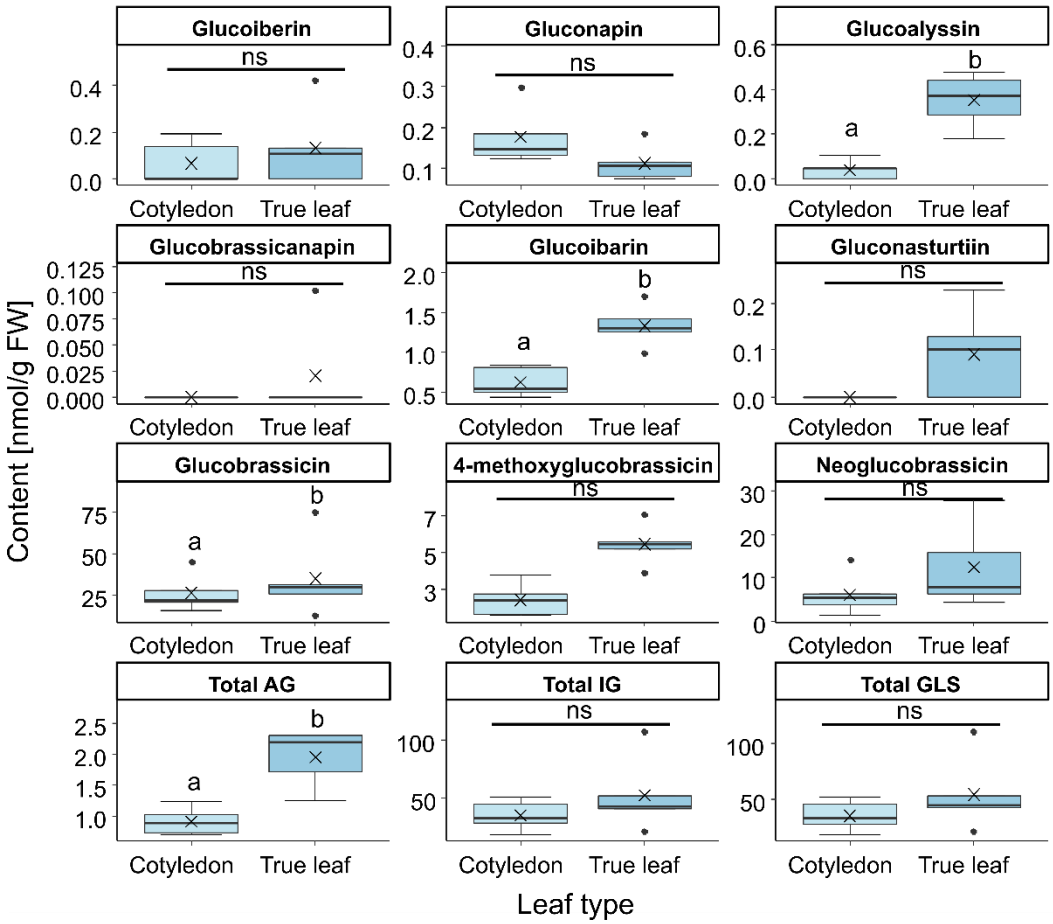

Figure 15. Glucosinolate (GLS) content in cotyledons and true leaves of oilseed rape. Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences ($P < 0.05$) between the leaf type; ns = not significant. Aliphatic compounds: glucoiberin, gluconapin, glucoalyssin, glucobrassicanapin, glucoibarin; aromatic/benzenic compounds: gluconasturtiin; aromatic/indole compounds: glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin. Statistical outputs are given for each compound and a group of compounds: gluconapin, $F_{1,8} = 3.82$, $P = 0.155$; glucoalyssin, $F_{1,8} = 12.81$, $P = 0.016$; glucoibarin; $F_{1,8} = 24.72$, $P = 0.005$; glucobrassicin, $F_{1,8} = 0.35$, $P = 0.569$; 4-methoxyglucobrassicin, $F_{1,8} = 20.83$, $P = 0.006$; neoglucobrassicin, $F_{1,8} = 2.07$, $P = 0.282$; total aliphatic glucosinolate (AG), $F_{1,8} = 22.33$, $P = 0.005$; total indole glucosinolate (IG), $F_{1,8} = 1.15$, $P = 0.353$; total GLS, $F_{1,8} = 1.31$, $P = 0.353$.

5.2.3.2 Inoculation of oilseed rape leaves with *Leptosphaeria maculans* changes the preference of *Plutella xylostella* caterpillars during the early stage of fungal infection

The mean consumption rate of *P. xylostella* was significantly influenced by the treatment ($F_{1,114}$ = 9.50, P = 0.002), but not by the time ($F_{1,114}$ = 0.13, P = 0.721) nor by the interaction between both ($F_{1,114}$ = 3.62, P = 0.06). Leaves inoculated with the fungal pathogen *L. maculans* were more palatable to *P. xylostella* caterpillars in comparison to control leaves 3 days post inoculation (3 dpi), after 48 h of feeding (Fig. 16A). At 3 dpi, and after 48 h of herbivory, the symptoms of fungal infection were not visible (Fig. 16C). In the subsequent stage of infection (7 dpi), caterpillar preference was no longer evident (Fig. 16A). At this stage of disease, symptoms start to be detectable: yellowing of the infected leaf and lesion formation (Fig. 16C).

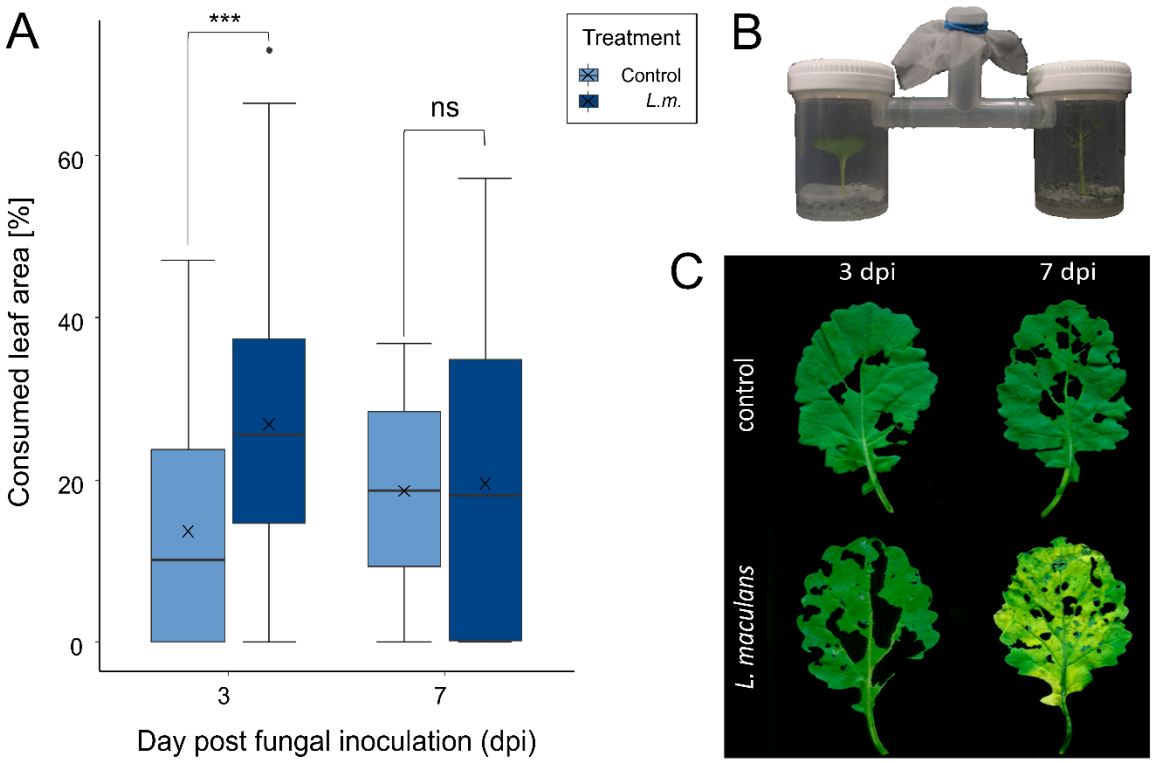

Figure 16. Feeding preference of *Plutella xylostella* caterpillars to control leaves of oilseed rape and leaves inoculated by *Leptosphaeria maculans* (*L.m.*) 3 and 7 days post inoculation (dpi). **A:** consumed leaf area (in %) caused by third-instar caterpillars after 48 h of feeding. Boxplot shows mean (´x´), median, 25th and 75th percentiles, and asterisks significant differences (P < 0.001) between treatments for a given time point, ns = not significant; **B:** vial system developed for performing larval feeding choice experiments with one non-inoculated leaf inside the left vial and one inoculated leaf inside the right vial. Three caterpillars were placed in the T-tube and were given a choice to feed on both leaves; **C:** scanned leaves after 48 h of caterpillar feeding; dpi = day post inoculation with *L. maculans*.

5.2.3.3 Inoculation of oilseed rape leaves with *Leptosphaeria maculans* changes the preference of *Brevicoryne brassicae* aphids during the later stage of fungal infection

The aphid preference was significantly influenced by the treatment (Chisq = 20.72, df = 1, P < 0.001) and by the interaction between the treatment and time (Chisq = 36.66, df = 1, P < 0.001), but not by the time alone (Chisq = 0.65, df = 1, P = 0.419). Aphids did not show any preference 3 days post *L. maculans* inoculation (Fig. 17A). At 3 dpi, and after 24 h of aphid feeding, the symptoms of fungal infection were not visible (Fig. 17C). However, leaves inoculated with the fungal pathogen *L. maculans* were less preferred by *B. brassicae* aphids in comparison to control leaves 7 days post inoculation (7 dpi; Fig. 17A), the time when symptoms start to be visible (Fig. 17C).

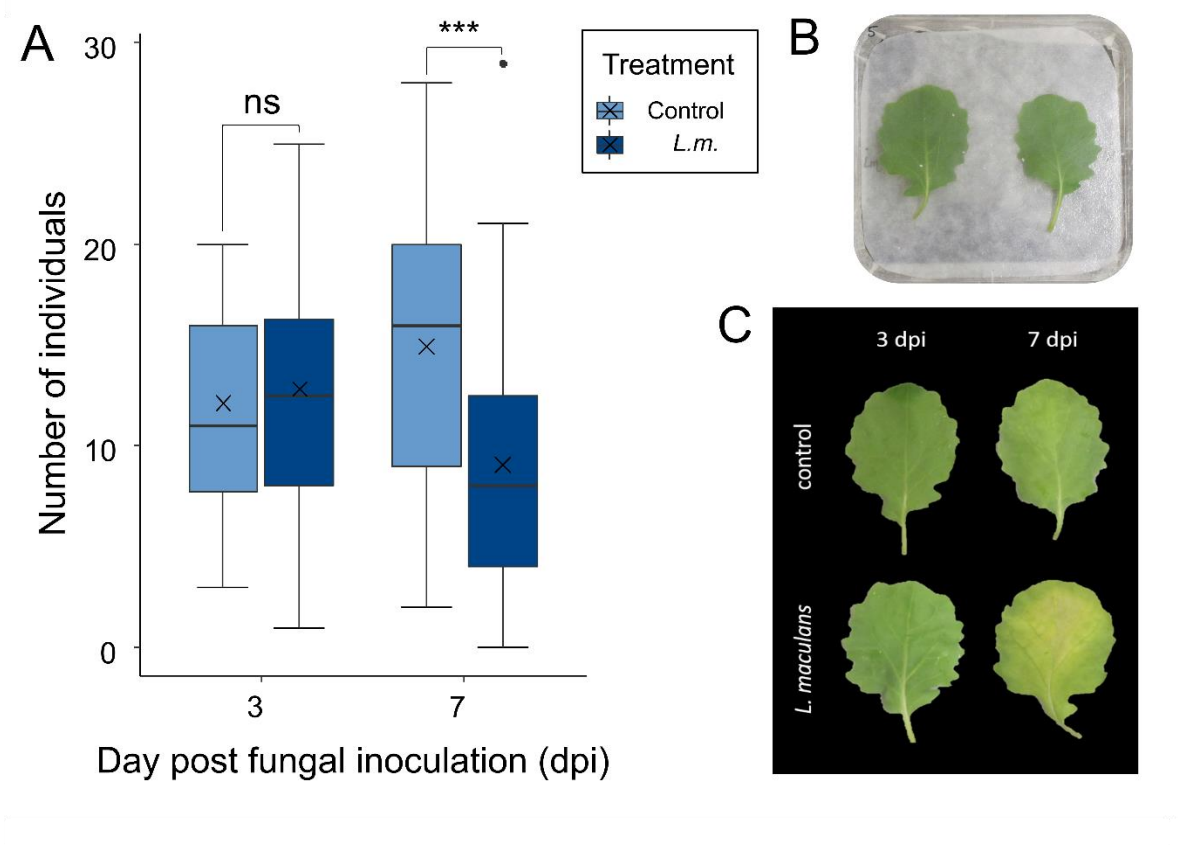

Figure 17. Feeding preference of *Brevicoryne brassicae* aphids to control leaves of oilseed rape and leaves inoculated by *Leptosphaeria maculans* (*L.m.*) 3 and 7 days post inoculation (dpi). **A:** number of individuals after 24 h of feeding. Boxplot shows mean (´x´), median, 25th and 75th percentiles, and asterisks significant differences (P < 0.001) between treatments for a given time point, ns = not

significant; **B:** Petri dish used for performing aphid choice experiments with one non-inoculated leaf on the right side and one inoculated leaf on the left side. Ten wingless aphids were placed in the middle of the dish and were given a choice to feed on both leaves; **C:** scanned leaves after 24 h of aphid feeding; dpi = day post inoculation with *L. maculans*.

5.2.3.4 Activation of signaling pathways in oilseed rape leaves inoculated with *Leptosphaeria maculans*

The activation of defence signaling pathways was monitored by gene transcription of marker genes. The activation of various phytohormone pathways was evaluated by examining specific biosynthetic and responsive genes: for SA, the biosynthetic genes *ICS1* and *PAL1* and the responsive gene *PR1*; for JA, the biosynthetic gene *LOX3* and the responsive gene *VSP*; for ET, the biosynthetic gene *ACS2*; and for both JA and ET, the responsive genes *βCHI* and *HEL*.

The SA pathway was activated by the pathogen *L. maculans* (Fig. 18, Tab. 2): both biosynthetic genes *ICS1* and *PAL1*, and a responsive gene *PR1* were significantly influenced by the treatment, *i.e.* upregulated in the inoculated compared to non-inoculated leaves; only *ICS1* was significantly influenced by the time point and the interaction between the treatment and the time point. The transcription of *ICS1* and *PAL1* increased by 2- and 5-fold, respectively, and of the responsive *PR1* gene by 74-fold compared to control treatment, at 3 dpi and in a similar way at 7 dpi.

The ET pathway was also activated in *L. maculans*-inoculated leaves (Fig. 18, Tab. 2): the biosynthetic *ACS2* gene was significantly influenced by the treatment, time point and the interaction between the treatment and the time point. The transcription of the *ACS2* gene was increased by 2- and 8-fold in *L. maculans*-inoculated leaves at 3 and 7 dpi, respectively (Fig. 18).

The JA pathway was not activated by *L. maculans* (Fig. 18, Tab. 2): the transcriptions of the biosynthetic *LOX3* gene, and of the responsive *VSP* gene were not significantly different from the control at both time points. The JA/ET responsive *βCHI* and *HEL* genes were significantly influenced by the treatment, *i.e.* upregulation was observed in *L. maculans*-inoculated compared to non-inoculated leaves (Fig. 18). The transcription of *βCHI* and *HEL* increased by 50- and 24-fold, respectively, at 3 dpi and similarly at 7 dpi.

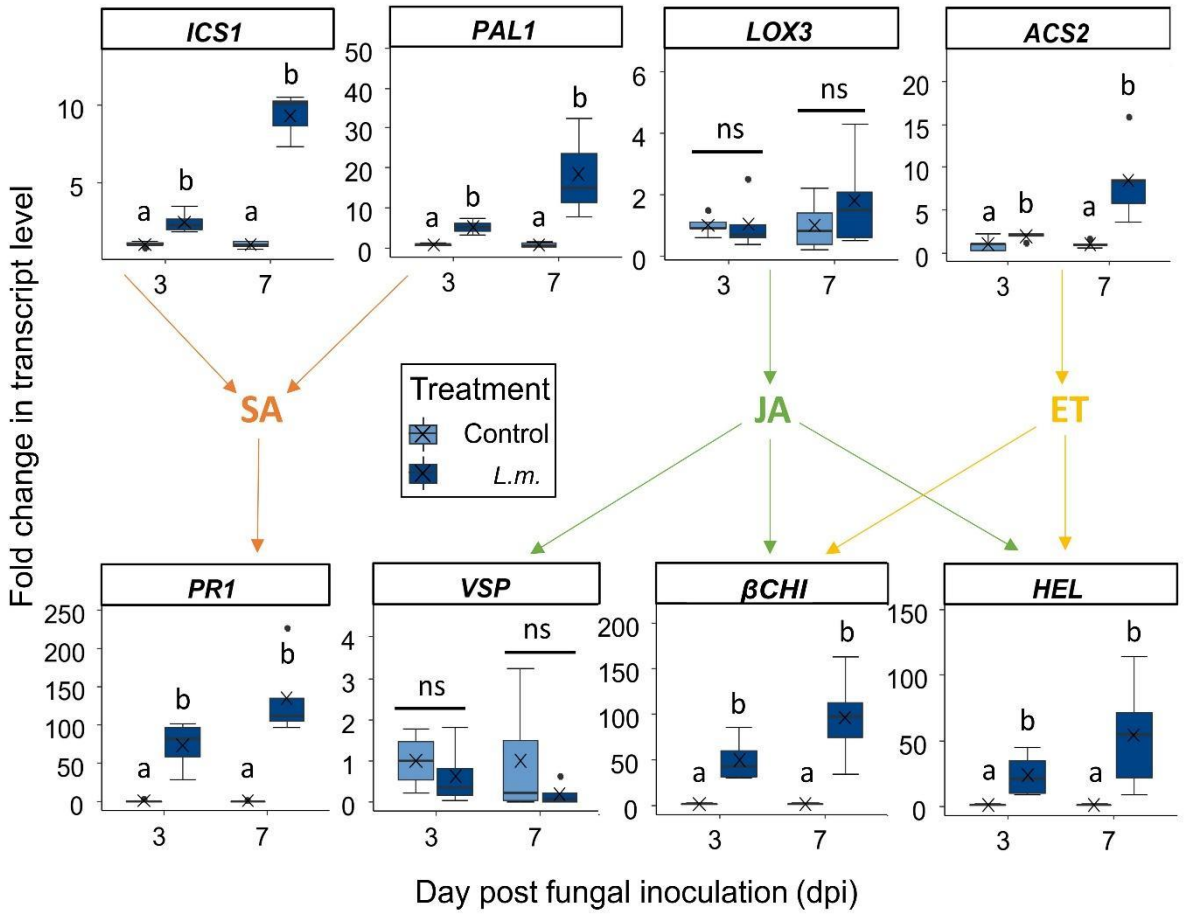

Figure 18. Activation of plant defence pathways in oilseed rape leaves inoculated by *Leptosphaeria maculans*. Gene transcriptions were detected in control and *L. maculans* (*L.m.*)-inoculated leaves 3 and 7 days post fungal inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences ($P < 0.05$) between treatments for a given time point. Four independent experimental runs per treatment (control vs. *L. maculans*-inoculated leaf) and time point (3 and 7 dpi) were performed. Abbreviations: *ICS1 = isochorismate synthase 1*; *PAL1 = phenylalanine ammonia lyase 1*; *LOX3 = lipoxygenase 3*; *ACS2 = ACC synthase 2*; *PR1 = pathogenesis related 1*; *VSP = vegetative storage protein*; *βCHI = β-chitinase*; *HEL = hevein-like*; SA = salicylic acid; JA = jasmonic acid; ET = ethylene; ns = not significant.

Table 2. Statistical output for each compound from analysis to determine differences in the transcript levels of defence genes between control oilseed rape leaves and *L. maculans*-inoculated leaves at 3 and 7 days post inoculation. ".": $P < 0.1$, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$; *treatment*: control vs. *L. maculans*-inoculated leaf; *time*: 3 vs. 7 days post inoculation.

Compound	Treatment	Time	Treatment:Time

	F value	df	P value		F value	df	P value		F value	df	P value		Residual df
isochorismate synthase 1, ICS1	201.95	1	0.00	***	37.74	1	0.00	***	58.89	1	0.00	***	13
phenylalanine ammonia lyase 1, PAL1	62.47	1	0.00	***	3.23	1	0.19		7.34	1	0.05	.	12
pathogenesis related 1, PRI1	277.79	1	0.00	***	3.33	1	0.19		3.38	1	0.17		15
lipoxygenase 3, LOX3	0.57	1	0.46		0.20	1	0.66		1.02	1	0.37		16
vegetative storage protein, VSP	1.72	1	0.24		1.75	1	0.28		0.03	1	0.87		13
ACC synthase 2, ACS2	34.88	1	0.00	***	12.10	1	0.01	*	8.23	1	0.04	*	16
β-chitinase, β-CHI	187.16	1	0.00	***	2.51	1	0.21		2.69	1	0.20		15
hevein-like, HEL	63.09	1	0.00	***	1.43	1	0.29		1.54	1	0.31		15

5.2.3.5 Inoculation with *Leptosphaeria maculans* and subsequent infestation by *Plutella xylostella* caterpillars alters the GLS profile in oilseed rape leaves

Six individual GLSs from the group of aliphatic/methionine-derived GLSs were analysed: glucoiberin, gluconapin, glucoalyssin, glucobrassicanapin, glucoibarin and glucoraphanin. In the analysis of control and *L. maculans*-inoculated leaves, glucoraphanin was not induced. One benzenic/phenylalanine-derived GLS, *i.e.* gluconasturtiin and three indole/tryptophan-derived GLS compounds, *i.e.* glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin were also analysed. The chemical names of all the compounds are listed in Tab. 3.

As for the 3 and 7 dpi, total GLS content was significantly influenced by the time and a total aliphatic GLS content by treatment and time, whereas there was no influence on the total indole level (Tab. 3). Total GLS level was higher in *L. maculans*-inoculated leaves compared

to control leaves at 3 dpi only, which was mainly driven by glucobrassicanapin (Fig. 19; Tab. 3). Contrary to that, the content of the aliphatic glucoiberin was higher in control than in *L. maculans*-inoculated leaves at 3 dpi but higher in *L. maculans*-inoculated than control leaves at 7 dpi (Fig. 19; Tab. 3). The content of glucoibarin did not differ between *L. maculans*-inoculated and control leaves at 3 dpi but was higher in *L. maculans*-inoculated leaves at 7 dpi (Fig. 19; Tab. 3). Indole 4-methoxyglucobrassicin was significantly increased in *L. maculans*-inoculated leaves only at 7 dpi, compared to control leaves (Fig. 19; Tab. 3). Amounts of aliphatic gluconapin and glucoalyssin, benzenic gluconasturtiin, and indole glucobrassicin and neoglucobrassicin were not significantly different between *L. maculans*-inoculated and control leaves at both observed time points (Fig. 19; Tab. 3).

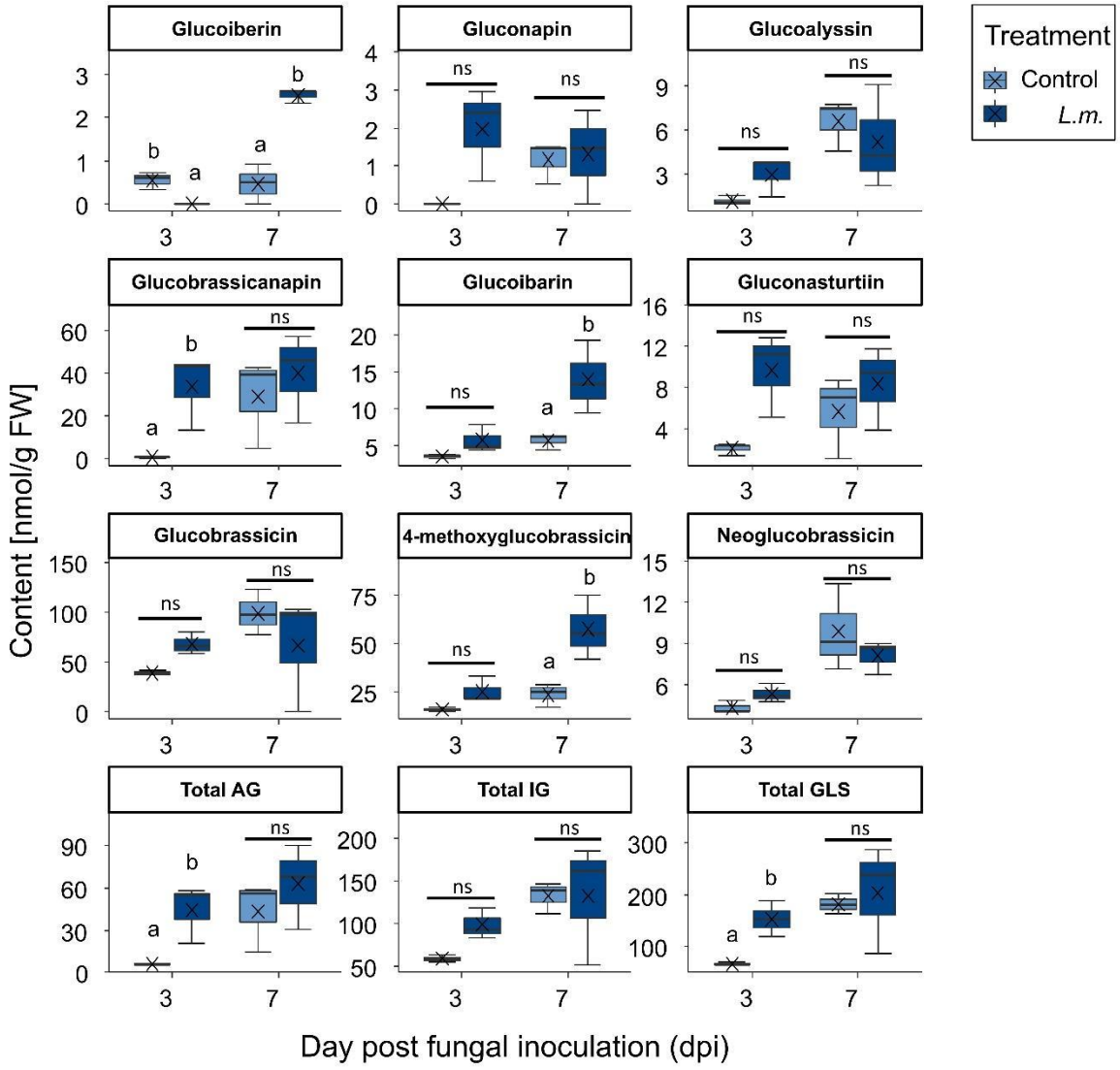

Figure 19. Glucosinolate (GLS) content in control and *Leptosphaeria maculans* (*L.m.*)-inoculated leaves of oilseed rape 3 and 7 days post fungal inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences ($P < 0.05$) between treatments for a given time point. Four independent experimental runs per treatment (control vs. *L. maculans*-inoculated leaf) and time point (3 and 7 dpi) were performed. Aliphatic compounds: glucoiberin, gluconapin, glucoalyssin, glucobrassicanapin, glucoibarin; aromatic/benzenic compounds: gluconasturtiin; aromatic/indole compounds: glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin. Abbreviations: AG = aliphatic glucosinolate; IG = indole glucosinolate; ns = not significant.

The redundancy analysis (RDA) showed that the experimental design explained 68 % of the total variance, with the axes 1 and 2 explaining 60 % and 27 % of the constrained variance, respectively (Fig. 20). Axis 1 separated the samples according to the time (3 vs. 7 dpi), while axis 2 according to the treatment (control vs. *L. maculans*-inoculated leaf). Samples that appear close to each other are chemically similar and vice versa. The GLS composition profile was significantly influenced by the treatment ($F_{1,8}$ = 5.16; P = 0.005), the time ($F_{1,8}$ = 7.39; P = 0.002), and their interaction ($F_{1,8}$ = 4.99; P = 0.013). For instance, RDA showed that gluconapin had a higher content in *L. maculans*-inoculated leaves at 3 dpi and glucoiberin in *L. maculans*-inoculated leaves at 7 dpi.

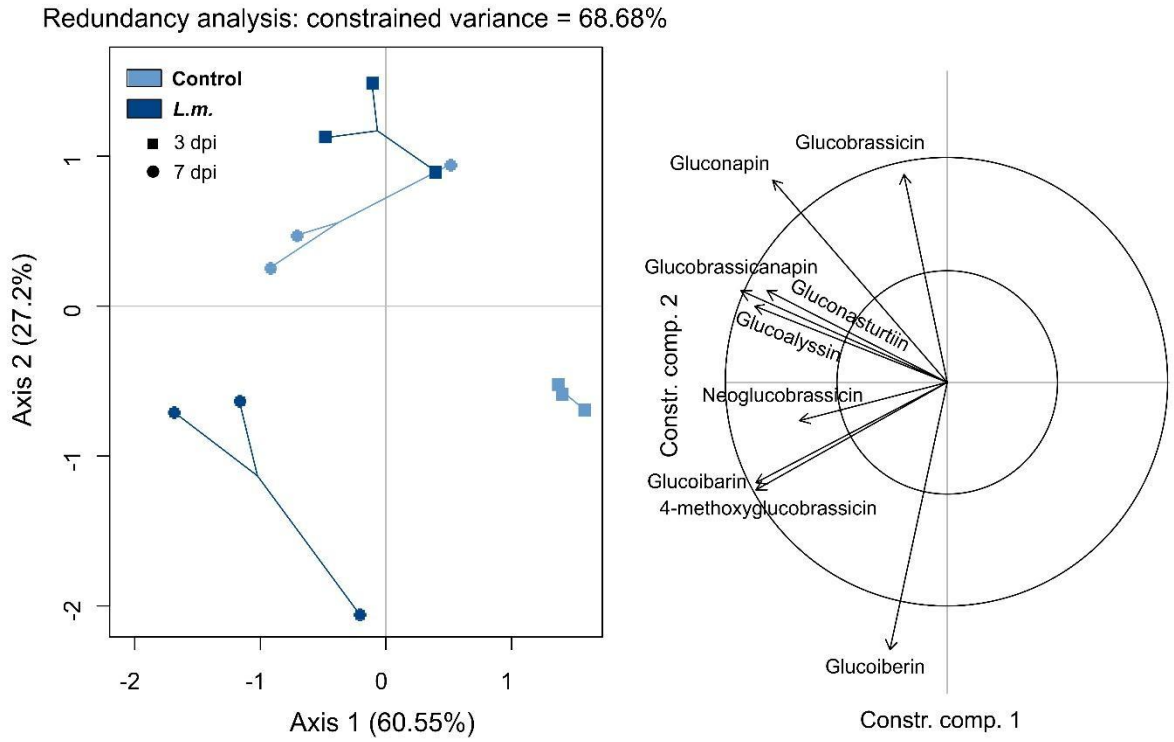

Figure 20. Redundancy analysis (RDA) of glucosinolate (GLS) composition in control and *Leptosphaeria maculans* (*L.m.*)-inoculated leaves of oilseed rape 3 and 7 days post inoculation (dpi). Score plot is shown on the left, with variances explained by the synthetic axes written in parentheses, and the correlation circle on the right.

Table 3. Statistical output for each compound from analysis to determine differences in glucosinolate (GLS) levels between control leaves of oilseed rape and *L. maculans*-inoculated leaves at 3 and 7 days post inoculation. ".": P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001; *treatment*: control vs. *L. maculans*-inoculated leaf; *time*: 3 vs. 7 days post inoculation.

Compound	Treatment			Time			Treatment:Time			Residual
	F value	df	P value	F value	df	P value	F value	df	P value	df
Glucoiberin (3-methylsulfinylpropyl, 3-msp)	0.43	1	0.60	10.68	1	0.03 *	23.86	1	0.01 *	8
Gluconapin (3-butenyl, 3-but)	4.56	1	0.13	2.40	1	0.19	10.40	1	0.07 .	8
Glucoalyssin (5-methylsulfinylpentyl, 5-msp)	0.41	1	0.60	14.04	1	0.02 *	3.95	1	0.20	8
Glucobrassicanapin (4-pentenyl, 4-pent)	14.22	1	0.02 *	9.68	1	0.03 *	7.43	1	0.10	8
Glucoibarin (7-methylsulfinylheptyl, 7-msh)	17.42	1	0.02 *	17.66	1	0.01 *	2.75	1	0.20	8
Total aliphatic (AG)	10.10	1	0.04 *	8.59	1	0.03 *	3.09	1	0.20	8
Gluconasturtiin (2-phenylethyl, 2-pe)	6.95	1	0.07 .	0.46	1	0.56	1.43	1	0.27	8
Glucobrassicin (indol-3-ylmethyl, I3M)	0.38	1	0.60	0.01	1	0.92	1.78	1	0.24	8
4-methoxygluco brassicin (4-methoxyindol-3-ylmethyl, 4MOI3M)	21.73	1	0.02 *	18.56	1	0.01 *	3.42	1	0.20	8
Neoglucobrassicin (N-methoxyindol-3-ylmethyl, NMOI3M)	0.00	1	0.99	25.91	1	0.01 *	2.52	1	0.20	8
Total indole (IG)	0.77	1	0.60	5.38	1	0.07 .	1.86	1	0.24	8
Total GLS	3.63	1	0.16	7.96	1	0.03 *	3.05	1	0.20	8

As for the 4 and 8 dpi, total GLS and total indole (IG) levels were significantly influenced by the treatment, whereas a total aliphatic (AG) content by the time only (Tab. 4). *L. maculans* inoculation alone increased glucoiberin at 4 dpi, and decreased gluconapin and 4-methoxyglucobrassicin at 8 dpi, compared to non-inoculated leaves (Fig. 21, Tab. 4). Feeding by *P. xylostella* alone and a combination of *L. maculans* inoculation and *P. xylostella* infestation significantly increased the total GLS amount and the total amount of indole GLSs in oilseed rape leaves at 4 and 8 dpi (Fig. 21, Tab. 4). All studied indole compounds, *i.e.* glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin were significantly induced in *P. xylostella*-infested leaves at 4 dpi, compared to non-inoculated leaves, whereas significant increase of glucobrassicin and neoglucobrassicin was also observed at 8 dpi. However, the level of 4-methoxyglucobrassicin was decreased at the later time point (8 dpi), compared to non-inoculated leaves. The combination of inoculation and infestation increased the level of glucobrassicin and neoglucobrassicin at 4 and 8 dpi; however, 4-methoxyglucobrassicin was not affected, compared to non-inoculated leaves (Fig. 21, Tab. 4). The total level of aliphatic GLSs was not significantly affected by any of the treatments (Fig. 21, Tab. 4). However, the infestation by *P. xylostella* caterpillars increased the individual content of aliphatic glucoiberin and glucoibarin and decreased the level of glucobrassicanapin at 4 dpi, compared to non-inoculated leaves, but did not affect their level at 8 dpi (Fig. 21, Tab. 4). The combination of *L. maculans* and *P. xylostella* significantly increased the amount of glucoiberin at 4 dpi, and decreased gluconapin at 8 dpi, compared to non-inoculated leaves. The content of gluconapin was not significantly influenced by any of the treatments at 4 dpi, and glucoalyssin and gluconasturtiin at both observed time points.

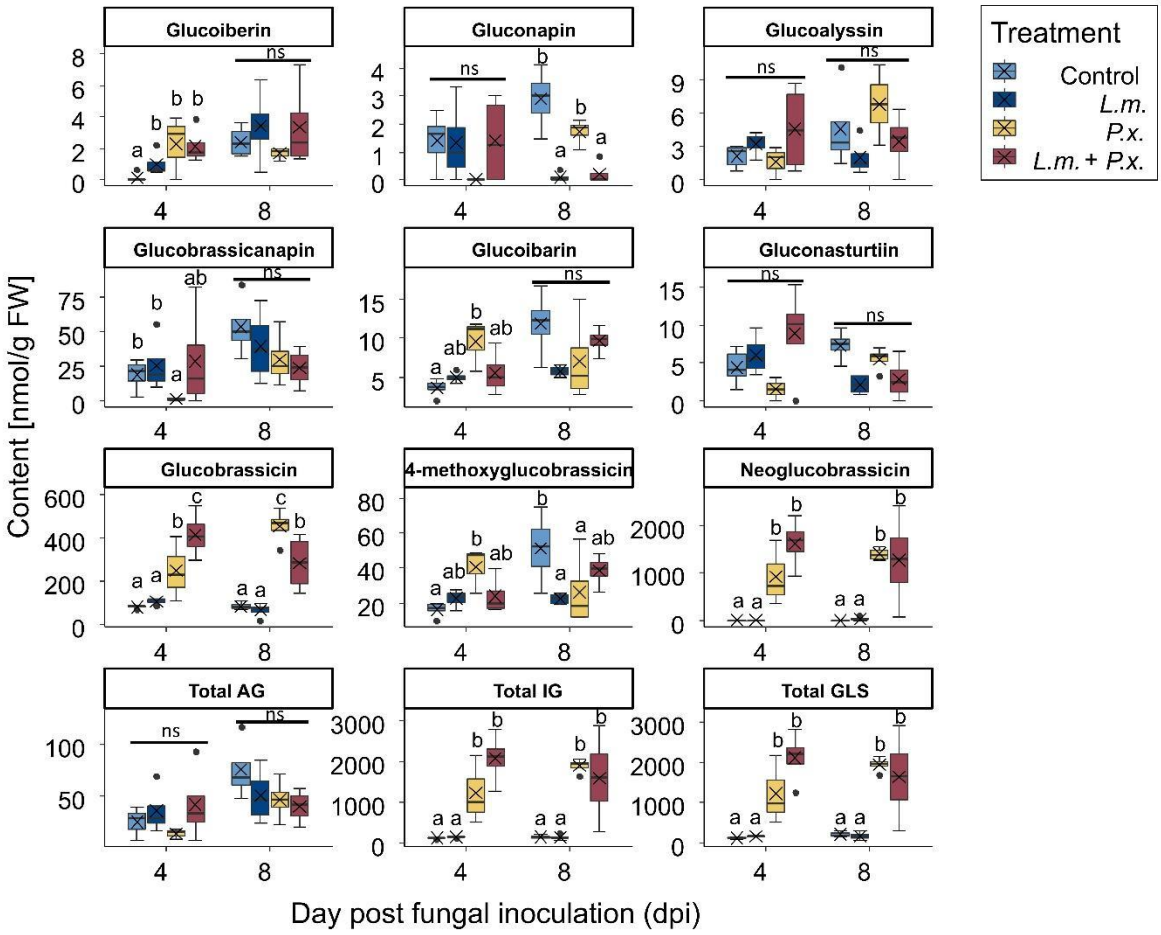

Figure 21. Total glucosinolate (GLS) content in control leaves, *Leptosphaeria maculans* (*L.m.*)-inoculated, *Plutella xylostella* (*P.x.*)-infested and a combination of *L. maculans* (*L.m.*)- and *P. xylostella* (*P.x.*)-treated leaves of oilseed rape 4 and 8 days post inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences ($P < 0.05$) between treatments for a given time point. Four independent experimental runs per treatment (control vs. *L. maculans*-inoculated leaf) and time point (3 and 7 dpi) were performed. Aliphatic compounds: glucoiberin, gluconapin, glucoalyssin, glucobrassicanapin, glucoibarin; aromatic/benzenic compounds: gluconasturtiin; aromatic/indole compounds: glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin. Abbreviations: AG = aliphatic glucosinolate; IG = indole glucosinolate; ns = not significant.

The RDA showed that the experimental design explained 55 % of the total variance, with the axes 1 and 2 explaining 42 % and 25 % of the constrained variance, respectively (Fig. 22). Axis 1 separated the samples according to the treatment (control and *L. maculans*-

inoculated leaf vs. *P. xylostella*-infested and the combination of *L. maculans* inoculation and *P. xylostella* infestation), while axis 2 according to the time (4 vs. 8 dpi). The GLS composition profile was significantly influenced by the treatment ($F_{3,24}$ = 4.77; P = 0.001), the time ($F_{1,24}$ = 4.52; P = 0.003), and their interaction ($F_{3,24}$ = 3.74; P = 0.001). For instance, RDA showed that glucobrassicin and neoglucobrassicin accumulated to a higher content in *P. xylostella*-infested leaves and *L. maculans*-inoculated and *P. xylostella*-infested leaves at 4 and 8 dpi.

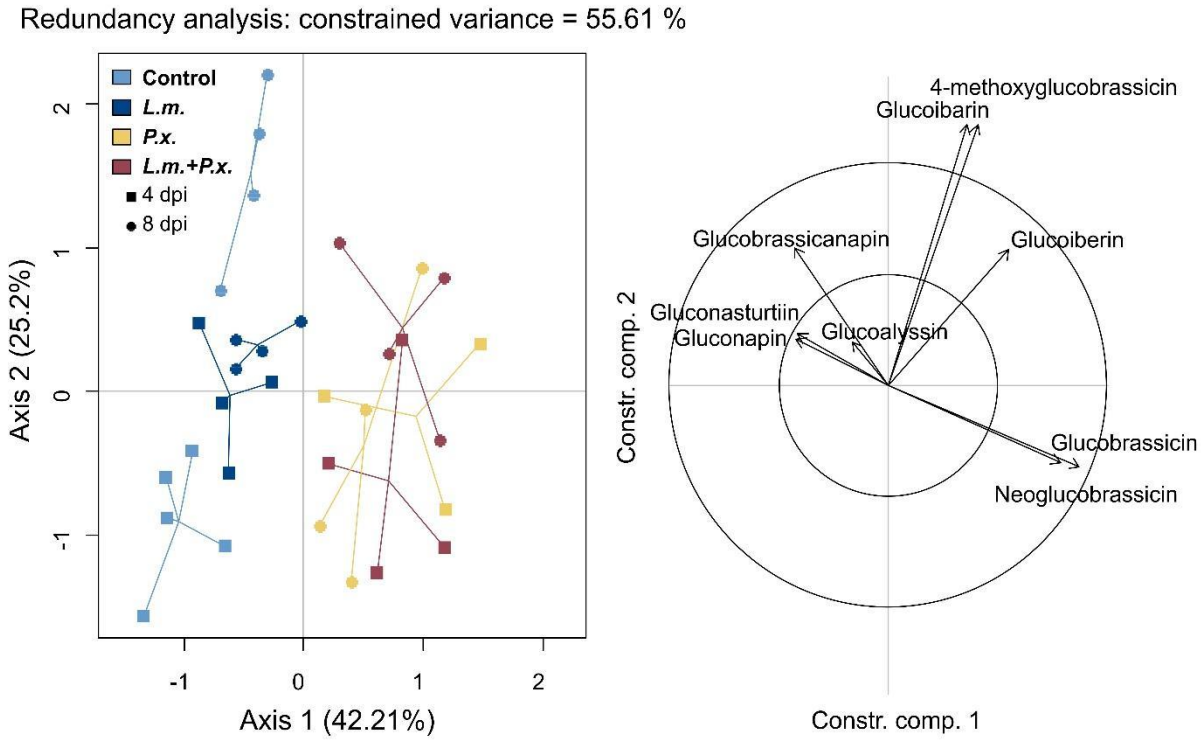

Figure 22. Redundancy analysis (RDA) of glucosinolate (GLS) composition in control, *Leptosphaeria maculans* (*L.m.*)-inoculated, *Plutella xylostela* (*P.x.*)-infested and a combination of *L. maculans* (*L.m.*)- and *P. xylostella* (*P.x.*)-treated leaves of oilseed rape 4 and 8 days post inoculation (dpi). Score plot is shown on the left, with variances explained by the synthetic axes written in parentheses, and the correlation circle on the right.

Table 4. Statistical output for each compound from analysis to determine differences in glucosinolate (GLS) levels between control leaves of oilseed rape and *L. maculans*-inoculated leaves at 4 and 8 days post inoculation. ".": P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001; *treatment*: control vs. *L. maculans*-inoculated leaf; *time*: 4 vs. 8 days post inoculation.

Compound	Treatment	Time	Treatment:Time

	F value	df	P value		F value	df	P value		F value	df	P value		Residual df
Glucoiberin (3-methylsulfinylpropyl, 3-msp)	4.86	3	0.02	*	15.17	1	0.01	*	3.63	3	0.06		24
Gluconapin (3-butenyl, 3-but)	3.45	3	0.06		0.31	1	0.79		5.80	3	0.02	*	24
Glucoalyssin (5-methylsulfinylpentyl, 5-msp)	0.04	3	0.99		0.61	1	0.79		2.54	3	0.16		24
Glucobrassicanapin (4-pentenyl, 4-pent)	2.78	3	0.11		10.74	1	0.01	*	1.70	3	0.22		24
Glucoibarin (7-methylsulfinylheptyl, 7-msh)	0.85	3	0.57		8.71	1	0.02	*	5.63	3	0.02	*	24
Total aliphatic (AG)	0.82	3	0.57		11.24	1	0.01	*	1.58	3	0.22		24
Gluconasturtiin (2-phenylethyl, 2-pe)	0.76	3	0.57		0.02	1	0.92		2.22	3	0.20		24
Glucobrassicin (indol-3-ylmethyl, I3M)	37.14	3	0.00	***	0.29	1	0.79		4.89	3	0.03	*	24
4-methoxygluco brassicin (4-methoxyindol-3-ylmethyl, 4MOI3M)	0.77	3	0.57		5.09	1	0.08		6.49	3	0.02	*	24
Neoglucobrassicin (N-methoxyindol-3-ylmethyl, NMOI3M)	71.50	3	0.00	***	0.01	1	0.92		1.68	3	0.22		24
Total indole (IG)	54.37	3	0.00	***	0.02	1	0.92		1.86	3	0.22		24
Total GLS	48.10	3	0.00	***	0.30	1	0.79		2.07	3	0.20		24

5.2.3.6 Inoculation with *Leptosphaeria maculans* alters the volatile organic compound (VOC) profile in oilseed rape leaves

A wide variety of VOCs was detected in the headspace of both control and *L. maculans*-inoculated oilseed rape leaves. Out of the 55 selected, which belong to distinct chemical classes (Liu et al., 2018), only 11 were statistically influenced either by treatment, by time or by their interaction (Tab. 5). However, after the P-value correction, only seven compounds were significantly different between control and *L. maculans*-inoculated leaves at 3 or 7 dpi (Fig. 23). A significant increase of some of the VOCs, *i.e.* '2-methyl-butanoic acid, hexyl ester', 'cyclohexanone', 'ethylbenzene' and '2,4,6-trimethyl-pyridine' was observed at both 3 and 7 dpi for *L. maculans*-inoculated leaves compared to control leaves (Fig. 23; Tab. 5). However, '2-methyl-butanenitrile', 'dimethyl disulfide' and 'nitro-methane' were significantly increased in the headspace of *L. maculans*-inoculated leaves at 7 dpi and not at 3 dpi, compared to control (Fig. 23; Tab. 5).

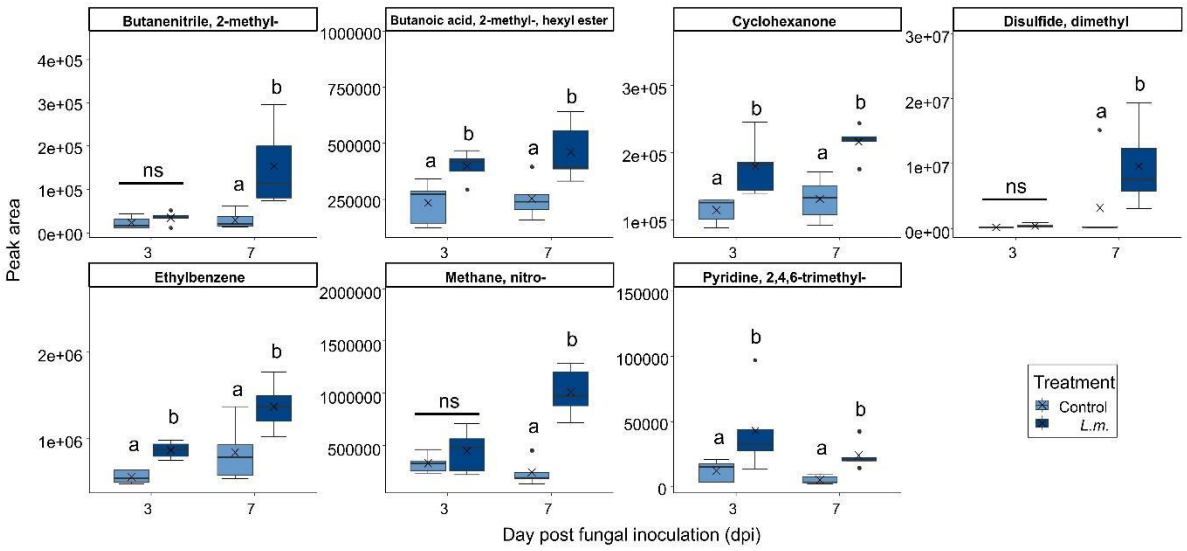

Figure 23. Selected volatile organic compounds (VOCs) with significantly different production in control and *Leptosphaeria maculans* (*L.m.*)-inoculated leaves of oilseed rape 3 and 7 days post fungal inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences ($P < 0.05$) between treatments for a given time point. Five biological replicates per treatment (control vs. *L. maculans*-inoculated leaf) were performed using the same leaves at 3 and 7 dpi; ns = not significant.

The RDA showed that the experimental design explained 30 % of the total variance, with axes 1 and 2 explaining 58 % and 32 % of the constrained variance, respectively (Fig. 24). Axis 1 separated samples according to the treatment (control vs. *L. maculans*-inoculated leaf) while axis 2 according to the time (3 vs. 7 dpi). The VOC composition profile was significantly influenced by the treatment ($F_{1,16}$ = 3.32; P = 0.005) and time ($F_{1,16}$ = 2.60; P = 0.025), but not by their interaction ($F_{1,16}$ = 0.99; P = 0.403). RDA confirmed that the peak area of most detected VOCs was higher in *L. maculans*-inoculated than control leaves at both time points, although not all were significantly different.

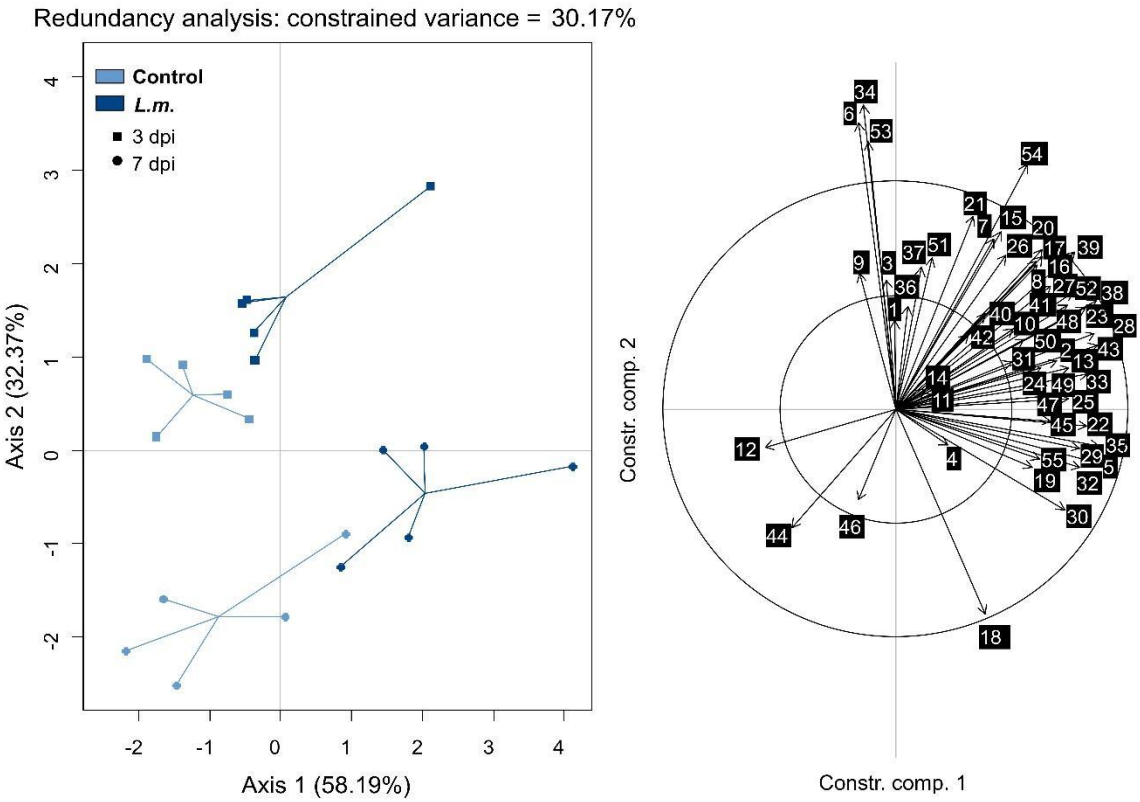

Figure 24. Redundancy analysis (RDA) of volatile (VOC) content in control and *Leptosphaeria maculans* (*L.m.*)-inoculated leaves of oilseed rape 3 and 7 days post inoculation (dpi). Score plot is shown on the left, with variances explained by the synthetic axes written in parentheses, and the correlation circle on the right. The numbers represent compounds, with their full name written in Table 5.

Table 5. Statistical output for each compound from analysis to determine differences in volatile organic compound (VOC) levels between control leaves of oilseed rape and *L. maculans*-inoculated leaves at 3

and 7 days post inoculation.. ".": P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001; *treatment*: control vs. *L. maculans*-inoculated leaf; *time*: 3 vs. 7 days post inoculation.

No.	Compound	Treatment			Time				Treatment:Time				Residual df
		F value	df	P value	F value	df	P value		F value	df	P value		
1	1,3-Diazine	0.59	1	0.56	0.32	1	0.77		0.45	1	0.93		16
2	2(3H)-Furanone, 5-ethyldihydro-	3.12	1	0.29	0.41	1	0.77		0.14	1	0.93		16
3	2(3H)-Furanone, dihydro-5-propyl-	0.47	1	0.57	1.60	1	0.53		0.34	1	0.93		16
4	2(5H)-Furanone	0.25	1	0.66	0.47	1	0.77		0.53	1	0.93		16
5	2-Decen-1-ol:2	2.40	1	0.30	7.94	1	0.07	.	1.99	1	0.86		16
6	2-Pentanol	0.01	1	0.92	37.11	1	<0.001	***	0.16	1	0.93		16
7	2-Pentanol, acetate	1.49	1	0.38	1.70	1	0.53		0.26	1	0.93		16
8	2-Pentanone	4.24	1	0.19	0.00	1	0.98		0.06	1	0.97		16
9	2-Propanone, 1-hydroxy-	0.56	1	0.56	4.37	1	0.27		2.08	1	0.86		16
10	3-Hexanone, 2,5-dimethyl-4-nitro-	0.99	1	0.49	0.04	1	0.93		0.02	1	0.97		16
11	3-Hexen-1-ol, acetate, (Z)-	0.55	1	0.56	0.00	1	0.98		0.01	1	0.97		16
12	4-Hexen-3-ol	1.76	1	0.36	0.28	1	0.77		0.16	1	0.93		16

13	5-Hepten-2-one, 6-methyl-	1.88	1	0.36		0.61	1	0.77		0.92	1	0.93	16
14	Acetic acid, hexyl ester	1.15	1	0.46		0.04	1	0.93		0.27	1	0.93	16
15	Acetic acid. pentyl ester	0.35	1	0.62		1.63	1	0.53		0.01	1	0.97	16
16	Allyl acetate	7.52	1	0.07	.	0.29	1	0.77		0.16	1	0.93	16
17	Alpha-limonene diepoxide	2.31	1	0.30		0.66	1	0.76		3.59	1	0.72	16
18	Alpha-thujene	0.89	1	0.51		13.84	1	0.02	*	0.71	1	0.93	16
19	Benzoic acid, 2-ethylhexyl ester	0.38	1	0.62		3.19	1	0.32		0.32	1	0.93	16
20	Benzoic acid, hexyl ester	4.20	1	0.19		0.29	1	0.77		2.67	1	0.86	16
21	Benzonitrile	8.31	1	0.07	.	0.96	1	0.70		0.02	1	0.97	16
22	Butanenitrile, 2-methyl-	15.89	1	0.01	**	11.21	1	0.03	*	6.81	1	0.52	16
23	Butanoic acid, 2-methyl-, hexyl ester	17.81	1	0.01	**	0.72	1	0.75		0.03	1	0.97	16
24	Butanoic acid, 2-methylpropyl ester	0.69	1	0.55		0.48	1	0.77		0.00	1	0.97	16
25	Butanoic acid, 3-methylbutyl ester	1.65	1	0.37		1.67	1	0.53		0.11	1	0.96	16
26	Butanoic acid, butyl ester	0.73	1	0.54		0.45	1	0.77		2.16	1	0.86	16

27	Cyclobutylamine	9.24	1	0.06	.	0.02	1	0.94		3.54	1	0.72	16
28	Cyclohexanone	29.51	1	<0.001	***	3.38	1	0.31		0.28	1	0.93	16
29	Decanal	0.50	1	0.57		3.45	1	0.31		0.45	1	0.93	16
30	Dimethyl sulfone	1.77	1	0.36		8.01	1	0.07	.	1.32	1	0.86	16
31	Dimethyl trisulfide	2.96	1	0.29		0.77	1	0.75		2.05	1	0.86	16
32	Disulfide, dimethyl	8.83	1	0.06	.	15.71	1	0.01	*	4.43	1	0.72	16
33	Dodecanal	1.52	1	0.38		1.21	1	0.61		0.83	1	0.93	16
34	Ethanol, 2-phenoxy-	2.58	1	0.30		59.27	1	<0.001	***	0.19	1	0.93	16
35	Ethylbenzene	21.20	1	<0.001	***	15.24	1	0.01	*	0.38	1	0.93	16
36	Ethylene glycol diallyl ether	2.31	1	0.30		2.51	1	0.42		0.63	1	0.93	16
37	Formamide, N,N-dibutyl-	0.02	1	0.90		1.73	1	0.53		0.47	1	0.93	16
38	Heptanal	7.04	1	0.08	.	0.73	1	0.75		0.00	1	0.97	16
39	Hexanal	2.91	1	0.29		0.26	1	0.77		1.13	1	0.93	16
40	Hexanoic acid, butyl ester	4.11	1	0.19		0.25	1	0.77		0.57	1	0.93	16
41	Hexanoic acid, hexyl ester:2	5.60	1	0.13		0.25	1	0.77		0.06	1	0.97	16

42	Isobutyl acetate	0.66	1	0.55		0.18	1	0.81		0.01	1	0.97	16
43	Methane, nitro-	28.50	1	<0.001	***	3.71	1	0.31		14.82	1	0.06	. 16
44	Methyl glyoxal	0.74	1	0.54		0.12	1	0.86		3.69	1	0.72	16
45	Methyl isobutyl ketone	5.53	1	0.13		2.43	1	0.42		1.40	1	0.86	16
46	N,N-dimethyl-glycine-(4-nitro-anilide)	2.30	1	0.30		0.03	1	0.93		1.38	1	0.86	16
47	Nicotine	2.80	1	0.30		1.23	1	0.61		0.77	1	0.93	16
48	Nonanal	2.18	1	0.31		0.00	1	0.98		1.56	1	0.86	16
49	Octanal	0.32	1	0.62		0.57	1	0.77		0.03	1	0.97	16
50	Octanoic acid, hexyl ester	1.56	1	0.38		0.11	1	0.86		1.03	1	0.93	16
51	Oxalic acid, cyclobutyl octyl ester	1.01	1	0.49		1.55	1	0.53		1.67	1	0.86	16
52	Pentanal	7.73	1	0.07	.	0.29	1	0.77		0.15	1	0.93	16
53	Phenylethyl alcohol	2.32	1	0.30		33.99	1	<0.001	***	0.52	1	0.93	16
54	Pyridine, 2,4,6-trimethyl-	21.38	1	<0.001	***	3.66	1	0.31		0.01	1	0.97	16
55	Undecanal	0.21	1	0.68		4.88	1	0.23		1.57	1	0.86	16

5.2.4 Discussion

Plant attractiveness to insect herbivores changes during the life span of the plant. According to the optimal defence theory, plants protect their most crucial parts with mechanical and chemical barriers to deter insect herbivores (Badenes-Perez et al., 2014). Thus, individual plant organs can differ in their attractiveness and resistance to insect herbivores, which can be demonstrated by plant damage caused by the attacker and/or reproduction rate. Feeding choice tests revealed that *P. xylostella* caterpillars and *B. brassicae* aphids strongly prefer true leaves to cotyledons when evaluated based on the leaf area eaten and a number of individuals feeding on the leaves. Therefore, true leaves were further used to study tripartite interaction between oilseed rape, *L. maculans* and *P. xylostella/B. brassicae*. This result is in line with the recent finding that *P. xylostella* larvae prefer to feed on true leaves of *Brassica oleracea* than cotyledons (Zhu et al., 2022)

To investigate changes in leaf palatability for *P. xylostella* larvae after the infection with the hemibiotroph *L. maculans*, non-inoculated (control) and inoculated detached leaves of oilseed rape were placed in separate vials interconnected by a T-tube, whereas to test the preference of *B. brassicae* aphids, control and inoculated detached leaves of oilseed rape were placed in Petri dishes. Although experiments with cut leaves do not provide field-realistic implications, *in vitro* experiments are a common method used in laboratories to test insect preference, and the results of insect choice are generally consistent with *in vivo* experiments (Robin et al., 2017). The results showed that caterpillars significantly prefer *L. maculans*-inoculated leaves to control leaves early after inoculation, and the palatability of *L. maculans*-inoculated leaves for caterpillars decreased at 7 dpi. On the other hand, aphids did not show any preference at 3 dpi but the significant avoidance for *L. maculans*-inoculated leaves was observed at 7 dpi. Regardless of *L. maculans*-inoculated leaves being still green and symptomless at 3 dpi, the previous research by Šašek et al. (2012b) in cotyledons of oilseed rape showed that conidia start to germinate at this early stage of the disease followed by the slow growth of mycelium, and symptoms start to be visually detectable (yellowing of infected leaf and first lesion formation) at 7 dpi when mycelium is already expanded. It should be noted that plants activate defence mechanisms when they come into contact with pathogens, which could alter their palatability to herbivores. Pathogenic microorganisms are primarily identified on the basis of PAMPs, which are represented by molecules present in their cell walls or spores.

Recognition of PAMPs is followed by the activation of defence responses. The second stronger wave of defence is activated by the effectors secreted by the pathogen. In case of *L. maculans*, oilseed rape response during the first wave is rather weak and later on, *i.e.* at 7 dpi, the hyphae colonize the leaf apoplast (Gay et al., 2021; Jones and Dangl, 2006). Thus, this may affect the feeding preference of *P. xylostella* caterpillars and *B. brassicae* aphids in the course of disease development.

Pathogen recognition triggers numerous defence mechanisms and phytohormone signaling pathways. The activation of SA, JA and ET signaling was examined and demonstrated that dominant signaling pathways in *L. maculans*-inoculated leaves were SA (based on *ICS1*, *PAL1* and *PR1* transcription) and ET (based on *ACS2*, *βCHI* and *HEL* transcription), and that the activation of JA signaling did not occur during the *L. maculans* infection in oilseed rape leaves. Therefore, *βCHI* and *HEL* were activated via the ET pathway at both time points. These results performed on detached true leaves correspond with previous work by Šašek et al. (2012b), which was done on intact *in vivo* cotyledons. As discussed previously, *L. maculans* activates the SA signaling pathway at the early stage of infection, during the biotrophic stage, whereas ET signaling starts its activity later on in connection with the development of necrotic symptoms (Lowe et al., 2014; Šašek et al., 2012b): this combination is quite uncharacteristic for plant pathogens and here reflects *L. maculans* transition from biotrophic to a necrotrophic phase. Since plant defence against chewing herbivores is generally regulated by JA and the activation of the JA signaling pathway was not observed (based on the lack of *LOX3* and/or *VSP* transcription), it cannot be assumed that *L. maculans* activated JA-dependent defence mechanisms against *P. xylostella* in leaves. Also, given the well-described antagonism between SA and JA signaling (Pieterse et al., 2012), a possible attenuation of the defence against chewing herbivores cannot be excluded. Thus, elevated SA-signaling could block production of MeJA, the molecule known as an activator of direct or indirect defence mechanisms against insect herbivores (Xiao et al., 2019). Unlike chewing herbivores, piercing-sucking aphids tend to activate SA and ET responses in plants (Khoshfarman-Borji et al., 2020). Although SA and ET signaling were activated at both time points in *L. maculans*-inoculated leaves, aphid deterrence towards inoculated leaves was observed only at 7 dpi. Nevertheless, the difference in plant palatability between 3 and 7 dpi is also dependent on other factors, due to the *L. maculans* transition to a necrotrophic stage between 7 and 9 dpi, as demonstrated by a wide

RNAseq study (Gay et al., 2021). The reported increased transcription of 148 genes by *L. maculans* during that time interval could affect oilseed rape physiology.

Caterpillar and aphid preference and performance is also mediated by restricted nutrition sources, the accumulation of products produced by secondary metabolites in the infected leaves, and changes in VOC composition (Babic et al., 2008; Merkx-Jacques and Bede, 2005; War et al., 2012). Results presented here show that the total content of specialized metabolites GLSs was highly elevated in *L. maculans*-inoculated leaves at 3 dpi compared to control leaves, especially aliphatic GLSs. Specific compounds play distinct roles in preference or performance of specialist pathogens and insect pests (Cole, 1997; Robin et al., 2020, 2017); for instance, specific GLS compounds can act as gustatory stimulants for *P. xylostella* larvae (Robin et al., 2017): when larvae were exposed to cabbage lines differing in GLS profile, a higher level of herbivory was observed on plant lines rich in aliphatic GLSs glucoerucin, glucoraphanin, progoitrin and gluconapin, and indole 4-hydroxyglucobrassicin; on the other hand, lower leaf damage was recorded on plants with high content of aliphatic sinigrin, glucoiberin and glucoiberverin, and indole glucobrassicin. On the other hand, *B. brassicae* aphids performed better on *Brassica* spp. with high content of aliphatic sinigrin and progoitrin, and worse on cauliflower cultivars rich in indole glucobrassicin and neoglucobrassicin (Cole, 1997). It could therefore be speculated that caterpillar and aphid preference to true leaves of oilseed rape may be due to the higher content of aliphatic GLSs and lower content of indole GLSs in the true leaves compared to cotyledons. Another reason for this speculation is that this preference seems not to be driven by the difference in the leaf surface between cotyledons and true leaves, such as in terms of waxy layer and trichome density (Zhu et al., 2022), as studies have shown that caterpillars and aphids generally prefer glabrous and/or less waxy leaves (Eigenbrode et al., 1991; Hao et al., 2020; Xuan et al., 2020), hence the cause could be the change in the leaf biochemical composition (Gruber et al., 2018). Moreover, *P. xylostella* induces high levels of indole GLSs in oilseed rape leaves (Fig. 21), which could be the plant mechanism to defend itself against the herbivory. Therefore, in accordance with the literature, one could speculate that *P. xylostella* preferred *L. maculans*-inoculated to control leaves at 3 dpi, for their lower content of glucoiberin and higher content of other tested aliphatic GLSs. Although not significant, gluconapin showed a similar trend as the increased glucobrassicanapin, considering they are both catalysed by the same enzyme (Augustine and Bisht, 2015).

Herbivores specialized to feed on Brassicaceae also use plant VOCs as cues for locating and selecting their host plant (Hussain et al., 2020; Liu et al., 2020; Renwick et al., 2006). Here, I demonstrated that *L. maculans* infection altered the spectrum of VOCs emitted from oilseed rape leaves. Both control and *L. maculans*-inoculated leaves of oilseed rape emitted sulfides (dimethyl disulfide, dimethyl trisulfide) at 3 and 7 dpi, with dimethyl disulfide being significantly higher in *L. maculans*-inoculated leaves at 7 dpi compared to control leaves. Interestingly, *B. brassicae* aphids induced significantly higher concentrations of dimethyl disulfide in *B. oleracea* compared to non-damaged plants (Najar-Rodriguez et al., 2015), and it can therefore be assumed that high levels of this compound at 7 dpi played a role in *B. brassicae* deterrence towards *L. maculans*-inoculated leaves. Also, sulfides were released from *A. thaliana* and *Brassica rapa* after *P. xylostella* infestation, which served as strong attractants to the parasitoid *Cotesia vestalis* in *B. rapa* (Kugimiya et al., 2010; Truong et al., 2014). As larvae are able to choose food and move from one spot to another (Zhu et al., 2022), their performance may be influenced by VOC levels: high concentrations of allyl ITC can be toxic to neonate *P. xylostella* (Li et al., 2000), and plant-produced (3*E*)-4,8-dimethyl-1,3,7-nonatriene can negatively affect the number of larval midgut microbiota which finally results in high larval mortality (Chen et al., 2021). However, no GSL-derived volatiles were detected in this study, likely due to the specific experimental setup.Numerous plant–insect–microorganism interactions have been investigated so far (Franco et al., 2017), which clearly show high variability in synergistic and antagonistic impacts on plant performance. A recent meta-analysis highlighted important information on the insect herbivore preference and performance on fungus-challenged plants (Fernandez-Conradi et al., 2018). Insect preference can be influenced by the plant species, the fungus lifestyle (biotrophic vs. necrotrophic), or the stage of infection, and therefore by various underlying defence mechanisms. Multiple interactions were reported on several members of Brassicaceae family, but to my knowledge, none concerned oilseed rape. In *Brassica rapa*, the infection with a biotrophic fungal pathogen *Erysiphe cruciferarum* did not affect the pupal weight and mortality of *Pieris brassicae* caterpillars; however, fungal infection with subsequent infestation indirectly and negatively affected the incidence of natural enemies by reducing the emission of VOCs (Desurmont et al., 2016). Another study on Brassicaceae plants demonstrated an interaction effect of an insect herbivore and a fungal pathogen, and how it differs on two resistant host cultivars of *Barbarea vulgaris* (Heimes et al., 2015): the insect-resistant genotype was severely infected by a biotrophic fungus *Albugo* spp.,

which reduced plant biomass loss when subsequently infested by *Phyllotreta nemorum* flea beetles. However, the reverse effect was observed in the insect-susceptible genotype, where *Albugo* spp. positively affected insect feeding, indicating some kind of a negative cross-talk towards plants. Furthermore, understanding the role of some VOCs is further complicated by the fact that they are produced by pathogens themselves, such as in the case here isobutyl acetate, which was reported as a volatile of *Ceratocystis platani* fungus (Brilli et al., 2020).

Multitrophic interactions are extremely complex and mainly context-dependent. While much work has been done to investigate plant-insect-fungi (biotrophic or necrotrophic) interactions, relatively little has been done to explore such relationships with hemibiotrophic fungi. This study brings novel findings regarding oilseed rape plants interacting with a destructive specialist hemibiotrophic fungal pathogen and a chewing insect herbivore, which lead to better understanding of plant resistance strategies and further development of enviromentally-friendly methods in terms of pest management. As a major crop, oilseed rape deserves more thorough exploration: its defence is controlled by different signaling pathways than in the model plant *A. thaliana* belonging to the same family (Šašek et al., 2012b), thus underlining the importance of carrying out phytopathological studies on relevant pathosystems.

5.3 Can resistance inducers alter palatability of oilseed rape for *Plutella xylostella* caterpillars?

5.3.1 Introduction

In modern agricultural practices, the effective management of insect herbivores poses a significant challenge, requiring sustainable and environmentally friendly solutions. Traditional methods often rely heavily on chemical pesticides, which can have adverse effects on ecosystems and human health, as well as contribute to the development of insect resistance. As a result, there has been a growing interest in alternative strategies, such as the utilization of resistance inducers in plant protection against insect herbivores. Resistance inducers are compounds or substances that trigger defence mechanisms within plants, enhancing their ability to withstand herbivore attacks. By stimulating the plant natural defence pathways, resistance inducers offer a promising strategy for reducing reliance on chemical pesticides while promoting sustainable pest management practices. Benzothiadiazole (BTH) functions by activating the SA signaling pathway (Blanco et al., 2009), whereas ulvan has been demonstrated to elicit plant defences through JA pathway (Přerovská et al., 2022). Hence, based on this dichotomy in their modes of action, I hypothesized that BTH and/or ulvan would influence the feeding preferences of the destructive insect herbivore *P. xylostella* in oilseed rape. To investigate this hypothesis, a series of choice test experiments were primarily conducted.

5.3.2 Experimental design

To investigate the behaviour of the plant elicitor BTH in oilseed rape, cotyledons were infiltrated by syringe with 32 µM of BTH solution. Subsequently, 24 h after treatment, infiltrated cotyledons and systemic true leaves were used for *PR1* gene transcription analysis to verify whether the signal was transferred to other parts of the plant. Systemic true leaves were then used for choice tests with *P. xylostella* caterpillars. Water-infiltrated cotyledons and systemic true leaves were used as control. Vacuum infiltration of detached leaves with BTH solution (32 µM) was also performed, in order to test the direct effect of the compound on caterpillar preference.

Ulvan solution (1 mg ml^{-1}) was sprayed evenly on attached plants of oilseed rape, and 48 h after detached leaves were used for choice tests with *P. xylostella* caterpillars. Water-sprayed leaves were used as control.

To determine the preference of *P. xylostella* caterpillars for different leaf treatments, a feeding choice test was performed on detached systemic true leaves of oilseed rape 24 h after the cotyledon treatment and 24 h after the vacuum leaf infiltration with BTH, and 48 h after ulvan spraying; with one leaf placed in each vial. Three third-instar caterpillars were starved for 3 h in a separate plastic container before being transferred to the T-tube using a fine paintbrush. T-tube connected vials were randomly placed in a controlled environment under the same conditions as plants used for experiments were growing. During the next 48 h, caterpillars could choose the vial and feed on the leaf within, and switch from one vial to another. After 48 h of exposure to herbivory, leaves were scanned and the percentage of the leaf area consumed by larvae was evaluated using BioLeaf (Machado et al., 2016).

At least three separate experiments, with approximately ten replicates per treatment (control vs. BTH/ulvan-treated leaf) within each biological repetition were performed. For the gene transcription analysis one leaf was placed in the individual non-connected plastic vial, and the samples were collected 24 and 48 h post infestation (hpi).

5.3.3 Results

5.3.3.1 Benzothiadiazole (BTH) induces *PR1* gene transcription in the systemic true leaves of oilseed rape

An upregulation of *PR1* gene marker was observed in the systemic true leaves of oilseed rape that did not come into direct contact with the BTH solution during treatment (Fig. 25). The increase in the *PR1* transcription was significantly higher in the systemic true leaves compared to cotyledons that were directly treated with the BTH solution ($F_{3,8} = 9596.56$, $P = 0.000$). The transcription in cotyledons treated with BTH increased to 200-fold, and in systemic true leaves up to 500-fold.

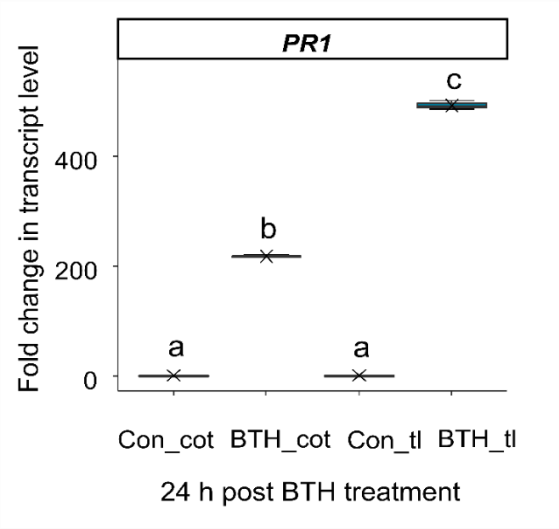

Figure 25. Systemic effect of benzothiadiazole (BTH) on *PR1* gene transcription in the true leaves of oilseed rape 24 h after cotyledon infiltration with 32 µM of BTH solution. Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences ($P < 0.001$) between treatments. Abbreviations: *PR1 = pathogenesis related 1*; Con_cot = control cotyledons; BTH_cot = BTH cotyledons; Con_tl = control true leaves; BTH_tl = BTH true leaves.

5.3.3.2 The method of oilseed rape leaf infiltration by BTH affects the palatability for *Plutella xylostella* caterpillars

No significant preference of *P. xylostella* caterpillars to detached systemic true leaves 24 h after infiltration of cotyledons with BTH solution, nor to the control leaves of oilseed rape was observed ($F_{1,98} = 0.316$, $P = 0.575$; Fig. 26). A different situation was observed when true leaves were directly treated with BTH: the results show significant preference of caterpillars to the control leaves, *i.e.* water-infiltrated leaves in comparison to BTH-infiltrated leaves ($F_{1,54} = 9.435$, $P = 0.003$; Fig. 27).

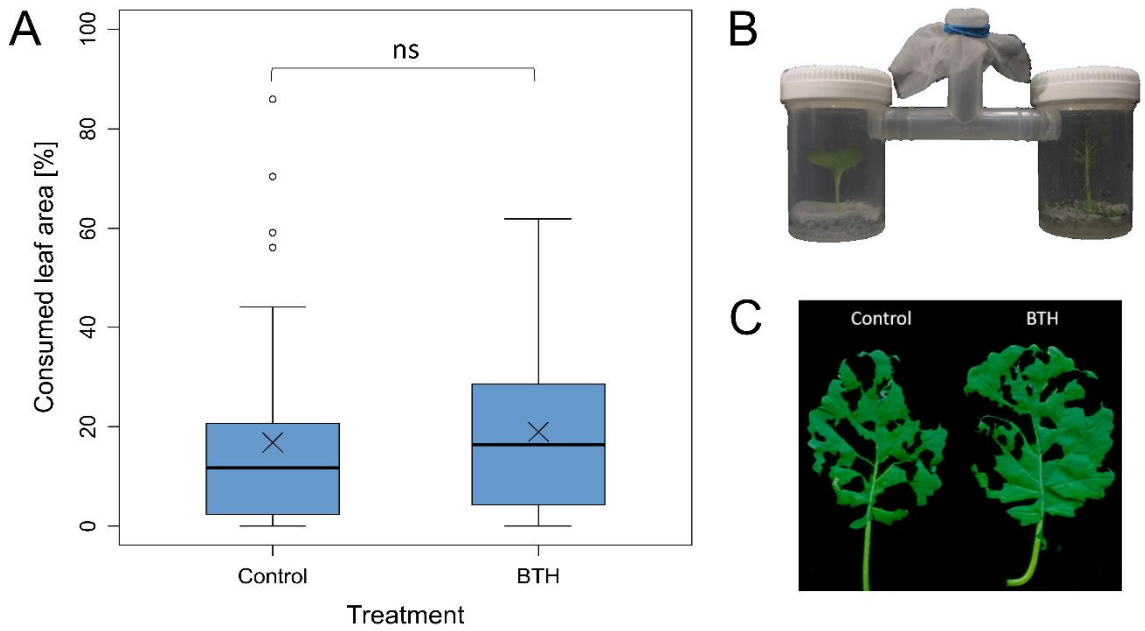

Figure 26. Feeding preference of *Plutella xylostella* caterpillars to systemic leaves of oilseed rape (cotyledons were previously treated with benzothiadiazole, 'BTH'). **A:** consumed leaf area (in %) caused by third-instar caterpillars after 48 h of feeding. Boxplot represents mean (´x´), median, 25th and 75th percentiles, ns = not significant; **B:** vial system developed for performing larval feeding choice experiments with one control leaf inside the left vial and one BTH-treated leaf inside the right vial. Three caterpillars were placed in the T-tube and were given a choice to feed on two types of leaves; **C:** scanned leaves after 48 h of caterpillar feeding.

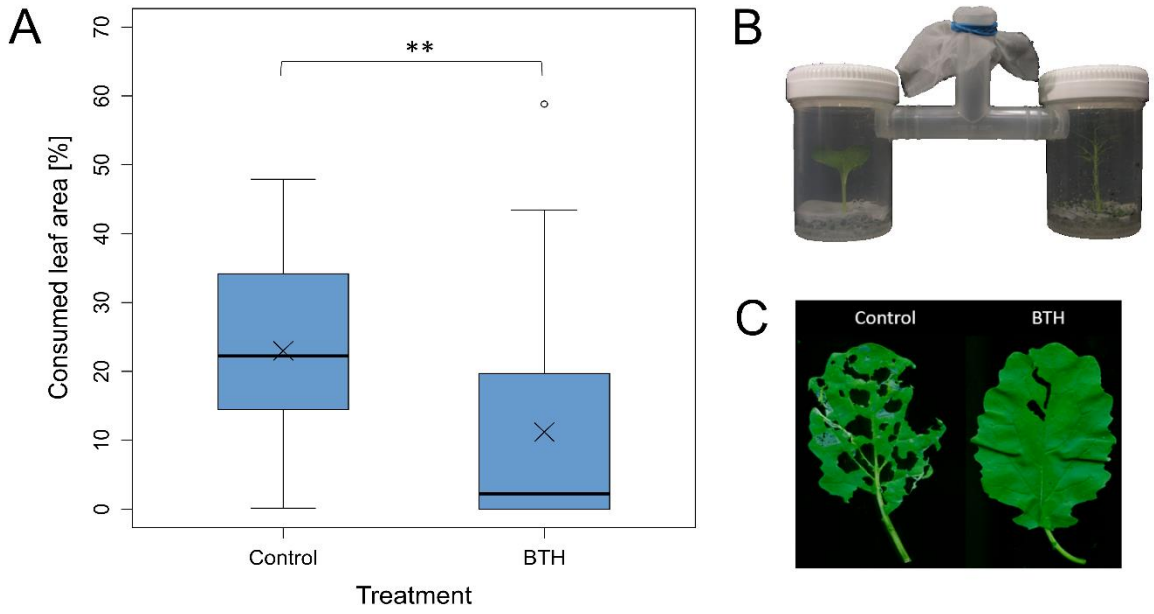

Figure 27. Feeding preference of *Plutella xylostella* caterpillars to detached leaves of oilseed rape infiltrated with benzothiadiazole (BTH). **A:** consumed leaf area (in %) caused by third-instar caterpillars after 48 h of feeding. Boxplot represents mean (´x´), median, 25th and 75th percentiles, and asterisks significant differences (P < 0.01); **B:** vial system developed for performing larval feeding choice experiments with one control leaf inside the left vial and one BTH-treated leaf inside the right vial. Three caterpillars were placed in the T-tube and were given a choice to feed on two types of leaves; **C:** scanned leaves after 48 h of caterpillar feeding.

5.3.3.3 Treatment of oilseed rape leaves with ulvan alters the preference of *Plutella xylostella* caterpillars

A clear preference of *P. xylostella* caterpillars to ulvan-treated leaves of oilseed rape was observed: significantly higher leaf consumption was recorded in control (*i.e.* water-sprayed) leaves compared to ulvan-sprayed leaves ($F_{1,52}$ = 8.146, P = 0.006; Fig. 28).

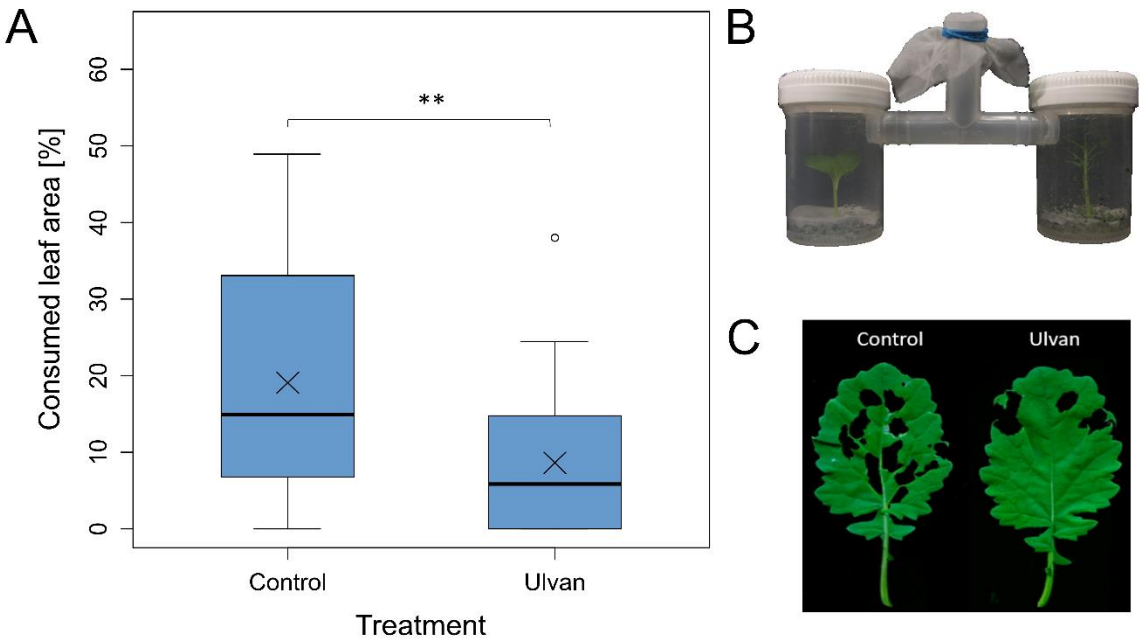

Figure 28. Feeding preference of *Plutella xylostella* caterpillars to detached leaves of oilseed rape treated with ulvan. **A:** consumed leaf area (in %) caused by third-instar caterpillars after 48 h of feeding. Boxplot represents mean (´x´), median, 25th and 75th percentiles, and asterisks significant differences (P < 0.01); **B:** vial system developed for performing larval feeding choice experiments with one control leaf inside the left vial and one ulvan-treated leaf inside the right vial. Three caterpillars were placed in

the T-tube and were given a choice to feed on two types of leaves; **C:** scanned leaves after 48 h of caterpillar feeding.

5.3.4 Discussion

Efficient, cheap, and environmentally friendly compounds, which could substitute synthetic pesticides, are of a high interest. Compounds inducing plant resistance to pathogens and pests pose a promising strategy. BTH is considered a powerful inducer of plant resistance to pathogens. Upon BTH perception, plants undergo activation of defence signaling pathways, and eventually develop the ability of resistance to pests (Li et al., 2018). Although it does not induce SA biosynthesis, BTH activates SA-dependent defence responses in plants and rather mimics SA function, which was proven in dicot plants (Blanco et al., 2009). Despite widely accepted opinion on nearly identical functions of BTH and SA, it has been reported recently that several upregulated and downregulated genes significantly differed in response to these two compounds in *Brachypodium distachyon* (Kouzai et al., 2018). Accordingly, these compounds may differ in their activity in individual host–pathogen interactions in inducing resistance (Sugano et al., 2010). Available data on BTH-induced plant resistance to insect herbivores are scarce; however, several studies are documenting none or weak effect (Gordy et al., 2015). Results presented here reveal that BTH activates both local and systemic *PR1* gene transcription in oilseed rape. Moreover, the method of BTH plant treatment affected caterpillar performance in distinct manner. While locally BTH-treated true leaves deterred *P. xylostella* caterpillars, the systemic non-treated leaves did not affect caterpillar choice. These results agree with previous findings by (Nombela et al., 2005), showing that BTH treatment negatively affected the egg-laying and development of *Bemisia tabaci* in tomato plants. Later, Li et al. (2018) observed that foliar application of BTH and BABA ultimately inhibited growth, development, and performance of geometrid larvae *Ectropis obliqua*, characterized by decreased body weight, prolonged development duration, and lower rates of survival, pupation and emergence of larvae compared to control. Several studies have described that peroxidase (PPO), polyphenol oxidase (POD) and phenylalanine ammonia-lyase (PAL) are involved in induced plant resistance to herbivores, and elicitors that trigger the activity of these enzymes could, therefore, induce plant resistance to insect herbivores (Li et al., 2018; War et al., 2012). Li et al. (2016) found that BTH and BABA may trigger the upregulation of PPO, POD, and

PAL accumulation in tea plants, which would indicate that BTH and BABA likely play a critical role in plant resistance to attackers. Moreover, BTH treatment causes changes in GLS levels of plants (Thakur et al., 2014) which might also be important in driving caterpillar preference.

Apart from having a biostimulative effect, extracts from *Ulva* spp. are triggering JA-dependent signaling in plants and are proven to have an effect in enhancing resistance to various pathogens (Přerovská et al., 2022; Shukla et al., 2021). There are no studies on the potential use of ulvan against insect herbivores; however, I observed that ulvan-treated leaves of oilseed rape were deterrent to *P. xylostella* caterpillars, which might be due to the JA-activated plant defence responses. Another reason could be the difference in GLS profile and/or VOC composition between ulvan-sprayed leaves and control leaves. Hence, further research is needed to pinpoint the exact cause of caterpillar choice.

5.4 Activation of defence signaling responses in *Arabidopsis thaliana* during *Brevicoryne brassicae* feeding

5.4.1 Introduction

Aphids, as phloem sap feeders, probe into leaf tissues and activate a complex network of plant defence responses. Phytohormonal signaling plays a major role in this network; however, the dynamics of the signal spreading are yet to be clarified. Despite the growing knowledge about transcriptomic changes upon infestation, results often differ due to sampling, and they vary strongly between the tissues collected at the single feeding site, individual leaves, pooled infested leaves, or whole plant rosettes. This study seeks to elucidate the activation patterns of SA and JA signals in *Arabidopsis* leaves upon infestation by *B. brassicae* with high spatio-temporal resolution. Employing genetically encoded fluorescent biosensors, histochemistry, and qRT-PCR, I rigorously map the activation of distinct branches of phytohormonal signaling. The results first show the dynamics of SA- and JA-related responses following stylet penetration of *Arabidopsis* leaves and bring a detailed insight into the spatio-temporal complexity of plant defence activation during specialist aphid attack.

5.4.2 Experimental design

To investigate the dynamics of *A. thaliana* defence responses and the role of individual cell reactions and cell-to-cell communication during infestation by a specialist aphid *B. brassicae*, a combination of biochemical and molecular biology methods with novel visualisation tools was used. Hence, to study the spatial progression of the signal induced by feeding aphids, the following approaches were combined: i) qRT-PCR of marker genes for different signaling pathways, sampling 6 mm-diameter leaf discs around the aphid feeding site; ii) monitoring activation of marker genes in vivo in the area surrounding the feeding site, using a set of visualisation tools, including plant lines expressing promoter-fluorescent reporter constructs for genes associated with SA, JA, and ET signaling pathways (Marhavý et al., 2019); and iii) monitoring the localization of *PR1* activation using plants expressing a PR1::GUS construct.

Non-winged adults (apterae) of *B. brassicae* were used in all experiments. Aphids were transferred on the leaves with a fine paintbrush. For gene transcription measurement by qRT-

PCR, five-week-old plants of *A. thaliana* Col-0 were infested with 20 non-winged aphids; for microscopic observation or histochemical studies four- to five-week-old transgenic plants of *A. thaliana* were infested with 15 non-winged adult aphids. Infested plants were kept individually in glass beakers covered with a fine mesh gauze, at 22°C, 70 % relative humidity, under 10 h light (100-130 $\mu E\ m^{-2}\ s^{-1}$) and 14 h dark regime. Aphid-free plants served as controls and were kept under the same conditions. Infested and non-infested plants were harvested 6, 24, 48 and 72 h post infestation (hpi) for gene transcription measurement, and 24, 48 and 72 hpi for imaging. Aphids were removed from the leaves using the brush and 6 mm leaf discs were used for microscopic imaging or were immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction.

5.4.3 Results

5.4.3.1 *Brevicoryne brassicae* regulates the transcription of genes involved in SA, JA and ET signaling and triggers callose deposition in *Arabidopsis thaliana*

Feeding by *B. brassicae* altered the transcription of genes involved in SA (*PR1*, *PR2*, *PR5*, *ICS1*, *ICS2*), JA (*AOS*, *LOX2*, *JAZ8*, *JAZ10*), ET (*ACS2*, *ACS6*) and JA/ET (*PDF1.2*, *HEL/PR4*) signaling in *A. thaliana* (Fig. 29). Aphid feeding normally caused upregulation of all the tested genes, rather than the downregulation. The upregulation of SA biosynthetic gene (*ICS1*) and SA downstream genes (*PR1*, *PR2*, *PR5*) around the aphid feeding sites was observed from 24 until 72 hpi, and biosynthetic *ICS2* was upregulated from 48 hpi. Interestingly, the transcription of *PR1* was over 100-fold higher in aphid-infested plants at 24 hpi compared to its respective control, and 1000-fold higher at 48 and 72 hpi. As for *PR2* and *PR5*, the pattern of induction was similar to that of *PR1* (gradual increase in transcription starting from 24 hpi). Moreover, the upregulation of JA biosynthetic genes *LOX2* and *AOS* at 24 and 48 hpi was significant, but fold change was not high. The transcription of JA-downstream gene *JAZ8* was significantly increased already at 6 hpi, indicating an early response to *B. brassicae* attack, and at 24 and 72 hpi, together with *JAZ10*. Moreover, genes involved in ET biosynthesis, *ACS2* and *ACS6* showed an early upregulation (6 hpi), with the strong induction at 48 and 72 hpi. In addition, genes involved in both JA and ET signaling (*PDF1.2*, *HEL/PR4*) were upregulated from 24 until 72 hpi.

Using fluorescent reporter lines of *A. thaliana*, an induced transcription of *PR1* and *JAZ10* was detected in the cells surrounding the stylet puncture at 24 hpi in comparison with non-infested plants (Fig. 30A, B). Notably, the induction started from the sites of stylet punctures (visible as an autofluorescent line in Fig. 30B) at 24 hpi, and, until 48 hpi, was spreading along the veins, whereas at 72 hpi, the reporter fluorescence was detected also in the leaf epidermis pavement cells outside the vein (Fig. 30B). The induction of *PR1* and *JAZ10* was evaluated by manual counting of the number of cells with nuclear fluorescence per leaf disc. The statistically significant induction of *PR1* and *JAZ10* was detected in the cells of aphid-infested plants at each time point (Fig. 30C). Unfortunately, high background noise was observed in non-infested leaves with reporter constructs for *ACS6, AOS* and *PR4/HEL*, which brought difficulties for data interpretation (Fig. 31).

To complement previous results regarding visualisation of spatio-temporal dispersion of *PR1* induction from the sites of aphid feeding, *Arabidopsis* plants expressing PR1::GUS were infested and analysed by X-GLUC assay sampling whole infested leaves. As expected, β-glucuronidase (GUS) activity was detected, as the *PR1* gene was slightly induced in infested plants at 24 hpi and more strongly in later time points (Fig. 32A, B) and was detectable around the sites of stylet puncturing (Fig. 32B, yellow arrows). Indeed, the strong induction of *PR1* seems to occur locally around the aphid feeding zones and when using the promoter-reporter line *pPR1::NLS3xVenus* (Fig. 30A, B). To study whether callose deposition is spatially connected with defence gene activation, I subsequently stained *PR1::GUS* leaves with aniline blue for callose deposition analysis after X-GLUC assay. Callose deposition was detected at all studied time points at the sites of *B. brassicae* stylet insertions; hence, it co-localized with *PR1* gene activation (Fig. 32B).

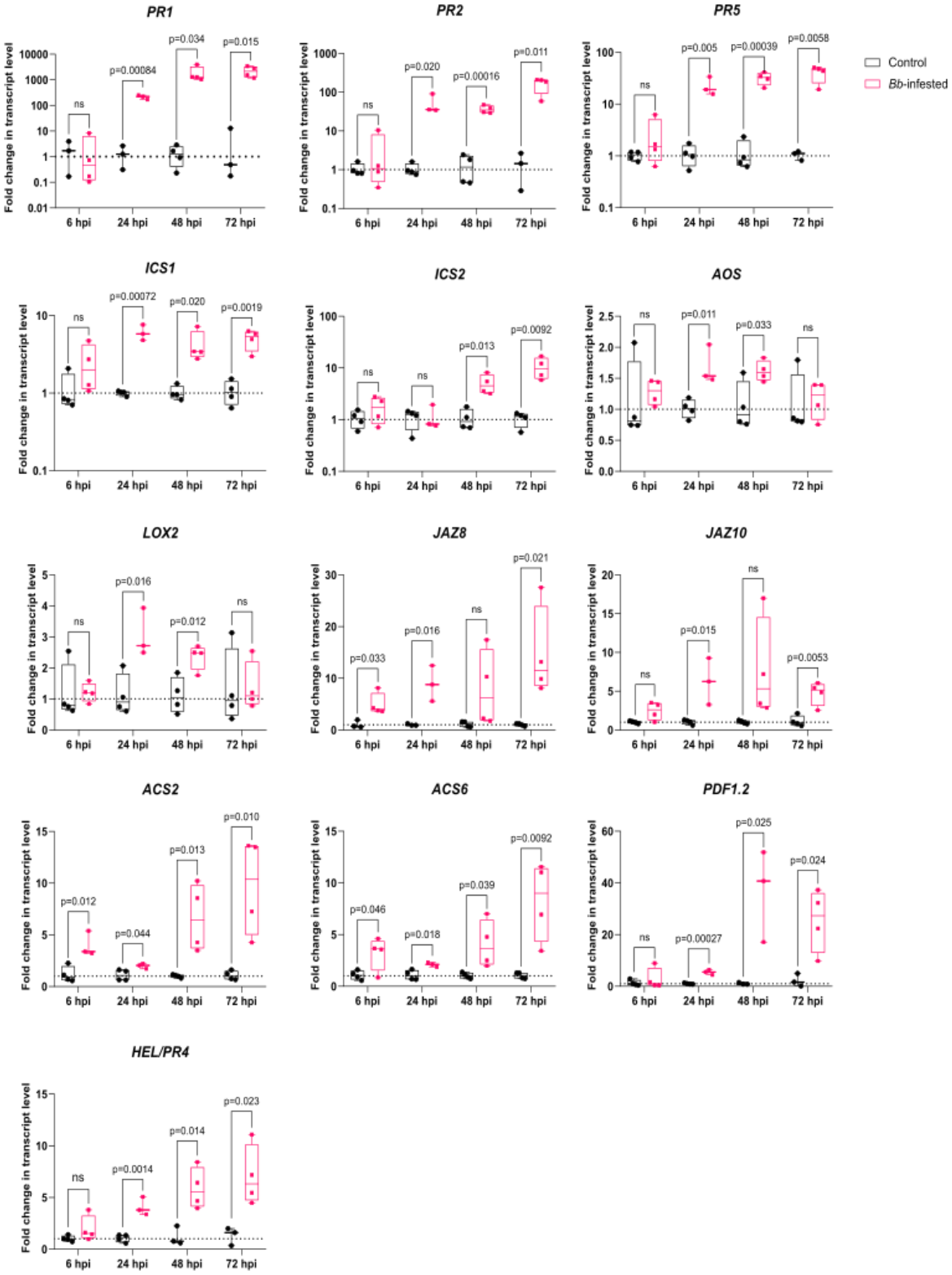

Figure 29. Gene transcription changes in leaf discs around feeding sites of the specialist aphid *Brevicoryne brassicae* (*Bb*) in WT plants of *Arabidopsis thaliana* (Col-0) 6, 24, 48 and 72 h post

infestation (hpi). Graphs represent genes involved in SA signaling pathway (*PR1*, *PR2*, *PR5*, *ICS1*, *ICS2*), JA signaling pathway (*LOX2*, *AOS*, *JAZ8*, *JAZ10*), ET biosynthetic pathway (*ACS2*, *ACS6*) and ET/JA signaling pathway (*PDF1.2*, *HEL/PR4*). CT values of target genes were normalised to the geometric mean of two housekeeping genes, namely *ACT8* and *SAND*. Control and infested samples were compared within each time point with Student's *t* test; P-value is indicated for comparisons that passed the significance threshold; n = 4. Error bars represent standard error of the mean (SE). Dashed line on y = 1 is set to a normalised mean of non-infested control for each time point. Abbreviations: *PR1,2,5 = pathogenesis-related 1,2,5*; *ICS1,2 = isochorismate synthase 1,2*; *AOS = allene oxide synthase*; *LOX2 = lipoxygenase 2*; *JAZ8,10 = jasmonate ZIM domain 8,10*; *ACS2,6 = ACC synthase 2,6*; *PDF1.2 = plant defensin 1.2*; *HEL/PR4 = hevein-like/pathogenesis-related 4*.

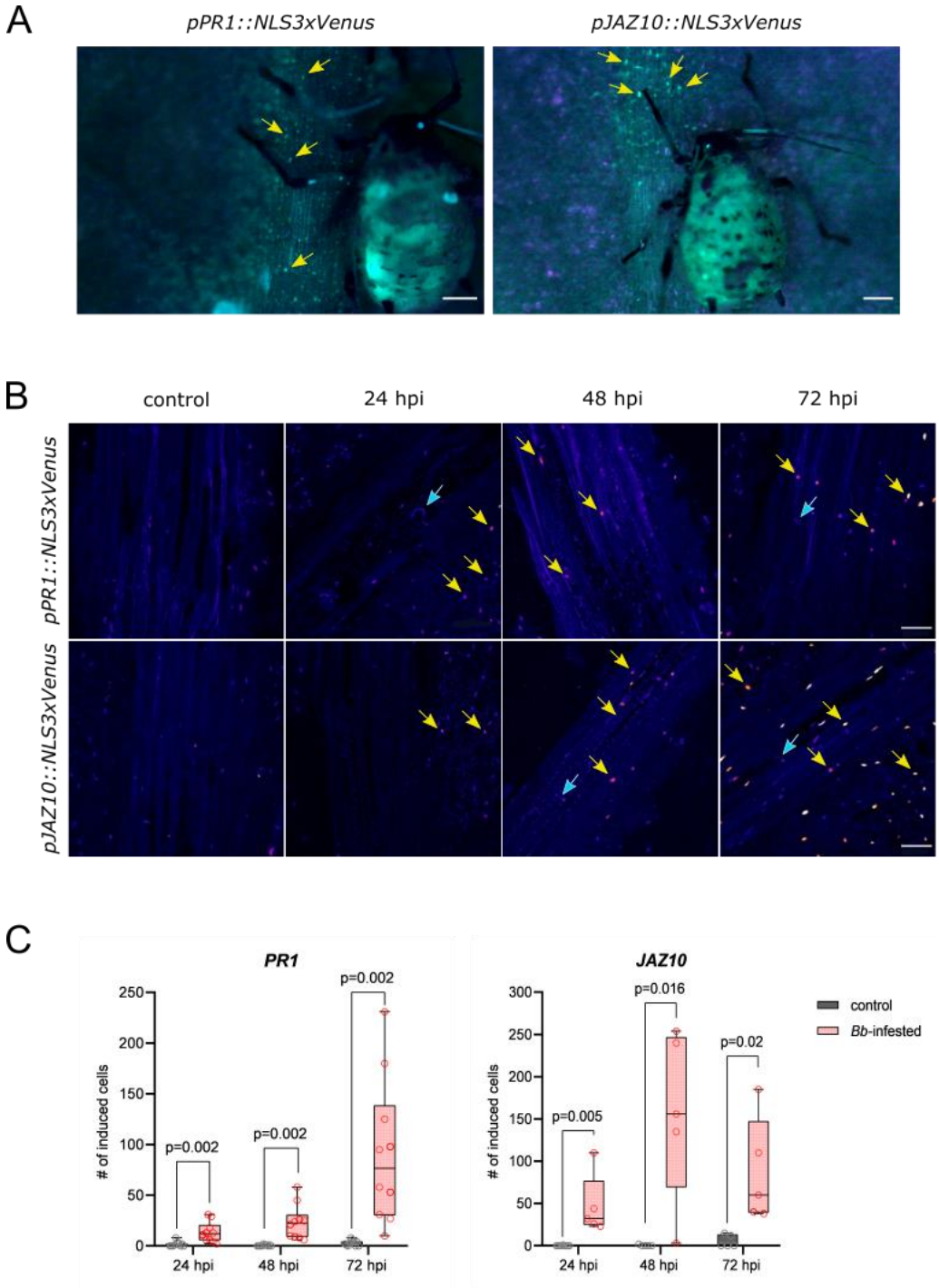

Figure 30. Local activation of *PR1* and *JAZ10* transcription in *Arabidopsis thaliana* veins upon infestation around the feeding sites of *Brevicoryne brassicae* (*Bb*). **A:** *B. brassicae* feeding on the leaves of *pPR1::NLS3xVenus* and *pJAZ10::NLS3xVenus* 48 h post infestation (hpi); scale bar = 500 µm; **B:** representative images of *PR1* and *JAZ10* induction; yellow arrows point to the nuclei with induced reporter construct expression, blue arrows point to the stylet puncture autofluorescence; scale bar 10

µm; **C:** *PR1* and *JAZ10* quantification of induced cells per 6 mm leaf disc; ** - variants are significantly different from non-infested control, P < 0.01.

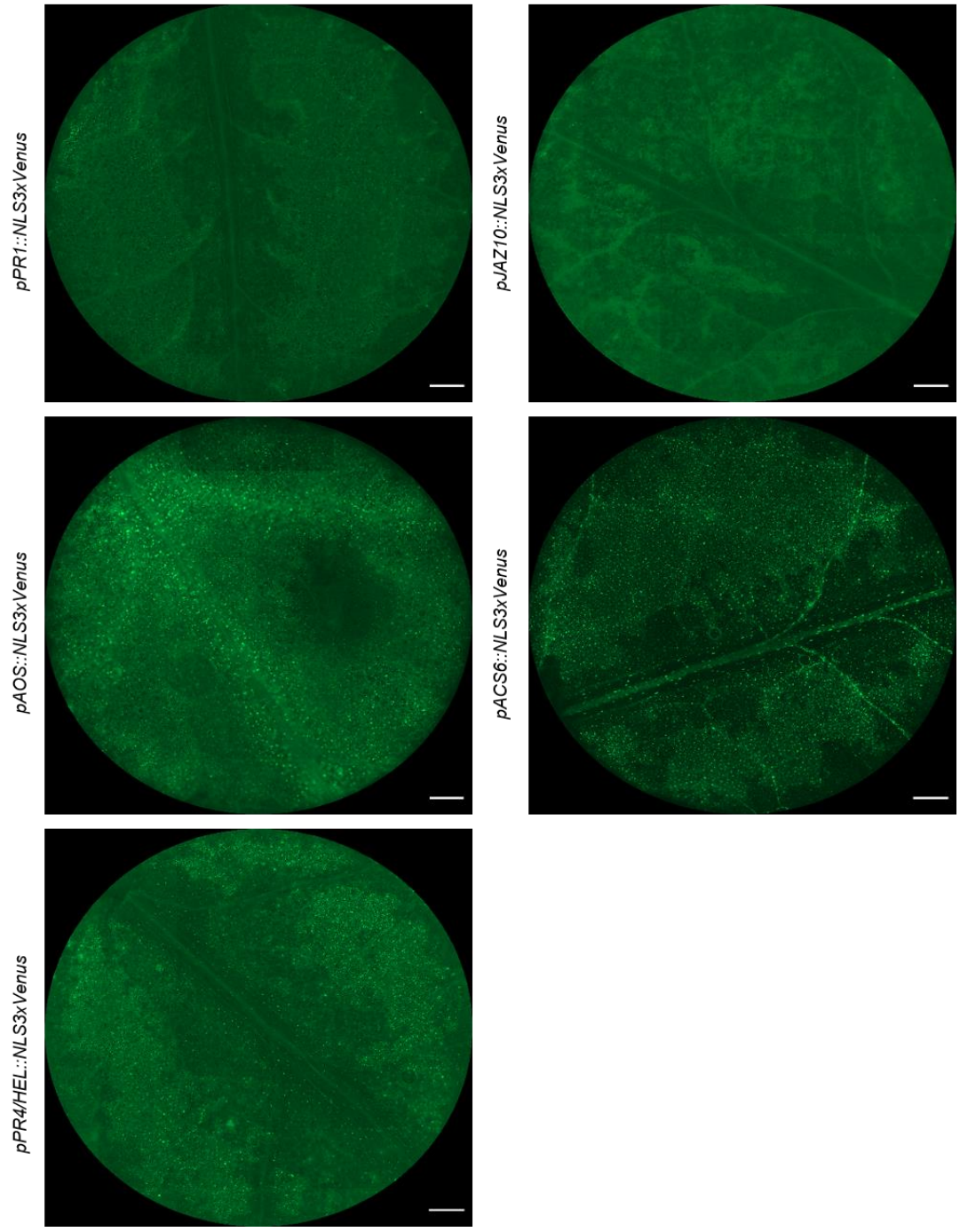

Figure 31. *Arabidopsis thaliana* plants with reporter constructs for *AOS, ACS6 and PR4/HEL* expressing high background noise compared to *PR1* and *JAZ10* genes in non-infested plants. Scale bar = 500 µm.

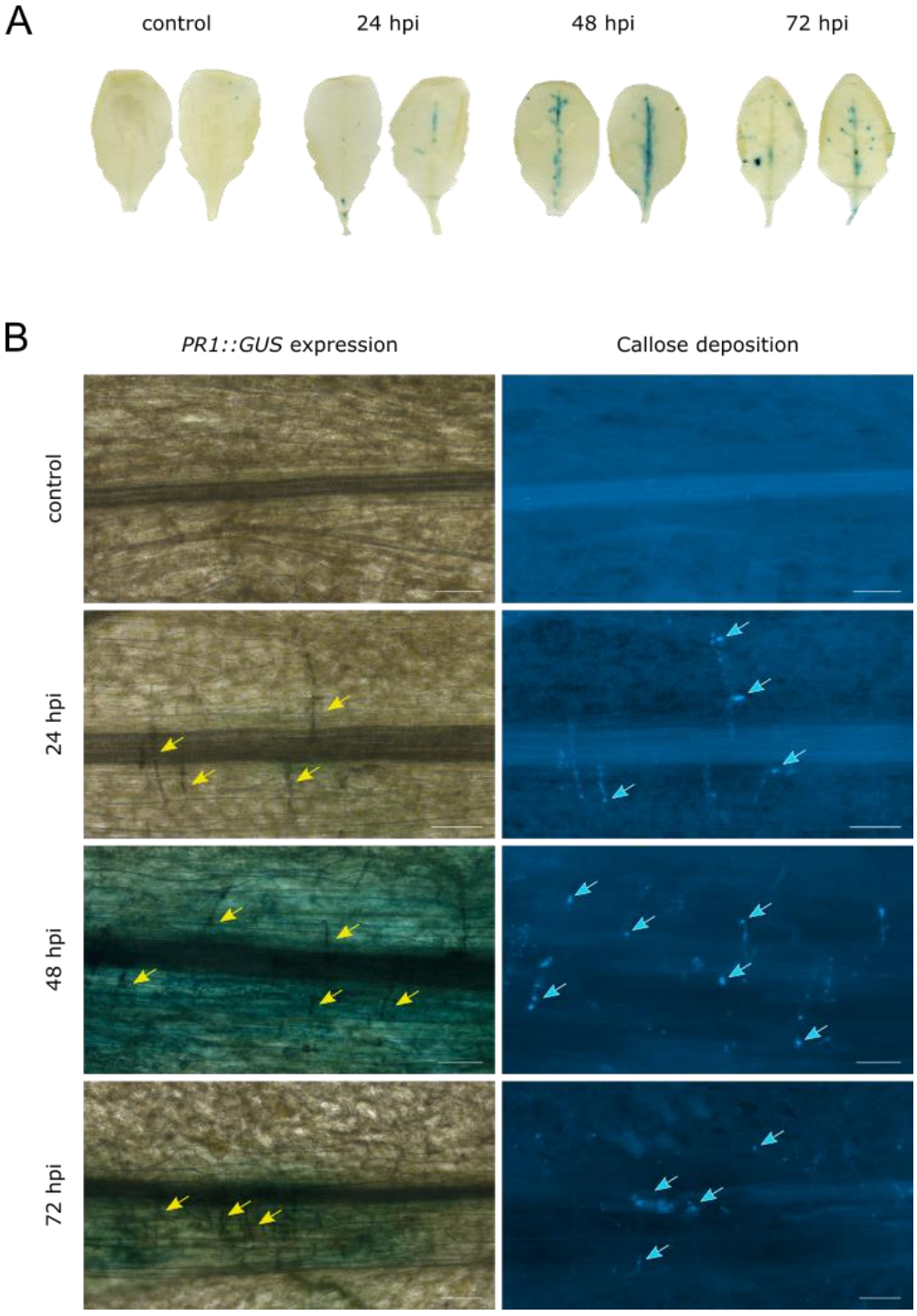

Figure 32. *Brevicoryne brassicae* triggers *PR1* expression and callose deposition. **A:** histochemical staining of β-glucuronidase (GUS) activity in leaves of *PR1::GUS* at 24, 48 and 72 h post infestation (hpi) by *B. brassicae*; **B:** dynamics of *PR1::GUS* expression and callose deposition in leaves at 24, 48 and 72 hpi by *B. brassicae*; yellow arrows indicate the sites of stylet insertion, blue arrows point to deposited callose; scale bar = 100 µm.

5.4.4 Discussion

The spatial dynamics of defence response triggering in leaf tissues in response to aphid feeding remains rather enigmatic. Despite an increasing amount of data, the sensitivity and accuracy of transcriptome studies is largely limited by various factors: (i) sample preparation, as a certain amount of leaf tissues needs to be homogenized, (ii) highly heterogeneous infestation due to the distinct number of individuals feeding on the leaf, (iii) different methods of sampling between studies. Several studies reported the induction of SA and JA signaling responses during the aphid infestation (De Vos et al., 2005; Moran and Thompson, 2001), while attackers differing in a range of host plants, *i.e.* specialists or generalists trigger distinct changes in plant transcriptome (Kuśnierczyk et al., 2007; Mewis et al., 2005; Moran et al., 2002). On the other hand, some of the studies reported changes in gene transcription when sampling the whole rosettes of *Arabidopsis* (Kuśnierczyk et al., 2008; Mewis et al., 2005; Onkokesung et al., 2019) or the individual aphid-infested leaves (Kuśnierczyk et al. 2007; Moran et al. 2002; Moran and Thompson 2001). However, feeding aphids are not homogenously dispersed on the leaf, but mostly localize along the veins, that might introduce a significant bias into sampling.

Changes in the transcription of the gene involved in SA synthesis (*ICS1)* and stress-responsive SA-related genes (*PR1, PR2, PR5*) were detected already at 24 h after *B. brassicae* infestation comparing to non-infested control; however, the values were quite heterogeneous, which might relate to different number of individuals feeding together on the same site. *ICS1* was significantly induced already at 24 hpi, and *ICS2* only from 48 hpi, suggesting that this gene is rather involved in maintaining than in the establishment of the response. Regarding the *PR1* gene, previous studies by Kuśnierczyk and colleagues also reported the strong induction at 72 hpi by a specialist aphid (Kuśnierczyk et al. 2008, 2007; Moran et al. 2002). However, *PR1* fold change presented here was considerably higher, likely due to the analysis of leaf discs closely surrounding the feeding sites, whereas other researchers focused on the analysis of whole leaves or even rosettes. Kuśnierczyk et al. (2007) infested three different ecotypes of *A. thaliana* (Ws, Cvi and Ler) with *B. brassicae* and after harvesting the whole leaves, the induction of *PR1* was five-fold higher in infested Ws ecotype compared to non-infested leaves, two-fold in Cvi ecotype and 21-fold in Ler ecotype, respectively. Moreover, Moran et al. (2002) reported 23-fold induction of *PR1* in infested leaves of *A. thaliana* ecotype Col-0 at 72 hpi. Surprisingly, Onkokesung et al. (2019) observed the induction of *PR1* when sampling whole

leaves of *A. thaliana* ecotype Col-0 (up to 50-fold) only at 6 hpi with *B. brassicae*, whereas there was no induction observed at 24, 48 and 72 hpi. The significant and strong induction at later time points presented here could be caused by the spatial sampling technique, as for the whole leaf sampling the signal may be masked by the background level in many non-induced cells. Indeed, the strongest induction of *PR1* seems to occur locally around the aphid feeding zones (Fig. 30A, 32A), as found when using reporter lines *pPR1::NLS3xVenus* and *PR1::GUS*.

In addition to the SA-related marker genes, an early induction of several JA and ET marker genes in leaf tissues surrounding feeding sites was detected by qRT-PCR. Infestation triggered the transcription of the gene coding for ZIM-domain protein, *JAZ8*, already at 6 hpi, and strongly increased the transcription of *JAZ8* and *JAZ10* genes at 24 and 72 hpi (Fig. 29). On the contrary, genes involved in the octadecanoid signaling pathway, or wound-inducible JA pathway (*LOX2*, *AOS*), were significantly upregulated in aphid-infested samples at 24 and 48 hpi. However, Kuśnierczyk et al. (2007) observed rather low induction of JA biosynthetic genes in the whole leaves of different ecotypes of *Arabidopsis* only at 72 hpi: a fold change in *LOX2* transcription ranged from three to six, and in *AOS* transcription from two to three. Later on, Kuśnierczyk et al. (2008) also reported lower, but still significant induction of *JAZ10* in the whole rosettes of *A. thaliana* ecotype Ler at 6, 24 and 48 hpi (up to 1.4-fold). My qRT-PCR analysis for the genes important for ET synthesis, *ACS2* and *ACS6*, showed the induction already at 6 hpi, indicating an early response to *B. brassicae* attack; then, low but significant induction was observed at 24 hpi, and it was followed by the strong upregulation at 48 and 72 hpi (Fig. 29). These results are in line with other studies reporting the involvement of ET responses during aphid infestations in whole leaves/rosettes (Kuśnierczyk et al., 2008, 2007; Smith and Boyko, 2007). Kuśnierczyk et al. (2007) also observed an increase in *ACS6* transcription in *B. brassicae*-infested leaves of *A. thaliana* ecotype Ws (up to two-fold), Cvi (up to 13-fold) and Ler (up to two-fold) at 72 hpi.

Genes involved in both JA and ET signaling pathways, *HEL/PR4* and *PDF1.2*, were also found to be induced in leaf discs: the infestation caused the strongest upregulation of *PDF1.2* at 48 h, and the transcription of *HEL* increased gradually. Interestingly, Moran et al. (2002) reported higher induction of *PDF1.2* in *B. brassicae*-infested leaves of *A. thaliana* ecotype Col-0 (up to 48-fold) after 72 h of aphid feeding, whereas Kuśnierczyk et al. (2008) reported lower induction of *PDF1.2* in infested rosettes of Ler ecotype at 48 hpi (up to two-fold). Similarly to results shown here, Kuśnierczyk et al. (2008, 2007) reported gradual upregulation of *HEL* gene

in the leaves and whole rosettes of *A. thaliana* ecotype Ler at 6, 24, 48 and 72 hpi; however, the induction was relatively low (up to 2.7-fold).

To visualize the activation of marker genes at a cellular level in different leaf tissues surrounding the stylet insertions, a set of promoter-reporter transgenic lines of *A. thaliana* (Marhavý et al. 2019) were used, expressing fusions of defence gene promoters with mVenus reporter and a nuclear localization signal (NLS). The lines were originally designed to study gene activation in roots during the infestation by nematode *Heterodera schachtii*. Hence, the authors monitored nematode performance from the first moment of root invasion until the feeding establishment and analysed SA, JA and ET-associated responses. During this cellular damage interval, the induction of SA and JA-associated markers was not detected, while the expression of ET marker line was significantly induced. This method was found applicable for experiments with aphids, as with this approach the single aphid "effect zone" can be studied. Using promoter-reporter lines the activation of SA and JA signaling during aphid infestation was confirmed.

The results of callose deposition confirmed previously observed callose formations (Kuśnierczyk et al. 2008) around the stylet insertion sites during *B. brassicae* wounding of *Arabidopsis* cells. Interestingly, generalist aphids seem to trigger similar response in *Arabidopsis*: De Vos et al. (2005) observed *PR1* gene induction around aphid feeding sites in PR1::GUS expressing leaves, but only after 72 h of *Myzus persicae* feeding. This could be explained by variation in protein concentration and composition of saliva between specialist and generalist aphid species that causes different response intensity from plant defence system (Will et al., 2009).

With the development of novel visualization tools, the research on plant/pest interactions is getting more precise and detailed. Here it was shown that the stylet penetration during attack by the *B. brassicae* aphid causes a rapid activation of plant defence signals in the cells surrounding the stylet puncture in *A. thaliana*. In time, the induction spreads along the veins and, to a lesser extent, radially to leaf tissues. Subtle, though biologically important changes in transcriptomic response can be detected by performing precise sampling procedures (*i.e.* collecting leaf discs around feeding sites instead of pooling material from the whole infested rosettes or leaves). Local transcriptomic remodelling seems to occur faster in tissues close to feeding sites than in the rest of the leaf when comparing to previously published studies. It can also be suggested that important dynamics in space and time soon after herbivore attack may

be missed if not using spatially explicit sampling, as also found for the reaction of leaf photosynthesis to herbivory (Moustaka et al., 2021). Attention should be directed to the need for a detailed description of sampling protocols that might influence the data output, especially for high-throughput methods such as transcriptomics or metabolomics. Thorough documentation of the starting material enhances the quality, reliability, and reproducibility of omics research, enabling scientists to draw more accurate and meaningful conclusions from their studies.

5.5 Can *Metarhizium robertsii* prime cauliflower for defence against *Leptosphaeria maculans*?

5.5.1 Introduction

Metarhizium robertsii, a renowned entomopathogenic fungus, has recently garnered attention for its potential as a root-beneficial microorganism. Traditionally recognized for its effectiveness in biocontrol against various insect pests, emerging research suggests that *M. robertsii* may also confer benefits to plants through symbiotic associations with their roots (Barelli et al., 2016; Cachapa et al., 2021). This shift in perspective highlights the multifaceted nature of microbial interactions within the rhizosphere and raises intriguing possibilities for agricultural applications. One particularly intriguing aspect of *M. robertsii* interaction with plants is its potential to modulate the host susceptibility to subsequent pathogen attacks. These alterations may lead to enhanced resistance against certain pathogens, thereby reducing the need for chemical interventions in crop protection strategies. However, the intricate mechanisms orchestrating this phenomenon remain incompletely understood, varying depending on the specific plant host and the type of pathogen involved. Understanding the mechanisms by which *M. robertsii* influences plant susceptibility to subsequent attacks holds significant implications for sustainable agriculture. By harnessing the beneficial effects of this fungus, farmers may be able to bolster crop resilience while minimizing reliance on synthetic pesticides. However, further research is needed to elucidate the intricacies of *M. robertsii*-plant-pathogen interactions and to optimize its utilization in agricultural settings. In this chapter, I try to elucidate the current understanding of *M. robertsii* as a root-beneficial fungus in cauliflower plants and explore its potential to alter plant susceptibility to subsequent infection by *L. maculans*. I examine the underlying mechanisms driving these interactions and discuss their implications for crop protection and sustainable agriculture. Additionally, I highlight areas for future research aimed at maximizing the beneficial effects of *M. robertsii* while minimizing any potential drawbacks.

5.5.2 Experimental design

Experiments with true leaves of cauliflower

Cauliflower seeds were sown individually in pots with non-sterilized soil or soil sterilized at 80°C for 72 h and covered with a plastic foil to increase the humidity for faster germination. Two to four days after, germinating seeds were inoculated with previously prepared spore suspension of *M. robertsii* KVL 12-35 (1 ml of 10^8 spore ml^{-1}). Distilled water and 0.05 % Triton X were used as controls. Three weeks after inoculation, when first true leaves have expanded, leaf discs were collected for the gene transcription and GLS analyses, and roots were harvested for performing the root colonization test (0 dpi – before *L. maculans* inoculation). At the same day, the rest of the plants were inoculated with the spore suspension of *L. maculans* JN3 or JN3-GFP by "puncture and drop" (10 µl of 10^7 spore ml^{-1}). Distilled water was used as control. Plants were covered for 48 h to increase the humidity and facilitate the infection. Leaf discs were collected 7 and 14 days after *L. maculans* inoculation (dpi), by sampling 5 to 6 mm-diameter discs around the puncture site, and later used for qRT-PCR and GLS analyses, and for microscopic observation of the hyphal spread. Roots were harvested at the same day for checking the establishment of *M. robertsii* (7 and 14 dpi – after *L. maculans* inoculation).

Experiments with cotyledons of cauliflower

Cauliflower seeds were sown in the soil previously sterilized on 80°C for 72 h, and the pots were covered with a plastic foil. Three days after, germinating seeds were used for inoculation with previously prepared spore suspension of *M. robertsii* KVL 12-35 (1 ml of 10^8 spore ml^{-1}). Distilled water and 0.05 % Triton X were used as controls. Two weeks after *M. robertsii* inoculation, cotyledons were inoculated with the spore suspension of *L. maculans* JN3-GFP by syringe infiltration (10^5 spore ml^{-1}), and roots were harvested for the root colonization test. Cotyledons were infiltrated with distilled water as a respective control. Then, 5, 7 and 11 days after *L. maculans* inoculation (dpi), whole cotyledons were detached and observed under fluorescent microscope and scanned subsequently. Roots were collected at the same day for analysis of fungal colonization. The hyphal growth was mostly not observed at 5 dpi (data not shown) but it started at 7 and excessively expanded at 11 dpi. Cotyledon discs (6 mm-diameter) were also harvested at 11 dpi for qRT-PCR analysis.

5.5.3 Results

5.5.3.1 *Metarhizium robertsii* successfully colonizes roots of cauliflower plants

The root colonization test confirmed that *M. robertsii* successfully colonizes cauliflower roots at 0, 5, 7, 11 and 14 dpi with *L. maculans*. Root samples of *M. robertsii*-treated plants showed characteristic dark green colonies and white hyphal growth on selective media, in contrast to control samples (distilled water- and 0.05 % Triton X-treated) which showed no fungal growth (Fig. 33).

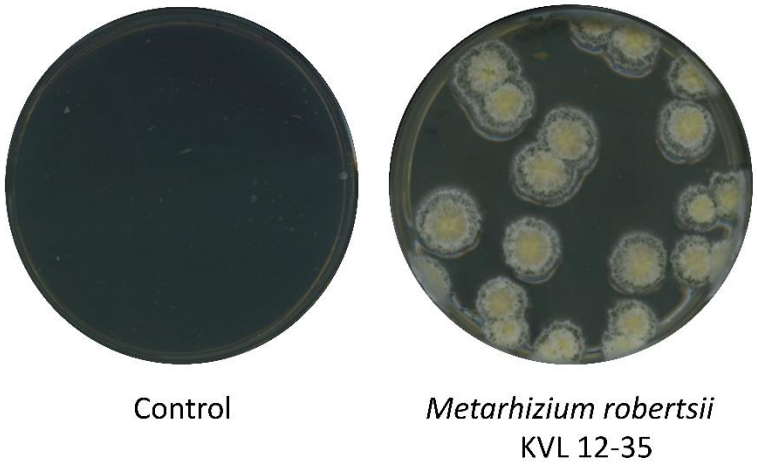

Figure 33. Root colonization test performed to confirm the presence of *Metarhizium robertsii* KVL 12-35 on treated roots of cauliflower and to exclude the contamination on the respective controls (distilled water- and 0.05 % Triton X-treated).

5.5.3.2 Successful disease development by *Leptosphaeria maculans* highly dependent on the inoculation method and leaf stage

5.5.3.2.1 *Leptosphaeria maculans* infection in the true leaves of cauliflower

The symptoms of *L. maculans* infection in the true leaves developed only in the first experimental repetition (Fig. 34A), whereas no symptoms were detected in the second repetition (Fig. 34B). Lesion development in the first repetition was quite weak, and only on several leaves. Nonetheless, the transcript level of *LmITS* gene (Fig. 35A, B; Tab. 6 and 7) and the microscopic imaging of GFP-tagged JN3 isolate (Fig. 36) confirmed that *L. maculans* was present in all *L. maculans* treatments around the puncture site.

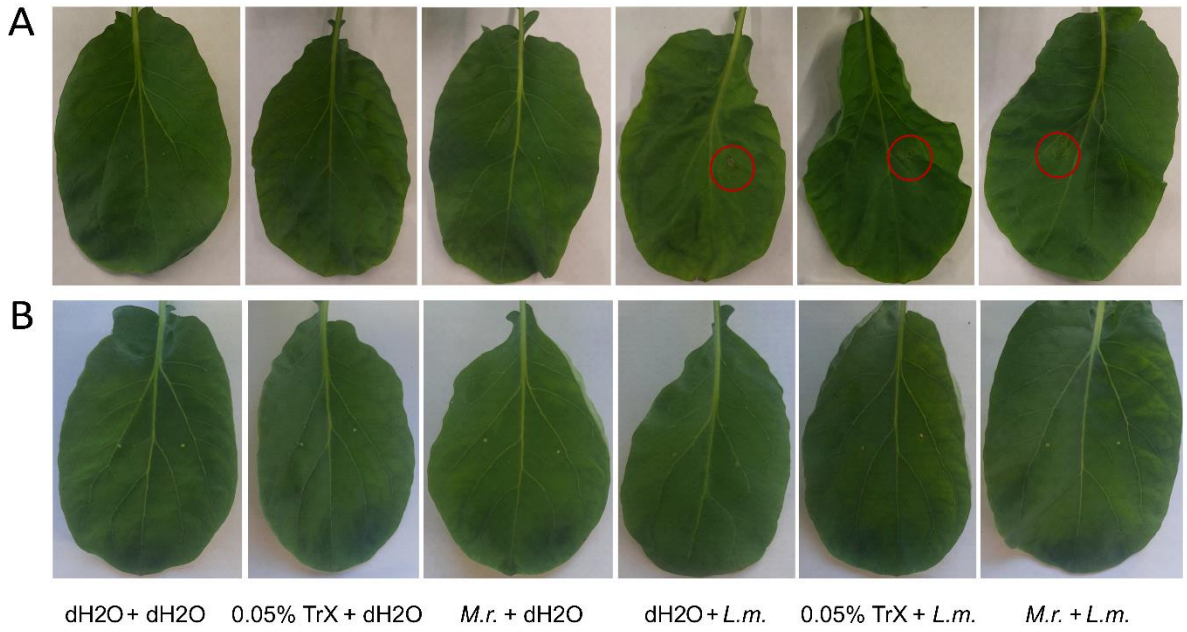

Figure 34. Lesion development in the true leaves of cauliflower 14 days post *Leptosphaeria maculans* (*L.m.*) inoculation by "puncture and drop". Roots were previously inoculated with *Metarhizium robertsii* (*M.r.*). Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % of Triton X (0.05% TrX). **A:** first biological repetition with developed symptoms marked with the red circle; **B:** second biological repetition with no visual symptoms.

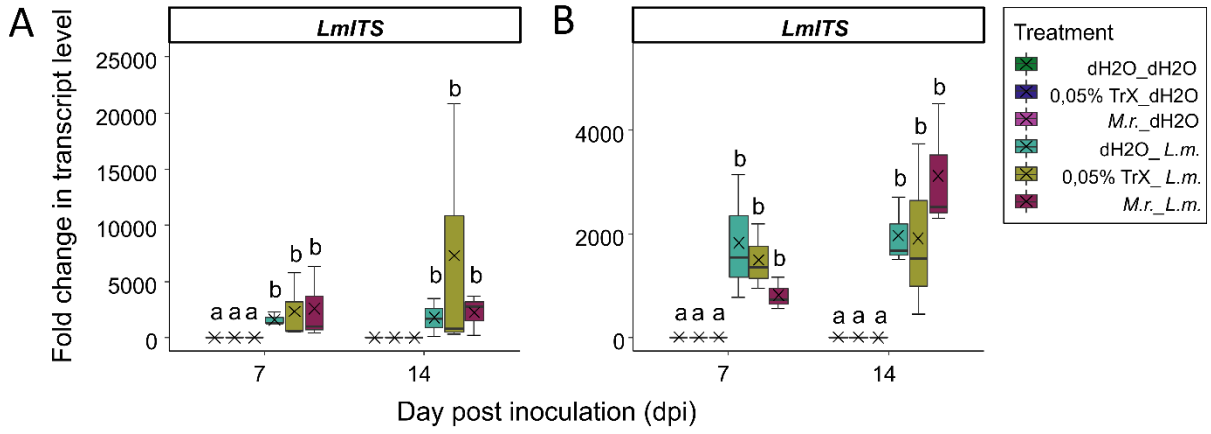

Figure 35. Transcription of *LmITS* (*Leptosphaeria maculans internal transcribed spacer*) gene in the true leaves of cauliflower inoculated with *L. maculans* (*L.m.*), and previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % Triton X (0.05% TrX). Gene transcriptions were detected 7 and 14 days post *L. maculans* inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles,

and lowercase letters significant differences ($P < 0.05$) between treatments for a given time point. **A:** first biological repetition with developed symptoms; **B:** second biological repetition with no recorded symptoms.

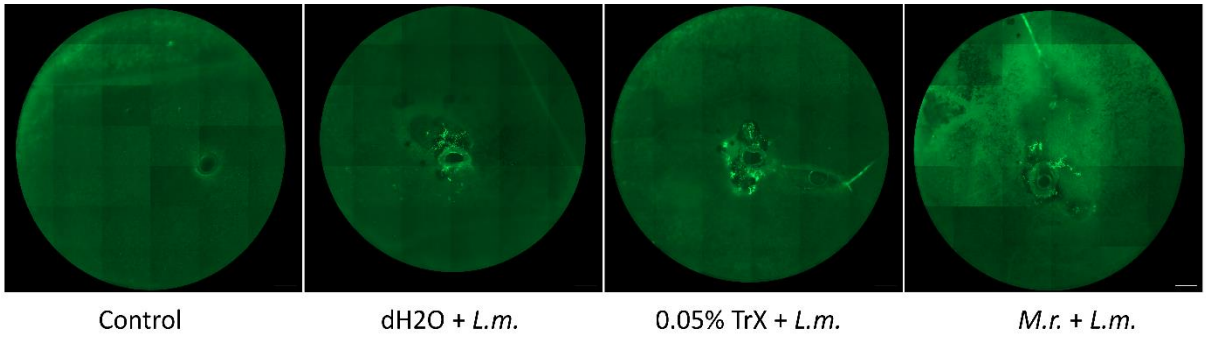

Figure 36. Microscopic imaging of 6 mm leaf discs of cauliflower14 days after *Leptosphaeria maculans* (*L.m.*) inoculation by "puncture and drop". Roots were previously inoculated with *Metarhizium robertsii* (*M.r.*). Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % of Triton X (0.05% TrX). Scale bar = 500 μm.

The activation of the SA, JA, ET, and combined JA/ET signaling pathways in the true leaves of cauliflower was monitored by analyzing the transcription of specific marker genes. For SA, the biosynthetic gene *ICS1* and the responsive gene *WRKY70* were assessed. JA signaling was evaluated through the biosynthetic genes *LOX3* and *AOS*. ET pathway activation was determined by the biosynthetic gene *ACS2*. The responsive genes *PDF3* and *PR4* were used to monitor both JA and ET pathways. Additionally, the genes *rbohC* and *rbohF*, which are involved in the oxidative stress response, were examined.

In the first biological repetition, the SA pathway was activated through *WRKY70* and the JA/ET pathway through *PR4* in all *L. maculans* (*L.m.*) treatments at 14 dpi compared to controls, whereas the rest of the tested genes did not show any significant alteration (Fig. 37; Tab. 6). None of the genes were significantly upregulated or downregulated at all time points in the second biological repetition compared to control treatments; however, *WRKY70* and *PR4* followed a similar trend as observed in the first repetition (Fig. 38; Tab. 7).

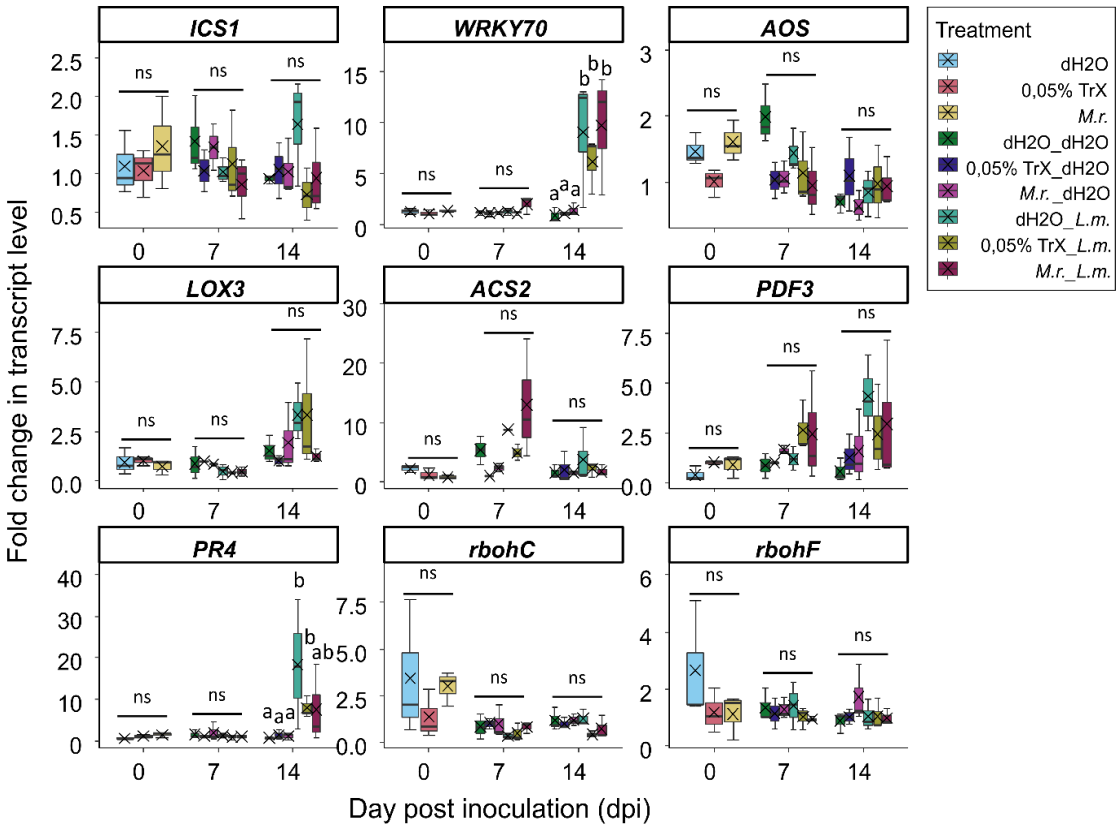

Figure 37. Activation of plant defence pathways detected in the first biological repetition in the true leaves of cauliflower inoculated with *Leptosphaeria maculans* (*L.m.*), and previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % Triton X (0.05% TrX). Gene transcripts were detected 0, 7 and 14 days post *L. maculans* inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences (P < 0.05) between treatments for a given time point. *ICS1 = isochorismate synthase 1*; *WRKY70 = WRKY70 transcription factor; AOS = allene oxide synthase; LOX3 = lipoxygenase 3*; *ACS2 = ACC synthase 2*; *PDF3 = plant defensin 3*; *PR4 = pathogenesis-related 4*; *rbohC = respiratory burst oxidase homolog C*; *rbohF = respiratory burst oxidase homolog F*; ns = not significant.

Table 6. Statistical values of defence genes in the first biological repetition. F values, df, P values and residual df for all the analysed compounds; ".": P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

	Treatment			Time			Treatment:Time			
Compound	**F value**	**df**	**P value**	**F value**	**df**	**P value**	**F value**	**df**	**P value**	**Residual df**

isochorismate synthase 1, ICS1	0.83	7	0.64		0.56	1	0.59		1.04	5	0,54		29
WRKY70 transcription factor, WRKY70	5.55	7	0.00	***	20.71	1	0,00	***	4,82	5	0.02	*	29
allene oxide synthase, AOS	1.08	7	0.54		10.15	1	0.01	**	1.92	5	0.27		29
lipoxygenase 3, LOX3	0.38	7	0.91		24.21	1	0.00	***	2.12	5	0.27		29
ACC synthase 2, ACS2	1.87	7	0.33		17.85	1	0.00	***	1.52	5	0.39		26
plant defensin 3, PDF3	1.70	7	0.33		0.09	1	0.76		1.03	5	0.54		29
pathogenesis-related 4, PR4	2.46	7	0.19		11.10	1	0.01	**	4.26	5	0,02	*	28
respiratory burst oxidase homolog C, rbohC	1.46	7	0.40		1.79	1	0.29		0.72	5	0.69		29
respiratory burst oxidase homolog F, rbohF	1.05	7	0.54		0.18	1	0.76		0.26	5	0.93		29
Leptosphaeria maculans internal transcribed spacer, LmITS	14.28	5	0.00	***	0.02	1	0.90		0.10	5	0.99		23

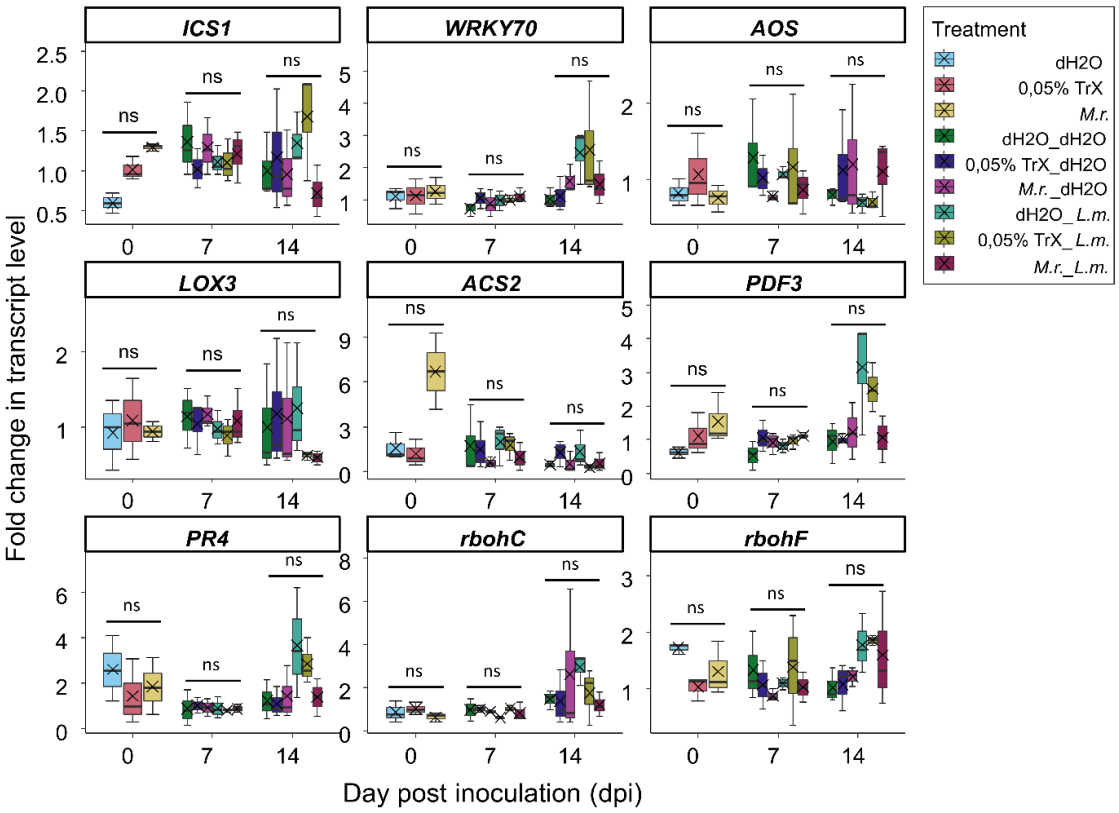

Figure 38. Activation of plant defence pathways detected in the second biological repetition in the true leaves of cauliflower inoculated with *Leptosphaeria maculans* (*L.m.*), and previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % Triton X (0.05% TrX). Gene transcripts were detected 0, 7 and 14 days post *L. maculans* inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences (P < 0.05) between treatments for a given time point. *ICS1 = isochorismate synthase 1*; *WRKY70 = WRKY70 transcription factor; AOS = allene oxide synthase; LOX3 = lipoxygenase 3*; *ACS2 = ACC synthase 2*; *PDF3 = plant defensin 3*; *PR4 = pathogenesis-related 4*; *rbohC = respiratory burst oxidase homolog C*; *rbohF = respiratory burst oxidase homolog F*; ns = not significant.

Table 7. Statistical values of defence genes in the second biological repetition. F values, df, P values and residual df for all the analysed compounds; ".": P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

	Treatment			Time			Treatment:Time			
Compound	**F value**	**df**	**P value**	**F value**	**df**	**P value**	**F value**	**df**	**P value**	**Residual df**

isochorismate synthase 1, ICS1	1.60	7	0.49		0.66	1	0.42		1.60	5	0.69	30
WRKY70 transcription factor, WRKY70	1.47	7	0.49		14.38	1	0.01	**	1.28	5	0.69	30
allene oxide synthase, AOS	0.24	7	0.97		0.73	1	0.42		0.95	5	0.69	30
lipoxygenase 3, LOX3	0.50	7	0.98		1.34	1	0.33		0.45	5	0.81	30
ACC synthase 2, ACS2	2.37	7	0.23		3.90	1	0.10		0.48	5	0.81	30
plant defensin 3, PDF3	2.34	7	0.23		7.15	1	0.04	*	1.84	5	0.69	30
pathogenesis-related 4, PR4	1.06	7	0.63		7.95	1	0.04	*	1.14	5	0.69	30
respiratory burst oxidase homolog C, rbohC	0.29	7	0.97		6.48	1	0.04	*	1.04	5	0.69	30
respiratory burst oxidase homolog F, rbohF	1.05	7	0.63		3.30	1	0.12		0.84	5	0.69	30
Leptosphaeria maculans internal transcribed spacer, LmITS	117.45	5	0.00	***	3.13	1	0.18		2.49	5	0.12	24

Three individual GLSs from the group of aliphatic-derived GLSs, *i.e.* glucoiberin, sinigrin and glucoibarin, and three indole-derived GLS compounds, *i.e.* glucobrassicin, 4-methoxyglucobrassicin and neoglucobrassicin were analysed. The chemical names of all the compounds are listed in Tab. 8 and 9.

Total GLS, AG and IG level was not significantly altered by the treatment and the interaction between time and treatment in the first biological repetition (Fig. 39; Tab. 8). The content of the aliphatic glucoiberin was significantly higher in 0.05 % Triton X-treated plants at 0 dpi than in *M. robertsii*-inoculated and distilled water-treated plants (Fig. 39; Tab. 8). The

content of glucoibarin differed significantly between treatments only at 14 dpi: higher content was detected in 0.05 % Triton X-treated plants (0.05% TrX_dH2O, 0.05% TrX_*L.m.*) compared to other treatments (Fig. 39; Tab. 8). From all the tested indole GLSs, only 4-methoxyglucobrassicin showed a significant alteration, and only at 14 dpi: the level was higher in 0.05 % Triton X and *L. maculans* (0.05% TrX_*L.m.*) samples compared to distilled water and *L. maculans* (0.05% dH2O_*L.m.*) (Fig. 39; Tab. 8).

Total GLS and IG level was not altered in the second biological repetition; however, the total AG level showed significant differences at 14 dpi, specifically sinigrin: the level of the compound was significantly higher in 0.05 % Triton X and distilled water-treated (0.05% TrX_dH2O) plants compared to other treatments. The level of the other analysed individual compounds was not changed (Fig. 40, Tab. 9).

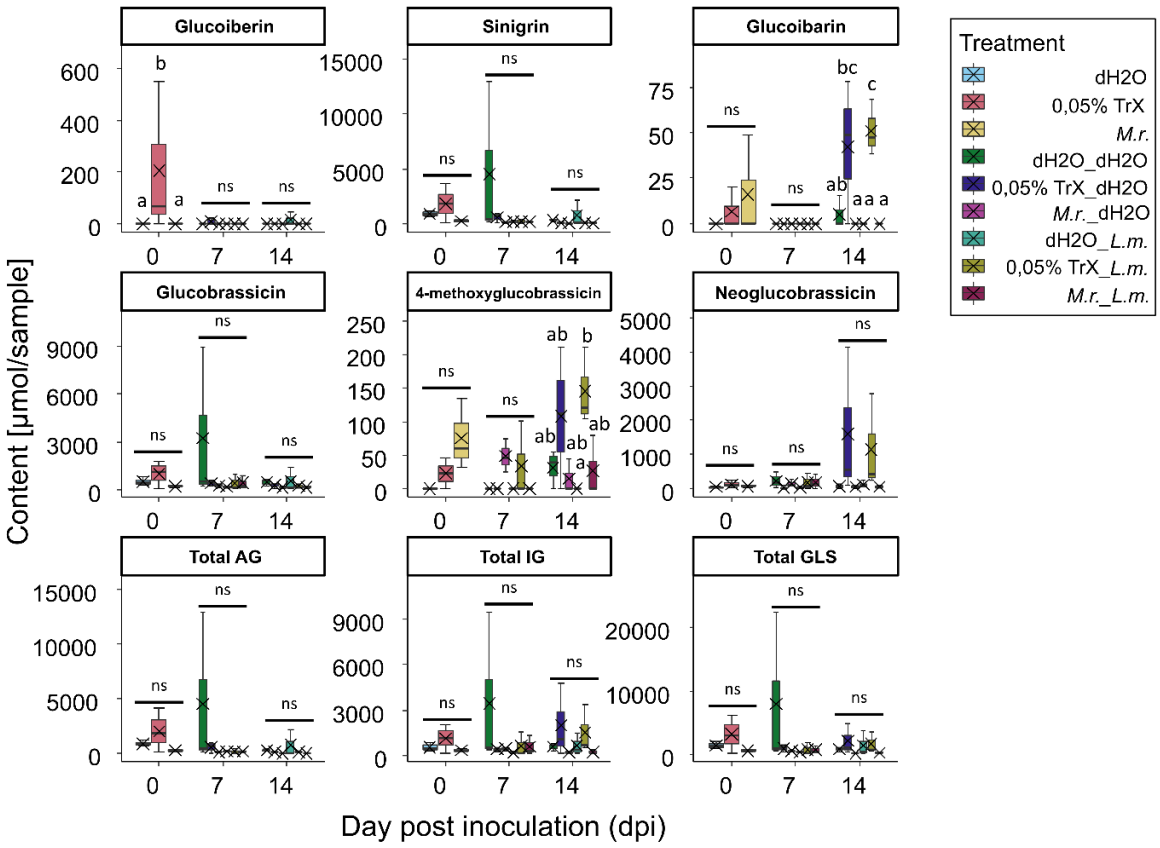

Figure 39. Total glucosinolate (GLS) content in the first biological repetition in the true leaves of cauliflower inoculated with *Leptosphaeria maculans* (*L.m.*), and previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as a control on the leaves,

and on the roots together with 0.05 % Triton X (0.05% TrX). GLSs were analysed 0, 7 and 14 days post *L. maculans* inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant differences (P < 0.05) between treatments for a given time point. Aliphatic compounds: glucoiberin, sinigrin, glucoibarin; aromatic/indole compounds: glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin. Abbreviations: AG = aliphatic glucosinolate; IG = indole glucosinolate; ns = not significant.

Table 8. Statistical values of glucosinolates (GLSs) in the first biological repetition. F values, df, P values and residual df for all the analysed compounds; ".": P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

Compound	Treatment			Time			Treatment:Time			
	F value	df	P value	F value	df	P value	F value	df	P value	Residual df
Glucoiberin (3-methylsulfinylpropyl, 3-msp)	3.18	7	0.05 *	0.01	1	0.93	0.59	5	0.79	30
Sinigrin (2-propenyl)	2.44	7	0.08	5.12	1	0.08	0.86	5	0.67	30
Glucoibarin (7-methylsulfinylheptyl, 7-msh)	3.04	7	0.05 *	12.94	1	0.01 **	3.75	5	0.05 *	30
Total aliphatic (AG)	2.47	7	0.08	4.14	1	0.09	0.92	5	0.67	30
Glucobrassicin (indol-3-ylmethyl, I3M)	1.22	7	0.42	1.24	1	0.41	0.46	5	0.80	30
4-methoxygluco brassicin (4-methoxyindol-3-ylmethyl, 4MOI3M)	3.95	7	0.04	7.05	1	0.06	3.27	5	0.05 *	30
Neoglucobrassicin (N-methoxyindol-3-ylmethyl, NMOI3M)	0.59	7	0.76	4.90	1	0.08	3.33	5	0.05 *	30
Total indole (IG)	0.99	7	0.51	0.07	1	0.89	1.38	5	0.59	30

Total GLS	1.26	7	0.42	0.17	1	0.88	1.06	5	0.67	30

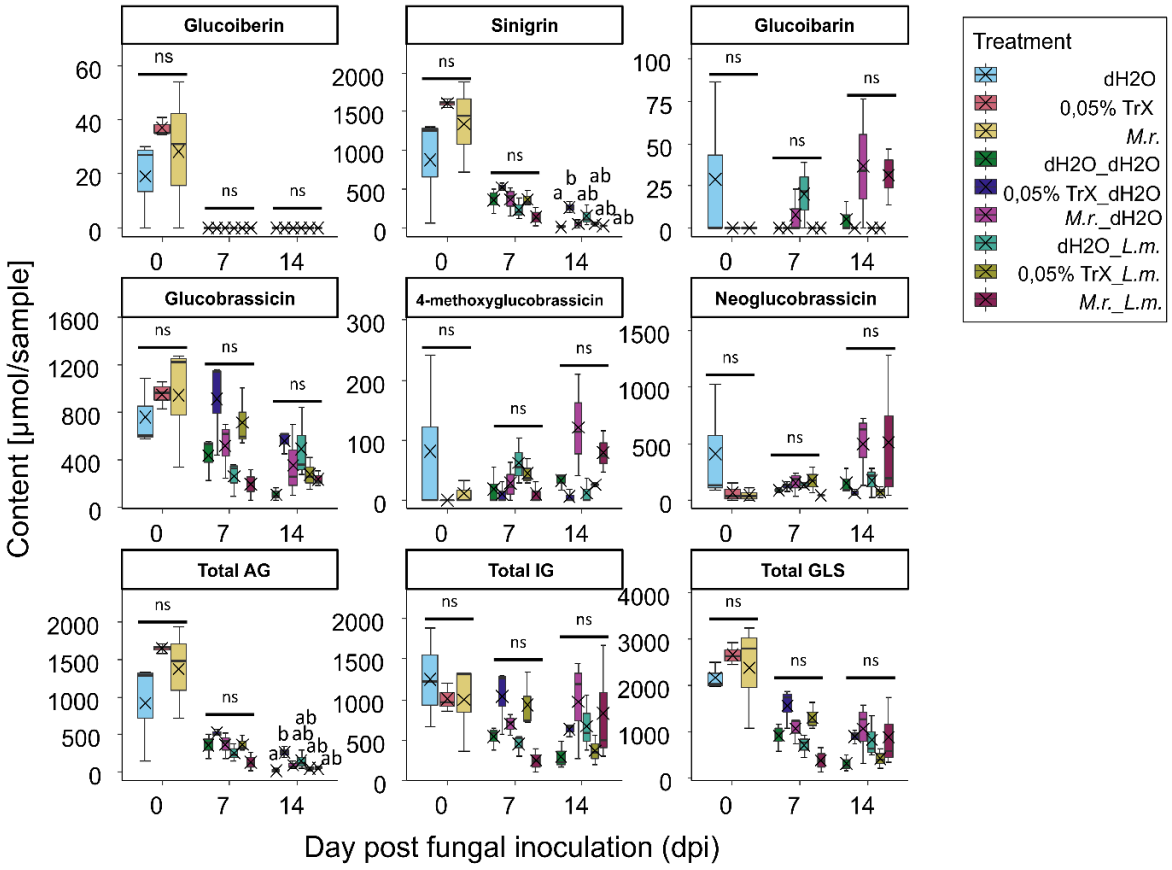

Figure 40. Total glucosinolate (GLS) content in the second biological repetition in the true leaves of cauliflower inoculated with *Leptosphaeria maculans* (*L.m.*), and previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % Triton X (0.05% TrX). GLSs were analysed 0, 7 and 14 days post *L. maculans* inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles. Aliphatic compounds: glucoiberin, sinigrin, glucoibarin; aromatic/indole compounds: glucobrassicin, 4-methoxyglucobrassicin, neoglucobrassicin. Abbreviations: AG = aliphatic glucosinolate; IG = indole glucosinolate; ns = not significant.

Table 9. Statistical values of glucosinolates (GLSs) in the second biological repetition. F values, df, P values and residual df for all the analysed compounds; ".": P < 0.1, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

Compound	Treatment			Time				Treatment:Time			Residual
	F value	df	P value	F value	df	P value		F value	df	P value	df
Glucoiberin (3-methylsulfinylpropyl, 3-msp)	0.80	7	0.59	0.00	1	1.00		0.00	5	1.00	30
Sinigrin (2-propenyl)	2.74	7	0.08	27.07	1	0.00	***	1.74	5	0.20	30
Glucoibarin (7-methylsulfinylheptyl, 7-msh)	2.35	7	0.11	2.23	1	0.26		3.57	5	0.09	30
Total aliphatic (AG)	3.30	7	0.07	29.10	1	0.00	***	2.33	5	0.10	30
Glucobrassicin (indol-3-ylmethyl, I3M)	3.02	7	0.07	4.45	1	0.10		2.54	5	0.09	30
4-methoxygluco brassicin (4-methoxyindol-3-ylmethyl, 4MOI3M)	1.54	7	0.25	1.54	1	0.34		2.65	5	0.09	30
Neoglucobrassicin (N-methoxyindol-3-ylmethyl, NMOI3M)	1.63	7	0.25	0.82	1	0.48		1.20	5	0.38	30
Total indole (IG)	1.39	7	0.28	0.23	1	0.72		2.72	5	0.09	30
Total GLS	2.03	7	0.15	4.37	1	0.10		3.12	5	0.09	30

The redundancy analysis (RDA) of 0 dpi showed that the experimental design explained 36 % of the total variance in the first biological repetition (Fig. 41A), and 21 % of the total variance in the second biological repetition (Fig. 41B). The axes 1 and 2 explained 57 % and 42 % of the constrained variance (Fig. 41A), and 89 % and 10 % of the constrained variance (Fig. 41B), respectively. The GLS composition profile was not significantly influenced by the treatment in the first biological repetition ($F_{2,3}$ = 1.71; P = 0.15), nor in the second biological

repetition ($F_{2,3}$ = 0.81; P = 0.62). Nonetheless, RDA points that glucoiberin, sinigrin and glucobrassicin were higher in 0.05 % Triton X-treated plants in both biological repetitions (Fig. 41A, B).

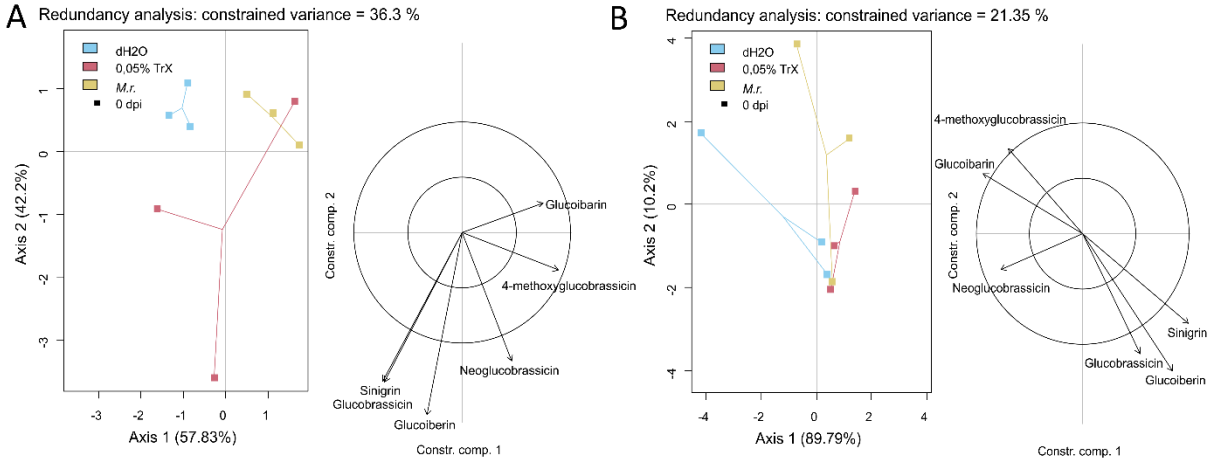

Figure 41. Redundancy analysis (RDA) of glucosinolate (GLS) content in the true leaves of cauliflower before inoculation with *Leptosphaeria maculans* (0 dpi), and previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % Triton X (0.05% TrX). Score plot is shown on the left, with variances explained by the synthetic axes written in parentheses, and the correlation circle on the right. **A:** first biological repetition; **B:** second biological repetition.

The RDA of 7 dpi showed that the experimental design explained 38 % of the total variance in the first biological repetition (Fig. 42A), and 47 % of the total variance in the second biological repetition (Fig. 42B). The axes 1 and 2 explained 51 % and 34 % of the constrained variance (Fig. 42A), and 55 % and 37 % of the constrained variance (Fig. 42B), respectively. The GLS composition profile was not significantly influenced by the treatment ($F_{5,3}$ = 1.47; P = 0.06) in the first biological repetition, contrary to the second biological repetition ($F_{5,3}$ = 2.20, P = 0.01). Glucobrassicin and sinigrin were higher in samples treated with 0.05 % Triton X and distilled water (0.05% TrX_dH2O) and distilled water and *L. maculans* samples (dH2O_*L.m.*) in both biological repetitions (Fig. 42A, B).

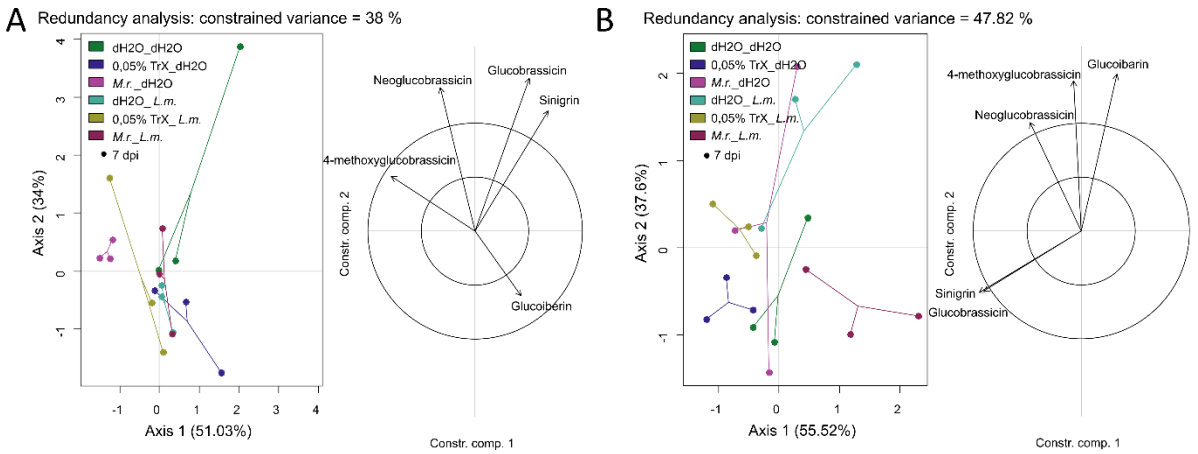

Figure 42. Redundancy analysis (RDA) of glucosinolate (GLS) content in the true leaves of cauliflower 7 days after inoculation with *Leptosphaeria maculans* (*L.m.*), previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % Triton X (0.05% TrX). Score plot is shown on the left, with variances explained by the synthetic axes written in parentheses, and the correlation circle on the right. **A:** first biological repetition; **B:** second biological repetition.

The RDA of 14 dpi showed that the experimental design explained 47 % of the total variance in the first biological repetition (Fig. 43A), and 56 % of the total variance in the second biological repetition (Fig. 43B). The axes 1 and 2 explained 58 % and 30 % of the constrained variance (Fig. 43A), and 75 % and 20 % of the constrained variance (Fig. 43B), respectively. The GLS composition profile was significantly influenced by the treatment in the first biological repetition ($F_{5,3}$ = 2.18; P = 0.01) and in the second biological repetition ($F_{5,3}$ = 3.16; P = 0.00). For instance, glucoibarin, 4-methoxyglucobrassicin and neoglucobrassicin were highly elevated in 0.05 % Triton X (0.05% TrX_dH2O, 0.05% TrX_*L.m.*) samples in the first biological repetition (Fig. 43A), whereas the higher content of the same compounds was observed in *M. robertsii* (*M.r.*_dH2O, *M.r._L.m.*) samples in the second biological repetition (Fig. 43B).

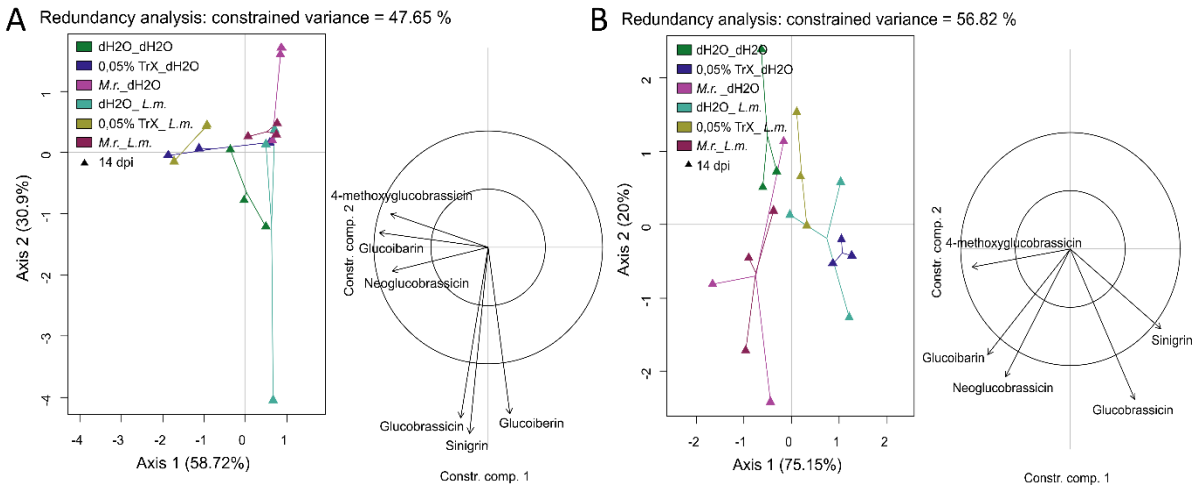

Figure 43. Redundancy analysis (RDA) of glucosinolate (GLS) content in the true leaves of cauliflower 14 days post inoculation with *Leptosphaeria maculans* (*L.m.*), previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as control on the leaves, and on the roots together with 0.05 % Triton X (0.05% TrX). Score plot is shown on the left, with variances explained by the synthetic axes written in parentheses, and the correlation circle on the right. **A:** first biological repetition; **B:** second biological repetition.

5.5.3.2.2 *Leptosphaeria maculans* infection in the cotyledons of cauliflower

Microscopic imaging of GFP-tagged JN3 isolate of *L. maculans* in the cotyledons of cauliflower confirmed the mycelium growth started to be visible 7 days post *L. maculans* inoculation (dpi) and was strongly expanded at 11 dpi (Fig. 44B). The fluorescence area of *L. maculans* mycelium was significantly higher in plants previously treated with 0.05 % Triton X (0.05% TrX) and *M. robertsii* (*M.r.*) compared to plants treated only with distilled water (dH2O), and even higher in *M.r.*-inoculated plants compared to 0.05% TrX-treated plants (Fig. 44A). Moreover, mycelium caused lesion development (Fig. 45B), and the transcript level of *LmITS* gene confirmed that *L. maculans* was present in higher amounts in plants previously treated with 0.05% TrX and *M.r.*, compared to plants treated only with dH2O (Fig. 45A).

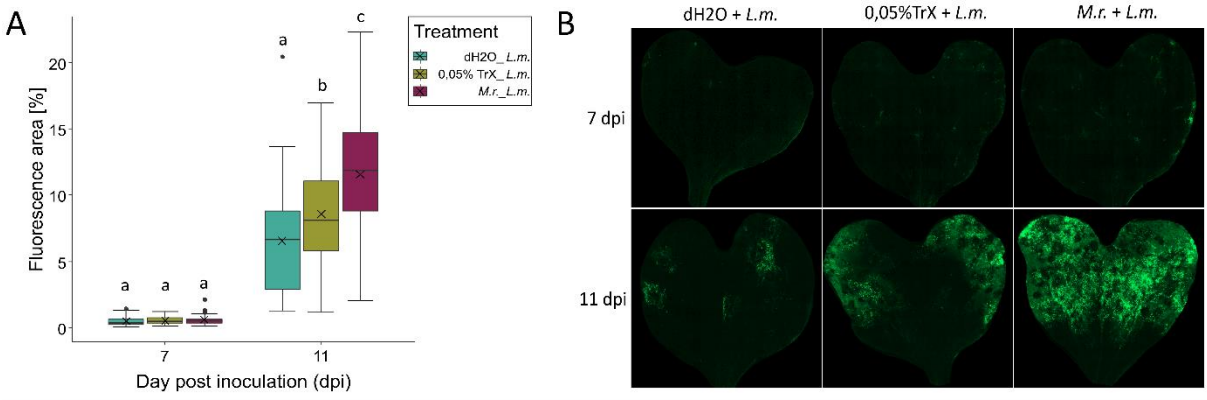

Figure 44. Fluorescence area of *Leptosphaeria maculans* (*L.m.*) mycelium formation 7 and 11 days post inoculation (dpi) by syringe infiltration in the cotyledons of cauliflower, previously treated with distilled water (dH2O), 0.05 % Triton X (0.05% TrX) or *Metarhizium robertsii* (*M.r.*) on the roots. **A:** percentage of fluorescence area of mycelium in cotyledons; **B:** representative images of mycelium development in cotyledons of cauliflower.

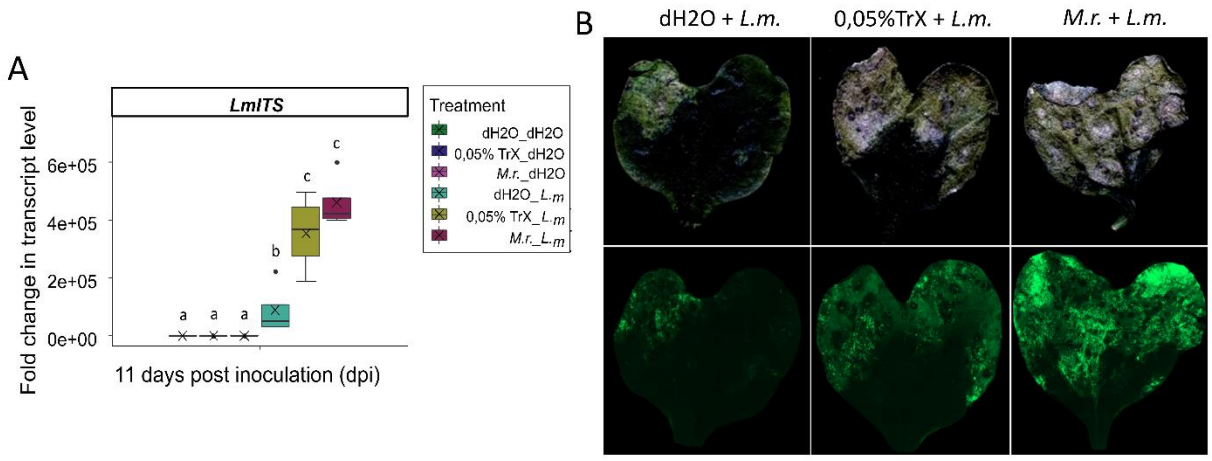

Figure 45. *Leptosphaeria maculans* (*L.m.*) development in the cotyledons of cauliflower 11 days post inoculation (dpi), previously treated with distilled water (dH2O), 0.05 % Triton X (0.05% TrX) or *Metarhizium robertsii* (*M.r.*) on the roots. **A:** *LmITS* transcription confirming the significantly higher presence of *L. maculans* in the samples treated with 0.05 % Triton X (0.05% TrX) and *Metarhizium robertsii* (*M.r.*) on the roots compared to the samples with distilled water treatment (dH2O); **B:** *L. maculans* lesion development and mycelium formation in the cotyledons of cauliflower.

Infection caused by *L. maculans* at 11 dpi caused an upregulation of genes involved in SA signaling (Fig. 46), *i.e.* biosynthetic *ICS1* and responsive *WRKY70*; however, previous root

treatment with 0.05 % Triton X (0.05% TrX_*L.m.*) caused downregulation of both genes compared to root treatment with water (dH2O_*L.m.*), and did not differ compared to root treatment with *M. robertsii* (*M.r._L.m.*). The gene involved in biosynthesis of JA, *AOS*, was significantly upregulated by *L. maculans*, but only in the combination with 0.05 % Triton X (0.05% TrX_*L.m.*) or *M. robertsii* (*M.r._L.m.*; Fig. 46). The combination of *M. robertsii* and *L. maculans* (*M.r._L.m.*) caused the induction of gene involved in ET signaling, *ACS2*, compared to dH2O_*L.m.* treatment (Fig. 46). Genes involved in JA/ET pathway, *i.e. PDF3* and *PR4*, and genes involved in oxidative stress responses, *i.e. rbohF* were significantly downregulated by 0.05% TrX_*L.m.* and *M.r._L.m.* compared to dH2O_*L.m.*, whereas *rbohC* only by *M.r._L.m.* compared to dH2O_*L.m.* treatment (Fig. 46).

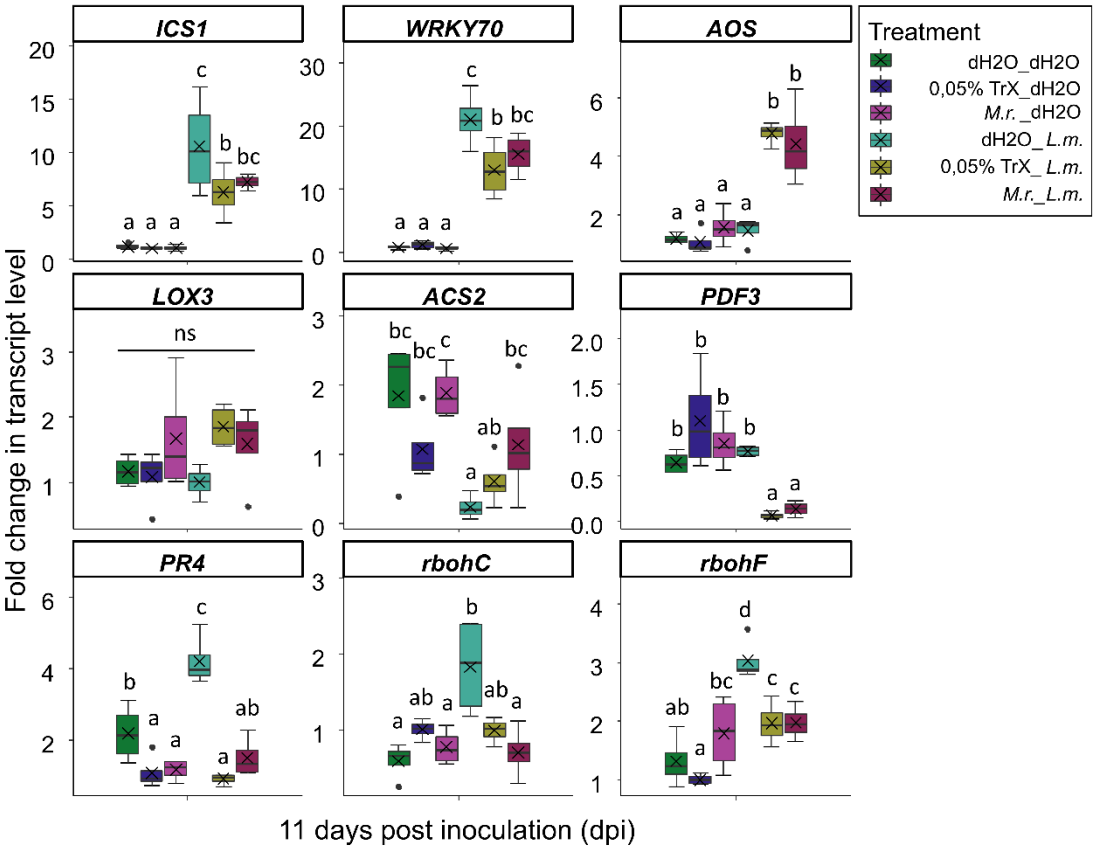

Figure 46. Activation of plant defence pathways detected in the cotyledons of cauliflower inoculated with *Leptosphaeria maculans* (*L.m.*), and previously inoculated with *Metarhizium robertsii* (*M.r.*) on the roots. Distilled water (dH2O) was used as a control on the leaves, and on the roots together with 0.05 % Triton X (0.05% TrX). Gene transcripts were detected 11 days post *L. maculans* inoculation (dpi). Boxplots show mean (´x´), median, 25th and 75th percentiles, and lowercase letters significant

differences (P < 0.05) between treatments for a given time point. *ICS1 = isochorismate synthase 1*; *WRKY70 = WRKY70 transcription factor; AOS = allene oxide synthase; LOX3 = lipoxygenase 3*; *ACS2 = ACC synthase 2*; *PDF3 = plant defensin 3*; *PR4 = pathogenesis-related 4*; *rbohC = respiratory burst oxidase homolog C*; *rbohF = respiratory burst oxidase homolog F*; ns = not significant.

5.5.4 Discussion

Entomopathogenic fungi have long been studied for their direct effect on insects as a sustainable approach in suppressing them; however, their role in plant associations drew favourable attention only recently. I have confirmed that *M. robertsii* successfully colonizes cauliflower roots, which is in line with the study by Qing et al. (2023). Plant-beneficial fungus *M. robertsii* mostly resides in the rhizosphere, but it can also act as an endophyte of roots only (Behie et al., 2015), or roots and leaves (Ahmad et al., 2020b, 2020a; Sasan and Bidochka, 2012). Here, I analysed its priming effect on subsequent infection by *L. maculans.* Unfortunately, the lesions of *L. maculans* did not develop well in the true leaves of cauliflower; but the opposite was observed in the cotyledons. Surprisingly, *M. robertsii* supported the infection by *L. maculans*, *i.e.* the mycelium and lesion development in cotyledons was much stronger compared to controls. Moreover, 0.05 % Triton X also supported the infection by *L. maculans* compared to water-treated plants. Many studies exist that focused on *M. robertsii* priming effect on insect herbivore infestation, with the results of insect growth or feeding suppression (Qing et al., 2023; Rasool et al., 2021b, 2021a). Several studies investigated the effect of *M. robertsii* on fungal disease development in the rhizosphere, showing the antagonism of *M. robertsii* towards pathogenic fungi and therefore reduced disease development (Ashmarina et al., 2022; Lozano-Tovar et al., 2017; Sasan and Bidochka, 2013). In contrast, only a limited number of studies analysed the effect of *M. robertsii* belowground effect on fungal disease development in aboveground level: for instance, Ahmad et al. (2022) showed that *M. robertsii* root colonization suppressed the disease development by *Cochliobolus heterostrophus* in maize leaves. The reason could be different lifestyles of pathogens, as *C. heterostrophus* is a necrotroph, and *L. maculans* hemibiotroph; therefore, different defence pathways are induced in the host plant during the infection. Hence, *C. heterostrophus* induced genes involved in SA and JA signaling (Ahmad et al., 2022), whereas I found no induction of JA-related genes by *L. maculans* alone. It was previously shown that plants exploit SA- and

ET-dependent responses as well as oxidative stress responses against hemibiotrophic *L. maculans* in the cotyledons of oilseed rape (Šašek et al., 2012b), and in the current study, the same effect was observed in the true leaves (first repetition) and cotyledons of cauliflower. Defence genes involved in SA (*i.e. WRKY70*) and ET (*i.e. PR4*) signaling were upregulated by *L. maculans* in the true leaves regardless of the previous root treatment. Defence genes involved in SA (*i.e. ICS1* and *WRKY70*) and ET (*i.e. PDF3* and *PR4*) signaling, and genes involved in oxidative stress (*i.e. rbohC* and *rbohF*) were upregulated by *L. maculans* infection in the cotyledons. However, the combination of 0.05 % Triton X and *L. maculans* downregulated genes involved in SA signaling relative to *L. maculans* alone. Moreover, 0.05 % Triton X or *M. robertsii* in the combination with *L. maculans* upregulated the tested biosynthetic gene of JA (*i.e. AOS*) and downregulated *PDF3*, *PR4* and *rbohF* relative to the treatment with *L. maculans*; additionally, *M. robertsii* and *L. maculans* also downregulated *rbohC.* These changes might play a role in plant susceptibility to *L. maculans* infection in the cotyledons of cauliflower. Cachapa et al. (2021) and Qing et al. (2023) also observed that root treatment by 0.05 % Triton X causes changes in the plant compared to water and *Metarhizium* spp. treatment; however, defence genes involved in phytohormonal pathways were not tested before in such system. For instance, Qing et al. (2023) detected less herbivory caused by *P. xylostella* larvae in *M. robertsii*-treated cauliflower compared to the control group; in addition, less consumption was observed in plants treated with 0.05 % Triton X compared to plants treated with water. The cause of this effect is still unclear, and it can only be speculated that it is due to differences in phytohormonal signaling responses or GLS production, as the changes in GLS profile patterns were also observed in the true leaves of cauliflower after 0.05 % Triton X treatment. Nonetheless, priming effects are highly context-dependent, and much is still to be learnt regarding such plant–fungus symbioses, *e.g.* the time course of priming effect and the range of antagonists, which highlights the importance of such phytopathological studies.

6 Conclusions and recommendation for scientific and technical development

Plants evolved a highly complex immune system to survive in a hostile world. The research presented in this thesis will advance the current knowledge on plant immunity during exposure to biotic stresses, with the following conclusions:

1. Inoculation of oilseed rape leaves by hemibiotrophic fungal pathogen *L. maculans* rendered plants more palatable for *P. xylostella* caterpillars in the early stage of infection (3 days post inoculation) compared to control plants. In the later stage (7 days post inoculation) the preference of caterpillars to infected leaves was no longer observed.

2. Inoculation of oilseed rape leaves by hemibiotrophic fungal pathogen *L. maculans* did not change the preference of *B. brassicae* aphids in the early stage of infection (3 days post inoculation), but made plants less palatable for aphids in the later stage of infection (7 days post inoculation) compared to control plants.

3. Plant inoculation by *L. maculans* activated SA and ET phytohormonal pathways in oilseed rape leaves.

4. Plant inoculation by *L. maculans* significantly upregulated the production of aliphatic glucosinolates (AGs) in leaves of oilseed rape 3 days post inoculation compared to non-inoculated leaves, and changed the spectrum of VOCs.

5. The infestation by *P. xylostella* caterpillars and a combination of infection and infestation significantly increased the production of indole glucosinolates (IGs) 4 and 8 days post *L. maculans* inoculation compared to control plants.

6. The application of the elicitor benzothiadiazole (BTH) and ulvan to oilseed rape leaves resulted in a reduction of leaf consumption by *P. xylostella* larvae.

7. *B. brassicae* significantly upregulated the genes involved in SA, JA, ET and JA/ET signaling around the aphid feeding zones in *Arabidopsis*; interestingly, the transcription of *PR1* was over 100 fold higher in aphid-infested leaves at 24 hpi, and 1000 fold higher at 48 and 72 hpi compared to its respective control. Additionally, *PR1* induction by aphid feeding was confirmed on mutant lines of *A. thaliana*. Moreover, significant

induction of *JAZ10* during aphid infestation was observed at all time points (24, 48 and 72 hpi) using a fluorescent promoter-reporter line of *A. thaliana*.

8. Callose deposition was detected at all timepoints around *B. brassicae* stylet insertions, and it co-localized with *PR1* gene activation.

9. Cauliflower root inoculation by *M. robertsii* supported the infection by *L. maculans* on the cotyledons of cauliflower compared to the control group; moreover, the infection was stronger in the plants previously treated with 0.05 % Triton X compared to water treatment.

10. The infection by *L. maculans* activated SA signaling regardless of the root treatment (water, 0.05 % Triton X, *M .robertsii*), and genes involved in ET signaling and oxidative stress responses were upregulated only with water treatment on the roots. However, the combination of *M. robertsii* or 0.05 % Triton X with *L. maculans* activated JA signaling and downregulated genes involved in ET signaling and oxidative stress, which might play a role in plant susceptibility to *L. maculans* infection.

The insights gained from this research hold the potential to inform the development of innovative agricultural practices that optimize plant interactions with both antagonists and mutualists, as well as potential resistance inducers, fostering resilient and sustainable agricultural systems. By advancing our understanding of these dynamic relationships, we can strive towards a future where agriculture coexists harmoniously with nature, ensuring food security, environmental sustainability, and ecosystem resilience. However, one has to keep in mind that the biological complexity of such plant interactions makes achieving these goals highly challenging.

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